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Control of invasive sea lampreys using the piscicides TFM and niclosamide: Toxicology, successes & future prospects

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ABSTRACT

The invasion of the Laurentian Great Lakes of North America by sea lampreys (*Petromyzon marinus*) in the early 20th century contributed to the depletion of commercial, recreational and culturally important fish populations, devastating the economies of communities that relied on the fishery. Sea lamprey populations were subsequently controlled using an aggressive integrated pest-management program which employed barriers and traps to prevent sea lamprey from migrating to their spawning grounds and the use of the piscicides (lampricides) 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide to eliminate larval sea lampreys from their nursery streams. Although sea lampreys have not been eradicated from the Great Lakes, populations have been suppressed to less than 10% of their peak numbers in the mid-1900s. The ongoing use of lampricides provides the foundation for sea lamprey control in the Great Lakes, one of the most successful invasive species control programs in the world. Yet, significant gaps remain in our understanding of how lampricides are taken-up and handled by sea lampreys, how lampricides exert their toxic effects, and how they adversely affect non-target invertebrate and vertebrates species. In this review we examine what has been learned about the uptake, handling and elimination, and the mode of TFM and niclosamide toxicity in lampreys and in non-target animals, particularly in the last 10 years. It is now clear that the mode of TFM toxicity is the same in non-target fishes and lampreys, in which TFM interferes with oxidative phosphorylation by the mitochondria leading to decreased ATP production. Vulnerability to TFM is related to abiotic factors such as water pH and alkalinity, which we propose changes the relative amounts of the bioavailable un-ionized form of TFM in the gill microenvironment. Niclosamide, which is also a molluscicide used to control snails in areas prone to schistosomiasis infections of humans, also likely works by uncoupling oxidative phosphorylation, but less is known about other aspects of its toxicology. The effects of TFM include reductions in energy stores, particularly glycogen and high energy phosphagens. However, non-target fishes readily recover from sub-lethal TFM exposure as demonstrated by the rapid restoration of energy stores and clearance of TFM. Although both TFM and niclosamide are non-persistent in the environment and critical for sea lamprey control, increasing public and institutional concerns about pesticides in the environment makes it imperative to explore other means of sea lamprey control. Accordingly, we also address possible "next-generation" strategies of sea lamprey control including genetic tools such as RNA interference and CRISPR-Cas9 to impair critical physiological processes (e.g. reproduction, digestion, metamorphosis) in lamprey, and the use of green chemistry to develop more environmentally benign chemical methods of sea lamprey control.

1. Introduction

1.1. Overview

Invasive sea lampreys (*Petromyzon marinus*) have been subjected to aggressive population control measures and extensively researched to find more effective means to limit their negative impact on fisheries in the Laurentian Great Lakes of North America. The piscicides, 3-trifluoromethyl-4-nitrophenol [TFM; IUPAC name = 4-nitro-3-(trifluoromethyl)phenol] and niclosamide (aka. Bayluscide®), which are applied to lamprey infested rivers and streams, have been critical to the success of the sea lamprey control program in the Great Lakes (Applegate et al., 1957, 1961; Lawrie, 1970; McDonald and Kolar, 2007). After 4,346 mostly organic compounds were tested, 15 were

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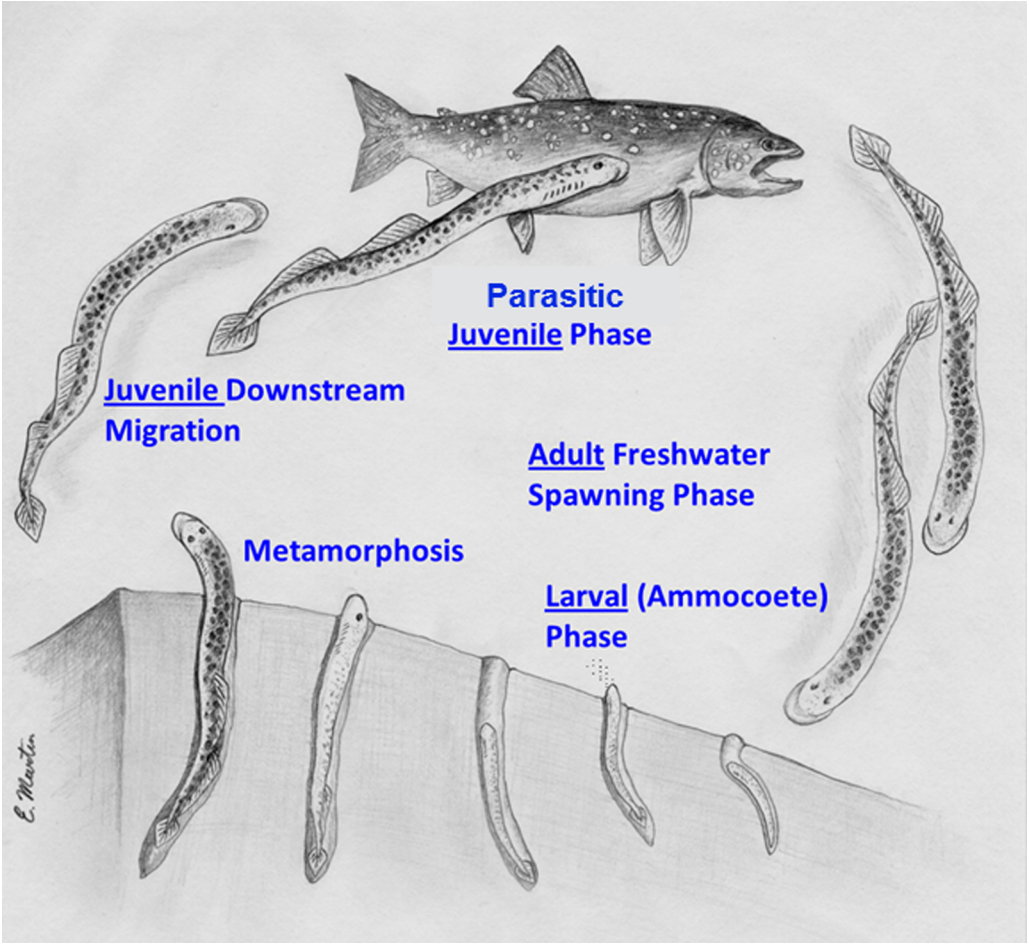


Fig. 1. Sea lamprey life cycle. Sea lamprey begin life as burrow-dwelling larvae (ammocoetes) in the substrate of rivers and streams draining into the Atlantic Ocean or the Laurentian Great Lakes. After 3–7 years, the larvae undergo metamorphosis, a non-trophic life stage lasting several months characterized by physiological and morphological changes that prepare them for the juvenile, parasitic life stage. Following metamorphosis, juvenile sea lamprey migrate downstream, and enter the Great Lakes or to the ocean in the case of anadromous populations. During the juvenile or parasitic phase, sea lamprey can feed on a wide range of host-fishes/prey, including large, economically important fishes such as lake trout and white fish in the Great Lakes. After 12–20 months, they cease feeding and migrate upstream, spawn and die. Refer to text for further details. Figure from Wilkie 2009. See text for further details.

identified that were selectively more toxic to sea lamprey than to rainbow trout (*Oncorhynchus mykiss*). All of these compounds were halogenated phenols containing a nitro group (mono-nitrophenols). Structural activity relationship analysis revealed that compounds with the nitro groups in the para position and the halogen in the meta-position were more toxic to lamprey than to non-target fishes. These compounds included those derived from dinitrophenols such as 5-chloro-2-nitrophenol, 3,4,6-trichloro-2-nitrophenol and 3-trifluoromethyl-2-nitrophenol, and those derived from 4-nitrophenol including 2,5-dichloro-4-nitrophenol, 3-bromo-4-nitrophenol and TFM, which had the greatest selectivity (Applegate et al., 1957, 1966). Other compounds and classes including phenol, mono- and poly-halo phenols, mononitrophenols, dinitrophenols and halo-dinitrophenols and poly-halo-mononitrophenols were either more toxic to non-target fishes or of similar toxicity in lamprey making them unsuitable as selective lampricides. While not the only potential lampricide identified, TFM was chosen because of its greater selectivity and potency to sea lamprey, and its suitable chemical properties including water solubility (Applegate et al., 1966; Thingvold and Lee, 1981). Niclosamide was subsequently incorporated into the sea lamprey control program because it reduces the amount of TFM needed to eradicate lampreys and it has a propensity to sink in its granular formulation, making it an excellent bottom-acting compound for large and/or fast flowing waters and for targetting lentic sea lamprey populations (Dawson, 2003).

Despite almost sixty years of use, many gaps remain in our understanding of how lampricides exert their toxic effects on sea lampreys, how/if they adversely affect non-target organisms, and how lampricides might be more effectively and safely applied to improve sea lamprey control and to protect the Great Lake's aquatic ecosystem. The overarching goal of this review will be to outline our current understanding of sea lamprey biology and control in the Great Lakes region, with a particular emphasis on the basic and applied toxicology of TFM and niclosamide. Our specific objectives will be to: **(i)** provide a brief account of sea lamprey biology and the events that led to their invasion of the Great Lakes, **(ii)** describe how TFM and niclosamide are used for sea lamprey population control, **(iii)** discuss the chemical properties and environmental fate of TFM and niclosamide, **(iv)** review how lampricides are taken-up, handled and eliminated by lampreys, with a focus on how water chemistry and events in the gill microenvironment effect lampricide bioavailablity and toxicity, **(v)** provide updated models addressing the mode of toxicity of TFM and niclosamide, **(vi)** explain our current state of knowledge of how lampricides affect non-target vertebrate and invertebrate organisms, and looking to the future, **(vii)** discuss emerging technologies that could improve the efficacy of sea lamprey control efforts, and/or even lead to the development of "next generation" lampricides that can contribute to the integrated pest management of sea lamprey in the Great Lakes. Because TFM is used for the vast majority of lampricide treatments and has been more intensively studied in recent years, it is the primary focus of this article.

1.2. The biology of sea lampreys

The sea lamprey is one of at least 39 species of jawless fishes belonging to the Order Petromyzontiformes (Renaud, 2011). Members of the family Petromyzontidae, they are found in waters of the Northern Hemisphere on both sides of the North Atlantic. While most sea lampreys are anadromous, there are landlocked, freshwater populations in the Great Lakes, the Finger Lakes, New York, and Lake Champlain, Vermont/New York. As a result of sea lamprey parasitism/predation

during the juvenile phase of their life cycle, sea lampreys have decimated commercial, recreational and culturally significant fisheries in these freshwater bodies. In the Great Lakes, a single juvenile sea lamprey may kill 10–20 kg of fish during the parasitic phase, when they attach themselves to large fishes and use their oral disc and rasping tongue to suck the blood from their hosts (Parker and Lennon, 1956; Farmer et al., 1975; Swink, 2003).

Sea lampreys spawn in freshwater rivers and streams giving rise to larvae that are also called ammocoetes (Fig. 1). The larval stage typically lasts 3–7 years, during which the relatively small (< 120 mm in length), functionally blind ammocoetes live burrowed in the soft substrate of streams, where they filter-feed on detritus, suspended organic matter and biofilm (Beamish and Potter, 1975; Sutton and Bowen, 1994). The feeding apparatus is comprised of a conspicuous oral hood connected to the oral cavity, and it functions to intercept water currents generated by a muscular velum found between the oral cavity and the pharynx. After entering the oral cavity, the water is then directed towards the pharynx where food particles are trapped on mucus secreted by an endostyle, and diverted to the gut (Rovainen, 1996). When body mass and body length are sufficient, typically greater than 2.5 g and 120 mm (Holmes and Youson, 1994), and lipid stores are sufficient, sea lampreys undergo a highly complex, multi-staged metamorphosis which takes place over 3–4 months (Lowe et al., 1973; Youson, 2003; Manzon et al., 2015). Metamorphosis is characterized by the appearance of eyes, a change of body color from light or dark brown to a metallic blue-black sheen, and the development of the multi-toothed, oral disc and rasping tongue that is used to attach to and feed on the blood of fishes when the animals enter the parasitic juvenile phase following metamorphosis (Fig. 1; Mallatt, 1996; Rovainen, 1996; Renaud et al., 2009). Because metamorphosis is a non-trophic (non-feeding) phase, energy is primarily provided by the lipid reserves accumulated during the larval phase (Lowe et al., 1973; Kao et al., 1997). Following metamorphosis, juvenile sea lampreys ingest large quantities of protein-rich blood from large salmonid fishes, other game fishes, sturgeons, as well as sharks and even cetaceans in marine environments (Bigelow and Schroeder, 1948; Beamish and Potter, 1975; Jensen and Schwartz, 1994; Wilkie et al., 2004, 2006; Gallant et al., 2006; Nichols and Hamilton, 2004; Renaud et al., 2009). The rate of blood ingestion by parasitic juvenile sea lampreys is typically 3–10% of their total body mass per day, but may approach 30% in the latter part of the parasitic life stage (Farmer et al., 1975; Farmer, 1980). These high rates of blood consumption, along with secondary infections of the wounds inflicted by the feeding juveniles, very likely explains why many fishes die following sea lamprey attacks. At the conclusion of the juvenile parasitic phase, the young adult lampreys cease feeding, migrate upstream, spawn and then die (Beamish and Potter, 1975).

1.3. Origin of sea lamprey in the Great Lakes

The origins of sea lampreys in the Great Lakes remains a subject of considerable debate (Eshenroder, 2009, 2014). Microsatellite DNA studies suggest that the Great Lakes and Atlantic populations are genetically distinct, supporting the hypothesis that sea lampreys were native to Lake Ontario, and perhaps the Finger Lakes and Lake Champlain in New York and Vermont (Bryan et al., 2005; Waldman et al., 2004, 2006; D'Aloia et al., 2015). However, a lack of historical documentation of sea lamprey in the region raises doubts about this theory. Instead, it has been proposed that sea lampreys gained access to Lake Ontario from the Hudson River in New York, following construction of the Erie Canal in the early 1800's (Lawrie, 1970; Eshenroder, 2009, 2014). Initially, sea lamprey were restricted to Lake Ontario due the presence of Niagara Falls, which prevented them from entering Lake Erie and the Upper Great Lakes. Following modifications to the Welland Canal in the early 1900s, sea lamprey were able to bypass Niagara Falls, with the first sea lamprey reported in Lake Erie in 1921 (Lawrie, 1970). In the ensuing years, they invaded the upper Great Lakes, and by the 1950s, parasitic sea lampreys, along with overfishing, had decimated native, culturally significant fisheries, as well as sport and commercial fisheries. The damage caused by sea lampreys was not restricted to fisheries, however. By targeting apex predators, populations of invasive alewife (*Alosa pseudoharangus*) and rainbow smelt (*Osmerus mordax*) exploded due to decreased predation, leading to seasonal die-offs, corresponding declines in water quality and extensive fouling of shorelines and beaches (GLFC, 2011).

1.4. A brief history of sea lamprey control

In response to the crisis, the Canadian and United States (US) governments signed the Convention on Great Lakes Fisheries in 1954, creating the Great Lakes Fishery Commission (GLFC) which was mandated to establish a "comprehensive program" to eradicate or control sea lamprey populations in the basin (GLFC, 2011). Vernon Applegate and his team (1957, 1966) subsequently identified 3-trifluoromethyl-4-nitrophenol (TFM) as a potential piscicide (lampricide) that specifically targeted larval sea lampreys. Field trials quickly followed, with the first treatments ocurring in Lake Superior in the early 1960s, followed by the other Great Lakes in subsequent years (Schnick, 1972; Smith and Tibbles, 1980).

In addition to TFM, 2′,5-dichloro-4′-nitrosalicylanilide, which is better known as niclosamide (aka. Bayluscide®), was incorporated into the lampricide program in the early 1960s (Howell et al., 1964). It should also be noted that niclosamide use is not restricted to sea lamprey control; it is also commonly used as a molluscicide applied to waters infested with snails that are the intermediate host for the parasite *Schistosoma japonicum* which causes shistosomiasis in humans (Lardans and Dissous, 1998; Joubert et al., 2001; Zhao et al., 2015). Niclosamide is also used in the treatment of cestode (tapeworms) and trematodes (flukes) infections in humans and other animals (Köhler, 2001; McKellar and Jackson, 2004). While the granular form of niclosamide is used for population surveys of larval sea lampreys, the wettable powder and liquid formulations are often co-applied (1–2%) with TFM to increase lampricide effectiveness in large, fast flowing or deep lentic waters, while maintaining the same treatment efficiency (Dawson, 2003). The addition of 1% niclosamide to the lampricide mixture can reduce the amount of TFM required for a treatment by 40% (Boogaard et al., 2003; Gutreuter and Boogaard, 2007).

In addition to lampricide applications, other methods of sea lamprey control including sea lamprey barriers (dams), trapping, and sterile male release, have been implemented and continue to be used as part of an integrated pest management program (Sower, 2003; McLaughlin et al., 2007). This integrated approach has reduced sea lamprey populations in the Great Lakes to 10% of their peak in the 1950s, making it one of the most successful aquatic invasive species programs in the world (Siefkes, 2017). In the last 10–20 years, lamprey specific pheromones have been studied for use as sea lamprey attractants/repellants to improve trapping effectiveness and/or to divert lamprey away from suitable spawning habitat (Wagner et al., 2006; Li et al., 2007, 2012; Sorensen and Hoye, 2007; Johnson et al., 2015; Siefkes, 2017). For now, chemical treatment with lampricides remains one of the most effective and widely used methods of sea lamprey control in the Great Lakes. However, gaps remain in our understanding of the mechanisms of action of these compounds, the physico-chemical and biological factors that affect lampricide effectiveness, and their potential adverse affects on non-target aquatic vertebrate and invertebrate populations (Boogaard et al., 2003; McDonald and Kolar, 2007).

2. Physico-chemical properties & environmental fate of lampricides

2.1. TFM

TFM is an aromatic phenol (Fig. 2A) that exists as a light yellow

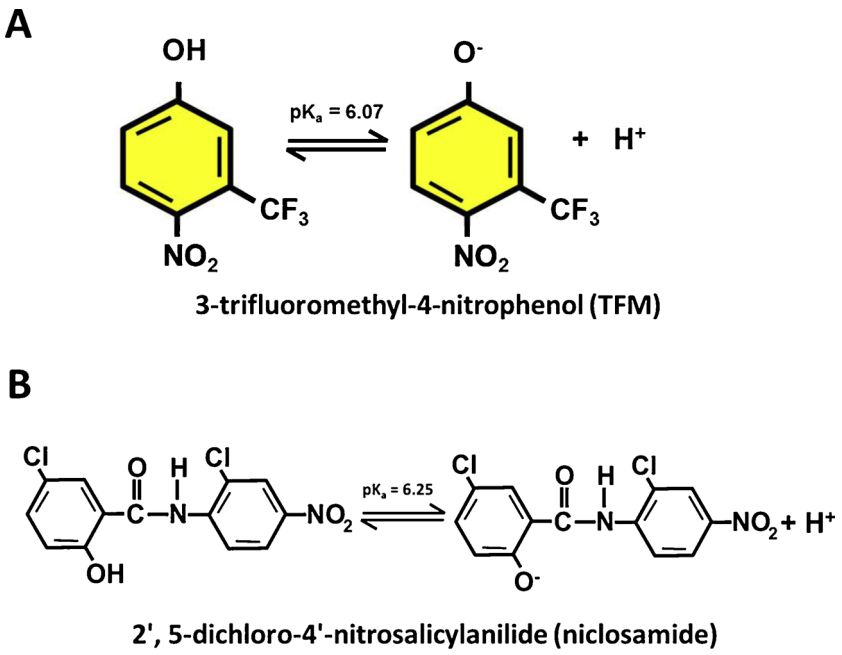


Fig. 2. Chemical structure of TFM and niclosamide. Both **(A)** TFM and **(B)** niclosamide are weak acids, with respective pKa's of 6.07 and 6.25. At lower pH, each exists as the more diffusable un-ionized (phenolic) form, but at higher pHs, the ionized (phenolate), less diffusable form of each lampricide predominates.

crystalline solid at room temperature, with the characteristic smell of phenolic, cresol-type compounds. In water it forms a light yellow or reddish-orange solution, depending on concentration. As a weakly acidic phenol, it will dissociate in water and has a pK_a of 6.07 (Hubert, 2003), which has important implications for understanding the pharmacokinetics of TFM and TFM treatment effectiveness in the field (see below). TFM is also hydrolytically stable, with half-lives ranging from 1444 days to over 4000 days depending on pH (Hubert, 2003). The enhanced stability is not unexpected because carbon-fluorine bonds are very strong and difficult to break, and aromatic rings are generally difficult to open.

TFM also binds to the sediment in streams, with a degree of binding proportional to the amount of organic content within the sediment (Kempe, 1973; Dawson et al., 1986). As might be expected, the un-ionized, or lipophilic form of TFM is more readily adsorbed, as silt type sediments with high organic content adsorb more TFM than sandy-type sediments (Dawson et al., 1986). However, recent evidence suggests that organic matter has little effect on the rates of TFM photodegradation (McConville et al., 2017a, b). TFM is also relatively resistant to aerobic microbial breakdown (Hubert, 2003), but not to anaerobic microbes, which rapidly degrade TFM into its constituent organic acids under laboratory conditions (Bothwell et al., 1973; Kempe, 1973).

Despite its chemical stability, TFM was thought to undergo relatively rapid photodegradation in aquatic ecosystems (Carey et al., 1988). In the lab, TFM rapidly photodegraded, with a half-life of 3–5 days in natural water and sunlight conditions, with rates increasing at higher pH (Carey et al., 1988; Ellis and Mabury, 2000). However, more recent work has revealed that the TFM undergoes virtually no photodegradation when it is applied to relatively small river systems, with reaches of only several km, before reaching the Great Lakes (McConville et al., 2017a, b). Neither organic TFM photo-degradation products such as gentisic acid or 4-hydroxycaetchol, or inorganic metabolites such as triflouroacetic acid, fluoride or nitrate/nitrite, were detected in stream water, suggesting the parent TFM was unchanged. Although some TFM was lost from the systems during passage downstream, this was attributed to factors such as dilution arising from upwellings, sorption to sediments and/or exchange with the hyporheic zone of the stream (McConville et al., 2017a). In larger, more complicated systems the likelihood of photodegradation was greater. Still, modelling exercises accounting for factors that can affect the intensity of solar radiation in the water (e.g. water depth, canopy cover, concentration of natural organic matter) indicated that photodegradation was only significant (> 50% decomposition of TFM) in about 12% of streams subjected to lampricide treatment (McConville et al., 2017a).

2.2. Niclosamide

Niclosamide (2′, 5-dichloro-4′-nitrosalicylanilide) is also a phenolic compound, but more properly classified as a salicylanilide comprised of two substituted aromatic rings bridged by an amide functional group, resulting in a much higher molecular weight than TFM (Fig. 2B). It is a pale yellow solid at room temperature, stable to hydrolysis, with a half-life of 19 days at 20 °C (El-Dib and Aly, 1976; Schultz and Harman, 1978). Like TFM, niclosamide is a weak acid (pKa ~6.25; Dawson, 2003) and it readily dissociates into its un-ionized form at higher pHs (Fig. 2B).

Niclosamide adsorbs much more strongly than TFM to organic matter, which leads to considerable binding and accumulation in sediments, particularly those with higher organic content (Dawson et al., 1986; McConville et al., 2016). Niclosamide is also much more lipophilic, with a log octanol:water partition co-efficient (log K_{ow}) of 10 (pH 9.6; Tomlin, 1994), which makes it more difficult to keep in aqueous solution than TFM. This factor, plus its greater toxicity (see below), is one reason why it is more often used as an additive (1–2%) with TFM. Due to the strength of its interactions with the sediments and its propensity to sink in its granular formulation, niclosamide may be used on its own for treating lamprey in large fast-flowing waters, or for targeting burrowed lamprey during lentic treatments (Dawson, 2003).

Niclosamide is thought to undergo rapid microbial degradation in natural waters and sediment, yielding less toxic CO_2 and low-molecular weight organic acids (Muir and Yarechewski, 1982; Graebing et al., 2004). However, the photodegradation of niclosamide is 2 orders of magnitude slower than it is for TFM, with values averaging 127 d, when continuously exposed to light that approximates noon-time light conditions (McConville et al., 2016). Unlike TFM, the rate of niclosamide photodegradation is faster in the presence of dissolved organic matter (McConville et al., 2017b). With its relatively long half-life, however, it

is unlikely that niclosamide undergoes significant photodecomposition within treated waters (McConville et al., 2017a) before reaching the Great Lakes. Although the fate of both niclosamide and TFM within the Great Lakes needs more study, the dilution of both compounds upon entering the lakes, and subsequent decomposition to less toxic products likely limits their persistence and adverse affects in aquatic ecosystems.

3. Toxicology

3.1. Factors influencing rates of lampricide uptake in fish

3.1.1. Water pH and alkalinity

The toxicity of phenols, like other xenobiotics, is strongly influenced by factors such as lipid and water solubility, molecular weight, and its ionization state (speciation), which in turn dictate movements across biological membranes (Hunn and Allen, 1974). With a log K_{ow} of 2.77 (Schmitt et al., 2000), TFM has moderate lipid solubility in its un-ionized form. Due to its weak acid properties (see above), the concentration of the un-ionized, lipophilic form of TFM therefore increases with decreases in water pH at a given concentration of total TFM. This can be demonstrated by calculating the relative amounts of un-ionized (TFM − OH) and ionized TFM (TFM-O^-) at different water pHs using the pKa of the compound and the Henderson-Hasselbalch equation. At the typical water pH of rivers and streams draining into the Great Lakes, pH 6.5–8.5, the majority of total TFM (Total TFM = TFM − OH + TFM-O-) is TFM-O-, as opposed to TFM − OH (Fig. 3A). Yet, at a given concentration of total TFM, uptake is higher at more acidic pH, as initially demonstrated in rainbow using ^{3}H-TFM (Hunn and Allen, 1974) and more recently in larval sea lamprey using ^{14}C-TFM (Fig. 3B; Hlina et al., 2017). This suggests that TFM is primarily taken-up in its un-ionized form, as depicted in the model of TFM uptake and toxicity in Fig. 4. This also illustrates why the minimal lethal concentrations (MLC) for TFM decrease in lower pH waters (Marking and Olson, 1975; Dawson et al., 1975). For this reason, water pH is measured before and throughout TFM applications to ensure that the application rates sustained are sufficient to kill larval sea lampreys, while protecting non-target organisms, that are generally much more tolerant to the lampricide (Bills et al., 2003; McDonald and Kolar, 2007). With a similar pKa of 6.25, niclosamide exhibits similar pH dependence based on similar tests (Marking and Hogan, 1967; Dawson, 2003).

The dependency of TFM accumulation on pH implies that it is mainly taken-up in the un-ionized form, but ionized TFM may also be taken-up, albeit to a lesser extent. For instance, when toxicity is expressed in terms of un-ionized TFM concentration, the 12-h minimum lethal concentration (MLC = $LC_{99.9}$, the TFM concentration required to cause 99.9% mortality) actually decreases disproportionaely with increasing pH (Hlina et al., 2017). If TFM uptake were solely in its un-ionized form, then the 12-h MLC for un-ionized TFM should be identical at all pHs tested. It is not. In fact, Saarikoski et al. (1986) noted that the while the accumulation of ^{14}C-labeled phenolic and carboxylic acids by the guppy (*Poecilia reticulata*) over a range of pHs was reduced at higher pHs, the changes were also disproportionate to the corresponding concentrations of the un-ionized species of each compound. Such disconnects may also simply be because the amount of un-ionized TFM in the bulk water is not necessarily reflective of its actual bioavailability in the gill microenvironment/gill surface, and/or it may because these phenolic compounds are also taken-up in the ionized-form.

Erickson et al. (2006a,b) modelled the uptake of other ionizable organic weak acid compounds, such as nitrophenols and chlorophenols, at the gills of rainbow trout. While the un-ionized forms of such substances are more readily taken-up across fish gills at low pH, like TFM, uptake rates and toxicity do not change proportionately. The authors provided a number of explanations: First, they point-out that compound speciation at the gill surface may be different than speciation in the surrounding (bulk) water. For instance, it is well-established that the pH at the gill surface (gill microenvironment) can be markedly different than the bulk water pH due to the excretion of CO_2 (Playle and Wood, 1989) and metabolic acid (H^+), which is pumped via apically-located V-ATPases (H^+-ATPases) and/or Na^+/H^+ exchange (NHE antiporters) found on the apical membrane of branchial mitochondria rich (MR) cells (e.g. Perry and Gilmour, 2006; Reis-Santos et al., 2008; Dymowska et al., 2012), which would acidify water as it is drawn across the gill surfacen (Fig. 4). Taking into account the counter-current flow of blood and water between the gill (secondary) lamellae, Erickson et al. (2006a) calculated that the pH of water with an alkalinity of 1 mM and inspired pH of 8.0, could drop by almost 1 full pH unit from lamellar base to tip. For a compound such as TFM, this would result in approximate several-fold increases in the un-ionized moiety at the gill surface, increasing the inwardly directed gradient relative to the bulk water and rates of TFM uptake (Fig. 5).

Second, diffusion trapping, in which the un-ionized moiety of an ionizable organic compound is converted to its ionized species as it crosses the gill epithelium from the water to the blood, is also key to maintaining the diffusion gradients across the gills, which in turn sustains uptake of the un-ionized species (Erickson et al., 2006a). Due to its relatively low pKa of 6.07, most un-ionized TFM entering the blood of lamprey or non-target fishes would be converted to the ionized form at physiological pH (Fig. 4; blood pH ~7.8 at 15 °C; Boutilier et al., 1993; Wilkie and Wood, 1991). Trapping would also be augmented by protein-TFM interactions, as well as by biotransformation and/or elimination of TFM by the fish (particularly non-target species). Of course the degree of diffusion trapping taking place would be influenced by water pH, which can vary substantially in freshwater ecosystems. For instance, at lower pH (i.e. ~pH 6.5) inwardly directed gradients due to diffusion trapping would be steeper at a given total TFM concentration than at more circumneutral pH (i.e. pH 7–8).

Finally, there is no a priori reason why significant amounts of ionized weak organic acids, particularly nitrophenols, cannot diffuse through the phospholipid bilayer. Indeed, the log K_{OW} for the un-ionized forms of nitrophenols, including TFM, are surprisingly close to the log K_{OW} of the corresponding ionized form (Erickson et al., 2006a). Interactions of TFM and niclosamide with organic matter and ions in the water, prior to reaching the fish gill, could also limit their uptake by the fish and impact their bioavailabilty and toxicity. Such interactions between TFM, niclosamide and the gill, however, have yet to be investigated. Another possibility is that ionized TFM could be taken-up via ion transport proteins on the gill, pharynx and/or intestine (Hlina et al., 2017). Possible candidates include organic anion transporters, such as the Mrp2 protein (multi-drug resistance-associated protein 2). Indeed, the genes for Mrp2 protein are present in the gills and liver of larval and adult sea lamprey where they are thought to be involved in bile salt homeostasis (Cai et al., 2013).

Unlike water pH, it is less clear why TFM toxicity generally decreases with increasing alkalinity (Bills et al., 2003). One possibility is that in waters of higher buffering capacity, which typify waters with higher alkalinity, there is less acidification of the water of the gill microenvironment, resulting in higher gill water pH and less un-ionized TFM. Thus, actual inward gradients for un-ionized TFM would be less, leading to less TFM accumulation in waters of higher alkalinity, at a given pH. Indeed, we have observed that TFM uptake rates by lake sturgeon (*Acinpenser fulvescens*) are lower in waters of higher vs lower alkalinity, as well as high vs low pH (S. Hepditch, O. Birceanu and M.P. Wilkie. Unpubl. observations). Niclosamide, which has a similar pKa (6.25) to TFM would be expected to behave similarly.

3.1.2. Water hardness

The effects of water hardness on TFM and niclosamide toxicity have received relatively little attention in recent years, so its importance remains unclear. It is well established that metal bioavailablity and uptake can be markedly influenced by the concentrations of Ca^{2+} and/or Mg^{2+} in the water, which can affect metal bioavailability through competition for Ca^{2+}-binding/uptake sites on the gills (Paquin et al., 2002; Niyogi and

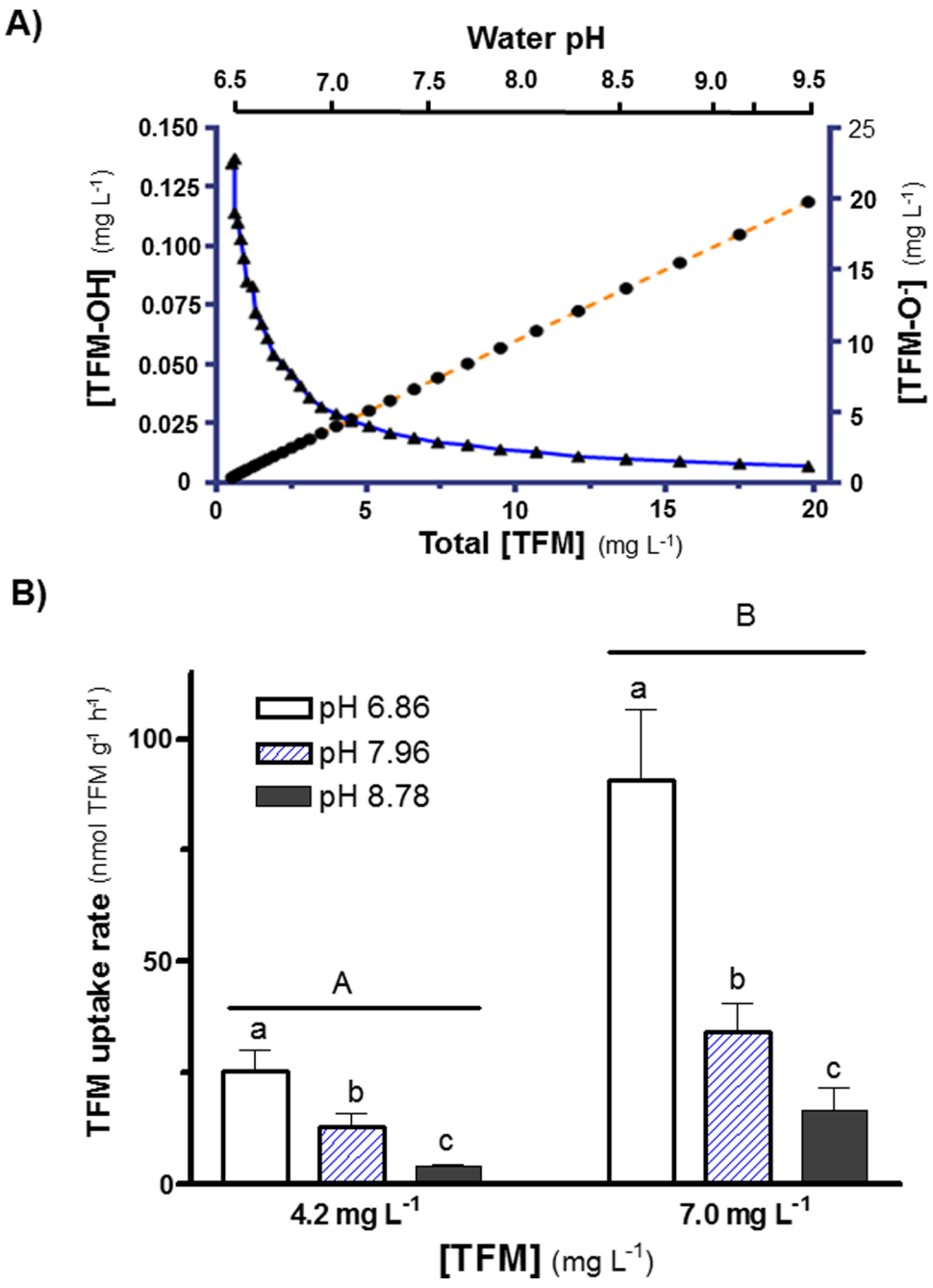


Fig. 3. Effects of pH on TFM speciation and uptake.
(A) At a given concentration of total TFM, the proportion of unionized TFM (TFM−OH; triangles, solid line) declines with increasing pH, with corresponding increases in the proportion of ionized TFM (TFM-O^-; circles, dashed line) in the water. **(B)** Decreases in the proportion of TFM−OH with water pH are accompanied by corresponding reductions in TFM uptake rate by larval sea lamprey. Lower case letters denote statistically significant differences in TFM uptake at different water pHs and at a given TFM concentration; upper case letters denote differences between lower and higher concentrations of TFM. TFM uptake data from Hlina et al. (2017). See text for further details.

Wood, 2004; Al-Reasi et al., 2011). Early studies suggested that TFM toxicity decreased with increasing water hardness in non-target fishes such as rainbow trout, common carp (*Cyprinus carpio*), bluegill (*Lepomis macrochirus*) and channel catfish (*Ictalurus punctatus*), but later studies indicated that larval sea lamprey exposed to TFM in soft water (low Ca^{2+} and Mg^{2+}) were no more susceptible to TFM than in hardwater (Marking and Olson, 1975; Marking et al., 1975; Dawson et al., 1977). However, it remains unclear if the differences in the TFM sensitivity of lamprey versus non-target fishes were due to species differences, and/or due to methodological differences. Because it was not clear how long the larval lamprey were acclimated to different water hardnesses in the latter study (if at all), interpretation of the data could have been confounded by the sudden changes in water Ca^{2+} and/or Mg^{2+}. In sediment dwelling soft-bodied invertebrates (*Chironomus* sp.), the rate of TFM uptake has been shown to be inversely proportional to water hardness (Kawatski and Bittner, 1975). A possible explanation is that the binding of hardwater cations (Mg^{2+}, Ca^{2+}) with the negatively-charged ionized (phenolate) form lowers the amount of total TFM (sum of ionized plus un-ionized TFM), which would result in decreased overall bioavailability of the un-ionized, more lipohilic TFM in the water. This would, in turn, reduce lampricide uptake rates and toxicity in hard waters. However, these aspects need further investigation.

3.1.3. Organic matter

Little is known about how organic matter influences TFM or niclosamide uptake. More measurements are clearly needed, particularly since studies have shown that both TFM and niclosamide tend to adsorb more readily to sediments high in organic content (Kempe, 1973; Dawson et al., 1986; McConville et al., 2016) and that natural organic matter (NOM) is known to influence the toxicity of other xenobiotics, particularly metals (see Paquin et al., 2002; Niyogi and Wood, 2004 for reviews).

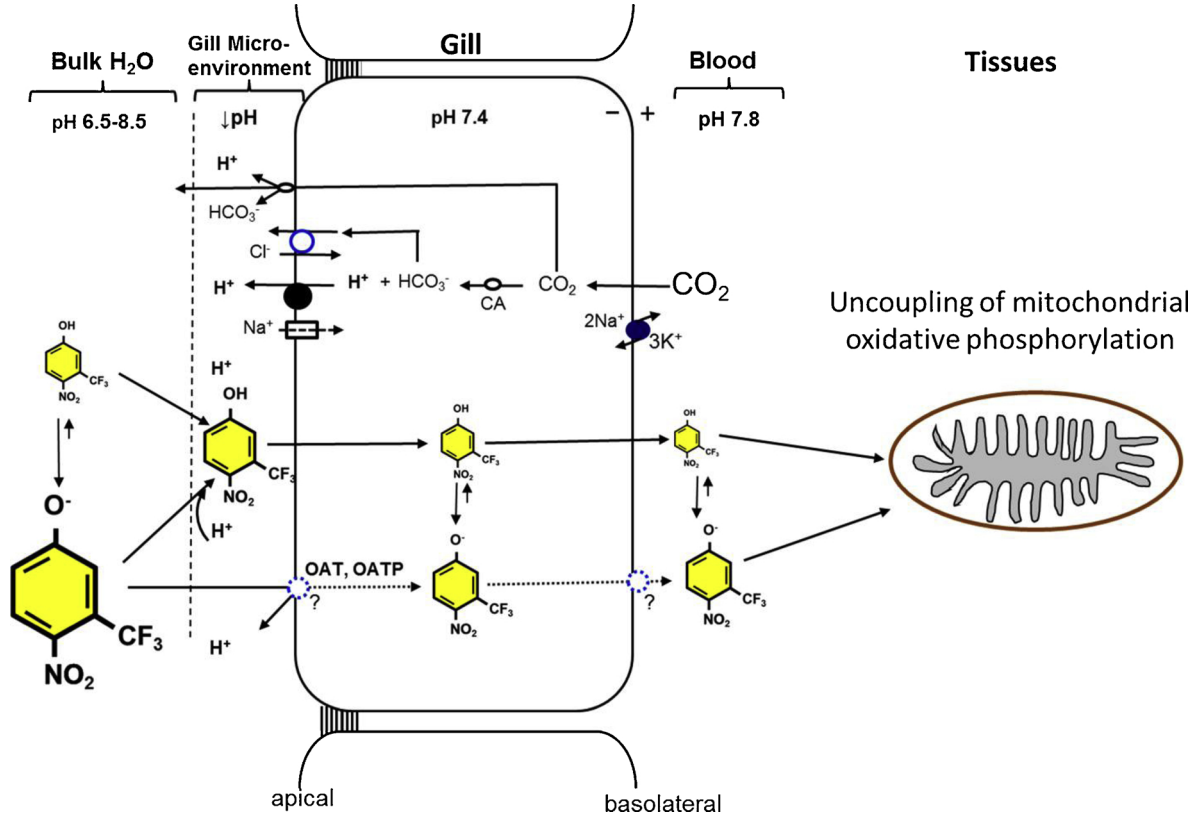


Fig. 4. Proposed model of TFM uptake and toxicity in sea lamprey and non-target fishes. The majority of TFM uptake takes place as un-ionized TFM − OH, which enters the fish via the gills down its corresponding diffusion gradient. Although the majority of total TFM (Total TFM = TFM − OH + TFM-O^-) is ionized (TFM-O^-), acidification of the gill micro-environment likely augments the inwardly directed TFM − OH diffusion gradient favouring greater rates of TFM uptake. A variety of physiological processes can promote acidification of the gill micro-environment including the excretion of H^+ arising from the carbonic anhydrase (CA) mediated hydration of CO_2 in the cytosol to HCO_3^- and H^+, which subsequently leads to H^+ excretion via proton-pumps (V-ATPase; solid circle) and/or Na^+/H^+ exchange (not shown). The hydration of CO_2 in the microenvironment, possibly via external CA, may also contribute to acidification. Uptake of ionized TFM-O^- is thought to be quatitatively less important than TFM − OH, but recent evidence demonstrated that there is appreciable TFM uptake by lamprey in the virtual absence of TFM − OH in alkaline water via unknown mechanisms that could include organic anion transporters (OAT, OATP). After entering the blood, TFM is delivered to the tissues where it exerts its toxicity by uncoupling oxidative phosphorylation, thereby lowering rates of ATP production. Also shown is the basolateral Na^+/K^+-ATPase, which pumps Na^+ out of the cell in exhange for K^+, sustaining low intracellular Na^+ to drive Na^+ uptake via apical Na^+ channels and/or Na^+/H^+ exchange; Cl^- uptake is likely driven by apical Cl^-/HCO_3^- exchange. See text for further details.

3.2. Distribution of TFM and niclosamide

As in water, the proportion of TFM in its un-ionized vs ionized form in the blood can be predicted using the pKa (6.07; Hubert, 2003) and the Henderson-Hasselbalch equation (Hunn and Allen, 1974; Clifford et al., 2012). While informative, such calculations only provide a rough estimation of TFM speciation internally because TFM-O^- binding to plasma proteins can alter the amount of free TFM in the plasma, and the pKa of ionizable compounds is variable. Cameron and Heisler (1983) demonstrated this principle for ammonia, which exists as either NH_4^+ or NH_3, with a pKa that can vary widely with changes in salinity and temperature. To more accurately predict how TFM is taken-up and distributed in the blood, it will be necessary to determine the pKa of this compound with greater accuracy at various salinities and temperatures.

Large amounts of TFM also accumulate in the liver of trout, followed by the heart, brain, blood and muscle following exposure to sublethal levels (Lech and Statham, 1975; M.P. Wilkie, S. Lantz, J. Bernardy et al. unpublished findings). Despite relatively low concentrations, a large proportion of TFM is stored in the muscle of fishes (Lech and Statham, 1975; Hubert et al., 2005; Birceanu et al., 2014), because it comprises over 60% of the total body mass (e.g. Stevens, 1968) and can therefore act as a sink for both TFM and its metabolites. However, it is rapidly cleared from the body within 24 h, with a $T_{1/2}$ of 0.8 to 1.4 h in trout (Lech et al., 1973). Studies on caged catfish and rainbow trout exposed to TFM for 12 h during lampricide treatments in the field corroborate such observations, with virtually all TFM and its metabolites being eliminated from edible muscle filets within 24–48 h of TFM exposure (Sills and Allen, 1975; Dawson et al., 2002; Vue et al., 2002). This rapid elimination of TFM by non-target fishes is likely due to its biotransformation to conjugated forms of TFM such as TFM-glucuronide and TFM-sulfate, which appear to be the major TFM-metabolites in non-target fishes (see below for further details). However, a detailed knowledge of the physico-chemical factors (e.g. lipid solubility, electrochemical gradients) that influence the partitioning of TFM and its metabolites in different body compartments is lacking at present. This information would also be useful for post-mortem determinations of tissue TFM concentrations, as well as its metabolites, to assist in the evaluation of causes of fish kills that may occur following treatments.

Also an ionizable phenol, niclosamide is handled in a similar manner to TFM by fishes. As with TFM, niclosamide was rapidly taken-up by rainbow trout, channel catfish and largemouth bass exposed to 0.05 mg L^{-1}, which is near concentrations typically applied to streams or benthic habitat in the field (Dawson et al., 1982). Not surprisingly, the greatest accumulation was in the bile, peaking at concentrations 60–80 times greater than measured in the plasma, and at 3–4 orders of magnitude higher than in the muscle. In addition to the gall bladder, niclosamide residues were also in the blood, heart, and fat tissue of rainbow trout, remaining in these tissues for at least 3 d following exposure to 0.05 mg L^{-1} of the piscicide (Statham and Lech, 1975). Unfortunately, little is known about how niclosamide is distributed,

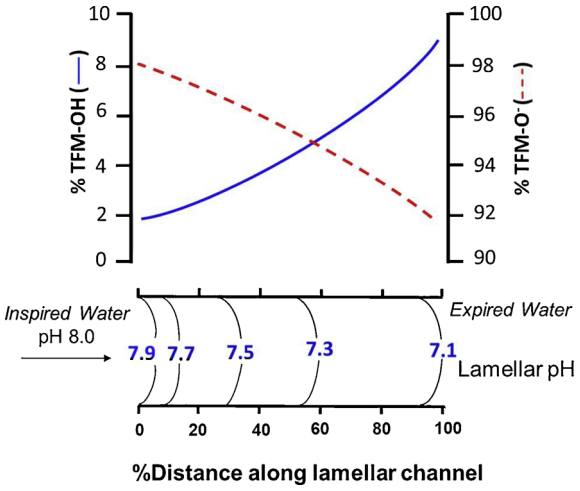


Fig. 5. Theoretical model of pH distribution and TFM speciation within the lamellar water channel of a fish gill. As water pH decreases, the proportion of un-ionized TFM (TFM−OH; solid blue line) in the water increases, with corresponding reductions in the amount of ionized TFM ($TFM\text{-}O^-$; red dashed line). Because the pH of inspired, circumneutral pH (pH ~7–8) water is gradually reduced due to branchial CO_2 and metabolic acid (H^+) excretion as it traverses the lamella, the pH of expired gill water in the gill microenvironment is more acidic. As a consequence, the proportion of the more difussable un-ionized TFM−OH present in the gill microenvironment will be greater than in surrounding bulk water, as depicted in bottom panel. Based on Erickson et al. (2006a).

metabolized and excreted by sea lampreys, the intended target of the piscicide.

3.3. Detoxification and elimination of lampricides

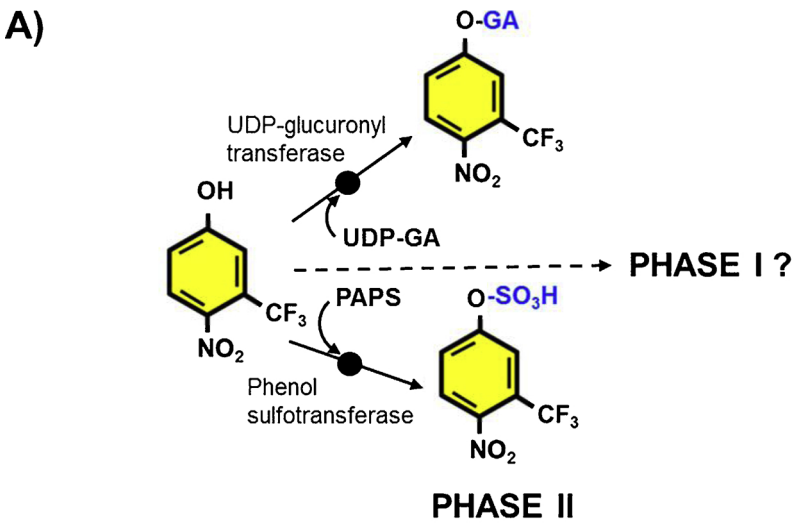
In teleost fishes, the majority of TFM accumulates in the liver, where it is subsequently detoxified using Phase II biotransformation processes (Fig. 6A; Lech and Costrini, 1972; Kane et al., 1994; Hubert et al., 2005; Bussy et al., 2018a; Foubister, 2018). Phase II biotransformation results in the addition of water soluble functional groups, such as glucuronic acid, acetyl groups, or sulfate esters to not only xenobiotics, but also to endogenous compounds such as steroids, making them more hydrophillic to facilitate excretion via biliary or renal routes (Clarke et al., 1991; Kalant and Roschlau, 1998). At least in trout, biotransformation appears to be restricted to the liver, and to a lesser extent the kidneys, where TFM-glucuronide also tends to accumulate (Lech and Statham, 1975). Glucuronidation was thought to be the predominate mechanism of TFM biotransformation within the liver in vivo (Lech and Costrini, 1972; Lech, 1974; Lech and Statham, 1975; Kane et al., 1994). However, a more complex picture of TFM metabolism has recently emerged suggesting that in addition to glucuronidation, TFM also undergoes sulfate conjugation (Fig. 6A) in non-target fishes including rainbow trout, lake sturgeon and bluegill (Bussy et al., 2018a,b; M. Wilkie, S. Lantz, J. Bernardy et al., Unpublished findings). Moreover, Bussy et al. (2018a,b) have shown that TFM undergoes phase I biotransformation to reduced 3-trifluoromethyl-4-aminophenol (TFMa) in liver homogenates of lampreys and non-target fishes, and in intact lamprey exposed to TFM. The accumulation of TFMa in intact lamprey is probably a consequence of their relatively low capacity to form TFM-glucuronide and TFM-sulfate, which would divert accumulated TFM to TFMa and its related metabolites. The importance of these pathways in non-target fishes is unknown, but the generation of TFMa is likely quantitatively and physiologically less important due to their ability to use glucuronide and sulfate congutation to biotransform TFM.

The relative inability to use glucuronidation and/or sulfation to detoxify TFM explains why this lampricide selectively targets larval sea lampreys. Unlike non-target fishes, TFM-glucuronide remains near or below levels of detection in the blood, muscle and liver of sea lampreys exposed to lethal concentrations of the lampricide (Lech and Statham, 1975; Bussy et al., 2018b). Inhibition of uridine diphosphate glucuronyltransferase (UDPGT) following the intra-peritoneal (IP) administration of salicylamide inhibits glucuronidation in trout treated with TFM in a dose-dependent manner (Lech, 1974) and it also reduces survival, but had no effect in sea lamprey (Lech and Statham, 1975). While some UDPGT activity was detected in sea lamprey, the efficiency of the enzyme was approximately 50% less than in rainbow trout and bluegill (Kane et al., 1994). At least two isoforms of UDPGT are likely present in sea lamprey, based on the recently assembled sea lamprey genome (Smith et al., 2013), in which two genes have been annotated for the enzyme (Ensembl transcript scaffold GL478009:1:108690:1 and GL479521:1:33375:1).

The greatest accumulation of niclosamide and its metabolites occurs in the liver, with concentrations up to 50–150 times higher in the bile (Statham and Lech, 1975). A large proportion of these residues were shown by mass spectroscopy (MS) to be glucuronidated-niclosamide (Statham and Lech, 1975; Fig. 6B), which would be excreted mainly via the urine. Sulfation also plays an important role in the metabolism of this lampricide (Fig. 6B), with significant amounts of the sulfated ester of niclosamide detected in the muscle of rainbow trout and catfish (Hubert et al., 2005). Like TFM, Phase I biotransformation appears to have little role in the metabolism of niclosamide. Munkittrick et al. (1994) reported no effect of niclosamide on mixed function oxidase enzymes in non-target fish. Surprisingly, there has been little additional work on niclosamide metabolism in sea lampreys or non-target species. Therefore the differences in the specificity between TFM and niclosamide towards sea lamprey and non-target fishes remains to be resolved.

The majority of TFM is excreted via renal routes in some non-target fishes such as salmon (Schultz et al., 1979), mainly as TFM-glucuronide (Hunn and Allen, 1975), but in others such as the channel catfish (*Ictalurus punctatus*) extra-renal routes (gills) predominate (Allen and Hunn, 1977). However, few additional pharmacokinetics studies have been completed. A combination of classical biochemical approaches (e.g. enzyme activity measurements) and surgical approaches (e.g. indwelling arterial and urinary catheters) are therefore needed to better characterize how different fish species detoxify and excrete the lampricides. Such studies could provide sea lamprey control agents, toxicologists and regulators with valuable insights to better predict and characterize how non-target fishes respond to lampricides.

In larval sea lamprey injected with toxicologically-relevant doses of ^{14}C-TFM labeled TFM (85 nmol g^{-1} ww), more than 95% of the load was cleared into the water within 24 h (Hlina et al., 2017). Because the capacity of sea lampreys to biotransform TFM via glucuronidation and sulfation is very limited, it seems likely that a larger proportion of the TFM was likely excreted at the gills in its un-ionized, hydrophobic form, with lesser amounts excreted via renal routes. The predominance of parent TFM in the blood and tissues of sea lampreys, with little to no TFM-glucuronide/sulfate or other hydrophilic metabolite(s), would preclude substantial renal filtration. This has been shown to be the case with ammonia elimination, where the majority of it is excreted across the gills in its unionized (NH_3) form, with less than 10% being excreted via the renal route (Read, 1968; Wilkie, 2002; Weihrauch et al., 2009). Similar to ammonia, we propose that the majority of the parent (un-ionized) TFM is excreted across the gills, down similar diffusion gradients during depuration in clean (TFM-free) water by sea lamprey (Clifford et al., 2012). During TFM-exposure it is likely that the diffusion gradients are directed into the fish, resulting in continual rises in total TFM in the early stages of exposure. It remains unclear whether or not the excretion of ionized TFM would involve any type of ion transport machinery in the gill or the kidneys. This possibility should be examined, however, because organic anion transporters (OATs) and organic anion transporter polypeptides (OATPs) involved in drug transport are found in numerous mammalian epithelia (see Kovacsics



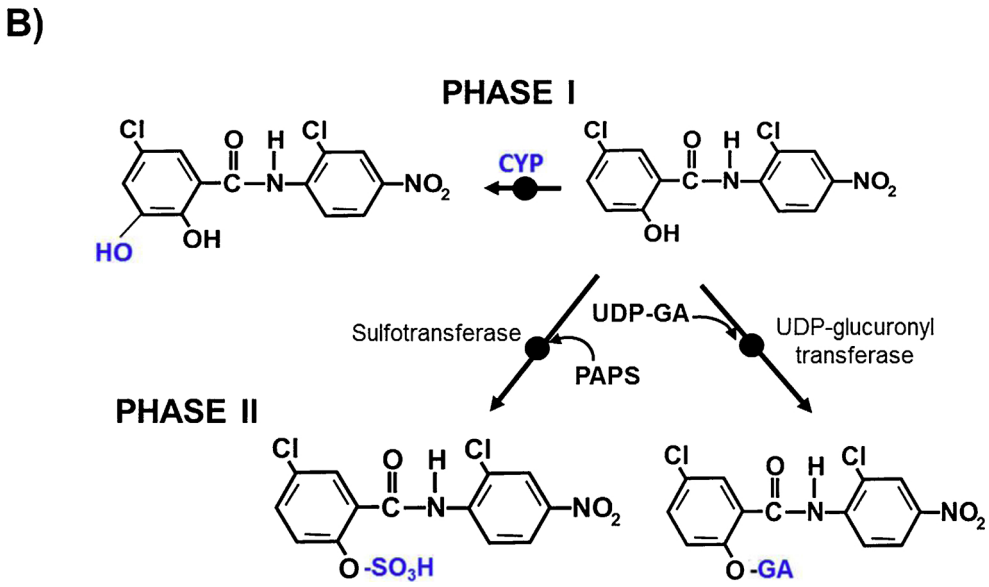


Fig. 6. Mechanisms of TFM and niclosamide detoxification. In non-target fishes **(A)** TFM and **(B)** niclosamide are primarily detoxified using Phase II biotransformation, with Phase I routes possibly playing a minor role. Glucuronidation is facilitated by UDP glucuronyl transferase, which leads to the transfer of a glucuronic acid functional group (GA) from UDP-glucuronic acid (UDP-GA) to the hydroxyl group of TFM or that found on the chlorophenol ring of niclosamide, yielding the respective glucuronide conjugates. Sulfation is facilitated by sulfonotransferase enzymes, resulting in the addition of a sulfate functional group to the same hydroxyl functional sites on TFM or niclosamide. The reaction is dependent upon the conversion of inorganic sulfate to the substrate 3′-posphoadenosine-5′-phosphosulfate (PAPS). The capacity of sea lampreys to use these pathways for TFM detoxification is limited compared to most non-target fishes. See text for further details.

et al., 2017; Huo and Liu, 2018 for recent reviews), and the gills of some fishes (Armitage et al., 2017), including sea lamprey (Cai et al., 2013). Niclosamide, which undergoes sulfation as well as glucuronidation, is primarily excreted renally in non-target fishes (Dawson, 2003), but little is known about how it is handled by sea lamprey.

4. Mechanism(s) of TFM and niclosamide toxicity

4.1. Uncoupling of mitochondrial oxidative phosphorylation

TFM and niclosamide have long been thought to be uncouplers of mitochondrial oxidative phosphorylation, due to the presence of a phenol ring and their weak acid properties (Moridani et al., 2003; McLaughlin and Dilger, 1980; Skulachev, 1998; Hollingworth and Gadelhak, 1998; Kadenbach, 2003; Ozaki et al., 2008; Solaini et al., 2011; Kuhn and Armes, 2012). Oxidative phosphorylation is the primary means of aerobic ATP generation, and it takes place in the mitochondria. As outlined in Fig. 7A, mitochondria are comprised of an outer and an inner membrane that separates the matrix from the intermembrane space. Oxidative phosphorylation begins with the oxidation of substrates generated from the citric acid cycle (electron donors; FADH2 and NADH) at the respiratory complexes comprising the electron transport chain (ETC) on the inner mitochondrial membrane. As a consequence, electrons are passed from one respiratory complex to the next (Complex I to IV), before combining with oxygen to generate water in the terminal step (complex IV) of the process. As electron transport proceeds, complexes I, III, and IV pump H^+ from the matrix into the inter-membrane space, resulting in the generation of an H^+ electrochemical gradient, the proton motive force. The low H^+ permeability of the inner mitochondrial membrane normally prevents non-specific H^+ diffusion from taking place, creating a transmembrane potential ($\Delta\Psi m$), restricting H^+ movements from the inter-membrane space into the mitochondrial matrix through the ATP synthase (aka. Complex V or F_0F_1-ATPase). This energy transduction is used to phosphorylate ADP to ATP. Thus, the oxidation of the different respiratory substrates along the ETC is *coupled* to the phosphorylation of ADP to ATP (see Brookes, 2005; Scatena et al., 2007 for reviews).

Uncouplers of oxidative phosphorylation "decouple" proton pumping from ATP synthesis by lowering $\Delta\Psi m$, usually by acting as proton shuttles or increasing the permeability of the inner

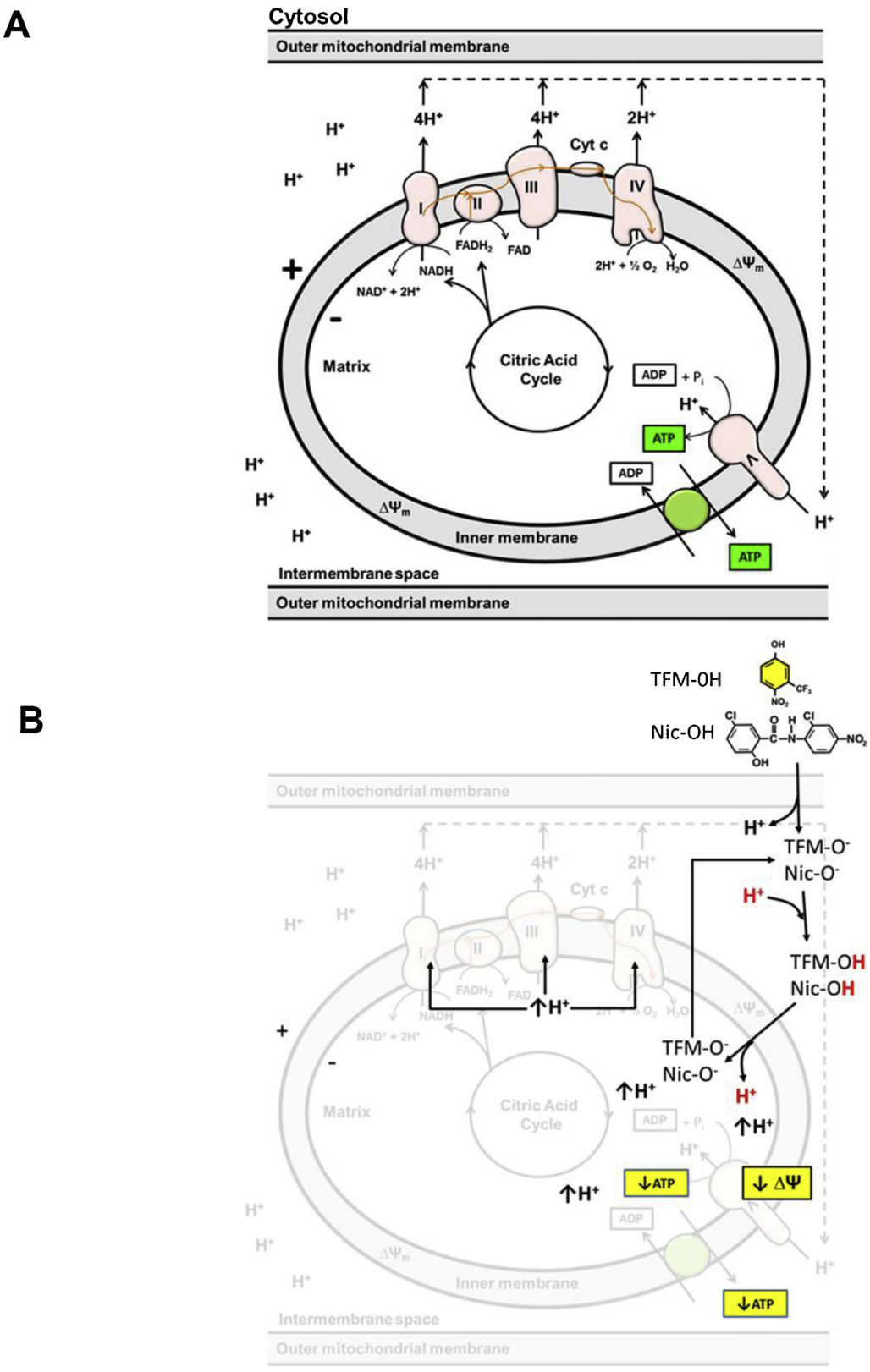


Fig. 7. Uncoupling of oxidative phosphorylation by TFM and Niclosamide.

(A) Oxidative phosphorylation takes place in the mitochondria, and is reliant on the generation of a proton (H^+) electrochemical gradient ($\Delta\Psi m = -200$ mV; matrix relative to intermembrane space) across the inner mitochondrial membrane to drive the formation of ATP. This "proton motive force" is generated by proton pumping by protein complexes I, III, and IV of the electron transport chain (ETC), which shuttle electrons from one complex to the other (orange arrows). As protons diffuse down their electrochemical through the ATP synthase (aka. Complex V), the energy released is used to phosphorylate ADP to ATP, which is then exported to the intermembrane space in exchange for ADP by the ATP/ADP antiporter. See text for further details.

(B) Both TFM and niclosamide appear to exert their toxicity by disrupting $\Delta\Psi m$, which interferes with ATP production by uncoupling oxidative phosphorylation by acting as protonophores. In both cases, TFM−OH or Nic−OH are generated in the more acidic intermembrane space, increasing their lipophilicity which allows each to diffuse down their respective diffusion gradients into the more alkaline mitochondrial matrix, where they are deproteinated, yielding the ionized TFM-O^- or Nic-O^- species. The ionized species are then shuttled back into the intermembrane space, down each' respective electrochemical gradient. As the cycle repeats, there is an accumulation of H^+ within the matrix, which collapses $\Delta\Psi m$, resulting in decreased ATP production. See text for further details.

mitochondrial membrane. This results in reduced H^+ flow through ATP-synthase and lower ATP production. While a great deal of research has focused on classic uncouplers, such as 2,4 dinitrophenol (see Skulachev, 1998; Wallace and Starkov, 2000 for reviews), surprisingly few studies have critically examined this issue with regard to TFM and niclosamide. Applegate et al. (1966) originally suspected that TFM was an uncoupler of oxidative phosphorylation due to its central phenolic ring, but other than early work on chironomid larvae that indicated greater oxygen consumption rates in the presence of TFM (Kawatski et al., 1974), there was no direct evidence in fishes or other vertebrates that TFM worked in this manner (Kawatski and McDonald, 1974). To help resolve this issue, Niblett and Ballantyne (1976) used micro-

respirometry to directly demonstrate that TFM reduced the ADP/O ratio in mitochondria isolated from rat liver. This finding confirmed that TFM increased oxidative metabolism (greater oxygen consumption) in the rat mitochondria, above that required to phosphorylate ADP to ATP.

More recently, Birceanu et al. (2011) used isolated mitochondria from adult sea lamprey and rainbow trout livers to establish that TFM uncoupled oxidative phosphorylation in fishes. As expected, they demonstrated that State III oxygen consumption was stimulated in the presence of ADP, but unaffected by the addition of TFM or 2,4-dinitrophenol to the reaction mixture. The expected decrease in oxygen consumption under State IV conditions, when all ADP had been consumed due to its phosphorylation to ATP under State III conditions, was observed in the absence of TFM or 2,4-dinitrophenol. However, State IV oxygen consumption was elevated in the presence of different concentrations of TFM or 2,4-dinitrophenol, with corresponding step-wise reductions in the respiratory control ratio (RCR = State III respiration/State IV respiration), a defining feature of uncoupled mitochondria. Using rhodamine 123 to measure mitochondrial membrane potential, the authors also demonstrated that TFM causes mitochondrial depolarization, which was indicative of a breakdown of the electrochemical H^+ gradient between the inter-membrane space and the mitochondrial matrix, the $\Delta_{\Psi m}$ (Fig. 7B). As a result, they surmised that reduced H^+ passage through the ATP synthase accounted for the decreased ATP production in the presence of TFM. The precise mechanism by which TFM depolarizes mitochondrial membrane potential has not been worked out, but as a weak acid that is lipophilic, it certainly suggests that it too acts as proton shuttle, similar to the mode of action of other uncoupling protonophores (Fig. 7B).

There is also evidence that salicylanililides such as niclosamide are uncouplers of oxidative phosphorylation in helminth parasites (Williamson and Metcalf, 1967; Weinbach and Garbus, 1969; Wilson et al., 1971; Van den Bossche, 1985), and that they act as protonophores, disrupting the proton gradient across the inner mitochondrial membrane and reducing ATP production (Wilson et al., 1971; Kaplay et al., 1972; Jurgeit et al., 2012). In their protonated (neutral) form, salicylanilides pass across the inner mitochondrial membrane into the mitochondrial matrix, where they release their ionizable H^+ before diffusing back into to the inter-membrane space, down their electrochemical gradient, where another H^+ is trapped (Terada, 1990). Evidence of such a process was recently demonstrated for niclosamide using cultured human cancer cells (HeLa cells), in which niclosamide depolarized mitochondrial membrane potential leading to apopotosis and cell autophagy (Park et al., 2011). To date, this has not been shown in aquatic organisms, but the weight of the evidence suggests that uncoupling is likely a consequence of niclosamide exposure as well (Fig. 7B).

It seems unlikely that niclosamide's effects are restricted to uncoupling oxidative phosphorylation. Recently, niclosamide has been shown to have anti-neoplastic effects that prevent cancer cell growth (Khanim et al., 2011; Park et al., 2011). Some salicylalinide drugs have also been reported to interfere with intracellular pH regulation and to inhibit glycolytic enzymes in helminths, thus impairing their ability to use glucose for anaerobic ATP production (Köhler, 2001). If this is indeed the case, then it could contribute to the greater toxicity of niclosamide compared to TFM in fishes. Indeed, the 24-h and 96-h LC_{50}'s of niclosamide are approximately 1 to 2-orders of magnitude less than TFM in various freshwater fishes including rainbow trout, white sucker, catfishes, bluegill, largemouth and smallmouth bass (Marking and Hogan, 1967; Marking and Olson, 1975). Such observations suggest that there remain unresolved fundamental differences in the pharmokinetics and/or pharmacodynamics of these two lampricides, despite some similarities in their mechanism of action.

4.2. Physiological consequences of uncoupling of oxidative-phosphorylation

The uncoupling of oxidative-phosphorylation by TFM results in a mismatch between ATP supply and demand, leading to marked reductions in the levels of high energy phosphagens (phosphocreatine), particularly in the brain and muscle of sea lampreys (Wilkie et al., 2007; Birceanu et al., 2009; Clifford et al., 2012; Henry et al., 2015) and rainbow trout (Birceanu et al., 2014). The metabolic effects of TFM on invertebrates are likely similar. Viant et al. (2001) demonstrated that in molluscs, owl limpets (*Lottia gigantean*) and red abalone (*Haliotus rufescens*), TFM exposure lead to substantial reductions (50%) in the high energy phosphagen, phosphoarginine, which, like phosphocreatine in vertebrates, buffers ATP concentrations in the body (Hochacka, 1991). Corresponding increases in inorganic phosphate, and increased arginine kinase activity, further supported the hypothesis that TFM uncoupled oxidative phosphorylation in these marine molluscs. Thus, the body of evidence now strongly suggests that high energy phosphagens could be reliable physiological markers of exposure and sensitivity of fishes and invertebrates to lampricides.

High energy phosphagens are generally short-lived sources of ATP when energy demands are increased or when ATP supply is limited. Once they are depleted, other anaerobic pathways, such as glycolysis, sustain ATP supply. Indeed, TFM exposure results in marked reductions in brain glycogen stores when larval sea lampreys (Birceanu et al., 2009; Clifford et al., 2012; Henry et al., 2015) and rainbow trout (Birceanu et al., 2014) are exposed to toxic concentrations of TFM. Because the brain depends on glucose, via glycogen, these findings strongly suggest that death is related to the depletion of glycogen reserves resulting in ATP starvation of the nervous system. However, cardiac failure cannot be completely ruled out either, because both TFM and niclosamide also accumulate in the heart (Lech and Statham, 1975; Statham and Lech, 1975).

5. Effects of lampricides on non-target organisms

5.1. Acute effects

5.1.1. TFM

In trout exposed to routine, sub-lethal concentrations of TFM, few adverse effects have been noted, other than transient increases in plasma cortisol after treatment (Birceanu and Wilkie, 2018). At higher concentrations of TFM, decreases in muscle and liver glycogen have been observed (Birceanu et al., 2014). It is unlikely that such disturbances would have any long-term impact on the animal's fitness, however, because liver glycogen fluctuates markedly in fishes with changes in food availability, food consumption and other stressors (Vijayan and Moon, 1992; Milligan, 2003; Miller et al., 2009). Muscle glycogen stores are also labile, as demonstrated by marked reductions following vigorous, exhaustive exercise (e.g. Milligan and Wood, 1986; Wang et al., 1994; Wilkie et al., 1997) and fasting (Scarabello et al., 1991). In the short-term, reductions in muscle glycogen could compromise burst or endurance swimming and negatively influence foraging or predator-evasion. However, there is a conspicuous lack of experiments investigating swim performance including burst, sustained and prolonged measures (see Farrell, 2008; Tierney, 2011 for reviews), in non-target fishes. It is likely that with feeding, glycogen stores would be restored within hours or days following TFM exposure. Brain glycogen concentrations also fluctuate markedly with fasting, followed by rapid replenishment following feeding (Soengas and Aldegunde, 2002; Polakof et al., 2007), suggesting that any neurophysiological energy deficits would be rapidly corrected in non-target fishes. It would be informative, however, to examine how altered energy charge in the brain following exposure to TFM influences behavior or sensory physiology in fishes. Sakamoto et al. (2016) noted that olfaction was impaired in lake sturgeon during exposure to TFM, but recovery following exposure was not examined. Nor were any behavioural effects,

including predator avoidance, noted in rainbow trout or fathead minnows exposed to TFM (Middaugh et al., 2014).

Damage to the gills, as suggested in earlier studies (Christie and Battle, 1963; Mallatt et al., 1985, 1994), does not appear to be a factor in TFM-induced physiological disturbances, at least in moderately hard to very hard water. Mallatt et al. (1994) suggested that if TFM reduced ATP supply, active ion transport by the gill would be impaired, leading to ionoregulatory disturbances and death in both lamprey and non-target fishes. However, exposure to TFM did not affect plasma ion balance or inhibit Na^+uptake in larval sea lamprey, although it did cause an increase in branchial Na^+/K^+-ATPase activity following a 9–12 h exposure (Birceanu et al., 2009). Similarly, disturbances to ion balance, Na^+/K^+-ATPase and V-ATPase (H^+-ATPase) activity were conspicuously absent in non-target fishes exposed to TFM, including lake sturgeon and rainbow trout (Birceanu et al., 2009, 2014; L. Sorensen and M.P. Wilkie, Unpublished findings).

5.1.2. Sensitivity of invertebrates and vertebrates to TFM

Due to the greater capacity of many fishes to detoxify TFM using glucuronidation and/or sulfation, their sensitivity to TFM is usually much less than observed in sea lampreys. There is, however, much intra- and inter-species variation. For instance, the sensitivity of lake sturgeon is life stage dependendent, with TFM sensitivity greatest when the animals are in their larval and juvenile stages, particularly when they are less then 100 mm in length (Boogaard et al., 2003; McDonald and Kolar, 2007; O'Connor et al., 2017). Of great concern is that the MLC of larval lake sturgeon overlaps with that of larval sea lamprey, which share similar habitat. The physiological basis for the greater sensitivity of larval lake sturgeon to TFM has not been determined, but it could be related to differences in their capacity to detoxify the lampricide, but they also appear to be more prone to TFM-induced mortality in waters of higher alkalinity (O'Connor et al., 2017).

Because water pH and alkalinity often vary amongst treatment sites, not to mention between laboratory studies, comparisons of the relative toxicity of TFM between species can be complicated and/or misleading. However, such complications can be minimized by expressing the sensitivity of different species to TFM (e.g. 12-h LC_{50}) relative to the MLC measured in larval sea lamprey under the same respective conditions. As the following species sensitivity distribution diagram (Fig. 8) illustrates, the most tolerant of teleosts to TFM is the American eel (*Anguilla rostrata*) followed by the centrarchid fishes [bluegill, smallmouth bass (*Micropterus dolomieu*)], the percid fishes [yellow perch (*Perca flavescens*)], salmonids, northern pike (*Esox lucius*) and musky (*Esox mascquinongy*), common carp (*Cyprinus carpio*), with the catfishes (Ictaluridae) and sturgeon amongst the most sensitive. Because tolerance to TFM varies amongst the most and least sensitive non-target fishes by almost an order of magnitude, protecting non-target species from TFM can be challenging. However, a better understanding of the environmental, developmental and physiological factors that contribute to the sensitivity and/or tolerance of non-target fishes to TFM could facilitate better management of this issue.

The sensitivity of amphibians to TFM also varies with life stage. Bullfrog (*Rana catesbeiana)* tadpoles are approximately ten-fold more sensitive to TFM than adults, following aqueous exposure to the lampricide (Kane et al., 1993). However, differences in sensitivity to TFM are likely due to greater rates of uptake in tadpoles, which require external gills to breathe, as their detoxification capacity appears to be similar to that of the adults (Kane et al., 1993). The sensitivity of mudpuppies (*Necturus maculosus*) to TFM and TFM/niclosamide mixtures has been of particular concern in recent years in the Great Lakes (Boogaard et al., 2003). The use of external gills likely provides a route for lampricide uptake by muduppies, but the well-vascularized, scaleless skin cannot be ruled out in these or other amphibians. Studies on mudpuppy vulnerability to TFM are restricted to toxicity studies, and suggest that the no observed effect concentrations (NOECs) are about 1.1–1.6 time higher than the MLC of sea lampreys tested under identical conditions (Boogaard et al., 2003), which may overlap with the concentrations used in the field, (1.2–1.5 times the MLC) (McDonald and Kolar, 2007). Unfortunately, little else is known about the uptake, distribution and handling of such compounds by these organisms.

Recently, considerable effort has been dedicated to evaluating the sensitivity of non-target invertebrates to lampricides, mostly among a number of listed unionid mussel species. Studies have evaluated the acute toxicity of TFM and the TFM/1% niclosamide mixture to a range of unionid mussel species and their sensitive life stages, including the glochidia and juvenile life stages of the federally endangered (U.S.A.) snuffbox (*Epioblasma triquetra*) and the glochidia, juvenile and adult life stages of the ellipse (*Venustaconcha ellipsiformis*) and sandshell (*Ligumia recta*) juveniles (Boogaard et al., 2015). Many of these studies were specifically requested by federal, provincial, state, or non-governmental agencies that had concerns regarding lampricide effects on individual species of interest. These studies have indicated that the sensitivity of most of these molluscs to TFM is well in excess of the MLC to larval sea lamprey. Lampricide levels up to 2.2 times those encountered during sea lamprey control operations had little effect on any of the unionid lifestages examined (Boogaard et al., 2015). However, tests conducted

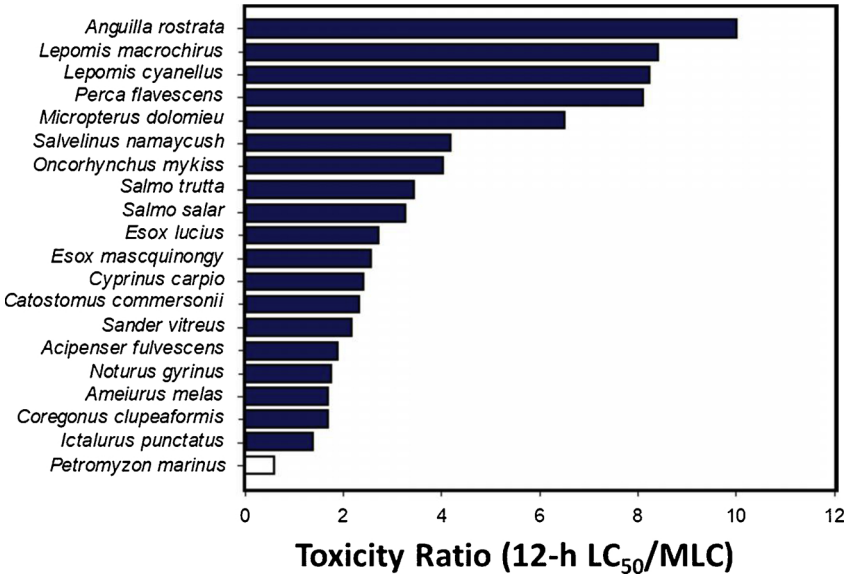


Fig. 8. Species sensitivity distribution (SSD) of TFM. Relative differences in the toxicity of 3-trifluoromethyl-4-nitriphenol (TFM) to selected fishes and larval sea lamprey during 12 h exposures to the lampricide. Sensitivity reported as a toxicity ratio defined as the calculated TFM concentration producing 50% mortality (12-h LC_{50}) in each respective non-target fish species, divided by the predicted sea lamprey minimum lethal concentration (MLC = 12-h $LC_{99.9}$) based on the pH and total alkalinity (mg L^{-1} as $CaCO_3$) of the test water as determined by Bills et al. (2003). Data compiled by M. Boogaard et al. (Upper Midwest Environmental Sciences Center, US Geological Survey, La Crosse, WI). See text for further details.

on adult logperch (*Percina caprodes*), the primary host fish for the snuffbox, resulted in substantial mortality at TFM concentrations typically applied to streams, and loss of host fish could adversely affect snuffbox reproduction.

5.1.3. Niclosamide

Niclosamide is much more toxic to non-target mussels (Boogaard et al., 2003; Newton et al., 2017) and fishes than TFM (Marking and Hogan, 1967), which is why it is usually used as an adjuvant with TFM, comprising only 1–2% of the mixture (Dawson, 2003; McDonald and Kolar, 2007). On its own, niclosamide shows little specificity for either gastropod snails (Andrews et al., 1982) or sea lamprey (Marking and Hogan, 1967). For instance, the niclosamide 24-h LC_{50}'s of several teleosts overlapped, and in some cases were less than, values reported for more than 20 species of snails (Andrews et al., 1982). A comprehensive analysis of niclosamide (2-amino ethanol salt of niclosamide) by Marking and Hogan (1967) on 18 species of freshwater fishes revealed that the LC_{50} for each species showed little variation in exposures lasting from 3 to 96 h, suggesting that it is highly potent and fast acting. The most sensitive species were Ictalurids **[brown** bullhead (*Ameiurus nebulosus*) and flathead catfish (*Pylodictis olivaris*)], and salmonids [rainbow trout, brook char (*Salvelinus fontinalis*)], while smallmouth bass (*Micropterus dolomieu*) and largemouth bass (*Micropterus salmoides*), sunfishes (*Lepomis* sp.), **yellow** perch (*Perca flavescens*), fathead minnow (*Pimephales promelas*), and **white** sucker (*Catostomus commersonii*) showed intermediate sensitivity. The most tolerant were common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*), possibly due to their high tolerance to low oxygen conditions. Indeed, the goldfish can survive anoxia for many days or weeks due to their ability sustain ATP production using anaerobic glycolysis (Shoubridge and Hochachka, 1980; Nilsson, 2001).

Because of such high variability in niclosamide tolerance among fish species, non-target effects may be an ongoing challenge for sea lamprey control. In the Great Lakes, the granular, rapidly sinking form of niclosamide is used for the control of lentic populations of larval sea lamprey, in population surveys and in very large, fast-flowing bodies of water such as the St. Marys River (Jones et al., 2015). The use of niclosamide, in combination with TFM, is often preferred under such conditions, because it significantly reduces the amount of TFM used, lowering the cost of the treatment and the amount of pesticide released in the environment (Gutreuter and Boogaard, 2007). As the use of niclosamide has risen in the past 15 years (S. Robertson, Fisheries and Oceans Canada, pers. comm.) it may be wise to develop a better understanding of its non-target effects, alone and in combination with TFM.

5.2. Chronic toxicity

The possibility that TFM negatively impacts vertebrates and invertebrates at the population level remains largely unexplored. Recently, Middaugh et al. (2014) reported that rainbow trout and lake sturgeon fingerlings experienced no adverse effects on growth in a two week period following a 12 h exposure to environmentally relevant concentrations of TFM in the lab. They further noted that predator avoidance of fathead minnows (to largemouth bass) was unaffected by exposure to the lampricide, implying that a typical treatment does not affect growth or susceptibility to predation.

There is some evidence that TFM has the potential to act as an endocrine disrupting compound, but there is currently no evidence to indicate that TFM negatively impacts the reproductive success of non-target fishes. Hewitt et al. (1998a,b) noted that TFM acted as an estrogen agonist in male rainbow trout hepatocytes and that it induced vitellogenin synthesis, a precursor of egg yolk protein and a well-established indicator of endocrine disruption, at concentrations of 10 mg TFM /L, which were not environmentally relevant. However, no significant changes in vitellogenin were observed in caged rainbow trout exposed to TFM for 18 h and 36 h following field TFM applications (Hewitt et al., 1998b).

Thus far with the studies and data available, TFM does not appear to have any ecologically relevant long-term effects. Wild fish are only episodically exposed to TFM for relatively brief periods (12 h) because it is not usually applied annually to most sites (McDonald and Kolar, 2007), nor is it persistent due to its relatively rapid biological degradation in natural waters (see above; Hubert, 2003; McConville et al., 2016) and its rapid dilution as it moves downstream and enters the lakes. With the advent of next generation sequencing approaches, and more sophisticated means to assess fish populations, it should be possible to predict with greater confidence whether or not TFM is adversely affecting non-target vertebrate and invertebrate populations. Because TFM and niclosamide are likely to remain key components of the sea lamprey control program in the Great Lakes, such knowledge would also provide sea lamprey control agents and regulators with the ability to better identify, predict and mitigate any potential adverse effects. Research on the sub-lethal effects of lampricides, particularly those effects that may adversely impact non-target populations, will be key to ensuring that lampricides remain part of the integrated pest management of sea lamprey in the Great Lakes for the foreseeable future.

6. The future of sea lamprey control in the Great Lakes

6.1. Green lampricides

Efforts to identify more species-specific lampricides with fewer adverse effects on non-target fishes and the environment are a desirable and necessary measure for 21st century invasive species control, particularly since there is a concern about the release of organic pesticides in the environment. The concept of green chemistry, aimed at producing chemical compounds with fewer adverse environmental effects, has been discussed for many years. Anastas and Williamson (1998) defined green chemistry as "the utilization of a set of principles that eliminates or reduces the use or generation of hazardous substances in the design, manufacture, and application of chemical products. The green effort starts with target molecule design and impacts all aspects of manufacture, production and use."

TFM and niclosamide were identified in the 1950s when there was an explosion in the synthesis of organic molecules to combat agricultural pests (Norris et al., 2003). The compounds came from a generation of chemicals that included substances that were responsible for serious negative environmental and human health impacts. Both TFM and niclosamide are fish toxicants and will cause fish mortality if used at concentrations outside of label guidelines. In addition to the lampricides, the only other piscicide currenly registered with the USEPA and Health Canada is rotenone, but there are efforts underway to re-register antimycin for use by state and federal fish management agencies (S. Lantz, US Geological Survey, personal communication). These two chemicals are mostly used in fishery reclamation projects where complete kill is necessary, followed by restocking of the desired species. Since these four chemicals were developed, there has been limited research conducted on identifying new fish toxicants. Over time, there have been numerous organic chemicals synthesized or isolated from natural sources. Given the increased scrutiny of lampricides by various public and environmental groups, it is prudent to search for "next generation" chemical alternatives to current lampricides. With the emphasis that has been placed on developing pesticides that are more environmentally benign, it is likely that "greener" lampricides can be found. Readers are referred to a recent review by Lantz et al. (2018) for further information on the strategies and research avenues that could be explored to identify and develop "next generation" approaches to control invasive species in the Great Lakes.

6.2. Genomic approaches

A detailed discussion of how genomic approaches will contribute to new methods of sea lamprey control is beyond the scope of this paper, but we would be remiss by not briefly discussing future applications of this promising, but potentially controversial, technology. The recently sequenced sea lamprey genome (Smith et al., 2013, 2018) and ongoing Japanese (Arctic) lamprey (*Lethenteron japonicum*) genome project (http://jlampreygenome.imcb.a-star.edu.sg/) could provide potentially fruitful avenues of inquiry that include mapping and identifying the gene(s) that control sea lamprey metamorphosis, reproduction, digestion or gill function. A key advantage of such approaches includes the potential to identify species-specific methods of sea lamprey control by targeting nucleotide or peptide sequences unique to lampreys, that will minimize the possibility of non-target effects.

RNA interference (RNAi; Fire et al., 1998), a gene silencing method, may hold the most promise as a "green lampricide". Fire et al. (1998) used the nematode *Caenorhabditis elegans* to first describe how gene silencing through RNAi works (also see Huvenne and Smagghe, 2010; Kim et al., 2015 for reviews). Briefly, short interfering RNA duplexes (siRNA) that are homologous to the mRNA being targeted are generated and incorporated into the silencing complex of the gene, thus preventing translation (Huvenne and Smagghe, 2010). The interfering RNA can be injected directly into cells or it can be introduced exogenously, through feeding or through gill uptake from the surrounding water. In nature, RNAi plays a key role in innate immunity by defending virtually all plants and animals against parasitic nucleotides arising from viral or bacterial infections, and it also has a role in regulating gene expression during development. Recently, Heath et al. (2014) explored the feasibility of using RNAi in sea lamprey control by first injecting embryonic sea lamprey with siRNA targetting 3 common proteins in vertebrates (elongation factor 1-α, calmodulin and α-actinin) which resulted in substantial reductions in their transcript levels. Most promisingly, they found that feeding the ammocoetes with the siRNA complexed to liposomes resulted in marked reductions in transcript of the target proteins, suggesting that siRNAs could be added to streams containing larval sea lamprey on a large scale. The small size of siRNAs (˜20 nucleotides) suggest that it may be possible to target gene sequences in sea lamprey in a highly specific manner to eliminate the risk of impacting non-target fishes and invertebrates. Unlike other molecular approaches such as "daughterless" technology (see Thresher et al., 2014, 2018; Teem and Gutierrez, 2014 for reviews), RNAi also has the advantage that its effects can be restricted to the Great Lakes, because the effects are non-hereditary.

A second emerging technique in pest control is the use of site-specific endonucelases that cleave targeted DNA sequences, disrupting the expression of specific genes of interest. Recent advances in the CRISPR-Cas9 [clustered, regularly interspaced short palindromic repeats (CRISPR); CRISPR associated system (Cas)] has simplified the process of creating such endonucleases. Studies on mosquito population control using CRISPR-Cas9 to target genes involved in female fertility have proven effective in reducing offspring numbers (Hammond et al., 2016). In the same study, the authors also created a CRISPR gene drive system for a recessive mutation in a gene essential for fertility, thus ensuring that the particular mutation would become established in the population. This increased the reproductive load on the mosquitos and led to reduced numbers. Similar approaches using the CRISPR system to disrupt gametogenesis or sexual maturation could be investigated in sea lamprey. In addition, a gene drive system targeting fertility, similar to that proposed by Hammond et al. (2016), could be developed in sea lamprey to alter sex ratios in the Great Lakes, thereby impairing recrutiment. While more research needs to be conducted on genome manipulation to silence genes or introduce mutations in lamprey, there is the potential that this technology could to be used as part of the integrated pest management program to control sea lamprey populations.

While promising, careful assesments of the potential effects of genetic approaches for sea lamprey control on non-target organisms will take time and money. Moreover, technical challenges will need to be overcome including the need to develop a sufficient and robust delivery vehicle(s) to facilitate the large scale administration of genetic materials such as siRNA to targeted lamprey populations before degrading in the microbial-rich waters in which the lamprey live. The identification of an appropriate delivery vehicle to ensure that the such material is readily taken-up across the gills or gut will be difficult, not to mention the development of processes to manufacture sufficient amounts of the genetic material for use on the large scale required for effective sea lamprey control. The concerns of the public, policy and decision makers and scientists about the deliberate introduction of genetic material into aquatic ecosystems also needs to be carefully considered, particularly in a climate where acceptance of such measures could be difficult to attain (Thresher et al. In Press). Such challenges may not be insurmountable, but we are likely years away from incorporating such measures into the sea lamprey control program. As a consequence, chemical control measures using TFM and niclosamide are likely to remain in place for the foreseeable future.

7. Conclusions

Invasive species, such as the sea lamprey, have the potential to cost the Great Lakes' economy billions of dollars per year (GLFC, 2011). In the Great Lakes, the value of the fishery has been estimated at more than 7 billion dollars (U.S.) (Krantzberg and De Boer, 2008; GLFC, 2011). Through an integrative pest management program, that relies heavily on chemical control, sea lamprey populations have been greatly suppressed, contributing to the rehabilitation of recreational, commercial and culturally important fisheries in the Great Lakes. For this reason, it is imperative to continue research aimed at better understanding the mechanism of action of TFM and niclosamide in sea lampreys and non-target organisms, alongside attempts to improve treatment effectiveness and to limit non-target adverse effects. The development of alternate control strategies, including pheromones, genetic-based technologies and next generation lampricides, should also be pursued further. Indeed, the lessons learned from such research efforts could be applied to control other invasive species (vertebrate, invertebrates, plants, micoorganisms), that are exerting pressure on numerous aquatic ecosystems worldwide.

When used appropriately, the adverse effects of TFM and niclosamide on aquatic ecosystems are minimal. However, instances of non-target mortality do occur, which are often highly publicized. Such incidents, along with greater antipathy towards the chemical control of pests in general, have led to increasing public and regulatory concern about the effects of lampricides on aquatic biota. It is therefore imperative to develop further knowledge of the biology and sensitivity of non-target species to lampricides, with a focus on species, differences in rates of uptake, detoxification, distribution and elimination. Such knowledge is needed to provide sea lamprey control agents, fisheries managers, decision makers, and regulators with the ability to better identify, predict and mitigate any potential adverse effects arising from lampricide applications.

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