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Review

At the intersection between toxicology and physiology: What we have learned about sea lampreys and bony fish physiology from studying the mode of action of lampricides ☆

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ABSTRACT

The lampricides 3-trifluoromethyl-4-nitrophemol (TFM) and niclosamide have been used for over 60 years to control the invasive sea lamprey (*Petromyzon marinus*) population in the Laurentian Great Lakes. In this review, we summarize these findings in the context of the mode of action of both lampricides, with a focus on: (1) the physiology of uptake, bodily distribution and mode of action, detoxification, and excretion of lampricides in lamprey and non-target fishes, (2) the development of an Adverse Outcome Pathway for TFM and niclosamide, and (3) the identification of novel avenues for future research that can be further explored to ensure continuous suppression of the sea lamprey population in the Great Lakes. We explored how research on the mode of action of lampricides has provided novel insights into the gill microenvironment and how this impacts lampricide toxicity; described new information on mitochondria and tissue physiology; and discussed how the activity of enzymes that are involved in detoxification pathways impacts the response of fishes to xenobiotics. Considering the information that has been generated over the years on sea lamprey and bony fish physiology from studying the mode of action of lampricides, here we propose an Adverse Outcome Pathway for TFM and niclosamide and identify novel avenues for research on the short and long-term effects of lampricide applications, either alone or in combination. Lastly, we discuss how the differences in physiology between sea lampreys and non-target fishes can be further exploited to ensure continuous suppression of the sea lamprey population in the Great Lakes.

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General overview

Sea lamprey (*Petromyzon marinus*) populations in the Great Lakes have been extensively controlled over the last 60 + years, to minimize their impact on the multibillion-dollar fishery. The most effective method of control has been the application of the piscicides 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide (Bayluscide®), known as lampricides (Fig. 1), which are applied to tributaries of the Great Lakes that contain larval sea lampreys, in both the US and Canada (Applegate et al., 1961; Lawrie, 1970; McDonald and Kolar, 2007). TFM was selected among 5000 + chemicals that were tested, because it is selectively toxic to the larval sea lampreys (Applegate et al., 1957). Niclosamide, a pesticide widely used as a molluscicide, was not incorporated into the sea lamprey control program until much later, due to a need to reduce the amount of TFM used in larger, faster flowing waters. While not as selective as TFM, applications of TFM/niclosamide mixtures reduce the amount of TFM needed, leading to overall lower treatment costs. In addition, because of the tendency of niclosamide to sink, the specificity of the treatment is maintained, with minimal non-target effects (Dawson, 2003). Lampricides are applied to streams and rivers sustaining larval sea lampreys on a 3–4 year cycle. TFM is applied at a concentration needed to kill 99.9% of the larval sea lampreys over 9 h, known as the minimal lethal concentration (MLC), which is dependent on water pH and alkalinity (Bills et al., 2003; for review on factors influencing TFM toxicity, see Wilkie et al., 2019).

While extensive research has been conducted to understand the mode of action of lampricides, most studies have been focused on TFM, because it is the most widely used of the two. The work has focused on both the target organism, the sea lamprey, and non-target fishes, to be able to better predict the short and long-term effects in fishes. We acknowledge that there is an extensive body of work done on the effects of lampricides in plants and invertebrates, but the overarching goal of this review is to provide a synopsis of how the studies on the

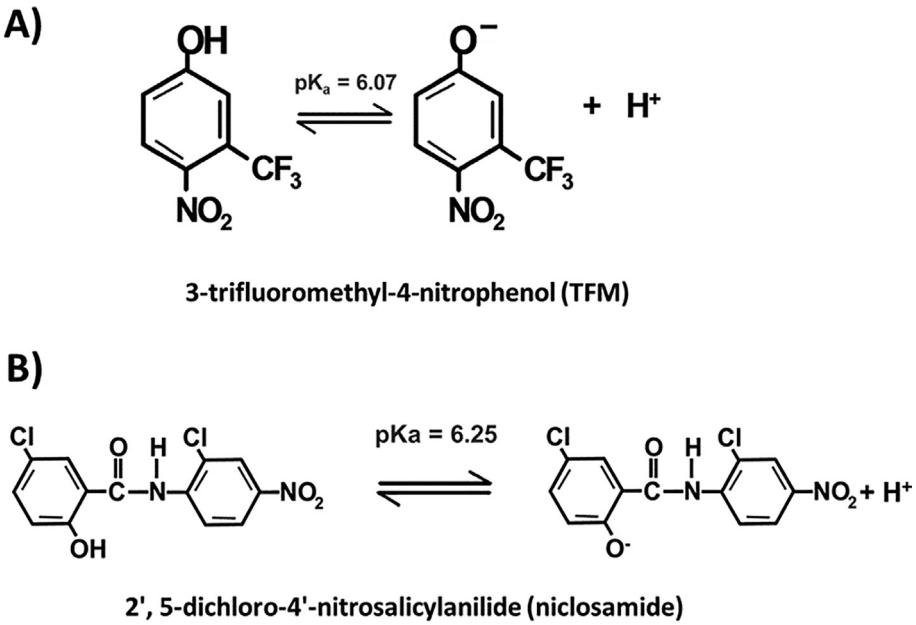

Fig. 1. Molecular structure of lampricides. The molecular structure of (A) TFM and (B) niclosamide, along with their dissociation constants.

mode of action of lampricides have led to novel understandings of the physiology of sea lampreys and bony fishes. To this end, we will explore the intersection of lampricide toxicity and fish physiology by (1) describing the physiology of uptake, bodily distribution and mode of action, detoxification, and excretion of lampricides in lampreys and non-target fishes, (2) proposing an Adverse Outcome Pathway for TFM and niclosamide, and (3) identifying novel avenues for research that can be explored to ensure continuous suppression of sea lampreys in the Great Lakes.

Studying lampricides offers insights into bony fish physiology

When determining the mode of action of any compound it is worth reflecting on the phrase "the dose makes the poison". The dose that an organism is exposed to following a lampricide treatment adheres to the pharmacological principles of ADME: absorption/uptake, distribution, metabolism (or detoxification) and excretion. In this section, we will present the information through the lens of the ADME principle, but will add another concept to the acronym, where we will be looking at the mode of action of the lampricides, in addition to the pharmacological principles of ADME.

In the case of lampricides, these are initially concentrated formulas that are applied to water bodies at concentrations between 1.0 and 2.5x the LC99.9 of the larval sea lampreys, depending on the chemistry of the water that they are applied to. Lampricides are taken up at the gill of both target and non-target fishes. Rates of lampricide uptake, and therefore the level of exposure, are mediated by the exposure time, the surface area of the organism, and whether the organism is passively or actively taking up the lampricide. Once inside the animal's body, lampricides will likely distribute to different tissues and organs based on the characteristics of the animal's digestive and/or circulatory systems and the chemical characteristics of the lampricides themselves (transport and tissue distribution; Fig. 2). The concentration of lampricide in each body compartment and/or tissue will not only depend on the deposition of the chemical but will also be influenced by the ability of the organism to detoxify and remove the chemical, which we address in the sections below. With regards to the dose of TFM that exerts negative effects on fish biology, it is likely that some of this will come down to the ability of the fish to detoxify the compound via detoxification processes and eliminate it effectively out of the body (Fig. 2).

The A in ADME – Routes and factors that impact lampricide uptake

The gill as the route of uptake for lampricides

The gill is the major site of uptake of chemicals from water by aquatic animals (Hayton and Barron, 1990). Lampricides are absorbed by passive diffusion at the gills, due to a concentration gradient between the water and the body. This absorption rate across the gills is heavily influenced by water pH, not only in sea lampreys but also in non-target species (Hepditch et al., 2019; Hunn and Allen, 1974; Marking and Olson, 1975). Bioavailability is higher when lampricides are in the unionized form (pKa for TFM is 6.07, while that for niclosamide is 6.25), as they are more lipophilic and able to cross the cell membranes more easily than the ionized form (Hughes, 1980). Hlina et al. (2017) showed that TFM uptake rates were approximately 5.5-fold greater at low water pH in larval sea lampreys, most likely due to the unionized, lipophilic form of TFM existing in greater amounts at a lower pH. This likely explains why TFM becomes less toxic to larval sea lampreys as water pH is increased (McDonald and Kolar, 2007; Hlina et al., 2017). Similar effect of pH on TFM uptake was recently observed in lake sturgeon, which had significantly greater TFM uptake rates in acidic (pH = 6.5) compared to more alkaline (pH = 9.0) waters (Hepditch et al., 2019). Uptake of TFM is also greatly influenced by pH in other non-target species, such as rainbow trout and channel catfish (Hubert, 2003; Sills and Allen, 1975), where higher uptake rates are consistently reported in more acidic waters. Uptake of niclosamide in fishes has been less studied, although with a pKa of 6.25 (Fig. 1), similar to that of TFM (pKa = 6.07), niclosamide is expected to be taken up in a similar way by the fish gill.

The studies on the effects of biotic and abiotic factors on the rates of uptake of TFM and niclosamide, have provided novel insights into the morphology and physiology of the fish gill (Fig. 3). Even though the lampricides are taken up at higher rates in acidic waters, increasing their toxicity, research shows that lampricides are also taken up in more alkaline waters, albeit at lower rates (Hlina et al., 2017; Wilkie et al., 2019). Indeed, while TFM uptake rates in lake sturgeon juveniles and larval sea lampreys

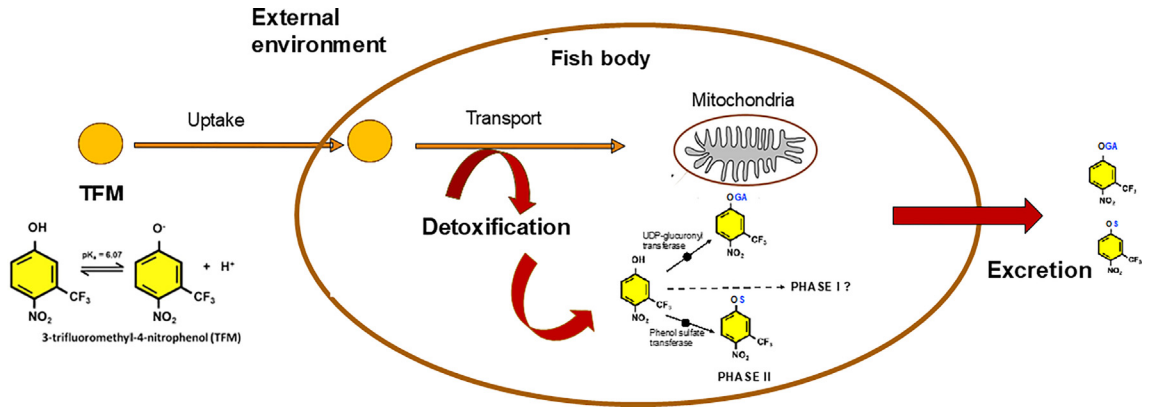

Fig. 2. Interaction of lampricides with the fish body. A broad overview of the interaction of lampricides with the fish body. Lampricides are taken up at the gills and transported in the fish body, where they target the mitochondria, leading to uncoupling of oxidative phosphorylation and reducing the ability of the mitochondria to produce ATP. This creates a mismatch between ATP supply and demand, forcing the fish to rely on their anaerobic energy reserves (such as glycogen and phosphocreatine) for survival. Some fishes can detoxify the lampricides (non-target fishes), through Phase I and Phase II detoxification processes, while others, like the sea lamprey, have a limited detoxification capacity. Our current understanding of lampricide elimination is that it is eliminated either through urine (non-target fishes) or at the gills (in larval sea lampreys). The figure also denotes that Phase I detoxification pathways for TFM still need to be elucidated (see question mark).

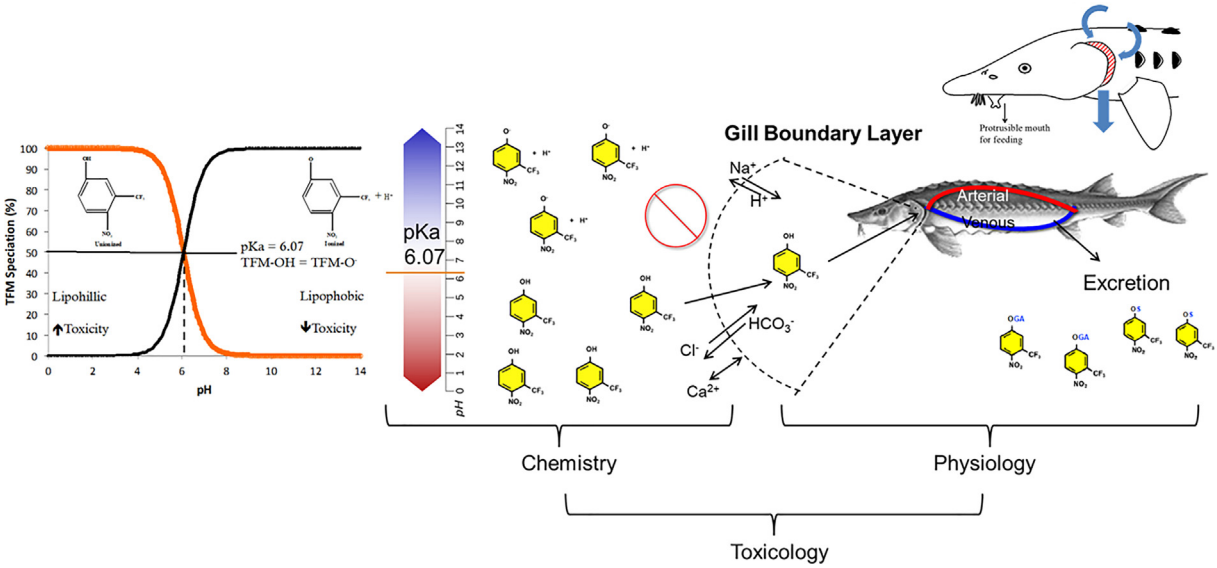

Fig. 3. The relationship between TFM chemistry in the water and fish gill physiology. TFM speciation is highly dependent on water pH and the physiology of the animal determines the acidity of the boundary layer (i.e., the layer of water that surrounds the gills filaments). Toxicity of TFM here is defined by the interaction of the two (see Fig. 4 for more details). The dotted line denotes the gill boundary layer, while the black arrows denote the movement of ions and unionized TFM in and out of the boundary layer. In high pH environments (pH > 6.07), the pH of the water and that of the gill boundary layer are higher, with fewer H^+ ions present; in addition, less unionized TFM is present, its bioavailability is lower and less is taken up at the gill (denoted by the red lined circle). In low pH environments (pH < 6.07), more unionized TFM is present in the water and the pH of the boundary layer is more acidic than that of the water. Therefore, more unionized TFM is present, its bioavailability is higher, and more is taken up at the gill. Figure . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) adapted from Paquin et al. (2002)

were approximately 6- to 10-fold lower in pH 9.0 water compared to the uptake rates in water of pH 6.5, animals in alkaline waters still took up TFM, when most of the compound was ionized, suggesting that active transport of the chemical, through transporters, may be a possibility (Hepditch et al., 2019). Therefore, the ionized TFM, and perhaps niclosamide, must be able to enter the gill cell through previously unexplored mechanisms, such as passive diffusion or organic anion transporters (Bills et al., 2003; Erickson et al., 2006; Hlina et al., 2017; see Wilkie et al. 2019 for review). Indeed, the water partition coefficient, log K_{ow}, for ionized (i.e., lipophobic or less toxic) nitrophenols is close to that of their unionized (i.e., lipophilic or more toxic) counterparts, suggesting that passive diffusion of the ionized form of the lampricides should not be ruled out (Erickson et al., 2006). Candidate proteins for active transport of lampricides could be Mrp2 (multi-drug resistance-associated protein 2) and OATPs (organic anion transporting polypeptides), which play significant roles in the uptake and detoxification of organic compounds. More importantly, Mrp2 has been found in the gills and liver of larval and adult sea lampreys (Cai et al., 2013), suggesting that it would be a good candidate protein to be explored as a gill transporter for the uptake of lampricides.

OATPs, which are part of the SLC21/SLCO solute carrier gene family, have been found in a variety of animals, including teleosts, and are involved in the uptake/absorption of a variety of anionic drugs (Verri et al., 2012; Kovacsics et al., 2017). In teleosts, there are currently 50 families and 338 members of the SLC gene series, performing major functions such as transport of ionorganic cations/anions, amino acids, oligopeptides, sugars and metal ions, performing mitochondrial cross-membrane transport, along with biotransformation and excretion functions, and many others (Hagenbuch and Stieger, 2013). Such proteins should be further explored as candidate transporters of TFM and niclosamide in the gills to provide a further understanding on how they interact with the fish body. There should be additional interest in pursuing studies on transporters for the lampricides, as they could be targets for exploring the potential development of resistance in sea lampreys.

The influence of the gill microenvironment on lampricide uptake and toxicity

In addition to water chemistry, the chemistry of the gill microenvironment may heavily influence the speciation of the lampricides, and, therefore, their rates of uptake and overall toxicity (Fig. 3). Recent work on the mode of action of lampricides has brought forth the importance of the gill boundary layer in TFM speciation (for a thorough review, see Wilkie et al., 2019). In addition, the gill microenvironment may also play a role in the relatively high TFM uptake rates in alkaline waters, as noted above in lake sturgeon and sea lampreys (Hlina et al., 2017; Hepditch et al., 2019). Below, we describe the chemistry of the gill boundary layer in detail and provide a theoretical model on how the speciation of TFM is affected by the changes in pH at the gill.

The chemistry of the gill boundary layer in fishes

Fishes take up oxygen by washing water over their gills using two methods: unidirectional and tidal ventilation. Unidirectional ventilation involves the use of buccal and opercular pumps, to move water over the gills in one direction: in from the mouth and out through the operculum. In this way, oxygenated water comes into contact with the gill filaments and the lamellae only once (McKim and Erickson, 1991; Evans et al., 2005). As water passes over the lamellae, oxygen is taken up by the blood in a counter-current exchange system. A second type of gill ventilation employed by some bony fishes and the adult life stage of the sea lampreys is known as tidal ventilation, which involves only the opercular pump or pumping muscles. Oxygenated water comes in through the gill pockets (in adult sea lampreys) or the opercular slits (in lake sturgeon), washes over the gills as it goes in, then it is expelled back through the same opercular slits. In this type of ventilation, the water washes over the gills twice: once when it goes in and again as it is expelled (Hunn and Allen, 1974; McKim and Erickson, 1991; Evans et al., 2005).

The amount of chemical that will be taken up by a fish is dependent on several factors, including: ventilation and respiration rate, contact time with the gill lamella, the ability to permeate biological membranes (which may change based on pH and speciation), and diffusion capacity into the blood (Larisch and Goss 2018). As oxygen diffuses into the blood, metabolic waste such as CO_2 is excreted with the aid of the catalyst carbonic anhydrase (CA). This process is used for acid-base regulation by the fish, which regulates H^+ and HCO_3^- at the gills via the reaction (Fig. 4):

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^- \quad (1)$$

This reaction can lead to changes in the pH of the gill microenvironment as HCO_3^- and H^+ are liberated during the hydration of CO_2 (Lloyd and Herbert, 1960; Szumski et al., 1982; Fig. 4 A, B). Hyperventilation may result in more CO_2 being excreted over the gills, whereas hypoventilation can allow for CO_2 to accumulate in the gill microenvironment. In soft water, the gill has an increased buffering capacity, and under acidic conditions water that moved across the gill becomes more basic, whereas under basic conditions the expired water is rendered more acidic in comparison to the bulk water (Playle and Wood, 1989; Wilkie et al, 2021; Fig. 4C). The discrepancies between the chemistry of the bulk water and the water that is directly at the gills of fish provides valuable information to sea lamprey control officials, aiding them to better predict lampricide toxicity to both sea lampreys and non-target fishes.

TFM toxicity is changed in the gill boundary layer

TFM is an ionizable chemical, with a pKa of 6.07 (Fig. 3). TFM is weakly acidic and will be present in greater proportions in its lipophilic unionized form in waters with lower pHs (Bills et al. 2003; McDonald and Kolar 2007). It is considered moderately lipophilic, with an octanol–water partition coefficient (log K_{ow}) of 2.77 (McKim and Erickson, 1991). The octanol–water partition coefficient is useful in predicting how a xenobiotic will interact with the gill boundary layer: the lipid membrane will allow lipophilic xenobiotics (log K_{ow} 1 – 6) to passively be taken up at the gills but may act as a barrier to those that are more lipophobic (log K_{ow} < 1) (Saarikoski et al., 1986; see Fig. 4). A thorough description of ionizable organic chemical uptake at the gills can be found in Armitage et al. (2017). Due to the chemical properties of TFM, a variety of factors, such as water pH and alkalinity, can influence its uptake rates and toxicity (Bills et al. 2003; McDonald and Kolar 2007; Hlina et al., 2017; Hepditch et al., 2019).

The mucus layer at the gill may play a role in the level of acidification of the water at the boundary layer (Saarikoski et al. 1986; Fig. 4), which, in turn, may impact the speciation of lampricides, as lower pHs favour their unionized, more toxic form. Erickson et al. (2006) describes one full pH unit difference across the gill lamella, with the expired water being rendered more acidic. This pH difference between inspired (more basic) and expired (more acidic)

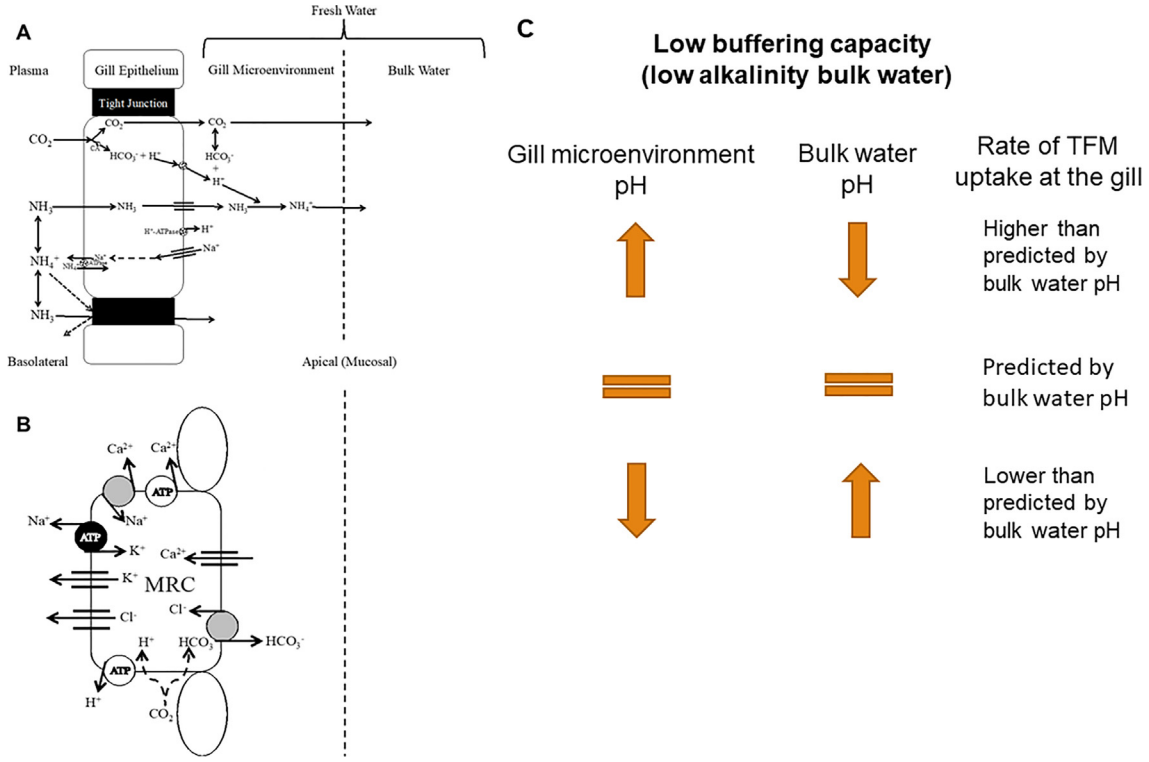

Fig. 4. The importance of the gill boundary layer on lampricide toxicity in bony fishes. A) As carbon dioxide (CO_2) diffuses from the blood, it becomes hydrated at the gill epithelium to form HCO_3^- and H^+. As this occurs, H^+ increases in the gill boundary layer, leading to a decrease in pH with increases in CO_2 excretion. The difference in pH between the microenvironment and the bulk water facilitates ammonia diffusion from the blood and into the water, where it becomes protonated (NH_4^+) and cannot cross back into the gill due to its positive charge. Figure adapted from Wilkie (2002) and Edwards and Marshall (2012). B) A model of a mitochondrial rich cell (MRC) for osmoregulation in freshwater fishes. A proton pump (V-type ATPase) drives Cl^- uptake via the Cl^-/HCO_3^- exchanger. Ca^{2+} is taken up both by a Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase. From Edwards and Marshall (2012). The two figures describe the chemistry of the boundary layer, which can have impacts on TFM toxicity (C), particularly in waters of lower buffering capacity, such as those of lower alkalinity. In such an environment, if the pH of the bulk water is acidic, the gill boundary layer will buffer the water at the gills, increasing its pH. A higher pH at the gill means that less TFM will be available for uptake than predicted by the bulk water pH (see panel C). In contrast, if the bulk water pH is high, the gill boundary layer will be acidified to maintain ion balance and ion homeostasis in the fish. A lower pH at the gill would increase the rate of TFM uptake, making it more toxic than it would have been predicted by the bulk water pH alone.

water is due to the differences between bulk water pH and pH of the blood (Wright et al. 1986). This can change the speciation of TFM at the gill boundary layer, as proposed by Wilkie et al. (2019), making predictions of TFM toxicity solely based on bulk water pH less accurate. However, there are inter-species differences in the amount of acidification and buffering that occurs at the gill. For example, CO_2 diffusion in teleost fish is limited to the entry of HCO_3^- into red blood cells; whereas, in cartilaginous fish the whole surface area of the gill can be used for CO_2 diffusion and thus it is limited by perfusion (Evans et al., 2005). These differences in CO_2 excretion of teleost vs cartilaginous fishes may indeed change the TFM speciation and uptake at the gills of non-target fishes.

In addition to tidal vs. unidirectional ventilation, fishes like the lake sturgeon are unique in that they can implement a quasi-directional ventilation strategy when feeding (Fig. 3). As the protrusible mouth feeds along the bottom of the lake/river, the sturgeon can use pressure differentials to draw water across the top of the gill filaments and expel it via the gill sieve (Burggren 1978). This unique ventilation strategy may result in changes to the chemistry of the gill microenvironment, depending on how efficient it is for removing metabolic waste (see Wilkie et al., 2019). This approach may increase the acidity of the gill boundary layer and, therefore, lead to higher lampricide uptake rates, increasing the overall sensitivity of sturgeon to lampricides.

The relationship between metabolic scaling and lampricide uptake

The correlation between oxygen and xenobiotic transfer at the gills of fishes and its relationship with metabolic rate has been well studied for a variety of organic compounds (Black and McCarthy, 1988; Brauner et al., 1994; Yang et al., 2000). Metabolic rate is known to dictate the kinetic uptake rate constant of several toxicants (Yang et al., 2000). TFM uptake in sea lampreys is dependent on biotic factors such as body size, life stage and metabolic rate (MO_2) (Tessier et al., 2018). MO_2 has an allometric scaling relationship with body size, where smaller organisms typically have a proportionally higher metabolisms compared to larger ones. This relationship can be described by the equation:

$$R = aM^b \quad (2)$$

where R is the metabolic rate (oxygen consumption or metabolism) of the animal, a is a species-specific constant, M is the mass of the individual organism and b is the (mass) scaling exponent (Kleiber, 1947; Goolish, 1991; Glazier, 2005). The scaling exponent for this relationship with the organism's mass is argued to be between the power of 0.67 – 0.75 (Goolish, 1991; Glazier, 2005). During situations which may increase ventilation, such as hypoxia, elevated temperature or pursuit stress, more water is being drawn across the gills which results in an increased amount of xenobiotic being in contact with the gill epithelium, leading to higher uptake rates (McKim and Goeden, 1982; Randall, 1982; Larisch and Goss, 2018).

TFM is hypothesized to be taken up passively at the fish gill (Larisch and Goss, 2018), down its concentration gradient. Because sea lampreys have a negligible capacity to detoxify TFM (Lech and Statham, 1975), the lampricide uptake rates are independent of the animal's ability to detoxify the compound. Consequently, TFM is taken up at the gill at a rate dependent on ventilation and metabolism alone, until a steady state is reached, known as equilibrium partitioning (Larisch and Goss, 2018). The most accurate method to measure TFM uptake rates is to rely on respiration rate at the gill, which is determined by the amount of water that comes into contact with the gill lamella for sufficient oxygen uptake to take place. This occurs because not all water that is pushed through the operculum actually comes into contact with the gill (Erickson and McKim, 1990; Larisch and Goss, 2018). Indeed, the excessive amount of water that flows over the gills is due to the low amount of oxygen that is diffused in the water compared to fish blood (Cameron and Davis, 1970). Thus, high volumes of water across the gills ensures that enough oxygen is taken up to meet the metabolic demands of the fish.

Oxygen consumption in larval sea lampreys scales according to the equation (Tessier et al. 2018):

$$MO_2 = 1.86M^{0.53} \quad (3)$$

In sea lampreys and in river lampreys (*Lampetra fluviatilis*), an increase in oxygen consumption was recorded after metamorphosis, while an increase in temperature also increased metabolic rate (Lewis and Potter, 1977). However, differences in metabolic rates between life stages in sea lampreys was not observed by Tessier et al. (2018), and neither late-stage metamorphosis nor juvenile parasitic lampreys had significantly higher rates of TFM uptake compared to the larvae.

TFM uptake scales according to the equation (Tessier et al., 2018):

$$\text{TFM uptake} = 7.24M^{0.34} \quad (4)$$

Oxygen consumption is tightly correlated to TFM uptake rates, as increased ventilation leads to move oxygen and, consequently, TFM being taken up. Therefore, animals with higher MO_2 rates have correspondingly higher rates of TFM uptake (Tessier et al., 2018). This relationship is not unique to lampreys or TFM; in rainbow trout, the uptake rate constant (k_1) for three different organic compounds was predicted based on the metabolic rate of the fish (Yang et al., 2000). The mass scaling exponent (b) for TFM of 0.34 indicates that uptake is disproportionally greater for smaller sea lampreys than larger ones (Tessier et al., 2018). The uptake efficiency of xenobiotics with log K_{ow} at or > 3 (TFM being at 2.77) is close to 100% (McKim et al., 1985; Larisch et al., 2017). As such, TFM is predicted to have a high uptake efficiency when respiration and ventilation rates are considered with the metabolic scaling relationship. Xenobiotics with higher K_{ow} values will diffuse more readily from the water into the blood, and thus their initial uptake rates will be highly dependent on the concentration gradient between the fish and bulk water (Yang et al., 2000). However, uptake is not the only factor to consider with the bioaccumulation of TFM, and its depuration (detoxification and excretion) rates must also be considered, as these also change allometrically. Previous studies have found that hydrophobic organic toxicants can be both taken up and excreted over the gills (Yang and Randall, 1995). The gills may be a site of excretion for TFM that has not been metabolized, but this would be dependent on passive diffusion and the concentration gradient of TFM between the animal and the bulk water. Further studies need to explore the exact sites of TFM excretion further, perhaps via divided chamber experiments, to separate excretion at the gill vs excretion through urine and feces.

The D in ADME – Lampricide distribution in the body

Lampricides have been measured in sea lampreys and non-target fish tissues. Schultz et al. (1979), measured TFM and niclosamide in several tissues from largemouth bass (*Micropterus salmoides*). The highest concentrations were found in bile, which presented increasingly higher concentrations over the 24 h exposure, while the lowest concentrations were detected in muscle (Schultz et al., 1979; Schultz and Harman, 1978). Both TFM and its glucuronide metabolite were also measured in rainbow trout muscle under controlled exposure (Lech, 1974), and in fillet tissues

from caged rainbow trout and channel catfish after a 12 h exposure in the field (Vue et al., 2002). Niclosamide was also rapidly accumulated in blood plasma, gallbladder bile, and muscle tissue of coho salmon (*Oncorhynchus kisutch*) and rainbow trout, and in bile and muscle of channel catfish and largemouth bass (Dawson et al., 1982). Niclosamide glucuronides and sulfate conjugates have been determined in rainbow trout muscle and channel catfish (Dawson et al., 2002; Hubert et al., 2005). Faster accumulation was observed for rainbow trout, reaching maximum concentrations after 12 h exposure, while channel catfish reached its maximum levels in fillet after 18 h (Dawson et al., 2002). The greatest accumulation of niclosamide in rainbow trout also occurred in the bile, suggesting that niclosamide is metabolized by the liver and collected in the gallbladder for excretion. Residues in the blood, heart, and fat tissue were also detected up to 3 days after the exposure. In contrast, residues in muscle, heart, and brain tissue were relatively low (Lech and Statham, 1975).

TFM and niclosamide do not accumulate in non-target fish tissues, as they are rapidly eliminated from the body post-exposure (i.e., TFM $T_{1/2}$ = 1.8 h in rainbow trout; 2.5 h in lake sturgeon) (Le Clair, 2014), likely due to detoxification processes, such as transformation to its glucuronide and sulfate conjugates, which will be discussed in the next section. In contrast, sea lampreys have higher accumulation rates and increased equilibrium levels than non-target species when exposed to identical water concentrations of TFM (Lech and Statham, 1975). For instance, Le Clair (2014) reported that concentrations of TFM in sea lampreys were 70% higher than in rainbow trout at nearly identical water TFM concentrations, while rainbow trout was able to maintain steady state internal concentrations between 15 and 20 nmol g^{-1}. Concentrations of TFM-glucuronide, a metabolite of TFM, reached a steady state after approximately 9 h in rainbow trout, while no TFM-glucuronide was detected in larval sea lampreys. The relatively slow elimination of TFM by larval sea lampreys ($T_{1/2}$ = 7–8 h) was attributed to the low capacity of transforming TFM to TFM glucuronide. Although sea lampreys were able to clear TFM from the body post-exposure, the elimination of TFM was probably restricted to passive diffusion of un-ionized TFM across the gills (Clifford et al., 2012; Le Clair, 2014).

Completing ADME with the mode of action of lampricides – Mitochondria as a target

TFM has been used for many years to reduce sea lamprey numbers in the Great Lakes fisheries, because it selectively targets larval sea lampreys. However, non-target fishes, such as whitefish, channel catfish, and lake sturgeon are relatively sensitive as well (Boogaard et al., 2003; Wilkie et al., 2019). At TFM concentrations used in the field, juvenile lake sturgeon less than 100 mm in size experience high mortality (Boogaard et al., 2003; Hepditch et al., 2019). In contrast, TFM did not reduce growth, cause avoidance behaviour, or increased predation on several fish species (Middaugh et al., 2014).

In the case of niclosamide, it is the most widely used molluscicide (Perrett and Whitfield, 1996). Niclosamide is toxic to all developmental stages of the snail life cycle and kills schistosome larvae in addition to fish (Lardans and Dissous, 1998). Niclosamide is toxic to *Australorbis glabratus* eggs at very low doses but is less toxic in the presence of chloride ions (Fox et al., 1967). It is often used to kill intermediate hosts of human parasites to reduce infection and outbreaks of these diseases. Clearly, differences exist in the response of target and non-target organisms to lampricides, but the reasons for these differences are still being explored.

TFM and its interaction with the mitochondria

We know that TFM and niclosamide have negative impacts on the function of the mitochondrial electron transport systems in fishes (Birceanu et al., 2011). The next question to answer is what mitochondrial pathways or cellular components are targeted by and disrupted by lampricides that result in this outcome. Multiple ideas must be considered. The first one to think about is the role that the concentration of lampricide might have on mitochondrial function. As stated above, several lines of evidence indicate that lampreys are not as effective at detoxifying TFM in comparison to other fishes. The effect of TFM on mitochondrial function might therefore be influenced by the fact that TFM is present at a critical threshold concentration in lamprey mitochondria, but that this does not occur in other fishes. If this is the case, then we should expect that the mechanism of TFM effects on mitochondrial function will be the same across different fish species, but that an effect will be evident at only at very different TFM concentrations in each species of fish.

The second consideration is how effectively TFM can cross the outer mitochondrial and inner mitochondrial membranes as that might help to determine its targets and mechanism of action. This might be dependent on the membrane phospholipid composition of different fishes. The outer mitochondrial membrane (OMM) is not very selective about what it allows to cross it. It is likely that the OMM does not pose as a barrier to TFM entering the intermembrane space of the mitochondrion. While it is possible that TFM is exerting its effects at the OMM, this seems unlikely. Once in the inner mitochondrial membrane (IMM), it might be able to bind protons and/or interact with various components of the membrane bilayer and/or complexes and carriers involved in electron transport systems (ETS). Wilkie et al. (2019) proposed that TFM acts as a shuttle, by binding and moving protons from the IMM into the matrix, therefore reducing the proton motive force and impairing the ability of the mitochondria to produce enough ATP to sustain vital processes. For example, one could imagine that the chemical characteristics of TFM might allow it to disrupt areas of the phospholipid bilayer which could lead to holes in the IMM that would allow for proton movement from the intermembrane space into the matrix, therefore impacting the transmembrane potential. Birceanu et al. (2011) did report that TFM disrupted the transmembrane potential in both sea lamprey and rainbow trout isolated liver mitochondria. This in turn would cause the disruption of the proton motive force and ATP biosynthesis. This disruption might also allow the premature release of the carrier cytochrome *c* and could lead to programmed cell death.

We could also envision a situation where TFM interacts with various complexes that are responsible for putting electrons into the system or removing electrons from the system. For example, perhaps TFM leads to a disruption in catalysis of enzymes that perform dehydrogenase reactions. A full understanding of the components present (e.g., complexes, uncoupling proteins, alternative proteins) is therefore very important. For example, TFM might target the proteins of Complex I, Complex II, Complex III, Complex IV, or Complex V. Using mitochondria isolated from the hearts of adult and parasitic sea lampreys, Huerta et al. (2020) found that mitochondrial energetics were affected as the TFM exposure levels were increased. Specifically, TFM appeared to target Complexes I and II from the ETS, and reduce the transmembrane potential across the IMM, leading to a mismatch between energy supply and demand. More research is required to examine the molecular diversity of ETS complexes present in animals, the epigenetic and post-translational regulation of ETS capacity, and protein turnover and redox homeostasis and the role that they play in surviving environmental stressors (Sokolova, 2018).

Such catalytic mechanisms are also subject to the influences of temperature and pH on the organism, enzymes, and mitochondrial characteristics and synergistic effects of multiple stressors are possible. For example, the seasonality and life history of the animal is also very important. It has been demonstrated that liver mitochondria in the river lampreys differ significantly in several key factors depending on whether they are isolated in winter (December to March) versus spring (April), with mitochondria isolated from winter animals being capable of exhibiting reversible metabolic depression (Savina et al., 2006). Measurements of membrane permeability indicated that the membrane permeability transition pore (MPT) was open and allowed protons, K^+, and Cl^- ions to diffuse in and out of the matrix in those mitochondria isolated from winter animals (Savina et al., 2006), further highlighting seasonal differences in mitochondria physiology in lampreys.

Work done in larval sea lampreys indicates that TFM likely disrupts oxidative phosphorylation (OXPHOS) and leads to a large reduction in fuel stores (Wilkie et al., 2007; Birceanu et al., 2009; Henry et al., 2015). While the precise mechanism of action remains to be determined, TFM treatment of lampreys results in ATP demand outstripping ATP supply (Birceanu et al., 2009, 2011; Huerta et al., 2020). In contrast, the mitochondria of animals that regularly experience changes in their environment have adapted to maintain operation (Sokolova, 2018). This includes many species of fishes that can maintain ETS activity, ATP biosynthesis, and mitochondrial coupling under adverse environmental conditions (Sokolova, 2018). The mechanisms that allow this may include: i) tight regulation of ETS capacity, ii) increases in antioxidant capacity prior to oxidative stress occurring, iii) removal and replacement of damaged mitochondrial proteins (Sokolova, 2018). Initially, mitochondrial dysfunction manifests as a decrease in ATP biosynthesis and an increase in reactive oxygen species (ROS) production leading to cellular damage. If left unchecked, ROS may damage amino acids and proteins, cellular and organelle membranes, leading to programmed cell death, and eventually organ/tissue failure and death of the organism (Sokolova, 2018).

Niclosamide interactions with mitochondria

With regards to the mode of action of niclosamide, little work has been done in fishes. Previous work in the cestode *Hymenolepsis diminuta* revealed that niclosamide does not exert its effects by acting on the ATPase, but that it is likely a protonophore (Park and Fioravanti, 2006). This protonophore activity of niclosamide has also been demonstrated using isolated membrane vesicles in *E. coli* as it dissipated the proton gradient that had been generated across the membrane (Fonseca et al., 2012). This effect was also seen for lysosome organelles (Fonseca et al., 2012). It is hypothesized that niclosamide's structure allows it to quickly bind and release protons and thereby dissipate proton gradients across membranes (Fonseca et al., 2012). When the effects of niclosamide were tested in human HeLa cells, it was demonstrated that it induced mitochondria fission, led to IMM depolarization, suppresses cell proliferation, and increased ROS (Park et al., 2011). Mitochondrial dynamics (i.e., mitophagy, proliferation, fission, and fusion) and the role that it plays in acclimation or adaptation to environmental stress remains an area ripe for exploration (Sokolova, 2018). These effects likely contributed to the programmed cell death and death by autophagy observed in the experiments (Park et al., 2011). This response is being exploited by investigating niclosamide as a cancer chemotherapy (Kumar et al., 2018). Niclosamide was found to upregulate genes encoding enzymes involved in detoxifying xenobiotics and stress responses, but in contrast downregulated the expression of a hemoglobin gene (Zhang et al., 2015). This indicates that niclosamide might negatively affect oxygen transport in the snail *Biomphalaria glabrata* (Zhang et al., 2015). However, none of these aspects have been explored in fishes, at levels of niclosamide that are applied to larval sea lampreys' nursery streams.

The M in ADME – Metabolism or detoxification of lampricides by the fish body

The much higher ability of non-target fishes to detoxify TFM relative to larval sea lampreys is what makes this lampricide so effective against the sea lampreys, with minimal to no effects on non-targets. Animals, fishes being no exception, detoxify compounds via Phase I and Phase II processes. Phase I involve the metabolism or biotransformation of chemicals vis cytochrome enzyme activity, transforming the in more soluble metabolites that can either be excreted or further processed in Phase II detoxification. Phase II processes involve further modifications of compounds via glucuronidation, sulfation, glutathione transferase activity and many more. Until recently, TFM has been known to be metabolized by Phase II processes, with little to no biotransformation via Phase I.

The role of phase I metabolism in TFM elimination has also been studied in sea lampreys (Bussy et al., 2018a), but to a lesser extent. The reduction of the nitro group to an amino residue had been previously identified *in vitro* using fish liver enzymes (Lech and Costrini, 1972). Bussy et al. (2018a) reported higher levels of reductive metabolites in the sea lampreys than in other fish species, after incubation of TFM with liver enzyme extracts from each species. The presence of the reductive metabolite amino TFM in fish liver incubates demonstrated that TFM undergoes phase I biotransformation via reduction to nitroso and hydroxylamine metabolites, not only in sea lampreys, but in non-target fish, although at a lower level in the latter (Bussy et al., 2018a).

Recent studies have shown that larval sea lampreys and non-target species are also capable of detoxifying TFM by sulfation, N-acetylation, glutathione conjugation and aromatic nitro group reduction (Bussy et al., 2018a), thus offering more insights into fish physiology. Phase II biotransformation processes are the main agents responsible in the metabolizing of lampricides in non-target fish (Fig. 5). These processes, which take place in the liver and, less significantly, in other tissues, increase the hydrophilicity of these compounds and makes them more susceptible of excretion via biliary or renal routes (Clarke et al., 1991; Lech and Statham, 1975). The effectiveness of TFM detoxification depends on the activity and kinetic characteristics of the enzyme UDP-glucuronyltransferase (UDPGT) (Kane et al., 1994), which is species-specific. UDPGT is much more active (higher V_{max} and lower K_m) in bluegill (*Lepomis macrochirus*), rainbow trout (*Oncorhynchus mykiss*), and channel catfish (*Ictalurus punctatus*) as compared to larval sea lampreys (Kane et al., 1994). Glucuronidation and sulfation processes have been demonstrated for TFM and niclosamide in non-target species such as rainbow trout, lake sturgeon and bluegill (Bussy et al., 2018a; Bussy et al., 2018b; Hubert et al., 1999; Kane et al., 1994; Lech and Statham, 1975). In contrast, the capacity of sea lampreys to use these Phase II processes for lampricide detoxification is limited (Fig. 5). The lower metabolization in sea lampreys via glucuronidation has been attributed to a lesser expression and lower affinity of the enzyme UDPGT for the lampricide. In larval sea lampreys, the efficiency of UDPGT is 50 % less when compared to most non-target species (Bock, 2016; Kane et al., 1994; Wang et al., 2014).

Niclosamide and its metabolites accumulate in the liver, and they are later excreted mainly via the urine (Statham and Lech, 1975). Studies on niclosamide metabolism demonstrated that niclosamide-glucuronide is the major metabolite of niclosamide in the bile of rainbow trout and largemouth bass (Dawson et al.,

1999; Graebing et al., 2004; Griffiths and Facchini, 1979; Schultz and Harman, 1978). Sulfation is also involved in niclosamide detoxification (Dawson, 2003; Dunlop et al., 2018), with significant amounts of the sulfated ester of niclosamide detected in the muscle of rainbow trout and catfish (Hubert et al., 2005). Niclosamide has been shown to have the potential to be degrade to amino niclosamide in aquatic systems (Graebing et al., 2004), but Phase I biotransformation appears to have a minor role in the metabolism of niclosamide in fish (Wilkie et al., 2019).

The effect of temperature and seasonal changes on the ability of enzymes to detoxify xenobiotics have been extensively studied in non-target fish. For instance, seasonal changes in UGT activity have been observed in rainbow trout, whitefish, and common carp, exhibiting decreased UGT activity during winter months and elevated activity during summer months (Curtis et al., 1990; Daidoji et al., 2006; Koivusaari, 1983; Koivusaari et al., 1981; Lindström-Seppä, 1985). Sea lampreys are more sensitive to TFM treatments in the spring months than during the summer, which was attributed initially to a lower UGT activity as well. Despite the general lower glucuronidation activity of sea lampreys (Kane et al., 1994; Lech and Statham, 1975), the changes in sensitivity to lampricide exposure led to the hypothesis that detoxification activity may vary seasonally (Scholefield et al., 2008; Muhametsafina et al., 2019). Although an increase in the UGT mRNA abundance in sea lampreys has been reported in different seasons, but not at different temperatures, it is still unknown if protein abundance and enzyme activity involved in detoxification could explain the differential sensitivity to TFM during the year (Hlina et al., 2021).

While most non-target fishes are more tolerant to TFM than sea lamprey, lake sturgeon (*Acipenser fulvescens*) juveniles are an exception. These fish displayed sensitivity to TFM that approached that of larval sea lampreys (Boogaard et al., 2003; Johnson et al., 1999; O'Connor et al., 2017), despite being able to eliminate TFM through transformation into TFM glucuronide (although with 50% less efficiently than rainbow trout) (Le Clair, 2014). This fact could indicate that some other factor could be involved in higher toxicity of TFM in lake sturgeon compared to other non-target species. O'Connor et al. (2017) conducted *in situ* toxicity tests using juvenile lake sturgeon exposed to TFM during field lampricide treatments and found that mortality increased in high alkalinity waters, which required more TFM to treat. While alkalinity is protective against TFM toxicity in both sea lampreys and lake sturgeon (Boogaard et al., 2003; Hepditch et al., 2019), the higher levels of lampricide in more alkaline waters is what likely accounted for the higher mortality in the juvenile sturgeon. There is yet more to learn about the various biotic and abiotic factors that affect the sensitivity of lake sturgeon to lampricides, particularly with respect to TFM/niclosamide mixtures, which are often applied in sturgeon nursery streams (Pratt et al., 2020).

The E in ADME – Excretion of lampricides in fishes

Excretion of lampricides in sea lampreys and non-target fish occur relatively fast. Elimination of TFM from tissues in non-target fish, such as largemouth bass, channel catfish, and rainbow trout, can take less than 12 h (Schultz et al., 1979). TFM elimination seems to be much faster (4-fold higher) in non-target fish such as rainbow trout than sea lampreys (Le Clair, 2014; Birceanu et al., 2014). Regardless, in larval and juvenile sea lampreys, 90 % of TFM removal is achieved within 24 h, with the highest elimination rates reached immediately after exposure (Hlina et al., 2017; Tessier et al., 2018). In the case of niclosamide, elimination periods fluctuate from 24 h in the case of rainbow trout to 96 h for channel catfish (Allen et al., 1979; Dawson, 2003).

Lampricides are excreted from the body in the form of their conjugates metabolites in non-target species, primarily via renal or biliary routes (Wilkie et al., 2019; Fig. 5). Largemouth bass exposed to TFM presented an increased concentration of TFM-glucuronide in bile (Schultz et al., 1979). In rainbow trout the glucuronide metabolite of both TFM and niclosamide was detected in bile and urine (Dawson et al., 1999; Lech, 1974; Statham and Lech, 1975). However, not all non-target fish studied excreted the lamp-

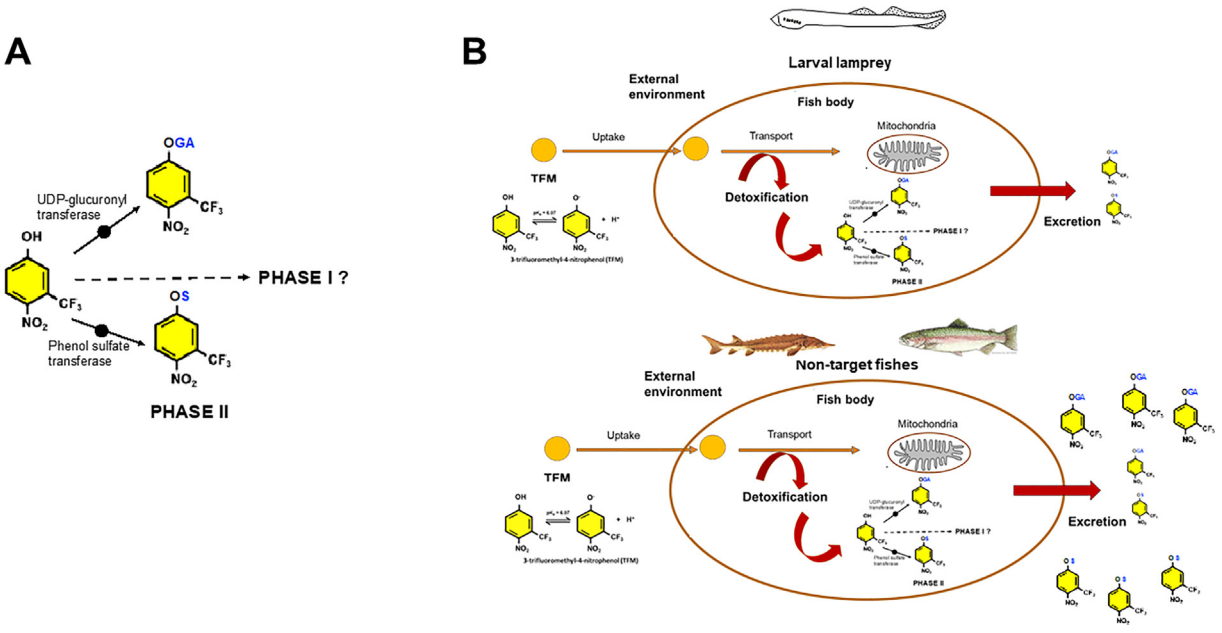

Fig. 5. General overview of TFM specificity. (A) Representative diagram of the main Phase II detoxification routes for TFM: glucuronidation and sulfation. The figure also denotes that Phase I detoxification pathways for TFM still need to be elucidated. (B) General overview of how TFM is handled by target (top, larval sea lampreys) and non-target (bottom, rainbow trout and lake sturgeon) fish species, with an emphasis on detoxification products. Larval sea lampreys have a limited capacity to detoxify TFM, while rainbow trout and lake sturgeon have a much higher detoxification capacity. These physiological limitations of the sea lamprey allows TFM to selectively target them, with minimal effects on non-targets.

ricides primarily through those routes. For instance, in the channel catfish, no TFM nor its conjugated metabolites were detected in either bile or urine, so it was assumed that gill excretion was the predominant excretion route (Allen and Hunn, 1977). Sea lampreys has a low capacity to detoxify TFM via conjugation (Kane et al., 1994; Lech and Statham, 1975). Phase I (reductive) metabolism may be a potential route for the TFM elimination, although to what magnitude is still uncertain (Bussy et al., 2018a; Bussy et al., 2018b). There is no information about the role that the renal and biliary excretion of TFM and its metabolites plays in the lampricide clearance (Hlina et al., 2021). It has been suggested that TFM excretion is mainly performed by passive diffusion of the neutral compound across the gills, with small amounts excreted via renal routes (Hlina et al., 2017; Tessier et al., 2018; Wilkie et al., 2019). Little is known about how niclosamide is excreted by sea lampreys (Wilkie et al., 2019).

Insights into tissue physiology gained from lampricide exposure studies

The gill

As the main site of lampricide uptake, the gill tissue is the first to be affected by the

exposure. There is some evidence that TFM can cause damage to the gills in rainbow trout and sea lampreys. Christie and Battle (1963) reported that exposure of rainbow trout to sub-lethal levels of TFM led to gill damage, such as vasodilation and edema, resulting in vascular collapse and increased permeability of the cells. However, it should be noted that in their study, the authors collected the gill tissue after death due to TFM exposure, which could have affected the tissue quality compared to tissue collection from surviving animals (i.e., cell apoptosis may induce membrane and organelle damage). In addition, Mallatt et al. (1994) reported that the respiratory lamellae of the gills of sea lampreys exposed to their respective 9-h TFM LC_{100} (TFM concentration that is lethal to 100% of the fish over a 9 h exposure period) appeared widened and the damage observed was confined to the ion uptake cells: vacuolization, enlarged nuclei, necrosis and a disorganization of the Golgi apparatus and mitochondrial cristae structure. Mallatt et al. (1994) exposed rainbow trout to their respective 9-h TFM LC_{100} and reported no effects on gill tissue and organelle structure. Our own work has investigated the possibility that TFM may induce gill damage, leading to a disruption in ion homeostasis in trout and lamprey. Birceanu et al. (2009) and Henry et al. (2015) found no effects on plasma ion balance, haematology and gill Na^+/K^+-ATPase (NKA) activity in larval, juvenile and adult sea lampreys exposed to their respective 12-h TFM LC_{50} over 12 h. In rainbow trout exposed to their respective 12-h TFM LC_{50}, Birceanu et al. (2014) reported transient changes in ion homeostasis, but the overall effects on ion balance and gill NKA were minimal, suggesting that disruption of ion regulation is not a mechanism of toxicity of TFM.

With respect to the effects of niclosamide on gill structure and function in fishes, little work has been done. Mallatt et al. (1994) exposed sea lampreys to a lethal dose of Bayer 73 (a formulation of niclosamide) and TFM/1.6% Bayer 73. The gill damage under both conditions was either similar to that induced by TFM exposure alone or more severe: the ionocytes were necrotic, detached from the lamellae and their numbers were reduced by 30% when compared to controls. These findings suggest that, while the effects of TFM on the gill morphology appear to be minimal, the presence of niclosamide induces gill damage. It is possible that induction of gill damage is an attribute of niclosamide alone, which may contribute to its overall higher toxicity and non-specificity when compared to TFM. Whether niclosamide or TFM/niclosamide mixtures impact ion regulation and homeostasis in fishes needs further exploring.

The brain

The brain in fishes is not a major energy storing organ, but its glycogen, glucose and phosphocreatine stores can be exhausted rapidly if energy demands are high. Glucose, the main energy source in the brain of vertebrates, can rapidly decrease in response to food deprivation, hypoxia and anoxia, ischemia and toxicant exposure (Lowry et al., 1964; Soengas et al., 1998, 2006; Polakof et al., 2007; Birceanu et al., 2009). Any disturbance to the glucose levels in the brain can profoundly impair the function of the central nervous system (CNS; Hochachka et al., 1993), leading to metabolic depression and death.

Studies on the mode of action of TFM in sea lampreys revealed that these animals have some of the largest brain glycogen levels among all vertebrates, yet this does not influence their sensitivity to lampricides. Compared to the trout brain glycogen, for example, measuring ~ 5–10 μmol glucosyl units g^{-1} wet tissue, the sea lamprey brain stands apart, with measured levels as high as 150–170 μmol glucosyl units g^{-1} wet tissue in larvae (Clifford et al., 2012), 60–100 μmol glucosyl units g^{-1} wet tissue in juveniles and 120–130 μmol glucosyl units g^{-1} wet tissue in adults (Henry et al., 2015). However, the glycogen present in the brain is mainly used to support CNS function, should energy reserves be compromised at some point (Rovainen, 1970; Rovainen et al., 1971; Murat et al., 1979; Foster et al., 1993). Following exposure to lethal concentrations of TFM, the fish rely first on the high energy phosphagen phosphocreatine (PCr) to temporarily meet the ATP demands of the body, before mobilizing anaerobic energy resources, like glycogen. However, PCr is quickly depleted, forcing the animals to access their glycogen reserves. Indeed, brain PCr and glycogen levels decreased, and lactate increased in larval sea lampreys as exposure time to TFM increased (Birceanu et al., 2009; Clifford et al., 2012; Henry et al. 2015). A similar trend was reported in rainbow trout (Birceanu et al., 2014) and juvenile and spawning sea lampreys (Henry et al., 2015). These findings highlight that the mode of action of TFM is the same, regardless of species.

Work from our group on the mode of action of TFM in larval, juvenile and adult sea lampreys has suggested that the effects of the lampricides may also be life-stage specific. Henry et al. (2015) reported that the adult sea lampreys were more sensitive to TFM compared to the larvae and the juveniles and that their brain glycogen levels were greatly reduced. Interestingly, the study found that in the adult sea lamprey brain, the stoichiometry of lactate:glycogen was less than 2:1 and brain lactate levels were lower than expected. Clifford et al. (2012) reported a similar finding in larval sea lampreys and suggested that the missing lactate may have been lost to the blood, via the monocarboxylate transporters (Halestrap and Price, 1999). Taken together, these two studies suggest that not only does the sea lamprey undergo massive morphological and -physiological changes as it undergoes metamorphosis into the parasitic juveniles, but that the way in which it accesses its energy reserves and handles biochemical waste also varies with life stage.

Our previous work has also shown that larval sea lampreys are able to recover their lost brain energy reserves following a TFM treatment. Clifford et al. (2012) found that brain energy reserves (i.e., glycogen and PCr) recover within 12–24 h after the animals were placed in clean water. This rapid recovery indicates that those animals that escape the TFM plume, either by leaving their burrows or seeking refuge near shore, where TFM levels may be lower, have a high chance of survival. In addition, the study proposed that

the mechanism by which the lampreys recover from TFM exposure is by passive diffusion of the lampricide at the gills, in its unionized form. The Clifford et al. (2012) study is one of the first to suggest the complexity with which TFM interacts with the fish body. As a weak acid with pKa of 6.07 (Fig. 3), most (~95%) of TFM would be in its ionized form at the physiological pH of ~ 7.85 (Boutilier et al., 1993). However, when calculating the distribution and speciation of TFM between in the plasma (pH 7.85) and the muscle (pH 7.3), Clifford et al. (2012) reported that the more acidic medium of the intracellular pH of the muscle would favour the formation of more unionized (TFM-OH) than in the plasma. This difference in concentration of TFM-OH between the two tissues would favour the passive movement of this unionized form into the plasma and from the plasma to the water once it reaches the gills. We now know more about the complex interaction of TFM with the fish body, from uptake, handling, target, detoxification and elimination, but it appears that the internal pH of various tissues impacts the distribution and speciation of the lampricides.

The liver

Studies on the mode of action of TFM have revealed that this lampricide leads to the mobilization of liver energy reserves, such as glycogen, glucose and phosphocreatine, to maintain vital functions. Our work has revealed that increases in exposure time to sub-lethal concentrations of TFM lead to time-dependent reductions in liver glycogen in larval (Birceanu et la., 2009), juvenile and adult (Henry et al., 2015) sea lampreys, as well as non-target fish species, such as rainbow trout (Birceanu et al., 2014). These studies have confirmed that liver glycogen reserves are mobilized first in these animals, following exposure to lampricides, but have also pointed to significant differences between the levels of tissue glycogen in sea lampreys and rainbow trout. Liver glycogen in rainbow trout can be as high as 100–150 μmol g^{-1} wet weight, whereas in larval, juvenile and adult sea lampreys, the levels are approximately 9, 2 and 5 μmol g^{-1} wet weight, respectively. In animals, liver glycogen is readily mobilized to cope with chemical stressors, whereas the glycogen in the brain and muscle is protected to sustain vital functions and burst exercise, respectively (Panserat et al., 2000; Polakof et al., 2012; Kieffer, 2000; Wilkie et al., 2001). Therefore, the lower levels of liver glycogen in the sea lampreys, irrespective of life stage, may contribute to their overall higher sensitivity to lampricides. Conversely, the higher levels of liver glycogen in the rainbow trout livers allow them to respond to chemical stressors more efficiently, making them more tolerant. Indeed, when trout were exposed to the 9-h LC99.9 of the larval sea lampreys, mimicking a field application, liver glycogen, along with the enzymes that are responsible for glycogen, glucose and protein breakdown (i.e., the liver metabolic capacity), remained unaffected (Birceanu and Wilkie, 2018).

Glycogen in the liver not only stores energy, but also acts as a pre-cursor for molecules involved in xenobiotic detoxification. Glycogen is a pre-cursor to the production of glucose-6-phosphate (G6P), a substrate required to produce glucuronic acid and fuel the Phase II detoxification process of glucuronidation (Bánhegyi et al., 1998), which is one of the main pathways through which TFM is detoxified (Bussy et al., 2018a; Bussy et al., 2018b). Vue et al. (2002) has identified TFM-glucuronide in various non-target fish tissues several hours following exposure to the lampricide, suggesting that the detoxification process occurs long after the lampricide has been removed from the water. Therefore, it indicates that higher levels of glycogen, particularly in the liver, which is responsible for detoxification, would significantly contribute to the overall tolerance of fishes to chemical stressors such as lampricides.

The muscle

The fish muscle is another important organ that has been the focus of studies that looked at the mode of action of lampricides over the past 20 years. The muscle contains protein and glycogen, as major components, but its glycogen reserves play a minor role in maintaining whole-body homeostasis (Panserat et al., 2000; Polakof et al., 2012), but are preferentially used to fuel glucose into glycolysis, to generate ATP during vigorous exercise (Kieffer, 2000; Wilkie et al., 2001). In addition, the muscle of the sea lamprey lacks glucose-6-phosphatase, preventing it from releasing glucose into circulation (Panserat et al., 2000). Therefore, it was quite surprising when reports from our group found that muscle glycogen levels were impacted by TFM, in both sea lampreys and non-target species exposed to the lampricide (Birceanu et al., 2009, 2014; Henry et al., 2015). In adult sea lampreys, muscle glycogen levels decreased by ~ 50% by 2 h of exposure to their respective 12-h $LC_{99.9}$ for TFM, a concentration required to kill 99.9% of the animals over 12 h, while lactate levels increased. This indicates that the animals were relying on anaerobic pathways to generate ATP (Henry et al., 2015). This effect, however, was not seen in the larval or juvenile parasitic individuals. Birceanu et al. (2009) did report marked decreases in muscle glycogen following exposure of the larval sea lampreys to TFM, but these effects were not seen until 9 h. The lamprey muscle is a mosaic of red (oxidative) and white (glycolytic) fibres (Peters and Mackay, 1961; Meyer, 1979; Boutilier et al., 1993), with high metabolic demands. Henry et al. (2015) proposed that these life-stage differences in the effects of TFM on muscle glycogen were due to the accumulation of TFM in the muscle, which would interfere with local ATP production. Due to the high metabolic demands of the muscle, glycogen reserves would be mobilized to ensure that ATP supply matches demand. Indeed, TFM does accumulate in the muscle of fishes (Hubert et al., 2005; Birceanu et al., 2014), where it interferes with oxidative phosphorylation. This finding highlights the differences in physiology between the sea lamprey life stages and is a one of the reasons why the adults were more sensitive to TFM than any life stage.

In rainbow trout, muscle glycogen reserves appeared to be less impacted by exposure to TFM when compared to the larval sea lampreys. Glycogen levels decreased by ~ 50% only after 12 h of exposure to their respective 12-h TFM LC_{50} (Birceanu et al., 2014). The study noted that this change could be due to the tissue essentially becoming anoxic as TFM levels increased in the body. Under this scenario, white muscle glycogen stores would be preferentially used over those in the liver, to support glycolysis (Mandic et al., 2008). Unlike lampreys, trout muscle has glucose-6-phosphatase, allowing it to release glucose into circulation and contribute to glucose homeostasis, but solely under specific stress conditions, such as ischemia/anoxia. This finding shed more light on the interactions of TFM with the fish muscle and provided additional insights into the role that this tissue plays in maintaining glucose homeostasis. Given this information, the possibility that TFM may impair swim performance in fishes should be investigated, as it can have long-term implications for burst swimming and predator avoidance behaviour. While this is less important for larval sea lampreys, the muscle physiology in non-target fish species may be impacted.

Less work has been done on the effects of niclosamide on the physiology of the muscle in fishes. This lampricide does accumulate in the muscle, much like TFM (Hubert et al., 2005), and studies on invertebrates and bacteria provide evidence that it exerts its toxicity by uncoupling oxidative phosphorylation (Park and Fioravanti, 2006; Fonseca et al., 2012). Wilkie et al. (2019) proposed that niclosamide has a similar mode of action as TFM once in the mitochondria. Therefore, if would be plausible that its effects

on the fish muscle would be similar to those of TFM. This aspect needs to be further investigated, for both niclosamide alone and in combination with TFM, to better understand how it impacts non-target species.

The kidney

The effects of lampricides on the kidney of sea lampreys and non-target species has not received much attention. The few studies into the mode of action of TFM conducted by our group have explored how kidney bioenergetics and steroidogenesis are impacted by the lampricide, with the first analysis being done on both sea lampreys and rainbow trout (Birceanu et al., 2014; Henry et al., 2015), while the latter was only done on rainbow trout (Birceanu and Wilkie, 2018). When exploring the impacts of TFM exposure on kidney bioenergetics in larval, juvenile and adult sea lamprey life stages, Henry et al. (2015) found that glycogen levels in the kidney of the larval sea lampreys was approximately 10–12 fold higher than in the juveniles and adults. This indicates that, perhaps, the larvae rely more on the kidney to maintain glucose homeostasis than the later life stages. Murat et al. (1979) reports that the kidney in adult river lampreys had higher glucose-6-phosphatase activity than the liver and the brain, further underscoring the important role that this organ plays in maintaining energy balance in the body. However, it should be noted that the juvenile and adult sea lampreys have a higher metabolic rate than the larvae, which may mean that their rates of glucose turnover are higher and could explain the lower glycogen stores. Henry et al. (2015) also noted that the larvae were most tolerant to TFM out of the three life stages explored and concluded that the kidney glycogen stores may play a more important role in this increased tolerance than previously believed. It should be noted that TFM exposure did not significantly impact kidney glycogen stores in either life stage, meaning that perhaps kidney glycogen is not readily mobilized unless the liver stores are severely impacted. ATP levels, however, were reduced in all life stages with an increase in exposure time, suggesting that the kidney energy reserves were indeed impacted by the lampricide. The role that the sea lamprey kidney plays in regulating glucose homeostasis, particularly during lampricide exposure, should be further investigated, as this organ plays an important role in stress response in these animals (Close et al., 2010).

In rainbow trout, kidney glycogen levels were not impacted by TFM exposure (Birceanu et al., 2014). Kidney phosphocreatine and ATP, however, were significantly reduced by 3 h of exposure, only to quickly recover by 9–12 h. Taken together with the previous findings in sea lampreys, these studies suggest that lampricide exposure affects numerous organs in fish, from the liver, brain, muscle and, of course, the kidney, which plays a key role in regulating the stress response in fishes, particularly the region known as the head kidney (Mommsen et al., 1999). Birceanu and Wilkie (2018) exposed rainbow trout to environmentally relevant concentration of TFM, then collected the head kidney and investigated its steroidogenic properties, independent of the hypothalamus and the pituitary. The study found that TFM exposure had no effect on *in vitro* stimulated cortisol production, but that fish still showed elevated plasma cortisol up to 36 h post-exposure. The authors could not exclude the possibility that the elevated cortisol could be due to either impaired cortisol breakdown or altered signaling from the. Further studies exploring the impacts of TFM and niclosamide on kidney function should be considered, with a focus on the steroid production pathway.

Lampricide interactions with the stress axis

To our knowledge, little work has been done on the interaction of lampricides with the stress axis [or the hypothalamic-pituitary-interrenal (HPI) axis] in fishes. Part of the kidney known as the head kidney houses the interrenal cells, which produce corticosteroids. The interrenals are part of the HPI axis, which regulates the production of stress hormones; of particular interest to energy regulation is the production of cortisol (in non-target fish species) or 11-deoxycortisol (in sea lampreys), hormones that mediate energy re-allocation and mobilize energy stores in the body during stress (Mommsen et al., 1999; Shaughnessy and McCormick, 2021).

Birceanu and Wilkie (2018) investigated the effects of a TFM exposure, mimicking a treatment, on the response of rainbow trout to a chasing stressor post-treatment, but no similar studies to date have been done on sea lampreys stress axis. The study found that TFM-exposed fish had higher basal cortisol levels than the controls and that their mRNA abundance profile of the steroidogenic genes

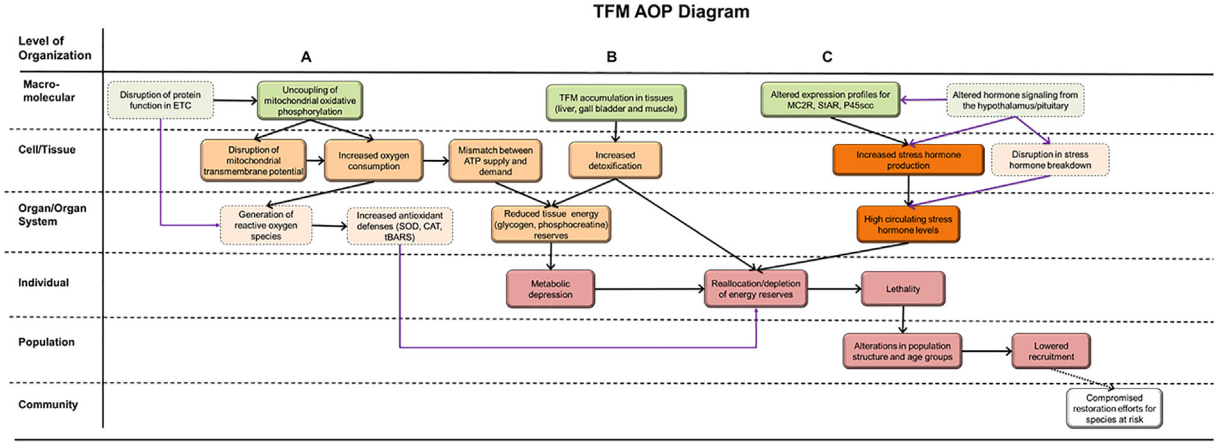

Fig. 6. The Adverse Outcome Pathway (AOP) for TFM. Note that dotted cases, in the lightest shade, represent endpoints that need investigating and are currently theoretical MIEs as part of this AOP. Light solid lines (or purple lines if in colour) represent relationships that need to be determined, while black solid lines represent established connections. This AOP framework assumes three molecular initiating events (MIE): two direct events [uncoupling of mitochondrial oxidative phosphorylation (A) and alterations in expression of genes involved in stress response pathway (C)] and one indirect (B), based on the accumulation of TFM in the tissues. The AOP moves through levels of organization, highlighting what is currently known (solid, medium shaded cases) and what needs further investigating as endpoints (dotted, lighter color cases). The darkest shaded cases (C) represent endpoints investigated only in non-target species. The impact of TFM on the community level, especially with respect to non-target sensitive fish species that are targeted for restoration, such as lake sturgeon, has been investigated and appears to be minimal (denoted by the dotted arrow and white case). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

melanocortin-2-receptor (MC2R), steroidogenic acute regulatory protein (StAR) and cytochrome p450 side-chain cleavage (p450scc) was altered when compared to controls. Surprisingly, the steroidogenic genes showed a profile similar to that of fishes exposed to chronic stress. This finding suggested that exposure to TFM impacts the stress response post-treatment, but whether this is by directly affecting the steroidogenic genes during the treatment or by impairing the hypothalamus or pituitary signaling is unknown. However, by exploring this interaction of TFM with the fish stress physiology, Birceanu and Wilkie (2018) suggested that perhaps the elevated cortisol post-treatment was beneficial, because it mobilized glycogen stores to glucose. These energy reserves are used when the mitochondria are unable to maintain ATP supply to the tissues, which is the case during TFM exposure, as this lampricide limits ATP production. However, the glucose generated from glycogenolysis is not only used for energy production, but it is also needed for detoxification (Bánhegyi et al., 1998); it generates glucose-6-phosphate, a substrate required to produce glucuronic acid, which is used to detoxify TFM to TFM-glucuronide in the liver.

Whether niclosamide interacts with the stress axis in a similar manner to TFM, it remains to be determined. However, glucuronidation does appear to be a main pathway by which this lampricide is detoxified (Dawson et al., 2002; Hubert et al., 2005), and, therefore, a similar effect would be expected. More work needs to be done to elucidate how niclosamide interacts with the HPI axis in fishes, particularly in those that are sensitive to lampricide treatments.

The development of an adverse outcome pathway (AOP) for TFM

In the last decade, numerous studies have explored the mode of action of TFM in fishes and have identified several pathways by which this lampricide may exert its toxic effects, either directly or indirectly. In this manuscript, we developed the first adverse outcome pathway (AOP; Fig. 6) for TFM in fishes, taking into consideration all the work that has been conducted to date on its mode of action in these organisms. The purpose of the TFM AOP is two-fold: (1) to provide a framework that summarizes the direct and indirect effects of exposure to TFM, in both target and non-target fishes and (2) to identify areas that need further exploration that can provide additional insights into the mode of action of this lampricide. An AOP may also guide our understanding of how the lampricide interacts with the fish body at all levels of organization and help identify potential biomarkers of TFM exposure. These biomarkers are molecules that are detected in the body and are typically produced following exposure to the lampricide.

The AOP framework starts with a molecular initiating event (MIE), which is the interaction of the chemical with the organism, leading to the initiation of the toxic process. In the case of TFM, we have identified two direct (Fig. 6 A and C) and one indirect (Fig. 6 B) MIEs. The two direct events that have been explored to date, in either sea lampreys, non-target species or both, identify the mitochondria (Fig. 6A) and the stress response pathway (Fig. 6C) as targets of TFM. This lampricide targets the mitochondria and enters the IMM by crossing the OMM. Once in the intermembrane space, TFM behaves like a protonophore (Birceanu et al., 2011), binding hydrogen ions and disrupting the proton motive force that drives ATP formation. Huerta et al. (2020) has shown that TFM targets the protein complexes in the ETS, leading to a disruption in the proton motive force.

How TFM precisely interacts with the mitochondria in fish is not entirely known, but Wilkie et al. (2019) proposed that TFM acts as a shuttle for protons: once TFM binds the hydrogen ions, it moves them in the matrix, bypassing the ATP synthase, uncoupling the oxidation reactions from the phosphorylation of ADP to ATP and disrupting the transmembrane potential (TMP; Birceanu et al. 2011). From the perspective of an AOP, this disruption in mitochondria TMP is a key event (KE), that triggers toxicity. However, there are additional KEs to the TFM AOP, such as an increase in mitochondria oxygen consumption to restore the proton motive force (Fig. 6A), a possible increase in ROS, leading to an increase in antioxidant defenses, which have yet to be explored in fishes. The compromised ability of the mitochondria to produce ATP leads to a mismatch between energy supply and demand, forcing the animal to rely on tissue energy reserves (such as glycogen and phosphocreatine) to maintain ATP demands for survival. A depletion of tissue energy reserves in fish exposed to TFM (Wilkie et al., 2007; Birceanu et al. 2009, 2014; Clifford et al., 2012; Henry et al., 2015) is an additional KE of the AOP, leading to adverse outcomes at the level of the individual, such as metabolic depression, reallocation of energy reserves to maintain vital bodily functions and eventually death. These adverse outcomes at the level of the individual also translate to adverse outcomes at the level of the population, such as a reduction in fish population. In the case of the sea

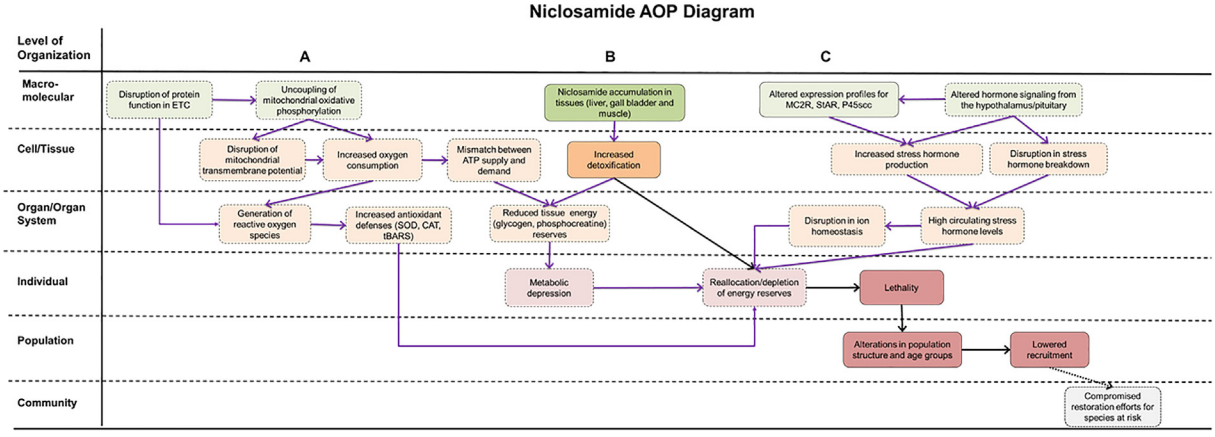

Fig. 7. The proposed Adverse outcome pathway (AOP) for niclosamide. This is only a proposed AOP, based on the knowledge that we currently have on the TFM AOP (see Fig. 6). Note that dotted cases, in the lightest shade, represent endpoints that need investigating and are currently theoretical molecular initiating events (MIEs) as part of this AOP. Light solid (purple if in colour) lines represent relationships that need to be determined, while black solid lines represent established connections. The purpose of this figure is to establish a framework around which the niclosamide AOP can be developed, while also highlighting potential avenues for research, endpoints and relationships that need to be investigated further. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lampreys, that is a desirable outcome in the Great Lakes. However, in the case of sensitive non-target species, such as young-of-the-year lake sturgeon, a reduction in population would not be desirable, but it must be considered when discussing and AOP.

An additional direct mode of action of TFM that our group has explored in rainbow trout is the effect on the stress response pathway following TFM exposure at environmentally relevant concentrations (Fig. 6C). In this context, the MIE represents an alteration in the mRNA abundance profile of the steroid genes in fish exposed to an additional stressor 12 h post-TFM treatment (Birceanu and Wilkie, 2018). This led to a KE where circulating levels of cortisol remained elevated up to 36 h post-TFM treatment, to reallocate energy reserves to the detoxification pathway. This additional KE contributes to the depletion of energy reserves, in addition to the effects of TFM on the mitochondria. Whether the effects on steroidogenic gene mRNA abundance are due to impaired signaling in the hypothalamus and/or pituitary, and whether these effects are also seen on the stress response axis in larval sea lampreys remain to be resolved.

A final, but indirect, MIE that we have identified that may contribute to the toxicity of TFM is the accumulation of the compound in the body of the fish (Fig. 6B). We called this MIE "indirect" because the accumulation of the lampricide in the body triggers the detoxification pathways in the fish liver, leading to a reallocation of energy reserves towards detoxification (KE). Taken together, the effects of TFM on mitochondria and the stress response pathway, in combination with the increased detoxification, increase energy demand on the body. Since the fish cannot maintain the supply, death ensues.

Future avenues for research - at the intersection of toxicology and physiology

Towards a niclosamide AOP: Identifying the mode of action of niclosamide in fishes

Although not as much work has been with respect to the mode of action of niclosamide in fishes, we have proposed a framework around which the AOP could be developed: it uses the TFM AOP to highlight what is known to date and it proposes additional avenues for research (Fig. 7). Similar to TFM, an indirect MIE for niclosamide would be the tissue accumulation, which has been studied to date (Lech and Statham, 1975; Kane et al., 1994; Dawson et al., 2002; Hubert et al., 2005), albeit not in as much detail as TFM. Research has shown that this lampricide is detoxified by fishes via glucuronidation and sulfation (Dawson et al., 2002; Hubert et al., 2005). Therefore, allocation of energy towards detoxification processes could be a key event on the AOP, much like it is for TFM. With respect to mechanism of toxicity of niclosamide, it is believed to target the mitochondria and behave as a protonophore, although this has not yet been shown in fish mitochondria. In other systems, however, such as cestodes and bacteria (Park and Fioravanti, 2006; Fonseca et al., 2012), niclosamide acts as a protonophore, binding hydrogen ions and dissipating the transmembrane potential. In addition, the effects of niclosamide on stress genes and hemoglobin (Zhang et al., 2015) suggest that the HPI axis and pathways of ion regulation should be explored as potential targets. Potential avenues for research with respect to the mode of action of niclosamide are further highlighted in Fig. 7. We encourage researchers to use this only as a guideline and consider adding additional MIEs, which could arise from the work done on the mode of action of this lampricide.

Furthering our understanding of the interaction of TFM/niclosamide on fish physiology

Another gap in our understanding on how lampricides interact with the fish body is knowledge on the effects of TFM/niclosamide mixtures. Few studies have explored this aspect and none have looked at the physiological effects in either sea lampreys or non-target species. Early studies indicated that TFM/niclosamide interactions were additive or less than additive, depending on the concentrations used, the duration of exposure and the species of fish tested (Bills and Marking, 1976; Marking and Bills, 1985), whereas others suggested that the interaction was greater than additive or synergistic (Marking and Dawson, 1975). These studies highlight that the response to lampricide exposure is species-specific, meaning that some fishes, while not sensitive to exposure to one lampricide alone, may be quite sensitive to the mixtures. These studies, however, only used mortality as an endpoint and did not look further into the physiological aspects of exposure to lampricide mixtures. Therefore, several questions remain that are worth exploring, in both sea lampreys and non-target sensitive species:

1. Can the interactions of lampricides in mixture be predicted?
2. What are the physiological effects of exposure to lampricide mixtures?
3. What are the long-term effects of TFM/niclosamide exposure on non-target fishes?
4. Does the mode of action of the lampricides change in mixture?
5. What are some of the biotic and abiotic factors that impact sensitivity to lampricide mixtures?
6. Can we identify a biomarker of exposure to lampricide mixtures?

We are also proposing that an adverse outcome pathway be developed for the mixtures as well, to add to the body of knowledge on the effects of lampricides in fishes.

Towards the next generation lampricides

The lampricides TFM and niclosamide have been used for over 60 years to control the invasive sea lamprey population in the Great Lakes. Considering the sea lamprey life cycle, where they spend 3–7 years as larvae that are exposed to lampricide treatments, the risk of resistance developing in this species cannot be ignored. Dunlop et al. (2018) have explored this aspect and concluded that resistance could develop through:

(i) avoidance behaviour – where the animal moves away from a treatment once it detects the lampricide in the water
(ii) target modification – where the lampreys increase their number of mitochondria or make them more resilient to lampricide exposure
(iii) increased detoxification – where the lampreys increase their detoxification capacity
(iv) excretion – where the routes of lampricide excretion are rendered more efficient

Recent work on the risk of resistance to lampricides developing in sea lampreys has suggested that perhaps such a phenomenon is unavoidable (Christie et al., 2019). A deeper understanding of how lampricides impact fish physiology, what they target and how the animals handle such challenges will allow us to make better predictions on how resistance could develop. Current practices on lampricide applications favour TFM alone because of its specificity to the lampreys, with mixtures of TFM/niclosamide and niclosamide applications being sparse. Could the application of mixtures, at TFM/1–2% niclosamide, which increases toxicity to lampreys without impacting specificity, be a way to decrease or delay the risk of resistance to TFM in larvae?

Our current use of lampricides makes it an efficient method of controlling the sea lamprey population because of the reduced ability of the sea lampreys to detoxify them compared to non-

target species. Exploring how lampricides interact with lampreys and non-target fish physiology will allow us to further identify and explore physiological, biochemical and genetic differences between lampreys and non-targets. Such differences could then be used to develop novel lampricides that could target only the lampreys. RNAi technology, targeting and blocking the onset of metamorphosis in the larvae, thus preventing them from transforming into parasitic juveniles, has been proposed, but numerous questions remain on its specificity (Dunlop et al., 2018). The CRISPR/Cas technology, to create sterile lampreys or skew the sex ratio towards males has been explored in theory, but it is far from being deployed (SLIS III group discussion). Physiological and biochemical differences between lampreys and other fishes appear to be preferred over genetic control/targets of lampricides; that is, until the feasibility of more specific genetic means of controlling sea lamprey populations, is further established.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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