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Effects of water pH on the uptake and elimination of the piscicide, 3-trifluoromethyl-4-nitrophenol (TFM), by larval sea lamprey

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ABSTRACT

Invasive sea lamprey (*Petromyzon marinus*) populations in the Great Lakes are controlled by applying the piscicide, 3-trifluoromethyl-4-nitrophenol (TFM), to infested streams with larval sea lamprey (ammocoetes). While treatment mortality is > 90%, surviving lamprey, called residuals, can undermine control efforts. A key determinant of TFM effectiveness is water pH, which can fluctuate daily and seasonally in surface waters. The objectives of this research were to evaluate the influence of pH on the uptake, elimination, and accumulation of TFM by larval sea lamprey using radio-labeled TFM (^{14}C-TFM), when exposed to a nominal concentration of 4.6 mg TFM L^{-1} or 7.6 mg TFM L^{-1}, 3 h or 1 h, respectively. TFM uptake rates were approximately 5.5-fold greater at low pH (6.86) compared to the high pH (8.78), most likely due to the unionized, lipophilic form of TFM existing in greater amounts at a lower pH. In contrast, elimination rates following the injection of 85 nmol TFM g^{-1} body mass were 1.7–1.8 fold greater at pH 8.96 than at pH 6.43 during 2–4 h of depuration in TFM-free water. Greater initial excretion rates at pH 8.96 were presumably due to predicted increases in outward concentration gradients of un-ionized TFM. The present findings suggest that TFM is mainly taken-up in its un-ionized form, more lipophilic form, but there is also significant uptake of the ionized form of TFM via an unknown mechanism. Moreover, we provide an explanation to how small increases in pH can undermine lampricide treatment success increasing residual lamprey populations.

1. Introduction

The piscicide, 3-trifluoromethyl-4-nitrophenol (TFM) has been used to control invasive sea lamprey (*Petromyzon marinus*) populations in the Laurentian Great Lakes since the early 1960s (Applegate et al., 1961; Hubert, 2003; Mcdonald and Kolar, 2007). Applied at regular intervals to streams containing larval sea lamprey, TFM specifically targets the animals in their burrows, where they live as sedentary suspension feeders (Beamish and Potter, 1975; Moore and Mallatt, 1980; Sutton and Bowen, 1994). The specificity of TFM compared to non-target fishes and other aquatic vertebrates is due to the relative inability of larval sea lamprey to detoxify TFM using glucuronidation (Kane et al., 1993; Lech and Statham, 1975). By uncoupling oxidative phosphorylation in the mitochondria (Birceanu et al., 2011; Niblett and Ballantyne, 1976), TFM causes death by interfering with oxidative ATP production leading to a depletion of anaerobic energy reserves, particularly glycogen and high energy phosphagens such as phosphocreatine in sea lamprey and other fishes (Birceanu et al., 2009, 2014; Clifford et al., 2012; Wilkie et al., 2007a), or phosphoarginine in molluscs (Viant et al., 2001).

The toxicity of TFM is also influenced by a variety of biotic and abiotic factors including life stage (Henry et al., 2015), season (Scholefield et al., 2008), and water chemistry (Bills et al., 2003). Ineffective treatments can lead to residual larval sea lamprey that survive TFM exposure, and subsequently complete metamorphosis into juvenile sea lamprey that go on to parasitize fishes in the Great Lakes (Hansen and Jones, 2009; Mcdonald and Kolar, 2007). Because TFM is a weak acid, with a pKa of ~6.07 (Hubert, 2003), its speciation is strongly influenced by water pH, which markedly influences TFM toxicity by changing the relative proportions of un-ionized (phenolic) and ionized TFM (phenolate ion), which changes bioavailability of TFM (Bills et al., 2003; Hunn and Allen, 1974; Kanayama, 1963). In its un-ionized form, TFM is more toxic than in its ionized form because it is more lipophilic and thought to cross the gills more readily (Hunn and Allen, 1974). Indeed, Hunn and Allen (1974) demonstrated that TFM accumulation was greater in rainbow trout (*Oncorhynchus mykiss*) at more acidic pHs, but they did not directly measure TFM uptake at different pHs, nor did they look at the effect of pH on TFM accumulation in larval sea lamprey. This question is not just of academic importance but one of management importance because the pH of streams in the Great Lakes

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varies widely (Mcdonald and Kolar, 2007), and rivers may also experience diurnal swings in water pH (Wetzel, 1983). Moreover, there is increasing evidence suggesting that organics including phenols can be taken-up in their ionized forms (see Erickson et al., 2006a, 2006b for review). Thus, the goal of the present study was to determine how changes in the speciation of TFM influenced rates of TFM uptake and accumulation to shed more light on the mechanism(s) of TFM accumulation in larval sea lamprey.

Changes in water pH could also affect rates of TFM elimination because of the sea lamprey's limited capacity to use glucuronidation to detoxify TFM (Kane et al., 1994; Lech and Statham, 1975). In its phenolic (un-ionized) form, TFM is likely eliminated via passive diffusion down its concentration gradient across the gills (Clifford et al., 2012). This could be particularly important if TFM were diluted (e.g. due to ground water seeps and small untreatable tributaries) or if application was interrupted, giving sea lamprey an opportunity to excrete the chemical, and potentially confound treatment success by increasing the numbers of surviving lamprey. At more alkaline water pH, a greater proportion of ionized TFM versus un-ionized TFM in the water could therefore promote greater TFM loss from the animal during a treatment, by trapping un-ionized TFM as TFM converts to its ionized form after it diffuses across the gills.

The primary goal of this study was to use ^{14}C-TFM to measure rates of TFM uptake in larval sea lamprey acclimated to acidic (target pH = 6.5), moderate (target pH = 7.8), and alkaline pH (target pH = 9.0). To test the hypothesis that TFM elimination does takes place via passive diffusion across the gills, and that TFM excretion is augmented by increases in external water pH, larval sea lamprey were also injected with ^{14}C-TFM, and the rates of TFM clearance measured during a 24 h depuration period in TFM-free water.

2. Methods

2.1. Experimental animals and set-up

Larval sea lamprey were provided courtesy of the US Fish and Wildlife Service (USFWS) and the Hammond Bay Biological Station (HBBS; US Geological Survey), Millersburg, Michigan, United States. The animals were captured by pulsed-DC electrofishing (ABP-2 Electrofisher, Electrofishing Systems, LLC, Madison, WI, USA) from streams and rivers draining into Lake Huron or Lake Michigan, and held an at the HBBS for several weeks before being shipped to Wilfrid Laurier University (WLU) in the spring of 2014 and 2015. At WLU, the larvae were housed in 110 L fiberglass aquaria, continuously receiving aerated well water (flow rate ~500 mL min^{-1}; pH 7.9 ± 0.4; hardness = 460 mg L^{-1} as $CaCO_3$; dissolved oxygen ≥ 80% saturation; T = 13 ± 2 °C), which was lined on the bottom with 3–5 cm of sand to provide the animals with burrowing substrate (Birceanu et al., 2009). The larvae were held under a 12 h dark: 12 h light cycle, and were fed a slurry of Baker's yeast (1 g of yeast per lamprey) once per week. All experiments were approved by the Wilfrid Laurier University Animal Care Committee, and followed the guidelines and principles of the Canadian Council of Animal Care (CCAC).

2.2. Experimental procedures

2.2.1. Acclimation to different water pHs

Larval sea lamprey were acclimated in groups of 40 to a target pH of low (6.5), moderate (7.8), or high (9.0) ± 0.2 in a glass aquaria (Volume = 40 L) for a period of one week. The bottom of each aquarium was lined with 3–5 cm of sand to provide burrowing substrate. The pH was maintained using a Radiometer PHM82 pH meter connected to a TTT80 autotitrator (Radiometer Copenhagen, Denmark) which regulated the drop-wise addition of 0.5 M HCl or 0.5 M KOH into the aquarium water using a solenoid valve (P/N: 01540-02, Cole Parmer, IL, USA). Additionally, the amount of KOH or HCl added to the system during both acclamation and experimental procedure was approximately 0.06 M and was not significant enough to increase the salinity of the water enough to create saline environment. One day prior to experimentation, the larvae were transferred in triplicate groups of 4 (N = 12 total) to darkened, plastic, 1 L containers which continuously received water of the appropriate pH at a rate of 100–200 mL min^{-1}, and were left to acclimate overnight. Containers did not have any burrowing substrate and lamprey were sedentary within their containers. The containers were placed in a recirculating system (150 L), also equipped with a pH-stat control system, in which the pH-corrected water drained from a head tank (volume = 50 L) into each container via a flow-splitter at approximately 100–200 mL min^{-1}, before draining into a lower reservoir (volume = 50 L) from which the water was returned to the head tank using a submersible pump. Immediately prior to each series of experiments, water flow to the containers was cut-off and the volumes adjusted to the desired level, for measurement of TFM uptake or excretion using ^{14}C-TFM (see below).

2.3. Experimental protocols

2.3.1. Rates of TFM uptake

Two series of experiments were completed to measure rates of TFM uptake. In the first series, larval sea lamprey (n = 12; 0.9 ± 0.02 g; 90 ± 0.44 mm) were exposed to a target concentration of 4.6 mg TFM L^{-1} (Birceanu et al., 2009). In these experiments, rates of TFM uptake were measured using ^{14}C-TFM over 3 h. In the second series, TFM uptake was measured in larval sea lamprey (n = 12; 1.1 ± 0.01 g; 90 ± 0.35 mm) exposed to a higher target concentration of TFM 7.6 mg TFM L^{-1} (Henry et al., 2015), but over 1 h. Test concentrations, both low and high, were previously determined by studies conducted in WLU well water that determined an LC_{50} and LC_{99} at approximately a pH of 8.0 (Birceanu et al., 2009; Henry et al., 2015). We decided to use these two concentrations as they were deemed as concentrations that are laboratory and field relevant. Additionally, the use of a low and a high concentration of TFM were used to assess if any dose dependent effects would occur during uptake experiments. Acclimation and exposure water quality parameters are reported in Table 1 for both exposures. Prior to each TFM uptake experiment, water flow to each container was shut-off, and the container volume adjusted to 750 mL. This was then immediately followed by the addition of 2.5 μCi of ^{14}C-TFM, from a 0.5 μC μL^{-1} ^{14}C-TFM working stock (^{14}C-TFM provided courtesy of Dr. T. Hubert, Upper Midwest Environmental Sciences Center, U.S.-Geological Survey, LaCrosse, WI, USA; DuPont/New England Nuclear, DE, USA) to each container, followed by sufficient non-radioactive (cold) field grade TFM (35% active ingredient dissolved in isopropanol; Clariant SFC GMBH WERK, Griesheim, Germany), provided courtesy of the Sea Lamprey Control Centre, Fisheries and Ocean Canada, Sault Ste. Marie, Ontario, to yield nominal concentrations of either 4.6 mg TFM L^{-1} or 7.6 mg TFM L^{-1}, depending upon the experiment. The containers were then covered, and left for 10 min to allow the non-radioactive and radioactive TFM to thoroughly mix. Water samples (10 mL) were subsequently taken at 0, 1 h, or 3 h as appropriate. The pH of each container was monitored hourly using a handheld pH meter and electrode (pH 11 meter, Oakton Instruments, IL, USA) and when necessary, pH was adjusted in a drop-wise manner using a Pasteur pipette using 0.1 M HCl or 0.1 M KOH. Reduced concentrations of HCl and KOH were used during the experiment allowing for a more fine adjustment of pH due to the smaller volume used to hold lamprey compared to acclimation. Following each experiment, survival of the animals was confirmed by lightly pinching the tail of the lamprey using forceps, and monitoring for movement. After which, the animals were euthanized with an overdose of tricaine methanesulfonate (1.5 g MS-222 L^{-1}; Syndel Labs, Port Alberni, BC, Canada; Birceanu et al., 2009) buffered with 3.0 g L^{-1} $NaHCO_3$. The animals were then thoroughly rinsed in non-radioactive water of the appropriate pH for 30 s, followed by 50 mg L^{-1} non-radioactive TFM for 30 s to remove

Table 1
Average water pH and temperature (°C) during a one week acclimation of larval sea lamprey prior to measurement of TFM uptake and elimination at three different pHs (low, moderate, and high pH). Data presented as the mean ± SEM.

TFM uptake experiments			
4.2 mg TFM L^{-1}		7.0 mg TFM L^{-1}	
pH	Temperature (°C)	pH	Temperature (°C)
Acclimation			
6.52 ± 0.04	20.6 ± 0.13	6.52 ± 0.04	20.6 ± 0.13
8.25 ± 0.02	19.1 ± 0.07	7.79 ± 0.02	20.4 ± 0.58
8.85 ± 0.06	20.7 ± 0.19	8.85 ± 0.06	20.7 ± 0.19
Exposure			
6.87 ± 0.14	17.7 ± 0.05	6.85 ± 0.18	17.6 ± 0.07
8.06 ± 0.03	18.3 ± 0.05	7.82 ± 0.15	17.3 ± 0.06
8.74 ± 0.07	17.4 ± 0.50	8.82 ± 0.06	17.2 ± 0.17

TFM excretion experiments	
pH	Temperature (°C)
Acclimation	
6.68 ± 0.09	19.9 ± 0.27
8.25 ± 0.02	19.1 ± 0.07
8.85 ± 0.06	20.7 ± 0.19
Experiment	
6.43 ± 0.03	19.6 ± 0.13
8.12 ± 0.03	16.9 ± 0.07
8.96 ± 0.04	18.8 ± 0.19

any surface-bound radioactive TFM, before a final rinse in non-radioactive water (~30 s). Each whole animal was blotted dry, weighed and then transferred to a 15 mL polypropylene centrifuge tube to which 10 mL of 1 N HNO_3 was added to digest the carcass prior to processing it for measurements of whole animal radioactivity (see below).

2.3.2. Rates of TFM excretion

After an overnight adjustment period (12 h) in their experimental chambers, measurements of TFM excretion were measured in separate groups (N = 8) of sea lamprey (mean mass = 1.0 ± 0.02 g; length = 100 ± 0.27 mm) at the appropriate acclimation pH (Table 1). The morning of experiments, the larval sea lamprey were first anaesthetized one at a time using 0.2 g L^{-1} of MS-222 buffered with 0.4 g L^{-1} $NaHCO_3$, followed by the intraperitoneal (IP) injection, using a1 mL syringe with a 28 gauge needle (BD B329424, BD, Franklin Lakes, New Jersey,USA), of a target of 100 nmol TFM g^{-1} fish containing 0.005 μCi ^{14}C-TFM g^{-1} fish in sea lamprey saline (Composition: 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 3.5 mM NaH_2PO_4, and 5.5 mM glucose, pH 7.4). The total volume of ^{14}C-TFM-labeled solution injected was equivalent to 2% of each larval sea lamprey's body mass. The mean specific activity (MSA) of the injection solution was 98.7 counts per min (CPM) nmol TFM^{-1}. The injection site was inspected for leakage of the solution, before returning the lamprey to its container. Immediately after returning the animal to its container, a water sample (10 mL) was collected to obtain background measurements of radioactivity, and the animals left for 1 h to allow the ^{14}C-TFM-labeled solution to uniformly distribute throughout the body. Water samples (10 mL) were then collected for determination of TFM excretion rates from 0 to 4 h (water samples collected at 0, 1, 2, 4 h), 4–12 h (4, 6, 8, 10, 12 h), and 24 h. After each flux measurement period (0–4 h, 4–12, 24 h), the water in each container was replaced with fresh non-radioactive water which was at the same pH (6.5, 7.8, or 9.0), before initiating the next measurement. Water samples were refrigerated until processed for measurement of ^{14}C-radioactivity and total TFM concentrations. The pH of each container was monitored every hour for 12 h, and was adjusted to the desired pH as described above (Table 1). After 24 h, lamprey were pinched on the tail to determine survivorship and subsequently euthanized using 1.5 g L^{-1} MS-222 buffered with 3.0 g L^{-1} $NaHCO_3$. As described above, the lamprey were then removed from their containers, thoroughly rinsed and the tissues digested for measurement of whole body radioactivity.

2.4. Analytical methods

2.4.1. Whole body beta radioactivity measurements

Tissues were processed and analyzed for whole body beta radiation following similar protocols to those described by Blewett et al. (2013) for mummichog (*Fundulus heteroclitus*). Here the lamprey were digested at 60 °C for 48 h in the same 15 mL polypropylene tubes described above, during which time the centrifuge tubes were vortexed at regular intervals to ensure thorough digestion of the carcass, and then centrifuged for 5 min at 1228 × *g* to separate the supernatant from digested carcass (IEC Medilite 12, Thermo Electron Corporation, MA, USA). Aliquots of supernatant (2 mL) were then removed and added to 7 mL scintillation vials in duplicate, followed by the addition of 4 mL of Ultima Gold™ AB organic scintillation cocktail (PerkinElmer, MA, USA), vortexed, and left overnight in the dark to minimize chemiluminescence prior to quantifying whole body beta radiation. Water samples (2 mL) were mixed with 4 mL of Optiphase Hisafe 2 scintillation cocktail (PerkinElmer, MA, USA), vortexed, and also left in the dark for 12 h prior to determining beta radioactivity. The total beta radiation of tissue and water samples were then measured in counts per minute (CPM) using a scintillation counter (LS6500, Beckman Coulter, CA, USA). Precision standards (0, 4, 8, 12 mg L^{-1} TFM provided courtesy of the Sea Lamprey Control Centre, Fisheries and Oceans Canada (DFO), Sault Ste. Marie, Ontario) were used to spectrophotometrically determine the non-radioactive TFM concentration in water samples using a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, England, UK) at a wavelength of 395 nm based on DFO Standard Operating Procedures (IOP: 012.4).

2.5. Calculations

2.5.1. TFM uptake

TFM uptake rates (nmol g^{-1} h^{-1}) were calculated as described by Blewett et al. (2014):

$$\text{TFM Uptake Rate} = CPM_{lamprey}/(MSA \times \Delta T) \quad (1)$$

where $CPM_{lamprey}$ is the CPM g^{-1} tissue in each larval sea lamprey, *MSA* is the measured mean specific activity of ^{14}C-TFM (CPM nmol TFM^{-1}) after a 3 h exposure to the 4.6 mg TFM L^{-1} or 1 h exposure at the 7.6 mg TFM L^{-1}, and ΔT is the duration of exposure to TFM (h).

2.5.2. Excretion

TFM-efflux rates were determined by calculating the difference in water radioactivity at the beginning and end of a sampling period. These data were then used to determine the TFM-efflux rate ($J^{14C\text{-}TFM}$) in CPM g^{-1} h^{-1} (Wilkie et al., 2007b) using Eq. (2) below:

$$J^{14C-TFM} = (CPM_f - CPM_i)/(T \times M) \quad (2)$$

where CPM_i and CPM_f denote the initial and final CPM in the water for a given flux period. *T* is the flux period duration (h), and M is the mass of the lamprey (g). After $J^{14C\text{-}TFM}$ was calculated, the TFM clearance rate (nmol TFM g^{-1} h^{-1};Wilkie et al., 2007b) was calculated using Eq. (3):

$$\text{TFM Clearance} = J^{14C\text{-}TFM}/MSA \quad (3)$$

where *MSA* is the measured mean specific activity (CPM g^{-1} nmol TFM^{-1}) of TFM injected into the larval sea lamprey.

Percent TFM excreted was determined by first calculating the amount of TFM remaining in the animal as described Eq. (4):

$$\text{TFM Remaining} = (CPM_{lamprey} \times \text{TFM}_{\text{injected}})/\text{CPM}_{\text{injected}} \quad (4)$$

where $CPM_{lamprey}$ is the CPM g^{-1} tissue in each larval sea lamprey, $\text{TFM}_{\text{injected}}$ is the concentration of TFM injected into the lamprey (nmol TFM g^{-1}), and $\text{CPM}_{\text{injected}}$ is the CPM of the TFM injected into the lamprey. TFM Remaining was then subtracted from the amount of TFM injected (TFM_{intial}; nmol TFM g^{-1}) and then dividing by the amount of TFM injected (TFM_{intial}; nmol TFM g^{-1}) as described in Eq. (5) below:

$$\text{Percent Excreted} = [(\text{TFM}_{\text{intial}} - \text{TFM}_{\text{Final}})/\text{TFM}_{\text{intial}}] \times 100 \quad (5)$$

^{14}C-TFM standards (0, 0.001, 0.005, 0.01, 0.05, and 0.1 μCi mL^{-1} ^{14}C-TFM) were prepared and used to determine beta counter efficiency (94.7%). Digested non-radioactive lamprey were also spiked with increasing ^{14}C-TFM concentrations and compared to spiked water samples to determine quench in the tissue digests. No quenching was observed.

2.6. Statistical analysis

All data are presented as the mean ± 1 standard error of the mean (SEM). The rates of TFM uptake, excretion, and percent TFM excreted were analyzed using one-way or two-way analyses of variance (ANOVA) followed by a Tukey Honest Significant Difference post-hoc test when data were normally distributed and homoscedastic. When assumptions were not met, even after data transformation (Log_{10} or power transformations), a Kruskal-Wallis rank sum test followed by a Dunn's multiple comparison test was used. Linear regression analyses were used to evaluate the relationship between uptake rates and the concentration of either un-ionized or ionized TFM at different pHs. For all statistical tests, the level of significance was set at $P < 0.05$. Statistical analyses and figures were produced using R version 3.3.3, RStudio version 1.0.136, and ggplot2, ISBN: 978-0-387-98140-6.

3. Results

3.1. Effect of pH on rates of TFM uptake

Rates of TFM uptake by larval sea lamprey at low, moderate, and high pH (Table 1) were measured at two different nominal TFM concentrations 4.6 mg TFM $L^{-1}$and 7.6 mg TFM L^{-1}.

When exposed to a measured TFM concentration of 4.2 ± 0.01 mg TFM L^{-1} for 3 h at pH 6.87 ± 0.06, the rates of TFM uptake averaged 25.3 ± 5.2 nmol TFM g^{-1} h^{-1}, and were significantly greater than the rates of 12.7 ± 2.5 nmol TFM g^{-1} h^{-1} and 4.4 ± 0.3 nmol TFM g^{-1} h^{-1} measured at pH 8.06 ± 0.03 ($P \leq 0.05$) and pH 8.74 ± 0.07 ($P \leq 0.001$), respectively (Fig. 2). When larval sea lamprey were exposed to a measured TFM concentration of 7.03 ± 0.2 mg TFM L^{-1} for 1 h at pH 6.85 ± 0.18, TFM uptake averaged 91.3 ± 16.4 nmol TFM g^{-1} h^{-1} which were significantly greater than uptake rates at pH = 7.82 ± 0.13, which were 34.0 ± 6.1 nmol TFM g^{-1} h^{-1} ($P \leq 0.05$; Fig. 2). Additionally, rates

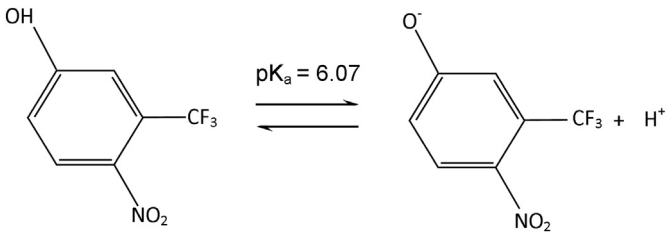

Fig. 1. Chemical structure of 3-trifluoromethyl-4-nitrophenol (TFM) and its dissociation equilibria. Because TFM is a weak acid with a pKa of 6.07, there is a higher proportion of total TFM in its un-ionized phenolic form at lower pH compared to more alkaline pHs, where the ionized (phenolate) form predominates (Hayton and Stehly, 1983; Hunn and Allen, 1974).

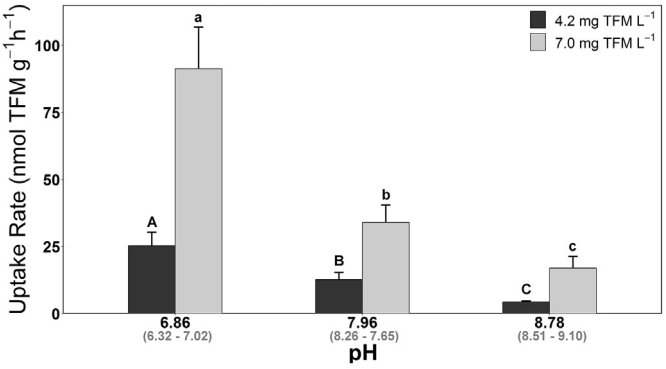

Fig. 2. Effects of pH on the rate of 3-trifluoromethyl-4-nitrophenol uptake. Changes in the TFM uptake rates of larval sea lamprey during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at 4.2 mg TFM $L^{-1}$for 3 h (black bars) and 7.0 mg TFM L^{-1}, for 1 h (light grey bars) at three different pHs (low, moderate, and high pH). Overall average pH between the two experiments for low, moderate, and high pH is on the x axis with the range indicated underneath. Data are presented as the mean + SEM (N = 9–12 fish at each pH). Statistically significant differences between the rates of TFM uptake or accumulation at each pH are denoted by upper case and lower case letters during exposure to 4.2 mg TFM L^{-1} and 7.0 mg TFM L^{-1}, respectively. The average pH between the two experiments is on the x-axis (black text) with the range of pH during the two experiments in parenthesis is underneath (light grey text).

were substantially lower at pH 8.82 ± 0.06, averaging 17.0 ± 3.76 nmol TFM g^{-1} h^{-1} which was significantly different from the measurements made at the two lower pHs ($P \leq 0.05$; Fig. 2).

The relative un-ionized TFM (TFM-OH) and ionized TFM (TFM-O^-) concentrations were also calculated at each pH using the Henderson Hasselbalch equation (Table 2, Supplementary Figs. 1 & 2), which was used to evaluate the relationship between average uptake rates of TFM by larval sea lamprey compared to the predicted TFM-OH and TFM-O^- concentrations in the water at each respective pH. This analysis indicated a positive relationship between uptake rates and TFM-OH during exposure to the total TFM concentrations of 4.2 mg TFM L^{-1} (Supplementary Fig. 1) and 7.0 mg TFM L^{-1} (Supplementary Fig. 2) as pH decreased.

Table 2
Distribution, speciation, diffusion gradients and Nernst potential calculations for different species of 3-trifluoromethyl-4-nitrophenol (TFM) at different pHs (low, moderate, and high pH following the determination of TFM uptake rates. Data calculated for larval sea lamprey exposed to 4.2 mg TFM L^{-1}. TFM-OH gradients with positive values denote an inwardly directed gradient favoring TFM uptake. Negative Nernst Potential values denote inward gradients for the ionized form of TFM (TFM-O^-).

	Blood	Water (pH 6.86)	Water (pH 7.96)	Water (pH 8.78)
pH	7.85[a]	6.87	8.06	8.74
Total TFM (nmol mL^{-1}; 4.2 mg TFM L^{-1})	0.038[b]	19.85	19.36	21.10
TFM-OH (nmol mL^{-1})[c]	0.006	2.71	0.196	0.05
TFM-O (nmol/mL^{-1})[d]	0.037	17.14	19.165	21.05
% TFM-OH	1.63	13.68	1.02	0.21
% TFM-O^-	96.70	86.32	98.98	99.79
Chemical gradient[e]		2.704	0.190	− 0.002
Nernst Potential (mV)[f]		− 153.22	− 156.01	− 158.35

[a] Arterial blood pH measured in adult sea lamprey (Boutilier et al., 1993).
[b] Whole body TFM burdens calculated by dividing the counts per minute of radiation in each fish (CPM g^{-1} fish) by the mean specific activity (MSA) of TFM in the water.
[c] $[\text{TFM-OH}] = [\text{TFM}]_{\text{total}}/[1 + \text{antilog}(\text{pH} - \text{pK}_a\ \text{TFM})]$, where pH is blood or bulk water pH and the pK_a of TFM = 6.07 (Hunn and Allen, 1974).
[d] $[\text{TFM-O}^-] = [\text{TFM}]_{\text{total}} - [\text{TFM-OH}]$.
[e] Chemical gradient equals $[\text{TFM}]_{\text{total}} - [\text{TFM}]_{\text{blood}}$.
[f] Nerst potential = (RT/zF)ln[TFM]o/[TFM]i, where R is the Ideal Gas Constant, T is temperature (°K), F is the Faraday constant, and z is the valence of ionized TFM (minus one), and [TFM] is the concentration outside (o) and inside the (i) the animal (e.g. Wilkie and Wood, 1995).

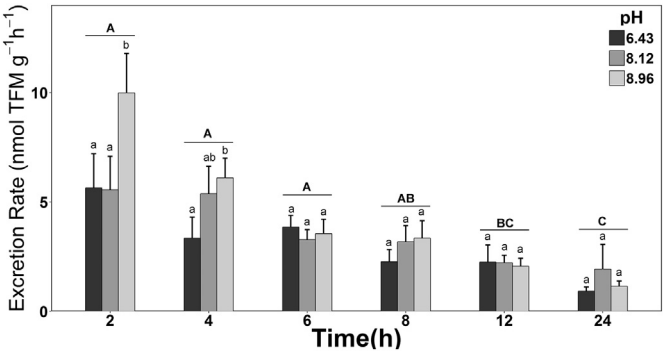

Fig. 3. Effects of pH on excretion rates of 3-trifluoromethyl-4-nitrophenol. Changes in excretion rates of 3-trifluoromethyl-4-nitrophenol (TFM) by larval sea lamprey at different pHs (low, moderate, and high pH) when injected with 85 nmol TFM g^{-1}. Data is presented as the mean + SEM, (N = 8 per treatment). Statistically significant differences between time periods are denoted by upper case letters while the excretion rates within each group are denoted by lower case letters at each time period. Rates observed at pH low, moderate, and high pH are resented by dark bars, grey bars, and light grey bars, respectively.

3.2. Effect of pH on rates of TFM excretion

Initial excretion rates (2 h) for lamprey injected with 85 nmol TFM g^{-1} were 5.2 ± 1.5 nmol TFM g^{-1} h^{-1} at pH 6.43 ± 0.07, 5.6 ± 1.4 nmol TFM g^{-1} h^{-1} at pH 8.12, ± 0.03, and 10.0 ± 1.7 nmol TFM g^{-1} h^{-1} at pH 8.96 ± 0.04. Excretion rates 4 h post injection for lamprey injected with 85 nmol g^{-1} TFM were 3.4 ± 0.9 nmol TFM g^{-1} h^{-1} at low pH, 5.4 ± 1.2 nmol TFM g^{-1} h^{-1} at moderate pH, and 6.1 ± 0.8 nmol TFM g^{-1} h^{-1} at high pH. The rate of TFM excretion measured over 2 h and 4 h of the depuration period at high pH were significantly greater than rates observed at low pH 6.5, but not moderate pH (Fig. 3; $P \leq 0.05$; one-way ANOVA). Beyond 4 h, however, no significant differences in TFM excretion were observed amongst the three different water pHs. Additionally, as time post injection proceeded the amount of TFM excreted decreased significantly regardless of pH treatment (Fig. 3: $P \leq 0.05$; one-way ANOVA). In fact, by 24 h approximately 95% of the injected TFM had been cleared by the larval sea lamprey at all three water pHs.

4. Discussion

4.1. The effect of water pH on TFM uptake

Despite being the target of lampricide treatments, no previous studies have quantified TFM uptake rates in larval sea lamprey. The present study demonstrated that higher rates of TFM uptake by larval sea lamprey at low compared to high pH contributes to their greater sensitivity to TFM at lower pH due to more rapid accumulation of the lampricide (Hunn and Allen, 1974). Hunn and Allen (1974) also demonstrated that at low pH, TFM accumulation was greater in rainbow trout and channel catfish (*Ictalurus punctatus*). TFM is a weak acid with an ionizable hydroxyl group, and a pKa of 6.07 (Hubert, 2003, Fig. 1). As pH increases, the equilibrium between unionized, phenolic TFM (TFM-OH) and the ionized, phenolate species (TFM-O^-) shifts towards the latter (Mcdonald and Kolar, 2007). Thus, the greater uptake of TFM at lower pH is likely because there is a greater proportion of TFM in its un-ionized form (TFM-OH) which is more lipophilic compared to the ionized, charged form of TFM (Hayton and Stehly, 1983; Hunn and Allen, 1974; Mcdonald and Kolar, 2007).

The size and structure of TFM is similar to 2,4-dinitrophenol (2,4 DNP), which also contains a central phenol ring and an ionizable hydroxyl group, which is known to diffuse across the gill epithelia of fishes (McKim et al., 1987; McKim and Goeden, 1982). Both 2,4 DNP and TFM are thought to be more diffusible across the gills in their un-ionized stage, which is thought to increase the lipid solubility and toxicity of each compound in fishes (Howe et al., 1994; Hunn and Allen, 1974). The more lipid soluble a substance, the greater its diffusibility across epithelial tissue (Hughes, 1980). Lipid solubility can be determined by calculating the Log of the octanol:water partition coefficient (log K_{OW}). Generally, compounds that have higher log K_{OW} values (> 3.0) are considered to be more lipophilic and diffuse more readily across epithelial membranes such as the gills (Cronin and Livingstone, 2004; Howe et al., 1994; McKim and Erickson, 1991; Saarikoski et al., 1986). With respective log K_{OW} values of 1.67 and 2.77 (Cronin and Livingstone, 2004; McKim and Erickson, 1991), 2,4-dinitrophenol and TFM only have moderate lipid-solubility. Yet, TFM accumulates rapidly in larval sea lamprey and non-target fishes, particularly at low pH, as demonstrated here (TFM) and in earlier studies (Hunn and Allen, 1974).

To better understand how TFM speciation influences TFM uptake rates in the present study, the proportions of TFM in its un-ionized (phenolic) and in its ionized (phenolate) forms were calculated using the Henderson-Hasselbalch equation (Hubert, 2003; Mcdonald and Kolar, 2007). At low pH, when the total TFM concentration was equal to 19.85 nmol TFM mL^{-1} (~4.2 mg TFM L^{-1}) the amount of un-ionized TFM in the water was 2.71 nmol TFM mL^{-1}, which represented about 13.7% of the total TFM in the water, with ionized-TFM comprising the remainder (Table 2). However, at high pH (~pH 8.74), the amount of un-ionized TFM in the water was > 98% lower, when concentrations were < 0.05 nmol TFM mL^{-1} or approximately 0.2% of the total TFM added to the water (Table 2). However, at high pH, where concentrations of un-ionized TFM were very low (and the ionized form of TFM very high > 99.8% (0.01 mg TFM L^{-1} vs. 2.71 mg TFM L^{-1}; Table 2), there was still appreciable TFM uptake and accumulation by the lamprey (Supplementary Figs. 1 & 2). Furthermore, at high pH where low amounts of un-ionized TFM were present, uptake of TFM still occurred at measurable rates (Supplementary Figs. 1 & 2). This suggested that TFM may be taken up, albeit to a lesser extent, in its ionized form.

Similar to TFM, the toxicity of ammonia in fishes is highly dependent on water pH and the proportion of un-ionized ammonia in the water (Ip et al., 2001; Thurston et al., 1981). With a pKa of approximately 9.5 in freshwater (at 15 °C; Cameron and Heisler, 1983), total ammonia is present as either un-ionized NH_3 or ionized NH_4^+. As a result, ammonia toxicity increases as the proportion of NH_3 increases with water pH (Ip et al., 2001; Wilkie, 2002). For this reason, ammonia toxicity is often based on the 48-h or 96-h LC_{50} of NH_3, rather than NH_4^+, with its lower solubility and positive charge (Thurston et al., 1981). Using similar rationale, if TFM uptake were solely in the un-ionized form, then the acute toxicity of un-ionized TFM should be more or less the same, regardless of water pH. To test this hypothesis, we plotted data generated by this study (Supplementary Figs. 1 & 2) and previously published TFM toxicity data at different water pHs (Bills et al., 2003). The LC_{99s} found by Bills et al. (2003) at different pHs were plotted against the concentrations of un-ionized TFM (Fig. 4), calculated using the Henderson-Hasselbalch equation (as described in Table 2). Unexpectedly, in both our study and Bills et al. (2003), the amount of un-ionized TFM decreased (Supplementary Figs. 1 & 2) or exponentially (Fig. 4) while pH increased,, further suggesting that TFM toxicity was not simply a function of the concentration of un-ionized TFM in the water (Fig. 4 & Supplementary Figs. 1 & 2). Rather, this analysis also suggested that there must be appreciable uptake of ionized TFM.

These observations challenge the assumption that TFM is taken up as only un-ionized TFM (Hunn and Allen, 1974; Marking and Hogan, 1967). In fact, the ionized form of TFM may not be as lipophobic as previously thought. Niclosamide is another nitrophenol with an ionizable hydroxyl group used for sea lamprey control in the Great Lakes (Mcdonald and Kolar, 2007), as well as a molluscicide to treat snail-infested waters in regions where they are vectors for schistosomiasis, a debilitating parasitic disease in much of Asia (Xia et al., 2014). After

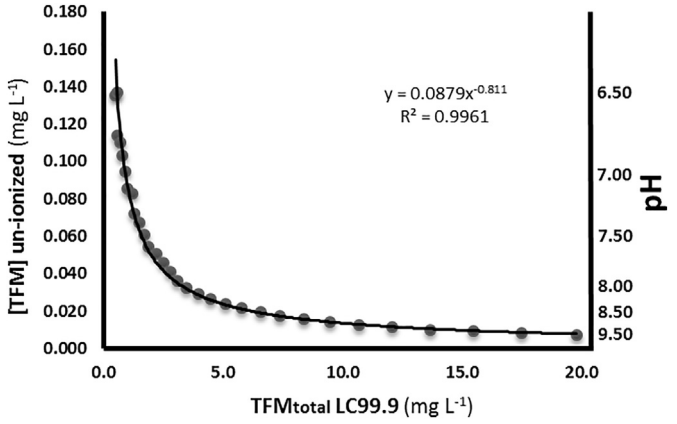

Fig. 4. Relationship between un-ionized 3-trifluoromethyl-4-nitrophenol (TFM-OH) concentration, water pH, and the 12-h $LC_{99.9}$ of total TFM concentration to larval sea lamprey. The concentrations of un-ionized (TFM-OH) were calculated using total TFM concentrations (sum of [TFM-OH] + [TFM-O^-]) toxicity data and water pH data provided by Bills et al. (2003). Calculations made using pKa of 6.07 for TFM and the Henderson-Hasselbalch equation.

reviewing Wu et al. (2011) noted that the log K_{OW} for the ionized form of niclosamide was surprisingly close to that of its un-ionized moiety. Taken together, these observations further support the thesis that niclosamide, and TFM, also enters the lamprey in its ionized form, albeit at lower rates than the un-ionized TFM. Possible routes of the entry of ionized TFM could be via passive diffusion of ionized TFM due to changes in the gill microenvironment which are complex and occur in response to changes in water chemistry (eg. high pH vs. low pH; Randall et al., 1991; Randall and Lin, 1993). Alternatively, transport of ionized TFM could be occurring via ion transport proteins on the gill, pharynx and/or gut. One group of candidates are the organic anion transporters, such the Mrp2 protein (multi-drug resistance-associated protein 2), for which the genes have been identified in the gills, liver and other tissues of larval and adult sea lamprey and are thought be involved in bile salt homeostasis (Cai et al., 2013). However, both pHs effect on gill microenvironment assisting in the uptake of ionized TFM and this intriguing possibility have not been investigated.

An additional/alternate explanation for the disconnect between rates of TFM uptake and the concentration of un-ionized TFM in the water, are differences between water chemistry in the microenvironment of the gills compared to the bulk water, upon which measurements of TFM and water chemistry are based. Indeed, when larval sea lamprey were exposed to both 4.2 mg TFM L^{-1} and 7.0 mg TFM L^{-1}, TFM uptake rates observed at low pH were statistically different from rates observed at both moderate and high pH (Fig. 2). This observation might be explained by the chemistry of the gill microenvironment which can be different than bulk water chemistry. Playle and Wood (1989) showed that the expired water pH was lower than that of the bulk water in rainbow trout acclimated to water pHs that were greater than pH 6.0 due to a combination of CO_2 and H^+ excretion across the gills. Thus, acidification of water adjacent to the gill could have shifted the TFM equilibrium towards un-ionized TFM-OH in that region, resulting in greater amounts of un-ionized TFM in the gill microenvironment, despite the low pH of the bulk water.

4.2. The effect of water pH on TFM excretion

Initial excretion rates during depuration at high pH were elevated compared to low pH, which suggests that TFM was cleared across the gills in its un-ionized form down favorable diffusion gradients and then trapped as ionized TFM as it entered the water. The net effect of such diffusion trapping would be to sustain the blood-to-water diffusion gradient for un-ionized TFM across the gills. Renal or biliary excretion via the gut seems less likely because of the sea lamprey's low capacity to biotransform TFM to TFM-glucuronide, which is used by non-target organisms including teleost fishes to detoxify and excrete this lampricide (Birceanu et al., 2014; Hubert et al., 2001; Kane et al., 1994; Lech and Statham, 1975; Lech and Costrini, 1972). Glucuronidation allows TFM, not to mention other xenobiotics or metabolites, to be more water soluble allowing the compound to be excreted via these extra-branchial routes (Clarke et al., 1991). Indeed, we were only able to detect trace amounts of TFM-glucuronide in larval sea lamprey exposed to TFM (B. Hlina and M.P. Wilkie, unpublished observations), whereas non-target fishes including largemouth bass (*Micropterus salmoides*), coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*) accumulated TFM-glucuronide in the bile following TFM exposure (Lech, 1974; Schultz et al., 1979). Significant amounts of TFM-glucuronide were also measured in the muscle of trout and Ictalurid fishes (catfishes) when exposed to TFM in the lab (Birceanu et al., 2014; Kane et al., 1994) and during field applications of TFM (Hubert et al., 2001; Vue et al., 2002).

If TFM is excreted via the gills, this process could be influenced by a combination of factors including free-TFM concentrations in the blood, boundary layer pH, and bulk water pH (Fig. 3; Hunn and Allen, 1974; Playle and Wood, 1989). Each of these factors would influence the blood-water TFM-OH diffusion gradient. In the blood (pH ~7.85; Boutilier et al., 1993), TFM would primarily persist as the ionized, phenolate ion but there would still likely be sufficient amounts of the un-ionized phenolic form which would more easily diffuse across the gill (Clifford et al., 2012), as has been reported for the NH_3 species of ammonia (e.g. Wilkie, 2002). It is likely that much of the ionized TFM was bound to blood plasma proteins (Escher et al., 2011) but even under such conditions however, a small but significant portion of TFM will be in this un-ionized form at physiological pH, which could diffuse across the gill into the water down favorable gradients following a lampricide treatment, when TFM is no longer present or present in very low concentrations. In this respect, the water could act as a "sink" into which surviving sea lamprey could unload the lampricide.

At a high pH or moderate pH, we predicted that this un-ionized TFM would be trapped as ionized TFM in the gill microenvironment, contributing to the generation of a larger gradient for TFM excretion into TFM free water. At a low pH we predicted that the outward gradient favoring un-ionized TFM should have been lower, resulting in lower TFM excretion rates. Indeed, this was observed over the first 4 h in TFM free water. Unexpectedly, TFM excretion rates were comparable beyond 4 h, suggesting that the differences in water pH only had a transient effect on TFM elimination. However, this could be important if TFM treatments were interrupted due to mechanical breakdowns, sudden weather events, or other adverse events. If water pH was higher, lamprey would likely excrete a greater proportion of their TFM body burden compared to lower pH, which could allow them to partially recover from treatment and increase the possibility of their survival if sufficient TFM was not used when the treatment resumes. However, the findings also clearly indicated that sea lamprey are able to rapidly clear TFM from their bodies in TFM free water. Indeed, by 24 h of depuration, > 95% of the loaded TFM was eliminated by sea lamprey at low, moderate, and high pH. Taken together with the findings of Clifford et al. (2012), which demonstrated that sea lamprey can rapidly (2–4 h) correct reductions in energy stores (phosphocreatine, glycogen) if they survive TFM exposure, suggests that if sea lamprey can survive TFM treatments, that their longer-term likelihood of survival is very high.

4.3. Conclusion

The present findings clearly suggest that TFM uptake decreases with increasing pH, which likely explains why TFM toxicity is lower in higher pH waters (e.g. Bills et al., 2003; Mcdonald and Kolar, 2007). The present findings suggest that TFM treatment effectiveness can be compromised by decreases in water pH which could take place due to high rates of photosynthesis due to macrophytes and phytoplankton (Wetzel, 1983), ground water seepage, or altered water flow. In

contrast, reductions in water pH due to increased respiration by aquatic plants and macrophytes, ground water seepage, or rainfall, could threaten non-target species by increasing the proportion of un-ionized TFM. Since TFM treatments are done over 9–12 h, diurnal changes in pH due to both photosynthesis and aerobic respiration can influence the pH of the stream causing stream pH to vary (Odum, 1956; Vannote et al., 1980; Wetzel, 1983). Thus, higher nutrient streams with an abundance of macrophytes, algae, and/or cyanobacteria would be more prone to such changes compared to less productive streams (Wetzel, 1983). In situations where diurnal changes influence pH greatly, TFM treatments become difficult to control potentially resulting in non-target species being more susceptible to TFM at different time points throughout the treatment. Such fluctuations in pH, even relatively small changes, could markedly affect TFM uptake and excretion through pH–dependent changes in TFM speciation, and ultimate affect toxicity when TFM concentrations are low. Sea lamprey control agents closely monitor pH and adjust TFM application rates accordingly (B. Stephenson, DFO, personal communication), but sudden increases in pH or lags in making corrections to TFM application rates, could conceivably result in less TFM accumulation by larval sea lamprey and a greater likelihood of residual larval sea lamprey that survive treatment. Thus, if stream pH fluctuates it may be prudent in some cases to extend treatment times to ensure that TFM uptake is sufficient to cause death of the larval sea lamprey being targeted. Additional factors including geomorphological features, rain events, runoff from agricultural, and timber harvests could also affect water quality and increase the susceptibility of rivers and streams to markedly fluctuating pH resulting in inefficient TFM treatments (Campbell and Doeg, 1989; Hynes, 1970; Mcdonald and Kolar, 2007; Poudel et al., 2013; Tiedemann et al., 1988).

Within our study, it is noted that the evaluation of both uptake and excretion were done in separate experiments, which both are unlikely to occur separately during a TFM treatment in the field. Both uptake and excretion of TFM are likely to occur simultaneously if concentration gradients favour uptake and excretion. However, when evaluating the concentration gradients that would hypothetical occur under field conditions (e.g. treatment water concentration of 7.0 mg TFM L^{-1}) the concentration found outside the larval sea lamprey is significantly greater than concentrations found within the lamprey (e.g. internal concentration of 0.03 mg TFM L^{-1}), resulting in little to no excretion of TFM via passive diffusion across the gills. Therefore, unless a treatment is abruptly stopped, it is unlikely that excretion of TFM via passive diffusion will assist in the survival of a lamprey exposed to lethal concentrations of TFM. It is important to note how uptake and excretion will influence TFM toxicity as sea lamprey control managers are likely to use information on the uptake and excretion of TFM to better plan and manage TFM treatments.

On a broader scale, it is likely that the uptake and excretion of other phenolic xenobiotics such as 2,4-dinitrophenol and similar compounds, are similarly affected by water pH, which may necessitate further studies on the effects that water pH has on the uptake and elimination of these compounds.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2017.05.005.

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