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# SENSITIVITY OF JUVENILE FRESHWATER MUSSELS (*LAMPSILIS FASCIOLA, VILLOSA IRIS*) TO TOTAL AND UN-IONIZED AMMONIA

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**Abstract**—This study evaluated the sensitivity of juveniles of two freshwater unionid mussel species (*Villosa iris* [Lea] and *Lampsilis fasciola* [Rafinesque]) to un-ionized and total ammonia. Five concentrations of ammonium chloride were tested using 96-h static-renewal toxicity tests at 12 and 20°C. Based on their respective mean 96-h lethal concentration to 50% (LC50s), *V. iris* (0.11 mg/L NH3-N) was more sensitive than *L. fasciola* (0.26 mg/L NH3-N). At 96 h, significant differences in sensitivity to un-ionized ammonia between the two temperatures were not observed for either species. Comparison of LC50s reported for other aquatic organisms to the 96-h LC50s calculated for juvenile *L. fasciola* and *V. iris* shows these two mussel species to be among the most sensitive to un-ionized ammonia. Based on reported levels of un-ionized ammonia in the aquatic environment from anthropogenic sources, un-ionized ammonia may be an important limiting toxicological factor to freshwater mussel populations.

**Keywords**—Juvenile freshwater mussels Ammonia toxicity

## INTRODUCTION

Freshwater mussel populations have declined significantly in recent decades, with over 70% of North America's mussel species considered to be endangered, threatened, or of special concern [1]. Declines have been attributed to many factors, including habitat alteration, competition with exotic species such as the Asian clam (*Corbicula fluminea*) and zebra mussel (*Dreissena polymorpha*), siltation, and degraded water quality. Considering the threatened and endangered status of many freshwater unionid mussels, it may be important to use these species as bioassay organisms to set water quality criteria. The sensitivity of juvenile freshwater mussels to various chemicals is not well understood. Sources of potentially harmful chemicals include agricultural runoff, industrial effluents, and organic waste discharges [2–4]. Ammonia is one contaminant of concern for which existing data are insufficient to facilitate conservation efforts [5]. These data are essential in order for regulators to set water quality standards protective of mussels and to identify sites where ammonia levels may limit survival or reproduction. Aqueous ammonia occurs in two forms, ionized ($NH_4^+$-N) and un-ionized ($NH_3$-N). The proportion occurring in each form is dependent on temperature and pH. Ammonia in its ionized form generally is relatively benign. The un-ionized form, however, can have substantial toxic effects to aquatic organisms. Freshwater bivalves are not routinely used as bioassay organisms to set water quality criteria for ammonia or to regulate wastewater treatment plant discharges [6,7].

Ammonia toxicity varies widely among invertebrate taxa, and uncertainty exists as to whether ammonia toxicity is attributable primarily to the un-ionized form and mechanisms responsible for toxicity [8–11]. Documented toxic effects of ammonia solutions to freshwater and marine bivalves include reduction in the amount of time valves are held open for respiration and feeding [12]; impaired secretion of the byssus thread [13]; reduced ciliary action [6,14]; depleted lipid, glycogen, and other carbohydrate stores; and altered metabolism [15] as well as acute toxicity [7,16,17].

Among invertebrates in general, relationships between the toxicity of un-ionized ammonia and temperature are not widely known [18,19]. Because data on ammonia–temperature relationships are limited for unionid freshwater mussels, related taxonomic groups are of interest. A review of toxicity studies on Asian clams, *Corbicula* spp., showed that greater sensitivity to toxic chemicals (including un-ionized ammonia) occurs at higher temperatures (above 20°C), possibly because of increased metabolism promoting uptake of the chemicals [20,21]. Contrary to this pattern, increased toxicity of un-ionized ammonia to fish has been documented at lower temperatures [22,23]. At temperatures tested to date, the fingernail clam, *Sphaerium novaezelandiae*, has not shown a temperature-related sensitivity to un-ionized ammonia [19]. Sensitivity to un-ionized ammonia is not affected by temperature in other invertebrates [19]. These research results suggest that toxicity mechanisms may differ between bivalves, invertebrates, and fish [19]. Such varying observations indicate the need for study of ammonia toxicity to invertebrates over a range of temperatures [19].

The first objective of this study was to evaluate and compare the sensitivity of juveniles of two freshwater unionid mussel species to un-ionized and total ammonia at two temperatures (12 and 20°C). An organism's early life stages are often the most sensitive to toxicants [24,25]; thus, the success of glochidia and juveniles is critical to the survival and persistence of mussel species [26]. The two species selected for testing,

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wavy-rayed lampmussel, *Lampsilis fasciola* (Rafinesque), and rainbow mussel, *Villosa iris* (Lea), are widely distributed species in Virginia.

A second objective was to compare juvenile freshwater mussel sensitivity with other aquatic organisms, including organisms used in standard toxicity tests such as fish and other invertebrates. The acute bioassay results were also used to estimate safe environmental levels of $NH_3$-N for juveniles of these two species. The third objective was to compare both the estimated safe levels and acutely toxic levels of $NH_3$-N with regulatory standards with existing data on ambient ammonia levels in three Virginia basins and with concentrations reported at wastewater treatment plant (WTP) outfalls.

## MATERIALS AND METHODS

### *Juvenile mussel bioassays*

Juvenile *V. iris* and *L. fasciola* were propagated at the Virginia Tech Aquaculture Center as described in Zale and Neves [27], except that the gravid mussels were not sacrificed. Host fish were largemouth bass (*Micropterus salmoides*) and smallmouth bass (*Micropterus dolomieu*) for *L. fasciola* and rock bass (*Ambolplites rupestris*) for *V. iris*. Newly transformed juvenile mussels between 2 and 5 d old were used in the toxicity tests.

Tests were conducted in an environmental control chamber under static-renewal conditions [26]. The maximum exposure concentration for both mussel species was approximately 0.6 mg $NH_3$-N/L, and a 50% dilution series was used. Dilution water for the chemical exposures was obtained from a spring at White Sulphur Springs National Fish Hatchery (White Sulphur Springs, WV, USA) and gravity filtered through a 30-μm sieve to remove potential predators. This water source was known to be of high quality as indicated by preliminary tests by the Seitz Water Quality Laboratory at Virginia Polytechnic Institute and State University (Blacksburg, VA, USA). An ammonia stock solution of 1,000 mg/L was prepared from reagent-grade ammonium chloride ($NH_4Cl$) that was oven dried at 105°C for several hours. Five exposure concentrations were tested, with four replicates per concentration. Ten juveniles were held in each 50-ml glass beaker [24]. Juvenile mussels were acclimated to test conditions over a 24-h period during which their holding water equilibrated with the environmental chamber temperature and was replaced with springwater from White Sulphur Springs in 20% increments [24]. A light:dark photoperiod of 16:8 h was maintained through all tests [24]. Tests were conducted at 12 ± 1°C and at 20 ± 1°C. Juvenile mussels were not fed during the duration of the test [2], and chemical solutions were renewed daily.

Juveniles were examined for mortality daily over 96 h. After water quality measurements were made in the test beakers, the majority of solution was decanted through a 130-μm nylon sieve to retain the juveniles. Water chemistry tests were performed on the decanted water. Each beaker was placed under dissecting microscope using base illumination, and juveniles were observed at ×40 magnification, which allowed internal anatomy to be viewed through their transparent valves. Juveniles were considered dead if they had gaping shells or lack of pedal or internal movement after 2 min of observation [3].

Ammonia concentrations and pH were monitored at the end of each day for the duration of the test (96 h), using an Orion (Beverly, MA, USA) ion-selective probe and an Orion model 290A pH meter. The pH was also measured directly in the test beakers. Dissolved oxygen, conductivity, and temperature also were measured within the test beakers, using a Yellow Springs Instruments (Dayton, OH, USA) model 85 dissolved oxygen and conductivity meter. Alkalinity and hardness were measured with HACH (Loveland, CO, USA) chemicals, and nitrite levels were determined with a LaMotte (Chestertown, MD, USA) test kit. At the start and end of each 24-h interval, temperature, pH, and ammonia were measured in two replicates of all concentrations and the control. Hardness, alkalinity, nitrites, dissolved oxygen, and conductivity were measured in all concentrations at the start and end of each 96-h test, and dissolved oxygen and conductivity were also measured at 24-h intervals in the lowest and highest concentrations.

Data were analyzed using the U.S. Environmental Protection Agency's (U.S. EPA) CT-TOX Program, and LC50 values with 95% confidence intervals were calculated for 24, 48, 72, and 96 h using the trimmed Spearman–Karber method [28]. Calculations were based on mean values of total or un-ionized ammonia for a given interval. Differences in LC50 values were considered significantly different if 95% confidence intervals did not overlap. Tests were considered valid only if control mortality did not exceed 20% [17].

### *Estimated safe levels, regulatory standards, and environmental ammonia*

To estimate a safe environmental level of un-ionized ammonia, the ratio of the concentration producing acute effects to the concentration producing chronic effects can suggest an application factor of 0.1, 0.01, or 0.001 [5,19]. Multiplying the application factor by the acute LC50 has been used to predict a safe environmental level [9,29]. Presently, no data exist on chronic effect levels of ammonia to juvenile freshwater mussels, so a surrogate species, the fingernail clam, *Musculium transversum*, was chosen to calculate an expected acute:chronic ratio for freshwater unionids. For *M. transversum*, chronic effect levels of 0.09 and 0.16 mg/L $NH_3$-N and acute effect levels of 0.93 and 1.29 mg/L $NH_3$-N were reported by Zischke and Arthur [30]. The ratio of mean chronic levels (0.125 mg/L) to mean acute levels (1.11 mg/L) produced a value of 0.113, suggesting an application factor of 0.1 to be applied to acutely toxic concentrations. This application factor is consistent with many studies that have suggested that chronic toxicity concentrations are often an order of magnitude lower than acute toxicity concentrations.

The Virginia Department of Environmental Quality (VA DEQ) measures total ammonia at ambient water quality monitoring stations located away from known point sources of pollution to obtain background levels. Temperature and pH are recorded at each sampling event such that the un-ionized fraction of ammonia ($NH_3$-N) can be calculated. Ambient ammonia data collected monthly (1996–2001) in the Clinch, Powell, and Holston drainages were reviewed to assess whether juvenile freshwater unionid mussels would be able to survive at un-ionized ammonia levels measured in the environment based on the results of this study. These river basins in southwestern Virginia and eastern Tennessee were chosen because they harbor a diverse assemblage of freshwater mussels, including rare and endangered species, and represent some of the best habitat still available for freshwater mussels in the southeastern United States.

Permitted WTP data were reviewed for the Cleveland WTP on the Clinch River, the Jonesville WTP on the Powell River, and the Saltville WTP on the North Fork Holston River (VA

DEQ. 2001. Data from Archive Table DMRARC, parameter 312 for permits VA0026808, Saltville, 1994–1997; VA0024015, Jonesville, 1999–2001; VA0021016, Cleveland, 1996–2000. Abingdon, VA, USA). The outfall at Cleveland is significant because of its proximity to a parcel of land recently purchased by The Nature Conservancy and intended as a protected site for mussel populations. The outfall at Jonesville is upstream of Fletcher Ford and Fletcher Cliffs, where a number of federally listed mussel species occur. Finally, the Saltville river reach on the North Fork Holston River is under consideration for restoration and reintroduction of mussels after long-term industrial inputs eliminated much of the aquatic fauna in the area.

The WTP data are reported for total ammonia. Un-ionized ammonia levels were calculated with pH and temperature ranges obtained for the same time period from the VA DEQ ambient monitoring station closest to the outfall (VA DEQ, 2001 Water Quality Monitoring Homepage, September 22, 2001 [http://www.deq/state/va.us/webapp/wqm.homepage]). To project a potential range of the un-ionized ammonia in the WTP effluent, the minimum and maximum temperature and pH values were used in the formulas of Emerson et al. [31] to project the least and greatest fractions of $NH_3$-N that might have occurred under these environmental conditions. Applying the greatest fractions to the maximum values for total ammonia represented a worst-case scenario for un-ionized ammonia where the maximum value co-occurs with the highest temperature and pH.

## RESULTS

### *Juvenile mussel bioassays*

For both the 12 and 20°C toxicity tests, *V. iris* and *L. fasciola* had concentration-dependent increases in the toxicity of total and un-ionized ammonia, and acceptable control survival of greater than 80% was observed (Fig. 1 and Table 1). At each 24-h interval, *V. iris* increased in sensitivity to total ammonia between 12 and 20°C but had similar LC50 values for un-ionized ammonia between 12 and 20°C (Table 1). This same pattern occurred for *L. fasciola* at 96 h. These results indicate that the ammonia toxicity to juveniles of these mussel species is attributable to the concentration of un-ionized ammonia.

Water quality parameters were fairly consistent within each test (Table 2). Ammonia concentrations and pH measurements were similar between replicates of each concentration and remained relatively stable over the course of each 96-h test, prior to and after solution renewals. Dissolved oxygen concentrations ranged from 7.23 to 11.48 mg/L. Nitrites were below the detection limit of 0.05 mg/L. Hardness ranged from 80 to 100 mg/L for the *V. iris* tests and from 240 to 260 for the *L. fasciola* tests. Measured pH was higher in the dilution water collected for the *L. fasciola* tests, while in the test solutions, pH for all tests decreased with increasing ammonia concentration. Conductivity increased with ammonia concentrations and reached a maximum of 1,500 μmhos/cm in the 12°C test with *V. iris*.

*Lampsilis fasciola* juveniles were more tolerant of un-ionized ammonia than *V. iris* juveniles. The 96-h LC50 values for *L. fasciola* juveniles at 12 and 20°C were 0.23 and 0.28 mg/L $NH_3$-N, respectively (Table 1). The 96-h LC50 values for *V. iris* juveniles at 12 and 20°C were 0.10 and 0.12 mg/L $NH_3$-N, respectively.

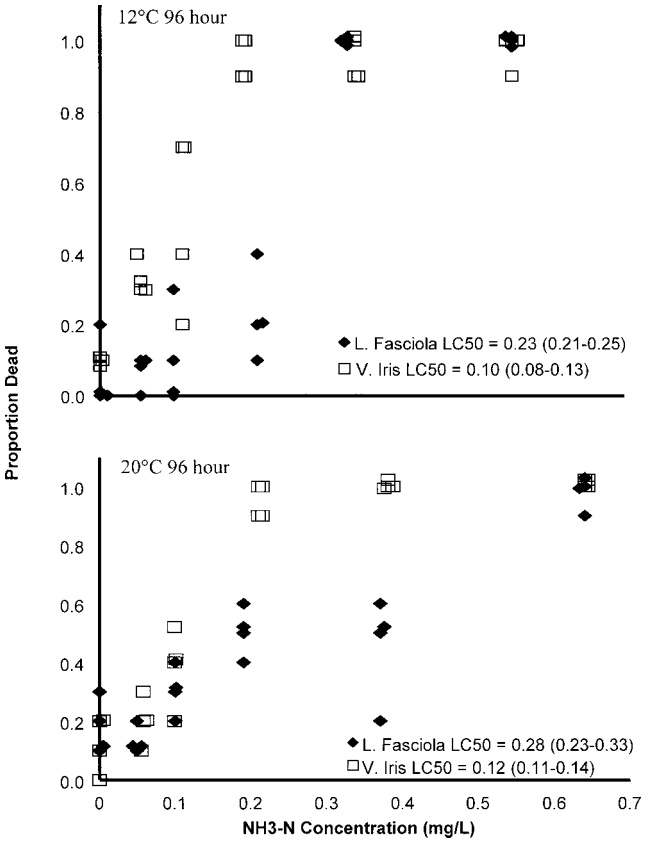


Fig. 1. Survival of juvenile *Vilosa iris* and *Lampsilis fasciola* exposed to test concentrations of un-ionized ammonia. Four replicate samples were conducted for each test concentration. In the graph, symbols were nudged in the case of overlapping results to fully present the data. Values in parentheses after the lethal concentration for 50% mortality (LC50) are 95% confidence limits.

### *Estimated safe levels, regulatory standards, and environmental ammonia*

Application of the 0.1 factor to the mean 96-h LC50 values found in this study yielded estimated safe environmental levels of 0.011 mg/L $NH_3$-N and 0.026 mg/L $NH_3$-N for juveniles of *V. iris* and *L. fasciola*, respectively. Comparison of the U.S. EPA acute 1-h limits with 24-h LC50s (the shortest interval for which LC50s were calculated) shows that the LC50 values are between 2.15 and 3.45 times higher than the concentration limits (Table 3). Comparison of the VA DEQ acute 1-h limit with 24-h LC50s indicated that the 1-h limits would be protective against a 24-h exposure, with the LC50 values between 1.77 and 2.83 times higher than the concentration limits. The VA DEQ chronic, 30-d limits provide protection against 96-h toxicity, with 96-h LC50 values between 3.83 and 9.02 times higher than the 30-d concentration limits.

For ambient water quality monitoring stations in the Clinch/Powell and Holston drainages, ammonia levels were below detection limits (<0.04 mg/L) in the majority of samples (93.6 and 88%, respectively). The mean concentration of un-ionized ammonia in measurable samples was 0.0019 mg/L in the Clinch/Powell basin and 0.0021 mg/L in the Holston River. Only one sample from the Clinch/Powell basin was above the 0.01-mg/L $NH_3$-N estimated safe level for juvenile *V. iris*, and no samples from the Holston basin were above this level. Ambient un-ionized ammonia levels in the Clinch River also were documented in a 1985 to 1986 study. Measurements

Table 1. The 24 through 96-h lethal concentration LC50 median values calculated with the Trimmed Spearman-Karber method for juveniles (age 5 d) of two freshwater mussel species, *Villosa iris* and *Lampsilis fasciola*, exposed to ammonium chloride at 12 ± 1°C and 20 ± 1°C, with 95% confidence interval (CI) [in brackets]

| | | Ammonia (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 24-h LC50 [95% CI] | | 48-h LC50 [95% CI] | | 72-h LC50 [95% CI] | | 96-h LC50 [95% CI] | |
| Species | Temp. (°C) | Total | Un-ionized | Total | Un-ionized | Total | Un-ionized | Total | Un-ionized |
| *V. iris*, juveniles | 12 ± 1 | 36.8 [27.7–48.9] | 0.22 [0.17–0.28] | 29.6 [24.7–35.6] | 0.17 [0.14–0.20] | 22.8 [19.4–26.9] | 0.12 [0.10–0.14] | 20.6 [16.6–25.6] | 0.10 [0.08–0.13] |
| | 20 ± 1 | 33.4 [28.0–40.0] | 0.32 [0.27–0.37] | 18.2 [15.6–21.3] | 0.18 [0.15–0.21] | 12.5 [10.8–14.6] | 0.14 [0.12–0.17] | 11.4 [10.1–12.9] | 0.12 [0.11–0.14] |
| Mean un-ionized ammonia LC50 | | | 0.27 | | 0.18 | | 0.13 | | 0.11 |
| *L. fasciola*, juveniles | 12 ± 1 | 21.1 [18.3–24.3] | 0.32 [0.28–0.36] | 16.1 [14.9–17.3] | 0.23 [0.21–0.24] | 15.4 [14.1–16.9] | 0.23 [0.21–0.24] | 14.9 [13.4–16.6] | 0.23 [0.21–0.25] |
| | 20 ± 1 | NA[a] | NA[a] | NA[a] | NA[a] | 16.5 [11.4–25.1] | 0.54 [0.39–0.74] | 7.74 [6.47–9.26] | 0.28 [0.23–0.33] |
| Mean un-ionized ammonia LC50 | | | NA[a] | | NA[a] | | 0.39 | | 0.26 |

[a] Not applicable. For the 20°C trials with *L. fasciola*, 24-h and 48-h LC50 values were not calculable because mortality did not reach 50% by 48-h in any of the toxicant concentrations.

ranged from 0.001 to 0.02 mg/L $NH_3$-N, and all but one of 22 total measurements were below the 0.01-mg/L estimated safe environmental threshold [32].

Data obtained for permitted WTP's on the Clinch, Powell, and North Fork Holston Rivers revealed ammonia levels reported as undetectable for 14.6% of the samples from Cleveland, 6.2% from Jonesville, and 8.8% from Saltville (Table 4). For the WTP samples in which ammonia was detectable, mean total ammonia concentrations ranged from 0.57 to 5.42 mg/L. Considering all data ranges for the three WTP outfalls, only two of the nine minimum un-ionized ammonia concentrations projected from mean total ammonia values were above the 0.01-mg/L estimated safe environmental threshold. However, all nine of the maximum projected values were not only greater than safe environmental levels but were also above or equal to 0.10 to 0.12 mg/L, which were the 96-h LC50s for *V. iris* juveniles. Five of the collected samples also were greater than the 0.23 and 0.28 mg/L 96-h LC50s for *L. fasciola*. Furthermore, considering the worst-case $NH_3$-N levels calculated from the maximum reported total ammonia values, all but one of the projected values (exception of 0.19 mg/L) were above the 24-h LC50s for both species of juvenile mussels, and six of the nine values were above 1 mg/L, which was approximately 10 times the LC50s found for juvenile *V. iris*.

Previously collected data from sites below WTPs (1985–1986) [31] include $NH_3$-N levels calculated from pH and temperature data collected at the same sampling event. Because the calculated un-ionized ammonia levels below WTPs in Table 5 were hypothetical values (projected from total ammonia measurements and pH and temperature ranges measured close to the site at different sampling times), it is useful to review the previously documented $NH_3$-N levels as a comparison. For 22 monthly measurements taken in 1985 and 1986, $NH_3$-N levels in the effluent plume 0.1 km below the outfalls at Cleveland and Richlands (Clinch River) ranged from 0.006 to 0.812 mg/L [32]. This maximum recorded value demonstrated that $NH_3$-N levels approaching 1 mg/L did occur in the environment. Eight of the 22 $NH_3$-N values were above the 0.10-mg/L 96-h LC50 found for *V. iris* juveniles in this study, and only two of 22 were below the 0.01-mg/L estimated safe environmental threshold.

## DISCUSSION

### *Relative sensitivities to un-ionized ammonia*

Comparison of the 96-h LC50 values calculated for juvenile *L. fasciola* and *V. iris* to the range of reported LC50s for a number of aquatic organisms shows these two mussel species to be among the most sensitive to un-ionized ammonia (Table 5). Salmonids, especially rainbow trout, were one of the most sensitive groups studied. Marine bivalves, crayfish, and aquatic insects tended to be relatively insensitive to ammonia. Some freshwater bivalves and snails had LC50 values similar to those observed for *V. iris* and *L. fasciola*. The only organisms with reported LC50 values lower than *L. fasciola* juveniles were rainbow trout, *Oncorhynchus mykiss*; juvenile Asian clams, *Corbicula fluminea*; juvenile mussels of *Utterbackia imbecillis*; glochidia of *V. iris*; the amphipod *Hyalella azteca*; and the cladoceran *Ceriodaphnia dubia*. The only organisms with reported LC50 values lower than those seen for *V. iris* juveniles were *C. dubia* and *H. azteca*.

Of the standard U.S. EPA test organisms, neither *P. promelas* nor *D. magna* were as sensitive to un-ionized ammonia as the two mussel species we tested. As a result, neither is

Table 2. Summary of water chemistry and mortality data recorded over each 96-h test for juveniles (age 5 d) of two freshwater mussel species, *Villosa iris* and *Lampsilis fasciola*

| | Ammonia (mg/L) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Target un-ionized concn. | Actual un-ionized mean ± SD[a] | Actual total mean ± SD[a] | Mean temp. (°C) min–max | Mean pH min–max | Mean dissolved oxygen (mg/L) min–max | Mean hardness (mg/L) min–max | Mean alkalinity (mg/L) min–max | Conductivity (μmhos/cm) min–max |
| *Villosa iris*, 12 ± 1°C | Control | 0.00 ± 0.0 | 0.00 ± 0.0 | 12.5 | 7.30 | 9.78 | 97.5 | 35 | 563.0 |
| | 0.0375 | 0.054 ± 0.01 | 11.5 ± 1.0 | 11.9–12.9 | 6.84–7.54 | 9.05–11.48 | 80–100 | 35[b] | 120–1,500 |
| | 0.075 | 0.11 ± 0.02 | 20.7 ± 1.2 | *n* = 96 | *n* = 96 | *n* = 72 | *n* = 24 | *n* = 24 | *n* = 72 |
| | 0.15 | 0.19 ± 0.03 | 42.0 ± 1.9 | | | | | | |
| | 0.3 | 0.34 ± 0.05 | 82.6 ± 2.9 | | | | | | |
| | 0.6 | 0.54 ± 0.11 | 162 ± 1.6 | | | | | | |
| *Villosa iris*, 20 ± 1°C | Control | 0.00 ± 0.0 | 0.00 ± 0.0 | 20.6 | 7.41 | 7.99 | 80 | 34.4 | 346.0 |
| | 0.0375 | 0.058 ± 0.004 | 5.05 ± 0.14 | 19.8–21.0 | 7.16–7.68 | 7.23–8.81 | 80[b] | 30–35 | 130–810 |
| | 0.075 | 0.099 ± 0.02 | 9.62 ± 0.33 | *n* = 96 | *n* = 96 | *n* = 72 | *n* = 24 | *n* = 24 | *n* = 72 |
| | 0.15 | 0.21 ± 0.04 | 19.0 ± 0.72 | | | | | | |
| | 0.3 | 0.38 ± 0.06 | 38.0 ± 0.51 | | | | | | |
| | 0.6 | 0.64 ± 0.09 | 75.5 ± 1.4 | | | | | | |
| *Lampsilis fasciola*, 12 ± 1°C | Control | 0 ± 0.0 | 0.00 ± 0.0 | 12.6 | 7.83 | 9.44 | 256.7 | 65.6 | 540.0 |
| | 0.0375 | 0.053 ± 0.003 | 3.11 ± 0.21 | 11.7–13.0 | 7.00–8.36 | 7.44 ± 10.40 | 240–260 | 65–70 | 400–820 |
| | 0.075 | 0.098 ± 0.005 | 5.70 ± 0.30 | *n* = 96 | *n* = 96 | *n* = 72 | *n* = 24 | *n* = 24 | *n* = 72 |
| | 0.15 | 0.21 ± 0.01 | 12.3 ± 0.37 | | | | | | |
| | 0.3 | 0.33 ± 0.02 | 24.4 ± 0.35 | | | | | | |
| | 0.6 | 0.55 ± 0.03 | 46.6 ± 1.9 | | | | | | |
| *Lampsilis fasciola*, 20 ± 1°C | Control | 0.00 ± 0.0 | 0.00 ± 0.0 | 20.6 | 7.96 | 8.5 | 255.8 | 69.4 | 467.4 |
| | 0.0375 | 0.049 ± 0.004 | 1.51 ± 0.31 | 19.5–21.0 | 7.73–8.25 | 7.87–9.45 | 240–260 | 65–75 | 340–570 |
| | 0.075 | 0.10 ± 0.003 | 2.51 ± 0.36 | *n* = 96 | *n* = 96 | *n* = 72 | *n* = 24 | *n* = 24 | *n* = 72 |
| | 0.15 | 0.19 ± 0.008 | 4.92 ± 0.66 | | | | | | |
| | 0.3 | 0.37 ± 0.02 | 10.3 ± 2.0 | | | | | | |
| | 0.6 | 0.64 ± 0.04 | 19.7 ± 2.1 | | | | | | |

[a] Standard deviation.
[b] Range not applicable, all measurements of the parameter were the same.

adequate for use as a surrogate for freshwater mussels in setting water quality standards for ammonia or mixed effluents containing ammonia. The range of reported LC50 values for the standard test daphnid, *C. dubia*, overlaps the LC50s calculated for the juvenile mussels, with the lowest reported values below those of the juvenile mussels, while the upper end of the LC50 range suggests a lesser sensitivity for this species. Further comparative study of *C. dubia* with additional mussel species and under a range of similar test conditions would be needed to assess whether this species is an appropriate surrogate for ammonia toxicity to juvenile freshwater mussels. Based on existing data showing overlapping LC50 ranges, ammonia standards based on the sensitivity of this daphnid would be only marginally protective of juvenile mussels.

Comparing the sensitivity of *V. iris* and *L. fasciola* in our toxicity trials, at 24 h no difference in species sensitivity was evident. At 48 h, a difference in sensitivity between the two species was observed in that, for the 20°C test with *L. fasciola*, 24- and 48-h LC50 values could not be generated because a level of mortality sufficient to calculate an LC50 did not occur until the 72-h time point. At 72 h, the two species had significantly different sensitivities, with *V. iris* emerging as the more sensitive species. The fact that *L. fasciola* had a slower rate of response in the 20°C test is in accordance with the higher LC50 values of this species. However, this slower response was observed only in the 20°C test. At 96 h, neither species exhibited a substantial difference in sensitivity to un-ionized ammonia between the two temperatures.

### *Juvenile mussel bioassays: Water chemistry*

Water quality conditions in the test chambers were not problematic. Dissolved oxygen concentrations remained well above the 5-mg/L threshold recommended for unionids [33]. The maximum recorded conductivity of 1,500 μmhos/cm was below the level (2,900 μmhos/cm) found to be safe for long-term exposures of *C. dubia* based on tests conducted in this lab. It is possible that variations in water chemistry parameters between the tests with *L. fasciola* and *V. iris* may have contributed to the observed differences in sensitivity. Hardness of the dilution water was substantially different for the two tests, ranging from 80 to 100 mg/L for the *V. iris* tests and from 240 to 260 mg/L for the *L. fasciola* tests. Either hardness range is suitable for juvenile survival, although higher hardness levels (≥250 mg/L) are recommended for long-term culture in order to provide adequate calcium for shell growth [34]. Documentation of whether increasing hardness or alkalinity affects ammonia toxicity is inconclusive. Substantially decreased ammonia toxicity at higher hardness has been reported in the amphipod *H. azteca* [35], while other researchers have found no effects of hardness on ammonia toxicity in the oligochaete *Lumbriculus variegatus* and midge *Chironomus tentans* [18] or in other species [19].

One mechanism by which higher hardness could ameliorate ammonia toxicity is by increasing the ionic strength of the toxicant solutions. Although pH and temperature are the primary factors that determine the fraction of ammonia occurring as $NH_3$-N, ionic strength also can play a role when total dissolved solids levels reach 200 to 300 mg/L, with higher ionic strength resulting in decreased proportions of $NH_3$-N [31]. It is possible, then, that in the tests with *L. fasciola*, hardness levels ranging from 240 to 260 mg/L may have increased the ionic strength of the solutions, thus decreasing the fraction of un-ionized ammonia and subsequent toxicity.

Table 3. Comparison between the U.S. Environmental Protection Agency (U.S. EPA) [6] and the Virginia Department of Environmental Quality (VA DEQ) (www.deq.state.va.us:411/webapp/wqm.homepage) standards for un-ionized ammonia and juvenile mussel sensitivities

| | 24-h $LC^b50$ value (mg/L $NH_3$-N) [temp °C, mean pH] | VA DEQ 1-h limit[a] (mg/L $NH_3$) [temp °C, pH] protective against 24-h acute toxicity | U.S. EPA 1-h limit[a] (mg/L $NH_3$) [temp °C, pH] protective against 24-h acute toxicity | 96-h $LC^b50$ value (mg/L $NH_3$-N) [temp °C, mean pH] | U.S. EPA 96-h limit[a] (mg/L $NH_3$-N) [temp °C, pH] protective against 96-h acute toxicity | Safe environmental level[c] (mg/L $NH_3$-N) | VA DEQ 30-d limit[a] (mg/L $NH_3$-N) [at temp °C, pH] Protective against 96-h acute toxicity | Acceptable as safe environmental level |
|---|---|---|---|---|---|---|---|---|
| *V. iris* | 0.22 | 0.0904 | 0.075 | 0.10 | 0.0109 | 0.01 | 0.0155 | |
| | [12 ± 1°C, pH 7.38] | [10°C, pH 7.5] yes | [10°C, pH 7.5] yes | [12 ± 1°C, pH 7.31] | [10°C, pH 7.5] yes | | [10°C, pH 7.5] yes | no |
| | 0.32 | 0.181 | 0.149 | 0.12 | 0.0153 | 0.012 | 0.0313 | |
| | [20 ± 1°C, pH 7.38] | [20°C, pH 7.5] yes | [20°C, pH 7.5] yes | [20 ± 1°C, pH 7.41] | [20°C, pH 7.5] yes | | [20°C, pH 7.5] yes | no |
| *L. fasciola* | 0.32 | 0.113 | 0.0929 | 0.23 | 0.0181 | 0.023 | 0.0255 | |
| | [12 ± 1°C, pH 7.86] | [10°C, pH 7.75] yes | [10°C, pH 7.75] yes | [12 ± 1°C, pH 7.83] | [10°C, pH 7.75] yes | | [10°C, pH 7.75] yes | no |
| | 0.54[d] | 0.259 | 0.214 | 0.28 | 0.0288 | 0.028 | 0.0590 | |
| | [20 ± 1°C, pH 7.94] | [20°C, pH 8.0] yes | [20°C, pH 8.0] yes | [20 ± 1°C, pH 7.96] | [20°C, pH 8.0] yes | | [20°C, pH 8.0] yes | no |

[a] Average concentration not to be exceeded more than once every three years.
[b] Lethal concentration.
[c] Estimated by application of 0.1 factor to acute 96 h LC50 value.
[d] 72 h LC50 value; 24 h value not calculable.

The differing pH levels for the two tests may also have had an effect on the two species' sensitivity. As with hardness and alkalinity, current data on whether pH affects the toxicity of un-ionized ammonia are inconclusive. Some researchers report lower toxicity of un-ionized ammonia toxicity with increasing pH, while in other cases no relationship is observed [8,19]. In our study, juveniles of *L. fasciola* were significantly more tolerant of un-ionized ammonia than those of *V. iris*; however, if higher hardness, alkalinity, or pH levels actually buffer the toxic action of total or un-ionized ammonia, the difference in sensitivity between the two species may be attributable to the differences in these water quality characteristics.

### *Estimated safe environmental levels and comparison with standards, ambient levels, and WTP outfalls*

Comparison of the U.S. EPA and VA DEQ water quality criteria for ammonia with our calculated LC50 values for *V. iris* and *L. fasciola* indicates that current standards should be adequate to protect juveniles of these two mussel species from acute exposures. However, it is noteworthy that the range of VA DEQ chronic, 30-d limits (0.0155–0.0590 mg/L for 10–20°C, pH 7.5–8.0) are above the ammonia levels estimated to be safe for long-term exposure and thus are not satisfactory for long-term protection (Table 3).

The high proportion of ambient samples from the Clinch, Powell, and Holston Rivers for which ammonia was undetectable, as well as the fact that the large majority of calculated un-ionized ammonia levels were well below the estimated safe environmental level, suggests that ambient ammonia conditions are within suitable bounds for these two common species. The hypothetical maximum $NH_3$-N values calculated for WTP effluent at discharge pipes demonstrate that these outfalls have the potential to result in un-ionized ammonia levels that not only are above estimated safe environmental levels but also would be acutely toxic to juveniles of these two mussel species. However, it should be noted that levels of un-ionized ammonia would be expected to be lower farther downstream from the WTP outfalls because of mixing and dilution of the effluent.

While the ambient water column measurements indicate relatively good conditions, two caveats bear consideration. One concern is that variability in ammonia levels may not be captured in monthly measurements. Agricultural discharges are noted to be highly variable, as in New Zealand streams receiving dairy-shed discharges, where streams with median concentrations of 2 mg/L total ammonia have spikes up to 100 mg/L [19]. Second, measurements of total and un-ionized ammonia in interstitial pore waters frequently exceed concentrations in overlying water and are strongly influenced by organic loading from the environment [19,36]. Total and un-ionized ammonia concentrations in sediment pore water from the Mississippi River frequently exceed water quality standards for ammonia [37]. Pore water ammonia has been found to be responsible for decreased richness and density among benthic macroinvertebrates in freshwater portions of the Potomac River (near Washington, DC, USA), with total ammonia ranging from 5.5 to 34.2 mg/L [38]. Other reported total ammonia concentrations for pore water are as high as 40 to 80 mg/L [36]. Limitations exist to using water-only tests to evaluate the toxic response of sediment-dwelling organisms, such as our test species. A water-only test does not represent a realistic environment and may present an added stress to the test organism. However, juveniles of these two mussel species show good survival in water-only controls, and studies of other ben-

Table 4. Total ammonia concentrations reported to Virginia Department of Environmental Quality (VA DEQ) (Archive Table DMRARC[a], Abingdon, VA, USA) from three permitted wastewater treatment plant outfall locations in potential freshwater mussel habitat. Projected un-ionized ammonia ranges calculated from temperature and pH ranges recorded at the ambient monitoring stations closest to the outfall site

| | | | Total ammonia (mg/L) | | | | Range calculated Total un-ionized ammonia (mg/L) | |
|---|---|---|---|---|---|---|---|---|
| Location | Date | No. samples | Min–max | Mean | pH | Temperature | Mean | Min and max |
| Cleveland, Clinch River | 1/96–12/97 | 41 | 0.02–5.2 | 1.6 | 7.37–8.61 | 2.2–25.5 | 0.0037–0.31 | $4.6\times10^{-5}$–1.0 |
| | 1/98–12/99 | 29 | 1.0–9.3 | 1.5 | | | 0.0036–0.30 | 0.0023–1.8 |
| | 1/00–6/01 | 5 | 0.21–1.0 | 0.57 | | | 0.0013–0.11 | $4.9\times10^{-4}$–0.19 |
| Jonesville, Powell River | 3/99–12/99 | 8 | 1.6–9.3 | 5.4 | 7.39–8.66 | 2.3–24.5 | 0.013–1.1 | 0.0039–1.9 |
| | 1/00–12/00 | 12 | 0.58–8.2 | 3.9 | | | 0.0096–0.78 | 0.0014–1.64 |
| | 1/01–6/01 | 6 | 2.5–7.2 | 5.0 | | | 0.012–0.95 | 0.0061–1.44 |
| Saltville, North Fork Holston River | 6/94–12/94 | 7 | 0.14–4.4 | 0.83 | 7.37–8.61[b] | 2.2–25.5[b] | 0.0019–0.16 | $3.3\times10^{-4}$–0.85 |
| | 1/95–12/95 | 11 | 0.19–6.5 | 1.6 | | | 0.0036–0.30 | $4.4\times10^{-4}$–1.25 |
| | 1/96–3/97 | 13 | 0.10–3.4 | 1.0 | | | 0.0024–0.20 | $2.3\times10^{-4}$–0.66 |

[a] Parameter 312, permits VAA0026808, Saltville, 1994–1997; VA0024015, Jonesville, 1999–2001, VA0021016, Cleveland, 1996–2000.
[b] Temperature and pH data taken from Clinch River (VA, USA), due to small number of samples available from Holston.

Table 5. Range of reported median lethal concentration LC50 values for standard test species, known sensitive species, and other aquatic invertebrates. Cited LC50 values are for tests conducted over a variety of temperatures, pH values, and other water quality parameters, as well as for multiple life stages and sizes, except where life stage is specified

| Species | Duration (hours) | Range of reported LC50 (mg/L NH3-N) | Reference |
|---|---|---|---|
| Standard Test Species | | | |
| Cladoceran spp., *Ceriodaphnia dubia* and *Ceriodaphnia acanthina* | 48 or 96 | 0.07–0.63 | [6, 16] |
| Cladoceran, *Daphnia magna* | 48 or 96[a] | 0.44–2.28 | [6] |
| Fathead minnow, *Pimephales promelas* | 96 | 0.37–2.83 | [6] |
| Rainbow trout, *Oncorhynchus mykiss* | 96 | 0.13–1.04 | [6, 10] |
| Freshwater clams | | | |
| Fingernail clam, *Musculium transversum* | 48 or 96[a] | 0.77–1.29 | [6, 10] |
| Fingernail clam, *Sphaerium novaezelandia* | | | |
| adult | 96 | 0.49 | [19] |
| Asian clam, *Corbicula fluminea* | | | |
| adults | 96 | 0.71–0.88 | [16] |
| juveniles | 96 | 0.09–0.28 | [16] |
| Freshwater mussels | | | |
| Paper pondshell, *Utterbackia imbecillis* | | | |
| juveniles | 96 | 0.13–0.77 | [17][b] |
| Freshwater mussel, *Pyganadon grandis* | | | |
| adult | 96 | 0.44–0.54 | [16] |
| Rainbow mussel, *Villosa iris* | | | |
| glochidia | 24 | 0.11–0.28 | [37, 16] |
| juveniles | 96 | 0.38–0.62 | [16] |
| juveniles | 96 | 0.10–0.11 | This study |
| Wavyrayed lampmussel, *Lampsilis fasciola* | | | |
| juveniles | 96 | 0.23–0.28 | This study |
| Marine bivalves | | | |
| Eastern oyster *Crassostrea virginica* | 96 | 8.3–37 | [12, 6] |
| Hard clam, *Mercenaria mercenaria* | 96 | 3.2–7.2 | [12, 6] |
| Other mollusks | | | |
| Snail, *Potamopyrgus antipodarum* | 96 | 0.31–0.36 | [19] |
| Snail, *Pleurocera unicale* | 96 | 0.742 | [37] |
| Snail, *Heliosoma trivolis* | 96 | 2.04–2.76 | [6, 10] |
| Snail, *Physa gyrina* | 96 | 1.59–2.49 | [6, 10] |
| Other invertebrates and aquatic insects | | | |
| Mayfly, *Ephemerella grandis* | 96 | 3.86–5.88 | [6] |
| Mayfly, *Callibaetis skokianus* | 96 | 3.15–4.82 | [10] |
| Stonefly, *Arcynopteryx paralleia* | 48 or 96[a] | 2.00–2.06 | [6] |
| Caddisfly (larvae), *Philartcus quaeris* | 96 | 10.07–10.2 | [6, 10] |
| Amphipod, *Hyalella azteca* | 96 | 0.04–9.2 | [19, 35] |
| Crayfish, *Oronectes nais* | 96 | 3.15 | [37] |
| Crayfish, *Oronectes immunis* | 96 | 14.72–33.83 | [6, 10] |
| Crayfish, *Cherax quadricans* | 96 | 0.98 | [40] |

[a] U.S. Environmental Protection Agency Water Quality Criteria for Ammonia does not distinguish 96- and 48-h tests for invertebrates.
[b] NH3-N LC50 values recalculated from LC50s reported as total ammonia with pH and temperature ranges.

thic macroinvertebrates demonstrate that toxic responses to ammonia found in water-only tests, such as our study, can be extrapolated to an interstitial environment [3]. As suggested by laboratory tests, interstitial pore-water concentrations of ammonia may be especially relevant to juvenile mussels because they do not filter overlying water but pedal feed in interstitial spaces [39]. Pore-water ammonia concentrations in the Clinch, Powell, and Holston Rivers have not been documented, but it is a topic worthy of investigation.

### *Implications*

While ambient ammonia levels appear to be largely within acceptable bounds for chronic exposure to juvenile mussels, differences in the proportion of detectable ammonia measurements and in calculated $NH_3$-N values between sampling sites indicates that in choosing potential reintroduction or protection sites, ambient ammonia levels should be a consideration. The hypothetical maximum values for $NH_3$-N below WTP effluent sites clearly are potentially harmful to freshwater mussels. Therefore, in regulating effluent discharges, mussel populations and habitat should be considered. Finally, in comparing the sensitivity of these two species of juvenile mussels to regulatory standards and environmental ammonia levels, it should also be noted that *L. fasciola* and *V. iris* are among the most common species in Virginia, and rarer species may be more sensitive.

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