The Effects of Metals and Ocean Acidification on Marine Invertebrates

A Thesis submitted
to the Graduate School
Valdosta State University

in partial fulfilment of requirements
for the degree of

MASTER OF SCIENCE

in Biology

in the Department of Biology
of the College of Arts and Sciences

May 2015

Samreen Siddiqui

BS, G.B. Pant University of Agriculture and Technology, India, 2009

This thesis, "The Effects of Metals and Ocean Acidification on Marine Invertebrates," by Samreen Siddiqui, is approved by:

Thesis Committee Chair

Gretchen K. Bielmyer-Fraser, Ph.D.
Associate Professor of Biology

Committee Member

Theresa J. Grove, Ph.D.
Associate Professor of Biology

Russell H. Goddard, Ph.D.
Professor of Biology

Interim Dean of the Graduate School

James T. LaPlant, Ph.D.
Professor of Political Science

ABSTRACT

Increasing use of metal oxide nanoparticles (NP) by various industries has resulted in substantial contributions of NP in aquatic systems. Additionally, ocean acidification (OA) is a growing concern due to its deleterious effects on aquatic organisms. The effects of OA and metal pollution on non-calcifying marine organisms are largely unknown. This research investigated the effects of copper oxide (CuO) NP on the sea anemone, *Exaiptasia pallida*, for 21 days. Sea anemones were measured for tissue copper accumulation as well as the activity of the enzymes: catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and carbonic anhydrase (CA). This study is useful in discerning the differences between CuO NP and dissolved copper exposure to sea anemones, as differences in copper accumulation pattern and enzymatic response were observed. In addition, we examined physiological effects of the sea anemone, *Exaiptasia pallida* after exposure to a global stressor, CO_2, and a local stressor, Cu, over 7 days. Cu accumulated in *E. pallida* in a concentration-dependent manner and activities of all anti-oxidant enzymes measured (CAT, GPx, GR) increased, with increasing Cu exposure. However, clear differences in only GR and to some degree GPx activity were observed due to increasing CO_2 exposure alone. Interactions between the two independent variables were also observed. Activity of the enzyme, CA, was significantly decreased with increasing Cu and the extent of CA inhibition was lessened with increasing CO_2. These results provide insight into toxic mechanisms of $CuCl_2$, CuO NP and CO_2 exposure to *E. pallida*.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

I would like to express the deepest appreciation to my committee chair Dr. G.K. Bielmyer-Fraser, for her supervision with my thesis.

I would like to thank my committee members, Dr. R.H. Goddard and Dr. T.J. Grove, whose work demonstrated to me that concern for global affairs supported by an “engagement” in comparative literature and modern technology, should always transcend academia and provide a quest for our times.

I would like to thanks Mr. Pratik P. Patel for support in the lab and my best thanks and regards to Mrs. Judith Cronk (Oma) and Miss Rachel Cronk, without whom I can’t make this thesis possible.

Chapter I

COMPARATIVE EFFECTS OF DISSOLVED COPPER AND COPPER OXIDE NANOPARTICLE EXPOSURE TO THE SEA ANEMONE, *EXAIPTASIA PALLIDA*

Introduction

In recent years, the use of nanoparticles (NP) in various industries has increased substantially, thereby increasing the input of NP into aquatic systems (Service, 2004; Moore, 2006). Copper oxide (CuO) NP, in particular, have biocidal, antibacterial, antiviral and anti-fungal properties, and are thus utilized in many fields (Srivastava, 2009; Grass et al., 2011; Santo et al., 2012). CuO NP are commonly used in antimicrobial drugs as well as in industrial applications like conductive films, lubrication, nanofluids, catalysis, and electronic devices, such as inkjet printing (Tilaki et al., 2007; Mitsudome et al., 2008; Jong & Borm, 2008; Raffi et al., 2010; Cady et al., 2011; Cheon et al., 2012; Longano et al., 2012; Chatterjee et al., 2012). At elevated concentrations, NP may interact with and potentially cause adverse effects to aquatic organisms (Griffitt et al., 2007). However, the environmental implications of increased NP use are largely unknown, particularly in marine systems.

NP are classified as less than 100 nm dimension in size (EPA, 2007), and their shape can vary among spherical, tubular, and irregular (Kelly et al., 2003). They can be found in fused, aggregated and agglomerated forms in the environment (Nowack & Bucheli, 2007). The surface properties of metal oxide NP are determined by their acidity constants and zero point of charge (Schindler & Stumm, 1987; Kormann et al., 1991; Hristovski et al.,

2007; Giammar et al., 2007), which in turn influences their interaction within aquatic systems.

The predicted risks of NP in aquatic environments can be better assessed by having an understanding of their mobility, bioavalability and toxicity to organisms (Nowack & Bucheli, 2007). The nano-size particles can cross biological cell membranes through diffusion, endocytosis and phagocytosis depending upon the cell type (Limbach et al., 2005; Lynch et al., 2006; Rothen-Rutishauser et al., 2006; Smart et al., 2006). NP can be stored inside vesicles, mitochondria and various other locations within the cell. Due to their smaller size, NP can generate reactive oxygen species (ROS), which in turn may result in denatured proteins, mutated DNA and lipid peroxidation (Klaassen, 1996; Richier et al., 2005) ultimately leading to cytotoxicity (Oberdörster et al., 2005; Nel et al., 2006). As a defence mechanism organisms can produce enzymes such as catalase (CAT) and glutathione peroxidase (GPx), oxidize monomeric glutathione, to convert harmful ROS, such as hydrogen peroxide (H_2O_2), to water (Forman et al., 1990; Asada 1992; Noctor & Foyer 1998; Sies, 1999). A subsequent reaction then uses glutathione reductase (GR) as a catalyst to reduce glutathione so that it can be recycled and used again in the previous reaction (Forman et al., 1990).

The sea anemone, *Exaiptasia pallida*, is indigenous to south-eastern United States and has been found to occupy near-shore rocky surfaces, mangrove roots, and coral reefs habitats (Voss 1976; Kaplan, 1988; Shick, 1991). *E. pallida* is a cnidarian which lives in a symbiotic relationship with dinoflagellate algae (*Symbiodinium* sp.), known as zooxanthellae (Ruppert & Fox, 1988). The zooxanthellae provide photosynthate to support the maintenance and growth of the host organisms (Falkowski et al., 1984). The algal pigments in zooxanthellae also give the host a golden-brown color. Their proximity to

anthropogenic disturbance and sensitivity to metal pollutants makes sea anemones ideally suited as bioindicators of stress in marine systems (Main et al., 2010; Brock & Bielmyer, 2013). Dissolved copper has been shown to cause toxicity to sea anemones (Mitchelmore et al., 2003a; Aruoja et al., 2009; Main et al., 2010; Brock & Bielmyer, 2013); however, fewer studies have assessed the toxicity of CuO NP to marine organisms, although its importance has been recognized (Griffitt et al., 2007, 2009; Gomes et al., 2011, 2012, 2013). The objectives of this study were to quantify tissue copper accumulation and compare sublethal effects in *E. pallida* exposed to CuO NP and dissolved copper in the form of copper chloride ($CuCl_2$).

Materials and Methods

Animal Culture

Over the last several years, *E. pallida* have been maintained in holding tanks at Valdosta State University at a temperature of 26.1 ± 0.4 °C and in 30 ppt synthetic saltwater, prepared by mixing Instant Ocean salt and reverse osmosis (RO) water. The anemones were fed brine shrimp (*Artemia* sp.) and libitum daily. A YSI meter (YSI® model 85, yellow springs, OH, USA) was used to measure salinity, temperature, and dissolved oxygen (DO) daily. Nitrite, nitrate and ammonia levels were measured weekly using a Red Sea Marine and Freshwater Test Kit® (detection limits = 2.5 ppm nitrate, 0.05 ppm nitrite and 0.25 ppm ammonia). Water quality parameters (mean ± standard error) were 6.75 ± 0.29 mg/L DO, 7.89 ± 0.5 pH, 29.5 ± 0.5 ppt salinity, 4.0 ± 1.5 ppm nitrate, 0.1 ± 0.1 ppm nitrite, and 0.33 ± 0.5 ppm ammonia.

Nanoparticle Basic Properties and Preparation

CuO NP were obtained from Nanophase Technologies Corporation (Romeoville, IL). The nano-crystalline metal oxides were chemically pure, less than 100 nm in size, non-

porous single crystals with a defined surface chemistry. They were prepared by vapor-phase coating and liquid phase processing techniques in Nanophase.

The CuO NP stock solution was observed under the Scanning Electron Microscope (SEM; JEOL-6480_LV) (Figure 1A). The slide was prepared by water film, dried in air for 1 hour, and high pressure mode with secondary electrons at 100,000 x magnification was used to observe particle shape. Then they were photographed and size was measured using analySIS imaging system GmbH® and the presence of copper was confirmed by energy dispersive spectroscopy systems (EDS) (oxford Aztec®, Inca; X-ford X-max 50 mm) (Figure 1B).

Solution Preparation

CuO stock solutions were prepared by adding 10 mg of CuO NP to 10 ml of ultra-pure 18 mΩ Milli-Q® water, vortexing for 30 s, sonicating for 30 min, dilution (1:10) with 30 ppt synthetic water containing 10 mg/L alginate (previously made in a 1 g/L stock in 18 mΩ Milli-Q® water) and vortexing again for 30 s. A 10 g/L copper, as $CuCl_2$, stock solution was prepared by adding $CuCl_2$ salt to 18 mΩ Milli-Q® water at least 24 h prior to use. Copper experimental solutions (1g/L) were prepared by mixing a 10 g/L copper stock solution with 30 ppt synthetic saltwater, 24 h prior to the start of experiment and 24 h prior to each water change to allow for equilibration (Bielmyer et al., 2004). Synthetic saltwater was made by mixing Instant Ocean salt and 18 mΩ Milli-Q® water 24 h before the copper solutions were prepared.

Experimental Design

E. pallida were exposed to a control, 10, 50, and 100 µg/L CuO NP in 2 L glass dishes for 21 days. On 0, 3, 7, 14 and 21 days, one sea anemones per replicate (n = 4) was removed, anaesthetised with Tricaine Methanesulfonate (MS-222), and cut in half through

the midsagittal plane with a scalpel. Half of the anemone was used for metal analysis and half for protein and enzyme analyses. Photosynthetic parameters were also measured in the zooxanthellae of the anemones prior to sampling using imaging pulse amplitude modulated (PAM) fluorometry. On each sampling day, water samples were collected for copper analysis. Every two days, 80% of the test solution was renewed in each replicate dish. A YSI meter (YSI® model 85, yellow springs, OH, USA) was used to measure salinity, temperature, and DO daily, and nitrite, nitrate and ammonia concentrations were measured weekly using a LaMotte salt water aquaculture test kit®, model AQ-4 (code 3635-03) (detection limits = 0.05 ppm nitrite, 0.25 ppm nitrate and 0.2 ppm ammonia). The pH of the saltwater was measured using a calibrated pH meter (Radiometer analytical meterLab®, PHM201) at every water change. Mean values ± standard deviations were 30.0 ± 0.5 ppt salinity, 24.3 ± 0.3 °C temperature, 7.8 ± 0.35 mg/L DO, 4.0 ± 1.3 mg/L nitrate, 0.1 ± 0.05 mg/L nitrite, and 0.43 ± 0.6 mg/L ammonia. The light cycle was maintained at 12 h light: 12 h dark and the light intensity was 36 µmol photons/m^2/s.

In a subsequent experiment, *E. pallida* were exposed to a control (salt water only), 50, and 100 µg/L copper, as $CuCl_2$ in 2 L glass dishes for 14 days. On 0, 5, and 14 days one sea anemones per replicate (n = 3) were removed, anaesthetised with MS-222 and cut in half through the midsagittal plane with a scalpel. The experiment was then performed as described above.

Metal Analysis

Water samples were filtered using 0.45 µm nylon filter, (Fisher Scientific, Pittsburg, PA), acidified with trace metal grade nitric acid (Fisher Scientific), diluted with 18 mΩ Milli-Q® water to minimize salt interference, and analyzed for copper using atomic absorption spectrophotometry with graphite furnace detection (AAS; Perkin Elmer AAnalyst 800;

detection limit = 1 µg/L). Sea anemone tissue samples were dried in an oven at 60 °C for 3-4 h, weighed using a microbalance, and then digested in a water bath at 80 °C for 12-14 h, by addition of trace metal grade nitric acid. After the tissues were digested fully, they were diluted with 18 mΩ Milli-Q® water and then measured for copper using AAS.

Protein Quantification and Enzyme Assays

The other half of the anemone tissue sample was homogenized using 5 ml of 50 mM KH_2PO_4/K_2HPO_4 buffer (pH of 7.0 and temperature of 25 °C) using a mortar and pestle on ice. The homogenate was centrifuged (Thermo IEC MultiRF) at 300 rpm for 10 min, and the supernatant was preserved at -80 °C for protein quantification. A Bradford assay (Bradford, 1976) was performed for each sample using BIO-RAD quick start kit. Total soluble protein was calculated using Bovine serum albumin (BSA) Bradford reagent, and a buffer. The absorbance was recorded for each sample and standard using a PerkinElmer Lamda 35 UV/vis spectrophotometer at a wavelength of 595 nm. All enzyme activity was expressed per mg holobiont protein. Positive controls were provided by Sigma® and the negative control was made from boiling the anemone tissue lysate for 1 h prior to use.

CAT, GR, and GPx assays were performed following modified Sigma protocols (EC 1.11.1.6, Sigma, 1994a; EC 1.6.4.2, Sigma, 1994b; EC 1.11.1.9; Sigma, 1994c) and spectrophotometric methods, previously described (Patel & Bielmyer-Fraser, 2015). CA activity in anemone supernatant was calculated using the delta pH method of Henry (1991). Briefly, the rate of change in pH was measured using CyberComm Pro Data Acquisition Software for Accumet basic Ab 15 Plus with a sensitive recording pH meter (Accumet® Basic, AB15 Plus pH meter, Fischer Scientific). CA activity in the sample was expressed as mmol/min/mg protein.

Zooxanthellae Photosynthetic Parameters

Every sampling day, PAM fluorometry (Imaging-PAM, M-Series, Walz, Germany) was utilized to determine the following parameters in one sea anemone from each treatment: effective quantum yield of photosystem II (YPSII), the relative electron transport rate (rETR), nonphotochemical quenching (YNPQ) of photosystem II, and the quantum yield of nonregulated energy dissipation (YNO). The YPSII provides an indication of the amount of energy used in photochemistry (photosynthesis). The rETR is closely related to the photosynthetic activity and is an approximation of the rate of electrons pumped through the photosynthetic chain. The YNPQ refers to the amount energy dissipated in the form of heat and the YNO is a measurement of the fraction of energy that is passively dissipated in the form of heat and fluorescence; indicative of inefficiency of both photochemical energy conversion and protective regulatory mechanisms (Schreiber, 2004; Klughammer & Schreiber, 2008).

Statistical Analysis

Statistical differences between treatments ($p < 0.05$; $n = 3$) were calculated using analysis of variance (ANOVA) followed by a post-hoc Tukey's test to determine differences between treatments with the software Sigma-plot 11.0. Data were assessed for normality and homogeneity of variances using the Shapiro-Wilk test and Bartlett's test, respectively.

Results

Copper Concentrations

The measured copper concentrations in each of the $CuCl_2$ treatments were similar to nominal values (Table 1). Alternatively, the copper concentrations in each of the CuO NP treatments were approximately 50% of the corresponding $CuCl_2$ concentrations. This suggests that leaching occurred from the CuO NP and that the CuO NP treatments were actually a mixture of dissolved and NP copper (Table 1). The presence of CuO NP was

verified using SEM and our results demonstrate that they were spherical in their shape and ranged from 40 nm to 100 nm (Figure 1A). EDS confirmed the presence of NP copper (Figure 1B).

Copper Accumulation

Copper accumulated in *E. pallida* in a concentration-dependent manner (Figure 2). Anemones exposed to 10 µg/L CuO NP demonstrated significantly increased tissue copper at 3 days, with a return to control levels by 7 days, followed by a significant increase in tissue copper at 14 days and 21 days. Anemones exposed to 50 and 100 µg/L CuO NP demonstrated a significant increase in tissue copper throughout the first 14 days, with concentrations leveling by 21- 37 days and 5 µg/g, respectively (Figure 2A). Anemones exposed to $CuCl_2$ accumulated higher copper concentrations than those exposed to the same concentrations of CuO NP (Figure 2). *E. pallida* exposed to 50 and 100 µg/L $CuCl_2$ had significantly higher tissue copper concentrations than controls with values reaching 65 and 93 µg/g, respectively, by 14 days (Figure 2B).

Anti-oxidant Enzyme Activity

In general, activity of the measured anti-oxidant enzymes in the anemones exposed to 50 and 100 µg Cu/L increased with increasing exposure of copper over 14 days, both as CuO NP and $CuCl_2$ with levels remaining elevated upto 21 days after exposure to CuO NP with one exception (GR activity, 50 µg/L CuO) (Figure 3). CAT activity in anemones exposed to 50 and 100 µg/L CuO NP or $CuCl_2$ increased over time; however, anemones exposed to 50 µg/L were significantly elevated within the first 4 days of exposure, while in those exposed to 100 µg/L were not significantly elevated until 14 days of exposure, as compared to controls (Figures 3A and D). Catalase activity in anemones exposed to 10 µg/L CuO NP increased over time with significant elevations observed at 7 and 14 days;

and then decreased back to control levels by 21 days (Figure 3A). *E. pallida* exposed to CuO NP generally had higher CAT activity (up to 50 units/mg protein) than those exposed to the same concentrations of $CuCl_2$ (up to 30 units/mg protein) (Figures 3A and D).

GPx activity in *E. pallida* generally followed a similar pattern as CAT activity, increased with increasing exposure to copper, as CuO NP or $CuCl_2$. Unlike the pattern of CAT activity observed, *E. pallida* exposed to all three CuO NP concentrations had significantly elevated GPx activity by 7 days (Figures 3B and E). After 7 days, GPx activity of anemones exposed to 10 µg/L returned to control levels, the GPx activity of those exposed to 50 µg Cu/L increased and then stabilized, and the GPx activity of anemones exposed to 100 µg/L CuO NP continued to increase over the 21 days (Figure 3B). Similarly, anemones exposed to 50 and 100 µg/L $CuCl_2$ demonstrated an increase in GPx activity over the 14 days exposure; however, the GPx activity was 2 and 1.6 times lower, respectively, than the GPx activity observed in anemones exposed to the same concentrations of CuO NP (Figures 3 B and E).

GR activity in the anemones exposed to CuO NP, followed a similar response pattern as CAT activity, with anemones exposed to the highest concentration of 100 µg/L CuO NP, only demonstrating a significant increase after 14 days of exposure (Figure 3C). The anemones exposed to the lower CuO NP concentrations, exhibited significantly elevated GR activity within the first three to seven days, both returning to control levels by 21 days (Figure 3C).

Carbonic Anhydrase Activity

E. pallida CA activity was significantly elevated after 3 days of exposure to 10 µg/L CuO NP (Figure 4A). At 7 days, *E. pallida* exposed to 50 µg/L CuO NP had significantly decreased CA and those exposed to 100 µg/L CuO NP had slightly (but not significantly)

reduced CA activity. By 14 days, CA activity was decreased in all copper treatments as compared to concurrent controls (Figure 4A). The decreased CA activity persisted throughout the 21 days exposure period. Anemones exposed to 50 and 100 µg/L $CuCl_2$ had significantly decreased CA activity at 7 days and the inhibition continued through the 14 days exposure period (Figure 4B). The degree of CA inhibition was similar when exposed to CuO NP as compared to exposure of the anemones to $CuCl_2$.

Behavioral Observations

Quantitatively, no bleaching was observed in any of the treatment; however, zooxanthllae density was not quantified. *E. pallida* exposed to 50 and 100 µg/L CuO NP collapsed, became dark in colour, and curled their tentacles over the exposure period. Mucous release from the peduncle region of the anemone was also observed. No marked bleaching was observed. Anemones exposed to 10 µg/L did not visibly appear different from the controls. *E. pallida* exposed to 50 and 100 µg/L $CuCl_2$ were decreased in size and had loss of their tentacles. Anemones exposed to $CuCl_2$ appeared to be severely affected by 14 days. Due to the severity of the anemones' response to $CuCl_2$, the anemones were all euthanized and sampled at 14 days. It did not appear that the aenmones would survive through 21 days of exposure.

Zooxanthellae Photosynthetic Parameters

Over the course of the experiment the average ± standard deviation for YPSII, rETR, YNPQ, or YNO from all treatments was 0.56 ± 0.02, 26.24 ± 3.70, 0.39 ± 0.04 and 0.52 ± 0.04, respectively. No significant differences ($p \leq 0.05$) were detected between treatments for any of the parameters in either experiment.

Discussion and Conclusion

Anemones exposed to $CuCl_2$ seemed severely stressed by 14 days of exposure; therefore, we were unable to carry out the experiment for 21 days, as we did the CuO NP experiment. In a previous study in our laboratory, a 96-h LC50 value for *E. pallida* exposed to copper was determined to be 148 µg/L (95% confidence interval = 126.4, 173.8). After 14 days of 50 and 100 µg Cu/L exposure, the visible stress and behavioural responses of the anemones indicated a poor likelihood for survival throughout 21 days of exposure.

The tissue copper concentrations observed in *E. pallida* in this study are similar to those reported in other studies (Harland & Ngarno, 1990; Mitchelmore et al. 2003a; Brock & Bielmyer, 2013; Patel & Bielmyer-Fraser, 2015). The control tissue copper values ranged from 4.94 µg/g in the $CuCl_2$ experiment to 9.56 µg/g in the CuO NP experiment. These levels are within the normal range of tissue copper concentrations reported in un-exposed (control) anemones from other studies (Brock & Bielmyer, 2013; Patel & Bielmyer-Fraser, 2015). *E. pallida* in this study accumulated 40 µg Cu/g and 50 µg Cu/g after 7 days exposure to 100 µg/L copper, as CuO NP and $CuCl_2$, respectively. The sea anemone *Anthopleura elegantissima* accumulated 20 and 40 µg Cu/g dw when exposed to 100 µg/L copper for 7 and 42 days, respectively (Mitchelmore et al., 2003a). Similarly, Harland & Ngarno (1990) reported tissue copper concentrations of 50 µg Cu/g dw in the anemone *Anemonia viridis* after exposure to 200 µg/L copper. Brock & Bielmyer (2013) reported a tissue copper concentration of 50 µg Cu/g dw in *A. palllida* after 7 days of combined exposure of copper, zinc (Zn), cadmium (Cd), and nickel (Ni), which is somewhat higher than the tissue copper concentrations of 24 µg Cu/g dw and 35 µg Cu/g dw observed in this study after 7 days exposure of the anemones to 50 µg/L copper, as CuO NP and $CuCl_2$, respectively.

In this study, the anemones exposed to 10 µg/L CuO NP had an initial increase in tissue copper at 3 days, followed by a return to control levels by 7 days, and then another increase in tissue copper at 14 and 21 days. Other studies from our laboratory have reported an initial increase in tissue copper after exposure to 10 µg/L dissolved copper followed by a return of tissue copper to control level by 7 days (Main et al., 2010; Brock & Bielmyer, 2013). The subsequent elevation in tissue copper after exposure to CuO NP could have resulted from changes in dissolution of copper into the exposure media from the NP over the course of the experiment. Gradual dissolution of metal oxide NP has been observed in other studies in our laboratory (Jarvis et al., 2013; Bielmyer-Fraser et al., 2014). Alternatively, there could be difference in copper uptake rate, dependent on the form of copper. The CuO NP exposure media has combination of dissolved copper and CuO NP and uptake of copper into the anemone could occur through different modes.

An increase in tissue copper in *E. pallida* generally coincided with increased activity of the enzymes, CAT, GPx, and GR, after exposure to both CuO NP and $CuCl_2$, although there were differences in the pattern of responses based on exposure concentrations. Additionally, the anemones exposed to $CuCl_2$ accumulated higher tissue copper concentrations, whereas, those exposed to CuO NP had a greater oxidative stress response. Oxidative stress and altered physiological responses has been a reported consequence of metal exposure to anemones (Gilbert & Guzman, 2001; Mitchelmore et al., 2003b, 2007; Main et al*.,* 2010; Brock & Bielmyer, 2013; Mosleh et al., 2006); as well as corals (Grant e al., 2003; Smith et al., 2003; Morgan et al., 2005; Venn et al., 2009; Bielmyer et al*.,* 2010; Downs et al., 2010; Negri & Hoogenboom, 2011; Schwarz et al., 2013). Increased CAT activity has been reported in *E. pallida* exposed to 50 µg/L copper for 7 days (Main et al., 2010). Likewise, Patel & Bielmyer-Fraser (2015) reported an

increase in CAT activity in *E. pallida* exposed to 5, 10, and 50 µg/L copper for 7 days; however, CAT activity returned to control levels by 21 days, a finding which differs from the anemones exposed to 10, 50, and 100 µg/L CuO NP in this study. The importance of CAT as a protective antioxidant in aquatic invertebrates, even after low metal exposure levels, has been demonstrated in several previous studies (Livingstone et al., 1992; Regoli et al., 2002; Anjos et al., 2014). In this study, CAT activity in *E. pallida* was approximately two fold higher after 14 days of exposure to 50 µg/L CuO NP, as compared to anemones exposed to 50 µg/L $CuCl_2$. Exposure of the anemones to 100 µg/L of either CuO NP or $CuCl_2$; however, resulted in similar CAT activities at 14 days, suggesting that the oxidative stress response is dependent on the form of copper, as well as, concentration and perhaps exposure time.

Glutathione is utilized for intracellular protection against metals using the enzymes glutathione S-transferase, GPx and GR (Valko et al., 2005; Buffet et al., 2011). The cysteine residues in glutathione tightly bind to metals as a means for detoxification (Meister & Anderson, 1983). Patel & Bielmyer-Fraser (2015) reported an increase in both GPx and GR activity in anemones exposed to 50 µg/L copper for 7 days in 25 ppt salinity water, with enzyme activity returning to control levels by 14 days. Similarly, GR activity reportedly increased in *E. pallida* exposed to 50 µg/L of Cu, Zn, Cd, and Ni for 7 days. With levels returning to control values by 14 days; whereas, the GR activity remained significantly elevated in anemones exposed to 100 µg/L of the mixture for up to 14 days (Brock & Bielmyer, 2013). Alternatively, in this study, both the GPx and GR activities remained significantly elevated in the anemones exposed to 50 µg/L CuO NP for 14 days, again suggesting that CuO NP (likely a combination of dissolved and NP copper) may cause more severe oxidative stress than solely dissolved copper.

To date, no CuO NP toxicity study using cnidarians has been reported (Baker et al., 2014). Gomes et al. (2012) reported oxidative stress, indicated by activity of SOD, CAT and GPx, in the mussel *Mytilus galloprovincialis* when exposed to 10 µg/L CuO NP for 15 days. CuO NP induced oxidative stress was also reported in the blue mussel, *Mytilus edulis* (Hu et al., 2014), marine invertebrates, *Scrobicularia plana*, *Hediste diversicolor*, (Buffet et al., 2011) and juvenile fish, *Epinephelus coioides* (Wang et al., 2014). Genotoxic effects of CuO NP in *M. galloprovincialis* have also been reported (Gomes et al., 2013).

In addition to oxidative stress enzymes, CA was also altered in this study. CA is considered as one of the most ubiquitous enzymes within living organisms, and primarily functions to catalyze the inter-conversion of CO_2 and water to bicarbonate and protons (Badger & Price, 1994). CA mainly helps in maintaining acid-base balance in the tissues and blood of organisms (Gilmour, 2010); however, CA may also play an important role in the detoxification process by scavenging ROS (Roesijadi & Robinson, 1994). In this study CA activity decreased when exposed to both forms of copper. Similarly, another study reported decreased CA activity in the sea anemone *Condylactis gigantea* (Weinland) and *Stichodactyla helianthus* after exposure to 10 µg/L copper for 48 h (Gilbert & Guzman, 2001). Bielmyer et al. (2010) reported a decreased CA activity in the corals *Acropora cervicornis* and *Montastraea faveolata* after exposure to 4-20 µg/L copper for five weeks. CA is a metalloprotein, and uses zinc as a co-factor on its active site (Vitale et al., 1999). Copper has been shown to bind to the enzyme at the zinc binding site, thus causing change in the conformation of the protein structure (Sun et al., 2011). Unlike the other enzymes measured, *E. pallida* exposed to CuO NP did not have a higher CA activity as compared to those anemones exposed to $CuCl_2$. These results suggest that NP may have a greater effect on primarily oxidative stress pathways.

The behavioural changes observed in this study are similar to those reported previously. Brown & Howard (1985) reported mucus secretion in cnidarians when exposed to metal concentration, consistent with the observations in this study. Loss of tentacles was another prominent observation in *E. pallida* exposed to $CuCl_2$, which is similar to the finding of Main et al. (2010) and Brock & Bielmyer (2013) in *E. pallida* exposed to dissolved copper. Alternatively, curling and retracting tentacles were observed in *E. pallida* exposed to CuO NP. The different responses of the anemones may have been due to the different forms of copper used. In both cases, it is clear that the anemone is trying to reduce contact with copper by reducing its surface area with surrounding environment. PAM fluorometry in this study indicated no significant photosynthetic impairment in the zooxanthellae after exposure to either form of copper, as compared to the controls. The oxidative stress responses observed in the holobiont after exposure to both forms of copper were more likely due to toxicity in the anemone than toxicity to the zooxanthellae.

The different forms of copper used in this study did lead to some different responses of the exposed anemones. Specifically, increased activity of anti-oxidant enzymes was observed in CuO NP-exposed anemones, as compared to those exposed to dissolved Cu. Bielmyer-Fraser et al. (2014) recently reported marked differences in cellular metal distribution in the marine diatom, *Thalassiosira weissflogii*, exposed to nano-metal oxides versus those exposed to dissolved metals. Metal concentrations were highest in the algal cell wall when cells were exposed to metal oxide NP, whereas algae exposed to dissolved metals had higher proportions of metal in the organelle and endoplasmic reticulum fractions (Bielmyer-Fraser et al., 2014). In this study, copper maybe distributing differently in *E. pallida* when exposed to CuO NP as compared to solely dissolved copper, although this has not been measured. Differences in distribution could cause differences

in toxic responses; however, more research is needed to elucidate this issue. In this study, copper may be distributing differently in *E. pallida* when exposed to CuO NP as compared to solely dissolved copper. These differences in distribution may contribute to the observed differences in toxic responses; however, more research is needed.

Copper accumulation in *E. pallida* was concentration-dependent after exposure to both forms of copper. However, the sea anemones exposed to $CuCl_2$ accumulated comparatively higher copper tissue burdens than those exposed to CuO NP, likely because copper was more available as $CuCl_2$ than as CuO NP. Alternatively, the oxidative stress response, as demonstrated by the activities of CAT, GPx, and GR, was observed to a greater degree in sea anemones exposed to CuO NP (actually a combination of dissolved and CuO NP) indicating clear differences in the toxicity of the two forms of copper. Independent of the form of copper exposed to the holobiont, the anemones appeared to be more susceptible to copper toxicity than their symbiotic zooxanthellae. This study presents new data concerning the toxicity of CuO NP in a symbiotic cnidarian and may have implications for NP exposure and toxicity in the environment.

Table 1: Dissolved Copper Concentrations

Treatment (µg/L)	$CuCl_2$ (µg/L)	CuO NP (µg/L)
Control	0.80 ± 0.09	0.10 ± 0.01
10	NT	4.43 ± 0.95
50	41.9 ± 0.14	14.3 ± 1.66
100	89.6 ± 0.21	39.6 ± 1.81

Note: All the values are reported as Mean ± Standard Error in testing waters over 14 and 21 days of exposure to $CuCl_2$ and CuO NP and NT indicates no treatment

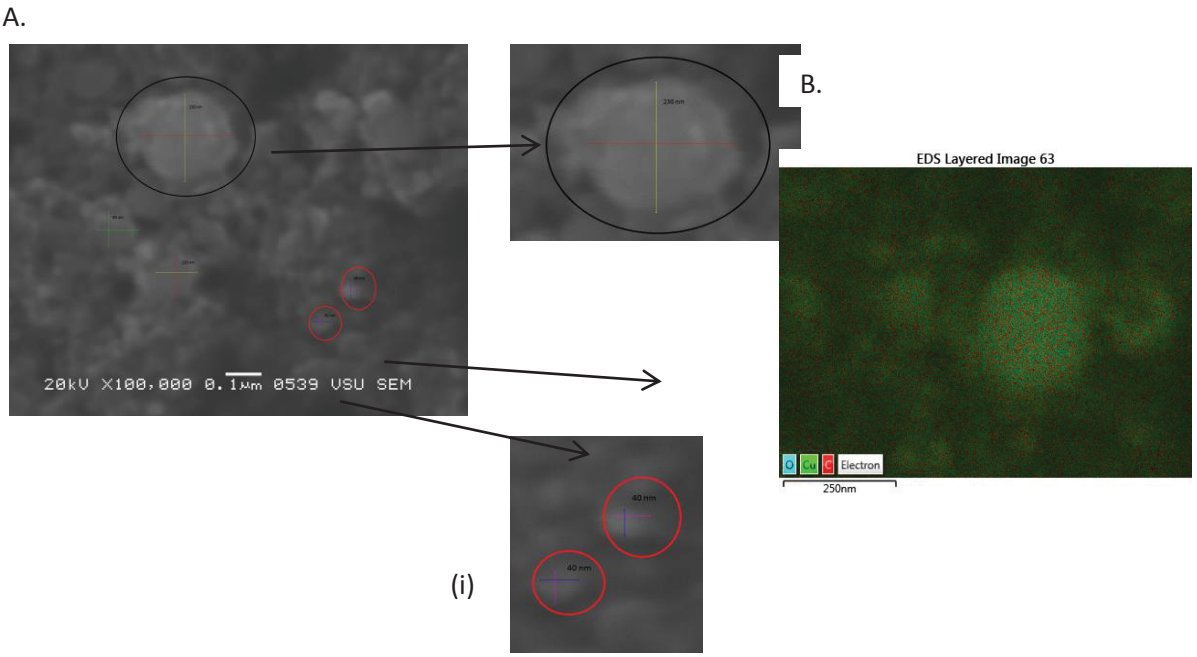


Figure 1. A) Scanning Electron Microscope (SEM) Image of CuO NP with their respective (i) shapes and size and B) Energy Dispersive Spectroscopy Systems (EDS) layered image.

Note: EDS layered image confirming copper with the green colour.

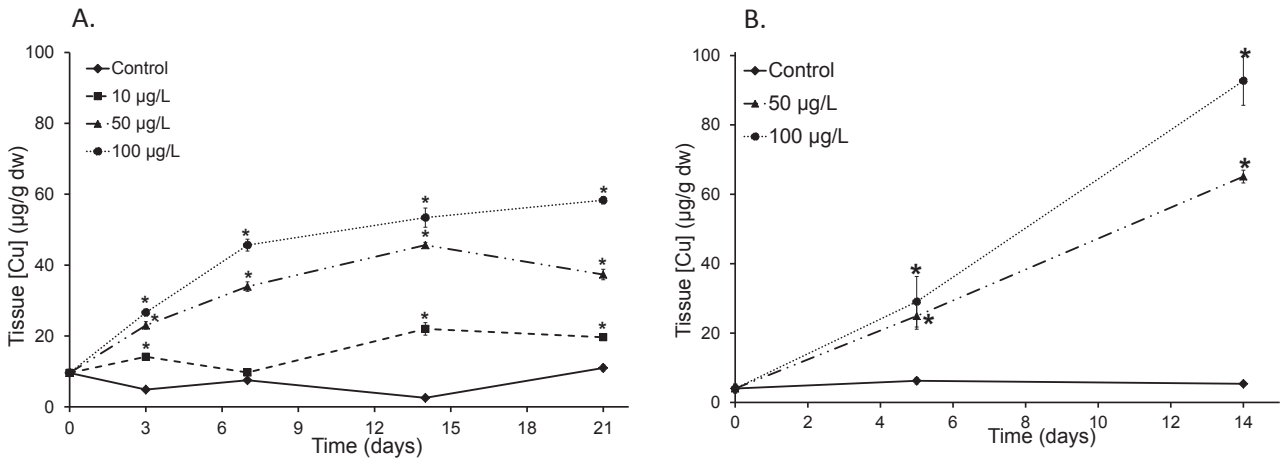
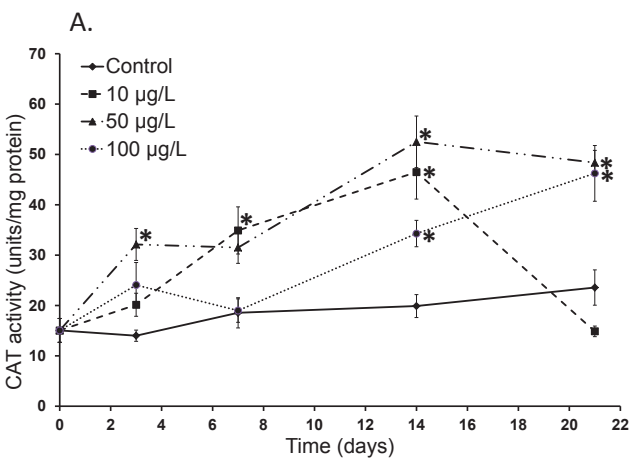
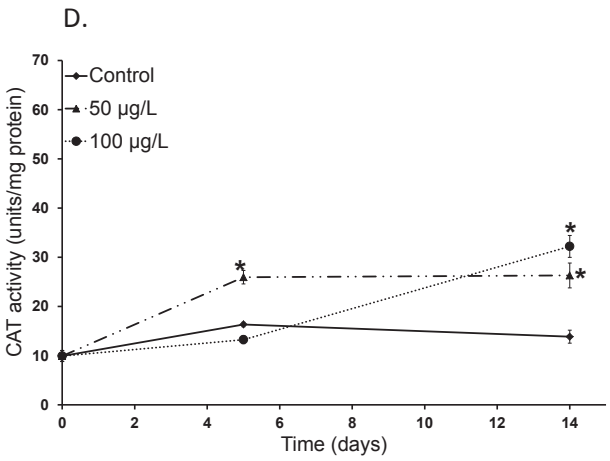
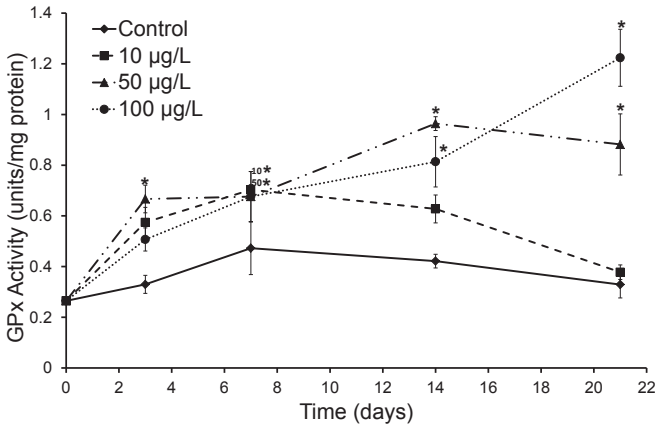


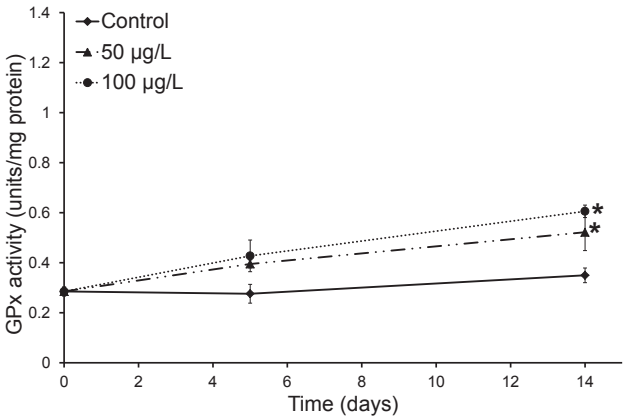
Figure 2. Tissue Copper Accumulation

Notes: All the values are reported as Mean ± Standard Error in *Exaiptasia pallida* after exposure to (A) Control, 10, 50 and 100 µg/L CuO NP for 21 days and (B) Control, 50 and 100 µg/L $CuCl_2$ for 14 days. * indicates a significant difference from concurrent control ($p \leq 0.05$; $n = 4$).


A.
Control
10 µg/L
50 µg/L
100 µg/L
CAT activity (units/mg protein)
Time (days)


D.
Control
50 µg/L
100 µg/L
CAT activity (units/mg protein)
Time (days)


B.
Control
10 µg/L
50 µg/L
100 µg/L
GPx Activity (units/mg protein)
Time (days)


E.
Control
50 µg/L
100 µg/L
GPx activity (units/mg protein)
Time (days)

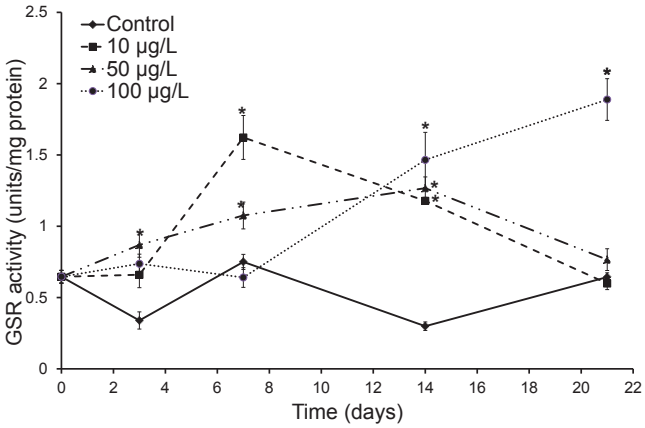

C.
Control
10 µg/L
50 µg/L
100 µg/L
GSR activity (units/mg protein)
Time (days)

Figure 3. A and D Catalase (CAT), B and E Glutathione Peroxidase (GPx) and C Glutathione Reductase (GR) Activity in after exposure to CuO NP (A-C) and $CuCl_2$ (D-E).

Notes: : All the values are reported as Mean ± Standard Error in *Exaiptasia pallida* after exposure to (A) Control, 10, 50 and 100 µg/L CuO NP for 21 days and (B) Control, 50 and 100 µg/L CuCl2 for 14 days in synthetic sea water. * indicates a significant difference from concurrent control ($p \leq 0.05$; $n = 4$).

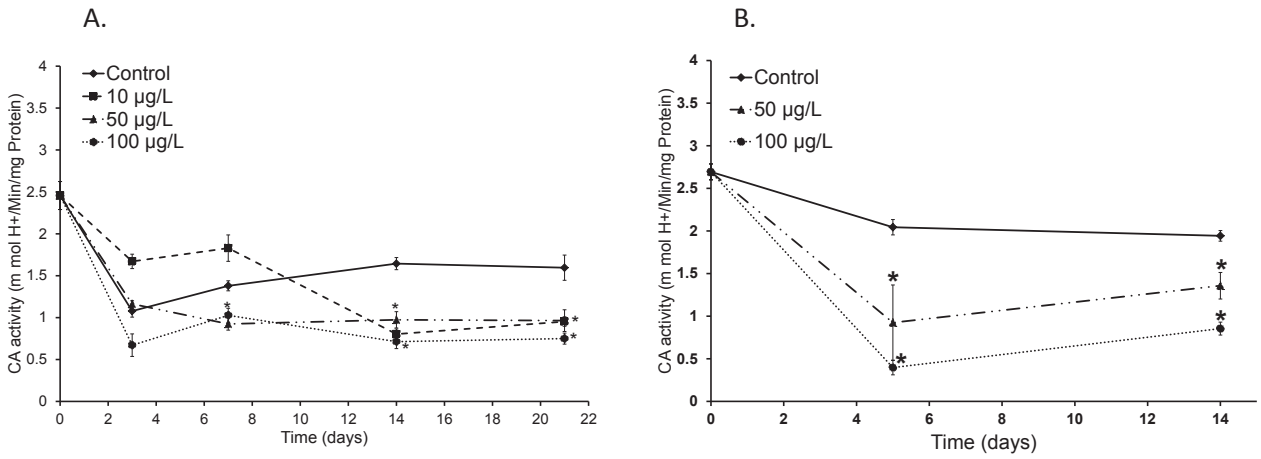


Figure 4. Carbonic Anhydrase (CA) Activity

Notes: All the values are reported as Mean ± Standard Error in *Exaiptasia pallida* after exposure to (A) Control, 10, 50 and 100 µg/L CuO NP for 21 days and (B) Control, 50 and 100 µg/L $CuCl_2$ for 14 days. * indicates a significant difference from concurrent control ($p \leq 0.05$; $n = 4$).

Chapter II

RESPONSES OF THE SEA ANEMONE, *EXAIPTASIA PALLIDA*, TO OCEAN ACIDIFICATION CONDITIONS AND COPPER EXPOSURE

Introduction

Advancing technology and industrial applications have resulted in increased metal inputs into aquatic ecosystems (Zhou et al., 2008; Luoma & Rainbow, 2008). At elevated concentrations, metals may cause toxicity to aquatic organisms (Bielmyer et al., 2010; 2012; Brock & Bielmyer, 2013; Patel & Bielmyer, 2015). The effects of these local stressors, in combination with global climate changes, such as ocean acidification (OA), are to a great extent unknown. Carbon dioxide (CO_2) levels have substantially increased since the industrial revolution (280 ppm to 400 ppm) mainly due to anthropogenic activities like fossil fuel combustion, cement production and deforestation (Canadell et al., 2007; Yool et al., 2013). In the next couple of decades CO_2 concentrations are predicted to increase to 1000 ppm (IPCC, 2001). Increased CO_2 in the ocean decreases the pH by increasing hydrogen ion concentration in water. With increasing CO_2 levels or concentrations in marine water, the pH has decreased by more than 0.1 units from the preindustrial era (Orr et al., 2005) and is predicted to decrease by another 0.14 to 0.35 units by the end of 21st Century (Caldeira & Wickett, 2005). At lower pH levels, metal speciation favors the metal ion, which has been shown to be the most bioavailable and toxic form (Tatara et al., 1997; Adamu et al., 2013). The fate of metals in the aquatic environment is altered by the presence and absence of hydroxyl (OH^-) and carbonate ions (CO_3^{2-}) (Millero et al., 2009). The combined effects from both stressors (CO_2 and metals), as well as the increased bioavailability of metals at lower pH levels, may have substantial biological effects at the

physiological level, which may in turn have repercussions for populations and perhaps even ecosystems (Agegian, 1985; Langdon et al., 2000; Leclercq et al., 2000; Le Quesne & Pinnegar, 2012).

Change in ocean pH can affect the efficiency of biological enzymes (Yamada & Suzumura, 2010) and the degradation of polysaccharides, which are a major component of marine organic matter (Piontek et al., 2010). Altered ocean chemistry can threaten many marine organisms, such as algae and protozoans. A study on a common crustose coralline alga in Hawaii demonstrated both declined calcification rates and recruitment rates at lower carbonate saturation state (Kuffner et al., 2008). Photosynthesis, calcification, acid-base homeostasis, respiration/gas exchange, and metabolic rate are some of the physiological processes in marine organisms that can be affected due to altered ocean chemistry by OA (Gattuso et al., 1999; Seibel & Walsh, 2003; Melzner et al., 2009). OA has been found to affect fishes like *Opsanus beta* resulting in intestinal base loss (Heuer et al., 2012) and disruption of respiratory gas exchange and acid-base balance (Esbaugh et al., 2012) after exposure to 1000 µatm CO_2. Behavioral responses to auditory stimuli, based on directional responses in juvenile clownfish have also been documented after exposure to 600 µatm CO_2 (Simpson et al., 2011).

Cu is a commonly used metal that can enter into marine systems through construction applications (Sundberg, 1998), heavy metal mining (Bryan, 1974), sewage treatment discharge, industrial effluent, leaching of anti-fouling paints, and copper refineries (Guzmán & Jiménez, 1992; Jones, 1997; Mitchelmore et al., 2003a). Relatively low levels of Cu have been shown to cause oxidative stress in marine cnidarians (Bielmyer et al, 2010; Brock & Bielmyer, 2013; Patel & Bielmyer-Fraser, 2015). Changes in ocean pH can affect Cu speciation, behavior and fate by changing Cu thermodynamics as well as

kinetics (Millero et al., 2009). According to some studies, the free-ion concentration of Cu may increase by 115% in coastal waters by the next 100 years as a result of decreased pH by OA (Pascal et al., 2010; Richards et al., 2011). However, the combined effects of altering CO_2 with Cu on marine organisms are to a large degree unknown (Roberts et al., 2013).

Many physiological effects have been well documented in marine cnidarians as a consequence of Cu exposure (Bielmyer et al., 2010; Main et al., 2010; Brock & Bielmyer, 2013; Patel & Bielmyer-Fraser, 2015). In particular, Cu has been shown to cause the generation of reactive oxygen species (ROS), which can lead to degradation of macromolecules like proteins, lipids and DNA (Chang et al., 1996; Luschak, 2011). Several anti-oxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx) (Luschak, 2011) are used to combat the harmful effects of ROS. The activity of these enzymes as well as carbonic anhydrase (CA), responsible for acid/base balance, were altered in cnidarians exposed to Cu in previous studies (Bielmyer et al., 2010; Main et al., 2010; Brock & Bielmyer, 2013; Siddiqui et al., 2015). In this study we examined the effects of Cu, a local stressor, and OA, a more global stressor, to determine their effects on cnidarian physiology. The sea anemone, *Exaiptasia pallida*, has been used in several past studies and has been shown to be sensitive to metals (Main et al., 2010; Patel & Bielmyer-Fraser, 2015; Siddiqui et al., 2015); therefore, it was chosen for this study. The objective of this research was to assess the combined effects of OA and Cu exposure on Cu accumulation as well as enzyme activity in *E. pallida* in a laboratory study using a pH/pCO_2 stat system.

Materials and Methods

Animal Culture

E. pallida was maintained in 30 L tanks with continuous filtration and aeration, in synthetic saltwater, prepared by mixing Instant Ocean salt and reverse osmosis (RO) water. A YSI meter (YSI® model 85, yellow springs, OH, USA) was used to measure salinity, temperature, and dissolved oxygen (DO) daily. Nitrite, nitrate, and ammonia levels were measured weekly using a Red Sea Marine and Freshwater Test Kit®. Water quality parameters in the culture tanks were (mean values ± standard error) were 6.72 ± 0.25 mg/L DO, 8.01 ± 0.4 pH, 29.3 ± 0.6 ppt salinity, 26.3 ± 0.5 °C, 4.0 ± 1.5 ppm nitrate, 0.1 ± 0.05 ppm nitrite, and 0.23 ± 0.5 ppm ammonia.

Experimental System

Because of the well understood relationship with the climate, partial pressure of CO_2 (pCO_2) has been considered a reliable method to calculate CO_2 levels by various scientists (Petit et al., 1999). This has widely been used in computer models like GEOCARB, PAL (Berner & Kothavala, 2001; Berner, 2006), COPSE (Bergman et al., 2004) and many others (Guttuso & Hansson, 2011). The pH/pCO_2 stat system used for this experiment is from Lolligo system with CAPCTRL software and set up by manual instruction. A standard curve for the calibration was prepared using a known CO_2/O_2 gas mixture of 2250 ppm. An automated negative feedback system was used to maintain the required amount of pCO_2 in the water of different tanks. The nominal pCO_2 values used in these experiments were 400 ppm and 1000 ppm, maintained through the system by continuously regulating the required pH over a 7 day experimental period using pure CO_2 gas. Each tank was also continuously aerated. Measured pCO_2 and Cu values are presented in Table 2.

Experimental Solutions

Synthetic saltwater was prepared by mixing Instant Ocean salt with 18 mΩ Milli-Q® water 24 h before experimental solutions were made. A 10 g/L Cu, as $CuCl_2$, stock solution

was prepared by adding $CuCl_2$ salt to 18 mΩ Milli-Q® water 24 h prior to use. Cu experimental solutions (10, 50 and 100 µg/L) were prepared by mixing a 10 g/L Cu stock with 30 ppt synthetic saltwater 24 h prior to the start of experiment and 24 h prior to each water change to allow for equilibration (Bielmyer et al., 2004).

Experimental Design

Three sequential experiments were performed. In each experiment there were eight tanks each with 12 L water. The CO_2 concentration has ranged from 180 to 300 ppm over the last 650,000 years, as measured at Dome Concordia ice core (Siegenthaler et al., 2005); and the CO_2 concentration in our culture tanks (290 ppm) was within this range. Since the recent global mean was ~398 ppm CO_2 (Dlugokencky & Tans, 2014), we chose 400 ppm CO_2 as the concentration for the control treatment in the experiments. The other CO_2 concentration (1000 ppm) used in these experiments was chosen based on the predicted increase in future pCO_2 in the next couple of decades (IPCC, 2001). There were four tanks at each CO_2 concentration, two without copper and two with copper (10 µg/L Cu in the first experiment). Each tank contained eight anemones, and two from each tank were used at each sampling period. Immediately following the preparation of the Cu solutions, the two pCO_2 concentrations (400 and 1000 ppm) were prepared and maintained in each tank 24 h prior to the start of the experiment. Sea anemones from the culture tanks were then transferred to the experimental tanks.

At the start of the experiment, five sea anemones from the culture tanks were selected randomly, anaesthetised with tricaine methanesulfonate (MS-222), and cut in half through the midsagittal plane with a scalpel. Half of the anemone was used for metal analysis and half for protein and enzyme analyses. For each treatment, two sea anemones per replicate tank (n = 2) were removed on 2, 5, and 7 days. The above method was

followed to sample anemones for metal analysis, protein, and enzyme assays. On 2, 5, and 7 days water samples were collected from each experimental tank for Cu analysis. A YSI meter (YSI® model 85, yellow springs, OH, USA) was used to measure salinity, temperature, and DO daily. Nitrite, nitrate, and ammonia concentrations were measured daily from each individual tank using a Red Sea Marine and Freshwater Test Kit®. Mean values ± standard deviations of all water quality parameters are provided in Table 3. The photoperiod for all the experiments was 12h light:12h dark with an average light intensity of 33.4 µmol photons m^{-2} s^{-1}. This experiment was repeated with 50 µg/L Cu, and then again using 100 µg/L Cu. In the 100 µg/L Cu experiment, after the start of the experiment, the sampling days were 2, 4, and 5 days (n =2) in the Cu treatments because of severe stress; and 2, 4, 5, and 7 days in the control treatments (n = 2) at each CO_2 level.

Metal Analysis

Tissue metal concentration and water concentration were analysed using the atomic absorption spectrophotometer with graphite furnace detection (AAS, detection limit 1 µg/L; Perkin Elmer AAnalyst 800). Sea anemone tissue samples were dried in an oven at 60 °C for 3-4 h, weighed using a microbalance, and then digested by addition of trace metal grade nitric acid followed by heating in a water bath at 80 °C for 12-14 h. After the tissues were digested fully, they were diluted with 18 mΩ Milli-Q® water and then measured for Cu using AAS.

Protein Quantification and Enzyme Assays

Protein was quantified in each sample using a BIO-RAD quick start kit following a Bradford assay (Bradford, 1976). Total soluble protein was calculated using bovine serum albumin (BSA) standards, Bradford reagent, and a 50 mM KH_2PO_4/K_2HPO_4 buffer (pH of 7.0 at 25 °C) (Siddiqui et al., 2015).

CAT, GR, and GPx assays were performed following modified Sigma protocols (EC 1.11.1.6, Sigma, 1994a; EC 1.6.4.2, Sigma, 1994b; EC 1.11.1.9; Sigma, 1994c) and spectrophotometric methods, as previously described (Patel & Bielmyer-Fraser, 2015; Siddiqui et al., 2015). Activity of these enzymes was normalized for protein content of the sample. CA activity in anemone supernatant was calculated using the delta pH method of Henry (1991). Briefly, the rate of change in pH was measured using CyberComm Pro Data Acquisition Software for Accumet basic Ab 15 Plus with a sensitive recording pH meter (Accumet® Basic, AB15 Plus pH meter, Fisher Scientific). CA activity in the sample was expressed as µmol/min/mg protein.

Statistical Analysis

Data were tested for normality and equal variance using Shapiro-Wilk's test and Bartlett's test. To identify interactions between the two independent variables and statistical significance between treatments, a 2-way ANOVA was performed using Sigma Plot 11.0 followed by a post-hoc Tukey's test, Holm-Sidak and Student-Newman-Keuls Method.

Results

Cu concentration and pCO_2 levels in all the treatment tanks were similar to the nominal values (Table 2). The pCO_2 levels were similar across the three experiments (Table 2).

Copper Accumulation

Cu accumulated in the sea anemones in a concentration dependent manner (Figure 5). Anemones exposed to 10 µg/L Cu at both CO_2 levels demonstrated significantly higher tissue Cu by 2 days, which persisted throughout the exposure (Figure 5A). Additionally, those anemones exposed to 10 µg/L Cu at 1000 ppm CO_2 had higher tissue Cu levels than

those at 400 ppm CO_2 at 2 days of exposure, after which time no significant differences were observed between the two treatments (Figure 5A). Anemones exposed to both 50 µg/L Cu and 100 µg/L Cu at both CO_2 levels demonstrated significantly increased tissue Cu throughout the exposure periods (Figures 5B, C). Those anemones exposed to 100 µg/L Cu at 1000 ppm CO_2 accumulated higher tissue Cu levels than those at 400 ppm CO_2 by 4 days of exposure; however, no differences in Cu accumulation were observed as a consequence of CO_2 level after exposure to 50 µg/L Cu (Figures 5B, C). There were statistically significant interactions between CO_2 and 10 µg/L Cu at 2 days and after 4 days in anemones exposed to 100 µg/L Cu.

Anti-oxidant Enzyme Activity

In general, activity of the anti-oxidant enzymes increased with increasing Cu exposure over 7 days (Figures 6-8). At the lowest Cu exposure concentration of 10 µg/L, CAT activity was significantly lower; whereas exposure levels of 50 and 100 µg/L generally resulted in increased CAT activity (Figure 6). There was a statistically significant interaction between CO_2 and Cu on CAT activity when anemones were exposed to10 µg/L Cu at 2, 5 and 7 days (Figure 6A). However there was only a statistically significant interaction between Cu and CO_2 on CAT activity at 2 days, when anemones were exposed to 50 and 100 µg/L Cu treatment (Figures 6 B, C). No significant differences in CAT activity were observed between the control groups at the two different CO_2 levels in the first two experiments; however, a slightly increased CAT activity was observed in the 1000 ppm CO_2 group as compared to the 400 ppm CO_2 group.

GPx activity in *E. pallida* increased in 50 and 100 µg/L Cu treatment at 1000 ppm CO_2 levels (Figures 7 B, C). A significant difference in anemone GPx activity was observed due to CO_2 alone (considering CO_2 treatments from all three experiments) at 7 d (Table 4;

Figure 7). Alternatively, anemones exposed to both Cu and CO_2 generally had at least slightly higher GPx levels than those exposed solely to Cu (Figure 7). There was a statistically significant interaction observed between CO_2 and Cu at 2, 5, and 7 days in the 10 µg/L Cu treatment (Figure 7 A), however in the 100 µg/L Cu treatment a significant interaction was only observed at 5 days.

GR activity generally increased with increased Cu exposure (Figure 8), with a few exceptions where a decrease in GPx activity was observed at 2 days of exposure (Figure 8A, C). Unlike the CAT and GPx activity responses, anemones in the 1000 ppm CO_2 control had significantly higher GPx activity than those in the 400 ppm CO_2 control at 2, 5, and 7 d (Table 4; Figure 8). Additionally, those anemones exposed to Cu and 1000 ppm CO_2 had the highest GPx activity of any of the treatments (Figure 8). There was a statistically significant interaction between CO_2 and 10 µg/L Cu at 2, 5 and 7 days and between CO_2 and 100 µg/L Cu at 4 and 5 days (Figure 8A).

Carbonic Anhydrase Activity

CA activity was significantly inhibited by Cu in all the treatments compared to the controls (Figures 9 A-C). CA activity did not significantly differ between the groups as a consequence of CO_2 alone (Table 4; Figure 9). CA activity in the sea anemones was significantly affected by interactions between 10 µg/L Cu and CO_2 at 2 and 5 days and between 50 µg/L Cu and CO_2 at 2 days. In the 10 and 50 µg/L Cu treatments at 1000 ppm CO_2, CA activity was generally higher (less inhibited) than in anemones exposed to Cu alone at 2 days; however, no differences were observed between the 100 µg/L Cu groups at the two CO_2 levels when compared to control (Figures 9).

Discussion and Conclusion

Cu has been shown to accumulate in the tissues of cnidarians as a consequence of Cu exposure (Brock & Bielmyer, 2013; Patel & Bielmyer-Fraser, 2015; Siddiqui et al., 2015). Harland & Ngarno (1990) exposed the anemone, *Anemonia viridis,* to 200 µg/L Cu and reported a tissue Cu concentration of 50 µg Cu/g dw. When the anemone, *Anthopleura elegantissima,* was exposed to 100 µg/L Cu for 7 and 42 days, 20 and 40 µg Cu/g dw tissue copper was accumulated, respectively (Mitchelmore et al., 2003a). Siddiqui et al. (2015) reported Cu accumulation of 40 and 50 µg/g dw in *E. pallida* after 7 days exposure to 100 µg/L Cu as CuO NP or $CuCl_2$, respectively. Similarly in present study, sea anemones accumulated 50-60 and 100-110 µg/g dw Cu as $CuCl_2$ after 5 days exposure to 100 µg/L Cu at 400 ppm and 1000 ppm CO_2, respectively. Brock & Bielmyer (2013) reported a tissue Cu concentration of 52 µg Cu/g dw in *A. pallida* after 7 days of combined exposure to 50 µg/L combined exposure of Cu, zinc (Zn), cadmium (Cd), and nickel (Ni), which is similar to the anemones in this study, exposed to 50 µg/L Cu. In a recent study, Patel & Bielmyer-Fraser (2015) reported a higher tissue Cu accumulation (52 µg/g dw) in *E. pallida* exposed to 50 µg/L Cu for 7 days in 20 ppt salinity saltwater (pH 7.7 ± 0.16) as compared to the tissue Cu (19 µg/g dw) in 25 ppt salinity (pH 8.07 ± 0.08) saltwater. In this study, the decrease in pH from 8.11 ± 0.12 to 7.81 ± 0.15, with the increase from 400 to 1000 ppm CO_2, also resulted in a higher tissue Cu accumulation in *E. pallida*. With increasing pCO_2 and lower pH, Cu can become more bioavailable due to speciation favouring the Cu ion (Richards et al., 2011; Roberts et al., 2013). At a lower pH, a greater degree of metal adsorption to aquatic organisms has been shown to occur in various aquatic systems (Graney et al., 1984; Albers & Camardese, 1993; Atkinson et al., 2007). Cu accumulation was increased with

decreasing pH in Rainbow trout (Lauren & Mc Donald, 2011), the marine algae, *Durvillaea potatorum* and *Ecklonia radiate*, (Matheickal & Yu, 1999) and Cod (Larsen et al., 1997).

Cu is known to cause oxidative stress and toxicity in cnidarians (Bielmyer et al., 2010; Main et al., 2010; Brock & Bielmyer, 2013; Anjos et al., 2014; Patel & Bielmyer-Fraser, 2015, Siddiqui et al., 2015). An increase in CAT, GPx, and GR activity, was observed with increasing tissue Cu in *E. pallida*, after exposure to $CuCl_2$ (at both CO_2 concentrations) consistent with other studies (Brock & Bielmyer, 2013, Patel & Bielmyer-Fraser, 2015; Siddiqui et al., 2015). Also, the increased Cu bioavailability at the higher CO_2 concentration (lower pH) resulted in a greater oxidative stress response. Earlier studies have reported oxidative stress and altered physiological responses as a result of metal exposure in anemones (Gilbert & Guzman, 2001; Mitchelmore et al., 2003b, Mosleh et al., 2006; Main et al*.,* 2010; Brock & Bielmyer, 2013; Patel & Bielmyer- Fraser, 2015; Siddiqui et al., 2015); and corals (Grant et al., 2003; Smith et al., 2003; Morgan et al., 2005; Venn et al., 2009; Bielmyer et al*.,* 2010; Downs et al., 2010; Negri & Hoogenboom, 2011; Schwarz et al., 2013).

Increased CAT activity has been reported in *E. pallida* exposed to 50 µg/L Cu for 7 days (Main et al., 2010); *E. pallida* exposed to 5, 10, and 50 µg/L Cu for 7 days (Patel & Bielmyer-Fraser, 2015); and *E. pallida* exposed to 10, 50, and 100 µg/L Cu, as $CuCl_2$ or CuO NP for 14 and 21 days, respectively (Siddiqui et al., 2015), comparable to this study. CAT is used to catalyze the conversion of H_2O_2 to H_2O and O_2 thus preventing the formation of hydroxyl ions (Chelikani et al., 2004). Increased CO_2 alone made very little difference in anemone CAT activity, but when increased CO_2 was combined with Cu (at the lowest exposure level in particular), a greater CAT activity was observed.

To recover from oxidative stress, living organisms develop nonenzymatic antioxidant GSH, which plays a key role in the intracellular protection against heavy metals using the enzymes glutathione S-transferase, GPx and GR (Valko et al., 2005; Buffet et al., 2011). Patel & Bielmyer-Fraser (2015), reported an increase in both GPx and GR activity in anemones exposed to 50 µg/L Cu for 7 days in 25 ppt salinity water, with enzyme activity returning to control levels by 14 days. Similarly, GR activity reportedly increased in *E. pallida* exposed to 50 µg/L of Cu, Zn, Cd, and Ni for 7 days with levels returning to control values by 14 days; whereas, the GR activity remained significantly elevated in anemones exposed to 100 µg/L of the mixture for up to 14 days (Brock & Bielmyer, 2013). Similarly, Siddiqui et al. (2015) reported significantly elevated GPx and GR activities in anemones exposed to 50 µg/L CuO NP and $CuCl_2$ for 14 days. Multiple studies in our laboratory have demonstrated that decreased salinity (from 35 to 20 ppt) and as a result decreased pH, caused an increase of all of these anti-oxidant enzymes to an extent greater than due to Cu (Brock & Bielmyer, 2013; Patel & Bielmyer-Fraser, 2015; Siddiqui et al., 2015). In the present study, GPx and GR activity increased with both Cu and CO_2 (Table 3); however, Cu elicited more of a response than did CO_2. The addition of the higher CO_2 level (1000 ppm) with Cu resulted in even greater activities of GPx (Figure 3) and GR (Figure 4) than either variable independently. Increasing pCO_2 (395 µatm – 3000 µatm) resulted in increased Cu toxicity by increasing the cellular stress responses in the marine bivalves, *Crassostrea virginica* and *Mercenaria mercenaria*, (Götze et al., 2014), and by decreasing early larval survivorship in the polychaete, *Arenicola marina* (Campbell et al., 2014).

The importance of the antioxidant enzymes measured in this study is very well documented (Livingstone et al., 1992; Regoli et al., 2002). At elevated levels, metal like Cu can overwhelm antioxidant defence mechanisms of an organism and can cause oxidative

damage to the cellular components such as proteins, lipids, and DNA (Krumschnabel et al., 2005; Luschak, 2011) or directly through the covalent binding of free ionic copper to macromolecules (Campbell et al., 2014). Acidification of seawater (pH 7.8 to 7.6) has been shown to affect SOD, CAT, peroxidase, alkaline phosphatase, and acid phosphatase in the brine shrimp, *Artemia sinica* (Zheng et al., 2015). In this study, GPx and GR played a greater role in the anti-oxidant response in *E. pallida* than CAT as a consequence of increasing CO_2 concentration, suggesting that pathway for conversion of peroxide to water is of greater importance in intracellular protection.

CA participates in the transport of inorganic carbon to actively photosynthesizing cells or away from actively respiring cells (Henry, 1996). Physiological functions involving carboxylation or decarboxylation reactions, including both photosynthesis and respiration require CA (Moroney et al., 2001). CA is also heavily involved in acid/base balance in many organisms (Gilmour, 2010). In this study, CA decreased with increasing Cu concentration, similar to that reported by Gilbert & Guzman (2001) in the sea anemones, *Condylactis gigantea* (Weinland) and *Stichodactyla helianthus* after exposure to 10 µg/L Cu for 48 h. CA activity also decreased in the corals *Acropora cervicornis* and *Montastraea faveolata* after exposure to 4-20 µg/L Cu for five weeks (Bielmyer et al., 2010). Increasing CO_2 generally lessened the degree of CA inhibition (increased CA) due to Cu exposure in this study; however, no significant differences between treatments were observed due to CO_2 alone.

Sea anemones live in a symbiotic relationship with dinoflagellate algae, which could influence the level of CO_2 in the holobiont (Weis, 1993). Zooxanthellae require CO_2 in higher quantity to maintain high rates of photosynthesis in their intracellular environment (Weis & Raynolds, 1999) and CA can facilitate this requirement by catalysing the

conversion of bicarbonate (HCO_3^-) to CO_2 (Weis et al., 1989). It has been suggested that efficient utilization of dissolved inorganic carbon under CO_2-limited conditions involves CA and biological adaptation to low CO_2 concentrations in the environment (Satoh et al., 2001). The average pH in the ocean is 8.2, where the majority of dissolved inorganic carbon (DIC) exists in the form of HCO_3^-. To complete photosynthesis external HCO_3^- must be converted into CO_2 (Bertucci et al., 2013). This may be the reason, in part, that the lower CO_2 treatments (400 ppm CO_2) in this study had comparatively lower CA activity, in the presence of Cu. Alternatively, the higher CO_2 treatments (1000 ppm CO_2) could have provided the necessary CO_2 even with minimal CA activity, which offset the CA inhibition caused by Cu alone (Main et al., 2012; Brock & Bielmyer, 2013; Patel & Bielmyer-Fraser, 2015; Siddiqui et al., 2015).

If acid-base compensation is not achieved by the organism, the pH in blood could be reduced, resulting in depressed metabolism (Hand, 1991; Pörtner & Reipschläger, 1996; Guppy & Withers, 1999) and disruption of both acid-base balance and respiratory gas exchange (Esbaugh et al., 2012). Some other physiological processes which may be affected by acidification include otolith development in seabass (*Atractoscion nobilis*) (Checkley et al., 2009), basal metabolic costs in Adult cardinalfish, *Ostorhinchus doederleini* and *O. cyanosoma* (Munday et al., 2009a), gamete maturation in the fat inkeeper worm, *Urechis caupo* (Holland et al., 1984), sperm activation in the polychaete *Arenicola marina* (Pacey et al., 1994), egg hatching in planktonic copepods (Kurihara et al., 2004), and olfactory behavioural responses in the orange clownfish, *Amphiprion percula* (Munday et al., 2009b).

In this study, physiological effects were observed in the symbiotic anemone, *E. pallida*, as a consequence of Cu and OA, both individually and in combination. Cu exposure

alone elicited more observed effects than did OA alone, but in combination, these stressors caused the most severe anti-oxidant enzyme responses measured in *E. pallida*. The Cu-induced inhibition of CA activity in *E. pallida*, however, was lessened by the increase in CO_2 level in some cases. Cu concentrations range from 1.1 to 50 µg/L in the polluted surface waters of marinas (Scarano et al., 1990; Hall et al., 1988; Sadiq, 1992; Eisler, 1998), 1 to 100 µg/L of Cu in storm water runoff (Georgopoulos et al. 2001), and 10 to 1800 µg/L in the water column of coral reef ecosystems (Peters et al., 1997). The Cu concentrations used, 10-100 µg/L, are within the range of those reported in more contaminated marine environments. OA is a growing concern and, as such, global CO_2 and ocean pH is being very well documented (Caldeira & Wickett, 2003). At the present rate of CO_2 increase, models predict that CO_2 will reach 1000 ppm by the end of this century. The results of this study demonstrate interactions between the two stressors and the findings have implications for aquatic environments with increasing metal pollution and a changing climate.

Table 2. Measured Dissolved Copper and pCO_2 Concentrations.

Treatment (µg/L)	pCO_2 (ppm)	$CuCl_2$ (µg/L)
Control	466.2 ± 33.8	0
	1198.1 ± 239.6	0
10	448.5 ± 67.2	9.3 ± 0.4
	1074.7 ± 241.5	9.5 ± 2.4
50	462.5 ± 79.2	46.8 ± 1.9
	1058.9 ± 221.1	47.9 ± 2.4
100	470 ± 65.2	94.6 ± 2.1
	1166.6 ± 228.5	95.9 ± 2.5

Note: All the concentrations are in Mean ± Standard Deviation over 7 days exposure period in Testing Solutions.

Table 3. Water Quality Parameters

Treatment (µg/L)	CO_2 (ppm)	Salinity (ppt)	Temp. (^oC)	DO (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)	Ammonia (mg/L)	pH
Control	400	30.0 ± 0.9	26.3 ± 0.5	7.8 ± 0.65	4.0 ± 1.3	0.1 ± 0.05	0.43 ± 0.06	8.17 ± 0.11
	1000	30.0 ± 0.6	26.3 ± 0.5	7.7 ± 1.05	4.0 ± 1.2	0.1 ± 0.03	0.41 ± 0.04	7.81 ± 0.14
10	400	30.0 ± 0.5	26.3 ± 0.5	7.5 ± 1.03	4.0 ± 0.9	0.1 ± 0.05	0.39 ± 0.06	8.15 ± 0.14
	1000	30.0 ± 0.3	26.3 ± 0.5	7.8 ± 0.45	4.0 ± 1.0	0.1 ± 0.02	0.42 ± 0.03	7.85 ± 0.14
50	400	30.0 ± 0.5	26.3 ± 0.5	7.7 ± 0.92	4.0 ± 1.1	0.1 ± 0.07	0.41 ± 0.05	8.09 ± 0.11
	1000	30.0 ± 0.4	26.3 ± 0.5	7.7 ± 0.84	4.0 ± 1.4	0.1 ± 0.04	0.38 ± 0.09	7.76 ± 0.18
100	400	30.0 ± 0.5	26.3 ± 0.5	7.8 ± 0.66	4.0 ± 0.9	0.1 ± 0.05	0.45 ± 0.08	8.06 ± 0.15
	1000	30.0 ± 0.5	26.3 ± 0.5	7.8 ± 0.64	4.0 ± 0.8	0.1 ± 0.03	0.39 ± 0.07	7.82 ± 0.07

Note: All the concentrations are in Mean ± Standard Deviation over 7 days exposure period at 400 and 1000 ppm pCO_2 in testing solutions.

Table 4. Measured Catalase (CAT); Glutathione Peroxidase (Gpx); Glutathione Reductase (GR) and Carbonic Anhydrase (CA) in the Control Groups (From all Three Experiments).

Measurement	CO_2 (ppm)	0 d	2 d	5 d	7 d
CAT (unit/mg Protein)	400	12.6 ± 0.44	10.18 ± 0.26	7.99 ± 0.36	9.14 ± 0.76
	1000	12.6 ± 0.44	10.73 ± 0.56	8.99 ± 1.05	10.34 ± 1.09
GPx (unit/mg Protein)	400	0.27 ± 0.02	0.24 ± 0.01	0.23 ± 0.03	0.29 ± 0.05 *
	1000	0.27 ± 0.02	0.28 ± 0.03	0.26 ± 0.01	0.22 ± 0.05 *
GR (unit/mg Protein)	400	0.25 ± 0.01	0.29 ± 0.01*	0.22 ± 0.02*	0.24 ± 0.03*
	1000	0.25 ± 0.01	0.35 ± 0.35*	0.41 ± 0.84*	0.41 ± 0.04*
CA (µmol H^+/min/mg Protein)	400	8.13 ± 1.33	6.31 ± 1	6.45 ± 0.53	5.63 ± 0.8
	1000	8.13 ± 1.33	6.11 ± 0.59	5.59 ± 0.52	5.23 ± 0.52

Note: CAT activity (unit/mg Protein); GPx (unit/mg Protein); GR (unit/mg Protein) and CA activity (µmol H^+/min/mg Protein) activities are reported as Mean ± Standard Error exposed to 400 and 1000 ppm pCO_2 Over 7 Days. * indicates a significant difference between the two pCO_2 treatments at a particular time point ($p < 0.05$; $n = 4$).

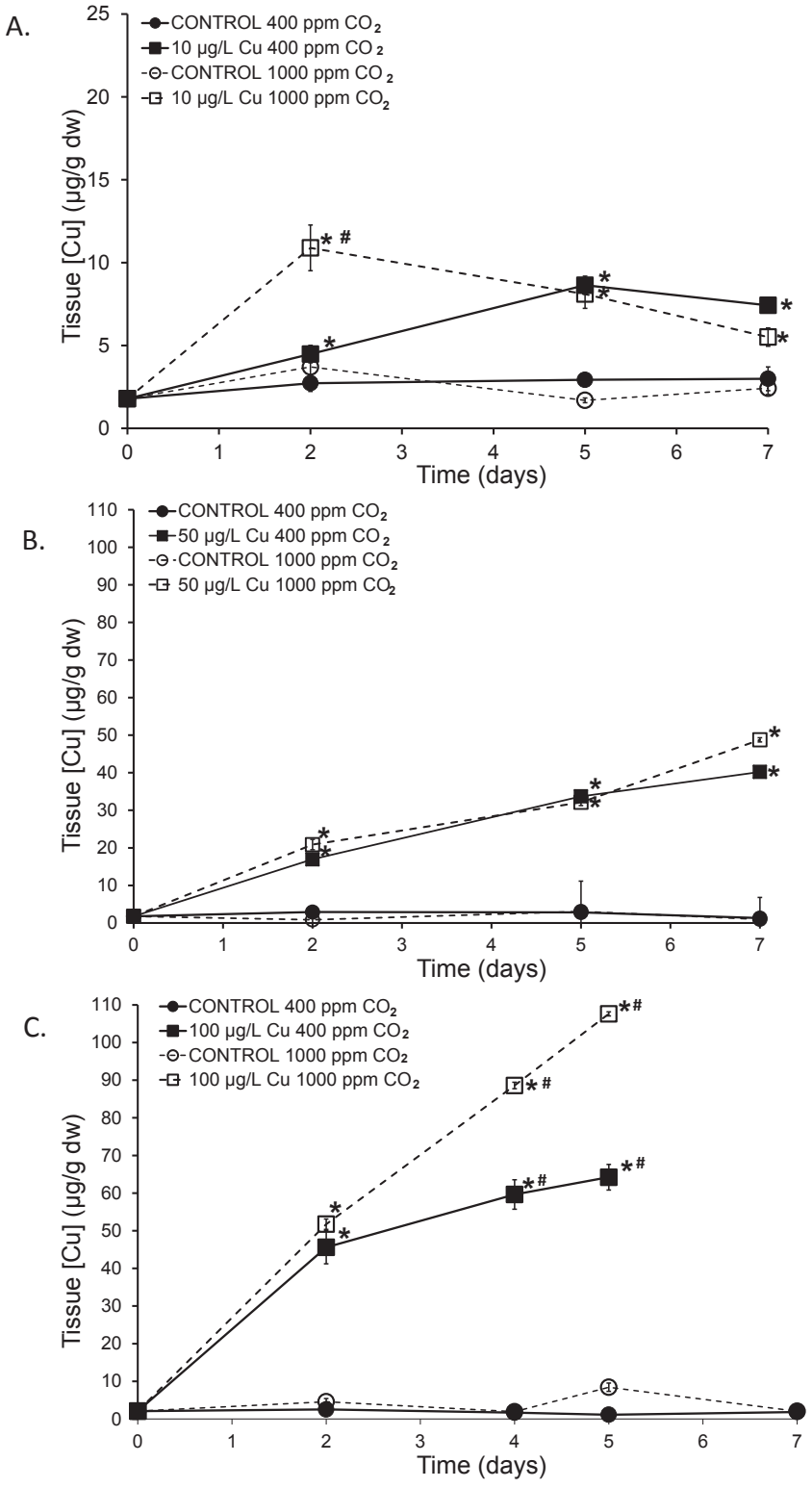


Figure 5. Tissue Copper Accumulation

Note: All the values are reported as Mean ± Standard Error with 400 ppm and 1000 ppm pCO_2 in *Exaiptasia pallida* After 7 Days Exposure to a Control, and A) 10, B) 50, And C) 100 µg/L Cu, As $Cucl_2$ at Two Different pCO_2 Values. * indicates a significant difference from the concurrent control at a particular pCO_2 level ($p < 0.05$; $n = 4$). # indicates a significant difference between pCO_2 level at a particular Cu concentration.

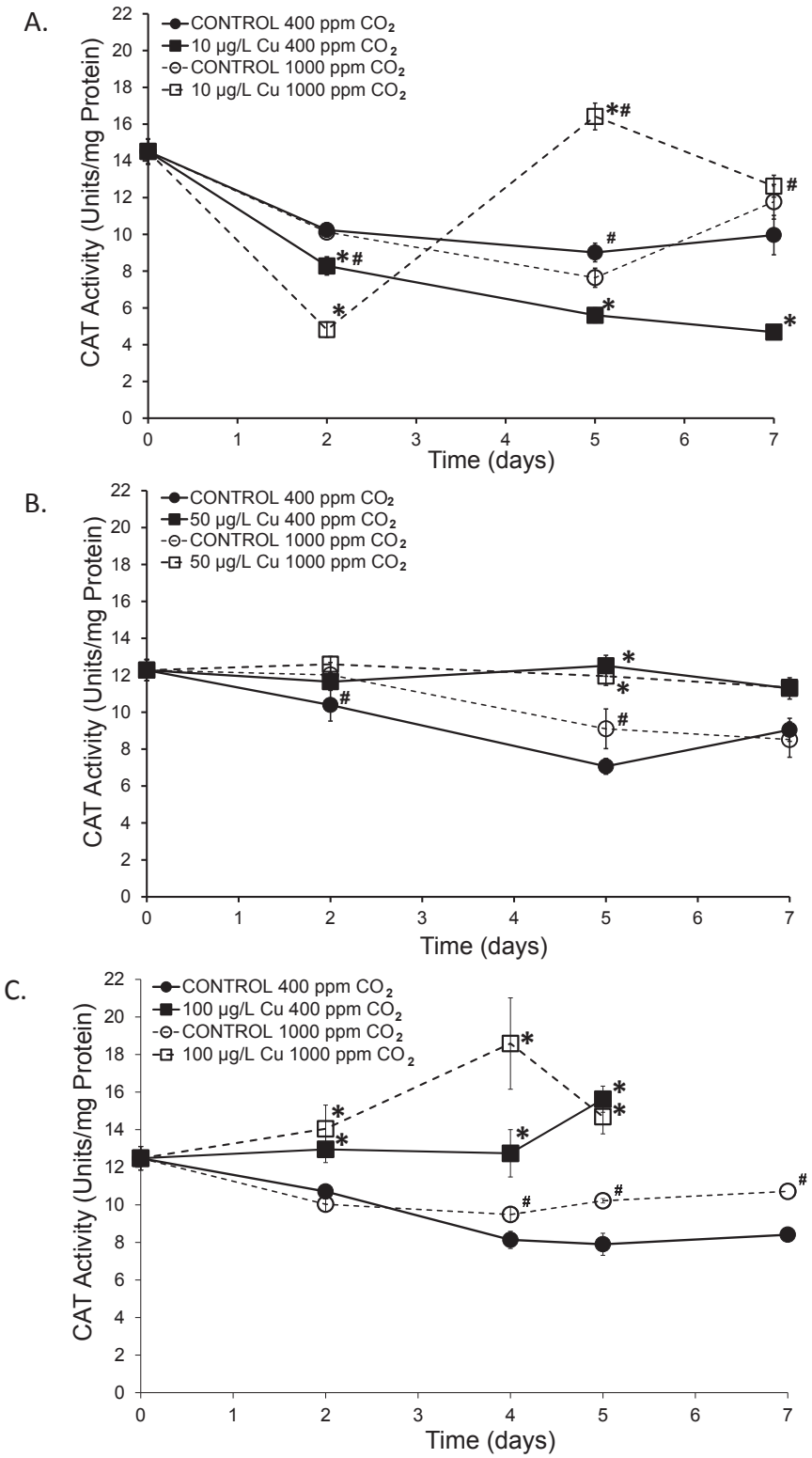


Figure 6. Catalase (CAT) Activity

Note: All the values are reported as Mean ± standard error in *Exaiptasia pallida* After 7 days exposure to a control, and A) 10, B) 50, and C) 100 µg/L Cu, as $CuCl_2$ at two different pCO_2 concentrations. 400 ppm and 1000 ppm pCO_2. * indicates a significant difference from the concurrent control at a particular pCO_2 level ($p < 0.05$; $n = 4$). # indicates a significant difference between pCO_2 level at a particular Cu concentration.

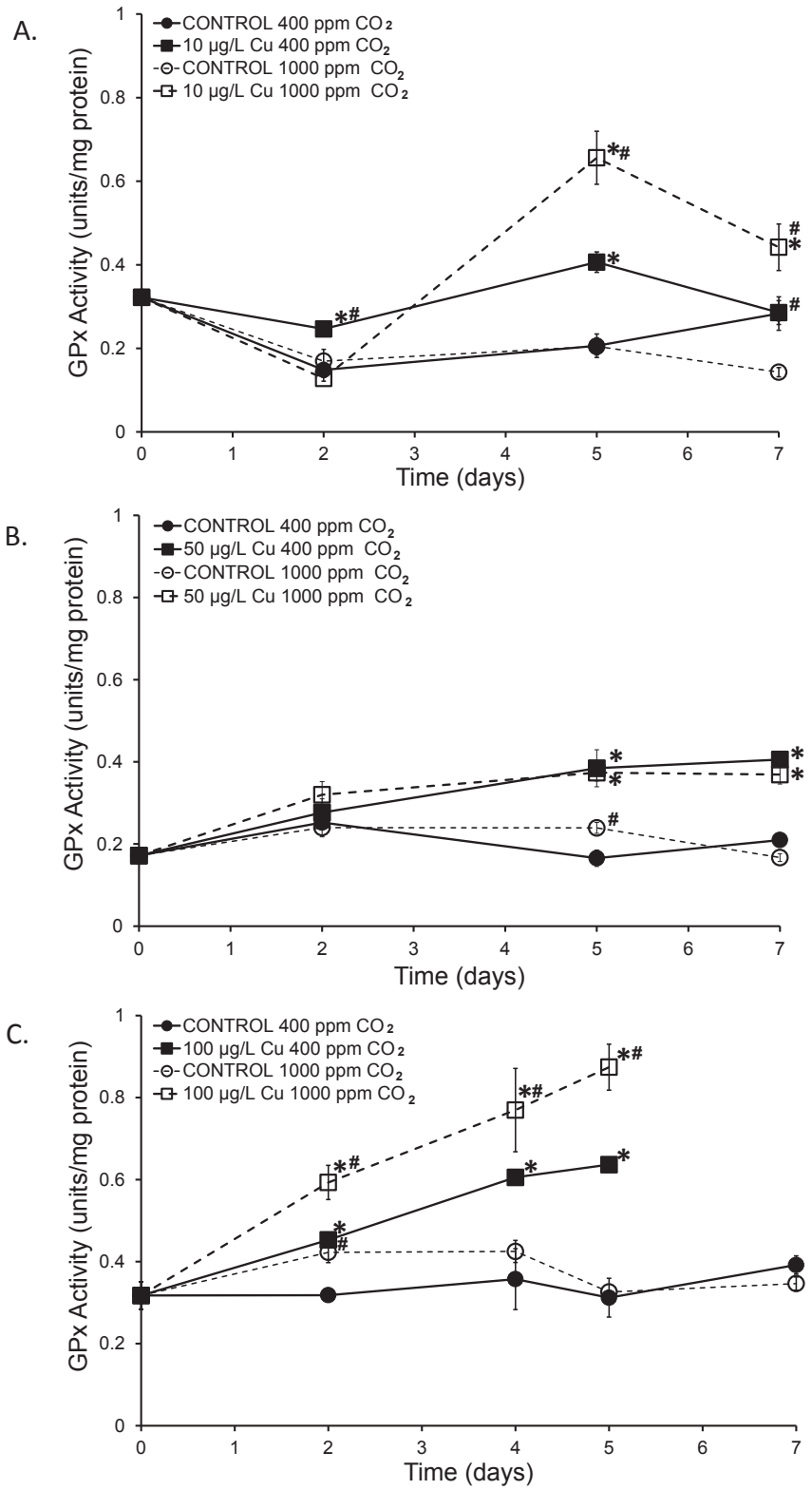


Figure 7. Glutathione Peroxidase (GPx) Activity.

Note: All the values are reported as Mean ± Standard Error in *Exaiptasia pallida* after 7 days exposure to a control, and A) 10, B) 50, and C) 100 µg/L Cu, as $CuCl_2$ at two different pCO_2 Concentrations 400 ppm and 1000 ppm pCO_2. * indicates a significant difference from the concurrent control at a particular pCO_2 level ($p < 0.05$; $n = 4$). # indicates a significant difference between pCO_2 level at a particular Cu concentration.

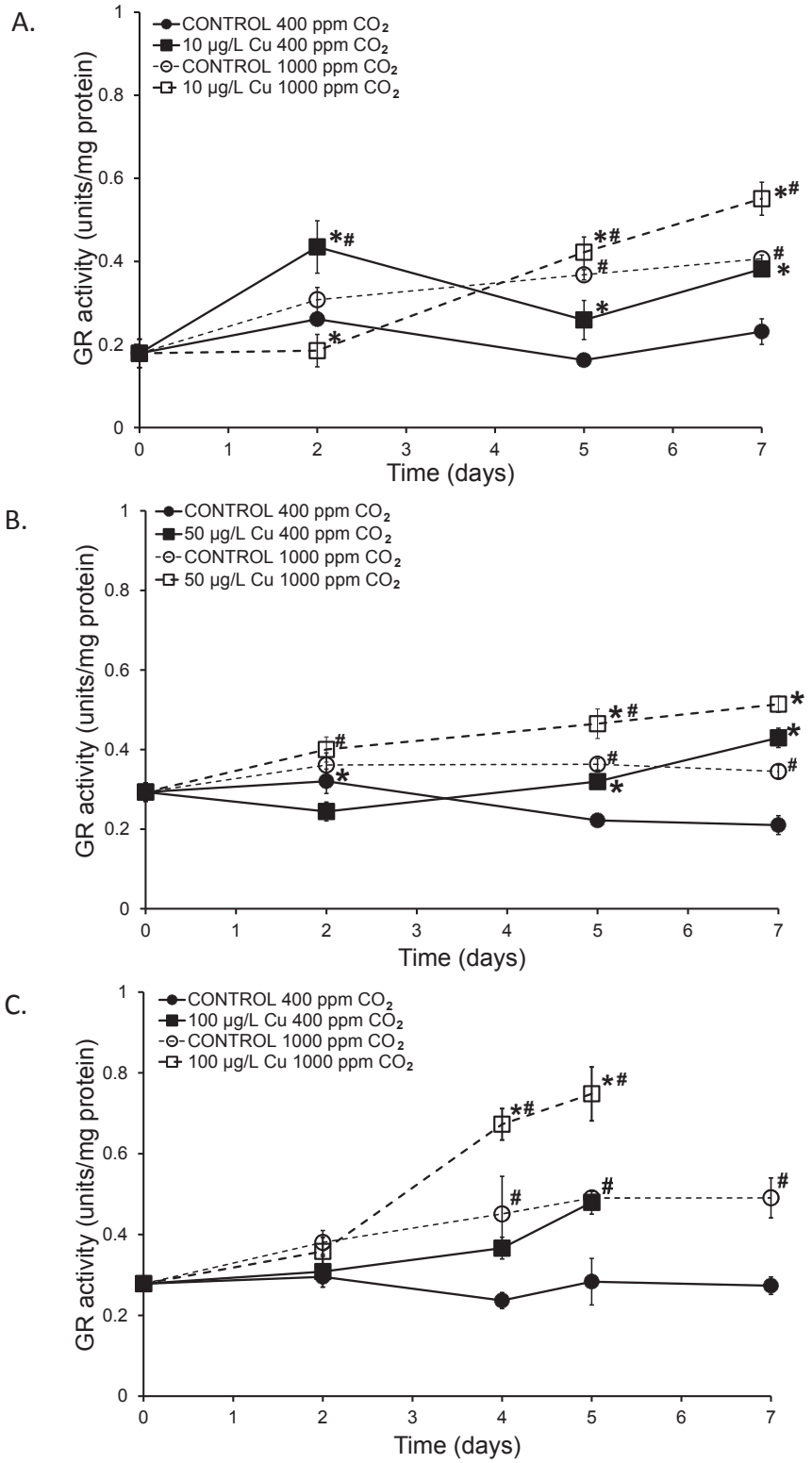


Figure 8. Glutathione Reductase (GR) Activity

Note: All the values are reported as mean ± standard error in *Exaiptasia pallida* after 7 says Exposure to a control, and A) 10, B) 50, and C) 100 µg/L Cu, as $CuCl_2$ at two different pCO_2 concentrations 400 ppm and 1000 ppm pCO_2. * indicates a significant difference from the concurrent control at a particular pCO_2 level ($p < 0.05$; $n = 4$). # indicates a significant difference between pCO_2 level at a particular Cu concentration.

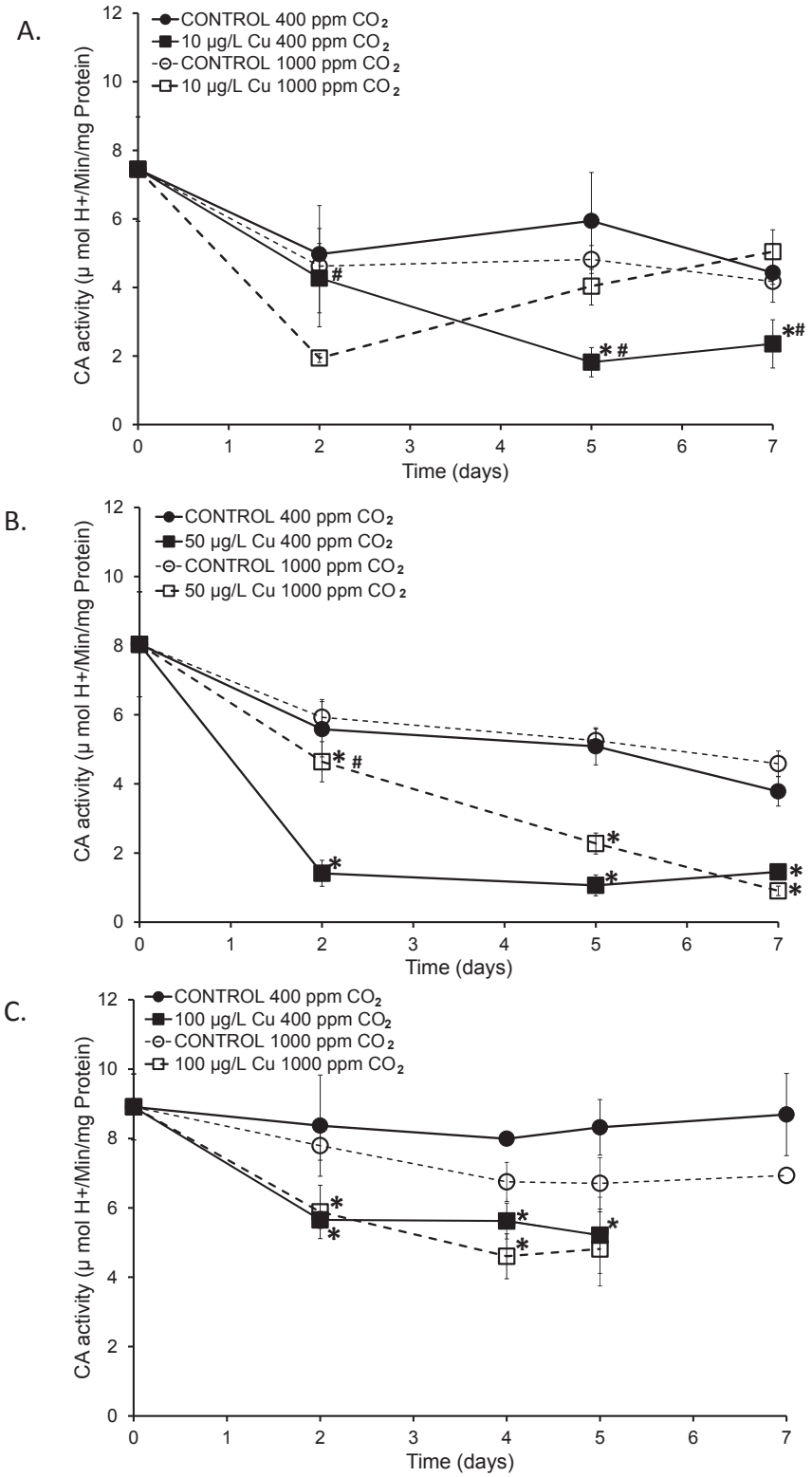


Figure 9. Carbonic Anhydrase (CA) Activity

Note: All the values are reported as mean ± standard error in *Exaiptasia pallida* after 7 days exposure to a control, and A) 10, B) 50, and C) 100 µg/L Cu, as $CuCl_2$ at two different pCO_2 concentrations (400 ppm and 1000 ppm pCO_2). * indicates a significant difference from the concurrent control at a particular pCO_2 level ($p < 0.05$; $n = 4$). # indicates a significant difference between pCO_2 level at a particular Cu concentration.

Chapter III

CONCLUSION AND OVERALL IMPLICATION

Metals are frequently used in various sectors of modern society, and as such, metal pollution is a common problem in many marine environments. The results from these studies demonstrated differences in metal accumulation and toxicity in marine organisms, dependent on the form of metal (dissolved versus nanoparticulate) exposed and dependent on other external variables, such as the global issue of increasing CO_2. Data on NP toxicity in marine organisms is scarce; therefore, new insight into mechanisms of NP toxicity to sea anemones should prove useful to both scientists and government regulators. Additionally, ocean acidification, caused by increasing CO_2, has been shown to substantially affect calcifying marine organisms; however, fewer studies have focussed on the effects of increasing CO_2 on non-calcifying organisms. This research provides new insight into the physiological effects of increasing CO_2 alone, and in combination with metal pollution, on sea anemones. Enzyme responses were detected which could be used as bioindicators for these stressors in the environment.

REFERENCES

Adamu, H., Luter, L., Lawan, M. M., Umar, B. A. 2013. Chemical Speciation: A Strategic Pathway for Insightful Risk Assessment and Decision Making for Remediation of Toxic Metal Contamination. *Environment and Pollution* 2, ISSN 1927-0909.

Agegian, C. R. 1985. The biogeochemical ecology of *porolithon gardineri* (Foslie), 178 PP. PhD thesis, university of Hawaii, Honolulu, HI.

Albers, P. H., Camardese, M. B. 1993. Effects of acidification on metal accumulation by aquatic plants and invertebrates. 1. Constructed wetlands. *Environ. Toxicol. Chem*. 12 (6), 959-967.

Anjos, V. A., da Silva-Junior, F. M. R., Souza, M. M. 2014. Cell damage induced by copper: An explant model to study anemone cells. *Toxicol. In Vitro* 28, 365-372.

Aruoja, V., Dubourguier, H-C., Kasemets, K., Kahru, A. 2009. Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*. *Sci. Total Environ*. 407, 1461-1468.

Asada, K. 1992. Ascorbate peroxidase - a hydrogen peroxide-scavenging enzyme in Plants. *Physiol. Plantarum* 85, 235–241.

Atkinson, C. A., Jolley, D. F., Simpson, S. L. 2007. Effect of overlying water pH, dissolved oxygen, salinity and sediment disturbances on metal release and sequestration from metal contaminated marine sediments. *Chemosphere* 69 (9), 1428-1437.

Badger, M. R., Price, G. D. 1994. The role of carbonic anhydrase in photosynthesis. *Annu. Rev. Plant Phys*. 45, 369–392.

Baker, T. J., Tyler, C. R., Galloway, T. S. 2014. Impacts of metal and metal oxide nanoparticles on marine organisms. *Environ. Pollut.* 186, 257-271.

Bergman, N. M., Lenton, T. M., Watson A. J. 2004. COPSE: a new model of biogeochemical cycling over Phanerozoic time. *Am. J. Sci.* 304, 397-437.

Berner, R. A. 2006. GEOCARBSULF: a combined model for Phanerozoic atmospheric O_2 and CO_2. Geoch. *Cosmochin. Ac*. 70, 5653-64.

Berner, R. A., Kothavala, Z. 2001. GEOCARB III: a revised model of atmospheric CO_2 over Phanerozoic time. *Am. J. Sci.* 301, 182-204.

Bertucci, A., Moya, A., Tambutte, S., Allemand, D., Supuran, C. T., Zoccola, D. 2013. Carbonic anhydrases in anthozoan corals—A review. *Bioorgan. Med. Chem*. 21, 1437-1450.

Bielmyer, G. K., Arnold, R., Tomasso, J., Klaine, S. J. 2004. Influence of salt source on synthetic saltwater quality. *Chemosphere* 57, 1707-1711.

Bielmyer, G. K., Grosell, M., Bhagooli, R., Baker, A. C., Langdon, C., Gillette, P., Capo, T. R. 2010. Differential effects of copper on three species of scleractinian corals and their algal symbionts (*symbiodinium* spp.). *Aquat. Toxicol*. 97, 125-133.

Bielmyer, G. K., Jarvis, T., Harper, B. T., Butler, B., Rice, L., Ryan, S. 2012. Metal Accumulation from Dietary Exposure in the Sea Urchin, *Strongylocentrotus droebachiensis*. *Arch. Environ. Contamin. Toxicol*. 63, 86–94.

Bradford, M. M. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem*. 72, 248–254.

Brock, J. R., Bielmyer, G. K. 2013. Metal accumulation and sublethal responses in the sea anemone, *Aiptasia pallida* after waterborne exposure to metal mixtures. *Comp. Biochem. Physiol*. C 158, 150-158.

Brown, B. E., Howard, E. S. 1985. Responses of coelenterates to trace metals: A field and laboratory evaluation. Proceeding of the fifth international coral reef congress, Tahiti. 6, 465 -470.

Buffet, P-E., Tankoua, O. F., Pan, J-F., Berhanu, D., Herrenknecht, C., Poirier, L., Amiard-Triquet, C., Berard, J-B., Risso, C., Guibbolini, M., Romeo, M., Reip, P., Valsami-Jones, E., Mouneyrac, C. 2011. Behavioural and biochemical responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles. *Chemosphere* 84, 166-174.

Buffet, P-E., Tankoua, O. F., Pan, J-F., Berhanu, D., Herrenknecht, C., Poirier, L., Amiard-Triquet, C., Berard, J-B., Risso, C., Guibbolini, M., Romeo, M., Reip, P., Valsami-Jones, E., Mouneyrac, C. 2011. Behavioural and biochemical responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles. *Chemosphere* 84, 166-174.

Cady, N. C., Behnke, J. L., Strickland, A. D. 2011. Copper-based nanostructured coatings on natural cellulose: nanocomposites exhibiting rapid and efficient inhibition of a multidrug resistant wound pathogen, *A. baumannii*, and mammalian cell biocompatibility in vitro. *Adv. Funct. Mater.* 21, 2506–2514.

Caldeira, K., Wickett, M. 2003 Anthropogenic carbon and ocean pH. *Nature* 425, 365.

Caldeira, K., Wickett, M. E. 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *J. Geophys. Res*. 110, C09S04.

Campbell, A. L., Mangan, S., Ellis, R. P., Lewis, C. 2014. Ocean acidification increases copper toxicity to the early life history stages of the polychaete *arenicola marina* in artificial seawater. *Environ. Sci. Technol*. 48, 9745−9753.

Canadell, J. G., Le Quere, C., Raupach, M. R., Field, C. B., Buitenhuis, E. T., Ciais, P., Conway, T. J., Gillett, N. P., Houhton, R. A., Marland, G. 2007. Contribution to accelerating atmospheric CO_2 growth from economic activity, carbon intensity, and efficiency of natural sinks. *PNAS USA*, 104, 18866-70.

Chang, L. W., Magos, L., Suzuki, T. (Eds)., 1996. *Toxicology of Metals*. Lewis/CRC Publishers, Boca Raton; pp 1-1198.

Chatterjee, A. K., Sarkar, R. K., Chattopadhyay, A. P., Aich, P., Chakraborty, R., Basu, T. 2012. A simple robust method for synthesis of metallic copper nanoparticles of high antibacterial potency against *E. coli*. *Nanotechnol.* 23, 85–103.

Checkley, D. M., Dickson, A. G., Takahashi, M., Radich, J. A., Eisenkolb, N., Asch, R. 2009. Elevated CO_2 enhances otolith growth in young fish. *Science* 324, 1683.

Chelikani, P., Fita, I., Loewen, P. C. 2004. Diversity of structures and properties among catalases. *Cell. Mol. Life Sci*. 61 (2), 192–208.

Cheon, J., Lee, J., Kim, J. 2012. Inkjet printing using copper nanoparticles synthesized by electrolysis. *Thin Solid Films* 520, 2639–2643.

Dlugokencky, E., Tans P. 2014. NOAA/ESRL (www.esrl.noaa.gov/gmd/ccgg/trends/) last access: January 2015.

Downs, C. A., Fauth, J. E., Downs, V. D., Ostrander, G. K. 2010. In vitro cell-toxicity screening as an alternative animal model for coral toxicology; effects of heat stress, sulphide, rotenone, cyanide and cuprous oxide on cell viability and mitochondrial function. *Ecotoxicology* 19, 171-184.

Eisler, R. 1998. Copper hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Geological Survey, Biological Resources Division, Biological Science Report USGS/BRD/BSR--1998-0002.

Esbaugh, A. J., Heuer, R. M., Grosell, M. 2012. Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*. *J. Comp. Physiol. B.* 182, 921-934.

Falkowski, P., Dubinsky, Z., Muscatine, L., Porter, J. 1984. Light and the bioenergetics of a symbiotic coral. *Bio Science* 34, 705–709.

Forman, H. J., Liu, R., Shi, M. M. 1990. Glutathione synthesis in oxidative stress. In: Packer L. Fuchs. J. (Eds.). Biothiols in Health and Diseases Marcel Dekker, NY. pp. 189-211

Gattuso, J-P., Allemand, D., Frankignoulle, M. 1999. Photosynthesis and calcification at cellular, organismal and community levels in coral reefs; a review on interactions and control by bicarbonate chemistry. *Am. Zoo*. 39, 160-83.

Georgopoulos, P. G., Roy, A., Yonone-Lioy, M. J., Opiekun, R. E. 2001. Environmental copper: Its dynamic and human exposure issues. *J Toxicol. Environ. Health B* 4 (4), 341-394.

Giammar, D. E., Maus, C. J., Xie, L. Y. 2007. Effects of particle size and crystalline phase on lead adsorption to titanium dioxide nanoparticles. *Environ. Eng. Sci.* 24, 85–95.

Gilbert, A. L., Guzmán, H. M. 2001. Bioindication potential of carbonic anhydrase activity in anemones and corals. *Mar. Pollut. Bull*. 42, 742-744.

Gilmour, K. M. 2010. Perspectives on carbonic anhydrase. *Comp. Biochem. Physiol. A* 157, 193-197.

Gomes, T., Araujo, O., Pereira, R., Almeida, A. C., Cravo, A., Bebianno, M. J. 2013. Genotoxicity of copper oxide and silver nanoparticles in the mussel *Mytilus galloprovincialis*. *Mar. Environ. Res*. 84, 51-59.

Gomes, T., Pinheiro, J. P., Cancio, I., Pereira, C. G., Cardosos, C., Bebianno, M. J. 2011. Effects of copper nanoparticles exposure in the mussel, *Mytillus galloprovincialis*. *Environ. Sci. Technol*. 45, 9356 – 9362.

Gomes, T., Pinheiro, J. P., Cancio, I., Pereira, C. G., Cardosos, C., Bebianno, M. J. 2012. Accumulation and toxicity of copper oxide nanoparticles in the digestive gland of *Mytilus galloprovincialis*. *Aquat. Toxicol*. 118–119, 72–79.

Götze, S., Matoo, O. B., Beniash, E., Saborowski, R., Sokolova, I. M. 2014. Interactive effects of CO_2 and trace metals on the proteasome activity and cellular stress response of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. *Aquat. Toxicol*. 149, 65-82.

Graney, R. L., Cherry, D. S., Cairns, J. 1984. The influence of substrate, pH, diet and temperature upon cadmium accumulation in the asiatic clam (*Corbicula fluminea*) in laboratory artificial streams. *Water Res.* 18 (7), 833–842.

Grant, A. J., Graham, K., Frankland, S., Hinde, R. 2003. Effect of copper on algal-host interactions in the symbiotic coral *Plesiastrea versipora*. *Plant Physiol. Biochem*. 41, 383–390.

Grass, G., Rensing, C., Solioz, M. 2011. Metallic copper as an antimicrobial surface. *Appl. Environ. Microbiol.* 77, 1541–1547.

Griffitt, R. J., Hyndman, K., Denslow, N. D., Barber, D. S. 2009. Comparison to molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. *Toxicol. Sci*. 107, 404-415.

Griffitt, R. J., Weil, R., Hyndman, K. A., Denslow, N. D., Powers, K., Taylor, D., Barber, D. S. 2007. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). *Environ. Sci. Technol*. 41, 8178-8186.

Guppy, M., Withers, P. 1999. Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biological Reviews* 74, 1–40.

Guttuso, J-P., Hansson, L. 2011. *Ocean acidification*. ISBN: 978-0-19-959108-4 Oxford University Press Inc. New York.

Guzmán, H. M., Jiménez, C. E. 1992. Contamination of coral reefs by heavy metals along the Caribbean coast of Central America. *Mar. Pollut. Bull*. 24 (11), 554–561.

Hall, W. S., Bushong S. J., Hall, L. W. Jr., Lenkevich M. S., Pinkney A. E. 1988. Monitoring dissolved copper concentrations in Chesapeake Bay, U.S.A. *Environ. Monitor. Assess*. 11, 33-42.

Hand, S. C. 1991. Metabolic dormancy in aquatic invertebrates. In *Advances in Comparative and Environmental Physiology*, 8, 1–50. Ed. by R. Gilles. Springer-Verlag, Heidelberg.

Harland, A. D., Ngarno, N. R. 1990. Copper uptake by the sea anemone *Anemonia veridis* and the role of Zooxanthellae in metal regulation. *Mar. Biol*. 104, 297-301.

Henry, R. P. 1991. Techniques for measuring carbonic anhydrase activity in vitro: the electrometric delta pH and pH stat methods. In: Dodgson, S. J., Tashien, R. E., Gros, G., Carter, N. D. (Eds.), *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*. Plenum Press, NY, pp. 119–125.

Henry, R. P. 1996. Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annu. Rev. Physiol*. 58, 523–538.

Heuer, R., Esbaugh, A., Grosell, M. 2012. Ocean acidification leads to counterproductive intestinal base loss in the gulf toadfish (*Opsanus beta*). *Physiol. Biochem. Zool.* 85(5), 450-459.

Holland, L., Gould-Somero, M., Paul, M. 1984. Fertilization acid release in *Urechis* eggs. I. The nature of the acid and the dependence of acid release and egg activation on external pH. *Develop. Biol*. 103, 337–342.

Hristovski, K., Baumgardner, A., Westerhoff, P. 2007. Selecting metal oxide nanomaterials for arsenic removal in fixed bed columns: from nanopowders to aggregated nanoparticle media. *J. Hazard. Mater*. 147, 265-274.

Hu, W., Culloty, S., Darmody, G., Lynch, S., Davenport, J., Ramirez-Garcia, S., Dawson, K. A., Lynch, I., Blasco, J., Sheehan, D. 2014. Toxicity of copper oxide nanoparticles in the blue mussel, *Mytilus edulis*: A redox proteomic investigation. *Chemosphere* 106, 289-299.

IPCC, 2001: Climate Change 2001: The Scientific Basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change [Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., van der Linden, P. J., Dai, X., Maskell, K., and Johnson, C. A. (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 881pp.

Jones, R. J. 1997. Zooxanthellae loss as a bioassay for assessing stress in corals. *Mar. Ecol. Prog. Ser*. 149, 163–171.

Jong, W. H. D., Borm, P. J. A. 2008. Drug delivery and nanoparticles: applications and hazards. *Int. J. Nanomedicine* 3, 133-149.

Kaplan, E. H. 1988. *A Field Guide to Southeastern and Caribbean Seashores*. Peterson Field Guides. Houghton Mifflin Company, Boston, MA 213.

Kelly, K. L., Coronado, E., Zhao, L. L., Schatz, G. C. 2003. The Optical Properties of Metal Nanoparticles: The Influence of Size, Shape, and Dielectric Environment. *J. Phys. Chem. B* 107, 668–677.

Klaassen, C. D. 1996. *Casarett and Doull's Toxicology: The Basic Science of Poisons*. McGraw Hill, NY, NY. 41.

Kormann, C., Bahnemann, D. W., Hoffmann, M. R. 1991. Photolysis of chloroform and other organic-molecules in aqueous TiO_2 suspensions. *Environ. Sci. Technol.* 25, 494–500.

Krumschnabel, G., Manzl, C., Berger, C., Hofer, B. 2005. Oxidative stress, mitochondrial permeability transition, and cell death in Cu exposed trout hepatocytes. *Toxicol. Appl. Pharmacol*. 209 (1), 62-73.

Kuffner, I. B., Andersson, A. J., Jokiel, P. L., Rodgers, K. S., Mackenzie, F. T. 2008. Decreased abundance of crustose coralline algae due to ocean acidification. *Nat. Geosci*. 1, 77–140.

Kurihara, H., Shimode, S., Shirayama, Y. 2004. Sublethal effects of elevated concentrations of CO_2 on planktonic copepods and sea urchins. *J. Oceanography* 60, 743–750.

Langdon, C., Takahashi, T., Sweeney, C., Chipman, D., Goddard, J., Marubini, F., Aceves, H., Barnett, H., Atkinson, M. J. 2000. Effect of calcium carbonate saturation state on the calcification rate of an experimental coral reef. *Global Biogeochem Cy*. 14(2), 639–654.

Larsen, B. K., Portner, H. O., Jensen, F. B. 1997. Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Mar. Biol.* 128, 337-346.

Lauren, D. J., Mc Donald, D. G. 2011. Influence of Water Hardness, pH, and Alkalinity on the Mechanisms of Copper Toxicity in Juvenile Rainbow Trout, *Salmo gairdneri*. *Canadian J.* Fish. *Aquat. Sci*. 43 (8), 1488-1496.

Le Quesne, W. J. F., Pinnegar, J. K. 2012. The potential impacts of ocean acidification: scaling from physiology to fisheries. *Fish and Fisheries* 13, 333-334.

Leclercq, N., Guttuso, J. P., Jaubert, J. 2000. CO_2 partial pressure controls the calcification rate of coral community. *Glob. Change Biol*. 6, 329-34.

Limbach, L. K., Li, Y., Grass, R. N., Brunner, T. J., Hintermann, M. A., Muller, M., Gunther, D., Stark, W. J. 2005. Oxide nanoparticle uptake in human lung fibroblasts: effects of particle size, agglomeration, and diffusion at low concentrations. *Environ. Sci. Technol*. 39, 9370–9376.

Livingstone, D. R., Lips, F., Garcia, M. P., Pipe, R. K., 1992. Antioxidant enzymes in digestive gland of the common mussel *Mytilus edulis. Mar. Biol*. 112, 265–276.

Longano, D., Ditaranto, N., Cioffi, N., Di Niso, F., Sibillano, T., Ancona, A., Conte, A., Del Nobile, M. A., Sabbatini, L., Torsi, L. 2012. Analytical characterization of laser-generated copper nanoparticles for antibacterial composite food packaging. *Anal. Bioanal. Chem*. 403, 1179–1186.

Luoma, S. N., Rainbow, P. S. 2008. *Metal Contamination in Aquatic Environments: Science and Lateral Management*. pp. xix + 573. ISBN 978-0-521-86057-4.

Luschak, V. I. 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat. Toxicol*. 101, 13-30.

Lynch, I., Dawson, K. A., Linse, S. 2006. Detecting cryptic epitopes created by nanoparticles. *Sci. STKE*. 327, pe14.

Main, W. P. L, Ross, C., Bielmyer, G. K., 2010. Copper accumulation and oxidative stress in the sea anemone, *Aiptasia pallida*, after waterborne copper exposure. *Comp. Biochem. Physiol. C* 151, 216-221.

Martin , J-M., Windom, H. L. 1991 “Present and future roles of ocean margins in regulating marine biogeochemical cycles of trace elements”. pp. 45-67. In R.F.C. Mantoura, J-M. Martin and R. Wollast (eds.). Ocean Margin Processes in Global Change. Dahlem Workshop. John Wiley & Sons, Chichester, U.K. 469 pp.

Matheickal, J. T., Yu, Q. 1999. Biosorption of lead(II) and copper(II) from aqueous solutions by pre-treated biomass of Australian marine algae. *Bioresource Technol*. 69 (3), 223-229.

Meister, A., Anderson, M. E. 1983. Glutathione. *Annu. Rev. Biochem*. 52, 711-760.

Melzner, F., Gutowska, M. A., Langenbuch, M. 2009. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* 6, 2313-2331.

Millero, F. N. J., Woosely, R., DiTrolio, B., Waters, J. 2009. Effects of ocean acidification on the speciation of metals in seawater. *Oceanography* 22, 72-85.

Mitchelmore, C. L., Ringwood, A. H., Weis, V. M. 2003b. Differential accumulation of cadmium and changes in glutathione levels as a function of symbiotic state in the sea anemone *Anthopleura elegantissima*. *Exp. Mar. Biol. Ecol*. 284, 71-85.

Mitchelmore, C. L., Verde, E. A., Ringwood, A. H., Weis, V. M. 2003a. Differential accumulation of heavy metals in the sea anemone *Anthopleura elegantissima* as a function of symbiotic state. *Aquat. Toxicol*. 64, 317–329.

Mitsudome, T., Mikami, Y., Ebata, K., Mizugaki, T., Jitsukawa, K., Kaneda, K. 2008. Copper nanoparticles on hydrotalcite as a heterogeneous catalyst for oxidant-free dehydrogenation of alcohols. *Chem. Commun*. 39, 4804–4806.

Moore, M. N. 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment. *Environ. Int*. 32, 967–976.

Morgan, M. B., Edge, S. E., Snell, T. W. 2005. Profiling differential gene expression of corals along a transect of waters adjacent to the Bermuda municipal dump. *Mar. Pollut. Bull*. 51, 524–533.

Moroney, J. V., Bartlett, S. G., Samuelsson, G. 2001. Carbonic anhydrases in plants and algae. *Plant Cell Environ*. 24, 141-153.

Mosleh, Y. Y., Paris-Palacios, S., Biagianti-Risbourg, S. 2006. Metallothioneins induction and antioxidative response in aquatic worms *Tubifex tubifex* (Oligocheta, Tubificidae) exposed to copper. *Chemosphere* 64, 121-128.

Munday, P. L., Crawley, N. E., Nilsson, G. E. 2009a. Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes. *Marine Ecology Progress Series* 388, 235–242.

Munday, P. L., Dixson, D. L., Donelson, J. M. Jones, G. P., Pratchett, M. S., Devitsina, G V., Døving, K. B. 2009b. Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *P NATL Acad Sci USA* 106, 1848–1852.

Negri, A. P., Hoogenboom, M. O. 2011. Water contamination reduces the tolerance of coral larvae to thermal stress. *PLoS One* 6, e19703.

Nel, A., Xia, T., Mädler, L., Li, N. 2006. Toxic potential of materials at the nanolevel. *Science* 311, 622–627

Noctor, G., Foyer, C. H. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Phys*. 49, 249–279.

Nowack, B., Bucheli, D. T. 2007. Occurrence, behaviour and effects of nanoparticles in the environment. *Environ. Pollut*. 150, 5-22.

Oberdörster, G., Oberdörster, E., Oberdörster, J. 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ. Health Perspect*. 113, 823–839.

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R. M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R. G., Plattner, G. K., Rodgers, K. B., Sabine, C. L., Sarmiento, J. L., Schlitzer, R., Slater, R. D., Totterdell, I. J., Weirig, M. F., Yamanaka, Y., Yool, A. 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* 437, 681-6.

Pacey, A. A., Cosson, J. C., Bentley, M. G. 1994. The acquisition of forward motility in the spermatozoa of the polychaete *Arenicola marina*. *J. Exp. Biol.* 195, 259–280.

Pascal, P-Y., Fledger, J. W., Galvez, F., Carman, K. R. 2010. The Toxicological interaction between ocean acidity and metal in coastal meiobenthic copeposs. *Mar. Pollut. Bull*. 60, 2201-2208.

Patel, P. P., Bielmyer-Fraser, G. K. 2015. The influence of salinity and copper exposure on copper accumulation and physiological impairment in the sea anemone, *Exaiptasia pallida*. *Comp. Biochem. Physiol. C* 168, 39–47.

Peters, E. C., Gassmann, N. J., Firman, J. C., Richmond, R. H., Power, E. A. 1997. Ecotoxicology of tropical marine ecosystems. *Environ. Toxicol. Chem*. 16, 12–40.

Petit, J. R., Jouzel, J., Raynaud, D., Barkov, N. I., Barnola, J-M., Basile, I., Bender, M., Chappellaz, J., Davis, M., Delaygue, G., Delmotte, M., Kotlyakov, V. M., Legrand, M., Lipenkov, V. Y., Lorius, C., Pepin, L., Ritz, C., Salzman, E., Stievenard, M. 1999. Climate and atmospheric history of the past 420,000 years from Vostoc ice core, Antarctica. *Nature* 399, 429-36.

Piontek, J., Lunau, M., Händel, N., Borchard, C., Wurst, M., Angel, A. 2010. Acidification increases microbial polysaccharide degradation in the ocean. *Biogeosciences* 7, 1615-1624. doi:10.5194/bg-7-1615-2010

Pörtner, H. O., Reipschläger, A. 1996. Ocean disposal of anthropogenic CO_2: physiological effects on tolerant and intolerant animals. In *Ocean Storage of Carbon Dioxide. Workshop 2— Environmental Impact*, 57 –81. Ed. by B. Ormerod, and M. V. Angel. IEA Greenhouse Gas R&D Programme, Cheltenham, UK.

Raffi, M., Mehrwan, S., Bhatti, T. M., Akhter, J. I., Hameed, A., Yawar, W., Hasan, M. M. 2010. Investigations into the antibacterial behavior of copper nanoparticles against *Escherichia coli*. *Ann. Microbiol*. 60, 75–80.

Regoli, F., Gorbi, S., Frenzilli, G., Nigro, M., Corsi, I., Focardi, S., Winston, G. W. 2002. Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. *Mar. Environ. Res*. 54, 419–423.

Richards, R., Chaoupka, M., Sano, M., Tomlinson, R. 2011. Modelling the effects of ''coastal'' acidification on copper speciation. *Ecol. Model*. 222, 3559-3567.

Richier, S., Furla, P., Plantivaux, A., Merle, P., Allemand, D. 2005. Symbiosis-induced adaptation to oxidative stress. *J. Exp. Biol.* 208, 277-285.

Roberts, D. A., Birchenough, S. N. R., Lewis, C., Sanders M. B., Bolam, T., Sheahan, D. 2013. Ocean acidification increases the toxicity of contaminated sediments. *Glob. Change Biol*. 19, 340-351.

Roesijadi, G., Robinson, W. E. 1994. Metal regulation in aquatic animals: mechanisms of uptake, accumulation and release. In: Malins DC, Ostrander GK, editors. *Aquatic Toxicology. Molecular, Biochemical and Cellular Perspectives*. Boca Raton, FL: Lewis Publishers, 387–420.

Rothen-Rutishauser, B. M., Schürch, S., Haenni, B., Kapp, N., Gehr, P. 2006. Interaction of fine particles and nanoparticles with red blood cells visualized with advanced microscope techniques. *Environ. Sci. Technol*. 40, 4353–4359.

Ruppert, E., Fox, R. 1988. *Seashore Animals of the Southeast: A Guide to Common Shallow-Water Invertebrates of the Southeastern Atlantic Coast*. University of SC Press. Columbia, SC. 429

Sadiq, M. 1992. *Toxic Metal Chemistry in Marine Environments*. Marcel Dekker, New York, p. 390.

Santo, C. E., Quaranta, D., Grass, G. 2012. Antimicrobial metallic copper surfaces kill *Staphylococcus haemolyticus* via membrane damage. *Microbiologyopen* 1, 46–52.

Satoh, A., Kurano, N., Miyachi, S. 2001. Inhibition of photosynthesis by intracellular carbonic anhydrase in microalgae under excess concentrations of CO_2. *Photosynth. Res.* 68, 215-224.

Scarano, G., Morelli, E., Seritti, A., Zirino, A. 1990. Determination of copper in seawater by anodic stripping voltammetry using ethylenediamine. *Anal. Chem*. 62 (9), 943-948.

Schindler, P. W., Stumm, W. 1987. The Surface Chemistry of Oxides, Hydroxides and Oxide Minerals. In: Stumm W (ed) *Aquatic Surface Chemistry*. Wiley, New York, 83–110.

Schreiber, U. 2004. Pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method: an overview. In: Papageorgiou, G.C., Govindjee (Eds.), Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Springer, Amsterdam, pp. 279–319.

Schwarz, J. A., Mitchelmore, C. L., Jones, R., O'Dea A., Seymour, S. 2013. Exposure to copper induces oxidative and stress responses and DNA damage in the coral *Motastraea franksi*. *Comp. Biochem. Phsiol. C* 157, 272-279.

Seibel, B. A., Walsh, P. J. 2003. Biological impacts of deep-sea carbon dioxide injection inferred from indices of physiological performance. *J. Exper. Biol*. 206, 641 –650.

Service, R. F. 2004. Nanotoxicology. Nanotechnology grows up. *Science* 304, 1732–1734.

Shick J. M. 1991. *A Functional Biology of Sea Anemones*. Chapman and Hall London, UK.

Siddiqui, S., Goddard, R. H., Bielmyer-Fraser, G. K. 2015. Comparative effects of dissolved copper and copper oxide nanoparticle exposure to the sea anemone, *Exaiptasia pallida*. *Aquat. Toxicol*. 160, 205-213.

Siegenthaler, U., Stocker, T. F., Monnin, E., Luthi, D., Schwan- ¨der, J., Stauffer, B., Raynaud, D., Barnola, J-M., Fischer, H., Masson-Delmotte, V., Jouzel, J. 2005. Stable carbon cycle-climate relationship during the late Pleistocene. *Science* 310, 1313 - 1317.

Sies, H. 1999. Glutathione and its role in cellular functions. *Free Radic. Bio. Med*. 27, 916-921.

Sigma, 1994a. Enzymatic Assay of Glutathione Reductase (EC 1.6.4.2).

Sigma, 1994b. Enzyme Assay of Catalase (EC1.11.1.6).

Sigma, 1994c. Enzymatic Assay of Glutathione Peroxidase (EC 1. 11. 1. 9).

Simpson, S. D., Munday, P. L., Wittenrich, M. L., Manassa, R., Dixson, D. L., Gagliano, M., Yan, H. Y. 2011. Ocean acidification erodes crucial auditory behaviour in a marine Fish. *Biol. Lett*. 7, 917–920.

Smart, S. K., Cassady, A. I., Lu, G. Q., Martin, D. J. 2006. The biocompatibility of carbon nanotubes. *Carbon* 44, 1034–1047.

Smith, L. D., Negri, A. P., Philipp, E., Webster, N. S., Heyward, A. J. 2003. The effects of antifoulant-paint-contaminated sediments on coral recruits and branchlets. *Mar. Biol.* 143, 651–657.

Srivastava, A. 2009. Antiviral activity of copper complexes of isoniazid against RNA tumor viruses. *Resonance* 14, 754–760.

Sun, X., Xiao, C. L., Ge, R., Yin, X., Li, H., Li, N., Yang, X., Zhu, Y., He, X., He, Q. Y. 2011. Putative copper- and zinc-binding motifs in *Streptococcus pneumoniae* identified by immobilized metal affinity chromatography and mass spectrometry. *Proteomics* 11, 3288-3298.

Sundberg, R. 1998. The fate of copper released from the Vasa shipyard museum. *Metall*. 52, 230–231.

Tatara, C. P., Newman, M. C., McCloskey, J. T., Williams, P. L. 1997. Predicting relative metal toxicity with ion characteristics: *Caenorhabditis elegans* LC_{50}. *Aquat. Toxicol*. 39, 279-290.

Tilaki, R. M., Iraji Zad, A., Mahadavi, S. M. 2007. Size, composition and optical properties of copper nanoparticles prepared by laser ablation in liquids. *Appl. Phys. A* 88, 415–419.

Valko, M., Morris, H., Cronin, M. T. 2005. Metals, toxicity and oxidative stress. *Curr. Med. Chem*. 12, 1161-1208.

Venn, A. A., Quinn, J., Jones, R., Bodnar, A. 2009. P-glycoprotein (multi-xenobiotic resistance) and heat shock protein gene expression in the reef coral *Montastraea franksi* in response to environmental toxicants. *Aquat. Toxicol*. 93, 188–195.

Vitale, A. M., Monserrat, J. M., Castilho, P., Rodriguez, E. M. 1999. Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda, Grapsidae). *Comp. Biochem. Physiol. C* 122, 121-129.

Voss, G. L. 1976. Seashore Life of Florida and the Caribbean: A Guide to the Common Marine Invertebrates of the Atlantic from Bermuda to the West Indies and the Gulf of Mexico. EA Seeman Publishing, Inc. Miami, FL. 168 pp.

Wang, T., Long, X., Cheng, Y., Liu, Z., Yan, S. 2014. The potential toxicity of copper nanoparticles and copper sulphate on juvenile *Epinephelus coioides*. *Aquat. Toxicol*. 152, 96- 104.

Weis, V. M. 1993. Effect of dissolved inorganic carbon concentration on the photosynthesis of the symbiotic sea anemone *Aiptasia pulchella* Carlgren: Role of carbonic anhydrase. *J Exp. Mar. Biol. Ecol.* 174, 209–225.

Weis, V. M., Reynolds, W. S. 1999. Carbonic anhydrase expression and synthesis in the sea anemone *Anthopleura elegantissima* are enhanced by the presence of dinoflagellate symbionts. *Physiol. Zool.* 72, 307–316.

Weis, V. M., Smith, G. J., Muscatine, L. 1989. A "CO_2 supply" mechanism in zooxanthellate cnidarians: role of carbonic anhydrase. *Mar. Biol.*100, 195.

Yamada, N., Suzumura, M. 2010. Effects of seawater acidification on hydrolytic enzyme activities. *J Oceanography* 66 (2), 233-241. DOI: 10.1007/s10872-010-0021-0

Yool, A., Popova, E. E., Coward, A. C., Bernie, D., Anderson, T. R. 2013. Climate change and ocean acidification impacts on lower trophic levels and the export of organic carbon to the deep ocean. *Biogeosciences* 10 (9), 5831-5854.

Zheng, C., Jeswin, J., Shen, K., Lablche, M., Wang, K., Liu, H. 2015. Detrimental effect of CO_2-driven seawater acidification on a crustacean brine shrimp, *Artemia sinica*. *Fish Shellfish Immunol*. 43, 181-190.

Zhou, Q., Zhang, J., Fu, J., Shi, J., Jiang, G. 2008. Biomonitoring: An appealing tool for assessment of metal pollution in the aquatic ecosystem. *Analytica Chimica Acta* 606, 135–150.