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Physiological responses of corals to ocean acidification and copper exposure

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ABSTRACT

Acidification and land-based sources of pollution have been linked to widespread declines of coral cover in coastal reef ecosystems. In this study, two coral species, *Acropora cervicornis* and *Pocillopora damicornis* were exposed to increased copper at two CO_2 levels for 96 h. Copper accumulation and anti-oxidant enzyme activities were measured. Copper accumulation only increased in *A. cervicornis* zooxanthellae and corresponded with photosynthetic toxicity. Enzyme activities in both coral species were affected; however, *A. cervicornis* was more sensitive than *P. damicornis*, and zooxanthellae were more affected than animal fractions of holobionts. Generally, activities of all anti-oxidant enzymes increased, with copper exposure in corals; whereas, activities of glutathione reductase and to some degree glutathione peroxidase were observed due to increasing CO_2 exposure alone. Exposure to copper in combination with higher CO_2 resulted in a synergistic response in some cases. These results provide insight into mechanisms of copper and CO_2 impacts in corals.

1. Introduction

Rising atmospheric carbon dioxide (CO_2), mainly due to anthropogenic activities, is rapidly changing the oceanic carbonate system (partial pressure of CO_2, pH, and alkalinity), resulting in ocean acidification (Hoegh-Guldberg, 1999; Anthony et al., 2011; Takahashi et al., 2012). Globally, oceanic CO_2 has increased by 30% over pre-industrial levels resulting in a 0.1 pH decrease (Caldeira and Wickett, 2003; Siegenthaler et al., 2005). At the current rate of change, atmospheric CO_2 levels are predicted to increase up to 1000 μAtm (~0.4 pH decrease) by the end of this century and up to 1900 μAtm (~0.8 pH decrease) by 2300 (Caldeira and Wickett, 2003; Orr et al., 2005). The carbonate system is such that CO_2 and water react to form carbonic acid, which then can dissociate to bicarbonate, and further dissociate to carbonate. Shifting the balance between these different carbon species may have dramatic effects on aquatic life, depending on which carbon species dominates. Corals and other marine calcifying organisms are particularly threatened due to the decreased calcium carbonate ($CaCO_3$) saturation state caused by ocean acidification (Orr et al., 2005; Fabry, 2008; Guinotte and Fabry, 2008; Anthony et al., 2011; Ateweberhan et al., 2013). Calcification involves precipitation of dissolved ions into solid $CaCO_3$ structures, which can be subsequently dissolved if seawater does not contain saturating carbonate ion concentrations. This problem may be exacerbated by increased pollution.

Coral reefs provide essential habitats for a wide array of marine life and are also among the world's most fragile and endangered ecosystems (Howard and Brown, 1984). Scleractinian (stony) corals have a mutualistic relationship with endosymbiotic dinoflagellates in the genus *Symbiodinium* (often referred to as “zooxanthellae”). The decline of coral reef ecosystems has been linked with global climate change and disease, ocean acidification, habitat destruction, pollution, and poor water quality (Hughes et al., 2003; Ateweberhan et al., 2013). Reefs in near shore environments close to heavily populated areas with substantial anthropogenic inputs are particularly threatened from combined exposure of multiple interacting stressors. Global climate change and other stressors have been found to disrupt the mutualistic relationship of corals and their endosymbiotic dinoflagellate (zooxanthellae), resulting in coral “bleaching” (loss of algal symbionts, or a reduction in their per-cell pigment concentrations) which causes visible paling of coral colonies (Brown and Howard, 1985; Guzman and Jimenez, 1992; Gardner et al., 2003; Baker et al., 2008). Corals may recover from bleaching, depending on the intensity and duration of the stress, but if the algal symbiont communities are not restored relatively quickly, corals may die.

The effects of ocean acidification on coral reefs has been extensively researched in the past two decades (Langdon et al., 2000; Leclercq et al., 2000; Schneider and Erez, 2006; Hoegh-Guldberg et al., 2007; Kuffner et al., 2008; Doney et al., 2009; Anthony et al., 2008, 2011; Albright and Langdon, 2011; Krief et al., 2010; Gómez et al., 2015). Reported effects include decreased.

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productivity and bleaching in corals and crustose coralline algae (Anthony et al., 2008; Albright et al., 2010); decreased coral recruitment and settlement (Anthony et al., 2008; Albright et al., 2010; Ateweberhan et al., 2013); reduced primary polyp growth in corals (Anlauf et al., 2010); and effects on early life history processes of coral (Albright et al., 2008; Albright and Langdon, 2011). In contrast, very little attention has been given to effects caused by both global changes in ocean acidification and local changes in water quality, such as metal pollution to coral reef organisms (Houlbrèque et al., 2011; Horwitz et al., 2014; Siddiqui and Bielmyer-Fraser, 2015; Biscéré et al., 2015; Marangoni et al., 2017).

Metals enter aquatic systems via industrial effluent, agricultural and stormwater runoff, sewage treatment discharge, fossil fuel combustion, mining processes, marine disposal of municipal solid waste, sacrificial anodes on boats, and shipwrecks (Bryan, 1974; Guzman and Jimenez, 1992; Howard and Brown, 1984; Brown and Howard, 1985; Peters et al., 1997; Ross and DeLorenzo, 1997; Knap et al., 1991; Richmond, 1993; Davis et al., 2001; Prego and Cobelo-Garcia, 2004; Flint and Davis, 2007). Copper is a commonly used metal in corrosion prevention and antifouling, therefore leachate from metal-based paints can be substantial in local areas such as marinas and ports (Reichelt and Jones, 1994; Evans et al., 2000; Voulvoulis et al., 2000). Waste disposal is also particularly problematic, especially in densely populated small islands, such as Bermuda (Jones, 2007, 2010). Much of the metallic bulk waste and municipal solid waste has been dumped directly into the ocean. As a result of this uncontained marine landfill, continuous contaminant leaching occurs onto nearby reefs, at levels exceeding water quality guidelines for copper in particular (Jones, 2010). Copper concentrations in seawater generally range from 0.13 to 9.5 μg/L (Kozelka and Bruland, 1998) but have been documented at nearly 30 μg/L in more polluted areas (Sadiq, 1992; Jones, 2010). The presence of heavy metals in coral tissue, water, and sediment samples collected from the coast of Florida and Hawaii (Glynn et al., 1984, 1989; Hanna and Muir, 1990), Puerto Rico (Pait et al., 2008), as well as Australia (Esslemont, 2000; Denton and Burdon-Jones, 1986a, 1986b; Haynes and Johnson, 2000) has been reported.

Copper is an essential element for all living organisms; however, at elevated concentrations copper may accumulate and cause toxicity in marine organisms (Bielmyer et al., 2005, 2010, 2012; Bielmyer and Grosell, 2011; Main et al., 2010; Patel and Bielmyer-Fraser, 2015; Siddiqui and Bielmyer-Fraser, 2015; Siddiqui et al., 2015). Copper generally exerts toxicity by altering enzyme function, causing oxidative stress, disrupting ionoregulation, and/or disrupting acid/base balance in aquatic organisms (Crespo and Karnaky Jr., 1983; McGeer et al., 2000; Bielmyer et al., 2005; Grosell, 2011; Patel and Bielmyer-Fraser, 2015; Siddiqui and Bielmyer-Fraser, 2015; Siddiqui et al., 2015). In the laboratory, copper has been shown to accumulate in corals and zooxanthellae and cause deleterious effects (Mitchelmore et al., 2007; Bielmyer et al., 2010; Marangoni et al., 2017). Biological effects in adult coral exposed to metals include reduced coral and zooxanthellae growth and coral bleaching (Howard and Brown, 1984; Goh and Chou, 1997; Jones, 1997; Peters et al., 1997; Brown, 2000; Bielmyer et al., 2010). Both metal pollution and ocean acidification have been shown to cause deleterious effects in aquatic organisms individually; however, the problem of dual exposure may be exacerbated because lower pH (increased acidification) causes changes in metal speciation, resulting in a shift to more toxic ionic metal species.

Physiological responses, such as reduced photosynthesis in zooxanthellae (Jones, 1997; Bielmyer et al., 2010; Siddiqui and Bielmyer-Fraser, 2015; Patel and Bielmyer-Fraser, 2015) and oxidative stress, in both host and symbiont have been reported consequences of metal exposure in cnidarians (Gilbert and Guzman, 2001; Mitchelmore et al., 2003; Main et al., 2010; Bielmyer et al., 2010; Brock and Bielmyer, 2013; Patel and Bielmyer-Fraser, 2015; Siddiqui and Bielmyer-Fraser, 2015). To neutralize the potentially harmful effects of reactive oxygen species (ROS) from metals and other stressors, cnidarians and other organisms produce antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) (Main et al., 2010; Brock and Bielmyer, 2013; Patel and Bielmyer-Fraser, 2015; Siddiqui et al., 2015). Carbonic anhydrase (CA) is another important enzyme that has been measured in recent studies to assess coral stress due to metal exposure (Bielmyer et al., 2010). CA catalyzes the interconversion of CO_2 to HCO_3 and is therefore important in respiration and metabolism processes (Weis et al., 1989; Bundy, 1977; Henry, 1996). Additionally, CA facilitates the formation of CO_3^{2-} which acts as a substrate for $CaCO_3$ formation.

Acropora cervicornis (Staghorn coral) and *Pocillopora damicornis* (cauliflower coral) are important reef building species and have been shown to be sensitive to copper in previous studies (Bielmyer et al., 2010). The goals of this project were to quantify copper accumulation and assess physiological responses in the corals, *A. cervicornis* and *P. damicornis*, and their symbionts after acute exposure to copper and increased CO_2 in the laboratory. We hypothesized that exposure to increasing atmospheric CO_2 would cause impairment to these organisms, which would further be affected by combined copper exposure.

2. Methods

2.1. Test organisms

In 2005, *A. cervicornis* was collected from Biscayne National Park, and in 2003, *P. damicornis* was purchased from The Coral Nursery. Since that time, all coral species have been maintained and cultured at the Coral Resource Facility at the University of Miami's Rosenstiel School of Marine and Atmospheric Sciences (RSMAS). The reef building clones are genetically identified, routinely fragmented, and housed in an isolated re-circulating seawater system. For each species, coral fragments were cut from a single colony and then mounted on tiles to facilitate handling. The fragments were allowed to recover from handling and observed for normal growth and performance such that only healthy coral fragments were used for experimentation. Coral fragments are uniquely suited for toxicological testing because they have > 90% survival using approved protocols (Shafir et al., 2002) and they have been shown to be sensitive to copper in previous studies (Bielmyer et al., 2010).

2.2. Experimental design

Coral fragments were exposed to a control or 20 μg/L copper (nominal concentration), as $CuCl_2$ at ambient or 1000 μAtm CO_2 levels in a flow-through system for 96 h. Measured copper values were 25 ± 11.2 μg/L and 17 ± 4.48 μg/L for the copper treatments in ambient and 1000 μAtm CO_2 respectively. Copper concentrations in the controls were below detection (< 2 μg/L).

There were three replicate tanks per treatment, each with two coral fragment of each species. Concentrated copper solutions and gravity fed natural filtered seawater (untreated or treated with CO_2) were continually mixed and then distributed into testing chambers (10 gal aquaria). A pH/pCO_2 stat system (Loligo system) was used to maintain the required amount of pCO_2 in the water of different tanks, where pH was continuously monitored and each tank was also aerated. The 1000 μAtm exposure CO_2 concentration was controlled using a commercially available PCO_2/pH feedback controller (DAQ-S; Loligo Systems Inc.) connected to a wtw pH 3310 meter and SenTix 41 pH electrode (Loligo Systems Inc.) and controlled using CapCTRL software (Loligo Systems Inc.). The automated system used measured pH data and an input PCO_2 – pH standard curve to determine water PCO_2 and has been employed previously for climate change relevant CO_2 exposures to marine organisms (Esbaugh et al., 2012; Heuer et al., 2012; Heuer et al., 2016; Heuer and Grosell, 2016). When PCO_2 in the aquaria drops below the set point, as determined by pH measurements, the system adds pure CO_2 gas via an airstone until the PCO_2 returns to the

desired level. The pH-PCO_2 standard curve was performed using air (380 ppm) and a commercial CO_2 gas mixture (3090 ppm). Temperature, dissolved oxygen (DO), and salinity were measured with a YSI meter, and pH with a calibrated pH meter daily throughout the experiment. Water quality conditions in the experimental chambers were as follows (mean ± standard deviation): 25.3 ± 0.40 °C, 6.32 ± 0.23 mg/L DO, and 30.9 ± 0.07 g/L salinity. The mean pH of the ambient and high CO_2 tanks was 8.06 ± 0.03 and 7.67 ± 0.05, respectively. At the beginning and end of the experiment water samples were collected, filtered (0.45 μm), and acidified with trace metal grade nitric acid for later metal analysis.

At 0 h (directly from holding tanks), 48 and 96 h, photosynthetic parameters of algal symbionts were measured in all coral fragments using imaging pulse amplitude modulated (PAM) fluorometry (Genty et al., 1989; Ralph et al., 1999). The fluorometer uses a range of flashing pulsed lights, and assessment of photosynthetic parameters is achieved by fluorescence quenching analysis (Beer and Björk, 2000). The parameters are measured after a saturation pulse is applied with 2 min illumination at 81 μmol quanta $m^{-2} s^{-1}$. PAM fluorometry assesses the interactions of photosystem II, membrane degradation, and photosynthetic electron transport efficiency (Rosenqvist and van Kooten, 2003) and has been used to assess toxicity in marine organisms in several other studies in this laboratory (Bielmyer et al., 2010; Jarvis and Bielmyer-Fraser, 2015; Patel and Bielmyer-Fraser, 2015; Siddiqui et al., 2015). Effective quantum yield of photosystem II (YII), relative electron transport rate (rETR) and the quantum yield of nonregulated energy dissipation (YNO) were measured. YII indicates the amount of energy used in photochemistry (photosynthesis); rETR is closely related to the photosynthetic activity and is an approximation of the rate of electrons pumped through the photosynthetic chain; and YNO indicates inefficiency of the system. Decreased YII, decreased rETR, and increased YNO indicates toxicity.

Three coral fragments were sampled at the start of the experiment directly from holding tanks (0 h) and then one coral of each species was sampled from each tank (n = 3 per treatment) at 48 and 96 h. Coral samples were preserved at −80 °C for later analysis of copper distribution and accumulation, and the activity of several antioxidant enzymes.

2.3. Coral fractionation

Coral fragments were weighed and then separated into coral soft tissue, algal symbiont, and coral skeletal fractions. The fragments were rinsed and then air brush blasted with 32 g/L synthetic seawater to separate the coral soft tissue and algal symbionts from the exoskeleton. The volume of seawater used was normalized in all samples. This “blastate” of combined soft tissue fractions was then mixed and homogenized using a Dounce-type glass homogenizer and the algal symbionts were then isolated from the coral soft tissue via centrifugation for 2 min at 50,000 × *g*.

2.4. Copper analysis

Soft tissue fractions (coral and zooxanthellae) were individually dried in pre-weighed aluminium weigh boats at 80 °F for 24 h to determine dry weight. Each fraction was then digested with trace metal grade nitric acid (1%; Fisher Scientific, Pittsburgh, PA) and analysed for copper using atomic absorption spectrophotometry (AAS) with graphite furnace or flame detection as appropriate (detection limits ≤2 μg/L). Water samples were diluted and analysed for copper using AAS. Certified standards were used with re-calibration every 40 samples.

2.5. Protein and enzyme assays

From all coral and zooxanthellae samples, an aliquot was homogenized with 10 mM potassium phosphate buffer pH 7.0 at 25 °C, and centrifuged at 3000 × *g* for 10 min. Supernatant was then assayed for total soluble protein, using a Bradford assay (Bradford, 1976) and bovine serum albumin was used as standards. Enzyme activity is expressed per mg protein.

UV/VIS spectrophotometric (Pye Unicam Ltd., Cambridge, England) methods were used to measure the enzymes CAT, GPx, and GR. CAT activity was measured in the samples by the decrease in absorbance at 240 nm for 90 s based on the decomposition of hydrogen peroxide following Sigma protocol EC 1.11.1.6 (Sigma, 1994a) with minor modifications. GR was measured in coral and zooxanthellae homogenate using Sigma protocol EC 1.6.4.2 (Sigma, 1994b) with minor modifications. The following reagents were added to a quartz cuvette: 2 mM oxidized glutathione, 100 mM potassium phosphate buffer, pH 7.5, with 1 mM EDTA, glutathione reductase (sample homogenate) and 2 mM reduced nicotinamide adenine dinucleotide phosphate (NADP), and change in absorbance (ΔA) was measured for 4 min at 340 nm. GPx activity was measured in the samples using Sigma method EC 1.11.1.9 (Sigma, 1994c), where the following reagents were mixed in a cuvette: 50 mM Tris HCl buffer, pH 8.0 containing 0.5 mM EDTA, NADPH assay reagent and sample homogenate. To initiate the reaction, 30 mM *tert*-butyl hydrogen peroxide was added and a decrease in absorbance was measured at 340 nm for 5 min.

Another aliquot of each coral and zooxanthellae sample was used for CA analysis using the delta pH method of Henry (1991, 1996), which uses a sensitive recording pH meter to directly measure the rate of pH change in a buffer containing CA upon the addition of substrate (e.g. CO_2 for hydration; Henry, 1991).

2.6. Statistical analysis

Data normality and equality of variance were analysed by Shapiro-Wilk's and Levene's tests, respectively. Comparisons of treatment data were performed using 1-way ANOVA followed by the multiple comparison test of Tukey. The variability explained by each factor and their interaction were derived from the sum of squares.

3. Results

Tissue copper concentrations in the two coral species are presented in Fig. 1. No significant differences in tissue copper concentration were observed among treatments in either coral species (Fig. 1). Similarly, copper concentrations in the zooxanthellae of *P. damicornis* did not significantly differ among treatments (data not shown). Alternatively, tissue copper significantly increased in the zooxanthellae of *A. cervicornis* after 24 and 48 h exposure to 20 μg/L Cu at both CO_2 levels, as compared to the respective controls (Fig. 2A). A trend of decreased YII and rETR from copper exposure was observed in the zooxanthellae of *A. cervicornis*; whereas, a trend of increased YII and rETR from elevated CO_2 exposure was observed in the zooxanthellae of *A. cervicornis* (Fig. 2). However, no significant differences were detected as compared to respective controls ($p \leq 0.05$). Zooxanthellae of *A. cervicornis* exposed to 20 μg/L Cu at 1000 μAtm CO_2 had significantly lower rETR as compared to the 1000 μAtm CO_2 control. Additionally, YNO in both copper-exposed groups of *A. cervicornis* zooxanthellae was significantly increased after 48 h (Fig. 2C). Effects on the photosynthetic parameters correlated with increased copper accumulation in these zooxanthellae. In contrast, no significant differences were observed in the photosynthetic parameters of the zooxanthellae in *P. damicornis* (data not shown).

CAT activity significantly increased over time in *A. cervicornis* exposed to 20 μg/L Cu as compared to the control at ambient CO_2 (Fig. 3A). Alternatively, CAT activity was not significantly different in *A. cervicornis* exposed to 20 μg/L Cu at 1000 μAtm CO_2, as compared to the control 1000 μAtm CO_2 treatment. However, both elevated CO_2 treatments (control and 20 μg/L Cu) had higher CAT activity than the ambient CO_2 control at 24 h; and CAT activity remained elevated at

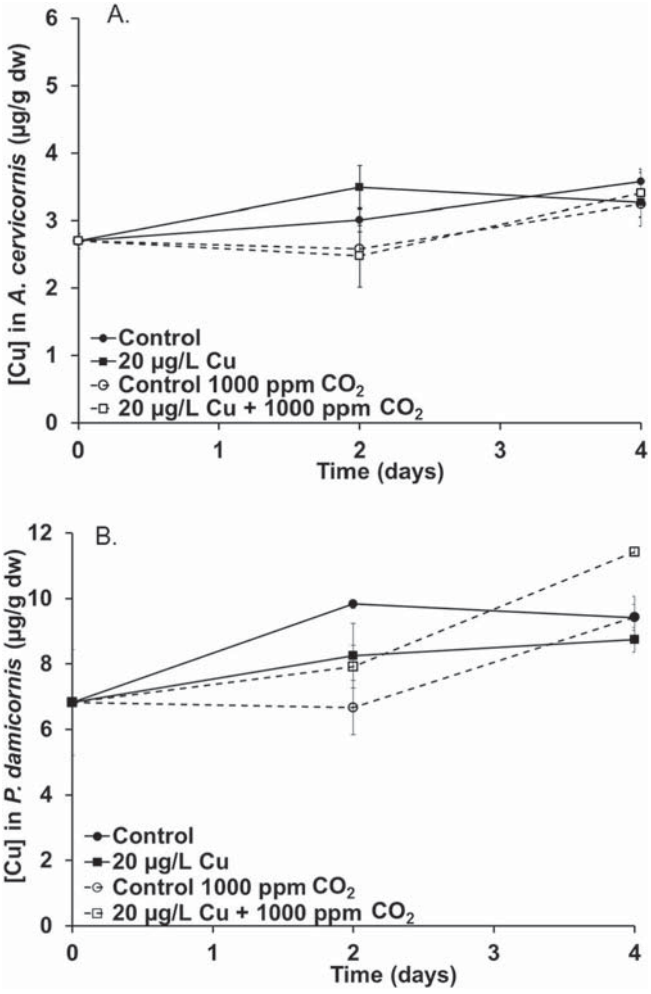

Fig. 1. Copper accumulation (µg/g dry weight) in coral tissue of A. *Acropora cervicornis* and B. *Pocillopora damicornis* exposed to control and 20 µg/L Cu at ambient CO_2 and elevated (1000 µAtm) CO_2.

48 h in the 20 µg/L Cu plus 1000 µAtm CO_2 treatment (Fig. 3A). No significant differences in CAT activity were observed in *A. cervicornis* zooxanthellae, *P. damicornis*, or *P. damicornis* zooxanthellae among treatments (Fig. 3B–D).

GPx activity was significantly elevated at 48 h in copper-exposed *A. cervicornis* at both ambient and elevated CO_2, as compared to respective controls; however, no significant differences in GPx activity were observed among treatments in *A. cervicornis* zooxanthellae (Fig. 4A, B). GPx activity in *P. damicornis* and their zooxanthellae exposed to 1000 µAtm CO_2 (without copper) was significantly elevated at 24 h and 48 h as compared to the ambient CO_2 control (Fig. 4C, D).

GR activity in *A. cervicornis* was significantly increased in copper-exposed groups at both ambient and elevated CO_2 as compared to respective controls (Fig. 5A). Additionally, there was a significant difference between the *A. cervicornis* exposed to 20 µg/L Cu and the *A. cervicornis* exposed to 20 µg/L Cu plus 1000 µAtm CO_2 (Fig. 5A). GR activity in the zooxanthellae of *A. cervicornis* was significantly increased in the copper-exposed groups and the 1000 µAtm CO_2 control group, as compared to the ambient CO_2 control (Fig. 5B). GR activity in *P. damicornis* was not significantly different among treatments (Fig. 5C); however, GR activity in the zooxanthellae in *P. damicornis* was significantly elevated in the 20 µg/L Cu plus 1000 µAtm CO_2 treatment as compared to the 1000 µAtm CO_2 control treatment, and as compared to the copper-exposed ambient CO_2 treatment (Fig. 5D).

CA significantly increased in *A. cervicornis* exposed to 20 µg/L Cu plus 1000 µAtm CO_2 at 24 h, as compared to the ambient CO_2 control, and at 48 h as compared to both controls (Fig. 6A). Additionally, CA activity in *A. cervicornis* was significantly elevated at 48 h in the 20 µg/L Cu plus 1000 µAtm CO_2 treatment, as compared to the 20 µg/L Cu at ambient CO_2 treatment (Fig. 6A). No significant differences were observed in CA activity in *P. damicornis* among treatments (Fig. 6B).

4. Discussion

Substantial metal accumulation in both symbiotic zooxanthellae and animal tissue fractions of branching corals has been previously demonstrated (Bielmyer et al., 2010; Bastidas and Garcia, 1999; Anu et al., 2007; Mitchelmore et al., 2007). Copper concentrations in the zooxanthellae and coral tissues of both *A. cervicornis* and *P. damicornis* in the present study were similar to those previously reported (Mitchelmore et al., 2007; Bielmyer et al., 2010). After exposure to 20 µg/L Cu for 96 h, copper accumulation was observed in the zooxanthellae (but not the host) of *A. cervicornis* at both current and elevated CO_2 levels, similar to the copper accumulation reported by Bielmyer et al., 2010. Increased CO_2 in the present study did not result in an observable difference in copper accumulation over 96 h between the copper-treated groups; however, a longer exposure time may have yielded different results. In several previous studies with cnidarians, the loss of the zooxanthellae resulted in reduced metal concentration (Main et al., 2010; Brown, 2000). Peters et al. (1997) suggest that the expulsion or loss of zooxanthellae may be a mechanism of metal detoxification since they have been found to accumulate heavy metals to a larger extent and thus be more tolerant than their symbiotic hosts. Bielmyer et al. (2010) reported significant copper accumulation in both the zooxanthellae and coral host of *A. cervicornis* after exposure to copper for five weeks; however, no copper accumulation was observed in *P. damicornis*, similar to the findings of the present study. In contrast, Mitchelmore et al., 2007 reported a slight but significant increase in *P. damicornis* after exposure to 5 and 15 µg/L Cu for 4 and 14 d.

It is well known that coral can host different types of *Symbiodinium* (clade A, B, or C); however, recent studies have reported a gradual shift in *Symbiodinium* communities present in corals worldwide as a consequence of global climate change (Rowan et al., 1997; LaJeunesse, 2002; Baker, 2003). *Symbiodinium* D is thermally tolerant and has been more commonly found on reefs where severe mortality and bleaching events have occurred (Baker, 2003). The consequence of this more stable host-symbiont relationship was reportedly an increased resistance to future bleaching events (Baker, 2003). Additionally, Bielmyer et al. (2010) demonstrated differences in copper accumulation and susceptibility of three species of scleractinian coral, each predominantly hosting different *Symbiodinium* clades (*Symbiodinium* A3, C1 and D1a) with the most metal tolerant coral species hosting *Symbiodinium* D1a. The same principle may hold true for metal exposure and ocean acidification, and perhaps host-symbiont relationships with more metal-tolerant zooxanthellae could decrease metal accumulation and toxicity to the host; thus accounting for differences among some studies.

Effects of metals on the symbiont's photosynthetic activity can be assessed before it dissociates from the coral (Bielmyer et al., 2010). PAM fluorometry was utilized to distinguish between photon energy captured by a chlorophyll-*a* pigment molecule used to drive photosynthesis versus the energy emitted as fluorescence or converted to heat (Warner et al., 1996; Hoegh-Guldberg and Jones, 1999; Ralph et al., 1999). This technique has been used in several recent studies to assess the effects of metals on seaweed, algae, and zooxanthellae (Jones et al., 1999; Nielsen et al., 2003; Bielmyer et al., 2010; Patel and Bielmyer-Fraser, 2015; Jarvis and Bielmyer-Fraser, 2015). In this study, copper accumulation in *A. cervicornis* zooxanthellae correlated with significantly lower rETR (at higher CO_2 concentration) and significantly higher YNO as compared to controls, after 96 h of copper exposure. A trend of lower YII was also observed in the copper-exposed groups. YII

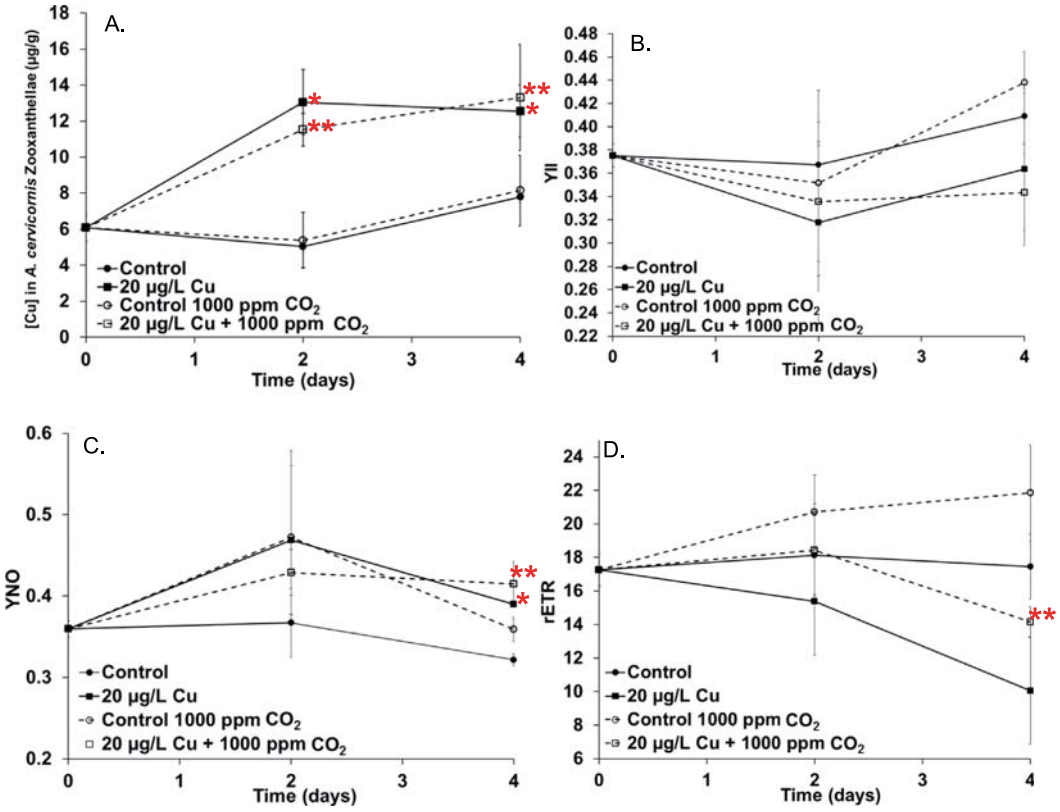

Fig. 2. A. Copper accumulation (µg/g dry weight), B. effective quantum yield (YII), C. relative electron transport rate (rETR) and D. quantum yield of nonregulated energy dissipation (YNO) in the zooxanthellae of *Acropora cervicornis* exposed to control and 20 µg/L Cu at ambient CO_2 and elevated (1000 µAtm) CO_2. Single asterisk represents a significant difference ($p \leq 0.05$) as compared to the ambient CO_2 control. Double asterisks represent a significant difference ($p \leq 0.05$) as compared to the 1000 µAtm CO_2 control.

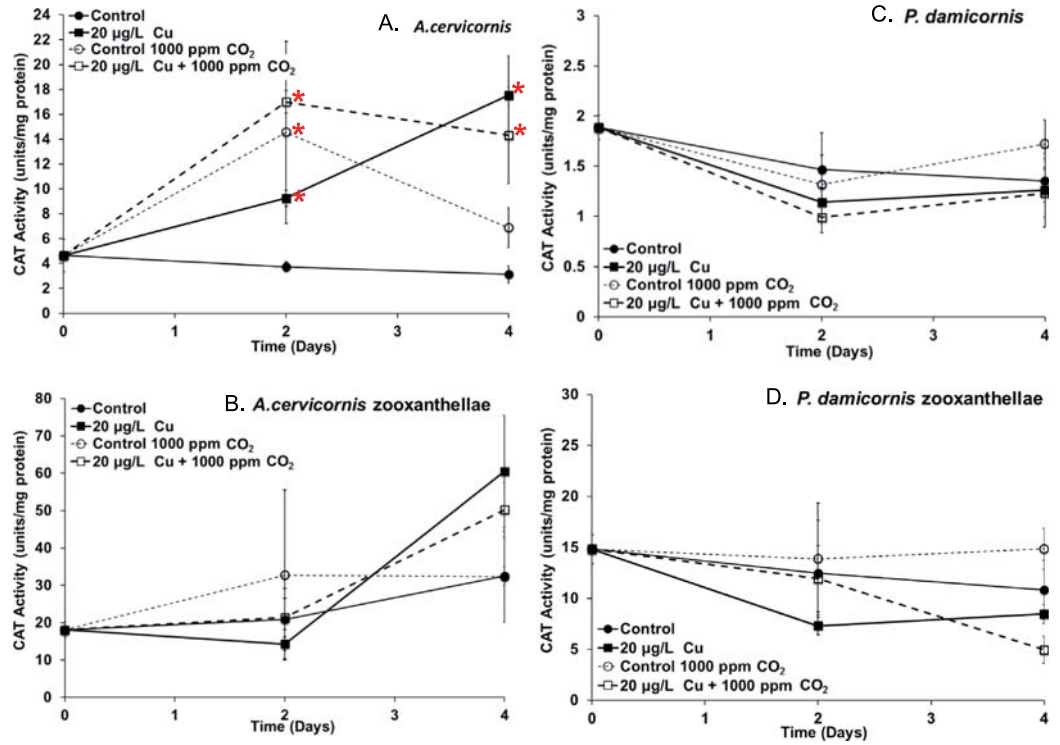

Fig. 3. Catalase (CAT) activity (units/mg protein) in A. *Acropora cervicornis*, B. *Pocillopora damicornis*, C. zooxanthellae of *Acropora cervicornis* and D. zooxanthellae of *Pocillopora damicornis* exposed to control and 20 µg/L Cu at ambient CO_2 and elevated (1000 µAtm) CO_2. Asterisks represent a significant difference ($p \leq 0.05$) as compared to the ambient CO_2 control.

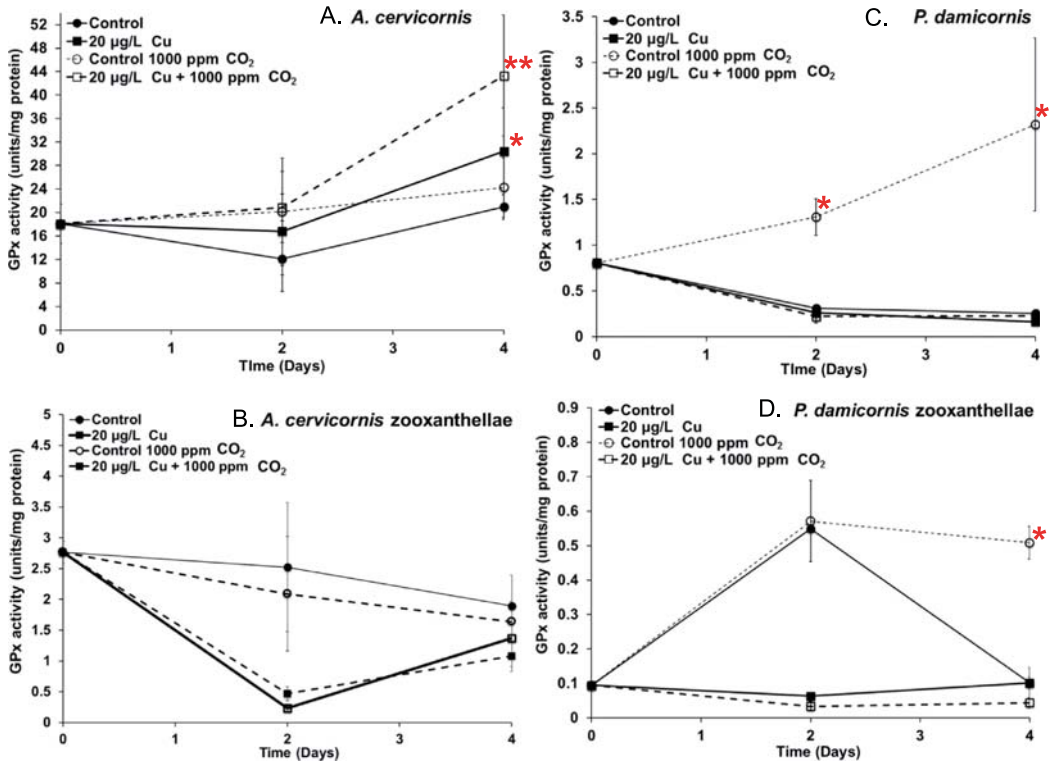

Fig. 4. Glutathione peroxidase (GPx) activity (units/mg protein) in A. *Acropora cervicornis*, B. *Pocillopora damicornis*, C. zooxanthellae of *Acropora cervicornis* and D. zooxanthellae of *Pocillopora damicornis* exposed to control and 20 µg/L Cu at ambient CO_2 and elevated (1000 µAtm) CO_2. Single asterisk represents a significant difference ($p \leq 0.05$) as compared to the ambient CO_2 control. Double asterisks represent a significant difference ($p \leq 0.05$) as compared to the 1000 µAtm CO_2 control.

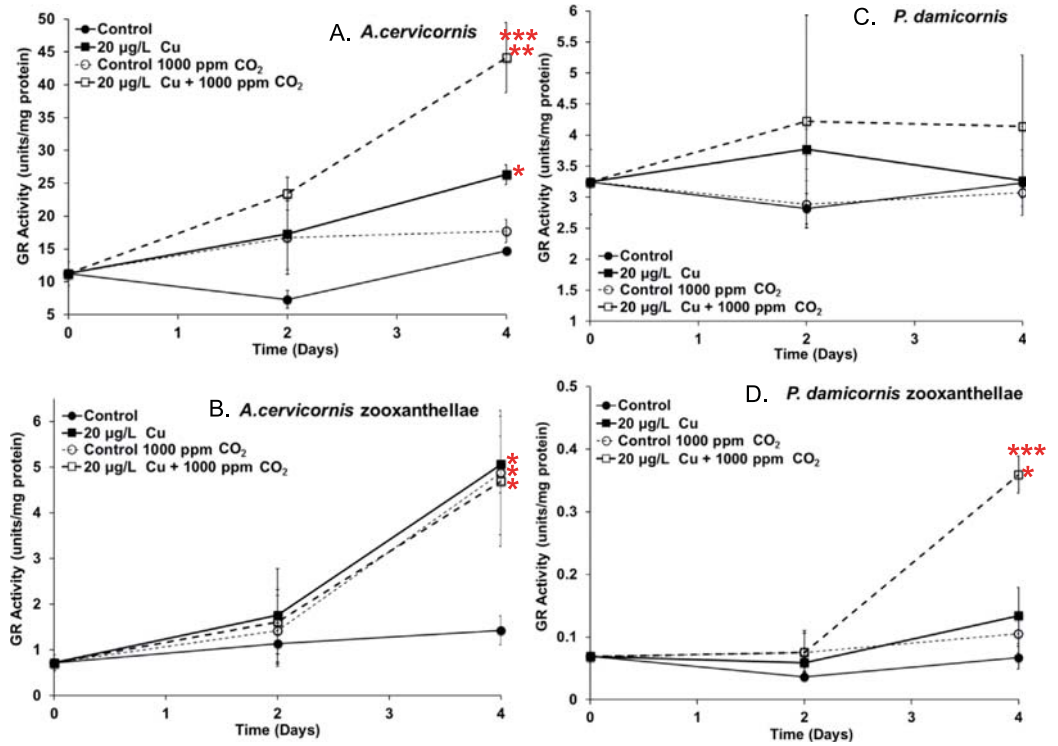

Fig. 5. Glutathione reductase (GR) activity (units/mg protein) in A. *Acropora cervicornis*, B. *Pocillopora damicornis*, C. zooxanthellae of *Acropora cervicornis* and D. zooxanthellae of *Pocillopora damicornis* exposed to control and 20 µg/L Cu at ambient CO_2 and elevated (1000 µAtm) CO_2. Single asterisk represents a significant difference ($p \leq 0.05$) as compared to the ambient CO_2 control. Double asterisks represent a significant difference ($p \leq 0.05$) as compared to the 1000 µAtm CO_2 control. Triple asterisks represent a significant difference ($p \leq 0.05$) from the ambient CO_2 and 20 µg/L Cu treatment.

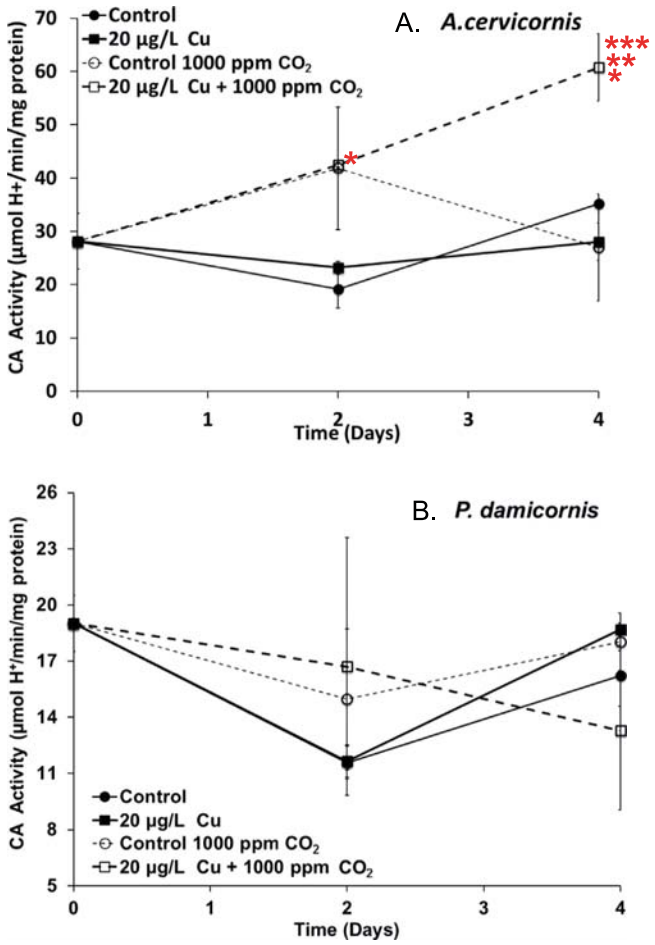

Fig. 6. Carbonic anhydrase (CA) activity (units/mg protein) in A. *Acropora cervicornis* and B. *Pocillopora damicornis* exposed to control and 20 µg/L Cu at ambient CO_2 and elevated (1000 µAtm) CO_2. Single asterisk represents a significant difference ($p \leq 0.05$) as compared to the ambient CO_2 control. Double asterisks represent a significant difference ($p \leq 0.05$) as compared to the 1000 µAtm CO_2 control. Triple asterisks represent a significant difference ($p \leq 0.05$) from the ambient CO_2 and 20 µg/L Cu treatment.

is indicative of the amount of absorbed quanta that is converted into chemically fixed energy by the photochemical charge separation at PS II reaction centers. Copper-exposed groups appeared to have lower YII and had a significantly lower amount of absorbed energy used for electron transport than the controls in the zooxanthellae of *A. cervicornis*. High YNO indicates that both photochemical energy conversion and protective regulatory mechanisms are inefficient. Increased CO_2 did not result in observable differences in copper accumulation in the zooxanthellae. However, increased CO_2 did appear to slightly increase YII and rETR, which is consistent with a higher CO_2 fixation rate in PSII dark reactions.

CA activity was concurrently increased in *A. cervicornis* zooxanthellae exposed to copper and elevated CO_2. It is likely that the activity of this enzyme was increased because of its importance in catalyzing interconversions between CO_2 and HCO_3^-. Weis et al. (1989) suggested that CA plays a vital role in photosynthetic metabolism of algal/cnidarian symbiosis in supplying the CO_2 necessary for zooxanthellae to maintain high photosynthesis rates. Other studies have shown short term increases in photosynthesis with elevated exposure to CO_2, which resulted in an eventual sharp decrease in photosynthesis. No significant differences were detected in any of the photosynthetic parameters of the zooxanthellae in *P. damicornis*, again demonstrating clear differences in the tolerance of the zooxanthellae population and likely their identity as compared to *A. cervicornis*. A longer exposure time to both variables could have affected the results. Significant reductions in photosynthetic parameters have been reported in *A. cervicornis* and *P. damicornis* exposed to 20 µg/L Cu for 5 weeks (Bielmyer et al., 2010) as well as sea anemones exposed to copper and elevated CO_2 for 7 d (Siddiqui and Bielmyer-Fraser, 2015). Bielmyer et al. (2010) also reported CA inhibition in the three coral species after copper exposure for five weeks. Likewise, Gilbert and Guzman (2001) reported a significant decrease in CA activity in the anemones, *Condylactis gigantea* and *Stichodactyla helianthus* exposed to elevated copper, nickel, lead, and vanadium in the laboratory and the coral, *Montastraea cavernosa* obtained from metal polluted sites as compared to those obtained from pristine sites. Although CA inhibition is a common response in organisms exposed to metals; the exposure of increased CO_2 results in an antagonistic response, at least temporarily, with an increase in CA activity. CA activity was increased even more in *A. cervicornis* exposed to copper and 1000 µAtm CO_2 combined.

In addition to reduced photosynthesis in zooxanthellae (Jones, 1997; Bielmyer et al., 2010; Siddiqui and Bielmyer-Fraser, 2015; Patel and Bielmyer-Fraser, 2015) oxidative stress has also been a reported effect of metal exposure in cnidarians (Gilbert and Guzman, 2001; Mitchelmore et al., 2003; Main et al., 2010; Bielmyer et al., 2010; Brock and Bielmyer, 2013; Patel and Bielmyer-Fraser, 2015; Siddiqui and Bielmyer-Fraser, 2015). Increased copper exposure can cause electron transport mechanisms (i.e., photosystems and mitochondrial transport chains) to become less efficient resulting in the formation of reduced ROS from the interaction of free electrons with diatomic molecular oxygen (Cabiscol et al., 2000). ROS can denature proteins, mutate DNA, and cause lipid peroxidation (Richier et al., 2005). Metals and other stressors can induce the formation of hydrogen peroxide (Cabiscol et al., 2000). The oxidation of Cu(I) in the Fenton reaction can then drive the conversion of hydrogen peroxide to hydroxide and hydroxyl radical (Klaassen, 1996). To neutralize the potentially harmful effects of ROS, antioxidant enzymes such as CAT, GPx, and GR are produced. CAT and GPx (using monomeric glutathione as the reducing agent) catalyze the conversion of hydrogen peroxide into water and oxygen (Forman et al., 1990; Sies, 1999a, 1999b; Sunagawa et al., 2008a, 2008b; Masella and Mazza, 2009). A subsequent reaction reduces the glutathione using NADPH and GR as the catalyst, so that it may be recycled for the previous reaction (Forman et al., 1990; Sies, 1999a, 1999b; Sunagawa et al., 2008a, 2008b; Masella and Mazza, 2009). In this study, anti-oxidant enzyme activities in both coral species were affected by both increased copper and CO_2; however, *A. cervicornis* was more sensitive than *P. damicornis*. Other studies have reported alterations in anti-oxidant enzyme activity with copper exposure (Brock and Bielmyer, 2013; Patel and Bielmyer-Fraser, 2015; Siddiqui et al., 2015) and copper with increased CO_2 (Siddiqui and Bielmyer-Fraser, 2015) to sea anemones. Increased copper, CO_2, and a combination of copper and CO_2, caused an increase of the anti-oxidant enzymes measured in many cases; however, differences were observed depending on the enzyme measured and the symbiotic partner of the holobiont (Table 1). CAT activity was increased due to both copper and CO_2 individually (Table 1) but no additive effects of the parameters were observed. GPx activity was increased in *A. cervicornis* with increasing copper exposure; whereas, GPx activity was increased in *P. damicornis* and their zooxanthellae with increasing CO_2 exposure (Table 1). GR activity was increased in *A. cervicornis* with increasing copper exposure and in their zooxanthellae by each copper and CO_2 (Table 1). There was an additive effect of both parameters on GR activity in *A. cervicornis* and *P. damicornis* zooxanthellae. An additive affect was also observed in CA activity in *A. cervicornis* after exposure to both parameters. Main et al. (2010) reported a significant increase in CAT activity after exposure of the symbiotic sea anemone, *Exaiptasia pallida* (formerly *Aiptasia pallida*) to copper exposure. An increase in both GPx and GR in *E. pallida* following copper exposure was reported in several studies (Brock and Bielmyer, 2013; Patel and Bielmyer-Fraser, 2015; Siddiqui et al., 2015).

Table 1
Increase (upward arrow), decrease (downward arrow), or no change (indicated by horizontal arrow or "No Effect") in activity of the following enzymes: catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), following exposure to Cu (20 μg/L) or increased CO_2 (1000 μAtm) or both Cu + CO_2 (20 μg/L Cu + 1000 μAtm CO_2). No additive effects on CAT or GPx were observed after combined exposure of Cu + CO_2.

Species	CAT		GPx		GR		
	Cu	CO_2	Cu	CO_2	Cu	CO_2	Cu + CO_2
A. cervicornis coral	↑	↑	↑	↔	↑	↔	↑
A. cervicornis zooxanthellae	No Effect		No Effect		↑	↑	No Additive Effect
P. damicornis coral	No Effect		↔	↑	No Effect	No Effect	No Additive Effect
P. damicornis zooxanthellae	No Effect		↔	↑	↔	↔	↑

Furthermore, Siddiqui and Bielmyer-Fraser (2015) reported an even greater increase in GPx and GR activity with dual exposure of copper and increased CO_2 to *E. pallida*. The enzymes measured in this study served as good stress indicators for both copper and CO_2 exposure.

5. Conclusion

This study has provided evidence that toxicity can occur in these corals after exposure for only 96 h to copper and increased CO_2. Additionally, clear differences in copper accumulation and sensitivity were observed between the two coral species, and between the coral and their zooxanthellae. These results suggests that copper accumulates first in the zooxanthellae and then in the coral fraction in *A. cervicornis*. Increased CO_2 did not affect the extent of the copper accumulation. Additionally, copper accumulation was not associated with effects in *P. damicornis*, since no copper accumulation was observed in the coral or zooxanthellae. Copper accumulation in *A. cervicornis* zooxanthellae was associated with photosynthetic toxicity at 96 h, unlike the zooxanthellae in *P. damincornis*; suggesting differences in sensitivity between the symbiont populations. Altered enzyme activity was observed in the zooxanthellae and coral fractions of both species; however, *A. cervicornis* was more affected by copper and *P. damicornis* seemed to be more affected by increased CO_2.

It is important to note that tissue copper did not correspond with effects, and differences were observed based on species, measured end point, and likely exposure time, as compared to other studies. Toxic effects were observed in these corals from exposure of copper and CO_2 individually; however, synergistic responses were limited (GR activity in *A. cervicornis* coral and *P. damicornis* zooxanthellae). Data concerning potential effects of both metals and ocean acidification, particularly on the holobiont (all symbiotic partners) of cnidarians is lacking. More laboratory studies are needed to better understand the physiological effects of combined ocean acidification and metal exposure. Results of this study provide new insight into the symbiont-host relationship of cnidarians and their responses to CO_2 and copper.

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