A Comparison of the Chemical Sensitivities between In Vitro and In Vivo Propagated Juvenile Freshwater Mussels: Implications for Standard Toxicity Testing

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Abstract: Unionid mussels are ecologically important and are globally imperiled. Toxicants contribute to mussel declines, and toxicity tests using juvenile mussels—a sensitive life stage—are valuable in determining thresholds used to set water quality criteria. In vitro culture methods provide an efficient way to propagate juveniles for toxicity testing, but their relative chemical sensitivity compared with in vivo propagated juveniles is unknown. Current testing guidelines caution against using in vitro cultured juveniles until this sensitivity is described. Our objective was to evaluate the relative sensitivity of juvenile mussels produced from both in vitro and in vivo propagation methods to selected chemicals. We conducted 96-h acute toxicity tests according to ASTM International guidelines with 3 mussel species and 6 toxicants: chloride, nickel, ammonia, and 3 copper-based compounds. Statistically significant differences between in vitro and in vivo juvenile 96-h median effect concentrations were observed in 8 of 17 tests, and in vitro juveniles were more sensitive in 6 of the 8 significant differences. At 96 h, 4 of the 8 statistically different tests for a given chemical were within a factor of 2, which is the intralaboratory variation demonstrated in a recent evaluation of mussel toxicity tests. We found that although differences in chemical sensitivity exist between in vitro and in vivo propagated juvenile mussels, they are within normal toxicity test variation. Therefore, in vitro propagated juvenile mussels may be appropriate for use in ASTM International-based toxicity testing.

INTRODUCTION

Freshwater mussels (family Unionidae) are an important ecological component in aquatic systems, and are a critically imperiled taxonomic group; approximately 70% of species in North America have experienced declines and high rates of imperilment (Williams et al. 1993; Haag and Williams 2014). A number of factors have contributed to these declines, including habitat alteration, stressors from introduced species, and chemical pollution of waterways (Bogan 1993; Strayer et al. 2004; Augspurger et al. 2007). For some pollutants, unionid mussels are known to be among the most sensitive faunal groups to chemical contaminants in aquatic systems and are exposed to toxicants through multiple routes (surface water, sediment, porewater, and diet; Augspurger et al. 2003; March et al. 2007; Cope et al. 2008). For some toxicants, the newly transformed juvenile is the life stage most sensitive to chemical contaminants, although this may vary by species and toxicant (Dimock and Wright 1993; Wang et al. 2007a; Raimondo et al. 2016). The most effective way to determine protective chemical thresholds is to test the chemical sensitivity at the most sensitive life stages. These tolerance thresholds are most often determined through toxicity testing with standard methods (e.g., ASTM International 2013) in a controlled laboratory setting. Thus, a consistent and high-quality supply of young juvenile mussels is needed for testing.

The concept of using mussels as bioindicators originated in the early 20th century (Ortmann 1909). In recent decades, toxicity testing with freshwater mussels has become more common (e.g., Bringolf et al. 2007; Wang et al. 2007a), due in
large part to the establishment of accepted testing guidelines for mussel early life stages (ASTM International 2006). This testing has indicated that mussels are among the most sensitive forms of aquatic life to toxicity from metals and major ions including ammonia, chlorine, copper, nickel, lead, potassium, sulfate, and zinc—all common water pollutants (Imlay 1973; Augspurger et al. 2003; Soucek 2006; Wang et al. 2007a, 2007b, 2007c, 2008, 2009, 2010, 2011a, 2011b, 2016, 2017; March et al. 2007; Besser et al. 2011, 2013; Gillis 2011; Ivey et al. 2013; US Environmental Protection Agency 2013). The US Environmental Protection Agency (USEPA) currently considers unionid sensitivity data from tests meeting the ASTM International data quality objectives when setting national water quality criteria (US Environmental Protection Agency 2013).

Because unionid mussels have an obligate parasitic life stage on fish (Lefevre and Curtis 1912; Kat 1983; Fritts et al. 2013), rearing mussels in a laboratory requires the maintenance of appropriate host fish for the duration of the transformation from larvae (glochidia) to juvenile. Rearing mussels using a host fish (in vivo propagation) provides a means to study mussel–host transformation efficiency and other interactions (Fritts et al. 2013; Douda 2015), and this method typically produces abundant, high-quality juveniles (Lima et al. 2012)—an important prerequisite for use in toxicity testing. However, in vivo culture presents several challenges. It is labor intensive, requiring the housing and care of host fish if the host species are known, and visual monitoring to collect transformed juveniles (Lima et al. 2012). Transformation success is often poor and highly variable, surviving juveniles come from a nonsterile environment, and they may be contaminated with xenobiotics from the host fish (Lima et al. 2012).

An alternative method of mussel propagation, in vitro culture, has been refined in recent decades and mitigates some of the problems associated with traditional host fish mussel propagation. Glochidia are placed into Petri dishes or flasks containing culture media supplemented with animal serum (often rabbit or horse). The glochidia complete the transformation process in the culture media, from which they derive protein and other nutrients, rather than from the host fish tissues (Isom and Hudson 1982; Dimock and Wright 1993; Lima et al. 2012). Culture techniques have greatly improved in recent years (Owen et al. 2010), and >40 species have been successfully transformed in culture media (Lima et al. 2012). The in vitro method removes uncertainty associated with the host fish, results in greater larval-to-juvenile transformation success, and is more cost effective than traditional, host–fish propagation. However, in vitro propagation requires specialized cell culture equipment, and there is risk of fungal or bacterial contamination of the culture media that can limit culture success (Owen et al. 2010; Lima et al. 2012). In addition, the relative health of in vitro juveniles is not well described compared with their in vivo counterparts, particularly with regard to chemical sensitivity (Fisher and Dimock 2006).

Few data are available on the relative health and chemical sensitivity of in vitro compared with in vivo juveniles (ASTM International 2006, 2013). In vitro juveniles tend to develop anatomically and physiologically at slower rates than in vivo juveniles of the same cohort and exhibit greater mortality post transformation (Fisher and Dimock 2006; Fox 2014). However, Summers (1999) did not observe statistically significant differences in sensitivity to copper between in vitro and in vivo propagated juveniles, although the in vitro juveniles generally died at lower concentrations of copper than their in vivo counterparts. Likewise, a review of copper toxicity studies on mussels by March et al. (2007) did not observe a consistent relationship in chemical sensitivity between in vitro and in vivo juveniles. The first ASTM International guidelines for conducting toxicity tests with juvenile mussels recommend not using in vitro propagated juveniles until their relative chemical sensitivities have been described (ASTM International 2006), and no comprehensive data have been generated to give guidance on more recent revisions (ASTM International 2013). Thus the purpose of the present study was to compare the relative chemical sensitivities of in vitro and in vivo cultured juvenile mussels during the first several weeks post transformation using compounds representing different chemical classes and modes of action, and to inform any future revisions to the ASTM International mussel testing guidelines.

MATERIALS AND METHODS

Test organisms

Three species of mussels were selected for testing based on availability, native range, and conservation status. Lampsis cardium, the plain pocketbook, is a relatively common species from the subfamily Ambleminae, tribe Lampslini, native to the US Interior and Great Lakes drainage basins (Parmalee and Bogan 1998). Lampsis abruptly, the pink mucket, co-occurs with L. cardium in the Interior basin, but is listed as federally endangered. Utterbackia imbecillis, the paper pondshell, is in the subfamily Unioniniae, tribe Anodontini. It is relatively common throughout the Interior and Atlantic Slope drainage basins and is used in toxicity testing (Parmalee and Bogan 1998; Summers 1999). For each species, gravid female mussels were collected from wild populations and held in hatchery conditions to serve as broodstock. Lampsis abruptly were collected in the lower Tennessee River (Tennessee drainage; TN, USA), L. cardium were collected from the lower Licking River (Ohio River drainage; OH, USA), and U. imbecillis were collected from small streams in the Cedar Creek watershed (Kentucky River drainage; KY, USA).

Propagation

Juvenile mussels were propagated at the Kentucky Center for Mollusk Conservation (Frankfort, KY, USA) using traditional host–fish (in vivo) and media culture (in vitro) propagation methods (Owen et al. 2010). Glochidia from 3 gravid females of a given species were extracted and mixed. Half were then used to inoculate the appropriate host fish for the given species, and half were placed in a culture media mixture of rabbit serum, antibiotics, amino acids, and lipids (Owen et al. 2010). After
transformation, mussels were held at the Kentucky Center for Mollusk Conservation under their standard rearing protocols until required for testing (Owen et al. 2010). Mussels were then shipped overnight in successive weekly batches to North Carolina State University (Raleigh, NC, USA) for use in toxicity testing. For transport, approximately 250 mussels were placed into a 50-mL centrifuge tube, which was then placed into an insulated Thermos® containing water from the culture facility for added temperature stability. The Thermos was then placed in a Styrofoam® cooler with hot or cold packs, depending on the ambient temperature.

**Juvenile assessment and acclimation**

On arrival at the laboratory, juvenile mussels were assessed for viability according to the ASTM International guideline (2013). After initial water temperature was measured, mussels were acclimated to the test temperature of 20°C at a rate of 2.5°C/d. In addition, mussels were acclimated to the test water (ASTM International hard water; ASTM International 2007) at a rate of 25% volume exchange/h. After 100% test water concentration and target temperature were reached, mussels were held for a minimum 24-h acclimation period.

Photographs were taken for a subset of mussels at the time of arrival, and the individual mussels were measured to the nearest micrometer using a Leica EZ4 D stereo microscope with integral digital camera and Leica Application Suite EZ digital photographic software. Individuals used in toxicity tests ranged in age from 0 to 8 wk post transformation at test digital photographic software. Individuals used in toxicity tests with integral digital camera and Leica Application Suite EZ containing a Leica EZ4 D stereo microscope for added temperature stability. The Thermos was then placed in a Styrofoam® cooler with hot or cold packs, depending on the ambient temperature.

**Test design**

Mussels were again assessed for viability (using foot movement) immediately prior to the start of the test, and if acceptable survival (>80%) was observed, mussels were allocated to test chambers. Tests were 96-h nonaerated static assays with 100% water renewal at 48 h, conducted according to ASTM International (2013) guidelines for juvenile mussels. Viability was assessed at 48 and 96 h.

For each chemical–species combination, a 96-h acute toxicity test was conducted for both in vitro and in vivo cultured juveniles. Each test had a control (no toxicant) and 6 treatment concentrations (Table 1), and 200 mL of the test solution was placed into a 250-mL evaporating dish. Each concentration was conducted in triplicate, with 10 mussels/replicate. Mussels were assessed for survival at 48 and 96 h, and the median effective concentration (EC50) causing immobility (absence of a heart beat or foot movement inside or outside the shell within a 5-min observation period/mussel) or mortality was calculated for each species–toxicant combination.

**Chemical-specific relative sensitivity**

Six chemicals with 3 modes of toxic action were selected based on known toxicity to mollusks and environmental relevance. Chloride (as sodium chloride: Sigma-Aldrich; 99% purity) is a well-described mussel toxicant (mode of action: respiratory stress; Wang et al. 2007b). Copper (as copper sulfate: Sigma-Aldrich; 99% purity) is another well-known mussel toxicant (mode of action: metallic stress; Wang et al. 2007b). Clearigate® (Applied Biochemists; 3.82% elemental copper) and Nautique® (SePRO; 9.1% metallic copper), both chelated copper aquatic herbicides, were also chosen. Nickel (as nickel chloride: Sigma-Aldrich; 99% purity; mode of action: metallic stress) and ammonia (as ammonium chloride: Fisher Scientific; 99% purity; mode of action: narcosis) are also well-described mussel toxicants selected for use in the comparison tests (Wang et al. 2007b, 2017). Target toxicity test concentrations were based on published 96-h EC50 or median lethal concentration (LC50) values (Wang et al. 2007b) or on range-finding tests.

**Age-related chemical sensitivity**

To determine the influence of age (as a proxy for development) on the relative chemical sensitivity of in vitro and in vivo juvenile mussels, juveniles from each propagation method were exposed to the copper sulfate. Test concentrations were 0, 12.5, 25, 50, 100, 200, and 300 µg Cu/L. Mussels were assessed for survival at 48 and 96 h, and the EC50 resulting in immobility or mortality was calculated. To assess the magnitude of any potential difference in survival between in vitro and in vivo mussels for a particular age, the ratio of the 2 96-h EC50s in each age pairing was calculated.

**Quality assurance**

All tests were conducted according to the ASTM International standard guide for conducting laboratory toxicity tests with freshwater mussels (ASTM International 2013). All but 3 of the 17 chemical-specific tests met the recommended >90% control survival at test termination (ASTM International 2013); 3 tests that had between 80 and 90% control viability (L. cardium in vivo chloride, L. cardium in vitro nickel, and U. imbecillis in vivo ammonia) at 96 h were retained in the analysis to facilitate matched comparisons. Water quality conditions of temperature, conductivity, dissolved oxygen, and pH were measured with a calibrated meter (YSI 566 MPS multiprobe) from a composite water sample at each treatment concentration (n = 310). Alkalinity and hardness were measured using standard titrimetric procedures (n = 48; American Public Health Association International 2005). Average water quality (and ranges) were: temperature 20.7°C (18.7–22.8); conductivity 560 µS/cm (443–905); dissolved oxygen 8.5 mg/L (7.8–9.1); pH 8.4 (8.0–9.4); alkalinity 111 mg/L CaCO3 (92–132); hardness 160 mg/L CaCO3 (128–180).

Toxicant exposure concentrations were verified with standard analytical chemistry instrumentation and USEPA-approved methods at RTI International (Research Triangle Park, NC, 2018).
USA) for copper and nickel; North Carolina State University Center for Applied Aquatic Ecology (a state-certified analytical laboratory in Raleigh, NC, USA) for ammonia; and SePRO (Whitakers, NC, USA) for Nautique and Clearigate according to their institutional standard operating procedures. Ammonia samples were verified at 0, 24, and 48 h of the tests to ensure that concentrations remained stable over time. In tests containing a copper-based toxicant, all glassware was pretreated with the appropriate test concentration 24 h prior to test initiation to minimize adsorptive loss of copper ions to the glass. Chloride concentrations were verified in the laboratory using a YSI conductivity probe. Chloride concentration was expressed as the volume of chloride ions (g/L) in addition to the salts required to make the ASTM International hard water, which reflects a background conductivity of approximately 550 μS/cm. A quality assurance protocol was followed for each of the analytes at the respective laboratories that included reagent blanks, spiked samples, duplicate samples, and internal standards. Briefly, the average percentage of recovery (and range) for each compound was as follows (all were within acceptable limits): ammonia 84.7% (77.2–100.9, n = 7); chloride 121.1% (105.6–168, n = 7); nickel 99.3% (97–102), Clearigate 72.6% (67.1–75.8, n = 3); copper 99.75% (96–104, n = 6); and Nautique 95.7% (90.0–98.8, n = 6).

Data are presented based on target concentrations rather than measured concentrations because of the high agreement in analytical recoveries.

### Statistical analysis

The EC50 was estimated with the trimmed Spearman–Karber method in the Comprehensive Environmental Toxicity Information Software package (CETIS, Ver 1.8.0.12; Tidepool Scientific). Confidence intervals (95%) were used to determine significant differences between EC50s. The ECs with overlapping confidence intervals were considered similar, whereas ECs with nonoverlapping confidence intervals were considered statistically different (Hamilton et al. 1977; American Public Health Association International 2005; Bringolf et al. 2007). Tests with copper sulfate from all species were pooled for an additional statistical analysis, and then split into 2 groups based on the age of the juveniles used: “younger” (<10 d old) and “older” (>10 d old). Normality of data was confirmed using a Shapiro–Wilk test, and a 2-way analysis of variance (ANOVA) was performed on the combined EC50s from all species to examine the effect of age (younger vs older) and propagation method (in vitro vs in vivo) and the potential interaction between the 2 independent variables.

The toxicity value ratio was calculated for both the chemical-specific tests and the age-related tests, using the method described in Raimondo et al. (2016). For chemical-specific tests, the ratio of the in vitro to in vivo 96-h EC50s for each species–toxicant combination was calculated by dividing the lower EC50 into the other. Ratios for each species were then averaged within a chemical to calculate the toxicity value ratio across species within a chemical. For age-related tests, the toxicity value ratio was calculated by dividing the smaller EC50 value into the larger EC50 value in an in vitro/in vivo age pair. All juvenile mussel ages are reported as post-transformation age at the start of the test, and age is expressed as “X days in vivo–Y days in vitro.” In addition, the toxicity value ratio was calculated within all in vivo tests and all in vitro tests for a species.

The coefficient of variation (CV) among EC50 values was calculated to quantify variation among tests for each chemical. We then employed Bartlett’s test for homogeneity of variances (Zar 1999; JMP Pro 14, SAS Institute) to compare the variation in EC50 values among the pooled, chemical-specific in vitro and in vivo tests from our experiments and the in vivo mussel toxicity data summarized by Raimondo et al. (2016; their Supplemental Data, summary provided on 10 March 2016 by S. Raimondo) for the chemicals used in our study (ammonia, chloride, copper, nickel).

### RESULTS

#### Chemical-specific relative sensitivity

Tests were conducted on 3 mussel species with each propagation method (in vitro and in vivo) with 6 chemicals, with the exception of *U. imbecillis* and Clearigate due to limited availability of mussels. In total, 17 pairs of tests were conducted. In vitro and in vivo tests (3 replicates of 10 mussels) for *L. abrupta* were not conducted concurrently due to constraints with mussel production; the in vitro and in vivo juveniles for this species were, on average, 1 wk different in age at the time of testing. Source broodstock was consistent across testing.

Nonoverlapping 95% confidence intervals (a statistically significant difference) for the EC50s between in vitro and in vivo.
TABLE 2: Median effect concentration (EC50) in in vitro and in vivo propagated juvenile mussels at 48 and 96 h, and toxicity value ratio (TVR) between in vitro and in vivo juveniles at 96 h

<table>
<thead>
<tr>
<th>Species</th>
<th>Toxin</th>
<th>Unit</th>
<th>In vivo 48 h EC50a</th>
<th>In vitro 48 h EC50</th>
<th>In vivo 96 h EC50b</th>
<th>In vitro 96 h EC50</th>
<th>TVR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lampsilis abrupta</td>
<td>Ammonia</td>
<td>mg NH4/L</td>
<td>17.5 (15.4–20.0)</td>
<td>16.2 (13.9–18.8)</td>
<td>6.2 (4.4–7.1)</td>
<td>7.7 (6.8–8.8)</td>
<td>1.10</td>
</tr>
<tr>
<td>Lampsilis cardium</td>
<td>Ammonia</td>
<td>mg NH4/L</td>
<td>26.9 (22.8–31.7)</td>
<td>&gt;32 (N/A)</td>
<td>10.1 (9.0–11.3)</td>
<td>7.7 (6.8–8.8)</td>
<td>2.32</td>
</tr>
<tr>
<td>Utterbackia imbecillis</td>
<td>Chloride</td>
<td>g NaCl/L</td>
<td>4.5 (4.1–4.9)</td>
<td>5.1 (4.2–5.0)</td>
<td>4.5 (4.2–4.9)</td>
<td>4.5 (4.2–4.9)</td>
<td>2.32</td>
</tr>
<tr>
<td>L. abrupta</td>
<td>Clearigate</td>
<td>μg Cu/L</td>
<td>296.3 (249.4–351.6)</td>
<td>722.9 (601.0–868.6)</td>
<td>176.0 (149.4–207.4)</td>
<td>480.1 (400.7–575.3)</td>
<td>2.73</td>
</tr>
<tr>
<td>U. imbecillis</td>
<td>Clearigate</td>
<td>μg Cu/L</td>
<td>&gt;300 (N/A)</td>
<td>128.1 (106.8–153.9)</td>
<td>130.0 (110.8–152.6)</td>
<td>168.0 (147.7–175.0)</td>
<td>2.42</td>
</tr>
<tr>
<td>L. cardium</td>
<td>Chloride</td>
<td>g NaCl/L</td>
<td>669.4 (451.2–993.3)</td>
<td>1293 (464.1–1030.2)</td>
<td>1169 (96.2–142.1)</td>
<td>1169 (96.2–142.1)</td>
<td>1.21</td>
</tr>
<tr>
<td>L. abrupta</td>
<td>Clearigate</td>
<td>μg Cu/L</td>
<td>&gt;8000 (N/A)</td>
<td>&gt;8000 (N/A)</td>
<td>&gt;8000 (N/A)</td>
<td>&gt;8000 (N/A)</td>
<td>1.01</td>
</tr>
<tr>
<td>U. imbecillis</td>
<td>Clearigate</td>
<td>μg Cu/L</td>
<td>&gt;1000 (N/A)</td>
<td>&gt;1000 (N/A)</td>
<td>&gt;1000 (N/A)</td>
<td>&gt;1000 (N/A)</td>
<td>1.01</td>
</tr>
<tr>
<td>L. abrupta</td>
<td>Nickel</td>
<td>μg Ni/L</td>
<td>1375.7 (1253.0–1510.0)</td>
<td>1383.7 (1226.7–1560.7)</td>
<td>1228.2 (1182.3–1275.8)</td>
<td>1228.2 (1182.3–1275.8)</td>
<td>1.01</td>
</tr>
<tr>
<td>U. imbecillis</td>
<td>Nickel</td>
<td>μg Ni/L</td>
<td>&gt;1500 (N/A)</td>
<td>&gt;1500 (N/A)</td>
<td>&gt;1500 (N/A)</td>
<td>&gt;1500 (N/A)</td>
<td>1.01</td>
</tr>
</tbody>
</table>

*EC50 causing 50% immobility or mortality (with 95% confidence intervals) in in vitro and in vivo propagated juvenile mussels at 48 h.

bEC50 causing 50% immobility or mortality (with 95% confidence intervals) in in vitro and in vivo propagated juvenile mussels at 96 h.

*No test run for U. imbecillis with Clearigate.

TVR = toxicity value ratio (in vivo:in vitro)

DISCUSSION

Chemical-specific relative sensitivity

In vitro propagated juveniles were statistically more sensitive to chemical toxicants than in vivo propagated juveniles in 8 of the 17 tests at 48 h, but in vivo juveniles were statistically more sensitive than their in vitro counterparts in 12 tests at 96 h. This may indicate that in vitro and in vivo juvenile mussels have different sensitivities to chemical toxicants, with in vitro juveniles generally being more sensitive than their in vivo counterparts.

Age-related chemical sensitivity

Eighth age-paired and 5 unpaired (4 in vitro and one in vivo) mussel groups were exposed to treatments at both 48 and 96 h, although these were not always the same in vitro group and in vivo group in paired tests. Eight paired tests were conducted with copper sulfate (Table 4) and control survival was 80% in all but one test (Raimondo et al. 2016; Table 5). For all chemicals except for the L. cardium 9/10-d test, five of the same age groups were tested for each mussel species (2016; Table 5). Bartlett’s test was not possible for the L. abrupta 300 ppm Cu/L test because of insufficient mortality.

Future work will involve examining the variation in in vivo juvenile mussel toxicity tests and the variation in EC50 values from Raimondo et al. (2016; Table 5). Bartlett’s test was not possible for the L. abrupta 300 ppm Cu/L test because of insufficient mortality. Among all chemicals, the average interspecies ratio of within- and among-laboratory variation for multiple chemicals is greater than that previously reported with juvenile unionids, for which age, mussel species, toxicant or formulation, and test conditions all contribute to variation (Table 3; Wang et al. 2007b, 2017; Raimondo et al. 2016). The variation in EC50 values for our pooled in vitro and in vivo group of tests was lower or no greater than the variation in the corresponding EC50 values among laboratories in the data from Raimondo et al. (2016), with the exception of nickel in L. abrupta. The interaction effect between age grouping of juveniles (older or younger than 10 d) and propagation method was not significant in the combined EC50 values from all species (p = 0.039), but age grouping was significant in the comparison between in vivo and in vitro juvenile mussels on nickel (p = 0.003) and copper sulfate (p = 0.020). For all chemicals except for the L. cardium 9/10-d test, five of the same age groups were tested for each mussel species (2016; Table 5). Bartlett’s test was not possible for the L. abrupta 300 ppm Cu/L test because of insufficient mortality. Among all chemicals, the average interspecies ratio of within- and among-laboratory variation for multiple chemicals is greater than that previously reported with juvenile unionids, for which age, mussel species, toxicant or formulation, and test conditions all contribute to variation (Table 3; Wang et al. 2007b, 2017; Raimondo et al. 2016).
toxicity value ratio across all species was 1.9 within laboratories and 3.6 among laboratories. Wang et al. (2007b) performed a collaborative series of chemical tests among 5 independent laboratories, each using mussels from the same propagation batch and the same test water for each test. For tests with copper, they found a within-laboratory toxicity value ratio of 1.2, and an among-laboratory toxicity value ratio of 1.5. In a recent study comparing the toxicity of newly transformed juveniles of 5 species to a number of chemicals, Wang et al. (2017) reported a within-laboratory variation factor of \( \frac{1}{20} \) for chloride and \( \frac{1}{20} \) for ammonia, copper, and nickel. The toxicity results for mussels from the 2 propagation types and test chemicals in the present study were within the 3.6 toxicity value ratio found in Raimondo et al. (2016), and all of the chemical-specific 96-h EC50 ratios are less than a factor of 2, similar to the ratios of both Raimondo et al. (2016) and Wang et al. (2007b; Table 3).

Coefficients of variation of EC50 values were lower in the present study than CVs of the data summarized by Raimondo et al. (2016) for 3 of 4 chemicals (Table 5). When interpreted with the Bartlett’s test result, the variance in our in vitro and in vivo comparison tests was significantly lower for ammonia and nickel and no greater than the variance from the in vivo summarized data for chloride and copper (Table 5). These findings indicate that variation within the pooled in vitro and in vivo juvenile mussel toxicity tests is lower or no greater than the variation in in vitro juvenile mussel toxicity tests.

Although the present study used mussel species from different subfamilies, tribes, and conservation status in testing, only 3 species were assessed, and future research would benefit from testing mussels of the 2 propagation methods with additional genera. However, approximately 70% of mussel species that have been used in toxicity testing vary in sensitivity from 2 commonly used surrogate species (\textit{Lampsilis siliquoidea} and \textit{U. imbecillis}) by a factor of \( \frac{1}{20} \) (Raimondo et al. 2016). In addition, as both types of propagation and culture methods continue to improve, any potential developmental gaps between in vitro and in vivo juvenile mussels should continue to be reduced.

### Table 3: Toxicity value ratios between median effect concentrations (EC50s) of in vitro and in vivo propagated juvenile mussels among 3 species

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>In vivo vs in vitro</th>
<th>Within-laboratory variation</th>
<th>Between-laboratory variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>1.6</td>
<td>ND</td>
<td>2.0</td>
</tr>
<tr>
<td>Chloride</td>
<td>1.0</td>
<td>ND</td>
<td>1.5</td>
</tr>
<tr>
<td>Clearigate</td>
<td>2.4</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Copper</td>
<td>1.9</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Nautique</td>
<td>1.1</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Nickel</td>
<td>1.0</td>
<td>ND</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Ratios from the present study are compared with interspecies ratios calculated by Wang et al. (2007b) and Raimondo et al. (2016) for within- and between-laboratory acute EC50 variation of tests with in vivo propagated juvenile mussels.

Average toxicity value ratio between in vitro and in vivo juveniles across the 3 species used in the present study.

ND = no ratio available.

### Table 4: Median effect concentration (EC50) of copper causing 50% immobility or mortality (with 95% confidence intervals) in juvenile mussels at 96 h

<table>
<thead>
<tr>
<th>Species</th>
<th>Age (d)</th>
<th>In vivo</th>
<th>In vitro</th>
<th>TVR</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Lampsilis abrupta}</td>
<td>6</td>
<td>___</td>
<td>20.7</td>
<td>___</td>
</tr>
<tr>
<td>\textit{L. abrupta}</td>
<td>12</td>
<td>115.7</td>
<td>26.6</td>
<td>___</td>
</tr>
<tr>
<td>\textit{L. abrupta}</td>
<td>18</td>
<td>100.9</td>
<td>21.2</td>
<td>___</td>
</tr>
<tr>
<td>\textit{L. abrupta}</td>
<td>22, 20</td>
<td>130.0</td>
<td>53.7</td>
<td>___</td>
</tr>
<tr>
<td>\textit{L. cardium}</td>
<td>3, 4</td>
<td>60.1</td>
<td>44.0</td>
<td>___</td>
</tr>
<tr>
<td>\textit{L. cardium}</td>
<td>7, 5</td>
<td>70.0</td>
<td>67.0</td>
<td>___</td>
</tr>
<tr>
<td>\textit{L. cardium}</td>
<td>9, 10</td>
<td>160.8</td>
<td>52.4</td>
<td>___</td>
</tr>
<tr>
<td>\textit{L. cardium}</td>
<td>16</td>
<td>___</td>
<td>84.9</td>
<td>___</td>
</tr>
<tr>
<td>\textit{L. cardium}</td>
<td>17</td>
<td>___</td>
<td>89.6</td>
<td>___</td>
</tr>
<tr>
<td>\textit{Utterbackia imbecillis}</td>
<td>3</td>
<td>33.7</td>
<td>41.0</td>
<td>___</td>
</tr>
<tr>
<td>\textit{U. imbecillis}</td>
<td>6</td>
<td>116.9</td>
<td>76.9</td>
<td>___</td>
</tr>
<tr>
<td>\textit{U. imbecillis}</td>
<td>17</td>
<td>62.1</td>
<td>52.6</td>
<td>___</td>
</tr>
<tr>
<td>\textit{U. imbecillis}</td>
<td>27</td>
<td>___</td>
<td>63.0</td>
<td>___</td>
</tr>
</tbody>
</table>

*Age is reported as “days in vivo, days in vitro,” unless in vivo and in vitro ages were the same.

**No test run.

EC50 at 96 h is significantly different between in vitro and in vivo juvenile (95% confidence intervals at this age do not overlap).

TVR = toxicity value ratio.
Overall, 96-h EC50s from both in vitro and in vivo mussels in the present study are similar to values published in the literature. The EC50s for ammonia in the present study ranged from 6.2 to 10.1 mg total ammonia nitrogen (TAN)/L for in vivo juveniles, and from 3.3 to 7.9 mg TAN/L for in vitro juveniles. Both are comparable to the results for freshwater mussels found in Wang et al. (2007a), which ranged from 2.3 to 11.1 mg TAN/L. Chloride values from the present study (in vivo: 1.7–2.9 g Cl/L; in vitro: 1.7–2.95) were also similar, but with a lower maximum value than those in the literature (e.g., 1.71–5.23 g Cl/L from Raimondo et al. 2016). Copper values determined in the present study (normalized to total water hardness of 100 mg/L CaCO3 for comparative purposes; in vivo: 116.9–178.1 μg Cu/L; in vitro: 53.7–84.9 μg Cu/L) were greater than those in the literature (e.g., 6.8–60.2 μg Cu/L at 100 mg/L mg/L CaCO3; Wang et al. 2007a). Copper sulfate was also the only toxicant that produced significant differences between in vitro and in vivo mussel EC50s among all species. However, Raimondo et al. (2016) found a between-laboratory toxicity value ratio of 4.8 for copper, so the apparent variation between propagation types in the present study is not beyond typical variation in toxicity tests. Nickel values in the literature were less (e.g., 96–377 μg Ni/L; Raimondo et al. 2016; Wang et al. 2017) than those generated in the present study (when normalized to a hardness of 50 mg/L as CaCO3; in vivo: 262–560 μg Ni/L; in vitro: 271–470 μg Ni/L).

**Overall relative chemical sensitivity**

In toxicity testing with freshwater mussels, <10 species are most commonly used (Raimondo et al. 2010, 2016) due to their ease of propagation, availability of gravid females, and propensity for long-term culture in the laboratory; the rarer species of conservation importance tend to be less well represented. In freshwater mussel toxicity testing, *L. siliquoidea* and *U. imbecillis* are commonly used as surrogates for other mussel species (Raimondo et al. 2016). Raimondo et al. (2016) found that the acute chemical sensitivity of approximately 70% species and age-pairs. There was no apparent systematic effect of age on the magnitude of variation between 96-h EC50s. This is similar to rates of variation that have previously been published for in vivo juvenile toxicity tests using copper (Wang et al. 2007b; Raimondo et al. 2016). Four age-pairs were within the factor of 1.2 within-laboratory variation found by Wang et al. (2007b), and 8 were within the within-laboratory variation of 2.1 reported by Raimondo et al. (2016). Six age-pairs were within the 1.5 between-laboratory variation found by Wang et al. (2007b), and all 8 age-pairs were within the 4.8 between-laboratory variation described by Raimondo et al. (2016).

Unionid chemical sensitivity can vary with age (Klaine et al. 1997; Wang et al. 2007a). If in vitro mussels are developmentally delayed relative to in vivo mussels (Dimock and Wright 1993; Fox 2014), their relative chemical sensitivities may depend on the time since their transformation, the developmental stage, or size during testing. Summers (1999) did not find statistically significant differences between in vitro and in vivo produced juveniles at 2 to 3 d post transformation, but their in vitro juveniles were generally more sensitive to copper.

The present study examined potential differences in 96-h EC50 for age-paired tests with juvenile mussels from the 2 propagation methods, but several study-design enhancements could improve the ability to draw conclusions from future experiments. First, our studies were conducted during the first 3 weeks of development, but no age-paired tests with individuals older than 20 d were done due to limited availability of juveniles. Moreover, no species used in the present study underwent tests at every time interval (1, 2, and 3 wk), which limited statistical and analytical chemistry comparisons. Future studies would benefit from testing with older individuals and additional age-pairs.
of tested mussel species varied from \textit{L. siliquoidea} and \textit{U. imbecillus} by no more than a factor of 2, which infers adequate representation. The toxicity value ratios between in vitro and in vivo juveniles in the present study ranged from 1 to 2.4, and all but the toxicity value ratio for Clearigate were below the variation associated with the aforementioned surrogate species (Table 3).

The present study demonstrates that variation in 96-h EC50s from toxicity tests conducted with in vitro propagated mussels is acceptable compared with the variation from the more routinely performed toxicity tests with in vivo propagated juveniles; however, opportunities remain to examine the health of in vivo juvenile mussels in future studies. For example, it would be valuable to measure sublethal biomarkers that indicate general physiological condition as well as exposure to and effect of toxicants, such as oxidative stress, reactive oxygen species, and lipid peroxidation, each of which would provide a measure of relative health or condition (Ringwood et al. 1998; Lushchak 2011). In addition to evaluating biomarker responses, studying the effect of the culture media recipe on the growth and condition of in vitro propagated mussels and its potential influence on 96-h EC50 variation in toxicity tests may be warranted. Such studies could examine potential health differences between juveniles raised on culture media compared with those raised on a host fish. In addition, it would be beneficial to perform chronic tests (e.g., 21–28 d in length) to examine differences in chemical sensitivity at lower toxicant concentrations over a longer time period.

In conclusion, we found that in vitro propagated juveniles were generally more sensitive to a chemical toxicant than their in vivo counterparts, and that the differences between 96-h EC50s were significant for approximately half of the tests ($n = 8$). However, even with statistically significant differences between in vitro and in vivo juvenile chemical sensitivities, variation in chemical sensitivity was no greater than the among-laboratory difference observed in previously reported studies conducted with in vivo propagated juveniles (Table 3). Therefore, in vitro propagated juvenile mussels may be considered functionally similar to in vivo propagated mussels, and the authors suggest that current ASTM International guidelines for conducting toxicity tests with juvenile mussels (ASTM International 2013) could be revised in the future to state that juvenile mussels cultured in vitro may be used to conduct toxicity tests. More research is needed to further define the health and condition relationship between in vitro and in vivo propagated mussels; however, the benefits of using in vitro cultured mussels may outweigh the drawbacks. Large numbers of mussels can be produced relatively inexpensively using in vitro propagation techniques. The use of in vitro propagated juvenile mussels in standard toxicity tests broadens opportunities for a multitude of private, state, and federal laboratories to generate data for mussel species that are of local conservation concern and require toxicity data. The present study indicates that it may be appropriate to use in vitro juveniles in freshwater mussel toxicity testing as functionally similar to in vivo propagated mussels with reasonable confidence.

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\textbf{Data Accessibility}—Data, associated metadata, and calculation tools are available from the corresponding author (apopp@ncsu.edu).

\textbf{REFERENCES}


