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Copper accumulation and oxidative stress in the sea anemone, *Aiptasia pallida*, after waterborne copper exposure

W.P.L. Main [a], C. Ross [a], G.K. Bielmyer [b,*]

[a] University of North Florida, USA
[b] Valdosta State University, 1500 N. Patterson St., Valdosta, GA 31698, USA

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ABSTRACT

Copper is a common marine pollutant yet its effects on symbiotic cnidarians are largely understudied. To further understand the impact of elevated copper concentrations on marine symbiotic organisms, toxicity tests were conducted using the model sea anemone, *Aiptasia pallida*, with and without its zooxanthellae symbiont. Symbiotic and aposymbiotic *A. pallida* were exposed to sublethal copper concentrations (0, 5, 15, and 50 μg/L) for 7 d and copper accumulation, behavior, and the activity of the oxidative stress enzymes, superoxide dismutase (SOD), and catalase (CAT) were measured. Additionally, acute 96-h toxicity tests were conducted to determine LC_{50} values of the organisms after copper exposure. Both symbiotic and aposymbiotic *A. pallida* rapidly accumulated copper in a time and dose dependent manner. However, higher copper concentrations accumulated in the aposymbiotic as compared to the symbiotic *A. pallida*. In response to the highest two copper exposures (15 and 50 μg/L) symbiotic *A. pallida* upregulated CAT activity to combat the damaging effects of hydrogen peroxide. Contrary to these results, SOD activity significantly decreased during the highest copper exposure, when compared to controls. CAT activity was not detected and SOD was substantially (>10 fold) reduced in aposymbiotic *A. pallida*, suggesting that the zooxanthellae are associated with the oxidative stress response. Copper exposure as low as 5 μg/L caused tentacle retraction and increased mucus production in both symbiotic and aposymbiotic anemones. The LC_{50} values for symbiotic and aposymbiotic *A. pallida* exposed to copper for 96 h were 148 μg/L (95% confidence interval = 126.4, 173.8) and 206 μg/L (95% confidence interval = 175.2, 242.2), respectively. Understanding the varying responses of symbiotic and aposymbiotic *A. pallida* to copper stress may advance our comprehension of the functional roles of zooxanthellae and host. Although the mechanism of copper toxicity has not been fully elucidated, it is clear that *A. pallida* accumulate copper and are sensitive, as effects were detected at environmentally relevant copper concentrations. Likewise, *A. pallida* may be useful in biomonitoring copper polluted environments.

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1. Introduction

Copper is a common pollutant found in marine ecosystems. Although copper is a trace mineral required by all organisms, it can be toxic in excess concentrations (Bryan and Hummerstone, 1971). Copper enters marine systems through runoff from rivers adjacent to heavy metal mining areas (Bryan, 1974); through sewage treatment discharge, industrial effluent, anti-fouling paints and refineries (Guzmán and Jiménez, 1992; Jones, 1997; Mitchelmore et al., 2003). These methods of pollution have a negative impact on coastal ecosystems and the various trophic levels contained within them.

One such coastal inhabitant, the sea anemone, *Aiptasia pallida*, is a native species of the southeastern United States (Kaplan, 1988). These symbiotic cnidarians reside on rocks, mangrove roots, coral, and other hard substrates in near shore, shallow coastal waters (Kaplan, 1988). Due to their close proximity to anthropogenic inputs, *A. pallida* may be a bioindicator of copper pollution. Healthy *A. pallida* are golden-brown in color due to their zooxanthellae (dinoflagellate algae of the genus *Symbiodinium*), which live in symbiosis with *A. pallida* (Cook et al., 1988). Zooxanthellae provide the anemone with photosynthate via carbon fixation pathways. In return, *A. pallida*, provide the zooxanthellae with nutrients (nitrogenous waste and CO_2) and protection (Cook et al., 1988). When this host–symbiont relationship becomes challenged by any number of environmental stressors, loss of the symbiont "bleaching" may occur (Brown, 2000).

Tissue copper accumulation has been reported in both corals and anemones after copper exposure (Mitchelmore et al., 2003, 2007; Bielmyer et al., in press). Harland and Nganro (1990) suggested that the pattern of copper accumulation in the tissues of symbiotic cnidarians may be influenced by the presence of the symbiont. These researchers showed that copper accumulation in the symbiotic sea anemone, *Anemonia viridis*, was not directly reflective of the copper concentrations in the environment, suggesting that detoxification and excretion mechanisms were used to combat increasing copper concentrations (Harland and Nganro, 1990). They suggested that copper accumulated to a greater extent in the zooxanthellae than host tissue and in a final detoxification

* Corresponding author. Tel.: +1 229 333 5766.
E-mail address: gkbielmyer@valdosta.edu (G.K. Bielmyer).

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attempt the anemone released their symbiotic algae to reduce internal copper concentrations (Harland and Nganro, 1990). The concept of zooxanthellae expulsion as a mechanism of metal detoxification has been suggested by several researchers, since zooxanthellae have been found to accumulate heavy metals to a larger extent and be more tolerant than their symbiotic hosts (Peters et al., 1997; Jones, 2004).

Although symbiotic cnidarians have been shown to accumulate copper, mechanisms of copper toxicity still remain unclear; however, recent studies have provided new data on copper and cadmium toxicity to scleractinian corals (Mitchelmore et al., 2007; Bielmyer et al., in press). Mitchelmore et al. (2007) reported copper and cadmium accumulation in both algal and animal tissue of the coral *Pocillopora damicornis* as well as a significant induction of the antioxidant, glutathione after exposure to either copper or cadmium. Copper accumulation in both algal and coral animal tissues, reduced zooxanthellae electron transport, reduced carbonic anhydrase activity, and reduced growth were reported in three species of scleractinian corals after five weeks of 4–20 μg/L copper exposure; however, the extent of the effects were species specific (Bielmyer et al., in press). The effect of ambient copper on anemones, in particular, is an understudied area of research; however, oxidative stress and reduced zooxanthellae density have been reported (Mitchelmore et al., 2003).

In many aquatic organisms, the accumulation of copper is typically associated with oxidative stress and subsequent macromolecular damage. The principle mechanism of copper toxicity involves the Haber–Weiss (Fenton) reaction (Gledhill et al., 1997). The oxidation of copper (I) in the Fenton reaction drives the conversion of hydrogen peroxide to hydroxide and hydroxyl radical (Klaassen, 1996). Copper can also react with superoxide anion to produce oxygen radicals (Craig et al., 2007). In times of increased abiotic stress, electron transport mechanisms (i.e., photosystems and mitochondrial transport chains) become less efficient. In turn, free electrons are more likely to interact with diatomic molecular oxygen ultimately resulting in a series of reduced reactive oxygen species (ROS). Due to photosynthesis, symbiotic anemones are subjected to very high levels of oxygen during the day and at night they experience a hypoxic internal environment (Plantivaux et al., 2004). This continuous transition between extreme oxygen environments can result in the increased production of ROS (Dykens et al., 1992; Nii and Muscatine, 1997). ROS can denature proteins, mutate DNA, and cause lipid peroxidation (Klaassen, 1996; Richier et al., 2005). To neutralize the potentially harmful effects of ROS, anemones produce enzymes such as SOD and CAT. SOD catalyzes the conversion of superoxide anion into hydrogen peroxide and oxygen; whereas CAT catalyzes the conversion of hydrogen peroxide into water and oxygen.

A. pallida are ideally suited for these toxicological studies because they are amenable to laboratory conditions, they are abundant and easy to collect, they reproduce quickly, especially compared to other symbiotic cnidarians such as corals, and they are found in near shore environments where copper contamination may be problematic. Conducting experiments with *A. pallida* at different symbiotic states may also contribute more knowledge about the role of the symbiont in copper accumulation, toxicity, and detoxification. The objectives of this study were to determine the sensitivity of the anemone, *A. pallida*, to acute copper exposure and to measure tissue copper accumulation and sublethal effects of copper exposure over a 7-d period in the symbiotic and aposymbiotic anemones.

2. Materials and methods

2.1. Animal holding

A. pallida used in the acute lethality experiments were collected from Whitney Laboratory (St. Augustine, FL, USA). The anemones were held in 10 gallon tanks filled with filtered (0.22 μm) seawater (SW; Whitney Laboratory) and maintained under constant filtration and aeration. Anemones (*A. pallida*) used in the 7-d sublethal experiments were obtained from the University of Miami (Key Biscayne, FL, USA) and acclimated in 10 gallon tanks filled with filtered (0.22 μm) SW for at least a month prior to testing.

Salinity, dissolved oxygen (DO), and temperature were measured weekly using a YSI® 85 Meter (YSI, OH, USA). Ammonia, nitrate and nitrite levels were measured weekly using API® Saltwater Master Test Kit (Aquarium Pharmaceuticals, PA, USA). Water quality in the holding system was maintained at an average salinity of 35 g/L (±1.3, mean±SD), DO of 6.8 mg/L (±0.2), temperature of 21 °C (±1.8), total ammonia-N concentration of 0.1 mg/L (±0.2), nitrate-N concentration of 0.5 mg/L (±1.4), and nitrite-N concentration of 0.3 mg/L (±1.6). The anemones were fed brine shrimp (*Artemia* sp.) daily. Symbiotic *A. pallida* were maintained under cool white fluorescent lighting (12 h light:12 h dark). Aposymbiotic anemones were obtained by placing symbiotic specimens in the dark for 3 months. The tanks housing the aposymbiotic *A. pallida* were completely covered so that the anemones were maintained in darkness and void of their zooxanthellae thus taking on a bleached white appearance.

2.2. Toxicity testing

Acute 96 h experiments to determine the concentration causing lethality to 50% of the organisms (LC_{50}) were performed using standard methods (USEPA, 2002). Concentrations of 0, 50, 100, 200, 400, and 800 μg/L copper were prepared 24 h prior to the start of the experiment using a 1 g/L copper, as $Cu(NO_3)_2$, stock solution (Fisher Scientific, PA, USA) and filtered (0.22 μm) SW. For each concentration there were three replicate 3 L plastic containers, each with 6 anemones. At 48 h, 75% of the solution in each replicate was renewed. Water samples were collected at 0, 48, and 96 h for later copper analysis. In all experiments, salinity, DO, and temperature were monitored daily using a YSI® 85 Meter (YSI). The average water quality values (mean±standard deviation) in the testing chambers for all experiments were as follows: 35 g/L ±0.4 salinity, 7.1 mg/L ±0.1 DO, and 23 °C ±0.3 temperature. Mortality (lack of responsiveness) was quantified daily. Behavioral observations of *A. pallida* were also noted daily, however, they were not quantified.

2.3. Sublethal experiments

Two sequential sublethal experiments were conducted with symbiotic and aposymbiotic *A. pallida*, respectively. Concentrations of 0, 5, 15, and 50 μg/L copper were prepared 24 h prior to the start of each experiment, using 1 g/L Cu, as $Cu(NO_3)_2$ (Fisher), and filtered (0.22 μm) SW. For each copper concentration, there were four replicate 3 L plastic containers. Six anemones were randomly placed in each of the 16 testing chambers. On day 4, anemones were fed brine shrimp for 1 h and then 75% of the solution was renewed. Water samples were taken at 0, 1, 4, and 7 d for later copper analysis. Behavioral observations, such as color and tentacle retraction of *A. pallida* were noted daily, however, they were not quantified. The sublethal experiment using symbiotic anemones was conducted under cool white fluorescent lighting with a light cycle of 12 h light:12 h darkness; whereas, the experiment with aposymbiotic anemones was performed in the dark.

At the start of each sublethal experiment, 3 anemones from the respective holding tanks were euthanized using tricaine methanesulfonate (Western Chemical, WA, USA), blotted, weighed, placed in plastic centrifuge tubes, acidified with 8 N trace metal grade analysis HNO_3 (Fisher) and later measured for copper. Three additional anemones from the respective holding tanks were concurrently flash froze using liquid nitrogen, placed in plastic centrifuge tubes and stored at −20 °C for later