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Sensitivity of the glochidia (larvae) of freshwater mussels to copper: Assessing the effect of water hardness and dissolved organic carbon on the sensitivity of endangered species

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ABSTRACT

The assessment of the potential impact of waterborne contaminants on imperilled freshwater mussels is needed. Acute copper toxicity was assessed in a standardized soft water (hardness 40–48 mg $CaCO_3$ equivalents L^{-1}) using the larvae (glochidia) from three common and six (Canadian) endangered mussel species. The resulting 24 h EC50s ranged from 7 to 36 μg Cu L^{-1}, with the EC50s of two endangered species <10 μg Cu L^{-1}. Acute copper sensitivity was also determined in *Ptychobranchus fasciolaris*, a species that employs conglutinates (packets of glochidia) in its reproductive strategy. Conglutinates were found to provide significant protection from acute copper exposure as the EC50 of the encased glochidia was more than four-fold higher than freed glochidia (72.6 μg Cu L^{-1} vs. 16.3 μg Cu L^{-1}). The glochidia from two endangered species, *Epioblasma triquetra* and *Lampsilis fasciola*, were used to examine the influence of water hardness and dissolved organic carbon (DOC) on copper sensitivity. Exposures in moderately-hard water (165 mg $CaCO_3$ L^{-1}) demonstrated that an increase in water hardness resulted in a significant reduction in copper sensitivity. For example, in *L. fasciola* the 24 h EC50s were 17.6 (14.2–22.6) μg Cu L^{-1} and 50.4 (43.5–60.0) μg Cu L^{-1} in soft water and moderately-hard water, respectively. The addition of DOC (as Aldrich Humic Acid) also resulted in a significant decrease in Cu sensitivity, such that a 10-fold increase in the EC50 of *E. triquetra* was observed when the reconstituted soft water was augmented with 1.6 mg DOC L^{-1}. To determine if current water quality regulations for copper would protect all glochidia, the USEPA's Biotic Ligand Model (BLM) was used to derive water quality criteria for these exposures. While BLM-derived criteria for the soft water exposures indicate that protection would be marginal for the sensitive endangered species, the criteria derived for the DOC exposures suggest that the natural complexity of most natural waters in Southern Ontario (Canada) will provide glochidia with protection from acute copper exposure.

1. Introduction

Freshwater unionid mussels are among the most imperilled groups both globally and in North America (Ricciardi and Rasmussen, 1999; Lydeard et al., 2004). Their decline has been attributed to a number of factors including loss of habitat, decline in fish host populations, predation, over-harvesting, invasive species, and exposure to environmental pollution (Bogan, 1993; Williams et al., 1993; Gillis and Mackie, 1994; Fleming et al., 1995). In fact, nearly 70% of all freshwater mussels in the United States are designated as either threatened, endangered, or in decline (U.S. Fish and Wildlife Service, 1990; Williams et al., 1993). Field surveys of unionid populations have found that the age classes of many species are skewed towards older individuals, indicating that reproductive failure and/or juvenile mortality is occurring (Zale and Neves, 1982; Neves and Widlak, 1987; Bruenderman and Neves, 1993). There have been suggestions that environmental contaminants may be responsible for failed reproduction or recruitment (Huebner and Pynnönen, 1992; Williams et al., 1993). As with most organisms, the early life stages of freshwater mussels are the most sensitive to contaminant exposure but until recently, adults have been the focus of studies addressing the effects of waterborne contaminants on mussels (Hart and Fuller, 1974; Keller and Zam, 1991).

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There are a number of potential chemical threats in the watersheds where many endangered species of mussels are found. Although many compounds are toxic to molluscs, freshwater mussels are known to be especially sensitive to copper (Huebner and Pynnönen, 1992; Jacobson et al., 1997; Wang et al., 2007). Recently, March et al. (2007) noted that ambient copper concentrations in some Oklahoma rivers exceeded those known to be harmful to mussels. In accordance with the Species at Risk Act in Canada (Canada, 2002), a Recovery Strategy must be produced for each species that is designated as Endangered. To date, all three of the recovery strategies developed for freshwater mussels in Canada, have noted the strong need for toxicological studies to assess waterborne contaminants as potential threats to recovery (Morris, 2006a,b; Morris and Burridge, 2006). Furthermore, Valenti et al. (2005) stated that the data provided by laboratory toxicity tests with freshwater mussels are critical to their conservation since such controlled exposures are not possible in the field. Although there have been studies that have assessed mussel sensitivity to a range of environmental contaminants including pesticides (Keller and Ruessler, 1997; Bringolf et al., 2007), ammonia (Augspurger et al., 2003; Newton and Bartsch, 2007), mercury (Valenti et al., 2005), cadmium (Markich et al., 2003), chlorine (Valenti et al., 2006), and copper (Huebner and Pynnönen, 1992; Jacobson et al., 1997; Wang et al., 2007), there is as yet no clear consensus as to the contribution of waterborne contaminants in the decline of freshwater mussels. An examination of the sensitivity of the mussel's early life stages (e.g. mussel larvae) should reveal whether toxicity-induced mortality of the young is contributing to their decline.

All aquatic organisms are directly and indirectly affected by the physical characteristics of their environment, especially the chemical composition of the water. The surrounding water may contain contaminants that can directly affect an organism's ability to survive in a particular environment, but in addition, the specific chemical composition of the water, namely the pH, organic carbon content, redox status, etc. will determine to what extent an organism will be affected by a given contaminant. The bioavailability and thus the toxicity of waterborne metals are strongly influenced by the exposure or receiving water. Major cations such as calcium, magnesium, and sodium compete with metal ions for binding sites on aquatic organisms and thus, based on their concentration, can provide 'protection' from metal toxicity (Pagenkopf, 1983; Playle, 1998). Also, waterborne ligands, such as dissolved organic matter, will bind to metals and prevent them from binding to the toxic site of action on aquatic organisms (Playle et al., 1993). This knowledge is the basis of the Biotic Ligand Model (BLM), which is used to predict the potential for site-specific metal toxicity (Di Toro et al., 2001; USEPA, 2007). Although the influence of water composition has been studied in a wide range of aquatic organisms, little is known about the effect of water composition on copper sensitivity on the early life stages of freshwater mussels.

Most freshwater mussels have a complex life cycle including a larval stage, called glochidia, which is an obligatory parasite on fish or other vertebrates (Pennak, 1989). In many species glochidia are released from the brooding chambers in the gills (marsupia) of the female mussel into the water column where upon contact they will encyst upon a host. The glochidium will take what nourishment it needs to metamorphose into a juvenile mussel before it drops off the fish to live freely in the benthic environment. The length of time that a given glochidium is present in the water column will depend on how long it takes to make contact with a host, although once they are released, glochidia typically survive somewhere between a day and a couple of weeks (Mackie, 1984; Ingersoll et al., 2006; ASTM, 2006). It is during this free-living stage that glochidia can be exposed to waterborne contaminants. Jacobson et al. (1997) examined copper sensitivity in many of the early stages of a mussel's life cycle, including glochidia still brooding inside the marsupium, free glochidia, and those that had encysted upon fish. They found that free glochidia were the most sensitive stage. Some species of mussels do not release their glochidia directly into the water column, instead they encase them in little packets called conglutinates before releasing them. These packets, containing 100–200 glochidia, mimic fish prey items in order to facilitate the transfer of glochidia to the host (Hartfield and Hartfield, 1996). Under natural conditions, glochidia that are encased in conglutinates are not directly exposed to the water column and thus any potential waterborne contaminants. To our knowledge it is not known if species that employ conglutinates are less vulnerable to undesirable water conditions.

We examined copper sensitivity in the glochidia of both endangered and common species of mussels including an endangered species that employs conglutinates in its reproductive strategy. Moreover, in order to examine the influence of water composition on copper sensitivity, we assessed the effect of water hardness and dissolved organic carbon (DOC) on the acute toxicity of copper using glochidia from two of the endangered species. The sensitivity of glochidia to copper is discussed in relation to current water quality regulations in North America.

2. Materials and methods

2.1. Mussel collection and laboratory care

Gravid female mussels were collected from streams, rivers, and lakes in Southern Ontario (see Table 1 for locations). The endangered species, *Epioblasma torulosa rangiana* (Lea 1838) (Northern Riffleshell), *Epioblasma triquetra* (Rafinesque 1820) (Snuffbox), *Lampsilis fasciola* (Rafinesque 1820) (Wavy-rayed Lampmussel), *Obovaria subrotunda* (Rafinesque 1820) (Round Hickorynut), *Ptychobranchus fasciolaris* (Rafinesque 1820) (Kidneyshell), and *Villosa fabalis* (Lea 1831) (Rayed Bean) were collected under the (Canadian) Species at Risk Permit Number SECT 06 SCI 007. Endangered species in Canada are designated as such by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC). COSEWIC defines Endangered Species as "a wildlife species that is facing imminent extirpation or extinction" (COSEWIC, 2007). Endangered species are listed under the Canadian Species at Risk Act (Canada, 2002). The common species used in this study were *Actinonaias ligamentina* (Lamarck 1819) (Mucket), *Lampsilis siliquoidea* (Barnes 1823) (Fatmucket), and *Ligumia recta* (Lamarck 1819) (Black Sandshell). Throughout the field seasons (May–October 2005 and 2006), the reproductive condition of the mussels was monitored in order

Table 1
Collection details including list of mussel species used, water body of origin (all sites in South-western Ontario), and the numbers of gravid mussels collected

Species	Site of origin	Number collected
Actinonaias ligamentina	Thames River	8
Lampsilis siliquoidea	Cox Creek (Grand R.) and Thames River	21
Ligumia recta	Grand River	3
Epioblasma torulosa rangiana[a]	Sydenham River	4
Epioblasma triquetra[a]	Sydenham River	4
Lampsilis fasciola[a]	Thames River	10
Obovaria subrotunda[a]	Lake Saint Clair	3
Ptychobranchus fasciolaris[a]	Sydenham River	6
Villosa fabalis[a]	Sydenham River	6

Note: Endangered, as designated and defined by the Committee on the Status of Endangered Wildlife in Canada. Endangered species are listed under the Canadian Species at Risk Act (Canada, 2002). All mussels were returned to their exact collection site following the experiments.

[a] Canadian Endangered Species.

to determine when the glochidia were mature. Once mature, the mussels (number varied with species, see Table 1) were brought to the University of Guelph Aqualab facility and maintained in a flow-through system using well water. The mussels were held at 10 ± 2 °C (in order to prevent the release of glochidia) and fed a commercial shellfish diet (Instant Algae Shellfish Diet 1800®, Richmond Hill, ON), which is a mixture of a *Nannochloropsis*, *Isochrysis*, *Pavlova*, *Thalassiosira*, and *Tetraselmis* algae, at a rate of approximately 1.2×10^{10} algae cells per mussel per day. Gravid females of endangered species were held in the laboratory for a maximum of 10 days prior to testing. Common species were held for up to six weeks.

Glochidia were collected by flushing the marsupia with water using a syringe. The viability of each mussel's glochidia was assessed prior to use and only gravid females whose glochidia exhibited greater than 90% viability were used in the exposures (viability procedure explained below). Glochidia were pooled from three, or if necessary, four gravid females for each experiment. For the endangered species used in this study, glochidia were only collected from one gill (marsupial), and each mussel was returned to the location from which they were collected to facilitate the release of the remainder of their glochidia in their natural habitat.

2.2. Toxicity testing in soft water

Acute toxicity tests in this study were modeled after the recently developed American Standard Testing Method (ASTM, 2006) for conducting toxicity tests with early life stages of freshwater mussels. In this method glochidia are exposed to various concentrations of a waterborne contaminant and then at the end of the exposure period, their viability is assessed with the aid of a dissecting microscope. In order to parasitize a fish, glochidia must be viable, which means that they must be able to close their valves and clamp down on a fish's gill in order to encyst. Glochidia viability (i.e., the ability to close valves) was examined by the addition of a saturated salt solution (NaCl 240 g L^{-1}) to a sub-sample of 100–200 of the exposed glochidia. Although results in this study are expressed as EC50s (the median effective concentrations) rather than LC50s (the median lethal concentrations), for all practical purposes non-viable glochidia can be considered 'dead' because they would not be able to attach to a fish host and complete their life cycle. In this study the viability of exposed glochidia was assessed after 24 and 48 h of exposure.

Toxicity was examined in each species in standard (ASTM, 2003) reconstituted soft water. Nominal components of ASTM soft water are: $NaHCO_3$ = 48 mg L^{-1}; $CaSO_4 \cdot 2H_2O$ = 30.0 mg L^{-1}; $MgSO_4$ = 30.0 mg L^{-1}; KCl = 2.0 mg L^{-1}; pH 7.3–7.5; hardness 40–48 mg $CaCO_3$ equivalents L^{-1}; and alkalinity 30–35 mg $CaCO_3$ equivalents L^{-1}. An aqueous copper stock made from certified ACS grade (Fisher Scientific) cupric sulphate ($CuSO_4 \cdot 5H_2O$) was used to create the copper exposures. Copper-spiked exposure water was held in the dark at 4 °C for 48 h before an experiment was initiated. Due to the varied availability of gravid mussels, the experiments were conducted over the course of two field seasons, 2005 and 2006. Nominal copper concentrations were 0, 6, 12, 25, 50, and 100 μg L^{-1} in the 2005 exposures involving *E. triquetra*, *L. fasciola*, and *L. recta*. The nominal copper concentrations were 0, 10, 20, 50, 100, and 200 μg L^{-1} in the 2006 exposures involving *A. ligamentina*, *E. torulosa rangiana*, *L. siliquoidea*, *O. subrotunda*, *P. fasciolaris*, and *V. fabalis*. Three replicates were used for each copper concentration tested (i.e. treatment). In exposures involving common mussel species, 500–1000 glochidia were added to each replicate, whereas in exposures where endangered species were employed, approximately 500 glochidia were used in each replicate. As indicated above, the ASTM method recommends that control survival be at least 90% for the standardized toxicity test to be considered valid. But because the purpose of this study was to assess copper sensitivity in a range of novel species, rather than undertake routine toxicity testing, we have calculated EC50s (24 and 48 h) for all species that exhibited greater than 80% control survival and have reported the corresponding control survival data. Since the ASTM method recommends using 24 h data rather than 48 h data if control survival falls below 90%, all EC50s presented in Section 3, the discussion, and the figures are derived using 24 h data (but 48 h data are presented in the tables).

Exposures were conducted in 250 mL glass beakers, under a 16:8 light:dark cycle in an environmental chamber maintained at 21 ± 2 °C. As per the ASTM method (ASTM, 2006), exposure solutions were not renewed nor were the glochidia fed during the exposure. Filtered (i.e. filtered through an Acrodisk™ 0.45 μm inline-syringe-tip filter) water samples (10 mL each) were taken at initiation and completion of the exposure to determine (i) the concentration of dissolved copper in each treatment (i.e. exposure concentrations), and (ii) the concentration of DOC in the exposure. Water hardness, pH, dissolved oxygen, and alkalinity were also measured in three of the treatments (control, middle, and highest exposure concentrations) at the beginning and end of each experiment. Finally, Ca, Mg, Na, K, Cl, and SO_4 concentrations were determined at the beginning of each experiment. Ca, Mg, Na, and K were measured using flame atomic absorption spectrometry, and Cl and SO_4 were measured using ion chromatography, all by the (Canadian) National Laboratory for Environmental Testing (Environment Canada, Burlington, ON, Canada). All glassware was acid washed with 10% nitric acid (Reagent Grade, Fisher Scientific) prior to use and all solutions were made with Millipore™ water.

Unlike the other species examined, gravid *P. fasciolaris* mussels produce conglutinates, therefore two types of exposures were conducted with this species. In the first, glochidia freed from their conglutinate casing were used, and in the second, intact conglutinates were used. Like glochidia, conglutinates were flushed from the marsupium using a syringe. A gentle stream of water injected into the gill between two conglutinates induces them to release and fall into the collection dish with the stream of water. *Note*: conglutinates will only 'fall' out of the marsupium if they are mature, forcing them out may cause damage to the mother and/or the conglutinate. Glochidia were freed from the conglutinate by dissection with fine forceps. Once a conglutinate's casing was pierced, the glochidia (100–200) would freely flow out. In order to conduct a toxicity test with freed glochidia, the glochidia from 60 conglutinates were combined (20–25 conglutinates from each of three gravid females). For toxicity tests conducted with intact conglutinates, two were used in each replicate. One conglutinate was opened after 24 h and the other after 48 h of exposure in order to assess the viability of the encased glochidia.

2.3. The influence of hardness and DOC on copper sensitivity

In order to assess the effect of water composition on copper sensitivity a second series of exposures were conducted using *E. triquetra* and *L. fasciola* in test water with higher hardness or DOC concentrations. Acute copper toxicity was assessed for each of the two species in (i) a standardized (ASTM, 2003) moderately-hard water (nominal components: $NaHCO_3$ = 192 mg L^{-1}; $CaSO_4 \cdot 2H_2O$ = 120 mg L^{-1}; $MgSO_4$ = 120 mg L^{-1}; KCl = 8 mg L^{-1}; pH 7.8–8.0; hardness 160–180 mg $CaCO_3$ equivalents L^{-1}; and alkalinity 110–120 mg $CaCO_3$ equivalents L^{-1}); and in (ii) the ASTM reconstituted soft water as described above, in which the concentration of DOC was augmented using a commercially available humic acid (Aldrich Humic Acid (AHA), Sigma–Aldrich, St. Louis,

MO, USA). An aqueous stock of AHA (100 mg L^{-1}) was used to create two DOC exposures, the first referred to as 'low' DOC, was created with the addition of 2 mg AHA L^{-1}, the second referred to as 'high' DOC, was created with the addition of 8 mg AHA L^{-1}. The DOC exposure water was then spiked with copper (as above) and held in the dark at 4 °C for 48 h before an experiment was initiated. Although the exposure water was not filtered before it was used in an experiment, the concentration of DOC in the exposure was determined in a filtered (<0.45 μm) water sample (see above). Test methods and conditions were as described above. The nominal concentration of copper in the exposures ranged from 0 to 100 μg L^{-1}. An exception was the 'high' DOC exposure with *E. triquetra*, in which the highest concentration of copper was raised to 300 μg L^{-1} in order to capture a response of the glochidia. This series of exposures employed glochidia collected from the same gravid females that were used in the soft water exposures.

2.4. Copper analysis

The concentration of dissolved copper in each exposure concentration was determined in a filtered (<0.45 μm) water sample. Copper was analyzed using a graphite furnace atomic absorption spectrophotometry using a Varian 220FS SpectraAA (Varian Techtron, Mulgrave, Victoria, Australia). Method blanks (3) and Fisher Scientific calibration standards (every 20 samples) were included in every run. A maximum of 5% difference between duplicates was accepted.

2.5. DOC analysis

The concentration of dissolved organic carbon in a sample of the filtered (<0.45 μm) exposure water was determined using a Shimadzu total organic carbon analyzer (model 5050A; Mandel Scientific, Guelph, ON, Canada). The concentration of total organic carbon in each sample was calculated automatically by subtracting inorganic carbon from total carbon.

2.6. Statistical analysis

EC50s were determined by Probit Analysis (SPSS version 11.0) using measured concentrations of dissolved copper at the beginning of the experiment ($t = 0$). EC50s are presented with 95% confidence intervals (CI) as EC50 (95% CI). EC50s were considered to be significantly different when their 95% CI did not overlap (Environment Canada, 2005). When the 95% CI of two EC50s overlapped, a simplified method (Litchfield and Wilcoxon, 1949) was applied to determine if they were significantly different.

2.7. Metal speciation analysis

The geochemical speciation program, WHAM VI was used to predict the concentration of free copper ion (i.e. Cu^{2+}) in the various exposures (i.e. soft water, hard water, and DOC augmented exposures).

2.8. Derivation of site-specific water quality criteria

In order to determine if the water quality regulations for copper in North America are protective of the glochidia of freshwater mussels, the acute toxicity data produced in this study were compared to current Canadian Water Quality Guidelines (CCME, 2005) and Water Quality Criteria (U.S. Environmental Protection Agency (USEPA), 2007). Canadian Water Quality Guidelines for copper are simply based on the hardness of the exposure/receiving water. In contrast, current American Water Quality Criteria for copper are derived on a site-specific basis using the USEPA Copper Biotic Ligand Model (BLM) (USEPA, 2007). The online version of the copper BLM (USEPA, 2007) was used to derive site-, or in this case, exposure-specific water quality criteria. The measured values for temperature, pH, DOC, Ca, Mg, Na, K, SO_4, Cl, and alkalinity were entered into the BLM. The BLM output includes a Final Acute Value (FAV) and a Criterion Maximum Concentration (CMC) for each exposure; these values were then compared to the 24 h copper EC50 values determined in this study. FAV and CMC values were derived for each of the four types of exposures (soft water, moderately-hard water, 'low' DOC, 'high' DOC) examined in this study. When a water chemistry parameter varied (e.g. pH 7.4–7.8) between experiments within a type of exposure (i.e. soft water), the full range of measured water chemistry values were entered into the BLM. The upper and lower limits of the FAV and CMC derived were reported in order to demonstrate the range of water quality criteria that would be derived based on the water chemistry in the exposures.

3. Results

3.1. Copper toxicity in soft water

In the 10 soft water exposures conducted, pH ranged from 7.4 to 7.8, dissolved oxygen ranged from 7 to 9 mg L^{-1}, hardness ranged from 40 to 48 mg $CaCO_3$ L^{-1}, and alkalinity ranged from 28 to 34 mg $CaCO_3$ L^{-1}. The range of measured values for other water chemistry parameters (e.g. DOC, Ca, Na, Cl) are presented in Table 2. In the majority of experiments, survival in the control treatment was above 90% (the minimum recommended by ASTM, 2006) after 24 h (Table 3). The exceptions were 89.5% for *E. torulosa rangiana*, 85.2% for *E. triquetra*, and 84.7% for *V. fabalis*. There was significant variation in the copper sensitivity among mussel species.

Table 2
Measured water composition from acute copper exposures with glochidia as well as the Final Acute Value (FAV) and Criterion Maximum Concentration (CMC) as computed using the USEPA Copper Biotic Ligand Model (BLM) (USEPA, 2007)

Exposure	Water chemistry (mg L^{-1})								Alkalinity (mg $CaCO_3$ L^{-1})	BLM derived (μg L^{-1})		Observed, 24 h EC50s
	pH	DOC	Ca	Mg	Na	K	SO_4	Cl		FAV	CMC	
Soft water ($n = 9$[a])	7.4–7.8[b]	0.2	7.0	6.0	13.0	1.1	41.5	0.95	28-34	1.5–2.4	0.7–1.2	7.4–36.1
M-Hard water ($n = 2$)	8.1–8.3	0.2	28.0	22.5	51.4	4.3	168	4.0	111–112	5.6–6.9	2.8–3.4	17.6, 50.4
Low DOC ($n = 2$)	7.7–8.0	0.3–0.6	7.0	5.9	13.2	1.1	40.5	0.95	28–30	3.2–8.8	1.6–4.4	30.9, 44.5
High DOC ($n = 2$)	8.0	1.6–1.7	7.0	6.0	13.5	1.1	40.5	0.95	29–32	23.3–24.7	11.6–12.4	79.7, >800

The range of observed 24 h EC50s from this study are shown for comparison. The corresponding range of FAV and CMC calculated via the BLM are reported. All exposures were conducted at 21 °C. Humic acid was set at 10% of dissolved organic carbon (DOC) in all exposures. Hardness in the soft water, low DOC, and high DOC exposures was 40–48 mg $CaCO_3$ L^{-1}. Hardness in the moderately-hard water (M-Hard) exposures was 165–167 mg $CaCO_3$ L^{-1}.

[a] Based on toxicity tests with free glochidia only.

[b] When a water chemistry parameter varied between toxicity tests within a group of exposures (i.e. in the soft water pH 7.4–7.8), then the full range of measured values were entered into the BLM (USEPA, 2007).

Table 3
Acute copper EC50s (95% confidence intervals) at 24 and 48 h, measured hardness of exposure water, and mean control survival (standard error) for glochidia of various species of freshwater mussels exposed to copper in reconstituted soft water

Species	Water hardness (mg $CaCO_3$ L^{-1})	24 h control survival (%)	24 h EC50 (μg L^{-1})	48 h control survival (%)	48 h EC50 (μg L^{-1})
Common species					
A. ligamentina	48	94.5 (1.7)	31.0 (26.0–38.0)	93.5 (1.9)	15.9 (13.8–19.3)
L. siliquoidea	48	97.2 (1.0)	36.1 (30.3–44.0)	96.5 (1.7)	21.6 (18.8–25.5)
L. recta	40	93.7 (1.7)	34.8 (29.4–39.9)	64.3 (5.4)	NC[a,b]
Endangered species					
E. torulosa rangiana	48	89.5 (1.7)	13.3 (11.7–15.1)	86.1 (1.6)	8.7 (6.8–10.7)
E. triquetra	40	85.2 (2.2)	7.4 (6.8–8.3)	85.8 (2.0)	5.2 (4.7–5.7)
L. fasciola	40	95.9 (0.6)	17.6 (14.2–22.6)	93.9 (1.1)	12.5 (11.6–13.6)
O. subrotunda	48	97.6 (0.4)	13.0 (9.5–21.0)	91.3 (1.3)	11.3 (9.3–13.9)
P. fasciolaris (freed glochidia)	48	92.8 (2.1)	16.3 (14.3–18.6)	76.8 (3.7)	NC
V. fabalis	48	84.7 (2.3)	6.9 (5.9–7.8)	80.2 (1.7)	4.6 (3.7–5.6)
P. fasciolaris (conglutinates)	48	92.5 (1.8)	72.6 (53.0–107.0)	95.9 (0.4)	54.4 (28.3–292.4)

[a] NC: an EC50 was not calculated since control survival was <80%.
[b] An unintentional increase in the temperature of the environmental chamber from 21 to 28 °C during the *L. recta* exposure was likely the cause of the reduced control survival at 48 h.

The 24 h EC50s for free glochidia ranged from 6.9 (5.9–7.8) to 36.1 (30.3–44.0) μg Cu L^{-1} (Fig. 1; Table 3); 48 h EC50s were comparable or lower (Table 3). The 48 h EC50s were not calculated for *L. recta* or *P. fasciolaris* (freed glochidia) because of reduced (<80%) control survival. In the case of *L. recta*, this was likely due to an unintentional increase in the temperature of the environmental chamber from 21 to 28 °C during the experiment. Overall, the endangered species were significantly more sensitive to copper (24 h EC50s) than the three common species tested (Table 3). *P. fasciolaris* glochidia were significantly more sensitive when they were exposed to copper as free glochidia (i.e. released from conglutinates) compared to glochidia that were encased in the conglutinate for the exposure. The 24 h EC50 of freed glochidia (16.3 (14.3–18.6) μg Cu L^{-1}) was significantly lower than the EC50 of glochidia encased in a conglutinate (72.6 (53.0–107.0) μg Cu L^{-1}).

3.2. The influence of hardness and DOC on copper sensitivity

In the two moderately-hard water exposures conducted, the pH ranged from 8.1 to 8.3, dissolved oxygen ranged from 8 to 9 mg L^{-1}, hardness ranged from 165 to 167 mg $CaCO_3$ L^{-1}, and alkalinity ranged from 111 to 112 mg $CaCO_3$ L^{-1}. The poor solubility of AHA led to much lower concentrations of DOC in the exposures than the nominal concentrations would suggest. The concentration of DOC was 0.3–0.6 mg C L^{-1} in the 'low' DOC (nominally 2 mg AHA L^{-1}) exposure and 1.6–1.7 mg C L^{-1} in the 'high' DOC (nominally 8 mg AHA L^{-1}) exposure. The range of measured values for other water chemistry parameters (e.g. Ca, Na, Cl) are presented in Table 2.

The composition of the exposure water had a significant impact on the sensitivity of glochidia to copper. For both species, the copper EC50s in the moderately-hard water were significantly higher than those in soft water (Table 4; Fig. 2). The addition of DOC as humic acid also resulted in significant decreases in copper sensitivity in both species (Table 4; Fig. 2). The addition of 2 mg AHA L^{-1} resulted in a significant increase in the 24 h EC50 compared to the standard soft water exposure (e.g. *E. triquetra*, EC50 = 30.9 μg Cu L^{-1} vs. 7.4 μg Cu L^{-1}). An even larger shift in sensitivity was observed in the 'high' DOC exposure, where the 24 h EC50 of *E. triquetra* increased to 79.7 μg Cu L^{-1}.

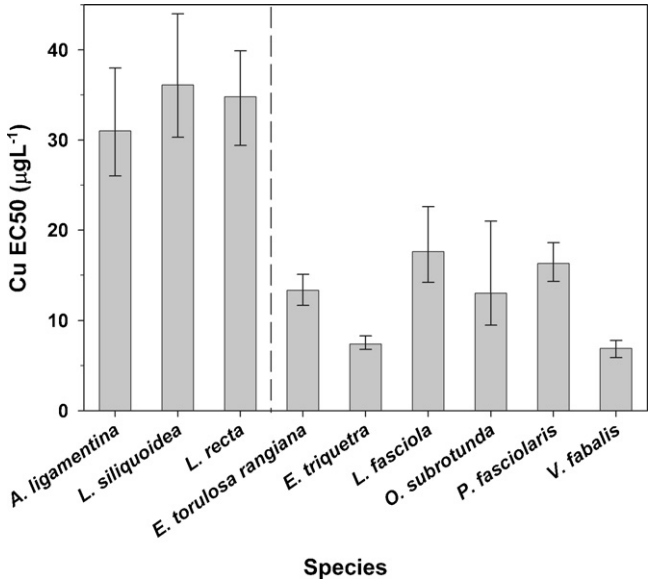

Fig. 1. Copper EC50s (24 h) for free glochidia from nine species of freshwater mussels. Error bars represent 95% confidence intervals around the EC50. Bars to the left of the dashed vertical line represent EC50s for common mussel species, bars to the right represent those for (Canadian) Endangered species.

3.3. Derivation of site-specific water quality criteria

The BLM-derived site-specific water quality criteria (FAV and CMC) along with the copper toxicity data from this study are presented in Table 2. These results are discussed below.

4. Discussion

4.1. Copper sensitivity in soft water

Acute copper toxicity testing in reconstituted soft water revealed that the glochidia of all species tested were sensitive to copper in the low microgram per litre range (24 h EC50s 7–36 μg L^{-1}; Fig. 1) and that endangered species were more significantly sensitive to copper than the three common species tested.

There have been a number of studies that have investigated copper sensitivity in the early life stages of freshwater mussels, but direct comparisons are difficult due to variation in exposure conditions. This is evident in a review by Naimo (1995), who reported just two Cu LC50s for the larvae of freshwater mussels, one for *Corbicula manilensis* (veliger) at 28 μg L^{-1} (a 24 h LC50 from Harrison et al., 1984) and one for *Lampsilis cardium* (glochidia) at >1000 μg L^{-1} (a 48 h LC50 from Lasse, 1991), but the hardness in the two studies was reported as 17 and 149 mg $CaCO_3$ L^{-1}, respec-

Table 4
Acute copper EC50s (95% confidence intervals), measured hardness of exposure water, and mean control survival (standard error) for glochidia of *E. triquetra* and *L. fasciola* exposed to copper in moderately-hard reconstituted water and reconstituted soft water augmented with dissolved organic carbon (DOC) as Aldrich Humic Acid

Species	Water hardness (mg $CaCO_3$ L^{-1})	24 h control survival (%)	24 h EC50 (μg L^{-1})	48 h control survival (%)	48 h EC50 (μg L^{-1})
Moderately-hard water					
E. triquetra	166	91.5 (0.6)	17.6 (15.8–19.9)	86.0 (1.3)	8.8 (7.3–10.6)
L. fasciola	165	96.8 (1.2)	50.4 (43.5–60.0)	93.6 (1.9)	22.0 (18.1–27.4)
Low DOC in soft water					
E. triquetra	41	85.1 (2.8)	30.9 (27.2–35.2)	80.4 (0.5)	19.0 (15.4–23.0)
L. fasciola	40	95.2 (1.3)	44.5 (38.9–51.7)	94.1 (1.7)	40.8 (35.4–47.6)
High DOC in soft water					
E. triquetra	42	86.0 (2.0)	79.7 (71.9–89.1)	51.8 (1.7)	NC[a]
L. fasciola	40	95.2 (0.5)	>800	95.4 (0.5)	127.2 (107.7–159.6)

The concentration of DOC was 0.3–0.6 and 1.6–1.7 mg CL^{-1} in the 'low' and 'high' DOC exposures, respectively.
[a] NC 48 h EC50 was not calculated for the *E. triquetra* 'high DOC' exposure due to low (<80%) control survival.

tively. Jacobson et al. (1997) examined acute copper toxicity in the glochidia of four species of freshwater mussels (including *L. fasciola*) in a moderately-hard (170 mg $CaCO_3$ L^{-1}), field-collected water and found that the 24 h LC50s ranged from 46 to 75 μg Cu L^{-1}. They reported a 24 h LC50 of 48 (45–50) μg Cu L^{-1} for *L. fasciola* in Cu-spiked field water, which is about three-fold higher than the 24 h EC50 reported here in reconstituted soft water but nearly identical to the 24 h EC50 in moderately-hard water in the current study 50 (44–60) μg Cu L^{-1}. Wang et al. (2007) surveyed copper sensitivity in glochidia of nine species and found that the 48 h EC50s in ASTM hard water (160–180 mg $CaCO_3$ L^{-1}) ranged from 7 to 86 μg L^{-1}. Although the exposure conditions vary between studies, the majority, including the current study, have found glochidia to be very sensitive to waterborne copper. In fact, Wang et al. (2007) compared the copper sensitivity of a number of commonly tested species (*Ceriodaphnia dubia* (cladoceran), *Daphnia magna* (cladoceran), *Hyalella azteca* (amphipod), *Pimephales promelas* (fathead minnow), and *Oncorhynchus mykiss* (rainbow trout)) to that of the early life stages of freshwater mussels. They reported that with the exception of *C. dubia* (EC50 = 15 μg Cu L^{-1}), that overall (10 of 11 mussel species tested), glochidia were more sensitive to copper than the commonly tested organisms (EC50s > 58 μg Cu L^{-1}).

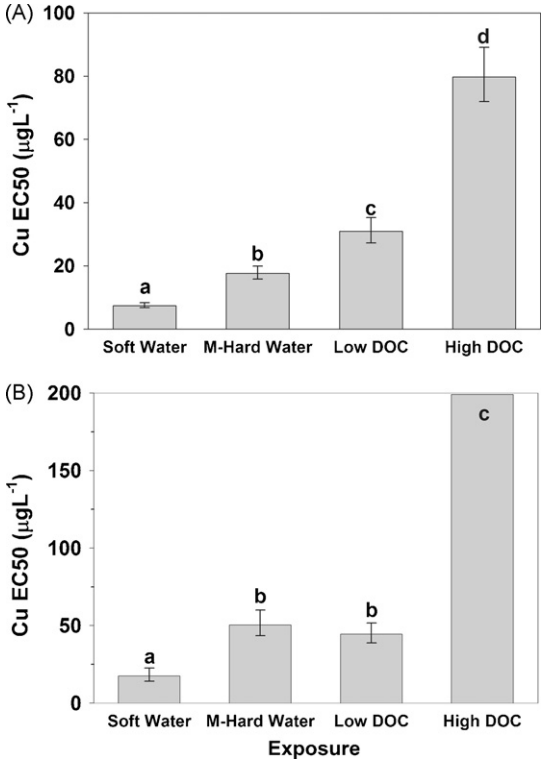

Fig. 2. Copper EC50s (24 h) for (A) *E. triquetra* and (B) *L. fasciola* in various waters. Hardness was approximately 40 and 165 mg $CaCO_3$ L^{-1} in the 'soft' and 'moderately-hard (M-Hard) water' exposures, respectively. The concentration of dissolved organic carbon (DOC) was 0.3–0.6 and 1.6–1.7 mg C L^{-1} in the 'low' and 'high' DOC exposures, respectively. Error bars represent 95% confidence intervals around the EC50. Bars labelled with different letters are significantly different from each other. The EC50 of *L. fasciola* in the high DOC exposure was predicted to be >800 μg Cu L^{-1}.

Of the nine species examined in this study, only *P. fasciolaris* employs conglutinates in its reproductive strategy. Our results demonstrate that glochidia inside a conglutinate can withstand copper exposures more than four-fold higher than free *P. fasciolaris* glochidia. The use of a minnow-shaped conglutinate to facilitate host transfer may also inadvertently protect *P. fasciolaris* glochidia from acute copper exposure. The conglutinates appear to act as barriers to metal toxicity, permitting the glochidia inside to remain viable at copper concentrations even two times higher than *L. siliquoidea*'s EC50, the most tolerant of the species tested. This finding is consistent with Jacobson et al. (1997) who demonstrated that encysted glochidia (i.e. those that have parasitized a fish gill) are at least 10 times more resistant to acute copper exposure than are released glochidia. Given that *P. fasciolaris* is an endangered species in Canada, it is evident that copper toxicity to the conglutinate stage is not the primary factor affecting this species. Although our results indicate that conglutinates provide protection from acute copper exposure, we cannot speculate as to whether conglutinates would provide protection from other classes of environmental contaminants that have very different modes of action (e.g. organics and ammonia), or whether Cu is more or less toxic to other stages in the life cycle of *P. fasciolaris*.

4.2. The influence of hardness and DOC on copper sensitivity

Copper sensitivity was strongly affected by water composition. Both *E. triquetra* and *L. fasciola* glochidia were significantly less sensitive to copper in moderately-hard, compared to soft water exposures. The protective effect of increased water hardness against metal toxicity has been documented in a wide range of aquatic species and thus many water quality regulations are adjusted for water hardness (CCME, 2005; USEPA, 2007). Although there has been limited study into the effect of exposure water composition on copper toxicity in glochidia, Keller and Zam (1991) examined acute (48 h) copper toxicity in juvenile *Anodonta imbecilis* and reported that the Cu LC50 (388 μg Cu L^{-1}) in moderately-hard (exact hardness not reported) water was more than twice that of the LC50 (171 μg Cu L^{-1}) in soft water (39 mg $CaCO_3$ L^{-1}) exposures.

This is comparable with the three-fold increase in the Cu EC50 that Jacobson et al. (1997) reported in hard water exposures compared to their soft water exposures.

An increase in the concentration of DOC in the exposure water also provided significant protection from acute copper toxicity. Even the small amount of DOC added (0.3–0.6 mg C L^{-1}) in the 'low' DOC treatment resulted in a two-fold increase in the EC50s. This is comparable with Glover and Wood (2005) who reported a five-fold decrease in toxicity when a similar amount of AHA (0.6 mg C L^{-1}) was added to the reconstituted water used to examine acute silver toxicity in *D. magna*. The 'high' DOC exposure (1.6 mg C L^{-1}) had a stronger protective effect, producing an EC50 for *E. triquetra* glochidia that was 10-fold higher than the EC50 in the similar soft water (without adding AHA). Hanstén et al. (1996) assessed the effect of a number of chelating factors on metal toxicity in the glochidia of *Anodonta anatine* and found that additions of humic acid (200 mg Pt L^{-1} as colour) reduced the toxicity of cadmium and copper. Markich et al. (2003) investigated the effect of DOC (as fulvic acid) on the response of adult *Hyridella depressa* mussels to metals. They reported an 18-fold decrease in copper sensitivity (as measured in terms of duration of valve opening) when the concentration of fulvic acid in the exposures was increased from 0 to 11 mg L^{-1}. A strong relationship between DOC concentration and reduced copper toxicity has also been shown in the larvae of marine mussels (Arnold, 2005). Overall, our results suggest that even though many of the lakes and rivers in which endangered mussels are found in Southern Ontario have been polluted by a variety of contaminants and agricultural runoff (Morris and Burridge, 2006), that the increased organic carbon load in these water bodies (6–15 mg C L^{-1}; Gillis, unpublished) may provide protection from acute copper exposure.

The differences in copper sensitivity between the various exposure types (i.e. soft, hard, and DOC augmented waters) can be partially explained by the concentration of free copper ion (Cu^{2+}) in each exposure. In the soft water exposures, 17–41% (depending upon the Cu exposure concentration) of the dissolved copper was present as Cu^{2+}. Whereas in the exposures augmented with DOC, the proportion of Cu existing as Cu^{2+} dropped to between 2 and 27% in the 'low' DOC exposures and 0 and 7% in the 'high' DOC exposures. In the exposures with moderately-hard water, although 6–12% of the copper was still present as Cu^{2+}, there would also be some 'protection' provided by the increased Ca and Mg in the water. The metal speciation predictions support our observed EC50s in that glochidia were significantly more sensitive to copper in the ion-poor soft water containing elevated levels of Cu^{2+} compared to the exposures with higher hardness or DOC that contained a much smaller proportion of the copper in the bioavailable free Cu ion form.

Even though our approach in this study was simplistic, employing only two levels of water hardness and two levels of DOC, we have demonstrated the significant ameliorating effects of complex waters on copper sensitivity in glochidia. Therefore, based on these findings we suggest that there is a need for a more in depth investigation into the effect of major ions and DOC on copper toxicity in glochidia. In this study a commercially available humic acid (AHA) was used as a source of DOC, therefore it is necessary to determine if the protection afforded by AHA is representative of the ameliorating effect that the natural dissolved organic matter found in the mussel's habitat will provide. Recently, Glover and Wood (2005) have reported that AHA is not necessarily representative of the natural organic matter found in natural waters. Therefore, direct comparisons between the concentrations of DOC reported in this study and those in the natural environment in terms of quantitative protection against copper toxicity (per unit DOC) are not necessarily appropriate.

Although using reconstituted waters in toxicity testing provides the consistency that is necessary for modeling and comparisons between various species and studies, the results from such tests cannot be directly applied to the natural environment. For instance the soft water used in the bulk of the toxicity testing in this study had a hardness of 40–48 mg $CaCO_3$ L^{-1}, whereas the hardness at the sites from which the mussels were collected ranged between 80 and 280 mg $CaCO_3$ L^{-1} (Gillis, unpublished). Similarly, the lack of natural organic matter in our standardized exposures contrasts with the concentration of inherent DOC (6–15 mg C L^{-1}) that is present in the site waters. The wide range in water composition in the mussels' habitats along with our results that demonstrate the significant effect of water chemistry on copper sensitivity reinforce the need to fully quantify the effects of water composition on copper sensitivity in the early life stages of freshwater mussels.

4.3. Copper sensitivity of glochidia in relation to water quality regulations

The Canadian Water Quality Guideline (WQG) for copper for waters with a hardness of less than 120 mg $CaCO_3$ L^{-1} is 2 μg Cu L^{-1} (CCME, 2005). Similarly, for waters with hardness between 120 and 180 mg $CaCO_3$ L^{-1} the guideline is 3 μg Cu L^{-1} (CCME, 2005). Although these guidelines are still in place, they may be outdated as they are based upon the science that was available at the time of their original derivation more than 20 years ago. Environment Canada is currently developing a new protocol to derive WQG, and copper will be one of the first metals to be updated once that protocol is in place (U. Schnieder, Environment Canada, personal communication, 2007). The most current and internationally recognized guidelines for copper are the USEPA water quality criteria for copper (USEPA, 2007). These criteria employ the BLM in order to make site-specific guidelines based on the water composition at that site, or for our purposes, a guideline can be derived for a particular exposure water. The USEPA derived the ambient water quality guideline for copper from a large data set of acute toxicity data for a variety of freshwater organisms. Briefly, and simplistically, the data were adjusted to a common water chemistry using the BLM and then sorted according to sensitivity of the organisms, with the most sensitive organism occupying the 1st percentile and the most tolerant organism, the 100th percentile. A Final Acute Value (FAV) could then be calculated. The FAV is based upon the 5th percentile of the acute toxicity data since USEPA's mandate is to protect 95% of the organisms 95% of the time. The FAV is then used to calculate the Criterion Maximum Concentration (CMC) which is the maximum allowable 1 h mean concentration (FAV/2). Finally, a Criterion Continuous Concentration (CCC), which is the maximum 4-day mean concentration that is 'allowed', over a three-year period, is derived using an acute-to-chronic ratio. The CMC and the CCC are subsequently adjusted for site-specific waters using the BLM again, and represent the actual Water Quality Criteria. In the case of glochidia where no acute-to-chronic ratio is possible because of their short lifespan, a CCC would not be appropriate.

Using the measured water chemistry (pH, hardness, DOC, Na, Ca, SO_4, etc.) from the soft water exposures in this study, the USEPA-BLM predicts FAVs from 1.5 to 2.4 μg Cu L^{-1} and thus CMCs ranging from 0.7 to 1.2 μg/L^{-1} (Table 2). These criteria (CMC) will be protective for all species tested, although for some of the endangered species with 24 h EC50s below 10 μg Cu L^{-1} (*E. triquetra* = 7.4 μg L^{-1}, *V. fabalis* = 6.9 μg L^{-1}), protection would be marginal. In the exposures in moderately-hard water (pH 8.1–8.3) the USEPA-BLM predicts FAVs that range from 5.6 to 6.9 μg Cu L^{-1} (depending upon the pH used in the model, Table 2). Even though an increase in water hardness resulted in a statistically significant reduction in the copper sensitivity of the two species tested, protec-

tion of glochidia of the very sensitive *E. triquetra* (moderately-hard water EC50 = 17.6 $\mu g\,Cu\,L^{-1}$) may still be a concern. The addition of DOC in the form of humic acid resulted in a significant decrease in copper sensitivity. When the measured water characteristics of the DOC treatments were used to calculate FAVs for these exposures, the 'allowable' copper value significantly increased. The predicted FAV range for the 'low' DOC (0.3–0.6 $mg\,C\,L^{-1}$) treatment was 3.2–8.8 $\mu g\,Cu\,L^{-1}$ (Table 2). The predicted FAV range for the 'high' DOC (1.6–1.7 $mg\,C\,L^{-1}$) treatment was 23.3–24.7 $\mu g\,Cu\,L^{-1}$. The EC50s of both species examined in the presence of AHA were well above the predicted FAVs ('low' DOC EC50s = 30.9 and 44.5 $\mu g\,Cu\,L^{-1}$, 'high' DOC EC50s = 79.7 and >800 $\mu g\,Cu\,L^{-1}$). Therefore, according to these USEPA-BLM-derived values, both of the endangered species tested would be protected by the current water quality criteria when exposed to copper in waters with elevated levels of DOC.

In conclusion, although glochidia were extremely sensitive to waterborne copper when exposed in reconstituted soft water, the BLM-derived FAVs for the DOC exposures suggest that the complexity of most natural waters in Southern Ontario (Canada) will provide protection from acute copper exposure.

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