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Effects of nitrate on freshwater mussel glochidia attachment and metamorphosis success to the juvenile stage☆

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ABSTRACT

Water quality and contaminants have been frequently identified as critical stressors for freshwater mussels, many species of which are highly imperiled throughout North America and the world. Nutrient pollution, specifically nitrate, has become one of the most prevalent causes of water quality degradation globally, with increasing anthropogenic input from suburban and agricultural runoff, municipal wastewater, and industrial waste. Nitrate acute toxicity is generally low for aquatic species, but the potential effects of nitrate exposure are largely unknown for freshwater mussels, particularly during the parasitic stage of their complex lifecycle. Therefore, this study was designed to determine the effects of short-term nitrate exposure at environmentally relevant concentrations on juvenile production in two freshwater mussel species. *Lampsilis siliquoidea* and *L. fasciola* glochidia were exposed to nitrate (0, 11, or 56 mg NO_3-N/L) for 24 h before inoculation on a primary host, Largemouth Bass (*Micropterus salmoides*). Glochidia attachment, metamorphosis success, and total number of juveniles produced were monitored on individual fish. Exposure of *L. siliquoidea* glochidia to 56 mg NO_3-N/L nitrate resulted in a significant ($p = 0.02$) 35% reduction of total juveniles produced, a combined result of moderate decreases in both glochidia attachment and metamorphosis success. A similar trend (28% reduction; $p = 0.06$) was evident with 11 mg NO_3-N/L. No effects were apparent for *L. fasciola,* suggesting species-specific differences in responses even among closely related species. These results are the first to suggest that glochidia exposure to nitrate may adversely affect juvenile recruitment in some species. Findings from these studies are important for improving characterization of the hazards of nitrate pollution to aquatic life and this work will help better define the role of water quality in assessing habitat suitability for mussel conservation efforts.

1. Introduction

Freshwater mussels play an important role in aquatic ecosystems, serving as a link between the water column and benthos, providing filtration and nutrient cycling, and serving as a food source for other aquatic animals (Vaughn, 2010). Their distribution and abundance has been declining in North America and globally (Lydeard et al., 2004; Ricciardi and Rasmussen, 1999). In the United States an estimated 10% of species are thought to be extinct and 65% of remaining species are considered to be threatened, endangered or vulnerable (Haag and Williams, 2014). Many factors have been implicated in these declines including pollution and invasive species, but there is limited evidence for effects of widespread pollution on mussel populations (Downing et al., 2010). Mussels are known to be sensitive to some ionic compounds such as ammonia, potassium, copper, and cadmium (Haag, 2012), but the toxicity of many common pollutants to this group is still unknown.

Over the last century increases in anthropogenic nitrogen inputs such as combustion of fossil fuels, application of fertilizers, and release of domestic wastewater, have substantially altered the nitrogen cycle and resulted in widespread aquatic nitrogen pollution (Galloway et al., 2003). Aqueous nitrogen pollution occurs in the forms of nitrate, nitrite, and ammonia. Nitrate is the most common aqueous form of nitrogen, with surface water concentrations reported as high as 120 mg/L NO_3-N (Iowa Department of Natural Resources, 2017). Due to its ubiquitous presence and increasing

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environmental concentrations, there has been increasing interest in the toxicity of nitrate to freshwater organisms. Nitrate generally has low acute toxicity to aquatic organisms (Baker et al., 2017; Scott and Crunkilton, 2000; Soucek and Dickinson, 2012), but recent evidence suggests that exogenous nitrate is biologically active and can affect hormone production, reproduction, secondary sex characteristics, growth, behavior, and development in aquatic organisms (see Edwards and Hamlin, 2018). Additionally, there is growing evidence that nitrate may be associated with freshwater mussel declines. For example, the probability of occurrence of freshwater mussel species was reduced in reaches of the Luznice River (Czech Republic) with elevated nitrate concentrations (Douda, 2010). Additionally, adult mortality in wild *Margaritifera margaritifera* populations in central Europe was positively correlated with nitrate concentrations (Bauer, 1988).

One of the challenges in assessing the role of nitrate pollution in freshwater mussel declines is the unique lifecycle of these animals. Freshwater mussels have a complex lifecycle that includes an obligate parasitic stage in which the larvae (glochidia) must attach to a host fish to metamorphose into the juvenile stage. Initially, fertilized eggs develop into glochidia, which are brooded in marsupial gills within a female mussel. Once the glochidia are ready for release, mussels utilize a variety of strategies to attract and infect an appropriate host fish, including lures, conglutinates, and broadcasting free glochidia into the water column (Barnhart et al., 2008; Haag, 2012). When glochidia encounter a host, they attach by clamping onto the gills or skin. Glochidia attached to a suitable host become encapsulated by host epithelial tissue (forming a cyst) and undergo an anatomical metamorphosis (typically 2–4 weeks) to develop the adult structures present in juvenile mussels before dropping from the host fish (Arey, 1932; Haag, 2012). Due to the variety of strategies utilized, the amount of time glochidia are exposed in the water column before being encapsulated on a host may vary from several hours to up to 14 days (Fritts et al., 2014; Haag, 2012).

Most toxicity studies evaluate the effects of contaminants on glochidia viability (a proxy for survival) or juvenile survival (Reviewed in Ingersoll et al., 2007) and fail to address other components of the mussel lifecycle. Few studies have addressed the effects of contaminants on the parasitic phase of the lifecycle, such as the ability of the glochidia to attach to fish and successfully undergo metamorphosis into juvenile mussels. Many declining mussel populations are characterized by the absence of juveniles, indicating a loss of recruitment (Gascho Landis and Stoeckel, 2016; Strayer and Malcom, 2012) and illustrating the need for more information to better understand factors that may limit recruitment. The increasing scale and severity of nitrate pollution, growing evidence of adverse nitrate effects in mussels and other aquatic organisms, and lack of information about contaminant effects on juvenile mussel recruitment, all lead to development of the present study to determine the effects of nitrate on the parasitic phase of the freshwater mussel lifecycle. We exposed glochidia of two species to environmentally relevant concentrations of nitrate and assessed the glochidia attachment and metamorphosis success to the juvenile stage.

2. Methods

2.1. Animal sourcing and holding

Twelve brooding female *Lampsilis siliquoidea* were obtained from a captive population maintained at the Kansas City Zoo by Dr. Chris Barnhart at Missouri State University. The mussels were transported in coolers of source water to the University of Georgia where they were acclimated over a period of 72 h to dechlorinated municipal tap water (Hardness: 40–50, Alkalinity: 35–45, pH: 7.4–7.8) and were held at 15 °C until the initiation of exposures. Eight brooding female *Lampsilis fasciola* were obtained from the Alabama Aquatic Biodiversity Center (Marion, AL), transported in coolers in moist towels to the University of Georgia and were acclimated over a period of 72 h to dechlorinated municipal tap water at 15 °C. Before initiation of exposures, adult mussels were acclimated to 20 °C over a period of 48 h. Juvenile (total length: 130–167 mm) Largemouth Bass (*Micropterus salmoides*) were obtained from American Sportfish Hatchery (Pike Road, AL) in March and October of 2016. The fish were held in dechlorinated municipal tap water and received pelleted food daily at a rate of 1–2% of body weight. Largemouth Bass are suitable primary hosts for both *L. siliquoidea* and *L. fasciola* (Trdan, 1981; Zale and Neves, 1982). All holding and experimentation occurred at the Aquatic Biology and Ecotoxicology Laboratory at the University of Georgia between March 2016 and July 2017.

2.2. Glochidia exposures – L. siliquoidea and L. fasciola

All test solutions were prepared in reconstituted moderately hard water (Hardness: 80–100 Alkalinity: 65–75 pH: 7.90–8.20) according to Smith et al. (1997). Nitrate solutions were prepared with sodium nitrate (CAS: 7631-99-4) dissolved directly in the reconstituted water. For *L. siliquoidea and L. fasciola*, nominal concentrations of 0 (control), 11 mg/L NO_3-N, and 56 mg/L NO_3-N were tested. These concentrations were selected to represent environmentally relevant concentrations. The 11 mg/L NO_3-N treatment was selected to be representative of the 10 mg/L NO_3-N U.S. drinking water criteria for human health (United States Environmental Protection Agency, 2009) while the 56 mg/L NO_3-N treatment is representative of high concentrations regularly measured in agriculturally intensive areas (U.S. Geological Survey, 2018).

For both species, glochidia were extracted from brooding females by gently opening their valves and using a gentle stream of water from a syringe to flush glochidia from the marsupial gills. Glochidia viability was determined by exposing a subsample (400–600) of glochidia to saturated NaCl solution and assessing the closure response as described by ASTM International (2013). The viability of a subsample of glochidia was calculated by subtracting the number of glochidia that did not close after the addition of NaCl from the number that were originally open, then dividing by the total number of glochidia (initially open and closed). Glochidia from female mussels ($n = 4–5$) with initial viability >90% were pooled, homogenized by gently mixing for 60 s, and randomly divided into replicate glass beakers (11,000–15,000 *L. siliquoidea*; 4700–11,000 *L. fasciola* glochidia per beaker) with 150 mL of test solution. For the exposure, the test beakers were maintained in a water bath for 24 h at 20 °C with 16L:8D photoperiod. For *L. siliquoidea* there were six replicates of the three nitrate treatments for a total of $n = 18$ experimental units (beakers). For *L. fasciola* there were five replicates of each nitrate treatment for a total $n = 15$ experimental units (beakers). *L. siliquoidea* exposures took place in March of 2016 and *L. fasciola* exposures took place in June of 2017. Temperature, pH, and dissolved oxygen were measured in a composite sample of each treatment at the initiation of exposures for both species. Nitrate concentrations were measured were measured at initiation and conclusion of exposures via nitrate Ion Selective Electrode (Thermo Scientific, Waltham, MA). Hardness and alkalinity were measured in the control composite at the initiation of exposures via titration. For the 24-h *L. siliquoidea* glochidia exposure, mean temperature was 19.3 °C ± 0.1 (average ± SD), pH was 8.18 ± 0.13 and dissolved oxygen was 9.1 ± 0.2 mg/L. Average nitrate concentration measured in the control treatment was 0.6 ± 0.0 mg/L NO_3-