

Contaminant Sensitivity of Freshwater Mussels

ACUTE AND CHRONIC TOXICITY OF TECHNICAL-GRADE PESTICIDES TO GLOCHIDIA AND JUVENILES OF FRESHWATER MUSSELS (UNIONIDAE)

ROBERT B. BRINGOLF,*† W. GREGORY COPE,† CHRIS B. EADS,‡ PETER R. LAZARO,†
M. CHRISTOPHER BARNHART,§ and DAMIAN SHEA††Department of Environmental and Molecular Toxicology, North Carolina State University, Campus Box 7633,
Raleigh, North Carolina 27695-7633, USA‡Department of Population Health and Pathobiology, North Carolina State University, Campus Box 8401,
Raleigh, North Carolina 27695-8401, USA

§Department of Biology, Missouri State University, Springfield, Missouri 65897, USA

(Received 17 October 2006; Accepted 7 February 2007)

Abstract—Chemical contaminants are among many potential factors involved in the decline of freshwater mussel populations in North America, and the effects of pesticides on early life stages of unionid mussels are largely unknown. The objective of this study was to determine the toxicity of technical-grade current-use pesticides to glochidia and juvenile life stages of freshwater mussels. We performed acute toxicity tests with glochidia (five species) and juveniles (two species) exposed to a suite of current-use pesticides including herbicides (atrazine and pendimethalin), insecticides (fipronil and permethrin), and a reference toxicant (NaCl). Because of limited availability of test organisms, not all species were tested with all pesticides. Toxicity tests with fungicides (chlorothalonil, propiconazole, and pyraclostrobin) were performed with one species (*Lampsilis siliquoidea*). *Lampsilis siliquoidea* glochidia and juveniles were highly sensitive to the fungicides tested but the technical-grade herbicides and insecticides, at concentrations approaching water solubility, were not acutely toxic to this or the other unionid species. In a 21-d chronic test with four-month-old juvenile *L. siliquoidea*, the 21-d median effective concentration (EC50) with atrazine was 4.3 mg/L and in atrazine treatments ≥ 3.8 mg/L mussel growth was significantly less than controls. The relatively high sensitivity of *L. siliquoidea* to chlorothalonil, propiconazole, and pyraclostrobin is similar to that reported for other aquatic organisms commonly used for toxicity testing. The relative risk associated with acute exposure of early life stages of mussels to technical-grade atrazine, pendimethalin, fipronil, and permethrin is likely low; however, survival and growth results with juvenile *L. siliquoidea* indicate that chronic exposure to high concentrations (≥ 3.8 mg/L) of atrazine may have the potential to impact mussel populations and warrants further investigation.

Keywords—Herbicides Insecticides Fungicides Early life stage Sodium chloride

INTRODUCTION

Nearly 70% of the freshwater mussel (Bivalvia: Unionidae) species in the United States are considered as endangered, threatened, or of special concern [1]. The decline in the abundance and diversity of North American mussels, especially evident in recent decades, largely has been attributed to anthropogenic activities resulting in habitat destruction and degradation; however, the exact causes of the declines, unless site-specific and catastrophic, generally are unknown [1,2]. Contributing factors are presumed to be long-term, low-level, and pervasive stressors and likely include siltation, dams, mining wastes, introductions of exotic bivalve species such as zebra mussels (*Dreissena polymorpha*) and Asian clams (*Corbicula fluminea*), and industrial and agricultural point and nonpoint source water pollution, among others [2].

The life history of most unionids includes a critical period between fertilization of ova in the adult female mussel and attachment of viable larvae (glochidia) to a suitable fish host for transformation to a juvenile mussel [3]. Female mussels release glochidia (thousands to millions) into the water where they must attach to the gills or fins of fish and become encysted by fish epithelial tissue for several weeks before dropping off

as transformed juveniles. Glochidia remain viable in the water column from hours to days depending on species [4]. The effects of glochidial exposure to contaminants while encysted on fish remain unresolved, but encystment may provide some measure of protection from at least some waterborne contaminants [5]. Any chemical or physical stressor that limits survival of released glochidia in the water column, success of glochidial transformation to juveniles, or juvenile survival could adversely impact recruitment to the population.

Increasingly, field studies are providing evidence of skewed age class distribution in unionid populations. Recent reports specifically indicate poor recruitment, suggesting impaired reproduction or mortality of early life stages [6,7]. Early life stages of aquatic organisms generally are regarded as more sensitive to toxicants than adults; thus, exposure to contaminants could account for the absence of early life stages in many mussel populations. Additionally, early life stages of freshwater mussels are known to be especially sensitive to certain contaminants, such as ammonia [8–10], metals [5,11], and some organics including pulp and paper mill effluents and aromatic hydrocarbons [12,13] relative to other aquatic species commonly used in toxicity testing, i.e., *Daphnia magna*, *Hyallela azteca*, *Pimephales promelas*, and *Oncorhynchus mykiss*. However, there is a paucity of information regarding the sensitivity of freshwater mussels to even the most widely used

* To whom correspondence may be addressed
(rbringolf@warnell.uga.edu).

pesticides. This may be, at least in part, because current U.S. Environmental Protection Agency (U.S. EPA) regulations do not require bivalve toxicity testing for pesticide registration. A recently approved standardized guideline for conducting toxicity tests with freshwater mussels [14] now enables a consistent and reliable method to assess toxicity of all chemicals, including pesticides, to freshwater mussels.

The objectives of this study were to determine the hazards of technical-grade (the high purity active ingredient on which pesticide registration is based), current-use pesticides to early life stages of native freshwater mussels. We report the results of standard acute toxicity tests with two herbicides (atrazine and pendimethalin), two insecticides (fipronil and permethrin), three fungicides (chlorothalonil, propiconazole, and pyraclostrobin), a reference toxicant (NaCl), and glochidia of five species and juveniles of two mussel species. Additionally, sublethal and lethal effects of an herbicide (atrazine) were evaluated in a 21-d chronic toxicity test with one species of juvenile mussel.

MATERIALS AND METHODS

Test chemicals

Technical-grade pesticides atrazine (99.9% purity), pendimethalin (99% purity), fipronil (99.7% purity), permethrin (44% *cis*, 55% *trans*), chlorothalonil (98% purity), propiconazole (98.2% purity), and pyraclostrobin (99% purity) were purchased from Chem Service (West Chester, PA, USA). Atrazine is among the most extensively used herbicides in the United States (<http://www.pestmanagement.info/nass/>) and commonly is detected in surface and groundwater surveys [15,16]. Pendimethalin is an herbicide used for control of annual grasses and certain broadleaf weeds in a variety of row crops. Fipronil is an unrestricted insecticide and miticide with agricultural and domestic applications. Permethrin is an unrestricted synthetic pyrethroid used as a broad-spectrum insecticide. Chlorothalonil is a broad-spectrum organochlorine (chloronitrile) fungicide used to control fungi that threaten vegetables, trees, small fruits, turf, ornamentals, and other agricultural crops. Propiconazole is a systemic foliar fungicide with a broad range of agricultural uses. Pyraclostrobin, a strobilurin fungicide, has various agricultural applications. Stock solutions of each chemical were prepared in acetone and stored at -20°C . Certified NaCl (American Chemical Society-grade, Fisher Chemical, Hampton, NH, USA) was used as the reference toxicant. Reconstituted hard water [14] was used as dilution water for all toxicity tests.

Test organisms

Brooding adult female mussels were collected from rivers and streams in generally forested, rural areas of North Carolina and Missouri, USA, from May through July 2004. Mussels were abundant at all sites from which they were collected. *Elliptio complanata* were obtained from Richland Creek, Wake County; *Lampsilis fasciola* were collected from the Little Tennessee River, Swain and Macon Counties; *Villosa constricta* were taken from Deep Creek, Person County; and *V. delumbis* were obtained from the Little River, Randolph County (all counties located in North Carolina). *Lampsilis siliquoidea* were collected from Silver Fork of Perche Creek, Boone County, Missouri, USA. None of these species are federally listed as endangered, but several share their genus with, and thus are surrogates for, listed species. *Lampsilis fasciola*, *V.*

constricta, and *V. delumbis* are listed in North Carolina as species of special concern. Roughly equal numbers of mature glochidia were obtained from the marsupial gills of at least three females by flushing the glochidia out of the gills with a syringe filled with water. Glochidia from Missouri were shipped in chilled coolers via overnight courier to North Carolina State University (Raleigh, NC, USA) for toxicity testing. Upon arrival at North Carolina State University, viability of all glochidia was assessed by exposing three subsamples of 50 to 100 glochidia (each) to a saturated NaCl solution, which initiates shell closure in viable glochidia. We used glochidia for toxicity tests only if initial viability exceeded 90%, in accordance with the standardized guidelines for toxicity tests with freshwater mussels [14]. Glochidia were acclimated to dilution water with a 50:50 mixture of culture water and dilution water for at least 2 h before toxicity tests began. Because the time that glochidia remain viable in the water column had not been reported previously for all of these species, viability was evaluated (as previously described) at 24 h and again 48 h after toxicity test initiation for each test.

Juvenile *L. fasciola* and *V. delumbis* were produced at the mussel propagation laboratory on the campus of North Carolina State University. Juvenile *L. fasciola* were transformed on juvenile largemouth bass (*Micropterus salmoides*) obtained from a pond owned by North Carolina State University. Juvenile *V. delumbis* were transformed on redbreast sunfish (*Lepomis auritus*) and green sunfish (*L. cyanellus*). The fish were anesthetized with tricaine methanesulfonate (Finquel®; Argent Laboratories, Redmond, WA, USA) and glochidia were pipetted on to the gills of the fish. Host fish were held in a recirculating system in 190- or 380-L tanks and fed daily. Once excystment of juveniles began, we siphoned tanks daily through a 150- μm sieve to collect the juvenile mussels, which were maintained in separate aquaria. Juvenile *L. fasciola* used in acute toxicity experiments were <7 d postmetamorphosis and juvenile *V. delumbis* were <2 d postmetamorphosis at the start of the tests. Neither species was fed before or during toxicity tests.

Juvenile *L. siliquoidea* were produced on the campus of Missouri State University (Springfield, MO, USA) by transformation on juvenile largemouth bass obtained from the Missouri Department of Conservation Chesapeake Hatchery (Chesapeake, MO, USA). The bass were infested with glochidia by swimming for 15 min in a suspension containing approximately 4,000 viable glochidia per liter. The infested fish then were transferred to a recirculating aquaculture system designed for the recovery of transformed juvenile mussels. After transformation and recovery from the host fish, the juvenile mussels were transferred to a culture system [17] where they were fed continuously with algal suspensions containing live *Neochloris oleoabundans* [18] and commercial preparations of *Nannochloropsis*, *Isochrysis*, *Pavlova*, *Tetraselmis*, and *Thalassiosira weissflogii* (Reed Mariculture, Campbell, CA, USA). Total algal cell concentration in the culture system was maintained at 5 to 10 $\times 10^4$ cells/ml. Temperature was maintained at 22 to 23°C and water was replaced weekly. Juveniles were shipped via overnight courier to North Carolina State University for toxicity testing. Upon arrival, viability of juvenile mussels was evaluated by assessment of foot movement. All juvenile mussels used in toxicity tests were acclimated to laboratory conditions by tempering into dilution water for 24 h prior to use in toxicity tests. Juvenile *L. siliquoidea* used for tests with atrazine, fipronil, pendimethalin, and per-

methrin were <one week posttransformation, whereas juvenile *L. siliquoidea* used for fungicide tests were eight weeks posttransformation at the start of the tests.

Not all species of glochidia could be tested with all pesticides because of limited availability of test organisms. *Lampisili siliquoidea* were most widely available and thus were used more extensively than the other species for both glochidia and juvenile tests. Availability of juveniles (species and number of organisms) was even more limited than glochidia; therefore pesticide tests with juveniles were limited to two species, *L. fasciola* and *L. siliquoidea*. Reference toxicant (NaCl) tests with juveniles were completed with these two species as well as *V. delumbis*.

Glochidia acute toxicity tests

Acute toxicity tests with glochidia were conducted with *V. delumbis*, *V. constricta*, *E. complanata*, *L. fasciola*, and *L. siliquoidea* according to standardized guidelines [14], with the exception that 150 to 200 glochidia were used in each replicate, rather than the 1,000 per replicate recommended by the American Society for Testing and Materials guideline [14]. Briefly, test chambers were 90 × 50 mm glass crystallizing dishes containing 100 ml of test solution. Three replicates were used for each of five or six test concentrations, dilution-water controls, and solvent controls. Reference toxicant (NaCl) tests were conducted by preparing a 0.5 serial dilution to create treatments ranging from 0.25 to 8.0 g NaCl/L; NaCl was chosen as the reference toxicant for quality assurance purposes because a solution of NaCl was used to evaluate viability of glochidia in toxicity tests [14]. Glochidia toxicity tests were conducted for 48 h and test solutions were not renewed. Viability was evaluated on subsamples of 50 to 75 glochidia from each replicate at 24 and 48 h according to standardized guidelines [14]. Digital photographs of glochidia were obtained before and after the NaCl response test to document glochidia response, thereby providing an additional measure of quality assurance/quality control for evaluation of glochidia survival in toxicity tests. Glochidia closed prior to addition of NaCl and open but not responding to NaCl were classified as nonviable based on the premise that they would not be able to attach to host fish for transformation into juveniles [5]. The test acceptability criterion was ≥90% control survival at termination of the test (48 h).

Juvenile acute toxicity tests

Acute toxicity tests with ≤two-month-old juvenile *L. siliquoidea*, *L. fasciola*, and *V. delumbis* were conducted according to standardized guidelines [14], with the exception that test chambers were 90 × 50 mm glass crystallizing dishes containing 100 ml of test solution rather than 50-ml beakers with 30 ml of test solution [14]. Three replicates, each with seven mussels, were used for each of five or six test concentrations, dilution-water controls, and solvent controls. Reference toxicant (NaCl) tests were conducted by preparing a 0.5 serial dilution to create treatments ranging from 0.5 to 8.0 g NaCl/L. Test duration was 96 h and test solutions were renewed (95%) at 48 h. Survival (based on movement inside or outside of the shell) was evaluated at 48 and 96 h. The test chamber (dish) was gently swirled to concentrate the mussels in a small area so all of the organisms in a dish could be observed simultaneously in the field of view under ×15 magnification. Test acceptability criterion was ≥90% control survival.

Juvenile chronic toxicity test

We adapted the standardized guidelines [14] to conduct a 21-d chronic toxicity test with four-month postmetamorphosis juvenile *L. siliquoidea* exposed to technical-grade atrazine. Test solutions were renewed (95%) every 48 or 72 h and survival (based on movement inside or outside of the shell) was assessed on days 7, 14, and 21. Shell length was measured on day 21 for determination of growth (% increase in shell length). Juvenile mussels were fed daily with a commercial mixture of nonviable microalgae prepared from Instant Algae® Shellfish Diet 1800 and *Nannochloropsis* (Nan 3600) concentrate (Reed Mariculture) according to standardized guidelines for conducting chronic tests with juvenile mussels [14]. At initiation of the chronic test, juveniles in each experimental unit were measured using QCapture PRO™ image analysis software (Ver 5.0, QImaging, Burnaby, BC, Canada) in conjunction with a stereomicroscope equipped with a digital camera. Mean shell length (±1 standard deviation) of juveniles at the start of the test was 1,227 ± 189 μm ($n = 180$). Lengths of surviving juvenile *L. siliquoidea* were evaluated on day 21. Growth was calculated for each experimental unit based on change in mean length from the start of the test. Mean ($n = 3$) daily growth was determined for each replicate with ≥30% survival; growth was not calculated for replicates with <30% survival. The test acceptability criterion was ≥80% control survival.

Pesticide analysis

Pesticide exposure concentrations were determined for at least two treatment concentrations of each pesticide in each toxicity test. A 30-ml aliquot from each replicate within a given treatment was collected and combined to form a composite water sample. Sample volume was determined and chrysened12 was added as a surrogate internal standard to monitor extraction efficiency for pesticides that required extraction (i.e., all except propiconazole and pyraclostrobin). Additionally, extraction efficiency for individual pesticides was determined from pesticide-spiked samples ($n = 3$). All water samples were extracted by liquid-liquid extraction with methylene chloride with the exception of chlorthalonil samples which were extracted with C-18 Empore disks (3M, St. Paul, MN, USA) and eluted with a 1:1 mix of acetone and methylene chloride. Liquid extracts were dried with anhydrous sodium sulfate and concentrated by use of rotary and nitrogen evaporation techniques. The concentrated extracts were analyzed on an Agilent 6890 gas chromatograph (Santa Clara, CA, USA) equipped with a Restek RTX-5MS capillary column and a 5973n mass selective detector (Bellefonte, PA, USA). Propiconazole and pyraclostrobin quantification was completed directly in water samples using liquid chromatography-tandem mass spectrometry on a Thermo LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The initial mobile phase consisted of 10% acetonitrile and 90% ammonium formate (0.1% v/v concentrated formic acid, 0.08% v/v concentrated ammonia in water) at 220 μl/min with a linear gradient programmed over 18 min to achieve a final composition of 10% v/v acetonitrile and 90% v/v isopropanol with ammonium formate (0.1% v/v concentrated formic acid, 0.08% v/v concentrated ammonia). Final analyte concentrations were not corrected for surrogate recovery or extraction efficiency.

Exposure accuracy (i.e., measured pesticide concentration compared to target concentration) was calculated for each pesticide as

$$\text{Exposure accuracy} = (P_m)/(P_t) \cdot 100$$

where P_m is the measured pesticide concentration and P_t is the target concentration.

Water chemistry

Standard methods were followed for measurement of all water quality parameters [19]. Calibrated meters were used for analysis of pH (Beckman Model ϕ 240, Beckman Instruments, Fullerton, CA, USA), dissolved oxygen, conductivity, and temperature (YSI Model 556 MPS, Yellow Springs Instruments, Yellow Springs, OH, USA). Alkalinity was determined by titration with 0.02 N H_2SO_4 to pH 4.5, and hardness by titration with 0.01 M ethylenediaminetetraacetic acid. For reference toxicant tests, NaCl concentrations were measured with a handheld meter (YSI Model 30, Yellow Springs Instruments).

Statistical analysis

Median effective concentration (EC50) estimates and 95% confidence intervals were calculated by the Trimmed Spearman-Kärber method [20] with ToxCalcTM statistical software (Ver 5.0.231, Tidepool Scientific Software, McKinleyville, CA, USA) with measured pesticide concentrations (for pesticide tests) and measured NaCl concentrations (for reference toxicant tests). Median effective concentration values were considered significantly different when 95% confidence intervals did not overlap [19]. Statistical analysis of growth was performed with JMP Statistical Analysis software (Ver 5.1, Statistical Analysis System Institute, Cary, NC, USA) using analysis of variance followed by Tukey's test for means comparison ($\alpha = 0.05$).

RESULTS AND DISCUSSION

General conditions and water chemistry

Control viability was >90% for all glochidia tests at 24 and 48 h, with the exception of tests with *E. complanata* at 48 h. Therefore, 48-h EC50s were not calculated for *E. complanata* glochidia tests. Control survival was >93% at 96 h for all juvenile acute tests and was 94.4% on day 21 of the chronic test.

Water chemistry values for test water were consistent within treatments and among tests and were similar to the values listed in the standardized guide for toxicity tests with mussels [14]. For all tests, temperature ranged from 20.1 to 21.9°C, pH ranged from 8.32 to 8.61, conductivity ranged from 523 to 625 $\mu\text{S}/\text{cm}$, alkalinity ranged from 116 to 130 mg CaCO_3/L , and hardness ranged from 170 to 192 mg CaCO_3/L . Dissolved oxygen was >80% of saturation at all times.

Pesticide exposure validation

Measured pesticide concentrations were 65 to 129% of target concentrations in all toxicity tests (Table 1). Surrogate (chrysene-d12) recovery (mean \pm standard deviation) was $83.9 \pm 10.0\%$ ($n = 23$). Extraction efficiency (mean \pm standard deviation) was $75.5 \pm 3.6\%$ for atrazine ($n = 3$), $85.2 \pm 6.0\%$ for fipronil ($n = 3$), $82.7 \pm 3.2\%$ for pendimethalin ($n = 3$), $81.5 \pm 2.6\%$ for permethrin ($n = 3$), and $106.1 \pm 8.2\%$ for chlorothalonil ($n = 3$). Pyraclostrobin and propiconazole did not require extraction from water samples before quantification. Measured pesticide concentrations were not corrected for surrogate recovery or extraction efficiency. None of the pesticides used in toxicity tests were detectable in water

Table 1. Summary of pesticide exposure accuracy (ratio of measured concentrations to target concentrations) for toxicity tests with glochidia and juvenile mussels. All pesticides were technical grade. Measured values were not corrected for surrogate or spike recovery

Lifestage	Pesticide	Mean exposure accuracy ^a (%)	Standard deviation	n
Glochidia	Atrazine	81.6	13.2	8
	Fipronil	64.6	20.1	8
	Pendimethalin	93.7	30.8	8
	Permethrin	79.6	26.3	8
	Chlorothalonil	107.9	10.5	3
	Propiconazole	100.1	37.4	3
	Pyraclostrobin	122.6	5.7	3
Juveniles	Atrazine	98.3	16.5	9
	Fipronil	70.5	25.0	4
	Pendimethalin	115.6	29.1	4
	Permethrin	86.5	14.4	4

^a Measured water concentration as a percentage of target concentration.

samples of the control ($n = 3$) and solvent control ($n = 3$) treatments.

Acute glochidia and juvenile toxicity

Reference toxicant. Sodium chloride is the recommended reference toxicant for bioassays with standard freshwater regulatory test organisms [21]. Moreover, NaCl is an extremely appropriate reference toxicant (for quality assurance purposes) in early life stage tests with freshwater mussels because its use is recommended in the American Society for Testing and Materials mussel testing guideline [14] to measure viability of glochidia. In the present study, mussel glochidia 48-h EC50s with NaCl ranged from 0.56 g/L for *L. siliquoidea* to 3.63 g/L for *V. delumbis* (Table 2). Juvenile mussel sensitivity to NaCl was less variable; 96-h EC50s ranged from 3.98 g/L for *L. fasciola* to 5.23 g/L for *V. delumbis* (Table 2). These glochidia EC50 values are comparable to those reported by Valenti [22], which ranged from 2.25 to 2.67 g NaCl/L for three species of unionid glochidia. Valenti [22] also reported NaCl toxicity values of 2.33 g NaCl/L for *C. dubia*, 4.96 g NaCl/L for *D. magna*, and 9.84 g NaCl/L for *P. promelas*. When reference toxicant tests with NaCl are conducted simultaneously with standard toxicant evaluations of early life stages of freshwater mussels, they provide a range of expected values for future tests that can be used to judge the relative health, condition, and sensitivity of a test species.

Pesticides. Technical-grade fungicides, particularly chlorothalonil and pyraclostrobin, were acutely toxic to early life stages of freshwater mussels (Table 2). Glochidia were more sensitive to chlorothalonil (48-h EC50 = 0.04 mg/L) than juveniles (96-h EC50 = 0.28 mg/L), whereas juvenile mussels were more sensitive than glochidia to pyraclostrobin and propiconazole (Table 2), and the juvenile 96-h EC50 (0.03 mg/L) for pyraclostrobin was the lowest EC50 of any in the present study. Conversely, technical-grade atrazine, fipronil, pendimethalin, and permethrin were not acutely toxic to the five species of glochidia or two species of juvenile mussels at the concentrations tested (Table 2). We were unable to calculate EC50 values for these pesticides with any of the glochidia or juveniles in acute tests because survivorship exceeded 50% in all treatments, even those approaching water solubility for the test compounds.

The acute toxicity data generated in the present study for

Table 2. Median effective concentrations (EC50s) and 95% confidence intervals (in parentheses) for early life stages of freshwater mussels exposed to technical-grade pesticides (mg/L) and NaCl (g/L), as a reference toxicant

Species	Toxicant	Glochidia EC50		Juveniles 96-h EC50
		24 h	48 h	
<i>Villosa delumbis</i>	NaCl	3.31 (3.12, 3.50)	3.63 (3.38, 3.88)	5.23 (4.64, 5.83)
	Atrazine	>30	>30	— ^a
	Permethrin	>0.2	>0.2	—
<i>Villosa constricta</i>	NaCl	2.76 (2.65, 2.88)	2.59 (2.41, 2.77)	—
	Atrazine	>30	>30	—
	Fipronil	>2.0	>2.0	—
	Pendimethalin	>0.3	>0.3	—
	Permethrin	>0.2	>0.2	—
<i>Elliptio complanata</i>	NaCl	2.67 (2.53, 2.81)	2.23 (2.02, 2.43)	—
	Atrazine	>30	NA ^b	—
	Fipronil	>2.0	NA	—
	pendimethalin	>0.3	NA	—
	Permethrin	>0.2	NA	—
<i>Lampsilis fasciola</i>	NaCl	1.84 (1.69, 2.00)	1.74 (1.58, 1.91)	3.98 (2.75, 5.21)
	Atrazine	>30	>30	>30
	Fipronil	>2.0	>2.0	>2.0
	Pendimethalin	>0.3	>0.3	>0.3
	Permethrin	>0.2	>0.2	>0.2
<i>Lampsilis siliquoidea</i>	NaCl	0.55 (0.52, 0.58)	0.56 (0.54, 0.59)	4.56 (3.92, 5.20)
	Atrazine	>30	>30	>30
	Fipronil	>2.0	>2.0	>2.0
	Pendimethalin	>0.3	>0.3	>0.3
	Permethrin	>0.2	>0.2	>0.2
	Chlorothalonil	0.09 (0.06, 0.15)	0.04 (0.02, 0.11)	0.28 (0.19, 0.41)
	Propiconazole	20.75 (14.10, 30.52)	19.21 (13.79, 27.03)	10.01 (7.47, 13.51)
	Pyraclostrobin	0.48 (0.19, 1.21)	0.08 (0.04, 0.19)	0.03 (0.03, 0.04)

^a — indicates no test performed.

^b NA = not applicable; control viability <90%.

chlorothalonil (EC50s 0.04–0.28 mg/L) and pyraclostrobin (EC50s 0.03–0.08 mg/L) indicate that these fungicides are highly to very highly toxic to freshwater mussels, consistent with reports for other aquatic invertebrates and fish. Spradley et al. [23] summarized the toxicity of several fungicides to aquatic organisms and reported median lethal concentrations of chlorothalonil as 0.07 mg/L for *D. magna* and 0.04 to 0.06 mg/L for fish. Ernst et al. [24] reported a chlorothalonil formulation median lethal concentration of 5.94 mg/L for blue mussel (*Mytilus edulis*), a saltwater mussel. Spradley et al. [23] report that pyraclostrobin median lethal concentrations were 0.0157 mg/L for *D. magna* and ranged from 0.0062 mg/L to 0.0114 mg/L for fish. Relatively little information exists regarding chlorothalonil and pyraclostrobin concentrations in surface waters; however, chlorothalonil was among the pesticides analyzed as a part of the National Water Quality Assessment program administered by the U.S. Geological Survey. The maximum chlorothalonil concentration detected was 0.0622 µg/L in an urban area; however, this was not a chlorothalonil-specific study, so sample sites and times do not necessarily represent worst-case scenarios adjacent to heavily treated areas [25]. The U.S. EPA [25] concluded that chlorothalonil has a low potential for acute or chronic toxicity to aquatic organisms because it rapidly dissipates in the aquatic environment. However, little is known about the role of sediment in exposure of freshwater mussels, particularly the juvenile stage, to these fungicides. Additionally, sublethal effects of fungicides have not been studied thoroughly. This information is critical for a more complete characterization of the risk of fungicides to freshwater mussels.

Toxicity to *L. siliquoidea* glochidia and juveniles was lower for propiconazole than for the other fungicides tested. Toxicity

values were again consistent with those from acute toxicity tests with standard aquatic test organisms: Rainbow trout 96-h median lethal concentrations range from 1.54 to 6.4 mg/L [23,26] and 3.2 to 10.2 mg/L for *D. magna* [23].

Few studies have examined the toxicity of current-use pesticides to early life stages of freshwater mussels but results to date suggest that broad generalizations regarding the sensitivity of unionids to pesticides are unfounded. Results of atrazine and permethrin acute toxicity tests in the present study largely were consistent with those of previous studies with unionid glochidia (Table 3). Water solubility of technical-grade atrazine is 33 mg/L; therefore, the highest concentration of atrazine in our tests was 30 mg/L. Similarly, water solubility of technical-grade permethrin is 200 µg/L at 20°C; therefore, we chose to limit the highest permethrin test concentration in our study to 200 µg/L. Permethrin data reported by Milam et al. [11] and that generated in the present study suggest that unionid glochidia sensitivity to permethrin is species-specific and highly variable (Table 3). To our knowledge, there are no other peer-reviewed published studies that report the toxicity of permethrin to juvenile unionids or toxicity of fipronil or pendimethalin to glochidia or juvenile unionids. Pendimethalin and fipronil concentrations in surface waters are generally <10 µg/L [27,28]. This, in addition to the data generated in the present study, suggests that the risk of adverse effects from acute exposure to these compounds is likely low for early life stages of unionid mussels.

Results of toxicity studies comparing the sensitivity of early life stages of unionids to standard species used for aquatic toxicity testing have not provided a clear indication of the relative sensitivity of unionids to pesticides. For example, Connors and Black [29] reported that *U. imbecillis* glochidia were

Table 3. Summary of acute toxicity of atrazine (mg/L) and permethrin ($\mu\text{g/L}$) to unionid glochidia

Pesticide	Test organism	EC50 ^a	Test duration (h)	Reference
Atrazine	<i>Utterbackia imbecillis</i>	241.3	24	Conners and Black [29]
	<i>Anodonta imbecillis</i>	>60	24	Johnson et al. [39]
	<i>Elliptio complanata</i>	>30	24	This study
	<i>Lampsilis cardium</i>	>30	48	This study
	<i>Lampsilis siliquoidea</i>	>30	48	This study
	<i>Villosa constricta</i>	>30	48	This study
	<i>Villosa delumbis</i>	>30	48	This study
	Permethrin	<i>Lampsilis cardium</i>	14.9	24
<i>Leptodea fragilis</i>		3,515	24	Milam et al. [11]
<i>Ligumia subrostrata</i>		1,740	24	Milam et al. [11]
<i>Utterbackia imbecillis</i>		1,714	24	Milam et al. [11]
<i>Elliptio complanata</i>		>200	24	This study
<i>Lampsilis fasciola</i>		>200	48	This study
<i>Lampsilis siliquoidea</i>		>200	48	This study
<i>Villosa constricta</i>		>200	48	This study
<i>Villosa delumbis</i>		>200	48	This study

^a EC50 = median effective concentration.

more acutely sensitive to formulations of carbaryl and glyphosate than surrogate organisms commonly used for aquatic toxicity testing. Interestingly, Milam et al. [11] and Conners and Black [29] reported that *U. imbecillis* was consistently among the least sensitive of six species of freshwater mussel glochidia in acute toxicity tests with 2,4-D, carbaryl, pentachlorophenol, and permethrin. Milam et al. [11] also reported that two unionids, *Leptodea fragilis* and *Megaloniais nervosa*, were more acutely sensitive to 2,4-D than surrogate aquatic test species *Ceriodaphnia dubia* and *D. magna*. However, Milam et al. [11] concluded that acute water quality criteria based on Daphnidae would be generally protective of Unionidae for the five organic chemicals (including four pesticides) tested in their study. Results of these and other [30,31] previous studies, in addition to those of our experiments, indicate that early life stages of unionids are not uniform in their sensitivity to pesticides compared to surrogate aquatic species commonly used for acute toxicity testing and that toxicity of pesticides to early life stages of mussels is species- and chemical- (or possibly chemical class) specific.

Chronic 21-d juvenile toxicity test

Atrazine exposure resulted in a 14-d EC50 of 15.8 mg/L (95% confidence interval = 12.0, 19.5) and a 21-d EC50 of 4.3 mg/L (95% confidence interval = 2.8, 5.8) for juvenile *L. siliquoidea*. Concentrations of atrazine as high as 0.102 mg/L have been measured in rivers in agricultural areas and up to 2.3 mg/L has been measured in edge-of-field run-off and tailwater pits in agricultural areas [16]. The no-observed-effect concentration for viability was 3.75 mg/L at 14 d and <1.9 mg/L (the lowest concentration tested) at 21 d. The 21-d effective concentration to 10% of test animals was 0.38 mg/L and the 21-d effective concentration for 25% of test animals was 0.903 mg/L. Although it is not likely that mg/L concentrations of atrazine persist in surface waters for extended periods, the proximity of environmental concentrations to the chronic no-observed-effect concentration, 10% effective concentration, and 25% effective concentration suggest that some juvenile mussels may be at risk to atrazine-induced toxicity. To our knowledge, this is the first peer-reviewed published study to examine the chronic toxicity of atrazine to native freshwater mussels.

Daily growth rate (over 21 d) decreased in a concentration-

dependent manner and the no-observed-effect concentration for growth was 1.9 mg atrazine/L; the lowest-observed-effects concentration was 3.8 mg/L (Fig. 1). We were not able to determine growth rate of mussels in the 15- and 30-mg/L atrazine treatments because >30% mortality occurred in these treatments by day 21. The growth rate of control mussels (5.0 $\mu\text{m/d}$) was similar to that reported for controls in other laboratory studies with juvenile unionids. Bringolf et al. [32] reported growth of 3.7 to 4.7 $\mu\text{m/d}$ for *L. siliquoidea* in 21- and 28-d experiments; Newton et al. [33] reported a growth rate of 5.9 $\mu\text{m/d}$ after 10 d for *L. cardium*; and Lasee [34] reported growth of 6.6 $\mu\text{m/d}$ after 7 d for *L. cardium*.

Juvenile mussel growth rate was not a robust indicator of sublethal effects of atrazine exposure in a 21-d static test; the 21-d lowest-observed-effects concentration (3.8 mg/L) for effects on growth was not significantly different than the 21-d EC50 (4.3 mg/L). Bringolf et al. [32] reported similar results for growth of juvenile *L. siliquoidea* exposed to glyphosate-based compounds. Previous toxicity studies with ammonia [33,35] and mercury [36] have demonstrated that growth rate of juvenile unionids was a robust indicator of sublethal effects; however, sediment was included in the test chambers in each of these studies. Additionally, growth rate may be more informative when a flow-through exposure apparatus is used [37]. The current American Society for Testing and Materials guideline for chronic toxicity testing with juvenile mussels

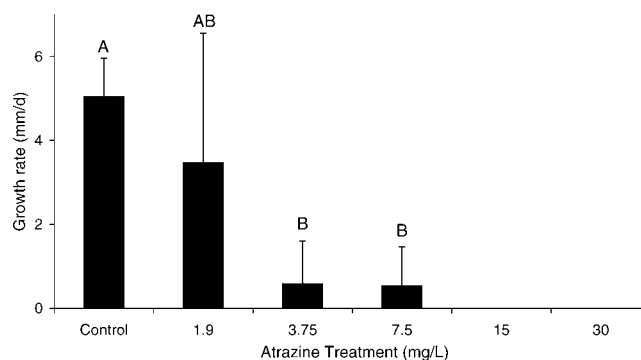


Fig. 1. Mean daily growth of juvenile *Lampsilis siliquoidea* exposed to technical-grade atrazine for 21 d. Data are presented as the mean \pm one standard deviation ($n = 3$). Treatments not sharing the same letters are significantly different (Tukey's test, $\alpha = 0.05$).

[14] recommends water-only exposures; therefore, the utility of determination of growth rate will require further evaluation, particularly for static-renewal tests. Additional research is needed to optimize dietary constituents and feeding rate for juvenile mussels in chronic static-renewal tests.

CONCLUSION

Data from this study has shown that *L. siliquoidea* glochidia and juveniles are among the most sensitive aquatic organisms tested to date with the technical-grade fungicides chlorothalonil, pyraclostrobin and, to a lesser extent, propiconazole. This and other species of unionid glochidia and juveniles in the present study were not sensitive to acute exposures of technical-grade atrazine, fipronil, pendimethalin, and permethrin at the concentrations tested; however, we have shown that a 21-d exposure to atrazine can be toxic to a juvenile unionid, *L. siliquoidea*. Our findings suggest that the acute ambient aquatic life water quality criteria of 1.5 mg/L for atrazine proposed by the U.S. EPA ([38]; <http://www.epa.gov/waterscience/criteria/atrazine/rev-draft.pdf>) would likely be protective of early life stages of *L. siliquoidea*, a unionid species that is not listed as threatened or endangered, but shares its genus with 10 others that currently are listed. However, these tests involved water-only exposures and acute effects, and additional research is needed to determine the role of sediment exposure and sublethal effects, particularly in chronic tests. Additional research also is needed to determine the hazards of formulations of the technical-grade pesticides described here and to determine the risk that additional current-use pesticides pose to early life stages of native freshwater mussels.

Acknowledgement—Funding for this project was provided by the Pesticide Environmental Trust Fund administered through the North Carolina Department of Agriculture and Consumer Services and the U.S. Environmental Protection Agency. We thank L. Humphries, S. Mosher, B. Kaiser, N. Deighton, and N. Glassbrook.

REFERENCES

- Williams JD, Warren ML Jr, Cummings KS, Harris JL, Neves RJ. 1993. Conservation status of freshwater mussels of the United States and Canada. *Fisheries* 18:6–22.
- Bogan AE. 1993. Freshwater bivalve extinctions (Mollusca: Unionoida): A search for causes. *Am Zool* 33:599–609.
- McMahon RF, Bogan AE. 2001. Mollusca: Bivalvia. In Thorpe JH, Covich AO, eds, *Ecology and Classification of North American Freshwater Invertebrates*, 2nd ed. Academic, New York, NY, USA, pp 331–416.
- Ingersoll CG, Kernaghan NJ, Gross TS, Bishop CD, Wang N, Roberts A. 2006. Laboratory toxicity testing with freshwater mussels. In Farris JL, Van Hassel J, eds, *Freshwater Bivalve Ecotoxicology*. Pensacola, FL, USA pp 97–137.
- Jacobson PJ, Neves RJ, Cherry DS, Farris JL. 1997. Sensitivity of glochidial stages of freshwater mussels (Bivalvia: Unionidae) to copper. *Environ Toxicol Chem* 16:2384–2392.
- Bruenderman SA, Neves RJ. 1993. Life history of the endangered fine-rayed pigtoe *Fusconaia cuneolus* (Bivalvia: Unionidae) in the Clinch River, Virginia. *Am Malacol Bull* 10:83–91.
- Neves RJ, Widlak JC. 1987. Habitat ecology of juvenile freshwater mussels (Bivalvia: Unionidae) in a headwater stream in Virginia. *Am Malacol Bull* 5:1–7.
- Augspurger T, Keller AE, Black MC, Cope WG, Dwyer JF. 2003. Water quality guidance for protection of freshwater mussels (Unionidae) from ammonia exposure. *Environ Toxicol Chem* 22:2569–2575.
- Mummert AK, Neves RJ, Newcomb TJ, Cherry DS. 2003. Sensitivity of juvenile freshwater mussels (*Lampsilis fasciola*, *Villosa iris*) to total and unionized ammonia. *Environ Toxicol Chem* 22:2545–2553.
- Black MC. 2001. Water quality standards for North Carolina's endangered mussels. Final Report. Department of Environmental Health Science, University of Georgia, Athens, GA, USA.
- Milam CD, Farris JL, Dwyer JF, Hardesty DK. 2005. Acute toxicity of six freshwater mussel species (glochidia) to six chemicals: Implications for daphnids and *Utterbackia imbecillis* as surrogates for protection of freshwater mussels (Unionidae). *Arch Environ Contam Toxicol* 48:166–173.
- McKinney AD, Wade DC. 1996. Comparative response of *Ceriodaphnia dubia* and juvenile *Anodonta imbecillis* to pulp and paper mill effluents discharged to the Tennessee River and its tributaries. *Environ Toxicol Chem* 15:514–517.
- Weinstein JE. 2001. Characterization of the acute toxicity of photoactivated fluoranthene to glochidia of the freshwater mussel, *Utterbackia imbecillis*. *Environ Toxicol Chem* 20:412–419.
- American Society for Testing and Materials. 2006. Standard guide for conducting laboratory toxicity tests with freshwater mussels. E2455-06. In *Annual Book of ASTM Standards*, Vol 11.06. Philadelphia, PA.
- Battaglin WA, Furlong ET, Burkhardt MR, Peter CJ. 2000. Occurrence of sulfonylurea, sulfonamide, imidazolinone, and other herbicides in rivers, reservoirs, and groundwater in the Midwestern United States, 1998. *Sci Total Environ* 248:123–133.
- Kolpin DW, Barbash JE, Gilliom RJ. 1998. Occurrence of pesticides in shallow groundwater of the United States: Initial results from the National Water-Quality Assessment Program. *Environ Sci Technol* 32:558–566.
- Barnhart MC. 2006. Buckets of muckets: A compact system for rearing juvenile freshwater mussels. *Aquaculture* 254:227–233.
- Gatenby C, Parker B, Neves RJ. 1997. Growth and survival of juvenile rainbow mussels, *Villosa iris* (Bivalvia: Unionidae) reared on algal diets and sediment. *Am Malacol Bull* 14:57–66.
- American Public Health Association. 1995. *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC.
- Hamilton MA, Russo RC, Thurston RV. 1977. Trimmed Spearman-Kärber Method for estimating median lethal concentrations in toxicity bioassays. *Environ Sci Technol* 11:714–719.
- U.S. Environmental Protection Agency. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. EPA/600/4-90/027F. Washington, DC.
- Valenti TW. 2006. Case study: Sensitivity of mussel glochidia and regulatory test organisms to mercury and a reference toxicant. In Farris JL, Van Hassel J, eds, *Freshwater Bivalve Ecotoxicology*. SETAC, Pensacola, FL, USA, pp 357–373.
- Spradley PCR, Goodwin AE, Selden GL Jr. 2005. Asian soybean rust fungicides and aquatic organisms. FSA7532-1M-10-05RV. University of Arkansas Division of Agriculture, Cooperative Extension Service, Little Rock, AR, USA.
- Ernst W, Doe K, Jonah P, Young J, Julien G, Hennigar P. 1991. The toxicity of chlorothalonil to aquatic fauna and the impact of its operational use on a pond ecosystem. *Arch Environ Contam Toxicol* 21:1–9.
- U.S. Environmental Protection Agency. 1999. Chlorothalonil: Reregistration eligibility decision (R.E.D.). EPA 738-R-99-004. Washington, DC.
- Matthiessen P, Whale GF, Rycroft RJ, Sheahan DA. 1988. The joint toxicity of pesticide tank-mixes to rainbow trout. *Aquat Toxicol* 13:61–75.
- Strandberg M, Scott-Fordsmand JJ. 2004. Effects of pendimethalin at lower trophic levels—A review. *Ecotoxicol Environ Saf* 57:190–201.
- Wirth EF, Pennington PL, Lawton JC, DeLorenzo ME, Bearden D, Shaddrix B, Sivertsen S, Fulton MH. 2004. The effects of the contemporary-use insecticide (fipronil) in an estuarine mesocosm. *Environ Pollut* 131:365–371.
- Conners DE, Black MC. 2004. Evaluation of lethality and genotoxicity in the freshwater mussel *Utterbackia imbecillis* (Bivalvia: Unionidae) exposed singly and in combination to chemicals used in lawn care. *Arch Environ Contam Toxicol* 46:362–371.
- Keller AE. 1993. Acute toxicity of several pesticides, organic compounds, and a wastewater effluent to the freshwater mussel, *Anodonta imbecillis*, *Ceriodaphnia dubia*, and *Pimephales promelas*. *Bull Environ Contam Toxicol* 51:696–702.
- Keller AE, Ruessler DS. 1997. The toxicity of malathion to union-

- id mussels: Relationship to expected environmental concerns. *Environ Toxicol Chem* 16:1028–1033.
32. Bringolf RB, Cope WG, Mosher S, Barnhart MC, Shea D. 2007. Acute and chronic toxicity of glyphosate compounds to glochidia and juveniles of *Lampsilis siliquoides* (Unionidae). *Environ Toxicol Chem* 26:2094–2100.
 33. Newton TJ, Allran JW, O'Donnell JA, Bartsch MR, Richardson WB. 2003. Effects of ammonia on juvenile unionid mussels (*Lampsilis cardium*) in laboratory sediment toxicity tests. *Environ Toxicol Chem* 22:2554–2560.
 34. Lasee BA. 1991. Histological and ultrastructural studies of larval and juvenile *Lampsilis* (Bivalvia) from the Upper Mississippi River. PhD thesis. Iowa State University, Ames, IA, USA.
 35. Bartsch MR, Newton TJ, Allran JW, O'Donnell JA, Richardson WB. 2003. Effects of pore-water ammonia on in situ survival and growth of juvenile mussels (*Lampsilis cardium*) in the St. Croix Riverway, Wisconsin, USA. *Environ Toxicol Chem* 22:2561–2568.
 36. Valenti TW, Cherry DS, Neves RJ, Schmerfeld J. 2005. Acute and chronic toxicity of mercury to early life stages of the rainbow mussel, *Villosa iris* (Bivalvia: Unionidae). *Environ Toxicol Chem* 24:1242–1246.
 37. Wang NIC, Ingersoll CG, Greer IE, Hardesty DK, Ivey CD, Kunz JL, Burmbaugh WG, Dwyer FJ, Roberts AD, Augspurger T, Kane CM, Neves RJ, Barnhart MC. 2007. Chronic toxicity of copper and ammonia to juvenile freshwater mussels (Unionidae). *Environ Toxicol Chem* 26:2048–2056.
 38. U.S. Environmental Protection Agency. 2003. Ambient aquatic life water quality criteria for atrazine—Revised draft. EPA-822-R-03-023. Office of Water, Washington, DC.