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The effect of copper-induced oxidative stress on regeneration in Anemonia viridis (Forskal, 1775)

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The effect of copper-induced oxidative stress on regeneration in *Anemonia viridis* (Forskäl, 1775)

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Abstract
The release of pollutants such as metals into the aquatic environment has raised concerns over the health of coral reefs, as elevated concentrations of metals are known to cause oxidative stress and bleaching. This is an important issue to address as metals are continuously released into the environment and there is evidence that metals such as copper can still be prevalent after historical mining activities, particularly in estuaries around the Plymouth Sound, UK. *Anemonia viridis* is commonly found around this area and is used in the literature as a model organism for coral species. *A.viridis* harbours symbiotic zooxanthellae and is also known to have strong regenerative abilities. The aim of this study was twofold: 1) to determine whether copper affected the regenerative abilities of *A.viridis*, using zooxanthellae density, glutathione content and lipid peroxidation products as indicators of oxidative stress, and 2) to generate new data on the regenerative abilities in *A.viridis* of different sizes. A new image analysis method was employed to quantify regeneration in tentacles. Results showed that copper induced oxidative stress as there was significant bleaching of zooxanthellae and an increase in total glutathione content ($P < 0.05$). Oxidative stress appeared to have an adverse effect on the initial growth rates of tentacles. Larger anemones had faster rates of regeneration compared to smaller anemones ($P < 0.05$) and the symbiont density varied in regenerating tentacles. This study demonstrated that copper had an adverse effect on the symbiotic status and regenerative ability in anemones, which may have implications for the health and growth of anemones and coral reefs worldwide as a result of metal pollution.

Introduction

Copper is present in low concentrations in aquatic environments due to natural processes such as rock erosion (Nriagu, 1979). However, copper concentrations are subject to anthropogenic input from sources such as mine drainage (Koski, 2012), agricultural run-off and anti-fouling paints (Turner, 2010). This has resulted in increased copper concentrations around estuaries and coastal regions (Sindermann, 1995) and this is particularly applicable to the estuaries around Plymouth, UK, as this area has been affected by mining activities in the past (Langston *et al.*, 2003). There is some evidence that copper is still prevalent in these areas, so consequently there are concerns over the effects of these residual concentrations on the organisms in the ecosystem (Mighanetara *et al.*, 2009).

In water, copper occurs as free metal ions or in ligand complexes. Ions can passively enter cells either by binding to surface membrane proteins or by permeating transport proteins, whereas ligand complexes adsorb onto the cell membrane and are then taken up by endocytosis (Simkiss & Taylor, 1995). Copper is an essential element in most organisms, as it is essential for the function of proteins and enzymes such as the respiratory pigment haemocyanin in crabs (Weser *et al.*, 1979), the anti-oxidant enzyme Cu-Zn superoxide dismutase found in most aerobic organisms (Weser *et al.*, 1979; Miller, 2012) and the plastocyanin electron transport protein found in photosynthetic systems (Raven *et al.*, 1999).

Although it is a necessary element, copper can become toxic at high concentrations. Copper ions are genotoxic and can destabilise the DNA structure by directly binding to nucleotide bases (Eichhorn *et al.,* 1966; Bagdonas & Vosyliene, 2006). Copper can also act as a catalyst in the formation of highly reactive free radicals known collectively as reactive oxygen species (ROS) (Figure 1). Increasing intracellular levels of copper and ROS stimulate the production of antioxidant molecules. There are different types of antioxidants including enzymic defences such as superoxide dismutase and non-enzymic molecules such as glutathione (GSH) and carotenoids (Davies, 1995, Pinto *et al.,* 2003). GSH is a tri-peptide that directly binds with copper and ROS to minimise damage to cells (Halliwell & Gutteridge, 2007). It is also utilised by the enzymes glutathione peroxidase and glutathione reductase to breakdown hydrogen peroxide formed from reactions between oxygen and copper (Figure 1). The binding capacity of these antioxidants can be overwhelmed by increasing levels of ROS and copper. If the antioxidants are not capable of detoxifying excessive ROS concentrations, damage to DNA, proteins and lipid membrane molecules can occur (Davies, 1995). Lipid membranes can be damaged through lipid peroxidation (Lushchak, 2011), which occurs when a hydrogen atom is lost to an ROS from the carboxyl end of polyunsaturated fatty acids, thereby forming lipid radicals (Davies, 1995). These lipid radicals then attract hydrogen atoms from other fatty acids and this consequently propagates peroxidation, ultimately causing loss of flexibility and the disintegration of membrane structure (Halliwell & Chirico, 1993; Davies, 1995). Damage to DNA, protein and lipids from ROS is known as oxidative stress which may result in cell death and subsequent inhibition of biological functions such as metabolism in fish (McGeer *et al.*, 2000) and photosynthesis in plants (Yruela *et al.*, 1996). Within the past decade there has been increasing interest in the biomolecular response to oxidative stress in corals caused by UV and thermal exposure due to concerns over global warming (Lesser, 2004). There is,

however, comparatively less literature on oxidative stress in coral and other cnidarian species in response to chemicals and metals in the environment.
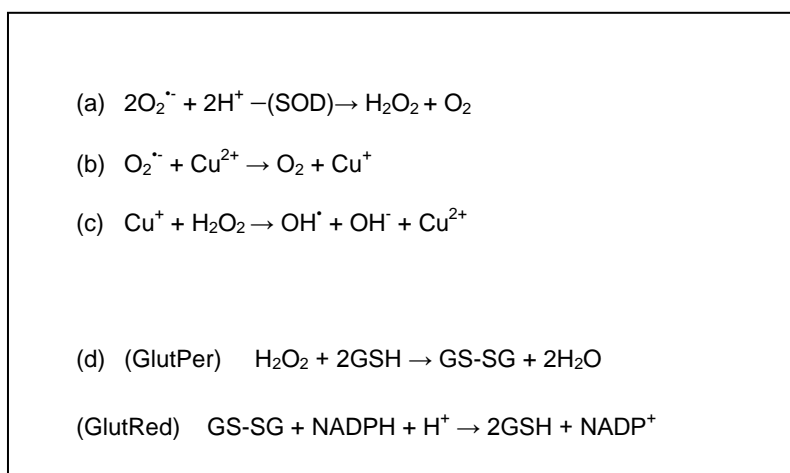
$$\text{(a)} \quad 2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{(SOD)}} H_2O_2 + O_2$$

$$\text{(b)} \quad O_2^{\cdot-} + Cu^{2+} \rightarrow O_2 + Cu^+$$

$$\text{(c)} \quad Cu^+ + H_2O_2 \rightarrow OH^{\cdot} + OH^- + Cu^{2+}$$

$$\text{(d)} \quad \text{(GlutPer)} \quad H_2O_2 + 2GSH \rightarrow GS\text{-}SG + 2H_2O$$

$$\text{(GlutRed)} \quad GS\text{-}SG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

Figure 1: Copper-induced formation of reactive oxygen species and antioxidant action of glutathione. (a) Superoxide ions ($O_2^{\cdot-}$) form hydrogen peroxide (H_2O_2) in a reaction catalysed by the antioxidant enzyme superoxide dismutase (SOD). (b) Copper reacts with superoxide ions and is reduced. (c) Copper donates electrons to hydrogen peroxide to form highly reactive hydroxyl radical ($OH^{\cdot}$) in the Fenton reaction (Davies, 1995; Lushchak, 2011). (d) Detoxification of hydrogen peroxide by reduced glutathione (GSH) and oxidised glutathione (GS-SG). This cyclic reaction is catalysed by antioxidant enzymes glutathione peroxidase (GlutPer) and glutathione reductase (GlutRed) and regenerates GSH for additional detoxification reactions (Halliwell & Gutteridge, 2007)

Cnidarians are a group of invertebrates that are mostly marine and contain around 10,000 species of Anthozoa (corals and anemones), Scyphozoa (jellyfish) and Hydrozoa (hydroids) (Ruppert *et al.*, 2004). Scyphozoan and hyrdrozoan life cycles comprise of two stages with different body forms known as the polypoid (polyp) and the medusoid (medusa) stages. Anthozoans are different in that they remain as a polyp, a radially symmetrical body form with a basal disc, column and oral disc surrounded by tentacles (Ruppert *et al.*, 2004). Most species of corals and anemones contain symbiotic dinoflagellates (*Symbiodinium* sp.) in their tentacles known as zooxanthellae (Whitehead & Douglas, 2003). These zooxanthellae supply the host with the majority of their nutritional requirements by releasing compounds such as glucose and amino acids (Whitehead & Douglas, 2003; Venn *et al.*, 2008). These symbionts are susceptible to environmental stress, which can lead to an increase in ROS production and oxidative stress in both symbiont and host cells. This often results in the subsequent release of zooxanthellae from the host cells, known as 'bleaching' (Venn *et al.*, 2008). Zooxanthellae density is a commonly used parameter to indicate oxidative stress in corals and anemones in response to stressors such as pollution and increasing temperature (Venn *et al.*, 2008), although the majority of literature has focused on the latter as part of climate change research.

Cnidarians have the ability to regenerate their tissues as part of asexual reproduction or in response to injury. Species such as *Anemonia viridis* and *Anthopleura elegantissima* reproduce through longitudinal fission where an individual tears

dorsoventrally and splits into two clones which then regenerate their torn tissue (Francis, 1973; Choresh, 2003). Regeneration is thought to be induced by signalling proteins released from damaged cells and the expression of particular genes that code for proteins which assist with the re-organisation of old cells and the proliferation of new ones (Figure 2). The mechanisms of regeneration have largely been determined from the classic model organism, the freshwater hydrozoan *Hydra*, but the cellular mechanisms of regeneration have only recently been established (Galliot & Chera, 2010). It is assumed that similar processes occur in *A.viridis* as they have similar cellular structures in their tentacles such as ectodermal, endodermal and mesogleal cell layers (Furla *et al.,* 1998). Currently there is only one study that has investigated the cellular regeneration process in anthozoans (Young, 1974) and the exact molecular mechanisms have yet to be established.
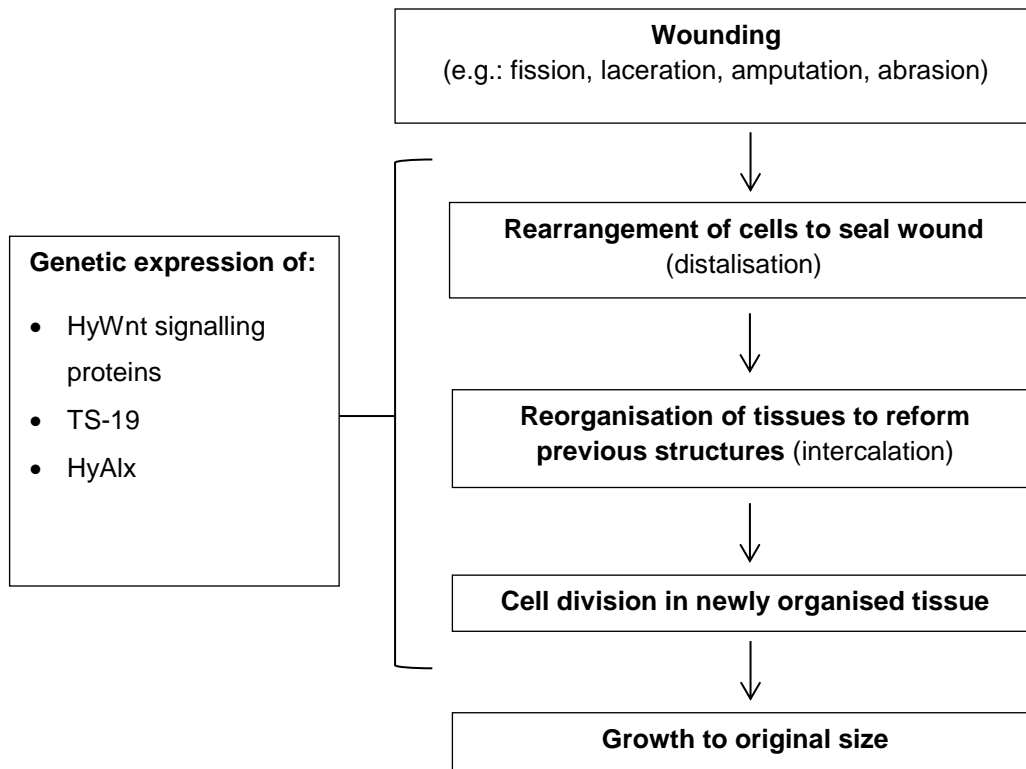

Figure 2: The process of wounding and morphallactic regeneration based on the *Hydra* model. Genetic expression of proteins are specific to tentacle amputation in *Hydra*. Mitogens signal the death of cells at wound site which then stimulate the expression of *Hydra* Wnt (HyWnt) signalling proteins to regulate tissue formation. Proteins TS-19 and HyAlx are expressed throughout the *Hydra* tentacle and are thought to assist with the regeneration of these areas. The degree of new growth in mature organisms is restricted to the previous condition of the old tissue (Galliot & Chera, 2010). Figure drawn based on information from Bode (2003) and Agata *et al.* (2007)

The snakelocks anemone *A.viridis* (Forskäl, 1775), previously *Anemonia sulcata*, is an example of a cnidarian that has strong regenerative abilities and employs a symbiotic relationship with photosynthetic zooxanthellae. It is commonly found on lower shorelines and sea beds in temperate regions (Muller-Parker & Davy, 2001). Green and brown colour morphs of this species exist and it is thought that the colour

difference may be due to the presence of green fluorescent protein and pink chromoproteins (Leutenegger *et al.,* 2007). Studies investigating the bleaching phenomenon and symbiotic relationships in corals use *A.viridis* as a model (Richier *et al.*, 2005, 2006; Leutenegger *et al.,* 2007; Merle *et al.*, 2007). It has been shown that *A.viridis* contains antioxidant enzymes including glutathione peroxidase (Hawkridge *et al.*, 2000) and demonstrates strong antioxidant responses. In contrast, the regenerative ability in *A.viridis* has been studied very little. There appears to be no data on the rate of growth, the extent of growth and the symbiotic state of regenerated parts after wounding. Anemones undergo wounding in the natural environment from abrasions or from foraging animals (Palmer *et al.*, 2011), but so far few studies have considered how regeneration in anemones may be affected by environmental contaminants. This is an important subject to address as toxicants such as metals may have an adverse effect on re-growth in *A.viridis*, particularly around the Plymouth estuary area where concentrations of heavy metals such as copper are still prevalent (Mighanetara *et al.*, 2009).

In this study two experiments were undertaken with two aims: 1) to investigate the effect of copper-induced oxidative stress on regeneration of tentacles and the symbiotic relationship in *A.viridis* and 2) to generate new data on the regenerative abilities in *A.viridis* of different sizes. Zooxanthellae density, glutathione content and lipid peroxidation in tentacles were analysed as indicators of oxidative stress from copper exposure. A new image analysis method was devised to quantify and monitor growth and regeneration rates of tentacles in *A.viridis* in both experiments.

Materials and methods

The effect of copper on regeneration

Animal collection, husbandry and experiment set-up
Individuals of *Anemonia viridis* were collected from relatively unpolluted sites at Wembury Bay (51°0'N, 48°4W) and Bovisand Beach (49°2'N, 49°9'W), Plymouth. Animals were transferred to the lab within two hours and held in 10 litre tanks containing filtered seawater (0.8 µm) with constant aeration in a 16°C temperature regulated room under a 12 hour light:dark routine. Seawater pH, temperature and salinity were measured during experimental treatments using a Hach HQ40d Portable Multi-Parameter Meter (Hach Company, Loveland, CO). The filtered seawater had an average pH of 8.25 (± 0.036), an average temperature of 17.01°C (± 0.54), and an average salinity of 33.4 ppt (± 0.37). Waters were changed every 96 hours and anemones in the holding tanks were fed with hatched *Artemia* sp on a weekly basis.

Individuals that had a pedal disc measuring less than or equal to 2 cm in diameter were selected for the experiment treatments. Four 2 litre tanks were used for all of the treatments and each tank contained one brown morph anemone and one green morph anemone (total of 16 tanks and 32 individual anemones). The tanks were filled with 1.5 litres of seawater from the same system as above and the anemones were added. The anemones were left to acclimatise for seven days and were starved for four days prior to and during treatments. Experimental tanks were continuously aerated and waters were changed every 96 hours throughout the 14 day duration of the experimental period.

Sampling
Samples of tentacles from anemones in each treatment were taken at the start of the experiment. For each anemone approximately 0.06 g wet weight of tentacle was placed into three Eppendorf tubes. The first two sample tubes were stored at -80°C and the third tube was retained for zooxanthellae analysis. At the end of 14 days tentacles from anemones in the control and copper treatment were sampled as above. Samples taken from regenerating tentacles were approximately 0.03 g wet weight, and results from these samples were doubled in order to obtain comparable data.

Treatments
In this experiment four treatments control, copper, regeneration and combined were conducted. The regeneration treatment refers to the anemones that had tentacles removed in the absence of copper. The combination treatment refers to anemones that had amputated tentacles that were also treated with copper.

For both the regeneration treatment and the combined treatment half of the tentacle mass was removed from individual anemones before the experiment. Preliminary trials of regenerative ability in *A.viridis* determined that individuals could not recover sufficiently within two weeks after full amputation of tentacles. *A.viridis* is flecked with white radial lines across the dorsal disc (Manuel, 1981), so these were used as a guide for the area of amputation (Figure 3). Individuals of *A.viridis* in the copper and the combined treatment were exposed to 50 µg l^{-1} of copper solution made from cupric sulphate salts ($CuSO_4 \cdot 5H_2O$, Sigma-Aldrich). This concentration of copper was chosen as it has been shown to induce stress without high levels of mortality in *A.viridis* (Harland & Nganro, 1990). The copper solution was steadily pipetted into tanks and stirred to ensure mixing. The tanks were re-dosed after water changes throughout the experiment period.

Tentacle length and zooxanthellae density analysis
Throughout the experiment duration tentacle lengths of anemones were measured using a new image analysis method. Tentacle lengths of anemones in all treatments were recorded by taking profile and overhead photographs every two days using an Acer CI-6330 digital camera. Tentacle lengths were then analysed using ImageJ software (Rasband 1997-2012) (Figure 3). Tentacle lengths were plotted against time and the initial rates of tentacle growth were calculated and expressed as mm d^{-1}. Zooxanthellae density was measured from tentacle samples taken before and after the experiment. The sample was homogenised with seawater and the zooxanthellae cells were counted on a Fuchs-Rosenthal haemocytometer. The remaining homogenate was centrifuged at 4000 g for 3 minutes and analysed for protein content as outlined in Bradford (1976). Zooxanthellae density was then calculated, normalised against protein content and expressed as cells mg^{-1} protein.
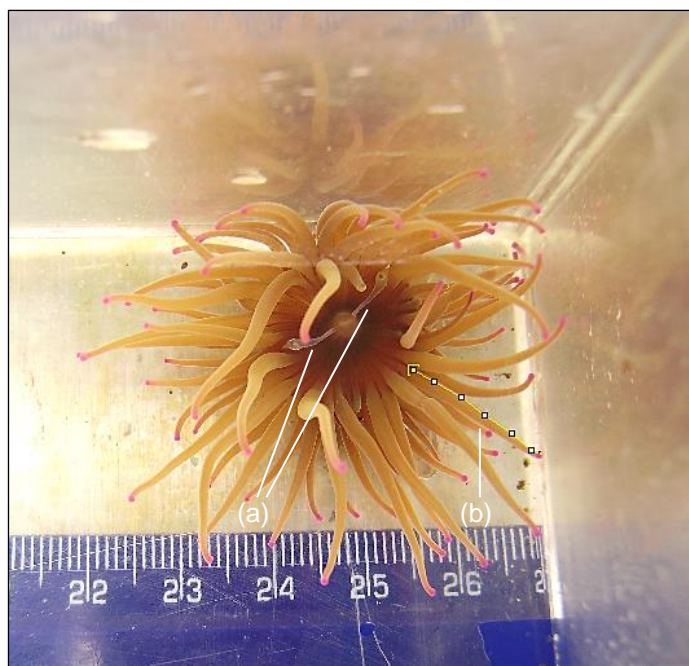
Figure 3: Screen shot of method of analysis for anemone regeneration in ImageJ software. (a) white radial lines used as guidelines for amputations, (b) tentacles were measured in ImageJ using the ruler as scale bar and the segmented line function to measure length of planar tentacles (determined from profile photographs that are not shown). The ruler was placed under the tank and used as a scale bar for analysis of tentacle length

Glutathione analysis

Total glutathione content (reduced GSH and oxidised GS-SG) in the tentacles were analysed using an assay outlined in Al-Subiai *et al*. (2009) with the following adaptations. Samples were thawed, homogenised in 1.0 ml seawater and centrifuged at 4000 g for 2 minutes before being treated in a 1:1 ratio with buffered DTNB solution (pH 7.5) buffer containing 50 mM potassium HEPES (Fisher Scientific), 5 mM potassium EDTA and 10 mM DTNB (Sigma Aldrich). Glutathione reductase (2.56 U/ml, Sigma Aldrich G3664 from *Saccharomyces cerevisiae*) was added to a pH 7.5 assay buffer containing 50 mM potassium HEPES and 5 mM EDTA to maintain an overall activity of 0.6 U. NADPH (Melford Laboratories, UK) was dissolved in assay buffer to give a final concentration of 1 mM. The DTNB-treated samples, blanks and standards (40 µl) were then transferred to the wells in the microplate (Sterillin Limited, UK). Glutathione reductase solution (210 µl) and NADPH solution (60 µl) were added to each well using a multipipettor. The colour change was then monitored in a microplate reader (Optimax, Molecular Devices, USA) to measure absorbance at 412 nm. Total glutathione content was calculated from the absorbance/time graphs and expressed as nmol mg^{-1} wet weight.

Lipid peroxidation analysis

Lipid peroxidation (LPO) was determined by the measurement of the molecular products of LPO that reacted with thiobarbituric acid (TBA, Sigma Aldrich). Malondialdehyde has been identified as one of these molecular products, but TBA also reacts with other compounds such as amino acids and carbohydrates (Lushchak, 2011). The levels of thiobarbituric acid reactive substances (TBARS) in tentacles were measured using the TBARS assay as outlined in the Animal Models

of Diabetic Complications Consortium protocol (AMDCC Protocols, 2004) with the following adaptations. The standard solution of 5 µl 1,1,3,3-tetramethoxypropane (Sigma Aldrich) was prepared in 1 ml of ethanol and 49 ml distilled water. Samples were processed as above for glutathione analysis, but seawater was added to the samples instead of RIPA buffer and the samples were not sonicated. The concentrations of the standards were prepared as 0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 µM 1,1,3,3-tetramethoxypropane. All samples and standards were incubated in Eppendorf tubes at 80°C for 30 minutes and then analysed in a plate reader to measure absorbance of 532 nm. LPO was expressed as nmol TBARS g^{-1} wet weight.

The effect of size on regeneration

Animal husbandry and experiment set-up
Individuals that were not used for the previous experiment were selected for this part of the study. The pedal disc diameters of green morphs of *A.viridis* were measured and anemones were segregated according to size into large (≥ 3 cm) and small (≤ 3 cm). A total of 16 anemones were kept in eight 2 litre tanks filled with 1.5 litres of seawater from the system described above with constant aeration. For this experiment the supply of food was minimised as a limiting factor by feeding anemones with *Artemia* sp. biweekly. Zooxanthellae density and tentacle regeneration were monitored as above during the 14 day experiment period.

Statistical analysis

Tentacle lengths over time in both experiments were modelled as exponential rise to maximum, linear or exponential decay regression using SigmaPlot (version 12.2). Initial rate was calculated by multiplying the amplitude by the rate constant obtained from the graphs. One-way analysis of variance (ANOVA) was used to determine statistical difference in tentacle growth over time between different treatments and sizes. Statistical differences in initial growth rate of tentacles between treatments and sizes were also determined using one-way ANOVA. Zooxanthellae density, total glutathione content and lipid peroxidation levels were compared using two-way ANOVA with Holm-Sidak post-hoc tests to determine significance between the start and end of the experiment within each colour morph. One-way ANOVA tests were carried out using Minitab (version 16), whereas two-way ANOVA tests were carried out using SigmaPlot (version 12.2). Graphs were also obtained using SigmaPlot. The level of probability accepted as significant was $P < 0.05$.

Results

The effect of copper on regeneration

The effect of copper-induced oxidative stress on regeneration was investigated in individuals of *Anemonia viridis*. Four treatments including control, copper-treated, regeneration and combination were set up and maintained for 14 days. The regeneration treatment refers to the anemones that regenerated tentacles in the absence of copper. The combination treatment refers to anemones that had tentacles amputated and were also treated with copper.

Tentacle length
Tentacle length changed in response to the treatments over time (Figure 4.a). Tentacle length did not change in anemones in the control treatment but decreased in the copper treatment. The tentacle length increased in both regeneration and combined treatments but growth in the combined treatment appeared suppressed

(Figure 4.b). The majority of tentacle lengths between the regeneration and combined treatments were not significantly different. Mean initial growth rate was significantly slower in the combined treatment (0.510 mm d^{-1}, n = 8) compared to the regeneration treatment (0.849 mm d^{-1}, n = 8) as confirmed by one-way ANOVA (P = 0.005).

Zooxanthellae density
Mean zooxanthellae density had decreased by the end of the experiment in both colour morphs in all treatments except controls (Figure 5). For green morphs there was significant decrease (P < 0.05) in the regeneration treatment between the start (1.60x10^{-7} cells mg^{-1} protein) and end of the experiment (0.369x10^{-7} cells mg^{-1} protein). There was also a decrease in the copper treatment before (1.62x10^{-7} cells mg^{-1} protein) and after the experiment (0.391x10^{-7} cells mg^{-1} protein). Anemones in the combination treatment also exhibited a decrease in zooxanthellae density before (1.32x10^{-7} cells mg^{-1} protein) and after the experiment (0.456x10^{-7} cells mg^{-1} protein). For the brown morphs there was a significant decrease (P < 0.05) in the regeneration treatment before (0.827x10^{-7} cells mg^{-1} protein) and after the experiment (0.214x10^{-7} cells mg^{-1} protein) and also in the combination treatment before (1.54x10^{-7} cells mg^{-1} protein) and after the experiment (0.354x10^{-7} cells mg^{-1} protein).

Glutathione content
Total glutathione content increased between the start and end of the experiment (Figure 6). In the green morphs there were statistically significant increases (P < 0.05) in the copper (from 2.25 nmol mg^{-1} wet weight to 4.59 nmol mg^{-1} wet weight) and combined treatments (from 2.17 nmol mg^{-1} wet weight to 4.01 nmol mg^{-1} wet weight). In the brown morphs there was also a significant increase in the copper treatment (from 2.28 nmol mg^{-1} wet weight to 4.63 nmol mg^{-1} wet weight) and the combined treatment (from 1.71 nmol mg^{-1} wet weight to 3.28 nmol mg^{-1} wet weight) for the brown morphs (P < 0.05).

Lipid peroxidation
There were mixed levels of LPO in response to the treatments (Figure 7). There were no statistically significant changes in green morphs, however anemones exposed to copper exhibited a decrease in LPO whereas anemones in control, regeneration and combined treatments exhibited an increase in LPO. There was a statistically significant decrease (P < 0.05) in the control treatment for the brown morphs between the start (237 nmol TBARS g^{-1} wet weight) and the end of the experiment (138 nmol TBARS g^{-1} wet weight). LPO in the anemones in the other treatments demonstrated similar patterns to green morphs.

The effect of size on regeneration

Tentacle length
Tentacle growth in response to amputation in small (≤ 3 cm) and large (≥ 3 cm) anemones were measured and analysed as above. Tentacle lengths at each time point between small and large anemones were significantly different (P < 0.05) except at day 9 and day 14 (Figure 8). One-way ANOVA determined that there was a significant difference in initial rates between small and large anemones (P = 0.041). Larger anemones had a faster mean initial rate of growth (1.25 mm d^{-1}, n = 8) than smaller anemones (0.67 mm d^{-1}, n = 8).

Zooxanthellae density
Zooxanthellae density varied between small and large anemones during regeneration of tentacles (Figure 9). Small anemones exhibited a decrease in mean zooxanthellae density (from 6.87×10^{-7} cells mg^{-1} protein to 5.44×10^{-7} cells mg^{-1} protein), whereas large anemones appeared to increase their mean density (from 3.92×10^{-7} cells mg^{-1} protein to 7.13×10^{-7} cells mg^{-1} protein). There were no significant differences in zooxanthellae densities between the start and end of the experiment for either size.

Observations
Anemones retracted their tentacles in response to amputation and did not fully project tentacles until several hours after wounding. During amputation anemones would alter their position so that the damaged side was against the side of the container. Clipped tentacles were observed to seal approximately 96 hours after amputation. Anemones that were exposed to copper also retracted their tentacles. Striping patterns of pink chromoprotein were observed along the tentacles which seemed to originate from the oral disc of the anemone, suggesting transferal of pink chromoprotein along the tentacles to the tips (Figure 10.a). 'Striping' occurred in less than half the total number of anemones used in the experiment, but 'striping' individuals had highly pigmented columns (Figure 10.b). 'Non-striping' anemones still regained pink colour in tips 14 days after amputation. There were cases of abnormal growth in untreated anemones. One brown morph in the control group exhibited a 'split' tentacle (Figure 11.a) and one large anemone did not start regeneration of tentacles in a small area of the oral disc until the last day of the experiment (Figure 11.b).
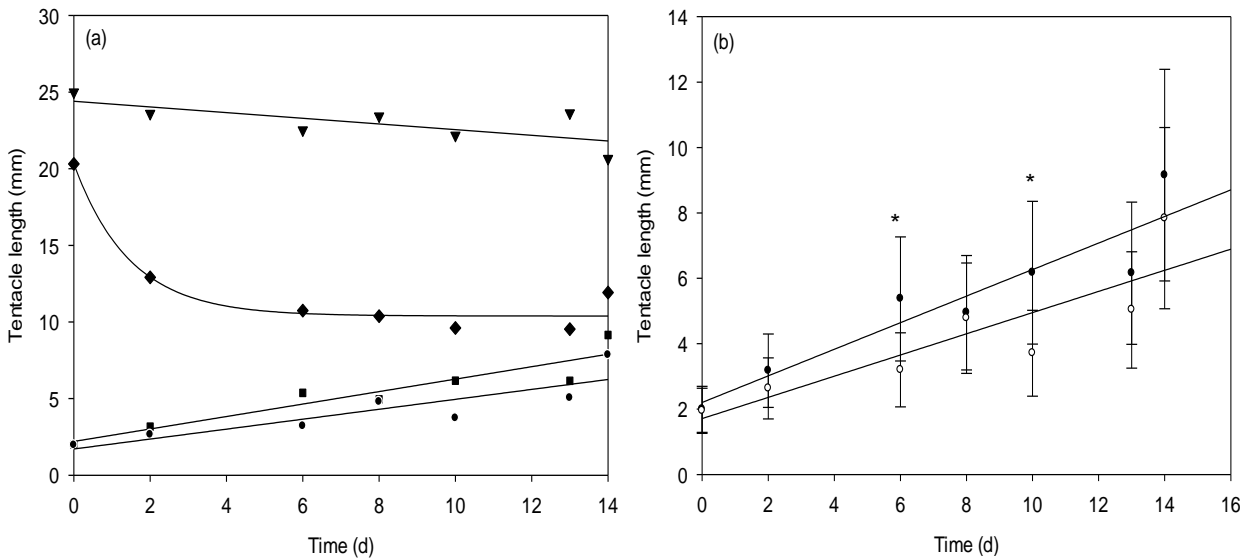
Figure 4: (a) mean tentacle length over time for all anemones in all treatments; control (▼), copper (♦), regeneration (■), and combination (●). (b) mean tentacle length for regeneration (●) and combination (○) treatments over time. Copper treatments were maintained at a concentration of 50 µg l^{-1} Cu. Bars indicate standard error. Asterisks indicate statistical significant differences in tentacle length as confirmed by one-way ANOVA ($P < 0.05$, n = 8 in each treatment)
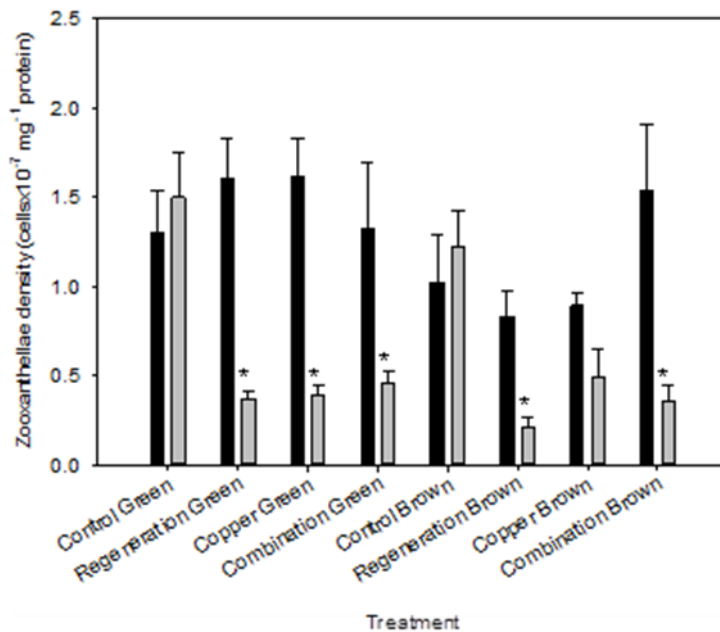

Figure 5: Zooxanthellae density before (black bars) and after (grey bars) the experiment in all treatments. Copper treatments were maintained at a concentration of 50 µg l^{-1} Cu. Brackets indicate standard error. Asterisks indicate significant difference as confirmed by two-way ANOVA and Holm-Sidak post-hoc test for each colour morph ($P < 0.05$, df = 3, n = 4)
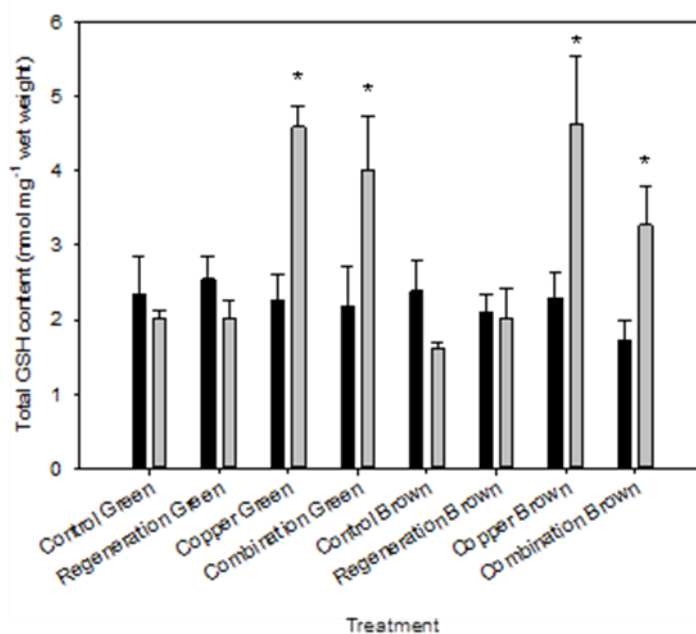
Figure 6: Total glutathione (GSH) content before (black bars) and after (grey bars) the experiment in all treatments. Copper treatments were maintained at a concentration of 50 µg l^{-1} Cu. Brackets indicate standard error. Asterisks indicate significant difference as confirmed by two-way ANOVA and Holm-Sidak post-hoc test for each colour morph ($P < 0.05$, df = 3, n = 4)
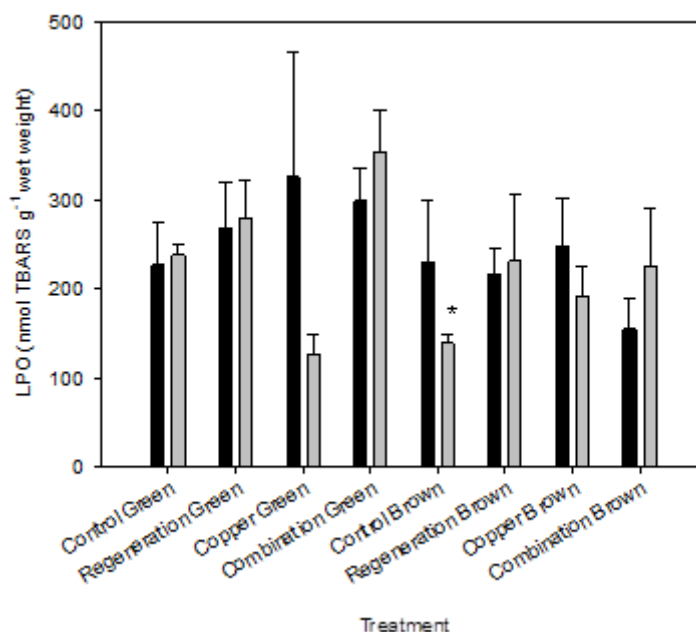

Figure 7: Lipid peroxidation (LPO) before (black bars) and after (grey bars) the experiment in all treatments. Copper treatments were maintained at a concentration of 50 µg l^{-1} Cu. Brackets indicate standard error. Asterisk indicates significant difference as confirmed by two-way ANOVA and Holm-Sidak post-hoc test for each colour morph ($P < 0.05$, df = 3, n = 4)
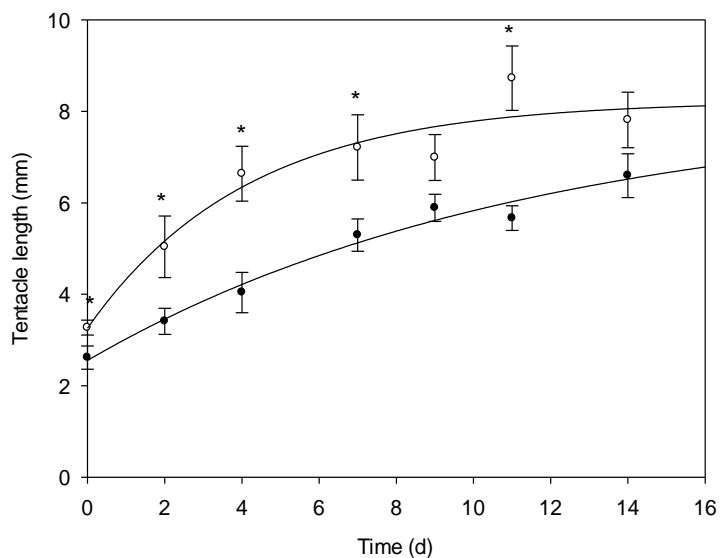
Figure 8: Mean tentacle length for large (○) and small (●) size anemones. Lines represent best fit with exponential rise to maximum. Brackets indicate standard error. Asterisks indicate where tentacle lengths are statistically significantly different as confirmed by one-way ANOVA ($P < 0.05$, n = 8)
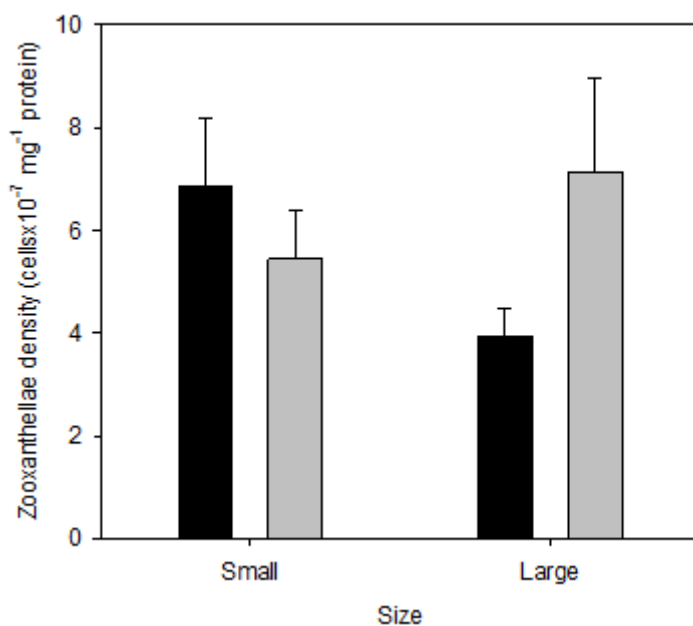

Figure 9: Zooxanthellae density for small (≤ 3 cm) and large (≥ 3 cm) green morph anemones before (black bars) and after (grey bars) the experiment. Brackets indicate standard error. There were no statistical significant differences in zooxanthellae density between times in small and large anemones as confirmed by two-way ANOVA ($P = 0.076$, df = 1, n = 8)
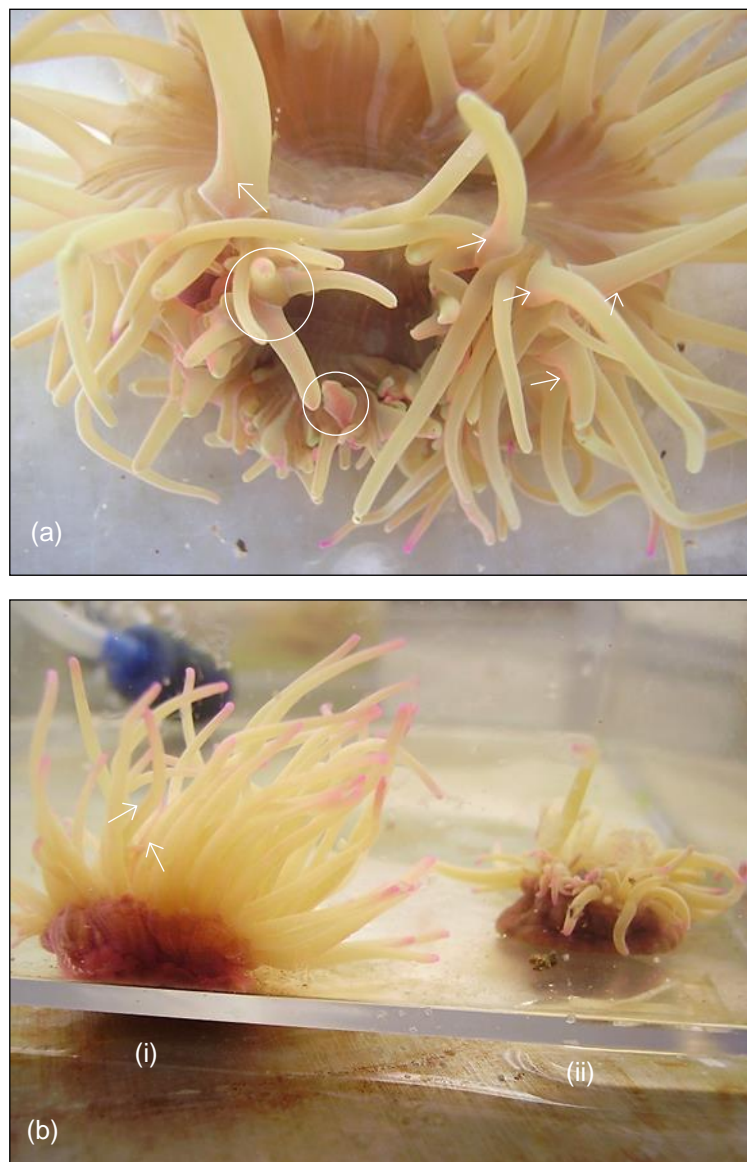
Figure 10: Examples of 'striping' anemones observed. Arrows indicate tentacles with pink stripes suggesting transport of chromoprotein up tentacle to the tips. (a) circles show evidence of origin of pink chromoprotein at base of amputated tentacles. (b) example of 'striping' anemone with pigmented column (i), compared to 'non-striping' anemone with less pigment present in the column (ii).

Figure 11: Examples of abnormal growth observed during the study. (a) single case of 'split' tentacle in a brown morph. (b) 'delayed' regeneration in a large anemone. Photograph taken on the second day of the experiment shows area of amputation within circle (i) and the same area is shown on the last day of the experiment (ii), where the majority of tentacles have regenerated apart from a small area of stunted growth

Discussion

The results of this study showed that exposure to copper caused significant bleaching of zooxanthellae in *Anemonia viridis* as well as significant increases in glutathione content, but there was a mixed response in levels of lipid peroxidation products. Initial growth rates in regenerating tentacles were adversely affected by copper. Tentacle growth was significantly different between larger and smaller anemones and larger anemones had significantly faster initial growth rates. Zooxanthellae density was found to differ within regenerating tentacles in each size before and after the experiment.

Oxidative stress

Environmental stress such as metal exposure and hyperthermia causes reactive oxidation species (ROS) to proliferate in the photosynthetic regions of the zooxanthellae, which can then trigger the elimination of symbionts from the host (Venn *et al.*, 2008). This is known as bleaching and is thought to be a generic stress response but is also thought to serve as a metal regulation mechanism (Harland *et al.*, 1990). The observed decrease in zooxanthellae density in this study coincides with previous reports on anemones exposed to copper (Harland & Nganro, 1990; Kaiser *et al.,* 2003; Mitchelmore *et al.*, 2003b) and UV or thermal stress (McCloskey *et al.,* 1996; Richier *et al.*, 2006; Leutenegger *et al.,* 2007; Moya *et al.*, 2012). These results are also in accord with those seen in corals in response to copper (Jones, 1997; Bielmyer *et al.,* 2010) and UV or thermal stress (Flores-Ramĺrez & Liñán-Cabello*,* 2007; Kuguru *et al.,* 2010). However, recent studies by Mitchelmore *et al.* (2003a, 2007) demonstrated no change in zooxanthellae densities in response to metal exposures. This is likely to be due to experimental design, as anemones used for zooxanthellae density analysis were segregated or kept in perforated containers within the main tank (Mitchelmore *et al.,* 2003a, 2003b). This may have had an impact on the flow of water within the tank and thus reduced the exposure of metal to the separated individuals. In addition, the symbiont density in coral species *Pocillopora damicornis* was not measured before exposure to copper and cadmium and subsequent measurements of algal cell density were compared to the control group (Mitchelmore *et al.,* 2007). It may be that a reduction in zooxanthellae occurred in the metal treated coral during the first four days but this was not reported.

Elevated levels of ROS are also thought to induce the up-regulation of antioxidants such as glutathione (GSH) in order to reduce cellular damage (Halliwell & Gutteridge, 2007; Lushchak, 2011). Currently there is only one study that has investigated GSH content in anemones before and after metal exposure, but the authors quantified the reduction of GSH instead of total content (Mitchelmore *et al.*, 2003a). The presence of GSH in *A.viridis* had been documented by Hawkridge *et al.* (2000) but as yet GSH content has not been quantified in this species. The increase in GSH content observed in this study is in accord with previous studies on anemones in response to oxidative stress induced from thermal and UV stress (Sunagawa *et al.*, 2008; Caparkaya *et al.*, 2010) and also in corals in response to metal and hyperthermia (Downs *et al.,* 2002; Mitchelmore *et al.*, 2007). However, accurate comparisons cannot be made as these studies quantify GSH in different units in various species of anthozoans in response the different stressors. For example, the reduction of GSH in *Anthopleura elegantissima* (Mitchelmore *et al.*, 2003a), the activity of glutathione enzymes in *A.viridis* (Caparkaya *et al.,* 2010) or GSH concentrations were normalised against protein content in *Pocillopora damicornis* and *Aiptasia pallida*

(Mitchelmore *et al.,* 2007; Sungawa *et al.,* 2008). This study utilised a method that quantified the GSH content per mg of wet weight of tentacle, as protein content varied significantly between the treatments and so normalising GSH content against protein would have introduced more variability into the data. It is recommended that future studies employ this method of quantifying GSH in order to obtain more comparable data.

Lipid peroxidation (LPO) and subsequent cellular damage is considered to be induced by elevated ROS levels (Davies, 1995). Previous investigations on oxidative stress in anemones have reported various responses in lipid peroxidation of either no significant change (Richier *et al.*, 2005; Caparkaya *et al.*, 2010) or increase (Downs *et al.*, 2002). One study by Jordão (2011) found that there was a decrease in lipid peroxidation in the anemone *Actinia equina* that had been exposed to mercury. This current study demonstrated mixed levels of LPO in response to copper. Copper treated anemones exhibited a decrease in LPO whereas there was an increase in anemones in the regeneration and combined treatments. This showed that cellular damage had occurred as a result of the amputation of tentacles, but it could not be concluded that cellular damage occurred from copper-induced oxidative stress. The decrease of LPO in copper treated anemones may be due to the presence of carotenoids and other antioxidants in the zooxanthellae of *A.viridis* (Caparkaya *et al.,* 2010), as they are known to be protective against ROS in algae and in corals (Pinto *et al.,* 2003; Flores-RamÍrez & Liñán-Cabello*,* 2007). The results of this current study highlight how the TBARs assay for lipid peroxidation analysis may be inappropriate as an indicator of oxidative stress.

Regeneration
The phenomenon of regeneration in organisms has been the subject of study for many years, yet until recently the cellular and molecular pathways (previously discussed) were largely unknown. In the model organism *Hydra*, it is thought that new tissue is regenerated from interstitial stem cells located between the ectodermal and endodermal cell layers that are co-ordinated by Wnt signalling proteins (Bode, 2003; Agata *et al.,* 2007; Galliot & Chera, 2010). It is not known whether interstitial cells are present in all cnidarians (Frank *et al.,* 2009), but a study on cellular regeneration in anthozoans by Young (1974) found that mesogleal cells have a similar role to interstitial cells during wound healing. Some aspects of regeneration remain poorly established, such as the exact mechanisms of the signalling pathways between cells (Tarrant, 2007) and the possible role of heat-shock proteins in tissue healing (Choresh, 2003). There is some doubt that enough is understood about these mechanisms in order to investigate how they might be affected by pollutants and oxidative stress (Tarrant, 2007).

To date there have been no studies on the effect of oxidative stress on regeneration in *A.viridis*. There appears to be very few studies on the toxicity of metals and other pollutants on regeneration in anemones or corals (Palmer *et al.,* 2011), however there have been some toxicity studies published on *Hydra* (Karntanut & Pascoe, 2002; Pachura-Bouchet *et al.,* 2006; Quinn *et al.,* 2008; Park & Yeo, 2012). These studies have demonstrated that there is an inhibition of regeneration or an adverse effect on the quality of newly regenerated tissues, but the mechanisms behind this are not clear and are often not discussed. It is thought that pollutants may inhibit cellular signalling in cnidarians, but few studies have been carried out to confirm this (Tarrant, 2007). It may be the case that pollutants have a multiple-level effect on the

regeneration process. Metals such as copper have been shown to have adverse effects on genetic, molecular and cellular structures (Davies, 1995; Bagdonas & Vosyliene, 2006; Lushchak, 2011) and these effects may also be applicable to regenerative processes. Copper and ROS may damage the DNA that code for the genes that are expressed in response to injury, which may subsequently lead to alterations in the production of signalling proteins. ROS may even directly modify the signalling proteins through oxidative damage, incurring further disruption of signalling pathways. This would have consequences for cell behaviour and tissue organisation as well as the formation of specialised cells such as nematocysts. Therefore, this may have an impact on the morphology and function of the organism. Observations during this study have demonstrated that abnormal growth of 'split' tentacles and 'delayed' regeneration occurs under normal conditions, so there may be a possibility that these occurrences could increase as a result of exposure to increasing levels of pollutants.

This experiment also demonstrated that there was an adverse effect on initial growth rates in regenerating tentacles of *A.viridis* during copper exposure. This may be exclusively due to oxidative stress and ROS formation interfering with cellular mechanisms mentioned above, but it may also be due to the lack of zooxanthellae. Symbionts provide the host with nutrients and, it is assumed, for the proliferation of new cells. If the symbionts are absent from the host as a result of oxidative stress and the host is not feeding then this may have implications for the ability to cope with tissue injury. It is not overly clear how symbionts function as part of regeneration and less is known about how symbiotic hosts, such as anemones and corals, can regenerate without symbionts under metal-induced oxidative stress. This is an important issue to consider particularly for reef-building coral species such as *Porites lutea* (Denis *et al.,* 2011).

There are no current studies that look into whether regeneration in anemones is size-dependent. The second experiment of this study determined that larger anemones had higher initial growth rates than smaller anemones. It may be that regenerative ability in anemones is subject to age and developmental stage, as it is known that this is the case for other regenerating organisms such as sea urchins (Galliot & Chera, 2010). It could also be speculated that this is because larger anemones have higher densities of photosynthetic zooxanthellae in their tissues to supply their host with nutrients for growth, however the density analysis in this study cannot support this assumption as there appeared to be fewer zooxanthellae in the tentacles of the larger anemones. The zooxanthellae density also appeared to increase in the regenerated tentacles of large anemones during the 14 day period. It may be that zooxanthellae are redistributed within larger anemones to compensate for loss of symbionts in regenerating tentacles and also to provide nutrients for new cells. There is evidence that aposymbiotic anemones can distribute newly acquired zooxanthellae within their tissues (Muller-Parker & Davy, 2001), but currently there is no evidence to suggest that zooxanthellae migrate within anemone tissues, although a study by Meszaros & Bigger (1999) demonstrated that zooxanthellae density increased at the site of injury in the coral *Plexaurella fusifera*. It is assumed that smaller anemones are unable to relocate their symbionts, as a decrease in zooxanthellae density was demonstrated in small anemones in both experiments of this study. It is recommended that further research is carried out to establish the role of symbionts in regeneration in anthozoa, in particular how the bleaching of zooxanthellae as a result of pollution may affect regeneration.

Limitations and future directions
This study had limitations in the analysis of the oxidative stress response in *A.viridis.* Zooxanthellae density, total GSH content and LPO were measured from samples taken before and after 14 days. It is thought that an initial decrease in total GSH content might have occurred after copper exposure, as the copper ions would have bound directly to the GSH initially present before GSH was up-regulated in response to oxidative stress. This pattern has been observed in unicellular algae (Pinto, 2003) and so may be applicable to the symbiotic algae in anthozoans. The fluctuation of GSH content may also have an impact on levels of LPO and the bleaching of zooxanthellae, so in order to gain more insight into the biochemical responses to copper, tentacle samples could be taken periodically throughout the experiment to monitor any fluctuations in these parameters. However it might not be possible to analyse the parameters regularly in regenerating tentacles, due to their slow growth and the introduction of additional stress from further amputation which could generate misleading data.

Some studies within the literature have attempted to use quantitative methods to analyse anemone regeneration (Bucklin, 1985), but these studies often use ranking systems which may incur problems with accurate characterisation of regenerative stages. This study devised a new image analysis method to accurately measure tentacle length over time. This worked well in practice but a lot of variation occurred within the data which was thought to be due to the retraction and extension in tentacles. This could be overcome by recording tentacle lengths more than once a day to gain an average. The new method could also be developed to investigate whether the growth of tentacles is restricted by individual size. Instead of obtaining a pedal disc diameter as a measure of size, column height could also be measured in order to obtain the volume of the body of the anemone. Regression analysis could then be used to establish a correlation between growth rate of tentacles and body volume which could help to determine whether regeneration in anemones is size-dependent.

This study has potential to be developed for further investigations into oxidative stress and anemone regeneration. Making comparisons between the results of this study with the wider literature has been difficult due to the lack of knowledge on the biochemical responses to pollutants in anemones. Cnidarian toxicology is a relatively new field that was established due to concerns over effects of pollutants on coral reefs (Rotchell & Ostrander, 2011) and as yet there is an absence of convergent information due to differences in experimental designs. There is less information on how pollutants that induce oxidative stress may affect regeneration in anthozoans, as the majority of information is derived from studies on *Hydra*. A regeneration assay using *Hydra* has been developed for use in toxicity testing, but this has often been used for the testing of the teratogenicity of chemicals in developing organisms (Pachura-Bouchet *et al.,* 2006) and has yet to be incorporated into environmental monitoring programs (Quinn *et al.,* 2012). A similar toxicological test for regeneration using anemones as a model for chronic exposures could be developed from methods utilised in this study in combination with methods employed by Jones (1997) and Main *et al.,* (2010). This could then be incorporated into environmental risk assessments and biomonitoring programs for local anemone populations such as those in the Plymouth Sound and for coral reefs worldwide.

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