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Baseline

A novel bioassay using the barnacle *Amphibalanus amphitrite* to evaluate chronic effects of aluminium, gallium and molybdenum in tropical marine receiving environments

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ABSTRACT

A need exists for appropriate tools to evaluate risk and monitor potential effects of contaminants in tropical marine environments, as currently impact assessments are conducted by non-representative approaches. Here, a novel bioassay is presented that allows for the estimation of the chronic toxicity of contaminants in receiving tropical marine environments. The bioassay is conducted using planktonic larvae of the barnacle *Amphibalanus amphitrite* and is targeted at generating environmentally relevant, chronic toxicity data for water quality guideline derivation or compliance testing. The developmental endpoint demonstrated a consistently high control performance, validated through the use of copper as a reference toxicant. In addition, the biological effects of aluminium, gallium and molybdenum were assessed. The endpoint expressed high sensitivity to copper and moderate sensitivity to aluminium, whereas gallium and molybdenum exhibited no discernible effects, even at high concentrations, providing valuable information on the toxicity of these elements in tropical marine waters.

1. Introduction

Tropical marine environments are hotspots for biodiversity and amongst the most productive systems in the world (Tittensor et al., 2010). Many communities depend on these systems as a source of income or resources (UNEP-WCMC, 2011), yet population growth accompanied by agricultural and industrial development place ever increasing pressures on estuaries and coastal seas (Lotze et al., 2006). Despite a clear need for environmentally sustainable development to maintain continued protection of marine resources, relevant techniques to predict hazards associated with marine contamination in tropical environments have long remained inadequate (van Dam et al., 2008), resulting in a lack of regionally-relevant data. Only in the past few years, a number of useful tools have been developed that facilitate the assessment of contaminant impacts in tropical marine waters (e.g. Howe et al., 2012; Trenfield et al., 2015a, 2015b; Wilkinson et al., 2015), yet standardised techniques and available datasets remain limited. Marine species from different climatic zones tend to exhibit dissimilar sensitivities to a range of contaminants, yet a consistent trend across regions is absent (Wang et al., 2014), demonstrating the necessity for regionally-specific data (van Dam et al., 2014). This is especially valid at sites with a potential for contamination, where public and conservation groups will demand site-relevant information (Warne et al., 2014a).

Bioassay toxicity testing is an important aspect of integrated environmental impact and risk evaluation. Toxicity test data constitute the basis of the models employed globally for the derivation of threshold concentrations for contaminants in the environment (e.g. ANZECC/ARMCANZ, 2000; CCME, 2007; European Commission, 2011; USEPA, 2015). These water quality guidelines (WQGs) present a reference tool for management actions targeting adequate protection of the receiving environment (Warne et al., 2014a). As WQGs are subject to agreed conservation standards, they support sustainable extraction/use of resources and management of industrial discharges. However, due to the scarcity of existing toxicity data and the limited availability of test protocols, currently no reliable regional or site-specific WQGs can be established for a range of common contaminants associated with industrial waste streams in Northern Australia, including several signature constituents of alumina refinery discharge waters such as aluminium (Al), gallium (Ga) and molybdenum (Mo). These elements have concentrations in effluents that are often elevated above background seawater (Harford et al., 2011; Negri et al., 2011) and therefore

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of potential concern for environmental health. The generic Australian WQG for Al in marine waters has recently been revised and adjusted from 0.5 to 24 μg L^{-1} for 95% species protection following an investigation by Golding et al. (2015). However, the dataset used consisted primarily of temperate test species and the question remains whether the WQG derived is equally relevant in tropical regions. In this paper, we aim to contribute to the development of more reliable, regionally-specific WQGs for Al, Ga and Mo by testing the chronic toxicity of these elements using a newly developed bioassay that may be used to assess effects of contaminants in tropical marine environments.

The purple striped acorn barnacle *Amphibalanus amphitrite* Darwin (Arthropoda: Cirripedia) is an important member of the macro-fouling community and occurs globally along almost every tropical and subtropical coastline. These barnacles can be found in the mid-intertidal to low intertidal zone, attached to hard surfaces such as rocks, oyster shells, mangrove roots and ships' hulls. The adults are iteroparous hermaphrodites, internally fertilizing proximate conspecifics, though self-fertilization may occur (Anderson, 1994). At tropical latitudes breeding occurs throughout the year, with individuals releasing broods as often as once a week (Holm, 1990). Embryos are brooded inside the mantle cavity and larvae released as free-swimming, heterotrophic nauplii. Drifting with the currents and tides, the nauplii moult several times before transitioning into non-feeding cyprid larvae that actively scout for suitable settlement substratum. Once located, the cyprids attach and metamorphose into juvenile barnacles (Anderson, 1994). Development and settlement success is highly dependent on external factors such as temperature, salinity and diet (e.g. Anil and Kurian, 1996; Harder et al., 2001), yet at 28 °C in the laboratory under optimal conditions, the six moults from nauplius stage I to cyprid are completed within 5 d.

Driven by its economic importance as a primary fouling species, *A. amphitrite* has been used for decades as a model species to test surfaces, chemicals and coatings for their antifouling capacities. Most of these tests employed acute (≤48 h) inhibition of either larval settlement (e.g. Rittschof et al., 1992; Wu et al., 1997) or swimming ability (e.g. Faimali et al., 2006; Rittschof et al., 1992) as endpoints to evaluate concentration-response relationships or screen compounds for antifouling properties. Qiu et al. (2005) reported on a chronic bioassay that allowed for a prolonged exposure duration and which assessed complete larval development from stage II nauplius to cyprid, however these resulted in poor control performance (i.e. only 40–60% of control animals reached cyprid stage after 8 d exposure) and the bioassay was never adopted for routine testing. In the current paper, we present a novel approach to evaluate the chronic effects of single toxicants or mixtures in a seawater matrix to newly hatched *A. amphitrite* nauplii, employing a 96 h bioassay. The assessment endpoint consisted of the ability of the nauplii to successfully complete 5 consecutive moults, from stage II to stage VI and subsequent transition to cyprid within the 96 h test duration. The sensitivity of the test method was validated by exposure of nauplii to copper (Cu), and the method was subsequently used to assess the toxicity of Al, Ga and Mo. The resulting toxicity data may be used by regulatory authorities and resource managers for the derivation of WQGs.

2. Materials and methods

2.1. Broodstock maintenance

Broodstock barnacles were collected on PVC pipes left suspended just below the water's surface in Darwin Harbour (12°26′57.48″S, 130°51′ 7.51″E). Non-desired biota were removed before the pipes were brought into the aquaria (Australian Institute of Marine Science, Darwin, Australia) and placed within 68 L polyethylene tubs which contained a submersible pump for water circulation and airstones for aeration. Tubs contained natural seawater filtered to 5 μm (FSW) and were maintained under static conditions at 29 ± 1 °C. Dual cool-white fluoro lights were suspended 60 cm over the water surface and 13: 11 h diurnal light: dark cycle introduced as broodstock were found to feed more actively in the light. Broodstock were fed freshly hatched brine shrimp (*Artemia salina*) daily. In addition, broodstock were fed an assortment of algae paste, prawn starter mix, live rotifers and live diatoms opportunistically. Care was taken that stock were not overfed in order to retain good water quality. Culture seawater was renewed twice a week and pipes sprayed down with salt water to remove debris, *A. salina* cysts and discarded moults. Once a week, tub, airstones, pump, pipes and barnacles were cleaned more thoroughly. Injured or diseased broodstock were discarded. Field-collected broodstock were observed in the laboratory for a minimum of 4 weeks in order to detect any signs of disease and allow for acclimatization to laboratory conditions before use.

2.2. Induction of spawning and collection of nauplii

Release of larvae in *A. amphitrite* can be controlled, which means the test may be started at any desired point in time. However, nutritional status of the adults needed to be optimal for breeding and larval release to occur. Spawning procedures were slightly modified from previously described methods (Rittschof et al., 1992). In the late afternoon, broodstock were cleaned and left exposed to (warm) air. The next morning, a clean tub was filled with FSW and broodstock pipes were resubmerged, triggering larval release. A point-source light was suspended over one corner of the water surface. The whole setup was covered with a dark cover and left for 1.5 h, after which the newly released, phototactic, stage II nauplii were collected using a wide-bore pipette. A typical spawning yielded many thousands of nauplii. Limited numbers released indicated broodstock were not in optimal condition and tests were not initiated.

2.3. General laboratory procedures

All equipment that came into contact with nauplii, dilution water or test solutions were made of chemically inert materials (PTFE, glass or plastic) and soaked in 5% v v^{-1} nitric acid for a minimum of 24 h before being rinsed with deionised, reverse-osmosis water and allowed to thoroughly dry. Glass funnels employed as test vessels had been treated with a silanizing agent (coatasil, Ajax Finechem) a maximum of 6 months prior to the test, to reduce sorption of test substance and food algae to the vessel surface. All reagents used were analytical grade and stock solutions were made using ultrapure water (18 MΩ Milli-Q; Millipore).

2.4. Preparation of test solutions

Test solutions consisted of UV-sterilized, natural seawater (32–35 psu), which was passed through a 0.5 μm spun polypropylene cartridge filter (dilution water) and spiked with a stock solution containing the element to be tested. For each metal, stock solutions of 2 g L^{-1} were prepared by dissolving analytical grade $CuSO_4 \cdot 2H_2O$, $AlCl_3 \cdot 6H_2O$, $GaCl_3 \cdot H_2O$ or $Na_2MoO_4 \cdot 2H_2O$ (Sigma-Aldrich) in ultrapure water. Where needed, these were further diluted. For each test concentration, 500 mL solution was made up in a HDPE bottle. Equal volumes of combined stock solution/Milli-Q were added to each bottle (final concentration in test solution <0.05% v v^{-1}). Fresh test solutions were prepared 1.5 times more concentrated than nominal values and made up the day prior to test initiation to allow for chemical equilibration.

2.5. Test apparatus and procedures

Several trials were conducted attempting to rear nauplii over multiple developmental stages in 10 mL and 50 mL static exposure vessels. Although these were successful at producing cyprids within a set time span, maintaining high survivorship and limited variability proved exceedingly difficult. In the absence of water movement, food algae and discarded moults settled to the bottom of the culture vessels, trapping

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