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Chronic Toxicity of Diphenhydramine Hydrochloride to a Freshwater Mussel, *Lampsilis siliquoidea*, in a Flow-Through, Continuous Exposure Test System

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Abstract Freshwater mussel populations are declining in North America. Potential anthropogenic stressors may be contributing to the declines and may include the continual presence of pharmaceutical compounds in waterways. Diphenhydramine hydrochloride (DH) is an over-the-counter antihistamine marketed under several name brand products including the common U.S. trademarked product, Benadryl^{TM}. The toxicity of DH to freshwater mussels was assessed by initiating an unprecedented 28 day, continuous exposure trial with 1 day old mussels. Results indicated that the survival and growth of *Lampsilis siliquoidea* was not impacted by DH concentrations $\leq$121 μg/L after 28 days of continuous exposure. With the successful completion of this study, the techniques are now verified to evaluate the toxicity of waterborne compounds initiating 28-day chronic exposures with 1 day old mussels.

Keywords Freshwater mussel · Toxicity · Diphenhydramine hydrochloride

Freshwater mussels are an imperiled organism in North America (Williams et al. 1993; Neves 1999; Neves 2004). The causes for the gradual loss of unionid abundance and diversity have not been well characterized. Potential anthropogenic stressors that could be contributing to the declines include siltation, dams, mining wastes, introduction of exotic bivalve species such as zebra mussels (*Dreissena polymorpha*) and Asian clams (*Corbicula fluminea*), industrial wastes, agricultural pollution, and pharmaceuticals and personal care products (PPCP).

Pharmaceuticals and personal care products are a group of compounds that include prescription and over-the-counter therapeutic drugs, detergent by-products, fragrances, cosmetics, sunscreen agents, and diagnostic agents. These compounds are continually introduced into the aquatic environment through wastewater treatment plants and runoff from domestic sewage sludge, farmyard manure, and landfills. Kolpin et al. (2002) documented the presence of many PPCP in surface waters across the United States. The ramifications of long-term PPCP persistence in the aquatic environment are largely unknown.

Diphenhydramine hydrochloride is an over-the-counter compound primarily used as an antihistamine that is marketed under several name brand products including the common U.S. trademarked product Benadryl^{TM} (any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.) To our knowledge, there was no publically available data describing DH toxicity to any chronically exposed aquatic invertebrate until Meinertz et al. (2010) reported that DH at relatively low concentrations significantly impacted on the survival and reproduction of *Daphnia magna*. Because DH, at a relatively low concentration, was toxic to what is considered a sensitive aquatic invertebrate organism, there was concern that DH was potentially toxic to the freshwater mussel. Therefore, this study was conducted to determine if continuous exposure to DH for 28 days would impact the survival and growth of 1 day old mussels.

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Materials and Methods

Diphenhydramine hydrochloride [Chemical Abstract Service (CAS) number, 147-24-0; molecular weight, 291.82; purity, 100 %] acquired from Sigma-Aldrich, Inc. (St. Louis, MO) was used to prepare a DH stock solution every 8 days or less by quantitatively transferring 0.020 ± 0.001 g to a 250 mL volumetric flask with deionized water (water deionized to a specific resistance of >17.8 mΩ cm^{-1} with a Barnstead E-pure water purification system, Dubuque, IA) and adjusting the flask volume to 250 mL with deionized water. Analytical standards used to generate high pressure liquid chromatography (LC)-mass spectrometry (MS) calibration curves were prepared by diluting the stock solution with deionized water.

Identification and quantification of the test chemicals were performed using an Agilent 1100 LC system (Agilent Technologies, Inc., Santa Clara, CA) with a Waters XBridge™ C18, 3.5 µm, 3.0 × 100 mm analytical column (Waters Corporation, Milford, MA) and an Agilent model G1946D mass selective detector. The LC parameters included an isocratic mobile phase of 85 % solvent A (59.8 % water, 40 % acetonitrile, and 0.2 % acetic acid) and 15 % solvent B (methanol), a flow rate of 0.5 mL/min, an injection volume of 20 µL, a column temperature of 35°C, and a run time of 6 min. Mass spectral detection was achieved using atmospheric pressure electrospray ionization in the positive mode. Mass spectrometer spray chamber operating parameters included a capillary voltage of 3,250 V, a nebulizer pressure of 2.76 bar, a drying gas (nitrogen) flow rate of 13 mL/min, and a temperature of 350°C. The single ion monitoring parameters are presented in Table 1. The acceptable retention time difference between the quantitation and confirmation ions was ≤0.03 min. The DH concentration in each sample was determined from the DH peak area and a quadratic regression equation developed from 6 analytical standard solutions with nominal DH concentrations ranging from 0.27 to 133 µg/L.

Largemouth bass were infested with glochidia from the freshwater mussel species, *Lampsilis siliquoidea*, according to procedures similar to those described in Meinertz et al. (2011). Sixteen days later, each test chamber was stocked with 40 newly transformed, 1 day old mussels, displaying foot movement. In addition, for an assessment of Day 0 length, 20 mussels, 1 day old and displaying foot movement, were transferred to a 20-mL glass vial and stored in a 10 % buffered formalin solution. One day later, those mussels were transferred to a 70 % ethanol solution.

Table 1 Parameters for determining DH concentrations using a LC–MS system under single ion monitoring conditions (electrometer gain, 2 V; tolerance, 20 %)

Product ion	Fragmentor voltage (V)	Ion assignments (*m/z*)	Confirmation/ quantitation ions ratio
DH quantitation	60	256.1[a]	89
DH confirmation	120	167.1	

[a] Mass + hydrogen

The experimental design included 6 treatment groups with 10 test chambers per treatment group. Treatment groups included a control group and 1 of 5 groups exposed to DH at 0.5, 2.5, 12.5, 50, and 100 µg/L. The test chamber was the experimental unit. Test chambers were configured in 10 blocks of 6 test chambers in a 2 × 3 configuration. Treatment was assigned to the test chambers according to a randomized block design so that each treatment group was represented only once in each block.

The test system was constructed with materials and operated with parameters described in the standard guide for conducting toxicity trials with freshwater mussels (ASTM 2006). The test system was described in Meinertz et al. (2011). Briefly, incandescent lighting provided 16 h of light and 8 h of darkness with 20 min transition periods for lights to fade on and off. There were 60 test chambers. Test chambers were modified 250-mL glass beakers containing 25 ± 1 g of 75 to 150 µm silica sand (blend FW 120, Badger Mining Corporation, Berlin, Wisconsin). Water pumped from facility wells (groundwater) flowed into a headbox, then through distribution boxes (1 box for each of the 6 treatment groups), then through the test chambers at 10.5 mL/min. The DH working solutions (1 for each treatment group and prepared every 4 days or sooner in water) and the food solution were metered into distribution boxes using a Masterflex® Digi Staltic pumping system with model 77310-01 pump drives, Masterflex® Easy-Load® II model 77202-60 heads, and model 77310-02 controllers. A food solution was prepared daily in 15 L of water with 9 mL of *Nannochloropsis* 3600 Instant Algae®, 4.5 mL of *Tetraselmis*, and 6 mL of *Chlorella* (all products of Reed Mariculture, Campbell, CA) into a 20 L Nalgene® vessel.

Each day, water samples (1 mL) from each test chamber of a common treatment group were pooled in a 20-mL glass vial. Pooled water was mixed and a portion filtered into a 2 mL amber glass LC vial (Agilent Technologies Inc.) through Pall GHP membrane, 0.2 µm, 13-mm, Acrodisc® syringe filter (Pall Corporation, East Hills, NY). Vials were stored at about 4°C until DH concentrations were determined by LC–MS.

Daily dissolved oxygen concentrations ranged from 7.46 to 8.68 mg/L. Daily pH ranged from 7.21 to 7.63. Daily temperature ranged from 20.0 to 20.7°C. Flow rates through test chambers ranged from 9.4 to 11.6 mL/min. Weekly measurements of alkalinity and water hardness

ranged from 118 to 130 mg/L as $CaCO_3$ and 166 to 178 mg/L as $CaCO_3$, respectively. Weekly light intensity measurements directly over the chambers ranged from 5 to 215 lux during the 16 h periods of light.

Twenty eight days after stocking the test chambers, the contents of each chamber were rinsed through a 202 μm sieve with well water at 21°C. The material collected on the sieve was rinsed into a petri dish. Live and dead mussels were enumerated with the aid of a dissecting scope. Mussels were classified as alive if foot movement or ciliary activity was observed. Live mussels were enumerated, transferred to a 20-mL glass vial containing a 10 % buffered formalin solution. One day later, mussels were transferred to a 70 % ethanol solution. Within 3 weeks, pictures of mussels were taken with a QImaging Micropublisher 3.3 RTV digital camera (QImaging, Canada) through a Nikon Eclipse model E600 microscope (Nikon Corporation). Shell length (distance across the shell parallel to the hinge) was determined using imaging software (Image Pro® Express, version 6.0.0.319, Media Cybernetics, Silver Spring, MD).

Statistical significance was declared when $P \leq 0.05$. The mussels could experience one of two outcomes, survival or death. Survival and death are therefore binary random variables that presumably follow Bernoulli distributions. The response data were categorized as the numbers of survivors or dead out of a fixed number of mussels in each test chamber. Test chambers were considered to be the fundamental experimental units. Exposure concentration was considered a categorical (classification) variable rather than a continuous variable in the data analysis because there was a limited number of concentrations whose range was exaggerated, making identification of a continuous response function impossible. The probability of survival at day 28 was modeled by a generalized linear mixed model fitted using the SAS GLIMMIX facility based on Wolfinger and O'Connell (1993). Overdispersion within the model was modeled using an R-side covariance structure. Comparison of least-square means was used to determine significant differences between treatment groups. Percent survival was determined by comparing the number of live mussels found with the number of mussels stocked into each chamber.

Shell length (μm) was determined only for mussels that survived until the end of the study. Data were evaluated to ensure assumptions of normality and equality of variance. Shell length data as a function of treatment group were fit to a generalized linear model (McCullagh and Nelder 1989) using the SAS GLM facility and model parameters and their 95 % confidence intervals were estimated using maximum-likelihood estimation. Likelihood-ratio F test was used to test hypotheses about the relation between shell length and treatment group. Determination of significant differences between treatment groups was accomplished by pair-wise comparison of least-square means using a Bonferroni adjustment for multiple comparisons.

Results and Discussion

The ASTM guide recommends that acute toxicity tests (<14 days) be conducted with juvenile mussels that are within 5 days post transformation and that chronic studies (>14 days) be conducted with mussels that are 60–120 days post transformation (ASTM 2006). The primary reason for the age designation was the substantial increases in mussel mortality that begin around 14 days post transformation (14 days old) and continued until about 42 days post transformation. The mortality has been attributed to an inappropriate food supply during this life stage. It is generally understood that the earliest life stages of aquatic organisms are regarded as the most sensitive. For example, Valenti et al. (2006) reported that there was a significant trend of declining chlorine toxicity with increased mussel age. Initiating chronic studies with 2–4 months post transformation mussels potentially overlooks critical life stages beginning immediately after transformation and continuing through the next 2 months.

In the current trial, the food solution offered continuously for 28 days resulted in >80 % survival not only in the unexposed group but also in the treatment group exposed to the highest DH concentration (Table 2). Survival was not significantly different among the treatment groups.

Because relatively low (≥71 μg/L) DH concentrations had negative impacts on *D. magna* (Meinertz et al. 2010) and the exposure duration for the current trial was to exceed the exposure duration of the *D. magna* trial, the concentration range for the current trial was reduced from what was used during the *D. magna* trial. As the results indicated, the reduction in maximum exposure concentration from about 850 μg/L to a nominal concentration of 100 μg/L (about 4,300 times the environmental concentration; Focazio et al. 2008) was too great of a reduction to identify toxic effect of DH exposure in mussels. Despite the lack of a toxic effect, the results from this study did indicate that *L. siliquoidea* is a more rugged organism than *D. magna* when exposed to DH. These results are consistent with the results of Keller et al. (2007) and Milam et al. (2005) who also reported that mussels are generally less sensitive to chemical exposures than *D. magna*.

Several factors may have contributed to an overall mean recovery (88 %) of live and dead mussels of less than 100 % (Table 2). Shells from newly transformed mussels are relatively fragile and may have become damaged during stocking procedures. Shells from fatally damaged mussels likely deteriorated through time and were most

Table 2 Mussels recovered from test chambers after continuous exposure to DH for 28 days

Treatment group (μg/L)	Mean recovered alive and dead	Mean recovered alive	Mean recovery (%)	Mean survival (%)	SD (%)	r.s.d. (%)
0.0	36.8	34.1	92.0	85.3	6.9	8.1
0.5	33.9	31.7	84.8	79.3	9.9	12
2.5	36.8	34.4	92.0	86.0	8.2	9.5
12.5	32.6	30.2	81.5	75.5	10	14
50	35.4	33.1	88.5	82.8	13	15
100	36.8	33.0	92.0	82.5	3.9	4.7

Table 3 Mean shell lengths of mussels after continuous exposure to DH for 28 days

Treatment group (μg/L)	n	Mean shell length (μm)	SD (μm)	r.s.d. (%)	Min (μm)	Max (μm)
1 day old	20	283	20	7.0	249	317
0.0	327	748	107	14	435	1,020
0.5	301	781	135	17	390	1,173
2.5	330	769	123	16	337	1,128
12.5	293	776	122	16	409	1,055
50	328	746	104	14	297	971
100	316	742	105	14	462	1,020

Table 4 DH concentrations in pooled water samples from all test chambers within a treatment group

	Treatment group (μg/L)				
	0.5	2.5	12.5	50	100
Mean	1.0	2.5	12.5	55.1	121
SD	0.6	0.37	1.8	10	23
r.s.d.	54	15	14	18	19

likely not recovered during reconnaissance procedures at the end of the study.

The mean shell length of 1 day post transformation mussels was 283 μm (Table 3). Mean shell lengths of mussels surviving to the end of the trial ranged from 742 μm (100 μg/L group) to 781 μm (0.5 μg/L group). Shell lengths were not significantly different among the treatment group.

Mean DH concentrations in pooled water from test chambers of each treatment group were within 21 % of the target concentrations with the exception of the 0.5–μg/L group (Table 4). DH was not present in water samples from the control group.

Previous to the current study, there has been no study published where the toxicity of a compound was evaluated by initiating a 28-day chronic exposure with 1 day old mussels. Results from this study indicated that continuous exposure of juvenile *Lampsilis siliquoidea* to DH concentrations $\leq$121 μg/L for 28 days did not significantly impact mussel survival or growth.

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