Effects of Temperature and Niclosamide on the Toxicity of the Lampricide TFM (3-trifluoromethyl-4-nitrophenol) to Sea Lamprey (*Petromyzon marinus*) and Rainbow Trout (*Oncorhynchus mykiss*)

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

This research was conducted at the University of Waterloo and Wilfrid Laurier University by Dejana Mitrovic under the supervision of Dr. Mark Servos and Dr. Michael Wilkie respectively. Dejana Mitrovic designed the studies in consultation with both supervisors.

Dejana Mitrovic was the sole author for Chapters 1 and 6, which were written under the supervision of Dr. Mark Servos and Dr. Michael Wilkie specifically for the thesis introduction and summary. As lead author of all four data chapters, Dejana was responsible for contributing to conceptualizing the study design, carrying out experiments, collecting and analyzing data, drafting and submitting manuscripts. The coauthors provided guidance during each step of the research and provided important feedback on the draft manuscripts/chapters.

This thesis consists, in part, of several manuscripts written for publication. Chapter 2 is published in Environmental Toxicology & Chemistry (ET&C). Chapter 3 is published in Aquatic Toxicology. Chapters 4 and 5 are prepared to be submitted for publication but were not yet submitted at the time of this thesis writing. Permission to use these manuscripts in the thesis was obtained from ET&C and Aquatic Toxicology. Contributor roles for the authorship contributions of each manuscript are detailed below.

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Abstract

The pesticide TFM (3-trifluoromethyl-4'-nitrophenol) is applied to rivers and streams that drain into the Laurentian Great Lakes to control populations of blood-feeding invasive sea lamprey (*Petromyzon marinus*), which continue to threaten its fisheries. Targeting larval sea lamprey, TFM impairs mitochondrial ATP production by uncoupling oxidative phosphorylation, leading to eventual energy depletion and death. TFM selectivity stems from the limited capacity of sea lamprey to detoxify the lampricide. In contrast, non-target fishes such as rainbow trout (*Oncorhynchus mykiss*) more efficiently detoxify TFM into more hydrophilic, readily excreted metabolites. TFM toxicity is affected by environmental factors such as pH and alkalinity, which alter the chemical speciation of TFM and its bioavailability. Water temperature also influences TFM toxicity, with larval TFM tolerance increasing at warmer temperatures, but the underlying mechanisms have not yet been resolved. During field treatments, TFM is often used in combination with niclosamide at 0.5-2.0% (w/w), which reduces the TFM concentrations required to eliminate 99.9% (12-h LC99.9) of larval sea lamprey by up to 40%, though the mechanism(s) of these interactions have not been resolved. This thesis therefore asked how water temperature or co-exposure to niclosamide alters TFM toxicity in sea lamprey relative to the non-target rainbow trout, and whether these effects can be explained by corresponding changes in detoxification capacity.

To test the hypothesis that an increased capacity to eliminate TFM explained the greater TFM tolerance in sea lamprey at warmer temperatures (Chapter 2), larvae were exposed to TFM over a range of temperatures, and liver and muscle were collected for TFM quantification. In sea lamprey, the 12-h LC_{50} of TFM increased steadily (~1.5-fold) as water temperature rose from 7°C to 28°C. When exposed to a common TFM concentration across temperatures, liver and muscle TFM concentrations were lowest in lamprey acclimated to 24°C, consistent with greater detoxification capacity at warmer temperatures. In contrast, toxicity was biphasic in rainbow trout exposed to TFM at 6-21°C (Chapter 4), with greatest tolerance at 6°C and 21°C, where the 12-h LC_{50} values were highest at intermediate temperatures of 11°C and 16°C. However, the effects of temperature on trout TFM tolerance were relatively small, with only a 30% difference between maximum and minimum 12-h

LC_{50} values, compared to sea lamprey, in which the 12-h LC_{50} value increased by 150% between 7°C and 28°C. Notably, tissue TFM accumulation in trout was highest at 21°C, indicating that TFM toxicity in rainbow trout, unlike in sea lamprey, is not directly tied to detoxification capacity but instead reflects interactions among multiple physiological processes. These findings indicate that water temperature, alongside water pH and alkalinity, should be considered when determining TFM application concentrations to optimize treatment efficacy and minimize non-target species risks.

Applications of TFM are regularly supplemented with 0.5-2.0% niclosamide (w/w), which substantially improves TFM efficacy. The concentration addition model was used to characterize the nature of TFM: niclosamide interactions using the toxic unit (TU) concept, in which 1TU = the 12-h LC_{50} of each chemical. Exposure of larval sea lamprey to TFM/1% niclosamide mixtures (Chapter 3) resulted in synergistic (greater than additive) interactions when the sum of TFM plus niclosamide ($TU_{Tot\ Exp}$) exceeded 1TU. However, interactions were antagonistic (less than additive) when the $TU_{Tot\ Exp}$ were less than 1TU, indicating a concentration-dependent shift in the nature of TFM/niclosamide interactions. Elevated TFM toxicity in the presence of niclosamide corresponded with greater TFM accumulation in the muscle but not in the liver, consistent with impaired hepatic metabolism of TFM by niclosamide. In rainbow trout (Chapter 5), the TFM/1% niclosamide mixture produced antagonistic interactions (< 50% mortality) across all concentrations tested. Co-exposure of the trout to TFM and niclosamide led to greater TFM accumulation in liver and brain, was consistent with competitive inhibition of enzymatically mediated detoxification by the liver. These divergent tissue-distribution patterns may indicate species-specific mechanisms underlying TFM toxicity and how toxicity is modified by niclosamide.

In conclusion, this thesis demonstrates that TFM toxicity is governed by water temperature in addition to pH and alkalinity. Thus, temperature is another abiotic variable that should be considered when planning and executing lampricide applications. Evidence was also provided that indicates that niclosamide interferes with TFM detoxification, accounting for enhanced toxicity to sea lamprey, trout and likely other non-target organisms when exposed to TFM/niclosamide mixtures. However, species-specific differences in modes

of TFM handling and detoxification need to be further elucidated between sea lamprey, trout, and other species. The improved mechanistic understanding generated by this research provides a foundation for more precise, evidence-based tailoring of lampricide treatments to prevailing environmental conditions. Thus, this thesis will help move the Sea Lamprey Control Program toward improved treatment efficacy, reduced chemical use and cost, and minimized effects on non-target species. More broadly, this work offers a framework for understanding how environmental conditions and chemical mixtures influence the toxicity of other phenolic compounds in other aquatic organisms in the environment.

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Dedication

To my dad: thank you for being the first and most steadfast wind in my sails, in this life and beyond it.

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List of Abbreviations

Abbreviation	Definition
CAN	Acetonitrile
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CF	Fulton's condition factor
CI	Confidence intervals
C_{SS}	Steady state concentration
DO	Dissolved oxygen
EC	Emulsifiable concentrate
GLFC	Great Lakes Fishery Commission
GLM	Generalized linear model
HPLC	High-performance liquid chromatography
IDL	Instrument detection limits
K_m	Michaelis Constant, enzyme binding affinity
LC_{10}	Lethal concentration affecting 10% of the population
LC_{25}	Lethal concentration affecting 25% of the population
LC_{50}	Lethal concentration affecting 50% of the population
$LC_{99.9}$	Lethal concentration affecting 99.9% of the population
LC-MS/MS	Liquid chromatography tandem mass spectrometry
log K_{ow}	Log octanol: water partition coefficient
MLC	Minimum lethal concentration, $LC_{99.9}$
MS-222	Tricaine methanesulfonate
Nic	Niclosamide or 2',5-Dichloro-4-Nitrosalicylanilide
Nic-^{13}C	Niclosamide-^{13}C
PCr	Phosphocreatine
S.E.M.	Standard error of the mean
SLCP	Sea Lamprey Control Program
$t_{1/2}$	Half-life
TFM	3-Trifluoromethyl-4-Nitrophenol
T_{opt}	Thermal optima temperature
TU	Toxic unit
$TU_{Tot\ Exp}$	Total toxic unit of exposure
UDP	Uridine diphosphate
UDPGT	Uridine diphosphate-glucuronosyltransferase
V_{max}	Maximum velocity (reaction rate)
ww	Wet weight
YOY	Young of the year

Chapter 1
General Introduction

1.1 Sea lamprey, an invasive species in the Laurentian Great Lakes

Invasive sea lamprey (*Petromyzon marinus*) have plagued fisheries in the Laurentian Great Lakes since the early 20th century, causing massive economic damage and declines in economically important commercial, recreational and Indigenous fisheries (Gaden et al., 2021; Siefkes, 2017). While there is ongoing debate whether sea lamprey are native to Lake Ontario (Bryan et al., 2005; Eshenroder, 2009, 2014; Waldman et al., 2004, 2006), there is a consensus that with the construction of the shipping canals in the late 1800s and early 1900s, sea lamprey were able to spread to all of the Great Lakes (Eshenroder, 2009, 2014; Hume et al., 2024; Smith & Tibbles, 1980). Peak sea lamprey numbers (above 1.5 million) during the 1960s (Siefkes, 2017) particularly impacted the populations of economically relevant species, including lake trout (*Salvelinus namaycush*) and lake sturgeon (*Acipenser fulvescens)*, each of which are also culturally important to many Indigenous communities surrounding the Great Lakes (Gaden et al., 2021; Siefkes, 2017; Smith & Tibbles, 1980).

As one of two surviving groups of ancient jawless fishes, called cyclostomes (round mouth; Docker & Potter, 2019), sea lamprey belong to the order Petromyzontiformes and are among at least 41 recognized lamprey species spanning freshwater and seawater habitats worldwide (Docker & Potter, 2019; Renaud, 2011). Lampreys exhibit an anti-tropical distribution, occurring primarily in temperate regions of both hemispheres (Renaud, 2011). Lampreys are believed to have diverged from the main vertebrate lineage roughly 450 million years ago (Gess et al., 2006), with recent fossil evidence suggesting that the predatory or parasitic lifestyle evolved much later, ~ 158-160 million years ago (Wu et al., 2023). Although all modern lampreys likely descended from an anadromous predatory/parasitic ancestor, most extant species are non-parasitic and complete their life cycle entirely in fresh water (Renaud, 2011). Of the at least 41 recognized lamprey species, only 18 retain a parasitic phase, including the sea lamprey (Docker & Potter, 2019; Potter et al., 2015).

Sea lamprey have a complex multi-stage and multi-year life cycle (Figure 1.1; Wilkie et al., 2019). For most of their life cycle (3-7 years), sea lamprey live buried in sediment as

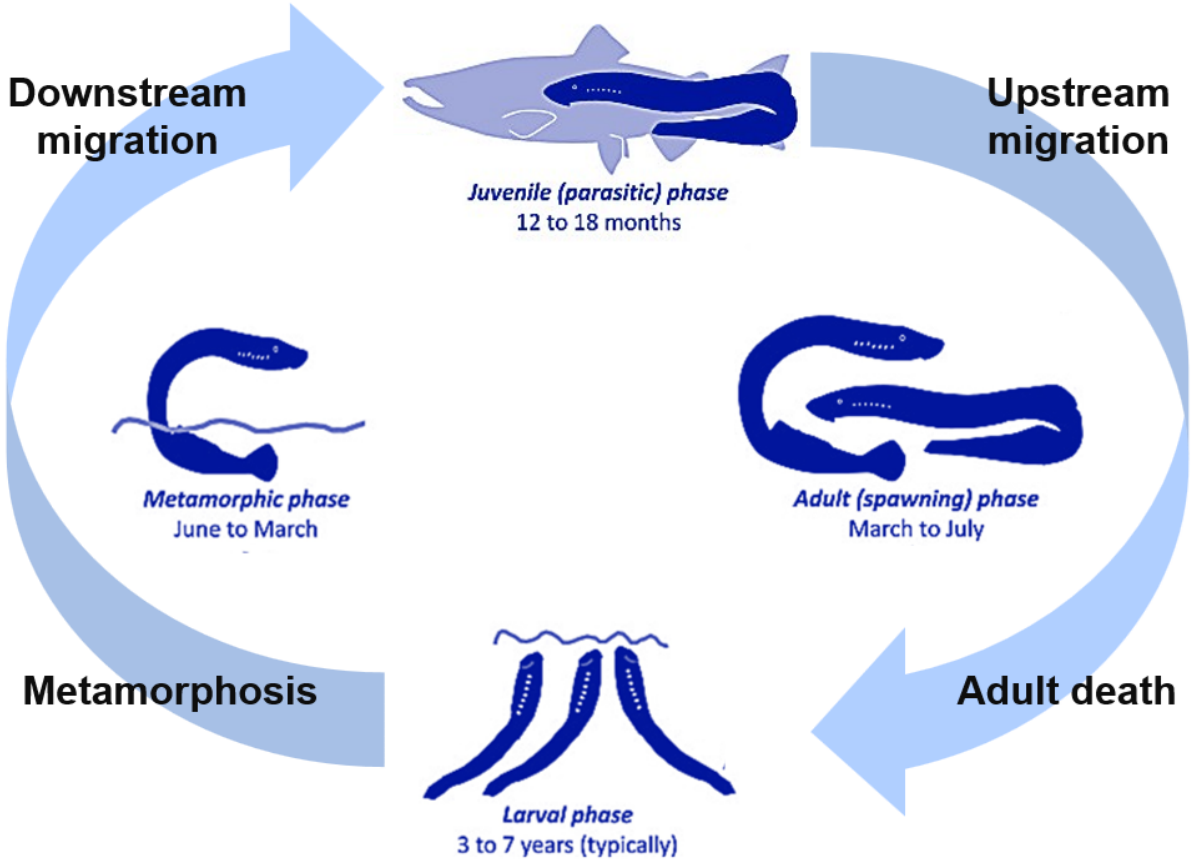


Figure 1.1 Sea lamprey (*Petromyzon marinus*) life cycle. Larval sea lamprey (ammocoetes) typically spend the first 3-7 years of life burrowed in the substrate of streams as filter-feeders. The larval phase is followed by a complex metamorphosis characterized by the formation of fully developed eyes, more pronounced dorsal fins and a multi-toothed oral disc and rasping tongue. The juvenile parasitic phase lasts 12-18 months, during which the juvenile sea lamprey attach to the bodies of various fish species, feeding on the blood of their hosts. Following the parasitic phase, sea lamprey enter the terminal spawning adult phase, when they cease feeding, migrate up rivers and streams, spawn and then die (Modified from Borowiec et al., 2021; Wilkie et al., 2019).

filter-feeding larvae, called ammocoetes (Beamish & Potter, 1975; Sutton & Bowen, 1994). Once they accumulate sufficient lipid reserves and reach a suitable size (length >120 mm; mass > 2.5 g), they undergo a prolonged metamorphosis into a parasitic juvenile lamprey (Youson, 1980; Manzon et al., 2015). This developmental period typically lasts 3-4 months and is characterized by significant changes in their internal and external body structure and

appearance (Youson, 1980; Manzon et al., 2015). Parasitic juvenile sea lamprey develop a multi-toothed oral disc and rasping tongue, which they use to attach to the side of fish and feed on the blood (Farmer et al., 1975; Farmer, 1980; Swink, 2003). The high rate of blood ingestion (3-30% of their total body mass per day), added to the possibility of secondary infections caused by attachment of the oral disc and rasping tongue, are the reasons why many fish die following a lamprey encounter (Farmer et al., 1975; Farmer, 1980). During its parasitic juvenile phase, a single sea lamprey could be responsible for killing the equivalent of up to 20 kg of lake trout (Swink, 2003). After 12-20 months, adult sea lamprey cease feeding, migrate upstream to spawn, and die shortly after (Beamish & Potter, 1975).

The historic range of the sea lamprey encompasses most of the north Atlantic Ocean, extending from Florida to Newfoundland and Labrador, and reaching into the Mediterranean and the Baltic Seas, as well as the Gulf of St. Lawrence and the upper St. Lawrence River (Hansen et al., 2016). The anadromous sea lamprey spends most of its life cycle in freshwater, with larval and metamorphosis phases and adult spawning occurring in tributaries to the Atlantic Ocean (Beamish & Potter, 1975; Manzon et al., 2015). The sea lamprey invasion of the Great Lakes resulted in a landlocked, entirely freshwater, and invasive parasitic/predatory population that had devastating economic, ecological and cultural consequences for Great Lakes fisheries (Figure 1.1; Gaden et al., 2021; Siefkes, 2017).

In response to the destruction caused by sea lamprey, the United States (US) and Canada signed the Convention on Great Lakes Fisheries in 1954, which led to the formation of the Great Lakes Fishery Commission (GLFC). The GLFC was given a mandate to find means to bring sea lamprey populations under control (Gaden et al., 2021). The GLFC subsequently initiated a Sea Lamprey Control Program (SLCP) and research agenda to suppress sea lamprey populations and protect vulnerable fish populations (Gaden et al., 2021; Sullivan et al., 2021). By combining physical barriers such as dams and traps, and the application of lampricides (pesticides more specific to lamprey), sea lamprey populations in the Great Lakes were reduced by 90% compared to the peak numbers of the 1960s (McLaughlin et al., 2007; Siefkes, 2017; Sullivan et al., 2021). While lampricide treatments

are the backbone of the current SLCP, the overall program would not be nearly as successful if not for the existence of physical dams and barriers that prevent adult sea lamprey from swimming upstream to reach the additional spawning grounds (Siefkes, 2017).

Lampricide treatments are typically applied every 2-3 years to streams and rivers draining into the Great Lakes, targeting larval sea lamprey before they complete metamorphosis into parasitic juveniles that prey on fishes (Smith & Tibbles, 1980; Sullivan et al., 2021). The most used lampricide, 3-trifluoromethyl-4-nitrophenol (TFM), is typically applied to larval sea lamprey infested streams at up to 1.5 times its minimum lethal concentration (MLC) to ensure maximum treatment efficiency (Bills et al., 2003; McDonald & Kolar, 2007; Sullivan et al., 2021). The MLC is defined as the concentration of TFM needed to kill 99.9% of larval lamprey over 9 h (McDonald & Kolar, 2007; Sullivan et al., 2021). Even so, approximately 10 percent of larval sea lamprey in streams are treatment survivors that manage to avoid death and potentially go on to metamorphose into parasitic juvenile sea lamprey that damage fisheries (Sullivan et al., 2021; Wilkie et al., 2019). The concentrations of TFM applied during a treatment depend on several environmental factors such as pH, water alkalinity and the time of the year (Bills et al., 2003; Hlina et al., 2021; Muhametsafina et al., 2019; Scholefield et al., 2008; Schueller et al., 2024; Wilkie et al., 2021).

TFM is often co-applied with niclosamide, usually as mixtures of 0.5-2.0% niclosamide (weight/weight), to increase TFM effectiveness and to reduce TFM consumption and costs (Dawson, 2003; Howell, 1980; Sullivan et al., 2021). Niclosamide (Nic, 2',5-dichloro-4-nitrosalicylanilide) is also a molluscicide primarily used to control snail populations, particularly in tropical regions where snails are vectors for the fluke that causes schistosomiasis in people (Joubert at al., 2001; Lardans & Dissous, 1998; McManus et al., 2018). It is also administered as an anthelmintic to control intestinal parasites in humans and livestock (Frayha et al., 1997; Sheth, 1975). When co-applied with niclosamide, its powder or emulsified concentrate form (0.5-2.0% w/w), TFM applications result in a decrease of up to 40% in the amount of TFM required in a treatment without compromising TFM selectivity

to larval sea lamprey (Boogaard et al., 2003; Sullivan et al., 2021). Thus, the presence of niclosamide reduces the treatment costs for the SLCP by requiring less TFM to maintain suppression of larval sea lamprey within the Great Lakes (Sullivan et al., 2021).

A granular niclosamide formulation (granular Bayluscide®), where granules of silica sand are coated with niclosamide (Sullivan et al., 2021), is also applied for population surveys and for the treatment of deep lentic waters or rivers with very high discharge, where the application of TFM is often impractical or less effective due to challenges in achieving and maintaining target concentrations (Dawson, 2003; Sullivan et al., 2021).

1.2 Lampricides used in the control of the sea lamprey population

1.2.1 TFM (3-trifluoromethyl-4-nitrophenol)

The identification of effective lampricides, which also limit non-target organism effects, is the primary reason behind the success of the SLCP. Among the more than 4,500 compounds tested, TFM was chosen due to its high selectivity and potency to sea lamprey (Applegate et al., 1966; Wilkie et al., 2019). TFM has also been highly effective because the applications target multiple generations of larval sea lamprey (due to their long larval stage, 3-7 years) in infested streams with relatively few adverse effects on non-target organisms (Wilkie et al., 2019). It has been hypothesized that TFM applications selectively affect target larval sea lamprey due to their relatively low biotransformation capacity to convert TFM into much less toxic and easier to excrete compounds (Kane et al., 1994; Lech & Statham, 1975; Lawrence et al., 2021). Although most non-target fish species are more tolerant to TFM due to greater capacity to detoxify it, sensitivity varies widely among species, ranging from high sensitivity in young-of-the-year lake sturgeon (*Acipenser fulvescens*) to very tolerant bluegill (*Lepomis macrochirus*; Boogaard et al., 2003; Kane et al., 1994; Lawrence et al., 2021).

TFM is an aromatic phenol (Figure 1.2), solid at room temperature with a yellow-orange colour in solution, that is very stable in water with a long half-life of up to 4,000 days, depending on pH (Hubert, 2003). Although laboratory studies have demonstrated TFM can photodegrade (Carey et al., 1988), during actual field treatments of only 9-12 hours, little to

no photodegradation products were found downstream of a treatment site (a few km long), indicating that loss in TFM concentration downstream is more likely due to dilution (McConville et al., 2017a, b).

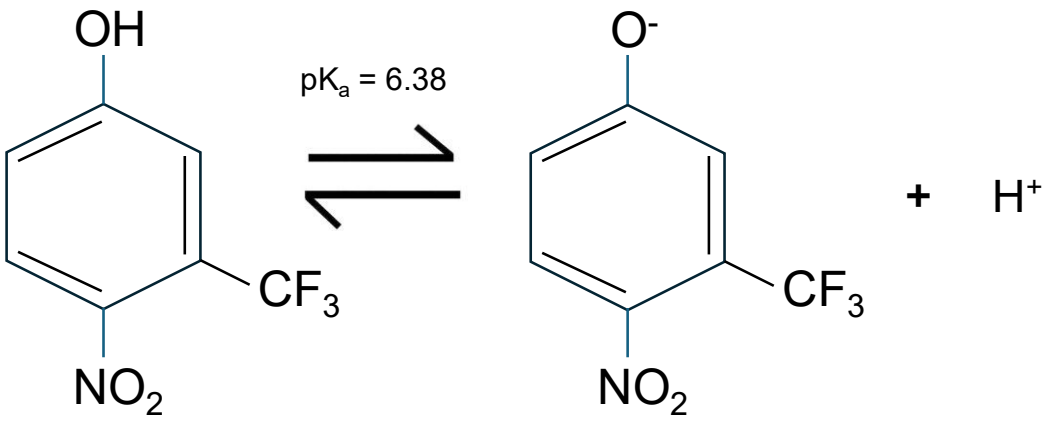


Figure 1.2 Chemical structure of 3-fluoromethyl-4-nitrophenol (TFM). Structure of un-ionized TFM and ionized TFM, and corresponding dissociation constant (pKa).

As a weak acid with a pKa = 6.38 (McConville et al., 2016), un-ionized TFM (TFM-OH) can partially dissociate in solution into ionized TFM (TFM-O^-), depending on water pH (Dawson, 2003; Hubert, 2003). By affecting the TFM ionization state, water pH can therefore alter the bioavailability and movements of TFM across gill membranes (Hunn & Allen, 1974; Wilkie et al., 2021). Decreasing water pH will increase TFM toxicity by increasing the concentrations of the more lipophilic un-ionized form of TFM (TFM-OH), making it easier to cross the gills than the ionized (O^-) form of TFM (TFM-O^-; Hlina et al., 2017; Hunn & Allen, 1974). As water pH increases, the proportion of ionized TFM increases, resulting in lower TFM uptake rates (Hlina et al., 2017; Hunn & Allen, 1974). Once in the blood, where the physiological pH is ~ 7.8, most of the un-ionized TFM would be converted to the ionized form TFM-O^-, thus trapping TFM within the animal and propagating the diffusion gradients across the gills and sustaining the un-ionized uptake of TFM (Erickson et al., 2006a, b; Wilkie et al., 2019). Higher alkalinity also leads to lower TFM toxicity,

possibly by increasing water buffering capacity that resists pH changes within the gill microenvironment and reduces acidification at the gill surface (Wilkie et al., 2021). The greater buffering capacity of high alkalinity water, therefore, results in reduced availability of the un-ionized form of TFM (TFM-OH) at the gill, leading to reduced uptake rates and lower TFM toxicity (Wilkie et al., 2021). While alkalinity does not directly influence TFM speciation, it can affect the pH of the gill microenvironment, thereby altering TFM bioaccumulation and toxicity (Wilkie et al., 2021).

It has been shown that TFM interferes with mitochondrial ATP production by uncoupling oxidative phosphorylation through the disruption of the H^+ electrochemical gradient (proton motive force) between the mitochondrial intermembrane space and the matrix (Birceanu et al., 2011; Huerta et al., 2020; Wilkie et al., 2019). Due to the lower pH of the intermembrane space relative to the mitochondrial matrix, H^+ ions associate with ionized TFM (TFM-O^-) within the intermembrane space to form the protonated species (TFM-OH), thereby increasing its membrane permeability and facilitating diffusion into the mitochondrial matrix (Wilkie et al., 2019). Once in the more alkaline matrix, TFM is deprotonated, and TFM-O^- moves back into the intermembrane space, repeating the cycle (Birceanu et al., 2021; Wilkie et al., 2019). The movement of TFM-OH across the inner mitochondrial membrane increases H^+ accumulation in the matrix, leading to the collapse of the proton gradient (Wilkie et al., 2019). Due to high proton levels in the matrix, H^+ movement through the ATP synthase decreases, thereby reducing ATP production (Wilkie et al., 2019).

The uncoupling of oxidative phosphorylation by TFM leads to an insufficient ATP supply relative to metabolic demands, resulting in the utilization of glycolysis and other anaerobic pathways to produce ATP (Wilkie et al., 2019). This eventually leads to marked tissue decreases in phosphocreatine and glycogen in sea lamprey (Wilkie et al., 2007; Birceanu et al., 2009, 2011; Clifford et al., 2012; Henry et al, 2015) as well as rainbow trout (*Oncorhynchus mykiss*; Birceanu et al., 2014) and invertebrates (Viant et al., 2001). As these

energy stores are finite, prolonged exposure to TFM ultimately leads to death due to ATP starvation (Birceanu et al., 2011; Clifford et al., 2012; Henry et al., 2015).

Once inside the fish, TFM accumulation is generally highest in the liver, which is also where Phase II detoxification occurs (Hubert et al., 2005; Kane et al., 1994; Lech & Statham, 1975; Wilkie et al., 2019). However, elevated concentrations of TFM can also be found in the blood and muscle (Dawson et al., 2002; Hubert et al., 2005; Lech & Statham, 1975; Vue et al., 2002). Despite relatively lower TFM concentrations in sea lamprey muscle, the muscle accounts for approximately 75% of total body mass as observed in ammocoetes of European brook lamprey (*Lampetra planeri*; Bull & Morris, 1967) and serves as a sink for circulating TFM (Lech & Statham, 1975; Wilkie et al., 2019). However, TFM does not stay within the fish very long, as most can be excreted/eliminated from the body within 24-48 h upon placement of fish in clean, TFM-free water (Dawson et al., 2002; Hlina et al., 2017; Lech et al., 1973).

The selectivity of TFM is due to the sea lamprey's limited ability to detoxify TFM compared to most non-target species (Bussy et al., 2018a, b; Kane et al., 1994; Lawrence et al., 2021; Lech & Statham, 1975). Larval sea lamprey have a relatively low capacity to convert the parent compound (TFM) into more water soluble, less toxic metabolites via Phase II detoxification (Figure 1.3), which are more easily excreted (Birceanu et al., 2021; Wilkie et al., 2019). Phase II metabolic pathways are generally considered to be a primary mechanism of detoxification for phenolic compounds such as TFM in fishes (Hubert et al., 2005; Kane et al., 1994; Lech & Costrini, 1972; Lech & Statham, 1975). Phase II detoxification attaches a water-soluble functional group to xenobiotics and endogenous compounds such as steroids (Clarke et al., 1991; Kalant & Roschlau, 1998). Glucuronidation is usually the predominant Phase II pathway and results in a glucuronic acid functional group being added to TFM to generate TFM-glucuronide (Kane et al., 1994; Lech & Stathan, 1975). Kane et al. (1994) showed that the activity of UDP-glucuronosyltransferase (UDPGT), enzymes required to convert parent TFM into TFM-glucuronide, was much higher in non-target fishes such as bluegill and rainbow trout, compared to sea lamprey. The lower

UDPGT activity in sea lamprey has been attributed to reduced expression of these enzymes and their lower affinity for TFM (Kane et al., 1994; Lawrence et al., 2022). In addition to glucuronidation, there is also evidence that sea lamprey can use another Phase II pathway enzyme, phenol sulphotransferase, that can transform parent TFM into TFM-sulphate (Bussy et al., 2018a, b). As the selective TFM toxicity to sea lamprey, relative to other species, is likely related to differences in Phase II metabolism, any factors that can influence this difference or capacity could, in turn, affect the relative TFM toxicity. Increasing water temperature or addition of another lampricide (e.g., niclosamide) could potentially result in altered activity and/or affinity of Phase II metabolic enzymes, altering the TFM blood and tissue accumulation.

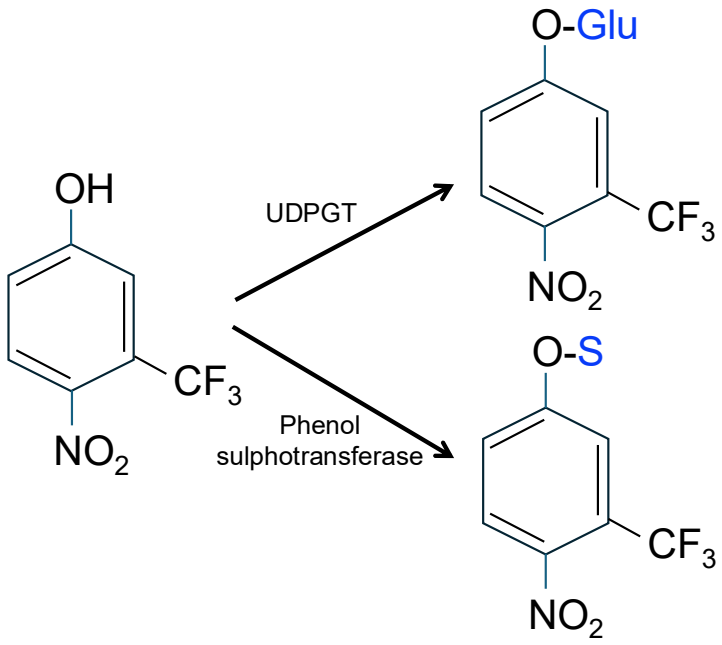


Figure 1.3 Biotransformation of TFM. TFM Phase II biotransformation to TFM-glucuronide via UDP-glucuronosyltransferase (UDPGT) or TFM-sulphate via phenol sulphotransferase using glucuronidation and sulphation (adapted from Bussy et al., 2018a).

There is some evidence that Phase I metabolism (reduction of the nitrophenol to an amino group) may play a relatively larger role in TFM detoxification in sea lamprey compared to other non-target fishes (Bussy et al., 2018a, b). Although Phase I metabolism of TFM would make the molecule slightly more water soluble and more susceptible to further metabolism (facilitating excretion), it remains unclear whether Phase I metabolism is physiologically relevant to the overall rates of TFM detoxification in sea lamprey.

1.2.2 Water temperature effects on TFM toxicity

During field applications, season has been shown to play a role in the sensitivity of larval sea lamprey to TFM, with greater tolerance in late summer compared to spring (Applegate et al., 1966; Hlina et al., 2021; Muhametsafina et al., 2019; Schueller et al., 2024). Recent studies suggest that these differences may be primarily related to higher water temperatures (Hlina et al., 2021; Muhametsafina et al., 2019), rather than differences in energy reserves as was previously suggested to explain the seasonal variation (Scholefield et al., 2008). Other phenolic compounds, including the oxidative phosphorylation uncoupler 2,4-dinitrophenol and pure phenol, are also less toxic to some fishes at warmer temperatures (Howe et al., 1994; Patra et al., 2015), but the underlying mechanisms remain unclear.

Muhametsafina et al. (2019) speculated that the seasonal differences in TFM toxicity were due to an increased capacity of sea lamprey to detoxify TFM at warmer temperatures. They suggested that at higher water temperatures, sea lamprey had lower TFM accumulation due to higher rates of TFM elimination, resulting in greater TFM tolerance. The lower TFM accumulation was attributed to the higher detoxification rates associated with the increased capacity of the available Phase II metabolic enzymes (Muhametsafina et al., 2019). Compared to TFM tolerant fish species with much higher detoxification capacities, higher temperatures could lead to marked increases in the rates of glucuronidation and sulphation in sea lamprey, making TFM less selective to lamprey at higher temperatures (Muhametsafina et al., 2019). The enzymes involved in this pathway could become more efficient at higher

temperatures, increasing the pathway's output and excretion rates (reducing tissue concentrations). The possibility of other metabolic pathways, including Phase I pathways that are thought to be present in sea lamprey (Bussy et al., 2018a, b), might also become relatively more important in sea lamprey at higher temperatures (increased metabolic rates), relative to other species. The possible influence of temperature on TFM toxicity is further supported by the observations that other phenolic toxins (known oxidative phosphorylation uncouplers) behave similarly, with decreasing toxicity in trout with increasing temperature (Howe et al., 1994), or exhibiting a decrease in phenol toxicity in the mid-range temperatures, compared to the outer limits in several fish species (Patra et al., 2015). The hypothesis that the changes in TFM toxicity at different water temperature are due to changes in detoxification capacity is still lacking evidence in both sea lamprey and non-target fishes.

1.2.3 Niclosamide (2',5-dichloro-4-nitrosalicylanilide)

Like TFM, niclosamide (2',5-dichloro-4-nitrosalicylanilide) is also a phenolic compound and a pale-yellow solid at room temperature (Figure 1.4). It consists of two aromatic rings connected by an amide group; its molecular weight (327.119 g mol^{-1}) is much higher than TFM (207.11 g mol^{-1}; Birceanu et al., 2021; Wilkie et al., 2019). Niclosamide is also a weak acid with a similar pKa (pKa niclosamide = 6.02) to TFM (pKa = 6.38), which results in greater proportions of the un-ionized (-OH) forms of each at lower pH (McConville et al., 2016), which are more diffusible in the lipid bi-layer and taken up across the gills (Figure 1.4; Wilkie et al., 2019). However, with a log octanol: water partition coefficient (log K_{ow}) of 4.56 (Lewis et al., 2016), niclosamide is also more lipophilic than TFM (log K_{ow} = 2.77; Schmitt et al., 2000). Thus, at the pH of natural waters, it is expected that niclosamide will more easily diffuse across the gills of fish than TFM, which could partly explain its greater relative toxicity (Birceanu et al., 2021; Borowiec et al., 2022).

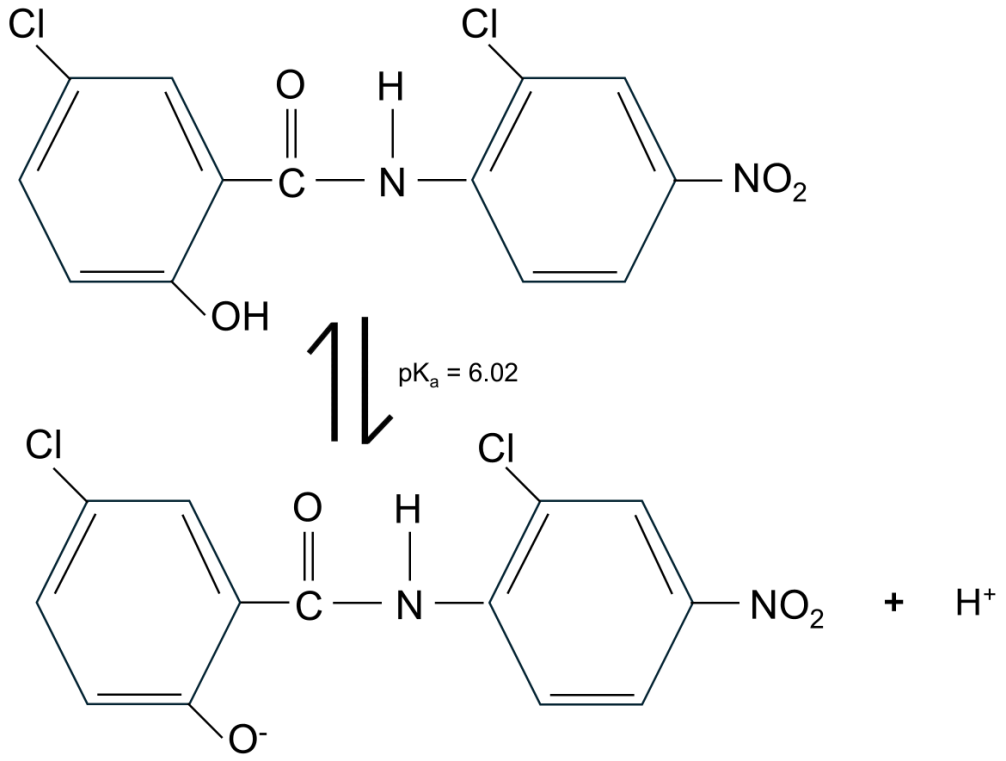


Figure 1.4 Chemical structure of 2,5-dichloro-4'-nitrosalicylanilide (niclosamide, Nic). Structural differences between un-ionized and ionized niclosamide and corresponding dissociation constant (pKa).

The information available on niclosamide's mode of action is primarily based on isolated mitochondrial respiration studies using liver from adult sea lamprey, demonstrating that, similar to TFM, niclosamide acts by uncoupling oxidative phosphorylation (Alasadi et al., 2018; Borowiec et al., 2022; Tao et al., 2014). However, the effects of niclosamide on larval sea lamprey, as well as its mechanisms of uptake, distribution, and excretion, still need to be fully explored. In addition to disrupting oxidative phosphorylation in a manner similar to TFM, niclosamide may act through an additional mechanism that could explain its higher toxicity. The involvement of niclosamide in mitochondrial fragmentation and the promotion of apoptosis has been observed in various organisms and cell types (Alasadi et al., 2018; Park

et al., 2011; Tao et al., 2014), which in turn could also account for more rapid effects and at much lower exposure concentrations.

1.2.4 Lampricide interactions

When TFM is applied in combination with niclosamide (0.5-2% w/w), TFM toxicity increases, reducing the amount of TFM required per treatment by up to 40% while maintaining effectiveness (Boogaard et al., 2003; Wilkie et al., 2019). The nature and the underlying mechanisms by which niclosamide enhances TFM toxicity are still unresolved.

There are several approaches used for evaluating the toxicological interactions between two chemicals or toxicants. One of the most widely used approaches is the concentration additivity model, in which toxicity is measured in toxic units (TU), which is used to evaluate the interactions between mixtures of substances with the same modes of action (Norwood et al., 2003; Playle, 2004). Any appropriate endpoint can be used to define a TU, but it is most frequently expressed as the concentration of each substance required to kill 50% of the test organism over a specified time, the LC_{50} (Playle, 2004). For instance, if 1 TU of TFM (TU_{TFM}) and 1 TU of niclosamide (TU_{Nic}) were set to the respective 12-h LC_{50} of each chemical, exposure to 0.5 TU_{TFM} and 0.5 TU_{Nic} would result in 50% mortality if the interactions were strictly additive (Newman, 2020). It would be less than additive (antagonistic) if the combined mortality was less than 50% and greater than additive (synergistic) if the combined mortality was greater than 50%.

The interaction relationship can also be explained in terms of expected vs. observed mortalities in the mixture exposures (Figure 1.5). A strictly additive interaction would produce observed mortalities that result in a linear relationship between the sum of the expected TU of mortalities and the sum of the TU of exposure of the two toxicants (TFM and niclosamide; Figure 1.5). If the observed mortalities fall below the strict additivity line, it would indicate less than expected mortality, meaning that the relationship between the two toxicants was antagonistic (less than additive). Higher than expected mortality numbers

would fall above the strict additivity line, indicating a synergistic (more than additive) relationship between the two toxicants.

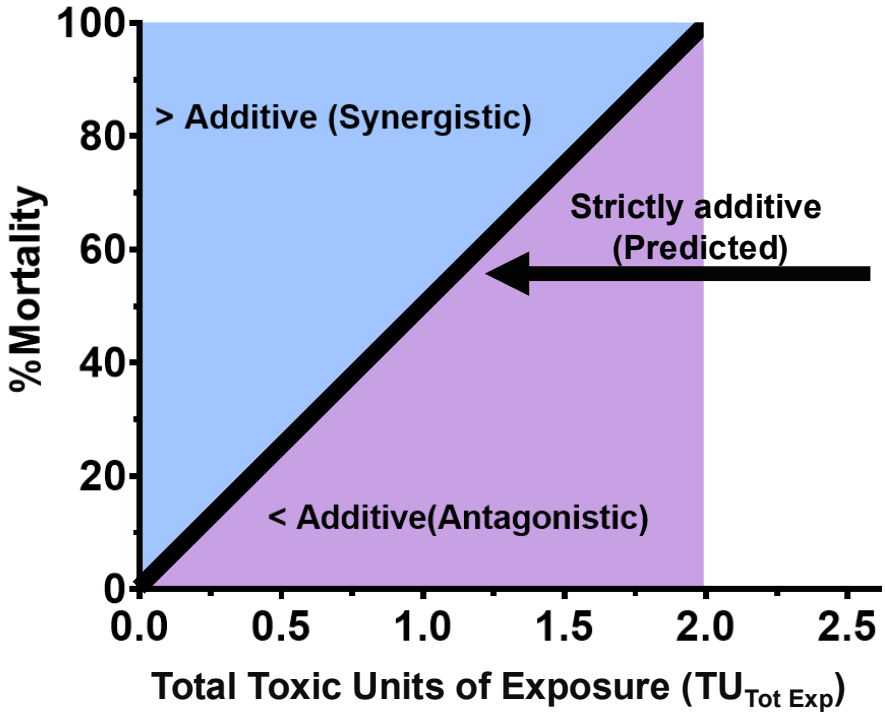


Figure 1.5 The theoretical concentration addition model using toxic units (TU). The concentration addition model determines the interaction of two chemicals by displaying percent mortality rates based on the combined effects of both chemicals (total toxic units of exposure). Strictly additive interactions between two chemicals would fall on the line of strict additivity (black line), antagonistic interactions would produce mortality rates lower than expected, falling below the black line (purple area), while synergistic interactions would produce higher than expected mortality rates and fall above the line of strict additivity (blue area). Figure adapted from Newman (2020).

Some previous studies in rainbow trout, brook trout (*Salvelinus fontinalis*) and larval sea lamprey indicated that mixtures of TFM and niclosamide were strictly additive or antagonistic (Bills & Marking, 1976; Dawson et al., 1977; Marking & Bills, 1985), while

others suggested that interactions were synergistic (Howell et al., 1964). However, these findings were based on the use of a wettable powdered niclosamide, Bayer 73, which is no longer used for sea lamprey control (Sullivan et al., 2021). The SLCP transitioned to an emulsifiable concentrate (EC) formulation of niclosamide, which is now the commonly used formulation co-applied with TFM, which was safer and proved easier to handle (Luoma et al., 2024; Sullivan et al., 2021). Most recently, Hepditch et al. (2021) employed the concentration addition model to elucidate the nature of interactions between TFM and niclosamide using the EC formulation. This latter study demonstrated that at relatively low concentrations of TFM (below one TU of total exposure; 1 $TU_{Tot\ Exp}$), TFM/1% niclosamide interactions were strictly additive, while concentrations above $1TU_{Tot\ Exp}$ produced a more synergistic interaction between TFM and 1% niclosamide in rainbow trout (Hepditch et al., 2021). Earlier studies (Bills & Marking, 1976; Dawson et al., 1977; Howell et al., 1964; Marking & Bills, 1985) based the interaction relationship between TFM and niclosamide across different exposure durations, rather than typical field application times. There also remains a lack of understanding about the toxic interactions between TFM and niclosamide on larval sea lamprey at environmentally relevant exposure concentrations. There is also a need for consistent experimental protocols in order to assess the TFM and niclosamide interactions within the lampricide mixture and across both sea lamprey and non-target species.

1.3 Non–target effects of lampricides

While there has been great success in controlling sea lamprey populations with lampricides, the effects of lampricide exposure on non-target organisms, such as lake sturgeon or lake trout (*Salvelinus namaycush*) remain a concern. Many, but likely not all, fishes have a greater capacity to detoxify TFM via glucuronidation and sulphation than sea lamprey (Lech & Statham, 1975; Kane et al., 1994; Lawrence et al., 2021). The relative tolerance of non-target fishes to TFM relative to sea lamprey can be illustrated using species sensitive distribution (SSD; Boogaard et al., 2003; Wilkie et al., 2019). The SSD analyses compare the relative toxicity of TFM of non-target species to larval sea lamprey by calculating the toxicity ratio in

which the 12-h LC_{50} of a respective non-target fish (or other animal) is divided by the MLC of the sea lamprey (Boogaard et al., 2003):

$$\text{Toxicity Ratio} = \text{12-h } LC_{50}/\text{MLC} \quad (1)$$

For most fishes, toxicity ratios reveal that the TFM tolerance of sea lamprey is 2-10 times higher than for larval sea lamprey (Bills et al., 2003; Boogard et al., 2003; Wilkie et al., 2019). The most TFM-tolerant teleosts are American eel (*Anguilla rostrata*) and centrarchid fishes (i.e., bluegill, smallmouth bass (*Micropterus dolomieu*)), while young of the year lake sturgeon and catfishes (family: Ictaluridae) are among the most sensitive (Wilkie et al., 2019). The protection of non-target species during lampricide field treatments is complicated by the inter-species differences as well as intra-species variation in their tolerance to lampricides. TFM toxicity may change depending on the life stage of the organism. This is true in young-of-the-year and juvenile lake sturgeon, which are more sensitive to TFM than the adult sturgeons (Boogard et al., 2003; McDonald & Kolar, 2007; O'Connor et al., 2017).

For most fishes, including rainbow trout, the effects of relatively short (< 12 h) lampricide exposure at sublethal concentrations that are typical of SLCP treatments are minimal and transient (Bills et al., 2003; Wilkie et al., 2019). When used in treatments, niclosamide concentrations do not exceed 2% of the TFM concentration (0.002 - 0.007 mg L^{-1}; Boogaard et al., 2003). These niclosamide concentrations are well below the LC_{50} and MLC concentrations that would cause physiological harm and mortality in most non-target fishes (Birceanu et al., 2021; Wilkie et al., 2019). The high capacity to biotransform and excrete TFM from the body would prevent concentrations of TFM from building up in tissues, thereby avoiding toxic effects in non-target fishes (Birceanu et al., 2021; Wilkie et al., 2019).

Another important aspect of non-target fishes that needs to be considered when evaluating the toxicity of TFM is the treatment duration (Birceanu et al., 2021; Wilkie et al., 2019). While acute TFM exposure affects non-target fish, most effects are temporary and resolve shortly after TFM exposure is terminated (Birceanu et al., 2021; Wilkie et al., 2019).

Reductions in fuel stores, such as glycogen, glucose and phosphocreatine (PCr), are frequently observed during lampricide exposures due to greater reliance on anaerobic metabolism when anaerobic ATP supply via the mitochondria no longer matches ATP demand (Birceanu et al., 2009, 2011; Clifford et al., 2012; Henry et al., 2015; Ionescu et al., 2021, 2025; Wilkie et al., 2007). Notably, these high energy stores are readily restored in surviving fish, including sea lamprey, rainbow trout and lake sturgeon, following lampricide exposure (Clifford et al., 2012; Ionescu et al., 2021, 2025).

There are several reasons why rainbow trout are often used as a representative model for non-target fishes. Their TFM sensitivity is about halfway between the most sensitive sea lamprey and the most tolerant fishes, such as American eel and bluegill (Boogaard et al., 2003; Wilkie et al., 2019). Setting the allowable maximum TFM treatment concentration below the toxic effects of a common fish like rainbow trout protects a wide range of the rarer and possibly sensitive fish species from accidental and catastrophic lampricide exposures, while ensuring the treatment is effective on sea lamprey populations. Rainbow trout are also commonly found in many streams and rivers of the Great Lakes, sharing the sea lamprey habitats and exposures to lampricides during SLCP treatments (Birceanu et al., 2021; Hepditch et al., 2021; Wilkie et al., 2019). While rainbow trout inhabit the same environments, they are generally considered a cold-water species, with the final temperature preferendum (FTP) of 15.5°C, an optimal growth temperature of 16°C, a critical thermal maximum (CTmax) of 22.1°C and an upper incipient lethal temperature (UILT) of 25°C (Hasnain et al., 2010). However, these values can vary widely among the populations (13-19°C) and can be altered by acclimation temperature (Chen et al., 2015; Verhille et al., 2016). Rainbow trout are also often used in toxicity studies with well characterized physiology, including what is already known about lampricide toxicity and modes of action, thus making rainbow trout a well-suited model for studying lampricide effects on fish metabolism.

1.4 Analysis of lampricides

It is essential to ensure that observed biological responses, including acute toxicity, can be accurately attributed to the intended chemical doses. There are several ways to measure the concentration of lampricides in water and fish tissues to confirm exposure. Commonly used methods to quantify lampricides in water include the use of spectrophotometry for TFM or high-performance liquid chromatography (HPLC) for niclosamide. HPLC is an analytical technique that separates compounds in a liquid sample based on their interactions with a stationary phase in a column and the liquid mobile phase, then the signal is quantified using a detector, such as a UV detector (Harris, 2016) or tandem mass spectrometry (MS/MS). The use of mass spectrometry can greatly increase sensitivity and selectivity but can be considerably more time-consuming and expensive. The greater selectivity and sensitivity of LC-MS/MS have led to it commonly being used to investigate drug metabolism in the pharmaceutical industry (Youdim & Saunders, 2010).

TFM in water can be quantified using a simple spectrophotometric procedure that can be performed in the field (Fisheries and Oceans Canada Instrument Operating Procedure (GLFC, 2024). A water sample (1 mL) collected straight from the exposure tank or directly from the treatment site is added to the glass cuvette and measured using a spectrophotometer at a wavelength of 395 nm. The sample absorbances are converted to concentrations using a standard curve calibration for known concentrations. Compared to TFM, niclosamide is much harder to measure accurately using a similar approach. The spectrophotometric method used to quantify TFM in water samples cannot be used for niclosamide measurements due to the low field concentrations of niclosamide ($\mu g\ L^{-1}$), which range from 0.5-2% of the TFM concentration (Johnson & Stephens, 2003). Detection of niclosamide in water samples usually requires more sensitive HPLC based methods to quantify niclosamide water concentrations (Dawson, 1982; Johnson & Stephens, 2003; Scholefield, 1987).

Analysis of lampricides in tissue is considerably more complex than in water, as the sample must be extracted with a solvent-based extraction (using sonication and/or centrifugation) before a solid phase extraction cleanup is performed to remove lipids and

other interference chemicals before detection can be achieved. Limited data exist on lampricide tissue measurements in sea lamprey or non-target fishes. However, studies using HPLC for separation followed by UV detection have provided reproducible results from rainbow trout and channel catfish (*Ictalurus punctatus*) fillets (Schreier et al., 2000). Bussy et al. (2018b) explored several extraction approaches, including liquid/liquid extraction (LLE), solid phase extraction (SPE), and protein precipitation (PPT). Although the SPE gave similar recoveries, they opted for protein precipitation for the identification of TFM metabolites in liver homogenates. They followed the extraction with ultra-HPLC separation using a C18 stationary phase (Acquity BEH C18) and detection using a triple quadrupole mass spectrometer on a Waters Xevo TQ-S MS/MS. These experiments provided a basis for development of the present method in this thesis to detect and quantify TFM and niclosamide tissue accumulation in sea lamprey and fish tissue. These previous studies focused on identifying and quantifying TFM metabolites by creating, purifying and quantifying TFM metabolic byproducts extracted from adult sea lamprey and non-target liver extracts (Bussy et al., 2018a, b). However, isotopically labelled standards, a similarly acting compound that aids in quantitation and compensates for matrix effects present in the analysis, are available for niclosamide but not for TFM or the metabolic products, making trace analysis difficult. Therefore, there was a need to develop and validate a sensitive method to quantify both TFM and niclosamide in fish tissues.

1.5 Research objectives

While lampricides have been used for the better part of the last 65 years as the primary tool for sea lamprey population control within the Great Lakes, knowledge gaps persist in our understanding of how lampricide effectiveness is influenced by water temperature and how TFM and niclosamide interact with one another. Although TFM is well studied, niclosamide as a lampricide has received much less attention, despite its widespread use as a molluscicide to control snail populations for schistosomiasis prevention (Joubert et al., 2001; Lardans & Dissous, 1998; McManus et al., 2018). As both lampricides are also pesticides which are added to the waters draining into the Great Lakes through SLCP treatments, a better

understanding of the mechanisms of action of TFM and niclosamide through continued research remains imperative. The differences observed between sea lamprey and non-target fishes in lampricide toxicity merit further study to improve treatment effectiveness and reduce adverse effects to non-target fishes. In particular, there is a need to understand why supplementing TFM applications with niclosamide makes TFM a more effective lampricide without increasing non-target sensitivity. Exploring how TFM-niclosamide mixtures interact with and affect sea lamprey and non-target fishes would provide valuable knowledge that could be used not only by the SLCP to tailor more effective treatments but also to shed light on how other organic compounds interact within a mixture and under differing conditions.

Recent evidence that water temperature influences TFM toxicity in sea lamprey helps explain why TFM effectiveness varies with season (Hlina et al., 2021; Muhametsafina et al., 2019; Schueller et al., 2024), but information on how higher water temperatures increase sea lamprey tolerance to TFM is lacking. Climate change may reduce the effectiveness of sea lamprey control strategies through shifts in phenological timing and physiological processes (Hume et al., 2024; Lennox et al., 2020). Warmer water temperature could lead to higher larval sea lamprey growth rates, thus increasing the treatment survival chances and increasing metamorphosis rates leading to the requirement of more frequent lampricide treatments (Wilkie, 2025). Further, little is known how warmer waters affect the TFM toxicity in non-target fishes, particularly those that are more sensitive to TFM.

The overarching aim of this PhD thesis was to address how water temperature and the presence of niclosamide modifed TFM toxicity in sea lamprey and rainbow trout (Figure 1.6). Accordingly, the specific research objectives of this thesis were to:

i. Characterize how temperature affects the toxicity and accumulation of TFM in larval sea lamprey.
ii. Determine how co-application of niclosamide with TFM affects the toxicity and tissue accumulation of TFM in larval sea lamprey.
iii. Characterize how temperature affects TFM toxicity and tissue accumulation in rainbow trout.

iv. Establish if niclosamide addition to TFM exposure affects the TFM toxicity and tissue accumulation in rainbow trout in a similar manner to that observed in sea lamprey.

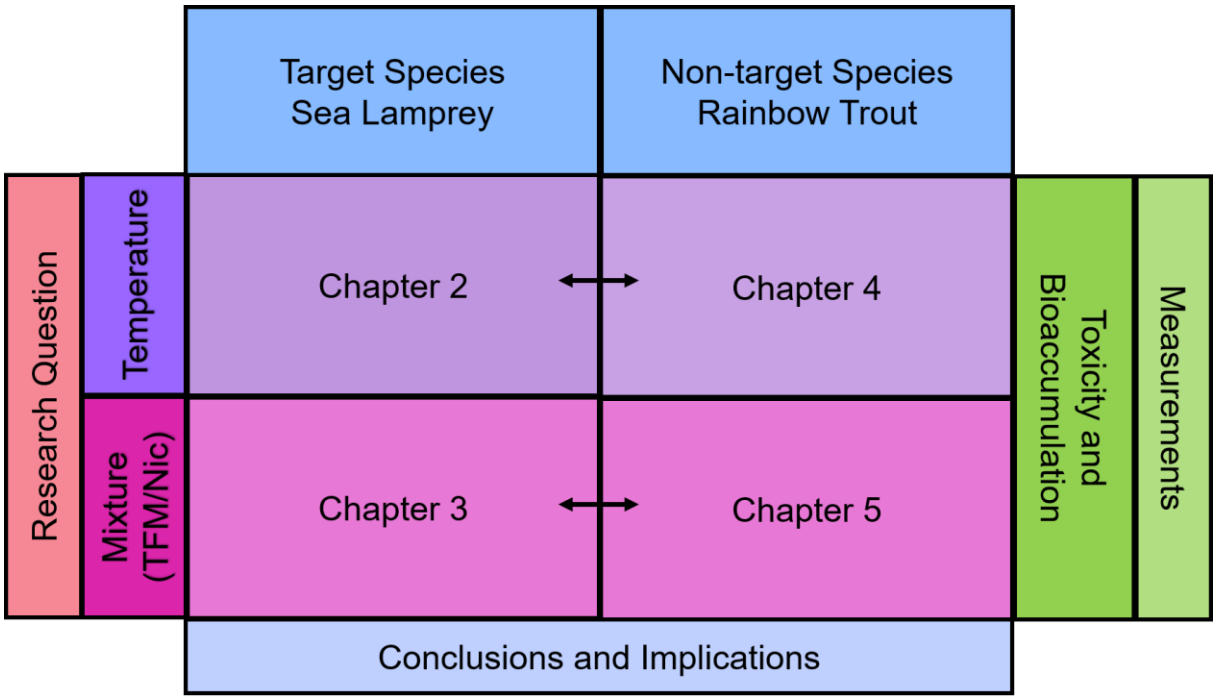


Figure 1.6 Graphical representation of the research studies. Each data chapter of this thesis focused on one research objective and one species, using toxicity and bioaccumulation measurements to evaluate the effects of temperature or niclosamide on TFM toxicity.

Before the specific objectives could be accomplished, a robust, rapid, and straightforward LC-MS/MS method for measuring concentrations of TFM and niclosamide in fish tissues, as well as niclosamide in water, had to be developed. Liquid chromatography with triple quadrupole (QQQ) tandem mass spectrometer (LC-MS/MS) was chosen and described as part of Chapters 2 and 3. The lack of available standards for metabolites limited the current studies to the quantification of the parent lampricides.

1.5.1 Research approach

The specific chapters evaluate the effects of elevated water temperature on the accumulation and toxicity of TFM in larval sea lamprey (Chapter 2) and in rainbow trout (Chapter 4). The hypotheses tested were that TFM toxicity decreases with increasing acclimation temperatures, due to lower TFM tissue accumulation. To determine the 12-h LC_{50} values for each species, the first set of experiments were conducted on larval sea lamprey acclimated to temperatures ranging from 7°C to 30°C, or rainbow trout acclimated to temperatures ranging from 6°C to 21°C. While the second set of experiments focused on sea lamprey or rainbow trout acclimated to different water temperatures and all were exposed to a single sublethal TFM concentration (the 12-h LC_{25}) up to 12 h.

Chapters 3 (sea lamprey) and 5 (rainbow trout) test the hypotheses that the addition of niclosamide results in increased TFM toxicity and higher TFM tissue accumulations due to increased competition for metabolic enzymes. To test the hypothesis that the presence of niclosamide increases TFM toxicity, toxicity tests were performed that exposed larval sea lamprey or rainbow trout to a range of concentrations of TFM, niclosamide, or a TFM/1% niclosamide mixture. To provide insight into the mechanisms that govern the TFM-niclosamide interactions in larval sea lamprey, liver and muscle tissues were collected for the measurement of TFM and niclosamide accumulation following exposure to a range of concentrations of each lampricide alone and to TFM/1% niclosamide mixtures.

An additional experiment was performed with rainbow trout exposed to a single sub-lethal concentration equivalent to the 12-h LC_{50} of the TFM/1% Nic mixture, of either TFM alone, niclosamide alone or TFM/1% niclosamide over a 9 h exposure. The collection and measurement of lampricide accumulation in tissues (liver, brain, and muscle) were conducted to determine how the mixture altered the accumulation of each lampricide, on its own or in the presence of the other.

Chapter 2

The Mechanism of Increased Tolerance to the Pesticide, 3-Trifluoromethyl-4-Nitrophenol (TFM), in Invasive Sea Lamprey (*Petromyzon marinus*) at Warmer Temperatures

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2.1 Introduction

The phenolic compound 3-trifluoromethyl-4-nitrophenol (TFM) has been used as a method to control invasive sea lamprey (*Petromyzon marinus*) populations in the Laurentian Great Lakes for over sixty years (Siefkes, 2017; Wilkie et al., 2019). Sea lampreys are jawless fish that invaded the Great Lakes in the mid-late 1800s and early 1900s via man-made canals (Eshenroder, 2014; Smith & Tibbles, 1980). Sea lamprey metamorphose from suspension-feeding larvae (also called ammocoetes) into blood sucking parasites (Manzon et al., 2015). Their parasitism of top predatory fishes in the Great Lakes resulted in massive reductions in the populations of commercial, recreational and Indigenous fisheries by the mid-twentieth century (Siefkes, 2017; Smith, 1980). Populations were subsequently brought under control following the implementation of a binational (USA and Canada) sea lamprey control program (SLCP) that uses a combination of barriers to block the upstream migration of adult lampreys and application of TFM to nursery streams containing larval lamprey (Hubert, 2003; Siefkes, 2017; Wilkie et al., 2019).

In the SLCP, field applications of TFM (typically < 12 h) target the larval sea lamprey, which have a greater sensitivity than non-target fishes to the compound due to their relatively low capacity to metabolize TFM using Phase II metabolism (e.g., glucuronidation) and eliminate the compound (Kane et al., 1994; Lawrence et al., 2021; Lech & Statham, 1975). TFM induces toxicity by interfering with aerobic (oxygen-dependent) ATP production through the uncoupling of oxidative phosphorylation in the mitochondria, resulting in ATP starvation and death (Birceanu et al., 2011; Huerta et al., 2020; Niblett & Ballantyne, 1976). As a weak acid, with a dissociation constant (pKa) of 6.38, the ionization state of TFM varies with changes in water pH (Hubert, 2003; McConville et al., 2016). Hence, the toxicity of TFM to both sea lamprey and non-target fishes changes with pH by altering its bioavailability. As the pH is lowered, the proportion of TFM in its lipophilic, un-ionized form (TFM-OH) increases, thereby increasing the transport across the gill membranes (Armitage et al., 2017; Figure 2.1). Similarly, higher alkalinity leads to lower TFM toxicity, most likely due to increased buffering capacity in the gill microenvironment, resulting in

reduced availability of the unionized form (TFM-OH) (Armitage et al., 2017; Wilkie et al., 2021).

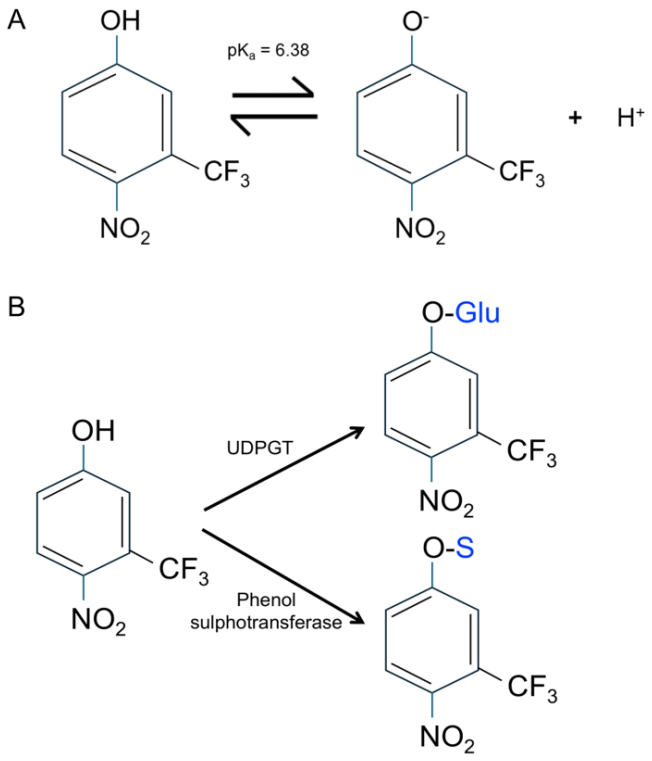


Figure 2.1 Structure and detoxification of 3-trifluoromethyl-4-nitrophenol (TFM). (A) Structure of un-ionized TFM and ionized TFM, and corresponding dissociation constant (pKa). (B) Predominant Phase II TFM biotransformation products, TFM-glucuronide and TFM-sulphate.

The season has also been shown to play a role in the sensitivity of larval sea lamprey to TFM, with greater TFM tolerance in summer compared to spring (Applegate et al., 1966; Muhametsafina et al., 2019; Scholefield et al., 2008; Schueller et al., 2024). Recent studies suggest that these differences may be primarily related to higher water temperatures (Hlina et al., 2021; Muhametsafina et al., 2019). Other phenolic compounds, including the uncoupler 2,4-dinitrophenol and pure phenol, have also been observed to be less toxic to fish at warmer

temperatures (Howe et al., 1994; Patra et al., 2015), but the underlying mechanisms remain unclear.

The present study aimed to assess the effects of temperature on the accumulation and toxicity of TFM in larval sea lamprey. Accordingly, 12-h LC_{50} values were determined for TFM in larval sea lamprey across a wide range of acclimation temperatures (7-30°C) to test the hypothesis that TFM tolerance increases with temperature. Another group of sea lamprey acclimated to 6°C, 12°C and 24°C, were subsequently exposed to a common concentration of TFM (LC_{25} at 12°C) to determine how patterns of TFM accumulation and elimination were affected by temperature alone, while minimizing TFM-induced mortality. We hypothesized that TFM toxicity would decrease with increased temperature and that its accumulation in the liver and muscle tissue would be reduced at warmer temperatures. A better understanding of how temperature alters the bioaccumulation and toxicity of TFM in sea lamprey will inform future management decisions for lamprey control and contribute to a better understanding of the implications of temperature for the assessment of phenolic organic contaminants.

2.2 Materials and methods

2.2.1 Experimental animals and holding

Larval sea lamprey (n = 595, mass = 1.94 ± 0.03 g and total length = 11.42 ± 0.6 cm at the time of the experiments) were provided by the US Geological Survey, Hammond Bay Biological Station (HBBS), Millersburg, Michigan, USA. The sea lamprey were held for approximately 2 months prior to experiments, under a 12-h light: 12-h dark photoperiod in aerated flow-through 75 L tanks fed with City of Waterloo dechlorinated tap water (temperature 12°C; pH 7.9 - 8.1; titratable alkalinity ~ 221 mg L^{-1} as $CaCO_3$) with a replacement rate of 0.5 L min^{-1}. The tanks were filled with 3-5 cm of Quikrete Play sand (Home Depot, Waterloo, ON, CA) to provide burrowing substrate (Birceanu et al., 2009). The larval lamprey larvae were fed a slurry of wet bakers' yeast (Fleischmanns®) once a week at a ration of 1 g per larva per week (Holmes & Youson, 1994). All experiments were approved by the Wilfrid Laurier University Animal Care Committee (Animal Use Protocol

Number R22003) and conducted in accordance with guidelines of the Canadian Council on Animal Care.

2.2.2 Chemicals and reagents

Field formulation TFM (35% dissolved in isopropanol; Clariant SFC GMBH WERK, Griesheim, Germany), provided courtesy of the Sea Lamprey Control Centre, Fisheries and Oceans, Canada, Sault Ste. Marie, Ontario, was used for all TFM exposure experiments. Working concentrations of TFM were prepared using dechlorinated laboratory water and verified against standards prepared using analytical-grade TFM (99%, CAS No: 88-30-2, Millipore Sigma, Oakville, ON). To ensure that TFM exposures during the experiments were stable, the appropriate amounts of working stock (1 g L^{-1}) were added to each aerated glass test aquarium approximately 12 h prior to each toxicity test. Water samples (10 mL) were collected from each tank for TFM measurements immediately after TFM working stock addition, the next day and at 0 h, 6 h and 12 h after sea lamprey were added to the tanks for toxicity tests.

2.2.3 Experimental protocols

Approximately 2 months after capture, the sea lamprey were acclimated to different temperatures ranging from 6°C to 28°C, two series of experiments were performed. The first series consisted of acute toxicity experiments to quantify the effect of temperature on larval sea lamprey sensitivity to TFM (Experiment 1). A second series subjected larval sea lamprey to an identical nominal TFM exposure concentration (the LC_{25} of TFM at 12°C) at one of three temperatures (6°C, 12°C, and 24°C) to determine how TFM accumulation in liver and muscle differed with temperature, and to measure muscle energy stores and energy metabolite concentrations (Experiment 2). All TFM exposures were conducted for 12 h to approximate typical field applications.

2.2.3.1 Experiment 1 – Acute toxicity tests

After an initial 2-month acclimation to laboratory conditions, larval sea lamprey were separated into seven aquaria (n = 80 animals per 70 L aquaria) with rate of partial water replacement of approximately 1 L min^{-1}. Each tank was equipped with either a heater (Finnex Titanium Heater TH-800 plus, JSK Merchandise Inc. USA) or a chiller (Coralife ¼ hp Aquarium Chiller, Franklin, WI, USA) and temperature controllers (Inkbird ITC-308 Temperature Controller, Ink Bird Tech. C.I., China). The water temperature of each tank was gradually adjusted at a rate of 2°C per 24 h to one of seven target temperatures: 7, 12, 18, 21, 25, 28 and 30°C. Lamprey larvae were acclimated at their respective target temperatures for at least three weeks (no more than 30 d), and fish were not fed for at least 48 h before beginning experiments.

Sea lamprey larvae (n = 70 - 80 per temperature, n = 10 per TFM concentration) were exposed to a range of nominal TFM concentrations (0.5 - 10.0 mg L^{-1}) to generate concentration-toxicity curves. From these curves, the 12-h LC_{50} and 12-h $LC_{99.9}$ (minimal lethal concentration, MLC) were calculated to compare the variation in TFM toxicity across seven temperatures (7, 12, 18, 21, 25 and 28°C). The tests followed American Standard and Testing Methods guidelines for static toxicity tests on aquatic organisms (ASTM, 2014). Each set of lamprey were tested at their respective acclimation temperature in glass aquaria filled with 8 L of the same well-aerated, dechlorinated water described above. The aquaria did not contain sand burrowing substrate, which can lead to greater TFM survival, presumably by interfering with TFM flow through the substrate (Bills et al., 2003), in keeping with previous TFM toxicity studies (e.g. Bills et al., 2003; Hlina et al., 2021; Schueller et al., 2024). Each aquarium was immersed in a recirculating water bath to maintain the appropriate temperature for the duration of the experiment.

On the day of the toxicity test, sea lamprey were collected from the appropriate acclimation tank using a dip net and arbitrarily distributed to each test tank containing TFM at one of eight concentrations (n = 5 lamprey per aquarium; n = 10 per TFM concentration). Lamprey acclimated to low temperatures (7°C, 12°C, and 18°C) were exposed to nominal

TFM concentrations ranging from 0.5 to 9.0 mg L^{-1}. In contrast, lamprey acclimated to high temperatures (21°C, 25°C, and 28°C) were exposed to nominal TFM concentrations ranging from 1 to 10 mg L^{-1} (Appendix A, Table A.1). TFM concentrations were then measured in water samples (10 mL) collected at 3, 6, and 12 h to determine whether TFM concentrations remained stable throughout each experiment (Appendix A, Table A.1).

Water temperature remained within 0.4°C of target values, while dissolved oxygen (DO) exceeded 90% saturation across treatments. The water pH was measured at the onset (0 h), 6 h and 12 h of the TFM exposures, and was relatively stable, with average water pH values falling between pH 8.0 and 8.1 for all treatments (Table 2.1). Water alkalinity ranged from 221 to 238 mg L^{-1} as $CaCO_3$ (Table 2.1).

Table 2.1 Measured water quality during 12-h toxicity tests of larval sea lamprey to a range of TFM concentrations at different temperatures. Data presented as the mean ± S.E.M. n denotes the number of total measurements per treatment. Nominal and measured TFM concentrations across temperature and associated mortalities are provided in Appendix A, Table A.1.

Temperature (°C)		pH	Alkalinity (mg L^{-1} as $CaCO_3$)	Dissolved O_2 (% saturation)	n
Nominal	Measured				
7	7.3 ± 0.1	8.08 ± 0.01	221	95.7 ± 1.8	28
12	11.9 ± 0.2	8.11 ± 0.03	221	99.1 ± 0.8	40
18	18.2 ± 0.1	8.08 ± 0.02	221	99.9 ± 0.4	40
21	21.3 ± 0.1	8.06 ± 0.01	221	98.1 ± 0.4	40
25	24.9 ± 0.1	8.08 ± 0.03	238	97.8 ± 0.3	30
28	27.0 ± 0.4	8.09 ± 0.03	238	96.5 ± 0.6	30

The larval sea lamprey (n = 10 per TFM concentration, in duplicate, 5 per tank) were monitored hourly during each toxicity test. When no movement or ventilation was visually observed, mortality was confirmed by a light tail pinch with forceps to ensure the animal was unresponsive. Mortalities were quickly removed, and the time of death, body length and mass were recorded (Appendix A, Table A.1). After the 12-h exposure, any survivors were euthanized in an aerated bath containing a lethal dose of buffered tricaine methanesulfonate

(MS-222, CAS No. 886-86-2, Syndel, Nanaimo, BC, Canada; 1.5 g L^{-1} MS-222 buffered with 3.0 g L^{-1} $NaHCO_3$).

2.2.3.2 Experiment 2 – Effects of temperature on TFM accumulation and tissue energy stores and metabolites

To quantify how temperature affected patterns of TFM tissue accumulation and energy reserves, separate groups of larval sea lamprey (n = 60 lamprey per temperature) were exposed to a single concentration of TFM (3.74 mg L^{-1}, the 12-h LC_{25} at 12°C determined in Experiment 1) at temperatures of 6°C, 12°C or 24°C. Water chemistry was measured over the 12-h exposure with minimal variations in measured temperature, pH, dissolved oxygen and alkalinity (Table 2.2). TFM concentration was stable over the 12 h of exposure across the temperatures (Table 2.2).

Table 2.2 Measured water quality and TFM concentrations during the 12-h exposure of larval sea lamprey to a nominal (3.74 mg L^{-1}) TFM concentration across temperatures. Data presented as the mean ± S.E.M. n = 22 total measurements per treatment.

Temperature (°C)		pH	Alkalinity (mg L^{-1} as $CaCO_3$)	Dissolved O_2 (% saturation)	[TFM] (mg L^{-1})	
Nominal	Measured				Nominal	Measured
6	6.2 ± 0.1	8.01 ± 0.02	221	98.4 ± 0.5	3.74	3.74 ± 0.04
12	12.3 ± 0.1	8.00 ± 0.02	204	94.9 ± 1.2	3.74	3.75 ± 0.04
24	23.9 ± 0.1	7.99 ± 0.01	204	94.2 ± 0.6	3.74	3.72 ± 0.02

For each temperature group, control lamprey (n = 10 - 15) were held under the same conditions, minus the TFM exposure with half the animals sampled within the first hour and half at the end of the 12-h exposure for measurement of baseline levels of energy stores and metabolites and to ensure there were no changes in these parameters over the duration of the experiment. At 1, 3, 6, 9 or 12 h, a sub-sample of lamprey (n = 8 - 10, per treatment) were euthanized as described above, followed by the rapid collection of liver and muscle, taken from both sides of the animal and ~ 3 cm below the gill arches, which were freeze-clamped

using liquid nitrogen-cooled aluminum tongs, and stored at -80°C until processed. Individual lamprey weight and lengths were recorded and used to calculate Fulton's condition factor (CF = mass (g)/length^3 (mm) x 10^6; Holmes & Youson, 1994; Schueller et al., 2024)

2.2.4 Analytical methods

Water TFM concentrations were confirmed spectrophotometrically using 1.5 mL polystyrene cuvettes on a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, UK) at a wavelength of 395 nm using established methods (USGS, Hammond Bay Biological SOP No. LAB 431.0. GLFC 2025). TFM standards and dilutions were prepared using experimental water.

The liver and muscle samples collected in Experiment 2 were processed for TFM quantification as initially outlined by Lawrence et al. (2021) with modifications and optimizations (for complete method details refer to Appendix A). Briefly, approximately 100 mg of finely ground tissue was extracted using Captiva EMR-Lipid 96-well plates (Agilent Technologies, Santa Clara, CA). The eluted sample was then analyzed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). The concentration of TFM in each sample was quantified using an Agilent 1260 HPLC coupled to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). An Agilent Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm) was used to separate the analytes chromatographically. All data were processed using Mass Hunter Quantitative Analysis software version 10.1 (Agilent, Santa Clara, USA), and TFM tissue concentration was expressed as nmol g^{-1} wet weight (ww). The calibration curve ranged from 0 µg L^{-1} to 5000 µg L^{-1}, and the instrument detection limit was 0.04 µg L^{-1}, while the method detection limit was 0.25 µg L^{-1}. The sample recoveries were between 85.9 and 103.8% based on our calibration curves.

2.2.5 Muscle energy metabolites

Tissue metabolite extraction procedures were described by Clifford et al. (2012). Briefly, sea lamprey muscle tissue samples were ground to a fine powder under liquid nitrogen and homogenized with 4 volumes of 8% PCA (perchloric acid) containing 1 mmol L^{-1} EDTA,

using a 30 sec pulses of a bead homogenizer (Bead Ruptor Elite, Omni International, Kennesaw, GA, USA). After centrifugation (at 4°C and 10,000g for 5 min), the resulting supernatant was divided into 2 aliquots: one for tissue glycogen measurements and the other for lactate, ATP, and phosphocreatine (PCr) analysis. After neutralization with potassium carbonate (glycogen aliquot) or potassium hydroxide (ATP/PCr aliquot), both aliquots were flash-frozen and stored at -80°C for later analysis.

The muscle tissue samples were analyzed using enzyme-linked assays outlined in Bergmeyer (1983) for ATP (hexokinase, HK), PCr (creatine kinase; CK), and lactate (lactate dehydrogenase; LDH). The second aliquot was used to enzymatically determine glycogen content by breaking it down into glucose using amyloglucosidase digestion, followed by enzymatic quantification of glucose concentrations using HK (Bergmeyer, 1983). All metabolite concentrations were determined spectrophotometrically at 340 nm wavelength and using a microwell plate spectrophotometer (SpectraMax 190, Molecular Devices, CA, USA). All concentrations were expressed as μmol g^{-1} wet weight of tissue.

2.2.6 Calculations and statistical analysis

2.2.6.1 Experiment 1 – Acute toxicity tests

Mortality data collected was analyzed in R (version 4.5.0, Code in Supplemental File 1), without transformation. The concentration-response relationship was determined by the generation of concentration-survival curves, where each tank (replicate or not) was treated as an individual exposure. A generalized linear model (GLM) with a Bernoulli distribution and a logit link was applied to determine the effects of TFM dose, acclimation temperature, and their interaction on the probability of death (mortality) as follows:

$$y = \alpha + \beta_1 \times \text{dose} + \beta_2 \times T + \beta_3 \times \text{dose} \times T \quad (1)$$

$$m = \exp(y) / (1 + \exp(y)) \quad (2)$$

Where $\alpha = -15.2984$, $\beta_1 = 3.8520$, $\beta_2 = 0.2004$, and $\beta_3 = -0.0846$. $R^2 = 0.7233$

For the R code, details of model generation and leave-one-out validation results are provided in Appendix A and Table A.2.

The above equations were then reworked to determine the 12-h LC_{50} and MLC (minimal lethal concentration; $LC_{99.9}$) for different temperatures, as follows:

$$\text{dose} = (\ln (m / (1 - m)) - \alpha - \beta_2 * T) / (\beta_1 + \beta_3 \times T) \quad (3)$$

Confidence intervals (CIs) for the LC doses were estimated from the model by determining the TFM concentrations with a 5% chance of achieving the desired mortality (lower concentration bound) and those with a 95% chance of achieving the desired mortality (upper concentration bound). Differences in the respective 12-h LC_{50} or MLC values were considered statistically significant if the 95% confidence intervals did not overlap (Finney, 1971). Toxicity data are reported as the 12-h LC_{50} or 12-h $LC_{99.9}$ (MLC) ± 95% confidence interval (CI).

2.2.6.2 Experiment 2 – TFM tissue accumulation

Larval length and weight, TFM tissue concentrations, and muscle energy metabolites were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests to compare values between temperatures and within treatment groups (time periods) at a given temperature. In cases where the assumptions of normality and homogeneity were not met, non-parametric (Brown-Forsythe and Welch) ANOVA was used, followed by Dunn's Test of Multiple Comparisons. Statistical analysis was performed using GraphPad Prism version 10.4.1 (GraphPad Software Inc, La Jolla, CA, USA). The morphometric and physiological data are reported as the mean ± S.E.M. The level of statistical significance was set at $\alpha = 0.05$.

2.3 Results

2.3.1 Effects of acclimation temperature on body condition

As acclimation temperature increased from 7°C to 28°C (Experiment 1), the CF of the larval sea lamprey steadily increased from 1.19 ± 0.02 at 7°C to 1.29 ± 0.02 at 28°C, with a peak

CF of 1.30 ± 0.02 at 25°C (Table 2.3). These differences in CF were primarily due to decreases in body length with acclimation temperature, which ranged from 117.2 ± 0.16 mm at 6°C to 109.2 ± 0.13 mm at 28°C ($p = 0.002$; Table 2.3). There were no significant changes in body mass across the six temperatures ($p > 0.05$), however.

Table 2.3 Effects of acclimation temperature on body length, mass and condition factor (CF). Larval sea lamprey were acclimated to each temperature for a minimum of 3 weeks. n (brackets) represents the number of larval sea lamprey per temperature treatment and variable. Data presented as mean ± S.E.M. Data sharing a common letter are not significantly different from one another ($p < 0.05$). CF = [Mass / (Length)3] x 10^6 (Holmes & Youson, 1994).

Temperature (°C)	CF	Mass (g)	Length (mm)
Experiment 1 - TFM Toxicity			
7	1.19 ± 0.02^{a} (75)	2.00 ± 0.08^{a} (80)	117.2 ± 0.16^{a} (75)
12	1.22 ± 0.01^{a} (80)	2.02 ± 0.07^{a} (80)	117.5 ± 0.15^{a} (80)
18	1.23 ± 0.02^{ab} (70)	1.91 ± 0.08^{a} (80)	114.4 ± 0.14^{a} (70)
21	1.26 ± 0.02^{ab} (70)	1.96 ± 0.09^{a} (80)	114.7 ± 0.15^{a} (80)
25	1.30 ± 0.02^{b} (70)	1.91 ± 0.09^{a} (79)	111.2 ± 0.14^{b} (70)
28	1.29 ± 0.02^{b} (69)	1.82 ± 0.08^{a} (80)	109.2 ± 0.13^{b} (69)
Experiment 2 - TFM Accumulation			
6	1.09 ± 0.02^{a} (48)	1.92 ± 0.07^{a} (48)	116.6 ± 0.15^{a} (48)
12	1.13 ± 0.02^{a} (60)	1.84 ± 0.09^{a} (60)	114.6 ± 0.17^{a} (60)
24	1.27 ± 0.03^{b} (60)	1.83 ± 0.07^{a} (60)	108.4 ± 0.13^{b} (60)

Similar observations were made in Experiment 2 (TFM accumulation), in which CF significantly increased from 1.09 ± 0.02 at 6°C to 1.27 ± 0.03 at 24°C ($p < 0.0001$). Again, these differences were due to the significantly lower ($p = 0.015$) body lengths, but not body mass, of the animals at 24°C (Table 2.3).

The measured mean TFM concentrations in exposure aquaria during acute toxicity tests were close to the nominal (target) concentrations (Appendix A, Table A.1). Water pH was relatively stable (8.08 ± 0.04) across treatment concentrations and temperatures, while

dissolved oxygen averaged 98.7 ± 0.5 and alkalinity during exposures was measured to be 221-238 mg L^{-1} as $CaCO_3$ (Table 2.1).

As water temperature increased incrementally from 7 to 28°C, the concentration-survival curves generated from the GLM model (see methods) underwent an overall rightward shift (Figure 2.2), reflecting a progressive increase in the 12-hr LC_{50} with temperature. The 12-hr LC_{50} increased from 4.3 mg L^{-1} (95% CI = 3.9 - 4.6 mg L^{-1}) at 7°C to 6.5 mg L^{-1} (6.1 - 7.2 mg L^{-1}) at 28°C, a more than 1.5-fold increase (Figure 2.3). Similarly, the MLC increased from an average of 5.7 mg L^{-1} (5.2 - 6.5 mg L^{-1}) at 7°C to 9.6 mg L^{-1} (8.5 - 12.1 mg L^{-1}) at 28°C, again a greater than 1.5-fold increase between the low and high temperature exposures (Figure 2.3). Acclimations at 30°C were also attempted; however, sea lamprey were not able to survive at this temperature for more than a few days (3-4).

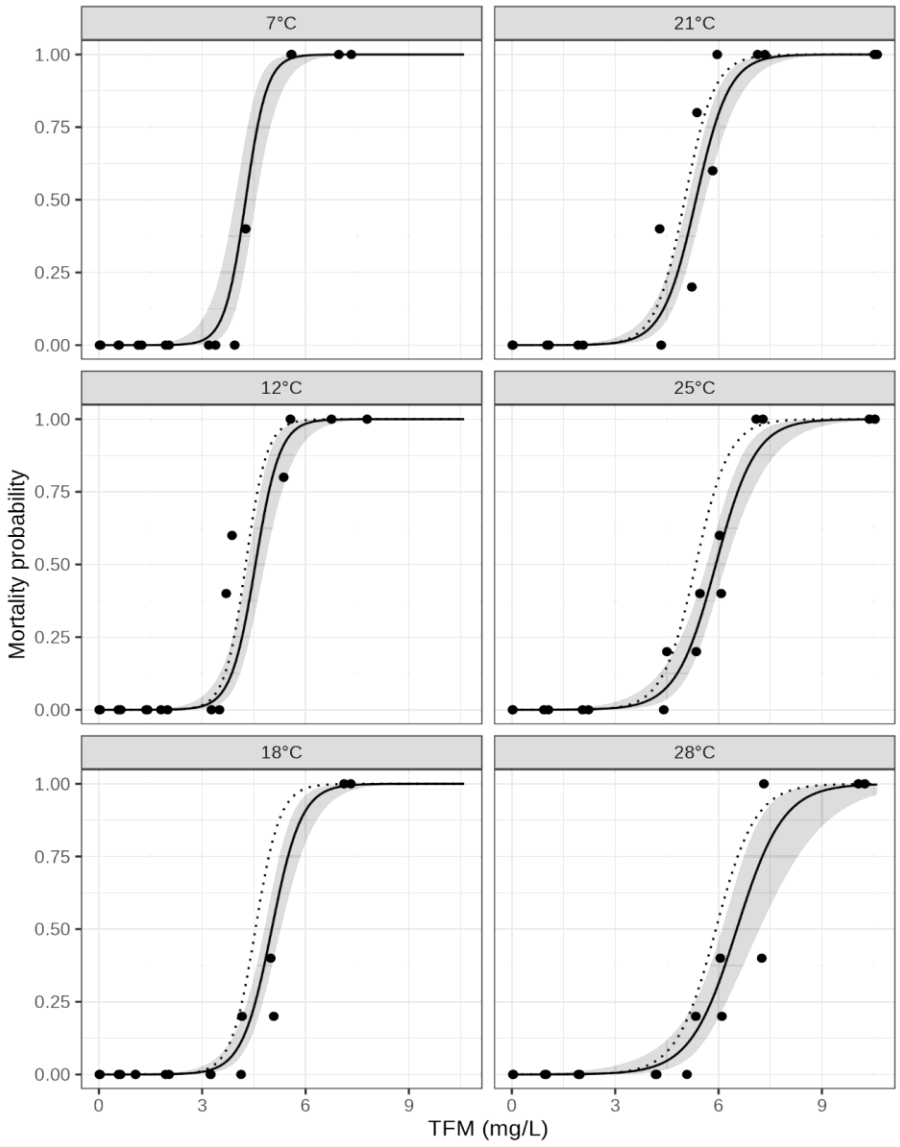


Figure 2.2 Shifts in the TFM concentration-mortality curves of sea lamprey with increasing temperature. Curves depicting changes in the toxicity of 3-trifluoromethyl-4-nitrophenol (TFM) with increasing temperature. Fitted survival curves (solid line) based on % mortality per test tank (n = 5 per tank; 2 tanks per concentration; solid dots). Grey (light) shading depicts 95% confidence intervals. Dotted lines represent the curve at the preceding temperature. n = 480 (n = 10 fish per concentration x 8 concentrations x 6 temperatures). Concentration-mortality curves were generated from a generalized linear model (GLM) with a Bernoulli distribution and a logit link (R code provided in Appendix A).

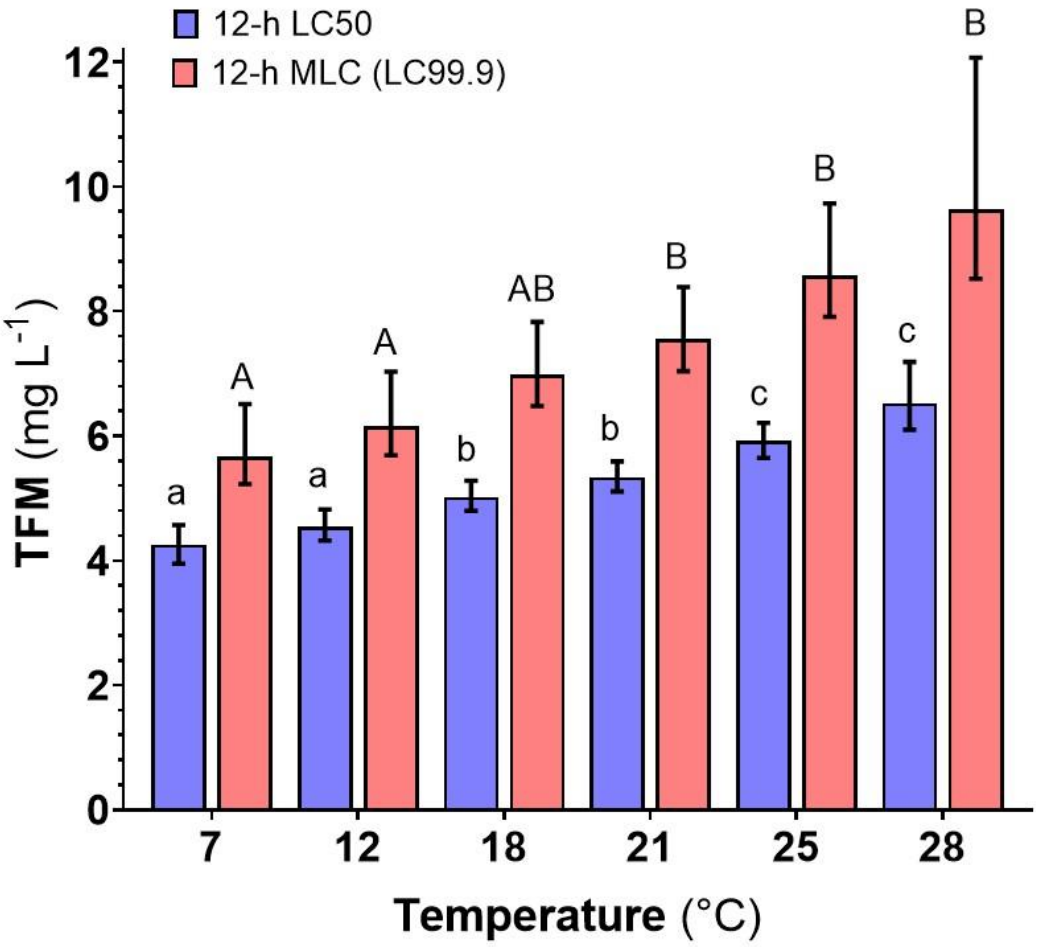


Figure 2.3 Effects of temperature on the acute toxicity of TFM to larval sea lamprey. Data depicted as the corresponding 12-h LC_{50} (blue bars) and minimum lethal concentration (12-h LC99.9; MLC, red bars) of TFM to larval sea lamprey (based on the results of Figure 2.2). Acclimation above 28°C was attempted, but survival was poor and required no further analysis. The 95% confidence intervals (CI) values sharing an identical lowercase letter are not significantly different between LC_{50} values, while identical uppercase letters denote no significant differences between MLC values across temperatures. Values where the 95% CI do not overlap are considered significantly different (Finney, 1971).

2.3.2 Effects of temperature on TFM tissue accumulation

All larvae survived the TFM exposure in cold (6°C) and warm (24°C) waters, but there were 2 mortalities out of 60 animals in the moderate 12°C treatment group (one at 9 h and one at

12 h), which were excluded from subsequent measurements and analysis. After the first hour of exposure, TFM accumulation in the liver was highest in the 12°C group, where liver TFM concentrations averaged 32.8 (± 3.9) nmol g^{-1} ww, which was significantly higher ($p = 0.003$) by approximately 39% than the TFM concentration measured at 24°C, averaging 19.9 (± 1.1) nmol g^{-1} ww. Accumulation was lowest in the 6°C group, which averaged 9.3 (± 1.0) nmol g^{-1} ww after 1 h (Figure 2.4A). TFM continued to accumulate in the liver at 12°C, before stabilizing after 6 h of exposure, at which time the mean TFM concentration was 52.6 (± 9.1) nmol g^{-1} ww and did not significantly change thereafter (Figure 2.4A). In contrast to the 12°C lamprey, the concentration of liver TFM stabilized after only 3 h in the 24°C group, at which time the concentration averaged 33.5 (± 2.1) nmol g^{-1} ww. The liver TFM concentration took the longest to stabilize at 6°C, reaching a concentration of 48.2 (± 6.3) nmol g^{-1} ww after 9 h (Figure 2.4A). At 12 h, the lowest TFM concentrations were observed in the 24°C lamprey (38.1 ± 2.5 nmol g^{-1} ww), which were 30% lower than in the 12°C lamprey ($p = 0.002$). However, the concentration of TFM in the liver of the 6°C lamprey (48.34 ± 6.4) fell between the measured concentrations of lamprey exposed to TFM at 12°C (54.73 ± 2.88) and 24°C lamprey, respectively, and were not different from either one ($p = 0.75$ and $p = 0.41$; Figure 2.4A).

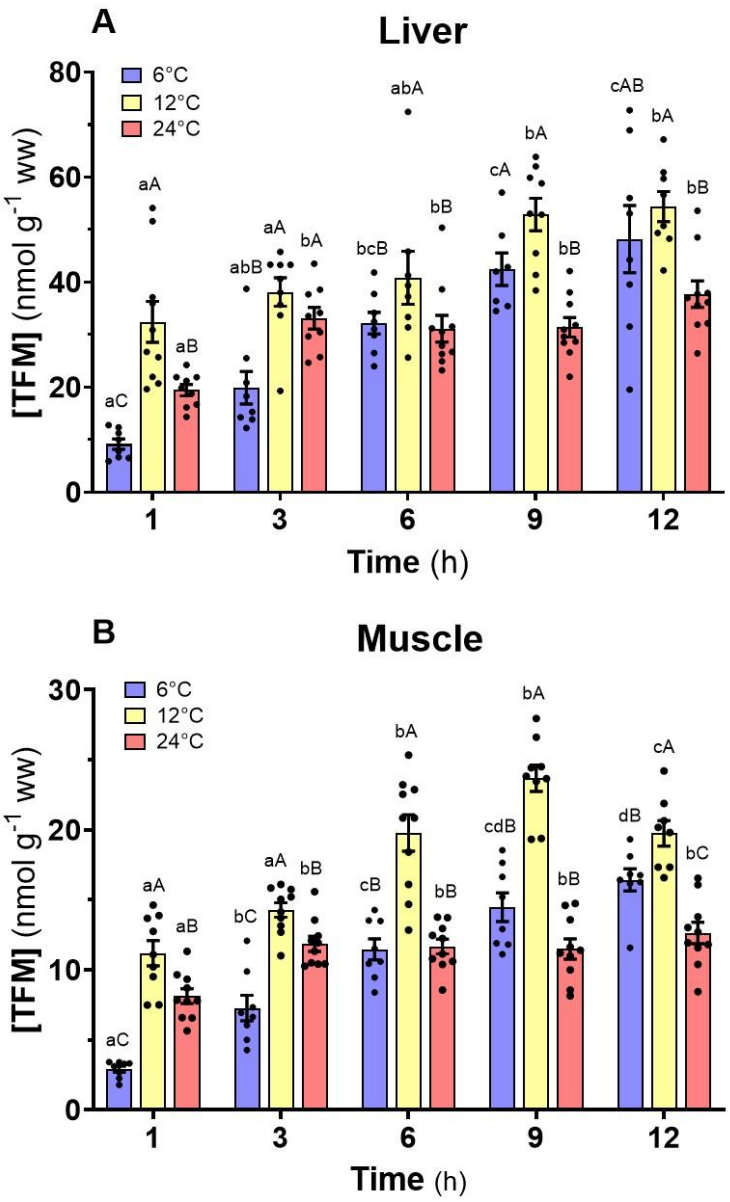


Figure 2.4 Effects of temperature on TFM accumulation in larval sea lamprey. The TFM concentration in the (A) liver and (B) muscle tissues of larval lamprey exposed to a TFM concentration (3.74 mg L^{-1} ± 0.02) at temperatures of 6°C (blue), 12°C (yellow) and 24°C (red). TFM concentrations in the tissues of the control fish (no TFM exposure) were non-detectable. Significantly different mean values within a single temperature are denoted by different lowercase letters. Significantly different mean values between the temperatures at a single time period are denoted by different uppercase letters. Data presented as mean ± S.E.M. (n = 8-10; $p < 0.05$).

Similar results were observed in the muscle tissue where, over the first hour of exposure, TFM accumulation was highest in the 12°C group, averaging 11.6 (± 0.9) nmol g^{-1} ww, while the TFM concentration in the 24°C group averaged 8.4 (± 0.5) nmol g^{-1} ww, which was significantly lower by approximately 28% ($p = 0.028$) than the moderate 12°C group (Figure 2.4B). As in the liver, muscle TFM accumulation was lowest in the 6°C group after 1 h, averaging 3.0 (± 0.2) nmol g^{-1} ww (Figure 2.4B). As observed in the liver, TFM concentration in the 24°C lamprey stabilized the fastest, after approximately 3 h of exposure, averaging between 11.8 and 12.9 nmol g^{-1} ww thereafter (Figure 2.4B). In the 12°C lamprey, TFM continued to increase in the muscle through 9 h of exposure, averaging 24.1 (± 1.0) nmol g^{-1} ww after 9 h, then falling slightly to 20.1 (± 0.9) nmol g^{-1} ww after 12 h (Figure 2.4B). TFM increased steadily in muscle at 6°C over the entire 12 h exposure period, but did not stabilize, reaching mean TFM concentrations of 16.5 (± 0.8) nmol g^{-1} ww after 12 h (Figure 2.4B). In the 12°C group, the TFM muscle concentration was 18 - 51% higher than in the 6°C and 24°C lamprey at all measured time points. Over the 12 h experimental period, the concentrations of TFM in the muscle at 6°C and 24°C overlapped at 6 h (Figure 2.4B). However, concentrations were higher after 1 h and 3 h of exposure at 24°C (8.4 ± 0.5 and 12.1 ± 0.5 nmol g^{-1} ww) than at 6°C (3 ± 0.2 and 7.4 ± 0.9 nmol g^{-1} ww; $p < 0.001$; Figure 2.4B). After 6 h of exposure at 6°C, TFM concentrations at 9 h and 12 h (14.6 ± 1.0 and 16.5 ± 0.8 nmol g^{-1} ww) were significantly higher ($p = 0.038$ and $p = 0.013$, respectively) than at 24°C (11.8 ± 0.7 and 12.9 ± 0.8 nmol g^{-1} ww). After 12 h exposure, the muscle TFM concentration in the 24°C lamprey (12.9 ± 0.8 nmol g^{-1} ww) was significantly lower by approximately 22% ($p = 0.001$) and 36% ($p < 0.001$) than at 6°C (16.5 ± 0.8 nmol g^{-1} ww) and 12°C (20.1 ± 0.9 nmol g^{-1} ww) lamprey, respectively. Unlike in the liver, TFM concentrations in the muscle at 6°C and 12°C after 12 h exposure were significantly different ($p = 0.018$) from one another (Figure 2.4B).

2.3.3 Effects of TFM exposure on muscle energy stores at different temperatures

Acclimation temperature had limited effects on the sea lamprey muscle energy stores during the 12-h LC_{25} TFM exposure, with minimal changes in glycogen and lactate concentrations within temperature treatments, while ATP and PCr concentrations were only affected at 6 and 24°C. Following acclimation, muscle glycogen concentrations were not significantly different amongst the control (no TFM) lamprey acclimated to 6°C, 12°C or 24°C, with respective control values averaging 16.7 (± 1.6) µmol g^{-1} ww, 15.9 (± 2.4) µmol g^{-1} ww and 10.3 (± 1.5) µmol g^{-1} ww (Table 2.4). The glycogen concentration in the 24°C group (5.9 ± 1.5) was significantly reduced ($p < 0.01$ and $p = 0.02$) from 6°C and 12°C (20.7 ± 3.6 and 15.1 ± 2.6 µmol g^{-1} ww) at 1 h and thereafter.

Glycogen concentration in the 6°C and 12°C treatments changed minimally during TFM exposure, and only statistically significant in the 6°C group by 12 h of exposure, when concentrations were 2.1 times higher than in muscle of the control animals (34.8 ± 4.8; Table 2.4). In contrast, at 24°C the muscle glycogen concentration trended downward from 10.3 (± 1.5) to 5.9 (± 1.5) µmol g^{-1} ww but was not significant ($p = 0.37$).

Initial (control, no TFM) lactate levels in 24°C sea lamprey muscle tissue were significantly different from those at 6°C ($p < 0.01$) and 12°C ($p = 0.02$), increasing from 0.9 (± 0.2) µmol g^{-1} ww in the 6°C group, to 2.2 (± 0.7) at 12°C, and to 5.6 (± 0.9) µmol g^{-1} ww in the 24°C group (Table 2.4). During the 12-h TFM exposure, there was an increasing trend in muscle lactate only observed in the 6°C group, approximately 5-fold at 1, 3, and 6 h, followed by a further and significant jump in lactate concentration at 9 h and 12 h when lactate was approximately 10-fold higher than the control values (both $p < 0.01$, Table 2.4). Notably, no significant changes in muscle lactate concentration were observed in the lamprey exposed to TFM at 12°C or 24°C (Table 2.4).

The concentrations of ATP in control (no TFM) larval sea lamprey at 12°C were significantly lower than measurements made at 6°C and 24°C (both $p < 0.01$), while they were not significantly different from one another ($p = 0.65$; Table 2.4). This pattern of

significant difference in ATP concentrations within the 12°C treatment when compared to the other 2 temperatures (6°C and 24°C) persisted over most of the exposure periods (Table 2.4). In the 24°C treatment, there was a significant ($p < 0.05$) downward trend in the ATP concentrations across the 12 h exposure. TFM exposure did not affect muscle ATP at 12°C, as none of the values obtained over the 12-h exposure period significantly differed from the controls.

In the control larvae (no TFM), PCr averaged 5.4 ± 0.2 μmol g^{-1} ww at 6°C, 8.9 ± 0.7 μmol g^{-1} ww at 12°C and 8.1 ± 0.9 μmol g^{-1} ww at 24°C, with only a significant ($p < 0.01$) change between 6°C and 12°C (Table 2.4). At 6°C, muscle PCr concentrations were significantly greater ($p = 0.04$ or $p < 0.01$) than those in the control animals but remained relatively stable throughout TFM exposure (Table 2.4). At 12°C, PCr concentration was relatively stable compared to controls except for a slight, but significant decrease at 1 h ($p = 0.03$; Table 2.4). At 24°C group, however, PCr gradually and significantly (p between < 0.05 and < 0.01) decreased during exposure and by 9 h, PCr concentrations were 50% lower than observed in control animals (Table 2.4).

Table 2.4 Changes in muscle energy stores and metabolite concentrations in larval sea lamprey exposed to TFM at different temperatures. Larval sea lamprey were exposed to the same concentration of TFM (3.74 mg L^{-1} ± 0.02) at 6°C, 12°C and 24°C. Asterisks denote significant differences within the temperature treatment compared to control values ($p < 0.05$), while a unique lower-case letter denotes significant differences between the treatment temperatures at a specific time point ($p < 0.05$). n = 8 - 10 (10 - 15 control) lamprey per timed exposure across temperatures.

[Glycogen] (µmol g^{-1} ww)

	6°C		12°C		24°C	
Time (h)	**Mean ± S.E.M.**	**Significance**	**Mean ± S.E.M.**	**Significance**	**Mean ± S.E.M.**	**Significance**
Control	16.65 ± 1.58[a]		15.86 ± 2.39[a]		10.33 ± 1.51[a]	
1	20.7 ± 3.61[a]	0.96	15.13 ± 2.26[a]	> 0.99	5.91 ± 1.54[b]	0.37
3	22.31 ± 3.36[a]	0.85	15.81 ± 2.25[a]	> 0.99	5.93 ± 1.24[b]	0.37
6	28.79 ± 3.31[a]	0.12	15.23 ± 2.83[b]	> 0.99	4.95 ± 1.34[c]	0.14
9	26.41 ± 3.75[a]	0.36	16.11 ± 2.64[b]	> 0.99	6.64 ± 1.55[c]	0.64
12	34.77 ± 4.82[*a]	< 0.01	20.73 ± 1.73[b]	0.71	6.59 ± 1.36[c]	0.58

[Lactate] (µmol g^{-1} ww)

	6°C		12°C		24°C	
Time (h)	**Mean ± S.E.M.**	**Significance**	**Mean ± S.E.M.**	**Significance**	**Mean ± S.E.M.**	**Significance**
Control	0.89 ± 0.18[a]		2.19 ± 0.68[a]		5.55 ± 0.86[b]	
1	4.23 ± 0.92[ab]	0.08	2.77 ± 0.79[a]	0.99	6.16 ± 0.86[b]	0.99
3	4.65 ± 1.16[a]	0.14	4.22 ± 0.89[a]	0.57	7.07 ± 1.05[a]	0.82
6	4.20 ± 0.81[*a]	0.05	5.29 ± 0.85[a]	0.14	6.39 ± 0.44[a]	0.98
9	8.82 ± 0.75[*a]	< 0.01	5.84 ± 1.06[a]	0.06	6.29 ± 0.84[a]	0.99
12	8.34 ± 1.45[*a]	0.02	5.74 ± 0.90[a]	0.07	6.53 ± 0.86[a]	0.97

[ATP] (µmol g^{-1} ww)

	6°C		12°C		24°C	
Time (h)	**Mean ± S.E.M.**	**Significance**	**Mean ± S.E.M.**	**Significance**	**Mean ± S.E.M.**	**Significance**
Control	3.54 ± 0.03^{a}		1.92 ± 0.18^{b}		3.29 ± 0.23^{a}	
1	3.23 ± 0.11^{a}	0.24	2.04 ± 0.30^{b}	0.91	$0.83 \pm 0.18^{*c}$	< 0.01
3	3.32 ± 0.05^{a}	0.04	1.86 ± 0.21^{b}	0.91	$0.94 \pm 0.24^{*c}$	< 0.01
6	$5.25 \pm 0.09^{*a}$	< 0.01	1.30 ± 0.20^{b}	0.16	4.46 ± 0.47^{a}	0.41
9	3.43 ± 0.08^{a}	0.32	1.21 ± 0.19^{b}	0.12	$0.84 \pm 0.14^{*b}$	< 0.01
12	$3.25 \pm 0.06^{*a}$	0.01	1.66 ± 0.30^{b}	0.27	$0.34 \pm 0.08^{*c}$	< 0.01

[PCr] (µmol g^{-1} ww)

	6°C		12°C		24°C	
Time (h)	**Mean ± S.E.M.**	**Significance**	**Mean ± S.E.M.**	**Significance**	**Mean ± S.E.M.**	**Significance**
Control	5.37 ± 0.16^{a}		8.37 ± 0.60^{b}		8.09 ± 0.88^{ab}	
1	$9.67 \pm 1.06^{*a}$	0.05	$5.93 \pm 0.33^{*b}$	0.03	$5.07 \pm 0.38^{*b}$	0.047
3	$10.43 \pm 0.72^{*a}$	< 0.01	7.99 ± 0.56^{a}	0.44	5.23 ± 0.76^{b}	0.12
6	$11.54 \pm 0.21^{*a}$	< 0.01	7.17 ± 0.63^{b}	0.44	$13.46 \pm 1.25^{*a}$	0.02
9	$8.21 \pm 0.64^{*a}$	0.04	7.18 ± 0.78^{a}	0.24	$3.60 \pm 0.32^{*b}$	< 0.01
12	$10.86 \pm 0.86^{*a}$	< 0.01	6.86 ± 0.77^{b}	0.18	$4.20 \pm 0.60^{*c}$	0.01

2.4 Discussion

The present study provides further evidence that the tolerance of larval sea lamprey to TFM increases with water temperature. Exposure to TFM in warmer water also led to less accumulation of TFM in the liver and muscle, suggesting that larval sea lamprey have a greater capacity to eliminate TFM from the body than at cooler temperatures. As we discuss below, we propose that the higher metabolic rates of larval sea lamprey at warmer temperatures (Holmes & Lin, 1994; Lewis, 1980) increased their ability to eliminate TFM. These findings provide further insight into why the tolerance of larval sea lamprey to TFM changes seasonally, with higher TFM tolerance in the late summer when water temperatures are typically much warmer than in the spring (Hlina et al., 2021; Muhametsafina et al., 2019; Scholefield et al., 2008; Schueller et al., 2024).

2.4.1 Relationship between TFM toxicity and water temperature in larval sea lamprey

The progressive increase in the TFM 12-h LC_{50} and 12-h MLC values of larval sea lamprey as water temperature progressively increased from 7°C to 28°C provides further evidence that greater TFM tolerances in the summer are mainly a function of temperature (Muhametsafina et al., 2019). The most important metric of TFM toxicity used in sea lamprey control is the 9-h $LC_{99.9}$ (MLC; Bills et al., 2003), at which TFM concentrations need to be sustained (or higher) for a minimum of 9 h in treated reaches of streams (Sullivan et al., 2021). Since the toxicity of TFM is dependent on water pH and alkalinity, treatment MLCs are routinely determined using pH-alkalinity charts based on prior comprehensive laboratory toxicity testing (Bills et al., 2003; Sullivan et al., 2021). More recently, lab and field studies have shown that the MLCs observed in the field typically exceed pH-alkalinity chart values at warmer temperatures, which was also the case in this study (Table 2.5; Muhametsafina et al., 2019; Hlina et al., 2021; Scholefield et al., 2008; Schueller et al.,

2024). These findings lend additional support to previous observations that reliance on the pH-alkalinity chart concentrations alone can greatly underestimate the MLCs needed for effective sea lamprey eradication. If strictly followed, use of these chart values would result in increased numbers of residual larval sea lamprey that survive treatment and subsequently metamorphose into parasitic sea lamprey (Muhametsafina et al., 2019; Schueller et al., 2024).

Table 2.5 Differences in the observed and predicted minimal lethal concentration (MLC) of TFM to larval sea lamprey acclimated to different water temperatures. Predicted 12-h MLC values obtained from charts depicting expected lethal concentrations of TFM to produce 99.9% mortality in larval sea lamprey at selected water pH and alkalinities (Bills et al., 2003).

Temperature (°C)	pH	Alkalinity (mg L^{-1} as $CaCO_3$)	Observed MLC (95% CI)	Predicted MLC	Difference (fold)
7	8.08 ± 0.01	221	5.67 (5.23 – 6.51)	3.4	1.67
12	8.11 ± 0.03	221	6.16 (5.69 – 7.03)	3.4	1.81
18	8.08 ± 0.02	221	6.99 (6.48 – 7.83)	3.4	2.06
21	8.06 ± 0.01	221	7.56 (7.04 – 8.39)	3.4	2.22
25	8.08 ± 0.03	238	8.57 (7.91 – 9.73)	3.6	2.38
28	8.09 ± 0.03	238	9.63 (8.52 – 12.07)	3.6	2.68

Previously, the tolerance of larval sea lamprey to TFM was shown to steadily increase as water temperatures increased from 6°C to 12°C to 24°C (Muhametsafina et al., 2019). Curiously, the relative increase in the respective 12-h LC_{50} and 12-h MLC of TFM over a similar temperature range (7 - 25°C) in the present study, increasing by approximately 1.3-fold and 1.5-fold, were lower than the 1.9-fold and 2.2-fold increases reported by Muhametsafina et al. (2019). At first glance, these apparent discrepancies suggest that the

effects of temperature on TFM toxicity may be less predictable than thought. However, such an interpretation is misleading because the actual concentration changes in the MLC and LC_{50} values we observed were similar to or greater than those reported by Muhametsafina et al. (2019; Table 2.6). For instance, the actual increase in the MLC between 7°C and 25°C in the present study was 2.9 mg L^{-1}, compared with approximately 2.2 mg L^{-1} observed between 6°C and 24°C in the earlier study. Thus, when interpreting temperature-TFM toxicity relationships, it is more appropriate to measure the actual (absolute) changes in MLC and LC_{50} at different temperatures rather than relative changes.

Table 2.6 Differences between the present study and Muhametsafina et al., 2019 in relative and actual changes of MLC (ΔMLC) and LC_{50} (ΔLC_{50}).

		ΔMLC		ΔLC_{50}	
Experimental Study	**Temp Range**	**Relative change (fold)**	**Actual change (mg L^{-1})**	**Relative change (fold)**	**Actual change (mg L^{-1})**
Present Study	7 - 25°C	1.3 - 1.6	2.9	1.3 - 1.5	1.7
Muhametsafina et al., 2019	6 - 24°C	1.8 - 2.5	2.2	1.8 - 2.0	1.5

A likely explanation for the differences in the relative toxicity of TFM were the marked differences in water (titratable) alkalinity between the two studies. Water pH was more or less similar (Muhametsafina et al. - pH ~ 7.9 - 8.2; Present study - pH ~ 8.0 - 8.1), but alkalinity was much lower in the earlier study (~ 85 mg L^{-1} as $CaCO_3$), resulting in relatively lower MLC and LC_{50} values at each respective temperature (Table 2.6). On the other hand, alkalinity was 3-4 times higher in the present study (221-238 mg L^{-1} as $CaCO_3$), resulting in much higher MLC and LC_{50} values. As noted above, the MLC and LC_{50} values for TFM are much smaller at lower alkalinity (Bills et al., 2003; Wilkie et al., 2021). Hence, the increases in actual MLC with temperature were proportionately greater at the lower alkalinities reported by Muhametsafina et al. (2019) than in the higher-alkalinity waters used in the present study.

The underlying mechanisms of why higher alkalinity attenuates TFM toxicity are unclear. One possibility is that the greater buffer capacity at higher alkalinity results in less acidification of the water next to the gill due to the excretion of respiratory CO_2 and metabolic acid (Lloyd & Herbert, 1960; Playle & Wood, 1989). This was demonstrated using rainbow trout (*Oncorhynchus mykiss*) fitted with opercular catheters, in which decreases in the pH of expired gill water were attenuated as water alkalinity increased (Wilkie et al., 2021). TFM uptake is directly related to water pH, with rates of TFM uptake increasing as water pH decreases due to increases in the relative amounts of the un-ionized species of TFM (TFM-OH) in the water (Figure 2.1; Hlina et al., 2018). Similarly, with less acidification, there would be less un-ionized TFM in the gill microenvironment than in bulk water of a given TFM concentration, which would result in a lower inwardly directed TFM-OH gradient across the gills and lower TFM accumulation (Wilkie et al., 2021). Similar acidification in the gill micro-environment of larval lampreys likely takes place, but further studies are needed to test this hypothesis.

Dietary factors or energy status of the lamprey may have also contributed to differences in sensitivity but were likely relatively minor (Hlina et al., 2021; Schueller et al., 2024). The animals in the present study had been in the lab for 2 - 3 months and were fed a maintenance diet of yeast prior to toxicity testing, as opposed to being held for 7 - 10 days following capture in mid-summer after feeding on their natural diet of detritus (Hlina et al., 2021; Sutton & Bowen, 1994). The latter had substantially higher condition factors, falling between approximately 1.6 and 1.7 (Hlina et al., 2021), compared to 1.2 and 1.3 in the present study, which is an important index of energy stores in fishes (e.g. Busacker et al., 1990; Schloesser & Fabrizio, 2017), and other studies have suggested that higher body condition and liver glycogen could be important determinants of TFM sensitivity in freshly caught sea lamprey (Scholefield et al., 2008; Schueller et al., 2024).

It is unlikely that the narrow differences in CF, which were due to shorter body lengths in the warm water larvae, had a significant impact on either TFM survival or TFM accumulation. Nalesnik et al. (2025) showed that there was a significant relationship between

survival time, body length and body mass in larval sea lamprey exposed to TFM. The present study, however, was designed to examine the LC_{50} and MLC, not survival time. Along with water, TFM concentration (dose) and temperature, we tested mass and length as co-variables in our toxicity curve models (Figure 2.2). However, the strongest relationship (goodness of fit) based on lower AIC (Akaike Information Criterion) values was for temperature and TFM concentration only. Inclusion of mass and length, alone or together, did not significantly improve the fit of our temperature vs survival model (Figure 2.2; Table S2). Moreover, the McFadden pseudo-R^2 values were virtually identical when body mass and length were included in the calculation, indicating no additional influence of these parameters on the TFM toxicity-temperature relationship. It is also worth noting that both length and mass decreased with increasing acclimation temperature in the present study, when sea lamprey were more tolerant to TFM. Given the information above, plus the narrow range in mean body mass and body length (less than 10% in both cases), we are confident that differences in TFM tolerance with temperature were not influenced by variation in body size in these experiments.

Differences in body size also likely had little effect on the TFM uptake rates in the present study. Rates of TFM uptake are inversely related to body size in larval sea lamprey, as described by the allometric power equation: TFM uptake rate = $7.24\ M^{0.34}$ (Tessier et al., 2018). Because this relationship was established in water with a similar chemical makeup as the present study, we were able to use this formula to calculate how much the mean TFM uptake rates could have varied in the present experiment using the mean body mass data from Table 2.3. As expected, the mean rates of TFM uptake would have varied little, between 4.55 and 4.88 nmol g^{-1} h^{-1}, or less than 10%, further suggesting that body mass had little effect on our findings.

The range of temperatures examined in the present study (7°C to 28°C) spans those observed in Great Lakes rivers and streams between April and November that are infested with larval sea lamprey and likely to be treated with TFM (e.g., Hlina et al., 2021; Schueller et al., 2024). However, it should be noted that the likelihood of larval sea lamprey being

present in streams in which water temperatures exceed their thermal niche (17.8 - 21.8°C) is reduced as water temperatures approach 25°C (Dawson & Jones, 2009; Holmes & Lin, 1994). It is unlikely that sea lamprey would be found living at 28°C, but temperatures approaching 24°C are not unusual in lamprey infested streams (Hlina et al., 2021).

A meta-analysis by Wang et al. (2019) demonstrated similar increases in tolerance with increasing temperature to phenol and pentachlorophenol in aquatic animals, including fishes, which was generally followed by a rapid decline as temperature surpassed the thermal optima (an inverse V-shaped toxicity curve with temperature). Similarly, the toxicity of 2,4-dinitrophenol, another well-known uncoupler of oxidative phosphorylation in mitochondria (Grundlingh et al., 2011), decreases with temperature in rainbow trout (Howe et al., 1994), as does the toxicity of phenol to trout before sharply declining as temperatures increase beyond the thermal preference zone (Patra et al., 2015).

2.4.2 Mechanisms of greater TFM tolerance in warmer waters based on TFM tissue accumulation

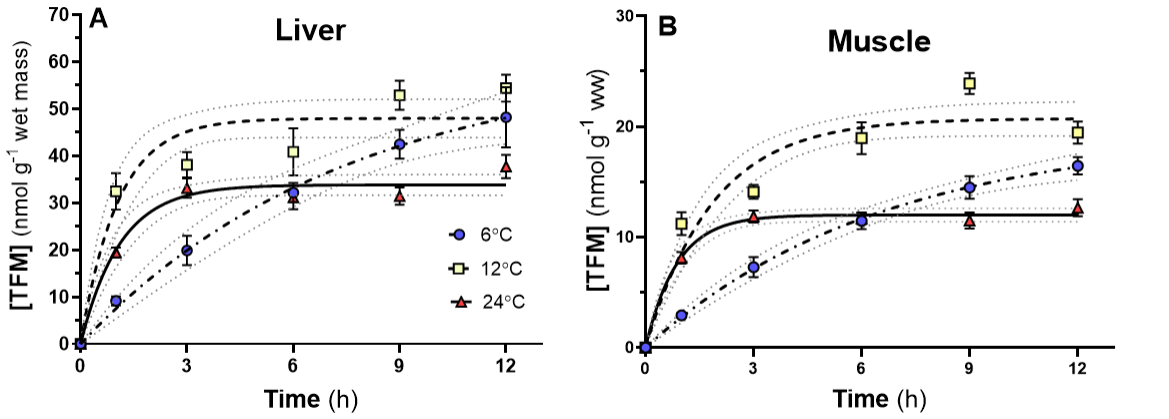
While several studies have shown that freshwater fishes exhibit increasing tolerance to phenolic compounds such as phenol, 2,4-dinitrophenol, and TFM with increasing temperature (e.g., Howe et al., 1994; Muhametsafina et al., 2019; Patra et al., 2015), the underlying mechanisms remain poorly understood. For this reason, patterns of TFM accumulation in the liver and the muscle of larval sea lamprey were measured at 6°C, 12°C and 24°C to provide insight into how TFM tolerance increases with temperature.

The primary site of TFM uptake is believed to be the gills, which (as noted above) is driven by the inward diffusion of TFM-OH down its water-to-blood concentration gradient (Wilkie et al., 2021). The rate of uptake is also proportional to metabolic rate, increasing as oxygen consumption rates increase, further demonstrating that the gill is the predominant site of TFM uptake (Tessier et al., 2018). As metabolic rate increases with ambient temperature in poikilothermic animals, including lampreys (Holmes & Lin, 1994; Lewis, 1980), it follows that elevated ventilation rates and gill irrigation also increase, resulting in higher uptake at

the gill as demonstrated in larval sea lamprey using ^{14}C-TFM (Hlina et al., 2021; Tessier et al., 2018). Yet, after 12 h of TFM exposure the measured concentration of TFM in the muscle and liver was lower in the larvae held at 24°C compared to those held at 12°C and 6°C (muscle only).

To explain these observations and provide further insight into how larval sea lamprey handled TFM at different temperatures we used a classical pharmacokinetics approach to calculate the steady state concentration (C_{SS}), or plateaus, at which the rated of accumulation is equal to the rate of elimination, and the half-life ($t_{1/2}$) of elimination (Gupta, 2016; Newman, 2020) for TFM from the liver and muscle at each temperature. Both of these organs are well perfused with blood (Percy & Potter, 1979; Peters & MacKay, 1961) in lamprey, and therefore representative of the relative TFM loads in the animals at the different temperatures. Each parameter was calculated using kinetic curves of the respective TFM accumulation versus time by liver and muscle using the data presented in Figure 2.4. The curves were fitted by non-linear regression using a one-compartment, least squares model (GraphPad Prism, version 10).

The analysis demonstrated that the C_{SS} in the liver at 24°C was 34.0 nmol g^{-1}, which was approximately 44% and 32% lower than the C_{SS} calculated at 6°C and 12°C, respectively (Figure 2.5A). The lower C_{SS} of the warm water lamprey, exposed to the same concentration of TFM as those at 6°C and 12°C, supports our hypothesis that their capacity to detoxify TFM increases with temperature. A similar pattern was observed in the muscle at 24°C, where C_{SS} was approximately 40% lower than 6°C and 12°C (Figure 2.5B).



Temp (°C)	C_{ss} (nmol g^{-1} ww)	$t_{1/2}$ Elimination (h)	k (h^{-1})	R^2
6°C	60.1 (48.1-109.9)	5.18 (2.95-13.04)	0.134 (0.053-0.235)	0.800
12°C	48.0 (43.7-52.7)	0.73 (0.42-1.25)	0.945 (0.341-1.660)	0.742
24°C	33.8 (31.7-36.1)	0.77 (0.55-1.07)	0.898 (0.647-1.271)	0.811

Temp (°C)	C_{ss} (nmol g^{-1} ww)	$t_{1/2}$ Elimination (h)	k (h^{-1})	R^2
6°C	19.5 (16.6-25.0)	4.55 (3.21-7.12)	0.152 (0.097-0.216)	0.906
12°C	20.7 (19.1-22.6)	1.29 (0.88-1.82	0.536 (0.380-0.788)	0.860
24°C	12.0 (11.4 – 12.6)	0.60 (0.46-0.78)	1.148 (0.882-1.536)	0.861

Figure 2.5 Relationship between temperature and TFM accumulation and elimination. The TFM concentration in the (A) liver and (B) muscle of larval sea lamprey exposed to a common concentration of TFM (3.74 mg L^{-1} ± 0.02) are plotted against time. Curves were fitted by non-linear regression using least squares analysis from which the apparent steady state concentration (C_{SS}) and elimination half-life ($t_{1/2}$) were calculated. The $t_{1/2}$ elimination was calculated from the rate constant of TFM accumulation (k) to reach C_{SS}, based on the assumption that at C_{SS}, the rate of TFM elimination was equal to its rate of accumulation. Calculations and curve fitting completed using GraphPad Prism 10.6. Data re-plotted from Figure 2.4. n = 7 - 10 samples at each time period. Light dashed lines: 95% confidence intervals.

Because the rates of absorption of xenobiotics is equal to the rate of elimination under C_{SS} conditions (Gupta, 2016; Kalant & Roschlau, 1998; Newman, 2020) we used the kinetic curves to calculate the half-life for TFM elimination ($t_{1/2}$), the time required to clear 50% of the TFM load by the animals, treating both the liver and muscle as single compartments. The $t_{1/2}$ of TFM in the liver was 5.18 h at 6°C, approximately 7-fold greater than that observed at 24°C, where $t_{1/2}$ was 0.77 h, indicating that the rate of elimination and uptake of TFM were much slower in colder water (Figure 2.5A). The $t_{1/2}$ of TFM in the liver at 12°C and 24°C

were not significantly different, suggesting that each had a similar rate of elimination. The differences in $t_{1/2}$ in the muscle were more clear-cut, decreasing in a stepwise manner as temperature increased (Figure 2.5B). The slower $t_{1/2}$ in the cold water also explains why there was no significant difference between the liver TFM concentration after 12 h of exposure at 6°C and 24°C (Figure 2.4A). With a slow $t_{1/2}$ (5.18 h – see above) at 6°C, the concentration of TFM in the liver would not have reached 95% of the C_{SS} of 60.1 nmol g^{-1} until approximately 22 h, after 4.3 half-lives would have elapsed (Gupta, 2016).

We propose that as waters warm that sea lamprey increase their capacity to eliminate TFM through temperature dependent increases in the rates of enzymatically mediated TFM-biotransformation in the liver. In poikilotherms, metabolic rates and enzyme activities are known to increase with temperature, as characterized by greater maximal enzyme activities (V_{max}) which can result from changes in protein structure (e.g., folding-unfolding), and either enhance or inhibit enzyme function through changes in substrate-enzyme affinity (Somero, 2004). Future enzyme kinetic studies and the quantification of mRNA and proteins would shed further light on the underlying mechanisms of increased TFM tolerance in warmer waters.

The Phase II metabolic pathways are thought to be the primary method of enzymatic TFM biotransformation in fishes, including sea lamprey (Kane et al., 1994; Lech & Statham, 1975). These include glucuronidation, during which a glucuronic acid functional group is added to TFM to generate TFM-glucuronide (Figure 2.1B; Kane et al., 1994; Lech & Stathan, 1975), and sulphation, in which a sulphate functional group is used to form TFM-sulphate (Figure 2.1B; Bussy et al., 2018a, b). Kane et al. (1994) demonstrated that the activity of UDP-glucuronosyltransferase (UDPGT), the enzyme required to convert parent TFM into TFM-glucuronide, was much higher in non-target fishes such as bluegill (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*), compared to sea lamprey. The efficiency of UDPGT (V_{max}/K_m) was also much lower in sea lamprey, which have since been found to also express fewer isoforms of the enzyme compared to bluegill (Kane et al., 1994; Lawrence et al., 2023). While the capacity of larval sea lamprey to produce TFM-

glucuronide is limited, there is also recent evidence that sea lamprey use phenol sulphotransferase to biotransform parent TFM into TFM-sulphate, which may be quantitatively more important for TFM detoxification than UDPGT (Bussy et al., 2018a, b). Hence, an increase in the activities of UDPGT and/or phenol sulphotransferase with water temperature may explain why TFM accumulation is lower in warmer waters.

2.4.3 Effects of warmer water on muscle energy stores

Reduced rates of mitochondrial oxidative phosphorylation during TFM exposures lead to the consumption of tissue anaerobic energy reserves (glycogen and PCr) to sustain ATP production (Birceanu et al., 2011). An increased reliance on anaerobic glycogen stores for ATP supply in the tissues of sea lamprey and non-target fish also results in the accumulation of lactate (Birceanu et al., 2014; Clifford et al., 2012). Consistent with greater reliance on anaerobic metabolism, significant reductions in PCr were observed at 24°C, but not at 6°C or 12°C, where no significant declines were observed (Table 2.4). The decrease in PCr at 24°C was likely related to the need to increase rates of PCr hydrolysis via the creatine phosphokinase reaction to buffer ATP stores (e.g., Clifford et al., 2012; Ionescu et al., 2021), compared to the colder 12°C and 6°C conditions, where ATP demands would be less. The significant increase in PCr with TFM at 6°C was unexpected, but we speculate that it may be due to their much lower metabolism at these temperatures, which in turn could be linked to lower ATP demand. The absence of significant changes in either glycogen or lactate at 12°C or 24°C were likely due to the relatively low concentration of TFM to which they were exposed (12-h LC_{25} of TFM at 12°C; 3.74 ± 0.02 mg L^{-1}) compared to previous studies where sea lamprey were exposed to the 12-h LC_{50} of TFM (Table 2.4). Had the TFM exposure periods been longer, particularly at 12°C or 24°C, we speculate we would have observed the anticipated drops in glycogen and higher lactate in the muscle (Wilkie et al., 2019). It should also be noted that the brain is more sensitive to TFM-induced reductions in glycogen and lactate, which were not examined in this study (Clifford et al., 2012).

2.5 Conclusion and implications

The present study supports the hypothesis that the underlying mechanisms of increased TFM tolerance in larval sea lamprey at warmer temperatures are related to an enhanced capacity to increase rates of TFM elimination stemming from either a greater rate of TFM metabolism and/or excretion. Future studies focused on measuring Phase II biotransformation products such as TFM-glucuronide or TFM-sulphate, enzyme kinetics, and enzyme gene and protein expression are needed to ascertain the underlying details that increase the sea lamprey's capacity to eliminate TFM at higher temperatures.

Our findings also provide further evidence of how warming temperatures later in the summer, or more sustained or transient elevations of water temperature due to climate change, could potentially undermine sea lamprey control measures in the Great Lakes (Lennox et al., 2020). We propose that temperature be considered when establishing TFM treatment concentrations, as routinely done with water pH and alkalinity (Bills et al., 2003; Schueller et al., 2024), to compensate for temperature-induced effects on TFM toxicity. Although most teleosts and invertebrate species of concern (e.g., native mussels) are less sensitive to TFM than the sea lamprey, it is not known how they will respond to TFM at higher temperatures, when metabolic demand is greater and oxygen availability is lower. How non-target species respond to TFM at warmer temperatures, and how to mitigate any adverse effects of lampricides under such conditions, should be a priority of future studies.

Beyond sea lamprey control, we propose that the simple phenolic structure of TFM may make it an ideal analog to model the uptake, distribution, detoxification and elimination of more toxic phenols in aquatic ecosystems. There are several phenolic compounds that are highly toxic even at very low concentrations and can be carcinogens (e.g., pentachlorophenol; International Agency for Research on Cancer, 2025), endocrine disruptors such as bisphenol A (e.g., BPA; Surana et al., 2022) or can cause genetic malformations (du Plessis et al., 2023). Further research into the effects of temperature on the pharmacokinetics of TFM and other phenolic compounds is needed to expand our understanding of how temperature influences their acute and chronic toxicity to various

aquatic organisms. This research may be particularly important as the average and peak temperatures of aquatic ecosystems increase due to climate change.

Chapter 3

Greater than Additive Interactions Between Two Phenolic Pesticides Used to Control Invasive Sea Lamprey (*Petromyzon marinus*): Underlying Mechanisms and Implications

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3.1 Introduction

Invasive sea lamprey (*Petromyzon marinus*) have plagued the Laurentian Great Lakes since the early 20th century, contributing to massive ecological damage and declines in the populations of economically important commercial, recreational and Indigenous fisheries (Gaden et al., 2021; Smith & Tibbles, 1980). In response, a sea lamprey control program (SLCP), coordinated by the Great Lakes Fishery Commission (GLFC), was launched in the 1950s with a mandate to control sea lamprey populations (Gaden et al., 2021). For over sixty years, the use of lampricides, pesticides that are relatively selective to sea lamprey, has formed the backbone of this program, along with barriers that block the upstream migration of adult spawning sea lamprey and the infestation of upstream habitat (McLaughlin et al., 2007; Sullivan et al., 2021). Since its initiation, the SLCP has reduced sea lamprey populations in the Great Lakes by 90% from their mid-20th-century peak (Robinson et al., 2021; Siefkes, 2017).

Lampricides are typically applied every 2-3 years to the streams and rivers draining into the Great Lakes, where larval sea lamprey are selectively targeted before they complete metamorphosis into destructive parasitic juvenile sea lamprey (Smith & Tibbles, 1980; Sullivan et al., 2021; Wilkie et al., 2019). The principal lampricide, 3-trifluoromethyl-4-nitrophenol (TFM, Figure 3.1A), is typically applied to streams infested with larval sea lamprey at concentrations that are up to 1.5 times its minimum lethal concentration (MLC = 12 h 99.9%) to ensure maximum treatment efficiency (Bills et al., 2003; McDonald & Kolar, 2007; Sullivan et al., 2021). The concentrations of TFM applied during treatment depend on environmental factors such as water pH and alkalinity, as well as the time of year (Bills et al., 2003; Sullivan et al., 2021; Wilkie et al., 2019).

Niclosamide (Nic, 2',5-dichloro-4-nitrosalicylanilide, Figure 3.1B), which is also used as a molluscicide and anthelmintic to control intestinal parasites, is often co-applied with TFM in large or fast-running streams (Dawson, 2003; Howell et al., 1980). The mixture of these lampricides, typically TFM and 0.5-2% niclosamide (w/w), results in a decrease of up

to 40% in the amount of TFM used in a single treatment without compromising TFM selective targeting of larval lamprey (Boogaard et al., 2003; Sullivan et al., 2021). This strategy reduces costs for the SLCP by requiring less TFM for treatments, without causing additional mortality to non-target fishes (Boogaard et al., 2003).

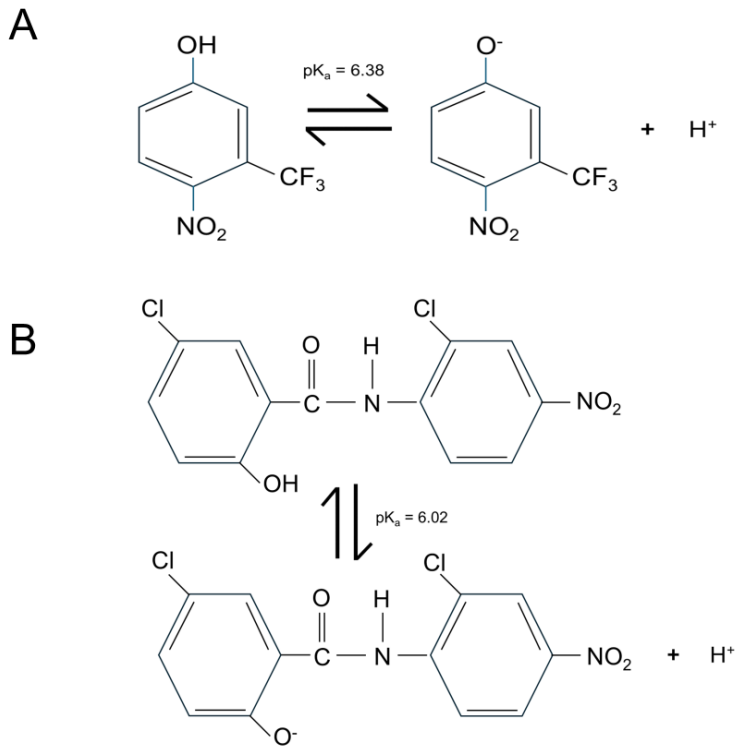


Figure 3.1 Chemical structure of (A) 3-fluoromethyl-4-nitrophenol (TFM) and (B) 2,5-dichloro-4'-nitrosalicylanilide (niclosamide, Nic) with their pKa values.

TFM applications selectively target the larval sea lamprey due to their relatively low capacity to use Phase II biotransformation processes of glucuronidation to convert TFM into much less toxic and easier to eliminate TFM-glucuronide (Lech & Statham, 1975; Kane et al., 1994; Lawrence et al., 2021, 2022). Most non-target fish species are more tolerant to TFM due to their greater capacity to use this process and detoxify TFM, although sensitivity varies widely among species, ranging from very sensitive young-of-the-year lake sturgeon to

very tolerant bluegill (Kane et al., 1994; Boogaard et al., 2003; Lawrence et al., 2021). In contrast to TFM, niclosamide is less selective toward sea lamprey, with broader toxic effects across fish species (Boogaard et al., 2003; Ionescu et al., 2022, 2025).

Both TFM and niclosamide have the same mechanism of action, interfering with mitochondrial ATP production by uncoupling oxidative phosphorylation in sea lamprey and other aquatic organisms (Birceanu et al., 2011; Borowiec et al., 2022; Huerta et al., 2020). This disruption of energy metabolism forces the animals to increase their reliance on anaerobic energy reserves, such as glycogen and phosphocreatine, to sustain ATP production, leading to eventual death when these sources of anaerobic ATP are insufficient (Birceanu et al., 2011; Clifford et al., 2012; Ionescu et al., 2022).

The underlying mechanisms by which niclosamide enhances TFM toxicity are unresolved. The nature of the interactions between two toxic substances with the same modes of action can be deduced using the concentration addition model, in which toxicity is measured in toxic units (TU; Norwood et al., 2003; Playle, 2004). Any appropriate endpoint can define one TU, but it is frequently expressed as the concentration of each substance required to kill 50% of the test organism over a specified time, e.g., 12-h LC_{50} (Playle, 2004). Thus, if 1 TU of TFM (TU_{TFM}) and 1 TU of niclosamide (TU_{Nic}) were set to the respective 12-h LC_{50} for each, exposure to 0.5 TU_{TFM} and 0.5 TU_{Nic} would result in 50% mortality if the interactions were strictly additive (Figure 3.2). It would be less than additive (antagonistic) if the combined mortality was less than 50% and greater than additive (synergistic) if the combined mortality was greater than 50%.

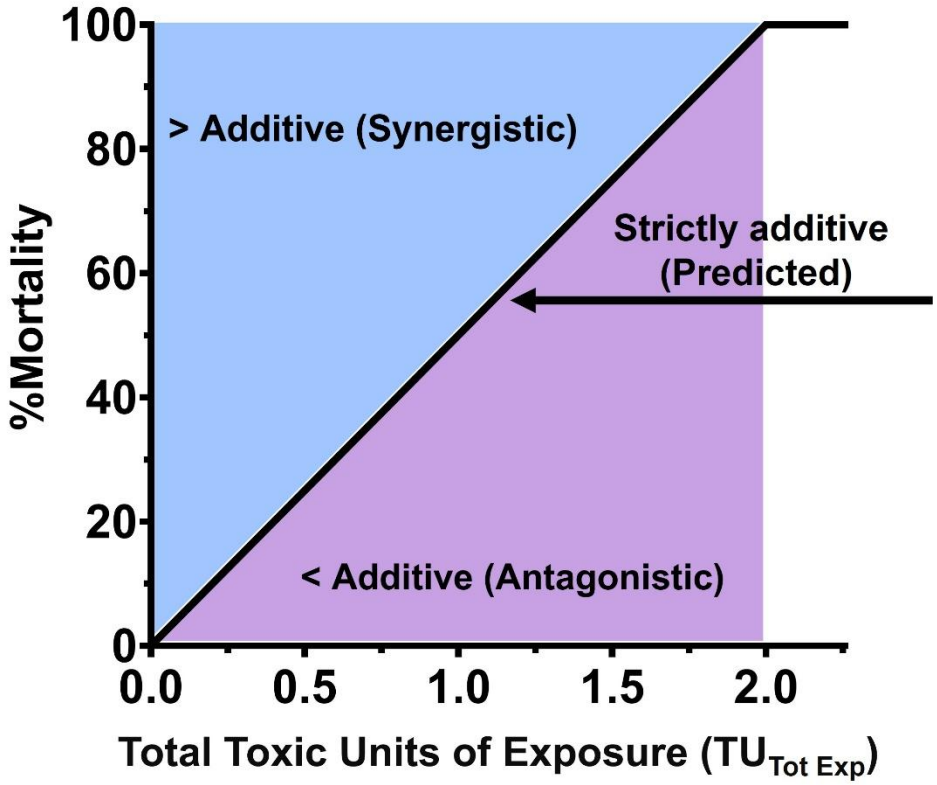


Figure 3.2 The concentration addition model. The theoretical concentration addition model using toxic units (TU). The concentration addition model determines the interaction of two chemicals by displaying percent mortality rates based on the combined effects of both chemicals (total toxic units of exposure). Strictly additive interactions between two chemicals would fall on the line of strict additivity (black line), antagonistic interactions would produce mortality rates lower than expected, falling below the black line (purple area), while synergistic interactions would produce higher than expected mortality rates and fall above the line of strict additivity (blue area). Figure adapted from Newman (2020).

Earlier studies in rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*) and larval sea lamprey indicated that mixtures of TFM and niclosamide (Bayer 73) were strictly additive or antagonistic (Bills & Marking, 1976; Dawson et al., 1977; Marking & Bills, 1985), while others suggested that interactions were synergistic (Howell et al., 1964). Most recently, Hepditch et al. (2021) employed the concentration addition model

to elucidate the nature of interactions between TFM and niclosamide in rainbow trout. This latter study demonstrated that at relatively low concentrations of TFM and 1% niclosamide, below 1 TU, the interactions were strictly additive but became greater than additive when the fish were exposed to concentrations above 1 TU (Hepditch et al., 2021). The goal of the present study was to examine TFM-niclosamide interactions via the concentration addition model in larval sea lamprey, using the now more commonly used emulsifiable concentrate (EC) formulation of niclosamide (Sullivan et al., 2021). To provide additional insight into the mechanisms that govern TFM-niclosamide interactions, liver and muscle tissue were also collected for the measurement of TFM and niclosamide accumulation following exposure to a range of concentrations of each lampricide alone and to TFM/1% niclosamide mixtures.

3.2 Materials and methods

3.2.1 Experimental animals and holding

Larval sea lamprey (n = 250, mass = 1.5 ± 0.1 g and total length = 112.7 ± 0.6 mm) were supplied by the Hammond Bay Biological Station (US Geological Survey), Millersburg, Michigan, USA, following their capture using pulsed-DC electrofishing by US Fish and Wildlife biologists. The larvae were placed into sealed plastic bags filled with oxygen saturated ice-cold water and shipped by overnight courier to Wilfrid Laurier University in hard-sided coolers (70 L). At Wilfrid Laurier University, the larvae were held under a 12 h light: 12 h dark photoperiod in aerated flow-through 75 L tanks fed with City of Waterloo dechlorinated tap water with the following parameters: temperature ~ 12°C; dissolved oxygen ~ 97%, pH ~ 8.02; titratable alkalinity ~ 208 mg L^{-1} as $CaCO_3$ with a replacement rate of 0.5 L min^{-1}. The bottom of each holding tank was filled with sand (5-10 cm depth, playbox sand, Home Depot, Canada) to provide burrowing substrate. The larvae were held under these conditions for 2 - 3 months before experiments, during which they were fed a slurry of wet bakers' yeast (Fleischmanns®, Baker's Select Fresh Yeast, Mississauga, CA) at a ration of 1 g per larva per week (Birceanu et al., 2009; Henry et al., 2015). Lamprey were not fed for 48 h before the experiments to minimize ammonia accumulation in the

experimental tanks. All experiments were approved by the Wilfrid Laurier University Animal Care Committee (Animal Use Protocol Number R22003) and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

3.2.2 Chemicals and reagents

Both TFM and niclosamide were kindly provided by GLFC through the Sea Lamprey Control Centre, Fisheries and Oceans Canada, Sault Ste. Marie, Ontario. Field formulation TFM (33.5% dissolved in isopropanol; Clariant SFC GMBH WERK, Griesheim, Germany) was used for all TFM exposure experiments. A stock solution of TFM (1 g L^{-1}) was prepared prior to the experiments, and an appropriate amount of working stock solution was added to each aquarium to yield the selected nominal TFM concentrations, which were quantified spectrophotometrically as described below.

Niclosamide (Bayluscide® Emulsifiable Concentrate; 16.9% niclosamide ethanolamine salt mixture; Coating Place Inc., Verona, WI, USA), diluted 10 times in 95% ethanol, was used as a stock solution for exposures. In each case, the appropriate concentrations of the stock solution were added to experimental aquaria to yield the desired exposure concentrations. Concentrations were confirmed by LC-MS/MS analysis at the beginning and end of the experiments (see Section 3.2.5 below).

3.2.3 Experimental protocols

All acute toxicity experiments were conducted in glass aquaria filled with aerated City of Waterloo dechlorinated tap water (temperature 12.0 ± 0.4°C, dissolved oxygen 97 ± 1% saturation, pH 8.02 ± 0.02, and alkalinity ranged from 204 to 212 mg L^{-1} as $CaCO_3$; Table 3.1) without sand to minimize niclosamide adsorption to sand particles, that could lead to reductions in the free concentration of niclosamide in the water (Ionescu et al., 2022). To maintain a constant water temperature, the aquaria were partially submerged in 12°C water.

Approximately 12 h before the experiments, larval sea lamprey were gently transferred from their sand-filled holding tanks to an aquarium of the same size but containing no burrowing substrate and left overnight to avoid excessive handling stress prior

to lampricide exposure the following day. At the same time, the experimental aquaria were dosed in duplicate with appropriate amounts of stock TFM or niclosamide to yield nominal concentrations of TFM only (1, 2, 3, 3.5, 4, 5 mg L^{-1}), niclosamide only (0.01, 0.05, 0.1, 0.25 mg L^{-1}) or TFM/1% Nic mixture (1/0.01, 2/0.02, 2.5/0.025, 3/0.03, 4/0.04 mg L^{-1}). Tanks containing no lampricides served as controls during experiments.

On the morning of the experiments, lampreys were transferred to each experimental tank (n = 5 per tank, with 2 tanks per nominal concentration). The 12-h exposure was initiated once all the lampreys were in their tanks. Water samples (20 mL) were collected at regular intervals (0, 6, and 12 h) for immediate measurement of TFM concentration, with an aliquot (10 mL) frozen for future niclosamide analysis (Appendix B, Table B.1). Sea lamprey survival was continuously monitored over the 12-h exposure. Mortality was assumed when the animals were immobile and unresponsive to a tail pinch using forceps, at which time they were promptly removed from the tanks, patted dry, and the time of death, weight and length were recorded. Liver and muscle tissue were then immediately sampled, placed onto aluminum foil and flash frozen using liquid nitrogen-cooled aluminum tongs. The liver and muscle samples were stored at -80°C for future TFM and niclosamide analysis. After 12 h, all remaining (surviving) lamprey were euthanized in an aerated bath containing a lethal dose of buffered tricaine methanesulfonate (MS-222 - Syndel, Nanaimo, BC, Canada; 1.5 g L^{-1} MS-222 plus 3.0 g L^{-1} $NaHCO_3$) and sampled as described above.

3.2.4 Concentration addition model

Toxicity data was analyzed using the concentration addition model, which uses the toxic unit (TU) concept to model interactions between mixtures of substances with the same modes of action (Norwood et al., 2003; Playle, 2004). 1 TU_{TFM} was set to equal its corresponding 12-h LC_{50} when exposed to TFM only, and 1 TU_{Nic} was set to equal its respective 12-h LC_{50} when exposed to niclosamide only. The individual 12-h LC_{50} values were then used to calculate the total toxic units of exposure using the equation below:

$$\text{Total TU of exposure } (TU_{Tot\ Exp}) = x\ TU_{TFM} + y\ TU_{Nic} \quad (1)$$

where x and y denote the proportion of each TU present in the mixture. This equation was then used to plot the total TU ($TU_{Tot\ Exp}$) of exposure against the expected % mortality as described below:

$$\text{Predicted \% mortality} = [(TU_{TFM} \times 0.5) + (TU_{Nic} \times 0.5)] \times 100\% \quad (2)$$

The corresponding line of equivalence of the relationship between the total $TU_{Tot\ Exp}$ versus predicted percentage mortality represented the line of strict additivity (Figure 3.2; Newman, 2020). For instance, if 0.5 TU_{TFM} and 0.5 TU_{Nic} were acting in a strictly additive manner, then the expected mortality would be 50%, falling directly on the middle of the line of strict additivity. If the observed mortality fell above the line of strict additivity, the interactions between TFM and niclosamide were considered greater than additive (synergistic) and if observed mortality fell below the line, the interactions were considered less than additive (antagonistic).

3.2.5 Analytical methods

Water TFM concentrations were measured spectrophotometrically on 1 mL water samples using 1.5 mL polystyrene cuvettes and a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, UK) at a wavelength of 395 nm following Fisheries and Oceans Canada Instrument Operating Procedure (IOP # 012.4; GLFC, 2024). TFM standard dilutions were made using experimental water and according to GLFC Standards Operating Procedure 43.1.0 (US Geological Survey, Hammond Bay Biological Station, MI, USA). Additionally, to further validate the spectrophotometrically measured TFM concentrations, an empty container was spiked with analytically confirmed TFM working stock, allowing for a comparison between the two measurements.

Niclosamide was analyzed in water following previously published protocols (Lawrence et al., 2021; Ionescu et al., 2022). Briefly, 5 mL of water samples were transferred to a clean glass test tube and spiked with 200 µg L^{-1} of an isotopically labelled internal standard, niclosamide (^{13}C-Nic, Millipore Sigma, Oakville, ON). In addition, duplicate matrix controls samples comprised of internal standard (^{13}C-Nic), plus 100 µg L^{-1} analytical

grade niclosamide, were prepared in Milli-Q water and included in each step of the analysis to account for possible matrix effects, which if left unchecked could lead to inaccurate results due to the presence of other compounds within the samples (Harris, 2016). After vacuum filtration (0.45 μm glass fibre filters, Pall Corporation, Michigan, USA), 1 mL of the eluent was transferred to amber glass vials, and LC-MS/MS analysis was performed immediately thereafter (details described below).

Tissue samples were processed for TFM and niclosamide concentration measurements using solid phase extraction prior to LC-MS/MS for lampricide analysis as initially outlined by Lawrence et al. (2021) with protocol optimization. Briefly, Captiva EMR-Lipid 96 well plates (Agilent Technologies, Santa Clara, CA, US) were used to extract TFM or niclosamide from approximately 100 mg of finely ground tissue. Due to the unavailability of isotopically labelled TFM standards, ^{13}C-niclosamide (^{13}C-Nic) was used as an internal standard by spiking all standards, samples and blanks with 200 μg L-1 of ^{13}C-Nic. Additionally, matrix spikes were prepared with control tissues spiked with ^{13}C-Nic and included 100 μg L^{-1} of TFM, niclosamide or both lampricides (depending on the experiment).

The eluted tissue (100-200 μL) or eluted water samples (1 mL) were then analyzed using an Agilent 1260 HPLC with a 6460 triple quad mass spectrometer (Agilent Technologies, Santa Clara, CA). An Agilent Eclipse XDB-C18 column (4.6 x 150 mm, 5 μm) was used to separate the analytes chromatographically. All data was processed using Agilent Mass Hunter Quantitative Analysis software version 10.1. The calibration curve ranged from 0-5000 μg L^{-1} for TFM and 0-500 μg L^{-1} for niclosamide. For both lampricides, the instrument detection limit was 0.04 μg L^{-1}, the method detection limit was 0.25 μg L^{-1}, and all sample recoveries were above 95%.

3.2.6 Statistical analysis

For the determination of the dose-response relationship of each lampricide and in the TFM/1% mixture, replicate tanks were treated as individual tanks when calculating the 12-h LC_{50} and 12-h $LC_{99.9}$ (minimal lethal concentration; MLC) lethal concentrations for each

lampricide and the TMF-1% Nic mixture. Dose-response curves, corresponding LC values and 95% confidence intervals (CI) were determined using non-linear regression (curve fit) using least squares analysis (GraphPad Prism version 10.6). Values were considered significantly different if 95% confidence intervals did not overlap (Finney, 1971).

Morphometric data, including larval length and weight, plus lampricide tissue concentrations, were presented as the mean ± S.E.M. Statistical significance between the means were evaluated using 1-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests. In cases where the assumptions of normality and homogeneity were not met, a non-parametric ANOVA (Kruskal-Wallis test), followed by Dunn's Test of Multiple Comparisons test, were used. Statistical analysis and figures were completed using GraphPad Prism version 10.6 (GraphPad Software Inc, La Jolla, CA, USA), and the significance level was set at $\alpha = 0.05$.

3.3 Results

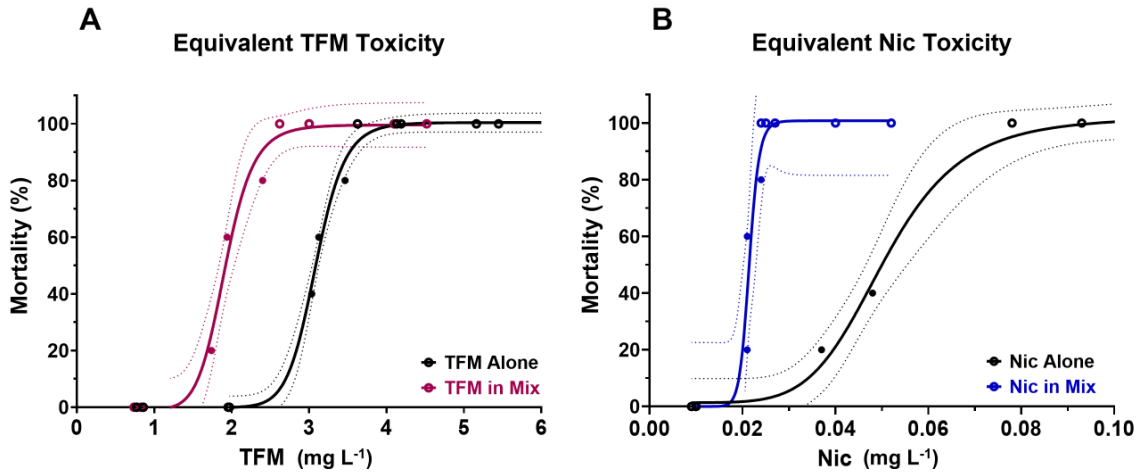
3.3.1 Lampricide toxicity: individual and in mixtures

Water pH was relatively stable across all treatment concentrations (8.05 ± 0.02), during which dissolved O_2 (97 ± 0.9% saturation), alkalinity (204 - 213 mg L^{-1} as $CaCO_3$) and temperature (12 ± 0.3°C) were relatively stable (Table 3.1). The measured water TFM concentrations from the individual lampricide and TFM/1% Nic mixture toxicity tests closely matched nominal target concentrations at 0, 6, and 12 h (Appendix B, Table B.1). Niclosamide, however, was more variable; thus, niclosamide water levels were measured at the beginning of the experiments, at 6 and 12 h post exposure and averaged over time (Appendix B, Table B.1). This allowed for the concentration correction, since niclosamide could precipitate from the water or adsorb to the animal over time, especially at lower exposure concentrations. Collection of water samples was stopped when complete mortality of all lamprey was observed in a given tank.

Table 3.1 Water quality parameters for each acute toxicity test. Measured mean water temperature, pH, alkalinity, and dissolved oxygen (DO) during acute toxicity tests of larval sea lamprey for TFM, niclosamide, and TFM/1% niclosamide. n denotes the number of total measurements per treatment. Data represented as mean ± S.E.M.

Acute toxicity test	Temperature (°C)	pH	Alkalinity (mg L^{-1} as $CaCO_3$)	Dissolved O_2 (% saturation)	n
TFM	12.6 ± 0.04	8.03 ± 0.02	213	96.6 ± 0.6	21
Niclosamide	11.8 ± 0.30	8.08 ± 0.04	204	98.8 ± 0.2	15
TFM/1% Nic	11.8 ± 0.14	8.05 ± 0.01	204	95.9 ± 0.8	18

Mixing niclosamide (~ 1% of mixture) with TFM (~ 99%) enhanced TFM toxicity, characterized by a leftward shift of the TFM dose-response curve, which corresponded to a 37% decrease in the 12-h LC_{50} of TFM from 3.10 (CI = 2.85 - 3.36) mg L^{-1} for TFM alone, to 1.96 (CI = 1.79 - 2.30) mg L^{-1} for the TFM/1% Nic mixture (Figure 3.3A). However, the 12-h $LC_{99.9}$ of TFM only treatment was not significantly different from the TFM/1% Nic mixture treatment because the 95% confidence intervals overlapped due to the higher variance of the measurements (Figure 3.3A). 12-h $LC_{99.9}$ of TFM only treatment was not significantly different from the TFM/1% Nic mixture treatment because the 95% confidence intervals overlapped due to the higher variance of the measurements (Figure 3.3A).



TFM (mg L^{-1})

LC	Concentration	95% CI
25	2.87	2.63 – 3.12
50	3.10	2.85 – 3.36
99.9	4.40	3.79 – 5.12

TFM in TFM/1% Nic Mixture (mg L^{-1})

LC	Concentration	95% CI
25	1.73*	1.45 – 2.06
50	1.92*	1.66 – 2.32
99.9	3.56	2.66 – 4.76

Niclosamide (mg L^{-1})

LC	Concentration	95% CI
25	0.040	0.031 – 0.051
50	0.048	0.038 – 0.061
99.9	0.112	0.077 – 0.162

Niclosamide in TFM/1% Nic Mixture (mg L^{-1})

LC	Concentration	95% CI
25	0.020*	0.018 – 0.022
50	0.021*	0.020 – 0.024
99.9	0.030*	0.026 – 0.034

Figure 3.3 Dose-response curves of larval sea lamprey. Comparing observed % mortality [± 95% confidence intervals (CI); dashed lines] of larval sea lamprey exposed to different concentrations of (A) TFM in the absence (TFM Alone) or presence of niclosamide (TFM in Mix), and (B) niclosamide in the absence (Nic Alone) or presence of TFM (Nic in Mix) over 12 h. n = 5 for each concentration shown, and solid circles denote partial mortalities. Corresponding tables summarize the calculated LC_{25}, LC_{50} and $LC_{99.9}$ for TFM or niclosamide concentrations ± 95% CI, determined by non-linear regression (curve fit) using least squares analysis (GraphPad Prism 10.6). Asterisks (*) indicate significant differences between the respective LC values of TFM or niclosamide and each corresponding mixture, as determined by lack of CI overlap.

There was also a leftward shift of the niclosamide dose-response curve in the presence of TFM, characterized by a 56% decrease in the 12-h LC_{50} of niclosamide from 0.048 (CI = 0.038 - 0.061) mg L^{-1} for Nic only treatment exposure to 0.021 (CI = 0.020 - 0.024) mg L^{-1} for the TFM/1% Nic mixture (Figure 3.3B). Notably, the niclosamide plus TFM mixture treatment dose-response curve was much steeper than the Nic only curve, as

the range of niclosamide concentrations producing no mortality, and complete mortality was very narrow (0.015 - 0.034 mg L^{-1}, respectively). A corresponding reduction was also observed for the respective 12-h $LC_{99.9}$, which decreased 73% from 0.112 (CI = 0.077 - 0.162) mg L^{-1} in the Nic only exposure treatment to 0.030 (CI = 0.067 - 0.034) mg L^{-1} when exposed to the TFM/1% Nic treatment (Figure 3.3B).

3.3.2 TFM and niclosamide interactions using the concentration addition model

Based on the respective 12-h LC_{50} of TFM and niclosamide (Figure 3.3), 1 TU of TFM was set to 3.10 mg L^{-1} and 1 TU of niclosamide was set to 0.048 mg L^{-1}. Accordingly, the predicted mortality if the lamprey were exposed to 0.5 TU of TFM and 0.5 TU of niclosamide would have been 50%, falling directly on the line of strict additivity of the corresponding TU of observed mortality vs TU of exposure relationship (Figure 3.4, diamond symbol). Exposure to the lowest TFM/1% Nic exposure concentrations (0.74/0.012 mg L^{-1} or 0.83/0.009 mg L^{-1}) corresponded to an average 0.25 TU of each lampricide, which totalled 0.50 TU of exposure, but resulted in no mortality. Exposure to 0.99 $TU_{Tot\ Exp}$ made up of 0.56 TU of TFM (measured [TFM] = 1.74 mg L^{-1}), and 0.43 TU of niclosamide (0.021 mg L^{-1}) resulted in 20% mortality, below the line of strict additivity (less than additive; Figure 3.4). At a slightly higher concentration equal to 1.06 $TU_{Tot\ Exp}$ observed mortality was 60% and above the strict additivity line, indicating the TFM and niclosamide were interacting in a greater than additive fashion (Figure 3.4). As the $TU_{Tot\ Exp}$ increased further to 1.27 and 1.37 TU, the observed mortality was 80 and 100%, again indicating that the interactions were greater than additive at higher exposure concentrations of TFM and niclosamide (Figure 3.4). Similarly, when $TU_{Tot\ Exp}$ equalled 1.47 and 1.55 TU, 100% mortality was observed, compared to the 75-80% mortality that was predicted based on the line of strict additivity (Figure 3.4). Not surprisingly, when the $TU_{Tot\ Exp}$ were greater than 2.0 TU, there was 100% mortality (Figure 3.4).

Tank	a	b	a	b	a	b	a	b	a	b
[TFM]	0.74	0.83	1.74	1.94	2.40	2.62	3.00	3.00	4.52	4.10
TU TFM	0.24	0.27	0.56	0.63	0.77	0.85	0.97	0.97	1.46	1.32
[Nic]	0.012	0.009	0.021	0.021	0.024	0.025	0.024	0.028	0.052	0.040
TU Nic	0.25	0.19	0.43	0.44	0.50	0.52	0.50	0.58	1.08	0.83
TU Total	0.49	0.46	0.99	1.06	1.27	1.37	1.47	1.55	2.54	2.16

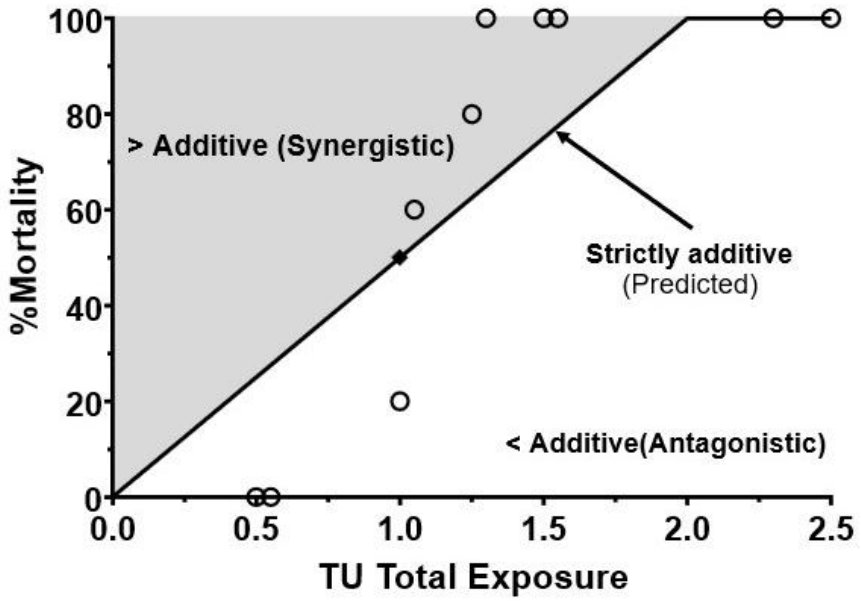


Figure 3.4 Interactive effects of TFM and niclosamide (Nic) mixture exposure on larval sea lamprey mortality expressed using the toxic unit (TU) concept. 1 TU is defined as the 12-h median lethal concentration (12-h LC_{50}) deduced from exposure to TFM or niclosamide alone. Observed mortalities would fall directly on the predicted mortality line (line of equivalence) if TFM plus niclosamide interactions were strictly additive, whereas interactions would be greater than additive (synergistic) if observed mortality fell above the line and less than additive (antagonistic) if observed mortalities fell below the line. Open circles: observed mortality when sea lamprey were exposed to increasing concentrations of TFM and niclosamide. Diamond: predicted mortality if exposure to 0.5 TU_{TFM} and 0.5 TU_{Nic} was strictly additive.

3.3.3 TFM and niclosamide accumulation in liver and muscle

To better understand the underlying interactions between TFM and niclosamide, liver and muscle tissue were collected immediately upon death or following the 12 h exposure period, and the concentrations of TFM and niclosamide were measured in each tissue. Because no significant differences (unpaired t-test at each exposure concentration; Appendix B Figure

B.1 and Figure B.2) were observed in TFM or niclosamide accumulation in either liver or muscle tissues of surviving (solid circles) vs non-surviving (open circles), the data were pooled in Figures 5 and 6 described below.

As the concentration of TFM in the water increased from 0.0 - 5.0 mg L^{-1}, in the absence of niclosamide, TFM concentrations in the liver gradually, but significantly, increased before stabilizing at a TFM tissue concentration of 42.9 ± 3.6 mg L^{-1} at a nominal 3.0 mg L^{-1} TFM exposure, beyond which no further significant changes were observed (Figure 3.5A). Similar TFM accumulation patterns were observed with exposure to the TFM/1% Nic mixture treatment. Initially, as the nominal TFM exposure concentration was increased to 1.0 and then 2.0 mg L^{-1}, the liver TFM concentration increased from 18.2 (± 1.9) to 31.8 (± 1.6) nmol g^{-1} ww and then stabilized between exposure concentrations of 2.5 to 4.0 mg L^{-1} (Figure 3.5A). When comparing TFM liver concentrations between the TFM only and TFM/1% Nic treatments, there were no significant differences between the TFM only and TFM/1% Nic treatments across all exposure concentrations ($p > 0.05$; Figure 3.5A).

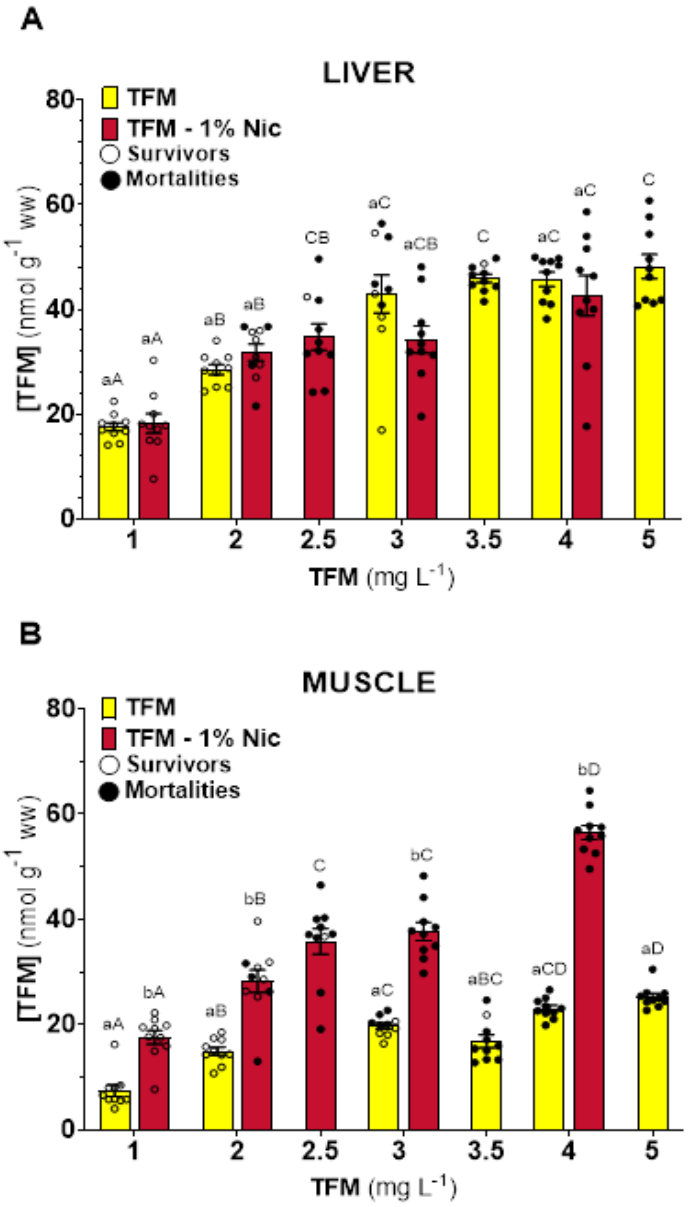


Figure 3.5 Tissue accumulation of TFM in sea lamprey. (A) liver and (B) muscle of larval sea lamprey exposed to increasing concentrations of TFM (yellow bars) or TFM/1% niclosamide (Nic; red bars) for up to 12 h. No between treatment comparisons were made at 2.5, 3.5 or 5 mg L^{-1} of TFM. Open circles depict survivors, and solid circles depict mortalities. TFM concentrations in the tissues of the control fish (no TFM exposure) were non-detectable. Data with different uppercase letters are significantly different within each treatment, and data with different lowercase letters are significantly different between the treatments ($p < 0.05$). Data presented as the mean ± S.E.M. (n = 8-10).

The TFM only treatment resulted in a gradual accumulation of TFM in the muscle, peaking at 25.3 (± 0.7) nmol g^{-1} ww at the highest TFM exposure concentration of 5.0 mg L^{-1} (Figure 3.6B). Exposure to the TFM/1% Nic mixture treatment resulted in greater TFM accumulation in the muscle compared to the TFM only treatment, steadily increasing as the TFM concentration in the TFM/1% Nic mixture treatment increased from 1 to 4 mg L^{-1}. Most notable was that at all TFM/1% Nic mixture concentrations, the concentration of TFM that accumulated in the muscle was 1.9- to 2.5-fold greater than observed in the TFM only exposure, peaking at 56.5 (± 1.4) nmol g^{-1} ww at the highest exposure concentration (4.0 mg L^{-1}) (Figure 3.6B).The measured niclosamide concentrations in both the liver and muscle closely resemble the patterns observed for TFM (Figure 3.6). As the concentration of niclosamide in water increased from 0.01 mg L^{-1} to 0.5 mg L^{-1}, the respective concentration of liver tissue niclosamide sharply increased from 1 nmol g^{-1} ww to 18 nmol g^{-1} ww. Liver niclosamide concentration subsequently peaked at 39.3 ± 4.3 nmol g^{-1} ww and did not significantly change thereafter (Figure 3.6A).

Exposure to 0.01 mg L^{-1} niclosamide in the TFM/1% Nic mixture resulted in a marked, significant increase in liver niclosamide concentration from approximately 9.0 ± 0.8 nmol g^{-1} (Figure 3.6A). As the niclosamide exposure concentration was increased to 0.02 mg L^{-1} in the TFM/1% Nic mixture, the liver niclosamide concentration further increased to 14.3 ± 1.5 nmol g^{-1} ww and did not significantly change as the niclosamide water concentration in the mixture increased further to 0.025, 0.03 and 0.04 mg L^{-1}.

In the muscle tissues, niclosamide concentrations in the Nic only treatment did not significantly increase until the water exposure concentration reached 0.1 mg L^{-1}, where tissue niclosamide increased from 1.1 ± 0.01 at an exposure concentration of 0.05 mg L^{-1} to 6.8 ± 0.8 at 0.01 mg L^{-1}. There was a further increase in muscle niclosamide, to 10.9 ± 1.0 ($p < 0.05$), when the Nic only exposure concentration reached 0.25 mg L^{-1} (Figure 3.6B).

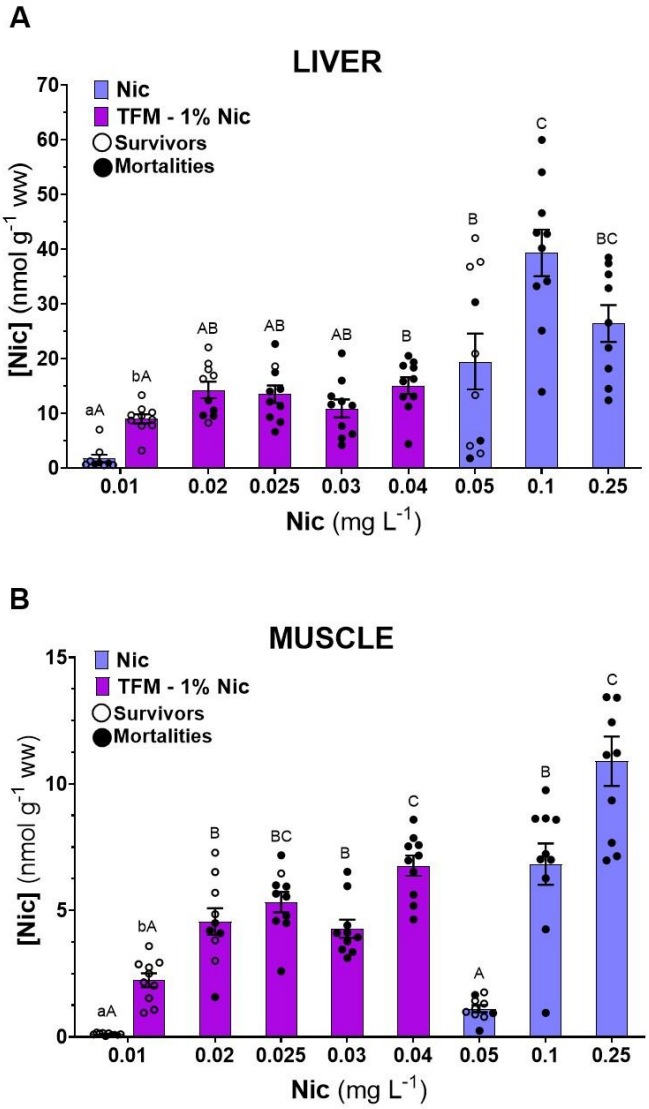


Figure 3.6 Tissue accumulation of niclosamide (Nic) in sea lamprey. (A) liver and (B) muscle tissues of larval sea lamprey exposed to increasing concentrations of Nic (blue bars) or TFM/1% Nic (magenta bars) for up to 12 h. The between treatments comparisons were made only at 0.01 mg L^{-1} of niclosamide, that is the only common concentration of niclosamide tested between the two treatments. Open circles depict survivors, and solid circles depict mortalities. Niclosamide concentrations in the tissues of the control fish (no TFM exposure) were non-detectable. Data with different uppercase letters within the treatment, whereas different lowercase letters denote a significant difference between the TFM vs TFM/1% Nic treatment ($p < 0.05$). Data presented as the mean ± S.E.M. (n = 8-10).

In the presence of TFM and 0.01 mg L^{-1} niclosamide, the amount of niclosamide in the muscle increased more than 20-fold, to 2.2 ± 0.3 nmol g^{-1} ww compared to 0.1 ± 0.01 nmol g^{-1} ww when exposed to Nic only ($p < 0.001$; Figure 3.6B). As the amount of niclosamide in the TFM/1% Nic mixture was increased further to 0.02 mg L^{-1}, the concentration of niclosamide in the muscle increased to 4.6 ± 0.5 nmol g^{-1} ww (Figure 3.6B). Muscle niclosamide concentration in the TFM/1% Nic mixture treatment did not significantly change as exposures increased further to 0.025 and 0.03 mg L^{-1}. However, at the highest exposure concentration of 0.04 mg L^{-1} in the TFM/1% Nic mixture, the muscle niclosamide concentration reached 6.7 ± 0.4 nmol g^{-1} ww (Figure 3.6B).

3.4 Discussion

Binary mixtures of TFM and niclosamide interact in a greater than additive (synergistic) manner to cause mortality in larval sea lamprey, but only when $TU_{Tot\ Exp}$ is greater than 1.0 $TU_{Tot\ Exp}$. However, when the $TU_{Tot\ Exp}$ was less than 1.0, less than additive (antagonistic) effects were observed (Figure 3.4). Marked increases in the muscle TFM concentration when sea lamprey were exposed to binary mixtures of TFM and niclosamide suggest that the greater than additive nature of the interactions was related to impaired TFM metabolism in the liver in the presence of niclosamide. Impaired TFM metabolism could have resulted from competitive inhibition by niclosamide of Phase II enzymes of biotransformation such as UDP-glucuronosyltransferases (UDPGT) or sulphotransferases, known to be involved in TFM biotransformation (Bussy et al., 2018a, b; Kane et al., 1994; Lech & Statham, 1975). It is less straightforward, however, why the interactions were less than additive when the larval sea lamprey were exposed to less than 1 $TU_{Tot\ Exp}$.

3.4.1 TFM and niclosamide interactions defined by the concentration addition model

Earlier studies across many different fish species, including sea lamprey, have demonstrated that TFM toxicity is enhanced with co-exposure to low concentrations of niclosamide (Boogaard et al., 2003; Marking & Dawson, 1975; O'Connor et al., 2017). Lampricide

mixture interactions were recently studied in rainbow trout (*Oncorhynchus mykiss*) using the concentration addition model, and a greater than additive relationship between TFM and niclosamide mixtures was also observed (Hepditch et al., 2021). However, the 12-h LC_{50} of TFM (6.4 mg L^{-1}) and 12-h LC_{50} of niclosamide (0.035 mg L^{-1}) needed to achieve the synergistic relationship between the lampricides in rainbow trout (Hepditch et al., 2021) far exceeded the concentrations that would be used (range 1.3 - 3.6 mg L^{-1} of TFM and niclosamide in 1-2% of TFM concentration) during a typical treatment at a similar water pH and alkalinity (Bills et al., 2003). Since lampricide stream treatments usually do not reach concentrations needed to observe the synergistic effects of lampricide mixtures in non-target fishes, the observations of extreme decreases in TFM concentration needed to achieve its toxic effects in the presence of niclosamide in less sensitive non-target fishes are often absent. The addition of niclosamide to TFM treatments in sea lamprey resulted in a modest 37% reduction in the TFM concentration required to maintain treatment effectiveness (12-h LC_{50}; Table 3.2). Similar values were reported for other TFM sensitive species, like young of the year (YOY) or 1+ lake sturgeon (Boogaard et al., 2003), where reductions in TFM concentration between the TFM only treatments and TFM/1% Nic treatments were 38 and 39%, respectively.

The reduction in 12-h LC_{50} of TFM concentration with the addition of niclosamide is much more pronounced in a lampricide tolerant fish, such as rainbow trout (Hepditch et al., 2021) or yellow perch and bluegill (Boogaard et al., 2003), where the concentration of TFM decreased by 64%, 68% and 59%, respectively (Table 3.2). The apparent relative difference (approximately double) between the TFM sensitive and TFM tolerant species, in the percent reduction of TFM needed to achieve comparable toxic effects, is visible only when comparing the relative LC_{50} (or similar) values. The considerable increase in TFM toxicity in the presence of niclosamide suggests that non-target TFM treatment species are more susceptible to TFM exposure in the presence of niclosamide than the sea lamprey and, as such, may be exposed to increased risks. Further studies on other non-target fish species at concentrations required to produce species specific toxicities will provide clarification on the extent of this claim.

Table 3.2 Differences between the sea lamprey (present study) and different fish species in 12-h TFM LC_{50} values in the presence or absence of niclosamide. Comparing the TFM concentration reductions (in %) of 12-h LC_{50} values between the TFM only (absence of niclosamide) and TFM/1% Nic Mixture (presence of niclosamide) with similar water chemistry between the TFM sensitive species (sea lamprey, young of the year (YOY) and 1+ (over 1 year of age) and TFM tolerant species (rainbow trout, yellow perch and bluegill). Values presented as mean values with confidence intervals (CI) in brackets.

Study	pH	Alkalinity (mg L^{-1} as $CaCO_3$)	12-h TFM LC_{50} (mg L^{-1}) (95% CI)	TFM in 12-h TFM/1% Nic mixture LC_{50} (mg L^{-1}) (95% CI)	Reduction of [TFM] in LC_{50}	Reference
Sea Lamprey	8.05	208	3.1 (2.85 - 3.36)	1.96 (1.66 - 2.32)	37%	Current Study
Rainbow Trout	8.1	210	17.6 (13.0 - 36.4)	6.37 (5.47 - 7.16)	64%	Hepditch et al., 2021
YOY Lake Sturgeon	7.9	102 - 129	1.78 (1.67 - 1.81)	1.10 (1.04 - 1.16)	38%	Boogaard et al., 2003
1+ Lake Sturgeon	7.9	102 - 129	3.53 (3.24 - 3.85)	2.15 (1.96 - 2.36)	39%	Boogaard et al., 2003
Yellow Perch	8.1	110 - 114	22.70 (20.9 - 24.7)	7.21 (6.48 - 8.02)	68%	Boogaard et al., 2003
Bluegill	8.1	110	24.60 (23.3 - 26.0)	10.20 (9.06 - 11.5)	59%	Boogaard et al., 2003

The results of the present study, analyzed in the same manner as Hepditch et al. (2021), demonstrate that the interactions between TFM and niclosamide in sea lamprey (i.e., 12-h LC_{50}) are concentration dependent when the proportion of niclosamide is approximately 1% of the TFM concentration. The less than additive interactions were observed when concentrations are below 1 $TU_{Tot\ Exp}$, whereas the interactions are greater than additive when the mixture concentrations exceed 1 $TU_{Tot\ Exp}$ (Figure 3.4). The present 12-h LC_{50} results are

comparable to those reported by Hepditch et al. (2021), who observed a similar concentration dependent relationship between TFM and niclosamide mixtures in rainbow trout.

Phenolic compounds such as TFM and niclosamide are detoxified using Phase II metabolic pathways, including the UDP family of enzymes or phenol sulphotransferase-mediated reactions (Birceanu et al., 2021; Wilkie et al., 2019), producing a more hydrophilic product that is easier to excrete via bile or urine (Bussy et al., 2018a, b; Clarke et al., 1991; Kalant & Roschlau, 1998). Larval sea lamprey have a lower capacity to use UDPGTs to form TFM-glucuronide compared to other fishes, possibly because they express fewer isoforms of the enzyme compared to more lampricide-tolerant fishes (Kane et al., 1994; Lawrence et al., 2023). This may explain why many non-target fishes have several-fold higher MLCs to TFM than sea lamprey (Boogaard et al., 2003; Wilkie et al., 2019). The lower UDPGT activity in sea lamprey was attributed to reduced expression of these enzymes and their lower affinity for TFM (Kane et al., 1994). Compared to non-target fishes, the efficiency of UDPGTs is up to 50% less in sea lamprey, as calculated by dividing the maximum velocity (V_{max}) of UDPGTs by their enzyme binding affinity (K_m) ratios (Kane et al., 1994).

Steric interactions at the UDPGT active sites may also explain the higher efficiency of the enzyme for niclosamide, as niclosamide lacks the trifluoromethyl side chain present in the TFM molecule, thereby allowing for more efficient binding of niclosamide to UDPGT (Applegate et al., 1966; Kane et al., 1994). Similarly, when comparing the efficiency ratios (V_{max}/K_m) associated with UDPGTs across fish species, Kane et al. (1994) found that UDPGT activity was significantly more influenced by the binding affinity (lower K_m), providing further support for the substrate binding influence on lampricide toxicity. Indeed, the presence of halogen atoms (i.e., Cl^-) interferes with substrate binding orientation and enzyme-catalyzed oxidation products of cytochrome P450 enzymes and halogenated substrates, resulting in different enzyme efficiencies based on the location of C-H bonds (aliphatic and aromatic vs para-halobenzoic bonds; Coleman et al., 2023). There are several ligand binding effects on the activity of cytochrome P450, including changes in ligand state or strength, the spin state of the heme iron, or interactions with electron transfer partners,

which can all influence the catalytic activity of cytochrome P450 (for a detailed review, please see Denisov et al., 2005). Likewise, mutations, both evolutionary and engineered, resulting in altered activity of cytochromes P450 have contributed to our understanding of substrate-enzyme binding properties (Bernhardt, 2006). We suggest that the presence of the tri-fluoro halide group of TFM, which is missing from niclosamide, may have similar effects on binding affinity to UDPGTs. Future studies should address this hypothesis using similar enzyme kinetics approaches to those used by Kane et al. (1993, 1994) to determine more precisely how TFM and niclosamide are interacting at the molecular level in sea lamprey and non-target fishes.

The observation that TFM and niclosamide interactions are less than additive below the 1 $TU_{Tot\ Exp}$, is more difficult to explain. Such antagonistic interactions amongst chemicals typically occur when actions of two different pesticides interfere with one another (Hernandez et al., 2017). Possible explanations include the induction of enzymes involved in TFM and/or niclosamide metabolism, which is a common mechanism underlying antagonistic drug and toxicant interactions (Hayes et al., 2020; Meech et al., 2019; Rowland et al., 2013). Some support for this hypothesis was provided by recent transcriptomics analysis in sea lamprey exposed to low doses of TFM (24-h LC_{10}), demonstrating an expression of genes coding for the Phase II enzymes sulphotransferase (*sult6b1*) and glutathione-S-transferase (*gstt3*), that may be involved in TFM detoxification (Lawrence et al., 2022). However, as noted above, more detailed enzyme kinetics analysis is required to investigate this possibility further. Another possibility may include altered physiology, as depressed respiration could lead to less TFM uptake at the gills, thus leading to a less toxic effect. However, recent studies indicated that exposure to TFM concentrations ranging from 2-5 mg L^{-1} resulted in greater rates of oxygen consumption (D'Souza et al., 2025), which would tend to argue against metabolic depression. Further, there is a strong relationship between oxygen consumption and TFM uptake by larval lamprey (Tessier et al., 2018).

The present experiments were performed over a 12-h exposure, which is a typical field treatment duration (McDonald & Kolar, 2007; Sullivan et al., 2021). This study and

field lampricide treatments are typically of shorter duration (up to 12 h) than previous studies in which strictly additive or less than additive interactions were reported for exposures over 24 h or 96 h (Bills & Marking, 1976; Marking & Bills, 1985). Hence, the less than additive interaction or strictly additive relationships between the TFM and niclosamide mixtures reported in these earlier studies may have been a reflection of the lower concentration of lampricides to which the fish were exposed, over longer time periods, and not necessarily misaligned with the present observations.

3.4.2 Niclosamide impairs TFM metabolism by the liver

The increased concentration of TFM in the tissues of larval sea lamprey exposed to the TFM/1% Nic mixture treatment (Figure 3.5) may help explain the greater than additive interactions observed between the two lampricides when the exposure concentration was greater than 1.0 TU. Although the liver is the primary site of TFM Phase II detoxification (Wilkie et al., 2019), TFM can be distributed throughout the body (Schultz et al., 1979), and TFM muscle concentrations can provide a more definitive explanation as to the lampricides' interactions and metabolism (Hubert et al., 2005; Lech, 1974; Vue et al., 2002).

In contrast to the liver, TFM accumulation in muscle was substantially higher when the lamprey were exposed to TFM/1% Nic mixture compared to the TFM only treatment. Thus, any impairment of TFM metabolism in the liver due to the presence of niclosamide could be reflected by significant TFM accumulation in the extracellular and intracellular compartments of other organs, including the blood and muscle. Although we were unable to obtain sufficient amounts of blood from the very small lamprey (~ 1.5 g), we were able to test whether there was a significant accumulation of TFM in the muscle. As the largest organ by mass, comprising approximately 75% of the body in ammocoetes of the European brook lamprey (*Lampetra planeri*; Bull & Morris, 1967), muscle could represent a large reservoir for lampricides and their metabolites circulating within the body of sea lamprey.

The accumulation of products of TFM metabolism (e.g., TFM-glucuronide or TFM-sulphate) could have potentially complicated the interpretation; however, it should be noted

that our LC-MS/MS method was able to resolve parent TFM from any TFM conjugates. For this reason, we suggest that relative TFM concentrations within the muscle of larval sea lamprey are directly influenced by the comparative detoxification rate reduction in the liver in the presence of niclosamide. Previously, studies reported that an increase in liver TFM levels was accompanied by higher TFM concentrations in other tissues (Lech, 1974; Lech & Statham, 1975). The approximately 3-fold greater concentrations of TFM observed in the muscle when the larvae were exposed to the TFM/1% Nic mixture compared to TFM only treatment (Figure 3.5B) support our hypothesis that impaired metabolism of TFM in the liver by niclosamide leads to greater toxicity when the lamprey were exposed to the lampricide mixture.

The TFM concentration trends in the liver and muscle tissues of the sea lamprey in the current study appear to differ from the findings reported by Lawrence et al. (2021), who found that liver concentrations of TFM were more than 5-fold greater when sea lamprey were exposed to TFM/1% Nic mixture compared to TFM only, with no change in muscle TFM concentration. However, Lawrence et al. (2021) measured TFM accumulation over a longer timeframe (24 h) at a lower, single dose equivalent to the 24-h LC_{10} of TFM, which may have led to less TFM accumulation when the lamprey were exposed to the TFM/Nic mixture. If the previous study had examined TFM accumulation over a range of TFM/1% niclosamide concentrations, as was done in the present study, it is possible that they would have observed similar results reported here.

3.5 Conclusions and implications

Exposure to a TFM/1% Nic mixture treatment produced an antagonistic (less than additive) interaction when exposures were below 1.0 $TU_{Tot\ Exp}$. However, interactions were greater than additive when the total $TU_{Tot\ Exp}$ exceeded 1.0 TU. The greater accumulation of TFM in the muscle in the presence of niclosamide suggests that niclosamide competitively inhibits TFM metabolism, possibly due to increased competition for Phase II detoxification enzymes, such as UDPGTs or sulphotransferases. To learn more about the underlying mechanisms of these interactions, future studies should characterize how the activities and protein expression

of detoxification enzymes change when sea lamprey are exposed to TFM in the presence and absence of niclosamide. The differences between TFM and niclosamide toxicity not only shed light on the possible mechanisms of phenol toxicity in general but could also provide insight into how binary mixtures of other phenolic compounds interact in many different fishes in aquatic ecosystems (Lech & Statham, 1975; Marking & Bills, 1985; Marking & Dawson, 1975; Statham & Lech, 1975).

The concentrations at which TFM and niclosamide interact in a greater than additive manner in larval sea lamprey (based on the 12-LC_{50}) correspond to the concentrations that would be used in the field to treat sea lamprey infested streams (Bills et al., 2003). The evidence presented in this paper can be directly extrapolated and applied by the Sea Lamprey Control Program (SLCP) to increase treatment effectiveness by further exploring the physiological effects of the binary lampricide mixture on larval sea lamprey and other non-target fishes. This will further enable better treatment tailoring, allow for increased use of low concentrations of niclosamide within TFM/niclosamide mixtures, increasing suppression of sea lamprey populations by reducing sea lamprey survivorship post treatment, all while minimizing non-target effects. Advancing the knowledge of how these lampricides interact in sensitive and tolerant fishes at species specific lampricide concentrations will continue to reduce non-target effects and ensure the continued effective control of sea lamprey populations within the Great Lakes. The information produced in the current and future similar studies on lampricide interactions may also inform risk assessments of similar phenolic chemicals entering the environment.

Chapter 4

The Influence of Water Temperature on the Bioaccumulation and Toxicity of the Lampricide, 3-Trifluoromethyl-4-nitrophenol (TFM) by Rainbow Trout (*Oncorhynchus mykiss*)

4.1 Introduction

Juvenile parasitic sea lamprey (*Petromyzon marinus*) have negatively impacted commercial, recreational and Indigenous fisheries of the Great Lakes since their introduction into Lake Ontario in the mid-1800s following the construction of the Erie Canal (Eshenroder, 2009, 2014; Smith & Tibbles, 1980; Wilkie, 2025). To protect and rehabilitate fisheries, the Great Lakes Fisheries Commission (GLFC) initiated a binational program to reduce sea lamprey populations across the Great Lakes (Siefkes, 2017; Gaden et al., 2021). Although several approaches (e.g., barriers, traps and sterilization) have been employed, treating the sea lamprey nursery streams with the phenolic pesticide 3-trifluoromethyl-4-nitrophenol (TFM) has been one of the most effective tools deployed to control sea lamprey populations (Siefkes, 2017; Sullivan et al., 2021; Wilkie et al., 2019).

In use for more than 60 years, TFM was selected from among > 4,500 chemicals tested because it was determined to be much more toxic to larval sea lamprey than to most non-target fishes (Applegate et al., 1966). The relatively lower capacity of the larval sea lamprey (jawless fish) to metabolize TFM into an easier to excrete compound using Phase II biotransformation likely explains its greater toxicity to sea lamprey relative to most other fishes (Kane et al., 1994; Lech & Statham, 1975; Lawrence et al., 2022). In contrast to sea lamprey, most fishes can rapidly metabolize phenolic compounds such as TFM via Phase II metabolism, conjugating TFM to TFM-glucuronide using the process of glucuronidation (Kane et al., 1994; Lech & Statham, 1975), and/or sulphation to produce TFM-sulphate (Bussy et al., 2018a, b). The activities of uridine diphosphate-glucuronosyltransferase (UDPGT) family of enzymes involved in glucuronidation are much higher in non-target fishes such as bluegill (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*) compared to sea lamprey, which have reduced expression of these enzymes and lower affinity for TFM (Kane et al., 1994; Lawrence et al., 2022). This is reflected by 12-h LC_{50}/MLC ratios (toxicity ratios) that are much greater in non-target fishes than in the sea lamprey (Boogaard et al., 2003; Wilkie et al., 2019). For instance, the toxicity ratio of rainbow trout is approximately midway (toxicity ratio ~ 4.5) between that of sea lamprey and

the most TFM-tolerant species, American eel (*Anguilla rostrata;* toxicity ratio ~ 10; Wilkie et al., 2019).

Typically, TFM is applied to sea lamprey infested rivers and streams draining into the Great Lakes every 2-3 years, targeting larvae before they complete metamorphosis into parasitic juveniles that are the threat to fisheries (McDonald & Kolar, 2008; Smith & Tibbles, 1980; Sullivan et al., 2021). Treatments generally are 9-12 h in duration (Sullivan et al., 2021), with TFM applied at concentrations needed to eliminate 99.9% of the larval sea lamprey (the minimum lethal concentration: MLC, or the 12-h $LC_{99.9}$), that are adjusted for environmental factors such as water pH and alkalinity, as well as the time of the year (Bills et al., 2003; Boogaard et al., 2003; Hlina et al., 2021).

As a weak acid with a pKa of approximately 6.38 (McConville et al., 2016), the speciation of TFM is strongly influenced by water pH, where the total TFM in solution is the sum of un-ionized (TFM-OH) vs ionized ($TFM\text{-}O^-$) TFM:

$$\text{Total TFM} = \text{TFM-OH} + \text{TFM-O}^- \quad (1)$$

The relative proportions of TFM-OH and $TFM\text{-}O^-$ are described by the following equilibrium reaction:

$$\text{TFM-OH} + H_20 \rightleftharpoons \text{TFM-O}^- + H_3O^+ \quad (2)$$

Thus, in lower-pH water, TFM will exist predominantly in its more lipophilic, un-ionized form (TFM-OH), which favours greater TFM uptake at a given concentration of TFM (Armitage et al., 2017; Hunn & Allen, 1974). Higher alkalinity results in greater buffering capacity of the gill microenvironment, which reduces acidification of expired water and therefore the bioavailability of TFM-OH at the gill surface and TFM toxicity (Wilkie et al., 2021).

The toxicity of TFM to larval sea lamprey is also known to vary seasonally, where TFM tolerance is known to be greater during summer months, compared to spring or fall (Applegate et al., 1966; Hlina et al., 2021; Muhametsafina et al., 2019; Scholefield et al., 2008; Schueller et al., 2024). Greater TFM tolerance in the summer has been shown to be

closely related to warmer temperatures, which, on its own, can increase the 12-h LC50 of larval sea lamprey by 1.5-2.0 times between 6°C and 24°C (Mitrovic et al., 2026a; Chapter 2; Muhametsafina et al., 2019). At warmer temperatures, increased tolerance to TFM in sea lamprey was demonstrated to be related to an enhanced TFM elimination capacity, characterized by lower steady state concentrations (C_{SS}) and faster half-lives of elimination (lower $t_{1/2}$) in the liver and muscle of larval lamprey at 24°C compared cooler temperatures (6°C and 12°C) either by increasing TFM metabolism and/or excretion rates (Mitrovic et al., 2026a; Chapter 2). Other phenolic compounds, including the mitochondrial uncoupler 2,4-dinitrophenol and pure phenol, have also been observed to be less toxic to fish at warmer temperatures (Howe et al., 1994; Patra et al., 2015).

The overarching goal of the present study was to determine if the relationship between temperature and TFM tolerance observed in sea lamprey extended to a non-target teleost fish, the rainbow trout (*Oncorhynchus mykiss*), which is widely used in toxicological studies and is distributed throughout the Great Lakes. Accordingly, the effects of water temperature on the toxicity of TFM and TFM tissue accumulation were examined in rainbow trout. To test the hypothesis that TFM toxicity would be lower at warmer temperatures, fish were acclimated to one of four environmentally relevant water temperatures (6°C, 11°C, 16°C, and 21°C) for 3 weeks, followed by an acute toxicity test to determine the 12-h LC_{50} at each temperature. A second set of experiments, in which the trout were exposed to a common sub-lethal TFM concentration at each respective acclimation temperature, was conducted to quantify how water temperature affected the accumulation of TFM in liver and muscle. It was hypothesized that exposure of rainbow trout to TFM at higher water temperature would result in less TFM accumulation by the fish and reduce acute toxicity.

4.2 Materials and methods

4.2.1 Experimental animals and holding

Rainbow trout fingerlings were purchased from Cedar Crest Trout Farms, West Grey, Ontario, CA (n = 428, mass ~ 15 g; and total length ~ 11 cm). The fish were acclimated in

two large 250 L fibreglass tanks filled with dechlorinated, municipal tap water with a replacement rate of ~ 1.0 L min^{-1} and a temperature of 16°C. The fingerlings were held under a 12 h light:12 h dark photoperiod and fed 3% of their weight daily with a standard 1.0 mm commercial floating pellet fish food (Corey Feed Mills, Fredericton, NB, Canada). The fish were allowed to acclimate to laboratory conditions (16°C) for at least one week before beginning experimental temperature acclimations. All experiments were approved by the Wilfrid Laurier University Animal Care Committee (Animal Use Protocol Number R22003), and Canadian Council of Animal Care guidelines were followed.

4.2.2 Chemicals and reagents

The Sea Lamprey Control Centre, Fisheries and Oceans, Canada, Sault Ste. Marie, Ontario, kindly provided the field TFM formulation (35% dissolved in isopropanol; Clariant SFC GMBH WERK, Griesheim, Germany) used for all TFM exposures. TFM working stock and all standards were prepared using dechlorinated laboratory water for all experiments. Analytical-grade TFM (99%, Millipore Sigma, Oakville, ON) was used to verify TFM experimental water concentrations. The concentration of TFM was spectrophotometrically measured prior to and during all TFM exposure experiments.

4.2.3 Experimental protocols

This study was composed of two experiments. Experiment 1 comprised acute toxicity tests to quantify how rainbow trout sensitivity to TFM was affected across 4 different water temperatures (6°C, 11°C, 16°C and 21°C). Experiment 2 exposed rainbow trout to a single nominal exposure concentration, using the 12-h LC_{25} of TFM determined at 16°C (measured in Experiment 1) to determine how TFM accumulation in liver and muscle differed with temperature.

4.2.3.1 Experiment 1 – Influence of temperature on acute toxicity of TFM in rainbow trout

One week after arrival, subsets of the trout were randomly transferred from the 16°C holding tanks to one of 4 individual tanks (~ 120 fish per tank) and allowed to acclimate to either 6, 11, 16 or 21°C for a period of 21-26 d. Other than the fish at 16°C, the water temperatures were either adjusted up or down at a rate of 1°C per day, until the target temperature was reached. Temperatures were controlled using submersible heaters (Finnex Titanium Heater TH-800 plus, JSK Merchandise Inc., USA) or aquarium chillers (Coralife ¼ hp Aquarium Chiller, Franklin, WI, USA) with temperature controllers (Inkbird ITC-308 Temperature Controller, Ink Bird Tech. C.I., China). Fish were not fed 48 h before beginning experiments.

For each temperature, fish were exposed to a range of TFM concentrations (7.5 to 25 mg L^{-1}). The mortalities from the toxicity tests were used to generate dose-response curves and calculate the 12-h LC_{50}. The American Standard and Testing Methods guidelines for static toxicity tests on aquatic organisms (ASTM, 2014) were followed when performing all toxicity tests.

Each toxicity test used the same experimental setup comprising 12 experimental 22 L glass aquaria filled with the same well-aerated, dechlorinated water described above at each temperature. Tanks were semi-immersed in a recirculating water bath to maintain the target temperature for the duration of the experiment. Fish were arbitrarily distributed to each tank (5 fish per aquarium in duplicate) and allowed to acclimate for at least 12 h before the beginning of the experiment. The next morning, TFM working stock (10 g L^{-1}) was added to each tank so that the desired range of nominal concentrations (0, 7.5, 10, 15, 20, 25 mg L^{-1}) was achieved for all temperatures except 16°C, where, due to high mortality numbers the TFM concentration range was 0, 7.5, 10, 12.5, 15 and 20 mg L^{-1}. Water samples (20 mL) collected at 15 min, 6 h and 12 h were used to measure TFM concentrations. General water chemistry (alkalinity, dissolved oxygen) was measured before and after the experiments, while water pH was measured before, 6 h after onset of exposure and after the experiments.

The addition of the lampricide(s) marked the beginning of the 12 h exposure during which the rainbow trout were continuously monitored for the first hour, followed by hourly checks thereafter. Mortalities were assumed when fish lost equilibrium and were unresponsive to a tail pinch, at which time they were quickly removed, and the time, body length and mass were recorded. After the 12 h exposure, any survivors were transferred into an aerated bath containing a lethal dose of buffered tricaine methanesulfonate (MS-222; Syndel, Port Alberni, BC, Canada; 0.5 g L^{-1} MS-222 with 1.0 g L^{-1} $NaHCO_3$).

4.2.3.2 Experiment 2 - Effects of temperature on TFM accumulation in rainbow trout liver and muscle

The effects of temperature on TFM tissue accumulation were quantified using separate groups of rainbow trout (n = 228), exposed to a single concentration of TFM (10.64 mg L^{-1}, the 12-h LC_{25} at 16°C determined in Experiment 1). Each temperature exposure (6, 11, 16 and 21°C) was done using glass aquaria filled with 22 L of the same water described above. There were 4 control tanks with 3 fish per tank (n = 12 for each temperature). At each sample period (1, 3, 6, 9 and 12 h) per temperature, fish exposed to TFM were sampled from triplicate tanks (3 fish per tank; n = 9 for each time period. All other experimental conditions were as described for Experiment 1. At each sample period, the trout were euthanized with an overdose of tricaine methanesulfonate (described above), followed by measurements of length and mass, and the immediate collection of liver and muscle, which were flash frozen in liquid nitrogen for future analysis. Water chemistry and temperature were measured at the start and at the end of the 12 h experiments, while pH was measured prior to TFM exposures and after each sample period (1, 3, 6 and 12 h after experimental onset).

4.2.4 Analytical methods

TFM exposure concentrations were confirmed spectrophotometrically using a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, UK) at a wavelength of 395 nm, based on a micro-modification of GLFC Standard Operating Procedure 431.0 (Hammond Bay

Biological Station, MI, USA), with standards and dilutions prepared using the experimental water.

The quantification of tissue TFM concentrations was initially described by Mitrovic et al. (2026a - Chapter 2 and Appendix A). Briefly, Captiva EMR-Lipid 96-well plates (Agilent Technologies, Santa Clara, CA) were used to extract approximately 100 mg of finely ground tissue. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to analyze the eluted samples. The TFM concentration in each sample was quantified using an Agilent 1260 HPLC coupled to a 6460 triple quadrupole mass spectrometer with an Agilent Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm). All data were processed using Agilent Mass Hunter Quantitative Analysis software version 10.1. The TFM calibration curve ranged from 0.5 µg L^{-1} to 5000 µg L^{-1}, and the instrument detection limit was 0.04 µg L^{-1}, while the method detection limit was 0.25 µg L^{-1}. The sample recoveries ranged from 85.9% to 103.8%. The TFM tissue concentration was expressed as nanomole per gram of wet weight (nmol g^{-1} ww).

4.2.5 Calculations and statistical analysis

Nonlinear regression (curve fitting) was used to determine the dose-response curves, and the corresponding LC values, and 95% confidence intervals (CI) at each temperature (GraphPad Prism version 10.6; GraphPad Software Inc, La Jolla, CA, USA). The absence of overlap between the 95% CI of values was considered significantly different from one another (Finney, 1971).

Individual fish weights, lengths and other morphometric data, as well as TFM tissue concentrations, were presented as the mean ± S.E.M. Fulton's condition factor (CF) was calculated as CF = mass (g)/length $(cm)^3$ x 100 (Schreck & Moyle, 1990).

Statistical significance was evaluated using a 1-way analysis of variance (ANOVA) followed by Tukey's post hoc tests to compare values across treatments. Non-parametric ANOVA (Kruskal-Wallis test) followed by Dunn's Test of Multiple Comparisons was used

when the assumptions of normality and homogeneity of variance were not met. GraphPad Prism version 10.6 was used for statistical analysis, with $\alpha = 0.05$ as the significance level.

To gain further comprehension into how water temperature influenced the patterns of TFM accumulation and handling by the liver, pharmacokinetic approaches were used to calculate the steady state concentration (C_{SS}) of TFM during the 12-h exposure period. This was done by non-linear regression using one-compartment least squares analysis (GraphPad Prism 10.6, Plateau followed by one phase association) to calculate the TFM accumulation constant (k), C_{SS} and half-life ($t_{1/2}$) of elimination from the measured liver TFM concentrations, under the assumption that at steady state the rate of elimination is equal to the rate of accumulation. It is also important to note that k does not represent the theoretical uptake rate constant defined under equilibrium conditions (Newman, 2020) but rather denotes the apparent uptake rate constant derived from the observed fish tissue concentrations over time and is therefore influenced by metabolic processes and it was only used to calculate half-life using the equation $t_{1/2} = \ln(2)/k$ (Mitrovic et al., 2026a; Chapter 2).

4.3 Results

4.3.1 Experiment 1 – Influence of temperature on acute toxicity of TFM to rainbow trout

Measured water chemistry in the exposure aquaria remained stable throughout the experiments and closely matched the nominal values (Table 4.1). Water temperatures remained within 0.2 - 0.6°C of the nominal. pH varied from 8.12 to 8.25, dissolved oxygen (DO) was ~ 92% saturation, and alkalinity was 170 - 187 mg L^{-1} as $CaCO_3$ during exposures (Table 4.1). Alkalinity was robust and stable over the course of each experimental series and was therefore measured only at the beginning of the experiment.

Table 4.1 Measured water quality during exposure of rainbow trout to TFM at different temperatures. Data presented as the mean ± S.E.M, with n denoting the number of total measurements per treatment. Alkalinity was only measured at the beginning of each experimental series.

Temperature (°C)		pH	Alkalinity (mg L^{-1} as $CaCO_3$)	Dissolved O_2 (% saturation)	n
Nominal	Measured				
6	6.1 ± 0.09	8.25 ± 0.02	187	90.7 ± 0.7	18
11	11.4 ± 0.2	8.12 ± 0.01	170	92.6 ± 0.9	18
16	16.1 ± 0.1	8.20 ± 0.04	170	90.7 ± 0.7	18
21	20.8 ± 0.1	8.10 ± 0.05	187	94.8 ± 0.7	18

4.3.1.1 Effects of acclimation temperature on body condition

Different acclimation temperatures between the treatments had an effect on trout body length, with the lowest length of 10.6 ± 0.1 cm reported at 21°C, which was significantly lower than the body length reported at the two intermediate temperatures, 11.7 ± 0.2 cm ($p < 0.01$) at 11°C and 11.3 ± 0.2 ($p < 0.01$) at 16°C (Table 4.2). No significant differences ($p > 0.05$) in rainbow trout mass were observed, which ranged from 14.4 ± 0.5 g to 16.8 ± 0.7 g among treatments (Table 4.2). The corresponding body CF varied with water temperature in rainbow trout, with a slightly but significantly lower CF of 1.09 at 6°C compared to 1.03 at 11°C ($p = 0.01$), and 1.08 at 16°C, which was not significantly different from either of the lower temperatures (Table 4.2). At 21°C, however, CF was significantly greater, averaging 1.19 ± 0.02 at 21°C ($p < 0.001$), which was likely related to the significantly shorter body length observed at 21°C (Table 4.2).

Table 4.2 Effects of acclimation temperature on body length, mass and condition factor (CF). Rainbow trout were acclimated to four different nominal temperatures for a minimum of 3 weeks. n represents the number of fish per treatment. Data presented as mean ± S.E.M. Data sharing a common letter are not significantly different from one another ($p < 0.05$).

Acclimation temperature (°C)	CF	Mass (g)	Length (cm)	n
6	1.09 ± 0.01^{a}	15.52 ± 0.70^{a}	11.1 ± 0.2^{ab}	50
11	1.03 ± 0.01^{b}	16.80 ± 0.72^{a}	11.7 ± 0.2^{a}	50
16	1.08 ± 0.01^{ab}	15.88 ± 0.61^{a}	11.3 ± 0.2^{a}	60
21	1.19 ± 0.02^{c}	14.44 ± 0.52^{a}	10.6 ± 0.1^{b}	60

4.3.1.2 Dose-response toxicity curves

TFM concentrations in each tank during the acute toxicity tests closely matched the nominal values and remained stable throughout the exposure period (Appendix C, Table C.1). As water temperature increased from 6°C to 11°C and 16°C, the toxicity of TFM increased, as characterized by the leftward shift of the dose-response curves (Figure 4.1). The corresponding 12-h LC_{50} at each temperature, calculated from dose-response curves, was 16.91 mg L^{-1} (CI = 14.29 - 20.07) at 6°C, falling 23% to 12.95 mg L^{-1} (CI = 10.56 - 15.88) at 11°C and 27% to 12.33 mg L^{-1} (CI = 10.93 - 13.91) at 16°C. Notably, TFM tolerance at 21°C matched that observed at 6°C, with both dose-response curves overlapping, with a corresponding 12-h LC_{50} of 17.29 mg L^{-1} (CI = 14.91 - 20.05) at 21°C (Figure 4.2).

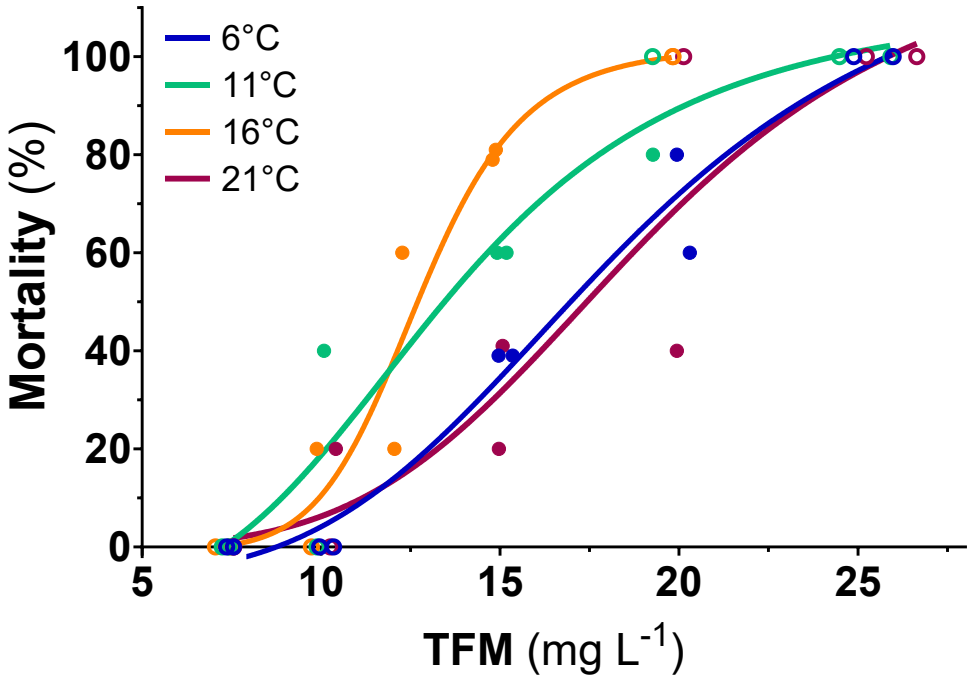


Figure 4.1 Dose-response curves depicting changes in toxicity of TFM over temperatures. Five fish per tank in duplicate for each exposure concentration; solid circles represent partial mortalities; n = 200 (10 fish per concentration x 5 concentrations x 4 temperatures). Dose-response curves were generated using nonlinear regression (curve fit) survival plot in GraphPad Prism 10.6.

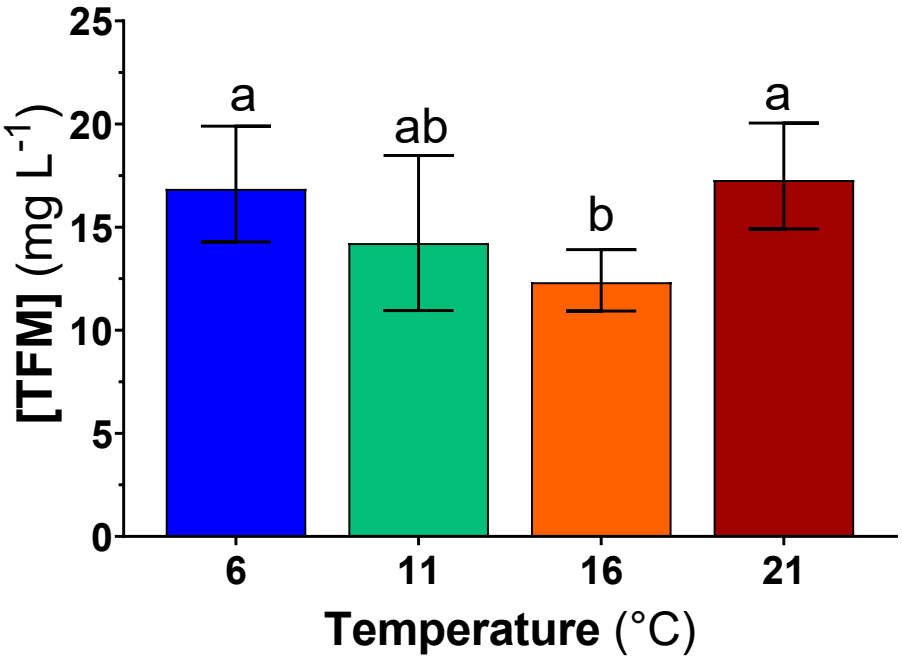


Figure 4.2 Effects of temperature on the acute toxicity of TFM to rainbow trout. Data depicted as the corresponding mean 12-h LC_{50} (± 95% confidence intervals) of TFM to rainbow trout (based on the results of Figure 4.1). Mean values sharing an identical lowercase letter are not significantly different from one another, where 95% CIs do not overlap, n = 200 (10 fish per concentration x 5 concentrations x 4 temperatures).

4.3.2 Experiment 2 – Effects of temperature on TFM tissue accumulation

During the second series of experiments, the measured water chemistry and TFM exposure concentrations were observed to be stable with minimal variations (Table 4.3). Water temperature varied slightly from 0.04°C to 0.3°C from the target temperature, pH ranged from 8.01 ± 0.01 to 8.17 ± 0.02 and DO averaged ~ 91.5% saturation, and alkalinity was 187 mg L^{-1} as $CaCO_3$ across all temperatures except at 6°C, where it measured slightly lower at 170 mg L^{-1} as $CaCO_3$. Measured TFM concentrations also remained relatively close to the nominal concentrations across all temperature treatments (Table 4.3).

Table 4.3 Measured water quality and TFM concentrations during 12-h exposure of rainbow trout to TFM across temperatures. Nominal exposure concentration of TFM was at the 12-h LC_{25} at 16°C of 10.64 mg L^{-1}. Data presented as the mean ± S.E.M. n denotes the number of total measurements per treatment.

Temperature (°C)		pH	Alkalinity (mg L^{-1} as $CaCO_3$)	Dissolved O_2 (% saturation)	[TFM] (mg L^{-1})	n
Nominal	Measured					
6	6.0 ± 0.1	8.04 ± 0.01	170	90.1 ± 0.9	10.71 ± 0.04	24
11	11.2 ± 0.3	8.10 ± 0.02	187	90.3 ± 1.2	10.68 ± 0.03	24
16	16.3 ± 0.04	8.17 ± 0.02	187	90.4 ± 0.8	10.69 ± 0.06	24
21	20.9 ± 0.1	8.01 ± 0.01	187	95.0 ± 0.5	10.66 ± 0.06	24

No fish mortalities were observed over the 12-h exposures to the nominal TFM concentration of 10.64 mg L^{-1} (12-h LC_{25} of 16°C treatment). After 1 h of TFM exposure, TFM accumulation was detected in all rainbow trout livers. There was a significantly greater TFM accumulation in livers of the 11°C and 21°C groups, 119.0 ± 12.4 and 128.8 ± 6.3 nmol g^{-1} ww ($p = 0.002$ and $p < 0.001$), which were approximately 2.5-fold greater than values measured in the 6°C fish (49.4 ± 5.6 nmol g^{-1} ww), where the TFM accumulation concentration was the lowest (Figure 4.3A). Notably, after 1 h of TFM exposure, the liver TFM concentration in the 16°C group was 93.8 ± 4.6 nmol g^{-1} ww and did not differ significantly from the concentrations at other temperatures. A similar trend was observed at the other measured time points (3, 6, 9, and 12 h; Figure 4.3A). The lowest TFM concentrations in the liver tissue were observed in the 6°C group (49.4 - 95.0 nmol g^{-1} ww), and the highest concentrations were observed in the 21°C group (128.8 - 288.1 nmol g^{-1} ww), encompassing a 1.8 to 3.2-fold increase in liver TFM concentrations. The TFM concentration in the livers of the fish at the two moderate temperature groups, 11°C and 16°C, were similar to each other across all the time points (Figure 4.3A). Compared to the 1 h sample period, there were no significant increases in TFM concentration in the 11°C group at any later time point, despite an apparent upward trend between 6 h and 9 h. For the 16°C group, significant increases over time were not seen until 9 h of exposure, when TFM concentration in the liver reached 211.8 ± 17.9 nmol g^{-1} ww (Figure 4.3A).

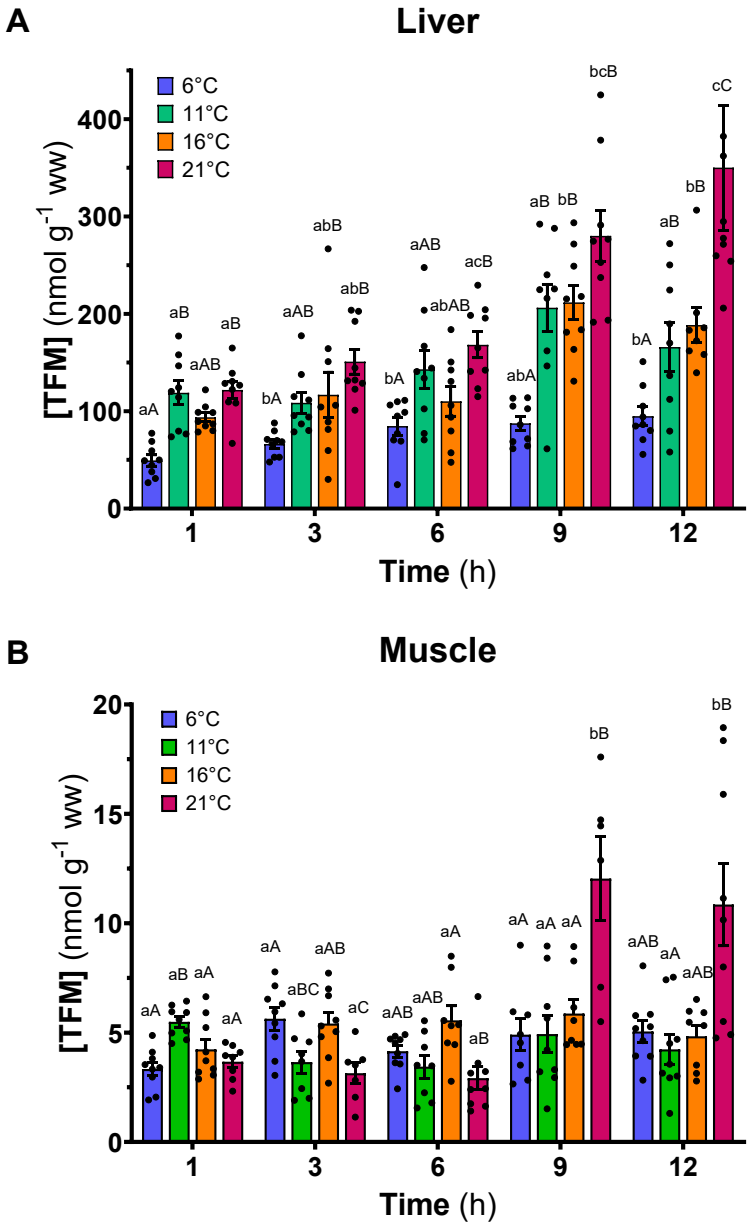
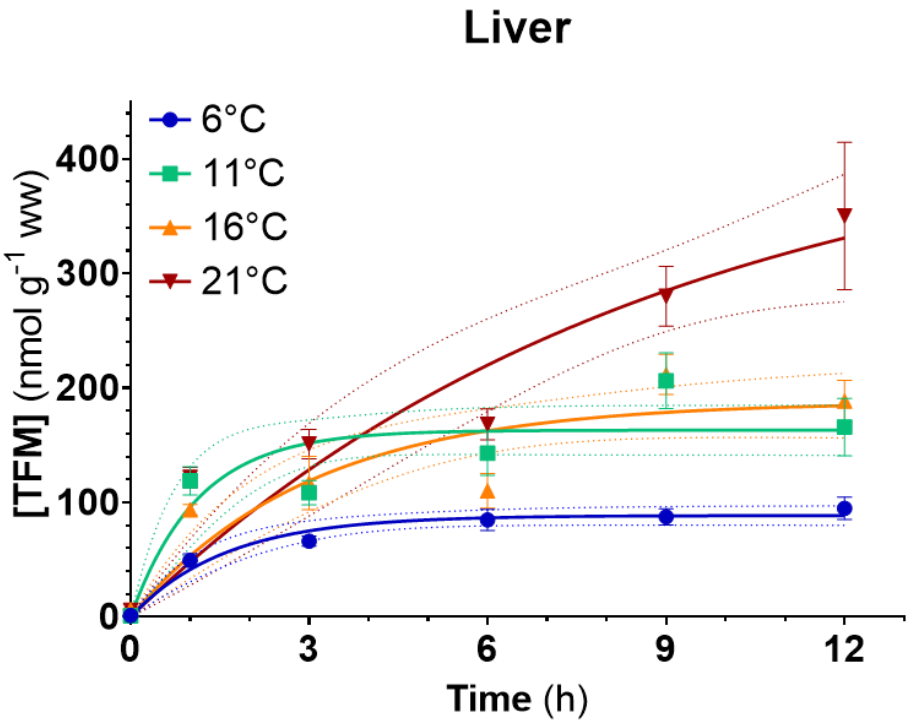


Figure 4.3 The TFM tissue concentration in the (A) liver and (B) muscle of rainbow trout. All fish were exposed to a nominal TFM concentration of 10.64 mg L^{-1} (12-h LC_{25} of 16°C) at temperatures of 6°C (blue), 11°C (green), 16°C (orange) and 21°C (red). There were no detectable TFM concentrations in the tissues of control fish (no TFM exposure). Data presented as mean ± S.E.M. (n = 7-9; $p < 0.05$). Significantly different mean values within a single temperature are denoted by different lowercase letters. In contrast, significantly different mean values between the temperatures at a single time period are denoted by different uppercase letters.

Concentrations of TFM in white muscle of trout were approximately 10 to 20 times less than those measured in the liver (Figure 4.3). Over the first hour of exposure, TFM accumulation was highest in the 11°C group, averaging 5.5 ± 0.2 nmol g^{-1} ww, while the other 3 temperature groups (6, 16 and 21°C) averaged ~ 3.5 nmol g^{-1} ww (Figure 4.3B). Over the 12 h exposure, TFM concentrations in the muscle of fish exposed to TFM at 6, 11 and 16°C remained relatively stable, fluctuating between 3.3 and 5.8 nmol g^{-1} ww, and were not significantly different from values measured after 1 h of exposure (Figure 4.3B). On the other hand, muscle TFM concentrations at 21°C were relatively low, fluctuating between 2.5 ± 0.3 and 3.7 ± 0.3 nmol g^{-1} ww, over the first 6 h of exposure, before spiking thereafter, when TFM significantly increased more than 4-fold to 11.4 ± 1.6 nmol g^{-1} ww (Figure 4.3B). This increase persisted through 12 h, at which time muscle TFM averaged 9.9 ± 1.9 nmol g^{-1} ww (Figure 4.3B). This TFM spike in the muscle coincided with abrupt TFM increases in the liver (comparison between Figure 4.3A and Figure 4.3B).

Kinetic curves illustrating the relationship between water temperature and TFM accumulation/elimination in the liver (Figure 4.4), were subsequently used to calculate the steady state concentration (C_{SS}), rate constant of TFM elimination (k) and elimination half-life ($t_{1/2}$) of TFM, defined as the time required to clear 50% of the TFM from the liver (Mitrovic et al., 2026a; Chapter 2). The C_{SS} for TFM in liver was lowest at 6°C, averaging 88.6 (95 % CI = 90.0 - 98.5) nmol g^{-1} ww, but was approximately 2-fold greater at 11°C and 16°C, with respective mean C_{SS} values of 163.1 (139.1 - 198.1) nmol g^{-1} ww and 188.0 (152.9 - 260.6) nmol g^{-1} ww. The calculated C_{SS} was not reached over the course of the 12 h TFM exposure at 21ºC, with a projected value of 444.1 nmol g^{-1} ww (Figure 4.4). The $t_{1/2}$ of TFM in the liver was estimated as 1.10 h at 6°C, reduced to 0.78 h at 11°C, before increasing to 2.08 h at 16°C and to 6.08 h at 21°C (see table in Figure 4.4).



Temp (°C)	Css (nmol g^{-1} ww)	$t_{1/2}$ Elimination (h)	k (h^{-1})	R^2
6°C	88.6 (80.0-98.5)	1.10 (0.64-1.79)	0.63 (0.39-1.09)	0.706
11°C	163.1 (139.1-198.1)	0.78 (NA-2.23)	0.89 (0.31-NA)	0.502
16°C	188.0 (152.9-260.6)	2.08 (0.78-4.90)	0.33 (0.14-0.89)	0.520
21°C	444.1 (282.7-NA)	6.08 (2.23-NA)	0.11 (NA-0.31)	0.546

Figure 4.4 Relationship between temperature and TFM. TFM accumulation and elimination in the liver of larval sea lamprey exposed to a common nominal concentration of 10.64 mg L^{-1} based on data presented in Figure 4.3A. Curves were fitted by non-linear regression using least squares analysis, from which the apparent steady state concentration (C_{SS}), elimination half-life ($t_{1/2}$) were calculated. The $t_{1/2}$ elimination was calculated from the rate constant of TFM accumulation (k) to reach C_{SS}, based on the assumption that at C_{SS} the overall rate of TFM elimination was equal to its overall rate of accumulation. Calculations and curve fitting were completed using GraphPad Prism 10.6. Data were re-plotted from Figure 4.3A. n = 7-9 measurements at each sample period. Light dashed lines show 95% confidence intervals.

4.4 Discussion

The sensitivity of rainbow trout to TFM was greatest at the moderate temperatures of 11°C and 16°C, the only temperatures where the 12-h LC_{50} of TFM was significantly lower compared to the other temperatures tested (6°C and 21°C). The highest tolerance to TFM were at the coldest (6°C) and warmest (21°C) exposure temperatures, which resulted in similar 12-h LC_{50} (Figure 4.2). When compared to the temperature effects in sea lamprey, which underwent 150% increase in their TFM 12-h LC_{50} as water temperature increased from 7°C to 24°C (Mitrovic et al., 2026a; Chapter 2) under similar water chemistry conditions, the effects of temperature on TFM toxicity were much less in rainbow trout, with the highest and lowest 12-h LC50 differing by only 27 - 29%. Thus, the present findings suggest that temperature has little influence on the temperature sensitivity of rainbow trout compared to sea lamprey.

The lower TFM tissue accumulation in the coldest (6°C) exposure, compared to higher temperatures, consistent with the greater TFM tolerance of the trout. The TFM concentrations in the liver generally increased over both time and temperature. However, TFM accumulations in muscle were much lower compared to the liver, and significantly greater muscle concentrations were observed only in the 21°C treatment at 9 and 12 h of exposure. Despite the higher liver and muscle tissue concentration at 21°C, TFM acute toxicity (12-h LC_{50}) was lower compared to 11°C or 16°C. This is also in contrast to sea lamprey, which have previously been shown to exhibit a stepwise increase in 12-h LC_{50} values with increasing temperature, accompanied by lower liver and muscle TFM accumulations at the highest temperature (24°C) treatment (Mitrovic et al., 2026a; Chapter 2). Thus, there appear to be marked interspecific differences in how rainbow trout handle and respond to TFM at different temperatures compared to sea lamprey.

4.4.1 Relationship between TFM toxicity and water temperature in rainbow trout

In the present study, a biphasic (U-shaped) pattern of TFM toxicity was observed, with the highest toxicities observed at mid-range temperatures (11°C and 16°C), as reflected by the lowest 12-h LC_{50} values (Figure 4.2). These observations do not align with the stepwise increase in TFM tolerance observed with increasing temperatures in larval sea lamprey (Mitrovic et al., 2026a; Chapter 2) or with phenol exposures in many different aquatic animals including fishes and observed with other well-known mitochondrial oxidative phosphorylation uncouplers such as 4-nitrophenol and 2,4-dinitrophenol (Petra et al., 2015; Wang et al., 2019). However, compared to sea lamprey, where the effects of temperature on TFM toxicity can be much more pronounced, with 150-250% increase in the mean 12-h LC_{50} (Mitrovic et al.,2026a; Chapter 2; Muhametsafina et al., 2019), the 12-h LC_{50} in rainbow trout varied by less than 30% over a similar temperature range (15°C compared to 18°C). The comparatively modest temperature dependence of TFM toxicity in rainbow trout implies that the physiological or biochemical processes underlying TFM uptake, metabolism, or tolerance in this species may be less thermally sensitive than those governing lamprey TFM tolerance, and that water temperature is therefore a less critical determinant of TFM vulnerability in rainbow trout.

The increase in TFM toxicity in rainbow trout, indicated by the significant decrease in 12-h LC_{50} (Figure 4.1 and Figure 4.2) as water temperature increased from 6°C to 11°C and 16°C, was reflected by a liver C_{SS} that was approximately 2-fold greater than in the coldwater fish (Figure 4.4). Colder water is associated with reduced gill ventilation and irrigation rates, driven by lower metabolic rates in poikilothermic animals due to reduced oxygen demand (Currie & Evans, 2021; Yang et al., 2000). TFM is believed to be passively absorbed across the gill epithelium down its concentration gradient into the blood, which distributes TFM to the tissues (Wilkie et al., 2021). Consequently, decreases in diffusion rates and gill ventilation could reduce the rate of TFM uptake, but this is not the entire story because the apparent rate constants (k) were similar at 6°C, 11°C and 16°C. Lower temperature is also

accompanied by an increase in lipid density of the gill membrane bilayer, imposing molecular-size limits across the membranes, which could further slow diffusion rates (Sijm et al., 1993). Lower diffusion rates can also result from increased viscosity of the aqueous diffusion layer at lower temperatures, leading to slower uptake rates (Gobas et al., 1986; Sijm et al., 1993). It was also notable that the $t_{1/2}$ of elimination was 2-fold and 6-fold higher at 16°C and 21°C, suggesting that, unlike in larval sea lamprey, TFM detoxification was not enhanced at warmer temperatures in the trout. Thus, the TFM tissue concentrations and C_{SS} at 6°C may therefore reflect differences in relative uptake and total detoxification rates compared to 11°C or 16°C during the short (12 h) exposure duration and require further study to tease apart temperature-induced effects on TFM bioaccumulation by rainbow trout.

Relative to the 6°C treatment, the temperature dependence of diffusion, membrane lipid density and aqueous viscosity, coupled with the increased energy demands that accompany rising water temperature, would be expected to produce higher TFM uptake rates at moderate temperatures, explaining the higher TFM toxicity at 11°C and 16°C. Previous studies using radio-labelled TFM (^{14}C-TFM) have reported that TFM uptake rates (per unit body mass) are inversely proportionate to water pH and body mass in larval sea lamprey (Hlina et al., 2017; Tessier et al., 2018), but similar studies have not yet been conducted to test the hypothesis that TFM uptake increases with temperature in lamprey or non-target fishes. Higher water temperatures affect a wide range of kinetic processes, including uptake, distribution, metabolism, and elimination rates, thereby directly influencing the toxicity of a chemical (Grenc et al., 2025). Increased uptake and elimination rate constants across many different aquatic species and organic chemicals have been reported at higher water temperatures (Grenc et al., 2025). An increase in water temperature from 6°C to 11 °C or 16°C would therefore be expected to substantially increase uptake rates. Although increases in uptake rates are generally accompanied by increases in elimination rates due to greater efficiency of metabolic enzymes, the corresponding rates of these two opposite processes may not be equivalent (Grenc et al., 2025; Raths et al., 2023), thus resulting in increased

TFM toxicity with increased liver and muscle TFM concentrations, as compared to 6°C fish (during short 12-h exposures).

At the highest acclimation temperature (21°C), TFM tolerance was greatest, with LC_{50} values comparable to those at the coldest treatment group, yet TFM concentrations were highest in the muscle and liver at this temperature. It therefore seems unlikely that TFM clearance was greater at 21°C, which is borne out by the 5.5-fold higher $t_{1/2}$ elimination at this temperature compared to 6°C. In other words, unlike in larval sea lamprey (Mitrovic et al., 2026a; Chapter 2), the decrease in TFM toxicity at the warmest temperature was not likely due to increased rates of biotransformation and/or excretion of TFM (Grenc et al., 2025). In fact, the highest-temperature treatment provided significantly higher liver and muscle TFM concentrations, suggesting that other processes or mechanisms may be involved in conferring TFM tolerance. One possibility is that the trout's capacity to provide ATP via anaerobic pathways such as glycolysis, or the creatine phosphokinase-mediated buffering of ATP using phosphocreatine, could have been enhanced at warmer temperatures. Indeed, previous studies have demonstrated the importance of these pathways in sustaining ATP supply due to TFM or niclosamide impairment of oxidative phosphorylation in rainbow trout (Birceanu et al., 2014; Clifford et al., 2012; Ionescu et al., 2025). However, further studies are needed to test this hypothesis.

The relationship between temperature and the physiological performance (metabolism) of an organism, also known as aerobic scope, can be described by thermal performance curves (TPC; Currie & Evans, 2021; Schulte, 2015; Whitney et al., 2016). TPCs are frequently left-skewed bell curves reflecting gradual increases in aerobic metabolic scope, the difference between maximum and standard metabolic rate, as temperature rises (Currie & Evans, 2021; Whitney et al., 2016). The peak metabolic performance of an organism occurs at the temperature where aerobic scope is maximal, the thermal optimum (T_{opt}), which is often followed by an abrupt decline in performance at higher temperatures, the pejus temperature (T_{pejus}; Currie & Evans, 2021; Schulte, 2015; Whitney et al., 2016). Several chemicals, including phenols, exhibit the lowest toxicity at the T_{opt} in different fishes,

with toxicity increasing as temperature deviates from this point in either direction (Wang et al., 2019). Although it can vary widely, rainbow trout are generally considered a cold-water species with a reported final preferred temperature of 15.5°C, a critical thermal maximum (CT_{max}) of 22.1°C and an upper incipient lethal temperature (UILT) of 25°C (Hasnain et al., 2010). The T_{opt} of rainbow trout can vary among populations (13 - 19°C) and can be altered by thermal acclimation (Chen et al., 2015; Verhille et al., 2016). Chen et al (2015) reported that above the maximum aerobic scope of rainbow trout (above 20°C), physiological performance was impaired when the fish were near their T_{pejus}. Other studies have shown that water temperature can have detrimental effects on the immunity and growth of rainbow trout at 21°C compared with 13°C and 17°C (Jiang et al., 2021). Rainbow trout acclimated to 21°C in the current study exhibited lower TFM toxicity than at lower acclimation temperatures, even though tissue concentrations were highest at 21°C. Future studies focusing on oxygen consumption and metabolic rates during TFM exposures, similar to those performed in sea lamprey (D'Souza et al., 2025), at different temperatures are needed to test the hypothesis that in rainbow trout TFM toxicity is lowest at the $T_{opt,}$ increasing as the temperature moves toward the T_{pejus}.

Higher water temperatures can have multilevel influences on metabolism, from molecular and cellular levels to tissue-specific to whole-organism performance (Currie & Evans, 2021). The cellular and molecular effects, such as DNA, RNA, and protein reaction rates, are often reflected in higher enzyme affinities and reaction rates (Schulte, 2015). These increases in enzyme activity and enzyme-substrate interactions, especially at temperatures near the T_{opt}, could therefore be a possible mechanism for the decreased TFM toxicity observed at higher temperatures. Similar increases in 12-h LC_{50} values were observed in larval sea lamprey as temperature increased from 6 to 24°C, likely due to increased metabolism resulting from higher biotransformation capacity (Mitrovic et al., 2026a; Chapter 2).

As the water temperature increased from 6°C to 11°C and 16°C, there was minimal and non-significant variability in the fish mass (g), while a significant increase in fish length

(cm) at 11°C resulted in a significant drop of condition factor (CF) at 11°C relative to 6°C but not 16°C (Table 4.2). However, CF at 21°C was significantly higher compared to all other temperatures, mostly due to significantly shorter bodies (Table 4.2). Higher CF is often an indicator of increased energy stores and overall fitness of the animal (e.g., Busacker et al., 1990; Schloesser & Fabrizio, 2017), which could affect TFM toxicity, as fish with higher CF can exhibit enhanced overall stress tolerance (Scholefield et al., 2008; Schueller et al., 2024). However, the narrow increase in the CF was unlikely to have had a considerable effect on TFM toxicity in these studies. Additionally, all fish were fed a 3% maintenance diet based on the average fish weight at the onset of temperature acclimations (i.e. 16°C). By feeding all the fish similar amounts (by weight) of food, the effects of food access across temperatures were minimized, thus avoiding confounding effects of food availability across temperatures on energy stores and, in turn, minimizing effects on TFM toxicity in the current study.

4.5 Conclusions and implications

A biphasic (U-shaped) relationship between water temperature and TFM toxicity was observed in fingerling rainbow trout, with greatest tolerance in cold (6°C) and warmer (21°C) water. Exposure to moderate temperatures of 11°C and 16°C increased TFM toxicity, compared to 6°C, before the 12-h LC_{50} increased markedly at 21°C, indicating lower toxicity. Paradoxically, both liver and muscle TFM concentrations spiked between 9 h and 12 h, suggesting that their hepatic biotransformation may have been compromised later in the exposure. This increase in tissue bioaccumulation could be related to increased enzyme denaturation at warmer temperatures, which would have compromised glucuronidation or other detoxification pathways (Bussy et al., 2018a, b; Kane et al., 1994; Lawrence et al., 2021; Lech & Statham, 1975). It is possible that slightly higher temperatures or exposure duration may have allowed the depletion of energy reserves and increased TFM toxicity. The presented results suggest that TFM toxicity is an intricate multilayered and species dependent process.

Clearly, differences in how different fish metabolize TFM appear to be playing a role in how temperature influences tissue accumulation and toxicity. Additional studies on the effects of temperature on rainbow trout and other non-target species, including metabolic performance, gene expression and enzyme kinetics, bioaccumulation and toxicity studies at different temperatures, are needed to better understand the differences in the sensitivity of fishes to TFM at different temperatures.

The observed decline in TFM toxicity to sea lamprey with increasing temperature has direct operational implications for the Sea Lamprey Control Program, as higher TFM concentrations will likely be required to achieve target mortality in warmer streams. This raises a critical question of whether the increased TFM dose will come at the cost of increased risk of toxicity to non-target species. At first glance, the relatively small changes in rainbow trout tolerance to TFM at warmer temperature compared to sea lamprey, suggests that they may be made less vulnerable to toxicity if higher concentrations of TFM are needed to control sea lamprey at warmer temperatures. However, it is important to note that there is no overlap between the concentrations of TFM needed to control sea lamprey an those that cause toxicity to trout, even at the warmest water temperatures where the trout are likely to be found during treatments (seldom > 20°C; Boogaard et al, 2003; Wilkie et al., 2019). In fact, calculations of the toxicity ratios (trout 12-h LC_{50}/lamprey MLC; refer to introduction for details) of rainbow trout, using MLC values measured for sea lamprey (Chapter 2) indicated little difference between 11°C and 21°C. The toxicity ratio at 11-12°C was 2.10 (12.95 mg L^{-1}/6.16 mg L^{-1}), compared to 2.29 at 21°C (17.29 mg L^{-1}/7.56 mg L^{-1}). Based on these analyses the higher TFM concentrations anticipated to be necessary for effective lamprey control under warming conditions would not be expected to compromise the safety margin for non-target rainbow trout. Nonetheless, warming temperatures may pose a greater risk to other non-target fish species, making it important to determine how temperature influences their toxicity ratios to inform sea lamprey control managers of the need to refine TFM application protocols in warmer waters.

Chapter 5

Exposure to Binary Mixtures of the Lampricides TFM and Niclosamide Alter Bioaccumulation and Toxicity in Rainbow Trout (*Oncorhynchus mykiss*)

5.1 Introduction

For over sixty years, populations of invasive sea lamprey (*Petromyzon marinus*) in the Great Lakes have been successfully controlled using lampricides (Sullivan et al., 2021; Wilkie et al., 2019), which has contributed to the rehabilitation of culturally and economically important fisheries such as lake trout (*Salvelinus namaycush*), lake whitefish (*Coregonus clupeaformis*) and walleye (*Sander vitreus*) (Gaden et al., 2021; Siefkes, 2017). The sea lamprey control program (SLCP) mainly relies on the lampricide 3-trifluoromethyl-4-nitrophenol (TFM, Figure 5.1A), which targets larval sea lamprey (ammocoetes) living in streams and rivers that drain into the Great Lakes (Smith & Tibbles, 1980; Sullivan et al., 2021; Wilkie et al., 2019).

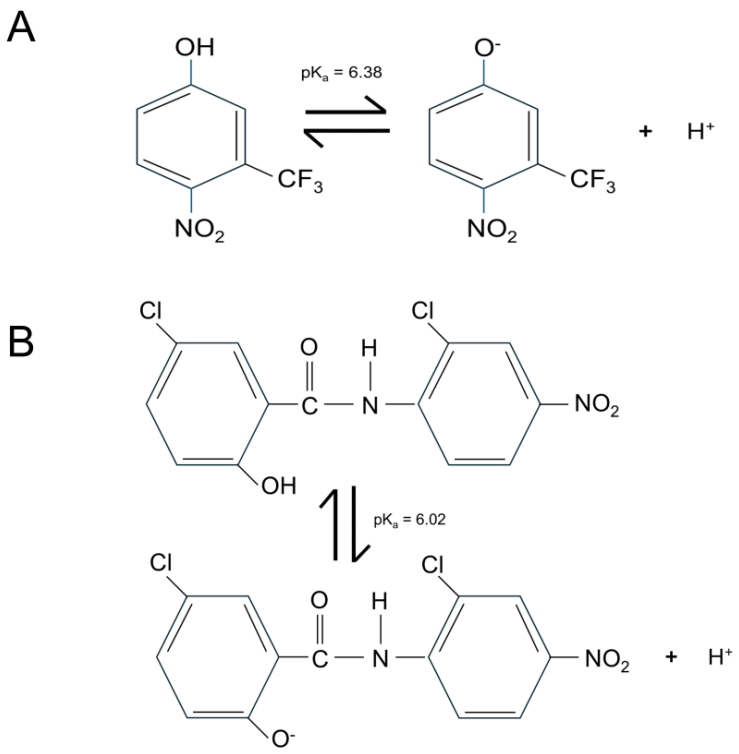


Figure 5.1 Chemical structure of (A) 3-fluoromethyl-4-nitrophenol (TFM) and (B) 2,5-dichloro-4'-nitrosalicylanilide (niclosamide, Nic) with their pKa values.

Lampricide treatments typically take place every 2-3 years, with treatments typically lasting approximately 12 h (McDonald & Kolar, 2007; Sullivan et al., 2021). TFM selectively targets larval sea lamprey, as they are more sensitive to the chemical compared to most other non-target fishes, which have a greater capacity to detoxify and excrete TFM (Bussy et al., 2018a, b; Howell et al., 1980; Kane et al., 1994; Lawrence et al., 2022; Lech & Statham, 1975). Environmental factors such as water pH and alkalinity can also influence the amount of TFM applied at a given site (Bills et al., 2003; Sullivan et al., 2021). The variation in water pH can alter TFM bioavailability by altering TFM speciation (Hunn & Allen, 1974; Wilkie et al., 2019; Figure 5.1A). At lower pH, a greater proportion of TFM exists in its un-ionized, more lipophilic form (TFM-OH), which readily diffuses across the gills, increasing uptake and toxicity. Similarly, elevated water alkalinity decreases TFM bioaccumulation and toxicity by buffering pH changes at the gill surface, which likely limits the availability of un-ionized TFM and thus decreases uptake (Wilkie et al., 2021). Temperature has also recently been shown to influence TFM effectiveness in sea lamprey, with tolerance increasing in warmer waters (Mitrovic et al., 2026a; Chapter 2; Muhametsafina et al., 2019).

Phase II metabolism is thought to be the primary biotransformation pathway for phenolic compounds such as TFM and niclosamide (Dawson, 2003; Hubert et al., 2005; Kane et al., 1994; Lech & Statham, 1975). Uridine diphosphate-glucuronosyltransferase (UDPGT) and phenol sulphotransferase enzymes can convert TFM to TFM-glucuronide or TFM-sulphate, respectively (Bussy et al., 2018a, b). Sea lamprey have a lower capacity to detoxify TFM to the easier-to-excrete TFM-metabolites compared to most other non-target species, such as rainbow trout (*Oncorhynchus mykiss*) (Bussy et al., 2018b; Hubert et al., 2005; Kane et al., 1994; Mitrovic et al., 2026a; Lawrence et al., 2021).

Previous studies in rainbow trout, brook trout (*Salvelinus fontinalis*) and larval sea lamprey indicated that mixtures of TFM and niclosamide (Bayer 73) acted in a strictly additive or less than additive manner (Bills & Marking, 1976; Dawson et al., 1977), while other studies have suggested that interactions were greater than additive (synergistic;

Marking & Dawson, 1975). More recently, Hepditch et al. (2021) employed the concentration addition model to elucidate the nature of TFM and niclosamide interactions in rainbow trout, in which the toxicity of TFM-niclosamide mixtures was quantified using the toxic unit (TU) concept (Norwood et al., 2003; Playle, 2004). Using this approach, with one TU defined as the 12-h LC_{50} of each chemical, they found that TFM/1% Nic interactions were strictly additive at lower TFM concentrations but had greater than additive (synergistic) effects at higher concentrations. However, there is little mechanistic understanding of how these TFM-niclosamide interactions take place.

Studies with larval sea lamprey have revealed that in the presence of niclosamide, exposure to TFM results in higher TFM concentrations in the muscle, suggesting potential impairment of hepatic TFM metabolism (Mitrovic et al., 2026b; Chapter 3). It remains unclear whether this is the case in non-target species such as rainbow trout, which have a greater TFM detoxification capacity and tolerance than larval lamprey (Kane et al., 1993; Statham & Lech, 1975). To better understand the nature and mechanisms of action of TFM-niclosamide interactions, a similar experimental approach to that used by Mitrovic et al. (2026b; Chapter 3) was applied to characterize how niclosamide might affect TFM toxicity in rainbow trout. Accordingly, acute toxicity tests were conducted to characterize TFM plus niclosamide toxicity in rainbow trout. This was followed by collection of liver, brain and muscle for subsequent TFM and niclosamide quantification from rainbow trout exposed to a single concentration of TFM in the absence (TFM only) or the presence of niclosamide (TFM/1% Nic Mix). As niclosamide may alter the hepatic Phase II metabolism of TFM, it was hypothesized that TFM accumulation would be greater in these tissues in the presence of niclosamide.

5.2 Materials and methods

5.2.1 Experimental animals and holding

Rainbow trout fingerlings were purchased from the Ontario Aquaculture Research Centre, ON, Canada (n = 420, mass = 18.9 ± 0.4 g and total length = 131.5 ± 6.2 mm). The fish were

held under a 12 h light:12 h dark photoperiod in a 250 L tank filled with aerated, dechlorinated municipal tap water (temperature ~13°C, pH ~8, and titratable alkalinity ~238 mg L^{-1} as CaCO3), which was replaced at a rate of 1.0 L min^{-1}. The fish were fed 3 times a week at 2% body weight with standard 1.0 mm commercial floating pellet fish food (Corey Feed Mills, Fredericton, NB, Canada). Fish were held a minimum of 3 weeks prior to experiments. Food was withheld for 48 h prior to the onset of experiments. All experiments were approved by the Wilfrid Laurier University Animal Care Committee (Animal Use Protocol No. R18004) and followed the Canadian Council on Animal Care guidelines.

5.2.2 Chemicals and reagents

The Sea Lamprey Control Centre, Fisheries and Oceans Canada, Sault Ste. Marie, ON, Canada kindly provided both the TFM and niclosamide field formulations. The field formulation TFM (35% dissolved in isopropanol; Clariant SFC GmbH, WERK Griesheim, Germany) was used in all TFM exposure experiments. A stock solution of TFM (10 g L^{-1}) was prepared prior to experiments, with appropriate amounts of stock solution added to each aquarium to yield the selected nominal concentrations of TFM.

Niclosamide (Bayluscide® Emulsifiable Concentrate; 16.9% niclosamide ethanolamine salt mixture; Coating Place Inc., Verona, WI, USA), diluted 10 times in 95% methanol, was used as a stock solution for exposures. In each case, the appropriate concentrations of the stock solution were added to experimental aquaria to yield the desired exposure concentrations. Exposure concentrations were confirmed by liquid chromatography – tandem mass spectroscopy (LC-MS/MS) analysis at the beginning and the end of the experiments using methods described in Mitrovic et al. (2026a, b; Chapter 2 and 3).

5.2.3 Experimental protocols

Two series of experiments were performed. Experiment 1 comprised 12 h acute toxicity tests to quantify the 12-h LC_{50} of TFM in the presence and absence of niclosamide, and to determine the 12-h LC_{50} of niclosamide alone. In Experiment 2, a separate group of rainbow trout was exposed for 9 h to either the 12-h LC_{50} concentration of the TFM plus niclosamide

(TFM/1% Nic; w/w) mixture (containing both lampricides) or to the equivalent mixture concentration of each lampricide individually. The 9-h exposure duration was selected to approximate the length of a typical lampricide application (Sullivan et al., 2021). Liver, muscle, and brain were collected at different time intervals of exposure (1, 3, 6, 9 h) for subsequent quantification of TFM and niclosamide concentration.

5.2.3.1 Experiment 1 - Acute toxicity tests

Rainbow trout fingerlings (n = 170, mean mass = 17.5 g ± 0.8 and mean length = 128.6 mm ± 1.8) were placed in test aquaria approximately 12-18 h prior to being exposed to a range of TFM, niclosamide (Nic), or a TFM/1% Nic mixture concentration to generate toxicity curves under static conditions. Toxicity tests followed American Standard and Testing Methods guidelines for static toxicity testing on trout (ASTM, 2014). Briefly, fish (n = 5) were placed in 25 L of aerated (~ 95% dissolved oxygen) dechlorinated water in a glass aquarium immersed in a recirculating water bath to maintain a stable temperature during the experiment, without water replacement. For the TFM only toxicity tests, trout were exposed to nominal TFM concentrations of 5.0, 7.5, 10.0, 12.5, 15, and 25 mg L^{-1}, plus non-exposed controls, in duplicate (i.e., n = 10 per concentration). In contrast, niclosamide only toxicity tests were conducted using nominal concentrations of 0.05, 0.1, 0.25, 0.5 and 1.0 mg L^{-1}, plus non-exposed controls. For the TFM/1% Nic mixture toxicity tests, fish were exposed to nominal concentrations of TFM/1% Nic in mg L^{-1}: 2.5/0.025, 5.0/0.050, 7.5/0.075, 10/0.1, 15/0.15, and 25/0.25 mg L^{-1}, plus non-exposed controls.

The morning of the toxicity tests, the targeted nominal concentrations of either lampricide in each test aquarium were achieved by dosing with specific volumes of TFM working stock (10 g L^{-1}) or niclosamide working stock (1 g L^{-1}) as described above. Each lampricide was added separately to each tank at the designated dose. The addition of the lampricide(s) marked the beginning of the 12 h exposure. Water samples (20 mL) were collected at the start of the experiment (0 h) and at 6 h and 12 h of exposure, for quantification of TFM and niclosamide exposure concentrations. Water chemistry, including

water temperature, pH, alkalinity, and hardness, was measured before and after the experiments to minimize disturbance to the fish.

During the exposures, fish were monitored hourly for the first four hours and every two hours thereafter. Mortality was assumed when fish lost equilibrium, and there was no visible ventilation (no opercular beats), at which time the fish were removed, and the time of death, body length and mass were recorded. After the 12 h exposure, any survivors were transferred into an aerated bath containing a lethal dose of buffered tricaine methanesulfonate (MS-222; Syndel, Port Alberni, BC, Canada; 0.5 g L^{-1} MS-222 with 1.0 g L^{-1} $NaHCO_3$) before sampling.

5.2.3.2 Experiment 2 – Effects of exposure to lampricide mixture on TFM and niclosamide tissue accumulation in liver, brain and muscle

Experiments (n = 168, weight = 19.4 ± 0.6 g, total body length = 124.5 ± 2.0 mm) were designed to compare TFM or niclosamide accumulation in the liver, muscle and brain of fingerling trout following 9 h exposure to either the 12-h LC_{50} concentration of the TFM/1% Nic mixture (containing both lampricides) or to the equivalent mixture concentration of each lampricide individually. There were four control tanks with 2 fish per tank (n = 8 controls). Treatment tanks compromised aquaria (volume = 8L) with n = 2 fish per tank, which were terminally sampled at 1, 3, 6 and 9 h of exposure to TFM, niclosamide or TFM + niclosamide (n = 12 for each of the four sample periods, plus control fish (n = 8 per treatment). All other experimental conditions were as described for Experiment 1. Water chemistry, temperature and pH were measured at the start and at the end of the experiment. Fish were sampled at 0, 1, 3, 6, and 9 h from the onset of lampricide(s) exposure. The fish were euthanized in an aerated MS-222 bath (0.5 g L^{-1} MS-222 with 1.0 g L^{-1} $NaHCO_3$), and the brain, liver and muscle tissues were removed and snap frozen in liquid nitrogen and stored at -80ºC until processed for TFM and niclosamide quantification.

5.2.4 Concentration addition model

Toxicity data were analyzed using the concentration addition model, which uses the toxic unit (TU) concept to model interactions between mixtures of substances with similar modes of action (Norwood et al., 2003; Playle, 2004). One TU was set to equal the corresponding 12-h LC_{50} of TFM or niclosamide when exposed to each chemical alone. In other words, 1 TU = 0.5 or 50% mortality for each lampricide. These individual 12-h LC_{50} values were then used to calculate the total TU of exposure ($TU_{Tot\ Exp}$) using the equation below:

$$TU_{Tot\ Exp} = \mathbf{x}\ TU_{TFM} + \mathbf{y}\ TU_{Nic} \quad (1)$$

where x and y denote the respective fraction of 1 TU contributed by TFM or niclosamide, respectively, as calculated using the following equation:

$$\text{Fraction TU} = \frac{\text{measured [TFM or Nic]}_{EXP}}{\text{12-h } LC_{50} \text{ of TFM or Nic}} \quad (2)$$

Where $[TFM\ or\ Nic]_{EXP}$ is the measured concentration in the water of TFM or Nic to which the fish were exposed. Equation 1 was then used to plot the total TU of exposure against the predicted % mortality as described below:

$$\text{Predicted \% mortality} = [(TU_{TFM} \times 0.5) + (TU_{Nic} \times 0.5)] * 100\% \quad (3)$$

The corresponding line of equivalence for the relationship between total $TU_{Tot\ Exp}$ and predicted percent mortality was the line of strict additivity (Newman, 2020). For instance, if 0.5 TU_{TFM} and 0.5 TU_{Nic} acted in a strictly additive manner, the expected mortality would be 50%, falling directly on the line of strict additivity. Accordingly, the observed percent mortalities obtained when the fish were exposed to different mixtures of TFM and niclosamide were plotted against the $TU_{Tot\ Exp}$. If the observed mortality fell above the line of strict additivity, the interactions between TFM and niclosamide were considered greater than additive (synergistic) and if observed mortality fell below the line, the interactions were considered less than additive (antagonistic).

5.2.5 Analytical methods

TFM concentrations in the water were measured spectrophotometrically using a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, UK) at a wavelength of 395 nm using a micro-modification of GLFC Standard Operating Procedure 431.0 (Hammond Bay Biological Station, MI, USA), with standard dilutions prepared using the experimental water.

Water concentrations of niclosamide were determined for each exposure tank prior to, during, and after the experimental exposures. The analysis followed previously published protocols (e.g. Ionescu et al., 2022). Briefly, 5 mL of completely thawed and vortexed water samples were transferred to a clean glass test tube, and each sample, internal control spikes, and blanks (used for validation of batch results) were spiked with isotopically labelled internal niclosamide standard (^{13}C-Nic, 200 µg L^{-1}). Samples were vacuum filtered through 0.45 µm glass fibre filters (Pall Corporation, Michigan, USA) and 1 mL of the collected eluent was transferred into a 2 mL amber glass vial for subsequent LC-MS/MS (described in detail below).

Extraction and quantification of lampricides from tissues were completed as described by Mitrovic et al. (2026a, b; Chapter 2 and 3). Briefly, approximately 100 mg of finely ground liver (121.5 ± 51.1 mg), muscle (150.0 ± 20.6 mg) or brain (80.9 ± 17.6 mg) tissue was placed into a 2 mL microcentrifuge tube and diluted with a 1:1 ratio of sample weight to solvent (acetonitrile (ACN) - 1% formic acid). Each sample contained 200 µg L^{-1} internal standard (^{13}C-Nic). Sample blanks were prepared with control tissues spiked with ^{13}C-Nic standard (200 µg L^{-1}). Additional quality control matrix spikes included 100 µg L^{-1} of TFM and niclosamide.

Eluted tissue extract and water samples (were analyzed using an Agilent 1260 HPLC coupled to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved using an Agilent Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm). Data were processed using Mass Hunter Quantitative Analysis software (version 10.1; Agilent). Calibration curves ranged from 0.1-5000 µg L^{-1} for TFM and 0.1-500 µg L^{-1} for niclosamide and were prepared with acetonitrile (Optima,

LC/MS grade, Fisher Scientific, Mississauga, Canada). For both lampricides, the instrument detection limit was 0.04 μg L^{-1}, the method detection limit was 0.25 μg L^{-1}, and sample recoveries exceeded 95%.

5.2.6 Calculations and statistical analysis

To determine the dose–response relationships for each lampricide and the TFM/1% mixture, replicate tanks were treated as independent exposures. Mortality data were analyzed using probit analysis to estimate the 12-h LC_{50} and $LC_{99.9}$ lethal concentrations for each lampricide and the mixture. Dose–response curves, corresponding LC values, and 95% confidence intervals (CI) were calculated using non-linear regression (curve fit) using least squares analysis (GraphPad Prism 10.6, GraphPad Software Inc., La Jolla, CA, USA). Differences among LC values were considered significant when 95% confidence intervals did not overlap (Finney, 1971).

Morphometric variables (length and weight) and lampricide tissue concentrations were reported as the mean ± 1 S.E.M. Differences among lampricide exposures within time periods were evaluated using one-way ANOVA followed by Tukey's post hoc tests. When assumptions of normality or homogeneity of variance were violated, a Kruskal–Wallis test with Dunn's multiple-comparisons test was applied. All analyses were performed using GraphPad Prism version 10.6 with statistical significance defined as $\alpha = 0.05$.

A pharmacokinetic approach was used to calculate TFM steady-state concentration (C_{SS}) in order to assess the effects of niclosamide presence on TFM accumulation in trout livers. Liver TFM accumulation data were reanalyzed using non-linear regression with a one-compartment least-squares model (GraphPad Prism 10.6). Plateau followed by one phase association analysis was used to calculate the TFM accumulation constant (k), C_{SS}, and elimination half-life ($t_{1/2}$). Note that k represents the apparent uptake rate constant based on actual fish concentrations over time and is influenced by metabolism (therefore, it is not equivalent to the theoretical equilibrium-based uptake rate constant). These calculations

assumed that, at steady state, the rate of elimination equals the rate of accumulation, such that the half-life ($t_{1/2}$) = ln(2)/k.

5.3 Results

5.3.1 Experiment 1 – Acute toxicity tests

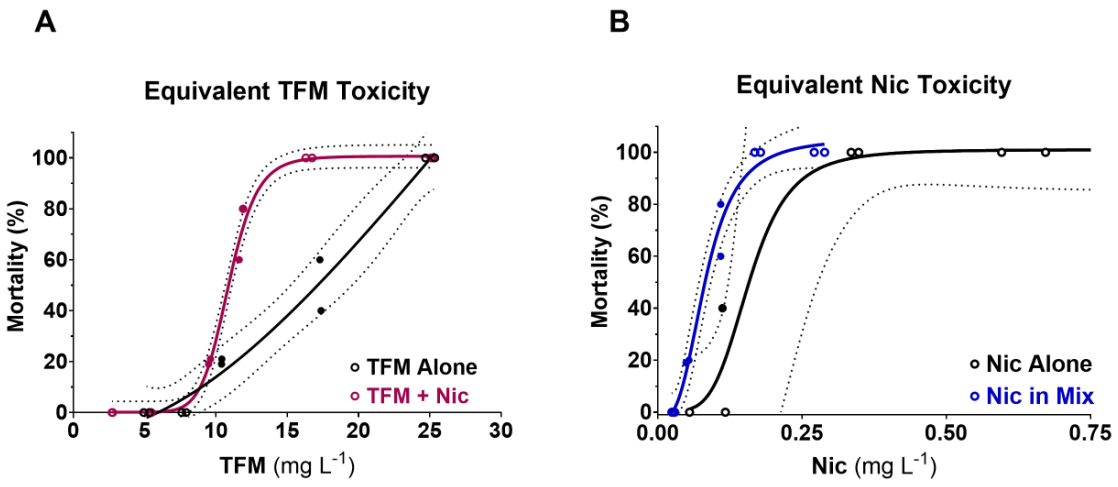
Most of the water chemistry parameters were relatively stable across treatments (Table 5.1), as dissolved oxygen was 95.8 ± 1.3 % and temperature averaged 12.1 ± 0.6°C. Water alkalinity was 238 mg L^{-1} as $CaCO_3$, and hardness averaged 221 mg L^{-1} as $CaCO_3$ across the 3 treatments (TFM alone, niclosamide alone and TFM/1% Nic mixture). The pH varied slightly over the course of the 12 h of exposure, changing by 0.09 - 0.20 during exposures that contained TFM (TFM only and TFM/1% Nic treatments), while pH in the niclosamide only treatment remained stable (Table 5.1). The measured TFM water concentrations in TFM alone and TFM/1% Nic mixture toxicity tests closely matched their nominal target concentrations at 0, 6, and 12 h, with minimal variation over time (Appendix D, Table D.1A and C). However, measured niclosamide concentrations were initially higher than nominal, but had declined by more than 40% over the 12 h exposure at the lowest exposure concentrations (Appendix D, Table D.1B and C). Once complete mortality of all trout was observed, water sample collection was stopped.

Table 5.1 Measured water quality during exposure of rainbow trout to TFM, niclosamide or TFM/1% niclosamide. n denotes the number of total measurements per treatment. Data presented as the mean ± S.E.M.

	Temperature (°C)	pH		Alkalinity (mg L^{-1} as $CaCO_3$)	Hardness (mg L^{-1} as $CaCO_3$)	Dissolved O_2 (% saturation)	n
		Start	End				
Experiment 1							
TFM	12.6 ± 0.1	8.15 ± 0.05	8.26 ± 0.04	238	215	97.1 ± 0.7	14
Niclosamide	12.3 ± 0.1	8.27 ± 0.05	8.24 ± 0.05	238	197	94.6 ± 1.1	12
TFM/1% Niclosamide	11.4 ± 0.1	8.22 ± 0.02	8.36 ± 0.03	238	245	95.6 ± 0.5	14
Experiment 2							
TFM	13.8 ± 0.2	8.29 ± 0.02	8.32 ± 0.03	221	233	94.8 ± 1.1	24
Niclosamide	14.2 ± 0.2	8.25 ± 0.03	8.24 ± 0.04	221	206	95.4 ± 0.8	24
TFM/1% Niclosamide	14.2 ± 0.4	8.21 ± 0.02	8.34 ± 0.02	221	239	95.1 ± 0.5	24

There was a leftward shift of the dose-response curve for TFM in the presence of niclosamide, characterized by a 31% decrease in the 12-h LC_{50} from 15.78 mg L^{-1} (CI = 12.21 - 20.39 mg L^{-1}) with exposure to TFM alone to 10.87 mg L^{-1} (CI = 9.90 - 11.94 mg L^{-1}) with TFM/1% Nic mixture exposure (Figure 5.2A). An even greater reduction was observed in the 12-h $LC_{99.9}$ (MLC) of TFM, decreasing 70% from 57.09 mg L^{-1} (CI = 21.43 - 152.10 mg L^{-1}) during TFM only exposure to 17.40 mg L^{-1} (CI = 14.79 - 20.48 mg L^{-1}) following the TFM/1% Nic mixture treatments (Figure 5.2A).

The corresponding reduction in the 12-h LC_{50} of niclosamide when exposed to the TFM/1% Nic mixture compared to niclosamide only values fell by more than 30%, from 0.118 (CI = 0.115 - 0.120) mg L^{-1} for niclosamide only to 0.081 (CI = 0.060 - 0.110) mg L^{-1} for the TFM/1% Nic mixture, also characterized by a leftward shift of the dose-response curves (Figure 5.2B).



TFM (mg L⁻¹)		
LC	Concentration	95% CI
50	15.78	12.21 - 20.39
99.9	57.09	21.43 - 152.10

TFM in TFM/1% Nic Mixture (mg L⁻¹)		
LC	Concentration	95% CI
50	10.87*	9.90 - 11.94
99.9	17.40*	14.79 - 20.48

Niclosamide (mg L⁻¹)		
LC	Concentration	95% CI
50	0.118	0.115 - 0.120
99.9	0.124	0.121 - 0.127

Niclosamide in TFM/1% Nic Mixture (mg L⁻¹)		
LC	Concentration	95% CI
50	0.081*	0.060 - 0.110
99.9	0.378*	0.149 - 0.964

Figure 5.2 Toxicity of TFM and niclosamide to rainbow trout exposed to TFM, niclosamide or TFM/1% niclosamide mixture. Rainbow trout were exposed to **(A)** TFM in the absence (TFM Alone) or presence of niclosamide (TFM in Mix), or **(B)** niclosamide in the absence (Nic Alone) or presence of TFM (Nic in Mix) for 12 h. n = 5 for each concentration shown, and solid circles are partial mortalities, while open circles indicate complete mortalities. Corresponding tables summarize the calculated LC_{50} and $LC_{99.9}$ for TFM or niclosamide concentration (± 95% confidence intervals), determined by non-linear regression (curve fit) using least squares analysis. Differences among LC values were considered significant when 95% confidence intervals did not overlap. Asterisks indicate significant differences between the respective LC values of TFM or niclosamide and each corresponding mixture, as determined by lack of CI overlap.

5.3.1.1 TFM-niclosamide interactions elucidated by the concentration addition model

Using the 12-h LC_{50} values generated from the acute toxicity test when the trout were exposed to TFM and niclosamide alone, 1 TU of TFM was set at 15.78 mg L^{-1}, and 1 TU of

niclosamide at 0.118 mg L^{-1}. The measured TFM and niclosamide mixture exposure concentrations from the acute toxicity test were then converted to $TU_{Tot\ Exp}$ (Equation 1), plotted against observed percent mortality and compared to the line of strict additivity to determine whether the interactions were strictly additive, less than additive, or greater than additive (Figure 5.3). Exposure to the lowest TFM/1% Nic mixture exposure concentrations, corresponding to 0.38 and 0.58 TU of total exposure, resulted in no mortality. At higher TU of exposure, the observed mortality fell below the line of strict additivity, with 1.04 TU of exposure only corresponding to approximately 20% mortality, rather than the 50% mortality that would have been observed had the interactions been strictly additive (Figure 5.3). Exposure to a higher concentration, corresponding to 1.67 TU, resulted in 70% mortality, slightly below the 75% mortality predicted by the line of strict additivity. Complete mortalities (100%) were observed as the total TU exceeded two (Figure 5.3).

[TFM]	2.73	5.39	9.55	11.76	16.52	25.13
TU TFM	0.17	0.34	0.61	0.75	1.05	1.59
[Nic]	0.024	0.028	0.052	0.109	0.173	0.280
TU Nic	0.205	0.240	0.437	0.921	1.464	2.373
TU Total	**0.38**	**0.58**	**1.04**	**1.67**	**2.51**	**>3.00**

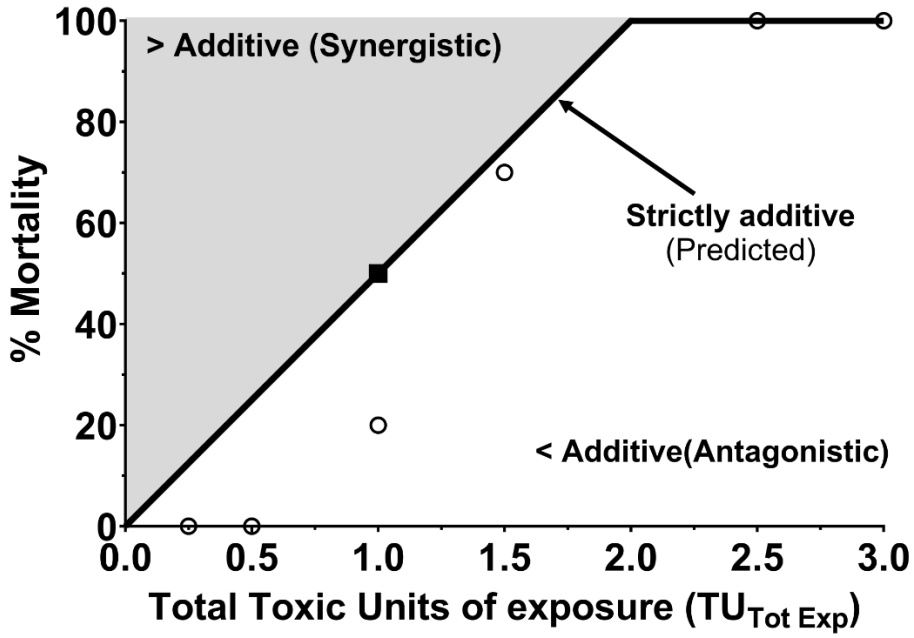


Figure 5.3 Interactions between TFM and niclosamide (Nic) lampricide concentrations in rainbow trout using the concentration-addition model. Plot comparing observed mortality to total toxic units of exposure ($TU_{Tot\ Exp}$), where 1 TU = 12-h LC_{50} of TFM or niclosamide. Strictly additive interactions between TFM and niclosamide would be expected to fall on the line of strict additivity (line of equivalence), whereas points below the line would indicate less than additive interactions (antagonistic) and points falling above the line would indicate greater than additive (synergistic) interactions. Open circles denote the observed mortality, and the diamond represents predicted mortality if exposure to 0.5 TU_{TFM} and 0.5 TU_{Nic} was strictly additive.

5.3.2 Experiment 2 – Effects of exposure to lampricide mixture on TFM and niclosamide accumulation

To characterize how TFM and niclosamide were distributed and handled by the different tissues, TFM and niclosamide concentrations were measured in the liver, brain and muscle of trout exposed to a TFM/1% niclosamide mixture, or TFM alone or niclosamide alone. The nominal TFM exposure concentrations, alone or in the TFM/1% niclosamide mixture, were equivalent to the 12-h LC_{50} of TFM when the trout were exposed to TFM/1% niclosamide mixtures in Experiment 1 (Table 5.2). The niclosamide concentration, alone or in the TFM/niclosamide mixtures were 1% of the TFM value (Table 5.2). Water pH ranged from 8.19 at the start of the exposures to 8.36 by the end of 9 h; temperature averaged 14.0 ± 0.08°C and dissolved oxygen was 95.1 ± 0.5% saturation, while alkalinity remained constant at 221 mg L^{-1} as $CaCO_3$ and hardness averaged 226 mg L^{-1} as $CaCO_3$ (Table 5.1). All fish survived the 9 h lampricide exposures, with average measured water concentrations of TFM only (10.94 ± 0.02 mg L^{-1}), niclosamide only (0.097 ± 0.003 mg L^{-1}), and TFM and niclosamide in the TFM/1% Nic mixture (TFM = 10.71 ± 0.04 mg L^{-1} and niclosamide = 0.102 ± 0.001 mg L^{-1}) treatments closely matching the nominal TFM and niclosamide values throughout (Table 5.2).

Table 5.2 Measured water lampricide concentrations during experiment 2. Rainbow trout were exposed to either TFM alone, niclosamide alone or a TFM/1% niclosamide mixture. Nominal exposure concentrations were 12-h LC_{50} of TFM determined from the TFM/1% niclosamide mixture from Experiment 1, with the niclosamide concentration being 1% of the TFM concentration. Lampricides were not detectable in control tanks or at time zero (pretreatment), and n denotes the number of total measurements per treatment. Data presented as the mean ± S.E.M.

Experimental Exposure	Nominal	Measured Concentration (mg L^{-1})		n
		TFM	Nic	
TFM only	10.87	10.94 ± 0.02	NA	24
Niclosamide only	0.109	NA	0.097 ± 0.003	24
TFM/1% niclosamide	10.87/0.109	10.71 ± 0.04	0.102 ± 0.001	24

Exposure to TFM only led to TFM accumulation in the liver, which averaged 64.2 ± 6.1 nmol g^{-1} ww at 1 h, but then significantly decreased to 42.0 ± 4.2 nmol g^{-1} ww ($p = 0.016$) and 37.5 ± 5.6 nmol g^{-1} ww ($p = 0.002$) after 6 h and 9 h, respectively (Figure 5.4A). Exposure to the TFM/1% Nic mixture resulted in an initial liver TFM concentration of 53.4 ± 7.7 nmol g^{-1} ww at 1 h ($p = 0.006$), which was not significantly different from that observed with exposure to TFM only (Figure 5.4A). However, there was 50 - 55% greater at 3 h and 6 h following exposure. By 9 h, the liver TFM concentration was approximately 2-fold greater than at 1 h ($p = 0.006$), at which time it averaged 103.2 ± 10.8 nmol g^{-1} ww (Figure 5.4A).

There was also significant TFM accumulation in the brain after 1 h of TFM exposure, at which time the concentration averaged 4.2 ± 1.2 nmol g^{-1} ww, before undergoing a significant increase to 8.1 ± 0.7 nmol g^{-1} ww at 3 h ($p = 0.12$) and to 10.0 ± 1.4 nmol g^{-1} ww at 6 h ($p = 0.002$) before falling slightly, but not significantly to 6.9 ± 0.6 nmol g^{-1} ww at 9 h (Figure 5.4B). Exposure to the TFM/1% Nic mixture resulted in greater accumulation in the brain at all sample periods, which were between 1.6- to 3.0-fold greater than concentrations measured in the brain of the fish exposed to only TFM (Figure 5.4B). Notably, the TFM concentration in the brains of fish exposed to TFM/1% Nic did not significantly change with time over the 9 h experiment (Figure 5.4B).

The concentration of TFM in the muscle was 5.6 ± 0.6 nmol g^{-1} ww following 1 h of TFM only exposure but, in contrast to liver and brain, did not significantly change with time over the ensuing 9 h (Figure 5.4C). Slightly lower amounts of TFM were present in muscle after 1 h with TFM/1% Nic treatment but were not significantly different from the TFM only concentrations (Figure 5.4C). However, TFM accumulation was up to 50%, and significantly lower in the TFM/1% Nic mixture treated animals than the TFM only treatment animals after 3 h ($p = 0.007$), but not at the other treatment times (Figure 5.4C).

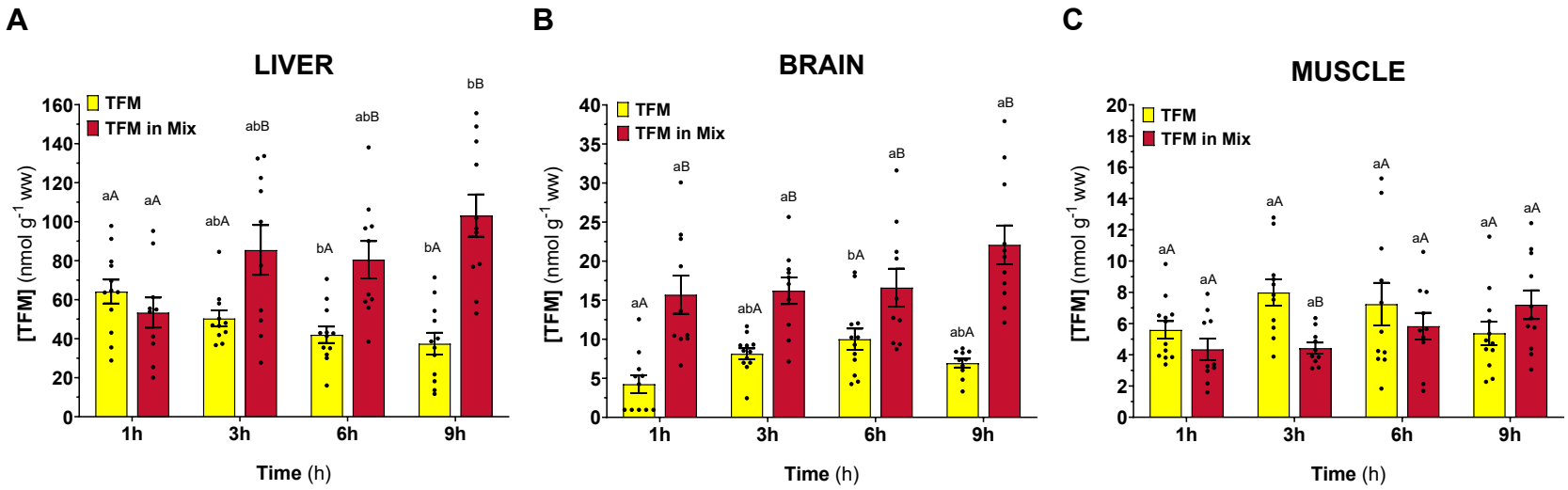


Figure 5.4 Accumulation of TFM in the tissues of rainbow trout. Fish were exposed to TFM only or a TFM plus niclosamide mixture. Changes in TFM concentration in (A) liver, (B) muscle, and (C) brain of rainbow trout exposed to nominal concentrations of TFM alone (yellow bars) compared to a TFM-niclosamide mixture (red bars). Nominal TFM exposure concentrations were equivalent to the 12-h LC_{50} of TFM alone or when exposed to the TFM/1% mixture, which were measured in Experiment 1. Different lowercase letters denote significant differences within the treatment vs time; different uppercase letters denote significant differences between TFM and TFM plus niclosamide treatments within each sample time. TFM was not detectable in fish at time zero (pretreatment). Data presented as mean ± 1 S.E.M. (n = 10-12; $p < 0.05$).

Niclosamide accumulation was quantified in liver, brain and muscle samples of the same trout exposed to the TFM/1% Nic mixture (where TFM was previously measured), or in the additional trout exposed to the equivalent TFM/1% Nic mixture niclosamide concentration. After 1 h of the niclosamide only exposure, the liver niclosamide concentration averaged 34.3 ± 4.8 nmol g^{-1} ww, which significantly increased by approximately 1.8-fold to 60.1 ± 4.0 nmol g^{-1} ww after 3 h ($p = 0.003$), where it remained relatively stable through the entire 9 h experiment, at which time the concentration averaged 71.3 ± 5.9 nmol g^{-1} ww (Figure 5.5A). The liver niclosamide concentration in the TFM/1% Nic mixture treatment after 1 h of exposure was 36.3 ± 4.6 nmol g^{-1} ww, which significantly ($p = 0.003$) increased to 77.6 ± 9.0 nmol g^{-1} ww after 3 h. Liver niclosamide remained relatively stable thereafter, averaging 83.5 ± 8.3 and 80.3 ± 8.8 nmol g^{-1} ww at 6 and 9 h, respectively (Figure 5.5A). Niclosamide concentrations increased more substantially in TFM/1% Nic mixture treatment compared to niclosamide only treatment, by more than a 1.5-fold increase at 6 h of exposure ($p = 0.04$; Figure 5.5A).

Within the white muscle tissue, niclosamide concentration in the niclosamide only treatment initially averaged 2.1 ± 0.4 nmol g^{-1} ww after 1 h before significantly ($p < 0.0014$) decreasing by approximately 70 % to 0.6 ± 0.06 nmol g^{-1} ww after 3 h and remained near this concentration for the remainder of the 9 h exposure (Figure 5.5C). Muscle niclosamide concentrations were initially 2-fold greater in the trout subjected to the niclosamide only exposure compared to the TFM-niclosamide mixture treatment, which averaged 1.1 ± 0.2 nmol g^{-1} ww (Figure 5.5C). The pattern was reversed afterwards, during which muscle niclosamide concentrations were 2-3-fold greater in the TFM-niclosamide treated compared to the niclosamide only animals (Figure 5.5).

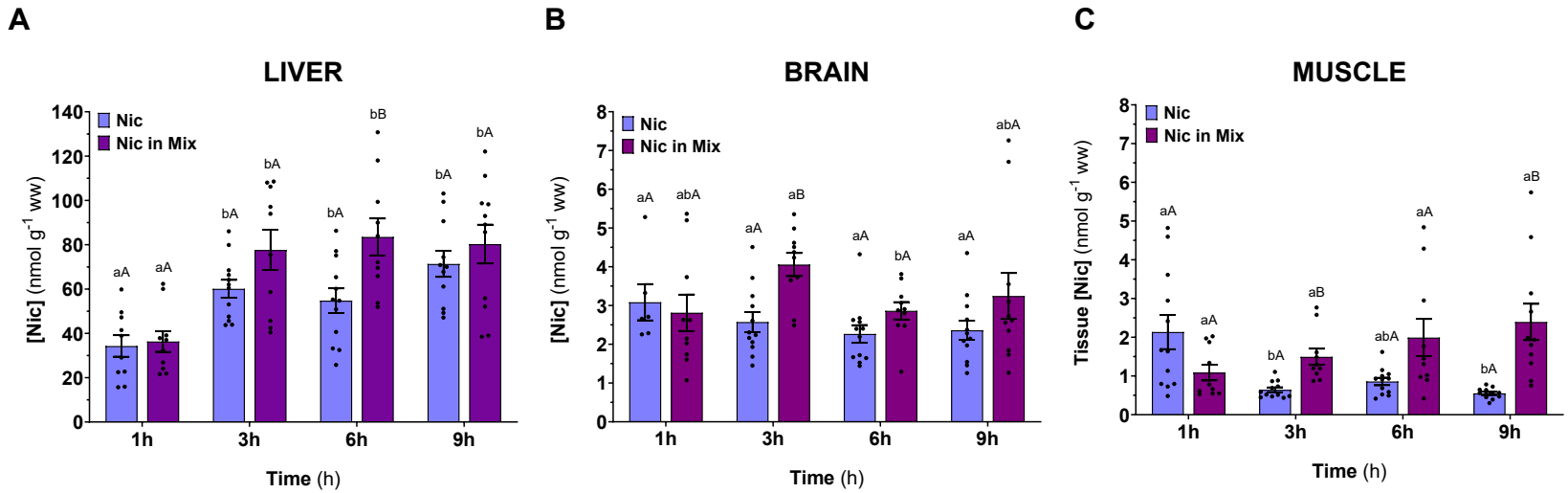
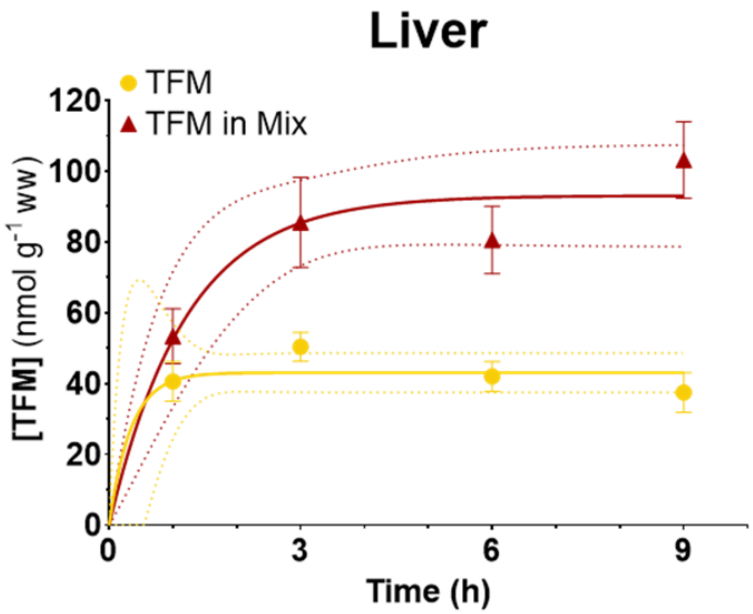


Figure 5.5 Accumulation of niclosamide in the tissues of rainbow trout. Fish were exposed to niclosamide only or a TFM plus niclosamide mixture. Changes in niclosamide concentration in (A) liver, (B) muscle, and (C) brain of rainbow trout exposed to the nominal concentrations of niclosamide alone (blue) compared to a TFM–niclosamide mixture (purple). Nominal niclosamide exposure concentrations were equivalent to 1% of the 12-h LC_{50} for TFM/1% niclosamide, which were measured in Experiment 1. Different lowercase letters denote significant differences within the treatment vs. time; different uppercase letters denote significant differences between niclosamide only and TFM plus niclosamide treatments within each sample time. Niclosamide was not detectable in fish at time zero (pretreatment). Data presented as mean ± S.E.M. (n = 10-12; $p < 0.05$).

The non-linear regression using one-compartment least squares analysis produced kinetic curves illustrating the effects of niclosamide on TFM accumulation in trout liver. The kinetic curves allowed calculations of steady state concentrations (C_{SS}), the TFM accumulation (elimination) rate constant (k), and accumulation/elimination half-life ($t_{1/2}$) (Figure 5.6). The rate constant (k) represents the apparent uptake rate constant based on the actual fish concentrations measured over time (equivalent to the total depuration rate constant at steady state). With the presence of niclosamide in the TFM/1% Nic mixture treatment, the C_{SS} increased more than 2-fold, averaging 93.06 nmol g^{-1} ww vs 43.07 nmol g^{-1} ww in the TFM only treatment (Figure 5.6). A notable 3.5-fold increase in half-time ($t_{1/2}$) from 0.24 to 0.84 h was observed with the presence of niclosamide in the TFM/1% Nic mixture treatment (Figure 5.6).



Treatment	C_{SS} (nmol g^{-1} ww)	$t_{1/2}$ (h)	k (h^{-1})	R^2
TFM	43.07 (37.64 - 48.62)	0.24 (NA - 0.71)	2.92 (0.98 - NA)	0.404
TFM in Mix	93.06 (79.14 - 110.10)	0.84 (0.38 - 1.72)	0.83 (0.40 - 1.84)	0.508

Figure 5.6 The relationship of TFM accumulation in the liver of rainbow trout exposed to TFM in the absence and presence of niclosamide. Data from Figure 5.4 were used to generate kinetic curves using least squares analysis, and to calculate the steady state concentration (C_{SS}) and the half-life ($t_{1/2}$) of elimination. Calculations and curve fitting were completed using GraphPad Prism 10.6 (Plateau followed by one phase association). It was assumed that at C_{SS}, the rates of accumulation and elimination were equal. Dashed lines represent 95% confidence intervals. n = 8-12 per data point.

5.4 Discussion

The present study demonstrates that niclosamide enhances the toxicity of TFM in rainbow trout by interfering with TFM elimination, resulting in greater internal TFM accumulation in the liver and in sensitive vital organs such as the brain. We propose that impairment of TFM metabolism in the liver by niclosamide, characterized by an increase in TFM elimination half-life ($t_{1/2}$), leads to increased overall body TFM concentrations and greater toxicity. Notably, the interactions between TFM and niclosamide in this study were less than or strictly additive, as reported in previous studies on TFM-niclosamide interactions (Bills and Marking, 1976), but in contrast to more recent findings of Hepditch et al. (2021).

5.4.1 Distribution, accumulation and elimination of TFM and niclosamide

Few studies have measured the concentrations of TFM or niclosamide in the brain of fishes (Chen et al., 2024; Lech & Statham, 1975; Zhu et al., 2022). These findings and the present study make it clear that these lampricides can cross the blood-brain barrier and accumulate in neural tissues. This is also the first study to reveal that TFM accumulation in the brain is increased when TFM is applied to water in the presence of niclosamide. Previous studies have shown that TFM exposure causes severe depletion of high energy stores such as glycogen and phosphocreatine (PCr) in the brain of rainbow trout (Birceanu et al., 2014) and sea lamprey (Birceanu et al., 2009; Clifford et al., 2021; Henry et al., 2015). More recently, significant decreases in brain glycogen and ATP levels were reported in lake sturgeon (*Acipenser fulvescens*) and rainbow trout exposed to niclosamide (Ionescu et al., 2022, 2025). The marked reductions in high energy stores were attributed to the proposed uncoupling of mitochondrial oxidative phosphorylation by TFM (Birceanu et al., 2011; Borowiec et al., 2022; Huerta et al., 2020) and by niclosamide (Alasadi et al., 2018; Jurgeit et al., 2012; Park et al., 2011; Borowiec et al., 2022).

The increased concentrations of TFM in the brain, along with the increased accumulation in the liver and muscle of rainbow trout exposed to the 12-h LC_{50} of TFM/1% Nic mixture treatment over 9 h (Figure 5.4) could have resulted from increased competition between the two lampricides for the Phase II metabolism enzymes in the liver. Compared to sea lamprey, more lampricide tolerant species such as rainbow trout have more efficient pathways for detoxifying and excreting toxicants such as TFM and niclosamide from the body (Bussy et al., 2018a, b; Lawrence et al., 2021, 2022; Lech & Statham, 1975; Kane et al., 1994; Statham et al., 1976). The Phase II biotransformation processes, glucuronidation and sulphation, are the main pathways for metabolizing lampricides into more hydrophilic compounds, thereby facilitating the excretion of TFM and niclosamide via the bile and urine (Allen et al., 1979; Bussy et al., 2018a, b; Clarke et al., 1991; Hubert et al., 2005; Kane et al., 1994; Lech & Costrini, 1972; Lech & Statham, 1975).

The main Phase II enzymes involved in TFM and niclosamide metabolism include the UDP-glucuronosyltransferase (UDPGT) enzyme family and the phenol sulphotransferases, both of which have been shown to be responsible for detoxifying TFM and niclosamide in rainbow trout exposed to lampricide treatments (Bussy et al., 2018a, b; Hubert et al., 2005; Kane et al., 1994; Lech & Statham, 1975). Compared to sea lamprey, many non-target species such as rainbow trout have a much higher capacity to use UDPGTs to form TFM-glucuronide, as demonstrated by higher maximum UDPGT activities (V_{max}) and TFM affinity (Kane et al., 1994). As a result, the efficiency of the TFM metabolizing UDPGTs, expressed as the ratio of the maximum velocity (V_{max}) to the enzyme binding affinity (K_m), can be up to 50% higher in trout than in sea lamprey (Kane et al., 1994).

Less is known about the sulphation pathways of Phase II detoxification of either lampricide. However, at least in rainbow trout, sulphation may be more important for niclosamide detoxification based on greater accumulation of sulphated niclosamide compared to the glucuronide metabolite in the muscle (Hubert et al., 2005). The ~ 17-fold greater concentration of Nic-sulphate relative to Nic-glucuronide supports this suggestion. However, enzyme kinetics experiments are needed to characterize the relative amounts of

these enzymes and their affinities for niclosamide, as well as TFM. Competitive enzyme inhibition assays would also shed light on the potential mechanisms of TFM and niclosamide interactions, as well as characterizing interspecific differences in TFM sensitivity.

To gain further insight into how TFM was handled by rainbow trout in the presence of niclosamide, non-linear regression using one-compartment least squares analysis was used to generate pharmacokinetic curves. The equations from these curves were then used to calculate the steady state concentrations (C_{SS}) and the half-life of TFM elimination ($t_{1/2}$) in the liver for trout subjected to the TFM only or the TFM-niclosamide mixture treatments, a classical pharmacokinetic approach (Gupta, 2016; Newman, 2020). The rate constant (k), upon which $t_{1/2}$ calculations were based (half-life ($t_{1/2}$) = ln(2)/k), represents the apparent rate constant using the measured values over time (GraphPad Prism version 10.6, Plateau followed by one phase association model). The C_{SS} values for TFM were more than 2-fold higher in the TFM/1% Nic mixture treatment (93.06 vs 43.07 nmol g^{-1} ww; Figure 5.6), indicating much higher TFM accumulation in the presence of niclosamide. A 3.5-fold longer half-life (Figure 5.6) in the TFM/1% Nic mixture treatment (0.84 h) than in the TFM only treatment (0.24 h) indicated that these fish take much longer to accumulate and clear the TFM body load. This data suggests that the presence of niclosamide has a large effect on the TFM metabolism in rainbow trout liver, providing further, if somewhat indirect, evidence that TFM toxicity is enhanced by the presence of niclosamide by reducing the rate of TFM biotransformation.

Compared to TFM, niclosamide is a much more potent piscicide than TFM (Boogaard et al., 2003; Borowiec et al., 2022; Hubert et al., 2005; Ionescu et al., 2021; Marking & Hogan, 1967). The selectivity of niclosamide for sea lamprey is much lower due to the high degree of overlap of relative 12-h LC_{50} values of niclosamide across many different fish species and sea lamprey (Boogaard et al., 2003; Ionescu et al., 2025; Lech & Statham, 1975). Niclosamide is much more toxic than TFM, with a 12-h LC_{50} concentration value that is more than 130-fold lower than the 12-h LC_{50} concentration value of TFM, matching previous studies (Boogaard et al., 2003; Lech & Statham, 1975; Marking &

Dawson, 1976). The basis for this greater sensitivity comes from experiments on isolated mitochondria in sea lamprey, which indicated that niclosamide is 40- to 60-fold more potent an uncoupler of mitochondrial oxidative phosphorylation compared to TFM (Borowiec et al., 2022).

The higher toxicity of niclosamide, compared to TFM, may also be attributed to the more efficient binding of niclosamide to UDPGT active sites, resulting from its simpler steric structure that lacks the bulky trifluoromethyl side chain present in the TFM molecule (Applegate et al., 1966; Kane et al., 1994). Similar variations in enzyme efficiency were observed with oxidation products of the cytochrome P450 enzyme and halogenated substrates, where enzyme efficiency depends on the location of C-H bonds (aliphatic or aromatic vs para-halobenzoic bonds; Coleman et al., 2023). Further studies will be needed to evaluate the enzyme kinetics of each lampricide and determine the extent to which they might affect interactions between the UDPGTs and each lampricide.

5.4.2 Characterization of TFM and niclosamide interactions

It is well established that mixing low concentrations of niclosamide (0.5-2.0 % w/w) with TFM increases TFM toxicity (Boogaard et al., 2003; Marking & Dawson, 1975), but there has been little consensus on whether these interactions were strictly additive, less than additive (antagonistic) or greater than additive (synergistic). TFM-niclosamide interactions have been elusive to characterize because of inter-species variation (target sea lamprey vs. non-target rainbow trout and other species) and differences in exposure duration (ranging from 9 h to 96 h; e.g. Bills & Marking, 1976; Marking & Bills, 1985), and water chemistry (pH, alkalinity, and hardness). Support for all three types of interaction have been reported: antagonistic (Bills & Marking, 1976), strictly additive (Marking & Bills, 1985), or greater than additive (Howell et al., 1964; Hepditch et al., 2021).

The results of the present study, which were analyzed using the concentration addition model (Norwood et al., 2003; Playle, 2004), demonstrated that TFM-niclosamide interaction in rainbow trout were less than additive (antagonistic) at relatively low

concentrations of TFM and niclosamide (< 1.0 $TU_{Tot\ Exp}$), and strictly additive at higher concentrations (~ 1.5 $TU_{Tot\ Exp}$; Figure 5.3), which is in agreement with earlier studies in which the interactions between TFM and a different formulation of niclosamide, powdered Bayer73®, were studied (Bills & Marking, 1976; Dawson et al., 1977; Marking & Bills, 1985). Only a few studies have examined interactions between TFM and the currently used emulsifiable concentrate (EC) formulation of niclosamide used for lampricide treatments to enhance TFM toxicity in sea lamprey (Hepditch et al., 2021; Mitrovic et al., 2026b; Chapter 3). TFM-niclosamide interactions were found to be greater than additive to larval sea lamprey, at concentrations falling within the ranges applied during lampricide applications (Mitrovic et al., 2026b; Chapter 3). However, the less than additive or strictly additive interactions observed in rainbow trout in the present study contrast with previous work on rainbow trout, where synergistic interactions were observed when the $TU_{Tot\ Exp}$ were $\geq$ 1.0 (Hepditch et al., 2021). However, there are several differences between the two studies that could have contributed to the different outcomes.

Differences in water hardness and varying pH, which may alter TFM and/or niclosamide bioavailability, may have contributed to the contrasting interpretation of TFM-niclosamide interaction between these studies (Table 5.3). Water hardness, defined as the concentration of dissolved divalent Ca^{2+} and Mg^{2+} ions, could have potentially altered the bioavailability of niclosamide or TFM by complexation of the negative anion (ionized) form of each chemical (Figure 5.1) with the positively changed hardness cations, leading to lower overall bioavailability of the lipophilic un-ionized form and to lower toxicity (higher 12-h LC_{50}) for both TFM and niclosamide (Ortega-Calvo et al., 2015; Shi et al., 2025). Indeed, studies performed on sediment-occupying invertebrates have shown decreased TFM uptake with increasing water hardness (Kawatski & Bittner, 1975). There is also evidence that greater water hardness protects against niclosamide toxicity in the snail *Biomphalaria alexandrina*, the vector for schistosomiasis (Kenawy & Rizk, 2004). However, no clear relationships have been elucidated between water hardness and niclosamide or TFM bioavailability or toxicity, alone or in combination (Bills and Marking, 1976). Moreover,

water hardness was substantially lower in the present study than in the Hepditch et al. (2021) study, which would have resulted in higher TFM and niclosamide bioavailability, in turn increasing toxicity, as evident from the lower 12-h LC_{50} values of TFM alone in the current study compared to Hepditch et al. (2021). Thus, the elevated TFM and niclosamide LC_{50} values of the mixtures observed in the present study are notable and may indicate that additional factors or underlying mechanisms contributed to the observed toxicity patterns.

Table 5.3 Measured water chemistry and 12-h LC_{50} values between the present study and Hepdtich et al., 2021.

Experimental Study	pH	Alkalinity (mg L^{-1} as $CaCO_3$)	Hardness (mg L^{-1} as $CaCO_3$)	12-h LC_{50} values (mg L^{-1})			
				TFM alone	TFM in Mix	Nic alone	Nic in Mix
Present Study	8.10-8.39	238	197 - 245	15.78	10.87	0.118	0.067
Hepditch et al., 2021	8.10	210	460	18.2	6.64	0.11	0.038

As both TFM and niclosamide are weak acids with pKa values of 6.38 and 6.25, respectively (Figure 5.1), water pH affects their dissociation chemistry, and even slight increases in pH could result in large increases in the lipophilic (easier to cross the cell membrane) form of the lampricides (TFM-OH or Nic-OH; Birceanu et al., 2021; Wilkie et al., 2019). The measured pH during the toxicity tests in the present study fluctuated slightly (8.10 - 8.39, Table 5.1), which could have been sufficient to lower the bioavailability, rate of uptake and toxicity of TFM to the trout (Hlina et al., 2017). However, it seems unlikely that the slight differences in observed water pH would have been sufficient to account for the significant changes in TFM or niclosamide toxicity, with pH values virtually overlapping. The LC_{50} values for TFM and niclosamide alone were very similar between the two studies (Table 5.3). Nevertheless, this point underscores the need to carefully track and record water pH in mechanistic studies addressing TFM, niclosamide or TFM-niclosamide mixture toxicity.

Differences in the nutritional state of the animals might have also explained the differences in TFM-niclosamide interactions between the two studies. Glucuronidation capacity is highly dependent on liver glycogen stores in vertebrates (Bánhegyi et al., 1988; Mandl et al., 2023), as it is needed to produce UDP-glucuronic acid, the substrate needed to conjugate glucuronic acid with various endogenous and exogenous compounds, including phenols such as TFM and niclosamide, to form various glucuronide (Phase II) metabolites (Clarke et al., 1991; Kane et al., 1994; Lech & Statham, 1975). As niclosamide exposure leads to hepatic glycogen depletion in rainbow trout (Ionescu et al., 2025), differences in liver glycogen stores of the fishes at the time of exposure could have potentially led to considerable variation in the nature of TFM-niclosamide interactions. We speculate that if liver glycogen was lower in the earlier study (Hepditch et al., 2021), these fish may have experienced a significant reduction in glucuronidation capacity, which would have greatly increased their sensitivity to TFM and/or niclosamide. Liver glycogen stores are highly labile in trout, so slight differences in feeding, diet, physiological status, or even strain of fish could have therefore accounted for the different TFM-niclosamide interactions reported by Hepditch et al. (2021) compared to the present study. Liver glycogen was not quantified in the present study, but it is notable that a recent study noted a significant correlation between hepatic glycogen stores and TFM tolerance in larval sea lamprey (Schueller et al., 2024).

Antagonistic interactions typically occur when the actions of two pesticides interfere with one another in some way, such as when one chemical promotes greater expression of Phase I or Phase II enzymes involved in detoxification or clearance or both (Hernandez et al., 2013). Therefore, the antagonistic relationship between TFM and niclosamide in rainbow trout exposed to lower concentrations of lampricide mixtures might be explained by increasing the activities of metabolic enzymes involved in the biotransformation of both lampricides, a common mechanism underlying drug and toxicant interactions (Rowland et al., 2013). Recent transcriptomic analysis demonstrated induction of genes encoding the Phase II enzymes sulphotransferase (*sult6b1*) and glutathione S-transferase (*gstt3*) when sea lamprey were exposed to low doses of TFM (24-h LC_{10}; Lawrence et al., 2022).

Additionally, TFM-niclosamide mixture exposure resulted in large increases of detoxification gene expression (*cyp1a1*, *ugt3*, *sult1st1*, *abcb6*) in bluegill (*Lepomis marcochirus*), most likely contributing to their exceptionally high lampricide tolerance (Lawrence et al., 2023). Further studies are needed to determine if this explains the less than additive effects of TFM-niclosamide mixtures at lower concentrations. These transcriptomics data also point to a role for Phase I metabolism, as was also suggested by Bussy et al. (2018a,b).

5.5 Conclusions and implications

The current study provides evidence that increased tissue accumulation of either lampricide (TFM or niclosamide) in the presence of the other (i.e., TFM/1% Nic mixture) is possibly due to competitive inhibition of Phase II enzymes, shedding light on detoxification mechanisms in non-target rainbow trout. In sea lamprey liver, TFM concentrations were not substantially different in the presence of niclosamide compared to TFM alone across a range of different TFM and TFM/1% Nic mixture concentrations (Mitrovic et al., 2026b; Chapter 3). In contrast, in rainbow trout there was a pronounced increase in TFM accumulation in the presence of niclosamide compared to TFM alone, suggesting a more pronounced role of niclosamide on Phase II metabolism and TFM biotransformation in rainbow trout.

Future studies are needed to more fully evaluate how the presence of niclosamide reduces TFM tolerance in non-target species such as rainbow trout in short-term exposures typical of the Sea Lamprey Control Program. Such studies, encompassing sea lamprey and non-target fishes, will be critical for future management of field lampricide applications by providing insights into the mechanisms of TFM and niclosamide metabolism. Furthermore, studies focusing on the expression and activity levels of Phase I and Phase II detoxification enzymes are needed to unravel the underlying mechanisms of TFM and niclosamide interactions. Finally, a better understanding of the toxicity mechanisms of related environmental contaminants, especially phenols, may allow a better understanding of the interactions of binary lampricide mixtures under different scenarios.

Chapter 6
Synthesis and General Discussion

6.1 Overall objectives and findings

For over 60 years, 3-trifluoromethyl-4-nitrophenol (TFM) treatments have been the most effective method for controlling invasive sea lamprey (*Petromyzon marinus*) populations within the Great Lakes (Gaden et al., 2021; Sullivan et al., 2021). Although the use of TFM has been supported by substantial research on target sea lamprey, for the sensitive life stages of lake sturgeon (*Acipenser fulvescens*) and other non-target fishes such as rainbow trout (*Oncorhynchus mykiss*) and bluegill (*Lepomis marcochirus)*, knowledge gaps persist in our understanding of TFM mechanisms of action and the influence of environmental factors on its toxicity. It is well established that TFM effectiveness varies with season (Hlina et al., 2021; Muhametsafina et al., 2019; Scholefield et al., 2008; Schueller et al., 2024) but exactly how temperature affects TFM toxicity has not been fully addressed. Also, since the early days of the Sea Lamprey Control Program (SLCP), it was noted that the addition of niclosamide makes TFM a more effective lampricide (Boogaard et al., 2003; Sullivan et al., 2021), but the underlying mechanism behind TFM-niclosamide interactions and the physiological effects of exposures to lampricide mixtures have not been fully explored.

This thesis aimed to bridge these knowledge gaps and enhance our understanding of how environmental factors influence TFM toxicity. As similar experiments were performed on both sea lamprey and rainbow trout, direct interspecies comparisons on how different fish species handle TFM exposures were possible, with particular emphasis on patterns of TFM bioaccumulation in different tissues. Changes in TFM toxicity were evaluated across a range of environmentally relevant water temperatures, or in the presence of niclosamide (Figure 6.1). The chapters that incorporated niclosamide also aimed to empirically characterize how binary mixtures of TFM plus niclosamide interacted to increase TFM toxicity in both sea lamprey and rainbow trout. Although each data chapter addressed a specific research question in a single fish species, together they demonstrate that TFM toxicity and bioaccumulation are modified by temperature and by the presence of niclosamide in sea lamprey and non-target rainbow trout, but in different ways.

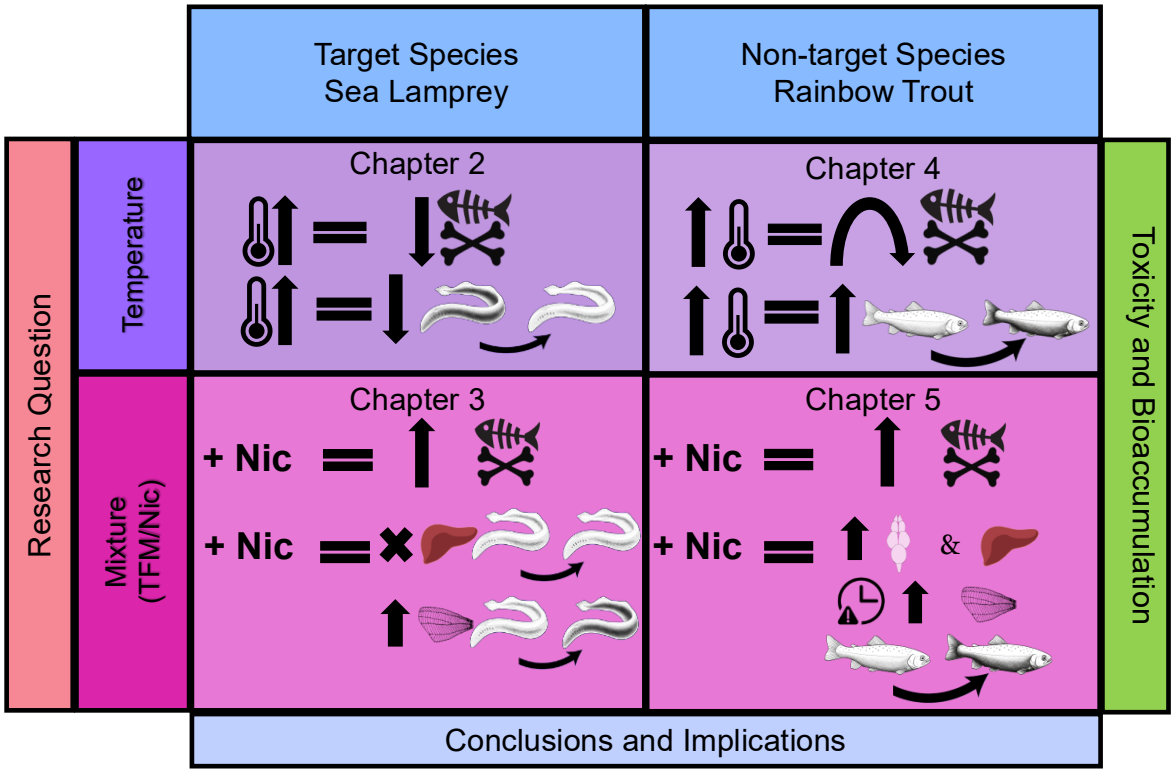


Figure 6.1 Graphical representation of thesis chapters with results. Each data chapter focuses on one research question and one species and uses toxicity and bioaccumulation measurements to evaluate the effects on TFM toxicity. Toxicity and bioaccumulation measurements were used to assess the effects of either temperature or mixture effects on each species separately. Increasing water temperature decreases TFM toxicity, most likely due to increased detoxification capacity. While addition of niclosamide increases TFM toxicity, most likely due to competitive inhibition. Both temperature and mixtures effects were species specific.

6.2 Temperature-dependent TFM toxicity: Divergent patterns in sea lamprey and rainbow trout

A central finding of this thesis is that temperature modifies TFM toxicity in a markedly species-specific manner. In larval sea lamprey (Chapter 2), 12-h LC_{50} values increased by approximately 1.5-fold across the temperature range tested (7-28°C), reflecting a consistent, stepwise increase in TFM tolerance at higher temperatures, consistent with prior observations

across a similar temperature range (6-24°C; Muhametsafina et al., 2019) and with field and laboratory studies demonstrating that observed minimal lethal concentrations (MLCs) routinely exceed pH-alkalinity chart predictions at warmer temperatures (Hlina et al., 2021; Scholefield et al., 2008; Schueller et al., 2024). This pattern was accompanied by differences in TFM bioaccumulation when lamprey at different temperatures were exposed to an identical TFM concentration, with those acclimated to 24°C having TFM concentrations that were approximately 30% lower in the muscle and 36% lower in the liver, relative to larvae held at cooler temperatures (Mitrovic et al., 2026a; Chapter 2). As depicted in the accompanying model (Figure 6.2), these findings suggest that rates of TFM uptake by larval sea lamprey increase with temperature, but so do rates of TFM elimination, with rates of elimination exceeding rates of uptake in warmer water. The net effect is greater TFM tolerance in warmer compared to colder waters.

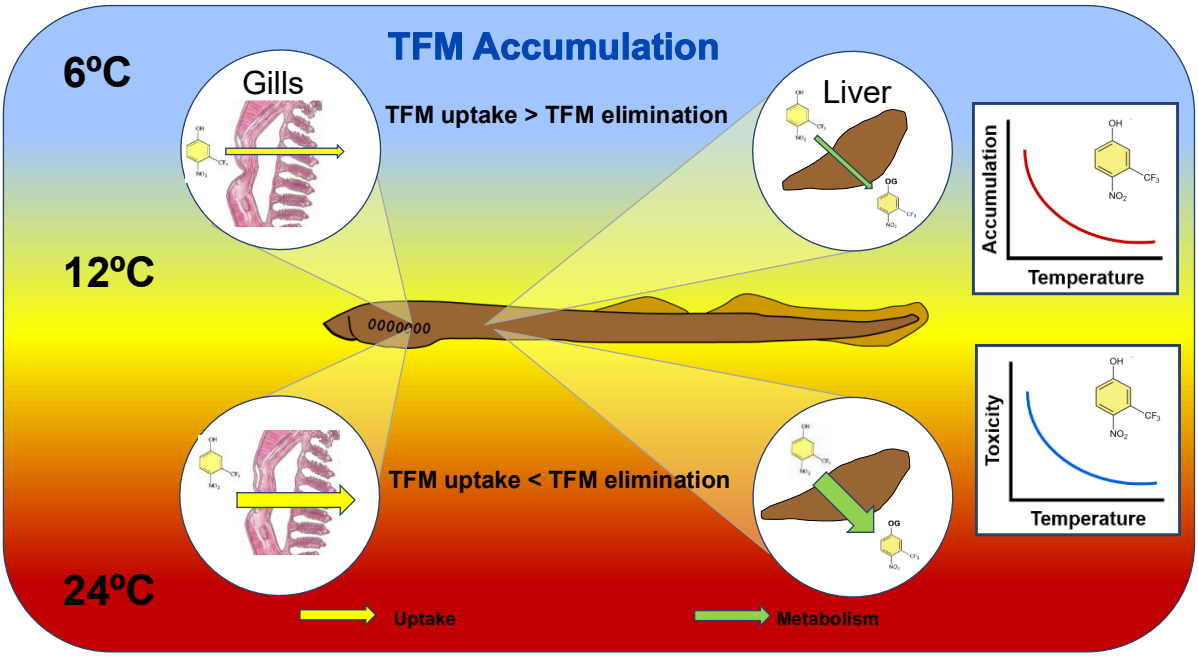


Figure 6.2. Model describing how warmer water temperatures enhance the TFM (3-trifluoromethyl-4-nitrophenol) tolerance of larval sea lamprey. At low temperature (6°C; blue shading), the rate of TFM uptake across the gill is relatively low but exceeds the rate of detoxification via Phase II metabolism of TFM to TFM-glucuronide (TFM-OG) by the liver, leading to net TFM accumulation and toxicity. As temperature increases (12-24°C; yellow-red shading), increased activities of detoxification enzymes (e.g., UDP-glucuronosyltransferase) enhance liver metabolism of TFM, increasing the rates of TFM elimination relative to uptake (uptake < elimination). The result is lower TFM accumulation and toxicity. Yellow arrows denote uptake; green arrows denote hepatic metabolism (glucuronide conjugation, TFM-OG); arrow width is proportional to the relative rate of TFM uptake and biotransformation. Attribution - Gill diagram including pore and inner gills in pouch - https://commons.wikimedia.org/wiki/File:Lamprey_Larva_x_sect_pharynx_labelled.png

Estimates of steady-state concentration (C_{SS}) and elimination half-life ($t_{1/2}$), derived using a one-compartment pharmacokinetic model (Gupta, 2016; Newman, 2020), further supported the interpretation that larval sea lamprey possess a greater capacity for TFM elimination at warmer temperatures. C_{SS} in the liver at 24°C was approximately 32 - 44% lower than at cooler temperatures, and $t_{1/2}$ of hepatic TFM elimination was approximately 7-

fold shorter at 24°C than at 6°C (Mitrovic et al., 2026a; Chapter 2). This pattern is consistent with the Q_{10} effect, whereby enzymatic reaction rates approximately double for every 10°C increase in temperature within the physiologically relevant range (Běhrádek, 1930; Mundim et al., 2020).

In contrast, rainbow trout (Chapter 4) exhibited a biphasic relationship between temperature and TFM toxicity. TFM toxicity was greatest at intermediate temperatures (11°C and 16°C) relative to the coldest exposure (6°C), before the 12-h LC_{50} increased at 21°C, indicating lower toxicity at the warmest temperature. This biphasic pattern of toxicity is difficult to reconcile with a straightforward Q_{10}-driven increase in detoxification capacity, and the tissue bioaccumulation data from Chapter 4 complicate interpretation further. At 21°C, the temperature at which trout showed greater tolerance, liver and muscle TFM concentrations were paradoxically elevated rather than reduced, suggesting a different bioaccumulation-toxicity relationship from that observed in sea lamprey (Mitrovic et al., 2026a; Chapter 2). This temperature-TFM relationship in rainbow trout is modelled in Figure 6.3, where TFM toxicity is low in cooler waters, likely due to low rates of TFM uptake, with toxicity increasing at moderate temperatures. The decrease in toxicity at 21°C could be related to other physiological processes in addition to detoxification pathways, such as an increased capacity to use anaerobic pathways of ATP production, such as glycolysis or phosphocreatine, to compensate for decreased ATP production caused by TFM, but further study is needed.

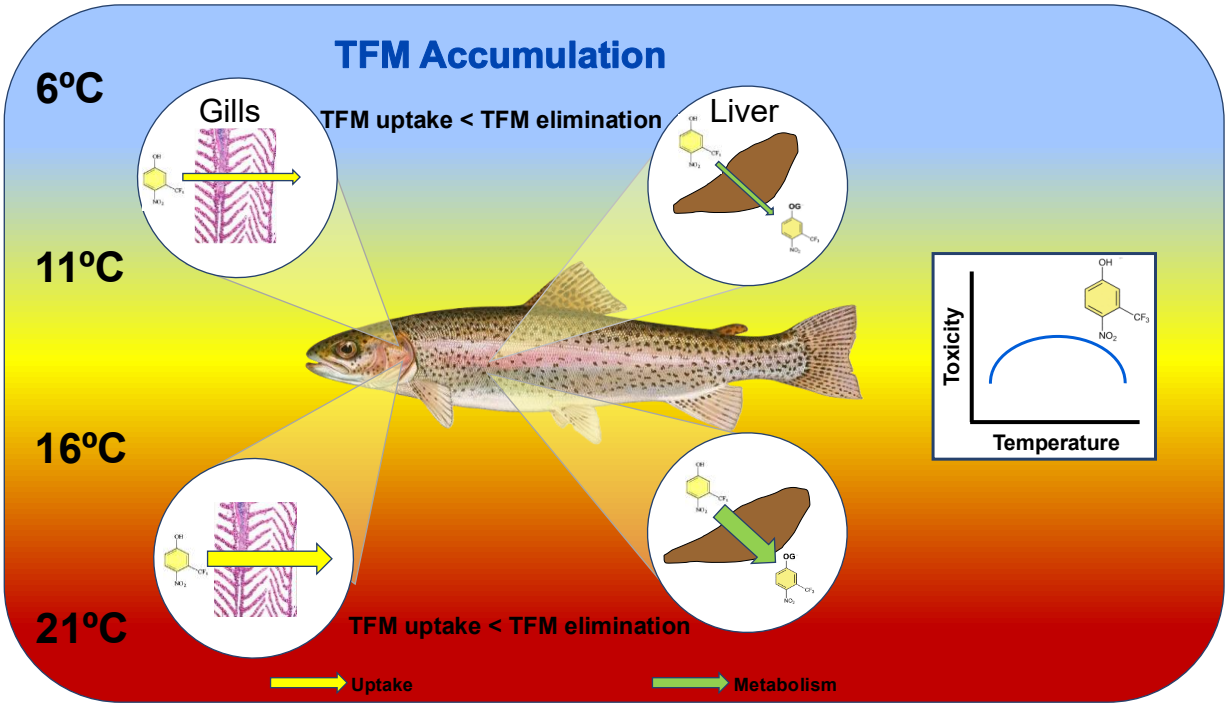


Figure 6.3. Working model illustrating the biphasic relationship between temperature and tolerance to TFM (3-trifluoromethyl-4-nitrophenol) in non-target rainbow trout. At low temperature (6°C; blue shading), TFM uptake rates are low due to lower overall metabolic demands and correspondingly low gill ventilation rates, resulting in slower TFM accumulation. However, metabolic ATP demands are also lower, leading to relatively high TFM tolerance at colder temperatures. At elevated water temperature (21°C; red shading), rates of TFM uptake are greater. However, rates of TFM metabolism in the liver are slightly greater, leading to higher rates of TFM elimination that exceed TFM uptake rates, lowering TFM toxicity. At intermediate temperatures (11-16°C; yellow-orange shading), rates of TFM accumulation are greater than rates of elimination, leading to higher toxicity. Yellow arrows denote uptake; green arrows denote hepatic metabolism (glucuronide conjugation, TFM-OG); arrow width is proportional to the relative rate of TFM uptake and biotransformation. Attribution - Gill diagram: https://www.researchgate.net/figure/Repair-of-the-gill-tissue-of-a-rainbow-trout-exposed-to-80-mg-L-of-chloramine-T-and_fig4_254319574

Taken together, the temperature data from Chapters 2 and 4 suggest that the metabolic basis of TFM selectivity is not simply a quantitative difference in biotransformation rate between species but reflects qualitative differences in the thermal sensitivity of the underlying enzymatic pathways. Sea lamprey appear to have a more

thermally labile and thus more temperature-responsive Phase II detoxification system, consistent with the proposal that temperature-dependent increases in UDPGT and/or phenol sulphotransferase activity drive enhanced TFM elimination in warmer water (Bussy et al., 2018a, b; Mitrovic et al., 2026a, b). In contrast, the relationship in rainbow trout is confounded by competing physiological factors, including energy substrate availability and the thermal tolerance limits of the species itself. The convergence of these findings with broader observations that tolerance to structurally related phenolic uncouplers, including phenol (Patra et al., 2015), 2,4-dinitrophenol (Howe et al., 1994), and pentachlorophenol (Wang et al., 2019) also increases with temperature in fishes before declining as thermal optima are exceeded, suggests that temperature-dependent modulation of Phase II biotransformation may be a general feature of phenol handling in aquatic vertebrates.

6.3 TFM-Niclosamide interactions: Concentration dependence of mixture toxicity

Chapters 3 and 5 demonstrated that the addition of niclosamide (at 1% of the total TFM formulation) modifies TFM toxicity in a manner that is both concentration-dependent and species-specific. In larval sea lamprey (Chapter 3), exposure to TFM/1% niclosamide mixtures produced antagonistic effects (less than additive toxicity) at total exposures below 1 toxic unit (TU, defined as the 12-h LC_{50}), but synergistic effects (greater than additive toxicity) when total exposure exceeded 1 TU (Mitrovic et al., 2026b; Chapter 3). These concentration-dependent interactions were assessed using the concentration addition model (Norwood et al., 2003; Playle, 2004), in which toxicity is expressed as the sum of toxic units contributed by each compound relative to its individual LC_{50}. This transition from antagonism to synergism with increasing exposure concentration is consistent with earlier observations of concentration-dependent mixture interactions between TFM and niclosamide in rainbow trout (Hepditch et al., 2021) and with the hypothesis that TFM and niclosamide compete for shared Phase II detoxification pathways in the liver that become saturated at higher exposure concentrations (Bussy et al., 2018a, b; Mitrovic et al., 2026b; Wilkie et al., 2019). Earlier studies in multiple fish species had reported strictly additive or antagonistic

interactions between these compounds (Bills & Marking, 1976; Dawson et al., 1977; Marking & Bills, 1985), while others had reported synergism (Howell et al., 1964), but none had systematically characterized the concentration-dependence of these interactions in sea lamprey using the toxic unit framework.

The mechanistic interpretation of these mixture effects is strongly supported by the tissue bioaccumulation data. In sea lamprey (Chapter 3), co-exposure with niclosamide resulted in 1.9- 2.5-fold greater TFM accumulation specifically in muscle tissue compared to TFM-only exposures, without a corresponding increase in liver concentrations (Mitrovic et al., 2026b). This pattern suggests that niclosamide competitively inhibits TFM metabolism at the level of Phase II conjugation enzymes, most likely UDPGTs and sulphotransferases, both known to be involved in TFM biotransformation (Bussy et al., 2018a, b; Kane et al., 1994; Lech & Statham, 1975), thereby reducing TFM clearance and increasing its whole-body retention. The absence of increased hepatic TFM accumulation despite presumed competitive inhibition of hepatic Phase II enzymes may reflect saturation of hepatic binding sites with niclosamide itself, or the kinetics of hepatic extraction and redistribution into peripheral tissues. The use of LC-MS/MS methods capable of resolving parent TFM from its glucuronide and sulphate conjugates confirmed that the elevated muscle concentrations reflected accumulation of parent TFM rather than metabolites (Mitrovic et al., 2026b), providing compelling evidence that niclosamide impairs TFM elimination. A model of TFM-niclosamide interactions is therefore proposed in which the biotransformation of TFM by the liver is impaired in the presence of niclosamide, leading to greater TFM bioaccumulation and greater toxicity (Figure 6.4). It is also important to note that the concentrations at which synergism occurred in these sea lamprey experiments, corresponded closely to those used in field treatments (Bills et al., 2003; Mitrovic et al., 2026b), underscoring the applied significance of these mechanistic findings.

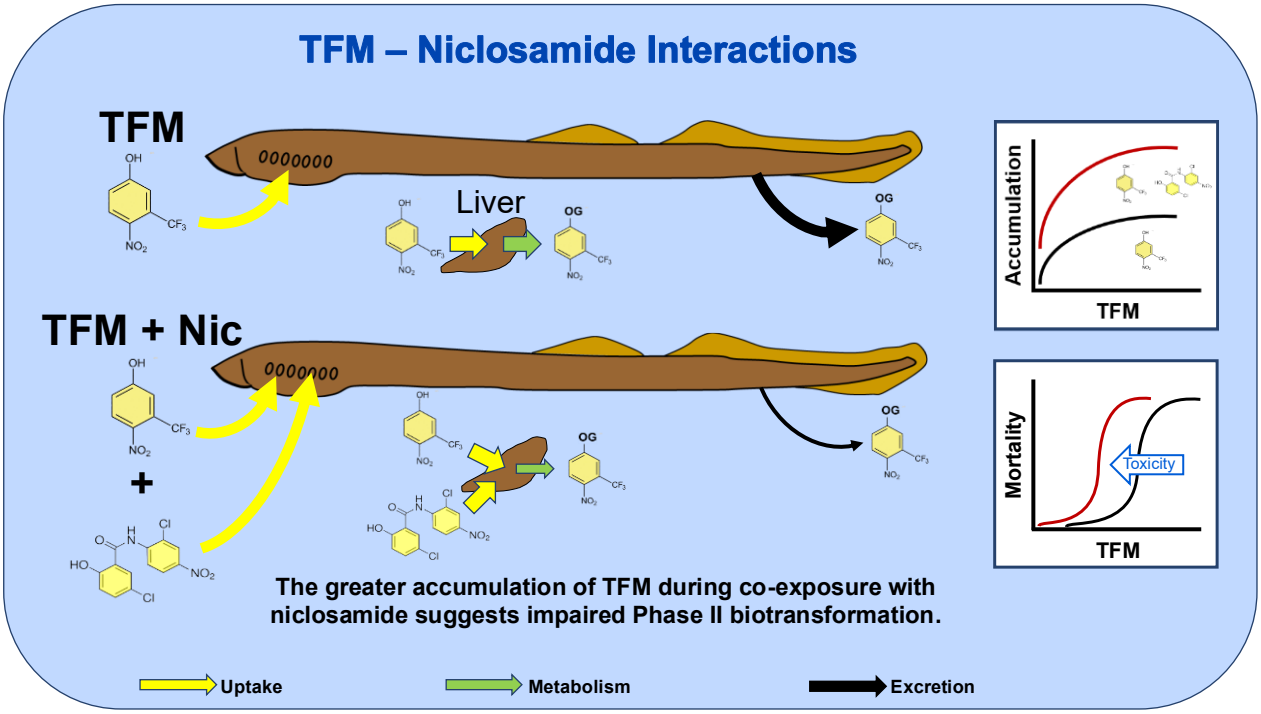


Figure 6.4. Working model of toxic interaction between TFM (3-trifluoromethyl-4-nitrophenol) and niclosamide (2′,5-dichloro-4′-nitrosalicylanilide) in larval sea lamprey. Following its uptake across the gills (yellow arrow; top panel), TFM is metabolized to TFM-glucuronide (TFM-OG; green arrow), and perhaps other metabolites, in the liver and subsequently excreted (black arrow). When co-applied with niclosamide (bottom panel), TFM metabolism in the liver is likely inhibited by niclosamide, leading to decreased TFM clearance, greater accumulation and greater toxicity. Note that arrow thickness is proportional to the relative rates of detoxification or elimination.

In rainbow trout (Chapter 5), TFM/niclosamide mixture exposures produced antagonistic effects when the combined, predicted toxicity was < 1.5 TU, with strictly additive interactions at 1.5 TU of exposure. These findings are more or less consistent with the strictly additive to less-than-additive interactions reported at < 1 TU concentrations in rainbow trout (Hepditch et al., 2021) and in less sensitive non-target species such as lake sturgeon, yellow perch, and bluegill at species-specific exposure concentrations (Boogaard et al., 2003). However, the tissue bioaccumulation data from Chapter 5 reveal a markedly different pattern of TFM distribution in this species compared to the sea lamprey. Co-

exposure with niclosamide produced significant increases in both liver and brain TFM concentrations in rainbow trout, while muscle TFM concentrations were not significantly altered. This distribution pattern is opposite of that observed in sea lamprey, where muscle accumulation was the dominant response (Mitrovic et al., 2026b). Both TFM and niclosamide are detoxified via Phase II metabolic pathways, including UGT-mediated glucuronidation and phenol sulphotransferase reactions (Birceanu et al., 2021; Bussy et al., 2018a, b; Wilkie et al., 2019), but the relative contributions of individual enzyme isoforms and their tissue-specific expression patterns appear to differ between species (Kane et al., 1994; Lawrence et al., 2023). The accumulation of TFM in the brain of trout during mixture exposure is particularly significant, given that both TFM and niclosamide disrupt mitochondrial ATP production by uncoupling oxidative phosphorylation (Birceanu et al., 2011; Borowiec et al., 2022), making brain energy supply especially vulnerable to combined exposure.

A further distinguishing feature of the rainbow trout response to mixture exposure was the rapid and pronounced accumulation of niclosamide in liver, brain, and muscle tissues, increasing more than 20-fold in muscle at the lowest niclosamide concentration tested in the TFM/1% Nic mixture relative to niclosamide alone. This contrasts with the more limited niclosamide bioaccumulation observed in sea lamprey (Mitrovic et al., 2026b). Because niclosamide is a considerably more potent uncoupler of mitochondrial respiration than TFM (Borowiec et al., 2022), these findings suggest that the toxic contribution of niclosamide per se may be greater in rainbow trout than previously though. Furthermore, the reduction in the TFM MLC observed in the presence of 1% niclosamide appears to be substantially greater in TFM-tolerant species than in sea lamprey. In sea lamprey, niclosamide may lower MLC by up to 40% with similar or greater reductions in the MLC of non-target species such as rainbow trout (64%), yellow perch (68%), and bluegill (59%) as well as more TFM-sensitive species such as lake sturgeon (38-39%; Boogaard et al., 2003; Hepditch et al., 2021; Mitrovic et al., 2026b). As a consequence, these findings suggest that non-target species, especially more TFM sensitive species (e.g., catfishes, sturgeon) may face

disproportionately elevated risks of mortality during TFM/niclosamide mixture treatments, compared to more tolerant fishes (e.g., bluegill, trout). More tolerant species are less likely to face an elevated risks during routine TFM-niclosamide applications, because their inherently high baseline TFM tolerance provides a sufficient buffer against this mixture-driven potentiation of toxicity.

6.4 Implications for the Sea Lamprey Control Program & beyond

The enhanced tolerance of sea lamprey to TFM exposure at warmer temperatures as demonstrated in the presented thesis, provide additional support to the conclusions of earlier studies that speculated that their greater TFM tolerance during the summer months was temperature related (Hlina et al., 2021; Muhametsafina et al., 2019). The evidence from these and the present study also suggest that that future climate-driven increases in water temperature of the rivers and streams infested with sea lamprey may complicate and potentially compromise the effectiveness of sea lamprey control efforts in the Great Lakes (Lennox et al., 2020; Wilkie, 2025). These conclusions are also anchored by observations that TFM accumulation was lower in the larval sea lamprey that exhibited the greatest tolerance to TFM at warm temperatures. This evidence suggests that water temperature should be considered when determining TFM treatment concentrations, as is currently done for pH and alkalinity (Bills et al., 2003; Sullivan et al., 2021), to account for the greater tolerance of larval sea lamprey to TFM in warmer waters.

Predicting how non-target organisms might be affected by exposure to higher temperature in warmer water may be more challenging, however. Although most teleosts and invertebrates of concern (e.g., native mussels) are less sensitive to TFM than sea lamprey (Boogaard et al., 2003; Wilkie et al., 2019), their responses to TFM at elevated temperatures when metabolic demand is higher and oxygen availability is reduced remain poorly understood. Indeed, the present thesis was notable for the markedly greater effects that temperature had on the TFM tolerance of larval sea lamprey compared to rainbow trout (Chapter 2 vs Chapter 4). Additional research evaluating how other fishes respond to TFM under warmer conditions will therefore be necessary to inform strategies that mitigate or

prevent non-target effects or mortality. These strategies may include restricting TFM applications to times of the year when water temperatures are cooler or depending on the species present. Another option could be to more frequently apply niclosamide-TFM mixtures during lampricide treatment in warmer waters which would lower TFM exposure concentrations.

The concentrations at which TFM and niclosamide exhibit synergistic interactions in larval sea lamprey (based on the 12-h LC_{50}) align with those typically used in field treatments (Bills et al., 2003; Boogaard et al., 2003) and could have direct implications for the Sea Lamprey Control Program (SLCP). The improved understanding of the physiological effects of binary lampricide mixtures, provided in this thesis, will enable the SLCP to more effectively control the sea lamprey populations within the Great Lakes. Specifically, the tissue-specific accumulation patterns identified for TFM and niclosamide of single compound versus mixture exposures provide a mechanistic explanation for differences in target-organ toxicity, which can inform how mixture ratios can be formulated to preserve efficacy against sea lamprey without disproportionately affecting non-target species. A more mechanistic perspective would allow for the development of increasingly targeted treatment protocols (very precise formulation ratios) that optimize efficacy while limiting unnecessary chemical exposure. By carefully adjusting mixture ratios, it may be possible to maintain effective sea lamprey control while reducing the total chemical burden introduced into treated systems. The species differences in Phase II biotransformation (UDPGT and sulphotransferase mediated glucuronidation and sulphation) proceeds at different rates and capacities between sea lamprey and rainbow trout further clarifies the biochemical basis of selective toxicity, giving the SLCP physiological rationale for why current TFM-niclosamide ratios are effective and a foundation for optimizing them further. Finally, delineating where toxic unit interactions shift from synergistic to antagonistic across the mixture's concentration range allows the SLCP to identify the mixture ratios that maximize lethality toward sea lamprey while staying within safety margins for co-occurring fish populations, supporting more precise, evidence-based treatment decisions in the field.

Beyond its application in sea lamprey control, the simple phenolic structure of TFM makes it a useful model compound for examining the uptake, distribution, detoxification, and elimination of other toxic phenols in aquatic ecosystems. Many phenolic compounds are highly toxic at low concentrations and include known carcinogens (e.g., pentachlorophenol (International Agency for Research on Cancer, 2025) or organochlorine pesticides (du Plessis et al., 2023)), endocrine disruptors such as bisphenol A (BPA; Surana et al., 2022), and compounds capable of inducing genetic malformations (du Plessis et al., 2023). Further investigation into the pharmacokinetics of TFM and related phenols at different temperatures is also needed to better understand how temperature influences acute and chronic toxicity in different aquatic organisms, which will be particularly important as climate change is likely to elevate average and peak water temperatures in many habitats (Collingsworth et al., 2017; Meisner et al., 1987).

A better mechanistic knowledge of TFM and niclosamide uptake kinetics, biotransformation, and elimination may also help clarify general principles governing the toxicity of other phenol compounds, alone or in mixtures, in different groups of aquatic organisms. Such knowledge is essential for predicting chemical interactions in natural aquatic ecosystems, where organisms are rarely exposed to single compounds in isolation and where interspecific variability can strongly influence ecological risk outcomes.

6.5 Limitations

The presented thesis provided evidence that, at least in sea lamprey, elevated water temperatures may increase detoxification capacity, as indicated by higher 12-h LC_{50} values for TFM and lower TFM tissues accumulation, thereby reducing TFM toxicity. The binary mixture interactions between TFM and niclosamide were species-specific, with synergistic effects observed in sea lamprey but not in rainbow trout. The higher TFM tissue accumulation in the presence of niclosamide suggested competitive inhibition of Phase II enzymes of biotransformation. However, due to the lack of TFM and niclosamide metabolic standards (e.g., TFM-glucuronide, Nic-glucuronide), tissue analysis had to focus only on the

parent (TFM and niclosamide) compounds and not on the Phase II metabolic products, which limited the interpretation.

Due to the very small liver sample sizes in sea lamprey (< 10 mg) which were used in their entirety in LC-MS/MS tissue analysis, energy metabolites (lactate, glycogen, ATP and phosphocreatine (PCr)) were not measured in this tissue. In all trout exposure experiments (both temperature and TFM-niclosamide mixture), sufficient liver and muscle tissue was available and collected to allow for analysis of energy metabolites, whose levels might indicate TFM effects on glucuronidation. Future studies should consider increasing sample sizes to allow for additional endpoints, although the availability of test organisms limits the scope of possible experiments. Although distinct differences were seen in the 12-h LC_{50} for TFM, additional organisms and replication would reduce uncertainty.

The combined effects of increasing water temperature and the presence of niclosamide on energy stores in rainbow trout warrant further investigation because both stressors independently increase metabolic demand while potentially impairing the compensatory mechanisms rainbow trout rely on to meet that demand, and their combined effect on already temperature-limited energy reserves could push sublethal toxicity into lethal territory in ways that single-stressor studies would fail to predict. Complementary data on energy stores from similar experiments in sea lamprey would also be valuable. Together, these data would provide important insight into how environmental conditions influence TFM toxicity.

Another limitation of the current thesis is the specific focus on rainbow trout as the non-target representative fish species. While trout is a common model used in many different toxicological and physiological studies, its TFM tolerance is not among the highest. Fish such as bluegill (*Lepomis macrochirus)* or American eel (*Anguilla rostrata*) have much higher lampricide tolerances, thus temperature and mixture experiments on these fish might provide additional clarification as to the environmental effects on TFM toxicity. Other more TFM sensitive fish species, such as lake sturgeon, designated as threatened within the Great Lakes-St. Lawrence drainage (COSEWIC, 2017; Hayes & Caroffino, 2012), could be

disproportionately impacted by lampricide treatments as larval and young of the year lake sturgeon share the larval sea lamprey habitats and MLC. Exploring the physiological differences between these species would advance our understanding of how diverse fish respond to toxic chemicals and their mixtures.

6.6 Outstanding questions

The development of methods for quantifying the tissue concentrations of TFM and niclosamide metabolites would greatly enhance our ability to understand how lampricides are detoxified at different temperatures and why niclosamide enhances TFM toxicity, when applied in mixtures. Measurements of lampricide metabolic byproducts could be more directly linked to changes in TFM toxicity to detoxification capacity or to the competitive inhibition of metabolic enzymes in TFM and niclosamide mixtures. Thus, an immediate future research priority should be the development of a method (e.g., LC-MS/MS) to quantify tissue Phase II TFM biotransformation products, such as TFM-glucuronide and TFM-sulphate. Similarly, quantification of niclosamide biotransformation products may allow measurement of Phase II detoxification rates of niclosamide and, in turn, possibly explain the effects of the TFM/niclosamide mixture on lampricide metabolism.

The sulphation of TFM and niclosamide in fishes remains poorly understood, and method development focusing on TFM-sulphate and niclosamide-sulphate will provide additional information about the importance of phenol sulphotransferases and the difference in reliance of each species (sea lamprey vs. rainbow trout) on this Phase II metabolic pathway. Additionally, LC-MS/MS method development can be extended to the TFM and niclosamide byproducts of the Phase I biotransformation. Exploring these pathways of lampricide metabolism and the difference between sea lamprey and non-target species may provide an additional layer of understanding about mechanisms of lampricide toxicity.

Currently, there are no isotopically labelled standards available for TFM, which limits the quality of existing methods for quantifying TFM tissue bioaccumulation. Internal standards provide increased accuracy by correcting for analyte losses and signal fluctuations,

resulting in a more reliable quantitative dataset. Development of a TFM standard that can account for sample preparation variation, instrument drift and matrix-induced ionization effects would greatly improve current and future quantifications of TFM tissue bioaccumulation, thus providing more robust data interpretation leading to more conclusive evaluations of TFM toxicity and species-specific sensitivity.

The lampricide tissue bioaccumulation results could be further supported by uptake and distribution/elimination experiments using radio-labelled TFM and niclosamide compounds (c.f. Hlina et al., 2017; Tessier et al., 2018) at different water temperatures and/or in the presence of niclosamide. Quantifying the appearance of radio-labelled lampricides in fish bodies to measure uptake rates or appearance in the water to measure rates of elimination rates could help with the interpretation of temperature effects on TFM pharmacokinetics and TFM toxicity. Similar experiments could be conducted with niclosamide, in the presence or absence of TFM to learn more about how these two compounds interact at the gill and in the body.

Enzyme kinetic analyses and quantification of mRNA and protein expression would be another avenue worth exploring to further characterize how TFM tolerance increases in warmer waters. Applying enzyme kinetic analysis, to determine how maximum enzyme reaction velocity changes relative to enzyme affinities of TFM metabolized enzymes (e.g. UDPGTs, sulphotransferases) could be used to compare how enzyme efficiency changes with temperature (e.g. Kane et al., 1993, 1994). Similar approaches could be used to better characterize how TFM and niclosamide interact with the biotransformation enzymes (UDPGTs or phenol sulphotransferases) at the molecular level to better understand how these two lampricides interact in both sea lamprey and non-target fishes.

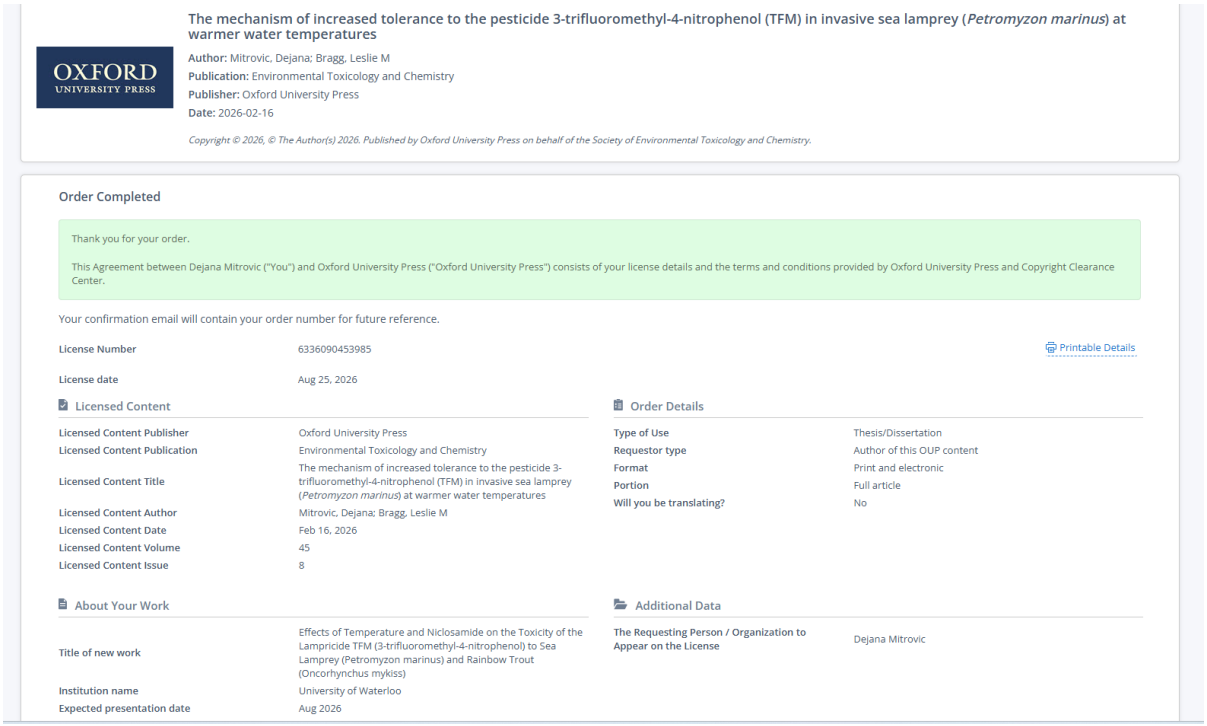
Recently, Lawrence et al. (2023), using RNA sequencing, showed that sea lamprey express fewer isoforms of the UDPGT enzyme compared to bluegill, adding to a better understanding of the limited TFM detoxification capacity of sea lamprey. The characterization of changes in detoxification enzyme activity and expression during exposure to TFM alone and in combination with niclosamide would further clarify the mechanisms

driving observed increases in TFM toxicity when mixed with niclosamide. Quantifying how the mRNA or protein expression of UDPGT and phenol sulphotransferase enzymes change with increasing water temperature also merits further study.

6.7 Conclusion

This thesis has implications for both aquatic toxicology and the control of invasive sea lamprey populations in the Great Lakes. By systematically integrating toxicity assessments with tissue bioaccumulation measurements across temperature gradients and TFM-niclosamide mixture exposure regimes, this research was able to provide mechanistic insight into how temperature and niclosamide influence TFM toxicity in both target sea lamprey and non-target rainbow trout. Furthermore, by demonstrating why TFM tolerance increases with temperature in sea lamprey, this work provides new critical, evidence-based insight into how the Sea Lamprey Control Program in the Great Lakes could be negatively impacted by rising water temperatures, both seasonally and due to environmental change. This integrated toxicity-bioaccumulation approach also led to better characterization of concentration-dependent interactions between TFM and niclosamide in sea lamprey and rainbow trout using the toxic unit framework. Finally, this research provide a framework around which the effects of temperature on the sensitivity of aquatic organisms to other, more toxic, phenolic compounds might be studied to better understand how such contaminants affect aquatic ecosystems.

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The mechanism of increased tolerance to the pesticide 3-trifluoromethyl-4-nitrophenol (TFM) in invasive sea lamprey (*Petromyzon marinus*) at warmer water temperatures

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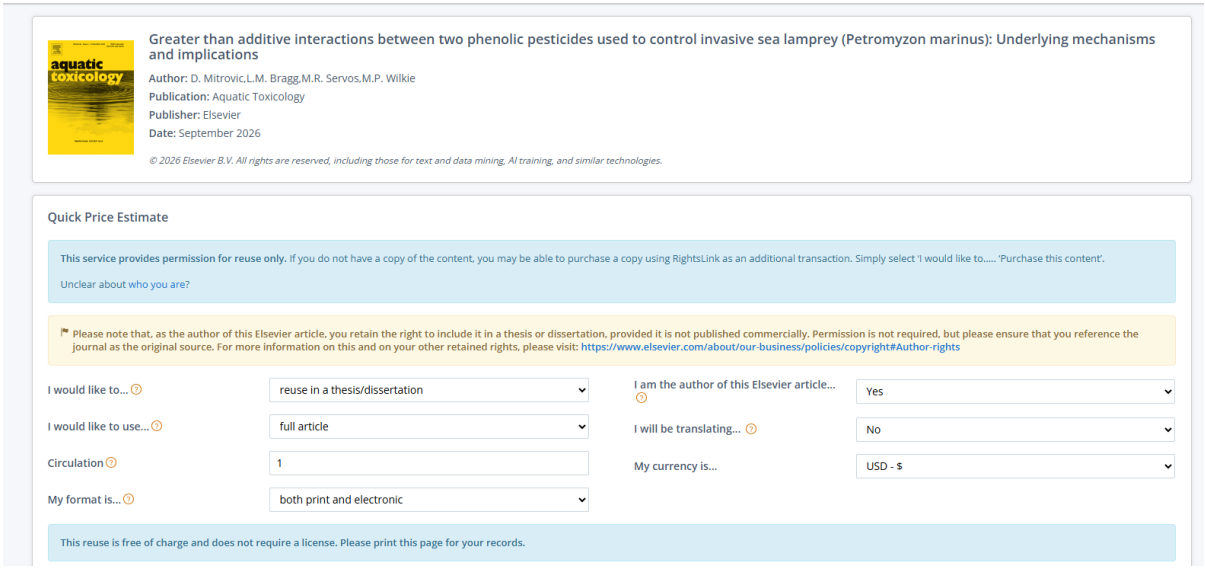
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Chapter 3



Greater than additive interactions between two phenolic pesticides used to control invasive sea lamprey (Petromyzon marinus): Underlying mechanisms and implications

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References

Alasadi, A., Chen, M., Swapna, G. V. T., Tao, H., Guo, J., Collantes, J., Fadhil, N., Montelione, G. T., & Jin, S. (2018). Effect of mitochondrial uncouplers niclosamide ethanolamine (NEN) and oxyclozanide on hepatic metastasis of colon cancer. *Cell Death & Disease*, 9, 1-15. http://doi:https://doi.org/10.1038/s41419-017-0092-6

Allen, J. L., Dawson, V. K., & Hunn, J. B. (1979). Excretion of the lampricide Bayer 73 by rainbow trout [Report](667). Aquatic Toxicology, Issue. T. American Society for & Materials. http://pubs.er.usgs.gov/publication/2001080

Applegate, V. C., Howell, J. H., Moffett, J. W., Johnson, B. G. H., & Smith, M. A. (1961). Use of 3-trifluoromethyl-4-nitrophenol as a selective sea lamprey larvicide. *Great Lakes Fishery Commission Technical Report No. 1*. Great Lakes Fishery Commission.

Armitage, J. M., Erickson, R. J., Luckenbach, T., Ng, C. A., Prosser, R. S., Arnot, J. A., Schirmer, K., & Nichols, J. W. (2017). Assessing the bioaccumulation potential of ionizable organic compounds: current knowledge and research priorities. *Environmental Toxicology and Chemistry*, 36(4), 882-897. https://doi.org/10.1002/etc.3680

ASTM International. (2014). Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates and amphibians. *ASTM International*. https://doi.org/10.1520/E0729-96R14

Bánhegyi, G., Garzó, T., Antoni, F., & Mandl, J. (1988). Glycogenolysis - and not gluconeogenesis - is the source of udp-glucuronic acid for glucuronidation. *Biochimica et Biophysica Acta (BBA): General Subjects*, 967(3), 429-435. https://doi.org/10.1016/0304-4165(88)90106-7

Beamish, F. W. H., & Potter, I. C. (1975). Biology of anadromous sea lamprey (*Petromyzon marinus*) in New Brunswick. *Journal of Zoology*, 177, 57-72. https://doi.org/10.1111/j.1469-7998.1975.tb05970.x

Běhrádek, J. (1930). Temperature coefficients in biology. Biological Reviews, 5(1), 30–58. https://doi.org/10.1111/j.1469-185X.1930.tb00892.x

Bergmeyer, H. L. (1983). Methods of enzymatic analysis. *Academic Press*, New York.

Bernhardt, R. (2006). Cytochromes P450 as versatile biocatalysts. *Journal of Biotechnology*, 124(1), 128-145. https://doi.org/10.1016/j.jbiotec.2006.01.026 Bills, T. D., & Marking, L. L. (1976). Toxicity of 3-trifluoromethyl-4-nitrophenol (TFM), 2′5-dichloro-4′-nitrosalicylanilide (Bayer 73) and a 98:2 mixture to fingerlings of seven fish species and to eggs and fry of coho salmon. *Investigations in Fish Control No. 69*. US Fish and Wildlife Service.

Bills, T. D., Boogaard, M. A., Johnson, D. A., Brege, D. C., Scholefield, R. J., Westman, R. W., & Stephens, B. E. (2003). Development of a pH/alkalinity treatment model for applications of the lampricide tfm to streams tributary to the Great Lakes. *Journal of*

Great Lakes Research, 29 (suppl.1), 510-520. https://doi.org/10.1016/S0380-1330(03)70512-7

Birceanu, O., McClelland, G. B., Wang, Y. S., & Wilkie, M. P. (2009). Failure of ATP supply to match ATP demand: the mechanism of toxicity of the lampricide, 3-trifluoromethyl-4-nitrophenol (TFM), used to control sea lamprey (*Petromyzon marinus*) populations in the Great Lakes. *Aquatic Toxicology*, 94(4), 265-274. https://doi.org/10.1016/j.aquatox.2009.07.012

Birceanu, O., McClelland, G. B., Wang, Y. S., Brown, J. C. L., & Wilkie, M. P. (2011). The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) uncouples mitochondrial oxidative phosphorylation in both sea lamprey (*Petromyzon marinus*) and TFM-tolerant rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*, 153, 342-349. https://doi.org/10.1016/j.cbpc.2010.12.005

Birceanu, O., Sorensen, L. A., Henry, M., McClelland, G. B., Wang, Y. S., & Wilkie, M. P. (2014). The effects of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) on fuel stores and ion balance in a non-target fish, the rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*, 160(1), 30-41. https://doi.org/10.1016/j.cbpc.2013.10.002

Birceanu, O., Tessier, L. R., Huerta, B., Li, W., McDonald, A., & Wilkie, M. P. (2021). At the intersection between toxicology and physiology: what we have learned about sea lampreys and bony fish physiology from studying the mode of action of lampricides. *Journal of Great Lakes Research*, 47, S673-S689. https://doi.org/10.1016/j.jglr.2021.07.007

Boogaard, M. A., Bills, T. D., & Johnson, D. A. (2003). Acute toxicity of TFM and a TFM/niclosamide mixture to selected species of fish, including lake sturgeon (*Acipenser fulvescens*) and mudpuppies (*Necturus maculosus*), in laboratory and field exposures. *Journal of Great Lakes Research*, 29(suppl 1), 529-541. https://doi.org/10.1016/S0380-1330(03)70514-0

Borowiec, B. G., Docker, M. F., Johnson, N. S., Moser, M. L., Zielinski, B., & Wilkie, M. P. (2021). Exploring the physiology of lampreys to refine methods of control and conservation. *Journal of Great Lakes Research*, 47, S723-S741. https://doi.org/10.1016/j.jglr.2021.10.015

Borowiec, B. G., Birceanu, O., Wilson, J. M., McDonald, A. E., & Wilkie, M. P. (2022). Niclosamide is a much more potent toxicant of mitochondrial respiration than TFM in the invasive sea lamprey (*Petromyzon marinus*). *Environmental Science and Technology*, 56(8), 4970-4979. https://doi.org/10.1021/acs.est.1c07117

Bryan, M. B., Zalinski, D., Filcek, K. B., Libants, S., Li, W., & Scribner, K. T. (2005). Patterns of invasion and colonization of the sea lamprey (*Petromyzon marinus*) in

North America as revealed by microsatellite genotypes. *Molecular Ecology*, 14(12), 3757-3773. https://doi.org/10.1111/j.1365-294X.2005.02716x

Bull, J. M., & Morris, R. (1967). Studies on freshwater osmoregulation in the ammocoete larva of *Lampetra planeri* (Bloch). I. ionic constituents, fluid compartments, ionic compartments, and water balance. *Journal of Experimental Biology*, 47(3), 485-494. https://doi.org/10.1242/jeb.47.3.485

Busacker, G. B., Adelman, I. R., & Goolish, E. M. (1990). Growth. In C. B. Schreck & P. B. Moyle (Eds.), *Methods for fish biology* (pp. 363–387). American Fisheries Society. https://doi.org/10.47886/9780913235584.ch11

Bussy, U., Chung-Davidson, Y. W., Buchinger, T., Li, K., Smith, S. A., Jones, A. D., & Li, W. (2018a). Metabolism of a sea lamprey pesticide by fish liver enzymes Part A: identification and synthesis of TFM metabolites. *Analytical and Bioanalytical Chemistry*, 410(6), 1749-1761. https://doi.org/10.1007/s00216-017-0830-8

Bussy, U., Chung-Davidson, Y. W., Buchinger, T., Li, K., Smith, S. A., Daniel Jones, A., & Li, W. (2018b). Metabolism of a sea lamprey pesticide by fish liver enzymes Part B: method development and application in quantification of TFM metabolites formed in vivo. *Analytical and Bioanalytical Chemistry*, 410(6), 1763-1774. https://doi.org/10.1007/s00216-017-0831-7

Carey, H. J., Fox, M. E., & Schleen, L. P. (1988). Photodegradation of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM). 2. Field confirmation of direct photolysis and persistence of formulation impurities in a stream during treatment. *Journal of Great Lakes Research*, 14(3), 338-346. https://doi.org/10.1016/S0380-1330(88)71563-4

Chen, X. L., Yang, S. Y., Zhu, B. R., Zhang, M. Y., Zheng, N., Hua, J. H., Li, R. W., Han, J., Yang, L. H., & Zhou, B. S. (2024). Effects of environmentally relevant concentrations of niclosamide on lipid metabolism and steroid hormone synthesis in adult female zebrafish. *Science of the Total Environment*, 910. https://doi.org/10.1016/j.scitotenv.2023.168737

Chen, Z., Snow, M., Lawrence, C. S., Church, A. R., Narum, S. R., Devlin, R. H., & Farrell, A. P. (2015). Selection for upper thermal tolerance in rainbow trout (*Oncorhynchus mykiss Walbaum*). *Journal of Experimental Biology*, 218(5), 803-812. https://doi.org/10.1242/jeb.113993

Clarke, D. J., George, S. G., & Burchell, B. (1991). Glucuronidation in fish. *Aquatic Toxicology*, 20(1-2), 35-56. https://doi.org/10.1016/0166-445X(91)90040-G

Clifford, A. M., Henry, M., Bergstedt, R., McDonald, D. G., Smits, A. S., & Wilkie, M. P. (2012). Recovery of larval sea lampreys from short-term exposure to the pesticide 3-trifluoromethyl-4-nitrophenol: implications for sea lamprey control in the Great Lakes. *Transactions of the American Fisheries Society*, 141, 1697-1710. https://doi.org/10.1080/00028487.2012.713887

Coleman, T., Podgorski, M. N., Doyle, M. L., Scaffidi-Muta, J. M., Campbell, E. C., Bruning, J. B., De Voss, J. J., & Bell, S. G. (2023). Cytochrome P450-catalyzed oxidation of halogen-containing substrates. *Journal of Inorganic Biochemistry*, 244, 112234, ISSN 0162-0134. https://doi.org/10.1016/j.jinorgbio.2023.112234

Collingsworth, P. D., Bunnell, D. B., Murray, M. W., Kao, Y., Feiner, Z. S., Claramunt, R. M., Lofgren, B. M., Höök, T. O., & Ludsin, S. A. (2017). Climate change as a long-term stressor for the fisheries of the Laurentian Great Lakes of North America. *Reviews in Fish Biology and Fisheries,* 27, 363-391. https://doi.org/10.1007/s11160-017-9480-3

Committee on the Status of Endangered Wildlife in Canada. (2017). COSEWIC assessment and status report on the lake sturgeon *Acipenser fulvescens*, western Hudson Bay populations, Saskatchewan-Nelson River populations, southern Hudson Bay-James Bay populations, Great Lakes-upper St. Lawrence populations in Canada. Committee on the Status of Endangered Wildlife in Canada.

Court, M.H., & Greenblatt, D.J. (2000). Molecular genetic basis for deficient acetaminophen glucuronidation by cats: UGT1A6 is a pseudogene, and evidence for reduced diversity of expressed hepatic UGT1A isoforms. *Pharmacogenetics*, 10, 355-369. https://doi.org/10.1097/00008571-200006000-00009

Currie, S., & Evans, D. H. (2021). *The Physiology of fishes* (5th ed.). CRC Press. https://doi.org/10.1201/9781003036401

Dawson, V. K., Cumming, K. B., & Gilderhuis, P. A. (1977). Efficacy of 3-trifluoromethyl-4-nitrophenol (TFM), 2′,5-dichloro-4′-nitrosalicylanilide (Bayer 73), and a 98:2 mixture as lampricides in laboratory studies. *Investigations in Fish Control No. 77*. US Fish and Wildlife Service.

Dawson, V. K., Schreier, T. M., Boogaard, M. A., Spanjers, N. J., & Gingerich, W. H. (2002). Rapid loss of lampricide from catfish and rainbow trout following routine treatment. *Journal of Agricultural and Food Chemistry*, 50, 6780-6785. https://doi.org/10.1021/jf020443h

Dawson, V. K. (2003). Environmental fate and effects of the lampricide Bayluscide: A review. *Journal of Great Lakes Research*, 29, 475-492. https://doi.org/10.1016/S0380-1330(03)70509-7

Dawson, V. K., Sills, J. B., & Luhning, C. W. (1982). Accumulation and loss of 2′,5-dichloro-4′-nitrosalicylanilide (Bayer 73) by fish: Laboratory studies. *Investigations in Fish Control No. 90*. US Fish and Wildlife Service.

Dawson, H. A., & Jones, M. L. (2009). Factors affecting recruitment dynamics of Great Lakes sea lamprey (*Petromyzon marinus*) populations. *Journal of Great Lakes Research*, 35(3), 353-360. https://doi.org/10.1016/j.jglr.2009.03.003

Denisov, I. G., Makris, T. M., Sligar, S. G., & Schlichting, I. (2005). Structure and chemistry of cytochrome P450. *Chemical Reviews*, 105, 2253-2277. https://doi.org/10.1021/cr0307143

Docker, M. F., & Potter, I. C. (2019). Life history evolution in lampreys: Alternative migratory and feeding types. In M. F. Docker (Ed.), *Lampreys: Biology, conservation and control: Vol. 2* (Fish & Fisheries Series, Vol. 38, pp. 287). Springer. https://doi.org/10.1007/978-94-024-1684-8_4

D'Souza, L., Flavio, H., & Wilkie, M. P. (2025). The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) stimulates oxygen consumption by larval sea lamprey in a dose-dependent manner. *Journal of Great Lakes Research*, 51(2), 102536. https://doi.org/10.1016/j.jglr.2025.102536

du Plessis, M., Fourie, C., Stone, W., & Engelbrecht, A. (2023). The impact on endocrine disrupting compounds and carcinogens in wastewater: Implications for breast cancer. *Biochemistry*, 209, 103-115. https://doi.org/10.1016/j.biochi.2023.02.006

Erickson, R. J., McKim, J. M., Lien, G. J., Hoffman, A. D., & Batterman, S. L. (2006a). Uptake and elimination of ionizable organic chemicals at fish gills: I. Model formulation, parameterization, and behavior. *Environmental Toxicology and Chemistry*, 25, 1512-1521. https://doi.org/10.1897/05-358R.1

Erickson, R. J., McKim, J. M., Lien, G. J., Hoffman, A. D., Batterman, S. L. (2006b). Uptake and elimination of ionizable organic chemicals at fish gills: II. Observed and predicted effects of pH, alkalinity, and chemical properties. *Environmental Toxicology and Chemistry*, 25, 1522-1532. https://doi.org/10.1897/05-359R.1

Eshenroder, R. L. (2009) Comment: Mitochondrial DNA analysis indicates sea lampreys are indigenous to Lake Ontario. *Transactions of American Fisheries Society*, 138, 1178-1189. https://doi.org/10.1577/T08-035.1

Eshenroder, R. L. (2014). The role of the Champlain Canal and Erie Canal as putative corridors for colonization of Lake Champlain and Lake Ontario by sea lampreys. *Transactions of the American Fisheries Society*, 143, 634-649. https://doi.org/10.1080/00028487.2013.879818

Farmer, G. J., Beamish, F. W. H., & Robinson, G. A. (1975). Food consumption of adult landlocked sea lamprey (*Petromyzon marinus*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 50, 753-757. https://doi.org/10.1016/0300-9629(75)90141-3

Farmer, G. J. (1980). Biology and physiology of feeding in adult lampreys. *Canadian Journal of Fisheries and Aquatic Sciences*, 37, 1751-1761. https://doi.org/10.1139/f80-220

Finney, D. J. (1971). *Probit Analysis* (3rd ed.). Cambridge University Press. https://doi.org/10.1016/0022-474X(72)90034-3

Frayha, G. J., Smyth, J. D., Gobert, J. G. & Savel, J. (1997). The mechanisms of action of antiprotozoal and anthelmintic drugs in man. *General Pharmacology*, 28, 273-299. https://doi.org/10.1016/s0306-3623(96)00149-8

Gaden, M. O., Brant, C., & Lambe, R. (2021). Why a Great Lakes Fishery Commission? The seven-decade pursuit of a Canada-U.S. Fishery Treaty. *Journal of Great Lakes Research*, 47, S11-S23. https://doi.org/10.1016/j.jglr.2021.01.003

Gess, R., Coates, M., & Rubidge, B. (2006). A Lamprey from the Devonian period of South Africa. *Nature*, 443, 981-984. https://doi.org/10.1038/nature05150

Gobas, F. A. P. C., Opperhuizen, A., & Hutzinger, O. (1986). Bioconcentration of hydrophobic chemicals in fish: relationship with membrane permeation. *Environmental Toxicology and Chemistry*, 5, 637-646. https://doi.org/10.1002/etc.5620050704

Grenc, L., Van den Brink, P. J., & Hendriks, A. J. (2025). Temperature effects on toxicokinetics of organic chemicals in aquatic organisms. *Environmental Science & Technology*, 59(44), 23984-23990. https://doi.org/10.1021/acs.est.5c00668

Grundlingh, J., Dargan, P. I., El-Zanfaly, M., Wood, D. M. (2011). 2,4-dinitrophenol (DNP): a weight loss agent with significant acute toxicity and risk of death. *Journal of Medical Toxicology*, 7, 205-212. https://doi.org/10.1007/s13181-011-0162-6

Gupta, P. K. (2016). Principles and basic concepts of toxicokinetics: Essential concepts and applications. In P. K. Gupta (Ed.), *Fundamentals of toxicology* (pp. 87–107). Elsevier. https://doi.org/10.1016/B978-0-12-805426-0.00009-3

Hansen, M. J., Madenjian, C. P., Slade, J. W., Steeves, T. B., Almeida, P. R., & Quintella. B. R. (2016). Population ecology of the sea lamprey (*Petromyzon marinus*) as an invasive species in the Laurentian Great Lakes and an imperiled species in Europe. *Reviews in Fish Biology and Fisheries*, 26, 509-535. https://doi.org/10.1007/s11160-016-9440-3

Harris, D. C. (2016). *Quantitative Chemical Analysis* (9th ed.). W. H. Freeman.

Hasnain, S. S., Minns, C. K., & Shuter, B. J. (2010). *Key ecological temperature metrics for Canadian freshwater fishes* (Climate Change Research Report No. CCRR-17). Ontario Forest Research Institute.

Hayes, D., & Caroffino, D. (2012). *Michigan's lake sturgeon rehabilitation strategy*. Michigan Department of Natural Resources.

Hayes, J. D., Dinkova-Kostova, A. T., & Tew, K. D. (2020). Oxidative stress in cancer. *Cancer Cell*, 38(2), 167-197. https://doi.org/10.1016/j.ccell.2020.06.001

Henry, M., Birceanu, O., Clifford, A. M., McClelland, G. B., Wang, Y. S., & Wilkie, M. P. (2015). Life stage dependent responses to the lampricide, 3-trifluoromethyl-4-nitrophenol (TFM), provide insight into glucose homeostasis and metabolism in the sea lamprey (*Petromyzon marinus*). *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, 169, 35-45. https://doi.org/10.1016/j.cbpc.2014.12.003

Hepditch, S. L., Birceanu, O., & Wilkie, M. P. (2021). A Toxic unit and additive index approach to understanding the interactions of 2 piscicides, 3-trifluoromethyl-4-nitrophenol and niclosamide, in rainbow trout. *Environmental Toxicology and Chemistry*, 40(5), 1419-1430. https://doi.org/10.1002/etc.4994

Hernandez, A. F., Gil, F., & Lacasana, M. (2017). Toxicological interactions of pesticide mixtures: an update. *Archives of Toxicology*, 91, 3211-3223. https://doi.org/10.1007/s00204-017-2043-5

Hlina B. L., Tessier L. R., & Wilkie, M. P. (2017). Effects of water pH on the uptake and elimination of the piscicide, 3-trifluoromethyl-4-nitrophenol (TFM), by larval sea lamprey. *Comparative Biochemistry and Physiology: Part C-Toxicology and Pharmacology*, 200, 9-16. https://doi.org/10.1016/j.cbpc.2017.05.005

Hlina, B. L., Birceanu, O., Robinson, C. S., Dhiyebi, H., & Wilkie, M. P. (2021). The relationship between thermal physiology and lampricide sensitivity in larval sea lamprey (*Petromyzon marinus*). *Journal of Great Lakes Research*, 47, S272-S284. https://doi.org/10.1016/j.jglr.2021.10.002

Holmes, J. A. & Lin, P. (1994). Thermal niche of larval sea lamprey, *Petromyzon marinus*. *Canadian Journal of Fisheries and Aquatic Sciences*, 51(2),253-262. https://doi.org/10.1139/f94-026

Holmes, J. A., & Youson, J. H. (1994). Fall condition factor and temperature influence the incidence of metamorphosis in sea lampreys, *Petromyzon marinus*. *Canadian Journal of Zoology*, 72(6), 1134-1140. https://doi.org/10.1139/z94-151

Howe, G. E., Marking, L. L., Bills, T. D., Rach, J. J., & Mayer, F. L. (1994). Effects of water temperature and pH on the toxicity of terbufos, trichlorfon, 4-nitrophenol and 2,4-dinitrophenol to the amphipod *Gammarus pseudolimnaeus* and rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry*, 13(1), 51-66. https://doi.org/10.1002/etc.5620130109

Howell, J. H., Everett, L. K. Jr, Smith, A. J., & Hanson, L. H. (1964). Synergism of 5,2′-dichloro-4′-nitro-salicylanilide and 3-trifluoromethyl-4-nitrophenol in a selective lamprey larvicide. *Technical Report No. 8*. Great Lakes Fishery Commission.

Howell, J. H., Lech, J. J., & Allen, J. L. (1980). Development of sea lamprey (*Petromyzon Marinus*) larvicides. *Canadian Journal of Fisheries and Aquatic Sciences*, 37(11), 2103-2107. https://doi.org/10.1139/f80-251

Hubert, T. D. (2003). Environmental fate and effects of the lampricide TFM: A Review. *Journal of Great Lakes Research*, 29(suppl 1), 456-474. https://doi.org/10.1016/S0380-1330(03)70508-5

Hubert, T. D., Bernardy, J. A., Vue, C., Dawson, V. K., Boogaard, M. A., Schreier, T.M., & Gingerich, W. H. (2005). Residues of the lampricides 3-trifluoromethyl-4-nitrophenol and niclosamide in muscle tissue of rainbow trout. *Journal of Agricultural and Food Chemistry,* 53, 5342-5346. https://doi.org/10.1021/jf050156o

Huerta, B., Chung-Davidson, Y. W., Bussy, U., Zhang, Y., Bazil, J. N., & Li, W. (2020). Sea lamprey cardiac mitochondrial bioenergetics after exposure to TFM and its metabolites. *Aquatic Toxicology*, 219, 105380. https://doi.org/10.1016/j.aquatox.2019.105380

Hume, J. B., Bennis, S., Bruning, T., Docker, M. F., Good, S., Lampman, R. Rinchard, J., Searcy, T., Wilkie, M. P., & Johnson, N. S. (2024). Evaluation of larval sea lamprey *Petromyzon marinus* growth frontiers in the laboratory: influence of temperature and diet. *Aquaculture Research*, 5547340. https://doi.org/10.1155/2024/5547340

Hunn, J. B., & Allen, J. L. (1974). Movement of drugs across gills of fishes. *Annual Review of Pharmacology and Toxicology*, 6, 15-18. https://doi.org/10.1146/annurev.pa.14.040174.000403

International Agency for Research on Cancer. (2025). *List of classifications agents classified by the IARC monographs, volumes 1–133*. International Agency for Research on Cancer. https://monographs.iarc.who.int/list-of-classifications.

Ionescu, R. A., Hepditch, S. L. J., & Wilkie, M. P. (2021). The lampricide 3-trifluoromethyl-4-nitrophenol causes temporary metabolic disturbances in juvenile lake sturgeon (*Acipenser fulvescens*): implications for sea lamprey control and fish conservation. *Conservation Physiology*, 9(1). https://doi.org/10.1093/conphys/coab069

Ionescu, R. A., Mitrovic, D., & Wilkie, M. P. (2022). Disturbances to energy metabolism in juvenile lake sturgeon (*Acipenser fulvescens*) following exposure to niclosamide. *Ecotoxicology and Environmental Safety*, 229, 112969. https://doi.org/10.1016/j.ecoenv.2021.112969

Ionescu, R. A., Mitrovic, D., Birceanu, O., McDonald, A. E., Wilson, J. M., Servos, M. R., & Wilkie, M.P. (2025). Rainbow trout rapidly recover from exposure to niclosamide: a piscicide and molluscicide used to control sea lamprey and snail populations. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, 294, 110199. https://doi.org/10.1016/j.cbpc.2025.110199

Jiang, X., Dong, S., Liu, R., Huang, M., Dong, K., Ge, J., Gao, Q., & Zhou, Y. (2021). Effects of temperature, dissolved oxygen, and their interaction on the growth performance and condition of rainbow trout (*Oncorhynchus mykiss*). *Journal of Thermal Biology*, 98. https://doi.org/10.1016/j.jtherbio.2021.102928

Johnson, D. A., & Stephens, B. E. (2003). Historical perspective on the development of procedures for conducting on-site toxicity tests and for measuring concentrations of lampricides in the sea lamprey control program during 1957 to 1999. *Journal of Great Lakes Research*, 29, S521-S528. https://doi.org/10.1016/S0380-1330(03)70513-9

Joubert, J. J., Evans, A. C., & Schutte, C. H. J. (2001). Schistosomiasis in Africa and international travel. *Journal of Travel Medicine*, 8, 92–99. https://doi.org/10.2310/7060.2001.24493

Jurgeit, A., McDowell, R., Moese, S., Meldrum, E., Schwendener, R., & Greber, U. F. (2012). Niclosamide is a proton carrier and targets acidic endosomes with broad antiviral effects. *PLoS Pathogens*, 8(10), 1-14. https://doi:10.1371/journal.ppat.1002976

Kalant, H. & Roschlau, W. H. E. (1998). *Principles of medical pharmacology* (6th ed.). Oxford University Press. https://doi.org/10.1017/S0317167100034508

Kane, A. S., Day, W. W., Reimschuessel, R., & Lipsky, M. M. (1993). 3-Trifluoromethyl-4-nitrophenol (TFM) toxicity and hepatic microsomal UDP-glucuronyltransferase activity in larval and adult bullfrogs. *Aquatic Toxicology*, 27, 51-60. https://doi.org/10.1016/0166-445X(93)90046-4

Kane, A. S., Kahng, M. W., Reimschuessel, R., Nhamburo, P. T., & Lipsky, M. M. (1994). UDP-glucuronyltransferase kinetics for 3-trifluoromethyl-4-nitrophenol (TFM) in fish. *Transactions of the American Fisheries Society*, 123, 217-222. https://doi.org/10.1577/1548-8659(1994)123%3C0217:UGKFTN%3E2.3.CO;2

Kawatski, J. A., & Bittner, M. A. (1975). Uptake, elimination, and biotransformation of lampricide 3-trifluoromethyl-4-nitrophenol (TFM) by larvae of aquatic midge *Chironomus tentans*. *Toxicology*, 4(2), 183-194. https://doi.org/10.1016/0300-483X(75)90097-9

Kenawy, E. R., & Rizk, E. S. (2004). Polymeric controlled release formulations of niclosamide for control of *Biomphalaria alexandrina*, the vector snail of schistosomiasis. *Macromolecular Bioscience*, 4(2), 119-128. https://doi.org/10.1002/mabi.200300003

Lardans, V. & Dissous, C. (1998). Snail control strategies for the reduction of schistosomiasis transmission. *Parasitology Today*, 14(10), 413-417. https://doi-org./10.1016/S0169-4758(98)01320-9

Lawrence, M. J., Mitrovic, D., Foubister, D., Bragg, L. M., Sutherby, J., Docker, M. F., Servos, M. R., Wilkie, M. P., & Jeffries, K. M. (2021). Contrasting physiological responses between invasive sea lamprey and non-target bluegill in response to acute lampricide exposure. *Aquatic Toxicology*, 237. https://doi.org/10.1016/j.aquatox.2021.105848

Lawrence, M. J., Grayson, P., Jeffrey, J. D., Docker, M. F., Garroway, C. J., Wilson, J. M., Manzon, R. G., Wilkie, M. P., & Jeffries, K. M. (2022). Variation in the transcriptome response and detoxification gene diversity drives pesticide tolerance in fishes. *Environmental Science and Technology*, 56(17), 12137-12147. https://doi.org/10.1021/acs.est.2c00821

Lawrence, M. J., Grayson, P., Jeffrey, J. D., Docker, M. F., Garroway, C. J., Wilson, J. M., Manzon, R. G., Wilkie, M. P., & Jeffries, K. M. (2023). Transcriptomic impacts and potential routes of detoxification in a lampricide-tolerant teleost exposed to TFM and niclosamide. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*. https://doi.org/10.1016/j.cbd.2023.101074

Lech, J. J. (1974). Glucuronide formation in rainbow trout - effect of salicylamide on the acute toxicity, conjugation, and excretion of 3-trifluoromethyl-4-nitrophenol.

Biochemical Pharmacology, 23, 2403-2410. https://doi.org/10.1016/0006-2952(74)90231-7

Lech, J. J. & Costrini, N. V. (1972). In vitro and in vivo metabolism of 3-trifluoromethyl-4-nitrophenol (TFM) in rainbow trout. *Comparative and General Pharmacology*, 3(10), 160-166. https://doi.org/10.1016/0010-4035(72)90021-3

Lech, J. J., Pepple, S., & Anderson, M. (1973). Effects of novobiocin on the acute toxicity metabolism and biliary excretion of 3-trifluoromethyl-4-nitrophenol in rainbow trout. *Toxicology and Applied Pharmacology*, 25, 542-552. https://doi.org/10.1016/0041-008X(73)90023-9

Lech, J. J. & Statham, C. N. (1975). Role of glucuronide formation in the selective toxicity of 3-trifluoromethyl-4-nitrophenol (TFM) for the sea lamprey: comparative aspects of TFM uptake and conjugation in sea lamprey and rainbow trout. *Toxicology and Applied Pharmacology*, 31, 150-158. https://doi.org/10.1016/0041-008x(75)90063-0

Lennox, R. J., Bravener, G. A., Lin, H., Madenjian, C. P., Muir, A. M., Remucal, C. K., Robinson, K. F., Rous, A. M., Siefkes, M. J., Wilkie, M. P., Zielinski, D. P., & Cooke, S. J. (2020). Potential changes to the biology and challenges to the management of invasive sea lamprey *Petromyzon marinus* in the Laurentian Great Lakes due to climate change. *Global Change Biology*, 26(3), 1118–1137. https://doi.org/10.1111/gcb.14957

Lewis, K. A., Tzilivakis, J., Warner, D. J., & Green, A. (2016). An international database for pesticide risk assessments and management. Human and Ecological Risk Assessment, 22(4), 1050–1064. https://doi.org/10.1080/10807039.2015.1133242

Lewis, S. V. (1980). Respiration of lampreys. *Canadian Journal of Fisheries and Aquatic Sciences*, 37(11), 1711-1722. https://doi.org/10.1139/f80-217

Lloyd, R., & Herbert, D. W. M. (1960). The Influence of carbon dioxide on the toxicity of un-ionized ammonia to rainbow trout (*Salmo gairdnerii Richardson*). *Annuals of Applied Biology*, 48(2), 399–404. https://doi.org/10.1111/j.1744-7348.1960.tb03536.x

Luoma, J.A., Schueller, J.R., Schloesser, N.A., Kirkeeng, C.A., & Wolfe, S.L. (2024). Comparative toxicity of emulsifiable concentrate and suspension concentrate formulations of 2′,5-dichloro-4′-nitrosalicylanilide ethanolamine salt: U.S. Geological Survey Open-File Report 2024–1037, 10 p., https://doi.org/10.3133/ofr20241037

Mandl, J. (2023). Glycogen-endoplasmic reticulum connection in the liver. *International Journal of Molecular Sciences*, 24(2), 1074. https://doi.org/10.3390/ijms24021074

Manzon, R. G., Youson, J. H., & Holmes, J. A. (2015). Lamprey Metamorphosis. In: Docker, M.F. (Ed.), Lampreys: Biology, Conservation and Control. Springer. https://doi.org/10.1007/978-94-017-9306-3_4

Marking, L. L., & Hogan, J. W. (1967). Toxicity of Bayer 73 on fish. *Investigative Fish Control*, 36, 1-13. US Fish and Wildlife Service.

Marking, L. L., & Dawson, V. K. (1975). Method for assessment of toxicity or efficacy of mixtures of chemicals. *Investigations in Fish Control No. 67*. US Fish and Wildlife Service.

Marking, L. L., & Bills, T. D. (1985). Effects of contaminants on the toxicity of the lampricides TFM and Bayer 73 to three species of fish. *Journal of Great Lakes Research*, 11, 171-178. https://doi.org/10.1016/S0380-1330(85)71756-X

McConville, M. B., Hubert, T. D., & Remucal, C. K. (2016). Direct photolysis rates and transformation pathways of the lampricides TFM and niclosamide in simulated sunlight. *Environmental Science and Technology*, 50, 9998-10006. https://doi.org/10.1021/acs.est.6b02607

McConville, M. B., Cohen, N. M., Nowicki, S. M., Lantz, S. R, Hixson, J. L., Ward, A. S., & Remucal, C. K. (2017a). A Field Analysis of lampricide photodegradation in Great Lakes tributaries. *Environmental Science: Processes & Impacts*, 19, 891-900. https://doi.org/10.1039/C7EM00173H

McConville, M. B., Mexyk, S. P., Lantz, S. R., & Remucal, C. K. (2017b). Indirect photodegradation of the lampricides TFM and niclosamide. *Environmental Science: Processes & Impacts*, 19, 1028-1039. https://doi.org/10.1039/c7em00208d

McDonald, D. G., & Kolar, C. S. (2007). Research to guide the use of lampricides for controlling sea lamprey. *Journal of Great Lakes Research*, 33, 20-34. https://doi.org/10.3394/0380-1330(2007)33[20:RTGTUO]2.0.CO;2

McLaughlin, R. L., Hallett, A., Pratt, T. C., O'Connor, L. M., & McDonald, D. G. (2007). Research to guide use of barriers, traps, and fishways to control sea lamprey. *Journal of Great Lakes Research*, 33, 7-19. https://doi.org/10.3394/0380-1330(2007)33[7:RTGUOB]2.0.CO;2

McManus, D. P., Dunne, D. W., Sacko, M., Utzinger, J., Vennervald, B. J., & Zhou, X. N. (2018). Schistosomiasis. *Nature Reviews Disease Primers* 4(1), 13. https://doi.org/10.1038/s41572-018-0013-8

Meech, R., Hu, D. G., McKinnon, R. A., Mubarokah, S. N., Haines, A. Z., Nair, P. C., Rowland, A., & Mackenzie, P. I. (2019). The UDP-glycosyltransferase (UGT) superfamily: new members, new functions, and novel paradigms. *Physiological Reviews*, 99(2), 1153-1222. https://doi.org/10.1152/physrev.00058.2017

Meisner, J. D., Goodier, J. L., Regier, H. A., Shuter, B. J., & Christie, W. J. (1987). An assessment of the effects of climate warming on Great Lakes basin fishes. *Journal of Great Lakes Research*, 13(3), 340-352. https://doi.org/10.1016/S0380-1330(87)71656-6

Mitrovic, D., Bragg, L. M., Flávio, H., Servos, M. R., & Wilkie, M. P. (2026a). The mechanism of increased tolerance to the pesticide, 3-trifluoromethyl-4-nitrophenol (TFM), in invasive sea lamprey (*Petromyzon marinus*) at warmer temperatures. *Environmental Toxicology and Chemistry*, vgag040. https://doi.org/10.1093/etojnl/vgag040

Mitrovic, D., Bragg, L. M., Servos, M. R., & Wilkie, M. P. (2026b). Greater than additive interactions between two phenolic pesticides used to control invasive sea lamprey

(*Petromyzon marinus*): underlying mechanisms and implications. *Aquatic Toxicology,* (Accepted with minor revision). https://dx.doi.org/10.2139/ssrn.6096173

Muhametsafina, A., Birceanu, O., Hlina, B. L., Tessier, L. R., & Wilkie, M. P. (2019). Warmer waters increase the larval sea lamprey's (*Petromyzon marinus*) tolerance to the lampricide 3-trifluoromethyl-4-nitrophenol (TFM). *Journal of Great Lakes Research*, 45(5), 921-933. https://doi.org/10.1016/j.jglr.2019.07.011

Mundim, K.C., Baraldi, S., Machado, H.G., & Vieira, F.M. (2020). Temperature coefficient (Q10) and its applications in biological systems: Beyond the Arrhenius theory. Ecological Modelling, 431, 109127. https://doi.org/10.1016/j.ecolmodel.2020.109127

Nalesnik, A. M., Martin, E. L., Kovacs, I. S., Johnson, C. S., Caroll, E. I., Jubar, A, Hemstrom, W., Wilkie, M. P., Dunlop, E. S., Sepulveda, M. S., Johnson, N. S., & Christie, M. R. (2025). Larger larval sea lamprey (*Petromyzon marinus*) have longer survival times when exposed to lampricide 3-trifluoromethyl-4-nitrophenol. *Journal of Great Lakes Research*. https://doi.org/10.1016/j.jglr.2025.102620

National Center for Biotechnology Information. (2025). *Niclosamide* (PubChem compound summary for CID 4477). PubChem. https://pubchem.ncbi.nlm.nih.gov/compound/Niclosamide

Newman, M. C. (2020). *Fundamentals of Ecotoxicology* (5th ed.). CRC Press, Taylor & Francis Group LLC. https://doi.org/10.1201/9781351133999

Niblett, P. D., & Ballantyne, J. S. (1976). Uncoupling of oxidative phosphorylation in rat liver mitochondria by the lamprey larvicide TFM (3-trifluoromethyl-4-nitrophenol). *Pesticide Biochemical Physiology*, 2, 103-120. https://doi.org/10.1016/0048-3575(76)90046-8

Norwood, W. P., Borgmann, U., Dixon, D. G., & Wallace, A. (2003). Effects of metal mixtures on aquatic biota: a review of observations and methods. H*uman and Ecological Risk Assessment*, 9, 795-811. https://doi.org/10.1080/713610010

O'Connor L. M., Pratt T. C., Steeves, T. B., Stephens, B., Boogaard, M., & Kaye, C. (2017). In situ assessment of lampricide toxicity to age-0 lake sturgeon. *Journal of Great Lakes Research*, 43,189-198. https://doi.org/10.1016/j.jglr.2016.10.011

Ortega-Calvo, J. J., Harmsen, J., Parsons, J. R., Semple, K. T., Aitken, M. D., Ajao, C., Eadsforth, C., Galay-Burgos, M., Naidu, R., Oliver, R., Peijnenburg, W. J. G. M., Römbke, J., Streck, G., & Versonnen, B. (2015). From bioavailability science to regulation of organic chemicals. *Environmental Science and Technology*, 49(17), 10255-10264. https://doi.org/10.1021/acs.est.5b02412

Park, S. J., Shin, J. H., Kang, H., Hwang, J. J., & Cho, D. H. (2011). Niclosamide induces mitochondria fragmentation and promotes both apoptotic and autophagic cell death. *Korean Society for Biochemistry and Molecular Biology*, 44 (8), 517-522. https://doi.org/10.5483/BMBRep.2011.44.8.517

Patra, R. W., Chapman, J. C., Lim, R. P., Gehrke, P. C., & Sunderam, R. M. (2015). Interactions between water temperature and contaminant toxicity to freshwater fish. *Environmental Toxicology and Chemistry*, 34(8), 1809-1817. https://doi.org/10.1002/etc.2990

Percy, R., & Potter, I. C. (1979). Intestinal blood-circulation in the river lamprey, *Lampetra fluviatilis*. *Journal of Zoology*, 187(Mar), 415-431. https://doi.org/10.1111/j.1469-7998.1979.tb03378.x

Peters, A., & Mackay, B. (1961). The structure and innervation of the myotomes of the lamprey. *Journal of Anatomy*, 4, 575-585.

Playle, R. C. & Wood, C. M. (1989). Water chemistry in the gill microenvironment of rainbow trout: experimental observations and theory. *Journal of Comparative Physiology Part B: Biochemical Systemic and Environmental Physiology*, 159(5), 527-537. https://doi.org/10.1007/BF00694377

Playle R. C. (2004). Using multiple metal–gill binding models and the toxic unit concept to help reconcile multiple-metal toxicity results. *Aquatic Toxicology*, 67, 359-370. https://doi.org/10.1016/j.aquatox.2004.01.017

Potter, I. C., Gill, H. S., Renaud, C. B., & Haoucher, D. (2015). The taxonomy, phylogeny, and distribution of lampreys. In: Docker, M.F. (Ed.), *Lampreys: Biology, Conservation and Control* (pp. 35-73). Springer. https://doi.org/10.1007/978-94-017-9306-3_2

Raths, J., Švara, V., Lauper, B., Fu, Q., & Hollender, J. (2023). Speed it up: how temperature drives toxicokinetics of organic contaminants in freshwater amphipods. *Global Change Biology*, 29, 1390-1406. https://doi.org/10.1111/gcb.16542

Renaud, C. B. (2011). *Lampreys of the world: An annotated and illustrated catalogue of lamprey species known to date* (FAO Species Catalogue for Fishery Purposes No. 5). Food and Agriculture Organization of the United Nations. https://openknowledge.fao.org/handle/20.500.14283/i2335e

Robinson, K. F., Miehls, S. M., & Siefkes, M. J. (2021). Understanding sea lamprey abundances in the Great Lakes prior to broad implementation of sea lamprey control. *Journal of Great Lakes Research*, *47*(Suppl. 1), S328–S334. https://doi.org/10.1016/j.jglr.2021.04.0

Rowland, A., Miners, J. O., Mackenzie, P. I. (2013). The UDP-glucuronosyltransferase: their role in drug metabolism and detoxification. *International Journal of Biochemistry and Cell Biology*, 45, 1121-1132. https://doi.org/10.1016/j.biocel.2013.02.019

Schloesser, R. W., & Fabrizio, M. C. (2017). Condition indices as surrogates of energy density and lipid content in juveniles of three fish species. *Transactions of the American Fisheries Society*, 146(5), 1058-1069. https://doi.org/10.1080/00028487.2017.1324523

Schmitt, H., Altenburger, R., Jastorff, B., & Schuurmann, G. (2000). Quantitative structure-activity analysis of the algae toxicity of nitroaromatic compounds. *Chemical Research in Toxicology*, 13(6), 441-450. https://doi.org/10.1021/tx9901635

Scholefield, R. J. (1987). *A comparison of two methods for the simultaneous determination of TFM and Bayer 73 concentrations* (Great Lakes Fishery Commission, Technical Report. No. 50). Great Lakes Fishery Commission.

Scholefield, R. J., Slaght, K. S., & Stephens, B. E. (2008). Seasonal variation in sensitivity of larval sea lampreys to the lampricide 3-trifluoromethyl-4-nitrophenol. *North American Journal of Fisheries Management*, 28(5), 1609-1617. https://doi.org/10.1577/M06-178.1

Schreck, C. B., & Moyle, P. B. (Eds.). (1990). Methods for fish biology. American Fisheries Society.

Schreier, T. M., Dawson, V. K., Choi, Y., Spanjers, N. J., & Boogaard, M. A. (2000). Determination of niclosamide residues in rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) fillet tissue by high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*, 48(6), 2212-2215. https://doi.org/10.1021/jf990695r

Schueller, J. R., Boogaard, M. A., Kirkeeng, C. A., Schloesser, N. A., Wolfe, S. L., Lettenberger, A. J., King-Heiden, T. C., & Luoma, J. A. (2024). Seasonal differences in larval sea lamprey (*Petromyzon marinus*) sensitivity to the pesticide TFM. *Journal of Great Lakes Research*, 50(1). https://doi.org/10.1016/j.jglr.2023.102248

Schulte, P. M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *Journal of Experimental Biology*, 218, 1856-2866. https://doi.org/10.1242/jeb.118851

Schultz, D. P., Harman, P. D., & Luhning, C. W. (1979). Uptake, metabolism, and elimination of the lampricide 3-trifluoromethyl-4-nitrophenol by largemouth bass (*Micropterus salmoides*). *Journal of Agriculture and Food Chemistry*, 27, 328-331. https://doi.org/10.1021/jf60222a008

Sheth, U. K. (1975). Mechanisms of anthelmintic action. *Progress in Drug Research*, 19, 147-157. https://doi.org/10.1007/978-3-0348-7090-0_19

Shi, G., Wang, J., Yu, J., Qin, S., Liu, R., & Wu, D. (2025). The impact of increased water hardness on pollutant toxicity in freshwater aquatic organisms. *Environmental Monitoring and Assessment*, 197, 1131. https://doi.org/10.1007/s10661-025-14625-y

Shrestha, B., Reed, J.M., Starks, P.T., Kaufman, G.E., Goldstone, J.V., Roelke, M.E., O'Brien, S.J., Koepfli, K.-P., & Frank, L.G. (2011). Evolution of a major drug metabolizing enzyme defect in the domestic cat and other felidae: phylogenetic timing and the role of hypercarnivory. *PLoS One,* 6, e18046. https://doi.org/10.1371/journal.pone.0018046

Siefkes, M. J. (2017). Use of physiological knowledge to control the invasive sea lamprey (*Petromyzon marinus*) in the Laurentian Great Lakes. *Conservation Physiology*, 5(1). http://dx.doi.org/10.1093/conphys/cox031

Sijm, D. T. H. M., Pärt, P., & Opperhuizen, A. (1993). The influence of temperature on the uptake rate constants of hydrophobic compounds determined by the isolated perfused gills of rainbow trout (*Oncorhynchus mykiss*), *Aquatic Toxicology*, 25(1-2), 1-14. https://doi.org/10.1016/0166-445X(93)90016-T.

Smith, B., & Tibbles, J. (1980). Sea lamprey (*Petromyzon marinus*) in Lakes Huron, Michigan, and Superior: history of invasion and control, 1936-78. *Canadian Journal of Fisheries and Aquatic Sciences*, 37, 1780-1801. https://doi.org/10.1139/f80-222

Somero, G. N. (2004). Adaptation of enzymes to temperature: searching for basic "strategies". *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 139(3), 321-333. https://doi.org/10.1016/j.cbpc.2004.05.003

Statham, C. N., & Lech, J. J. (1975). Metabolism of 2',5-dichloro-4'-nitrosalicylanilide (Bayer-73) in rainbow trout (*Salmo gairdneri*). *Journal of the Fisheries Research Board of Canada*, 32, 515-522. https://doi.org/10.1139/f75-063

Sullivan, W. P., Burkett, D. P., Boogaard, M. A., Criger, L .A., Freiburger, C. E., Hubert, T. D., Leistner, K. G., Morrison, B. J., Nowicki, S. M., Robertson, S. N. P., Rowlinson, A. K., Scotland, B. J., & Sullivan, T. B. (2021). Advances in the use of lampricides to control sea lampreys in the Laurentian Great Lakes, 2000-2019. *Journal of Great Lakes Research*, 47, S216-S237. https://doi.org/10.1016/j.jglr.2021.08.009

Surana, D., Gupta, J., Sharma, S., Kumar, S., & Ghosh, P. (2022). A review on advances in removal of endocrine disrupting compounds from aquatic matrices: future perspectives on utilization of agri-waste based absorbents. *Science of the Total Environment*, 826, 154129. https://doi.org/10.1016/j.scitotenv.2022.154129

Sutton, T. M. & Bowen, S. H. (1994). Significance of organic detritus in the diet of larval lampreys in the Great Lakes Basin. *Canadian Journal of Fisheries and Aquatic Sciences*, 51, 2380-2387. https://doi.org/10.1139/f94-239

Swink, W. D., 2003. Host selection and lethality of attacks by sea lampreys (*Petromyzon marinus*) in laboratory studies. *Journal of Great Lakes Research*, 29, 307-319. https://doi.org/10.1016/S0380-1330(03)70496-1

Tao, H. L., Zhang, Y., Zeng, X. G., Shulman, G. I., & Jin, S. K. (2014). Niclosamide ethanolamine-induced mild mitochondrial uncoupling improves diabetic symptoms in mice. *Nature Medicine*, 20(11), 1263–1269. https://doi.org/10.1038/nm.3699

Tessier, L. R., Long, T. A. F., & Wilkie, M. P. (2018). Influence of body size, metabolic rate and life stage on the uptake and excretion of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) by invasive sea lamprey (*Petromyzon marinus*). *Aquatic Toxicology*, 194, 27-36. https://doi.org/10.1016/j.aquatox.2017.10.020

Verhille, C. E., English, K. K., Cocherell, D. E., Farrell, A. P., & Fangue, N. A. (2016). High thermal tolerance of a rainbow trout population near its southern range limit suggests local thermal adjustment. *Conservation Physiology*, 4(1). https://doi.org/10.1093/conphys/cow057

Viant, M. R., Walton, J. H., & Tjeerdema, R. S. (2001). Comparative Sublethal actions of 3-trifluoromethyl-4-nitrophenol in marine molluscs, as measured by in vivo P-31 NMR. *Pesticide Biochemistry and Physiology*, 71(1), 40-47. https://doi.org/10.1006/pest.2001.2554

Vue, C., Bernardy, J. A., Hubert, T. D., Gingerich, W. H., & Stehly, G. R. (2002). Relatively rapid loss of lampricide residues from fillet tissue of fish after routine treatment. *Journal of Agriculture and Food Chemistry*, 50, 6786-6789. https://doi.org/10.1021/jf0204708

Waldman, J. R., Grunwald, C., Roy, N. K., & Wirgin, I. I. (2004). Mitochondrial DNA analysis indicates sea lampreys are indigenous to Lake Ontario. *Transactions of the American Fisheries Society*, 133(4), 950-960. https://doi.org/10.1577/t03-104.1

Waldman, J. R., Grunwald, C., & Wirgin, I. I. (2006). Evaluation of the native status of sea lampreys in Lake Champlain based on mitochondrial DNA sequencing analysis. *Transactions of the American Fisheries Society*, 135(4), 1076-1085. https://doi.org/10.1577/t05-055.1

Wang, Z., Lui, G. C. S., Burton Jr., G. A., & Leung, K. M. Y. (2019). Thermal extremes can intensify chemical toxicity to freshwater organisms and hence exacerbate their impact to the biological community. *Chemosphere*, 224, 256-264. https://doi.org/10.1016/j.chemosphere.2019.02.103

Whitney, J. E., Al-Chokhachy, R., Bunnell, D. B., Caldwell, C. A., Cooke, S. J., Eliason, E. J., Roger, M., Lynch, A. J., & Paukert, C. P. (2016). Physiological basis of climate change impacts on North American Inland Fishes. *Fisheries*, 41(7), 332-345. https://doi.org/10.1080/03632415.2016.1186656

Wilkie, M.P., Holmes, J.A., Youson, J.H., 2007. The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) interferes with intermediary metabolism and glucose homeostasis, but not ion balance, in larval sea lamprey (Petromyzon marinus). *Canadian Journal of Fisheries and Aquatic Sciences*, 64, 1–9. https://10.1139/F07-082.

Wilkie, M. P., Hubert, T. D., Boogaard, M. A., & Birceanu, O. (2019). Control of invasive sea lampreys using the piscicides tfm and niclosamide: toxicology, successes and future prospects. *Aquatic Toxicology*, 211, 235-252. https://doi.org/10.1016/j.aquatox.2018.12.012

Wilkie, M. P., Tessier, L. R., Boogaard, M., O'Connor, L., Birceanu, O., Steeves, T. B., & Sullivan, W. P. (2021). Lampricide bioavailability and toxicity to invasive sea lamprey and non-target fishes: the importance of alkalinity, pH, and the gill microenvironment. *Journal of Great Lakes Research*, 47, S407–S420. https://doi.org/10.1016/j.jglr.2021.09.005

Wilkie, M. P. (2025). How resilient ecophysiology of the sea lamprey allowed them to invade the Laurentian Great Lakes and could protect them from climate change. *Frontiers in Fish Science*, 3, 1591515. https://doi.org/10.3389/frish.2025.1591515

Wu, F. X., Janvier, P., & Zhang, C. (2023). The rise of predation in Jurassic lampreys. *Nature Communications*, 14, 6652. https://doi.org/10.1038/s41467-023-42251-0

Yang, R., Brauner, C., Thurston, V., Neuman, J., & Randall, D. J. (2000). Relationship between toxicant transfer kinetic processes and fish oxygen consumption. *Aquatic Toxicology*, 48, 95–108.

Youdim, K. A., & Saunders, K. C. (2010). A review of LC–MS techniques and high-throughput approaches used to investigate drug metabolism by cytochrome P450s. *Journal of Chromatography B*, 878(17-18), 1326-1336. https://doi.org/10.1016/j.jchromb.2010.02.013

Youson, J. H. (1980). Morphology and physiology of lamprey metamorphosis. *Canadian Journal of Fisheries and Aquatic Sciences*, 37, 1687-1710. https://doi.org/10.1139/f80-216

Zhu, B. R., Lei, L., Sun, Y. M., Shi, X. J., Fu, K. Y., Hua, J. H., Martyniuk, C. J., Han, J., Yang, L. H., & Zhou, B. S. (2022). Niclosamide exposure at environmentally relevant concentrations efficaciously inhibited the growth and disturbed the liver-gut axis of adult male zebrafish. *Environmental Science & Technology*, 56(16), 11516-11526. https://doi.org/10.1021/acs.est.2c02712

Appendix A
Supplementary Information for Chapter 2

A.1 LC-MS/MS method development and optimization

A.1.1 Tissue sample preparation and extraction

Each batch of extracted samples included processing a blank sample as a negative control along with quality assurance/quality control (QA/QC) matrix spiked samples. Sample blanks were prepared with control (no TFM exposure) tissues spiked with ^{13}C-Nic standard (200 µg L^{-1}), while the quality control matrix spikes additionally contained 100 µg L^{-1} of TFM. Because no adequate internal standard for TFM exists, ^{13}C labelled niclosamide (^{13}C-Nic, Sigma Aldrich) was used (from a combined method developed to analyze TFM and Nic mixtures in water and tissue samples) as it demonstrated reliable TFM results (data obtained during method development) compared to spectrophotometric numbers (SpectraMax 190, Molecular Devices, Sunnyvale, California) at a wavelength of 395 nm using precision TFM standards.

Approximately 100 mg of finely ground muscle tissue sample was collected into a 2 mL microcentrifuge tube and diluted with acetonitrile (ACN) with 1% formic acid (1:1 ratio of sample weight to solvent volume). Each sample tube included two stainless steel beads (2.4 mm, Omni International; Kennesaw, GA, USA) to aid tissue homogenization and 200 µg L^{-1} internal standard (^{13}C-Nic). All tissue samples were homogenized using a Bead Ruptor Elite (Omni International) for 2 min at 2.4 m s^{-1}. The final volume of each tube was adjusted to 500 µL by adding ACN with 1% formic acid and vortexed for 15 sec to ensure sample mixing. After incubating for 20 min at 4°C for protein precipitation, the sample tubes were centrifuged at 10,000 g for 10 min at 4°C to separate the supernatant.

Solid-phase extraction (SPE) was performed using Captiva EMR-Lipid 96-well plates (Agilent Technologies, Santa Clara, CA) to further clean up the samples and remove lipids.

The sample supernatant (~ 300 µL) was passed through an inactivated, unconditioned SPE cartridge using a 96-well plate vacuum manifold (Agilent Technologies) and eluted into a 2 mL reservoir well plate at a rate of 1 drop per 3-5 sec. The eluted sample was then transferred into a 200 µL glass insert within a 2 mL amber glass vial and placed within the LC-MS/MS instrument for analysis.

Liver tissues were analyzed similarly; however, due to the size limitations of the lamprey liver (22.3 ± 6.2 mg), the protocol had to be micro-adjusted. The final volume was reduced to 250 µL, and only ~ 100 µL was placed in a vial for injection. All other steps were as described above.

A.1.2 LC-MS/MS analysis

Samples were analyzed for the analytes of interest using an Agilent 1260 HPLC (high-performance liquid chromatography) equipped with a high-performance autosampler, binary pump, and a 6460 triple quadrupole mass spectrometer with an Agilent Jetstream electrospray ionization source. An Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm) was used to separate the analytes chromatographically. Samples (2 µL) were injected at a constant temperature of 35°C and a flow rate of 0.8 mL min^{-1}, to provide chromatographic separation using a gradient. Mobile phase A was Milli-Q water and mobile phase B was acetonitrile. The gradient used was 0 min: 80/20; 1 min: 80/20; 10 min: 0/100; 12 min: 0/100; 12.1 min: 80/20 (percentiles A/B). The post-run re-equilibration time was 5 min.

Following separation, the identity and quantification of analytes were obtained using a dynamic multiple reaction monitoring (MRM) method optimized with negative polarity on the triple quadrupole. The instrument source parameters were a gas temperature of 230°C, a gas flow of 12 L min^{-1}, a nebulizer at 40 psi, a sheath gas temperature of 400°C, a capillary voltage of 2500 V and a nozzle voltage of 2000 V. The compound parameters for Nic-^{13}C were precursor ion at 332.07, product ions at 296 and 177.1, fragmentor at 133 V, collision energy at 9 and 25 V. For niclosamide, precursor ion was 325, while products were 289 and 171 with fragmentor at 125 V and collision energy of 10 and 22 V. For TFM, precursor ion

was 206, while products were 176 and 160 with fragmentor at 132 V and collision energy of 18 and 22 V. Cell accelerator was at 4 V for all compounds.

The calibration curve ranged from 0 - 5000 μg L^{-1} for TFM, and 0 - 500 μg L^{-1} for niclosamide. Standards and spikes were dissolved in ACN (Optima, LC/MS grade, Fisher Scientific, Ottawa, Canada) to facilitate protein precipitation and avoid gelation. Every batch of samples included matrix blank and matrix spiked samples which were always within acceptable ranges.

Repeated injections (n = 10) at low concentrations were used to determine the instrument detection limits (IDLs). In contrast, method detection limits (MDLs) were determined by measuring matrix samples spiked at various low concentrations (n = 7). The instrument detection limit was determined to be 0.04 μg L^{-1} for TFM and 0.05 μg L^{-1} for niclosamide. The method detection limit in water was 0.25 μg L^{-1} for TFM and 0.08 μg L^{-1} for niclosamide. In liver, the MDL was 0.030 nmol g^{-1} for TFM and 0.018 nmol g^{-1} for Nic while in muscle the MDL was 0.013 nmol g^{-1} for TFM and 0.029 nmol g^{-1} μg L^{-1} for Nic. The sample recoveries for TFM and niclosamide were 85.9 - 103.8%. All data was processed using Agilent Mass Hunter Quantitative Analysis software version 10.1.

Table A.1 TFM concentrations during 12-h TFM toxicity measurements across 6 different temperatures and mortality numbers at each concentration. TFM concentrations were measured spectrophotometrically at a wavelength of 395 nm and confirmed against standards prepared using analytical-grade TFM and the same dechlorinated water used during the experiments.

Temperature (Actual)	Nominal	Tank	TFM Concentration (mg L^{-1})	Mortalities
7°C	0.5	a	0.57	0
(7.2 ± 0.1)		b	0.59	0
	1	a	1.15	0
		b	1.24	0
	2	a	1.94	0
		b	2.04	0
	3	a	3.39	0
		b	3.19	0
	4	a	3.94	0
		b	4.26	2
	5	a	5.58	5
		b	5.6	5
	7	a	6.97	5
		b	7.33	5
12°C	0.5	a	0.64	0
(11.7 ± 0.7)		b	0.57	0
	1	a	1.37	0
		b	1.41	0
	2	a	1.81	0
		b	2	0
	3	a	3.5	0
		b	3.27	1
	4	a	3.7	2
		b	3.87	3
	5	a	5.37	4
		b	5.56	5
	7	a	6.75	5
		b	7.79	5

Temperature (Actual)	Nominal	Tank	TFM Concentration (mg L^{-1})	Mortalities
18°C	0.5	a	0.57	0
(18.3 ± 0.1)		b	0.63	0
	1	a	1.07	0
		b	1.06	0
	2	a	1.94	0
		b	2.04	0
	3	a	3.24	0
		b	3.25	0
	4	a	4.16	1
		b	4.13	0
	5	a	5.08	2
		b	4.99	1
	7	a	7.31	5
		b	7.12	5
21°C	1	a	1.08	0
(21.3 ± 0.1)		b	1.02	0
	3	a	1.92	0
		b	2.07	0
	4	a	4.29	2
		b	4.34	0
	5	a	5.38	4
		b	5.23	1
	6	a	5.84	3
		b	5.97	5
	7	a	7.14	5
		b	7.35	5
	10	a	10.54	5
		b	10.62	5

Temperature (Actual)	Nominal	Tank	TFM Concentration (mg L^{-1})	Mortalities
25°C	1	a	0.93	0
(24.9 ± 0.1)		b	1.05	0
	3	a	2.05	0
		b	2.22	0
	4	a	4.41	0
		b	4.50	1
	5	a	5.46	2
		b	5.36	1
	6	a	6.03	3
		b	6.08	2
	7	a	7.10	5
		b	7.30	5
	10	a	10.55	5
		b	10.39	5
28°C	1	a	0.97	0
(27.0 ± 0.2)		b	1.01	0
	3	a	1.95	0
		b	1.99	0
	4	a	4.19	0
		b	4.22	0
	5	a	5.10	0
		b	5.35	1
	6	a	6.07	2
		b	6.11	1
	7	a	7.27	2
		b	7.33	5
	10	a	10.26	5
		b	10.07	5

A.2 R Code

Table A.2 Leave-one-out validation (LOOV) of the generalized linear model (GLM) with a Bernoulli distribution, of dose and temperature interaction with and without a CF, a weight variable, length variable and the effects of the LOOV. McFadden pseudo R^2, AIC values and model prediction accuracy (40 errors over 480 data points) across the models.

				Significance of the terms				
Model	**n**	**McFadden pseudo R^2**	**AIC**	**Dose * temperature**	**Weight**	**Length**	**Condition**	**LOOV Accuracy (%)**
dose * temperature + condition	434	0.7111	169.77	0.0229	-	-	0.815	90.80
dose * temperature + weight + length	434	0.7161	168.98	0.0145	0.323	0.154	-	90.55
dose * temperature + length	434	0.7146	167.95	0.0127	-	0.172	-	90.32
dose * temperature + weight	480	0.7249	171.81	0.0047	0.342	-	-	91.04
dose * temperature	480	0.7233	170.72	0.0055	-	-	-	91.67

A.2.1 Script 1: support_functions.R

```
#' This script is part of the analysis pipeline for the following paper:
#' The Mechanism of Increased Tolerance to the Pesticide
#' 3-Trifluoromethyl-4-Nitrophenol (TFM) in Invasive Sea Lamprey
#' in Warmer Waters
#'
#' Journal: Environmental Toxicology and Chemistry
#' DOI: https://doi.org/10.1093/etojnl/vgag040


#' This script was ran in R 4.5.0, on Kubuntu 24.04
#'
convert_link <- function(P, link = c("identity", "sqrt", "inverse",
"invsqrt",
                                     "log", "logit", "cloglog")) {
```

```
link <- match.arg(link)
if (any(grepl("se.fit", names(P)))) {
  output <- data.frame(
    fit = rep(NA_real_, length(P$fit)),
    upper = rep(NA_real_, length(P$fit)),
    lower = rep(NA_real_, length(P$fit)))
  calculate.CI <- TRUE
} else {
  output <- data.frame(fit = rep(NA_real_, length(P$fit)))
  calculate.CI <- FALSE
}
if (link == "identity") {
  output$fit <- P$fit
  if (calculate.CI) {
    output$upper  <- P$fit + 1.96*P$se.fit
    output$lower <- P$fit - 1.96*P$se.fit
  }
}
if (link == "sqrt") {
  output$fit <- (P$fit)^2
  if (calculate.CI) {
    output$upper  <- (P$fit + 1.96*P$se.fit)^2
    output$lower <- (P$fit - 1.96*P$se.fit)^2
  }
}
if (link == "inverse") {
  output$fit <- (P$fit)^-1
  if (calculate.CI) {
    output$upper  <- (P$fit - 1.96*P$se.fit)^-1
    output$lower <- (P$fit + 1.96*P$se.fit)^-1
  }
}
if (link == "invsqrt") {
  output$fit <- (P$fit)^(-1/2)
  if (calculate.CI) {
    output$upper  <- (P$fit + 1.96*P$se.fit)^(-1/2)
    output$lower <- (P$fit - 1.96*P$se.fit)^(-1/2)
  }
}
if (link == "log") {
  output$fit <- exp(P$fit)
  if (calculate.CI) {
    output$upper  <- exp(P$fit + 1.96*P$se.fit)
    output$lower <- exp(P$fit - 1.96*P$se.fit)
  }
}
if (link == "logit") {
  output$fit <- exp(P$fit) / (1 + exp(P$fit))
  if (calculate.CI) {
    output$upper <- exp(P$fit + 1.96*P$se.fit) /
                    (1 + exp(P$fit + 1.96*P$se.fit))
    output$lower <- exp(P$fit - 1.96*P$se.fit) /
```

```
                    (1 + exp(P$fit - 1.96*P$se.fit))
    }
  }
  if (link == "cloglog") {
    output$fit <- 1 - exp(-exp(P$fit))
    if (calculate.CI) {
      output$upper <- 1 - exp(-exp(P$fit + 1.96*P$se.fit))
      output$lower <- 1 - exp(-exp(P$fit - 1.96*P$se.fit))
    }
  }
  return(output)
}

find_LC <- function(model, target, temp, precision) {
  x <- 0
  increase <- 1

  keep_going <- TRUE
  low_done <- FALSE
  fit_done <- FALSE
  up_done <- FALSE


  while(keep_going) {
    aux <- data.frame(temperature = temp, dose = x)
    P <- predict(model, newdata = aux, se = TRUE, type = "link")
    fit <- exp(P$fit) / (1 + exp(P$fit))
    SeUp <- exp(P$fit + 1.96*P$se.fit) / (1 + exp(P$fit + 1.96*P$se.fit))
    SeLow <- exp(P$fit - 1.96*P$se.fit) / (1 + exp(P$fit - 1.96*P$se.fit))

    if (!up_done) {
      if (round(SeUp, precision) == target) {
        dose_up <- x
        up_done <- TRUE
        x <- 0
        increase <- 1
        # message('Found lower bound')
      } else {
        if (round(SeUp, precision) > target) {
          x <- x - increase
          increase <- increase / 10
        } else {
          x <- x + increase
        }
      }
    }

    if (!fit_done & up_done) {
      if (round(fit, precision) == target) {
        dose_fit <- x
        fit_done <- TRUE
        x <- 0
```

```
        increase <- 1
        # message('Found mean value')
      } else {
        if (round(fit, precision) > target) {
          x <- x - increase
          increase <- increase / 10
        } else {
          x <- x + increase
        }
      }
    }

    if (fit_done & up_done) {
      if (round(SeLow, precision) == target) {
        dose_low <- x
        low_done <- TRUE
        x <- 0
        increase <- 1
        # message('Found high bound')
      } else {
        if (round(SeLow, precision) > target) {
          x <- x - increase
          increase <- increase / 10
        } else {
          x <- x + increase
        }
      }
    }


    keep_going <- !(low_done & fit_done & up_done)
  }

  return(c(dose_low, dose_fit, dose_up))
}
```

A.2.2 Script 2: tfm_analysis.R

```
#' This script is part of the analysis pipeline for the following paper:
#' The Mechanism of Increased Tolerance to the Pesticide
#' 3-Trifluoromethyl-4-Nitrophenol (TFM) in Invasive Sea Lamprey
#' in Warmer Waters
#'
#' Journal: Environmental Toxicology and Chemistry
#' DOI: https://doi.org/10.1093/etojnl/vgag040
#'
#'
#' This script was ran in R 4.5.0, on Kubuntu 24.04
#'
library('ggplot2')
source("support_functions.R")
```

```
tfm <- read.csv('lamprey_temperature_tfm_tox_test_2022-02_INDIVIDUALS.csv')

head(tfm)
str(tfm)

# Build mortality model
# -------------------------------------
  # model mortality rather than survival
  tfm$died <- !tfm$survived

  m1 <- glm(died ~ dose * temperature + weight.g,
        family = binomial(link = 'logit'),
        data = tfm)

  summary(m1)
  car::Anova(m1, type = "III")

  # remove weight as it is not significant

  m2 <- glm(!survived ~ dose * temperature,
        family = binomial(link = 'logit'),
        data = tfm)

  summary(m2)
  car::Anova(m2, type = "III")

  coefs <- as.data.frame(summary(m2)$coefficients)
# -------------------------------------

# Equation to calculate LC50
# -------------------------------------
  #   # y = a + bx
  #   a = coefs$Estimate[1]
  #   b = coefs$Estimate[2:4]
  #   T = 7
  #   m = 0.5
  #
  #   # solve for dose
  #   y = a + b[1] * dose + b[2] * T + b[3] * dose * T
  #   b[1] * dose + b[3] * dose * T = y - a - b[2] * T
  #   dose * (b[1] + b[3] * T) = y - a - b[2] * T
  #   dose = (y - a - b[2] * T) / (b[1] + b[3] * T)
  #
  #   # deal with the model's link to use
  #   # mortality directly.
  #   # solve the below for y
  #   m = exp(y) / (1 + exp(y))
  #   y = ln(m / (1 - m))
  #
  #   # Replace y with m
  #   dose = (ln(m / (1 - m)) - a - b[2] * T) / (b[1] + b[3] * T)
# -------------------------------------
```

```
# prepare values for plots
# ------------------------------------
  pred <- data.frame(temperature = rep(unique(tfm$temperature), each =
100),
                     dose = rep(seq(from = min(tfm$dose),
                                    to = max(tfm$dose),
                                    length = 100),
                                times = 6))

  P <- predict(m2, newdata = pred, se = TRUE, type = "link")
  pred <- cbind(pred, convert_link(P, link = "logit"))

  tmp_labs <- paste0(unique(pred$temperature), '°C')

  pred$label <- factor(paste0(pred$temperature, '°C'),
                               levels = tmp_labs)
  tfm$label <- factor(paste0(tfm$temperature, '°C'),
                             levels = tmp_labs)

  # prepare dashed lines
  dashes <- pred[pred$temperature != 28, ]

  # push all dashed levels up by one
  dashes$temperature <- as.factor(dashes$temperature)
  levels(dashes$temperature) <- c(12, 18, 21, 25, 28)
  dashes$temperature <- as.numeric(as.character(dashes$temperature))

  dashes$label <- droplevels(dashes$label)
  levels(dashes$label) <- levels(pred$label)[2:6]

  dashes$label <- factor(dashes$label,
                         levels = levels(pred$label))
# ------------------------------------

# plot model with aquaria mortality points
# ------------------------------------
  list_tfm <- split(tfm, paste0(tfm$temperature, "_", tfm$aquarium))
  names(list_tfm)

  aux <- lapply(list_tfm, function(i) {
    output <- data.frame(temperature = i$temperature[1],
                         dose = i$dose[1],
                         pMort = sum(!i$survived)/nrow(i))
    return(output)
  })
  aq_mort <- data.table::rbindlist(aux)

  aq_mort$label <- factor(paste0(aq_mort$temperature, '°C'),
                          levels = paste0(unique(aq_mort$temperature),
'°C'))
```

```
  p <- ggplot()
  p <- p + geom_line(data = pred,
                     aes(x = dose, y = fit, group = temperature))
  p <- p + geom_line(data = dashes,
                     aes(x = dose, y = fit, group = temperature),
                     linetype = 'dotted')
  p <- p + geom_ribbon(data = pred,
                       aes(x = dose, ymax = upper,
                           ymin = lower, group = temperature),
                       alpha = 0.2)
  p <- p + geom_point(data = aq_mort, aes(x = dose, y = pMort))
  p <- p + facet_wrap(label ~ ., scales = "fixed", ncol = 3)
  p <- p + labs(x = expression('[TFM] (mg L'^'-1'~')'),
                y = "Mortality probability")
  p <- p + theme_bw()
  p

  ggsave('tfm_model_mort_by_aquarium.png', width = 8, heigh = 5)
# -------------------------------------

# Calculate LC as a function of temperature, and plot it
# -------------------------------------
  pred <- data.frame(temperature = rep(seq(from = 7, to = 28, length = 50),
                                       times = 1),
                     LC = rep(c(0.5, 0.999), each = 50))

  # note: this takes a bit to run
  output <- apply(pred, 1, function(r) {
    find_LC(model = m2, target = as.numeric(r[2]),
            temp = as.numeric(r[1]), precision = 5)
  })

  pd <- cbind(pred, t(output))
  colnames(pd)[3:5] <- c('upper', 'fit', 'lower')
  pd$LC <- pd$LC*100
  pd$LC <- factor(pd$LC, levels = c(99.9, 50))

  p <- ggplot(data = pd, aes(x = temperature,  group = LC))
  p <- p + geom_line(aes(y = fit, colour = LC))
  p <- p + geom_ribbon(aes(ymax = upper, ymin = lower, fill = LC),
                       alpha = 0.2)
  p <- p + theme_bw()
  p <- p + labs(x = 'Temperature (°C)',
                y = expression('[TFM] (mg L'^'-1'~')'),
                fill = "12-h LC", colour = "12-h LC")
  p <- p + scale_y_continuous(limits = c(0, 15),
                              breaks = 0:7*2)
  p

  # ggsave('LC_curves_axis_to_0.png', width = 5, height = 4)
# -------------------------------------
```

Appendix B

Supplementary Information for Chapter 3

Table B.1 Differences in the measured and nominal lampricide tank concentrations. Water lampricide concentrations for (A) TFM, (B) niclosamide, and (C) TFM/1% niclosamide, measured in water at the start (0 h), middle (6 h), and end (12 h) of exposure. The time-averaged measured values, along with the recorded mortalities per treatment tank, were used to calculate LC_{50} and MLC values across treatments.

A – TFM only		**Measured TFM Concentration (mg L^{-1})**				
Nominal	**Tank**	**Initial**	**Middle**	**End**	**Averaged**	**Mortalities**
1.0	a	0.71	0.89	0.72	0.77	0
	b	0.69	1.08	0.81	0.86	0
2.0	a	1.95	2.03	1.88	1.95	0
	b	1.93	2.13	1.85	1.97	0
3.0	a	3.16	3.14	3.08	3.13	2
	b	3.11	3.03	2.96	3.03	3
3.5	a	3.40	3.34	3.65	3.46	4
	b	3.55	3.70	empty	3.63	5
4.0	a	4.11	4.27	empty	4.19	5
	b	4.11	4.16	empty	4.14	5
5.0	a	5.27	5.06	empty	5.17	5
	b	5.40	5.50	empty	5.45	5
B – Nic only		**Measured Niclosamide Concentration (mg L^{-1})**				
Nominal	**Tank**	**Initial**	**Middle**	**End**	**Averaged**	**Mortalities**
0.01	a	0.011	0.010	0.007	0.009	0
	b	0.013	0.010	0.007	0.010	0
0.05	a	0.040	0.062	0.043	0.048	1
	b	0.036	0.035	0.039	0.037	2
0.1	a	0.087	0.069	empty	0.078	5
	b	0.093	empty	empty	0.093	5
0.25	a	0.298	empty	empty	0.298	5
	b	0.283	empty	empty	0.283	5

C – TFM/1% Nic		Measured TFM/Niclosamide Concentration (mg L^{-1})				
Nominal	**Tank**	**Initial**	**Middle**	**End**	**Averaged**	**Mortalities**
1.0/0.010	a	0.75/0.018	0.75/0.009	0.72/0.009	0.74/0.012	0
	b	0.84/0.012	0.84/0.007	0.80/0.009	0.83/0.009	0
2.0/0.020	a	1.87/0.024	1.69/0.018	1.67/0.021	1.74/0.021	1
	b	1.95/0.026	1.92/0.021	1.95/0.017	1.94/0.021	2
2.5/0.025	a	2.45/0.030	2.39/0.022	2.35/0.020	2.40/0.024	4
	b	2.67/0.028	2.57/0.022	empty	2.62/0.025	5
3.0/0.030	a	3.04/0.015	2.95/0.013	empty	3.00/0.014	5
	b	3.07/0.030	2.92/0.024	empty	3.00/0.027	5
4.0/0.040	a	4.52/0.052	empty	empty	4.52/0.052	5
	b	4.20/0.040	empty	empty	4.20/0.040	5

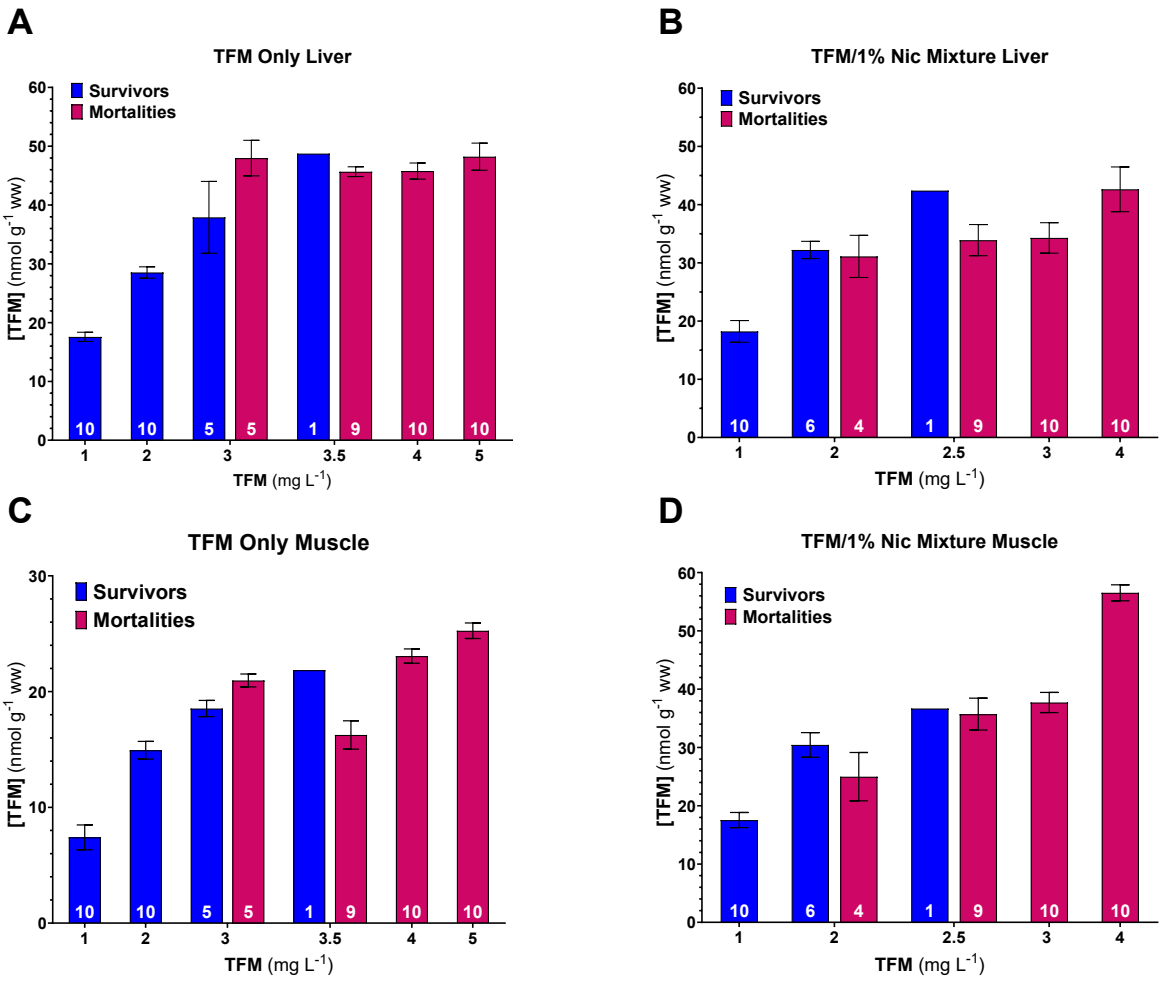


Figure B.1 TFM concentrations in the liver (A, B) and muscle (C, D) of sea lamprey that survived or suffered mortality following TFM ± niclosamide exposure. No significant differences among groups. n = 10, the number of larval sea lamprey per group.

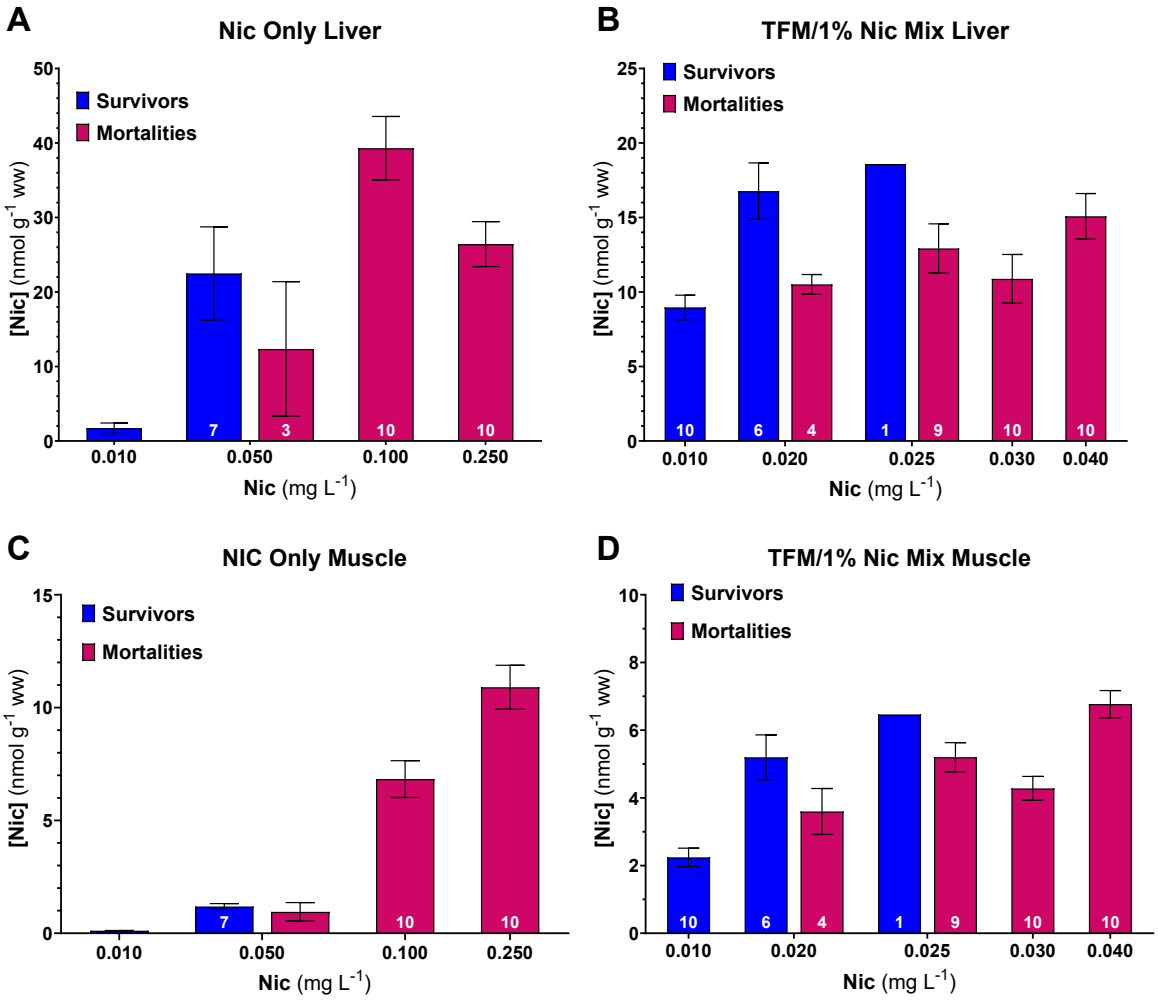


Figure B.2 Niclosamide concentrations in the liver (A, B) and muscle (C, D) of sea lamprey that survived or suffered following niclosamide ± TFM exposure. No significant differences among groups. n = 10, the number of larval sea lamprey per group.

Appendix C

Supplementary Information for Chapter 4

Table C.1 TFM concentrations during 12-h TFM toxicity measurements across 4 different temperatures and mortality numbers of rainbow trout at each concentration. TFM concentrations were measured spectrophotometrically at 395 nm and confirmed against standards prepared from analytical-grade TFM and the same dechlorinated water used during the experiments. The starting number of fish in each tank was 5.

Temperature (°C)	Nominal	Tank	[TFM] (mg L^{-1})	Mortalities
6	7.5	a	7.55	0
		b	7.36	0
	10	a	10.35	0
		b	9.96	0
	15	a	15.35	2
		b	14.96	2
	20	a	20.3	3
		b	19.94	4
	25	a	25.98	5
		b	24.88	5
11	7.5	a	7.45	0
		b	7.24	0
	10	a	9.73	1
		b	10.08	0
	15	a	15.18	1
		b	14.92	3
	20	a	19.27	4
		b	19.26	5
	25	a	24.48	5
		b	25.92	5

Temperature (°C)		Tank	[TFM] (mg L^{-1})	Mortalities
16	7.5	a	7.45	0
		b	7.05	0
	10	a	9.73	1
		b	9.88	0
	12.5	a	12.05	1
		b	12.27	3
	15	a	14.88	4
		b	14.79	4
	20	a	19.83	5
		b	19.82	5
21	7.5	a	7.55	0
		b	7.56	0
	10	a	10.41	1
		b	10.26	0
	15	a	14.97	1
		b	15.07	2
	20	a	20.13	4
		b	19.94	3
	25	a	25.23	5
		b	26.64	5

Appendix D

Supplementary Information for Chapter 5

Table D.1 The nominal and measured (observed) lampricide tank concentrations for acute toxicity tests (Experiment 1). (A) TFM, (B) Nic and (C) TFM/1% Nic Mix. The lampricide water concentrations were measured at the start (0 h), middle (6 h), and end (12 h) of exposure, and averaged across time, along with the recorded mortalities per treatment tank, were used to calculate LC_{50} and MLC values across treatments.

A – TFM only		**Measured TFM Concentration (mg L^{-1})**				
Nominal	**Tank**	**Initial**	**Middle**	**End**	**Averaged**	**Mortalities**
5.0	a	5.42	5.32	5.26	5.33	0
	b	5.03	4.89	4.86	4.93	0
7.5	a	7.98	7.86	7.86	7.90	0
	b	7.56	7.65	7.59	7.60	0
10.0	a	11.24	10.00	9.94	10.39	1
	b	10.30	10.46	10.46	10.41	1
17.0	a	17.58	17.53	17.00	17.37	2
	b	17.51	17.44	16.92	17.29	3
25.0	a	24.77	24.62	empty	24.70	5
	b	25.40	25.28	empty	25.34	5
B – Nic only		**Measured Niclosamide Concentration (mg L^{-1})**				
Nominal	**Tank**	**Initial**	**Middle**	**End**	**Averaged**	**Mortalities**
0.05	a	0.069	0.057	0.038	0.055	0
	b	0.068	0.060	0.038	0.055	0
0.10	a	0.143	0.127	0.081	0.117	0
	b	0.132	0.126	0.077	0.112	2
0.25	a	0.339	0.330	empty	0.335	5
	b	0.361	0.334	empty	0.348	5
0.5	a	0.596	empty	empty	0.596	5
	b	0.672	empty	empty	0.672	5
1	a	1.308	empty	empty	1.308	5
	b	1.346	empty	empty	1.346	5

C- TFM/1% Nic		Measured TFM/Nic Concentration (mg L^{-1})				
Nominal	**Tank**	**Initial**	**Middle**	**End**	**Averaged**	**Mortalities**
2.5/0.025	a	2.98/0.024	2.41/NA	2.73/0.14	2.71/0.019	0
	b	3.07/0.025	2.49/NA	2.7/0.013	2.75/0.019	0
5/0.05	a	5.46/0.028	5.36/NA	5.55/0.019	5.46/0.023	0
	b	5.04/0.029	5.16/NA	5.76/0.023	5.32/0.026	0
7.5/0.075	a	9.44/0.049	9.32/NA	9.69/0.037	9.48/0.043	1
	b	9.72/0.054	9.51/NA	9.64/0.031	9.62/0.046	1
10/0.1	a	11.67/0.109	11.6/NA	11.62/0.061	11.63/0.085	3
	b	12.00/0.109	11.84/NA	11.85/0.060	11.90/0.085	4
15/0.15	a	16.30/0.178	empty	empty	16.30/0.178	5
	b	16.74/0.168	empty	empty	16.74/0.168	5
25/0.25	a	25.07/0.289	empty	empty	25.07/0.289	5
	b	25.19/0271	empty	empty	25.19/0.271	5