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Toxicity of noradrenaline, a novel anti-biofouling component, to two non-target zooplankton species, *Daphnia magna* and *Ceriodaphnia dubia*

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ABSTRACT

Noradrenaline (NA) is the active component of novel antifouling agents and acts by preventing attachment of fouling organisms. The goal of this study was to examine the toxicity of NA to the non-target zooplankton *D. magna* and *C. dubia*. Neonates were exposed to one of five concentrations of NA and effects on survival, reproduction and molting were determined. Calculated LC_{50} values were determined to be 46 and 38 μM in *C. dubia* and *D. magna*, respectively. A 10-day *C. dubia* study found that reproduction metrics were significantly impacted at non-lethal concentrations. In *D. magna*, concentrations greater than 40 μM significantly impacted molting. A toxicity test was conducted with *D. magna* using oxidized NA, which yielded similar results. These data indicate that both NA and oxidized NA are toxic to non-target zooplankton. Results obtained from this study can be used to guide future ecological risk assessments of catecholamine-based antifouling agents.

1. Introduction

Biofouling is the unwanted accumulation of bacteria, algae, plants and animals on submerged structures. This includes the accumulation of both native and non-native species in marine and freshwater environments. Biofouling causes increased drag on ships, can threaten water intakes, and, if left unchecked, lead to structural disintegration, corrosion and, ultimately, failure of immersed structures. Furthermore, biofouling can facilitate transport and introduction of non-native species (Yebra et al., 2004).

Previous antifouling compounds have resulted in major environmental impacts. In the 1950s, triorganotin biocides, such as tributyltin (TBT) based paints, were introduced and are estimated to cover 70% of today's world fleet (Yebra et al., 2004). Numerous species have been shown to be sensitive to low concentrations of TBT that may leach from these paints. Exposure to TBT and other organotins may result in imposex (Folsvik et al., 1999), shell deformity (Giltrap et al., 2009), and altered immune function (Harford et al., 2007) in fish and shellfish. In 2008, the United Nations Law of the Sea banned the use of TBT based antifouling paints on maritime vessels (Yebra et al., 2004). Today, TBT paints have largely been replaced by copper based ablative antifouling coatings. These paints incorporate cuprous oxide along with organometallic co-biocides (Yebra et al., 2004). Typically, the commercial products release copper anywhere from 4 to 48 μg/cm2/day (Valkirs et al., 2003; Schiff et al., 2004; Finnie, 2006). It has been estimated that upwards of 3000 t of copper is released per year from ships at sea (Almeida et al., 2007). This amount poses a significant environmental threat to aquatic ecosystems, particularly in ports and harbors where the released copper accumulates in sediments. Copper has been shown to have a variety of detrimental effects on aquatic organisms at relatively low concentrations (μg/L), depending on water chemistry, chemical speciation and/or differences in physiology (Perrett et al., 2006; Grosell et al., 2007; Yamada, 2007; Christiansen et al., 2011).

More recent research of antifouling compounds has focused on bioactive surface coatings that interact directly with the sensory organs of marine invertebrates to prevent initial settling activity (Yebra et al., 2004). One such approach using catecholamines, specifically adrenoreceptor agonists and antagonists including noradrenaline (NA) has been promising (Holm, 2012; Imbesi et al., 2012). Work by Gohad et al. demonstrated that exposure to NA-conjugated coatings resulted in apoptosis in oyster hemocytes and deterred settling behavior in both oyster pediveligers and cyprid larvae of barnacles (Gohad et al., 2010). However, the toxicity of leached material from these coatings to non-target organisms is largely unknown.

The crustaceans *Daphnia magna* (Straus, 1820) and *Ceriodaphnia dubia* (Richard, 1804) are well-studied model organisms for use in environmental toxicology (Davenport et al., 1994; Hansen et al., 2002; Lampi et al., 2006; Goulet et al., 2007; Roberts et al., 2007; Stoeckel

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et al., 2008). Typical endpoints include mortality, reproduction, and growth but some authors have suggested that endpoints such as heart rate and metabolism may also be sensitive, particularly in the assessment of pharmacological compounds (Campbell et al., 2004; Dzialowski et al., 2006a). Although specific neurotransmitters/receptors may not be shared between taxa, analogues may exist which allow for shared modes of action (Huggett et al., 2002). For example, it has been reported that receptors for octopamine, the crustacean equivalent of dopamine in vertebrates, are similar to adrenergic receptors (Roeder et al., 2003; Dzialowski et al., 2006a). These similarities allow for potential modes of action to be conveyed across taxa. Studies utilizing the β-adrenergic receptor drugs propranolol (Campbell et al., 2004; Dzialowski et al., 2006a) and metoprolol (Dzialowski et al., 2006a) significantly reduce heart rate in *Daphnia sp.* within minutes as would be expected in a vertebrate model. Campbell et al. (2004) also report that treatment of *Daphnia pulex* with adrenaline or the β-adrenergic receptor agonist isoproterenol increased heart rate within minutes; again similar to what would be expected in a vertebrate model. Thus, although relatively little is known regarding the role of noradrenaline in *Daphnia*, it is likely that exposure to noradrenaline containing materials will exert some effects on daphnid physiology. The concentration ranges at which those effects may be elicited are unknown.

The goal of this study was to develop a non-target species toxicity-testing model for NA-based antifouling materials using two species of daphnids, *D. magna* and *C. dubia*. These species were selected as they are both recommended for use by various environmental enforcement organizations, are sensitive to a variety of environmental contaminants, and can be easily used in acute and chronic toxicity tests. Endpoints tested in this study include survival, reproduction, and molting. Concentration ranges for both acute and chronic effects of waterborne NA were determined for use in hazard assessments concerning the use of NA-based antifouling materials.

2. Materials and Methods

2.1. Reagents

Unless otherwise noted, all reagent-grade chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Stocks of NA were made by dissolving NA powder in reconstituted freshwater and serially diluting to achieve nominal test concentrations.

2.2. Test conditions

For all studies, neonates of *C. dubia* and *D. magna* less than 24 hours old were obtained from cultures maintained at the University of North Texas (Denton, TX, USA). Tests were conducted in a 20 ° C room with a 16:8 hour light: dark photoperiod. Test chambers for *C. dubia* consisted of 25 mL plastic cups containing 20 mL of test solution, whereas 250 mL beakers containing 150 mL of test solution were used for *D. magna.* All studies were run in accordance with US EPA protocols in reconstituted freshwater (reverse osmosis water reconstituted to a pH of 7.8, hardness of 140 mg/L $CaCO_3$, and alkalinity of 50 $CaCO_3$) (EPA, 1993; U.S.EPA, 2002).

2.3. Survival assays

For acute toxicity studies, ten neonates of either *C. dubia* or *D. magna* were placed into each test chamber that contained one of five concentrations of NA (0, 2, 4, 8 and 16 mg/L) with four replicate chambers per concentration. Test solutions were renewed every 24 hours with freshly prepared NA and each test chamber was fed a suspension of the green algae, *Selenastrum capricornutum*, and YTC following daily renewals. Survival was recorded every 24 hours and the tests were concluded at 96 hours.

To test the toxicity of oxidized NA, a study was conducted using NA that was allowed to oxidize. The study was run in the same manner as the NA toxicity studies with the exception that the NA was allowed to oxidize prior to exposure. NA powder was added to a volumetric flask (16 mg/L) and brought to volume with reconstituted freshwater and allowed to sit at room temperature (21 °C for 24 hours) for oxidation to occur. This high stock NA was then diluted to the desired concentrations for the exposure. Fresh NA solution was made daily and allowed to sit for 24 hours prior to renewals.

2.4. Molting test

A molting test was performed using *D. magna*. One neonate was placed into each test chamber that contained one of five concentrations of NA (0, 1.7, 5, 6.8 and 8.5 mg/L) with five replicate chambers per concentration. Each test chamber was fed a suspension of the green algae, *Selenastrum capricornutum*, following daily renewals. The number of molts for each individual was recorded every 24 hours and tests were concluded after 96 hours.

2.5. Reproduction test

Because of the length of the *D. magna* test (at least 21 days), a reproduction test was run using only *C. dubia*. One neonate was placed into each test chamber that contained one of five concentrations of NA (0, 1.7, 5, 6.8 and 8.5 mg/L) with ten replicate chambers per concentration. Each test chamber was fed a suspension of the green algae, *Selenastrum capricornutum*, following daily renewals. Reproduction (number of new neonates produced) was recorded every 24 hours for each individual and tests were concluded after 10 days.

2.6. Noradrenaline stability

In preliminary experiments, we observed that NA was rapidly oxidized over a few hours to a reddish-brown metabolite. Because stability is an important exposure metric for ecotoxicology, we determined the rate of oxidation using a basic spectrophotometric assay (Roston, 1960; Powell and Heacock, 1970). Briefly, flasks of five concentrations of NA were made as described above. An aliquot (200 μl) was taken from each flask at specified time points over the course of 24 hours and run in triplicate on a BioTek Plate Reader ($\lambda = 300$ nm). The half-life of the material was determined based on increased absorbance over time using a simple linear regression.

2.7. Statistics

All statistical analyses were conducted using JMP 11.1.1 (SAS, Cary, NC). To determine significant differences from controls, an analysis of variance (ANOVA) followed by Dunnett's post-hoc test was performed on data from all toxicity tests. Additionally, 96-hour LC_{50} and oxidation rate half-life values were determined using a nonlinear logistic 3P model with an inverse prediction set at 50%. Results were deemed significant if $p < 0.05$.

3. Results

3.1. NA exposure

C. dubia controls exhibited less than 10% mortality. Overall, *C. dubia* survival was NA concentration dependent. Significant mortality ranging from 30% to 100% was observed at concentrations ≥ 4 mg/L NA (4 mg/L NA $p = 0.02$; 8 and 16 mg/L NA $p < 0.01$, Fig. 1A). The calculated LC_{50} value was determined to be 7.8 mg/L (6.4–8.9 95% CI). Acute toxicity results were similar for *D. magna. D. magna* controls exhibited less than 3% mortality. Overall, *D. magna* survival was NA concentration dependent with mortality ranging from 79% to 91% at concentrations ≥ 8 mg/L