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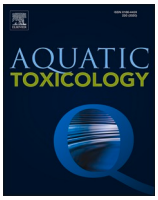

Contrasting physiological responses between invasive sea lamprey and non-target bluegill in response to acute lampricide exposure

M.J. Lawrence [a,*], D. Mitrovic [b], D. Foubister [b], L.M. Bragg [c], J. Sutherby [a], M.F. Docker [a], M. R. Servos [c], M.P. Wilkie [b], K.M. Jeffries [a]

[a] Department of Biological Sciences, University of Manitoba, Winnipeg, MB, R3T 2N2, Canada
[b] Department of Biology and Laurier Institute for Water Science (LIWS), Wilfrid Laurier University, Waterloo, ON, N2L 3C5, Canada
[c] Department of Biology, University of Waterloo, Waterloo, ON, N2L 3G1, Canada

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ABSTRACT

Control of invasive sea lamprey (*Petromyzon marinus*) in the Laurentian Great Lakes of North America uses lampricides, which consist of 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide. Lampricides are thought to inhibit aerobic energy synthesis, with TFM having a relatively greater selective action against lampreys. While the toxicity and physiological effects of TFM are known, the impacts associated with exposure to niclosamide and TFM:niclosamide mixtures are poorly characterized in fishes. Therefore, focusing on energy metabolism, we quantified the physiological responses of larval sea lamprey and bluegill (*Lepomis macrochirus*), a non-target, native species. Exposures consisted of each lampricide alone (TFM at the species-specific 24 h LC_{10}; niclosamide at 1.5% of the mixture's TFM concentration) or a mixture of the two (larval sea lamprey at TFM 24 h LC_{10} + 1.5% niclosamide; bluegill at sea lamprey's TFM 24 h $LC_{99.9}$ + 1.5% niclosamide) for 24 h. Tissues (brain, skeletal muscle, and liver) were sampled at 6, 12, and 24 h of exposure and assayed for concentrations of ATP, phosphocreatine, glycogen, lactate, and glucose and tissue lampricide levels. In larval sea lamprey, TFM had little effect on brain and skeletal muscle, but niclosamide resulted in a depletion of high energy substrates in both tissues. Mixture-exposed lamprey showed depletion of high energy substrates, accumulation of lactate, and high mortality rates. Bluegill were largely unaffected by toxicant exposures. However, bluegill liver showed lower glycogen and lactate under all three toxicant exposures suggesting increased metabolic turnover. Bluegill also had lower concentrations of TFM and niclosamide in their tissues when compared to lamprey. Our results indicate that lampricide toxicity in sea lamprey larvae is mediated through a depletion of high energy substrates because of impaired aerobic ATP synthesis. We also confirmed that non-target bluegill showed high tolerance to lampricide exposure, an effect potentially mediated through a high detoxification capacity relative to lampreys.

1. Introduction

In the early 20th century, the construction of waterways linking the Atlantic Ocean and the Laurentian Great Lakes of North America permitted the invasion and range expansion of the sea lamprey (*Petromyzon marinus*; Eshenroder 2014). By the 1930′s, populations of sea lamprey had become widespread throughout the Great Lakes (reviewed in Smith and Tibbles 1980; Siefkes 2017) resulting in severe ecological damage, and along with over-fishing, caused massive declines in the populations of a number of culturally, commercially, and recreationally important native fishes (Sonzogni et al., 1983; Hansen et al., 2016). In response, the Great Lakes Fishery Commission (GLFC) established a sea lamprey control program in which 3-trifluoromethyl-4-nitrophenol (TFM) and 2′,5-dichloro-4′-nitrosalicylanilide (niclosamide) were applied to larval lamprey-infested tributaries of the Great Lakes beginning in the late 1950′s (Siefkes 2017; Marsden and Siefkes, 2019; Wilkie et al., 2019). An advantage of TFM is that it selectively targets lampreys due to their relative inability to detoxify the agent (Lech and Statham 1975; Kane et al., 1994). Niclosamide, in its powdered form and more recently as an emulsifiable concentrate, was introduced in the 1960′s and co-applied with TFM in a 1–2% niclosamide mixture, which reduced the amount of lampricide required to cause lamprey mortality without loss of TFM specificity (Bills and Marking 1976; Marking and Bills 1985; Dawson 2003). A bottom acting formulation of niclosamide, granular

* Corresponding author.
E-mail address: m_lawrence27@live.ca (M.J. Lawrence).

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Bayluscide®, is used for treating large lentic areas and deeper rivers with high rates of discharge and for population surveys (Dawson 2003; Wilkie et al., 2019).

Structurally, TFM is a phenol-based weak acid compound with an ionizable hydroxyl group and a negative log acid dissociation constant (pKa) of 6.07–6.38 (Hubert 2003; McConville et al., 2016), that primarily exists in the ionized state at typical environmental pH's (i.e., pH 6.5–8.5). In fishes, the branchial epithelium is believed to be the primary route of uptake of TFM (Hunn and Allen 1974; Hlina et al., 2017; Hepditch 2018; Tessier et al., 2018), with deposition of the toxicant in tissues such as the muscle, blood, liver, and brain of teleosts and sea lamprey (Lech and Statham 1975; Shultz et al. 1979; Vue et al., 2002; Hubert et al., 2005). Its mode of action is by interfering with ATP production by uncoupling oxidative phosphorylation in the mitochondria (Kawatski et al., 1974; Niblett and Ballantyne 1976; Birceanu et al., 2011; Huerta et al., 2020). In fishes, metabolic processes are greatly perturbed during acute TFM exposure, with animals exhibiting an increased dependency on anaerobic metabolic pathways with a corresponding reduction in tissue stores of glycogen and high energy phosphates (e.g., Phosphocreatine [PCr], ATP) resulting in mortality when these reserves are depleted after 9–12 h of exposure (Wilkie et al., 2007; Birceanu et al., 2009, 2014). However, most of the physiological profiling of TFM exposure to date has been assayed in sea lamprey and in domestic rainbow trout (*Oncorhynchus mykiss*). As such, the physiological responses to lampricide exposure in native teleost species in the Great Lakes is sparse despite there being considerable evidence of a high degree of variation in TFM sensitivity amongst native species (see Table S1; Wilkie et al., 2019). This variation in sensitivity to TFM may stem from a differential capacity of taxa to detoxify via phase II biotransformation, specifically glucuronidation via UDP-glucuronosyltransferase (Lech and Statham 1975; Kane et al., 1994; Bussy et al., 2018a,b) and sulfation via phenol sulfotransferase (James 1987; Bussy et al., 2018a,b).

Niclosamide is a chlorinated salicylanilide that, like TFM, exists predominantly in the ionized state in most water bodies (pKa = 6.03–6.25; McConville et al., 2016). Uptake is presumed to occur across the branchial epithelium (Dawson 2003; Wilkie et al., 2019, Wilkie et al., 2021). Deposition of niclosamide has been observed in the plasma, brain, liver, heart, white muscle, and bile of teleost fishes (Statham and Lech 1975; Shultz and Harman 1978; Dawson et al., 1982; Hubert et al., 2005), with elimination via urinary routes and biliary (faecal) excretion. Surprisingly, no equivalent information is available for sea lamprey, nor is there much information pertaining to the physiological effects of niclosamide exposure on fishes. Further, there are knowledge gaps in its specific mechanism of action in lampreys and fishes. Historically, niclosamide was used to treat parasitic cestode (tapeworms) and trematode (flukes) infections in humans, pets, and livestock (Köhler 2001; McKellar and Jackson 2004), and is still used as a molluscicide to kill snails that serve as vectors for the parasitic flatworms that cause schistosomiasis in humans (Lardans and Dissous 1998; Joubert et al., 2001; Zhao et al., 2015). Like TFM, niclosamide is believed to uncouple mitochondrial oxidative phosphorylation (Weinbach and Garbus 1969; Jones 1979; Pampori et al., 1984; Pearson and Hewlett 1985). In tapeworms (*Cotugnia digonopora*), niclosamide exposure resulted in lactate accumulation, reduced CO_2 production, and reduced glycogen content suggesting impaired aerobic ATP production (Pampori et al., 1984). Niclosamide is also hypothesized to uncouple oxidative phosphorylation in fishes with physiological effects that are less well-defined, but likely to be similar to those of TFM (reviewed in Wilkie et al., 2019). While information is scarce, a recent study on embryonic zebrafish (*Danio rerio*) chronically exposed to niclosamide (5 d) exhibited transcriptome profiles that suggested impaired lipid metabolism and steroidogenesis, which corresponded with lower whole animal ATP and lipid concentrations (Zhu et al., 2020).

The purpose of this study was to determine if niclosamide interferes with aerobic energy metabolism in a similar manner to TFM. We predicted that, like TFM, niclosamide would lead to the depletion of high energy stores such as ATP and PCr, and glycogen in larval sea lamprey (i.e., the target species) and in a non-target teleost, the bluegill (*Lepomis macrochirus*). Bluegill have a wide distribution throughout the Great Lakes basin where lampricides are applied (Scott and Crossman 1973), and are amongst the most tolerant fishes to lampricides (see Table S1; Fig. 8 in Wilkie et al., 2019), making it an excellent comparative model against sea lamprey. We predicted that larval sea lamprey would be more physiologically perturbed under lampricide exposure, such as lower glycogen, ATP, and PCr reserves, and higher lactate levels, when compared to bluegill, which has a higher capacity to detoxify TFM than lamprey (Kane et al., 1994; Bussy et al., 2018a). In testing this, we exposed each species to sublethal concentrations of niclosamide and TFM (the respective 24 h LC_{10}'s; see Table 1 for summary) over 24 h to allow us to better characterize the response of bluegill and lamprey to each agent separately. We then addressed how each species responded to a mixture of the two compounds, to more accurately mimic TFM application and exposure scenarios in Great Lakes tributaries and to better understand how these lampricides interact.

2. Materials and methods

2.1. Animal collection and holding

Larval sea lamprey ($n = 568$; mass = 1.5 ± 0.03 g; total length [TL] = 104.9 ± 0.69 mm) were collected by United States Fish and Wildlife Service personnel, using pulsed DC electrofishers (ABP-2 Electrofisher, Electrofishing Systems, LLC, Madison, WI, USA), from tributaries draining into Lake Huron and held at the U.S. Geological Survey's Hammond Bay Biological Station, Millersburg, Michigan. After 2–4 weeks, the animals were shipped in oxygen saturated water in 70 L coolers to Wilfrid Laurier University (Waterloo, ON, Canada) in May 2018, where they were held in large, flow-through and aerated tanks supplied with dechlorinated tap water (~100 L; replacement rate ~ 0.5 L min^{-1}; pH 8.1–8.2, alkalinity ~255 mg l^{-1} as $CaCO_3$, temperature ~14°C), under a 12 h light:12 h dark photoperiod. Stocking density of larvae was kept at 150 individuals per tank per 100 L. As larval lamprey naturally burrow in soft sediments (Scott and Crossman 1973; Dawson et al., 2015), there was a layer of sand in the bottom of each tank (~8–10 cm deep) (Birceanu et al., 2009). The larval lamprey were

Table 1
Nominal and measured concentrations of the lampricides in the water, averaged across all time points, used in the physiological exposure series in this study in both sea lamprey and bluegill at their 24 h LC_{10}. The exception to this is for the bluegill mixture group where these concentrations represent the sea lamprey's 24 h $LC_{99.9}$. All values are expressed in mg L^{-1}. Measured concentrations are as mean ± SEM. Note, as all sea lamprey perished before 24 h of exposure, TFM:niclosamide mixture concentrations were only measured over the first 12 h of exposure for this treatment group.

Species	Toxicant							
	TFM		*Niclosamide*		*Mixture*			
					TFM		*Niclosamide*	
	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured
Sea lamprey	2.21	2.18 ± 0.02	0.033	0.0224 ± 0.0012	2.21	2.54 ± 0.07	0.033	0.0197 ± 0.0012
Bluegill	22.06	21.19 ± 0.07	0.068	0.0583 ± 0.0049	4.5	4.47 ± 0.01	0.068	0.0432 ± 0.0025

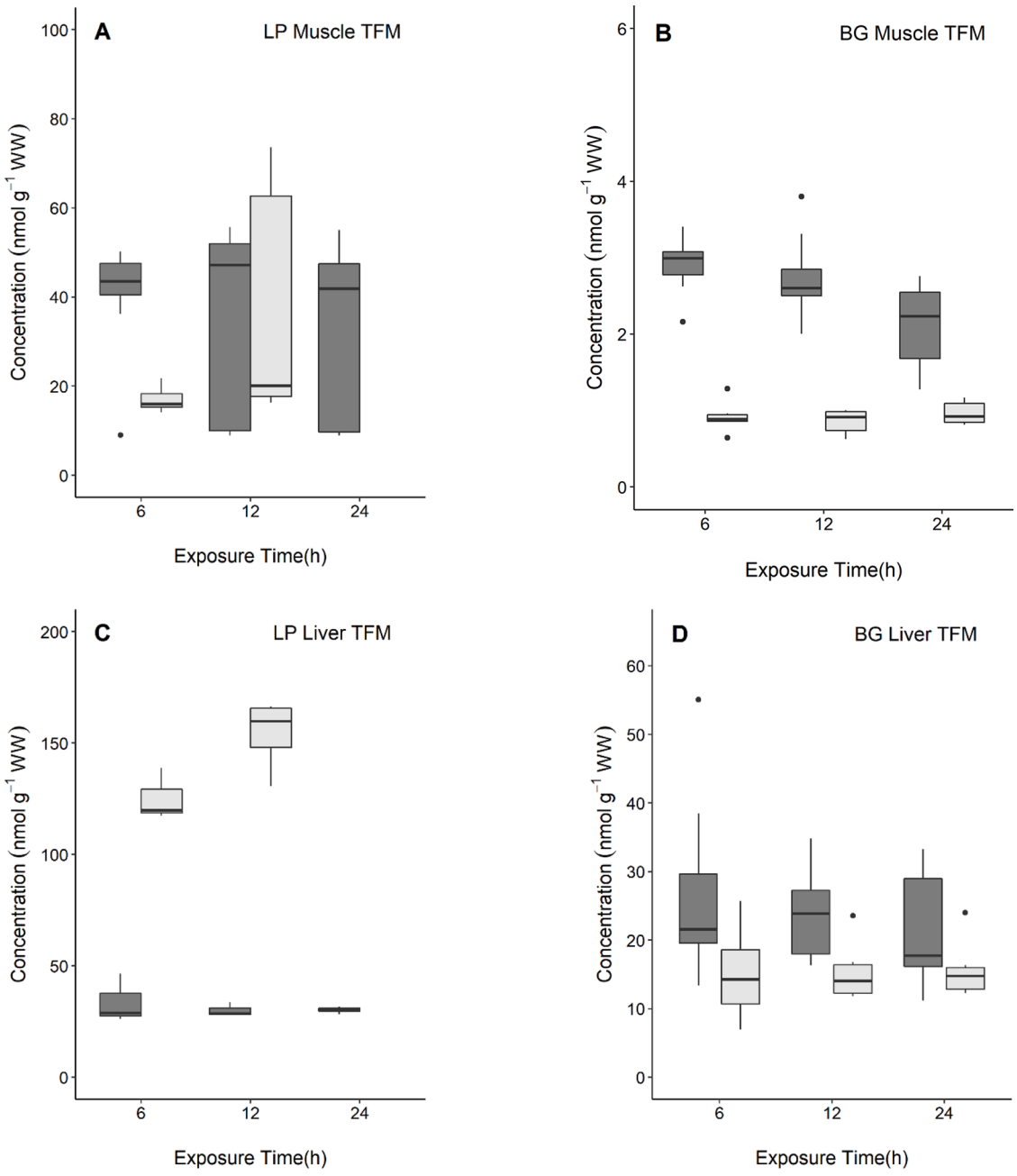

Fig. 1. Concentrations of TFM in the skeletal muscle (A,B) and liver (C,D) of sea lamprey (LP) and bluegill (BG) exposed to TFM (dark grey), and a mixture of TFM-niclosamide (1.5%; light grey) at 6, 12, and 24 h of exposure. Note the different scales on the Y axes between the two species. Box plots show the median value delineated by the interquartile range (1st to 3rd quantile), and an accompanying whisker represents 1.5x beyond this range. Suspected statistical outliers are presented as black circles outside of the interquartile range. Data were analysed using 95% bias-corrected and accelerated bootstrap (BCa) confidence intervals with pairwise comparisons being made against control values for an individual treatment group. All values presented were statistically different from control values. No comparisons were made for TFM concentrations in niclosamide-exposed fish. 24 h values for sea lamprey exposed to the TFM:niclosamide (1.5%) mixture are not presented here as the fish did not survive beyond 12 h of exposure.

acclimated to laboratory conditions for at least 2 months prior to experimental trials, during which they were fed weekly doses of commercial baker's yeast (1 g of yeast per larva; Holmes and Youson 1994; Wilkie et al., 1999).

Juvenile bluegill ($n = 200$; mass = 25.5 ± 0.8 g; TL = 97.3 ± 0.9 mm) were purchased from Kinmount Fish Farm (Kinmount, ON, Canada) in September 2018 and held in a single large tank (~1000 L) in dechlorinated tap water at 12–14 °C, and were fed ad libitum daily with commercial fish feed (EWOS #1, Cargill, ON, Canada) and bloodworms. All experimental series and animal care procedures were conducted in accordance with Canadian Council of Animal Care guidelines and were approved by the Wilfrid Laurier University Animal Care Committee (Animal Use Protocol No. R18001).

2.2. Lampricides

All experiments were conducted using field grade TFM (35% active ingredient dissolved in isopropanol; Clariant, Griesheim, Germany) and/or niclosamide (emulsifiable concentrate containing 16.9% niclosamide ethanolamine salt mixture; Coating Place Inc., Verona, WI, USA).

2.3. TFM acute toxicity tests

The 24 h LC_{10} (lethal concentration for 10% of the individuals) of TFM for both sea lamprey larvae and bluegill were determined using a similar approach to that outlined in Birceanu et al. (2009). Briefly, exposures were conducted in glass aquaria (10 L for sea lamprey larvae, 14 L for bluegill) filled with aerated, dechlorinated water, and maintained at ~ 14–15 °C by immersion in a water bath (Coralife ¼ HP Aquarium Chiller, Franklin, WI, USA). In each aquarium, stocking density was set at nine and four individuals per test tank for lamprey and bluegill, respectively. Sea lamprey larvae were exposed to 6 concentrations of TFM (0.5, 1, 1.5, 2, 2.5, 5 mg L^{-1}) in triplicate (i.e. $n = 27$ per concentration). Bluegill were exposed to seven TFM concentrations (5, 15, 20, 22.5, 25, 40, 60 mg L^{-1}) in triplicate ($n = 12$ per concentration). Concentrations of TFM were measured using water samples collected at 0 and 24 h of exposure to ensure that the concentrations were stable throughout the experiment (see below for details). The number of mortalities was recorded hourly over the first 8h of TFM exposure, and again at 10, 12, and 24 h.

2.4. Part I: Effects of sublethal toxicant exposure to TFM and niclosamide

In the sublethal exposure, both species of fish were exposed to one of three experimental treatments: a control (i.e., no toxicant, dechlorinated tap water), TFM, or niclosamide. Niclosamide in all physiological exposures was set at 1.5% of the mixture's TFM concentration, which is consistent with proportions currently used when TFM and niclosamide are used in combination in the field (1–2% niclosamide; Dawson 2003; Boogaard et al., 2007). Briefly, nominal niclosamide concentrations used in niclosamide-exposures were 0.033 mg L^{-1} and 0.068 mg L^{-1} for sea lamprey larvae and bluegill, respectively. More detailed information concerning the target and measured concentrations of each of the toxicants is in Table 1.

All exposures were conducted in glass aquaria using the same experimental setup as described in the TFM toxicity tests (see above). Sea lamprey larvae and bluegill were kept in groups of 6 and 2–3 individuals per tank, respectively, in triplicate ($n = 18$, $n = 9$, respectively). Sampling occurred at 6, 12, and 24 h of TFM or niclosamide exposure, at which time each individual fish was quickly netted from the tank and placed into a bath containing a lethal dose of buffered tricaine methanesulfonate (MS-222; Syndel, Port Alberni, BC, Canada; 1.5 g L^{-1}

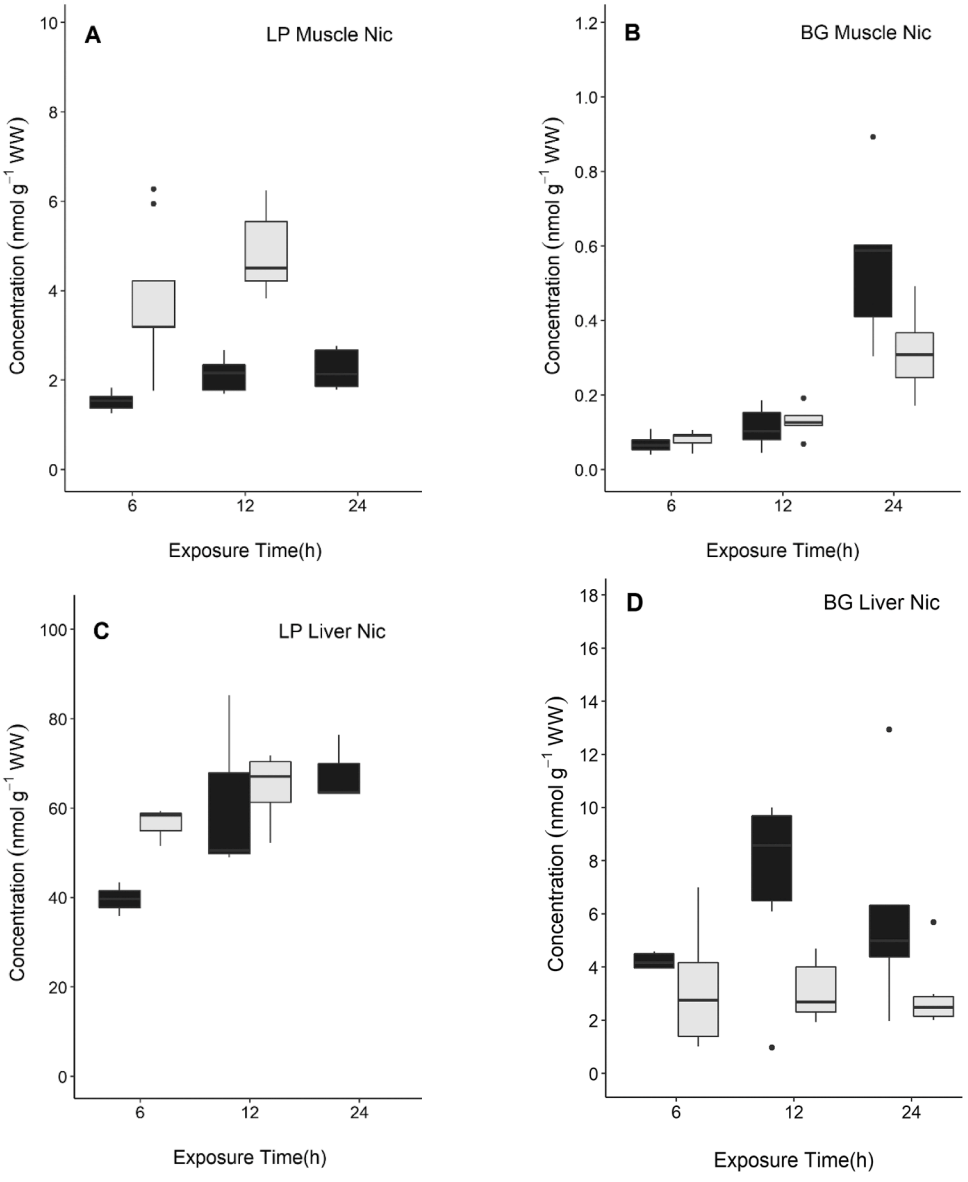

Fig. 2. Concentrations of niclosamide (Nic) in the skeletal muscle (A,B) and liver (C,D) of sea lamprey (LP) and bluegill (BG) exposed to niclosamide (black), and a mixture of TFM-niclosamide (1.5%; light grey) at 6, 12, and 24 h of exposure. Note the different scales on the Y axes between the two species. Box plots show the median value delineated by the interquartile range (1st to 3rd quantile), and an accompanying whisker represents 1.5x beyond this range. Suspected statistical outliers are presented as black circles outside of the interquartile range. Data were analysed using 95% bias-corrected and accelerated bootstrap (BCa) confidence intervals with pairwise comparisons being made against control values for an individual treatment group. All values presented were statistically different from control values. No comparisons were made for niclosamide concentrations in TFM-exposed fish. 24 h values for sea lamprey exposed to the TFM: niclosamide (1.5%) mixture are not presented here as the fish did not survive beyond 12 h of exposure.

MS-222 with 3.0 g L^{-1} $NaHCO_3$). Gill, brain, liver, and skeletal muscle tissue were then quickly excised, flash frozen in liquid nitrogen, and stored at −80 °C for later analysis of tissue energy stores (ATP, PCr, glycogen, glucose), and lactate, and TFM and niclosamide concentrations in the muscle and liver only. Sea lamprey livers were not assessed for any of the energy metabolites in this study as liver tissue was prioritized towards whole transcriptome analyses as part of a complementary study and the quantification of parent TFM and niclosamide (Fig. 1 and 2). Furthermore, the responses of the sea lamprey liver under acute lampricide exposure have been characterized previously (e.g., Birceanu et al., 2009; Clifford et al., 2012; Ionescu et al., 2021).

2.5. Part II: Exposure to the TFM:niclosamide mixture

To address the physiological responses of bluegill and larval lamprey to a mixture of TFM and niclosamide, we exposed bluegill to the 24 h $LC_{99.9}$ (lethal concentration for 99.9% of the individuals) of TFM to sea lamprey larvae, mixed with 1.5% niclosamide (i.e., target concentrations = 4.5 mg L^{-1} TFM + 0.068 mg L^{-1} niclosamide). This was done to more closely mimic the concentrations likely to be encountered by bluegill during lampricide applications in which the TFM:niclosamide dose is based on the corresponding 9 h $LC_{99.9}$, which is also referred to as the minimum lethal concentration (MLC) of TFM (Brege et al., 2003). Sea lamprey larvae were retained at their LC_{10} for TFM as described above. Experimental series were conducted in the same manner as outlined in Part I involving tissue sampling at 6, 12, and 24 h, except that sampling in the 12 h sea lamprey larvae group was modified as mortality began occurring after ~9–10 h of exposure. Mortality likely has little bearing on tissue metabolites given prior work has indicated these metrics are slow to change post-mortem (at our time scales; Thomas et al., 1999; Forgan et al., 2010; Wosnick et al., 2017) and that linear modelling showed that metabolite concentration and time were largely unrelated to one another in our study (Fig. S1, S2).

2.6. Energy reserves and lactate analyses

Analyses for tissue (brain, muscle, liver) energy stores and lactate were conducted similarly to that outlined previously (Wilkie et al., 2001; Birceanu et al., 2014; Clifford et al., 2012). Briefly, the frozen tissues were first ground to a fine powder under liquid nitrogen using a mortar and pestle and the resulting homogenate deproteinized using 8% perchloric acid (PCA) solution containing 1 mmol L^{-1} EDTA (Lawrence et al., 2015), with the resulting supernatants buffered back to physiological pH (pH ~ 8.0) using either 3 mol L^{-1} K_2CO_3 solution (glycogen assays only) or a 2 mol L^{-1} KOH solution (lactate, ATP, and PCr). All assays were conducted in duplicate at room temperature (~22 °C), followed by quantification on a microplate spectrophotometer (SpectraMax 190; Molecular Devices, San Jose, CA, USA) at a wavelength of 340 nm. Unless otherwise noted, all reagents were sourced from Sigma-Aldrich (Oakville, ON, Canada). Due to the small mass of larval sea lamprey brain (2–5 mg), the brains were pooled from two individuals.

Glycogen concentrations were determined using tissue supernatants that had been incubated at 37 °C for 2 h in 1% amyloglucosidase plus acetate buffer (2 mol L^{-1}, pH 4.5). Following incubation, the reaction was terminated using a 70% PCA solution and re-balanced to physiological pH (3 mol L^{-1} K_2CO_3). Total glucose content of the hydrolysed samples was then determined using a hexokinase-linked glucose assay (Bergmeyer, 1974, 1985) with glycogen concentration expressed in μmol glucosyl units g^{-1} wet mass. Free tissue glucose was determined on tissue supernatants to which no amyloglucosidase was added, which was then subtracted from the total glucose determined in the amyloglucosidase treated samples. Whole tissue lactate, ATP, and PCr were determined by enzyme linked assays as outlined in previous works (Bergmeyer 1985; Wilkie et al., 1997; Clifford et al., 2012).

2.7. Water sample collection and quantification of lampricides

Water samples were collected from all experimental tanks containing niclosamide or a mixture of niclosamide and TFM at 0 h (right after the addition of lampricides), followed by sampling at 3, 6, 12, and 24 h for most of the exposures. Water samples were collected in 20 mL glass scintillation vials and stored at −20 °C until analysis.

Water TFM concentration was measured spectrophotometrically on unmodified water samples at a wavelength of 490 nm (NovaSpec II spectrophotometer, Pharmacia Biotech, Cambridge, UK; Fisheries and Oceans Canada IOP # 012.4). Niclosamide concentrations were measured using liquid chromatography mass spectroscopy (LC-MS/MS). Defrosted samples were vortexed for 20 s. From each sample, 5 mL of water was transferred to a new clean glass test tube and spiked with niclosamide-(2-chloro-4-nitrophenyl-13C6) hydrate (Sigma-Aldrich, St Louis, USA; NIC—C13) as the internal standard (200 μg L^{-1}). Quality control samples (matrix spike) were prepared with 5 mL of MilliQ water in a glass test tube spiked with NIC—C13 standard (200 μg L^{-1}) and with 100 μg L^{-1} of NIC. Blanks were prepared with 5 mL of MilliQ water spiked with 200 μg L^{-1} NIC—C13. All samples were vortexed again for an additional 20 s after spiking. A vacuum filtration apparatus (250 mL flask with 15 mL reservoir, Sigma-Aldrich) was then used to filter samples (0.45 μm glass fibre filters; Pall Corporation, Michigan, USA). From each of the filtered samples, 1 mL of eluent was transferred to a labelled 2 mL amber glass vial and analysed by LC-MS/MS as detailed below. The left-over sample volume was stored at −20 °C.

2.8. Tissue sample preparation and extraction of lampricides

The liver and muscle samples were processed for metabolite quantification by grinding the tissue to a fine powder under liquid nitrogen using a mortar and a pestle. Approximately 100 mg of sample (108.9 ± 13.8 mg) was collected into a 2 mL microcentrifuge tube and diluted with acetonitrile (ACN) with 1% formic acid (1:1 ratio of sample to solvent). Each tissue sample was then spiked with 200 μg L^{-1} of the internal standard (NIC—C13). Quality control samples (matrix spike) and blanks were prepared with unexposed control tissues spiked with NIC—C13 standard (200 μg L^{-1}) and with 100 μg L^{-1} of NIC and TFM (omitted in blanks). When processing muscle tissues, two stainless steel beads (2.4 mm, Omni International: Kennesaw, GA, USA) were added and tissues were homogenized using a Bead Ruptor Elite (Omni International, Kennesaw, GA, USA) for 2 min at 2.6 m s^{-1}. Liver tissues were homogenized using a handheld motorized homogenizer (Gerresheimer Kimble Kontes LLC, Dusseldorf, Germany). The volume of each tube was topped up to 500 μL with ACN with 1% formic acid and refrigerated at 4 °C for 20 min to facilitate protein precipitation. Afterwards, the tubes were centrifugated at 12,000 RCF at 4 °C for 10 min. Solid phase extraction (SPE) was performed using Captiva EMR-Lipid 96 well plates (Agilent Technologies, Santa Clara, CA) with a 96-well plate vacuum manifold (Agilent Technologies, Santa Clara, CA). Samples were eluted into a 2 mL reservoir well plate at a rate of 1 drop per 3–5 s. The eluted sample was then transferred into a 200 μL glass insert in a 2 mL amber glass vial and analysed by LC-MS/MS. Due to size limitations of lamprey liver, the samples had to be pooled (3 livers per sample; average weight 20.8 ± 2.8 mg) with the total volume being corrected to 250 μL and only 100 μL were placed in a vial for injection. All other steps were as described above.

2.9. LC-MS/MS analysis

Samples were measured for the analytes of interest using an Agilent 1260 HPLC with 6460 triple quad mass spectrometer with an Agilent Jetstream ESI source in negative ionization mode. The calibration curve ranged from 0 μg L^{-1} to 1000 μg L^{-1} for each standard. Standards and spikes for water analysis were made up with methanol (Optima, LC/MS grade, Fisher Scientific, Ottawa, Canada). Standards and spikes for the

Table 2
LC-MSMS QQQ mobile phase parameters used in the analysis of water niclosamide and tissue niclosamide and TFM concentrations. Mobile phase A represents MilliQ water and mobile phase B represents acetonitrile.

Time (min)	% Mobile Phase A	% Mobile Phase B
0	80	20
1	80	20
10	0	100
12	0	100
12.1	80	20

tissue analysis were made using ACN (Optima, LC/MS grade, Fisher Scientific, Ottawa, Canada) instead of methanol in order to facilitate protein precipitation and avoid gelation.

An Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm) was used to chromatographically separate the analytes. Samples were injected at 10 μL sample volume, at 35 °C constant temperature and a flow rate of 0.8 mL min^{-1}. Information concerning the phase gradients can be found in Table 2. The post run re-equilibration time was 5 min. The instrument source and compound parameters can be found in Supplemental Materials. All data was processed using Mass Hunter Quantitative Analysis software (Agilent, Santa Clara, USA) version B.05.02.

2.10. Statistical analyses

All statistical analyses were conducted in R (Version 3.5.1) and R Studio (Version 1.1.463; RStudioTeam 2020). Unless otherwise noted, statistical significance was accepted at $\alpha = 0.05$ with data presented as mean ± 1 SEM (n). For determining the 24 h LC_{10}, we used a log probit analysis (Sprague 1969) through the R package 'ecotox' (Hlina et al., 2019; Muhametsafina et al., 2019). For the physiological data, validity of model assumptions pertaining to normality and homoscedasticity were assessed using a Shapiro Test and a Levene's Test, respectively. Plots of the residuals (homoscedasticity) and QQ plots (normality) were also used to visually verify test results. Data were analysed using a two-way analysis of variance (ANOVA; Type I) model with the main effects of treatment group (i.e., toxicant; 2 levels) and exposure duration (3 levels), and the interaction of these two factors. A single toxicant was only compared against controls with no toxicants being compared to one another (i.e., TFM vs control, niclosamide vs control, etc.). In instances where the data violated model assumptions, data was transformed using $\log_{10}$, x^2, square root, or 1/x transformations, where appropriate. When statistical significance was detected in the base model, a Tukey's honestly significant difference (HSD) was used. Tissue levels of TFM and niclosamide were statistically assessed using a different approach as the data routinely violated normality and homogeneity of variance. Rather, data were analysed using visual inspection of the 95% confidence intervals for which control-treatment comparisons that had non-overlapping intervals were considered to be statistically different from one another (Weerahandi 1995; Cumming and Finch 2005). Our analyses were restricted to looking at only the main effect of treatment as both time and the interaction term were not of interest in this context. Comparisons were not made for concentrations of TFM in niclosamide-treated fish and for concentrations of niclosamide for

Table 3
Statistical output for the measurement of toxicant concentrations (TFM or niclosamide [Nic]) in the skeletal muscle and liver of sea lamprey larvae and bluegill exposed to either a control, TFM, niclosamide, or a TFM:niclosamide (1.5%) mixture. Analyses were conducted using exposure group as the sole main effect and made use of 95% bias-corrected and accelerated bootstrap (BCa) confidence intervals. Statistically significant effects of exposure group are denoted by bolded text and were accepted when control and treatment groups had non-overlapping confidence intervals.

Species	Tissue	Toxin Measured	Exposure Group	N	Mean	Bootstrap Mean	Bca Lower	Bca Upper
Sea lamprey								
	Muscle							
		[TFM]	*Control*	24	0.23	0.23	0.15	0.36
			TFM	27	36.80	36.80	**29.70**	**42.20**
			Control	16	0.24	0.24	0.15	0.42
			Mixture	20	27.50	27.50	**20.40**	**38.70**
		[Nic]	*Control*	24	0.05	0.05	0.03	0.09
			Niclosamide	26	1.95	1.95	**1.79**	**2.14**
			Control	16	0.05	0.05	0.02	0.11
			Mixture	20	4.39	4.39	**3.86**	**4.89**
	Liver							
		[TFM]	*Control*	11	0.43	0.43	0.26	0.69
			TFM	9	31.30	31.40	**28.90**	**37.60**
			Control	7	0.45	0.45	0.21	0.82
			Mixture	7	142.00	142.00	**128.00**	**156.00**
		[Nic]	*Control*	11	0.36	0.36	0.19	0.68
			Niclosamide	8	58.40	58.40	**48.60**	**69.80**
			Control	7	0.38	0.38	0.17	0.84
			Mixture	7	61.10	61.10	**55.70**	**66.70**
Bluegill								
	Muscle							
		[TFM]	*Control*	24	0.02	0.02	0.02	0.03
			TFM	26	2.61	2.61	**2.37**	**2.82**
			Control	24	0.02	0.02	0.02	0.03
			Mixture	17	0.92	0.92	**0.84**	**1.00**
		[Nic]	*Control*	24	0.00	0.00	0.00	0.00
			Niclosamide	17	0.23	0.23	**0.14**	**0.38**
			Control	24	0.00	0.00	0.00	0.00
			Mixture	17	0.18	0.18	**0.13**	**0.25**
	Liver	*[TFM]*	*Control*	23	0.37	0.37	0.29	0.49
			TFM	25	24.10	24.10	**21.10**	**28.90**
			Control	23	0.37	0.37	0.29	0.49
			Mixture	18	15.40	15.40	**13.30**	**18.00**
		[Nic]	*Control*	23	0.02	0.02	0.01	0.04
			Niclosamide	16	5.99	6.01	**4.63**	**7.65**
			Control	23	0.02	0.02	0.01	0.04
			Mixture	18	3.08	3.08	**2.49**	**3.96**

TFM-treated fish. Our approach used bootstrap confidence intervals, specifically bias corrected and accelerated confidence (BCa) intervals, which are rather robust to traditional assumption violations (Carpenter and Bithell 2000). Analyses were conducted using the R package 'rcompanion' (version 2.3.26; Mangiafico 2020) and used 10,000 sampling iterations.

3. Results

3.1. Part I: effects of sublethal toxicant exposure to TFM and niclosamide

3.1.1. TFM and niclosamide accumulation in liver and muscle

The summary statistics for tissue TFM and niclosamide accumulation can be found in Table 3. In sea lamprey exposure to a TFM concentration of 2.2 mg L^{-1}, the 24-h LC_{10}, resulted in marked accumulation in both the muscle and liver, in which the TFM concentration averaged 30–41 nmol g^{-1} ww in both tissues (Fig. 1A, C). Despite being exposed

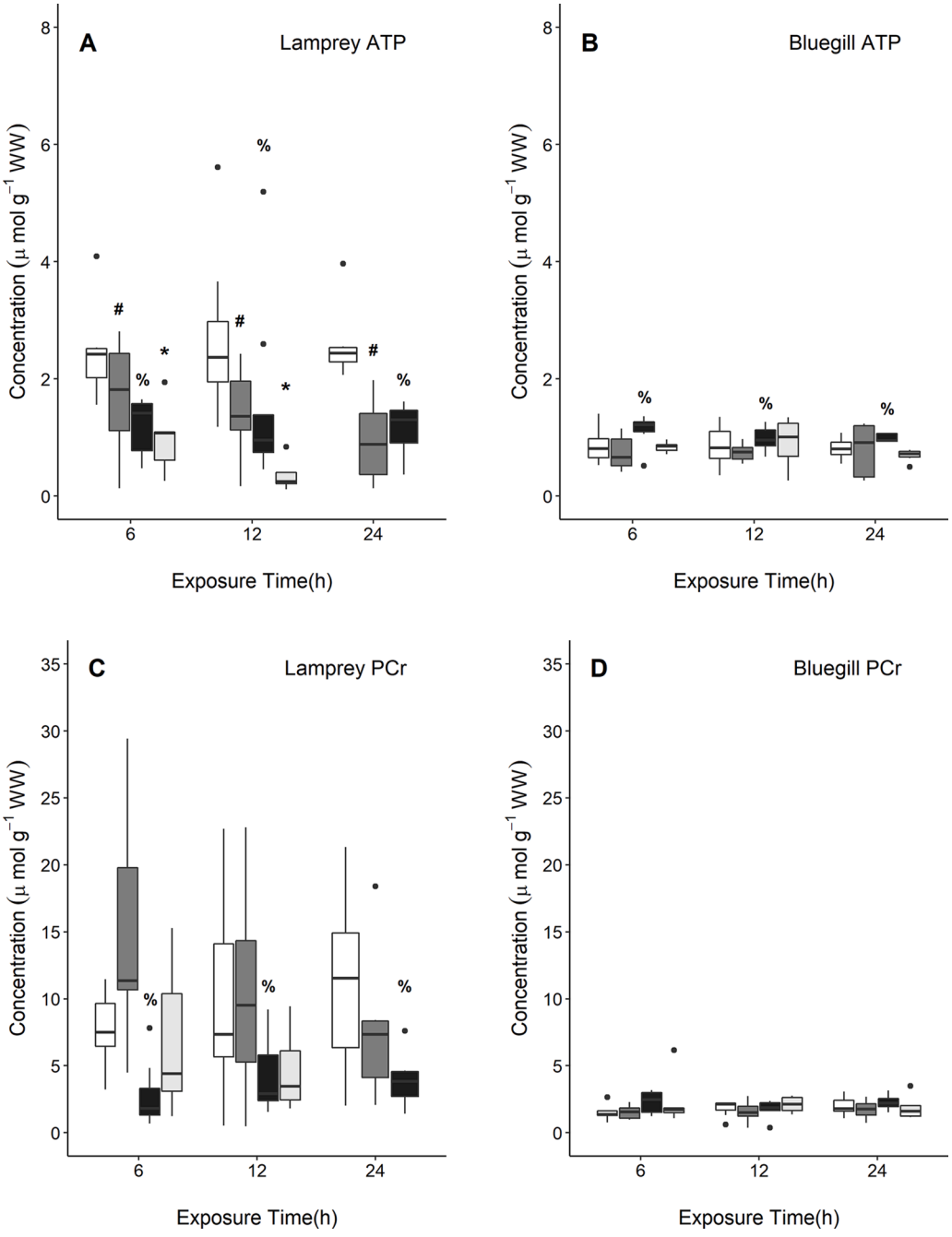

Fig. 3. Concentrations of brain ATP (A, B) and PCr (C, D) in larval sea lamprey and bluegill exposed to, from left to right, control (white bars), TFM (dark grey), niclosamide (black) and a mixture of TFM-niclosamide (1.5%; light grey) at 6, 12, and 24 h of exposure. Box plots show the median value delineated by the interquartile range (1st to 3rd quantile), and an accompanying whisker represents 1.5x beyond this range. Suspected statistical outliers are presented as black circles outside of the interquartile range. Data were analysed using a type I two-way ANOVA coupled with a Tukey HSD post-hoc test with pairwise comparisons being made against control values only. Significant treatment level effects ($\alpha = 0.05$) within a toxicant-control comparison are denoted by unique characters (# TFM vs control, % niclosamide vs control, * mixture vs control). 24 h values for sea lamprey exposed to the TFM:niclosamide (1.5%) mixture are not presented here as fish did not survive beyond 12 h of exposure.

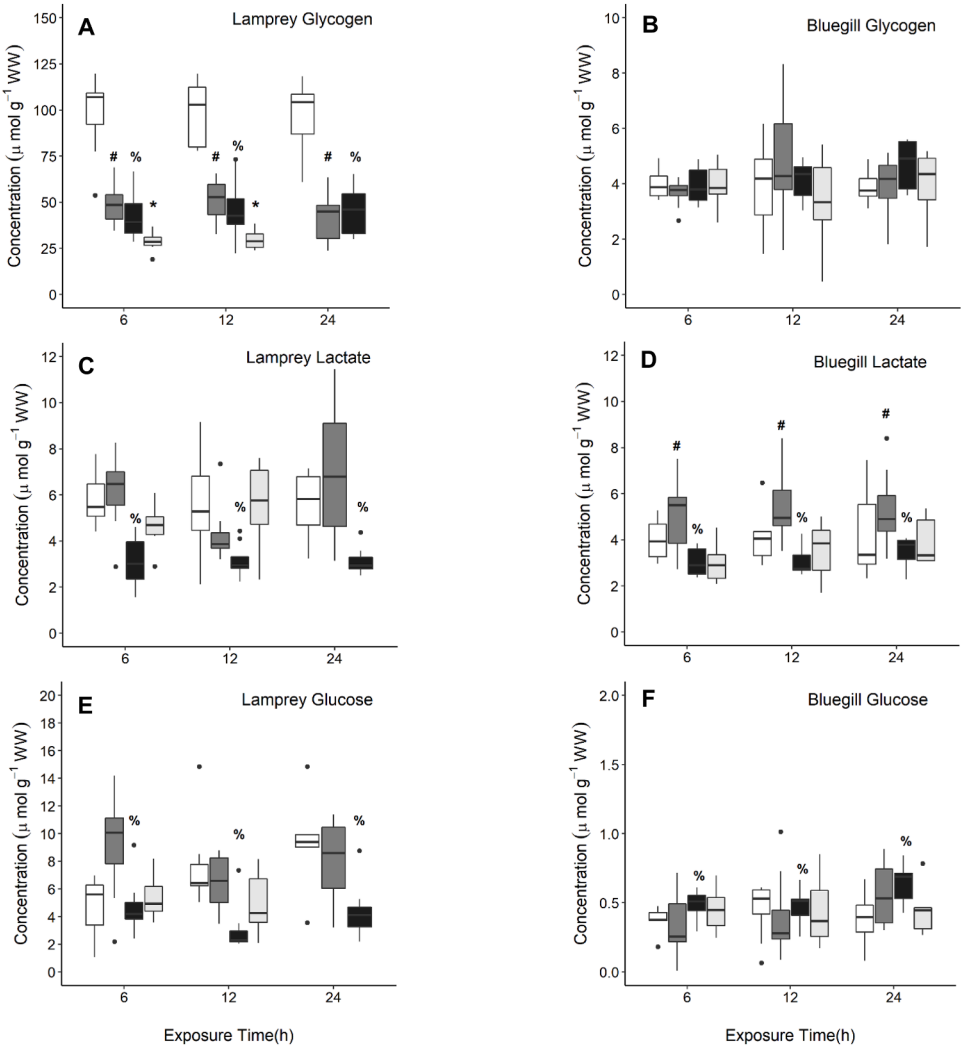

Fig. 4. Concentrations of brain glycogen (A, B), lactate (C, D), and glucose (E, F) in larval sea lamprey and bluegill exposed to, from left to right, control (white bars), TFM (dark grey), niclosamide (black) and a mixture of TFM-niclosamide (1.5%; light grey) at 6, 12, and 24 h of exposure. Box plots show the median value delineated by the interquartile range (1st to 3rd quantile), and an accompanying whisker represents 1.5x beyond this range. Suspected statistical outliers are presented as black circles outside of the interquartile range. Data were analysed using a type I two-way ANOVA coupled with a Tukey HSD post-hoc test with pairwise comparisons being made against control values only. Significant treatment level effects ($\alpha = 0.05$) within a toxicant-control comparison are denoted by unique characters (# TFM vs control, **%** niclosamide vs control, * mixture vs control). A significant interactive effect (not shown) was observed for sea lamprey brain glucose when exposed to TFM ($F = 3.88$; $P = 0.029$), niclosamide ($F = 5.42$; $P = 0.008$), and the mixture ($F = 4.84$; $P = 0.037$). In sea lamprey brain glucose, a significant main effect of niclosamide alone was observed as well ($F = 17.29$; $P < 0.001$). 24 h values for sea lamprey exposed to the TFM: niclosamide (1.5%) mixture are not presented here as fish did not survive beyond 12 h of exposure.

to a 10-fold higher concentration of TFM, 21.2 mg L^{-1} (Table 1), muscle TFM concentrations were more than 90% lower in bluegill muscle, ranging from 2.1 to 2.9 g^{-1} ww (Fig. 1B). Unlike in the sea lamprey, TFM concentrations were 9–10-fold greater in the liver than in the muscle, fluctuating around 23 nmol g^{-1} ww (Fig. 1D). Notably, these concentrations are about 20–28% lower than measured in the lamprey liver, despite the much higher TFM concentration.

Similar trends were noted following exposure to niclosamide, in which the accumulation of niclosamide in the muscle of lamprey was approximately 20-fold greater than observed in the bluegill muscle over the first 12 h of exposure. In lamprey exposed to 0.02 mg L^{-1} niclosamide, muscle niclosamide concentrations fluctuated around 2 nmol g^{-1} ww whereas in the bluegill, niclosamide concentrations were less than 0.2 nmol g^{-1} ww at 6 and 12 h of exposure, before increasing to 0.6 nmol g^{-1} ww at 24 h, but still well below concentrations measured in the lamprey muscle (Fig. 2A, B). In the liver, niclosamide concentrations were 8 to 11-fold higher in the lamprey than in the bluegill at similar time points (Fig. 2C,D).

3.1.2. *TFM effects*

In larval sea lamprey, TFM treatment resulted in 31–64% lower brain ATP concentration (Fig. 3A; $df = 1$; $F = 16.01$; $P < 0.001$) and 48–57% lower brain glycogen concentration (Fig. 4A; $df = 1$; $F = 98.44$; $P < 0.001$) relative to the controls. In contrast, TFM treatment did not affect brain PCr concentration (Fig. 3C), and there were likewise no significant differences in lactate concentration, which fluctuated between ~4 and 7 μmol g^{-1} ww (Fig. 4C). With respect to brain glucose concentrations, there was a significant interaction between time and treatment (Fig. 4E; $df = 2$; $F = 3.88$; $P = 0.029$), but no significant differences were observed after the post-hoc test.

In bluegill, baseline brain ATP and PCr concentrations were much lower than in sea lamprey larvae (average fold differences: lamprey ATP = 3.1x greater; lamprey PCr = 5.3x greater), and neither was affected by TFM exposure (Fig. 3B, D). Control brain glucose and glycogen concentrations were also noticeably lower than in control sea lamprey larvae (e.g., ~98 μmol glycogen g^{-1} ww for larval sea lamprey, ~4 μmol g^{-1} ww for bluegill), and levels were likewise unaffected by TFM exposure (Fig. 4B, F). Only brain lactate concentrations were significantly affected by TFM treatment, with levels 25–35% higher under TFM treatment compared to the controls (Fig. 4D; $df = 1$; $F = 7.34$; $P = 0.010$).

In sea lamprey larvae, concentrations of metabolites in the muscle were unaffected by TFM treatment (Fig. 5, 6). In bluegill, the only muscle metabolite affected by TFM treatment was PCr, which was significantly higher in TFM-treated bluegill relative to the controls (Fig. 5D; $df = 1$; $F = 4.96$; $P = 0.031$). However, PCr concentrations were highly variable in bluegill muscle in both control and experimental fishes and were very low overall at ~1/10 or less the concentrations measured in larval lamprey. Muscle ATP concentrations, which were likewise very low in bluegill compared to larval sea lamprey, were the only muscle metabolite for which an effect of time was observed (Fig. 5B; $df = 2$; $F = 5.80$; $P = 0.006$).

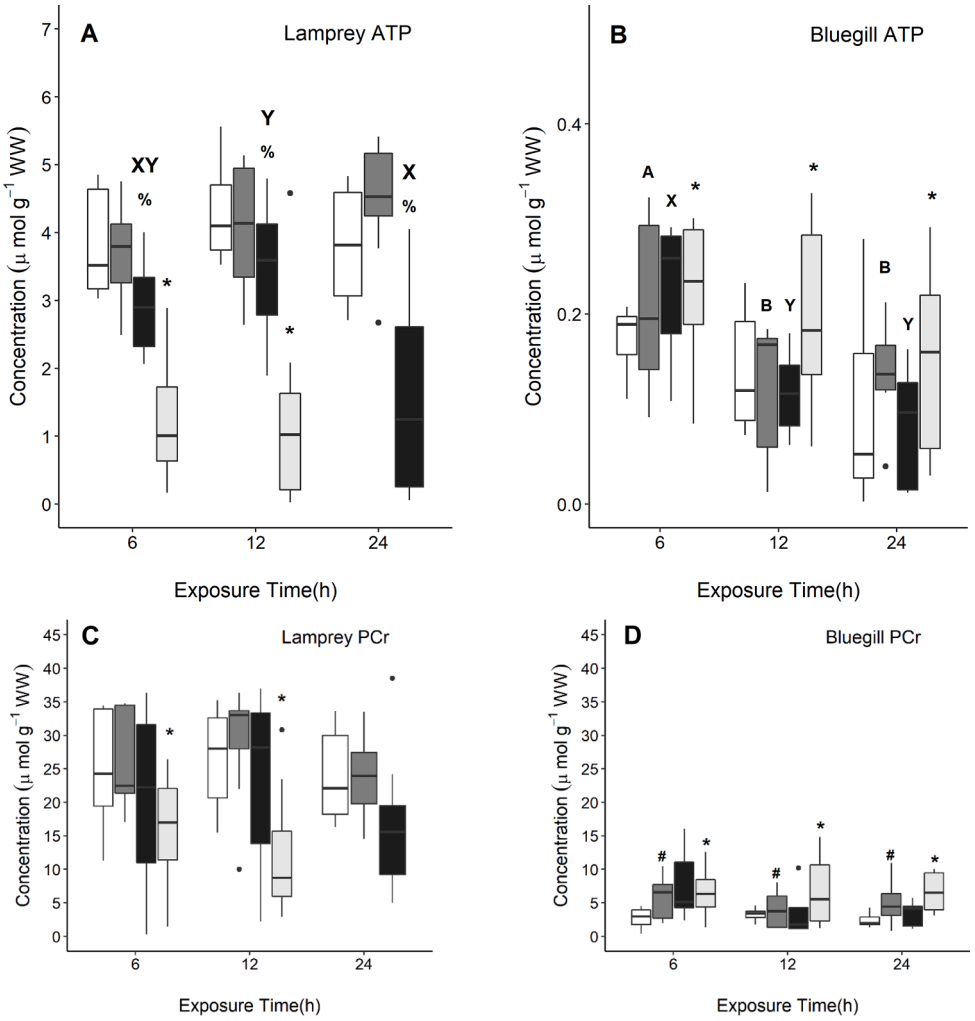

Fig. 5. Concentrations of skeletal muscle ATP (A, B) and PCr (C, D) in larval sea lamprey and bluegill exposed to, from left to right, control (white bars), TFM (dark grey), niclosamide (black) and a mixture of TFM-niclosamide (1.5%; light grey) at 6, 12, and 24 h of exposure. Box plots show the median value delineated by the interquartile range (1st to 3rd quantile), and an accompanying whisker represents 1.5x beyond this range. Suspected statistical outliers are presented as black circles outside of the interquartile range. Data were analysed using a type I two-way ANOVA coupled with a Tukey HSD post-hoc test with pairwise comparisons being made against control values only. Significant treatment level effects ($\alpha = 0.05$) within a toxicant-control comparison are denoted by unique characters (# TFM vs control, **%** niclosamide vs control, * mixture vs control) whereas pairwise differences in time periods for a given toxicant-control comparison are indicated by unique capital letters (*ABC*-TFM vs control, *XYZ*-niclosamide vs control). 24 h values for sea lamprey exposed to the TFM:niclosamide (1.5%) mixture are not presented here as fish did not survive beyond 12 h of exposure.

Metabolites were not analysed in the lamprey liver, due to insufficient amounts of tissue. In bluegill liver, TFM treatment had no effect on ATP (Fig. 7A) and PCr concentrations (Fig. 7B) but resulted in 26–29% lower glycogen concentrations (Fig. 8A; $df = 1$; $F = 21.05$; $P < 0.001$) and 29–38% lower lactate concentrations (Fig. 8B; $df = 1$; $F = 39.34$; $P < 0.001$) when compared to control fish. The decrease in liver glycogen concentrations corresponded with significantly higher liver glucose concentrations in which there was a significant interaction term (Fig. 8C; $df = 2$; $F = 4.18$; $P = 0.022$). TFM-exposed fish had a higher glucose concentration at 12 h of exposure, being 130% higher than the corresponding control.

3.2. Niclosamide effects

In the brains of sea lamprey larvae, niclosamide resulted in lower concentrations of ATP (Fig. 3A; $df = 1$; $F = 26.41$; $P < 0.001$), PCr (Fig. 3C; $df = 1$; $F = 17.24$; $P < 0.001$), glycogen (Fig. 4A; $df = 1$; $F = 106.04$; $P < 0.001$), and lactate (Fig. 4C; $df = 1$; $F = 45.93$; $P < 0.001$), relative to control lamprey. A significant interaction between treatment and time was detected for brain glucose concentrations (Fig. 4E; $df = 2$; $F = 5.42$; $P = 0.008$), with niclosamide-treated fish differing from controls at 12 and 24 h of exposure. In contrast to the situation in larval sea lamprey, brain ATP concentrations were 14–30% higher in niclosamide-exposed bluegill when compared to controls (Fig. 3B; $df = 1$; $F = 4.88$; $P = 0.034$). Brain PCr and glycogen concentrations were comparable to controls (Fig. 3D, 4B), but brain lactate concentrations decreased by 18–25% (Fig. 4D; $df = 1$; $F = 6.54$; $P = 0.015$) and brain glucose concentrations increased by 5–67% compared to controls (Fig. 4F; $df = 1$; $F = 5.94$; $P = 0.020$). In niclosamide-treated bluegill, there were no effects of time or significant interaction terms detected in brain metabolite concentrations.

In the muscle of sea lamprey larvae, niclosamide treatment resulted in 21–59% lower ATP concentrations (Fig. 5A; $df = 1$; $F = 20.51$; $P < 0.001$) and 36–65% lower glycogen concentrations (Fig. 6A; $df = 1$; $F = 17.88$; $P < 0.001$), compared to controls, while PCr (Fig. 5C) and muscle lactate concentrations were unaffected (Fig. 6C). A time-dependant effect of niclosamide treatment on larval sea lamprey muscle ATP concentrations (Fig. 5A; $df = 2$; $F = 5.11$; $P = 0.011$) was the only significant influence on muscle metabolite concentrations.

Niclosamide exposure did not have any significant effects on the concentrations of muscle metabolites in bluegill relative to the controls (Fig. 5,6). There was a significant effect of time on bluegill ATP concentrations where fish exposed for 6 h had higher concentrations of ATP than 12 h- and 24 h-exposed fish (Fig. 5B; $df = 2$; $F = 8.62$; $P < 0.001$), but no significant interaction terms were detected for muscle metabolites of niclosamide-treated bluegill.

Niclosamide treatment had no effect on liver ATP or glucose concentrations in bluegill (Fig. 7A and Fig. 8C, respectively), but resulted in

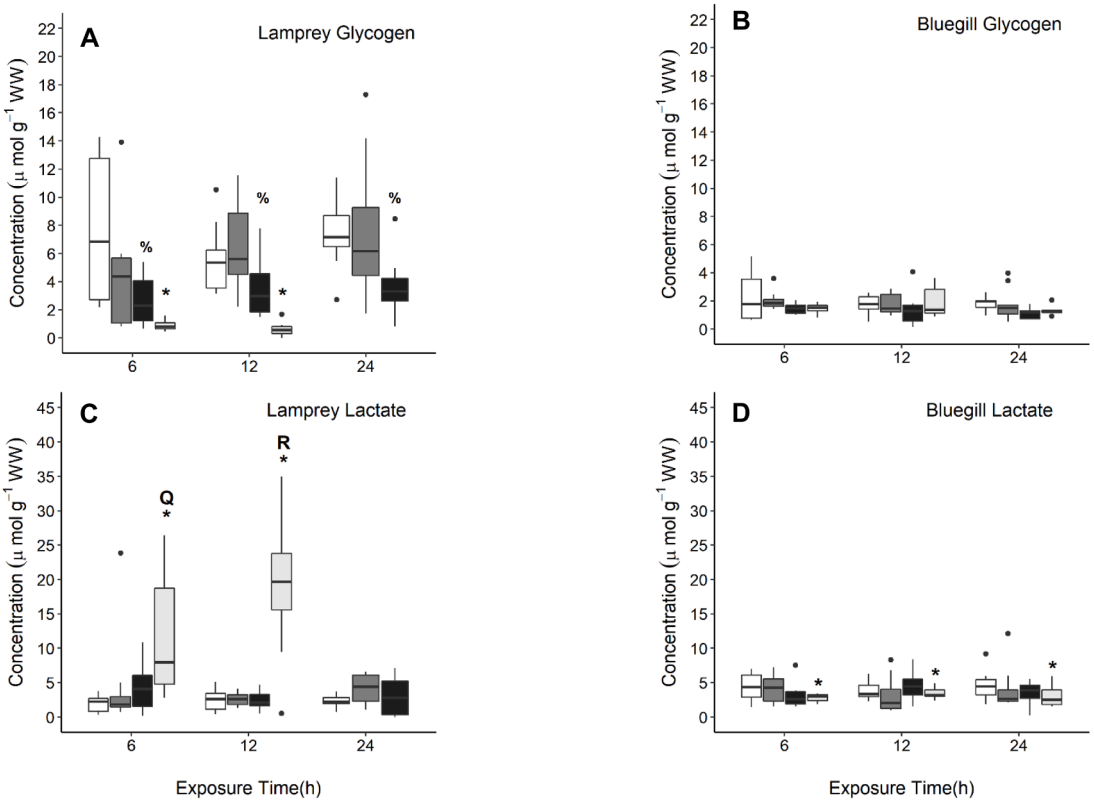

Fig. 6. Concentrations of skeletal muscle glycogen (A, B) and lactate (C, D) in larval sea lamprey and bluegill exposed to, from left to right, control (white bars), TFM (dark grey), niclosamide (black) and a mixture of TFM-niclosamide (1.5%; light grey) at 6, 12, and 24 h of exposure. Box plots show the median value delineated by the interquartile range (1st to 3rd quantile), and an accompanying whisker represents 1.5x beyond this range. Suspected statistical outliers are presented as black circles outside of the interquartile range. Data were analysed using a type I two-way ANOVA coupled with a Tukey HSD post-hoc test with pairwise comparisons being made against control values only. Significant treatment level effects ($\alpha = 0.05$) within a toxicant-control comparison are denoted by unique characters (% niclosamide vs control, * mixture vs control) whereas pairwise differences in time periods for a given toxicant-control comparison are indicated by unique capital letters (*QRS*-mixture vs control). 24 h values for sea lamprey exposed to the TFM:niclosamide (1.5%) mixture are not presented here as fish did not survive beyond 12 h of exposure.

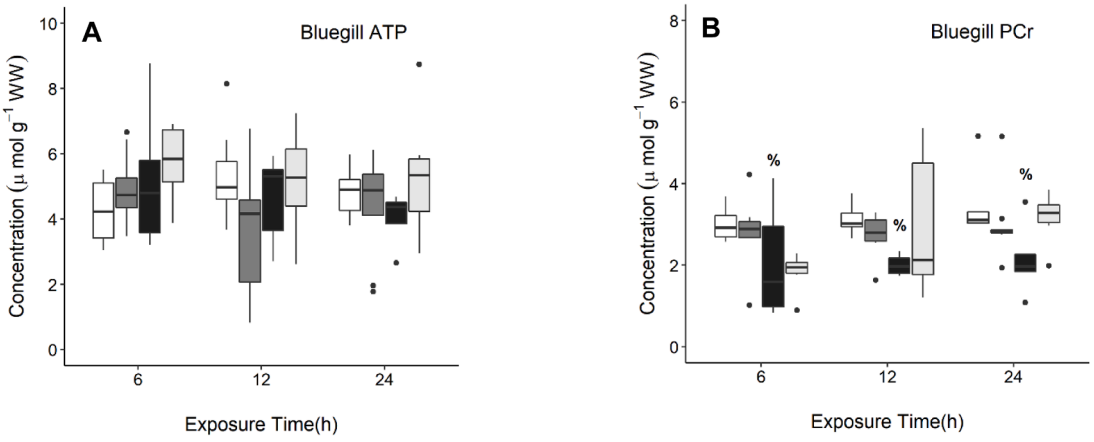

Fig. 7. Concentrations of liver ATP (A) and PCr (B) in bluegill exposed to, from left to right, control (white bars), TFM (dark grey), niclosamide (black) and a mixture of TFM-niclosamide (1.5%; light grey) at 6, 12, and 24 h of exposure. Box plots show the median value delineated by the interquartile range (1st to 3rd quantile), and an accompanying whisker represents 1.5x beyond this range. Suspected statistical outliers are presented as black circles outside of the interquartile range. Data were analysed using a type I two-way ANOVA coupled with a Tukey HSD post-hoc test with pairwise comparisons being made against control values only. Significant treatment level effects ($\alpha = 0.05$) within a toxicant-control comparison are denoted by unique characters (% niclosamide vs control).

lower concentrations of liver PCr (Fig. 7B; $df = 1$; $F = 22.95$; $P < 0.001$), glycogen (Fig. 8A; $df = 1$; $F = 19.18$; $P < 0.001$), and lactate (Fig. 8B; $df = 1$; $F = 16.11$; $P < 0.001$) relative to controls. No significant effects of time were observed for liver metabolite concentrations.

3.3. Part II: Exposure to the TFM:niclosamide mixture

3.3.1. TFM and niclosamide accumulation in liver and muscle

In larval sea lamprey, exposure to a TFM:niclosamide mixture, which was near concentrations that could be encountered in field applications,

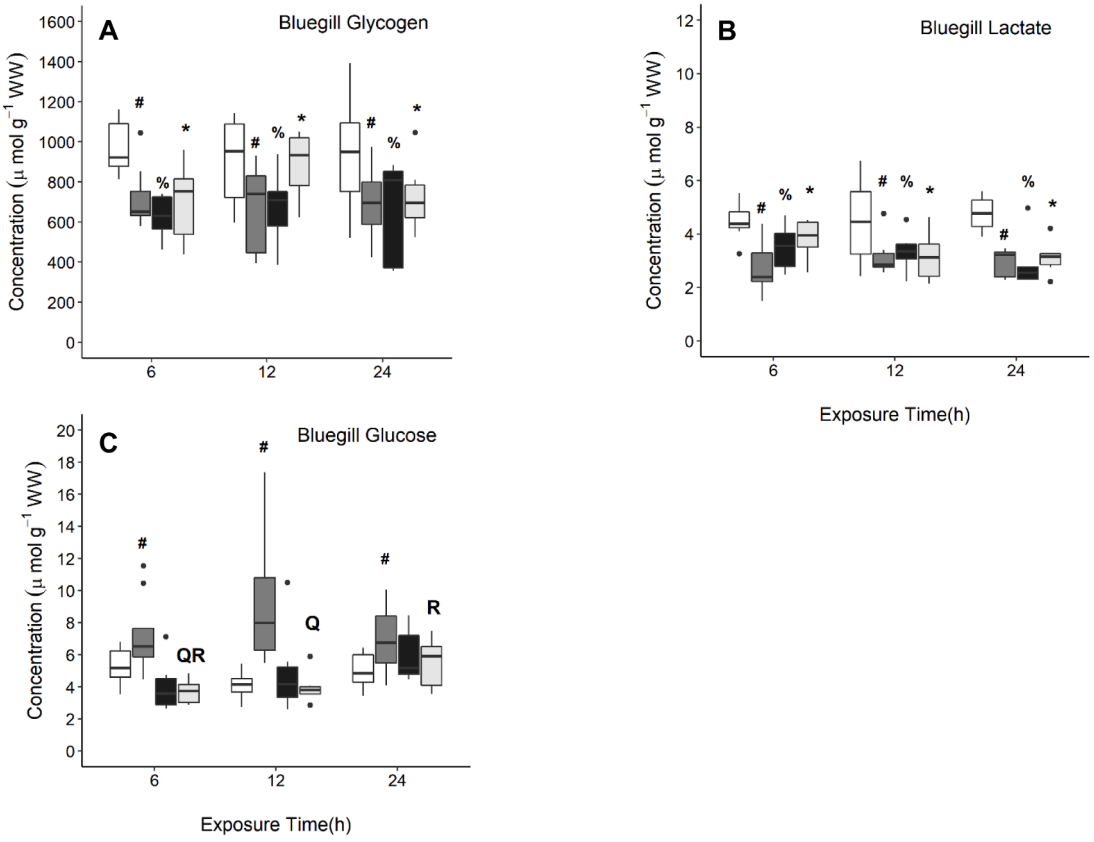

Fig. 8. Concentrations of liver glycogen (A), lactate (B), and glucose (C) in bluegill exposed to, from left to right, control (white bars), TFM (dark grey), niclosamide (black) and a mixture of TFM-niclosamide (1.5%; light grey) at 6, 12, and 24 h of exposure. Box plots show the median value delineated by the interquartile range (1st to 3rd quantile), and an accompanying whisker represents 1.5x beyond this range. Suspected statistical outliers are presented as black circles outside of the interquartile range. Data were analysed using a type I two-way ANOVA coupled with a Tukey HSD post-hoc test with pairwise comparisons being made against control values only. Significant treatment level effects ($\alpha = 0.05$) within a toxicant-control comparison are denoted by unique characters (# TFM vs control,% niclosamide vs control, * mixture vs control) whereas pairwise differences in time periods for a given toxicant-control comparison are indicated by unique capital letters (*QRS*-mixture vs control). While not indicated on the plot explicitly, a significant interactive effect was observed for bluegill liver glucose when exposed to TFM ($F = 4.18$; $P = 0.022$). Liver glucose was also affected by TFM alone ($F = 30.57$; $P < 0.001$).

resulted in 93.3% mortality between 6 and 12 h of exposure. Consequently, only data collected at 6 and 12 h are presented here. Exposure to a mixture of TFM and niclosamide resulted in elevated muscle TFM and niclosamide concentrations in both sea lamprey and bluegill. In sea lamprey, muscle TFM was 16.9 and 36.1 nmol g^{-1} ww at 6 and 12 h, before the animals died Fig. 1A. Notably, niclosamide concentration increased from 3.9 nmol g^{-1} ww to 4.8 nmol g^{-1} ww over the same period (Fig. 2A). In contrast to sea lamprey, muscle TFM concentrations were much lower in the muscle of the bluegill, despite being exposed to higher concentrations of TFM and niclosamide (Table 1). Muscle TFM concentrations were close to 1 nmol g^{-1} ww (Fig. 1B).

Both TFM and niclosamide concentrations were much greater in the liver than observed in the muscle. In the lamprey, TFM concentrations were approximately 125 nmol g^{-1} ww after only 6 h exposure, surpassing 150 nmol g^{-1} ww by 12 h before the fish died (Fig. 1C). The niclosamide average fluctuated around 60 nmol g^{-1} ww over the same 12 h interval (Fig. 2C). On the other hand, there was much less TFM and niclosamide accumulation in the liver of bluegill, despite being exposed to higher concentrations of the lampricides than lamprey. Moreover, both liver TFM and niclosamide concentrations were relatively stable, fluctuating around 15 nmol g^{-1} ww and 3 nmol g^{-1} ww, respectively (Fig. 1D, 2D).

3.3.2. *Physiological effects of TFM:niclosamide exposure*

In lamprey, exposure to the TFM:niclosamide (1.5%) mixture resulted in markedly lower brain ATP concentrations, which were reduced by 62–87% relative to the controls (Fig. 3A; $df = 1$; $F = 36.21$; $P < 0.001$), as well as large (~70%) decreases in brain glycogen concentrations (Fig. 4A; $df = 1$; $F = 159.47$; $P < 0.001$). There were no observed effects on concentrations of brain PCr (Fig. 3C) or lactate (Fig. 4C). While there was a significant interaction term for brain glucose concentrations (Fig. 4E; $df = 1$; $F = 4.84$; $P = 0.037$), there were no pairwise differences detected in the post-hoc test.

In contrast to lamprey, all bluegill survived the exposure to the TFM:niclosamide (1.5%) mixtures, and no significant changes were observed in brain ATP, PCr, glycogen, lactate or glucose concentrations, with all values comparable to control fish (Fig. 3,4). There were no significant effects of time or significant interaction term on the concentrations of brain metabolites in bluegill.

Muscle physiology was greatly altered in sea lamprey larvae exposed to the TFM:niclosamide (1.5%) mixtures. The mixture treatment resulted in lower muscle ATP (Fig. 5A; $df = 1$; $F = 67.89$; $P < 0.001$), PCr (Fig. 5C; $df = 1$; $F = 16.33$; $P < 0.001$), and glycogen concentrations (Fig. 6A; $df = 1$; $F = 50.32$; $P < 0.001$) and higher muscle lactate concentrations (Fig. 6C; $df = 1$; $F = 61.15$; $P < 0.001$) when compared to control fish. Only muscle lactate concentrations showed significant effects of time ($df = 1$; $F = 4.15$; $P = 0.049$). In bluegill, exposure to the mixture resulted in higher muscle ATP (Fig. 5B; $df = 1$; $F = 4.21$; $P = 0.048$) and PCr concentrations (Fig. 5D; $df = 1$; $F = 12.33$; $P = 0.001$), but lower lactate concentrations (Fig. 6D; $df = 1$; $F = 5.15$; $P = 0.029$) when compared to controls. Muscle glycogen concentrations (Fig. 6B) were unaffected by the mixture treatment. No significant

effects of time were observed.

Bluegill liver ATP and PCr concentrations were unaffected by exposure to the lampricide mixture (Fig. 7A, B). Glycogen, which was very high in the controls (approaching 1000 μmol g^{-1} ww), was reduced by 2–28% (Fig. 8A; $df = 1$; $F = 6.87$; $P = 0.013$). Similarly, liver lactate concentrations were 15–34% lower in bluegill exposed to the lampricide mixture (Fig. 8B; $df = 1$; $F = 16.19$; $P < 0.001$), although lactate concentrations in the control bluegill were already relatively low (3–5 μmol g^{-1} ww). Liver glucose concentrations, while being significantly affected by time (Fig. 8C; $df = 2$; $F = 3.90$; $P = 0.029$), were comparable to control levels; no other significant effects of time were observed.

4. Discussion

4.1. Overview

This work characterized how energy metabolism was affected in larval sea lamprey and bluegill exposed to TFM, niclosamide, and a TFM: niclosamide (1.5%) mixture. In larval sea lamprey, TFM exposures led to a reduction of high energy substrates (brain ATP & glycogen) consistent with an uncoupling of oxidative phosphorylation, which is the proposed mechanism of TFM toxicity (Niblett and Ballantyne 1976; Birceanu et al., 2011; Huerta et al., 2020). In contrast, bluegill were generally unaffected by TFM exposures but this was likely due to a much higher capacity to detoxify TFM rather than differences in the sensitivity of the mitochondria to TFM. This conclusion is supported by the much lower concentrations of TFM that accumulated in the muscle and the liver of bluegill relative to lamprey, and in line with previous work demonstrating much higher activities of the UDP-glucuronosyltransferase (UDP-GT), which mediates the phase II biotransformation of TFM to TFM-glucuronide (Kane et al., 1994).

Like TFM, niclosamide resulted in a depletion of energy substrates (Brain: ATP, PCr, glycogen, lactate, glucose; Muscle: ATP, glycogen), but even during exposure to much higher concentrations of niclosamide, energy substrates in the bluegill were generally unaffected. This too likely reflects a much higher capacity of bluegill to detoxify TFM, in which niclosamide accumulation in the muscle and liver was approximately 90% less than observed in the sea lamprey, despite being exposed to much higher concentrations of the lampricide. Less is known about how niclosamide is handled by fishes, but it is thought to involve phase II biotransformation to niclosamide glucuronide and niclosamide sulfate (Dawson 2003; Wilkie et al., 2019). Indeed, bluegill are amongst the most tolerant fishes to both niclosamide and TFM, with 5- to 10-fold higher LC_{50} values than in sea lamprey (Marking and Hogan 1967; Marking and Olsen 1975; Wilkie et al., 2019). Future transcriptomic and pharmacological analyses should reveal more about the underlying mechanism(s) of the bluegill's high tolerance to both lampricides.

Exposure to the TFM/niclosamide mixture resulted in high rates of mortality in larval sea lamprey, which corresponded with more severe depletions in tissue energy substrates and lactate accumulation than observed for either lampricide alone. These observations support the idea that increasing reliance on anaerobic pathways of ATP production characterizes the responses of these animals to both lampricides, and that death ensues when anaerobic reserves are no longer sufficient to maintain ATP supply (Wilkie et al., 2007; Birceanu et al., 2009). Survival may also be compromised by profound acid-base disturbances, as recently suggested by Ionescu et al. (2021), who demonstrated that intracellular muscle pH was decreased by 0.6 units in larval lamprey exposed to the 12-h LC_{50} of TFM for 9-h. Again, bluegill suffered no mortality and tissue indices were comparable to control levels under TFM:niclosamide mixture exposure reflecting their very high tolerance to these lampricides.

4.2. Sublethal TFM toxicity

In larval sea lamprey, exposure to low (24 h LC_{10}) sublethal doses of TFM generated disturbances consistent with previous studies demonstrating an increased dependence on anaerobic metabolism resulting in a depletion of high energy substrates including ATP and glycogen in the brain (Birceanu et al., 2009; Clifford et al., 2012; Henry et al., 2015). Due to the very high ATP demands of the brain (Soengas and Aldegunde 2002), the uncoupling of oxidative phosphorylation by TFM (Birceanu et al., 2011; Huerta et al., 2020) likely led to a greater reliance on glycolysis leading to the reduced brain glycogen content (Birceanu et al., 2009). Compared to other fishes, sea lamprey have extremely high concentrations of glycogen in the meninges of the brain (Rovainen 1970; Rovainen et al., 1971; Foster et al. 1993; Clifford et al., 2012), with the liver playing a comparatively minor role in glucose homoeostasis (O'Boyle and Beamish 1977). Our data supports Rovainen's (1970) hypothesis that the sea lamprey brain is the main glucose reservoir for the central nervous system during environmental perturbations which contrasts teleosts and higher vertebrates (Moon 2001; Soengas et al., 1996).

We observed no changes in PCr concentrations in the brain, unlike previous studies (Birceanu et al., 2009, 2014; Clifford et al., 2012). Likely, the lower TFM exposure concentrations used here (24 h LC_{10} versus 12 h LC_{50} or 12 h $LC_{99.9}$) resulted in lower TFM uptake and accumulation in the lamprey (Hlina et al., 2017), and less impairment of oxidative phosphorylation in the mitochondria. Indeed, several studies have demonstrated that isolated mitochondria from rats, lamprey, and trout respond to TFM in a dose dependant manner (Niblett and Ballantyne 1976; Birceanu et al., 2011; Huerta et al., 2020). At higher concentrations of TFM, we expected decreases in brain PCr, larger drops in glycogen, and an accumulation of lactate. Rather, lactate was likely metabolised as it is an important metabolic fuel in lamprey central nervous system (CNS) tissue (Foster et al., 1993; reviewed in Soengas and Aldegunde 2002).

Exposure to relatively lower concentrations of TFM also likely explains why muscle tissue was unaffected by TFM exposure in sea lamprey larvae in the current study, contrasting previous works (Wilkie et al., 2007; Birceanu et al., 2009). This result was unexpected, given that both this work (Fig. 1,2) and prior research (Lech and Statham 1975) demonstrated significant TFM deposition in the muscle. While TFM concentrations are somewhat comparable between TFM- and TFM: niclosamide mixture-exposed lamprey (Fig. 1A), there was a distinct difference in tissue level responses suggesting that the addition of niclosamide is required to exert a toxic effect in this tissue at these TFM concentrations. This interpretation is supported by previous research demonstrating that TFM and niclosamide interact in a greater than additive (synergistic) manner in fishes when applied in combination (Marking and Dawson 1975; Hepditch et al., 2021). It is also important to acknowledge that variation in some of the metabolites was quite high, which may reflect biological variation from the use of wild-sourced fishes and intraindividual variation in tissue energy contents (Hislop et al. 1991; Lambert and Dutil 1997). These findings also indicate that, compared to the brain, the muscle is rather resilient to TFM likely because of its relatively low metabolic demands under resting (routine) conditions (Moran et al., 2015; Sukhum et al., 2016).

Bluegill were very tolerant to the sublethal effects of TFM and experienced few changes in metabolite concentrations. Bluegill liver was the only site where multiple effects of sublethal TFM exposure were observed, with lower glycogen and lactate concentrations, and higher glucose, compared to controls. Unlike lamprey, the liver is the major organ of glucose homoeostasis in teleost fishes, maintaining systemic glucose levels via glycogenolysis (Moon 2001; Polakof et al., 2012). The decrease in liver glycogen and higher glucose concentrations likely reflected increased mobilization of liver glycogen to help maintain glucose supply to the brain and other organ systems to help sustain ATP production via aerobic and/or anaerobic glycolysis. The latter may explain the lactate accumulation observed in the brain of bluegill during TFM exposure, where higher rates of anaerobic glycolysis may have been necessary to offset decreases in aerobic ATP production due to impaired

oxidative phosphorylation.

Studies using isolated mitochondria indicate that TFM acts on the mitochondria in a dose-dependant manner (Niblett and Ballantyne 1976; Bireanu et al. 2011; Huerta et al., 2020), suggesting that even at the low concentrations of TFM measured in the bluegill that there may have been some impairment of mitochondrial ATP synthesis necessitating increased reliance on anaerobic ATP production using glycolysis. In addition, reduced liver glycogen may have also indicated a higher ATP demand for supporting TFM detoxification (Kawatski and McDonald 1974). Indeed, the glycolytic intermediate, glucose-6-phosphate, is the substrate used to generate UDP-glucuronide, which is used to metabolize TFM to TFM-glucuronide via the enzyme UDP-glucuronosyltransferase in fishes (Kane et al., 1994; Birceneau et al. 2014; Bussy et al., 2018b).

Interestingly, liver glycogen levels in the bluegill in our study were substantially higher than previously reported for wild caught sunfish (Heath and Pritchard 1965; Du et al., 2019; Lawrence et al., 2019). Liver glycogen levels in centrarchids can be influenced by temperature (Heidinger and Crawford 1977), with liver energetic status varying on a seasonal basis (Bulow et al., 1978; Dehn 1992) such that time of year may dictate available hepatic energy stores for responding to TFM. This high glycogen content may be the result of collection time (i.e., early autumn) which could help them prepare for over-wintering to cope with poor food abundance and/or episodic hypoxia (van den Thillart et al. 1980; Nilsson 1990). Presumably, these greater glycogen stores may have also conferred a greater tolerance to TFM toxicity by providing the animals with a larger glucose pool to meet their systemic ATP demands, as well as providing substrate for glucuronidation.

4.3. Sublethal niclosamide toxicity

Niclosamide is a more potent piscicide than TFM (Wilkie et al., 2019), but it causes similar homoeostatic disturbances, including markedly lowering ATP and PCr in the brain, consistent with the known uncoupling effects of salicylanilides on oxidative phosphorylation in other animals (Leader and Whitehouse 1966; Weinbach and Garbus 1969; Kaplay et al., 1972; Park et al., 2011). Indeed, in mitochondria isolated from the cestode *Cotognia digonophora*, niclosamide, which is used as an antihelmintic, was demonstrated to inhibit oxidative phosphorylation (Pampori and Srivastava 1987). The corresponding reductions in brain glycogen reserves observed here suggests increased glycogenolysis made up for the ATP shortfalls, as previously described for TFM (e.g., Clifford et al., 2012). Surprisingly, there was no corresponding increase in lactate, as has been observed previously in sea lamprey larvae exposed to much higher doses of TFM (e.g.,12 h LC_{50} or $LC_{99.9}$; Birceanu et al., 2009; Clifford et al., 2012). These results are in line with the actions of niclosamide in cestodes and in other invertebrates (i.e., reduced glycogen & ATP; Strufe 1964, as cited in Andrews et al. 1982; Rusak and Kovchur 1972; El Gindy and Mohamed 1978, as cited in Andrews et al. 1982; Pampori et al., 1984) and in teleosts (i.e., reduced glycogen, ATP, and lipid contents; Shoman 2001; Zhu et al., 2020). However, prior works have also reported that, as in the case of TFM, niclosamide treatment can cause an increase in lactate production as the animal increasingly relies upon anaerobic metabolism (Pampori et al., 1984). In contrast, we saw reduced brain lactate under niclosamide treatment, which indicates that lactate oxidation was continuing despite an uncoupling of ATP synthesis (see Wilkie et al., 2019). This highlights the need for a greater insight into the specific mechanism(s) by which niclosamide affects mitochondrial respiration and ATP synthesis (e.g., Birceanu et al., 2011). Unlike lamprey, bluegill were resilient to the sub-lethal effects of niclosamide exposure, which had no general effect on brain or muscle metabolites. However, as seen with TFM, both glycogen and lactate concentrations in the liver were reduced, which was also likely related to the need to maintain glucose homoeostasis and/or to support niclosamide detoxification.

4.4. Mixture toxicity: the lethal effects of TFM and niclosamide

TFM is often applied in combination with 1–2% niclosamide, which lowers the TFM 9 h $LC_{99.9}$, also referred to as the minimum lethal concentration (MLC), in sea lamprey larvae, without decreasing its selectivity (reviewed in McDonald and Kolar 2007; Wilkie et al., 2019). In the present study, bluegill were exposed to a nominal TFM:niclosamide (1.5%) mixture concentration that was equivalent to the TFM 24 h MLC of larval lamprey to provide us with better temporal resolution of the physiological responses that lampreys and non-target fishes experience when exposed to both lampricides in the field. In the field, 1.2–1.5x the 9 h MLC is typically applied to ensure that lamprey are exposed to the MLC for at least 9 h of TFM, which can be diluted as it moves downstream (Barber and Steeves 2004).

The TFM:niclosamide mixture resulted in a much faster and greater magnitude of physiological disturbance in the sea lamprey brain. This was characterized by an almost complete depletion of ATP and large drops of glycogen in the brain, which culminated in the death of most sea lamprey larvae by 12 h. The greater severity of the disturbances were reflected more so in the muscle, in which there was a near depletion of ATP and glycogen, along with marked reductions in PCr and large elevations in lactate. The magnitude of these disturbances was likely because much more niclosamide accumulated in muscle tissue when the lamprey were exposed to the TFM:niclosamide mixture than when treated with niclosamide alone (Fig. 3C). This suggests that the bioavailability of niclosamide was higher in the animal, possibly due to a reduced ability to detoxify niclosamide in the presence of TFM. In fact, liver TFM concentrations were 3.7 to 5.1-fold higher in lamprey exposed to the TFM:niclosamide mixture compared to niclosamide alone, suggesting that the higher TFM may have interfered with the liver's ability to biotransform niclosamide. This would have resulted in the accumulation of more toxic niclosamide in the muscle, impairing this normally more robust tissue's ability to maintain homoeostasis, a situation which likely extended to other organs leading to the death of most lamprey by 12 h.

To date, we know relatively little about the nature of the interactions between TFM and niclosamide. The present findings indicate that, on their own, they each interfere with ATP generation, likely via the mitochondria. In combination, TFM and niclosamide undermine ATP supply to the CNS to a far greater extent than during TFM exposure alone (e.g., Clifford et al., 2012). Marking and Dawson (1975) suggested that the interactions of TFM and niclosamide were greater than additive, but they used ratios of TFM:niclosamide which were not reflective of current lampricide application packages. However, the greater than additive nature of the TFM:niclosamide interactions were confirmed in rainbow trout exposed to increasing concentrations of TFM and 1% niclosamide (Hepditch et al., 2021). It is not yet known if such synergistic effects are due to non-competitive or competitive effects of TFM and niclosamide on their rates of metabolism by phase II biotransformation using sulfation or glucuronidation in the liver. Sea lamprey have very low activities of the sulfotransferase and UDP-glucuronyltransferase that mediate these conjugation reactions (Lech and Statham 1975; Kane et al., 1994; Dawson 2003; Bussy et al., 2018a,b), which could quickly become saturated in the presence of either TFM or niclosamide, thus increasing bioavailability and magnifying the magnitude of the physiological effect. This is supported here by the apparent accumulation of both TFM and niclosamide in the tissues, which suggested a reduced ability to detoxify in this species. Further studies on the pharmacokinetics and pharmacodynamics of TFM and niclosamide are clearly needed to identify the underlying mechanisms of the synergistic actions of these two agents in sea lamprey.

Notably, there was little evidence of similar interactive effects in the bluegill. That bluegill were largely unaffected by exposure to both compounds at doses lethal to sea lamprey larvae across all tissue types except for the liver was likely a result of a higher capacity to detoxify TFM using glucuronidation, and possibly sulfation (Kane et al. 1994; Bussy et al., 2018a). Indeed, bioassays on bluegill exposed to mixtures of

TFM and niclosamide indicated that they are one of the most tolerant species of teleosts at doses far greater than what was used here (i.e., 12 & 96 h LC_{50}; Bills and Marking 1976; Boogaard et al., 2003). The absence of physiological perturbations and relatively low concentrations of parent TFM and niclosamide measured in both the muscle and liver when the fish were exposed to the TFM:niclosamide mixture strongly supports the hypothesis that the high tolerance of bluegill to lampricide is due a high capacity to detoxify these agents. Based on these findings, we conclude that under typical field conditions, the use of TFM:niclosamide mixtures for lampricide treatments likely poses very little risk to the physiological functioning of bluegill and other centrarchids at large (Boogaard et al., 2003; Table S1). However, further work needs to address the mechanism(s)/pathways underlying taxonomic variation in lampricide detoxification.

5. Conclusions

In this study, we characterized the physiological consequences to acute, sub-lethal exposures to TFM and niclosamide, and a mixture of the two in a target and non-target species. This was done to elucidate the potential underlying mechanisms of toxicity and provide insight into how a non-target fish in the Laurentian Great Lakes responds to exposure to such compounds. Overall, the results of this study provide some of the first characterizations of the physiological effects of niclosamide and a TFM:niclosamide mixture across several tissues of both target and non-target species. We showed that native bluegill are resilient to the effects of both lampricides (Boogaard et al., 2003; Barber and Steeves 2004), which likely corresponds to a high capacity to detoxify the agents. In contrast, the tissues of sea lamprey larvae experienced significant physiological perturbations when exposed to all three treatment groups. Furthering our understanding of the toxicological actions of TFM and niclosamide is important because the effects of lampricide application in the Laurentian Great Lakes are largely unknown for the broader fish community despite widespread use of such agents (see McDonald and Kolar 2007). Understanding the toxicological mechanisms of TFM and niclosamide will allow us to make more informed management decisions that can be tailored to enhance sea lamprey control while simultaneously minimizing impacts on local fish communities.

CRediT authorship contribution statement

M.J. Lawrence: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **D. Mitrovic:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **D. Foubister:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **L.M. Bragg:** Methodology, Writing – review & editing. **J. Sutherby:** Methodology, Writing – review & editing. **M.F. Docker:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **M.R. Servos:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **M.P. Wilkie:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **K.M. Jeffries:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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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Supplementary materials

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