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Development of a chronic, early life-stage sub-lethal toxicity test and recovery assessment for the tropical zooxanthellate sea anemone *Aiptasia pulchella*

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ABSTRACT

There is an urgent need to identify additional tropical marine species and develop sensitive sub-lethal and chronic toxicity test methods for routine ecotoxicology. The tropical symbiotic sea anemone *Aiptasia pulchella* is a suitable species for use in ecotoxicology and here we have assessed the effects of trace metal exposures on the development of asexually produced *A. pulchella* pedal lacerates to a juvenile stage. Concentrations of 55 μg/L for cadmium, 262 μg/L for cobalt, 5 μg/L for copper, and 269 μg/L for zinc were estimated to inhibit normal development by 50 percent after 8-d exposures, and are among the most sensitive available toxicity estimates for marine organisms. This work illustrates the potential value of this species and sub-lethal toxicological endpoint for routine ecotoxicology in tropical marine environments.

1. Introduction

The paucity of tropical marine species used in routine ecotoxicology has been criticised for more than a decade and results in a reliance on temperate toxicity data for derivation of water quality guidelines (Lacher and Goldstein, 1997; Peters et al., 1997; Van Dam et al., 2008). This practise continues despite the effects that temperature may have on toxicity (Cairns et al., 1975; Chapman et al., 2006; Sokolova and Lannig, 2008), biological differences in tropical compared to temperate marine species and ecosystems (Canning-Clode et al., 2011), and the overall unreliability of extrapolating toxicity data between climatic regions (Chapman et al., 2006; Kwok et al., 2007). A lack of regionally-relevant toxicity data for commonly detected contaminants such as trace metals (Markich and Camilleri, 1997) and pesticides (GBRMPA, 2010) has been discussed, and the need for additional tools for ecotoxicological assessment of an array of newly-emerging contaminants has been emphasised (*e.g.* engineered nano-particles; Matranga and Corsi, 2012; Van Dam et al., 2008). It is necessary to identify a number of tropical marine species and to provide representation from a range of ecologically-relevant taxa (Van Dam et al., 2008), and to ensure that both filter-feeding and suspension-feeding organisms are included in routine ecotoxicological testing (Matranga and Corsi, 2012).

One ecosystem potentially at high risk due to these knowledge gaps are coral reefs (Van Dam et al., 2008). These ecosystems support areas of the highest biodiversity on earth (Reaka-Kudla, 1997), affect global climate (Jones and Ristovskil, 2010) and provide invaluable economic, ecological and subsistence value for humans (Birkeland, 1997). Globally, the greatest risks to coral reefs come from increases in sea surface temperature (SST; Hoegh-Guldberg, 1999), and ocean acidity (Veron et al., 2009). However, contaminants have also been recognised as primary threats to coral reefs in Australasia and the Indo-Pacific (Australian Government, 2013; Reichelt-Brushett, 2012). Furthermore, there is evidence that global-stressors may act additively, synergistically or sequentially with local-stressors (which are much more easily mitigated) including synthetic contaminants such as biocides (Negri and Heyward 2001), and excess naturally occurring contaminants (*e.g.* trace metals; Negri and Hoogenboom, 2011; Sokolova and Lannig, 2008; Yaroslavtseva and Sergeyeva, 2008).

Sea anemones are cnidarians which usually house intracellular photosynthetic dinoflagellates from the genus *Symbiodinium* sp. (Birkeland, 1997). Anemones have been used in laboratory research for many decades, including in studies of cnidarian physiology (Muller-Parker, 1984), algal-host symbioses (Steen, 1986), metal uptake and accumulation (Mitchelmore et al., 2003), and stress responses such as zooxanthellae loss (Dunn et al., 2002; Perez, 2007). In corals, higher metal accumulation has been reported in zooxanthellae and soft tissue

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compared to the skeleton (Reichelt-Brushett and McOrist, 2003), hence anemones may be useful proxies in studies of metal toxicity, uptake and accumulation (Main et al., 2010) and would at the very least provide some representation from the phylum Cnidaria. Unlike corals, many anemone species are easy to culture in large numbers in laboratory conditions, and reliably reproduce both sexually and asexually throughout the year (Howe et al., 2012; Ladriere et al., 2008b; Lehnert et al., 2012). Similarly to corals, anemones will appear bleached, produce excess mucus, and retract their tentacles and tissue in response to stressors such as extreme temperatures (Dunn et al., 2002) and contaminants (Bao et al., 2011; Howe et al., 2012; Main et al., 2010).

The tropical zooxanthellate anemone *Aiptasia pulchella* (Carlgren, 1943) has a wide range throughout the tropics and sub-tropics and is therefore a regionally-relevant species for ecotoxicology throughout Australasia, including areas where marine biodiversity 'hotspots' are increasingly exposed to anthropogenic contaminants (Reichelt-Brushett, 2012). *A. pulchella* has previously been identified as a useful cnidarian species for laboratory studies (Hoegh-Guldberg, 1988; Hong et al., 2009; Sawyer and Muscatine, 2001), and relatively simple and inexpensive methods of laboratory husbandry reliably produce sufficient numbers of organisms to run replicated toxicity tests (Howe et al., 2012). However, prior to our recent work the sensitivity of *A. pulchella* to a range of contaminants, and hence its suitability as a toxicity test species, had not been investigated.

The aims of this study were to investigate the sensitivity and efficacy of a newly-developed chronic sub-lethal laboratory toxicity test using the zooxanthellate tropical sea anemone *A. pulchella*. The contaminants used to investigate this test method were cadmium, cobalt, copper, nickel and zinc, for which sub-lethal chronic toxicity estimates are provided. Results are compared to toxicity estimates for other tropical marine species, and demonstrate the relevance and suitability of this species and sub-lethal endpoint as a routine test method for ecotoxicology relevant to tropical marine environments.

2. Methods

2.1. Test organisms

Original stocks of *A. pulchella* were obtained from the flow-through seawater system at the National Marine Science Centre, Charlesworth Bay, NSW, Australia and housed at Southern Cross University, Lismore, NSW, Australia (Howe et al., 2012). This species reproduces asexually by 'pedal laceration', whereby a small piece of parent pedal disc tissue separates from the adult and subsequently develops into a genetically-identical anemone (Clayton, 1985; Hunter, 1984; Lin et al., 1992). The offspring is termed a 'lacerate' until such time as eight tentacles have developed and it is defined as a 'juvenile', as described for *Aiptasia pallida* (Cary, 1911).

In this study new lacerates were collected from culturing tanks and lacerates with no tentacles were selected using a stereo microscope (Olympus SZ51®). Four lacerates were placed in each of five replicate 2 M HCl washed and 75-mL seawater-aged polyethylene test containers for all metals, with the exception of copper where two lacerates were used in each of five replicates. Each test container was filled with 73 mL of solution. Initial observations of lacerate development showed that the time for first tentacle appearance was short (1–2 d) and that subsequent development to a juvenile stage was often complete within 3 d. Hence, a short 3-h acclimation period was used to ensure that test organisms had no tentacles at test commencement; all lacerates adhered to test containers within 24 h.

2.2. Toxicity tests

Five metal concentrations and a seawater control were used in all tests. Metal stock solutions (500 mg/L) were prepared using Analar grade metal salts of $CuCl_2$, $CdCl_2$, $CoCl_2$, $NiCl_2$ and $ZnCl_2$ (99 percent purity, Sigma-Aldrich©) and reverse osmosis water. All test solutions were prepared using these stocks and natural seawater no more than 1 h prior to test commencement and 48-h test solution renewals. The seawater used as a diluent was collected from the same location (28.49°S, 153.55°E) on an incoming tide, and was aerated at ambient room temperature for use within 48 h of collection. Seawater had been collected from this location for use in culturing and other experiments for approximately 18 months prior to this study, during which time there was no evidence of contamination in culturing tanks, fresh dilution water, or associated with test procedures (see Howe et al., 2012; Howe et al., in press; Howe et al., unpublished data). Regular water quality measurement within 48 h of collection showed variations in pH and electrical conductivity less than 0.5 units and 5 percent, respectively, and a minimum of 85 percent DO saturation during this 18 month period. In addition, periodic analysis of dissolved concentrations identified negligible background levels of cadmium, cobalt, copper, lead, nickel and zinc.

All tests were conducted in water baths at 25 ± 1 °C with a 12: 12-h photoperiod (50–60 $\mu E\ m^2\ s^{-1}$) using 30 W white (Sylvinia Aquastar©) and blue (Coralstar©) fluorescent aquarium lighting. Electrical conductivity, pH, and temperature were measured using a Eutech Cyberscan PC300 © combined metre at midday every 24 h and dissolved oxygen (DO) concentrations were measured using a YSI© metre at test commencement, and in old and new solutions at each 48-h renewal. At test commencement and four renewal times, 10 mL aliquots of each new solution were acidified and filtered to 0.45 μm before measurements of dissolved metal concentrations using inductively coupled plasma mass spectrometry (ICP-MS) as per American Public Health Association method 3120 for analysing metals (1 μg/L detection limit; APHA, 2005). Aliquots were also taken from spent test solutions and analysed to provide an indication of the changes in metal concentrations after 48-h in static test conditions. As the metal concentrations of old solutions was only tested once, the mean measured dissolved metal concentrations from new solutions at 0, 2, 4, 6 and 10 d were used to calculate toxicity estimates.

The number of visible tentacles on each anemone was counted using a stereo microscope (Olympus SZ51©) prior to solution renewal at 4, 6, 8, 10 and 14 d. A juvenile was recorded if eight tentacles of any length were visible (Cary, 1911). Mortality was recorded if necrotic tissue was observed, which occurs rapidly following death (authors personal observation). After 14 d, test solutions were decanted and test containers were rinsed three times and filled with clean seawater for a 7-d recovery period with the same experimental conditions as the test period.

2.3. Data analyses

The probit method (SPSS© statistical software) was used to estimate 8-d and 14-d EC10 and EC50 values using the mean metal concentrations. For tests where lacerate mortality occurred, the probit method was used to calculate 8- and 14-d lethal metal concentration estimates for 10 percent (lethal concentration, 10 percent; LC10) and 50 percent of the test population (lethal concentration, 50 percent; LC50). Estimated LC/EC10 values are the preferred alternatives to no-observed effect concentrations (NOECs) or lowest-observed effect concentrations (LOECs) (Chapman et al., 1996).

3. Results

3.1. Quality assurance and control

The temperature of 25 ± 1 °C was maintained, and the pH ranged between 7.98 and 8.4 and the DO concentration between 7.6 and 8.5 mg/L. Electrical conductivity varied between 52.1 and 53.8 mS/cm in new test solutions, and increased by up to 15 percent in 48-h old solutions. Analysed dissolved metal concentrations of new solutions varied by < 20 percent over the duration of the test. Metal concentrations in 48-h old solutions were at least 88 percent of the original concentrations, and there was a general tendency for concentrations to increase after 48 h in test conditions (Table 1). This was likely due to evaporation of test solutions and the fact that test containers were not washed between renewals, and was generally more pronounced in lower treatment concentrations.

3.2. General observations

There was no mortality or obvious loss of condition in any control in any test, and all had developed to a juvenile stage within 8 d (Figs. 1A and 2A). Since the exact time since separation from the parent was unknown, variability was seen in the tentacle development rate in controls during the initial 8 d (Fig. 1A). Table 2 provides the concentrations estimated to cause 10 percent (EC10) and 50 percent (EC50) inhibition of normal lacerate development to a juvenile stage, and 10 and 50 percent mortality (LC10 and LC50 values). The order of toxicity for inhibition of normal lacerate