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Contaminant Sensitivity of Freshwater Mussels

ACUTE AND CHRONIC TOXICITY OF GLYPHOSATE COMPOUNDS TO GLOCHIDIA AND JUVENILES OF *LAMPSILIS SILIQUOIDEA* (UNIONIDAE)

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Abstract—Native freshwater mussels (family Unionidae) are among the most imperiled faunal groups in the world. Factors contributing to the decline of mussel populations likely include pesticides and other aquatic contaminants; however, there is a paucity of data regarding the toxicity of even the most globally distributed pesticides, including glyphosate, to mussels. Therefore, the toxicity of several forms of glyphosate, its formulations, and a surfactant (MON 0818) used in several glyphosate formulations was determined for early life stages of *Lampsilis siliquoidea*, a native freshwater mussel. Acute and chronic toxicity tests were performed with a newly established American Society of Testing and Materials (ASTM) standard guide for conducting toxicity tests with freshwater mussels. Roundup®, its active ingredient, the technical-grade isopropylamine (IPA) salt of glyphosate, IPA alone, and MON 0818 (the surfactant in Roundup formulations) were each acutely toxic to *L. siliquoidea* glochidia. MON 0818 was most toxic of the compounds tested and the 48-h median effective concentration (0.5 mg/L) for *L. siliquoidea* glochidia is the lowest reported for any aquatic organism tested to date. Juvenile *L. siliquoidea* were also acutely sensitive to MON 0818, Roundup, glyphosate IPA salt, and IPA alone. Technical-grade glyphosate and Aqua Star® were not acutely toxic to glochidia or juveniles. Ranking of relative chronic toxicity of the glyphosate-related compounds to juvenile mussels was similar to the ranking of relative acute toxicity to juveniles. Growth data from chronic tests was largely inconclusive. In summary, these results indicate that *L. siliquoidea*, a representative of the nearly 300 freshwater mussel taxa in North America, is among the most sensitive aquatic organisms tested to date with glyphosate-based chemicals and the surfactant MON 0818.

Keywords—Pesticides Growth *Lampsilis siliquoidea* Early life stage Herbicides

INTRODUCTION

Greater than 70% of the nearly 300 native freshwater mussel (Unionidae and Margaritiferidae) taxa in North America are listed as endangered, threatened, or of special concern [1] and freshwater bivalves comprise the largest group of invertebrates listed under the Endangered Species Act, placing them among the most imperiled faunal groups in the world. Environmental contaminants have been cited among the primary factors suspected as contributing to the decline of mussel species [1,2]. Published toxicological data indicate that early life stages of freshwater mussels (glochidia and juveniles) are among the most sensitive aquatic organisms to contaminants such as ammonia [3–5], metals [6], and some pesticides [7]. In contrast, early life stages of mussels have been reported to be less sensitive to some pesticides compared to other aquatic invertebrates such as amphipods, chironomids, and cladocerans [7–9]. Because spring/summer agricultural pesticide applications often coincide with the reproductive period of native freshwater mussels, early life stages of mussels may be exposed to high concentrations of pesticides due to overspray or runoff. Clearly, more research is needed to understand the impact of current-use pesticides on native freshwater mussels.

Glyphosate is the most widely used herbicide in the world [10]. In the United States, glyphosate use increased more than fivefold (4.7 mil kg to 26.2 mil kg) from 1997 to 2005 [11,12] and can be attributed to increased use of genetically altered glyphosate-resistant plants that allow glyphosate to be applied over row crops, killing weeds but leaving the crops unharmed. Glyphosate concentrations in streams have been measured at mg/L concentrations in agricultural regions [13] and, recently, Kolpin et al. [14] reported that urban use of glyphosate also contributes to glyphosate concentrations in streams in the United States. Risk of adverse effects of glyphosate to aquatic organisms generally is considered to be low; however, an increasing number of studies with fish, amphibians, and aquatic invertebrates report that some glyphosate formulations are substantially more toxic due to surfactants in the formulation [15–19]. Additionally, limited evidence [7] suggests that juvenile mussels (*Utterbackia imbecillis*) are more sensitive to a glyphosate formulation (Roundup®; Monsanto, St. Louis, MO, USA) than are other aquatic organisms commonly used for toxicity testing such as *Daphnia magna* and *Gammarus pseudolimnaeus*. Because current pesticide registration toxicity testing is not commonly performed with bivalves, the impact of globally widespread pesticides such as glyphosate on freshwater mussels largely is unknown. Thus, the need for more information about the toxicity of glyphosate and its formulations to freshwater mussels is of paramount importance.

The purpose of this study was to describe the toxicity of glyphosate and its formulations to early life stages of a native freshwater mussel (fatmucket, *Lampsilis siliquoidea*). This species of freshwater mussel is a member of the same genera as 10 other species listed as endangered (five species), threatened (three species), or of concern (two species) in the United States, but itself is not listed federally. Chemicals evaluated

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in this study included technical-grade glyphosate acid, the technical-grade isopropylamine (IPA) salt of glyphosate, IPA, Roundup (a glyphosate IPA formulation that contains MON 0818 surfactant blend), Aqua Star® (Albaugh, Ankeny, IA, USA; a glyphosate IPA formulation without MON 0818), and MON 0818, a polyethoxylated tallowamine (POEA) surfactant blend in Roundup that enables the active ingredient to penetrate the waxy surfaces of plant leaves. We determined the acute toxicity of these six chemicals to *L. siliquoidea* glochidia and juveniles in 48- and 96-h toxicity tests, respectively. Additionally, we evaluated the chronic effects of five of the six chemicals (all except IPA) on juvenile *L. siliquoidea* survival and growth in 21- or 28-d toxicity tests.

MATERIALS AND METHODS

Test chemicals

Technical-grade glyphosate acid (98% purity) and technical-grade glyphosate IPA salt (>95% purity) were purchased from Chem Service (West Chester, PA, USA). Roundup Ultramax™ (active ingredient: 50.2% glyphosate IPA salt) and Aqua Star (active ingredient: 53.8% glyphosate IPA salt) were purchased from retail suppliers. MON 0818, obtained from Monsanto Company, is a code designation for the proprietary commercial blend of POEA surfactants used in Roundup and other glyphosate-based herbicide formulations marketed by the Monsanto Company. Technical-grade IPA (99.5% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A working solution of IPA (10,000 mg/L) was prepared by dissolving 100 mg of neat IPA in deionized water to a final volume of 10 ml. The working solution of MON 0818 (10,000 mg/L) was prepared by dissolving 250 mg of MON 0818 in deionized water to a final volume of 25 ml. Concentration units for all glyphosate-containing test compounds were based on the acid equivalent (a.e.) concentration of glyphosate.

Test organisms

Brooding adult female *L. siliquoidea* were collected from Silver Fork of Perche Creek, Boone County (MO, USA) on April 4, 2005. Mussels were transported to the aquatic laboratory at Missouri State University (MO, USA) in coolers and held unfed in an aquarium at 10°C for 24 h. 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 using a syringe filled with well water. Exposing approximately 100 individuals to a saturated sodium chloride (NaCl) solution, which initiates shell closure in viable glochidia, tested glochidia viability. Glochidia immediately were shipped in chilled coolers via overnight courier to North Carolina State University (Raleigh, NC, USA) for toxicity testing. Upon arrival, viability of the glochidia was assessed by exposing three subsamples of 50 to 100 glochidia (each) to a saturated NaCl solution as described previously. Glochidia were used for toxicity tests only if initial viability ≥90%, in accordance with established guidelines [20]. Consistent with the standardized guideline [20], glochidia were acclimated to a 50:50 mixture of culture:dilution water for at least 2 h before toxicity tests began.

Juvenile *L. siliquoidea* were produced by transforming glochidia on young-of-year largemouth bass (*Micropterus salmoides*) obtained from the Missouri Department of Conservation Chesapeake Hatchery (Mount Vernon, MO, USA). Bass were infested with glochidia by swimming for 15 min in a suspension containing approximately 4,000 viable glochidia per liter of water. The infested fish then were transferred to a recirculating 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 [21] where they were fed continuously with algal suspensions containing live *Neochloris oleoabundans* [22] and commercial larviculture 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 × 10^4 cells/ml. Water was replaced weekly and temperature was maintained at 22 to 23°C. Transformed juveniles (1 to 8 weeks postmetamorphosis) were shipped via overnight courier to North Carolina State University for toxicity tests. Upon arrival, viability of all juvenile mussels was evaluated by assessing movement of the foot and shell closure response to a stimulus (blunt probe) within a 5-min period. Juvenile mussels were acclimated to laboratory conditions by tempering into dilution water for 24 h prior to use in toxicity tests. Juveniles used in acute experiments were two (average shell length = 732 ± 96 μm; n = 150) to eight weeks (average shell length = 2,196 ± 432 μm; n = 150) postmetamorphosis and juveniles used in chronic toxicity tests were three (mean shell length = 1,012 ± 118 μm; n = 150) to eight weeks (mean shell length = 2,057 ± 416 μm; n = 150) postmetamorphosis.

Acute toxicity tests

Water-only acute toxicity tests with *L. siliquoidea* glochidia and juveniles were performed from June through September 2005, according to standard methods [20]. Briefly, there were five or six treatments of each test chemical plus a control and three replicates of each treatment. Test solutions were prepared in reconstituted hard water [20] and water chemistry (temperature, pH, dissolved oxygen, conductivity, alkalinity, and hardness) was measured according to standard methods [23] in one replicate of at least two treatments at the beginning and end of each test. A calibrated multiprobe was used for analysis of pH, 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. Initial glyphosate (in the form of glyphosate acid) exposure concentrations were verified with standard analytical methods [24] in each of three replicates from the control, low, intermediate, and high test concentrations by Research Triangle Institute (Research Triangle Park, NC, USA).

Glochidia were exposed to test chemicals for 48 h in 150-ml glass beakers. An experimental unit consisted of a beaker containing 100 ml of test solution and approximately 200 glochidia. Test conditions were consistent with standard test guidelines [20]. Test solutions were not renewed during the 48-h glochidia toxicity tests. A subsample of 50 to 100 glochidia was evaluated for survival (via shell closure response to a saturated NaCl solution as described previously) at ×10 to 20 magnification under a stereomicroscope after 24 and 48 h. 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.

Acute toxicity tests with juvenile mussels were conducted

for 96 h in 90 × 50 mm glass crystallizing dishes containing 100 ml of test solution. Each experimental unit contained seven or 10 juveniles. Age of juveniles used in acute tests was one week posttransformation for tests with glyphosate IPA and Roundup; one month posttransformation for tests with technical-grade glyphosate, IPA, and Aqua Star; and two months posttransformation for the MON 0818 test. All environmental and laboratory conditions for juvenile acute tests were similar to glochidia tests [20]. Test solutions were renewed (90%) after 48 h of exposure. At 48 and 96 h, viability of juveniles (based on foot movement within a 5 min period) was assessed under a stereomicroscope at ×20 to 40 magnification.

Consistent with standardized guidelines [20], median effective concentrations (EC50s), as opposed to median lethal concentrations (LC50s), were calculated for all toxicity tests because the death of mussels (and other invertebrates) is not easily discerned from immobility [25]. The endpoint for glochidia viability was shell closure and the endpoint for juvenile viability was foot movement [20].

Chronic toxicity tests

Water-only chronic (21- or 28-d) toxicity tests were performed from August through October 2005, with juvenile *L. siliquoidea*. Age of juveniles used in the chronic tests was one month posttransformation in tests with technical-grade glyphosate and Roundup and two months post-transformation in tests with Aqua Star, glyphosate IPA, and MON 0818. Test conditions during chronic tests were similar to those of the acute toxicity tests [20]; however, test solutions were renewed (~90%) at 48- or 72-h intervals and juvenile mussels were fed daily with a solution of microalgae concentrates prepared from Instant Algae® Shellfish Diet and *Nannochloropsis* concentrate (Reed Mariculture, Campbell, CA, USA) according to standard guidelines for conducting chronic tests with juvenile mussels [20]. At initiation of each test, juveniles in each experimental unit were measured with QCapture PRO™ image analysis software (Ver 5.0, QImaging, Burnaby, BC, Canada) in conjunction with a stereomicroscope equipped with a digital camera. Survival of juvenile *L. siliquoidea* was evaluated on days 7, 14, 21, and 28, except for the glyphosate test, which was terminated after 21 d. Mean growth was calculated for each experimental unit with ≥50% survival and was based on change in shell length from time 0 to termination of the test (day 21 or 28). Growth was not calculated for treatments in which mean viability was <50%.

Statistical analysis

Median effective concentration estimates and 95% confidence intervals were calculated by the Trimmed Spearman-Karber method with ToxCalc™ statistical software (Ver 5.0.231, Tidepool Scientific Software, McKinleyville, CA, USA). Estimates of EC50 values were considered significantly different among the two life stages and six chemicals tested when 95% confidence intervals did not overlap [23]. Statistical analysis of growth was evaluated at the a = 0.05 level and performed with JMP Statistical Analysis software (Ver 5.1, Statistical Analysis Systems Institute, Cary, NC, USA) using analysis of variance followed by Dunnett's test to compare treatments to controls.

RESULTS

General conditions, water chemistry, and recovery of test chemicals

Control viability was >90% in all acute and chronic tests. Water chemistry was consistent among treatments within and among experiments: Temperature ranged from 21.1 to 21.8°C, pH ranged from to 8.22 to 8.76, conductivity ranged from 500 to 616 μS/cm, alkalinity ranged from 114 to 128 mg $CaCO_3$/L, hardness ranged from 158 to 170 mg $CaCO_3$/L, and dissolved oxygen was >83% of saturation at all times. The pH values were slightly greater than the range of 7.8 to 8.0 listed for standard reconstituted hard water [20], but similar pH values for reconstituted hard water also have been reported in studies performed at other laboratories [26,27]. Measured glyphosate concentrations in water samples collected at initiation of exposure ranged from 82.2 to 104.4% of target concentrations (mean 94.2%, $n = 12$). Therefore, all subsequent EC50 calculations were expressed based on initial target test concentrations of glyphosate (in terms of acid equivalents).

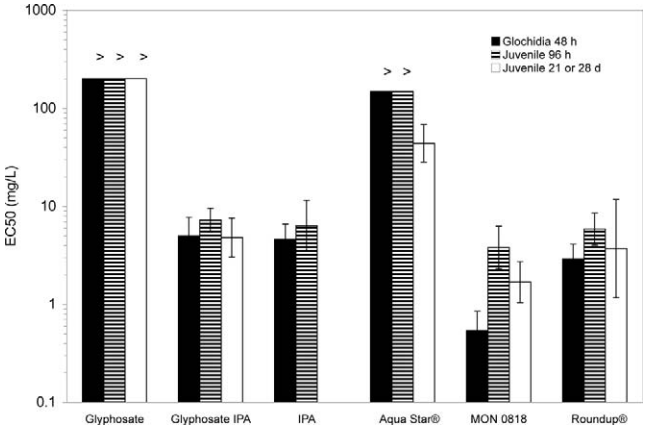

Fig. 1. Median effective concentration estimates (EC50s) of glyphosate-based chemicals, isopropylamine (IPA), and the surfactant blend MON 0818 for *Lampsilis siliquoidea* glochidia (24 h) and juveniles (96 h and 21 or 28 d). All glyphosate concentrations are expressed in acid equivalents. Juveniles in acute tests ranged in age from one week to two months post-transformation at the start of the test; juveniles in chronic tests were one to two months post-transformation. Bars represent 95% confidence intervals for EC50s. A > symbol indicates that the EC50 was greater than the highest test concentration for a particular chemical.

Acute tests—glochidia and juveniles

Of the six chemicals tested, the surfactant MON 0818 was most toxic to *L. siliquoidea* glochidia, and Roundup, technical-grade glyphosate IPA salt, and IPA were also acutely toxic (Fig. 1). *Lampsilis siliquoidea* glochidia were not acutely sensitive to technical-grade glyphosate or Aqua Star, even at test concentrations approaching water solubility limits for glyphosate (Fig. 1). Less than 63% of test organisms were affected in the 200 and 150 mg a.e./L treatments of technical-grade glyphosate and Aqua Star, respectively, with both glochidia and juveniles; therefore, according to standard toxicity testing guidelines [20], an EC50 was not calculated for these compounds. Glochidia EC50s for individual chemicals did not differ significantly at 24 and 48 h for any of the chemicals tested (Table 1).

Juvenile *L. siliquoidea* 96-h EC50 values were similar for MON 0818, Roundup, technical-grade glyphosate IPA salt, and IPA (Fig. 1). However, like acute tests with glochidia, technical-grade glyphosate and Aqua Star were not acutely toxic to juveniles at test concentrations approaching glyphosate water solubility limits, thus EC50s were not calculated (Table 1). Juvenile EC50s for individual chemicals at 48 and 96 h were not significantly different except for the MON 0818 test, in

Table 1. Median effective concentration estimates ([EC50s]; and 95% confidence intervals) for glyphosate-based chemicals, isopropylamine (IPA), and the surfactant blend MON 0818 for early life stages of the freshwater mussel *Lampsilis siliquiodea*. The EC50s for all glyphosate-based chemicals are expressed in glyphosate acid equivalents

	EC50 (mg/L)							
	Glochidia		Juvenile (acute)		Juvenile (chronic)			
Chemical	24 h	48 h	48 h	96 h	7 d	14 d	21 d	28 d
Glyphosate (technical grade)	>200	>200	>200	>200	>200	>200	>200	NA[a]
Glyphosate IPA (technical grade)	5.9 (4.0, 8.6)	5.0 (3.3, 7.6)	8.3 (5.4, 9.6)	7.2 (5.5, 9.6)	7.6 (3.8, 15.3)	6.9 (2.6, 18.5)	5.4 (3.5, 8.3)	4.8 (3.0, 7.6)
IPA	6.0 (4.5, 8.0)	4.6 (1.9, 11.1)	9.9 (7.1, 13.6)	6.3 (3.5, 11.5)	NA[b]	NA[b]	NA[b]	NA[b]
Aqua Star®[c]	>148	>148	>148	>148	>148	>148	m957.3 (37.3, 88.0)	43.8 (28.2, 68.1)
Roundup®[d]	3.0 (2.2, 4.3)	2.9 (2.1, 3.9)	5.9 (4.0, 8.5)	5.9 (4.0, 8.5)	>7.4	>7.4	6.0 (1.3, 27.8)	3.7 (1.2, 11.7)
MON 0818	0.6 (0.4, 1.0)	0.5 (0.4, 0.8)	>6.4	3.8 (2.3, 6.2)	2.8 (1.8, 4.3)	1.9 (1.2, 3.1)	1.7 (1.0, 2.7)	1.7 (1.0, 2.7)

[a] Test terminated at day 21.
[b] NA = not applicable; no test performed.
[c] Albaugh (Ankeny, IA, USA).
[d] Monsanto (St. Louis, MO, USA).

which less than 63% of juveniles were affected in the highest test concentration (6.4 mg/L) at 48 h, but an EC50 of 3.8 mg/L was obtained at 96 h (Table 1).

Chronic tests—juveniles

Viability of controls was >90% for all chronic tests, consistent with standard guidelines [20]. Like the results from the acute toxicity tests with glochidia and juveniles, chronic toxicity tests with juvenile *L. siliquoidea* showed that MON 0818 was most toxic of the five chemicals tested. Roundup and technical-grade glyphosate IPA salt were slightly less toxic than MON 0818, and technical-grade glyphosate and Aqua Star had the least impact on viability of juvenile mussels. Viability in the chemical treatment groups generally decreased throughout the 21- or 28-d exposure period for all five chemicals and, as survival decreased, the 95% confidence intervals for the EC50s became narrower (Table 1). As in the acute tests, we were unable to calculate an EC50 for technical-grade glyphosate in the chronic tests because less than 63% of test organisms were unresponsive in any of the treatments [20]. For all chemicals but technical-grade glyphosate, the highest treatment concentrations had viability <50% by 21 or 28 d. In treatments with <50%, viability growth was not calculated. Viability for the highest treatment concentration for which growth was calculated averaged ($n = 3$) 90.5% for technical-grade glyphosate, 90.5% for glyphosate IPA, 76.2% for Aqua Star, 85.7% for Roundup, and 71.4% for MON 0818.

Glyphosate-containing compounds and MON 0818 had mixed effects on juvenile mussel growth during the chronic tests. A reduction in growth (compared to controls) occurred for the high treatment concentrations of technical-grade glyphosate and MON 0818 (Fig. 2). The concentration-response trends for growth of mussels exposed to technical-grade glyphosate IPA salt, Aqua Star, and Roundup were consistent with an inverted U-shape as opposed to a linear concentration-response relationship; however, differences in growth were not significantly different from controls (Fig. 2). Growth of mussels in control treatments was consistent among the five chronic tests, ranging from 3.7 to 4.7 mm/d.

DISCUSSION

To our knowledge this is one of only two published studies that describe the toxicity of glyphosate to early life stages of native freshwater mussels. In the present study, we assessed toxicity of the different forms and components of technical-grade chemical (i.e., glyphosate, glyphosate IPA salt, and IPA alone), formulations (i.e., Roundup and Aqua Star), and the surfactant used in Roundup (i.e., MON 0818) to identify the component(s) of glyphosate-containing compounds responsible for toxicity to freshwater mussels. We found that Roundup was acutely toxic to early life stages of freshwater mussels, whereas technical-grade glyphosate was not. The toxicity of Roundup to *L. siliquoidea* glochidia (48-h EC50 = 2.9 mg a.e./L) was greater than the only other report for another unionid, *U. imbecillis* (24-h EC50 = 13.5 mg a.e./L) [7]. Our finding that Roundup is more toxic to a unionid than technical-grade glyphosate is consistent with reports for other aquatic organisms [15,17,28].

Toxicity tests with various components of Roundup demonstrated that the surfactant MON 0818 was the most toxic constituent and likely was responsible for much of the toxicity of Roundup to *L. siliquoidea* glochidia and juveniles. Previous studies also indicated that the toxicity of Roundup was due to MON 0818, the POEA surfactant blend used in the formulation [15,17,28]. However, results of the present study indicate that technical-grade glyphosate IPA salt, the active ingredient in Roundup, also is toxic to freshwater mussels. Technical-grade glyphosate IPA EC50s for *L. siliquoidea* glochidia and juveniles were similar and are the lowest reported for any species in the peer-reviewed literature, as determined through a search (1975 to 2006) of databases including Agricola (http://agricola.nal.usda.gov/), Biological Abstracts (http://www.ovid.com/site/catalog/DataBase/25.jsp), and Toxicology Abstracts (http://www.csa.com/factsheets/toxicology-set-c.php).

The acute toxicity of technical-grade glyphosate IPA was similar to that of IPA alone. Because technical-grade glyphosate was not toxic to *L. siliquoidea* glochidia or juveniles, the toxicity of technical-grade glyphosate IPA salt likely was caused by the IPA component, specifically due to the liberation

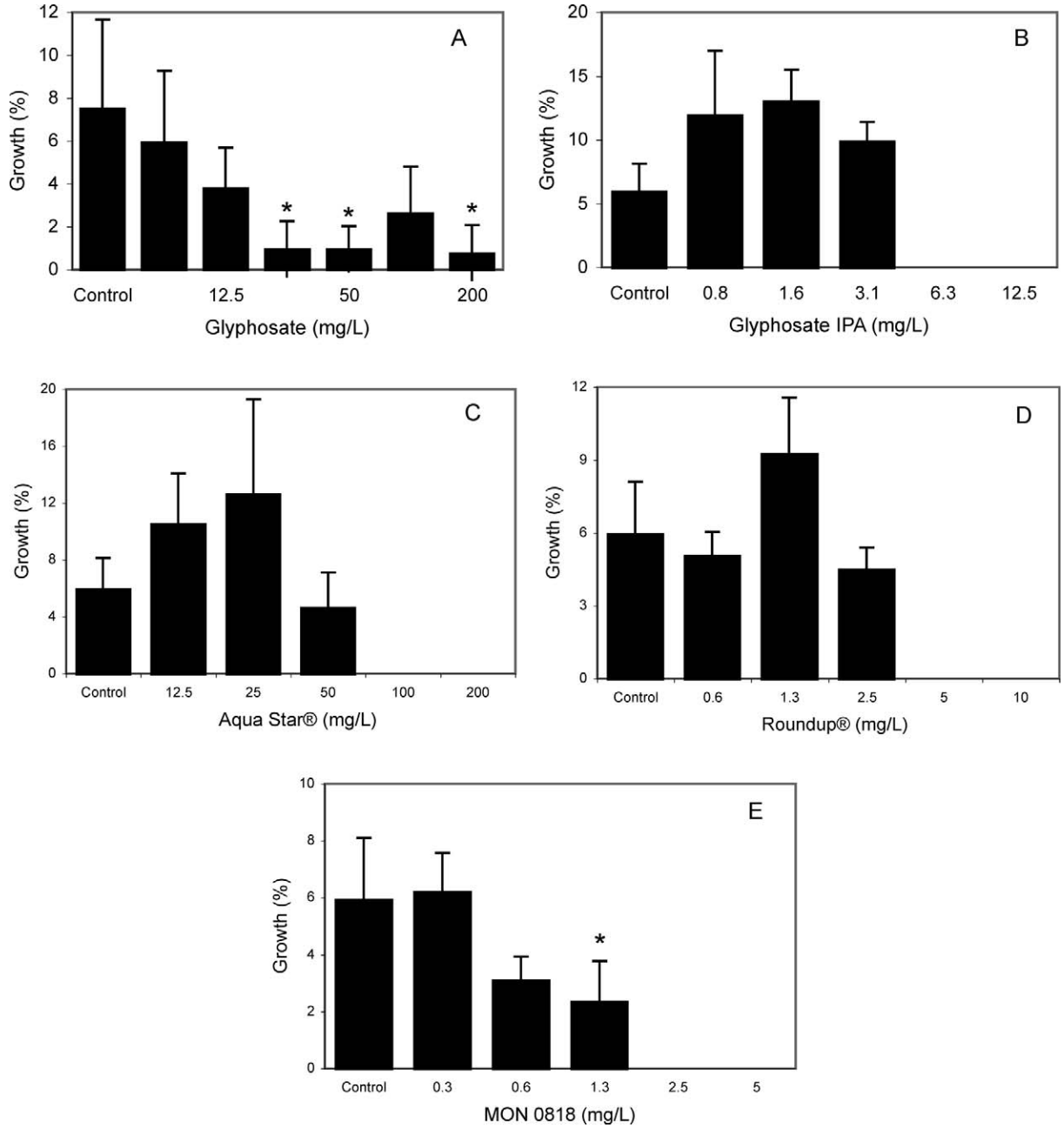

Fig. 2. Average (n = 3) growth (percent change in shell length) of juvenile *Lampsilis siliquoidea* (one to two month post-transformation) during a 21-d exposure to (**A**) glyphosate (technical grade), (**B**) glyphosate isopropylamine (IPA) salt (technical grade), (**C**) Aqua Star® (Albaugh, Ankeny, IA, USA), (**D**) Roundup® (Monsanto, St. Louis, MO, USA), and (**E**) the surfactant blend MON 0818. All glyphosate concentrations are expressed in acid equivalents. Growth was not calculated for treatments with <50% viability. Significant differences (α = 0.05, Dunnett's test) in growth between treatment groups and controls are noted with an asterisk (*).

of ammonia from the amine group of the IPA upon addition to water. The concentration of ammonia in the technical-grade glyphosate IPA treatments (up to 0.18 mg N/L; data not shown) was correlated strongly (r^2 = 0.994) with glyphosate IPA concentration, but was somewhat less than the range of ammonia 96-h LC50 values reported for juvenile mussels [3,5,29]. Interestingly, Aqua Star, a glyphosate formulation labeled for aquatic application that contains glyphosate IPA salt as its active ingredient but does not contain MON 0818, was not acutely toxic to *L. siliquoidea* glochidia or juveniles. Further research is needed to understand why technical-grade glyphosate IPA was toxic but a formulation based on the same active ingredient was not. Other components of the formulation may have influenced the liberation of ammonia, which resulted in the low toxicity of Aqua Star.

Giesy et al. [13] recently reviewed the toxicity of glyphosate-based chemicals and found a wide range of sensitivity among invertebrates. For example, LC50s ranged from 2.59 mg a.e./L in a crayfish (*Orconectes nais*; 96-h) to 5,600 mg a.e./L in a midge larvae (*Chironomus riparius*; 48-h). Additionally, Folmar et al. [15] reported LC50s for fish and invertebrates exposed to Roundup ranging from 1.7 mg a.e./L for *Pimephales promelas* to 32 mg a.e./L for *Gammarus pseudolimnaeus*. Therefore, the Roundup 48-h EC50 (2.9 mg

a.e./L) for *L. siliquoidea* glochidia reported in the present study places it among the most sensitive of invertebrates tested to date to a glyphosate-based chemical.

The 48-h EC50 (0.5 mg/L) for MON 0818, the POEA surfactant blend used in Roundup, for *L. siliquoidea* glochidia, to our knowledge, is the lowest reported for any species tested to date. MON 0818 was as toxic, or more toxic, than the glyphosate-based chemicals in acute and chronic tests with *L. siliquoidea*. Folmar et al. [15] described MON 0818 LC50s for four species of fish and a midge larvae ranging from 1.0 mg/L for *Oncorhynchus mykiss* to 13 mg/L for *Ictalurus punctatus* and *Chironomus plumosus*. Giesy et al. [13] reported a range of 48-h LC50s for MON 0818 from 2.0 to 4.1 mg/L for daphnids. Similarly, Wang et al. [30] reported an LC50 of 2.9 mg/L for *D. magna* in water-only toxicity tests with MON 0818. Roundup is not registered for use over water; however, overspray and runoff have led to measured concentrations of glyphosate in streams in agricultural regions as high as 2.3 mg/L [13]. As outlined by Wang et al. [30], direct application of Roundup with 30% glyphosate a.e. and 15% MON 0818 to shallow water bodies (1.0 m or less) at the maximum recommended application rate of 4.2 kg glyphosate a.e./ha (i.e., 2.1 kg MON 0818/ha) would result in an instantaneous MON 0818 concentration greater than 0.2 mg/L. The U.S. Environmental Protection Agency [31] assumes that a pesticide poses an acute risk to nontarget aquatic biota when the expected environmental concentration exceeds one-tenth of the acute LC50 or EC50 for the most-sensitive species. Based on the findings of the present study, the acute threshold concentration for MON 0818 in an aquatic environment would be 0.05 mg/L, which is exceeded by the expected environmental concentration (0.2 mg/L) by fourfold (thus posing an acute risk to nontarget biota). No reports were found in the literature of environmental MON 0818 or POEA concentrations; however, assuming a 2:1 ratio of glyphosate:MON 0818, environmental concentrations of MON 0818 could be as high as 1.15 mg/L, greater than 20× the acute threshold concentration (0.05 mg/L). Wang et al. [30] reported that the toxicity of MON 0818 to *D. magna* declined rapidly in the presence of sediment due to adsorption of the surfactant to sediment particles. Therefore, more research is needed to determine the toxicity of MON 0818 and POEA surfactants to benthic dwelling organisms, including freshwater mussels, in the presence of sediment.

Growth was significantly less in the highest test concentrations of technical-grade glyphosate (compared to other treatments) and followed a similar trend with MON 0818, but we were unable to evaluate growth in the highest test concentrations of the other three chemicals because substantial mortality (i.e., >50% in all three replicates of the treatments) had occurred by the end of the test. Interestingly, an inverted U-shaped concentration-response curve, in contrast to a traditional linear toxicity response curve, was evident for growth of mussels exposed to Roundup, technical-grade glyphosate IPA salt, and Aqua Star; however, the difference in growth of treatments compared to controls was not statistically significant for any of these compounds. The similarity in concentration-responses for these three compounds is consistent with the common active ingredient in each; Aqua Star and Roundup both contain technical-grade glyphosate IPA salt as the active ingredient, suggesting that a component of glyphosate IPA salt may be contributing to growth. Increased growth of mussels in low and intermediate treatment concentrations partially may be explained by the additional carbon or nitrogen from test compounds available for microorganisms, which in turn could provide a supplemental food source for the mussels [32,33]. At higher test concentrations, toxicity would preclude any beneficial effects. Additional research is needed to definitively determine the effects of glyphosate-based chemicals on growth.

Few published studies report the growth of juvenile mussels in static conditions in the laboratory; however, growth rates of mussels in control treatments in the present study (3.7–4.7 μm/d) are consistent with those reported for juvenile *L. cardium* (5.9 μm/d after 10 d) by Newton [34]. Similarly, Lasee [35] reported that juvenile *L. cardium* grew an average of 6.6 μm/d in the control treatment after 7 d. However, Wang et al. [36] reported 28-d growth (as percent increase in shell length) of 47 to 71% for juvenile *Villosa iris* in a flow-through lab study. Additional research is required to determine the optimal diet and feeding rate for juvenile mussels in static laboratory tests and to determine the utility of growth as an endpoint for static toxicity tests with juvenile mussels.

The age of juveniles in the present study ranged from one to eight weeks posttransformation. Although this is a very short time period relative to life expectancy (several decades) of most mussel species, limited evidence suggests that there may be age/size-related differences in sensitivity of juvenile mussels to some contaminants [37]. Newly-transformed juveniles were more sensitive to copper than juveniles that were two months posttransformation. Because earliest life stages may be most sensitive, we recommend that future toxicity tests with juvenile mussels be performed with newly-transformed juveniles.

CONCLUSION

In conclusion, these tests indicate that native freshwater mussels are among the most sensitive invertebrates tested to date with glyphosate-containing compounds and the surfactant blend MON 0818. The toxicity of Roundup could not be attributed to surfactant alone, because glyphosate IPA salt, the active ingredient in Roundup, also was toxic to both mussel glochidia and juveniles likely due to liberation of ammonia from the amine group of the glyphosate IPA salt. Future research should examine further the toxicity of glyphosate-based herbicides and MON 0818 to native freshwater mussels in the presence of sediment and efforts should be made to determine environmental concentrations of POEA surfactants.

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