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Acute effects of salinity exposure on glochidia viability and host infection of the freshwater mussel *Anodonta anatina* (Linnaeus, 1758)

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HIGHLIGHTS

- Effects of salinity on endangered mussels are largely unknown.
- *Anodonta anatina* release their larvae in the winter during elevated salt runoff.
- Chloride concentrations above 727 mg·L^{-1} affected *A. anatina* glochidia viability.
- Glochidia attachment to hosts was negatively correlated with chloride concentration.

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ABSTRACT

Freshwater mussels of the Unionida are globally in decline and knowledge of the sensitivity of their vulnerable life stages to stressors is crucial for their conservation. Increasing salinity, e.g., due to road deicing, has been proposed as an important stressor, but its impact on the complex interaction between mussel larvae and their obligate attachment to fish hosts remains largely unknown. This study tested the acute toxicity effects of environmentally relevant chloride concentrations on larvae of European *Anodonta anatina* mussel as well as the impacts on attachment rates of the larvae to their fish host. Chloride concentrations above 727 mg·L^{-1} significantly affected glochidia viability and the 24 h EC_{50} value was determined at 2505 mg·L^{-1}. Successful attachment of glochidia to the host fish *Phoxinus phoxinus* was negatively correlated with increasing chloride concentration and became significant at concentrations >2909 mg·L^{-1}. Comparable responses could be observed by separately counting fin and gill attached glochidia, while gill attachment showed the highest correlation with overall attachment rates. These results indicate a potential threat from short-term elevated chloride concentrations during runoff events on sensitive life stages of freshwater mussels. Consequently, we propose additional chloride sensitivity tests on other mussel species as well as the reduction of salt peak input loading into freshwater bodies through a 3R-principle (restriction of use, retention of runoff for peak concentration avoidance and replacement by alternatives) in areas where endangered mussels occur.
Capsule: Freshwater mussels of the Unionoida are globally in decline and knowledge on the sensitivity of the most vulnerable larval stages to salinity is crucial for their conservation.

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1. Introduction

Salinization of freshwater ecosystems has recently been recognized as a major threat to ecosystem health (Gillis, 2011; Schulz, 2011; Canedo-Arguelles et al., 2013). Sources of salinization comprise agricultural irrigation, mining activities, sewage and industrial effluents and the usage of road deicing salts in cold climate regions (Marsalek, 2003; Canedo-Arguelles et al., 2013). Road deicing salts have been shown to increase ambient concentrations of chloride in surface waters in several areas (Thunqvist, 2004; Kaushal et al., 2005; Novotny et al., 2008). Winter runoff from highways with high concentrations of road deicing salt typically causes a significant increase in the salinity levels of surrounding waters (Koryak et al., 2001; Marsalek, 2003; Norrström, 2005). The region-specific background concentrations, originating from natural sources, can be critically exceeded during winter. As summarized by Novotny et al. (2008), the reported chloride concentrations in the northeastern United States reach values as high as 5000 mg·L^{-1} in urban streams. Surveys conducted in Germany and Sweden reported chloride concentrations in road runoff up to 3490 mg·L^{-1} in winter compared to average background concentrations of 15.6 mg·L^{-1} during summer (Bäckström et al., 2003; Schmidt, 2010).

Changes in salinity can lead to disturbance of ecosystem patterns, processes and species communities. Several studies reported impacts

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on microbial, algal and macroinvertebrate communities, functional feeding group composition, leaf-litter processing rates and osmoregulatory stress in individual species in river systems (Dickmann, 1978; Blasius and Merritt, 2002; Porter-Goff et al., 2013). The present study focused on freshwater mussels, as they are known to play a key role in ecosystem processes through their influence on nutrient cycling, bioturbation and deposition of suspended materials (Vaughn, 2010). Additionally, freshwater mussels belong to the most threatened taxonomic group in freshwater systems in the northern hemisphere (Bogan, 1993; Geist, 2011). Due to the high conservation priority of freshwater mussels, there is an urgent need to understand the reasons for their decline (Strayer et al., 2004; Geist, 2010). In particular, the larval stages are supposed to be more vulnerable compared to many other species (e.g., Wang et al., 2007), and knowledge of their sensitivity to environmental stressors is thus urgently needed.

Freshwater mussels of the Unionoida have a complex life cycle which includes the release of glochidia, mussel larvae that need to attach to a host fish for further development (Barnhart et al., 2008). The attachment of glochidia to either the skin or gill tissue of the fish host is controlled by an ion gradient (Lefevre and Cutis, 1912). As such, the use of saturated salt solutions is used to assess valve closure responses of glochidia as a measure of viability in toxicity studies (ASTM E2455–06, 2006) and for host infections in conservation programs (Taeubert et al., 2012, 2013a). Premature clamping of glochidia induced by increased salinity in the wild may ultimately reduce the numbers of larvae able to encyst on fish hosts and thus be a factor in the observed decline of freshwater mussels. Knowledge of the ecotoxicological relevance of salinity on the most sensitive life stages of freshwater mussels is particularly relevant to establish scientifically based threshold values for environmental regulation. Quantitative relationships between toxicity and glochidia viability are rarely investigated. A recent study by Fritts et al. (2014) reported for the first time the relationship of glochidia viability and infectivity. NaCl toxicity data for glochidia from a range of species have been included in species sensitivity distributions used to derive Water Quality Guidelines in Canada (CCME, 2011). However, negative effects of NaCl to unionid mussels have so far been reported almost exclusively for North American species (Bringolf et al., 2007; Gillis, 2011; Pandolfo et al., 2012; Blakeslee et al., 2013; Fritts et al., 2014). Gillis (2011) reported significant variation in glochidia EC_{50} values, ranging from 100 to 1400 $mg \cdot L^{-1}$ chloride. Another study by Bringolf et al. (2007) reported 48 h EC_{50} values ranging from 339 $mg \cdot L^{-1}$ for *Lampsilis siliquoidea* to 2202 $mg \cdot L^{-1}$ chloride for *Villosa delumbis*. Elevated ion concentration caused by anthropogenic sources of salinity cannot only lead to death of the glochidia but also induce a clamping response. Those glochidia are typically considered as ecologically and functionally dead since the clamping prevents the obligate attachment to their hosts, but only one study, to our knowledge, addressed this mechanism so far in a North American species (Fritts et al., 2014). The European pond mussels *Anondonta anatina* and *Anodonta cygnea* release glochidia during winter months and early spring, when the highest load of road deicing salts are applied and expected to increase salt input into rivers (Aldridge, 1999). We thus chose *A. anatina* as a model species. *A. anatina* is still widespread in Europe, distributed from the Iberian Peninsula in the Southwest, to Scandinavia in the North and Russia in the East (Zettler et al., 2006), but the species already disappeared from several parts of the continent and is listed as threatened and protected in Germany (IUCN, 2014). In Europe, the species has a region-specific conservation status, ranging from “critically endangered” to “vulnerable” (IUCN, 2014).

We determined the acute (24 h) toxic effect of increased chloride concentrations on *A. anatina* glochidia viability, testing the hypothesis that there is a negative effect on the glochidia–host interaction. In order to quantify the effect of acute elevated salt exposure on *A. anatina*, we investigated their attachment rates on gills, fins, and skin of *Phoxinus phoxinus* immediately after the 24 h toxicity test, allowing for an evaluation of the ecological performance of surviving glochidia.

2. Material and methods

2.1. Test species

Mussels (*A. anatina*, Linnaeus, 1758) were collected from a fishpond near Reinhardswinden (Bavaria, Germany) and an artificial lake near Hof (Bavaria, Germany) in March and October 2013. Mussels were transported to the institute in Freising (Bavaria, Germany) in aerated containers and maintained in an 8000 L artificial fishpond supplied with local groundwater for 3 months prior to glochidia harvesting. Upon arrival at the institute, all of the collected mussels were genetically validated to be *A. anatina* following the RFLP method as described by Zieritz et al. (2012) using hemolymph samples for DNA extraction (Geist and Kuehn, 2005). In the weeks prior to the period of glochidia release, the mussels were gently checked for ripe glochidia on a regular basis by visual examination of their gills.

A. anatina carrying ripe glochidia were transferred to the laboratory to harvest the glochidia. The glochidia were isolated following the ASTM standard guideline E2455-06 (ASTM E2455–06, 2006). Mature glochidia were gently flushed from the marsupia of the adult mussels into a 1 L Duran glass beaker using a squirt bottle. Maturity and viability of the isolated glochidia were evaluated as recommended by ASTM standard guideline E2455-06 (ASTM E2455–06, 2006). Mature glochidia from 4 females were pooled for further experiments. Prior to the toxicity test, glochidia were held in filtered local well water for 1 h in a 1 L Duran glass beaker. The mean viability of glochidia before toxicity testing was 94.2% (standard deviation 3.9). To account for the typical winter stream water temperatures, when salt peak input from the application of road deicing salts is highest, the test temperature was adjusted to 11 °C (±0.1 °C) instead of the 20 °C according to the standard guideline (ASTM E2455–06, 2006).

Eurasian Minnow (*P. phoxinus*), a common and suitable host for *A. anatina* (Bauer et al., 1991), were obtained from a local fish farm (Fischereibetrieb Rösch, Bavaria, Germany). To avoid the possibility of an immune response due to previous contact with glochidia, the fish originated from a hatchery where previous contact with freshwater mussels could be excluded. Fish were acclimatized to the same environmental conditions as used during the experiment for several weeks. Fish had a mean size of 8.6 ± 1.2 cm (total length (TL) ± standard deviation (SD)) and a mean weight of 8.4 ± 2.1 g (wet weight ± SD).

2.2. Glochidia acute exposure

NaCl 24 h acute toxicity was tested according to ASTM standard guideline E2455-06 (ASTM E2455–06, 2006) with the exception that a lower number of 150 to 350 glochidia were used in each replicate, as suggested by Bringolf et al. (2007). Local well water, filtered through a 4 μm filter (Machery-Nagel, Germany), was used for preparation of test solutions. The specific ion composition of the exposure water is listed in Table 1. Standard grade NaCl (99.5% purity, Merck, Germany) was used for the preparation of test concentrations between 130 and 6000 $mg \cdot L^{-1}$ chloride. Test concentrations were chosen to yield a suitable range for EC calculations to cover both known effect concentrations from the literature for other mussel species and excessive concentrations based on published maximum chloride measurements in road runoff. The lowest test concentration of 130 $mg \cdot L^{-1}$ chloride served as a control in this study, as it equals the background chloride concentration of the local groundwater source which also feeds natural aquatic ecosystems where *A. anatina* are found. Chloride concentrations were verified photometrically by using the Spectroquant® chloride test kit (Merck, Germany) analogue to the methods EPA 325.1 and APHA 4500-Cl-E with a detection range of 10 - 250 $mg \cdot L^{-1}$ (standard deviation (SD) ± 2.8, accuracy ± 10). Test concentrations exceeding

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