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Establishing mussel behavior as a biomarker in ecotoxicology

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ABSTRACT

Most freshwater mussel species of the Unionoida are endangered, presenting a conservation issue as they are keystone species providing essential services for aquatic ecosystems. As filter feeders with limited mobility, mussels are highly susceptible to water pollution. Despite their exposure risk, mussels are underrepresented in standard ecotoxicological methods. This study aimed to demonstrate that mussel behavioral response to a chemical stressor is a suitable biomarker for the advancement of ecotoxicology methods that aids mussel conservation. Modern software and Hall sensor technology enabled mussel filtration behavior to be monitored real-time at very high resolution. With this technology, we present our method using *Anodonta anatina* and record their response to de-icing salt pollution. The experiment involved an environmentally relevant 'pulse-exposure' design simulating three subsequent inflow events. Three sublethal endpoints were investigated, Filtration Activity, Transition Frequency (number of changes from opened to closed, or vice versa) and Avoidance Behavior. The mussels presented a high variation in filtration behavior, behaving asynchronously. At environmentally relevant de-icing salt exposure scenarios, *A. anatina* behavior patterns were significantly affected. Treated mussels' Filtration Activity decreased during periods of very high and long de-icing salt exposure ($p < 0.001$), however, increased during short de-icing salt exposure. Treated mussels' Transition Frequency increased during periods of very high and long de-icing salt exposure ($p < 0.001$), which mirrored the Avoidance Behavior endpoint observed only by mussels under chemical stress. Characteristics of Avoidance Behavior were tighter shell closures with repeated and irregular shell movements which was significantly different to their undisturbed resting behavior ($p < 0.001$). Additionally, we found that mussels were sensitive to a chemical stressor even when the mussel's valves were closed. Due to the effects of de-icing salt pollution on freshwater mussel behavior, we suggest better management practices for de-icing salt use be implemented. Our experimental method demonstrated that, with the application of current technologies, mussel behavioral response to a chemical stressor can be measured. The tested sublethal endpoints are suitable for mussel ecotoxicology studies. Avoidance Behavior proved to be a potentially suitable endpoint for calculating mussel behavior effect concentration. Therefore we recommend adult mussel behavior as a suitable biomarker for future ecotoxicological research. This method could be applied to other bivalve species and for physical and environmental stressors, such as particulate matter and temperature.

1. Introduction

Pollution is a major driver towards the endangerment of freshwater bivalves (Unionoida) which are the fastest diminishing taxa with all of the European species listed on the International Union for Conservation of Nature's (IUCN) 'Red List' (Bogan, 1993; Geist, 2011; IUCN, 2014). Unionoida conservation is a high priority. As keystone species they contribute to a number of essential functions in aquatic ecosystems (Geist, 2010; Vorosmarty et al., 2010). Due to their high exposure pathway as filter feeders and their limited evasion capabilities, mussels are especially susceptible to organic and inorganic chemicals (Strayer et al., 2004). The number of registered chemical substances is over 100 million (CAS, 2015). This therefore presents a substantial risk to Unionoida (Dudgeon et al., 2006). The consequences of further decline in filter-feeder biodiversity is likely to have far reaching implications towards the failure of lotic and lentic ecosystem services (Cardinale et al., 2012; Chapin et al., 2000; Vaughn, 2010).

In recognition of this, Eggen et al. (2004) appealed for the ecotoxicology research community to develop new concepts, tools,

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and approaches to assess the effects of complex chemical mixtures on all levels of the ecosystem, i.e., organisms, communities and ecosystem functions. The use of standard ecotoxicology methods, exposing standard organisms (i.e., algae, daphnia, and fish) to measure standard endpoints (i.e., mortality, growth and fertility) is known to often underestimate the toxic effects on the diverse range of aquatic organisms, particularly for filter feeders (Connon et al., 2012). The sublethal effects of chemical exposure, such as behavior response, can be 10–100 times more sensitive than standard lethal exposure parameters (Hasenbein et al., 2015; Robinson, 2009). The behavior response of mussels is the result of physiological and environmental factors. Behavioral measurements reflect the combination of conditions representing a series of acute cumulative effects (Bae and Park, 2014). Thus, the development of sublethal ecotoxicological methods, by monitoring mussel filtration behavior, may provide the opportunity to measure the consequence of chemical stress on bivalves. This could offer an important addition to the suite of currently used ecotoxicological tools and explain an element causing freshwater mussel decline.

The measurement of mussel valve movements (valvometry) using remote sensing technologies were first implemented by Kramer et al. (1989) as a tool for early warning alarms for aquatic pollution. Recent technology developments (e.g., Halls sensor technology and software) provide the ability to collect valvometric measurements with very high resolution in real-time (Robson et al., 2009; Sow et al., 2011). A Hall sensor is a transistor that, with the application of a controlled current, creates an output voltage proportional to the strength of a magnetic field (Nagai et al., 2006). The attachment of a Hall sensor and a magnet to the valves of an adult mussel allows the recording of their valve gap movements as a change in voltage output. The recording of gap changes provides evidence of a mussel's response to disturbances as the closing of a mussel's shells is an indicative evasion behavior (Lorenz et al., 2013). To our knowledge, Hall sensor technologies have not been used to improve the understanding of bivalves' filtration behavior or evasive behavioral response to environmentally relevant chemical exposure scenarios. Mussel behavior could be established as a biomarker in ecotoxicology studies and provide the tool to assess mussel filtration behavior as a sublethal endpoint.

To discuss and demonstrate the use of mussel behavior as a biomarker, we use the example of de-icing salt pollution. Sodium chloride (NaCl) is the most common component of de-icing salt and a substantial contaminant to freshwater ecosystems, particulary during winter and spring (Kaushal et al., 2005). Across the northern hemisphere millions of tons of NaCl are dispersed on hard surfaces to maintain community safety and economic activity during periods of freezing temperatures (Environment_Canada, 2004; Thunqvist, 2004; US_EPA, 2010). Chloride mass balance studies by Howard and Haynes (1993) and Perera et al. (2013) of the Highland Creek watershed east of Toronto (Canada) revealed that up to 60% of the de-icing salt, applied to hard surfaces, runs into surface water bodies almost immediately after application. Consequently, Cl^- concentrations in urban freshwater streams have been reported to peak as high as 5000 mg/L in North America from de-icing salt contaminated snow melt (Corsi et al., 2010; Kaushal et al., 2005). This Cl^- concentration is in the order of brackish conditions (i.e., Cl^- concentrations between 500 mg/L to 5000 mg/L). The effects of de-icing salt on mussel larvae (glochidia) mortality has been investigated in a few studies, but not for adult mussel behavior (Beggel and Geist, 2015; Blakeslee et al., 2013; Gillis, 2011). NaCl is used in toxicology as a reference toxicant (ASTM, 2006), making it a suitable chemical to demonstrate our methodology. In our study, we use the European Unionoida species *Anodonta anatina* (Linnaeus 1758) as the model species.

The purpose of this study was to establish mussel behavior patterns as a biomarker for ecotoxicology research for Bivalvia. The objective was to test the suitability of three sublethal endpoints; Filtration Activity, Transition Frequency (change in activity status) and, Avoidance Behavior, in an innovative ecotoxicological experiment using environmentally relevant potential exposure scenarios simulating winter storms. With a 'pulse-exposure' experimental design and Hall sensor technology, we tested the hypothesis that exposure of adult *A. anatina* to de-icing salt (NaCl) alters their filtration behavior patterns.

2. Materials and methods

2.1. Study animals and experimental setup

Anodonta anatina mussels were collected from ponds in Bavaria (Germany) and were kept outdoors in an 8000 L tank supplied with groundwater until experimentation. Following DNA extraction as described by Geist and Kuehn (2005), the mussels were genetically validated as *A. anatina* following the molecular identification method in Zieritz et al. (2012). Thirty mussels were used in the experiment with the mean length, height, and thickness of 9.02 ± 0.73 cm (mean ± SD), 5.26 ± 0.47 cm, and 3.2 ± 0.42 cm, respectively. Five mussels were placed randomly in one of six 25 L aquaria (25 cm × 40 cm × 25 cm with 4 cm of gravel substrate). Three aquaria were used for the treatment replicates and three aquaria for control replicates. All aquaria were kept in a water bath 12.5 °C ± 0.5 °C and filled with local groundwater (pH: 8.03, electrical conductivity at 20 °C: 1072 μS/cm, and 460 mg/L $CaCO_3$). The mussels were fed 5 mL of Shellfish Diet 1800™ (Reed Mariculture Inc., U.S.A.) per aquarium once a week.

A semi-closed system was designed to control the Cl^- concentration within each aquarium without directly disturbing the mussels. A peristaltic pump (Ismatec MCP Standard, IDEX Health & Science GmbH, Germany) with Tygon® tubing (ID 3.2 mm, Saint-Gobain Performance Plastics Corporation, France) was used to create a flow-through exposure system. A stock solution of 10 g/L NaCl (99.5% purity, Merck KGaA, Germany) or fresh groundwater was pumped into the aquaria to achieve the desired Cl^- concentration. Control aquaria received fresh groundwater during treatment phases. Two aeration stones were placed in each aquarium to ensure even mixing of the saline solution and to maintain dissolved oxygen levels.

2.2. NaCl exposure scenarios

The mussels in the treatment aquaria were exposed to three dynamic NaCl exposure events designed to simulate possible de-icing salt runoff events, each a week apart (Fig. 1). The three scenarios included:

- I A simulated single heavy runoff event after extensive application of road salts. Exposure duration of 30 h and a peak Cl^- concentration of 3125 mg/L.
- II Three simulated runoff events within a short time frame. Total exposure duration of 48 h, with three peak Cl^- concentrations up to 1750 mg/L and with low concentrations of 1000 mg/L between peaks.
- III A simulation of a period of cold conditions with salt application but without runoff of de-icing chemicals followed by a heavy runoff event with numerous de-icing salt applications. Exposure duration of 72 h and a peak Cl^- concentration of 3840 mg/L.

2.3. Hall sensors, data measurement and data collection

Hall sensor technologies were used in this experiment similar to the methods described by Wilson et al. (2005) and Robson et al.