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The effects of metals on embryo-larval and adult life stages of the sea urchin, *Diadema antillarum*

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Abstract

Since the massive population decline of the long-spined sea urchin, *Diadema antillarum*, in the early 1980s, the dynamics of coral reef ecosystems in the Caribbean have changed tremendously. The absence of *D. antillarum*, once a keystone herbivore, has led to macroalgal dominance in many of these reef communities. *D. antillarum* is not only important ecologically, but may also be a sensitive bioindicator species for toxicant exposure. Echinoderm larval development tests were conducted with *D. antillarum* exposed to elevated levels of aqueous copper (Cu), silver (Ag), nickel (Ni), or selenium (Se). All metals significantly affected larval development, based on normal development to the pluteus stage. The EC50s based on dissolved metal concentrations were 11 μg/L Cu, 6 μg/L Ag, 15 μg/L Ni, and 26 μg/L Se. Adult sea urchins were exposed to aqueous copper under flow through conditions for 96 h. The 96-h LC50 for this exposure was 25 μg/L dissolved Cu. Additionally, behavioral and physiological disturbance was observed. The physiological responses included both acid–base balance disturbance, as evidenced by reduced coelomic fluid pH and apparent ionoregulatory effects. In addition, behavioral effects included spatial orientation within the exposure tank, spine closure, and loss of spines. The high sensitivity of both adult and larval *D. antillarum* to these metals supports the use of this organism as an important biological indicator for metal exposure in marine environments.

Keywords: Copper; Silver; Nickel; Selenium; *Diadema antillarum*

1. Introduction

Sea urchins have been extensively used as bioindicators of marine pollution over the last several decades (Kobayashi, 1971; Flammang et al., 1997; Phillips, 1990). The two main life stages of the sea urchin most generally studied and used in testing are the embryo-larval and adult stages.

Sea urchin embryo-larval development has been studied since the late 19th century (Hertwig and Hertwig, 1887) and this life stage has been used to monitor pollutants in marine environments since the 1950s (Tabata, 1956; Okubo and Okubo, 1962; Kobayashi, 1971, 1984, 1994). In particular, the early life stages

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of several different species of sea urchins have been shown to be sensitive to metals (Kobayashi, 1973, 1980; Kobayashi and Fujinaga, 1976; Phillips et al., 2003).

The accumulation of pollutants in adult sea urchins has been used to monitor contaminants of many coral reef habitats (Flammang et al., 1997; Phillips, 1990). Studies have demonstrated metal accumulation in sea urchins adequately reflects abundance and bioavailability in contaminated waters (Augier et al., 1989; Ablanedo et al., 1990; Flammang et al., 1997). The urchins *Diadema setosum* and *Paracentrotus lividus* have been used as bioindicators for assessing heavy metal contamination in coral reef ecosystems of the Indo-West Pacific and the north-western Mediterranean, respectively (Flammang et al., 1997; Warnau et al., 1995).

Coral reef ecosystems appear particularly sensitive to metal pollutants (Howard and Brown, 1984). The long-spined sea urchin *Diadema antillarum* is a keystone species in healthy coral reef ecosytems of the Caribbean and its ecological importance to coral reef communities has been well documented (Carpenter, 1988; Lessios, 1988; Edmunds and Carpenter, 2001). Active grazing by *D. antillarum* in coral reef ecosystems has been shown to reduce macroalgal cover, thereby increasing abundance of juvenile corals on the reef (Edmunds and Carpenter, 2001). A significant part of research conducted on *D. antillarum* was initiated by a mortality event, which started in 1983 presumably caused by a waterborne pathogen (Lessios et al., 1984). Only 1 year later, more than 97% of the population was decimated leading to unregulated macroalgal growth in these coral reef ecosystems (Lessios et al., 1984). Although sea urchin populations have started to recover in some parts of the Caribbean (Edmunds and Carpenter, 2001; Miller et al., 2003), unfortunately, populations in the Florida Keys are still very low compared to historical levels (Lessios et al., 1984; Bauer and Agerter, 1994; Chiappone et al., 2002).

The Rosenstiel School of Marine and Atmospheric Sciences (RSMAS) at the University of Miami has been successfully culturing *D. antillarum* for the last 4 years as part of a recovery effort for this organism (Capo et al., 2003). In September 2003, mass mortality occurred in one recirculating culture system over a 24-h time period. Subsequent water analysis revealed elevated copper concentrations (~100 μg/L), due to a newly installed pump containing brass fittings, which prompted a study of Cu toxicity in adult *D. antillarum*. *D. antillarum* exhibited high sensitivity to Cu based on both sublethal endpoints and mortality. Although sea urchins in general are commonly used test organisms, little work has been done on representatives of the family Diadematidae. Given its ecological importance and the apparent sensitivity of the adult life stage to Cu, the objectives of this study were to characterize the responses of adult *D. antillarum* to Cu exposure and to determine the effects of copper, silver, nickel, and selenium on embryo-larval development.

2. Methods

2.1. Embryo-larval testing

Adult *D. antillarum* (~2 years old), which were field captured from coastal waters near Haiti and held for approximately 1 year at the RSMAS University of Miami hatchery, were induced to spawn by 0.5 M KCl injection (1 mL) into the coelomic cavity. Embryo-larval tests were performed in general accordance with standard methods developed for other sea urchin species (Dinnel and Stober, 1985; Kobayashi, 1990). In brief, sperm and eggs were collected by Pasteur pipette and added to separate beakers containing seawater (~100 mL). The eggs were counted using a Sedgewick-rafter counting cell and a dissecting microscope, diluted to a density of 200 eggs/mL and then combined with sperm for an incubation period of 15 min. The fertilized eggs were then added to 9 mL of seawater containing varying concentrations of copper, silver, selenium, or nickel to attain a final density of 20 fertilized eggs/mL. The metal solutions were equilibrated for 24 h prior to use. Three replicates containing fertilized eggs were fixed to verify the initial density. All other treatments, each with three replicates, were incubated at 20 °C and a salinity of 33 g/L until control larva reached the pluteus stage (~40 h). The formation of the pluteus stage has been shown to be more sensitive than any of the earlier developmental stages (Kobayashi, 1990). Lighting was continuous throughout the exposure period. The nominal metal concentrations used in the experiments were 0, 5, 10, 20, and 40 mg/L Cu as $CuSO_4 \times 5H_2O$; 0, 15, 30, 60, and

120 μg/L dissolved $AgNO_3$ labeled with ^{110m}Ag; 0, 5, 10, 25, 50, and 75 μg/L dissolved Ni as $NiCl_2 \times 6H_2O$; and 0, 5, 10, 20, 40, and 80 mg/L dissolved Se as Na_2SeO_4. After incubation, each replicate was scored for normal and abnormal pluteus development following ASTM guidelines (ASTM, 1995). Water samples for metal analysis were collected at the initiation of each embryo-larval test and acidified to 1% with trace metal grade nitric acid (Fisher Scientific, Pittsburgh, PA, USA).

2.2. Adult sea urchin Cu exposures

Adult *D. antillarum* (~1 year old) were field captured in the Bahamas and held for approximately 6 months at the RSMAS University of Miami hatchery. Sea urchins (n = 8–9/tank) were exposed to 0, 1, 1.5, 2, 4, 6, 13, 25, or 48 μg/L dissolved Cu for 96 h in 60-L tanks using a flow through system (flow rate = 0.5 mL/min.). The salinity was maintained at 33 g/L in all tanks. Each individual tank was photographed at 0, 48, 72, and 96-h to monitor behavioral and mortality. Behavioral end points were quantified using the following three criteria: spatial orientation within the tank (specifically falling to the tank bottom), spine closure, and loss of spines. At the end of the exposure, coelomic fluid samples were collected by a 1-mL syringe fitted with a gage 23 needle from surviving urchins.

2.3. Analytical chemistry

Copper concentrations were measured by first coprecipitating with Fe (Weisel et al., 1984) while nickel was measured by solvent extraction using chelating agents (Kinrade and Van Loon, 1974) both followed by graphite furnace atomic absorption spectrophotometry (GFAAS; Varian 220FS Mulgrave, Victoria, Australia). Selenium was measured by hydride generation (Cutter, 1986). Silver was measured by ^{110m}Ag radioisotopic dilution, as follows. An atomic absorption spectrophotometer was used to measure the silver concentration of a stock solution nominally containing 10 mg/L Ag with 250 μCi of ^{110m}Ag/L and a gamma counter (Tm Analytic) was used to measure radioactivity (total counts per minute, cpm) of ^{110m}Ag using appropriate counting windows (Hansen et al., 2002). The total silver concentration in each treatment was calculated from the ^{110m}Ag activity of the water sample and the measured specific activity of the stock solution.

For adult *D. antillarum*, coelomic fluid was analyzed for pH using a combination glass electrode (pHC3005-8 Radiometer, Villeurbanne Cedex, France) coupled to a meter (PHM220 MeterLab™, Radiometer, Copenhagen), osmolality via an osmometer (5520 WESCOR Inc., Logan, UT, USA), total CO_2 via a total CO_2 analyzer (965 Corning Limited, Halstead, Essex, UK), and Mg^{2+}, Ca^{2+}, K^+, Na^+, Cl^-, SO_4^{2-} via GFAAS (Varian 220FS Mulgrave, Victoria, Australia) and ion chromatography (DX-120; Dionex Corp., Sunnyvale, CA, USA). Total and dissolved copper, as $CuSO_4{\cdot}5H_2O$, was measured in the exposure media at 0, 48, and 96 h via AAS.

2.4. Data analysis

Embryo-larval data was normalized using Abbott's correction and the effect concentration at which 50% of the embryos had abnormal development to the pluteus stage (EC50) values were determined using ToxCalc (Mckinleyville, CA, USA). Trimmed Spearman Karber analysis was used to estimate the lethal concentration at which 50% of the sea urchins died (LC50) for adult *D. antillarum* exposed to copper. Student's t-test (two-tailed) was used to determine significant differences ($p < 0.05$) between measured physiological parameters.

3. Results

Measured dissolved metal concentrations in the embryo-larval tests were 1.9, 4.6, 9.7, 21, 40, 80 μg/L Cu; 0, 15, 32, 65, 126 μg/L Ag; 0, 8, 11, 37, 74, 101 μg/L Ni; and 0, 5, 10, 20, 36, 73 μg/L Se. All of the metals tested significantly affected larval development of *D. antillarum*. Sea urchin larva exposed to the lower treatments of Cu, Ag, Ni, or Se had abnormal development to the pluteus stage, particularly stunted arm development, and at higher concentrations did not develop past the blastula/gastrula stages (Fig. 1A–D). The resulting dissolved EC50s were 11 μg/L Cu, 6 μg/L Ag, 15 μg/L Ni, and 26 μg/L Se.

Significant mortality of adult *D. antillarum* exposed to waterborne Cu occurred in the two highest Cu

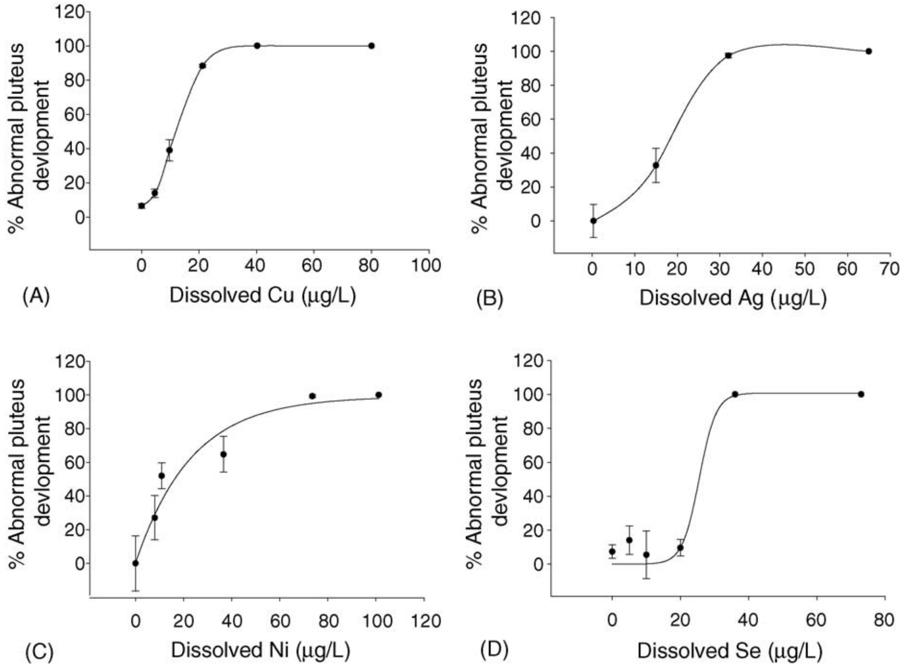

Fig. 1. *D. antillarum* embryos exposed to waterborne metals ((A) Cu; (B) Ag; (C) Ni; and (D) Se) until pluteus stage (40 h). Each data point represents the mean of three replicates and vertical bars represent standard error.

treatments resulting in a 96-h LC50 of 25 μg/L dissolved Cu (95% CI = 21.0, 29.5). Behavioral or physiological disturbance was also observed in all Cu treatments. Behavioral responses included spine closure at low concentrations, animal positioning on the bottom of the tank, and eventually complete loss of spines (Fig. 2A and B). The sea urchins first started positioning themselves on the bottom of the tank at low Cu concentrations, followed closely by spine closure at slightly higher levels. Spine loss occurred in the two highest treatments. Physiological responses included a significant increase in coelomic fluid total CO_2 concentration (Fig. 3A) in the three lowest treatments relative to the control ($p < 0.05$) leading to a significant decrease in coelomic fluid pH in the highest Cu concentration (Fig. 3B). Significant reduction in coelomic fluid osmolality also occurred in the sea urchins exposed to the middle range of Cu concentrations tested; however, no significant changes were observed in the highest two treatments (Fig. 3C). Despite change in coelomic fluid osmolality, ion concentrations in the coelomic fluid showed little if any change (Table 1).

Table 1
D. antillarum coelomic fluid ion concentrations at 96 h

Treatment (μg/L Cu)	Ca (mM)	Mg (mM)	K (mM)	Cl (mM)	SO_4 (mM)	Na (mM)
0	8.6 ± 0.3	46 ± 16.4	10 ± 3.6	544 ± 25.1	28 ± 1.0	444 ± 15.4
1	8.8 ± 0.3	46 ± 16.1	10 ± 3.6	518 ± 8.50	29 ± 0.2	445 ± 4.42
1.5	9.4 ± 0.2	50 ± 17.8	10 ± 3.6	481 ± 45.2	29 ± 1.1	500 ± 17.4
2	9.3 ± 0.4	53 ± 18.6[a]	11 ± 3.9	542 ± 8.04	28 ± 0.5	456 ± 5.49
4	9.2 ± 0.2	49 ± 17.4	10 ± 3.5	513 ± 16.2	28 ± 0.3	473 ± 11.4
6	9.3 ± 0.5	50 ± 18.8	11 ± 4.2	536 ± 8.58	29 ± 0.5	455 ± 7.56
13	8.8 ± 0.3	45 ± 18.4	8.9 ± 3.6[a]	541 ± 43.0	30 ± 1.1	494 ± 13.1
25	9.5 ± 0.3	51 ± 19.3	11 ± 4.1	550 ± 26.6	27 ± 1.6	488 ± 18.9

[a] Indicates statistical significant difference ($p < 0.05$).

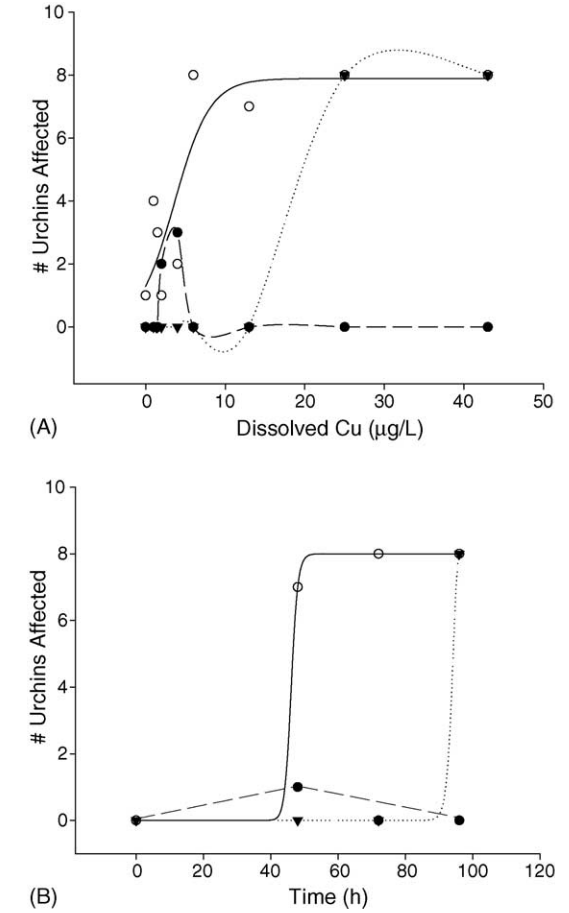

Fig. 2. Behavioral responses of *D. antillarum* exposed to: (A) varying levels of dissolved Cu for 96 h and (B) 25 µg/L dissolved Cu over 96 h. Solid circles and a long-dashed line represent spine closure, open circles, and a solid line represent orientation on the bottom of the tank, and solid triangles and a dotted line represent loss of spines.

4. Discussion

Sea urchin larvae have been shown to be highly sensitive to metals and therefore have been extensively used in marine pollution bioassays (Kobayashi and Fujinaga, 1976; Kobayashi, 1977, 1980, 1981, 1994; Phillips et al., 2003; Kobayashi and Okamura, 2004).

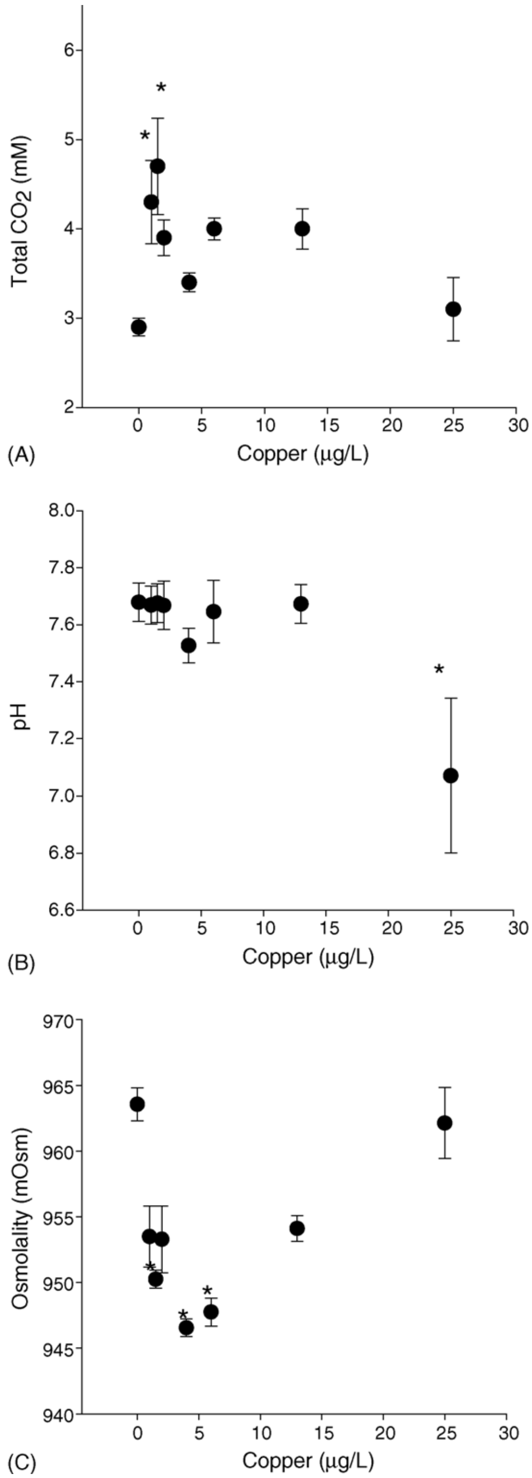

Fig. 3. (A) Total CO_2; (B) pH; and (C) osmolality of adult *D. antillarum* coelomic fluid after exposure to waterborne copper for 96 h. Each data point represents the mean values of eight to nine individuals. Asterisks indicate statistical significant difference ($p < 0.05$).

Metal concentrations in seawater generally range from 0.13 to 9.5 μg/L Cu (Kozelka and Bruland, 1998), from 0.1 to 2 ng/L Ag (Kramer et al., 2002), from 0.016 to 4.74 μg/L Se (Cutter and Cutter, 2004; Sherrard et al., 2004), and from 0.2 to 130 μg/L Ni (WHO, 1991; DETR, 1998) with the highest concentrations a result of significant anthropogenic inputs. However, in most cases, the concentration of organic ligands, such as humic substances and low molecular weight amino acids, as well as the concentration of inorganic ligands in seawater exceed metal concentrations thus rendering the metals less bioavailable to aquatic organisms (Jones et al., 1980; Turner et al., 1981; Donat and van den Berg, 1992; Campbell, 1995; Morgan and Stumm, 1991; Ma et al., 1999; Kramer et al., 2002; Lorenzo et al., 2002). In natural environments, high levels of metal complexing substances (DOC, humic substances, etc.) co-occur with elevated metal levels (Turner et al., 1981; Campbell, 1995) mainly because the dominant source for both metals and complexing agents are freshwater inputs in coastal regions.

Copper and silver have been shown to be toxic to several different species of marine invertebrates (Sosnowski et al., 1979; Kobayashi and Okamura, 2004; Hook and Fisher, 2001). The EC50 of 11 μg/L Cu measured in this study is at the low end of the range (11–120 μg/L Cu) of reported EC50s for other species of sea urchin larvae (Kobayashi, 1977, 1980, 1981, 1994; Rumbold and Snedaker, 1997; Phillips et al., 2003). *D. antillarum* larvae were also very sensitive to Ag with an EC50 below the range (14–100 μg/L) of EC50s reported for other urchins (Dinnel et al., 1982, 1983, 1989; Warnau et al., 1996). Current U.S. EPA Ambient Water Quality Criteria (AWQC) for Cu and proposed AWQC for Ag in marine systems are 3.1 and 2.9 μg/L, respectively (U.S. EPA, 1980, 2003). For these two metals, the most sensitive taxa tested to date is *Mytilus* spp. (U.S. EPA, 1980, 2003). Both EC50 values for Cu and Ag measured in this study are above current or proposed AWQC indicating that this organism would be protected by these criteria.

Previously measured Ni EC50s for sea urchins range from 200 to 350 μg/L (Kobayashi and Fujinaga, 1976; Kobayashi, 1994; Phillips et al., 2003). The EC50 for Ni measured in the present study was much lower than these reported values and five times lower than the existing AWQC (75 μg/L) for Ni in marine waters, demonstrating that the saltwater criterion is not sufficient to protect this keystone organism. Other researchers reported similar findings of effects below WQC after testing the following marine species: topsmelt, *Atherinops affinis*, red abalone, *Haliotis rufescens*, and mysid, *Mysidopsis intii* (Hunt et al., 2002).

Selenium exists as two different forms, selenate and selenite, dependent on redox state of the surrounding environment (Cutter, 1986, 1992; Oremland et al., 1990). In more reducing environments, selenite is the dominant form and in oxidizing environments selenium predominantly exists as selenate (Cutter, 1986, 1992; Oremland et al., 1990). Selenium has been shown to be a developmental toxicant in both marine invertebrates and vertebrates (DeForest et al., 1999). The selenate form was used in these studies and *D. antillarum* larvae were highly sensitive to dissolved Se with an EC50 of 26 μg/L. No AWQC currently exists for selenate because only one species, the striped bass, has been tested (96-h LC50 = 9790 μg/L). However, a number of species have been tested for selenite toxicity and an acute AWQC of 127 μg/L has been derived. The EC50 generated in these experiments is much lower than any other species tested suggesting that the current AWQC for selenium (based on selenite) is under-protective.

With the exception of *Diadema setosum*, all other urchins commonly used in toxicity testing are phylogenetically distant from *D. antillarum* as they are not in the same superorder. Therefore, it is not surprising that their sensitivities to metals are considerably different. The toxicity values generated after exposure to Cu and Ag were near the range reported in the literature; however, *D. antillarum* were much more sensitive to Ni and Se exposures than any other organism tested. This may at least in part reflect the limited toxicity data for Ni and Se as well as the apparent hypersensitivity of *D. antillarum* to these metals.

Adult *D. antillarum* exposed to Cu demonstrated both behavioral and physiological responses. All sampling was conducted at 96 h; however, the sea urchins in the highest Cu treatments likely experienced the physiological changes earlier than those observed in the lower treatments towards the end of the 96 h. This suggestion is supported by observed behavioral responses in the sea urchins. Those exposed to the highest Cu concentrations closed their spines within the first 24 h, whereas spine closure in the lower Cu treatments occurred between 48 and 96 h.

From our observations of ultimately reduced coelomic fluid pH at the highest Cu concentrations and elevated total CO_2 (largely HCO_3^-) at the lowest concentrations, we suggest that copper exposure induces respiratory acidosis (elevated PCO_2 in coelomic fluids) in these animals. This acidosis appeared to be compensated at lower copper concentrations by increased coelomic total CO_2, presumably by retention of HCO_3^- (metabolic alkalosis). Impaired CO_2 excretion (respiratory acidosis) could be the result of Cu induced inhibition of the enzyme carbonic anhydrase as previously suggested by other researchers (Wang et al., 1998; Pilgaard et al., 1994). Supporting the idea are demonstrations of carbonic anhydrase inhibition caused by copper in invertebrates (Vitale et al., 1999) although in the latter case, acid–base parameters were not recorded.

An osmoregulatory disturbance in response to Cu exposure was apparent from reduced coelomic fluid osmolality at the lower Cu concentrations; however, this effect did not exhibit traditional Cu concentration dependence. Interestingly, the drop in plasma osmolality was not explained by corresponding changes in the concentrations of coelomic fluid inorganic osmolytes, suggesting that an organic osmolyte (perhaps amino acid or polypeptide) contributes significantly to the osmotic pressure of the coelomic fluids and that this constituent is influenced by Cu exposure. It should be noted that even though the sum of the measured inorganic osmolytes exceed the measured osmotic pressure, additional osmolytes might still be present. The osmotic coefficient of main electrolytes in biological fluids can be expected to be <1, which means that only a fraction of the concentration measured by anion chromatography or atomic absorption spectrophotometry contributes to the osmotic pressure of the sample.

Considering both the acid–base balance disturbance and the osmoregulatory response combined, one might be tempted to suggest that the acidosis is secondary to an osmoregulatory disturbance (increased strong cation–anion difference made up by H^+). Admittedly, the resolution of our inorganic anion measurements may not have been sufficient to detect such subtle shifts. However, we note that the acidosis is fully developed at the highest copper concentrations whereas osmolality in the same samples was similar to control values. This demonstrates that the acid–base balance disturbance and the osmoregulatory disturbance are uncoupled.

Respiratory impairment leading to acid–base balance disruption appears to be the primary cause of acute toxicity in these invertebrates. The apparent respiratory acidosis occurred at lower copper concentrations but is compensated by metabolic alkalosis (evident from elevated total CO_2). The compensatory capacity appears to be exceeded at the highest Cu concentrations, leading to significantly reduced coelomic fluid pH and mortality.

Interestingly, behavioral responses mimicked the impaired physiology of the organism. Typically, the control organisms sorbed to the sides of the tank whereas the Cu-exposed sea urchins fell to the bottom of the tank in a time and dose-dependent manner (Fig. 2A and B). In addition, sea urchins closed their spines at lower Cu concentrations (Fig. 2A). Sea urchins exposed to higher Cu concentration initially closed their spines and then lost them entirely (Fig. 2B). These behavioral responses may have useful application as a non-invasive biomonitoring tool in polluted marine environments.

5. Conclusions

Sea urchin larval development to the pluteus stage was affected by Cu, Ag, Ni, and Se at low levels. To the best of our knowledge, *D. antillarum* larvae is the most sensitive marine species tested to date for Ni and Se and the second most sensitive species for Ag and Cu. Adult sea urchins were also highly sensitive to waterborne Cu with both behavioral and physiological responses occurring at low dissolved Cu concentrations. A respiratory acidosis rather than an osmoregulatory disturbance may be the mechanism of acute Cu toxicity in *D. antillarum*. Both embryo-larval and adult *D. antillarum* appear to be highly sensitive bioindicators for metal pollution in marine environments and should be considered when determining ecological risk in coral reef environments.

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