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SHORT COMMUNICATION

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Increasing copper concentrations do not affect *Myxobolus cerebralis* triactinomyxon viability

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The cold, high-gradient mountain streams of Colorado provide ideal habitat for salmonid populations, including brook trout (*Salvelinus fontinalis*, Mitchill), brown trout (*Salmo trutta*, Linnaeus), cutthroat trout (*Oncorhynchus clarkii*, Richardson), mountain whitefish (*Prosopium williamsoni*, Girard) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). Salmonid habitat often co-occurs with both high aqueous metal concentrations (copper: 0.15–935 μg/L, cadmium: 0.01–7.92 μg/L, zinc: 0.25–1,940 μg/L; Schmidt et al., 2010) associated with the metal rich geology of the Colorado Mineral Belt (Church et al., 2012; Tweto & Sims, 1963) and the parasite that causes salmonid whirling disease (*Myxobolus cerebralis*, Hofer). *M. cerebralis* was first detected in North America in 1956, spread through the transfer of live fish (Hoffman, 1970, 1990), and subsequently found in 22 states (Bartholomew & Reno, 2002) and Canada. The disease caused widespread population-level declines, especially in rainbow trout populations throughout the Intermountain West (Nehring & Thompson, 2001; Nehring & Walker, 1996; Schisler, Bergersen, & Walker, 1999; Schisler, Walker, Chittum, & Bergersen, 1999; Vincent, 1996). Aqueous copper concentrations in Colorado's surface waters are often artificially elevated because historical mining activity has the potential to accelerate the weathering of metal rich ore (Younger, Banwart, & Hedin, 2002) exposed during the twentieth and twenty-first centuries.

Although copper is an essential element (Grosell, 2011), chronic copper toxicity concentrations for brook trout, brown trout and rainbow trout range between 16.25 and 31.15 μg/L (U.S. Environmental Protection Agency, 2007). Dissolved copper affects several fish physiological processes resulting in reductions in growth (10–140 μg/L; Buckley, Roch, McCarter, Rendell, & Matheson, 1982; Heydarnejad, Khosravian-hemami, Nematollahi, & Rahnama, 2013; McKim & Benoit, 1971), reduced viable egg production and hatchability (32.5 μg/L; McKim & Benoit, 1971), reduced olfactory responses (0.18 μg/L; Hecht et al., 2007), and reductions in swimming ability (5 μg/L; Beaumont, Butler, & Taylor, 1995). Damage to the gills and opercula of rainbow trout can also occur following exposure to copper (500 μg/L; Kirk & Lewis, 1993; Wilson & Taylor, 1993), which can be stressors and/or pathways for infection and diseases. Copper can also affect biochemical parameters (as low as 10 μg/L; Heydarnejad et al., 2013) and gene expression (as low as 3.2 μg/L; Santos et al., 2009), and inhibit the fish immune system (3.82–290 μg/L; Anderson, Dixon, Bodammer, & Lizzio, 1989; Dethloff & Bailey, 1998; Dethloff, Bailey, & Maier, 2001; Elsasser, Roberson, & Hetrick, 1986; Mushiake, Nakai, & Muroga, 1985; O'Neill, 1981). As such, copper exposure can increase susceptibility to bacterial pathogens such as *Vibrio anguillarum*, Pacini (3.2–8 μg/L; Baker, Knittel, & Fryer, 1983) and *Yersinia ruckeri*, Ewing (7.0 μg/L; Knittel, 1981), infectious hematopoietic necrosis virus (3.9 μg/L; Hetrick, Knittel, & Fryer, 1979), and fungal infections from *Saprolegnia parasitica*, Coker (250 μg/L; Carballo, Muñoz, Cueller, & Tarazona, 1995).

Following establishment in the 1990s, *M. cerebralis* was found in 11 of 15 of Colorado's major river drainages. The progression of disease in young fish results in skeletal deformities that can affect behaviour (El-Matbouli, Fisher-Scherl, & Hoffman, 1992) and mortality before reproductive age. Little is known about the interactions between copper and whirling disease in fish populations, likely because of the complicated life cycle of the parasite which includes two hosts and two free-living life stages (Markiw & Wolf, 1983; Wolf & Markiw, 1984). Toxic effects on the immune system at copper levels sublethal to the two hosts, salmonid fishes and *Tubifex tubifex* (Müller) worms, have the potential to increase spread and virulence of the disease. *T. tubifex* worms have a lower sensitivity to copper than other benthic invertebrates (Roman, De Schamphelaere, Nguyen, & Janssen, 2007). Although the effect of copper on *T. tubifex* susceptibility to whirling disease has not been directly studied, Shirakashi and El-Matbouli (2010) showed that cadmium exposure did not affect worm survival or reproduction, but exposed worms exhibited higher infection prevalence and produced a higher number of triactinomyxons, the waterborne infectious actinospore, than unexposed worms.

Toxic effects of copper on the free-living life stages of *M. cerebralis* are under studied. The myxospore and triactinomyxon are potentially susceptible to copper toxicity, thus reducing risk of the disease in aquatic ecosystems with trace copper. However, copper sulphate exposure to the myxospores resulted in a survival of 38%–96% of the spores, similar to myxospores maintained in a control source (Hoffman & Hoffman, 1972). The objective of this study was to determine whether aqueous copper exposure would decrease triactinomyxon viability, thus breaking the life cycle of *M. cerebralis* in aquatic systems where copper is present.

Triactinomyxons were obtained from lineage III *T. tubifex* cultures maintained in 76-L static tanks at the Colorado Parks and Wildlife (CPW) Parvin Lake Research Station (Red Feather Lakes, Colorado, USA). In addition to feeding the worms following the methods of Nehring et al., (2014) and Nehring, Schisler, Chiaramonte, Horton, and Poole (2015), worms were fed *M. cerebralis*-infected fish from previous laboratory exposure experiments. Myxospores from infected fish are ingested by the worms and undergo transformation within the intestinal epithelial cells, eventually becoming the infectious triactinomyxon (El-Matbouli & Hoffman, 1998; El-Matbouli, Holstein, & Hoffman, 1998; El-Matbouli, McDowell, Antonio, Andree, & Hedrick, 1999). Triactinomyxons are then released into the water column by the worms where they can remain viable from 6–15 days post-release in water temperatures between 7–15°C (El-Matbouli et al., 1999; Markiw, 1992) and attach to and infect the salmonid host (Hedrick & El-Matbouli, 2002; Markiw, 1986). To obtain triactinomyxons from the *Tubifex* tanks, the entire volume of the 76-L tank was filtered through a 20-μM screen. The contents of the screen were gently rinsed into a 1,000-ml jar containing clean, filtered lake water and transported to the CPW Aquatic Toxicology Laboratory for enumeration and experimentation.

Viable triactinomyxons, identified by the presence of a compact and intact sporoplasm, and non-viable triactinomyxons, identified by the absence of a sporoplasm (Figure 1), were counted following the methods of Fetherman, Winkelman, Schisler, and Antolin (2012) and Fetherman, Winkelman, Schisler, and Myrick (2011). Ten counts were conducted on the filtrate obtained from the *Tubifex* tanks to account for a possible uneven distribution of triactinomyxons, and an average of the 10 counts was used to obtain the number of viable and non-viable triactinomyxons per ml. Triactinomyxon viability was calculated as a percentage of viable triactinomyxons over the total number of triactinomyxons present.

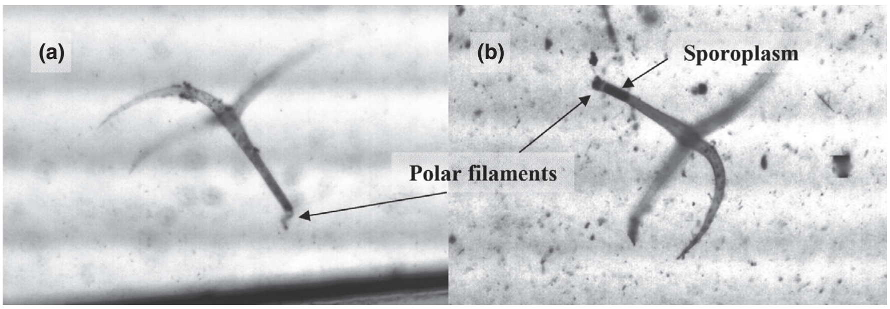

FIGURE 1 (a) Non-viable triactinomyxon with absence of a sporoplasm and extruded polar filaments, and (b) viable triactinomyxon with a compact and intact sporoplasm, and non-extruded polar filaments

TABLE 1 Target (nominal), dissolved and total copper (Cu) concentrations (μg/L; [SE]), and total hardness concentrations (mg/L; [SE]) for the six treatments before and after the 24-hr triactinomyxon exposure period

		Dissolved Cu		Total Cu		Total hardness[a]	
Treatment	Target Cu	Before	After	Before	After	Before	After
Control	0	1.25 [0.08]	2.38 [0.20]	1.77 [0.35]	6.44 [1.61]	48.63 [0.86]	58.67 [3.96]
Low	7.5	9.13 [0.15]	16.3 [0.46]	10.27 [0.15]	19.17 [0.18]	50.59 [0.77]	50.40 [0.14]
Medium	15	16.3 [0.46]	28.73 [0.42]	19.03 [0.17]	35.43 [0.19]	51.19 [0.35]	56.89 [3.64]
High	30	30.37 [1.34]	51.37 [1.49]	36.03 [0.32]	68.07 [3.44]	50.45 [0.34]	62.93 [0.19]
HH	60	63.47 [2.84]	99.67 [5.81]	72.00 [0.51]	145.33 [14.84]	50.65 [0.83]	63.97 [0.86]
HHH	120	96.93 [5.75]	178.67 [7.83]	134.67 [2.33]	277.33 [28.67]	48.13 [1.06]	64.21 [0.83]

[a]Hardness was estimated using the following equation: Total hardness = ([Mg mg/L] × 4.116) + ([Ca mg/L] × 2.497) + ([Al mg/L] × 5.564) + ([Fe mg/L] × 1.729) + (Mn mg/L] × 1.822) + ([Sr mg/L] × 1.142) + ([Zn mg/L] × 1.531).

Triactinomyxons were exposed to five levels of aqueous copper plus a control (Table 1), with three replicates per concentration. Nominal copper concentrations ranged from 0 (control) to 120 μg/L, spanning copper concentrations commonly found in Colorado rivers (Schmidt et al., 2010). Within the 18 experimental units, stock solutions of copper sulphate ($CuSO_4$) were diluted with dechlorinated municipal tap water (Fort Collins, Colorado, USA) to the appropriate concentrations for each treatment level. This water source provided water chemistry similar to oligotrophic mountain streams common in the Mineral Belt of Colorado. Low alkalinity (30–40 mg/L), soft hardness (40–53 mg/L $CaCO_3$), low dissolved organic carbon (1–3 mg/L), low sulphate (8–13 mg/L) and neutral pH are historically observed (2012–2015; CPW unpublished data). 40 ml of filtrate, gently stirred to keep triactinomyxons in suspension, was distributed to experimental beakers, randomized in blocks, with 20 ml delivered to each beaker on each of two passes to account for possible uneven triactinomyxon distribution within the filtrate. Based on the assessment of 770 viable triactinomyxons per ml in the filtrate, each experimental unit contained 30,830 viable triactinomyxons, or 154 viable triactinomyxons per ml. Beakers were gently mixed and stored in an incubator at 12°C for 24 hr.

At the beginning and end of the 24-hr period, copper concentrations were assessed for each beaker using inductively coupled plasma-optical emission spectrometry (ICP-OES). After a 24-hr exposure, the percentage of viable triactinomyxons was assessed using methods described above. Three quantitative assessments were conducted from each replicate beaker. Dissolved and total copper concentrations, hardness and percentage of viable triactinomyxons were compared before and after the 24-hr period using a repeated measures analysis of variance (RM ANOVA) implemented in SAS Proc Mixed (SAS Institute, 2018).

Dissolved and total copper concentrations were close to the target concentrations for all treatments at the beginning of the experiment. Copper concentrations increased over the 24-hr period as a result of evaporation (Table 1). Triactinomyxon viability dropped significantly within the 24-hr period from the percentages observed in the filtrate at the beginning of the experiment (Figure 2). However, despite the range of target copper concentrations within treatments, and the increase in copper concentrations during the 24-hr period, triactinomyxon viability did not differ among treatments at the end of the period (p = 0.86; Figure 2). The overall decrease in triactinomyxon viability is similar to that observed by Kallert and El-Matbouli (2008) at two days of age and stored at 12°C.

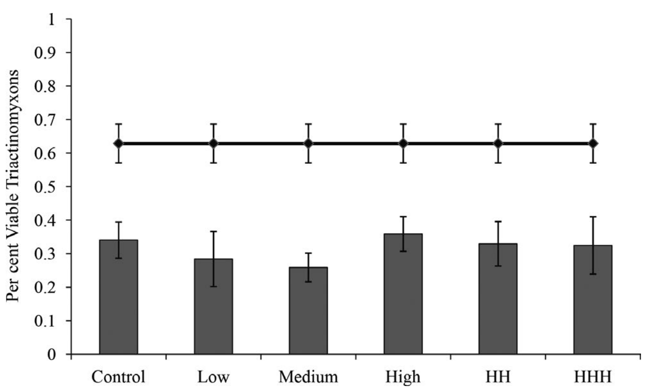

FIGURE 2 Percent viable *Myxobolus cerebralis* triactinomyxons in the six treatments before (line ± 2 SE; counts obtained from filtrate prior to copper exposure) and after (bars ± 2 SE) the 24-hr triactinomyxon copper exposure period

These results, and those of Hoffman and Hoffman (1972), suggest that both free-living life stages of *M. cerebralis* are likely unaffected at most environmentally relevant concentrations, those found in Colorado streams (Schmidt et al., 2010) and spanning the range of chronic toxicity to brook trout, brown trout and rainbow trout (U.S. Environmental Protection Agency, 2007). Thus, copper likely provides no protective effect against whirling disease. Additionally, triactinomyxon production could increase in the presence of metals (Shirakashi & El-Matbouli, 2010). Because sublethal concentrations of copper can reduce immune function and increase susceptibility to diseases in salmonids (Anderson et al., 1989; Dethloff & Bailey, 1998; Elsasser et al., 1986; Mushiake et al., 1985; O'Neill, 1981), the effect and spread of whirling disease are potentially greater in fish populations stressed by copper, and further research investigating this scenario is needed. At lethal levels of copper, the absence of either host ensures that the parasite could be extirpated from an ecosystem within 14 months (Nehring, Alves, Nehring, & Felt, 2018). Such disease-free locations may be priority candidates for mine reclamation efforts. Results also suggest that exposure rates of salmonids to triactinomyxons will not be reduced if copper is present, which enables future laboratory studies examining the disease-toxicant interactions in fish stressed by both whirling disease and aqueous copper.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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