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EFFECT OF ANTHROPOGENIC POLLUTANTS ON ESA CORAL HEALTH

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Abstract

Genetics and differences in physiological performance among genotypes of coral species have been of particular interest recently in coral reef restoration endeavors as a means of optimizing restoration efforts. Dose-response profiles for three genotypes of the Endangered Species Act listed Caribbean coral *Acropora cervicornis* (staghorn coral) were compared when exposed to copper (II) chloride, a common marine toxicant. Results indicate differential responses in photosynthetic activity and wound healing among the three genotypes tested.

Executive Summary

Coral nurseries and the outplanting of coral propagules are being used extensively in the U. S. Caribbean to mitigate and restore degraded *Acropora cervicornis* populations. Differentiating among susceptible and hardy nursery stocks is an important factor to increase success in restoration practices. Three genotypes of *A. cervicornis* were subjected to varying concentrations of copper over a 96 h exposure period. Dose-response effects were determined from three endpoints: coral health scores (visual observations), coral tissue regeneration (image analysis of photo-macrographs), and photosynthetic activity (pulse-amplitude modulated, PAM, fluorometry). The no observable effect concentration (NOEC) for tissue regeneration was 100 µg/L (100 ppb) for all genotypes, while the lowest observable effect concentration (LOEC) was 200 µg/L of copper chloride, which showed differential responses among genotypes. Both the photosynthetic activity and health scoring indicated an adverse effect (LOEC) at 50 µg/L CuCl₂ for two genotypes, suggesting copper had a deleterious effect on the algal symbionts.

Introduction

Coral reefs are among the most diverse ecosystems on Earth, containing almost a quarter of the world's marine life in less than one percent of the ocean's area (Porter and Meier 1992; Sheppard et al. 2009; Plaisance et al. 2011). Yet they are fragile, being sensitive to minute changes in their environment (Achituv and Dubinsky 1990; Kleypas et al. 1999; Precht et al. 2002). Reefs worldwide are faced with severe threats as the marine environment changes, driven by anthropogenic and natural factors; Caribbean coral reefs alone have experienced an estimated 80 percent decrease in cover since the 1990s (Hughes et al. 2003; Bellwood et al. 2004; Hoegh-Guldberg et al. 2007). The negative effects of overfishing as well as pollution from terrestrial run-off and agriculture have been exacerbated by environmental changes and severe outbreaks of disease, decimating global coral populations (Harvell et al. 1999; Watson and Team 2001; Prouty et al. 2008). Of these factors, the effects of anthropogenically-introduced heavy metals into coral reef ecosystems have been studied among the least (Howard and Brown 1984), though present a growing threat to reef ecosystems around the globe (Bielmyer et al. 2010).

Heavy metal contamination of the oceanic environment stems from various sources, including but not limited to industrial discharges, urban and agricultural run-off, sewage discharges, and anti-fouling paints (Jones 1997; Reichelt-Brushett and Harrison 2000; Mitchelmore et al. 2007). One of the most toxic of the heavy metals is copper, particularly the free copper ions Cu^+ and Cu^{2+} (Campbell 1995; Reichelt-Brushett and Harrison 2005; Neira et al. 2014). Ship groundings result in significant exposure of coral reefs to copper-based anti-fouling paints (Negri et al. 2002), while new industries on coastal and island nations such as metal mining and smelting (Brown 1987), food processing, and manufacturing of batteries, fertilizers, cosmetics, and pharmaceuticals all contribute to significant copper contamination of their surrounding coastal waters (Guzmán and Jiménez 1992; Ross and DeLorenzo 1997). Copper concentrations have been measured in healthy oceanic ecosystems to be approximately 8.0×10^{-4} $\mu\text{g/L}$ (parts per billion) Cu^{2+} (Sadiq 1992), but have been found up to 36.5 $\mu\text{g/L}$ Cu^{2+} at highly polluted sites, such as in the coastal waters surrounding mining operations in Taiwan (Hung et al. 1990; Webster et al. 2001).

Mechanisms of copper toxicity on coral reef communities include oxidative stress through the production of free radicals (and subsequent macromolecular damage) (Mason and Jenkins 1995; Main et al. 2010), primarily through the Haber-Weiss reaction (Main et al. 2010; Zeeshan et al. 2016). Inhibition of growth and metabolic processes has been demonstrated in *Porites cylindrica* (Nyström et al. 2001), as well as decreased production of the enzyme carbonic anhydrase in *Montastraea cavernosa* and a resulting decrease in calcification (Gilbert and Guzmán 2001), an increase in larval mortality in *Pocillopora damicornis* (Esquivel 1986), a decrease in larval settlement success (Reichelt-Brushett and Harrison 1999,2000,2005), and a bleaching response

(Reichelt and Jones 1994; Jones 1997; Baird et al. 2009). Copper exposure has also been shown to induce a decrease in effective quantum yield of photosystem II in the zooxanthellae of *Aiptasia* (Main et al. 2010). Some genotypes of coral species (such as *Orbicella faveolata* and its dinoflagellate symbiont *Symbiodinium* D1a) have been shown to be more resistant to heavy metal exposure, and are theorized to have a different copper requirement and potentially more metal binding proteins (Bielmyer et al. 2010).

Mitigation of the harmful effects of copper toxicity has been shown in both host corals as well as symbiotic zooxanthellae. Increased mucus production by *Anemonia viridis* has been demonstrated with increasing exposure to copper (Harland and Nganro 1990). Mucus has been theorized to bind heavy metals in harmless complexes, and sequestration of these metals into mucus could prevent macromolecular damage to host tissues (Reichelt and Jones 1994; Mitchelmore et al. 2003; Mitchelmore et al. 2007). Symbiotic bacterial communities in coral tissues are suspected to have a protective role against toxicants, due to their capacity to bind heavy metals at the cell surface or transport it into the cell for a number of cellular functions (Ehrlich 1997; Webster et al. 2001). Sequestration of metals into the zooxanthellae, and later sacrificial exposure of these symbionts (Harland and Nganro 1990) is understood to be a mechanism for protection of the host coral, as well as in regulation of trace metals (Webster et al. 2001; Reichelt-Brushett and McOrist 2003; Main et al. 2010).

Acropora cervicornis (commonly known as staghorn coral) is a fast-growing, branching Caribbean coral with growth rates ranging from 10-20 cm per year (Gladfelter et al. 1978; Reichelt-Brushett and Harrison 2004; Bielmyer et al. 2010). It has played an essential role in the growth and development of Caribbean reef habitats, occurring in back and fore reef environments with a depth profile ranging from 2-30 m (Reichelt-Brushett and Harrison 2004; Sheppard et al. 2009; Lirman et al. 2014). Changing ocean conditions, both naturally- and anthropogenically-induced, including ocean acidification, warming, sedimentation, disease, and increased hypoxic and anoxic zones (Hughes et al. 2003; Hoegh-Guldberg et al. 2007; Lewis et al. 2016) have contributed to a significant decrease in *A. cervicornis* coral cover in the Caribbean, leading to its listing as “threatened” under the Endangered Species Act (ESA) of 1973 (Hogarth 2006).

In recent years, it has become evident that coral reef ecosystems will not recover from or suitably adapt to anthropogenic stress without the use of active restoration methods to encourage reef development (Rinkevich 2005). For example, the Coral Restoration Foundation (CRF) maintains a well-established coral nursery several miles off the coast of Tavernier, FL. This nursery maintains 150 known genotypes of *A. cervicornis*, identified through microsatellites developed by Baums et al. (2009), and utilizes the Coral Tree Nursery[®] method, in which asexually fragmented corals from the same parent colony are suspended from floating PVC-framework trees by monofilament line (Nedimyer et al. 2011). This method has been successful in the mariculture of *A. cervicornis*

and *A. palmata*, both corals exhibiting increased growth rates due to increased water flow and lack of substrate holdfast, and has been recommended for the mariculture of other stony, branching corals including *Stylophora* and *Montipora* species (Johnson et al. 2011; Nedimyer et al. 2011).

Genetics and differences in phenotypic expression among genotypes of coral species have been of particular interest in restoration science since the early 2000s (Baums 2008; Lohr and Patterson 2017). Some nursery-grown genotypes of *A. cervicornis* have been shown to display significantly elevated growth rates above other genotypes (Bowden-Kerby 2008; Lirman et al. 2014; O'Donnell et al. 2017), and some carry a higher bleaching resistance (Edmunds 1994). A study carried out by Libro and Vollmer (2016) indicated that more than 5% of *A. cervicornis* genotypes are resistant to White Band Disease, a highly pathogenic disease that has decimated Acroporid populations since its first observation in the 1970s (Gladfelter 1982; Vollmer and Kline 2008). Recent restoration efforts have attempted to identify these resilient, or vigorous genotypes for use in selections for nursery stocks and outplantings (Lohr and Patterson 2017), as it is highly unlikely that all known genotypes are well-suited for reef restoration due to increasing stressors (Bowden-Kerby 2008; van Oppen et al. 2015). Understanding phenotypic differences between genotypes of threatened coral species, and how these differences may influence the success of outplants, will be critically useful in increasing the frequency of positive restoration outcomes. Furthermore, developing a trait-based system for identifying hardy genotypes within nurseries has the potential to increase success in restoration over the long-term (van Oppen et al. 2015).

We conducted exposure-response toxicity testing to determine the effects of copper exposure on the threatened *A. cervicornis* coral (staghorn). In response to copper (II) chloride, we measured three endpoints: tissue regeneration, photosynthetic activity through quantum yield, and visual observations using coral health scores. We also examined whether *A. cervicornis* genotypes displayed differential responses to the copper (II) chloride exposures. To test if there were differences in genotype vigor, we used three nursery-grown genotypes from CRF. These genotypes were selected based on CRF outplanting history and noted differences in phenotype, such as genotype U44 displaying higher bleaching resistance, while K2 and U41 displayed greater total length extension based on previous studies (Lohr and Patterson 2017).

Materials and Methods

Test organisms

Three genotypes of asexually-fragmented *A. cervicornis* (U44, U77, and K2) were obtained from the Coral Restoration Foundation (CRF) nursery in Tavernier, Florida in May 2016 and transported

to the NOAA Center for Coastal Environmental Health and Biomolecular Research's coral culture facility in Charleston, South Carolina. The corals were held under permit (South Carolina Department of Natural Resources permit NI16-0401 and Florida Keys National Marine Sanctuary permit FKNMS-2016-021) in a closed-artificial seawater system at a salinity of 35 parts per thousand (ppt) and temperature of 26°C. The corals were cultured under an Aqualife light fixture with photosynthetically active radiation (PAR) measurements of 109.5 μE . Corals were allowed to recover after handling and transport by acclimating at these conditions for three weeks prior to experimentation. Prior to acclimation, coral branches were glued to notched Teflon (polytetrafluoroethylene, PTFE) pegs using cyanoacrylate gel Super Glue.

Test conditions

All artificial seawater used was prepared using Tropic Marin reef salt in Type 1 (Milli-Q) water at a salinity of 35 ppt and left overnight to acclimate to a temperature of 26°C within the temperature-controlled dosing room before use. Temperature and salinity of artificial seawater solutions was kept constant across acclimation solutions and copper treatment solutions. Prior to copper dosing, coral branches were cross-sectioned by removing the apical branch tips using shears to yield coral fragments each approximately 1.75 cm in length and 0.75 cm in diameter. Each fragment was placed vertically into a Teflon PTFE stand in its own 300 mL treatment beaker filled with 200 mL treatment solution under the same overhead Aqualife lighting fixture used in acclimation. Photosynthetically active radiation (PAR) measurements ranged from 100-120 μE . Photoperiod was 10 h light and 14 h darkness. Treatment beakers were completely randomized on a grid situated on a bench top under the lighting fixture to minimize variability in light exposure, and each beaker had its own Teflon (PFA) airline for aeration.

Concentrated stock solutions were prepared in artificial seawater from copper (II) chloride (CuCl_2 , Sigma-Aldrich, CAS# 7447-39-4) prior to the initiation of the experiment and were then diluted daily to the final concentrations: 0, 10, 50, 75, 100 and 200 $\mu\text{g/L}$ CuCl_2 . The exposures were carried out in a block design. Block 1 included concentrations 0, 10 and 50 $\mu\text{g/L}$ in which each treatment concentration had three independent replicates for each genotype. Block 2 included concentrations 0, 75, 100 and 200 $\mu\text{g/L}$ CuCl_2 in which each treatment concentration had three independent replicates for each genotype. Transfer of wounded coral fragments from acclimation seawater into the treatment solution using Teflon-coated tongs (PFA) initiated each 7-day experimental trial. All experimental variables were tested on each individual coral fragment over the trial. Water was changed (100%) daily for all treatment concentrations following macroscope photography.

Tissue regeneration

The ability and rate at which coral are able to heal wounds is an indication of the overall physiological condition of the coral. Wound healing requires multiple physiological processes to function properly in order to lay down new tissue, to heal damaged tissue margins and then cover exposed skeleton with new tissues. Wound healing rate of *A. cervicornis* genotypes as exposed to copper was determined to analyze the effect of heavy metals on the regenerative potential and energy allocation of the coral.

A uniform wound on each coral fragment was inflicted through cross sectioning, exposing the bare coral skeleton on each apical fragment tip. All coral fragments were photographed individually within their treatment beakers under bright field illumination daily using an Olympus MVX10 Research Macro Zoom System Microscope and an Olympus DP71 digital camera, equipped with Olympus CellSens imaging software. Magnification and objective were kept constant throughout the experiment at 1.25 and 0.63x, respectively. Photos were analyzed using Adobe Photoshop by measuring the area (cm²) of exposed skeleton on the cut coral face, which decreased over the course of the experiment as the tissue regenerated over the wound. A Teflon (PTFE) ruler, leveled with the cut surface, was used to calibrate the scale in Photoshop. Percent tissue regeneration was calculated by dividing the day 4 (96-hr) remaining bare skeleton area by the day 0 (immediately following placement into the copper treatment) skeleton area, and multiplying by 100 to determine percent of remaining bare skeleton. The percent of newly regenerated tissue was determined by subtracting this value from 100% (a healed wound with tissue fully regenerated).

The experiment was continued another 3 days for a total of 7 days of exposure to determine effects of longer-term exposure. Newly generated tissues were stained with the live-cell stain, Toluidine Blue O (Sigma-Aldrich, CAS# 92-31-9) to provide greater contrast between bare skeleton and regenerated tissue at the end of the experimental trial (day 8). A 1% stock solution was prepared by dissolving 0.03 g Toluidine Blue O in 3 mL dimethyl sulfoxide (DMSO, Sigma-Aldrich, CAS# 67-68-5). The stock solution was diluted to a final concentration of 0.1% in artificial seawater (35ppt) and pipetted onto the exposed cut face of each coral fragment for 6 min, rinsed in artificial seawater and then photographed following the same procedures described above.

Effective quantum yield

The effective quantum yield is a measure of photosynthetic activity and an indirect measure of the ability of the symbiont to fix carbon in the form of photosynthate, an energy source for the coral. Photosynthetic activity was determined by measuring fluorescence and calculating light adapted quantum yield of the algal symbionts using a maxi-imaging pulse-amplified modulated (IMAGING-PAM *M*-Series) fluorometer (Walz, Effeltrich, Germany) on all coral fragments

immediately prior to dosing (time 0) and at day 7 of the experiment. Three coral fragments of the same treatment concentration and genotype were measured at a time by orienting each fragment horizontally in a glass dish containing sufficient copper-free artificial seawater to cover the coral fragments completely. Orientation of the coral fragments was maintained for repeated measurements by aligning a notch cut into the Teflon PFTE peg with a notch cut into a rectangular Teflon PFTE stand.

Fluorescence measurements were taken 4 h into the light cycle and placed in the dark for 5 min prior to the initiation of a saturation pulse (intensity=10). The Walz software ImagingWin was then used to determine the light adapted photosynthetic efficiency [effective quantum yield $Y(II) = (F_m' - F) / F_m'$] from measured values of F (fluorescence yield briefly before saturation pulse), F_m' (maximum fluorescence yield of illuminated sample with all PSII centers closed) for all corals at each copper concentration and time point. Three areas of interest were measured on images of each replicate coral fragment, to obtain an average effective quantum yield along the length of the fragment. Data were represented as percent of control effective quantum yield.

General Coral Health Scoring

Overall coral health score (Fig 1) was based on a cumulative, eleven-point scale of three variables modified from a scoring rubric by Woodley et al. (2014). "Tissue health" was scored on a scale from 0-5, with one corresponding to a significant loss or "sloughing" of tissue and five corresponding to intact, healthy tissue. "Color" was scored on a scale from 0-5 with zero being completely bleached, while intact tissue showing normal golden-brown, consistent color received a score of 5. "Polyp behavior" was scored as 0 or 1, with zero indicating polyps were retracted and one, polyps were extended. Coral health scores were recorded for each coral fragment daily (at the same time of day) and representative photos for each condition were taken.

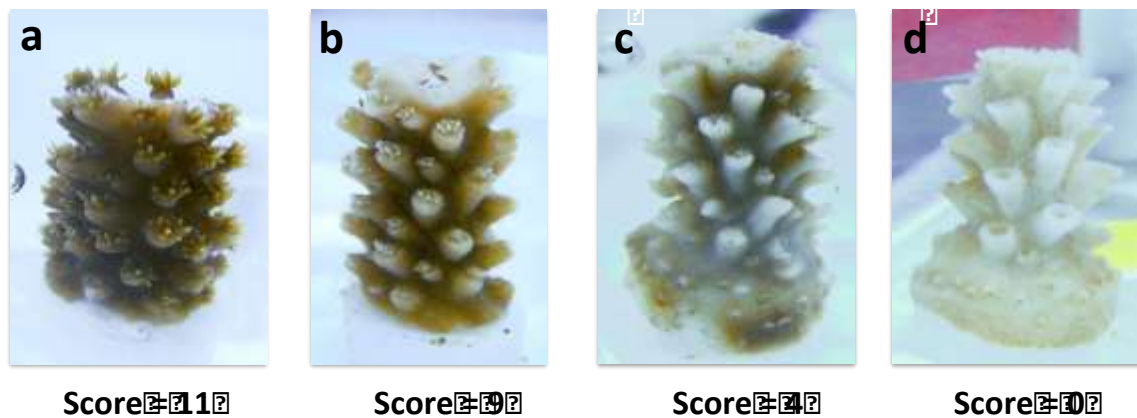


Figure 1 Examples of *A. cervicornis* Health Scoring Criteria. Normal control coral (a) with a maximum health score of 11. Panels b-d show degrees of change in morphology with increasing concentrations of $CuCl_2$ and accompanying health score.

Statistical Analyses

Control comparisons were first completed to test if experimental blocks could be compared. Comparisons between controls showed that the differences in both blocks were not statistically significant, which allowed for the comparison of data across both blocks ($p = 0.1471$). All data were tested for normality using the Shapiro Wilks W test, and are presented as the mean \pm standard error of the mean. All statistical tests were conducted using a significance level of $\alpha = 0.05$.

SAS software (v9.4) was used to perform two-way ANOVA (across concentration and genotype) on two of the three data variables: tissue regeneration and coral health score. When there were no significant differences across genotypes in the two-way ANOVAs, one-way ANOVAs were performed on concentrations within each genotype. For the tissue regeneration data, a multiple comparisons Dunnett post hoc test was applied to aid in the determination of a no observable effect concentration (NOEC) and a lowest observable effect concentration (LOEC). These values are commonly derived in environmental toxicology studies to identify toxicity thresholds to various toxicants. For coral health score data, one-ANOVAs were unnecessary, and all-pairwise comparison Dunnett post hoc tests were applied to identify NOEC and LOEC values.

Photosynthetic efficiency data grossly failed the Shapiro Wilks normality test due to mortality of select individuals in the highest copper concentration. Non-parametric, single-factor Kruskal-Wallis tests were subsequently run for all concentrations of each genotype, and post hoc Dunnett (non-parametric) tests were applied to each genotype for comparisons versus control. Single-factor Kruskal-Wallis ANOVAs were also run on all genotypes across each concentration, and post hoc multiple comparison analyses were applied to each concentration through the Dwass, Steel, Critchlow-Fligner method to determine if there were significant differences between each genotype.

Results

Tissue regeneration

A no observable effect concentration (NOEC), i.e., the highest tested concentration at which the response did not differ from the control, was determined to be 100 $\mu\text{g/L}$ CuCl_2 ($p = 0.9616$) (Table 1). At 96h for concentrations lower than 200 $\mu\text{g/L}$, tissue regeneration was estimated at 73.25 percent across all treatments. Data indicated that 200 $\mu\text{g/L}$ CuCl_2 inhibited tissue regeneration in genotype U44, while causing tissue loss in genotypes U77 and K2 (Fig 2). There was no significant genotype effect ($p = 0.0796$, $df = 2$) observed with percent tissue regeneration of *A. cervicornis*; however, a significant concentration effect was observed at 200 $\mu\text{g/L}$ CuCl_2 , which was significantly different from the control ($p < 0.0001$, $df = 5$). Through the Dunnett post hoc test, this concentration was determined to be the lowest observable effect concentration (LOEC) ($p < 0.0001$), or the lowest concentration at which the response significantly differed from the control, at -15.74 percent regeneration.

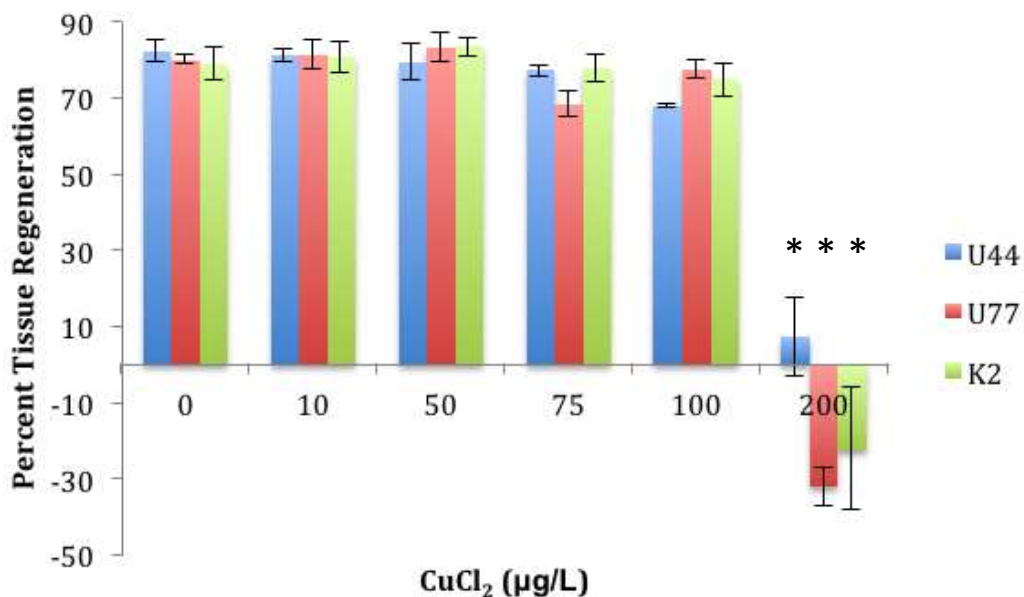


Figure 2 Mean 96 h percent tissue regeneration for *A. cervicornis* genotypes U44, U77, K2. Genotypes U77 and K2 showed marked tissue loss at 200 $\mu\text{g/L}$ concentrations, while U44 maintained tissue but was unable to heal ($n=3$). Error bars represent SE. Asterisks denote concentrations at which percent tissue regeneration significantly differed from the control concentration as identified by two-way ANOVA ($p < 0.0001$).

Effective Quantum Yield (Y(II))

Single-factor Kruskal-Wallis ANOVAs on each genotype (across all concentrations) allowed for the determination of NOEC and LOEC values with respect to this variable (Fig 3). For genotype U44, a significant concentration effect was seen ($p = 0.0128$, $df = 5$), and a post hoc Dunnett test for

comparison versus control determined a NOEC to be 10 $\mu\text{g/L}$ CuCl_2 ($p = 0.3968$) at 102.59 percent of control and a LOEC to be 50 $\mu\text{g/L}$ CuCl_2 ($p = 0.0498$) at 97.86 percent of control (Table 1). For genotype U77, a significant concentration effect was seen ($p = 0.0445$, $df = 5$), and the same Dunnett test determined a NOEC to be 100 $\mu\text{g/L}$ CuCl_2 ($p = 0.0905$) at 103.41 percent of control and a LOEC to be 200 $\mu\text{g/L}$ CuCl_2 ($p < 0.0001$) at 65.39 percent of control. For genotype K2, a significant concentration effect was seen ($p = 0.0048$, $df = 5$), however NOEC and LOEC values could not be calculated because Dunnett test results determined all concentrations to be significantly different from control ($p = 0.0036$). Single-factor Kruskal-Wallis ANOVAs on each concentration (across all genotypes) allowed for determination of differences in genotype. Significant genotype differences were observed between genotype U77 and K2 in the 0 $\mu\text{g/L}$ CuCl_2 ($p = 0.0157$) and 100 $\mu\text{g/L}$ CuCl_2 ($p = 0.0319$) treatments.

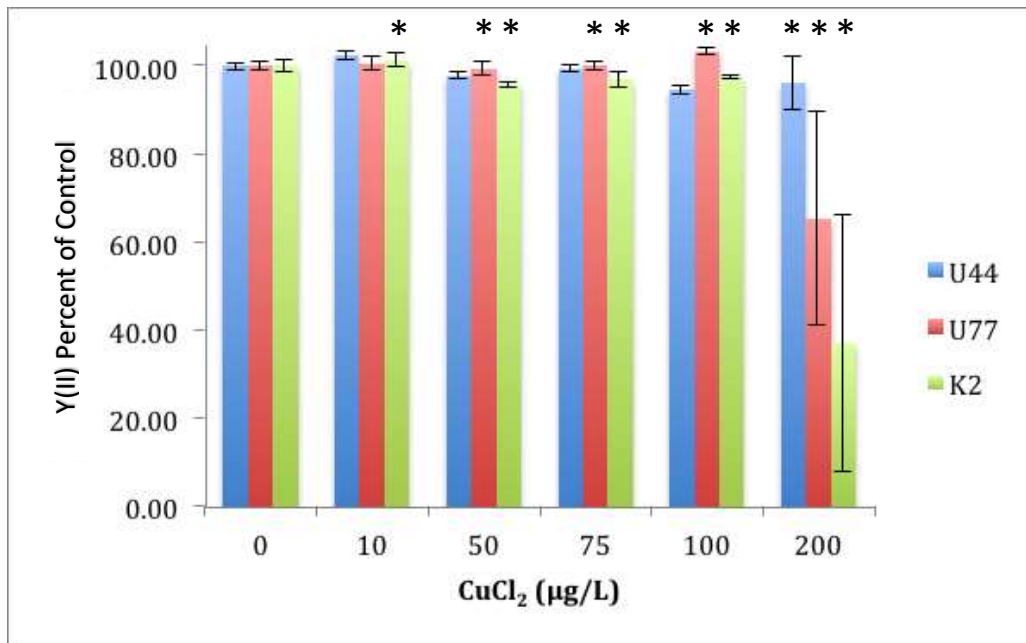


Figure 3 Effective Quantum Yield ($Y(II)$) of PS II for *A. cervicornis* genotypes. Effective quantum yield is represented as the percent of control, for each of the three *A. cervicornis* genotypes exposed to increasing concentrations of CuCl_2 over 7 days ($n=3$). Error bars represent standard error of the mean. Asterisks denote concentrations at which $Y(II)$ (percent of control) significantly differed from the control concentration as identified by two-way ANOVA ($p = 0.0128$ for U44, $p = 0.0445$ for U77, $p = 0.0048$ for K2)

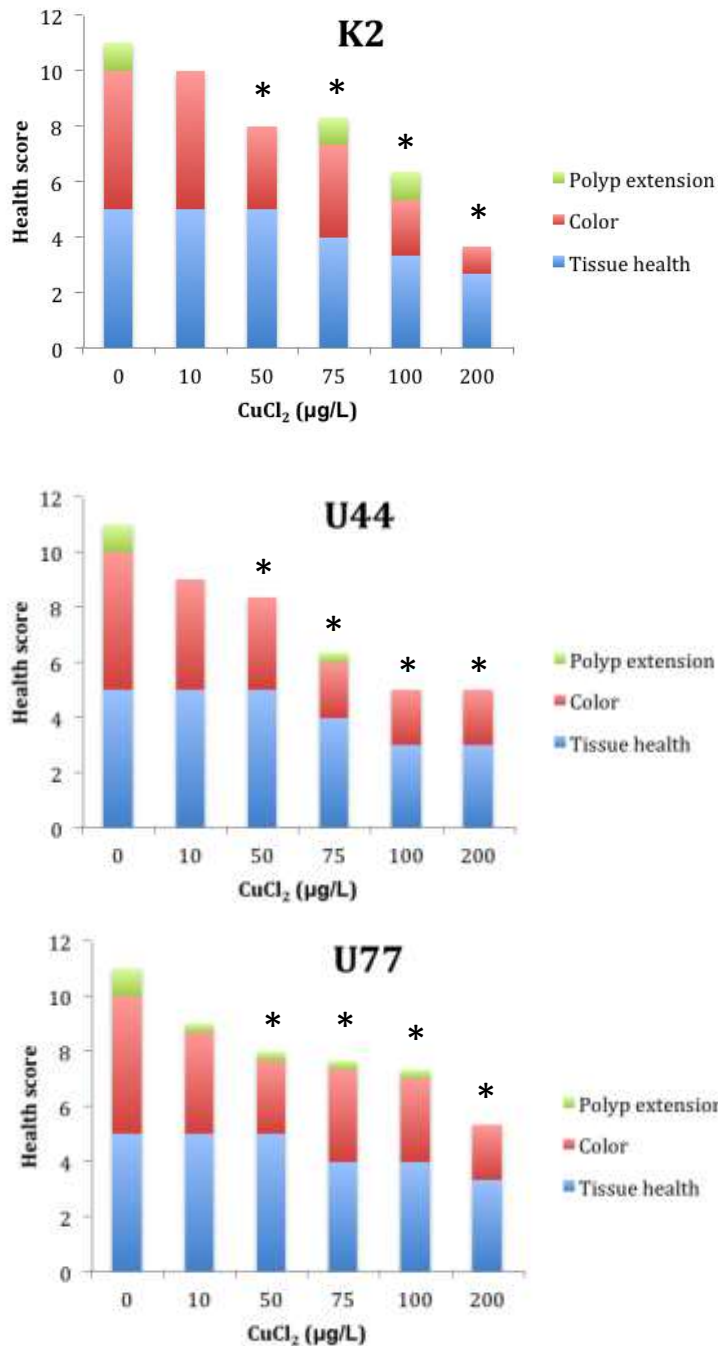


Figure 4 Health Scores of *A. cervicornis* Genotypes Exposed to CuCl₂. Coral fragments were exposed concentrations at 96-h of exposure (n=3). Error bars represent SE. Asterisks denote concentrations at which health scores significantly differed from the control concentration as identified by two-way ANOVA ($p < 0.0001$). Genotypes U44 and U77 displayed differential responses to the exposures ($p=0.0023$).

Overall Coral Health

Gross morphological features were scored daily according to an 11 point rubric. At 96h the coral health score, demonstrated a significant concentration effect ($p < 0.0001$, $df = 5$) at much lower concentrations as compared with control (Fig 4). A post hoc Tukey Kramer test determined a NOEC to be 10 µg/L CuCl₂ with an average score of 9.33 and a LOEC to be 50 µg/L CuCl₂ ($p < 0.0001$), with an average score of 8.11 (Table 1). A significant genotype effect was also seen with respect to coral health score, with genotypes U44 and U77 significantly differing from each other ($p = 0.0023$, $df = 2$), however, genotype K2 did not significantly differ from either U44 or U77 ($p = 0.0779$) (Fig 4).

The experimental exposures were extended to 7 days (Fig 5) to determine whether health scores continued to degrade at the lower concentrations. Genotypes U77 and K2 appeared to experience increased adverse effects compared to U44 (Fig 5 A-C).

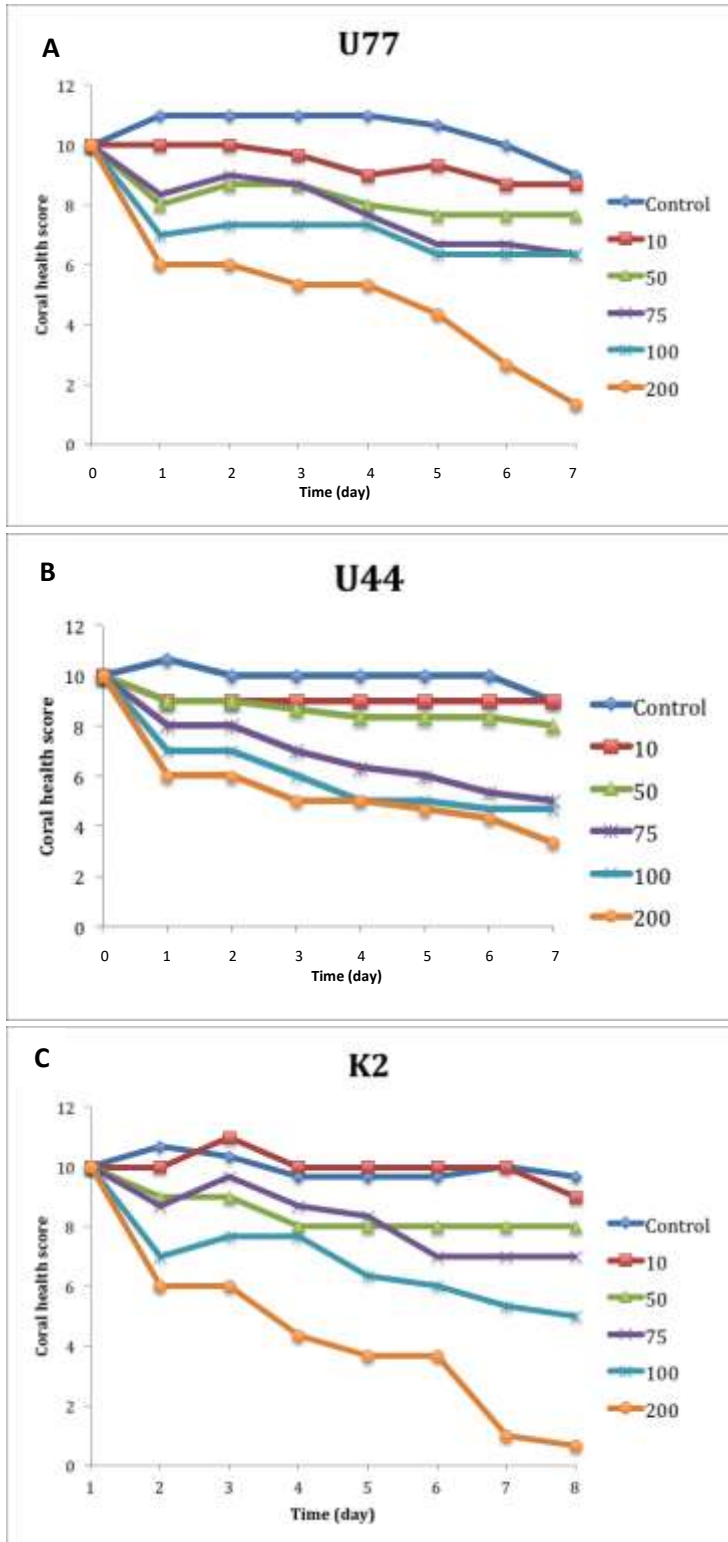


Figure 5 Exposure Response Profiles Over Time. Overall health score performance over 7 days exposure for three genotypes of *A. cervicornis*: a) U77, b) U44, and c) K2 as exposed to increasing concentrations of CuCl_2 ($n=3$).

Table 1. No observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) of *A. cervicornis* genotypes for each variable as exposed to copper (II) chloride (n=3).

Variable	NOEC ($\mu\text{g/L CuCl}_2$)			LOEC ($\mu\text{g/L CuCl}_2$)		
	U44	U77	K2	U44	U77	K2
Tissue Regeneration	100	100	100	200	200	200
Photosynthetic Effective Quantum Yield	10	100	--	50	200	--
Coral Health Score	10	10	10	50	50	50

Discussion

Coral reef ecosystems, often in close proximity to developed coastal zones, are under direct threat by increasing copper contamination in coastal waters from industrial discharges, urban and agricultural run-off, sewage discharges, and antifoulant paints. The 1988 U.S. ban on tributyltin (TBT) based anti-fouling paints due to its severe toxicity to aquatic life has resulted in a sharp increase of copper-based paints in the Caribbean (Inoue et al. 2004). A study by Saphier and Hoffmann (2005) forecasted an increase in copper levels in the ocean of $0.037 + 0.014$ ppb due to copper-based antifouling paints; in fact, leaching from ship hulls that carry these paints has been estimated to contribute 93% of total copper entering the Shelter Island Yacht Basin in San Diego, California (Bosse et al. 2014). Marinas, lagoons and harbors are areas where water is not consistently flushed and have been found to experience the most severe contamination (Saphier and Hoffmann 2005).

The effects of copper on the marine environment are expected to increase dramatically as the ocean experiences anthropogenically-induced changes. Changes in pH are predicted to alter the bioavailability of waterborne metals due to changes in their speciation in seawater at a lower pH (Millero 2009). In the next century, concentrations of the toxic free ion Cu^{2+} is expected to increase by 115% in coastal waters due to this phenomenon (Pascal et al. 2010; Richards et al. 2011; Lewis et al. 2016). In addition, a study by Lewis et al. (2016) found that damage to DNA and lipids by copper exposure was exacerbated under simulated low pH conditions compared to control conditions in two key species of marine invertebrates. Considering metals can remain in the water column or collect in marine sediments without degrading almost indefinitely (Millero

2009), we can expect that copper can have a long-lived significant, negative effect on marine ecosystems, including coral reefs. The treatment concentrations used in this experiment were prepared from copper chloride dihydrate, of which the toxic cupric ion is only 37% by mass, corresponding to much lower actual concentrations of the free copper ion (Table 2). Considering copper concentrations in the marine environment have been found to exceed 36.5 $\mu\text{g/L}$ Cu^{2+} at highly polluted sites (Hung et al. 1990), the concentrations used in this experiment are highly relevant environmentally with effects levels falling within levels reported in polluted areas (Hung et al. 1990).

Table 2. CuCl_2 concentrations used in this study, as well as calculated, corresponding concentrations of the free cupric ion, Cu^{2+} .

CuCl_2 concentration	Cu^{2+} concentration
0 $\mu\text{g/L}$	0 $\mu\text{g/L}$
10 $\mu\text{g/L}$	3.7 $\mu\text{g/L}$
50 $\mu\text{g/L}$	18.5 $\mu\text{g/L}$
75 $\mu\text{g/L}$	27.75 $\mu\text{g/L}$
100 $\mu\text{g/L}$	37 $\mu\text{g/L}$
200 $\mu\text{g/L}$	74 $\mu\text{g/L}$

Despite the growing interest in identifying and propagating resilient genotypes of threatened, nursery-reared coral genotypes, it is essential that restoration efforts strive to maintain genetically diverse populations in nurseries and maintain overall genetic diversity in outplanted, monoclonal populations (Baums 2008; Shearer et al. 2009; Lohr et al. 2015). The poor recovery of *A. cervicornis* populations in the Caribbean has been partly attributed to its reliance on asexual fragmentation (Tunncliffe 1981; Neigel and Avise 1983) and its rare sexual reproduction (Tunncliffe 1981; Vollmer and Palumbi 2007). Populations with high genetic diversity have been associated with higher resilience (Van Oppen and Gates 2006; van Oppen et al. 2015; O'Donnell et al. 2017), enhanced natural fertilization rates (Bowden-Kerby 2008) and higher sexual recruitment (Johnson et al. 2011), as well as overall healthier reefs (Selkoe et al. 2016). A recent study by O'Donnell et al. (2017) suggests that successful reef restoration should focus on maximizing levels of genetic diversity in coral outplants rather than selecting high-performing genotypes, but recognizes the importance of identifying and investigating genetic characteristics that increase outplant success at the individual, genotypic level for use in nursery management.

Previous studies have shown the importance of how phenotypes can vary among nursery stocks of *A. cervicornis* (Lohr and Patterson 2017). The genotypes used in this study (U44, U77, and K2) were among those phenotypically characterized by the Lohr and Patterson (2017). This study evaluated growth and bleaching-potential over a 390 day period under field conditions. Their work showed that genotype U44 exhibited lowest frequency of high temperature-induced bleaching. Susceptibility to bleaching due to heavy metal exposure (this study) was shown to be low, compared to genotypes K2 and U77, with no change in photosynthetic activity at concentrations lower than 200 ug/L copper (II) chloride and only slight color change. Lohr and Patterson (2017) showed that genotype K2 exhibited low prevalence of high temperature-induced bleaching and elevated total linear extension (TLE) while having low fecundity and low fertilization (M. Miller, pers. comm.). In contrast, K2 was more sensitive than U44 when exposed to copper (II) chloride as demonstrated by significant adverse changes in photosynthetic activity as compared to the control at 50 ug/L (similar response to U77). Genotype U77 demonstrated the lowest TLE phenotype (Lohr and Patterson 2017) with variable fertility (M. Miller, pers. comm.). With copper (II) chloride exposure genotype U77 performed similar to genotype K2 in photosynthetic activity and tissue regeneration, both exhibiting total tissue loss at the highest concentration (200 ug/L).

Results of our study show that differential responses to copper exposure can occur among known, nursery-grown genotypes of *A. cervicornis*. Furthermore, these data provide evidence that overall health of *A. cervicornis* is negatively impacted at low concentrations of copper (II) chloride, with LOEC values as low as 50 $\mu\text{g/L}$ CuCl_2 in the photosynthetic efficiency and overall coral health variables. Though tissue regeneration was affected by copper exposure, there was no significant differences in this response observed among genotypes. The changes in photosynthetic activity and color observed in copper exposed specimens, points to the symbiotic algae being the component that is differentially affected vs the coral animal. This observation brings into question whether *A. cervicornis* genotypes host algal symbiont variants and whether these components of the coral holobiont are responsible for the phenotypic and physiological variations observed in this and the Lohr and Patterson (2017) studies.

Conclusions

Copper, in concentrations high enough, is toxic to scleractinian corals in the marine environment, possessing the capacity to inhibit tissue regeneration, cause declines in the photosynthetic efficiency of the symbiotic dinoflagellates, and cause bleaching. With increased copper input, the overall health of corals are likely to be adversely affected, as will their ability to recover from natural perturbations in the marine environment. While it is now clear that intraspecific variation exists among *A. cervicornis* genotypes, observation of their various phenotypic responses to

differing stressors should be taken into consideration in selecting nursery stocks for propagation and outplanting. Further investigation into the role host and symbiont genotype play in coral restoration will become critical as global coral health continues to decline, and experiments like this are used to better characterize pre-dispositions of certain genotypes to either tolerate or succumb to environmental conditions and in so doing, improve restoration outcomes.

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