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INFLUENCE OF pH ON THE ACUTE TOXICITY OF AMMONIA TO JUVENILE FRESHWATER MUSSELS (FATMUCKET, *LAMPSILIS SILIQUOIDEA*)

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Abstract—The objective of the present study was to evaluate the influence of pH on the toxicity of ammonia to juvenile freshwater mussels. Acute 96-h ammonia toxicity tests were conducted with 10-d-old juvenile mussels (fatmucket, *Lampsilis siliquoidea*) at five pH levels ranging from 6.5 to 9.0 in flow-through diluter systems at 20°C. Acute 48-h tests with amphipods (*Hyalella azteca*) and 96-h tests with oligochaetes (*Lumbriculus variegatus*) were conducted concurrently under the same test conditions to determine the sensitivity of mussels relative to these two commonly tested benthic invertebrate species. During the exposure, pH levels were maintained within 0.1 of a pH unit and ammonia concentrations were relatively constant through time (coefficient of variation for ammonia concentrations ranged from 2 to 30% with a median value of 7.9%). The median effective concentrations (EC50s) of total ammonia nitrogen (N) for mussels were at least two to six times lower than the EC50s for amphipods and oligochaetes, and the EC50s for mussels decreased with increasing pH and ranged from 88 mg N/L at pH 6.6 to 0.96 mg N/L at pH 9.0. The EC50s for mussels were at or below the final acute values used to derive the U.S. Environmental Protection Agency's acute water quality criterion (WQC). However, the quantitative relationship between pH and ammonia toxicity to juvenile mussels was similar to the average relationship for other taxa reported in the WQC. These results indicate that including mussel toxicity data in a revision to the WQC would lower the acute criterion but not change the WQC mathematical representation of the relative effect of pH on ammonia toxicity.

Keywords—Juvenile mussels Unionidae Ammonia pH Water quality criteria

INTRODUCTION

Ammonia is one of the most pervasive contaminants in aquatic environments. In aqueous solution, ammonia primarily exists in two forms in equilibrium: un-ionized ammonia and ammonium ion. Un-ionized ammonia is more toxic than ammonium ion, and the relative distribution of the two forms depends on pH and temperature. The acute toxicity of ammonia increases with pH for many aquatic organisms as the proportion of un-ionized ammonia increases, and the magnitude of pH effects can vary among test species [1]. Based on the toxicity data for commonly tested organisms (e.g., cladocerans and fish), the U.S. Environmental Protection Agency (U.S. EPA) used a sigmoidal model to describe the pH dependence of total ammonia toxicity in the ambient acute water quality criterion (WQC) for ammonia [1].

Toxicity data generated from freshwater mussels have not been used in the derivation of the WQC for ammonia, although some laboratory studies have indicated that the early life stages of freshwater mussels are more sensitive to ammonia than are some commonly tested organisms and the current WQC might not adequately protect freshwater mussels from ammonia exposure [2–7]. In these previous mussel studies, the equation in the WQC document was used to normalize ammonia toxicity data for mussels to a common pH end point (e.g., total ammonia nitrogen [N] at pH 8.0) for comparing toxicity data with other species. However, little is known about the influence of pH on ammonia toxicity to mussels [8].

Specifically, the objective of the present study was to evaluate the influence of pH on the acute toxicity of ammonia to juvenile mussels (fatmucket, *Lampsilis siliquoidea*) at five pH levels ranging from 6.5 to 9.0. In addition, the relationship between pH and ammonia toxicity to juvenile mussels was compared to the average relationship for other taxa reported in the U.S. EPA WQC for ammonia. Acute ammonia toxicity tests with an amphipod (*Hyalella azteca*) and an oligochaete (*Lumbriculus variegatus*) were conducted concurrently with the mussel tests to determine the sensitivity of mussels relative to these two commonly tested benthic invertebrate species.

MATERIALS AND METHODS

Test organisms

Gravid female fatmuckets were collected from Silver Fork of Perche Creek (Boone County, MO, USA) in March 2006, where the species was abundant and apparently healthy and reproduction and recent recruitment were evident. The adult mussels were delivered in a cooler to Missouri State University (Springfield, MO, USA), and held in American Society for Testing and Materials (ASTM)–reconstituted hard water (hardness 160–180 mg/L as $CaCO_3$, pH 8.0 [9]) at 10°C. In April 2006, roughly equal numbers of glochidia were removed from each of six female mussels. Glochidia were flushed from the

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marsupial gills by injecting water from a syringe. The viability of glochidia isolated from each mussel was determined by exposing about 100 individuals to a concentrated saline solution. Healthy glochidia closed their valves immediately in response to the saline solution [10]. The viability of glochidia from all samples was >95%. The remaining glochidia isolated from the six mussels were pooled for the production of juvenile mussels.

Glochidia of most freshwater mussel species require a period of obligate parasitism on host fish for successful transformation to the juvenile stage. For the present study, host fish were hatchery-reared largemouth bass (*Micropterus salmoides*, approximately 8 cm total body length) that were obtained from the Missouri Department of Conservation, Chesapeake Fish Hatchery (Chesapeake, MO, USA). The bass were infested with glochidia for 15 min in the ASTM hard water containing about 4,000 glochidia per liter. Afterward, the infested fish were transferred to a recirculating aquarium system designed for the recovery of the transformed juvenile mussels. Water temperature was maintained at 23°C. After approximately two weeks of fish infestation, newly transformed juvenile mussels were collected daily. Juvenile mussels that were derived from a single collection day during the peak of drop-off (excystment) from the host fish were fed continuously with live algae (*Neochloris oleoabundans*) and commercially available nonviable algae (Instant Algae® products Shellfish Diet, Reed Mariculture, Campbell, CA, USA) in a compact culture system [11]. Total algal concentration in the culture system was maintained at about 50,000 to 100,000 cells per milliliter. Juvenile mussels were reared at 22 to 23°C for one week then shipped overnight to the Columbia Environmental Research Center, U.S. Geological Survey (Columbia, MO, USA) for testing.

Amphipods and oligochaetes were mass cultured at the Columbia Environmental Research Center in flow-through glass aquaria containing well water (hardness 280 mg/L, pH 7.8) at 23°C [12,13]. Amphipods were fed maple leaves and ground Tetramin® flake fish food (Zeigler Bros., Gardners, PA, USA), and oligochaetes were fed flake fish food ad libitum. Approximately 8-d-old amphipods and adult oligochaetes were used to start the toxicity tests.

The temperature of the water used to hold the test organisms was adjusted to test temperature by submerging the containers into a water bath at 20°C. The organisms of each species were divided into five groups, and about 200 organisms were placed into each of two 300-ml beakers for each pH treatment. Test organisms were acclimated to each pH treatment by replacing 50% of the holding water with pH-adjusted dilution water in a diluter system at each pH (see the section on the pH-control exposure system for detail descriptions of the beakers and diluter system). Afterward, each beaker received 120 ml of dilution water every 4 h over a 3-d acclimation period. During the acclimation period, juvenile mussels in each acclimation beaker were fed 2 ml of a nonviable algal mixture [6] twice daily, and amphipods and oligochaetes were fed approximately 1 ml of the ground flake fish food once daily.

Dilution water and pH-control exposure system

Reconstituted ASTM hard water was used as dilution water. The major anions and cations in the dilution water measured at the beginning of tests were sodium at 48 mg/L, potassium at 4.4 mg/L, magnesium at 23 mg/L, calcium at 30 mg/L, chloride at 7.9 mg/L, and sulfate at 189 mg/L. Nitrite nitrogen was not detected at a detection limit of 0.005 mg/L in water samples collected from low, medium, and high ammonia exposure concentrations of each pH level at the beginning of the tests. All of these analyses were made in basic accordance with standard methods [14] by Engineering Surveys and Services Testing Laboratories (Columbia, MO, USA).

Five intermittent flow-through diluter systems [13] were used to conduct the ammonia toxicity tests at five nominal pH levels (6.5, 7.5, 8.0, 8.5, and 9.0). Before any pH adjustment, the pH of the reconstituted dilution water was approximately 8.3. The dilution water was first adjusted to a pH of 8.0 in a 700-L polypropylene mixing tank. The water was then adjusted to the other target pH levels in 39-L polypropylene mixing tanks before the water was delivered to the diluters. The pH was adjusted by injecting dilute hydrochloric acid or sodium hydroxide into each mixing tank by use of a pH-pump-control system with proportional output (Barnant HD PH-P1, Barrington, IL, USA). Each mixing tank was aerated to mix the hydrochloric acid or sodium hydroxide with the dilution water.

Each diluter system delivered five ammonia concentrations with a dilution factor of 0.5 (i.e., 50% serial dilution) plus a control and provided approximately 120 ml of test solution to each replicate beaker every 4 h. Each beaker had a 2.5-cm hole in the side covered with 50-mesh (279-μm-wide opening) stainless-steel screen and contained 200 ml of water. Beakers were placed in temperature-controlled water baths at 20°C. Ammonia stock solution was delivered with each cycle of the diluter by a Hamilton syringe pump (Hamilton, Reno, NV, USA). The ammonia stock solutions consisted of a mixture of ammonium chloride (NH_4Cl; Fisher Scientific, Houston, TX, USA; more acidic pH) and ammonium hydroxide (NH_4OH; Fisher Scientific; more basic pH). The proportion of these two chemicals was adjusted to provide a pH of the ammonia stock solution that was similar to the target pH of the exposure water (e.g., higher percentage of ammonium hydroxide in ammonia stocks for higher pH levels to maintain a consistent pH within each level).

Toxicity testing

Experiment 1. Ammonia toxicity tests were conducted concurrently, at each pH level and ammonia concentration, with amphipods for 48 h and with oligochaetes and juvenile mussels for 96 h in accordance with standard methods outlined in ASTM [9,10,15]. Water temperature was maintained at 20 ± 1°C. At the beginning of the tests, 10 organisms were exposed in each of three replicate beakers for mussels or two replicate beakers for amphipods and oligochaetes. A thin layer of fine sand was added into beakers with amphipods [15]. Test organisms were not fed during the toxicity tests.

Ambient laboratory light of approximately 200 lux was used with a photoperiod of 16:8 h light:dark. Total ammonia and pH were measured once daily at all of the exposure concentrations. Total ammonia nitrogen was determined by use of an Orion ammonia electrode and Orion EA940 meter (Thermo Electron, Beverly, MA, USA). The meter for total ammonia analyses was calibrated each time before measuring samples with 0.1 and 1 mg N/L, 1 and 10 mg N/L, or 10 and 100 mg N/L independent calibration verification standards, depending on the range of total ammonia concentrations to be measured. The percentage recovery of the standards ranged from 90 to 100%. A minimum reporting limit of 0.1 mg N/L was selected based on a method detection limit of 0.02 mg N/L and a method quantitation limit of 0.06 mg N/L. The

pH was determined using an Orion glass pH electrode and the Orion EA940 meter. The meter was calibrated daily. In addition, dissolved oxygen, conductivity, hardness, and alkalinity were measured on composite samples of test solutions collected from the control, medium, and high ammonia exposure concentrations at each target pH using standard methods [14] at 0, 48, and 96 h.

Survival of mussels at the end of the toxicity tests was determined using a dissecting microscope. Juvenile mussels that exhibited foot movement within a 5-min observation period were classified as alive [10]. The criterion for death of amphipods or oligochaetes was lack of movement following gentle prodding [9]. The acceptability criterion for a toxicity test was at least 90% control survival [9,10]. As a further measure of the health of mussels in the tests, surviving mussels in control beakers at the five pH levels were held for an additional 3 d after the toxicity test [10].

Experiment 2. The 96-h ammonia toxicity test with juvenile fatmucket at pH 6.5 was retested one month after experiment 1 because high pH variation occurred during the test and less than 50% mortality was found at any ammonia exposure concentration by the end of the test. An ammonia toxicity test with juvenile mussels at pH 8.0 was tested concurrently. The age of juvenile mussels, test conditions, and procedure in both tests in experiment 2 were the same as those in experiment 1, except that exposure concentrations were higher (up to 32 mg N/L in experiment 1 and up to 128 mg N/L in experiment 2) and a different batch of juvenile mussels was tested, which were produced from adult mussels collected at the same location and time for experiment 1.

Data analysis

Median effective concentrations (EC50s) for survival at the end of tests were calculated with TOXSTAT® software [16] using a Probit model [17]. If the data did not fit the Probit model, either a Spearman-Karber or a trimmed Spearman-Karber method was used [17]. Mean measured concentrations of total ammonia were used for the EC50 calculations.

Based on these observed EC50s, parameters were estimated for the sigmoidal logEC50 versus pH relationship specified by the log transformation of equation 8 in the U.S. EPA WQC for ammonia [1]:

$$\log(EC50) = \log(EC50_8) + \log\left(\frac{\frac{R}{1 + 10^{pH_T - pH}} + \frac{1}{1 + 10^{pH - pH_T}}}{\frac{R}{1 + 10^{pH_T - 8}} + \frac{1}{1 + 10^{8 - pH_T}}}\right)$$

where $EC50_8$ denotes the EC50 at the reference pH of 8.0, R equals $EC50_{HI}/EC50_{LO}$ (limiting values for the sigmoidal relationship at high and low pH), and pH_T is the pH in the transition region of the sigmoidal relationship at which the EC50 is the arithmetic average of $EC50_{HI}$ and $EC50_{LO}$. To accommodate apparent differences in sensitivity between the two experiments, separate values for the parameter $\log(EC50_8)$ were estimated for each test set, but the shape of the relationship determined by R and pH_T was assumed to be the same. Parameters for this equation ($\log(EC50_8)_{Exp1}$, $\log(EC50_8)_{Exp2}$, $\log(R)$, and pH_T) were estimated by least-squares, nonlinear regression with SigmaPlot® software [18], specifying the equation by use of the user-defined regression option.

The results of these analyses for the mussel data were compared to the pooled acute toxicity relationship in the ammonia criteria by, first, examining the similarity of the values for the shape parameters ($\log(R)$ and pH_T) from the mussel and criteria analyses and, second, by visually comparing the regression lines from the analysis of the mussel data to the lines that use $\log(EC50_8)_{Exp1}$ and $\log(EC50_8)_{Exp2}$ from the mussel analysis but use the shape parameters from the U.S. EPA WQC for ammonia [1].

RESULTS AND DISCUSSION

In experiment 1, mean measured water hardness in each test ranged from 160 to 170 mg/L as $CaCO_3$, which was within the range of values for the ASTM hard water (hardness of 160–180 mg/L as $CaCO_3$ [9]). The alkalinity of dilution water was within the range of values for the ASTM hard water (alkalinity of 110–120 mg/L as $CaCO_3$ [9]) before any pH adjustment. However, the addition of hydrochloric acid to the dilution water for the pH adjustment to 8.0 in the 700-L mixing tank lowered the alkalinity. The mean alkalinity of the dilution water at pH 8.0 ranged from 60 to 70 mg/L as $CaCO_3$. After the additional pH adjustment to other target pH levels in the 39-L mixing tank, the mean alkalinity of the dilution water slightly increased at pH 8.5 and 9.0 (70–80 mg/L as $CaCO_3$) or slightly decreased at pH 7.5 (60 mg/L as $CaCO_3$) but substantially decreased at pH 6.5 (8 mg/L as $CaCO_3$). Mean dissolved oxygen was more than 8.1 mg/L across all tests.

The measured water quality values in experiment 2 were similar to those in experiment 1. Mean hardness was 170 mg/L as $CaCO_3$ for both tests at pH 6.5 and 8.0. Mean alkalinity was 11 mg/L as $CaCO_3$ at pH 6.5 and 80 mg/L as $CaCO_3$ at pH 8.0. Details of water quality measures are presented in Table S1 (see *Supplemental Data*; http://dx.doi.org/10.1897/07-193.S1).

The measured pH values were consistent throughout the tests and close to target values (Table 1). The pH variation over the exposure period was less than 0.1 pH unit except for the test at pH 6.5 in experiment 1, in which the pH at the higher ammonia concentration declined from 6.4 to 6.1 between 24 and 48 h of the test, probably due to the effect of low pressure associated with a storm during that period. The mean pH values across ammonia concentrations within each test also were relatively consistent; the difference within a test was no more than 0.1 pH unit (Table 1). Measured ammonia concentrations were relatively constant over exposure periods (Table 1). The coefficient of variation for ammonia concentrations ranged from 2 to 30% (median value 7.9%), and ranged from 2 to 15% at concentrations bracketing the EC50. The daily measured pH and ammonia concentration at each test concentration and the coefficient of variation for ammonia concentrations over the exposure period are presented in Table S2 (*Supplemental Data*; http://dx.doi.org/10.1897/07-193.S1).

Control survival was at least 90% at the end of all toxicity tests with the three test species (Table 1). Control survival of juvenile mussels at the five pH levels did not change over the extended 3 d after the 96-h toxicity tests, indicating the good quality of test organisms [10]. Appreciable mortality (>50%) of juvenile mussels occurred at higher exposure concentrations at all pH levels except for the pH 6.5 treatment in experiment 1, whereas mortality of amphipods was low in all exposure concentrations except for the pH 9.0 treatment (Table 1), and no mortality was observed in all treatments with oligochaetes. Total ammonia EC50s for juvenile mussels varied by a factor of 92, ranging from 88 mg N/L at pH 6.6 to 0.96 mg N/L at pH 9.0, and the EC50s generally decreased with increasing pH (Table 2). The EC50s for amphipods and oligochaetes could

Table 1. Mean pH and total ammonia concentrations (standard deviation in parentheses; $n = 5$), and survival of juvenile mussels (*Lampsilis siliquoidea*) and amphipods (*Hyalella azteca*) in acute ammonia toxicity tests[a]

Treatment	pH	Total ammonia nitrogen (mg/L)[b]	Survival (%)[c] Mussel	Amphipod
Experiment 1				
pH 6.5	6.5 (0.06)	Control	100	90
	6.5 (0.04)	1.9 (0.1)	100	100
	6.5 (0.05)	4.2 (0.2)	100	100
	6.4 (0.06)	7.4 (0.4)	100	95
	6.4 (0.09)	17 (0.9)	100	90
	6.4 (0.20)	36 (1.7)	93	95
pH 7.5	7.6 (0.03)	Control	93	95
	7.6 (0.03)	1.1 (0.1)	93	100
	7.6 (0.01)	2.0 (0.2)	90	95
	7.6 (0.02)	3.8 (0.1)	92	95
	7.6 (0.02)	7.7 (0.6)	73	85
	7.7 (0.05)	19 (1.8)	7	90
pH 8.0	8.1 (0.03)	Control	100	95
	8.1 (0.03)	1.0 (0.1)	100	85
	8.1 (0.03)	1.9 (0.1)	100	75
	8.1 (0.03)	3.8 (0.1)	83	85
	8.1 (0.02)	8.6 (0.7)	3	95
	8.1 (0.02)	19 (1.0)	0	100
pH 8.5	8.5 (0.03)	Control	100	100
	8.5 (0.04)	1.1 (0.1)	90	100
	8.5 (0.05)	1.9 (0.2)	93	100
	8.5 (0.02)	3.9 (0.2)	37	100
	8.5 (0.01)	8.5 (0.6)	0	100
	8.5 (0.01)	19 (1.7)	0	100
pH 9.0	9.0 (0.02)	Control	100	95
	9.1 (0.03)	0.3 (0.1)	100	70
	9.1 (0.02)	0.5 (0.2)	83	40
	9.1 (0.02)	1.0 (0.1)	60	75
	9.0 (0.03)	1.9 (0.2)	0	55
	9.0 (0.06)	4.4 (0.5)	0	50
Experiment 2				
pH 6.5	6.6 (0.07)	Control	100	NT[d]
	6.6 (0.06)	7.1 (1.0)	100	NT
	6.6 (0.06)	15 (0.6)	100	NT
	6.6 (0.05)	30 (1.5)	100	NT
	6.6 (0.06)	60 (2.6)	100	NT
	6.6 (0.10)	130 (2.5)	0	NT
pH 8.0	8.1 (0.05)	Control	97	NT
	8.1 (0.05)	1.0 (0.2)	100	NT
	8.1 (0.05)	2.0 (0.1)	100	NT
	8.1 (0.04)	4.1 (0.2)	90	NT
	8.1 (0.04)	9.0 (0.4)	90	NT
	8.1 (0.05)	19 (0.7)	0	NT

[a] Survival of oligochaetes (*Lumbriculus variegatus*) was 100% in all treatments, and the data are not shown in this table.

[b] Minimum reporting limit was total ammonia nitrogen (N) at 0.1 mg/L. Mean ammonia concentration was 0.1 mg N/L in all control treatments.

[c] Survival was calculated from pooled data of three replicates for mussels ($n = 30$) and of two replicates for amphipods ($n = 20$) at each exposure concentration.

[d] Not tested.

not be calculated due to high survival in all of the exposure concentrations except for amphipods at pH 9.0 (Table 1), where the EC50 was with 2.5 mg N/L with 95% confidence intervals of 0.90 to 6.8 mg N/L. Therefore, the results indicate that ammonia toxicity to juvenile mussels was affected by pH and that juvenile mussels were more sensitive to total ammonia than these two commonly tested benthic invertebrate species.

Relatively high-effect concentrations have been reported in previous pH–ammonia toxicity studies with amphipods and oligochaetes. For example, Ankley et al. [19] observed that 96-h median lethal concentrations for amphipods ranged from 40 mg N/L at pH 8.3 to 105 mg N/L at pH 6.4 in moderately hard water (~100 mg N/L as $CaCO_3$). Schubauer-Berigan et al. [20] reported that 10-d median lethal concentration for oligochaetes ranged from 6.6 mg N/L at pH 8.6 to 390 mg N/L at pH 6.3.

Table 2. Median effective concentrations (EC50s) and 95% confidence interval (CI) for total ammonia nitrogen (N) in 96-h toxicity tests with juvenile fatmucket (*Lampsilis siliquoidea*) at various pH levels

Treatment	pH[a]	EC50 (mg N/L; 95% CI)
Experiment 1		
pH 6.5	6.5	>36
pH 7.5	7.6	11 (8.7–13)
pH 8.0	8.1	5.2 (4.6–5.8)
pH 8.5	8.5	3.4 (2.9–4.0)
pH 9.0	9.0	0.96 (0.83–1.1)
Experiment 2		
pH 6.5	6.6	88 (NC[b])
pH 8.0	8.1	11 (9.9–13)

[a] pH values were the average of the mean pHs at six exposure concentrations in each treatment (Table 1).

[b] NC = 95% CI could not be calculated because no partial kills occurred. Based on the assumption of a log-normal tolerance distribution and independence among test organisms, 88 mg N/L is the maximum likelihood estimate for the EC50, and the concentrations in parentheses (60 and 130 mg N/L) are more than 99.9% CI for the EC50. Based on Probit analysis slopes from other treatments, the 95% CI for this EC50 would be substantially narrower than these bracketing concentrations.

In the repeated testing at pH 8.1, the EC50s were with 5.2 and 11 mg N/L (Table 2). The EC50 variation of a factor of two was likely due to the between-test variability. In the authors' previous ammonia toxicity tests with juvenile fatmucket at pH 8.3, the 96-h EC50s were with 4.9 and 10 mg N/L from two static-renewal tests [5] and 4.6 mg N/L from one flow-through test [6]. It is unlikely that the higher EC50 in experiment 2 was because glochidia used for the production of juvenile mussels for experiment 2 had a one-month-longer brooding period in mussel marsupial gills than those used for experiment 1. In previous acute toxicity tests with ammonia, copper, or chlorine, Wang et al. [21] observed that glochidia, collected from adult fatmucket held in the laboratory between April and July, had similar sensitivity.

The relationship between EC50 and pH in experiments 1 and 2 with mussels was accurately described by the model in the U.S. EPA WQC for ammonia [1]. Least-squares regression of the observed EC50s versus pH using the equation previously shown resulted in an adjusted R^2 of 97% and estimates for pH_T of 7.16 (standard error [SE] = 0.26), log(R) of −2.11 (SE = 0.46), $\log(EC50_8)_{Exp1}$ of 0.81 (SE = 0.08), and $\log(EC50_8)_{Exp2}$ of 1.15 (SE = 0.11). The visual fit to the data is good (Fig. 1) and supports the assumption of similar shape parameters between the two experiments. The estimated shape parameters for the mussel data are very close to those for the pooled acute toxicity data in the U.S. EPA WQC for ammonia [1], for which pH_T was 7.20 (SE = 0.05) and log(R) was −2.15 (SE = 0.06). With such small differences in the shape parameters, the EC50 versus pH relationships from the mussel and criteria analyses are virtually indistinguishable when they go through the same estimated mussel EC50s at pH 8.0 (Fig. 1). These results indicate that the generic relationship of acute ammonia toxicity

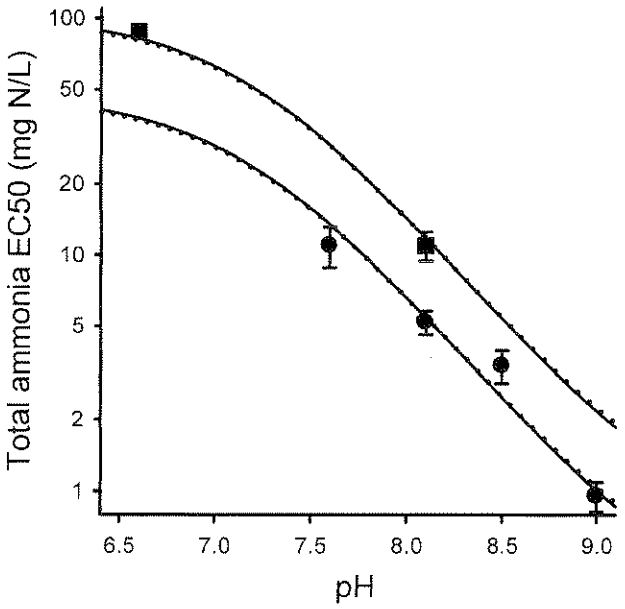

Fig. 1. Median effective concentrations (EC50s) and 95% confidence intervals (error bar) for total ammonia versus pH for experiments 1 (●) and 2 (■) with juvenile mussels (fatmucket, *Lampsilis siliquoidea*). The solid lines denote the estimated EC50 versus pH relationships from a pooled regression analysis of both mussel experiments, assuming the same line shape, but different values for EC50 at pH 8.0 for the two experiments. The dotted lines use the shape of the acute toxicity versus pH relationship from the U.S. Environmental Protection Agency water quality criteria for ammonia [1], fitted to coincide with the same EC50s at pH 8.0 as the solid lines.

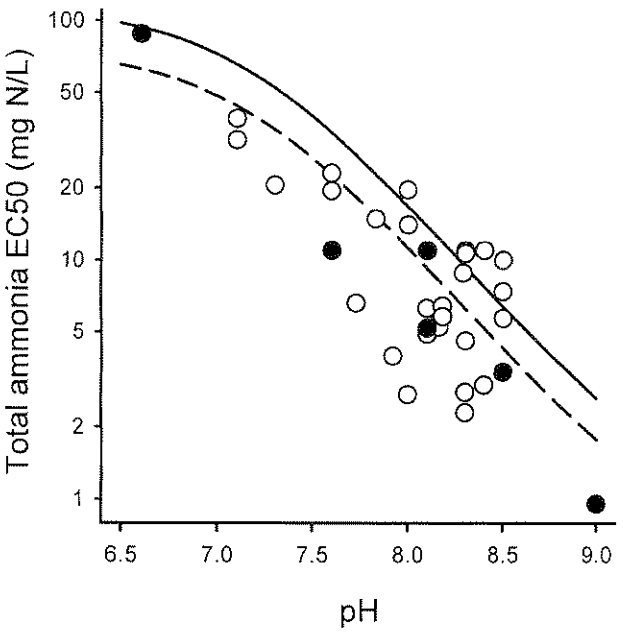

Fig. 2. Comparison of median effective concentrations (EC50s) for total ammonia at various pH levels in the present study with juvenile mussels (fatmucket, *Lampsilis siliquoidea*; ●) and in other studies with juveniles of 11 mussel species (○; species names and references of the previous studies are listed in Table S3) to final acute values in the U.S. Environmental Protection Agency water quality criteria for ammonia [1] with (– –) and without (—) protection for the presence of salmonid fish.

to pH used to derive the current U.S. EPA WQC is appropriate for fatmucket and might be useful for other species of mussels as well.

The total ammonia EC50s for juvenile fatmucket in experiments 1 and 2 across a broad range of pH levels are consistent with the EC50s for juveniles of 11 mussel species tested in previous studies over a narrower range of pH levels (Fig. 2; Table S3; supplemental data found at http://dx.doi.org/10.1897/07-193.S1). The total ammonia EC50s for the juvenile mussels are at or below the U.S. EPA final acute values [1] calculated without protection for salmonid fish (Fig. 2). Furthermore, more than half of the EC50 values were up to 76% (average 39%, $n = 23$) below the final acute values calculated with protection for the salmonids (Fig. 2). Therefore, the current U.S. EPA acute WQC may not adequately protect the early life stages of freshwater mussels from exposure to ammonia. In 28-d ammonia toxicity tests with juveniles of three mussel species (fatmucket; rainbow mussel, *Villosa iris*; wavy-rayed lampmussel, *Lampsilis fasciola*), Wang et al. [6] found that ammonia chronic values (geometric mean of the no-observed-effect concentration and the lowest-observed-effect concentration) for survival and growth were below the U.S. EPA chronic WQC for ammonia, indicating the chronic WQC also may not be protective of mussels.

In conclusion, juvenile mussels were more sensitive to ammonia toxicity than were other tested organisms, and mussel sensitivity to ammonia increased with increasing pH. The pH–ammonia relationship for juvenile mussels is similar to the pooled relationship for various test species used for the derivation of the U.S. EPA WQC for ammonia. Hence, including mussel toxicity data for revising the WQC would result in a lower acute criterion but would not likely change the generic relationship of ammonia toxicity to pH used for the derivation of the WQC.

SUPPORTING INFORMATION

Table S1. Water quality characteristics during acute ammonia toxicity tests with juvenile mussels (*Lampsilis siliquoidea*), amphipods (*Hyalella azteca*), and oligochaetes (*Lumbriculus variegatus*). Values are means (standard deviation; $n = 3$).

Table S2. Daily measured pH and total ammonia concentration, and the means and coefficients of variance (CV) during the acute ammonia toxicity tests with juvenile mussels (*Lampsilis siliquoidea*), amphipods (*Hyalella azteca*), and oligochaetes (*Lumbriculus variegatus*).

Table S3. The 96-h ammonia median effective concentrations (EC50s) for juvenile freshwater mussels. Only those tests that met the American Society for Testing and Materials criteria for toxicity testing with mussels are listed [10]. The acute values reported in original references have been converted to milligrams per liter of total ammonia as N.

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On-line Supplemental Information for the manuscript (MS #07-193) "Influence of pH on the toxicity of ammonia to juvenile mussels (fatmucket, *Lampsilis siliquoidea*)" by Ning Wang, Russell J. Erickson, Christopher G. Ingersoll, Christopher D. Ivey, Eric L. Brunson, Tom Augspurger, and M. Chris Barnhart.

Table S-1. Water quality characterizes during acute ammonia toxicity tests with juvenile mussels (*Lampsilis siliquoidea*), amphipods (*Hyalella azteca*), and oligochaetes (*Lumbriculus variegatus*). Values are means (standard deviation; n=3)

Treatment	Exposure concentration	Temperature (°C)	Hardness (mg/L as $CaCO_3$)	Alkalinity	Conductivity (µs/cm)	Dissolved oxygen (mg/L)
Experiment 1						
pH 6.5	Control	20.8 (0.9)	163 (11)	8.0 (2.0)	657 (8.6)	8.1 (0.3)
	Medium	20.5 (1.1)	171 (10)	8.7 (2.3)	742 (9.8)	8.2 (0.2)
	High	20.8 (1.0)	165 (6.4)	7.2 (2.4)	1029 (5.5)	8.2 (0.2)
pH 7.5	Control	20.7 (0.9)	168 (1.2)	58 (11)	629 (10)	8.1 (0.4)
	Medium	20.3 (1.0)	176 (21)	60 (13)	662 (6.0)	8.1 (0.3)
	High	20.5 (1.2)	171 (8.1)	60 (13)	817 (6.5)	8.2 (0.2)
pH 8.0	Control	20.6 (1.2)	170 (10)	64 (6.9)	631 (6.0)	8.1 (0.3)
	Medium	20.6 (1.4)	168 (3.5)	67 (11)	664 (3.6)	8.2 (0.2)
	High	20.5 (1.2)	174 (8.1)	64 (8.7)	813 (8.0)	8.1 (0.1)
pH 8.5	Control	20.6 (1.2)	166 (11)	76 (10)	652 (6.4)	8.2 (0.2)
	Medium	20.6 (1.2)	166 (11)	79 (12)	686 (7.8)	8.1 (0.3)
	High	20.6 (1.2)	160 (1.2)	82 (7.2)	831 (7.6)	8.1 (0.3)
pH 9.0	Control	20.8 (1.4)	157 (14)	71 (1.0)	650 (1.7)	8.2 (0.2)
	Medium	20.5 (10)	162 (18)	72 (2.0)	651 (5.7)	8.1 (0.2)
	High	20.6 (1.2)	154 (9.2)	73 (1.2)	687 (5.7)	8.1 (0.1)
Experiment 2						
pH 6.5	Control	19.6 (1.0)	168 (4.2)	10 (0)	672 (4.0)	8.7 (0.1)
	Medium	19.6 (1.0)	171 (2.3)	11 (1.2)	947 (3.5)	8.7 (0.1)
	High	19.5 (1.1)	170 (0)	11 (2.3)	1974 (3.6)	8.7 (0.1)
pH 8.0	Control	19.4 (1.2)	172 (3.5)	77 (5.8)	630 (5.5)	8.7 (0.1)
	Medium	19.3 (1.2)	171 (1.2)	81 (2.3)	664 (3.1)	8.7 (0.1)
	High	19.4 (1.0)	171 (1.2)	79 (4.2)	814 (6.1)	8.6 (0.1)

Table S-2. Daily measured pH and total ammonia concentration, and the means and coefficient of variance (CV) during the acute ammonia toxicity tests with juvenile mussels (*Lampsilis siliquoidea*), amphipods (*Hyalella azteca*), and oligochaetes (*Lumbriculus variegatus)*

Treatment	Nominal exposure concentration (mg/L)	pH							Total ammonia (mg N/L)						
		Day 0	Day 1	Day 2	Day 3	Day 4	Mean	% CV	Day 0	Day 1	Day 2	Day 3	Day 4	Mean	% CV
Experiment 1															
pH 6.5	Control	6.50	6.58	6.54	6.43	6.52	6.51	0.9	0.14	0.16	<0.1[a]	0.11	0.13	0.13	19
	2	6.49	6.52	6.46	6.43	6.52	6.48	0.6	1.9	2.0	1.7	2.0	2.0	1.9	6
	4	6.47	6.54	6.41	6.42	6.48	6.46	0.8	4.2	4.3	4.4	4.2	3.8	4.2	6
	8	6.45	6.49	6.34	6.41	6.46	6.43	0.9	7.8	7.8	6.9	7.5	7.1	7.4	6
	16	6.47	6.44	6.26	6.46	6.45	6.42	1.4	17	18	18	18	16	17	5
	32	6.54	6.40	6.05	6.49	6.46	6.39	3.1	36	39	37	35	35	36	5
pH 7.5	Control	7.57	7.59	7.63	7.57	7.61	7.59	0.3	0.14	0.16	0.13	0.12	<0.1	0.13	19
	1	7.59	7.64	7.63	7.58	7.62	7.61	0.3	1.2	1.0	1.2	1.0	1.1	1.1	8
	2	7.59	7.60	7.61	7.58	7.58	7.59	0.2	2.1	1.9	2.2	1.8	1.8	2.0	10
	4	7.57	7.61	7.61	7.58	7.59	7.59	0.2	3.6	4.0	3.9	3.8	3.9	3.8	4
	8	7.61	7.64	7.66	7.62	7.60	7.63	0.3	8.5	7.8	7.4	8.0	6.9	7.7	8
	16	7.73	7.74	7.74	7.71	7.62	7.71	0.7	20	22	17	19	19	19	9
pH 8.0	Control	8.08	8.11	8.15	8.09	8.09	8.10	0.3	0.15	0.14	0.17	0.12	<0.1	0.14	20
	1	8.11	8.14	8.18	8.12	8.11	8.13	0.4	1.1	0.87	0.93	1.06	0.87	0.96	11
	2	8.12	8.14	8.17	8.12	8.10	8.13	0.3	2.1	1.8	2.0	1.8	1.7	1.9	7
	4	8.12	8.15	8.18	8.13	8.11	8.14	0.3	3.8	4.0	3.9	3.9	3.7	3.8	2
	8	8.10	8.16	8.15	8.14	8.13	8.14	0.3	9.7	8.7	8.3	7.8	8.5	8.6	8
	16	8.11	8.13	8.15	8.10	8.10	8.12	0.3	19	21	18	18	18	19	5
pH 8.5	Control	8.53	8.53	8.59	8.52	8.57	8.55	0.4	0.15	0.16	0.11	0.13	0.10	0.13	20
	1	8.52	8.54	8.58	8.48	8.55	8.53	0.4	1.2	0.8	1.1	1.1	1.0	1.1	14
	2	8.45	8.52	8.57	8.53	8.55	8.52	0.5	2.1	1.8	2.2	1.7	1.9	1.9	10
	4	8.52	8.53	8.56	8.51	8.52	8.53	0.2	3.8	4.0	4.2	3.7	3.7	3.9	5
	8	8.46	8.48	8.50	8.48	8.48	8.48	0.2	9.4	8.4	8.0	8.5	8.3	8.5	6
	16	8.45	8.45	8.48	8.45	8.45	8.46	0.2	20	20	19	16	19	19	9
pH 9.0	Control	9.04	9.02	9.07	9.05	9.02	9.04	0.2	0.18	0.19	<0.1	0.13	<0.1	0.14	30
	0.25	9.02	9.03	9.10	9.05	9.05	9.05	0.3	0.35	0.29	0.24	0.30	0.23	0.28	18
	0.5	9.04	9.04	9.09	9.05	9.05	9.05	0.2	0.78	0.42	0.54	0.51	0.39	0.53	29
	1	9.04	9.03	9.09	9.06	9.04	9.05	0.3	1.1	1.0	1.1	0.95	0.78	1.0	15
	2	8.95	8.98	9.04	9.00	9.00	8.99	0.4	2.2	1.8	1.9	2.0	1.6	1.9	12
	4	8.91	8.97	9.07	9.00	8.98	8.99	0.6	4.5	5.1	4.6	4.2	3.7	4.4	12
Experiment 2															
pH 6.5	Control	6.68	6.72	6.56	6.64	6.57	6.63	1.0	<0.1	0.14	<0.1	0.10	0.13	0.11	19
	8	6.68	6.65	6.55	6.62	6.56	6.61	0.9	7.1	6.6	6.3	8.8	6.6	7.1	14
	16	6.69	6.63	6.53	6.58	6.55	6.60	1.0	16	14	15	14	15	15	4
	32	6.67	6.61	6.62	6.55	6.55	6.60	0.8	32	29	28	29	29	30	5
	64	6.69	6.62	6.60	6.52	6.58	6.60	0.9	64	60	58	60	58	60	4
	128	6.71	6.52	6.53	6.45	6.60	6.56	1.5	132	127	130	128	133	130	2
pH 8.0	Control	8.08	8.12	8.00	8.09	8.10	8.08	0.6	0.13	0.11	0.11	0.11	0.11	0.11	8
	1	8.10	8.13	8.00	8.10	8.03	8.07	0.7	0.98	1.0	0.79	1.2	1.0	1.0	16
	2	8.09	8.14	8.02	8.11	8.05	8.08	0.6	2.1	2.1	1.9	2.2	1.9	2.0	6
	4	8.09	8.14	8.02	8.11	8.08	8.09	0.5	4.4	4.1	3.8	4.1	4.0	4.1	6
	8	8.09	8.13	8.03	8.09	8.06	8.08	0.5	9.7	9.2	8.7	8.8	9.0	9.0	4
	16	8.09	8.14	8.01	8.06	8.04	8.07	0.6	20	19	18	18	19	19	4

[a] The value <0.1 is treated as 0.1 for mean and CV calculations.

Table S-3. The 96-h ammonia median effective concentrations (EC50s) for juvenile freshwater mussels. Only those tests that met the ASTM criteria for toxicity testing with mussels (ASTM 2007)[a] are listed. The acute values reported in original references have been converted to mg/L total ammonia as N

Species	pH	EC50 (mg N/L)	Reference
Actinonaias pectorosa	8.0	14	[1]
Epioblasma capsaeformis	8.5	5.7	[2]
Lampsilis abrupta	8.3	2.3	[2]
Lampsilis abrupta	8.3	2.8	[3]
Lampsilis cardium	7.6	23	[4]
Lampsilis cardium	7.1	39	[4]
Lampsilis fasciola	8.5	7.4	[2]
Lampsilis fasciola	7.8	15	[5]
Lampsilis higginsii	7.6	20	[4]
Lampsilis higginsii	7.1	32	[4]
Lampsilis rafinesqueana	8.4	11	[2]
Lampsilis rafinesqueana	8.3	11	[2]
Lampsilis siliquoidea	8.5	10	[2]
Lampsilis siliquoidea	8.1	4.9	[2]
Lampsilis siliquoidea	8.3	4.6	[3]
Lampsilis siliquoidea	6.6	88	This study
Lampsilis siliquoidea	7.6	11	This study
Lampsilis siliquoidea	8.1	5.2	This study
Lampsilis siliquoidea	8.1	11	This study
Lampsilis siliquoidea	8.5	3.4	This study
Lampsilis siliquoidea	9.0	0.96	This study
Lasmigona subviridis	7.7	6.6	[6]
Lasmigona subviridis	7.7	6.6	[6]
Lasmigona subviridis	7.9	4.0	[6]
Utterbackia imbecillis	8.0	2.7	[6]
Utterbackia imbecillis	8.3	8.8	[6]
Utterbackia imbecillis	8.2	5.8	[6]
Utterbackia imbecillis	8.2	5.3	[6]
Utterbackia imbecillis	8.0	20	[1]
Villosa iris	8.1	6.3	[2]
Villosa iris	8.4	3.0	[2]
Villosa iris	8.3	11	[2]
Villosa iris	7.3	21	[5]
Villosa iris	8.2	6.4	[7]
Villosa iris	8.2	5.8	[7]

[a] American Society for Testing and Materials. 2007. Standard guide for conducting laboratory toxicity tests with freshwater mussels. E2455-06. In *Annual Book of ASTM Standards*, Vol 11.06. West Conshohocken, PA, pp 1378-1429.

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