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The effect of alkalinity on lampricide effectiveness and gill physiology in invasive sea lamprey (Petromyzon marinus)

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Abstract

The pesticides, 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide are used to control populations of invasive sea lamprey (*Petromyzon marinus*) in the Laurentian Great Lakes of North America. Added to streams infested with larval sea lamprey, the effectiveness of these pesticides, commonly called lampricides, are strongly influenced by water pH, with greater toxicity for both TFM and niclosamide in lower pH than higher pH water. However, the TFM and niclosamide sensitivity of sea lamprey are also greater in poorly buffered, low alkalinity water than in high alkalinity water but it is unclear why. One goal of my thesis was to propose a model that explained why TFM and niclosamide toxicity to larval sea lamprey was greater in lower versus higher alkalinity water. Based on toxicity tests , the model proposed contends that at low alkalinity there is greater acidification of the gill boundary layer water due to CO_2 and H^+ excretion by the larval sea lamprey as they breathe. The acidification increases the bioavailability of TFM and niclosamide at the gill surface, increasing their sensitivity to the lampricides. Another goal of my thesis was to determine if changes in gill function also contributed to the sea lamprey's greater sensitivity to lampricides in low versus high alkalinity water. To examine this possibility, total ATPase and Na^+/K^+-ATPase activity in the gills, and plasma ion concentrations (Na^+, Cl^-) were measured in larval sea lamprey exposed to TFM (3.5 mg L^{-1}) alone, a TFM/1 % niclosamide mixture (2.9 mg L^{-1}/ 29 µg L^{-1}) or niclosamide alone (78 µg L^{-1}) in waters of low (~50 mg L^{-1} as $CaCO_3$), moderate (~150 mg L^{-1} as $CaCO_3$) and high alkalinity (~250 mg L^{-1} as $CaCO_3$), at a common water pH of ~ 8.3. At low and moderate alkalinity, total ATPase activity decreased with exposure to TFM and TFM/1 % niclosamide, but Na^+/K^+-ATPase activity was unimpaired. Blood plasma Na^+ and Cl^- concentration were not compromised following exposure to TFM, TFM/1 % niclosamide or niclosamide alone at low, moderate or high alkalinity. I

conclude that any disturbances to gill function caused by exposure to lampricides, regardless of alkalinity, are insufficient to cause severe reductions in plasma Na^+ or Cl^- balance in larval sea lamprey. Instead, I propose that the greater sensitivity of sea lamprey to lampricides in waters of lower alkalinity is primarily a function of greater lampricide bioavailability in the gill boundary layers due to increased acidification of the water crossing the gills compared to higher alkalinities.

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Chapter 1

Introduction and Literature Review

Introduction

Sea lamprey (*Petromyzon marinus*) that inhabit North America can be divided into an anadromous population, native to the Atlantic coast and an invasive, landlocked population living in the Laurentian Great Lakes (Bryan et al., 2005; Smith and Tibbles, 1980). The life cycle of the sea lamprey begins in freshwater streams and rivers, where the eggs hatch and subsequently develop into larvae called ammocoetes. They remain in this larval stage for 3-7 years, buried in the substrate and filter feeding on organic material by intercepting the streams current using their oral hood (Sutton and Bowen, 1994). When they reach a sufficient body size, typically greater than 2.5 g in mass and a length greater than 120 mm, they begin a 3–4-month complex metamorphosis (Holmes and Youson, 1994). After metamorphosis, juvenile sea lamprey enter their parasitic juvenile phase, feeding on the blood of other fishes. The high rate of blood consumption, and possible infection post feeding often leads to the death of the parasitized fish (Farmer, 1980). After 12-20 months of parasitic feeding, the maturing adults stop-feeding, migrate upstream, spawn and die (Beamish and Potter, 1975).

Sea lamprey gained entry to the upper Great Lakes from Lake Ontario to Lake Erie in the early 20th century via the Welland Canal, which created a bypass around the Niagara Falls (Eshenroder, 2014, 2009). By the mid-twentieth century parasitism of large-bodied sport and commercial fishes by juvenile sea lamprey helped cause significant socioeconomic and ecological damage to the Great Lakes (Gaden et al., 2021; Siefkes, 2017; Smith and Tibbles, 1980). Indeed, one parasitic lamprey is capable of killing almost 21 kg of fish (Kitchell and Breck, 1980; Swink, 2003). Because they selectively feed on larger fishes such as lake trout (*Salvelinus namaycush*) (Hansen et al., 2016), the invasion, along with over-harvesting, contributed to the near crash of the lake trout fishery and the extirpation of some species of cisco (*Coregonus spp.*). The loss of top predators also contributed to an explosion in populations of

invasive alewife (*Alosa psuedoharengus*), a fish native to the Atlantic Ocean, which eventually lead to large die-offs that frequently littered the shorelines with dead fish, negatively impacting local economies dependent on recreational fishing and tourism (Tanner and Tody, 2002). In response to the crisis, a Canada and the United States formed a partnership that led to the creation the Great Lakes Fisheries Commission (GLFC) in 1955 (Gaden et al., 2021). The GLFC was given the responsibility to oversee fisheries management and to eradicate sea lamprey in the Great Lakes (GLFC, 2011).

Following the formation of the GLFC, a sea lamprey control (SLC) program was implemented which included low head barrier dams, traps, and the release of sterile male lamprey to control sea lamprey populations (GLFC, 2011). Chemical control with the lampricides 3-trifluoromethyl-4- nitrophenol (TFM) and 2′,5-dichloro-4′-nitrosalicylanilide (niclosamide) was also implemented in the 1960s and remains highly effective at suppressing sea lamprey populations by targeting larval sea lamprey in their nursery streams (Wilkie, 2019). Together, barriers and lampricides have helped reduce the sea lamprey population to 10% of their peak in the 1950's (Siefkes, 2017).

Due to their relative low capacity to detoxify TFM, sea lamprey are much more vulnerable to TFM than most non-target fishes (Bussy et al., 2018a, b; Kane et al., 1994; Lech, 1974; Statham and Lech, 1975). At first, TFM was used alone, but it is now often mixed with niclosamide (1-2 % of the TFM concentration) which increases TFM toxicity and can lower TFM requirements by 40%, and therefore help lower treatment costs and the amount of chemical released into the environment (Boogaard et al., 2003; Wilkie et al., 2019). While TFM normally has relatively low toxicity to non-target species, the same cannot be said for niclosamide which has greater potency and less selectivity than TFM (Boogaard et al., 2003; Wilkie et al., 2019). It

is therefore important to better understand the physiological impacts of niclosamide in both sea lamprey and non-target species in comparison to TFM.

TFM is a phenolic compound with an aromatic ring and a yellow-brownish colour with a viscous consistency. It is a weak acid with a low pKa of 6.07 to 6.38 (Hubert, 2003; McConville et al., 2016), which means that it can easily lose or gain a H^+ ion from its hydroxyl (-OH) functional group, depending on water pH (Figure 1.1A.). It is well established that TFM toxicity increases in acidic environments (Bills et al., 2003). The water pH affects the relative amounts of un-ionized and ionized forms of the lampricide. In an acidic environment, there will be greater amounts of the phenolic (un-ionized) form of TFM, but at higher pH there will be more of the ionized form of TFM (Figure. 1.1A; McDonald and Kolar, 2007; Wilkie et al., 2019). When TFM is in this un-ionized form, it is more lipophilic and it can pass across the gill and into the blood more easily when compared to its ionized form, rendering it more toxic (Hlina et al., 2017; Hunn and Allen, 1974).

Niclosamide shares similar properties to TFM. It is also a phenolic compound and is of a yellow-brownish colour with a viscous consistency, but it has two aromatic rings (Hubert, 2003; Wilkie et al., 2019). Niclosamide is also a weak acid with a low pKa of 6.25, and an ionizable hydroxyl group (Figure 1.1.B.). Based on this, it is predicted that a greater proportion of niclosamide, like TFM, would be in its un-ionized form at lower water pH, leading to greater rates of uptake and toxicity (Wilkie et al., 2019). However, few studies have addressed how differences in pH and alkalinity affect niclosamide uptake and toxicity, which would be expected to decrease as water pH increases.

The toxicity of TFM and niclosamide also decrease as alkalinity increases (Bills et al., 2003), but the underlining mechanisms are not clear. Alkalinity is a measure of the capacity of an aqueous solution (e.g. water) to resist changes in pH by buffering acids by binding hydrogen ions

(H^+). Alkalinity per say will not influence TFM or niclosamide speciation, but by influencing the pH near the gill surface in the gill microenvironment it could alter lampricide bioavailability. Classic studies by Wright et al. (1986) and Playle and Wood (1989) demonstrated that as inspired water passed across the gills, its pH was altered by the excretion of ammonia, metabolic acid (H^+) and CO_2 by the gills. Rainbow trout held under circumneutral pH conditions resulted in a more acidic (lower pH) gill microenvironment due to direct H^+ excretion that was coupled to NH_3 excretion, and the hydration of CO_2 to H^+ and HCO_3^- in comparison to the surrounding bulk water. Subsequent studies demonstrated that this could have important implications for fishes exposed to toxicants such as metals and to compounds that were weak acids or bases with ionizable functional groups because it could alter their chemical speciation and therefore change their bioavailability relative to the bulk water (Erickson et al., 2006; Playle, 1998). In the case of larval sea lamprey, it would be expected that in waters of higher alkalinity, but of comparable pH, mortality would be expected to be lower when exposed to the same concentration of TFM or niclosamide. This was recently shown to be true in juvenile lake sturgeon exposed to TFM, in which mortality and the rates of TFM uptake were higher in low (50 mg L^{-1} as $CaCO_3$) compared to higher alkalinities (150 and 250 mg L^{-1} as $CaCO_3$), but this has not yet been demonstrated in larval sea lamprey. Hence, a major goal of my M.Sc. was to determine how exposure of larval sea lamprey to TFM, a TFM plus niclosamide (1% of the TFM concentration) solution (TFM/Nic), or niclosamide (Nic) alone in varying alkalinities influenced toxicity.

The mode of action of TFM in sea lamprey and other fishes has been studied more than niclosamide. After it is taken-up, TFM uncouples mitochondrial oxidative phosphorylation in the cells, resulting in a decrease in ATP production (Birceanu et al., 2011; Huerta et al., 2020). Oxidative phosphorylation is the primary process by which the aerobic production of ATP (adenosine triphosphate) occurs (Terada, 1990). By preventing the phosphorylation of ADP to

ATP, TFM causes a shortfall in ATP supply leading to greater reliance on anaerobic energy reserves such as phosphocreatine and glycogen (Birceanu et al., 2009; Clifford et al., 2012; Wilkie et al., 2019). Phosphocreatine, or high energy phosphagens, are a temporary solution to meet energy demands when ATP supply is limited (Hochachka and Matheson, 1992). Anaerobic glycolysis can sustain ATP supply for longer than phosphocreatine but are also finite. Once these anaerobic energy reserves are depleted and ATP demands can no longer be met, death results, possibly by starving critical organ systems of ATP. The nervous system appears to be particularly vulnerable to TFM (Birceanu et al., 2009), but other critical organ systems such as the cardiovascular system could also be compromised (Statham and Lech, 1975).

Recent findings have shown that niclosamide also inhibits ATP production by lamprey, rainbow trout, and lake sturgeon (Ionescu et al., 2022a, 2022b). Additionally, niclosamide has shown to also uncouple oxidative phosphorylation (Weinbach and Garbus, 1969) in cancerous colon cells in mice(Alasadi et al., 2018), zebrafish (Zhu et al., 2022), and most recently in larval sea lamprey (Borowiec et al., 2022). *In vitro* experiments using human multiple myeloma cell cultures have also shown that niclosamide is not limited to un-coupling oxidative phosphorylation, since it can also prevent cancer cell growth by killing multiple myeloma cells through mitochondria fragmentation (Khanim et al., 2011). Niclosamide can also interfere with intracellular pH regulation and impair glycolytic enzymes, which are needed to convert glucose-6-phosphate and nicotinamide adenine dinucleotides (NAD+) to pyruvate and NADH by producing two molecules of ATP, thus decreasing the ability of cells to use glucose for anaerobic ATP production (Köhler, 2001). Niclosamide has also been shown to alter the structure of larval lamprey gills causing cell necrosis and swelling (Figure 1.2.; Mallatt et al., 1994). In contrast, TFM alone was shown to have little to no negative impacts on gill structure or function such as osmoregulation and acid-base regulation (Birceanu et al., 2009; Mallatt et al., 1994). This

combination of the toxicological effects may at least partially explain the greater potency of niclosamide compared to TFM.

The gills have four primary functions: gas exchange, nitrogenous waste excretion, acid-base regulation, and ionoregulation (Evans et al., 2005). Each is critical for a fish's survival, but ion and acid-base regulation could be vulnerable to impaired ATP production due to their reliance on active transport processes to mediate ion uptake by the gills in fresh water, particularly the ions Na^+, Cl^- and Ca^{2+} (Evans et al. 2005; Ferreira-Martins et al. 2021). Mitochondrion-rich cells (MRC's) in the gills are the key sites of ion-regulation and do so via pumps located on the MRC's. V-ATPase is a vacuole type electrogenic H^+-pump that are typically located on the apical membrane of the MRC. In teleost fishes, V-ATPases pump H^+ ions across the epithelial membrane into the water, which is hypothesized to help create an electrochemical gradient for Na^+ uptake by making the inner apical more negative, in turn helping to drive the uptake of Na^+ through an acid-sensing ion channel (Dymowska et al., 2015). The Na^+/K^+ ATPase (NKA) also contributes to the generation of the Na^+ electrochemical gradient through negative membrane potential, by pumping Na^+ across the basolateral membranes of the MRCs into the extracellular fluid bathing the cells. A similar set-up is proposed to exist in the sea lamprey gill, in which apical V-ATPase proteins have been localized to the apical membrane of lamprey MRCs (Reis-Santos et al., 2008; Sunga et al., 2020), and mRNA work indicates than an epithelial Na^+ channel (eNac) is likely present which could serves route of Na^+ entry (Ferreira-Martins et al., 2021). On the apical surface of the MRC's there are also Cl^-/HCO_3^- exchanger sites. Acidification within the apical microvilli by V-ATPase lowers the HCO_3^- activity causing the uptake of Cl^-. Cl^- enters the blood via basolateral anion channels, most likely through cystic fibrosis transmembrane conductance regulator (CFTR) channels (Evans, 2011; Marshall et al., 2002).

The electrogenic NKA pump which moves $3Na^+$ and $2K^+$ against their respective electrochemical gradients is located on basolateral membranes of MRC's (Blanco and Mercer, 1998). As the NKA is critical for gill function, it is hypothesized that in the presence of niclosamide, lower ATP production in the gills would impair the uptake of Na^+ by larval sea lamprey with a corresponding disturbance to plasma Na^+ and Cl^- balance. Although TFM has not been shown to cause major disturbances to ion balance in larval sea lamprey or rainbow trout (Birceanu et al., 2014, 2009), it was predicted that the NKA would be more sensitive to niclosamide due to its much more potent effects on mitochondrial ATP production compared to TFM (Borowiec et al., 2022). Accordingly, the second major aim of my M.Sc. thesis was to determine if exposure to niclosamide and/or TFM-niclosamide mixtures inhibits gill-mediated ion transport processes and/or causes damage to the gills that could result in disturbances to plasma ion balance in larval sea lamprey.

Objectives and Hypothesis

The overarching goal of my thesis was to determine how the sensitivity of larval sea lamprey to TFM or niclosamide, alone and in combination, was affected by differences in water alkalinity, and to establish if any differences in survival were related to gill-mediated ion regulation. The specific objectives were to:

1. Determine how water alkalinity altered the sensitivity of larval sea lamprey to TFM, and to establish if any observed differences in survival could be explained by changes in gill total ATPase activity, NKA activity and ion balance.
2. Determine how water alkalinity altered the sensitivity of larval sea lamprey to niclosamide alone or a TFM plus niclosamide (1%) mixture, and to relate any differences in survival to possible differences in gill total ATPase activity, NKA activity, and ion balance.

Accordingly, larval sea lamprey were acclimated to waters of low (~ 50 mg L^{-1} as $CaCO_3$), moderate (~ 150 mg L^{-1} as $CaCO_3$) and high (~250 mg L^{-1} as $CaCO_3$) alkalinity for 3 weeks. To quantify how water alkalinity affected the lampricide sensitivity of larval sea lamprey, survivorship tests were conducted by exposing them to TFM (4.2 mg L^{-1}), a TFM-niclosamide mixture (1%) (2.9 mg L^{-1}/ 29 µg L^{-1}) or to niclosamide alone (29 µg L^{-1}) at each alkalinity. Gills were collected from sea lamprey exposed to TFM (Objective 1) or to niclosamide and TFM/niclosamide (1%; Objective 2), for measurement of total and NKA activity. Blood samples were also analysed for measurement of plasma Na^+ and Cl^- to determine if internal ion balance was disturbed following lampricide exposure at each of the three alkalinities.

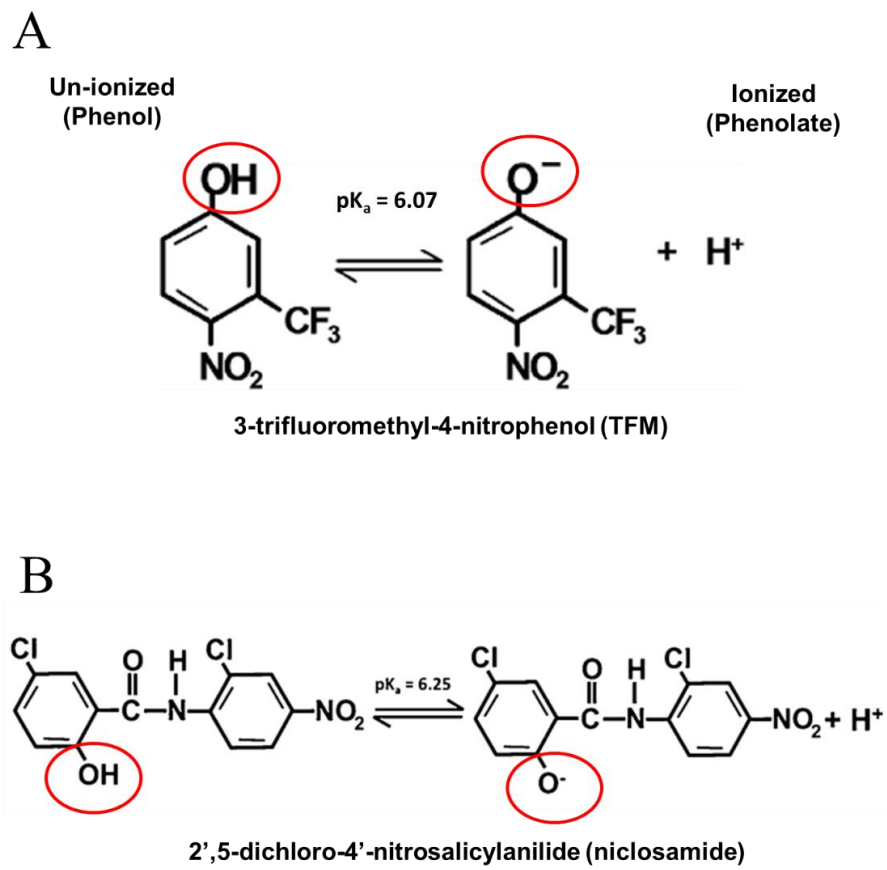

Figure 1-1. Structure and Dissociation of TFM and Niclosamide.

(A) TFM in its un-ionized (phenolic) form (TFM-OH) is more lipophilic and can pass across the gill into the blood more easily compared to its ionized form (TFM-O^-). With a pKa of 6.07 (Hubert 2003), there would be a larger concentration of the phenolic form in a more acidic environment (pH < 6.07), whereas in a more basic environment (pH > 6.07), there would be more of the phenolate form. (B) It is predicted that niclosamide would have similar characteristics as TFM given its phenolic structure and similar pKa of 6.25 (Wilkie et al. 2019). In a more acidic environment (pH < 6.25) there would therefore be a larger proportion of the phenolic form of niclosamide, whereas in a more basic environment (pH > 6.25) there would be more of the phenolate form.

Chapter 2

Effects of different water alkalinities on survival and gill function in larval sea lamprey (*Petromyzon marinus*) exposed to 3-trifluoromethyl-4-nitrophenol (TFM).

Introduction

Sea lamprey gained entry to the upper Great Lakes from Lake Ontario to Lake Erie in the early 20th century via the Welland Canal, which created a bypass around the Niagara Falls, preventing sea lamprey from moving between the two lakes (Eshenroder, 2014, 2009). By the mid-twentieth century, parasitism of large-bodied sport and commercial fishes by juvenile sea lamprey, along with overfishing, had caused significant socioeconomic and ecological damage including the crash of the lake trout (*Salvelinus namaycush*) fishery and the extinction of some species of ciscoes (*Coregonus spp.*) (Siefkes, 2017; Smith and Tibbles, 1980). Following the formation of the Great Lakes Fishery Commission (GLFC) by Canada and the United States in 1954, a highly successful sea lamprey control (SLC) program was implemented including low head barrier dams, traps, and sterile male release in later years (GLFC, 2011). However, the key to the success of the SLC program was chemical treatment with the lampricide 3-trifluoromethyl-4- nitrophenol (TFM), which targeted larval sea lamprey in their nursery streams (Siefkes, 2017; Wilkie et al., 2019). The sea lamprey were more sensitive to TFM compared to most non-target fishes due to their relatively low capacity to detoxify TFM (Bussy et al., 2018a; 2018b, Kane et al., 1994; Lech, 1974; Statham and Lech, 1975). Combined with barriers and trapping, TFM treatment contributed to a 90 % reduction in Great Lakes sea lamprey populations from their peak in the mid-twentieth century (Siefkes, 2017).

TFM is a phenolic compound with an aromatic ring and a yellow-brownish colour with a viscous consistency. It is a weak acid with a low pKa of 6.07, which means that it can easily lose or gain a H^+ ion depending on water pH (Hubert, 2003). Below pH 6.07, the majority of TFM is un-ionized (TFM-OH), with the ionized form (TFM-O^-) predominating at water pHs greater than pH 6.07 (McDonald and Kolar, 2007; Wilkie et al., 2019). As a result, the bioavailability of TFM is dependent upon water pH, with greater amounts of the more lipophilic, phenolic (un-ionized)

form present at lower water pHs, compared to higher pHs (Wilkie et al., 2019). Therefore, the relative toxicity of TFM is greater at lower pHs (Bills et al., 2003) due to much higher rates of uptake of the un-ionized form of TFM (Hlina et al., 2017; Hunn and Allen, 1974; Wilkie et al., 2021). The uptake of TFM may be further complicated by acidification of the gill boundary layer microenvironment, where acidification of the water due to the excretion of CO_2 and metabolic H^+ leads to more un-ionized TFM crossing the gills (Wilkie et al. 2019).

The toxicity of TFM also decreases as alkalinity increases in both sea lamprey and non-target fishes (Bills et al., 2003). This may be because higher alkalinity increases the buffer capacity of the water near the gills, decreasing the capacity of lamprey and non-target fishes to acidify the gill microenvironment (Wilkie et al. 2021). At lower alkalinity and a set water pH, more TFM-OH would therefore be present in the gill microenvironment, leading to greater TFM accumulation by the animal and greater toxicity. This could also result in greater TFM uptake across the gill, with greater effects on ATP-mediated ion transport processes, which are critical for maintaining ion homeostasis in fishes including lampreys (Dymowska et al., 2015; Ferreira-Martins et al., 2021; Reis-Santos et al., 2008; Zydlewski and Wilkie, 2013).

Studies using isolated mitochondria from sea lamprey and trout liver, and from sea lamprey heart, have shown that TFM interferes with ATP production by uncoupling oxidative phosphorylation (Birceanu et al., 2011; Borowiec et al., 2022; Huerta et al., 2020), leading to decreased ATP production and ultimately death. It remains unclear, however, whether or not TFM interferes with ATP production in the gills, in which ion transport processes are heavily reliant on ATP (Evans et al., 2005; Ferreira-Martins et al., 2021).

The gills have four primary functions: gas exchange, nitrogenous waste excretion, acid-base regulation, and ionoregulation (Evans et al., 2005). Each is critical for a fish's survival, but ion and acid-base regulation could be vulnerable to impaired ATP production due to their

reliance on primary and secondary active transport processes to mediate ion uptake by the gills in fresh water, particularly the ions Na^+, Cl^- and Ca^{2+} (Evans et al., 2005; Ferreira-Martins et al., 2021). Mitochondrion-rich cells (MRC's) in the gills are the key sites of ion-regulation and do so via different ATPase pumps located on the apical or basolateral membrane. The apically located V-ATPase pumps H^+ ions across the epithelial membrane into the water, making the inner apical membrane more negative, which is hypothesized to contribute to the generation of the electrochemical gradient needed for uptake of Na^+ through an epithelial sodium channel (ENaC) which has been found in lamprey (Dymowska et al., 2015; Ferreira-Martins et al., 2021; Reis-Santos et al., 2008; Bartels and Potter, 2004, Wilkie et al., 1998). The Na^+/K^+ ATPase (NKA) is an electrogenic pump, which consumes the largest amount of ATP in the gill (Evans, 2011; Marshall, 2002; Marshall et al., 1997; Skou, 1957). The NKA transports two K^+ ions into the cell in exchange for three Na^+ which are transported into the extracellular fluid and plasma per ATP (Blanco and Mercer, 1998). On the apical surface of the MRC's of teleost fishes there are also thought to be Cl^-/HCO_3^- exchangers, which take-up Cl^- in exchange for HCO_3^-, and the Cl^- then entering the blood via basolateral anion channels (cystic fibrosis transmembrane conductance regulator (CFTR) channel; Evans, 2011; Marshall, 2002), which has yet to be confirmed in sea lamprey gills.

Alkalinities in the Great Lakes can range from 13 to 122 mg L^{-1} as $CaCO_3$ in Lake Superior, and from 16 to 196 mg L^{-1} as $CaCO_3$ in Lake Michigan (Kanayama, 1963; O'Connor et al., 2017). Lake Ontario has an average alkalinity of 92 mg L^{-1} as $CaCO_3$, Lake Erie averages 88 mg L^{-1} as $CaCO_3$, and the alkalinity of Lake Huron water averages 79 mg L^{-1} as $CaCO_3$ (Urban and Desai, 2009). Not surprisingly, the alkalinity of streams containing larval sea lamprey can also vary greatly, ranging from 25 to 241 mg L^{-1} as $CaCO_3$ in different Upper Great Lakes rivers

and streams treated with lampricides, similar to the range covered in the present study (O'Connor et al., 2017).

Given the predicted effects of alkalinity on TFM toxicity, one goal of the present study was to quantify TFM sensitivity of larval sea lamprey acclimated to low, moderate, or high alkalinity water, which encompasses the wide variation of alkalinity in the Great Lakes. Another goal was to determine if ATP-mediated gill ion transport processes were greater in low compared to higher alkalinity waters due to greater TFM bioavailability in the gill microenvironment due to the lower buffer capacity of lower alkalinity water. To test this hypothesis, larval sea lamprey were exposed to sublethal concentrations of TFM (12-h LC_{25}) for 8-24 h in low, moderate or high alkalinity water, accompanied by the collection of gills for measurements of total ATPase and Na^+/K^+-ATPase (NKA) activity, and blood for the measurements of plasma Na^+ and Cl^- concentrations at different time intervals of exposure.

Methods

Experimental animals and holding

All experiments followed Canadian Council of Animal Care guidelines and were approved by the WLU Animal Care Committee under AUP R18004. Larval sea lamprey (*Petromyzon marinus*) were collected by pulsed DC electrofishing by US Fish and Wildlife personnel from tributaries draining into Lakes Michigan and/or Huron and were held at the Hammond Bay Biological Station (HBBS), Millersburg, Michigan, United States, before shipment to Wilfrid Laurier University. Once received, the larval sea lampreys were housed in 110 L fiberglass aquaria, lined with ~ 5 cm of sand to provide the animals with burrowing substrate, for a minimum of two weeks in continuously flowing aerated well water (pH ~ 8.3; alkalinity ~ 250 mg L^{-1} as $CaCO_3$; temperature ~ 13-15^oC; dissolved oxygen > 80% saturation; flow rate ~ 500

mL min^{-1}). The larval lamprey were fed Baker's yeast (1 g of yeast per lamprey) on a weekly basis and were held under a 12 h dark:12 h light cycle.

Acclimation to different alkalinities

A minimum of two weeks prior to experiments, sub-sets of larval sea lamprey (N ~ 70) where acclimated to water of low, medium, and high alkalinity (nominal = 50, 150, 250 mg L^{-1} as $CaCO_3$; see Table 2-1 for detailed water chemistry). The reconstituted water was made-up using reverse osmosis water to which appropriate amounts of $NaHCO_3$ were added to yield nominal water alkalinities 50, 150, 250 mg L^{-1} as $CaCO_3$, plus appropriate amounts of other salts (KCl, $CaSO_4 \cdot 2H_2O$, $MgSO_4$ (BioShop Canada Inc., Burlington, Ontario) to ensure that water ion content and pH were comparable among the different tanks and to natural freshwaters (Table 2-1). The larvae were acclimated in three 37 L glass aquaria (N = 54 per aquaria) containing diffuse cotton to simulate burrowing substrate and to calm the animals (Wilkie et al., 2007). The water supplying each tank was recirculated as a closed looped system from an overhead 100 L head tank. Water alkalinity was measured once a day using a commercial kit (Hach, Alkalinity Test Kit, Model AL-AP, Hach Canada, Mississauga, ON), pH was measured using a handheld meter (pH11 meter, Oakton Instruments, Vernon Hills, IL, U.S.A.), and dissolved oxygen (DO) and temperature measured using a DO meter (YSI Pro 2030, Xylem Water Solutions Inc., Cincinnati, Ohio, U.S.A.). Additional $NaHCO_3$ was added or diluted as needed to maintain appropriate water alkalinity, and pH was controlled by the drop-wise addition of 0.1M of HCl or NaOH to maintain the pH at approximately 8.3 during experiments. Flame atomic absorption spectroscopy (AAS, PinAAcle 900T, Perkin Elmer, Waltham, MA, USA) was used to measure plasma Na^+, and a Cole-Parmer Chloride Analyzer (Chloride Analyser 926, Cole Parmer, Vernon Hills, Il, USA) was used to measure water Cl^- concentrations.

The sea lamprey were exposed to field grade TFM (Clariant SFC GMBH WERK, Griesheim, Germany; 32.5 % active ingredient dissolved in isopropanol), provided courtesy of the Sea Lamprey Control Centre, Fisheries and Oceans Canada (DFO), Sault Ste. Marie, ON.

Experimental protocols

Effects of water alkalinity on larval sea lamprey tolerance to TFM

To test the hypothesis that exposure to TFM is more toxic in low versus higher alkalinity water, time of survival tests were conducted to determine the relative tolerance of lamprey to different concentrations of the lampricide. The experiment was preceded by an acute toxicity test to determine the LC_{50} of TFM to the sea lamprey at moderate alkalinity (150 mg L^{-1} as $CaCO_3$) water, serving as range-finder to select a common TFM exposure concentration for the subsequent time of survival tests. 12 h before the range finder experiments, groups of lampreys (N = 10 per tank; N = 70 total) were transferred to one of seven tanks filled with 5 L of water at an alkalinity of 150 mg L^{-1} as $CaCO_3$ (moderate), pH of 8.3, temperature of 12.0°C ± 0.1, and left overnight to acclimate to their surroundings. The next morning (12 h after transfer), sufficient amounts of TFM were added to each individual aquarium to yield nominal concentrations of TFM in the water of 0, 3.0, 3.5, 4.0, and 4.5 mg L^{-1}. As this was a range-finder test, replicate tanks were not used at each concentration in order to minimize sea lamprey mortality and to conserve animals for subsequent experiments. The sea lamprey were monitored hourly, with an end-point of death determined by a lack of buccal movement and responsiveness to a pinch of the tail with a pair of forceps (Hlina et al., 2017). For unresponsive animals, the time of death was recorded, the specimen weighed, and then disposed. Parameters that were recorded and monitored included dissolved oxygen (DO), pH, alkalinity, water temperature, and TFM treatment concentrations measured before, during and after experiments. The dissolved

oxygen was monitored to ensure a minimum of 80 % saturation, which was archived for all experiments which maintained an average of 95.59 ± 0.21 %.

Using the 12-h TFM LC_{50} calculated from the preliminary acute toxicity trials in moderate alkalinity water, time of survival tests were then conducted by exposing sea lamprey to a TFM concentration of 4.23± 0.03 mg L^{-1} in either low, moderate or high alkalinity for 24 h. 12 h prior to the experiment, the lamprey were transferred into 10 L glass aquaria filled with 5 L of water (to prevent lamprey from escaping) at one of the three alkalinities (nominal alkalinities: 50 mg L^{-1} as $CaCO_3$, 150 mg L^{-1} as $CaCO_3$, and 250 mg L^{-1} as $CaCO_3$) in triplicate (N = 10 lamprey per aquaria; N = 30 total per alkalinity). Each tank was placed in a water bath to maintain the temperature at 12.0°C ± 0.1. The following morning (12 h after transfer), tanks were dosed with TFM, and the animals were monitored hourly for the first 12 h of the experiment, at 18 h, and finally at 24 h when surviving animals were euthanized using tricaine methanesulfonate (TMS; 1.5 g L^{-1}, Syndel Labs, Nanaimo, BC, Canada) buffered with 3.0 g L^{-1} of $NaHCO_3$ and the carcasses disposed. Water samples were collected at the beginning and end of the experiment for quantification of TFM as described above. Control animals (N = 10 at each alkalinity) were treated in an identical manner, but not exposed to TFM.

Effects of alkalinity and TFM on gill function of larval sea lamprey

To test the hypothesis that TFM exposure interfered with gill-mediated ion regulation in quantifiably different ways at low, moderate, and high alkalinity, plasma ion concentration and Na^+/K^+ -ATPase activity was measured in sea lamprey exposed to the same pre-determined TFM concentration described above, at low, moderate or high alkalinity.

Larval lamprey were acclimated for at least one week to the appropriate alkalinity. Twelve hours prior to the experiments, the lamprey (N = 3 per aquaria) were transferred into 10 L glass aquaria, continuously receiving water of low, moderate or high alkalinity (N = 54 at each

alkalinity). The next morning, water flow was cut-off and the lamprey were exposed to 3.5 ± 0.03 (12) mg L^{-1} of TFM at each of the three alkalinities, which was equivalent to the 12-h LC_{25} determined in the TFM range-finder tests at moderate alkalinity, described above. The 12-h LC_{25} was used to ensure that sufficient numbers of sea lamprey would be alive for sampling, particularly at low alkalinity, where the animals were much more susceptible to TFM than at moderate and high alkalinity (as noted in the results below). After 1, 2, 4, 8 h of TFM exposure in low alkalinity water, and after 4, 12, 24, and 48 h of TFM exposure (N = 12 per sample period) at moderate and high alkalinity, the sea lamprey were euthanized with anaesthetic (described above) followed by gill tissue and blood collection. It was noted during the trials in low alkalinity water, that the time to death was much faster than in animals exposed to the same concentration of TFM in moderate and high alkalinity. Accordingly, the sampling times were more frequent (1, 2, 4, and 8 h), and the total duration of exposure (8 h), shorter than at moderate and high alkalinity. At each sample period, gill samples (corresponding to branchiopores 1, 3 and 5) were collected by making cross sections (~ 4 mm wide) through the entire branchial (gill) region, which were then transferred to 1.5 mL polypropylene centrifuge tubes, snap frozen in liquid N_2 and stored at -80°C until analyzed for NKA and total ATPase activity (McCormick, 1993; Reis-Santos et al., 2008). Whole blood was then collected using heparinized haematocrit tubes from an incision behind the last branchiospore, spun for three minutes at 10,000 *g* using a microcentrifuge (Thermo Scientific, 75002492, MA, USA), and the plasma drawn off and transferred into 0.5 mL polypropylene centrifuge tubes, and snap frozen in liquid N_2 to be stored at -80 °C until analysis for plasma Na^+ and Cl^- ion concentration.

Analytical techniques

TFM concentrations

Water TFM exposure concentrations were quantified, using a Novaspec II spectrophotometer (Pharmicia Biotech) at a wavelength of 395nm (Barber and Steeves, 2019), and verified using precision TFM standards (0, 4, 8, 12 mg L^{-1} TFM) provided courtesy of the Sea Lamprey Control Centre, Fisheries and Oceans Canada (DFO) (Sault Ste. Marie, Ontario).

Na^+/K^+-ATPase and Total ATPase Activity Assay

Gill NKA activities were measured using a kinetic microassay (McCormick, 1993). Briefly, gill tissues (1,3,5) were homogenized in sodium deoxycholic acid (SEI) buffer (250 mM sucrose, 10 mM Na^2EDTA, 50 mM imidazole) with sodium deoxycholate added to 0.1% volume of SEI buffer, using a Precellys 24 bead homogenizer (Bertin Corporation, Maryland, USA), and centrifuged at 12,000 *g* for 5 min at 4 °C. Specific Na^+/K-ATPase activity was then determined on 10 µL aliquots of supernatant (in triplicate) were transferred to the well of a 96-well microplate, followed by the addition of 200 µL of one of two assay mixtures. Assay mixture 1 (AM 1) contained 50 mM imidazole buffer, 2 mM phosphoenolpyruvate, 0.16 mM nicotinamide adenine dinucleotide (NADH), 0.5 mM adenosine triphosphate (ATP), 2.86 U mL^{-1} lactic dehydrogenase, and 3.57 U mL^{-1} pyruvate kinase. The second assay mixture (AM 2) also contained 0.5 mM of ouabain, which used to inhibit NKA activity. The microplates were shaken for 1 minute and then read on a microplate spectrophotometer at 340 nm, every 47 s, for 20 min (Epoch 2, BioTek Instruments, Inc., VT, U.S.A.). Standard curves were produced for ADP to determine the amount of ATP (nmol) converted to ADP during each enzyme measurement. The difference between the measured total ATPase activity (AM1, no oubain) minus the ATPase activity in the presence of oubain (AM2), equaled the wet tissue NKA activity, expressed in nmol ADP mg wet $tissue^{-1}$ h^{-1}. Specific total ATPase and specific NKA activity were then

expressed in nmol ADP μg protein^{-1} h^{-1} following determination supernatant protein concentration, which was determined using a BCA protein assay (G-Biosciences, St. Louis, MI, U.S.A.).

Plasma Na^+ and Cl^- analysis

Plasma Na^+ concentration were determined in triplicate using atomic absorption flame spectrophotometry (AAS; PinAAcle 900T, Perkin Elmer, Waltham, MA, USA), after diluting the plasma 1000 times in deionized water, acidified with a matrix modifier of 1%HNO_3/1%$CaCl_3$/0.1%CsCl. Stock Na^+ standards had a concentration of 1000 mg L^{-1}, which was diluted to create a standard curve with a range of 0, 0.6, 1.2, 1.8, 2.4, and 3 mg L^{-1}. Plasma Cl^- concentration was measured in 5 μL undiluted sample using a Cole-Parmer Chloride Analyzer (Chloride Analyser 926, Cole Parmer, Vernon Hills, IL, USA).

Calculation and statistics

Determination of the 12-h LC_{50} and 12-h $LC_{99.9}$ of TFM were calculated using an online R software program (Adams, 2016), based on the Litchfield Wilcoxon fitted model, including 95% confidence intervals. Differences in the survivorship of larval sea lamprey exposed to TFM at each of the three alkalinities were subjected to Log-rank (Mantel-Cox) tests to determine if the family of resulting TFM survival curves were significantly different from one another at the $P<0.05$ level. Pairwise comparisons between different curves were then made using a Bonferroni corrected threshold value of $P<0.0083$, determined by dividing the overall level of significance ($P<0.05$) by the total number of comparisons ($K= 6$, three treatment groups and one control). To compare differences in the rates of mortality in the different treatment groups, a hazard-risk ratio (relative slope of the survival curves) was calculated using the Mantel–Haenszel method. This statistical analysis was done using GraphPad Prism (version 9. San Diego, CA).

Physiological data (plasma ions, Na^+/K^+ -ATPase and total ATPase specific activity) was presented as the mean ±1 standard error of the mean (SEM). After confirming the data validity and the absence of collinearity among the explanatory variables, a Bernoulli generalised linear model (GLM) with logit link function (i.e. a logistic regression) was applied. Then a stepwise goodness-of-fit model selection was performed to determine which covariate combination would produce the best model. When significant variability was observed, statistical significance between the means was assessed using the Tukey post-test at the $p< 0.05$ level. Outliers were tested and removed using the Grubb's test, also known as a maximum normalized residual test. The Grubbs test detects a single outlier at either end of the data distribution. If an outlier is detected, the data point is removed, and the test is tested again until there are no more outliers (Tessier et al., 2018).

Results

Effects of alkalinity on the TFM tolerance in larval sea lamprey

The range-finder test conducted in water of moderate alkalinity (151.3 ± 0.82 mg L^{-1} as $CaCO_3$), yielded a 12-h TFM LC_{50} of 4.2 mg L^{-1} (CI = 4.194 - 4.284 mg L^{-1}), a 12-h TFM LC_{25} of 3.5 mg L^{-1} (CI = 3.070 - 3.805 mg L^{-1}) and 12-h $LC_{99.9}$ of 4.5 mg L^{-1} (CI = 4.474 - 4.576 mg L^{-1}). The 12-h LC_{50} value ultimately served as the nominal concentration of TFM to which the sea lamprey were exposed in the subsequent time of survival tests when sea lamprey were exposed to TFM in low, moderate or high alkalinity water.

The larval sea lamprey exposed to a nominal TFM concentration of 4.2 mg L^{-1} (measured [TFM] = 4.15 ± 0.04 mg L^{-1}) were most sensitive to the TFM in low alkalinity water (measured alkalinity = 59.4 ± 1.02 mg L^{-1} as $CaCO_3$; pH = 8.31 ± 0.01), in which % survival reached zero between 3 and 6 h of exposure (Figure 2-1). In contrast, exposure to the same nominal concentration of TFM (measured [TFM] = 4.26 ± 0.03 mg L^{-1}) at moderate alkalinity (measured

alkalinity = 151.3 ± 0.82 mg L^{-1} as $CaCO_3$; pH = 8.27 ± 0.01), was characterized by a slower onset of mortality between 8 and 12 h, when the observed mortality was 30%, with no further mortality for the remainder of the 24 h exposure (P = <0.0001). Survival was 100% in the sea lamprey exposed to TFM (measured [TFM] = 4.27 ± 0.03 mg L^{-1}) at high alkalinity (251.3 ± 2.55 mg L^{-1} as $CaCO_3$; pH = 8.36 ± 0.02), and in the control (non-exposed) animals at all three alkalinities (Figure 2-1).

Effects of alkalinity and TFM on gill function and plasma ion balance

Specific Na^+/K^+-ATPase Activity and Total ATPase Activity

In the absence of TFM, there were no significant differences in gill total ATPase activity between lamprey acclimated to low, moderate or high alkalinity water (Figure 2-2A). Additionally, total ATPase activity was not affected by TFM exposure (measured [TFM] = 3.55 ± 0.09 mg L^{-1}) at low alkalinity (Figure 2-2A). However, total gill ATPase activity was significantly reduced by approximately 30 % after 48 h of exposure to TFM (measured [TFM] = 3.47 ± 0.12 mg L^{-1}) in moderate alkalinity (Figure 2-2A; P = 0.0470). Similarly, 30-35 % reductions in total ATPase activity were observed during exposure to TFM (measured [TFM] = 3.50 ± 0.16 mg L^{-1}) in high alkalinity water after 4 h (P = <0.001), and 12 h (P = 0.0365) of exposure (Figure 2.2A).

In the absence of TFM, there were no significant differences in gill NKA activity between lamprey acclimated to low, moderate, or high alkalinity water (Figure 2-2B). However, at low alkalinity, exposure to TFM resulted in a transient 60 % reduction in gill Na^+/K^+ -ATPase activity after 1 h (P = 0.031), but subsequently recovered to pre-exposure activities after 2 h, where it remained through the complete 8 h exposure (Figure 2-2B). No significant changes in gill NKA activity were observed at any time during exposure to TFM at moderate alkalinity. The NKA activity was reduced by approximately 60 % in the sea lamprey exposed to the same

concentration of TFM at high alkalinity after 4 h (P = 0.00281), returning to pre-exposure values at 12 h and 24 h, before decreasing again after 48 h (Figure 2-2B; P = 0.01189).

Plasma ions and haematocrit

Water alkalinity had no affect on plasma Na^+ concentrations with a plasma Na^+ concentration ranging between 105.7 ± 2.39 mmol L^{-1}, 98.2 ± 2.75 mmol L^{-1}, and 109.9 ± 6.49 mmol L^{-1} in control larval sea lamprey acclimated to low, medium, and high alkalinity water respectively (Fig. 2-3A). Exposure to TFM had little effect on plasma Na^+ concentration at high alkalinity. However, after 8 h TFM exposure in low alkalinity water, plasma Na^+ was significantly reduced by 15 % (Figure 2-3 A; P = 0.048). There were also time dependent changes in plasma Na^+ over time in moderate alkalinity, during which plasma Na^+ concentrations significantly differed from the controls after 24 h (P = 0.003) and 48 h (P = 0.037) of exposure. Additionally, the respective Na^+ concentrations in moderate alkalinity were 30 to 40 % greater at the 12, 24, and 48 h interval when compared to high alkalinity (Figure 2-3A). Plasma Cl^- concentrations were not affected by alkalinity, ranging from 76 ± 1.62 mmol L^{-1}, 76.6 ± 2.68 mmol L^{-1}, and 78.5 ± 2.61 mmol L^{-1} in control larval sea lamprey acclimated to low, medium, and high alkalinity water respectively. However, in low alkalinity water there was a 12 % reduction of plasma Cl^- after 8 h, but no other significant differences were observed (Figure 2-3 B; P = 0.022). The haematocrit of larval sea lamprey was affected by TFM treatments in low and moderate alkalinity but not high alkalinity. In low alkalinity water, haematocrit increased by 17 %, 25 %, and 42 % after 2 h, 4 h and 8 h, respectively (Table 2-2). Moderate alkalinity yielded one significant value, a 10 % decrease in haematocrit after 12 h.

Discussion

The present study demonstrates that when sea lamprey are exposed to the same concentration of total TFM, higher water alkalinity protects them from TFM toxicity,

independent of the bulk water pH. However, these protective effects in higher alkalinity water appear to be unrelated to differences in the capacity of the gills to maintain internal ion balance via ATP-dependent ion exchange processes. Despite some inhibition and variation in total ATPase and Na^+/K^+ -ATPase activity at low, moderate, and high alkalinity, plasma Na^+ and Cl^- concentrations were, for the most-part, unaffected over 48 h of sub-lethal TFM exposure.

TFM toxicity is inversely related to water alkalinity

It has long been known that the acute toxicity of TFM, as measured by its LC_{50} or $LC_{99.9}$, decreases as alkalinity is increased (Bills et al., 2003), but the underlying mechanisms are not as well established. The toxicity of TFM is inversely related to water pH, increasing as water pH decreases due to increases in the amount of the more bioavailable TFM-OH in the water, which is more lipid soluble and more readily taken up across the gills than TFM-O^- (Bills et al., 2003; Hlina et al., 2017; Hunn and Allen, 1974). The higher buffer capacity of water at the gill surface likely explains the greater protection against TFM toxicity observed in sea lamprey exposed to TFM at higher alkalinities (Wilkie et al. 2021). In teleost fishes, the water pH at the gill surface, also referred to as the gill microenvironment, tends to differ from the bulk water pH due to the hydration of respiratory CO_2 as it crosses the gills (Playle and Wood, 1989; Wright et al., 1986) and the excretion of metabolic acid or base by modulating branchial H^+ and/or HCO_3^- excretion using branchial H^+-ATPase (V-ATPase) or Na^+/H^+ antiporters, and Cl^-/HCO_3^- exchangers, respectively (Dymowska et al., 2015; Wright and Wood, 2012). Similar events likely occur in the gill microenvironment of lampreys, in which the gills have very similar structure and function to those of teleost fishes, with an abundance of mitochondrion rich ionocytes (MRCs; Bartels and Potter, 2004; Ferreira-Martins et al., 2021). As with teleost fishes, the MRCs of sea lamprey are characterized by an abundance of basolateral NKA transporters, and apical V-ATPases, and possibly apical Cl^-/HCO_3^- exchange proteins (Dymowska et al., 2015; Ferreira-Martins et al.,

2021). By influencing the pH of the gill microenvironment, the bioavailability of ionizable compounds such as TFM will change based on their acid-base chemical properties, as reflected by their pKa values (see above; Erickson et al., 2006; Wilkie et al., 2021).

With a better knowledge of how the speciation of TFM changes at the gill surface of sea lampreys, not to mention non-target fishes, it may be possible to predict with greater accuracy how the bioavailability and toxicity of TFM changes in waters of different alkalinity. Calculations of TFM speciation based on bulk-water and gill microenvironment pH may help develop a more accurate in field application of TFM, rather than underestimating the bioavailability of TFM which could lead to non-target impacts. It may be also worth investigating how the pH is changed or maintained in the gill microenvironment in varying alkalinities using methods similar to previous studies done by Wright et al. (1986), Playle and Wood (1989) and, most recently, Wilkie et al. (2021) using opercular catheters. The opercular catheters would be able to determine if there is actual changes to the pH in the gill microenvironment when exposed to various alkalinities in adult sea lamprey, however it may prove to be difficult to do so with larval sea lamprey.

Unlike pH, alkalinity does not directly affect the ionization of weak acids or bases. Alkalinity refers to the capacity of water to neutralize acids and bases and to maintain a relatively stable pH level (US Geological Survey, 2020). In other words, the higher the alkalinity, the greater the buffer capacity of the water in the gill microenvironment pH. Hence, in low alkalinity, poorly buffered waters, the gill microenvironment would be expected to be more prone to acidification compared to higher alkalinity, well buffered waters (refer to model depicted in Figure 2-4). As a result, the pH in the gill microenvironment would be expected to be less in lower than higher alkalinity water at a given bulk water pH. Thus, at a given concentration of TFM in the bulk water, the bioavailability of TFM would also be greater in the

gill microenvironment leading to higher rates of TFM uptake and mortality in low versus high alkalinity waters (Figure 2-4). Indeed, support for this model was recently demonstrated in lake sturgeon (*Acipenser fulvescens*), exposed to similar amounts of TFM, in which the rates of ^{14}C-labelled TFM uptake were greatest in low compared to high alkalinity water (Hepditch et al., 2019).

Effects of alkalinity on the sensitivity of gill ionoregulatory processes

There was a significant decrease in NKA activity in low alkalinity after 8 h, which was also accompanied by decrease in both plasma Na^+ and Cl^-, suggesting the ionoregulatory disturbances may have played a role in TFM toxicity. This interpretation is supported by the marked increases in haematocrit also observed at low alkalinity, which could be indicative of cell swelling due to water uptake caused by osmotic and ion disturbances (Milligan and Wood, 1982). Although exposure of sea lamprey to TFM at moderate and high alkalinity affected gill NKA activity, and to a greater extent total gill ATPase activity, there was no evidence that it negatively altered ion balance. Further, haematocrit was only slightly affected by TFM exposure, a transient decrease at 12 h at moderate alkalinity.

The mode of toxic action for TFM is through the uncoupling of oxidative phosphorylation within the mitochondria of cells, which results in decreased ATP production and ultimately a breakdown in physiological homeostasis and death (Birceanu et al., 2011, 2009). Mallatt and colleagues (1985; 1994) proposed that TFM could potentially compromise gill function and ion balance by interfering with ATP-dependent ionoregulatory processes in the gills. However, more recent studies conducted in hard waters (~ 450 mg L^{-1} as $CaCO_3$) of high alkalinity (~ 200 mg L^{-1} as $CaCO_3$) indicated that disturbances to plasma Na^+ and Cl^- balance and Na^+/K^+ -ATPase activity were relatively minor or absent over 12 h exposure to the 12-h LC_{50} of TFM (Birceanu et al., 2011; Hlina et al., 2017).

The lack of disturbances to plasma Na^+ and Cl^- concentrations could be attributed to differences in the ion permeability of the gills in waters of different alkalinities. A major route of ion loss is through paracellular tight junction (TJ) proteins located between the PVCs and MRCs (Chasiotis et al., 2012; Ferreira-Martins et al., 2021; Kolosov et al., 2017a). These TJ complexes join adjacent cells to one another, separating the extracellular fluid (e.g. plasma, interstitial fluid) from the environment, serving as scaffolding between adjacent cells (Chasiotis et al., 2012; Kolosov et al., 2017; Ferreira-Martins et al., 2021). It is possible that when the larval sea lamprey were exposed to TFM, the composition and/or abundance of TJ protein complexes was altered to make the gills less ion permeable to compensate for possible decreases in Na^+ and Cl^- uptake. The plasticity of the TJ network for this purpose was recently demonstrated in larval lamprey exposed to ion poor water for a 2 week period, in which passive ion losses through the gill epithelium were mitigated by an increase in the TJ proteins Tric and *cldn-3B, -4, -10, and -19* in the gill (Kolosov et al., 2020, 2017a, 2017b). The recruitment of these proteins may have further minimized the loss of ions by tightening the gill epithelium (Ferreira-Martins et al., 2021), compensating for any decreases in Na^+ or Cl^- uptake due to TFM exposure.

Given the greater susceptibility of sea lamprey to TFM in lower alkalinity water and its greater bioavailability at a given concentration of total TFM, it is hypothesized that that there would be a greater prevalence of ionic disturbances in sea lamprey exposed to TFM in low versus higher alkalinity water. Surprisingly, there were no significant differences in total ATPase activity, NKA activity, and plasma ion concentration between the control animals acclimated to low, moderate, and high alkalinity water. This suggests that ATP demands of the gill may be more or less stable over a wide range of alkalinities.

In these experiments, an overall decrease in total ATPase activity was observed, in some cases significant, in moderate and high alkalinity water when exposed to TFM. Total ATPase

activity is the sum of all ATPase activity in the gills. One such ATPase is the H^+-ATPase, which is present in sea lamprey gills and thought to be involved in acid-base regulation and possibly Na^+ uptake as described above (Reis-santos et al., 2008; Sunga et al. 2020). Another ATPase that contributes to Total ATPase that was not investigated due to the limitation in lamprey size, is Ca^{2+} ATPase, which is involved in maintaining Ca^{2+} homeostasis and of particular importance for fishes living in waters of low hardness (Flik and Verbost, 1993; Perry and Wood, 1985; Reis-santos et al., 2008). Studies have yet to investigate whether Ca^{2+} ATPase activity is affected during TFM exposure and compared the activity to the amount of Ca^{2+} ions in the blood plasma.

While there were was greater variation in total ATPase and NKA activity in the gills of the larval sea lamprey exposed to TFM, it should be noted that the *in vitro* assays used to measure activity used gill homogenates provided with an excess of ATP (McCormick,1996). Thus, an effect of TFM on ATP supply, and hence ion uptake during TFM exposure *in vivo*, cannot be completely ruled out. In other words, further experiments are needed to determine if a limited supply of ATP results in lower ATPase activity and decreased ion uptake in the presence of TFM.

It was anticipated that by uncoupling oxidative phosphorylation, exposure to TFM could stimulate the ventilatory hypoxic response in the larval lamprey, leading to enhanced loss of ions across the gills (Gonzalez and Mcdonald, 1994). When ATP supply is limited or demand increases, fishes increase their rate of ventilation and the vascular area of the gills, in order to promote increased oxygen consumption (Booth, 1979; Wood and Eom, 2021). A corresponding cost, however, is that it also promotes ion loss by making the gills more ion permeable, which is known as osmoregulatory compromise (Gonzalez and Mcdonald, 1994; Nilsson, 1986). Ion loss likely increase due to increased hydrostatic pressure with greater blood flow during exercise (Gonzalez and Mcdonald, 1994). However, the absence of reductions in plasma Na^+ and Cl^-

concentration suggests that even if there were increased ventilation, due to the known effects of TFM on mitochondrial O_2 consumption (Birceanu et al., 2011; Borowiec et al., 2022), they were insufficient to cause measurable reductions in plasma ions.

It is interesting that that despite the uncoupling effects of TFM on mitochondrial ATP production (Birceanu et al., 2011, 2009; Clifford et al., 2012; Ionescu et al., 2022b), there was no impairment of ion balance, which would have been expected had TFM accumulated in the gills, particularly MRCs. This lack of effect on plasma ion balance, and presumably by extension, ATP supply, could be related to the gill's potential ability to detoxify TFM. Recent transcriptomics studies of the gills of larval sea lamprey, in the presence and absence of TFM, have identified the mRNA coding for key enzymes of TFM detoxification including UDP-glucuronyl transferases (UGTs) and sulfotransferases, which are involved in the biotransformation of TFM via Phase II detoxication processes (Lawrence et al. 2022). A recent study by Lawrence and colleagues (2022) demonstrated that while the mRNA (transcripts) for these enzymes were present, the transcriptional responses in the gills of sea lamprey were limited to upregulation of the P450 mRNA but not the mRNA coding for UGT genes. It is not known if the corresponding proteins (enzymes) were produced in sufficient quantities to help detoxify TFM, but measurements of these enzymes in the gills using enzymatic activity measurements and/or western blots, along with the measurement of TFM and its metabolites could be used to test this hypothesis. If the gills had the ability to endogenously detoxify TFM, it could explain why there was little to no disturbances in the ATPase activity and or decreases in plasma ion concentration.

Summary and Conclusion

The present study supports the hypothesis that the greater sensitivity of larval sea lamprey to TFM at lower alkalinity is due to a greater bioavailability of TFM in the gill

microenvironment compared to the bulk water. Here, I propose a model (Figure 2-4) in which alkalinity affects the toxicity of TFM indirectly by buffering the pH in the gill microenvironment. In high alkalinity water, the acidification of the gill microenvironment is buffered to a greater extent, lowering the bioavailability TFM readily available for uptake.

There was some evidence of ionoregulatory of ionoregulatory and osmotic disturbance in larval sea lamprey exposed to TFM in lower alkalinity water, but not at higher alkalinity. However, it is unclear if the slight decreases in plasma ion concentrations, and increased haematocrit contributed to the greater sensitivity of larval sea lamprey to TFM at low alkalinity. The absence of any physiologically relevant changes observed in total ATPase or NKA activities, or plasma Na^+ or Cl^- balance at moderate and high alkalinities is consistent with greater TFM bioavailability and uptake by larval sea lamprey in lower compared to higher alkalinity waters. Because it is well-established that TFM interferes with the mitochondrial ATP production, upon which the gills depend to maintain ion balance, larger physiological disturbances were expected. This lack of response could be due to compensatory changes in TJ abundance or composition which could, or could be related to the recent findings that suggest that gills have a endogenous phase 1 and 2 detoxification mechanisms that protect the gills from xenobiotics including TFM (Lawrence et al., 2021).

This study could help sea lamprey control agents understand more about how alkalinity affects TFM bioavailability at the gill surface, which may help better predict how sea lamprey and non-target fishes will respond to TFM in waters of different alkalinities and pH. In turn, this could be used to apply TFM with greater accuracy over the wide-range of water alkalinities found in the Great Lakes drainages that are infested with larval sea lamprey and minimize any adverse affects to non-target species. This could also help agents to better refine TFM application procedures by allowing them to use less TFM in waters of lower alkalinity, saving

resources and application efforts, but still effectively managing the sea lamprey population. Lastly, this study suggests that stream side toxicity testing may be necessary take into account differences in the sensitivity of larval sea lamprey to TFM in lower compared to higher alkalinity waters.

Table 2-1. Nominal chemical composition of low, moderate, and high alkalinity reconstituted water used for larval sea lamprey acclimation TFM exposures.

Treatment	Low Alkalinity	Moderate Alkalinity	High Alkalinity
Target Alkalinity (mg L^{-1} as $CaCO_3$)	50	150	250
$CaSO_4$ (mmol L^{-1})	0.41	0.41	0.41
$MgSO_4$ (mmol L^{-1})	0.58	0.58	0.58
KCl (mmol L^{-1})	0.06	0.06	0.06
$NaHCO_3$ (mmol L^{-1})	0.98	2.92	4.86

Table 2-2. Blood haematocrit of larval sea lamprey exposed to a nominal concentration of TFM of 3.5 mg L^{-1}. Data presented as the mean ± 1 SEM (N). Data with an asterisk are significantly different from the control.

Alkalinity	TFM exposure time (h)	Haematocrit (%)	P-Value & Significance
Low (50 mg L^{-1} as $CaCO_3$)	Control	28 ± 1 (21)	-
	1	30 ± 1 (11)	0.814
	2	33 ± 1 (12)	0.029*
	4	35 ± 2 (10)	0.009*
	8	40 ± 2 (11)	< 0.001*
Moderate (150 mg L^{-1} as $CaCO_3$)	Control	28 ± 1 (19)	-
	4	31 ± 1 (12)	0.803
	12	25 ± 4 (11)	0.006*
	24	26 ± 2 (11)	0.708
	48	37 ± 4 (11)	0.346
High (150 mg L^{-1} as $CaCO_3$)	Control	28 ± 1 (20)	-
	4	32 ± 1 (11)	0.355
	12	30 ± 2 (11)	0.857
	24	32 ± 1 (10)	0.287
	48	29 ± 2 (11)	0.940

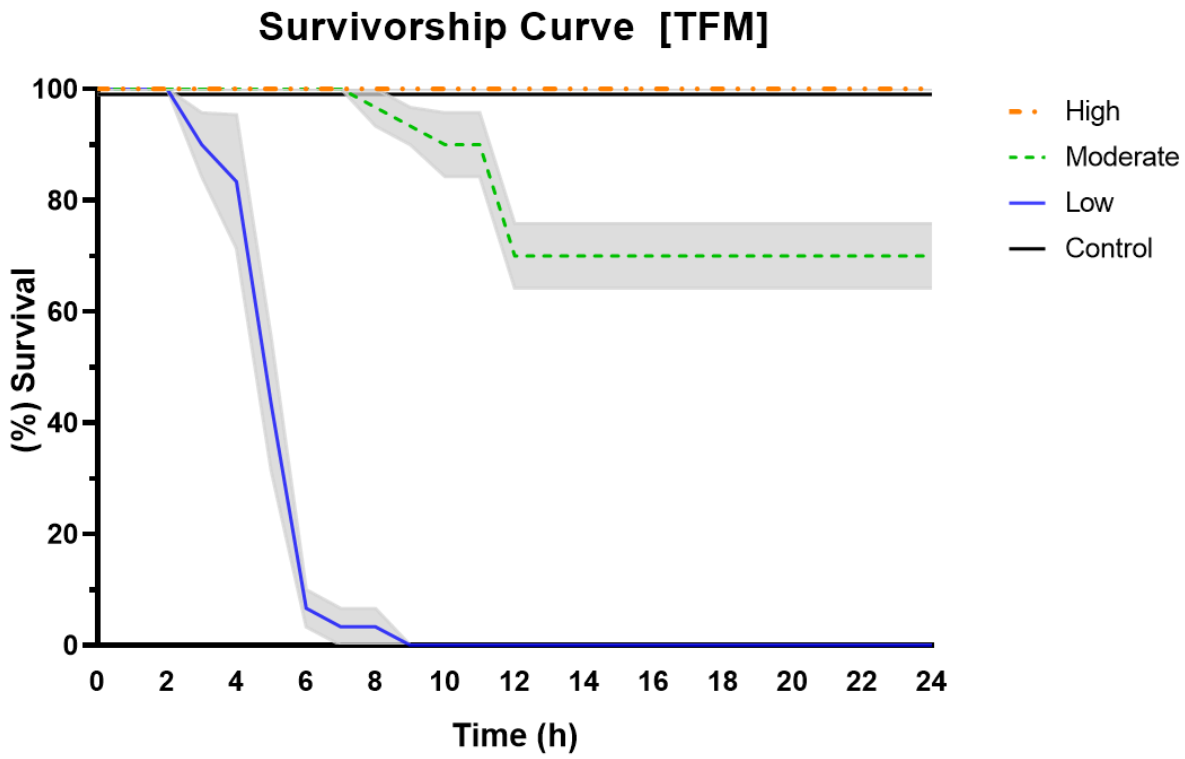

Figure 2-1. Effect of water alkalinity on time of survival of sea lamprey exposed to 3-trifluoromethyl-4-nitrophenol (TFM).

Groups of larval sea lamprey were exposed to a nominal TFM concentration of 4.2 mg L^{-1} for 24 h in water of low alkalinity (59.4 ± 1.02 mg L^{-1} as $CaCO_3$), moderate alkalinity (151.3 ± 0.82 mg L^{-1} as $CaCO_3$) or high alkalinity (251.3 ± 2.55 mg L^{-1} as $CaCO_3$). Survival tests at each alkalinity were done in triplicate, with N =10 larval sea lamprey per replicate, and the mean survival rate was plotted over time. A separate group of control animals (N = 30 in triplicate; N = 10 per replicate) were treated in an identical manner, in the absence of TFM. Shaded areas denote the SEM.

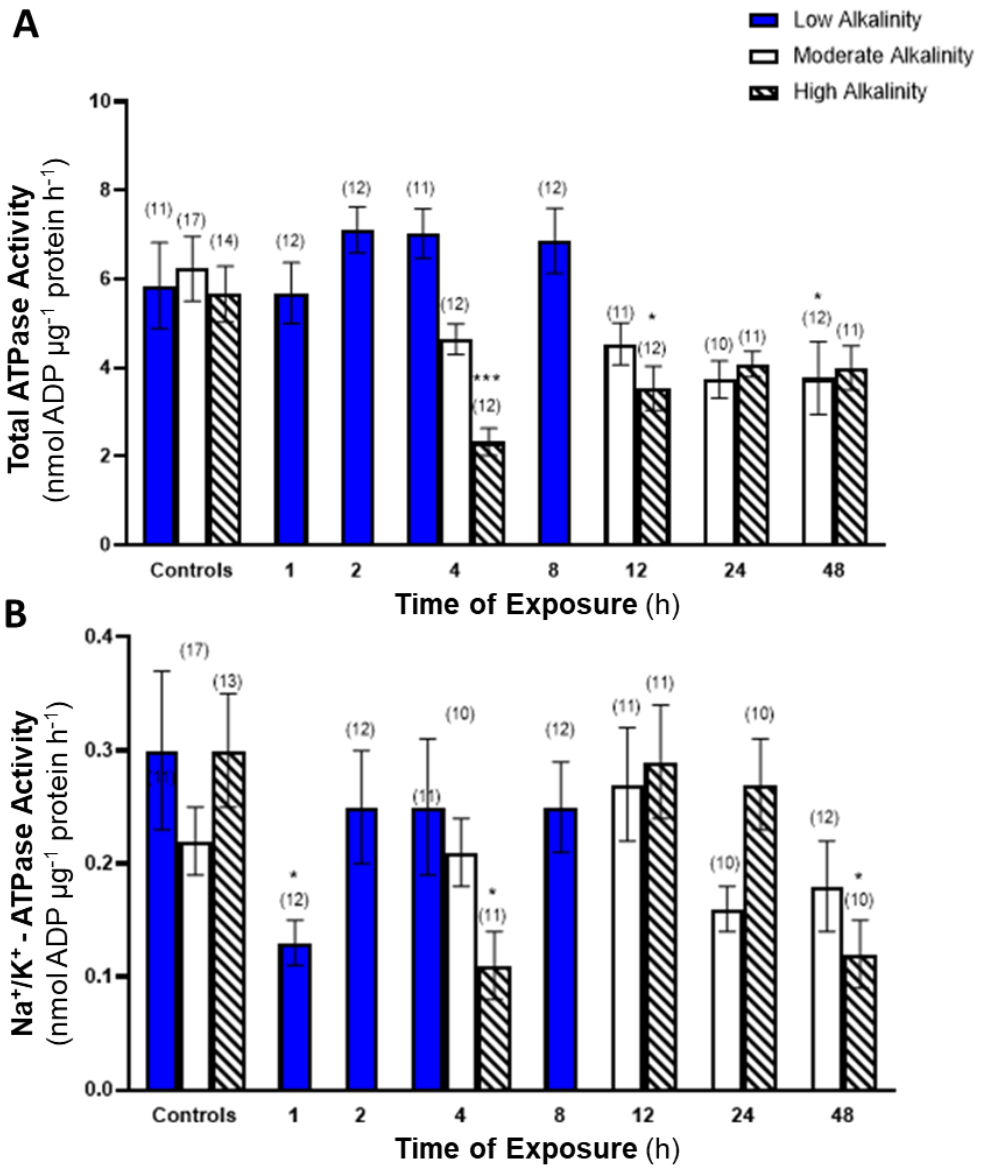

Figure 2-2. Effects of TFM exposure on gill Na^+/K^+-ATPase activity in larval sea lamprey. Changes in (A) total ATPase activity and (B) NKA activity in the gills of larval sea lamprey (*Petromyzon marinus*) under control (no TFM exposure) or following 1, 2, 4, 8, 12, 24, and 48 h exposure to a nominal TFM concentration of 3.5 mg L^{-1} at low alkalinity (blue bars; 54.4 ± 2.2 mg L^{-1} as $CaCO_3$), moderate alkalinity (open bars; 149.6 ± 0.2 mg L^{-1} as $CaCO_3$) or high alkalinity (cross-hatched bars; 255 ± 0.01 mg L^{-1} as $CaCO_3$) at a pH of 8.3. Data presented as the mean ± 1 SEM. Sample sizes (N) indicated over each sample period.

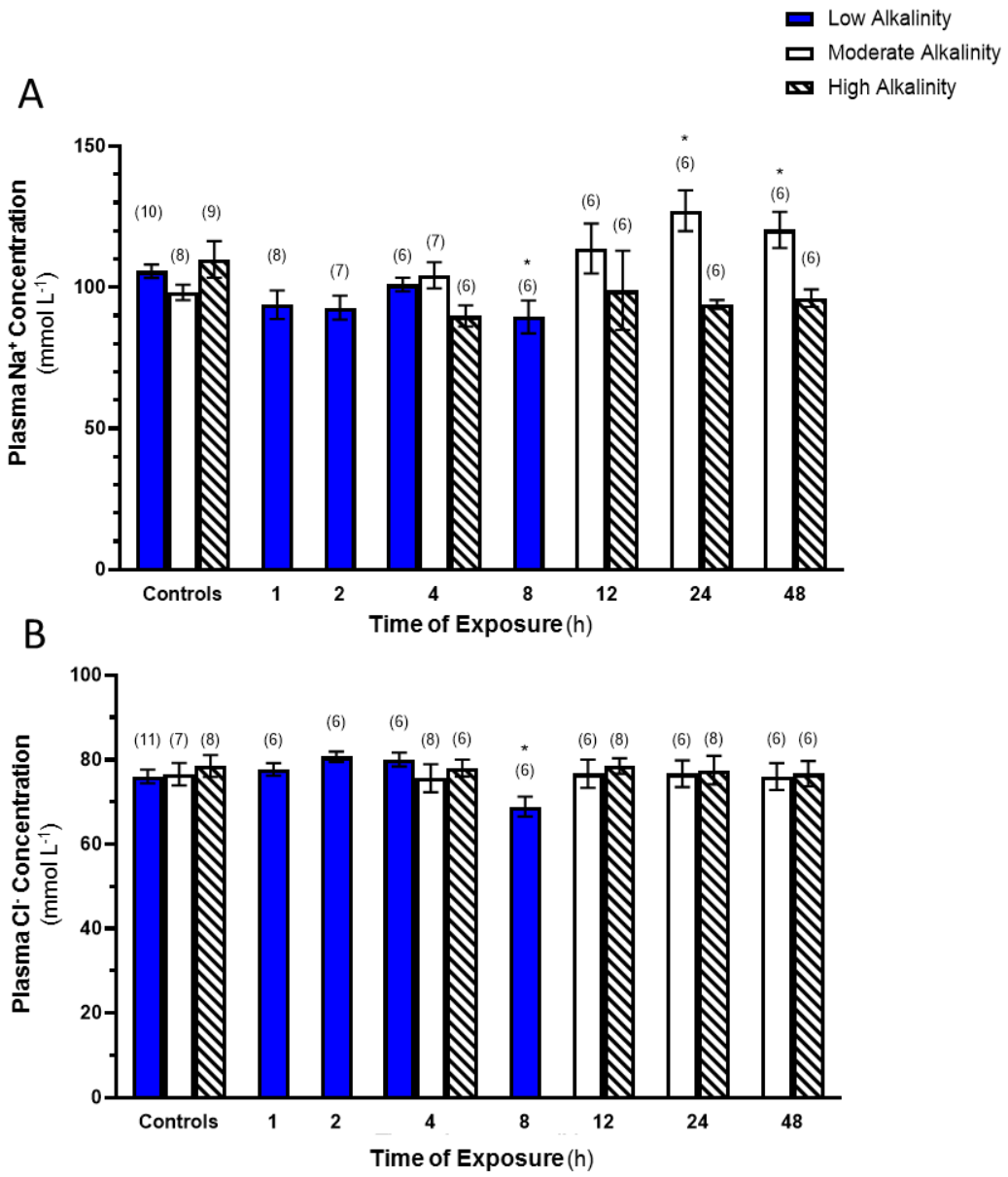

Figure 2-3. Effects of TFM on blood plasma Na^+ and Cl^- in larval sea lamprey.

Changes in (A) plasma Na^+ concentration, and (B) plasma Cl^- concentration of larval sea lamprey under control (no TFM exposure) or following exposure to a nominal TFM concentration of 3.5 mg L^{-1} at low alkalinity (blue bars; 54.4 ± 2.2 mg L^{-1} as $CaCO_3$), moderate alkalinity (open bars; 149.6 ± 0.2 mg L^{-1} as $CaCO_3$) or high alkalinity (cross-hatched bars; 255 ± 0.01 mg L^{-1} as $CaCO_3$) at pH of 8.3. Data presented as the mean ± 1 SEM. Sample sizes (N) indicated over each sample period.

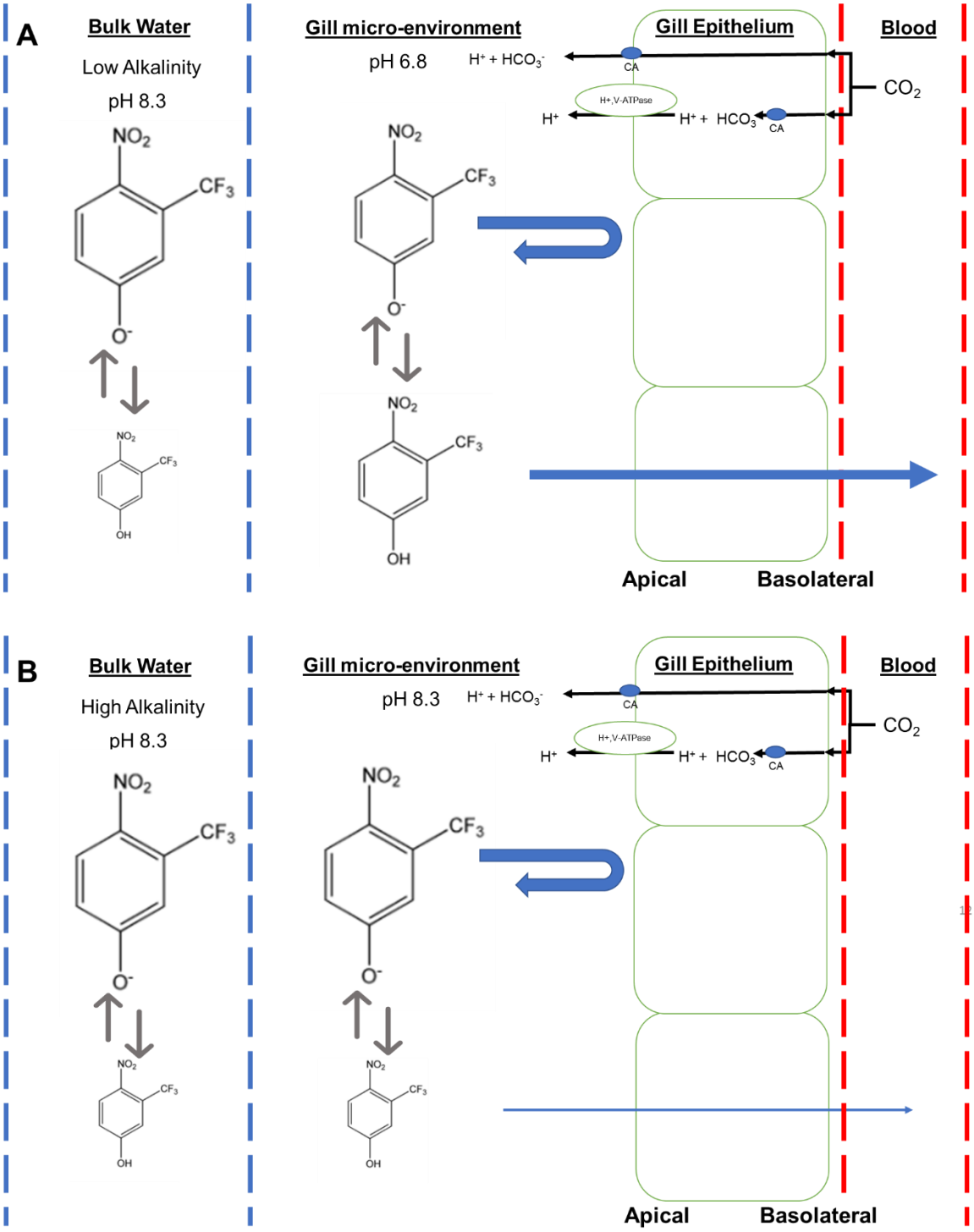
A
Bulk Water
Low Alkalinity
pH 8.3
Gill micro-environment
pH 6.8
Gill Epithelium
Blood
H+ + HCO3-
CA
H+,V-ATPase
H+
H+ + HCO3
CA
CO2
NO2
CF3
O-
OH
Apical
Basolateral
B
Bulk Water
High Alkalinity
pH 8.3
Gill micro-environment
pH 8.3
Gill Epithelium
Blood
H+ + HCO3-
CA
H+,V-ATPase
H+
H+ + HCO3
CA
CO2
NO2
CF3
O-
OH
Apical
Basolateral

Figure 2-4. Proposed model depicting how TFM uptake and toxicity to larval sea lamprey is affected by water alkalinity.

It is proposed that TFM uptake by larval sea lamprey and other fishes occurs mainly in TFM-OH, entering the animal via the gills down its diffusion gradient. A known variable that influences TFM uptake and toxicity is pH, in which the concentration of the more bioavailable TFM-OH is greater at low compared to higher pH, at a given total TFM concentration (Total TFM = TFM-OH + TFM-O-) concentration. The bioavailability of TFM can also be altered at the gill surface (gill microenvironment) due to acidification caused by metabolic H^+ excretion and the hydration of respiratory CO_2 to H^+ and HCO_3^- (Panel A). The amount of acidification taking place near the gill surface is influenced by water alkalinity, which determines he water buffer capacity. At higher water alkalinity, the buffering capacity of water is higher, which attenuates acidification of the gill microenvironment, resulting in less TFM-OH compared to lower alkalinity at a given concentration of TFM, and lower rates of TFM uptake (compare panel A to B).

Chapter 3

Effects of water alkalinity on the toxicity of TFM/1% niclosamide and niclosamide alone to larval sea lamprey (*Petromyzon marinus*)

Introduction

Although TFM is the primary lampricide used to control sea lamprey populations in the Great Lakes, it is often applied in combination with 1-2 % niclosamide in large rivers with high flow rates to enhance TFM toxicity (Dawson, 2003; Wilkie et al., 2019). Niclosamide shares similar properties to TFM, in that is also a phenolic compound, but with two aromatic rings (Hubert, 2003; Wilkie et al., 2019). Like TFM, niclosamide is also a weak acid with a pKa of 6.25, with an ionizable hydroxyl group (Dawson, 2003). Based on this, it is predicted that a greater proportion of niclosamide, like TFM, would be in its un-ionized form at the gill surface at lower water pH and in lower alkalinity water, leading to greater rates of uptake and toxicity (Wilkie et al., 2019). Accordingly, one goal of this study was to determine how water alkalinity alone affected the sensitivity of sea lamprey to TFM-niclosamide (1 %) mixtures or niclosamide.

Both TFM and niclosamide interfere with ATP production by uncoupling mitochondrial oxidative phosphorylation (Niblett and Ballantyne, 1976; Birceanu et al., 2011; Huerta et al., 2020; Borowiec et al., 2022) which ultimately leads to death in sea lamprey and, at sufficiently high concentrations, other fishes. In the absence of sufficient ATP production due to TFM exposure, the body's cells can no longer maintain cellular homeostasis including intracellular ion and osmotic balance, acid-base balance and nutrient levels (Birceanu et al., 2009; Clifford et al., 2012; Ionescu et al. 2020a,b). Compared to TFM, niclosamide is 60 times a more potent uncoupler of oxidative phosphorylation (Borowiec et al., 2022), suggesting that physiological disturbances arising from TFM-niclosamide mixtures could be much more severe than with TFM alone.

Niclosamide (20 µg L^{-1}) alone or TFM-niclosamide mixtures (1-2 % niclosamide) (1.25 mg TFM L^{-1} and 20 µg niclosamide L^{-1}) has also been shown to alter the structure of larval sea lamprey gills causing cell necrosis and swelling (Mallatt et al., 1994). In contrast, TFM alone

was shown to have little to no negative impacts on gill structure or function (Mallatt et al., 1994; Birceanu et al., 2011, 2014). Although TFM apparently has little effect on gill function (Chapter 2 results), it is possible that TFM-niclosamide mixtures or niclosamide alone could significantly interfere with gill-mediated functions due to its greater potency compared to TFM.

Mitochondrion-rich cells (MRC's) in the gills are the key sites of ion-regulation and do so via pumps located on the MRC including V-ATPase pumps that transport H^+ ions across the epithelial membrane into the water and basolateral Na^+/K^+ ATPase (NKA) pumps, which consume the largest amount of ATP (Skou, 1957). Accordingly, another goal of this study was to determine if niclosamide and/or TFM-niclosamide mixtures inhibit gill-mediated ion transport processes and/or cause damage to the gills that could result in disturbances to plasma ion balance in larval sea lamprey.

The overarching aim of this study was to determine whether exposure of larval sea lamprey to a TFM + niclosamide (1 %) mixture (TFM/Nic) or niclosamide (Nic) alone at varying alkalinities influenced the toxicity of TFM/Nic or Nic to the animals and/or increased their sensitivity to ion balance disturbances in the blood by interfering with gill structure or function. Accordingly, larval sea lamprey underwent time of survival toxicity tests when exposed to niclosamide alone or TFM-Nic in waters of low, moderate or high alkalinity. To determine how water alkalinity affected gill function and internal ion balance, larval sea lamprey were exposed to sub-lethal concentrations of niclosamide or TFM-Nic (1 %) at each alkalinity for 24 h, during which gill and blood samples were collected for respective measurements of gill total ATPase and NKA activity, plasma Na^+ and Cl^- concentration, and haematocrit.

Methods

Experimental animals, acclimation, and working solutions

Experimental animals

All experiments followed Canadian Council of Animal Care guidelines and were approved by the Wilfrid Laurier University (WLU) Animal Care Committee under AUP R18004. Larval sea lamprey (*Petromyzon marinus*) were collected by pulsed DC electrofishing by US Fish and Wildlife personnel from tributaries draining into Lakes Michigan and/or Huron and were held at the Hammond Bay Biological Station (HBBS), Millersburg, Michigan, United States, before shipment to WLU. Once received, the larval sea lamprey were housed in 110 L fiberglass aquaria, lined with ~ 5 cm of sand to provide the animals with burrowing substrate, for a minimum of two weeks in continuously flowing aerated well water (pH ~ 8.3; alkalinity ~ 250 mg L^{-1} as $CaCO_3$; temperature ~ 13-15^{o}C; dissolved oxygen > 80% saturation; flow rate ~ 500 mL min). The larval lamprey were fed Baker's yeast (1 g of yeast per lamprey) on a weekly basis and were held under a 12 h dark:12 h light cycle.

Acclimation to different alkalinities

As described in Chapter 2, sub-sets of larval sea lamprey (N = 54) were removed from their holding tanks and acclimated to water of low, medium, and high alkalinity (nominal = 50, 150, 250 mg L^{-1} as $CaCO_3$) in one of three 37 L glass aquaria (= 45 per aquaria). Each aquarium contained diffuse cotton to simulate burrowing substrate, which calms the animals (Wilkie et al., 1999). The water was made-up using reverse osmosis water and the appropriate amounts of potassium chloride (KCl), calcium sulfate ($CaSO_4 \cdot 2H_2O$), sodium bicarbonate, and magnesium sulfate ($MgSO_4$) (Refer to Chapter 2 - Table 2-1). Each aquarium was supplied with reconstituted water of the appropriate alkalinity from an overhead 100 L head tank, which was part of a closed-loop recirculating system. Water alkalinity was measured once a day using a

commercial kit (Hach, Alkalinity Test Kit, Model AL-AP, Hach Canada, Mississauga, ON), the pH was measured using a handheld meter (pH11 meter, Oakton Instruments, Vernon Hills, IL, U.S.A.), and the dissolved oxygen (DO) and temperature were measured using a DO meter (YSI Pro 2030, Xylem Water Solutions Inc., Cincinnati, Ohio, U.S.A.). Additional sodium bicarbonate was added or diluted as needed to maintain appropriate water alkalinity, and pH was controlled by the drop-wise addition of 0.1M of HCl or NaOH to maintain the pH at approximately 8.3 during experiments.

Field grade TFM [Clariant SFC GMBH WERK, Griesheim, Germany (32.5 % active ingredient dissolved in isopropanol)] and niclosamide (Bayluscide ® 16.9 % active ingredient emulsifiable concentrate; Coating Place Inc., Verona, WI, USA) were provided courtesy of the Sea Lamprey Control Centre, Sault St. Marie, Ontario, Canada.

Experimental protocols

Effects of water alkalinity on larval sea lamprey sensitivity to niclosamide or TFM-niclosamide mixtures.

To test the hypothesis that exposure to TFM/Nic or niclosamide is more toxic in low versus higher alkalinity water, time of survival tests were conducted to determine the relative tolerance of lamprey to different concentrations of the lampricide or mixtures. The experiment was preceded by a range finder test to determine the 12-h LC_{50} of TFM/Nic or niclosamide for lamprey exposed in moderate alkalinity (150 mg $CaCO_3\,L^{-1}$) water, which was then used as the TFM/Nic or niclosamide exposure concentration for the time to survival tests and subsequent studies on the effects of alkalinity on gill function and structure (section 2.2.2 below). The night before the range finder experiments, groups of lampreys (N = 10) were transferred to one of seven tanks filled with 5 L of water at an alkalinity 150 mg $CaCO_3\,L^{-1}$ (moderate), pH of 8.3, and temperature of 12°C ± 0.1, and left overnight to acclimate to their surroundings. The next

morning, sufficient amounts of TFM/Nic or niclosamide were added to each individual aquarium to yield nominal concentrations of TFM/Nic in the water of 0, 1.50/ 0.015, 1.75/0.0175, 2.5/0.025, 3.5/0.035, and 4.5/0.045 mg L^{-1}, or a nominal concentration of niclosamide in the water of 0, 0.05, 0.08, 0.1, 0.15, 0.2, and 0.3 mg L^{-1}. As this was a range-finder test, replicate tanks were not used at each concentration to minimize sea lamprey mortality and to conserve animals for subsequent experiments.

The sea lamprey were monitored hourly, with an end-point of death determined by a lack of buccal movement and movement following a pinch of the tail with a pair of forceps (Hlina et al., 2017). For all mortalities, time of death was recorded, the specimen weighed, and then disposed. Parameters that were recorded and monitored included dissolved oxygen (DO), pH, alkalinity, water temperature, and TFM treatment concentrations measured before, during and immediately after experiment. Niclosamide treatment concentrations were verified in water samples that were frozen and subsequently quantified using LC-MS/MS at a later time.

To determine how water alkalinity affected lampricide toxicity, time of survival tests were conducted by exposing sea lamprey to a single TFM/Nic or niclosamide concentration at either low, moderate, or high alkalinity for 24 h. For the TFM/nic mixture, the corresponding target TFM exposure concentration was the TFM 12-h LC_{50} measured in moderate alkalinity in the range-finder tests described above. The 12-h LC_{50} of niclosamide, measured in moderate alkalinity water was the target exposure concentration for the niclosamide alone time of survival test.

The night prior to each time of survival test, the lamprey were transferred into 10 L glass aquaria filled with 5 L of water (to prevent lamprey from escaping) at one of three alkalinities (nominal alkalinities: 50 mg L^{-1}, 150 mg L^{-1}, and 250 mg L^{-1} as $CaCO_3$) in triplicate (N = 10 lamprey per aquaria; N = 30 total per alkalinity), which was placed in a water bath to maintain

the temperature at 12.0°C ± 0.1. The following morning, the tanks were dosed with the appropriate concentration of TFM and niclosamide ([TFM] = 2.94 mg L^{-1} ± 0.03 (12); [niclosamide] = 29.31 µg L^{-1} ± 1.94 (12)), and or niclosamide only ([niclosamide] = 78.26 µg L^{-1} ± 1.94 (12)). Animals were monitored hourly for the first 12 h of the experiment and again at 18 h and 24 h, at which time surviving animals were euthanized using tricaine methanesulfonate (TMS; 1.5 g L^{-1}, Syndel Labs, Nanaimo, BC, Canada) buffered with 3.0 g L^{-1} of $NaHCO_3$ and the carcasses disposed. Water samples were collected at the beginning and end of the experiment for quantification of the lampricides as described above. Control animals (N = 10 at each alkalinity) were treated in an identical manner but not exposed to either of the lampricides.

Effects TFM-niclosamide mixtures or niclosamide only on larval sea lamprey gill function at different water alkalinities.

To test the hypothesis that TFM/Nic or niclosamide only exposure interfered with gill-mediated ion regulation at low, moderate and high alkalinity, plasma ion (Na^+, Cl^-) concentration, haematocrit, total ATPase activity, and Na^+/K^+ -ATPase activity were measured in larval sea lamprey exposed to the 12-h LC_{25} of TFM in the TFM/Nic mixture, or the 12-h LC_{25} of niclosamide only, at low, moderate or high alkalinity.

Larval lamprey were allowed to acclimate for one week to the appropriate alkalinity. Twelve hours prior to the experiments, the lamprey (N = 3 per aquaria) were transferred into 10 L glass aquaria, each receiving water of low, moderate or high alkalinity (as described above). The next morning, water flow was cut-off and the lamprey were exposed to the 12-h LC_{25} of the TFM/Nic mixture (measured [TFM] = 2.02 ± 0.02 mg L^{-1}; measured [Nic] = 20.73 ± 0.60 µg L^{-1}), or the 12-h LC_{25} of niclosamide only (measured [Nic] = 70.53 ± 2.21 µg L^{-1}) at each of the three alkalinities. The 12-h LC_{25} was used to ensure that sufficient numbers of sea lamprey would be alive for sampling, particularly at low alkalinity, where the animals were much more

susceptible to lampricides than at moderate and high alkalinity (as noted in the results below). Gills were collected, after euthanization with 1.5 g L^{-1} MS222 (tricaine methane sulfonate; Syndel, Nanaimo, B.C.) neutralized with 3 g L^{-1} of $NaHCO_3$, at 1, 2, 4, 8 h in low alkalinity water, and at 4, 12, 24, and 48 h, plus (non-exposed) controls (n = 12 lamprey per sample period) at moderate and high alkalinity. One set of gills (corresponding to branchiopores 1, 3 and 5) were collected by making cross sections (~ 4 mm wide) through the entire branchial (gill) region, transferred to 1.5 mL polypropylene centrifuge tubes, snap frozen in liquid N_2 and stored at -80°C until analyzed for NKA and total ATPase activity (McCormick 1993; Reis-Santos et al. 2008).

Whole blood was collected using heparinized hematocrit tubes from an incision behind the last branchiopore, centrifuged for three minutes at 10,000 *g* using a microcentrifuge with hematocrit rotor (Thermo Scientific, 75002492, MA, USA), and the plasma drawn off and transferred into 0.5 mL polypropylene centrifuge tubes, snap frozen in liquid N_2 and then stored at -80 °C until analyzed for plasma Na^+ and Cl^- concentration (see below).

Analytical techniques

TFM and niclosamide concentrations

Water TFM exposure concentrations were quantified, using a Novaspec II spectrophotometer (Pharmicia Biotech) at a wavelength of 395nm (Barber and Steeves, 2019), and verified using precision TFM standards (0, 4, 8, 12 mg L^{-1} TFM) provided courtesy of the Sea Lamprey Control Centre, Fisheries and Oceans Canada (DFO) (Sault Ste. Marie, Ontario).

Water niclosamide exposure concentrations were quantified by Dejana Mitrovic in the lab of Dr. Mark Servos, Department of Biology, University of Waterloo, using the following protocol. Prior to niclosamide analysis, the water samples were taken out to thaw a room temperature, vortexed for 20 s, and a 5 mL aliquot of each sample, standard or blank transferred

to a new, clean glass test tube and spiked with 100 µg L^{-1} niclosamide as additional matrix. 200 µg L^{-1} of niclosamide-(2-chloro-4-nitrophenyl-^{13}C6) hydrate (VETRANAL®) was also added to each test tube as the internal standard, then vortexed for another 20 s. The samples were filtered through 0.45 µm glass fiber filters (Pall Corporation, Michigan, USA) using a vacuum filtration tool (15 mL, Sigma Aldrich), and 1 mL of the eluent transferred into a 2 mL amber glass vial for subsequent LC-MS/MS analyses (Agilent 1260 HPLC with 6460 Triple Quad and Agilent Jetstream ESI source in negative ionization mode). To chromatographically separate the analyte, 10 µL of sample was injected onto an Agilent Eclipse XDB-C18 column (4.6 ×150 mm, 5 µm) at 35 ˚C, at a flow rate of 0.8 mL min^{-1}. Elution was done via gradient flow, with the following combinations of de-ionized water (mobile phase A) and acetonitrile (mobile phase B): 0 min: 80, 20; 1 min: 80, 20; 10 min: 0, 100; 12 min: 0, 100; 12.1 min: 80, 20 (numbers expressed in percentages of mobile phase A, B). The instrument source parameters were as follows: temperature =400 ˚C for evaporation, gas temperature =230 ˚C, gas flow =12 L min^{-1}, nebulizer set to 275.8 kPa, and capillary voltage =2500 V. The calibration curve for niclosamide ranged from 0 µg L^{-1} to 500 µg L^{-1} of each standard, made up in HPLC grade methanol. Niclosamide concentrations were calculated, after adjusting for background noise, by linear regression (y =1.0001x --0.0008; R2 =0.9998). Recovery rates were always greater than 95 %.

Na^+/K^+-ATPase and Total Protein Activity Assay

Gill NKA activities were measured using a kinetic microassay (McCormick, 1993) as described in Chapter 2. Briefly, gill tissues were homogenized in 0.1% sodium deoxycholic acid (SEID) buffer and centrifuged. The supernatant (10 µL) for each gill homogenate sample was transferred to a 96- well microplate and activity was measured using a microplate spectrophotometer (Epoch 2, BioTek Instruments, Inc., VT, U.S.A.).

Plasma Na^+ and Cl^- analysis

Plasma Na^+ concentrations were determined using atomic absorption flame spectrophotometry and plasma Cl^- concentrations was determined using the Cole-Parmer Chloride Analyzer (120/220 VAC, 50/60 Hz, Item # RK-02656-20). Refer to chapter 2 for the complete methods.

Calculation and statistics

Determination of the 12-h LC_{50} for larval lamprey exposed to each lampricide was done using an online R software program which uses the Litchfield Wilcoxon fitted model on the log10-probit scale (Adams, 2016).

Differences in the survivorship of larval sea lamprey exposed to the 12-h LC_{50} of the TFM/Nic mixture (measured [TFM] = 2.94 ± 0.03 mg L^{-1}; measured [Nic] = 29.31 ± 1.94 ug L^{-1}), or the 12-h LC_{50} of niclosamide only (measured [Nic] = 78.26 ± 3.56 ug L^{-1}) at each of the three alkalinities were plotted against each other on the same axes. The survival data were subjected to Log-rank (Mantel-Cox) tests to determine if the family of resulting TFM survival curves were significantly from one another at the $P<0.05$ level.

Physiological data (plasma ions, Na^+/K^+ -ATPase and total ATPase specific activity) was presented as the mean ±1 standard error of the mean (SEM). After confirming the absence of collinearity among each of the variables, a Bernoulli generalised linear model (GLM) with logit link function (i.e. a logistic regression) was applied to the data set, followed by a stepwise goodness-of-fit model to determine which covariate combination would produce the best model. When significant variability was observed, statistical significance between the means was assessed using the Tukey post-test at the $p< 0.05$ level. Outliers were tested and removed using the Grubb's test, also known as a maximum normalized residual test. The Grubbs test detects a

single outlier at either end of the data distribution. If an outlier is detected, the data point is removed, and the test is tested again until there are no more outliers (Tessier et al., 2018).

Results

Effects of alkalinity on TFM/Nic and niclosamide tolerance in larval sea lamprey

The range-finder test completed in water of moderate alkalinity (151.3 ± 0.8 mg L^{-1} as $CaCO_3$) yielded a TFM 12-h LC_{50} of 2.9 mg L^{-1} (CI = 2.7 – 3.2 mg L^{-1}). For niclosamide alone the 12-h LC_{50} was 78 µg L^{-1} (CI = 72 – 85 µg L^{-1}) (data not shown). The corresponding 12-h LC_{50} of TFM for the TFM/niclosamide mixture, and the niclosamide 12 h-LC_{50}, for the niclosamide alone treatment in moderate alkalinity water, then served as the respective exposure concentrations to which the sea lamprey were exposed to during the survivorship tests conducted in low, moderate and high alkalinity water.

The larval sea lamprey were most sensitive to the TFM/Nic mixture (measured [TFM] = 3.0 ± 0.1 mg L^{-1}; measured [Nic] = 26.7 ± 1.2 µg L^{-1}) in low alkalinity water (measured alkalinity = 59.4 ± 1.1 mg L^{-1} as $CaCO_3$; pH = 8.09 ± 0.01), in which there was rapid and complete mortality between 3 and 6 h of exposure. In contrast, exposure to the same concentration of TFM/Nic (measured [TFM] = 2.9 ± 0.1 mg L^{-1}; measured [Nic] = 25.5 ± 2.8 µg L^{-1}) at moderate alkalinity (measured alkalinity = 151.3 ± 0.8 mg L^{-1} as $CaCO_3$; pH = 8.13 ± 0.02), was characterized by a slower onset of mortality, with 15 % mortality after 6 h, and 75 % mortality between 8-10 h, and complete mortality by 12 h (Figure 3-1; P = <0.0001). Survival was 80% in the sea lamprey exposed to TFM/Nic (measured [TFM] = 2.93 ± 0.02 mg L^{-1}; measured [Nic] = 25.35 ± 3.3 µg L^{-1}) at high alkalinity (251.3 ± 2.6 mg L^{-1} as $CaCO_3$; pH = 8.19 ± 0.04), with 100% survival in the control (non-exposed) animals at all three alkalinities (Figure 3-1).

The larval sea lamprey were most sensitive to the niclosamide alone (measured [Nic] = 64.40 ± 3.7 µg L^{-1}) treatment in low alkalinity water (measured alkalinity = 59.4 ± 1 mg L^{-1} as $CaCO_3$; pH = 8.1 ± 0.02), in which there was a steep increase in mortality at 8-12 h of exposure, and complete mortality by 24 h (Figure 3-2). In contrast, the onset of mortality was slower during exposure to the same concentration of niclosamide (measured [Nic] = 84.9 ± 4.2 µg L^{-1}) at moderate alkalinity (measured alkalinity = 151.3 ± 0.8 mg L^{-1} as $CaCO_3$; pH = 8.17 ± 0.02), with 25% mortality after 24 h (Figure 3-2; P = <0.0001). Survival was 100% in the sea lamprey exposed to niclosamide (measured [Nic] = 85.47 ± 3.3 µg L^{-1}) at high alkalinity (251.3 ± 2.6 mg L^{-1} as $CaCO_3$; pH = 8.18 ± 0.02), and in the control (non-exposed) animals at all three alkalinities (Figure 3-2).

Effects of alkalinity and TFM/Nic on gill function of larval lamprey

Na^+/K^+-ATPase and Total Protein Activity Assay

TFM/1%Nicosamide

In the absence of TFM/Nic, there were no significant differences in gill total ATPase or gill NKA activity between lamprey acclimated to low, moderate, or high alkalinity (Figure 3-3 A, B). Although, the exposure to TFM/Nic yielded significant differences in total ATPase activity over time with varying alkalinities. At low alkalinity, exposures to TFM/Nic (measured [TFM] = 1.98 ± 0.04 mg L^{-1}; [Niclosamide] = 21.3 ± 2.6 µg L^{-1}) resulted in a 40 % reduction in total ATPase activity at 4 h (P = 0.011) and was 60 % lower after 8 h (P < 0.001; Figure 3-3A). No animals survived beyond 8 h of exposure to TFM/Nic in low alkalinity water. In moderate alkalinity, exposure to TFM/Nic (measured [TFM] = 2.03 ± 0.09 mg L^{-1}; [Niclosamide] = 19.31 ± 2.3 µg L^{-1}) resulted in a 40 % decrease NKA activity at 48 h (P = <0.001), preceded by a slight, but non-significant drop in activity at 24 h (Figure 3-3A). There were 6 mortalities in moderate alkalinity, where 3 died at 24 h and 3 died after 48 h. Lamprey exposed to TFM/Nic

(measured [TFM] = 2.05 ± 0.04 mg L^{-1}; [Niclosamide] = 21.6 ± 2.3 µg L^{-1}) at high alkalinity showed a similar trend to those exposed in moderate alkalinity where there was a 15 % decrease in NKA activity after 24 h ($P = 0.035$) and 48 h ($P = 0.015$). No mortalities occurred throughout the sampling experiment in high alkalinity.

The specific NKA activity of larval sea lamprey was approximately 95 % lower than total ATPase activity in the non-exposed controls at all three alkalinities. Exposure to the TFM/Nic mixture resulted in no significant differences in specific NKA activity at either low, moderate or high alkalinity (Figure 3-3B).

Niclosamide

In the absence of niclosamide, there were no significant differences in gill NKA or total ATPase activity between lamprey acclimated to low, moderate, or high alkalinity (Figure 3-4A,B). Exposures to niclosamide in low (measured [Niclosamide] = 66.57 ± 7.6 µg L^{-1}), moderate (measured [Niclosamide] = 69.69 ± 6.7 µg L^{-1}), and high alkalinity (measured [Niclosamide] = 73.0 ± 2.2 µg L^{-1}) did not yield significant differences over time for total ATPase (Figure 3-4A).

Exposure to niclosamide resulted in no significant differences in gill NKA activity at low alkalinity. However, NKA activity was significantly elevated at moderate alkalinity, in which measurements were 2.0 to 2.5-fold higher after 4 h ($P = 0.02$), 12 h ($P = 0.04$), and 48 h ($P = 0.007$) of niclosamide exposure (Figure 3-4B). In contrast, NKA activity was unchanged following niclosamide exposure at high alkalinity.

Effects of TFM/niclosamide and niclosamide alone on haematocrit, plasma Na^+ and Cl^- concentration

TFM/1%Niclosamide

There were no significant effects of alkalinity alone on plasma ion balance, in which the plasma Na^+ concentrations fluctuated around 100-110 mmol L^{-1}, and Cl^- concentrations averaged 75-80 mmol L^{-1}, in larval seal lamprey acclimated to low, moderate and high alkalinity (Figures 3-5, 3-6).

Exposure to the TFM/Nic mixture at low alkalinity caused plasma Na^+ to increase by approximately 25% at low alkalinity following 2 h, 4 h, and 8 h (Figure 3-5A; $P < 0.001$ at all time periods). The concentration of Cl^- remained relatively stable during TFM/Nic exposure at all three alkalinities, with one exception; there was a slight, significant 12 % decrease, after 8 h of TFM/Nic exposure at low alkalinity (Figure 3-5B; $P = 0.003$). Note that there was no data for moderate alkalinity at the 24 h sampling interval due to the small size of the animals and the insufficient yields of blood sample.

The haematocrit of larval sea lamprey was for the most part unaffected by TFM/Nic exposure at all 3 alkalinities, except at 4 h of exposure at high alkalinity, when there was slight, but significant increase of 6 % (Table 3-1).

Niclosamide

Exposure to niclosamide had significant effects on plasma Na^+ concentrations at low and high alkalinity, but no effect at moderate alkalinity. At low alkalinity, exposure to niclosamide resulted in rapid rise of plasma Na^+ by roughly 25% after the 1 h interval, followed by another 25 % increase at 2 h, 4 h, and 8 h (Figure 3-6A; $P < 0.001$ at all time periods). Exposure to niclosamide at moderate alkalinity did not yield any significant differences in plasma Na^+. However, at high alkalinity, exposure to niclosamide resulted in similar trends to those observed

at low alkalinity, during which there was a rapid and sustained rise of plasma Na^+ of approximately 40 % from 4-48 h (Figure 3-6A; $P < 0.001$ at all time periods). Neither alkalinity, nor exposure to niclosamide, resulted any significant differences in plasma Cl^- concentration (Figure 3-6B).

The hematocrit of larval sea lamprey was affected by niclosamide treatments in low, moderate, and high alkalinity. In low alkalinity, niclosamide exposure resulted in an increase of hematocrit of 21 % and 39 %, respectively (Table 3). In contrast, niclosamide exposure at moderate and high alkalinity resulted in a decrease in hematocrit. Exposure at moderately alkalinity was characterized by 25 % decrease in hematocrit after 48 h, whereas it decreased by 39 % after 48 h exposure at high alkalinity (Table 3-2).

Discussion

The present findings demonstrate that as water alkalinity increases, the toxicity of TFM-niclosamide (1 % niclosamide) mixtures and niclosamide alone decreases. In other words, as with TFM alone (Bills et al., 2003; Chapter 2), at a given concentration of total TFM (sum of un-ionized plus ionized TFM), higher water alkalinity protects larval sea lamprey from TFM and niclosamide toxicity. This is likely because TFM and/or niclosamide bioavailability decreases in the water of the gill microenvironment as the acid buffer capacity of the water increases with alkalinity. In agreement with previous studies (Birceanu et al. 2009; Henry et al. 2015; Wilkie et al., 2007), the relative stability of plasma Cl^- concentration, and stable or elevated plasma Na^+ concentrations, appear to rule out direct interference with branchial Na^+ and Cl^- uptake as an underlying contributor to death in larval sea lamprey exposed to niclosamide plus TFM, or niclosamide alone. Notably, the effects of the TFM/niclosamide mixture and niclosamide on total ATPase activity were distinctly different than observed in lamprey exposed to niclosamide alone, complicating interpretation. However, the fact that NKA activity was sustained during exposure

to TFM/Nic and niclosamide alone, may explain why there were no decreases in plasma Na^+ and Cl^- concentration despite the known inhibitory effects of niclosamide and TFM on mitochondrial ATP production (Birceanu et al. 2011; Borowiec et al. 2022; Huerta et al. 2017; Niblett and Ballantyne 1976).

TFM/1% niclosamide and niclosamide toxicity is inversely related to water alkalinity

Water pH is the most important factor for determining the bioavailability of TFM and the corresponding target concentrations of TFM to add to the water during field applications of lampricide for sea lamprey control (Bills et al., 2003; Wilkie et al., 2021). As previous studies have shown, at a given concentration of total TFM, TFM toxicity increases as pH decreases, which affects both sea lamprey and non-target fishes (Bills et al., 2003; McDonald and Kolar, 2007; Wilkie et al., 2019). This relationship also holds for TFM-niclosamide mixtures, during which niclosamide is often added to the water at concentrations between 1-2 % of the total TFM concentration, reducing the quantity of TFM required for treatments by 30-40 % because niclosamide reduces the MLC of TFM (Boogaard et al., 2003; Dawson, 2003). Niclosamide is usually co-applied to the treated waters at the same time as TFM as an emulsifiable concentrate (EC), which contains surfactants that act as a delivery vehicle to help keep niclosamide in solution due to its lower water solubility compared to TFM (GLFC, 2021). The toxicity of niclosamide alone is also profoundly influenced by water pH (Bills et al., 2003; Boogaard et al., 2003), for similar reasons to those that explain TFM toxicity, and as the present study shows, alkalinity also strongly affects the sea lamprey's sensitivity to TFM/Nic mixtures and niclosamide alone.

Water pH is so important for determining the bioavailability of TFM and niclosamide because it directly influences their speciation and how readily available each is for uptake by both sea lamprey and non-target species (Hlina et al., 2017; Hunn and Allen, 1974; Wilkie et al.

2019). Like TFM, niclosamide is weak acid with an ionizable hydroxyl group, from which the H^+ ion dissociates as water pH moves away from the pKa. The pKa, defined as the negative log of the acid dissociation constant, Ka, for an acid-base reaction, falls between 6.07 and 6.38 for TFM, and is 6.25 for niclosamide (Dawson, 2003; Hubert, 2003; McConville et al., 2016). At lower pHs, each lampricide will exist as its more diffusible un-ionized (phenolic) form. However, at higher pHs the ionized (phenolate), less diffusible form of each lampricide would be dominant (Chapter 1 - Figure 1-1).

There are several environmental factors that could lead to the variation of the daily pH levels in an aquatic environment. These factors can include underlying bedrock, temporal changes, and respiration and photosynthesis processes (Acton et al., 2015; Cole and Weihe, 2015). As already mentioned, alkalinity can influence the pH through the buffering of acids using carbonates and bicarbonates. The release of carbonates is dependant on the bedrock present such as limestone which is rich in carbonates or Precambrian shield bedrock which is poor (Acton et al., 2015). Rainwater which is naturally acidic can lower the pH in an aquatic environment, however if it flows over limestone bedrock, the acidity of the rain will dissolve the limestone creating bicarbonate salts resulting in and increase of pH and alkalinity in the water (Wurts and Durborow, 1992). Aquatic pH can also vary due to respiration and photosynthesis processes. After sunset there is a decline of photosynthesis which results in a decrease in the amount of O_2 being released in the environment, where plant, animal, and microbial respiration continues to occur consuming oxygen and releasing CO_2 (Choi et al., 1998; Wurts and Durborow, 1992). The release of CO_2 reacts with water creating carbonic acid, which leads to the decrease in pH. However, during the day, when photosynthesis occurs, CO_2 is removed increasing the pH in the water system (Choi et al., 1998; Wurts and Durborow, 1992). Given the

daily changes of pH that can occur, it underlines the need of continuous monitoring of the pH throughout the entirety of lampricide application.

Another factor that can influence TFM and niclosamide speciation are events taking place in the gill microenvironment, which can be very different from bulk water pH (Playle and Wood, 1989; Wright et al., 1986). Depending on the bulk water pH, the excretion of CO_2, metabolic acid or base by the gill, can raise or lower the pH of gill microenvironment near the gill surface (Playle and Wood, 1989; Wright et al., 1986). This could have profound implications for fishes exposed to lampricides, where acidification at the gill surface could change the speciation of the lampricides, resulting in higher concentrations of the phenolic form of each lampricide and increased uptake (e.g. Figure 2-4). There are a variety of physiological processes that could lead to the acidification of the gill microenvironment, which includes the excretion of H^+ via proton-pumps (V-ATPase) from carbonic anhydrase hydration of CO_2 (Bartels and Potter, 2004; Erickson et al., 2006; Playle and Wood, 1989; Reis-Santos et al., 2008; Wright et al., 1986). Depending upon water buffer capacity, this process may ultimately lower the pH at the gill surface, potentially converting more TFM and niclosamide into their un-ionized forms during lampricide exposure.

Alkalinity, like pH, influence the toxicity of TFM to sea lamprey and non-target fishes (Bills et al., 2003; Hepditch et al., 2019; Kanayama, 1963). Bills et al. (2003) created a model that incorporates both pH and alkalinity showing that both have a relation in lampricide effectiveness, and how the two factors are correlated. In general, waters with high alkalinities typically have higher pHs, which makes it difficult to separate the effects of alkalinity on TFM and niclosamide bioavailability from those of pH's. In the present study, the effects of water alkalinity were separated from pH in the bulk water by preparing artificial waters of low, moderate, and high alkalinity with similar water pH, as described above (Hepditch et al. 2019).

As a result, it was demonstrated that water alkalinity alone affected the toxicity of both the TFM/niclosamide mixture and niclosamide in larval sea lamprey. It is unlikely that these differences were due to direct affects of water alkalinity on TFM speciation in the bulk water, because the pH was more or less the same. Rather, the results suggest that events taking place in the gill microenvironment, next to the gill surface are changing the water chemistry and altering the bioavailability of TFM and niclosamide. It is proposed that the effects of alkalinity on both TFM and niclosamide toxicity are indirect and related to differences in the buffer capacity of the water in the gill microenvironment, which influences the degree to which the fish can change the water pH in this region and therefore speciation for each of these lampricides.

The importance of water buffer capacity on gill microenvironment acid-base chemistry was recently shown in rainbow trout (*Oncorhynchus mykiss*) fitted with opercular catheters and used to measure the pH of the expired water crossing the gills at different alkalinities (Wilkie et al., 2021). By measuring the pH of the water within the opercular chamber, this method makes it possible to separate the chemistry in the gill microenvironment in relative isolation from the bulk water (Playle and Wood 1989; Wright et al. 1987).

The experiments were conducted in waters of low, moderate and high alkalinity, to determine how the buffering capacity of water influences the amount of acidification taking place in the gill microenvironment. As predicted, the pH of the expired water was much less (change of 0.5 to 1.5 in pH) than that of the bulk water at low alkalinity (50 mg L^{-1} as $CaCO_3$) compared to the bulk water, whereas the differences were much less in moderate and high alkalinity (Wilkie et al., 2021). In other words, the fish were able to acidify the water to a much greater extent in poorly buffered low alkalinity water compared to higher alkalinities. Theoretical calculations of the speciation of TFM, demonstrated that the bioavailable form of TFM at the gill-surface was much higher in the gill microenvironment than in the bulk water at low

alkalinity, whereas in moderate and high alkalinity, where there was little (if any) acidification, there were lower amounts of bioavailable TFM at the gill-surface (Wilkie et al., 2021). These findings likely explain the greater sensitivity of larval sea lamprey when exposed to both the TFM-niclosamide mixture and niclosamide alone in lower compared to higher alkalinity water in the present study. However, a limitation to both studies is that the actual rates of uptake of TFM and niclosamide in varying alkalinities have not yet been measured. Future studies using ^{14}C-labeled TFM and niclosamide should therefore be used to better quantify how alkalinity effects the rate of uptake of these lampricides at various water alkalinities by calculating the amount of ^{14}C-TFM and niclosamide accumulation in the bodies of sea lamprey and non-target fishes (Hlina et al., 2017; Tessier et al., 2018).

Based on their respective LC_{50} values, the toxicity of niclosamide (12-h LC_{50} ~ 70 µg L^{-1}) was much greater than for TFM (LC_{50} ~ 4 mg L^{-1}), and TFM toxicity was significantly lower when combined with 1% niclosamide by mass (TFM 12-h LC_{50} ~ 2.9 mg L^{-1}). These observations were expected because niclosamide has a far greater potency than TFM, which may be related to the much higher lipid-solubility of niclosamide (Borowiec et al., 2022). The lipid solubility of different substances is determined by measuring the log octanol-water co-efficient, called the log K_{ow}. The log K_{ow} measures how readily different substances are distributed between octanol, a nonpolar substance, and water, which is polar (Borowiec et al., 2022). Substances that are highly lipid soluble will have a higher higher log K_{ow} because more will be distributed in the octanol layer of the octanol-water solution, whereas a substance with a lower log K_{ow} is more soluble in water. TFM has a log K_{ow} = 2.87 for TFM, which is far less than that of niclosamide with a log K_{ow} = 10 (PubChem, 2022). Due to the more lipophilic nature of niclosamide compared to TFM, Borowiec et al. (2022) predicted that it more easily interferes with mitochondrial ATP production because it would more easily penetrate the inner

mitochondrial membrane to enact the uncoupling of mitochondrial oxidative phosphorylation, which is known to be the mechanisms of toxicity for both lampricides (Borowiec et al., 2022; Terada, 1990). For this reason, it was predicted that this property of niclosamide made gill-mediated physiological processes of sea lamprey more sensitive to niclosamide than to TFM.

Alkalinity and lampricide cause minimal disturbance to gill-mediated ionoregulatory processes

The gills have various functions that include, but are not limited to, gas exchange, nitrogenous waste excretion, acid-base regulation, and ion exchange (Evans et al., 2005; Wilkie et al., 2002; Wilson and Laurent, 2002). The present investigation focused on the potential impacts of TFM plus niclosamide on ionoregulatory processes in larval sea lamprey, which was premised on the likelihood that greater amounts of TFM and niclosamide would be more bioavailable, with greater accumulation in the gills at lower compared to higher alkalinity water. Similar to teleost fishes, larval sea lamprey gills are composed of various MRCs in the lamellar epithelium and interlamellar space (Ferreira-Martins et al., 2021; Reis-Santos et al., 2008). In fresh water, sea lamprey are in a hypo-osmotic environment, resulting in a need to actively uptake ions through their skin and gills (Evans et al., 2005; Ferreira-Martins et al., 2021). The MRCs are the main sites of active uptake of ions through their gills, which is a ATP intensive process (Bartels and Potter, 2004, Evans et al., 2005; Ferreira-Martins et al., 2021).

Given that the gills of the larval lamprey contain MRCs, or ionocytes, and their heavy reliance on ATP to actively uptake and transport ions from ion poor fresh waters (Evans et al., 2005; Ferreira-Martins et al., 2021; Kolosov et al., 2020; Wilkie et al., 2019), and that niclosamide is much more potent than TFM alone, it was predicted that exposure to a TFM-niclosamide (1 %) mixture and niclosamide alone would result in greater disturbances to plasma Na^+ and Cl^- balance than observed with TFM (see Chapter 2). Previous work had shown that

niclosamide and TFM/Nic caused MRC swelling, loss of apical microvilli, vacuolization, and necrosis, which further suggested that ion balance was more likely to be compromised than in larval sea lamprey exposed to TFM alone (Mallatt et al., 1994). However, this study provided no evidence of gill NKA impairment in larval sea lamprey exposed to niclosamide, nor did the animals undergo reductions in plasma Na^+ or Cl^-, further suggesting that neither TFM/niclosamide mixtures nor niclosamide alone impair ion uptake. In fact, during niclosamide alone exposures, in low and high alkalinity water, plasma Na^+ concentration increased.

At all three alkalinities, exposure to TFM/niclosamide mixture resulted in a decrease in total ATPase activity by the end of each respective exposure period. However, this effect was not seen with niclosamide alone, which remained unchanged. Na^+/K^+ ATPase activity was more or less unaffected by lampricide exposure, but more variable, following exposure to the mixture of both lampricides, only accounting for approximately 5 % of the total ATPase activity as reported previously (Reis-Santos et al., 2008). This could suggest that the TFM-niclosamide mixture could be damaging the gills more than niclosamide alone, potentially causing necrosis as observed by Mallatt et al. (1994). The absence of significant decreases in NKA following exposure to the TFM-niclosamide at each of the three alkalinities may explain why blood plasma Na^+ and Cl^- concentrations balance was maintained. However, the reduction in total (residual) ATPase activity at all three alkalinities by the end of the TFM-niclosamide exposure period, raises the possibility that the concentrations of other important plasma ions such as Mg^{2+} and Ca^{2+} could have been altered due to direct effects on Mg^{2+}-ATPase and Ca^{2+}-ATPase activity, which were not specifically examined. In addition to NKA, other ATPases present in the gills of fishes that could have been impacted include V-ATPases which mediate H^+ excretion by the gill and important for extracellular pH regulation in fishes (Bijvelds et al., 1996; Ferreira-Martins et al., 2021; Hwang et al., 1990, Perry and Wood, 1985).

Plasma Na^+ concentration was either maintained or higher following exposure to the TFM-niclosamide mixture, rather than the decrease that was predicted. Similarly, there was a slight increase in the gill NKA activity and plasma Na^+ concentrations with niclosamide alone. One explanation for these findings, could have been haemoconcentration due to a build-up of lactate in the muscle, which arises following both TFM and niclosamide exposure (Birceanu et al., 2014; Lech, 1974; Statham and Lech, 1975; Wood and Graham, 1983). This would draw water away from the plasma and into the muscle, effectively decreasing the plasma volume, concentrating the ions. Another possibility is that gill permeability decreased due to an upregulation or changes in tight junctions (TJ) composition in the gills, preventing the loss of Na^+ ions (Chasiotis et al., 2012; Ferreira-Martins et al., 2021; Gonzalez and Mcdonald, 1994; Kolosov et al., 2017a, 2017b). The TJs play a critical role in restricting ion loss across the gills in fresh water (Chasiotis et al., 2012). Alterations to the TJ protein complexes when exposed to TFM/niclosamide or niclosamide alone may have therefore made the gills less ion permeable, restricting or lower the loss of ions, specifically Na^+ and Cl^-, in the face of lower rates of uptake due to a lack of ATP generation for ion pumps.

Another possible explanation for the unexpectedly greater NKA and plasma Na^+ concentrations could have been an actual upregulation or increased turnover of gill NKA protein. Although NKA activity appears to be maintained in the larval sea lamprey exposed to the TFM/Nic mixture and niclosamide alone, it should be noted that this may not reflect the gills actual responses to lampricide exposure. If the lampricides were interfering with ATP production, actual enzyme NKA activity would be lower *in situ* due to less available fuel (ATP) during exposure. In response, the fish may have increased NKA activity or turnover to compensate, resulting in a stabilization or even an elevation of plasma Na^+ concentration. As a result, gill NKA activities measured *in vitro* would have been higher than *in situ*, masking any

impairment of NKA function that took place when the animals were exposed to lampricide. The analytical methods (McCormick, 1993) used in this study measured maximum total ATPase and NKA activity in the gills after the larval lamprey were exposed to the lampricide. Under these conditions the gills homogenates were provided excess ATP when making our determinations of maximal activity. It also seems unlikely that NKA was directly inhibited by TFM or niclosamide because Na^+ or Cl^- balance was maintained throughout the exposures in varying alkalinities. Future studies using isolated larval lamprey gill cells or cultured gill cells and directly adding TFM/ niclosamide or niclosamide alone to the homogenate (as per the McCormick methods), followed by the measurement of ATPase activities may help resolve this question. These approaches would be complimented by conducting western blot analysis, to directly quantify the amount of NKA protein in the gills (e.g. Reis-Santos et al., 2008) and shed more on how NKA function is influenced by exposure TFM-niclosamide mixtures and niclosamide.

Summary and Conclusion

This study has demonstrated that the sensitivity of larval sea lamprey to TFM/Nic mixtures and niclosamide alone is greatest in waters of low alkalinity, decreasing as water alkalinity and buffer capacity increases. This is likely due to the indirect effects that alkalinity has on the bioavailability of the lampricides. At lower alkalinities, the bioavailability of TFM and niclosamide in the gill microenvironment would be greater than at high alkalinities due to the lack of buffering capacity, makes this region more sensitive to reductions in water pH as a result of CO_2 and H^+ excretion by gills. In contrast, the greater buffer capacity of higher alkalinity waters, would tend to buffer H^+ excreted by the gill microenvironment, which would make the gill microenvironment less sensitive to changes pH, and more similar to the pH of the bulk water, which would result in less differences between the more bioavailable phenolic forms of TFM and niclosamide in the gill microenvironment compared to the bulk water. To measure this impact,

future studies should use opercular catheters similar to methods used on rainbow trout by Wilkie et al. (2021) to quantify the changes occurring in the gill microenvironment

Exposure of larval sea lamprey to TFM/Niclosamide or niclosamide alone did not cause ion loss as predicted. Instead, there was a lack of impaired function of NKA, suggesting that the cause of death in larval lamprey when exposed to these lampricides is unlikely related to the impairment of gill-mediated ion uptake function. However, the effects of TFM or niclosamide on other ions such as Ca^{2+} or Mg^{2+}, or acid-base balance to impaired V-ATPase activity cannot be ruled out. It was also predicted that the greater potency of niclosamide alone would impact the gills far greater than the TFM/Nic mixture, but there were no distinguishable differences in ATPase activity and blood plasma ion concentrations.

This study could help sea lamprey control agents understand more about how alkalinity affects the bioavailability of TFM/niclosamide and niclosamide alone at the gill surface, which may help better predict how sea lamprey and non-target fishes will respond to these lampricides in waters of varying alkalinity and pH. Ultimately, this would help with the accuracy of lampricide application in the Great Lakes drainages, most of which fall within the alkalinity ranges studied here. Such knowledge could help sea lamprey control agents fine-tune lampricide dosages, resulting in greater treatment effectiveness (more dead sea lamprey), while minimizing the potential for adverse affects to non-target species. A need for less lampricide in low alkalinity waters treated with TFM-niclosamide mixtures or niclosamide would also result in cost saving and reduced pesticide use, while still effectively managing the sea lamprey population. Lastly, this study highlights the need to conduct stream side toxicity tests prior to lampricide applications to better gauge and adjust for differences in the sensitivity of larval sea lamprey to TFM and niclosamide in waters of different alkalinity throughout the Great Lakes.

Table 3-1. Blood haematocrit of larval sea lamprey exposed to a nominal concentration of TFM and Niclosamide mixture of 2 mg $^{-1}$ and 20 µg L^{-1}. Data presented as the mean ± 1 SEM (N). Data with asterisk are different from the controls.

Alkalinity	TFM exposure time (h)	Haematocrit (%)	P-Value & Significance
Low	Control	28 ± 1 (21)	-
(50 mg L^{-1} as $CaCO_3$)	1	24 ± 2 (8)	0.674
(pH = (8.31 ± 0.01))	2	26 ± 2 (10)	0.938
	4	29 ± 1 (12)	0.997
	8	31 ± 3 (11)	0.742
Moderate	Control	28 ± 1 (19)	-
(150 mg L^{-1} as $CaCO_3$)	4	24 ± 2 (12)	0.365
(pH = (8.30 ± 0.01))	12	30 ± 2 (11)	0.983
	24	32 ± 2 (12)	0.639
	48	33 ± 3 (10)	0.405
High	Control	28 ± 1 (20)	-
(150 mg L^{-1} as $CaCO_3$)	4	34 ± 1 (11)	0.017*
(pH = (8.31 ± 0.01))	12	34 ± 3 (11)	0.521
	24	29 ± 2 (12)	0.984
	48	31 ± 1 (8)	0.314

Table 3-2. Blood haematocrit of larval sea lamprey exposed to a nominal concentration of Niclosamide of 70 µg L^{-1}. Data presented as the mean ± 1 SEM (N). Data with asterisk are different from the controls.

Alkalinity	TFM exposure time (h)	Haematocrit (%)	P-Value & Significance
Low	Control	28 ± 1 (21)	-
(50 mg L^{-1} as $CaCO_3$)	1	34 ± 2 (10)	0.012*
(pH = (8.26 ± 0.01))	2	32 ± 2 (10)	0.252
	4	30 ± 2 (12)	0.871
	8	39 ± 1 (11)	< 0.001*
Moderate	Control	28 ± 1 (19)	-
(150 mg L^{-1} as $CaCO_3$)	4	32 ± 2 (12)	0.520
(pH = (8.27 ± 0.01))	12	28 ± 1 (10)	0.999
	24	25 ± 2 (12)	0.547
	48	21 ± 2 (12)	0.004*
High	Control	28 ± 1 (20)	-
(150 mg L^{-1} as $CaCO_3$)	4	31 ± 1 (9)	0.795
(pH = (8.31 ± 0.01))	12	29 ± 2 (11)	0.990
	24	25 ± 3 (12)	0.767
	48	17 ± 1 (8)	< 0.001*

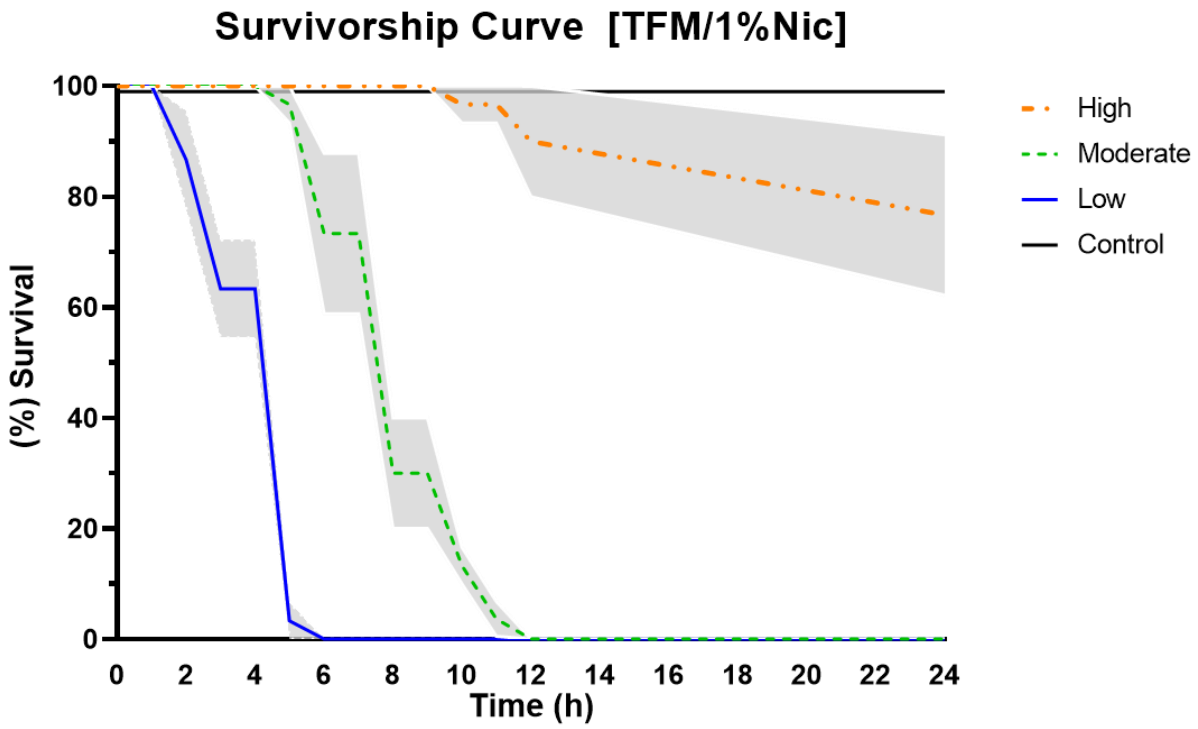

Figure 3-1. Effect of water alkalinity on time of survival of sea lamprey exposed to a mixture of 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide (1 %) mixture.

Three groups of larval sea lamprey were exposed to a nominal TFM concentration of 2.9 mg L^{-1} (12-h LC_{50} of TFM) mixed with niclosamide (1 % the TFM concentration) for 24 h in water of low alkalinity (59.4 ± 1.02 mg L^{-1} as $CaCO_3$), moderate alkalinity (151.3 ± 0.82 mg L^{-1} as $CaCO_3$) or high alkalinity (251.3 ± 2.55 mg L^{-1} as $CaCO_3$). Survival tests at each alkalinity at a pH of 8.3 were done in triplicate, with N = 10 larval sea lamprey per replicate, and the mean survival rate was plotted over time. A separate group of control animals (N = 30 in triplicate; N = 10 per replicate) were treated in an identical manner, in the absence of the lampricides. Shaded areas denote the SEM.

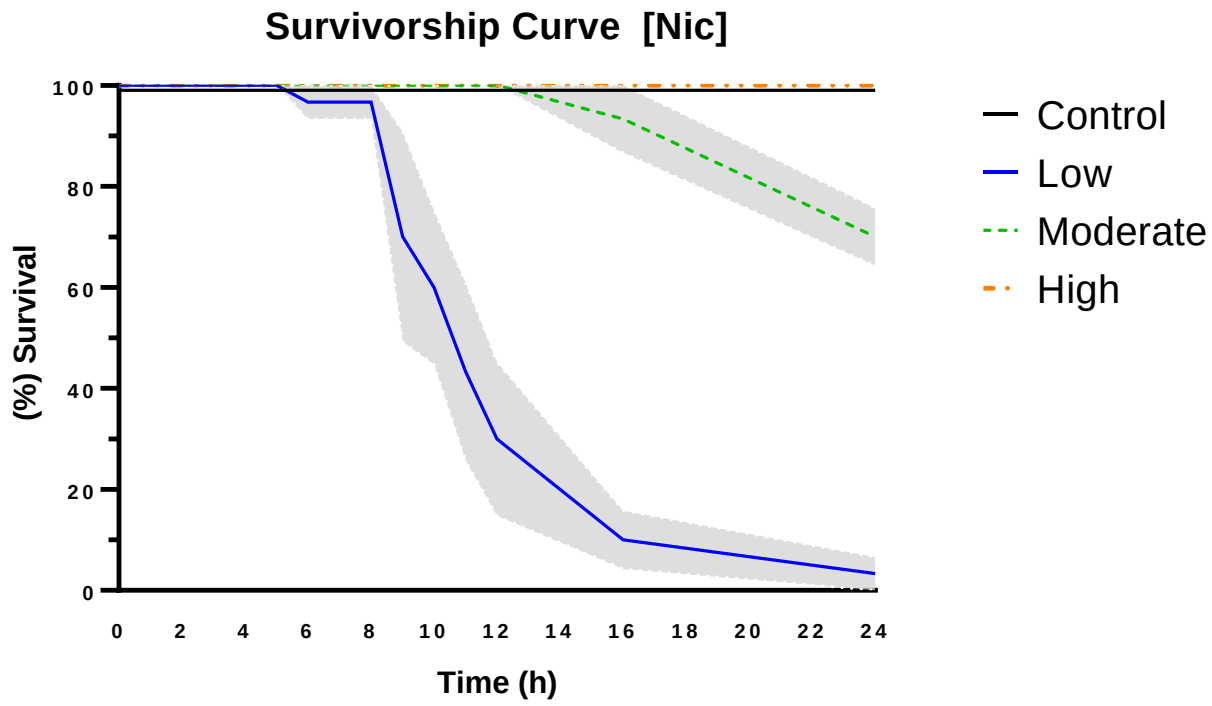

Figure 3-2. Effect of water alkalinity on time of survival of sea lamprey exposed to 2′,5-dichloro-4′-nitrosalicylanilide (niclosamide).

Groups of larval sea lamprey were exposed to a nominal niclosamide concentration of 78 µg L^{-1} for 24 h (24-h LC_{50} of niclosamide) in water of low alkalinity (59.4 ± 1.02 mg L^{-1} as $CaCO_3$), moderate alkalinity (151.3 ± 0.82 mg L^{-1} as $CaCO_3$) or high alkalinity (251.3 ± 2.55 mg L^{-1} as $CaCO_3$). Survival tests at each alkalinity were done in triplicate, with N =10 larval sea lamprey per replicate, and the mean survival rate was plotted over time. A separate group of control animals (N = 30 in triplicate; N = 10 per replicate) were treated in an identical manner, in the absence of niclosamide. Shaded areas denote the SEM.

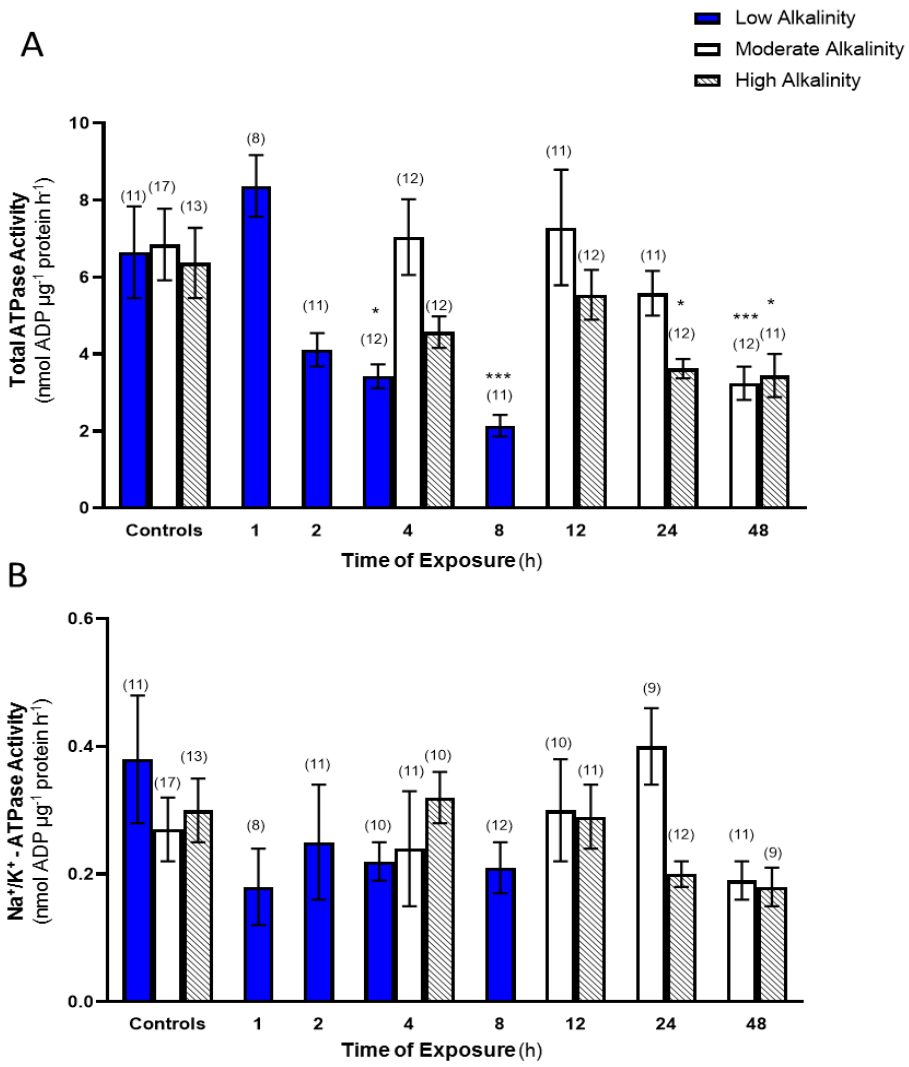

Figure 3-3. Effects of exposure to a mixture of 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide (1 %) on gill Na^+/K^+-ATPase activity in larval sea lamprey.

Changes in (A) NKA activity and (B) total ATPase activity in the gills of larval sea lamprey (under control (no lampricide exposure) or following 1, 2, 4, 8, 12, 24, and 48 h exposure to a nominal TFM concentration of 2.0 mg L^{-1}, mixed with niclosamide (1 % the TFM concentration) at low alkalinity (blue bars; 54.4 ± 2.2 mg L^{-1} as $CaCO_3$), moderate alkalinity (open bars; 149.6 ± 0.2 mg L^{-1} as $CaCO_3$) or high alkalinity (cross-hatched bars; 255 ± 0.01 mg L^{-1} as $CaCO_3$) at pH 8.3. Data presented as the mean ± 1 SEM. Sample sizes (N) indicated in brackets over each sample period).

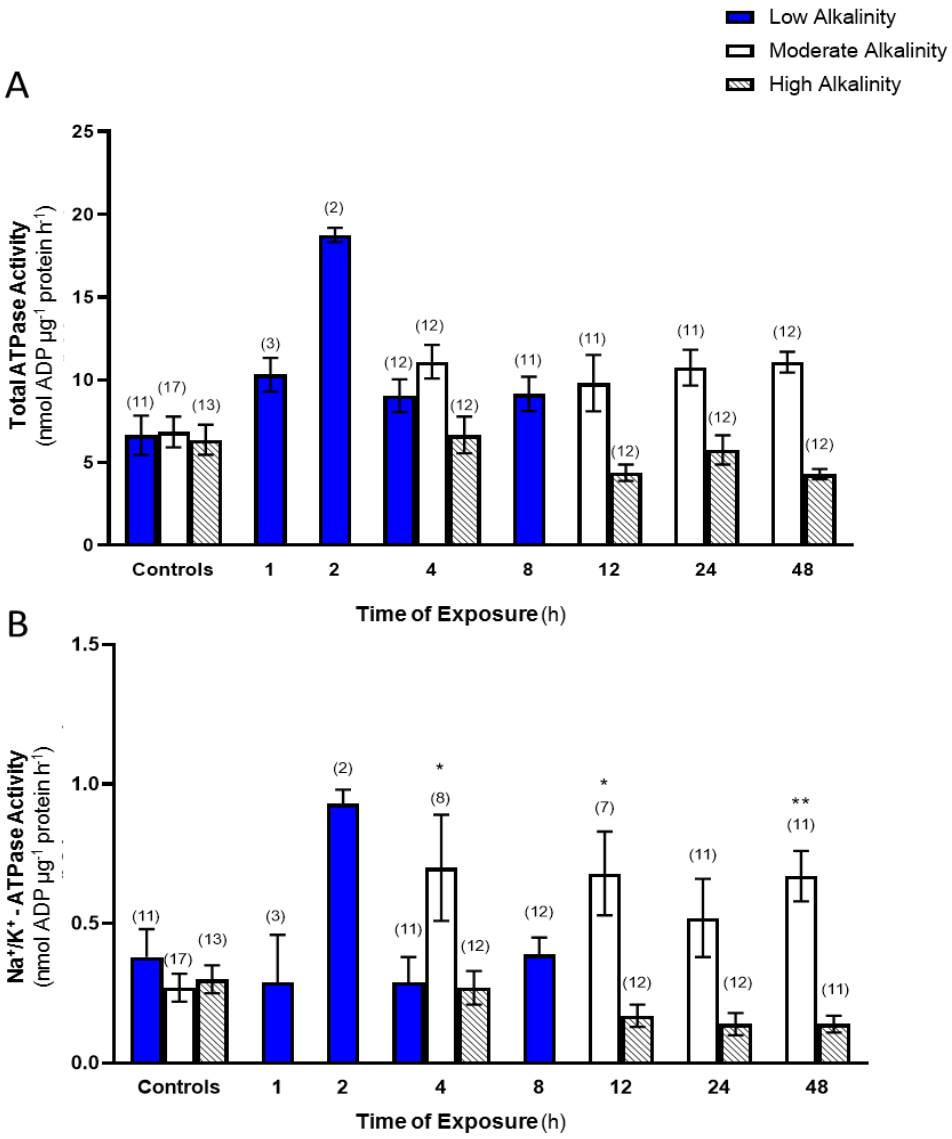

Figure 3-4. Effects of niclosamide exposure on gill Na^+/K^+-ATPase activity in larval sea lamprey.

Changes in (A) NKA activity and (B) total ATPase activity in the gills of larval sea lamprey) under control (no niclosamide exposure) or following 1, 2, 4, 8, 12, 24, and 48 h exposure to a nominal niclosamide concentration of 70 µg L^{-1} at low alkalinity (blue bars; 54.4 ± 2.2 mg L^{-1} as $CaCO_3$), moderate alkalinity (open bars; 149.6 ± 0.2 mg L^{-1} as $CaCO_3$) or high alkalinity (cross-hatched bars; 255 ± 0.01 mg L^{-1} as $CaCO_3$) at pH of 8.3. Data presented as the mean ± 1 SEM. Sample sizes (N) indicated in brackets over each sample period.

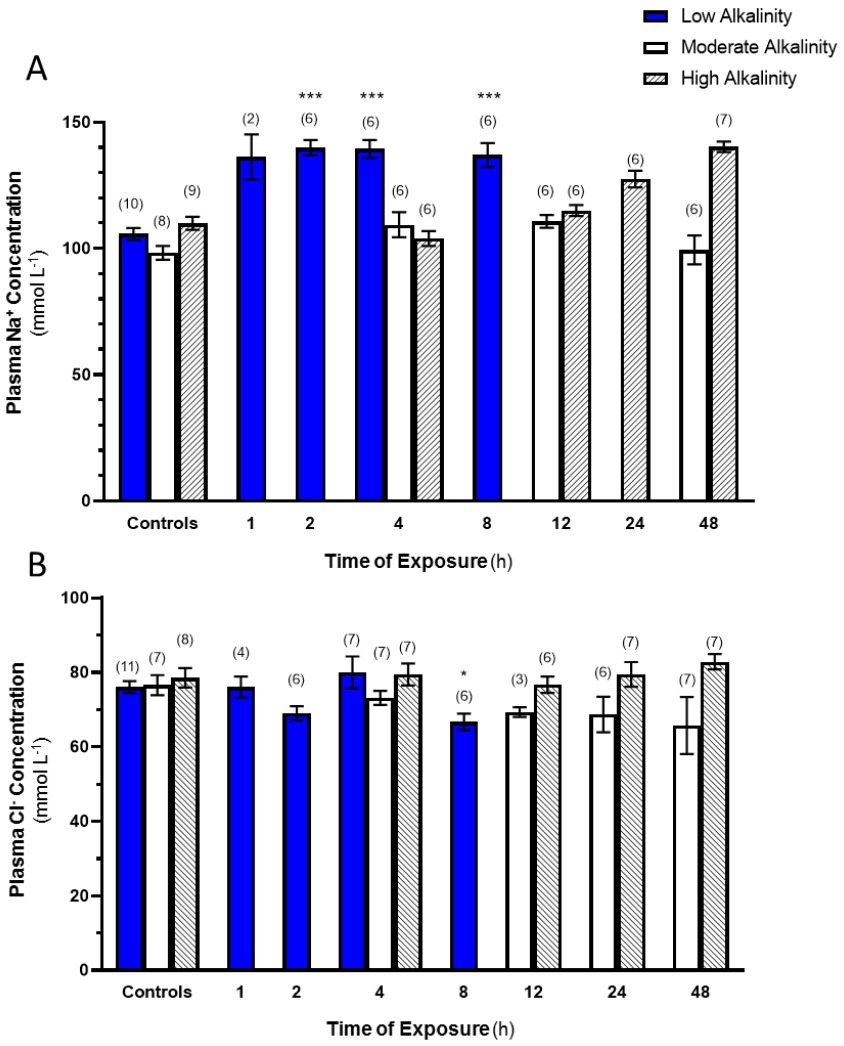

Figure 3-5. Effects of exposure to a mixture of 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide (1 %) on blood plasma Na^+ and Cl^- concentration in larval sea lamprey.

Changes in (A) plasma Na^+ concentration, and (B) plasma Cl^- concentration of larval sea lamprey under control (no lampricide exposure) or exposure to a nominal TFM concentration of 2.0 mg L^{-1}, mixed with niclosamide (1 % the TFM concentration) at low alkalinity (blue bars; 54.4 ± 2.2 mg L^{-1} as $CaCO_3$), moderate alkalinity (open bars; 149.6 ± 0.2 mg L^{-1} as $CaCO_3$) or high alkalinity (cross-hatched bars; 255 ± 0.01 mg L^{-1} as $CaCO_3$) at pH 8.3. Data presented as the mean ± 1 SEM. Sample sizes (N) indicated in brackets over each sample period.

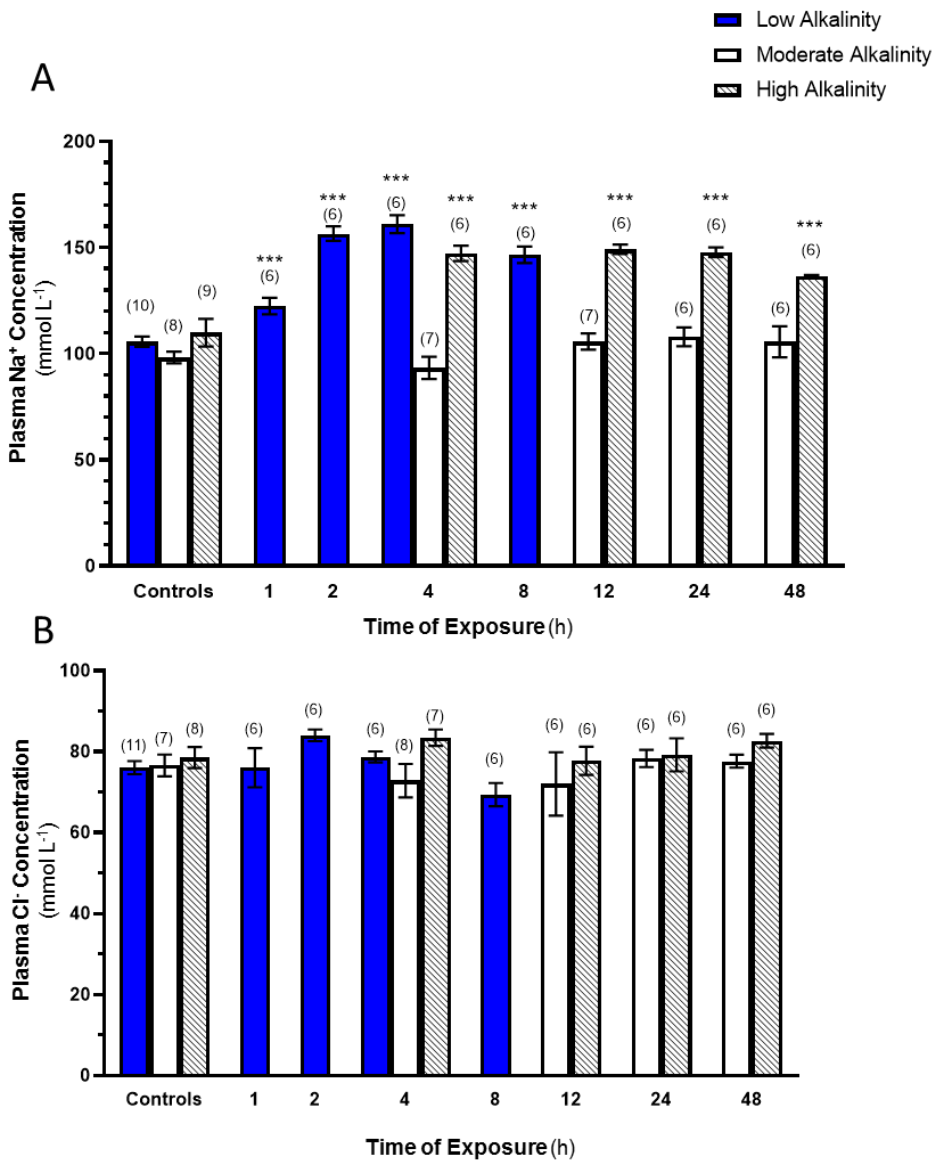

Figure 3-6. Effects of niclosamide exposure on plasma Na^+ and Cl^- concentration of larval sea lamprey.

Changes in (A) plasma Na^+ concentration, and (B) plasma Cl^- concentration of larval sea lamprey under control (no niclosamide exposure) or following exposure to a nominal niclosamide concentration of 70 µg L^{-1} at low alkalinity (blue bars; 54.4 ± 2.2 mg L^{-1} as $CaCO_3$), moderate alkalinity (open bars; 149.6 ± 0.2 mg L^{-1} as $CaCO_3$) or high alkalinity (cross-hatched bars; 255 ± 0.01 mg L^{-1} as $CaCO_3$) at pH of 8.3. Data presented as the mean ± 1 SEM. Sample sizes (N) indicated in brackets over each sample period.

Chapter 4

An Integrated Model Describing the Influence of Water Alkalinity on the Toxicity of TFM and Niclosamide in Larval Sea Lamprey

Introduction

Sea lamprey control in the tributaries surrounding the Laurentian Great Lakes relies heavily on the application of TFM and niclosamide (GLFC, 2011). However, there still remains a need to understand how TFM and niclosamide is affected by abiotic factors and how it can be used more effectively (McDonald and Kolar, 2007). It is well known that TFM sensitivity is influenced by abiotic factors such as water pH (McDonald and Kolar, 2007; Scholefield et al., 2003), alkalinity (Bills et al., 2003; Hepditch et al., 2019; O'Connor et al., 2017), and temperature (Muhametsafina et al., 2019), but the underlying reasons for this are poorly understood. The present study provides insight on the processes that influence the toxicity of TFM and niclosamide due to variation in alkalinity when the variables of pH and temperature are kept constant.

My thesis used various integrative scientific approaches to study how alkalinity affected the sensitivity of larval sea lamprey to lampricides, including methods commonly used in toxicology, biochemistry, physiology, and limnology. Toxicology was used to conduct a series of range finder toxicity test in different water chemistries which provided dosage concentrations for subsequent survivorship experiments which also fell under this field of study. Physiology helped me understand the various and complex functions and structures related of the larval sea lamprey anatomy, such as enzymes (proteins) pertaining to ion transport (Evans et al., 2005; Ferreira-Martins et al., 2021; Reis-Santos et al., 2008). Additionally, physiology provided insight on how the sea lamprey are able to detoxify chemicals, such as the lampricides, and determine possible effects to various gill functions when exposed to the lampricides (Birceanu et al., 2009; Borowiec et al., 2022; Ionescu et al., 2022a). Finally, limnology was used to determine how variation in the water chemistry of the streams that drain into the Great Lakes and the streams, particularly alkalinity, affects lampricide effectiveness and their mode of action. Alkalinity may

vary throughout the Great Lakes which depending on the type of underlying bedrock upon which the rivers and lakes are found. In rivers draining the Canadian shield, buffer capacity and water pH can be very low, but if the underlying bedrock is predominantly limestone, alkalinity and thus buffering capacity will be much higher (Acton et al., 2015). This understanding of fundamental limnology was very important in helping me select the range of alkalinities used in this study (O'Connor et al., 2017).

In this chapter, the results of this research study are discussed to develop more effective methods of TFM and niclosamide applications in the integrated pest management of sea lamprey control in the Great Lakes.

Integrated Pest Management of the Sea Lamprey in the Great Lakes

In the 1900s the invasive sea lamprey became a threat to the fisheries in the Great Lakes, contributing to its crash by the 1940s and 1950s (Gaden et al. 2021; Siefkes, 2017; Smith and Tibbles, 1980; Wilkie et al., 2019). Over the years, the methods used to control the sea lamprey involved an integrated approach that relied on several different techniques which included velocity barriers, electrical barriers, adjustable-crest barriers, and traps to catch and remove adult spawning sea lamprey and/or to block their upstream migration (GLFC, 2011; Siefkes, 2017). Chemical treatment with TFM and niclosamide was and remains the most effective and widely used method of control in the tributaries surrounding the Great Lakes, with applications typically occurring every 2 - 4 years (McDonald and Kolar, 2007; Wilkie et al., 2019). The combined effects of the sea lamprey control program have resulted in a decrease of parasitic juvenile lamprey in the Great Lakes. Despite these efforts, there are residual sea lamprey that manage to avoid or survive the efforts mentioned above, which undermines the effectiveness of the sea lamprey control efforts. This thesis reveals some of the underline mechanisms that explain why

alkalinity is a key variable that needs to be considered when applying TFM, as well as niclosamide to streams or rivers containing invasive sea lamprey.

Influence of pH and alkalinity on TFM and Niclosamide Bioavailability

It has been well established that pH strongly influences TFM due to the changes in speciation of the chemical (Bills et al., 2003; McDonald and Kolar, 2007; Wilkie et al., 2019). However, there is a paucity of research correlating the effects of pH on niclosamide toxicity. There has been assumption that the effects of pH on niclosamide would be similar to that of TFM due to similarities in chemical properties (Dawson, 2003), but there has been virtually no research on this matter. Based on research on TFM and the assumptions of similarities to niclosamide, at a given concentration of lampricide, there is greater un-ionized, phenolic TFM or niclosamide present in more acidic waters, resulting in greater uptake compared to higher pH environments where the relative amounts of the ionized or phenolate chemical species of the lampricides predominate (Bills et al., 2003; Hepditch et al., 2019; Hlina et al., 2017; Hunn and Allen, 1974). Alkalinity has been shown to have a protective affect on TFM toxicity for sea lamprey (Bills et al., 2003), and for the first time this relationship is the same for niclosamide. Further, there is a paucity of previous studies that have researched the effects of alkalinity on TFM and niclosamide toxicity in larval sea lamprey when pH is held constant. Hepditch et al. (2019) demonstrated the protective effect of alkalinity on TFM toxicity in sturgeon, that was independent of pH. In that study, there was significant decrease in the rates of uptake of TFM in waters of higher alkalinity (> 150 mg L^{-1} as $CaCO_3$). In this study, similar findings were observed in the survivorship tests, in which there was much lower survivorship of sea lamprey when exposed to TFM, TFM/Nic or niclosamide alone at low compared to moderate and high alkalinity (Figure 2-1, 3-1, 3-2).

In Chapter 2 a model was proposed that explains the protective effect of alkalinity is due to the greater buffer capacity of higher alkalinity waters, which results in relatively lower TFM bioavailability compared to lower alkalinity waters (Figure 2-4). A similar model (Figure 4-1) applies to niclosamide. As for TFM, I propose that in higher alkalinity waters, there is less acidification of the gill micro-environment by metabolic H^+ excretion and the hydration of respiratory CO_2, decreasing the amount of bioavailable lipophilic un-ionized niclosamide (Figure 4-1).

Influence of alkalinity and TFM/Niclosamide exposure on the gill function of larval sea lamprey

The gills are known to be the primary location for TFM uptake (Hunn and Allen, 1974), and previous studies have found physiological damage to the gills of larval sea lamprey following the exposure of TFM, TFM/1% niclosamide and niclosamide alone (Mallatt et al., 1994). It is hypothesized that the lampricides, particularly niclosamide, would interfere with mitochondria rich cells (MRC) function leading to disturbances in ion balance. It was also hypothesized that disturbances would be greater in lower alkalinity water, due to higher relative amounts of TFM-OH and niclosamide (Nic-OH). However, in larval sea lamprey water alkalinity did not inhibit NKA or Total ATPase activity in the gills, in the presence or absence of TFM or niclosamide. In addition, with the exception of TFM exposure in low alkalinity waters (Chapter 2), were few physiologically relevant differences of blood plasma Na^+ and Cl^- ions observed during lampricide exposure at any of the alkalinities studied. This finding suggests that the toxic effects of both TFM and niclosamide are restricted to the mitochondria and, at least in the gills, do not directly target the ion transport machinery of the gills. However, further studies are needed to determine ATP supply in the gills is compromised *in situ* during actually lampricide exposure, rather than post-hoc experiments and analysis used in my thesis.

Future Directions, implications for sea lamprey control and Conclusions

The sea lamprey control program in the Great Lakes has successfully reduced their population by 90% from their peak abundance, however there is still a need to increase the efficiency of treatments by reducing the amounts of lampricides required, increasing their effectiveness, all while mitigating non-target impacts (GLFC, 2011; Heinrich et al., 1980). This study highlights the important role that alkalinity plays in determining the toxicity of TFM, TFM/1% niclosamide, and niclosamide alone. It was not very surprising that alkalinity affects TFM sensitivity, but no previous studies have shown how pronounced the effects of alkalinity are on the niclosamide sensitivity of larval sea lampreys. It may therefore be very helpful to consider the effects that alkalinity when applying niclosamide, not only in combination with the TFM, but when much higher concentrations of niclosamide are used when it is used alone in its granular formulation when treating high velocity and high discharge streams (Dawson 2003). Monitoring how the alkalinity changes throughout the targeted tributaries may also reduce "overdosing" systems, especially in low alkalinity environments, to minimize the risk of non-target mortality. Additionally, the toxicity and survivorship test done in this study highlights and confirms the effectiveness of the TFM/1% niclosamide, where there was a 40 – 50% increase in toxicity compared to when TFM was used alone. Consideration might therefore be given to treating more waters with the TFM-niclosamide mixtures, increases effective use of lampricides, reducing the effort and increasing the number of streams to be treated, and ultimately saving on costs.

Future investigations are also needed to better define and quantify how the speciation of TFM and niclosamide changes in the gill microenvironment of sea lamprey and non-target fishes. This could allow researchers to predict with more confidence how much TFM and niclosamide to use in treatments, and to better identify fishes that might be susceptible to non-

target mortality. The calculations of TFM and niclosamide speciation using only bulk water pH measurements, may lead to underestimates in the actual amount of bioavailable, un-ionized TFM and niclosamide in the gill micro-environment, particularly at low alkalinities where bulk water pH is much more variable. By homing in the ability to accurately predict dosing requirement in the streams through better understanding of the influence of alkalinity on toxicity and accurately calculating the true amount of bioavailable TFM and niclosamide in the bulk water and gill micro-environment, will more effectively control sea lamprey number while mitigating non-target species impact. may lead to new measures for controlling other invasive species, parasites, and carriers of diseases. Such examples where niclosamide is being used include the controlling molluscs in African and Asian countries (Dai et al., 2008; Yang et al., 2010; Zhu et al., 2022) which are straining aquatic ecosystems globally, mitigating snail populations that are intermediate host for a parasite that causes schistosomiasis in humans (Lardans and Dissous, 1998; Zhao et al., 2015), treatment for cestodes and trematodes infections in humans (Köhler, 2001; McKellar and Jackson, 2004), as well as potentially providing insight on abiotic factors that affect the speciation of niclosamide, which could potentially be used for cancer treatment (Khanim et al., 2011).

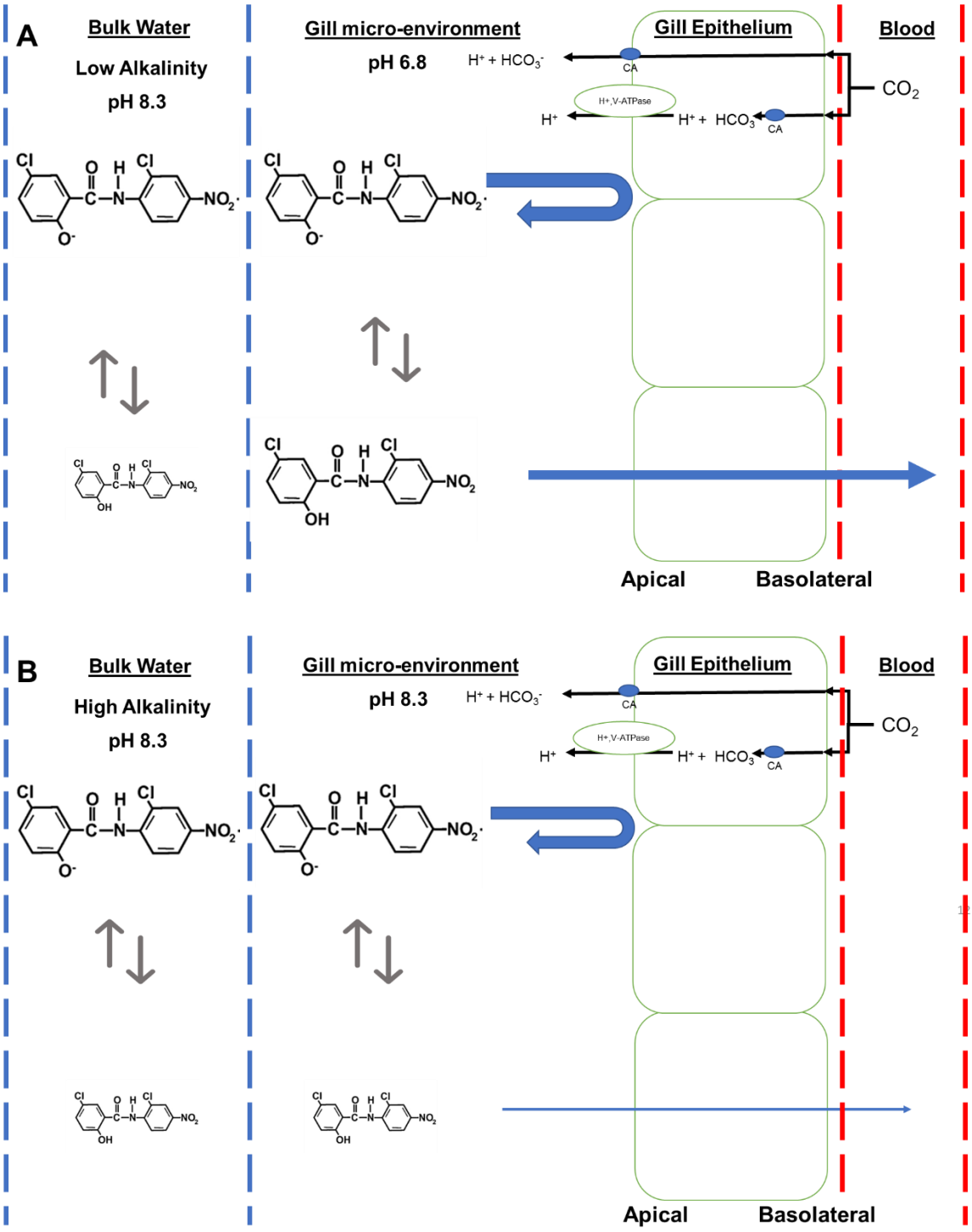
A
Bulk Water
Low Alkalinity
pH 8.3
Gill micro-environment
pH 6.8
Gill Epithelium
Blood
CA
CO2
H+,V-ATPase
Apical
Basolateral
B
Bulk Water
High Alkalinity
pH 8.3
Gill micro-environment
pH 8.3
Gill Epithelium
Blood
CA
CO2
H+,V-ATPase
Apical
Basolateral

Figure 4-1. Proposed model depicting how niclosamide uptake and toxicity to larval sea lamprey is affected by water alkalinity.

It is proposed that niclosamide uptake by larval sea lamprey and other fishes occurs mainly in Nic-OH, entering the animal via the gills down its diffusion gradient. A key variable that influences niclosamide uptake and toxicity is pH, in which the concentration of the more bioavailable Nic-OH is greater at low compared to higher pH, at a given total niclosamide concentration (Total Niclosamide = Nic-OH + Nic-O^-) concentration. The bioavailability of niclosamide can also be altered at the gill surface (gill microenvironment) due to acidification caused by metabolic H^+ excretion and the hydration of respiratory CO_2 (Panel A). The amount of acidification taking place near the gill surface is influenced by water alkalinity, which determines the water buffer capacity. At higher water alkalinity, the buffering capacity of water is higher, which attenuates acidification of the gill microenvironment, resulting in less Nic-OH compared to lower alkalinity at a given concentration of total niclosamide (compare panel A to B).

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