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The effects of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) on fuel stores and ion balance in a non-target fish, the rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The pesticide 3-trifluoromethyl-4-nitrophenol (TFM) is used to control sea lamprey (*Petromyzon marinus*) populations in the Great Lakes through its application to nursery streams containing larval sea lampreys. TFM uncouples oxidative phosphorylation, impairing mitochondrial ATP production in sea lampreys and rainbow trout (*Oncorhynchus mykiss*). However, little else is known about its sub-lethal effects on non-target aquatic species. The present study tested the hypotheses that TFM exposure in hard water leads to (i) marked depletion of energy stores in metabolically active tissues (brain, muscle, kidney, liver) and (ii) disruption of active ion transport across the gill, adversely affecting electrolyte homeostasis in trout. Exposure of trout to 11.0 mg l^{-1} TFM (12-h LC_{50}) led to increases in muscle TFM and TFM-glucuronide concentrations, peaking at 9 h and 12 h, respectively. Muscle and brain glycogen was reduced by 50%, while kidney and muscle lactate increased with TFM exposure. Kidney ATP and phosphocreatine decreased by 50% and 70%, respectively. TFM exposure caused no changes in whole body ion (Na^+, Cl^-, Ca^{2+}, K^+) concentrations, gill Na^+/K^+ ATPase activity, or unidirectional Na^+ movements across the gills. We conclude that TFM causes a mismatch between ATP supply and demand in trout, leading to increased reliance on glycolysis, but it does not have physiologically relevant effects on ion balance in hard water.

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1. Introduction

The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) is used to control sea lamprey (*Petromyzon marinus*) populations in the Great Lakes, where it is applied to nursery streams containing larval sea lampreys (Smith and Tibbles, 1980; Bills et al., 2003; Boogaard et al., 2003; McDonald and Kolar, 2007). TFM is the major component in the integrated pest management program of the Great Lakes Fisheries Commission, which was established in 1955 as a partnership between Canada and the United States to coordinate fisheries research and management, and to control sea lamprey populations in this region (Great Lakes Fishery Commission, 2011). The use of TFM has contributed to the restoration of fisheries that were decimated in the mid-20th century due to the combined effects of overfishing and lamprey predation (i.e. parasitism; Lowry, 1970; Christie et al., 2003; McDonald and Kolar, 2007). Treatments with TFM have proven effective mainly because of the specificity of TFM for the larval lampreys (Applegate and King, 1962; Lech and Costrini, 1972; Lech and Statham, 1975) and the relatively sedentary life style of these animals, which mainly restricts them to streams and rivers in this life stage. Despite its success in sea lamprey control, little is known about the potential physiological effects that TFM has on non-target fishes (McDonald and Kolar, 2007).

It is known that the concentrations of TFM tolerated by most fishes are 3–5 times higher than that required to kill larval sea lampreys (Applegate and King, 1962; Bills et al., 2003; Boogaard et al., 2003). This tolerance is related to the greater capacity of most non-target fishes to

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biotransform TFM to TFM-glucuronide via the process of glucuronidation (Olson and Marking, 1973; Lech, 1974; Lech and Statham, 1975; Kane et al., 1993, 1994), making TFM more water soluble and easier to excrete via renal pathways or the gastrointestinal tract (Clarke et al., 1991).

However, there is some evidence that suggests that TFM exposure can have negative effects on non-target fish species. Christie and Battle (1963) demonstrated that TFM can damage the gills in trout (*Oncorhynchus mykiss*) and larval sea lampreys, but Mallatt et al. (1994) detected no changes in gill ultrastructure in trout exposed to their respective 9-h TFM LC_{100} (TFM concentration that is lethal to 100% of the trout over a 9 h exposure period). Kane et al. (1993) reported that bullfrog (*Rana catesbiana*) tadpoles were approximately 13 times more sensitive to TFM than adults due to a lower glucuronidation capacity in the tadpole phase (larval LC_{50} = 0.95 mg l^{-1} vs. adult LC_{50} = 12.99 mg l^{-1}).

Evidence that TFM exerts its toxic effects by creating a shortfall in ATP supply in sea lampreys was provided by Wilkie et al. (2007a), who observed significant decreases in plasma glucose and whole body phosphocreatine (PCr) levels in larval lamprey exposed to TFM (12-h LC_{50} = 2.0 mg l^{-1}). In addition, Birceanu et al. (2009) and Clifford et al. (2012) reported that as the exposure time increased, ATP and glycogen levels in the brains and in the livers of larval sea lampreys were reduced in a step-wise fashion following exposure to sub-lethal (12-h LC_{50}) and lethal (12-h $LC_{99.9}$) concentrations of TFM, respectively. Recently, Birceanu et al. (2011) used isolated liver mitochondria to demonstrate that TFM causes such shortfalls in ATP supply by impairing oxidative phosphorylation in both rainbow trout and sea lampreys. This would subsequently force the fish to rely more on their glycogen supplies and anaerobic glycolysis in order to maintain the ATP demand in the body. Viant et al. (2001) suggested that TFM tolerance could be related to capacity for sustained anaerobic glycolysis in two marine molluscs, limpets (*Lottia gigantean*) and abalone (*Haliotis rufescens*).

While the proximate mechanism of TFM toxicity is the same in rainbow trout and sea lampreys, it is the trout's high glucuronidation capacity (Olson and Marking, 1973; Lech, 1974; Lech and Statham, 1975) that likely prevents the buildup of free-TFM to levels that significantly impair mitochondrial function, and thus aerobic ATP production. Under these conditions, the fish would be forced to rely more on their anaerobic energy stores (i.e. glycogen, phosphocreatine) to compensate for the shortfall in ATP. This shortage of ATP in the body could also indirectly affect ion homeostasis by reducing the ATP supply to ATP-dependent ion pumps in the kidneys and in the gills. Thus, the overarching goal of the present study was to determine if TFM exposure is interfering with ATP supply in rainbow trout, and whether or not such disturbances also impair ion homeostasis.

To test the hypothesis that TFM toxicity results in a mismatch between ATP supply and ATP demand in the trout, we exposed the fish to their respective 12-h TFM LC_{50}, and measured changes in tissue (brain, liver, muscle, kidney) glycogen, ATP, phosphocreatine (PCr) and lactate over the 12 h exposure period, which approximates the length of time that a typical TFM treatment lasts in the field (B. Stephens, DFO — Sea Lamprey Control Center, pers. comm.). The rates of Na^+ uptake, gill Na^+/K^+ ATPase activity, plasma ion (Na^+, Cl^-) and whole body ion (Na^+, Cl^-, Ca^{2+} and K^+) concentrations, and muscle tissue water were also quantified in rainbow trout, following exposure to TFM to determine if the lampricide interfered with gill-mediated ion exchange.

2. Material and methods

2.1. Experimental animals and holding

Rainbow trout (*O. mykiss*, Salmonidae; mass = 5–10 g; N = 62 for the metabolite experiments; mass = 30 ± 2.8 g; N = 48 for the ion flux experiments) were purchased from Rainbow Springs Hatchery (Thamesford, ON, Canada) and held in 120 l polyethylene tanks receiving hard Wilfrid Laurier University well water on a flow-through basis (pH ~ 8.0; titratable alkalinity ~ 200 mg $CaCO_3$ l^{-1}; hardness ~ 450 mg $CaCO_3$ l^{-1}; $[Na^+]$ ~ 1.1 mmol l^{-1}; temperature ~ 10–13 °C). The fish were held under a 12 h light:12 h dark photoperiod, and fed 3 times per week with ground 3.0 commercial floating pellets for the small fish and whole pellets for the larger fish (Corey Feed Mills, Fredericton, NB, Canada). The animals were held in the laboratory for at least 2 weeks before experiments commenced, and were starved for 72 h prior to the experiments, to decrease the amount of ammonia that could accumulate in the water during the 12-h static TFM exposure period. All experiments and fish husbandry were approved by the Wilfrid Laurier University Animal Care Committee and followed Canadian Council of Animal Care guidelines.

2.2. Experimental protocol

2.2.1. Determination of the acute toxicity of TFM

To determine the 12-h LC_{50} of TFM in Wilfrid Laurier well water for rainbow trout, a range finder experiment was conducted by exposing trout to either control conditions (no TFM) or to nominal TFM concentrations of 8.0, 10.0, 12.0, 16.0, 20.0 and 25.0 mg l^{-1}, for 12 h, in 15 l of well water. All fish were acclimated to their respective treatment containers for 12 h prior to the addition of the chemical. A total of 5 fish were exposed to each concentration, at pH 8.14 ± 0.03. The experiments were conducted in the dark, since TFM is sensitive to photodegradation (Carey and Fox, 1981; Hubert, 2003) and the containers were placed in a flow-through tank supplied with well water to ensure the temperature remained constant during the exposure. For unidirectional ion flux experiments, larger rainbow trout (30 ± 2.8 g) were exposed to their respective TFM 12-h LC_{100} in the same well water to determine if higher TFM exposure concentrations interfered with unidirectional Na^+ movements (influx, efflux, net flux) across the gill. These experiments were therefore preceded by a second range finder experiment during which the trout (N = 10 per concentration) were exposed to the same nominal concentrations as above (measured [TFM] = 8.0, 9.7, 11.6, 13.9, 17.8 and 27.1 mg l^{-1}, plus non-exposed controls) to determine the LC_{100} of TFM in the same water.

Field formulation TFM (35% active ingredient in isopropanol; Clariant SFC Gmbh Werk Griesheim, Germany) was used for all range finder and TFM exposure experiments and provided courtesy of the Sea Lamprey Control Center, Fisheries and Oceans Canada (Department of Fisheries and Oceans [DFO]; Sault Ste. Marie, ON, Canada). To verify water TFM concentrations, the absorbance of water samples was measured at a wavelength of 395 nm using a 96-well plate spectrophotometer (SpectraMax 190, Molecular Devices, CA, USA), and using the precision TFM standards and the Standard Operating Procedures (Instrument Operating Procedure, IOP012.3, DFO, Sault Ste. Marie, ON, Canada) provided by the Sea Lamprey Control Center and modified for the 96-well plate spectrophotometer (SpectraMax 190, Molecular Devices, CA, USA) used for the current measurements.

2.2.2. TFM accumulation and its effects on energy stores, metabolites and whole body ions

To test the hypothesis that TFM toxicity was associated with an energy imbalance in rainbow trout, the effects of TFM on fuel stores [ATP, PCr, glycogen and glucose] and lactate were measured in the liver, brain, muscle and blood at different intervals (1, 3, 6, 9, 12 h) during exposure to the pre-determined 12-h LC_{50} of TFM (nominal [TFM] = 11.0 mg l^{-1}, n = 8 at each time point in TFM-exposed fish; n = 11 controls with n = 5 sampled at the beginning of the experiment and 6 sampled 12 h later). Each of the fish (control and TFM-exposed trout) were contained in static 1.0 l containers filled with 1.0 l of Wilfrid Laurier aerated well water and left to acclimate for 12–24 h prior to the beginning of the experiment. Immediately prior to the addition of TFM to the treatment containers, approximately 75% of the water was replaced with fresh well water in all containers, including the controls. At each sample interval (0, 1, 3, 6, 9, 12 h), sub-sets of live fish were

anesthetized with 0.5 g l^{-1} tricainemethanesulfonate (MS222) buffered with 1.0 g $NaHCO_3$, blotted dry with a paper towel, and their mass and lengths were measured. Blood was then collected by cardiac puncture using a 1 ml insulin syringe that was previously rinsed with 50 IU heparin solution to prevent clotting. Blood for subsequent lactate and hemoglobin determination, along with tissues (brain, muscle, kidney and liver) for metabolite and fuel store analysis were processed as described by Birceanu et al. (2009). Briefly, a sub-sample of blood (10 μl) was mixed with two parts 7% perchloric acid (20 μl) for later determination of lactate concentration. Another blood sub-sample (10 μl) was added to 2.5 ml of Drabkin's reagent for blood hemoglobin determination. The remaining blood sample was centrifuged at 10,000 *g* for 3 min, and the plasma was collected, frozen in liquid nitrogen and saved for later determination of plasma glucose concentration. Immediately following blood sampling (within 1 min), the brain, liver, kidney, muscle and the gill baskets were collected from each fish, and snap frozen in liquid nitrogen (Wang et al., 1994a). Control fish were sampled identically to the treatment fish, except they were sampled at 0 h ($n = 5$) and 12 h ($n = 6$) only, to ensure there were no changes in the metabolite, fuel stores and ion levels due to the fish being held in the containers for an additional 12 h on top of the 12–24 h acclimation period. All tissues were kept at −80 °C until processed for later quantification of energy stores, ions, metabolites, and muscle TFM concentration.

To measure whole body ions, fish were anesthetized as previously described and whole bodies were collected at 0, 3, 6, 9 and 12 h of TFM (nominal 11.0 mg l^{-1}) exposure, weighed, and placed in tubes containing 5 times the fish' mass in 1 N HNO_3. The whole bodies were digested for 48 h at 60 °C; the homogenates were then mixed with a vortex mixer, and a sub-sample (1.5 ml) withdrawn and centrifuged at 10,000 *g* for 2 min. The supernatant was then diluted with deionized water, and analyzed using atomic absorption spectroscopy for Na^+, K^+, Mg^{2+}, and Ca^{2+} concentration using well established protocols. The remainder of the homogenate was frozen at −20 °C.

2.2.3. Unidirectional Na^+ fluxes in the presence of TFM

The unidirectional movements of Na^+ across the gills (influx, efflux and net flux) in the presence of TFM were measured on fish that were exposed to their respective TFM 12 h-LC_{100}. Approximately 12 h prior to the experiment, 9 rainbow trout were transferred to 1200 ml individual, aerated, darkened plastic containers (1 fish per container) receiving Wilfrid Laurier University well water on a flow-through basis. At the beginning of the experiment, the volume in the flux chambers was adjusted to 1000 ml. Next, 2.5 μCi $^{24}Na^+$ was added to each chamber and allowed to equilibrate for 10 min. During the first 4 h flux (control conditions), 20 ml water samples were collected. At the end of this flux period, the chambers were not flushed, but the water volume was readjusted to 1000 ml, and extra isotope was added to the container to compensate for ^{24}Na loss due to radioactivity decay (the half-life of $^{24}Na^+$, $T_{1/2}$–$^{24}Na^+$, is 15 h), dilution and sampling. Following this 4 h control period, a nominal TFM concentration of 17.8 mg l^{-1} (measured 17.5 mg l^{-1}) was added to the chambers. The rates of Na^+ influx, efflux and net flux were then measured after 0–2, 2–4, 4–6, 6–8, and 8–10 h of TFM exposure, after which the experiment was stopped because of mortalities caused by the TFM. Accordingly, any TFM-treated fish that were still alive at the end of the 10 h exposure period were then euthanized by a blow to the head. There were no mortalities observed in control fish over the exposure period.

2.3. Analytical techniques

2.3.1. Extraction and quantification of TFM from the muscle tissue

Muscle tissue was selected for analysis because previous studies have shown that TFM accumulates in trout muscle following routine TFM application to streams, as well as in the laboratory (Hubert et al., 2001; Dawson et al., 2002; Hubert et al., 2005). All samples were randomized to reduce bias during processing. The TFM was extracted from the muscle tissue according to the protocol described by Hubert et al. (2001), with minor changes. Briefly, a sub-set of control muscle samples were spiked with 100 ng ml^{-1} TFM prior to extraction to determine TFM recovery during processing. This muscle tissue and experimental samples (approx. 0.3 g) were then ground into a fine powder under liquid nitrogen and shaken for 10 min with 4.0 ml of 80% methanol on a MaxQ 2000 orbital shaker (Barnstead Lab Line, Dubuque, Iowa). The samples were then centrifuged at 3000 rpm for 10 min using a clinical centrifuge and the supernatant was collected and transferred to a new tube. The process was repeated twice more, after which the total supernatant was evaporated to ~8.0 ml in a nitrogen evaporator (N-EVAP Analytical Evaporator, Organomotion, Berlin, MA, USA), in a 55 °C waterbath. Samples were then passed through pre-conditioned (100% methanol, followed by 70% methanol) solid phase extraction (SPE) columns (Agilent Bond Elut C18/OH) containing 1.0 cm high-density glass filter beads (3 M Empore Filter Aid 400). Captured effluents were evaporated as above, and the pH of the samples was adjusted to 9.5 ± 0.2 with 1 N NaOH. A second set of SPE cartridges (Empore SDB-XC) were eluted with water at pH 9.5, and the effluent was captured and similarly placed in the water bath to evaporate the residual methanol. The pH of the solutions was then adjusted to 4.0 ± 0.2 with 6 N HCl. Samples were processed through a third set of SPE columns (Agilent Bond Elut C18/OH) standardized with 24.6 mM acetate buffer (pH 4.0). These columns captured TFM and TFM-glucuronide, while undesired constituents were discarded in the effluent. Glucuronidated-TFM was eluted first from the columns with 60% 24.6 mM acetate buffer (pH 4.0):methanol solution and the effluent was evaporated to approximately 6.0 ml. Non-glucuronidated TFM (TFM) was eluted last from the third set of SPE columns into a separate tube with 6.0 ml of 75% methanol. To the G-TFM samples, 1.0 ml of β-glucuronidase solution (0.02% β-glucuronidase w/v; Sigma Aldrich β-glucuronidase, Type B-1; from bovine liver) in 400 mM potassium phosphate buffer (pH 6.8 ± 0.2), was added and the samples were incubated for 18 h in a 35 °C waterbath to allow the enzyme to digest TFM-glucuronide to TFM. Enzyme activity was stopped by adjusting pH to 2.5–3.0 with 6 N HCl. TFM was eluted from the digested TFM-glucuronide samples with 6.0 ml of 75% methanol. Both TFM and TFM-glucuronide samples were then evaporated to 1.5 ml. Lastly, 0.5 ml of 40 mM sodium borate buffer (pH 8.5 ± 0.2) was added to each tube, and the final volume was adjusted to 2.0 ml using deionized water (MilliQ synthesis system, Millipore, Billlerica, MA, USA).

Concentrations of TFM in the muscle were determined using an HPLC fitted with a reverse phase HPLC column (Kinetex 2.6u XB-C18 100A; Phenomenex) configured with a Varian Prostar 230 Solvent Delivery Module, Varian Prostar 310 UV–vis Detector, and Varian Prostar 410 Autosampler. TFM was injected (1.0 ml) and then eluted through the column using an 83% sodium borate and 17% acetonitrile solution mobile phase with a 6.16-minute retention time. The column was washed between each sample with an 83% e-pure:17% acetonitrile solution. Areas under the curve of each sample were determined and integrated against 0.015, 0.05, 0.25, 0.5, 1.5, and 5.0 μg ml^{-1} TFM standards made from 99% analytical grade TFM (product number N2780; Sigma-Aldrich) using the Varian Star Chromatography Workstation Version 5.51 software. The percent recovery of TFM, determined in control rainbow trout muscle samples that were spiked with 100 ng ml^{-1} TFM, averaged 56 ± 6%. The values reported here, however, represent the uncorrected measured concentrations of TFM and TFM-glucuronide in the muscle of the surviving fish.

2.3.2. Blood and tissue processing and analysis

Unless noted, all enzymes and reagents were purchased from the Sigma-Aldrich Chemical Company. Blood hemoglobin concentration was determined using a spectrophotemetric method (cyanomethemoglobin method) at a wavelength of 540 nm on a

SpectraMax 190, plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Blood lactate and plasma glucose were determined enzymatically as previously described in Bergmeyer (1974, 1985), but modified for analysis using 96-well microplates on the plate spectrophotometer. Whole body Na^+, K^+, and Ca^{2+}, and water Na^+ were quantified using atomic absorption spectrophotometry (GTA100 atomizer, SpectrAA 880, N2 gas; Varian, 171 Mississauga, ON, Canada). Whole body Cl^- concentrations were determined colorimetrically using the mercuric thiocyanate assay (Zall et al., 1956).

Tissue processing for ATP, PCr, and glycogen, along with metabolite determination are outlined in Wilkie et al. (2001) and Birceanu et al. (2009). Briefly, frozen tissue (liver, brain and kidney) and carcasses (whole body with the head, viscera and kidneys removed, referred to as muscle) were initially pulverized using a mortar and a pestle, under liquid nitrogen. Approximately 50 mg of muscle was dried to constant weight at 60 °C for 48 h, and the difference used to calculate the percentage muscle water, which was then expressed as ml water per g dry tissue (e.g. Wilkie et al., 2007b; Birceanu et al., 2009). The remaining frozen muscle pieces, as well as whole liver, kidney and brain, were then ground to a fine powder under liquid N_2, followed by deproteination in 4 volumes of 8% perchloric acid (PCA) containing 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA). The resulting homogenate was then placed on ice for 10 min, and then split into two sub-samples. One sub-sample was neutralized with 3 mol l^{-1} K_2CO_3, frozen in liquid N_2, and saved at −80 °C until processed for glycogen. The second sub-sample was processed for ATP, PCr, and lactate determination as follows. First, it was centrifuged at 10,000 *g* for 2 min, and the supernatant drawn off, weighed and neutralized with 0.5 vol. of 2 mol l^{-1} KOH cocktail (composed of 0.4 mol l^{-1} imidazole and 0.4 mol l^{-1} KCl). This solution was then mixed using a vortex mixer, centrifuged again, and the supernatant removed and stored at −80 °C. The same procedure was used for the brain and the liver samples, except that, due to the small size of these tissues, the PCA solution was added directly to the microcentrifuge tube and the samples were homogenized on ice using a hand-held motorized pestle (Gerresheimer Kimble Kontes LLC, Düsseldorf, Germany).

Tissue glycogen was determined in the first neutralized extract after converting the glycogen to glucose using amyloglucosidase (40 units per sample) in acetate buffer (2 mol l^{-1}, pH 4.5) and incubating the resulting digest at 37 °C for 2 h. The incubation was terminated by adding 70% PCA to the digest, and the solution was then neutralized with 3 mol l^{-1} K_2CO_3. Samples were stored at −80 °C for later analysis of glucose, and glycogen concentration was expressed as μmol g^{-1} wet tissue. The second sub-sample was used to enzymatically measure ATP (hexokinase, using glucose-6-phosphatase as coupling enzyme), PCr (creatine kinase), and lactate (lactate dehydrogenase) based on methods in Bergmeyer (1974, 1985). ATP, PCr, and lactate were each expressed as μmol g^{-1} wet tissue.

2.3.3. Gill Na^+/K^+–ATPase activity

Gill Na^+/K^+–ATPase activity determination followed McCormick (1993), in which the activity of the Na^+/K^+–ATPase was calculated from the difference between the uninhibited total ATPase activity and ouabain inhibited ATPase activity. Quantification of Na^+/K^+–ATPase activity was done using approximately 25 mg gill tissue (whole gill minus the gill arch, ground to a fine powder under liquid nitrogen), to which 4 vol. of a salt solution were added (composition in mmol l^{-1}: [imidazole buffer] = 50, [NaCl] = 190, [$MgCl_2 \cdot 6H_2O$] = 10.5, [KCl] = 42, pH 7.5). The gill tissue was then homogenized on ice using a hand-held motorized pestle. For each gill, 25 μl of homogenate were then added to individual microplate wells in triplicate, followed by 200 μl of reaction media–salt solution mixture (composition in mmol l^{-1}: [imidazole] = 50, [phospho*enol*pyruvate] = 2.8, [ATP] = 3.5, [NADH] = 0.3, pH 7.5) containing lactate dehydrogenase (4 U ml^{-1}) and pyruvate kinase (PK; 5 U ml^{-1}). The Na^+/K^+–ATPase activity was then determined by following the decrease in absorbance (at 340 nm) of the mixture over 10 min, in the presence or absence of ouabain. The protein concentration in each gill homogenate was determined by the Bradford (1976) assay, and gill specific Na^+/K^+–ATPase activity was expressed in μmol ADP formed min^{-1} mg^{-1} protein.

2.3.4. Determination of $^{24}Na^+$ radioactivity of water samples

The $^{24}Na^+$ radioactivity (counts per minute = CPM) in water samples collected for the determination of unidirectional Na^+ flux rates was determined after adding 2 ml water to 4 ml aqueous counting scintillant (Amersham Biosciences, USA) and leaving the samples overnight to minimize chemiluminescence. The radioactivity was then determined on triplicate samples at each sample period using a Beckman–Coulter Multi-Purpose Scintillation Counter (Model LS6500, USA). The remaining water samples were saved for quantification of non-radioactive (cold) Na^+.

2.4. Calculations and statistical analysis

The net Na^+ (J^{Na}_{Net}) was calculated from changes in the cold Na^+ concentration at the beginning and end of a flux measurement period using the following equation (e.g. Wood, 1988; Wilkie et al., 1999):

$$J^{Na}_{Net} = \left[\left(\left[Na^+_i\right] - \left[Na^+_f\right]\right) \times V\right] / (M \times T) \quad (1)$$

where $[Na^+]_i$ and $[Na^+]_f$ represent the respective concentrations (μmol ml^{-1}) of total non-radioactive (cold) Na^+ in the water at the beginning and end of a flux period; *V* is the total volume of water (ml) in the container, *M* is the mass of the fish (kg) and *T* is the duration of the flux interval (h). Using this approach, positive values represent net *inward* movements or gains of Na^+, while negative values indicate net *outward* movements or loss of ions.

Rates of Na^+ influx (J^{Na}_{In}) were determined from reductions in water radioactivity during each flux period, using the following formula:

$$J^{Na}_{In} = \left[\left(CPM_i - CPM_f\right) \times V\right] / (MSA \times M \times T) \quad (2)$$

where CPM are counts per minute at the start (*i*) and end (*f*) of a flux period, and MSA (CPM μmol^{-1}) is the mean specific activity of $^{24}Na^+$ in the external water relative to the respective "cold" concentrations of $^{24}Na^+$ during a given flux period, and *V*, *M* and *T* are as previously stated.

Efflux, or outward movements, of Na^+ (J^{Na}_{Out}) were based on the fact that the net Na^+ flux (J^{Na}_{Net}) is the sum of the Na^+ influx (J^{Na}_{In}) plus Na^+ efflux. Accordingly, Na^+ efflux rates were calculated using the following equation:

$$J^{Na}_{Out} = J^{Na}_{Net} - J^{Na}_{In}. \quad (3)$$

Determination of the 12-h LC_{50} and LC_{100} for rainbow trout exposed to TFM was done by Probit Analysis (Sprague, 1969) with Two-Point Interpolation using the analytical program CETIS (Tidepool Scientific Software, Version 1.6.1, CA, USA).

Data are presented as the mean + or ± 1 S.E.M. Unpaired comparisons of tissue ion, water, energy stores, metabolites, TFM and TFM-glucuronide versus time were done using one-way analysis of variance (ANOVA) followed by a Tukey–Kramer post-test where justified. A one-way ANOVA was conducted on control fish sampled at 0 h and control fish sampled at 12 h, to determine if there were any changes in parameters (metabolites, fuel stores and whole body ions) due to confinement of the fish in the exposure containers for 12 h. Since there were no statistical differences among the control fish, the values obtained for each parameter were pooled to yield n = 11 for all controls. In instances where the standard deviations between each group were significantly different, a non-parametric ANOVA (Kruskal–Wallis) and Dunn's post-test was used to test for statistical significance. For unidirectional Na^+ flux data, repeated measures ANOVA, followed by a

Student–Newman–Keuls post-test was used to determine if Na^+ influx, efflux or net flux rates were significantly different from control rates following exposure to TFM. All statistical differences were determined at the $p < 0.05$ level.

3. Results

3.1. Determination of the acute toxicity of TFM in rainbow trout

The nominal 12-h LC_{50} for TFM was 11.0 mg l^{-1} (CI = 10.6–11.5), which was subsequently established as the target concentration for experiments investigating the effects of TFM on energy balance and whole body ion homeostasis. The 12-h TFM LC_{100} was 17.8 mg l^{-1} (CI = 17.0 to 18.6), which was used as the nominal TFM exposure concentration (measured 17.5 mg l^{-1}) in the unidirectional Na^+ flux experiments.

3.2. Determination of TFM in the muscle of rainbow trout

During the 12 h exposure to TFM, there was 25% mortality in the exposed fish, beginning with 3 h of exposure. In rainbow trout surviving exposure to the 12-h LC_{50} of TFM (nominal of 11.0 mg l^{-1}, measured water [TFM] of 11.0 ± 0.1 mg l^{-1}) there was a rapid accumulation of TFM in the muscle, which peaked after 3 h of TFM exposure at a concentration of 14.7 ± 3.2 nmol g^{-1} wet tissue (Fig. 1). The TFM levels then significantly decreased to 6.2 ± 0.7 nmol g^{-1} wet tissue by 12 h of exposure. The concentration of TFM-glucuronide rose less than that of TFM over the first hour of exposure, but by 6 h, there were no statistical differences between the two compounds (Fig. 1). After 6 h of exposure, TFM-glucuronide concentrations exceeded muscle TFM concentrations, which steadily declined despite the persistent exposure to TFM in the water. By 12 h, muscle TFM-glucuronide concentrations were significantly higher than TFM concentrations (16.9 ± 2.3 nmol g^{-1} vs. 6.2 ± 0.7 nmol g^{-1} for TFM-glucuronide and TFM, respectively; Fig. 1).

3.3. Effects of TFM on rainbow trout energy stores and metabolites

Exposure of rainbow trout to the 12-h LC_{50} (measured concentration = 11.0 ± 0.1 mg l^{-1}) of TFM markedly lowered energy

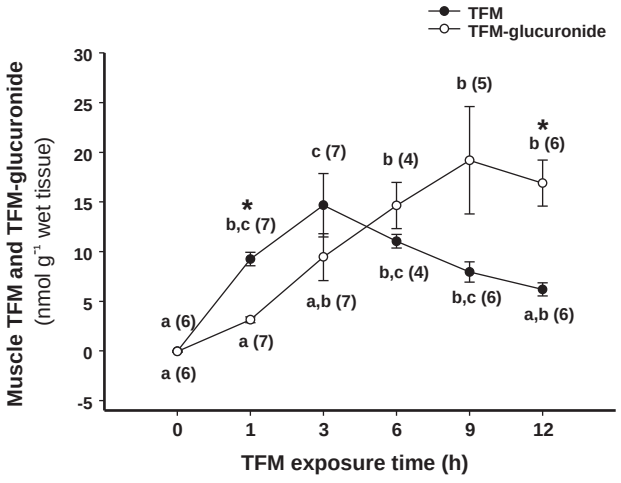

Fig. 1. TFM and TFM-glucuronide accumulation in muscle filet of rainbow trout. Accumulation of free-TFM and TFM-glucuronide in the muscle of rainbow trout over 12 h of exposure to TFM at a measured concentration of 11.0 ± 0.1 mg l^{-1}. Solid circles represent free-TFM (non-glucuronidated), while open circles represent TFM-glucuronide. Data are expressed as the mean ± S.E.M. (N). For each chemical measured individually, data points sharing the same letter are not significantly different. Statistically significant differences between the two chemicals are denoted by an asterisk (*).

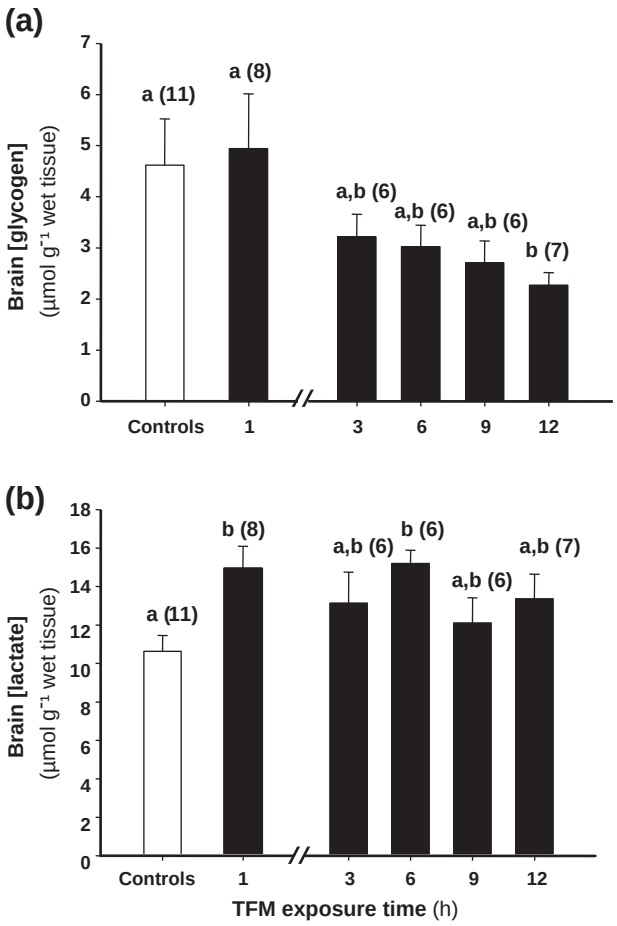

Fig. 2. Effects of TFM on brain glycogen and lactate in rainbow trout. Changes in brain concentrations of (a) glycogen and (b) lactate in resting rainbow trout (*Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM; solid bars) at a measured concentration of 11.0 ± 0.1 mg l^{-1} for 1, 3, 6, 9 and 12 h, or held under control conditions (no TFM; open bars). Data are expressed as the mean + S.E.M. (N). Mean values sharing the same letter are not significantly different from one another.

stores in the brain, kidney and muscle, but not in the liver. Brain glycogen levels were approximately 4.6 ± 0.9 μmol g^{-1}, and decreased by approximately 50% after 12 h of exposure (Fig. 2a). This was accompanied by a significant increase in brain lactate at 1 h and 6 h, after which lactate levels returned to control values of 10.6 ± 0.8 μmol g^{-1} (Fig. 2b). Brain ATP levels were also adversely affected by TFM, decreasing by approximately 75% after 1 h of exposure, from control values of 0.6 ± 0.1 μmol g^{-1} (Fig. 3a). PCr values were just above levels of detection, highly variable, and not significantly different from control levels of 3.0 ± 1.2 μmol g^{-1} (Fig. 3b).

The changes noted in brain fuel store and metabolite levels were not reflected in the liver. Liver glycogen appeared to decrease by 3 h of exposure to TFM, but the decrease was not significantly different from controls. After 3 h, liver glycogen concentrations began to recover towards control values of 47.9 ± 9.5 μmol g^{-1} (Fig. 4a). No significant changes were noted in liver lactate values, which were similar to the control concentration of 4.2 ± 0.6 μmol g^{-1} (Fig. 4b). Liver ATP and PCr levels were unaltered by exposure to TFM, at approximately 1.1 ± 0.1 μmol g^{-1} and 8.4 ± 0.1 μmol g^{-1}, respectively (data not shown).

Kidney glycogen levels were unaffected by TFM, with controls averaging 8.5 ± 1.2 μmol g^{-1} (Fig. 5a), but kidney lactate was 40% higher after 1, 3, and 6 h of exposure compared to control lactate concentrations of 6.4 ± 0.5 μmol g^{-1} (Fig. 5b). Kidney ATP decreased by almost 50% after 1, 3 and 6 h of TFM exposure, but by 9 h, ATP concentrations in the surviving animals were similar to the

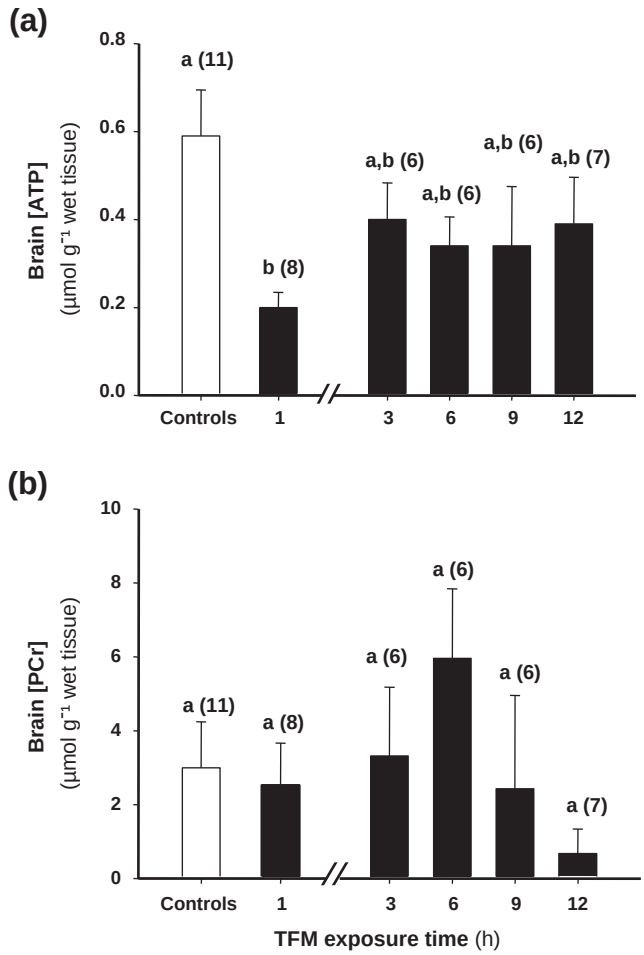

Fig. 3. Effects of TFM on brain ATP and PCr in rainbow trout. Changes in brain concentrations of (a) adenosine triphosphate (ATP) and (b) phosphocreatine (PCr) in resting rainbow trout (*Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM; solid bars) at a measured concentration of 11.0 ± 0.1 mg l^{-1} for 1, 3, 6, 9 and 12 h, or held under control conditions (no TFM; open bars). Data are expressed as the mean + S.E.M. (N). Mean values sharing the same letter are not significantly different from one another.

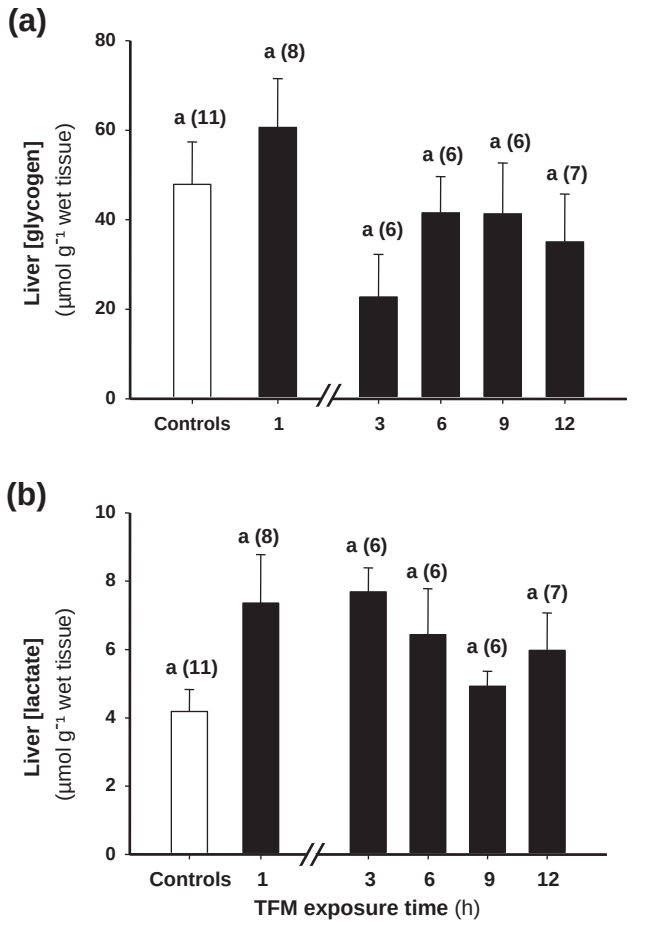

Fig. 4. Effects of TFM on liver glycogen and lactate in rainbow trout. Changes in liver concentrations of (a) glycogen and (b) lactate in resting rainbow trout (*Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM; solid bars) at a measured concentration of 11.0 ± 0.1 mg l^{-1} for 1, 3, 6, 9 and 12 h, or held under control conditions (no TFM; open bars). Data are expressed as the mean + S.E.M. (N). Mean values sharing the same letter are not significantly different from one another.

control values of 0.4 ± 0.03 μmol g^{-1} (Fig. 6a). These changes were accompanied by 70% lower concentrations of PCr after 3 h of TFM. However, PCr concentrations were comparable to the control values of 1.2 ± 0.3 μmol g^{-1} from 6 to 12 h in those fish that survived TFM exposure (Fig. 6b).

Disturbances were also observed in the muscle where glycogen fluctuated between 2 and 5 μmol g^{-1} over the first 9 h of TFM exposure compared to control values of 5.4 ± 0.9 μmol g^{-1} (Fig. 7a). At 12 h, however, muscle glycogen had significantly decreased by more than 60% compared to the non-TFM exposed controls (Fig. 7a). The effect of TFM exposure on muscle glycogen was accompanied by a significant 40% increase in muscle lactate after 3 h, which was sustained at 6 h (Fig. 7b). However, lactate declined slightly between 9 and 12 h to levels that were not significantly different from the control values of 0.8 μmol g^{-1} wet tissue. Muscle ATP, although variable, remained unaltered from control values of 0.7 ± 0.3 μmol g^{-1} (Fig. 8a), and muscle PCr was unaffected by TFM, fluctuating around 31.7 ± 2.0 μmol g^{-1} (Fig. 8b).

The metabolic disturbances noted in the brain, muscle and kidney were reflected by minimal changes in blood lactate concentrations, which increased by 50% at 3 h and 6 h of TFM exposure. However, this increase was not significant ($p = 0.08$) from the control values of 1.6 ± 0.3 mmol l^{-1}. Blood glucose levels remained unaltered throughout the exposure, averaging 9.2 ± 1.2 mmol l^{-1} (Table 1).

3.4. Effects of TFM on hematology and whole body ion balance

TFM had some transient effects on hematology (Table 1). Hemoglobin (Hb) concentration in the blood increased by 35% after 3 h of exposure to TFM. However, Hb values returned to control levels of 68 ± 7 g l^{-1} by 6 h (Table 1). Whole body Na^+ levels varied slightly with TFM exposure, increasing by 13% compared to control values of 47.8 ± 1.5 mmol l^{-1} after 12 h. However, whole body Cl^- and Ca^{2+} remained unaltered from the control concentrations of 35.6 ± 2.0 mmol l^{-1} and 111.2 ± 0.9 mmol l^{-1}, respectively. Whole body K^+ however, decreased by 18% after 3 h of exposure, and then increased by 23% after 6 h, when compared to the control value of 79.1 ± 1.4 mmol l^{-1} (Table 2).

3.5. Effects of TFM on unidirectional Na^+ movements in rainbow trout

Prior to TFM exposure, the rainbow trout were in net ion balance, with a J^{Na}_{net} of −57 ± 64 μmol kg^{-1} h^{-1}, which was not significantly different from zero (Fig. 9). At this time, J^{Na}_{in} averaged 246 ± 34 μmol kg^{-1} h^{-1}, and J^{Na}_{out} was approximately −312 ± 68 μmol kg^{-1} h^{-1} (Fig. 9). From 0 to 2 and 2 to 4 h of TFM exposure, rainbow trout continued to lose Na^+ at rates comparable to control conditions. Although the net Na^+ flux was inwardly directed, at a rate of 165 ± 40 μmol kg^{-1} h^{-1} during the 4–6 h flux period, this was not significantly different from controls. No significant differences in Na^+ uptake were noted, which fluctuated between

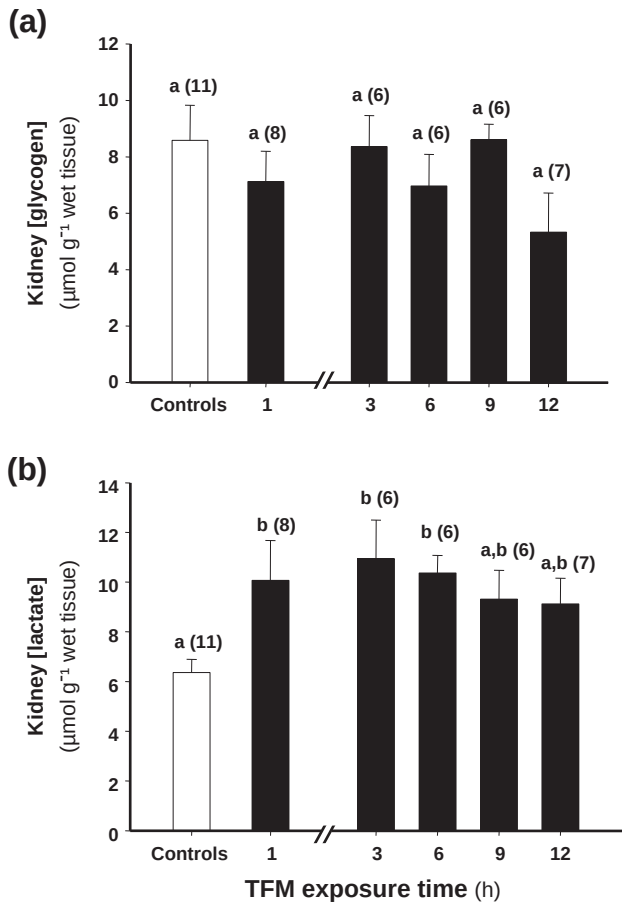

Fig. 5. Effects of TFM on kidney glycogen and lactate in rainbow trout. Changes in kidney concentrations of (a) glycogen and (b) lactate in resting rainbow trout (*Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM; solid bars) at a measured concentration of 11.0 ± 0.1 mg l^{-1} for 1, 3, 6, 9 and 12 h, or held under control conditions (no TFM; open bars). Data are expressed as the mean + S.E.M. (N). Mean values sharing the same letter are not significantly different from one another.

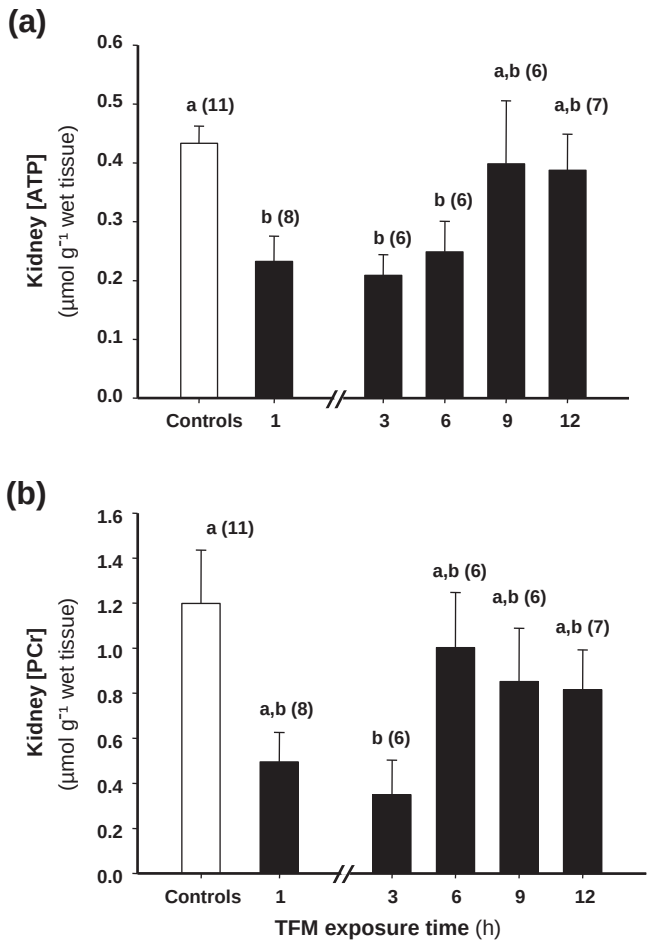

Fig. 6. Effects of TFM on kidney ATP and PCr in rainbow trout. Changes in kidney concentrations of (a) adenosine triphosphate (ATP) and (b) phosphocreatine (PCr) in resting rainbow trout (*Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM; solid bars) at a measured concentration of 11.0 ± 0.1 mg l^{-1} for 1, 3, 6, 9 and 12 h, or held under control conditions (no TFM; open bars). Data are expressed as the mean + S.E.M. (N). Mean values sharing the same letter are not significantly different from one another.

200 and 400 kg^{-1} h^{-1} during the TFM exposure period (Fig. 9). Gill Na^+/K^+ ATPase specific activity was also relatively stable in the presence of the TFM, with activities fluctuating around 3.3 ± 0.5 nmol ADP μg $protein^{-1}$ h^{-1} (Table 2).

Despite the absence of significant differences in the unidirectional Na^+ flux (influx, efflux or net flux), one-way ANOVA revealed that there was significant variation between the controls and TFM treated animals in the net Na^+ flux, which led to cumulative net ion losses in some fish. Accordingly, the cumulative net loss or gain of Na^+ by each fish was determined by calculating the total Na^+ lost or gained during each flux period (0–2 h, 2–4 h, 4–6 h, 8–10 h) and multiplying the corresponding net Na^+ flux rate by the duration of each corresponding flux period (2 h), and then adding all the values together for each fish. This analysis revealed that on average the fish exposed to TFM lost 750.0 ± 526.0 μmol Na^+ kg^{-1} over the 10 h TFM exposure, which was not significantly different from a net loss of 569.0 ± 641.0 μmol Na^+ kg^{-1} in the control fish over the same time period (Table S1 — Supplementary data). However, in some individuals, the total loss rates during TFM exposure approached 3000 μmol Na^+ kg^{-1} over the 10 h period (data not shown).

4. Discussion

4.1. TFM accumulation in the muscle of rainbow trout

Our results indicate that TFM adversely affects ATP supply in the rainbow trout in a similar manner to that seen in the larval sea lampreys. However, in trout this effect was tissue-specific, with brain and kidney showing the greatest response to this lampricide. The response was also transient in trout that survived TFM exposure, likely due, in part, to the relatively high capacity of the trout to biotransform TFM to the more hydrophilic TFM-glucuronide (Fig. 1). TFM had only modest effects on ion homeostasis in trout, with no disruption in whole body ions, except for a significant rise in Na^+ after 12 h of exposure.

Exposure of trout to TFM (12-h LC_{50}) resulted in significant lampricide accumulation in the muscle, as shown previously (Lech and Costrini, 1972; Lech and Statham, 1975; Dawson et al., 2002; Vue et al., 2002; Hubert et al., 2005). It should be noted that the concentration of TFM to which the trout were exposed (11.0 mg l^{-1}), was approximately 4 times greater than what these fish would likely encounter during an actual TFM treatment in the field in hard waters of similar alkalinity (Bills et al., 2003; pH ~ 8.0; titratable alkalinity 220 mg l^{-1}). Thus, any adverse physiological effects that would be manifested in rainbow trout during an actual TFM treatment in hard, circumneutral pH waters would be minimal, provided there were no sudden decreases in water pH, which is known to increase the toxicity of TFM (Bills et al., 2003; McDonald and Kolar, 2007). At lower pH, a greater proportion of TFM exists as the more lipophilic, un-ionized form of the compound (Hubert, 2003), which presumably allows it to be taken up at a faster rate and leads to greater TFM accumulation (Hunn and Allen, 1974).

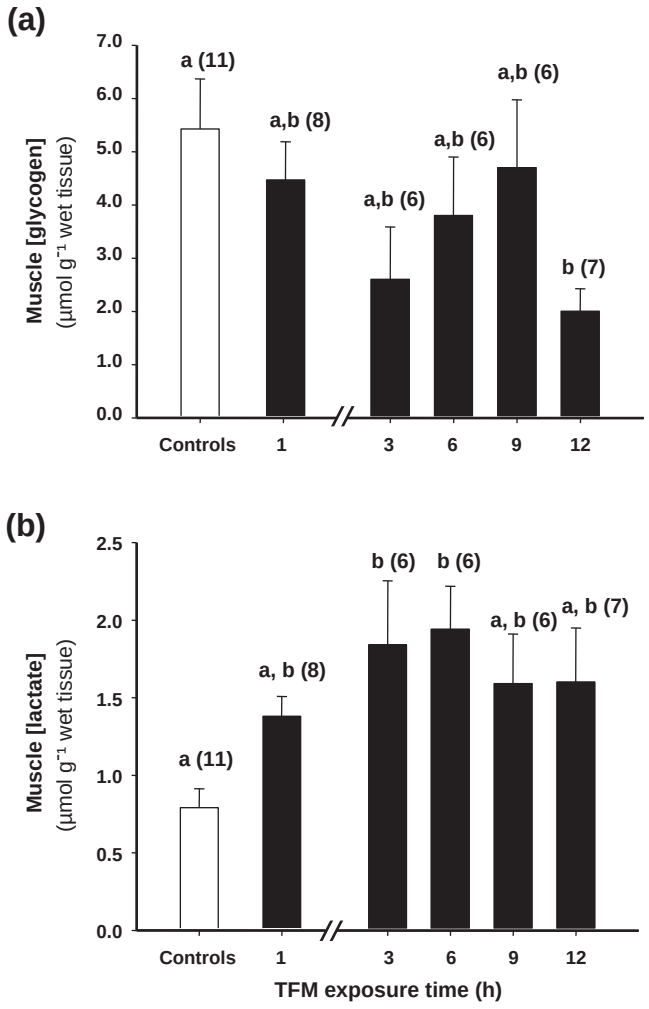

Fig. 7. Effects of TFM on muscle glycogen and lactate in rainbow trout. Changes in muscle concentrations of (a) glycogen and (b) lactate in resting rainbow trout (*Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM; solid bars) at a measured concentration of 11.0 ± 0.1 mg l^{-1} for 1, 3, 6, 9 and 12 h, or held under control conditions (no TFM; open bars). Data are expressed as the mean + S.E.M. (N). Mean values sharing the same letter are not significantly different from one another.

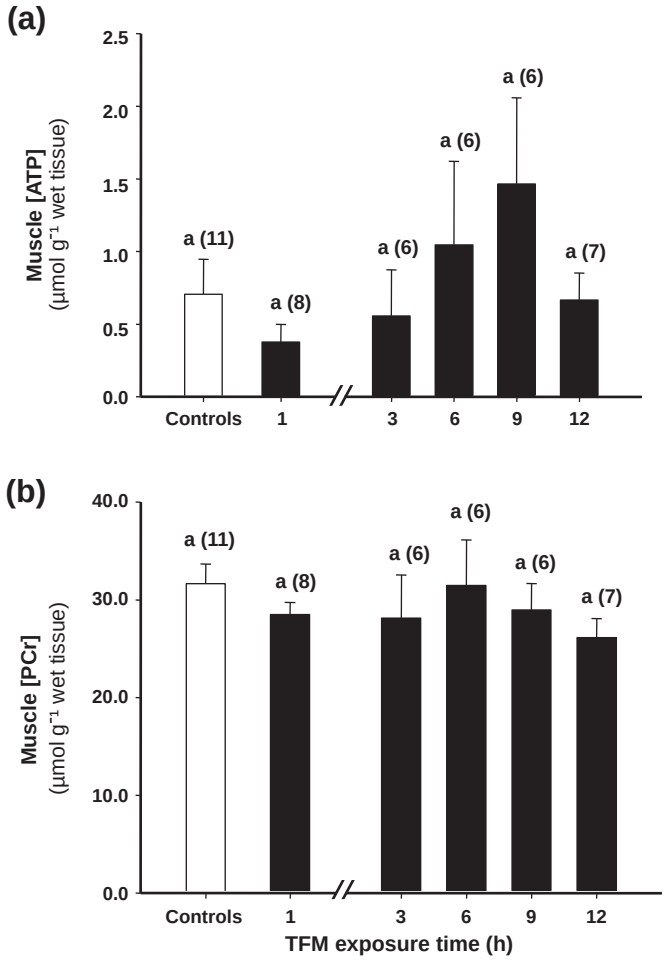

Fig. 8. Effects of TFM on muscle ATP and PCr in rainbow trout. Changes in muscle concentrations of (a) adenosine triphosphate (ATP) and (b) phosphocreatine (PCr) in resting rainbow trout (*Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM; solid bars) at a measured concentration of 11.0 ± 0.1 mg l^{-1} for 1, 3, 6, 9 and 12 h, or held under control conditions (no TFM; open bars). Data are expressed as the mean + S.E.M. (N). Mean values sharing the same letter are not significantly different from one another.

The steady rise in TFM-glucuronide beyond 1 h of TFM exposure, followed by the subsequent decline in muscle TFM concentration, despite no change in the external TFM levels, suggests the fish had additional reserve capacity to detoxify TFM. It was also notable that the physiological disturbances observed in the brain, kidneys and muscle were less severe by 6–9 h, during which time the observed increases in lactate concentration, and decreases in ATP and phosphocreatine levels (kidney only) had been corrected.

The restoration of lactate, ATP and phosphocreatine to pre-exposure levels is reliant upon the oxidative generation of ATP in the mitochondria (Hochachka, 1991; Wang et al., 1994b; Moyes and West, 1995; Richards et al., 2002). These observations suggest that the capacity of the mitochondria to generate ATP by oxidative phosphorylation was restored as the TFM burden in the body was reduced beyond 6 h of TFM exposure due to its conversion to TFM-glucuronide. Clifford et al. (2012) recently suggested that an identical process likely explained the rapid restoration of glycogen, ATP and phosphocreatine, and the elimination of lactate, in larval sea lampreys recovering from short-term (up to 6 h) TFM exposure. These findings and the present study therefore indicate that the sub-lethal effects of TFM on metabolic processes in non-target rainbow trout and sea lampreys are completely reversible.

4.2. Effects of TFM on energy stores in rainbow trout

Exposure of rainbow trout to TFM caused marked decreases in brain and muscle glycogen, followed by corresponding increases in lactate levels in both tissues. These findings lend further support to the hypothesis that trout may have increased their reliance on glycolysis to meet their ATP demands during TFM exposure. Liver glycogen was not affected by TFM, although it is known to be involved in maintaining glucose homeostasis in the circulatory and nervous systems of vertebrates (Panserat et al., 2000). However, trout have a much larger reserve of glycogen in their livers compared to other tissues (Vijayan and Moon, 1992; Bleau et al., 1996; Soengas et al., 1998; Shanghavi and Weber, 1999; Begg and Pankhurst, 2004). The trout rely on this large glycogen pool for their immediate energy requirements, such as maintaining blood glucose levels during starvation or during chronic exposure to stressors (Vijayan and Moon, 1992; Bleau et al., 1996). Vijayan and Moon (1992) determined that handling had no effect on liver glycogen in fed trout, but liver glycogen reserves were approximately 50% lower in fish that had been starved for 30 days and then decreased further during the 8 h post-handling period. This suggests that liver glycogen is a robust energy store, but it is negatively impacted by prolonged exposure to a stressor. In the case of TFM exposure, however, it appears

Table 1
Hemoglobin, blood glucose and lactate concentrations, and muscle water content in rainbow trout exposed to toxic concentrations of TFM (nominal 12-h LC_{50} of 11.0 mg l^{-1}). Data presented as the mean ± S.E.M. (N). Data points sharing the same letter are not significantly different.

TFM exposure time (h)	Hemoglobin (g l^{-1})	Blood glucose (μmol ml^{-1})	Blood lactate (μmol ml^{-1})	Muscle H_2O (ml g^{-1} dry tissue)
Control	68 ± 7 (10)^{a,b}	9.2 ± 1.2 (12)^{a}	1.6 ± 0.3 (11)^{a}	3.8 ± 0.1 (11)^{a}
1	75 ± 9 (7)^{a,b}	10.1 ± 1.8 (8)^{a}	2.4 ± 0.3 (8)^{a}	3.6 ± 0.2 (8)^{a}
3	105 ± 11 (6)^{b}	7.5 ± 2.2 (6)^{a}	3.2 ± 0.6 (6)^{a}	3.7 ± 0.1 (6)^{a}
6	61 ± 7 (5)^{a}	9.4 ± 1.8 (6)^{a}	3.0 ± 0.6 (6)^{a}	4.0 ± 0.3 (6)^{a}
9	57 ± 8 (6)^{a}	8.3 ± 1.5 (6)^{a}	2.2 ± 0.3 (6)^{a}	3.8 ± 0.1 (6)^{a}
12	68 ± 13 (7)^{a,b}	5.7 ± 1.6 (7)^{a}	1.7 ± 0.5 (6)^{a}	4.0 ± 0.1 (7)^{a}

Table 2
The effects of toxic concentrations of TFM (nominal 12-h LC_{50} of 11.0 mg l^{-1} TFM) upon whole body ion concentrations and gill Na^+/K^+ specific activity in rainbow trout. Data presented as the mean ± S.E.M. (N). Data points sharing the same letter are not significantly different.

TFM exposure time (h)	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Ca^{2+} (mmol l^{-1})	K^+ (mmol l^{-1})	Na^+/K^+ ATPase specific activity (nmol ADP μg^{-1} prot. h^{-1})
Controls	47.8 ± 1.5 (10)^{a}	35.6 ± 2.0 (12)^{a}	111.2 ± 0.9 (9)^{a}	79.1 ± 1.4 (12)^{a}	3.29 ± 0.48 (11)^{a}
3	52.1 ± 1.8 (8)^{a,b}	36.5 ± 2.0 (8)^{a}	110.2 ± 1.3 (8)^{a}	64.8 ± 1.7 (8)^{b}	2.80 ± 0.41 (6)^{a}
6	52.7 ± 1.1 (4)^{a,b}	35.4 ± 1.4 (4)^{a}	115.1 ± 2.0 (4)^{a}	103.3 ± 1.5 (4)^{c}	2.78 ± 0.34 (6)^{a}
9	53.0 ± 1.3 (6)^{a,b}	39.4 ± 1.5 (6)^{a}	112.8 ± 1.9 (6)^{a}	83.8 ± 1.1 (6)^{a}	3.75 ± 0.59 (6)^{a}
12	54.8 ± 1.5 (9)^{b}	42.2 ± 1.7 (9)^{a}	114.35 ± 1.0 (9)^{a}	76.2 ± 1.5 (9)^{a}	3.67 ± 0.36 (7)^{a}

that the 12 h exposure was not long enough to alter liver glycogen stores, as it did in the brain and the muscle of the fish in the present study.

The pronounced decrease in brain glycogen (Fig. 2a) suggests that glycogen depletion contributes to death in rainbow trout by depleting the nervous system of its main fuel source, glucose, as previously suggested by Wilkie et al. (2007a) and reported by Birceanu et al. (2009) in larval sea lampreys. Glucose is the main energy source in the brain of vertebrates, and it decreases in response to food deprivation, hypoxia/anoxia, ischemia and toxicant exposures (e.g. Lowry et al., 1964; Soengas et al., 1998, 2006; Polakof et al., 2007; Birceanu et al., 2009). Soengas et al. (1998) reported that brain glycogen significantly decreased in trout that had been starved for 14 days, while Lowry et al. (1964) determined that glycogen was the most rapidly consumed energy store in the brain of ischemic mice. Brain glycogen concentrations decreased in the brains of larval sea lampreys by 70–85% after 6–12 h of exposure to TFM

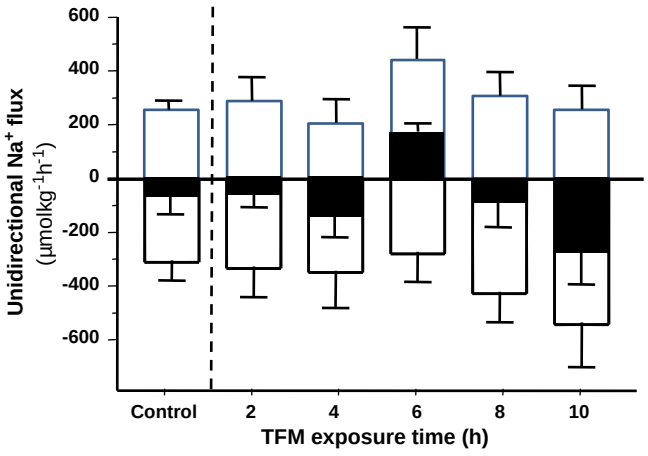

Fig. 9. Effects of TFM on Na^+ influx (upward facing open bars), efflux (downward facing open bars) and net flux (solid bars) rates in rainbow trout. Changes in Na^+ influx, efflux and net flux rates in resting rainbow trout (*Oncorhynchus mykiss*) following exposure to 3-trifluormethyl-4-nitrophenol (TFM) at a measured concentration of 17.5 mg l^{-1}, which is the LC_{100} of rainbow trout over 12 h. N = 9 individuals per flux period. Data are expressed as the mean + S.E.M. There were no statistically significant changes in Na^+ movement across the gills.

(Birceanu et al., 2009; Clifford et al., 2012). The current study shows that TFM does cause a decrease in brain glycogen in trout, but not to the same degree as it does in larval lampreys exposed to doses of TFM with equivalent toxicity (i.e. the respective 12-h LC_{50} for each species). This difference is likely related to the much lower capacity of larval lampreys to detoxify TFM to TFM-glucuronide compared to rainbow trout (Lech and Statham, 1975; Kane et al., 1993), even though larval sea lamprey have very high brain glycogen reserves compared to trout. These glycogen reserves of sea lampreys are stored in the meninges at concentrations that can exceed 100 μmol g^{-1} wet weight (Rovainen et al., 1969; Rovainen, 1970; Rovainen et al., 1971; Clifford et al., 2012).

Similar to liver glycogen, kidney glycogen levels were unaffected by TFM exposure, but lactate increased slightly. The kidney and the liver are the two most important organs that maintain glucose levels in the body of most vertebrates (Shanghavi and Weber, 1999). The finding that kidney lactate increased with exposure to TFM also suggests that trout were relying more on glycolysis in this and other tissues, to meet the body's energy needs. Although the liver and the kidney are the sites of toxicant detoxification in fish, the fact that TFM had little impact on kidney glycogen was not surprising, as previous studies found that kidney glycogen did not appear to be affected by exercise or starvation. For instance, Shanghavi and Weber (1999) demonstrated that steady exercise had little impact on kidney glycogen in rainbow trout, and that fish were able to maintain a steady glucose level in the body by matching the rate of hepatic glucose production with the rate of peripheral glucose utilization.

The 50% decrease in muscle glycogen concentration after 12 h of exposure to TFM might appear surprising at first, since the trout muscle glycogen is not involved in maintaining glucose homeostasis in the nervous and circulatory systems (Panserat et al., 2000; Polakof et al., 2012). Instead, muscle glycogen is used to generate ATP by glycolysis during vigorous exercise, such as during burst swimming (Wang et al., 1994b; Kieffer, 2000). However, Mandic et al. (2008) reported a 60% decrease in white muscle glycogen levels immediately following hypoxia in goldfish, and provided evidence that muscle glycogen stores were preferentially used over liver glycogen stores to support glycolysis under hypoxic conditions. This provides an explanation as to why muscle glycogen decreased, but liver glycogen reserves remained unaffected in the presence of TFM in the current study. Since mitochondrial ATP production was impaired by TFM (Birceanu et al., 2011), the

fish were no longer able to use oxygen efficiently as the final electron acceptor in the electron transport chain to generate ATP and, therefore, they were essentially becoming hypoxic. This would have forced them to rely on their muscle glycogen reserves, as suggested by Mandic et al. (2008). Moreover, the trout used in this study had low muscle glycogen reserves, compared to those reported by Ferguson et al. (1993) for similarly sized trout. These lower glycogen reserves might have made the muscle more susceptible to TFM-induced perturbations, thus leading to the decrease in glycogen noted in this study after a prolonged (12 h) TFM exposure.

Although starvation can affect glycogen stores in liver, kidney and brain, the relatively short (72 h) period of fasting prior to TFM exposure did not likely influence our results. This period of fasting was to ensure that the build-ups of ammonia due the protein and amino acid catabolism did not lead to post-prandial increases in ammonia excretion (e.g. see Wood, 2001 for review) by the fish that would have led to the excess accumulation of ammonia in the 12 h TFM exposure period. Recent studies have shown that this period of starvation does not significantly change the energy stores and metabolite levels in rainbow trout when compared to fed fish (Pottinger et al., 2003; López-Luna et al., 2013). Plasma glucose levels in rainbow trout and channel catfish (*Icatalurus punctatus*) only begin to fall after 7–14 days of starvation (Pottinger et al., 2003; Peterson and Small, 2004). In addition, metabolic rate in fasted rainbow trout only begins to decline beyond 7 days of starvation (MacIntyre and Wang, unpublished findings).

Additional evidence that TFM causes a mismatch between ATP supply and ATP demand in trout was provided by the initial decreases in brain and kidney ATP after 1 h exposure to the lampricide (Figs. 3a and 6a). Phosphocreatine buffers ATP in response to decreases in ADP/ATP ratio, thus temporarily maintaining homeostatic ATP levels in the body (Moyes and West, 1995; McLeish and Kenyon, 2005). In the kidney, the decrease in phosphocreatine also coincides with a decrease in ATP levels (Fig. 6), with levels recovering by 6–9 h of TFM exposure, suggesting that phosphocreatine reserves were sufficient to maintain ATP levels in that tissue.

4.3. Detoxification of TFM by rainbow trout

The ability of surviving rainbow trout to maintain or restore fuel stores as TFM exposure continued might be related to an ability to enhance their glucuronidation capacity during the exposure period. Glucuronidation is a major pathway of detoxification of endogenous and xenobiotic compounds, and it is catalyzed by the enzyme UDP-glucuronyltransferase (UDPGT; Clarke et al., 1991). In fishes, it is the major route by which phenolic compounds such as TFM are detoxified (as opposed to the mixed function oxidases; Clarke et al., 1991). Several studies have shown that the selective toxicity of TFM is related to the higher glucuronidation capacity of non-target species to detoxify this lampricide (Lech, 1974; Lech and Statham, 1975; Kane et al., 1993). Lech et al. (1973) reported that the concentration of TFM-glucuronide excreted in trout bile increased by more than 50% after 4 h of exposure to TFM, while Lech and Statham (1975) could not detect any TFM-glucuronide formation in larval sea lamprey exposed to lethal concentrations of TFM.

The rainbow trout might have increased their glucuronidation capacity during the TFM exposure period by increasing the UDPGT DNA transcription rate, UDPGT synthesis, and/or allosteric activation of the enzyme activity or a combination of all three. Because the liver and kidney are major sites of glucuronidation in fishes (Lech and Costrini, 1972; Kawatski and McDonald, 1974), high endogenous UDPGT activities, combined with an ability to increase their capacity to detoxify TFM might therefore explain why lesser changes in glycogen concentrations occurred in these, and other organs, compared to larval lampreys, which have a very low glucuronidation capacity (Kane et al., 1993) and experience pronounced reductions in glycogen, ATP and PCr in the brain, liver and, to a lesser extent, muscle (Birceanu et al., 2009). Such findings could also have implications for TFM applications in the field, where it may be possible to enhance the glucuronidation capacity, and TFM tolerance, of non-target fishes by exposing them to low levels of TFM, before boosting the concentrations to levels that can effectively eliminate larval sea lampreys. However, further studies are warranted to determine whether the liver and kidney do indeed increase their detoxifying capacity in the presence of TFM, and to what degree TFM impacts the detoxification process in rainbow trout and other non-target fishes.

4.4. Effects of TFM on hematology, whole body ion balance, gill Na^+/K^+ ATPase and gill Na^+ uptake

The increase in hemoglobin concentration at 6 h of exposure suggests that TFM led to either hemoconcentration due to osmotic disturbances, or increased hemoglobin production to compensate for the deficit in ATP production, as they would during prolonged hypoxia (Steffensen and Farrell, 1998). The absence of substantive changes in muscle water and ions (Na^+, K^+, Cl^-, Ca^{2+}), and gill specific Na^+/K^+ ATPase activity, suggests that internal ionic or osmotic homeostatic disturbances were minimal (Tables 1 and 2). In addition, the fact that TFM had no effect on Na^+ movement across the gills of the rainbow trout (Fig. 9) further confirmed that TFM had little impact on gill mediated-ion exchange in trout exposed to their TFM LC_{100} over 10 h. These findings are in agreement with Mallatt et al. (1994), who reported that mitochondria rich cell ultrastructure was not affected in rainbow trout exposed to the 9-h TFM LC_{100} of larval sea lampreys. Collectively, these findings suggest the TFM does not likely interfere with gill-meditated ion exchange in hard waters.

To further determine if TFM interfered with ion balance in the trout, the cumulative net Na^+ losses for the trout over the entire experiment (10 h) were calculated. This analysis indicated that the trout exposed to TFM would have lost on average the equivalent of 750 μmol kg^{-1} Na^+ compared to approximately 570 μmol kg^{-1} Na^+ if the trout had not been exposed to TFM (Table S1 — Supplementary Data). A net loss of 750 μmol kg^{-1} Na^+ would only represent a 1.8% decrease of the exchangeable internal Na^+ pool of 42 mmol kg^{-1} in trout (Wood, 1988), which would induce only minimal disturbance in the fish. Some individual fishes did experience larger net Na^+ losses approaching 3 mmol kg^{-1} over the 10 h exposure period, but even in these fish it seems unlikely that such ionic disturbances would make a significant contribution to TFM toxicity because the total Na^+ losses would have only represented 7% of the total exchangeable internal Na^+ pool. Grosell et al. (2002) have pointed out that it is only when Na^+ losses approach 30% of the internal Na^+ pool that mortality can be expected in fish exposed to toxic copper and silver loads. Thus, TFM-induced ionic disturbances are an unlikely mechanism of TFM toxicity in the rainbow trout, at least in the hard waters in which these experiments were conducted. The absence of an effect of TFM on the Na^+/K^+–ATPase activity also suggests that TFM did not damage the fish's ion exchange machinery, either directly or indirectly. It remains to be determined, however, if the sensitivity to TFM-induced ionic disturbances is greater in softer waters, or in earlier life stages or in smaller fishes, or if there are interspecific differences in the vulnerability of fishes to electrolyte imbalances due to TFM.

4.5. Mechanism of TFM toxicity in rainbow trout — relevance for field applications and risk assessment

The current study provides evidence that TFM interferes with ATP production in rainbow trout, a representative non-target species that resides in many streams treated with this lampricide. The effects of TFM in trout, although less pronounced, appear to be similar to those in larval sea lampreys (Wilkie et al., 2007a; Birceanu et al., 2009; Clifford et al., 2012), which indicates that TFM causes a mismatch between ATP supply and ATP demand in non-targets as well, forcing the

fish to rely more on glycolysis and phosphocreatine to generate ATP. As previously shown by Birceanu et al. (2011), TFM does uncouple mitochondrial oxidative phosphorylation in trout *in vitro*, suggesting that if fish are faced with high enough concentrations of the lampricide during routine treatments, they would be forced to rely more on their glycogen reserves for survival.

Although non-target fish will not normally encounter lethal concentrations of TFM during a regular stream treatment (McDonald and Kolar, 2007), they may be inadvertently exposed to toxic/lethal concentrations of TFM due to sudden drops in stream pH, which could occur due to rainfall, or changes in plant/algal respiration that generate acidifying CO_2. Lower pH would significantly increase TFM toxicity to non-target fishes and to larval sea lampreys by changing the speciation of TFM to its more lipophilic, un-ionized form (Hunn and Allen, 1974; McDonald and Kolar, 2007). At pH 8.0, a pH typical of many streams in the Great Lakes (E. Koon, U.S. Fish and Wildlife Service, Ludington, Michigan, pers. comm.), 2% of total TFM is in its phenolic or un-ionized form. But, at pH 7.0 the amount of un-ionized TFM increases to 20% of the total TFM in solution (McDonald and Kolar, 2007), which increases the susceptibility of target and non-target organisms to the lampricide (Bills and Johnson, 1992; Boogaard et al., 2003).

In addition to the brain, the current study also noted decreases in muscle glycogen, which could adversely impact burst and/or endurance swimming, two essential processes for foraging, migration, reproduction and predator evasion by fishes. The sensitivity of non-target fishes to TFM could also be greater as they emerge from the over-wintering periods, when food supply is low, and their glycogen reserves are limited. As previously shown (Soengas et al., 1998; Soengas and Aldegunde, 2002; Soengas et al., 2006; Polakof et al., 2007), trout glycogen reserves, especially brain glycogen, are quite labile when the fish are starved. Therefore, starvation could increase the sensitivity to TFM of non-target fishes, but further work is needed to test this hypothesis. Moreover, glycogen reserves vary with life stage and size in non-target fishes (Ferguson et al., 1993), which implies that the sensitivity of non-target fishes could also be life stage or body size dependent. Further studies analyzing the TFM sensitivity of fishes at different life-stages and seasons are therefore needed to better evaluate the sub-lethal effects that transient TFM exposure has on non-target fishes. Such knowledge could be used to alter the timing and amounts of TFM used for treatments at different times of the year or when fish are at life stages where they may be particularly sensitive to TFM. Together with a better understanding of the factors that influence the sensitivity of larval sea lampreys to TFM, such information would enable the sea lamprey control personnel to minimize the risk of TFM toxicity to non-target fish species, while continuing to protect the Great Lakes fisheries from sea lampreys.

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