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# Differential accumulation of heavy metals in the sea anemone *Anthopleura elegantissima* as a function of symbiotic state

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**Abstract**

The accumulation of metals by the North American Pacific Coast temperate sea anemone *Anthopleura elegantissima*, and its dinoflagellate-algal symbiont *Symbiodinium muscatinei* was examined following laboratory metal exposures. Both, naturally occurring symbiotic and symbiont-free (aposymbiotic) anemones were used in this study to investigate differences in metal uptake due to the symbiotic state of the animal. The effects of metal exposures on the anemone-algal symbiosis were determined using measures of algal cell density and mitotic index (MI). Anemones were exposed to either cadmium, copper, nickel or zinc chloride (0, 10, 100 $\mu g\,l^{-1}$ for Cd, Cu and Ni; 0, 100, 1000 $\mu g\,l^{-1}$ for Zn) for 42 days followed by a 42-day recovery period in ambient seawater. Anemones were analyzed for metal content using inductively coupled plasma mass spectroscopy (ICP-MS) at various time points during the study. Symbiotic anemones accumulated Cd, Ni and Zn to a greater extent than aposymbiotic anemones. A dramatically different pattern of Cu accumulation was observed, with aposymbiotic anemones accumulating higher levels than symbiotic anemones. Following recovery in ambient seawater, all tissue metal levels were reduced to near pre-exposure control levels in most cases. No changes in algal cell density or MI were observed in symbiotic anemone tentacle clips at any dose or time point in the Cd and Cu exposures. However, significant reductions in algal cell densities were observed in the Ni-exposed and some Zn-exposed animals, although levels returned to control values following recovery. There were no changes in mitotic index (MI) following Ni or Zn exposures. These results demonstrate that the extent of heavy metal accumulation depends upon cnidarian symbiotic state and the heavy metal in question.


*Keywords:* Anemone; Cadmium; Copper; Nickel; Zinc; Cnidarian; Symbiosis

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## 1. Introduction

Heavy metals are common marine pollutants that emanate from such sources as industrial and sewage treatment discharges and anti-fouling paints. Extensive research has been directed towards determining the levels of metal accumulation and their toxic effects in fish and some invertebrate species, such as mussels (e.g., Mussel Watch Program, U.S. EPA, 1989). However, metal accumulation in cnidarians and metal effects on the symbiotic relationship have received relatively little attention, despite the importance of cnidarians in marine communities worldwide.

In temperate coastal communities, anemones are often conspicuous members of the fauna. Tropical coral reefs are dominated by a wide variety of stony corals and anemones that form the trophic and structural foundation of the entire reef ecosystem (Birkeland, 1996). Some temperate and most tropical cnidarians engage in a mutualistic endosymbiosis with photosynthetic dinoflagellates in the genus *Symbiodinium*. These important and complex symbioses exist as a sensitive balance between the two partners. Under adverse conditions to either or both organisms, breakdown of the symbiosis can occur, usually resulting in loss of algae from the host. This phenomenon, known as bleaching, has become increasingly prevalent in coral reefs since the 1970s. Bleaching has been associated with a variety of "natural" and anthropogenic perturbations (Glynn, 1993; Jones, 1997; Meehan and Ostrander, 1997; Wilkinson, 1999; Brown, 2000; Pandolfi and Jackson, 2001), including heavy metals (see Brown, 1997; Meehan and Ostrander, 1997; Brown, 2000) that may act in isolation or synergistically.

Studies to date have documented metal accumulation in symbiotic cnidarians (Howard and Brown, 1984; Brown, 1987; Scott, 1990; Guzman and Jimenez, 1992; Harland et al., 1990; Harland and Nganro, 1990; Mitchelmore et al., 2003). However, only a few studies have addressed the possible role of the symbiosis in metal accumulation. One study compared copper accumulation in symbiotic and artificially bleached specimens of *Anemonia viridis*, a temperate European anemone, and it was found that bleached individuals accumulated more copper than symbiotic anemones (Harland and Nganro, 1990). In a field study, the symbiotic anemone *A. viridis* had higher tissue levels of cadmium in comparison to the aposymbiotic species, *Actinia equina* (Harland et al., 1990). We have found similar results in a previous study that symbiotic *Anthopleura elegantissima* accumulates Cd to a greater extent compared with aposymbiotic anemones of this species (Mitchelmore et al., 2003). Further studies, from both the field and laboratory, have examined the effects of various metals to symbiotic corals (Esquivel, 1986; Howard et al., 1986; Harland and Brown, 1989) and anemones (Harland and Nganro, 1990) as measured by bleaching. In all these studies, metal exposure resulted in the loss of algae from host tissues although this was not the case in our previous studies (Mitchelmore et al., 2002, 2003). Studies in freshwater hydra species have demonstrated that green hydra, *Hydra viridissima*, symbiotic with zoochlorellae are more susceptible to the toxic effects of metals (Cd, Cu and Zn) compared with the pink non-symbiotic species, *Hydra vulgaris* (Holdway et al., 2001). To date, besides our previous investigations (Mitchelmore et al., 2002, 2003) no studies have examined accumulation and effects of heavy metals as a function of symbiotic state on a 'single' species of cnidarian.

The abundant North American Pacific Coast temperate sea anemone *A. elegantissima* is an important member of the rocky intertidal community from Alaska to Baja California (Sebens, 1981; Fitt et al., 1982). In high light habitats this anemone harbors the symbiont *Symbiodinium muscatinei*. Unlike corals however, in low light locations, such as in caves and rock crevices *A. elegantissima* occurs in a symbiont-free (aposymbiotic) state. The existence of a naturally occurring symbiotic and aposymbiotic state within a single species is a powerful comparative tool for the study of symbiosis and the role of symbiosis in a variety of biological processes. Therefore, *A. elegantissima* makes an ideal model for the examination of cnidarian-algal symbioses (Weis and Reynolds, 1999; Reynolds et al., 2000).

In this study, the effects of exposure to four different heavy metals (cadmium, copper, zinc and nickel) on symbiotic and aposymbiotic *A. elegantissima* are reported. Cu, Ni and Zn are essential elements required by organisms in trace amounts, which can cause toxic effects at elevated concentrations, whereas Cd serves no essential function in biological organisms (Goering et al., 1995; Mason and Jenkins, 1995). During a 42-day exposure and 42-day recovery period, metal accumulation as a function of symbiotic state was evaluated. In parallel, loss of algae from symbiotic anemones and algal MI were evaluated.

## 2. Materials and methods

### 2.1. Animals

Naturally occurring symbiotic and aposymbiotic specimens of *A. elegantissima* were collected from Indian Point, Ecola State Park, OR and transferred to a laboratory facility at the Environmental Protection Agency (EPA) at Oregon State University's Hatfield Marine Science Center in Newport, OR. Anemones were placed in seasoned 12.5 l plastic tanks (as detailed below) in flowing ambient seawater at 14($\pm$1 °C). Metal halide lamps (5000 W) were used to provide optimal lighting conditions for algal photosynthesis (200–250 μmol quanta $m^{-2}$ $s^{-1}$, 12/12 h light/dark cycle; Weis and Levine, 1996). Two collections of anemones (May and August, 2000) were made for experiment 1 (Cu and Cd exposures) and experiment 2 (Ni and Zn exposures), respectively. Anemones were acclimatized for 2 weeks before the addition of metals in their respective tanks (see below).

### 2.2. Experimental design

Anemones were placed in experimental tanks as follows. In each tank, 32 symbiotic anemones were placed on the left side of the tank and 32 aposymbiotic anemones were placed on the right separated by a plastic grid down the center of each tank. An additional, four symbiotic anemones per tank were placed in perforated plastic containers and repeatedly sampled for algal cell densities. To maintain temperature at 14($\pm$1 °C) the experimental tanks were placed in a larger tank containing running ambient seawater. Metal exposures were carried out using nominal concentrations in two experiments as follows. In experiment 1, anemones were exposed to three concentrations of cadmium or copper chloride (0, 10 and 100 μg $l^{-1}$ Cd or Cu in ambient seawater; 10.5 l static fill), with tanks in duplicate for 10 tanks total. For experiment 2, anemones were exposed to three concentrations of nickel or zinc chloride (0, 10 and 100 μg $l^{-1}$ Ni or 0, 100 and 1000 μg $l^{-1}$ Zn in ambient seawater; 10.5 l static fill), with tanks in duplicate for 10 tanks total. For both experiments metal exposures were carried out for 42 days. Water was changed every 2 or 3 days and tanks were randomly repositioned to offset any position effects. Following metal exposures, anemones were allowed to 'recover' for a total of 42 days post-exposure. This recovery period was conducted with flowing ambient seawater (at 14$\pm$1 °C) to the tanks, which were cleaned every 2–3 days. Four anemones of both types were sampled from each tank on Days 0, 3, 7, 21 (Ni and Zn) or 28 (Cd and Cu), and 42 during the metal exposures; and also sampled on 14 and 42 days post-exposure (recovery). To sample, anemones were removed from the tanks, cleaned of mucus and patted dry to remove excess water. Animals were then cut into quarters with a Teflon-coated razor blade, flash frozen in liquid nitrogen and stored at −80 °C until analyzed. At each time point, the four anemones in the perforated plastic containers in each tank were anesthetized by placing them in 50% 0.37 M $MgCl_2$ and 50% seawater solution. Three to five tentacles were excised from each anemone, frozen in liquid nitrogen and stored at −80 °C for subsequent algal cell counts.

### 2.3. Metal analysis

Anemone samples were prepared for metal analysis as follows. Anemone quarters were weighed (wet weight), homogenized in a Teflon glass tissue grinder in 1 ml of a 50 mM ammonium

bicarbonate buffer (pH 7.8). The resulting homogenate was placed in a pre-weighed acid washed 10 ml polypropylene tube. The grinder was rinsed twice with 1 ml of buffer and the rinsate was added to the homogenate. All samples were first frozen at −80 °C, lyophilized and weighed (dry weight). Blanks were processed in the same way using the buffer alone for all processing steps. We calculated that both symbiotic and aposymbiotic anemones contained approximately 75% water by comparing wet and dry weight values (using regression analyses; $y = 4.2795x$ ($r^2 = 0.98$) and $y = 4.221x$ ($r^2 = 0.99$) for symbiotic and aposymbiotic anemones, respectively).

For the digestion of tissues for metal analysis, 2 ml of metal-analysis grade nitric acid was added to all tubes and samples were then incubated overnight in a 60 °C oven, and the following day heated to 90 °C for 2 h. One ml of ultra-pure grade hydrogen peroxide (30%) was gradually added to each sample and then incubated for an additional 2 h. The tubes were cooled to room temperature and 4 ml of ultra-high-quality water was added to all tubes. Analysis of metal content was carried out using a VG Elemental PQ ExCell ICP-MS machine as follows. For Cd, Cu and Ni analysis, 500 μl of sample was added to 100 μl of internal standard (containing beryllium ($^{9}$Be) and indium ($^{115}$I)) and diluted with 5 ml of 1% ultra-pure double-distilled nitric acid. For Zn analysis, 50 μl of sample was added to 100 μl of internal standard (containing $^{9}$Be and $^{115}$I) and diluted with 5.49 ml of 1% ultra-pure double-distilled nitric acid. Metal concentrations were calculated using the following isotopes: $^{111}$Cd, $^{65}$Cu, $^{58}$Ni and $^{66}$Zn isotopes. A set of calibration standards was added to the analysis together with appropriate blanks. A standard reference material (NIST Oyster Tissue, 1566a) was processed and analyzed with the samples to verify the measurements (expected concentrations: Cd, $4.15 \pm 0.38$ μg g$^{-1}$; Cu, $66.3 \pm 4.3$ μg g$^{-1}$; Ni, $2.25 \pm 0.44$ μg g$^{-1}$; Zn, $830 \pm 57$ μg g$^{-1}$). The mean measured concentrations of standard material were Cd, $3.99 \pm 0.42$ μg g$^{-1}$; Cu, $66.1 \pm 3.3$ μg g$^{-1}$; Ni, $2.3 \pm 0.65$ μg g$^{-1}$; and Zn, $821 \pm 67$ μg g$^{-1}$ dry weight of oyster tissue.

### 2.4. Algal cell density and mitotic index

A common response of cnidarians to stressful conditions is the loss of algal symbionts and/or changes in rates of mitosis (MI) in the algal symbionts. To assess algal cell density and MI, tentacles were thawed and homogenized in sterile filtered (0.2 μm) seawater (FSW). For experiment 1 (Cd and Cu) homogenates were centrifuged at $1500 \times g$ for 10 min to pellet the algae and the pellet was re-suspended in FSW and counted using a hemocytometer. The supernatant was placed at −20 °C for subsequent protein determination. In experiment 2 (Ni and Zn) tentacles were homogenized in FSW and counted using a hemocytometer without first pelleting the algae. Following cell counts the homogenate was placed at −20 °C for later protein determination. Protein assays were carried out in triplicate for all samples using the Bradford assay (Bradford, 1976). Data for cell counts were expressed as number of algal cells per mg protein (animal protein in experiment 1 and total protein for experiment 2). Concurrently with density estimates, the MI of the zooxanthellae was measured as described by Wilkerson et al. (1988). The number of algae undergoing cytokinesis per 1000 cells was noted and the resultant percentage taken as the MI.

### 2.5. Calculations and statistical design

Results from replicate tanks in all assays were compared, and significant differences from treatments compared ($n = 3$–4) using ANOVA ($\alpha = 0.05$) to determine if samples could be pooled. Since there were no observed replicate tank differences, samples were then pooled for further statistical analysis ($n = 6$–8; means ± S.D.) using ANOVA ($\alpha = 0.05$) coupled with the Bonferroni test for multiple comparisons. Statistical analyses was carried out comparing each dose over time compared with their respective Day 0 values (details in figures) and also at each time point in comparison with the respective control tanks (referred to in the following section where appropriate).

## 3. Results

In this study, heavy metals were accumulated in a time- and dose-dependent manner in both symbiotic and aposymbiotic *A. elegantissima*, to varying extents depending upon the metal type and anemone symbiotic state. The levels accumulated were higher than could be attributed to the water flux between the animal and seawater. Approximately, 75% of the anemone wet weight was water (see Section 2), so it is possible that by simple water exchange between the anemone and the seawater a maximal accumulation of 0.3 ng heavy metal $mg^{-1}$ dry weight anemone tissue in 100 μg $l^{-1}$ metal exposures or 3 ng heavy metal $mg^{-1}$ dry weight anemone tissue in 1000 μg $l^{-1}$ exposures could be observed. The accumulation values measured were an order of magnitude or greater than these values.

Visual observations were also made during the course of these experiments. In all experiments, there were no visual signs of bleaching and no mortalities were observed over the exposure periods. Anemones exposed to Cu produced excessive amounts of mucus, particularly in the high doses. A thick layer of mucus remained closely associated with the anemones. Symbiotic anemones appeared to produce more mucus than aposymbiotic animals, although the mucus was not quantified. In addition, at the highest Cu dose anemones spent the entire exposure period with their tentacles retracted, although as soon as these anemones were placed in ambient seawater for the recovery period, tentacles were fully extended. Exposure to Cd, particularly at the higher doses, resulted in visual swelling of both anemone types. This was apparent up to Day 7, after which anemones appeared to recover and were not visibly different from the controls.

Different responses in metal accumulation were observed depending on the metal type and the anemone symbiotic state. Fig. 1 illustrates the time- and dose-dependent accumulation of Cu in symbiotic and aposymbiotic anemones. Levels of metals in control animals did not change over time ($P > 0.05$) and were not significantly different between symbiotic and aposymbiotic anemones ($P > 0.05$). Mean control values over the duration

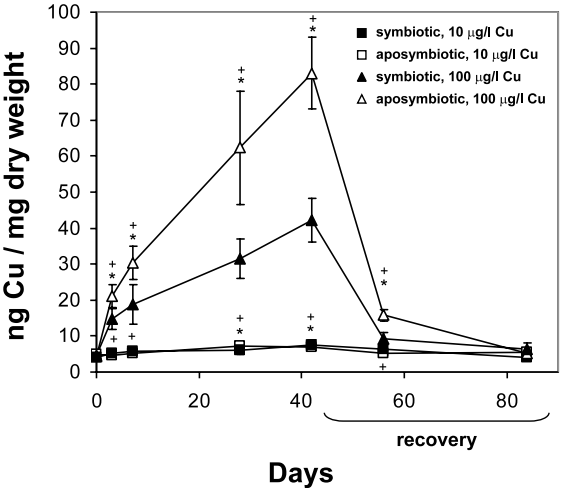


Fig. 1. Cu accumulation in symbiotic and aposymbiotic anemones exposed to 10 and 100 μg $l^{-1}$ Cu. Anemones were exposed for 42 days followed by a 42-day recovery. Data are presented as means (±S.D.; $n = 6–8$) and expressed as ng Cu $mg^{-1}$ dry weight anemone tissue. Statistically significant differences for symbiotic (*) and aposymbiotic (+) anemones were observed within treatments over time when compared to their respective Day 0 values ($P$ at least $< 0.05$). For clarity purposes significance symbols (* and +) are grouped together by dose with the higher dose above the lower dose. Significantly lower levels of Cu accumulated in the symbiotic compared to aposymbiotic anemones following exposure to 100 μg $l^{-1}$ Cu at all time points except Day 0 and Day 42 recovery ($P < 0.001$).

of the experiment were 4.82 ± 0.52 ng Cu $mg^{-1}$ dry weight for symbiotic and 4.44 ± 1.01 ng Cu $mg^{-1}$ dry weight for aposymbiotic animals. Following exposure to 100 μg $l^{-1}$ Cu, lower levels of Cu in symbiotic compared with aposymbiotic anemones were observed, with maximum accumulations for both types achieved following 42 days exposure. Significant differences ($P < 0.001$) between symbiotic and aposymbiotic anemones following 100 μg $l^{-1}$ Cu exposure were observed at all time points except at Days 0 and 42 recovery. A similar trend of increasing accumulation with time was observed at the lower Cu exposure (10 μg $l^{-1}$) in symbiotic and aposymbiotic anemones, although no significant differences ($P > 0.05$) between anemone types were observed.

In animals exposed to 100 μg $l^{-1}$ Cu, significant differences from the controls at Day 0 ($P < 0.001$) were observed in symbiotic and aposymbiotic anemones at all time points except Day 42

recovery. However, following exposure to 10 μg $l^{-1}$ Cu, significantly higher ($P < 0.05$) Cu concentrations were only observed at Days 28 and 42 in symbiotic anemones compared with Day 0 controls whereas aposymbiotic anemones contained significantly higher levels ($P < 0.01$) of Cu compared with Day 0 controls at all time points except Day 42 of recovery. Following recovery in ambient seawater both the symbiotic and aposymbiotic anemones contained metal levels similar to the original Day 0 pre-exposure levels (Fig. 1).

In contrast to the accumulation patterns observed with the Cu exposures, symbiotic anemones accumulated more Cd than aposymbiotic anemones, particularly in the 100 μg $l^{-1}$ Cd exposures. Fig. 2 illustrates the time- and dose-dependent accumulation of Cd in symbiotic and aposymbiotic anemones following 10 and 100 μg $l^{-1}$ Cd exposures. There were no significant differences ($P > 0.05$) in Cd levels in either symbiotic or aposymbiotic control animals through

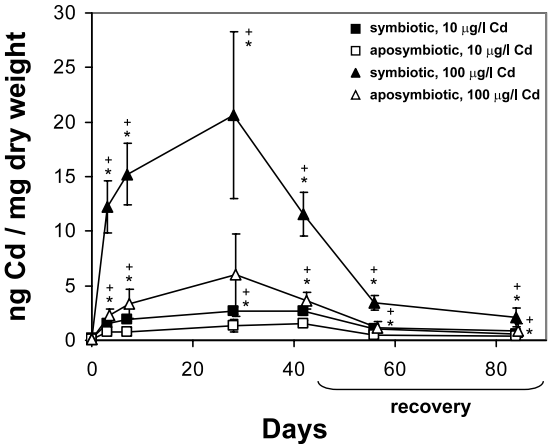


Fig. 2. Cd accumulation in symbiotic and aposymbiotic anemones exposed to 10 and 100 μg $l^{-1}$ Cd. Anemones were exposed for 42 days followed by a 42-day recovery. Data are presented as means (±S.D.; $n = 5–8$) and expressed as ng Cu $mg^{-1}$ dry weight anemone tissue. Statistically significant differences for symbiotic (*) and aposymbiotic (+) anemones were observed within treatments over time when compared to their respective Day 0 values ($P$ at least $< 0.05$). For clarity purposes significance symbols (* and +) are grouped together by dose with the higher dose above the lower dose. Significantly greater levels of Cd accumulated in the symbiotic compared to aposymbiotic anemones following exposure to 100 μg $l^{-1}$ Cd at all time points except Day 0 ($P < 0.01$).

time or between treatments. Mean control values across all time points were 0.11 ± 0.05 and 0.08 ± 0.02 ng Cd $mg^{-1}$ dry weight for symbiotic and aposymbiotic anemones, respectively. There were significant differences ($P < 0.01$) between the two types of anemones at both dosages and at all time points except Day 0. Exposure to 10 μg $l^{-1}$ Cd resulted in a time-dependent accumulation with maximal levels in symbiotic and aposymbiotic anemones occurring at Day 42. Interestingly, maximal accumulation in both anemone types following exposure to 100 μg $l^{-1}$ Cd occurred at Day 28, but the levels were reduced at Day 42, before removal from the metal. Following recovery in ambient seawater, the Cd levels of both the symbiotic and aposymbiotic anemones were significantly reduced although the levels were still statistically higher ($P < 0.05$) compared with Day 0 levels.

Algal cell densities in excised tentacle clips did not change throughout the experiment in control animals and averaged 3.6 ± 1.0 algal cells × $10^6$ $mg^{-1}$ protein over the six time points (Fig. 3). We did observe a significant decrease in algal cell density at Day 14 of recovery in anemones exposed to 100 μg $l^{-1}$ Cu. In the remaining time points and exposures, no significant changes in densities were observed, although there were trends in all exposures of decreasing cell densities through the exposure and 14-day recovery period, with apparent return to control levels by 42 days of recovery. No significant changes in MI were observed (average MI for all Cd and Cu exposures of 0.3 ± 0.06%) over the duration of the experiment.

In comparison to the marked accumulation of cadmium and copper in anemones, Fig. 4 demonstrates the relatively low amount of Ni accumulation in symbiotic and aposymbiotic anemones following 10 and 100 μg $l^{-1}$ Ni exposures. Control values did not change over time and no significant differences ($P > 0.05$) were seen between symbiotic and aposymbiotic anemones in the controls or at Day 0 (mean control values were 0.47 ± 0.48 and 0.57 ± 0.42 ng Ni $mg^{-1}$ dry weight for symbiotic and aposymbiotic animals respectively over the seven time points). At 100 μg $l^{-1}$ Ni exposures, significant differences between the two anemone types were observed at Days 3, 7, 28 and 42 ($P <$

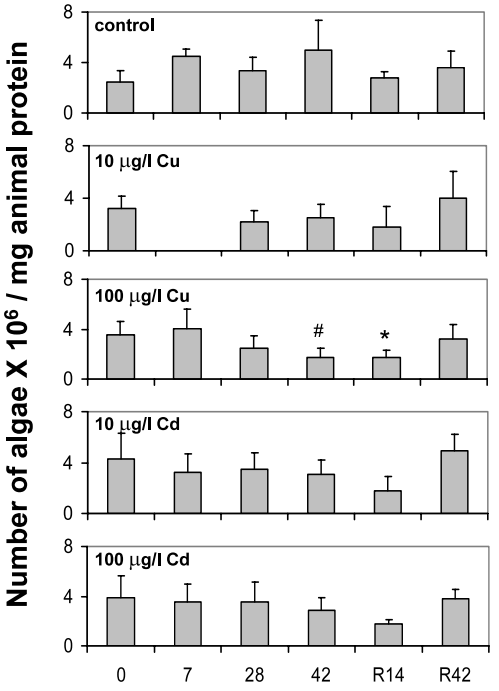


Fig. 3. Algal cell density in excised symbiotic anemone tentacles in control, Cd and Cu treatments (number of algal cells $\times 10^6$ mg$^{-1}$ animal protein). Data are expressed as means ($n = 6$) $\pm$ S.D. No statistically significant differences between the treatments and Day 0 values were observed except a decrease at Day 14 of recovery ($P < 0.05$, indicated by an asterisk, *). No statistically significant differences between treatments at each time point were observed ($P > 0.05$).

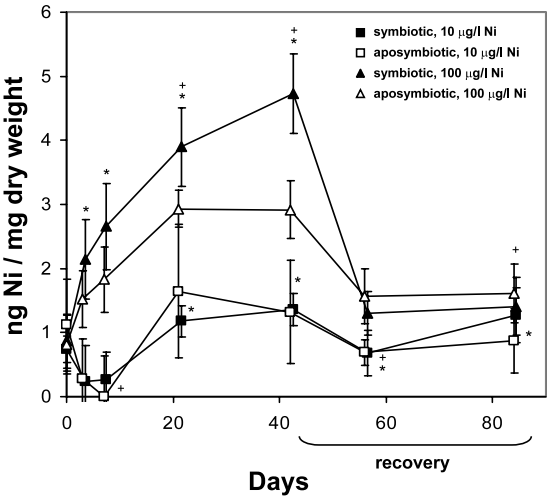


Fig. 4. Ni accumulation in symbiotic and aposymbiotic anemones exposed to 10 and 100 μg l$^{-1}$ Ni. Anemones were exposed for 42 days followed by a 42-day recovery. Data are presented as means ($\pm$ S.D.; $n = 7$–8) and expressed as ng Cu mg$^{-1}$ dry weight anemone tissue. Statistically significant differences for symbiotic (*) and aposymbiotic (+) anemones were observed within treatments over time when compared to their respective Day 0 values ($P$ at least $< 0.05$). For clarity purposes significance symbols (* and +) are grouped together by dose with the higher dose above the lower dose. Significantly greater levels of Ni accumulated in the symbiotic compared to aposymbiotic anemones following exposure to 100 μg l$^{-1}$ Ni at Days 3, 7, 21 and 42 ($P < 0.01$).

0.01) with the symbiotic anemones exhibiting a greater accumulation. However, no significant differences ($P > 0.05$) were seen in 10 μg l$^{-1}$ exposures between the anemone types. Following 100 μg l$^{-1}$ Ni exposures both anemone types significantly accumulated levels of Ni above Day 0 values. Following 100 μg l$^{-1}$ Ni exposures and then recovery in ambient seawater both the symbiotic and aposymbiotic anemones had significantly lower metal levels. Levels in symbiotic anemones were not significantly different ($P > 0.05$) to control values, however, levels in aposymbiotic animals were significantly higher ($P < 0.05$) compared with the original Day 0 pre-exposure levels.

The Zn concentrations of control anemones were higher in comparison with the other three metals, and over the time course of the experiment, no significant differences ($P > 0.05$) in controls were observed and levels were the same in both anemone types. Mean control values were 249.6 $\pm$ 33.4 and 215.6 $\pm$ 39.8 ng Zn mg$^{-1}$ dry weight for symbiotic and aposymbiotic anemones, respectively, over the seven time points. Zinc levels were frequently significantly higher in symbiotic compared with aposymbiotic anemones (Fig. 5). Maximal Zn levels were observed at Day 42 for both dose regimes in both anemones. Following recovery in ambient seawater, Zn concentrations of both the symbiotic and aposymbiotic anemones did decrease, but were significantly higher ($P < 0.05$) compared with original pre-exposure levels.

No statistically significant changes in algal cell densities in excised tentacle clips were observed in control anemones over the duration of the exposures (Fig. 6; average over seven time points 1.30 $\pm$ 0.21 algal cells $\times 10^6$ mg$^{-1}$ total protein). Statistically significant differences from Day 0

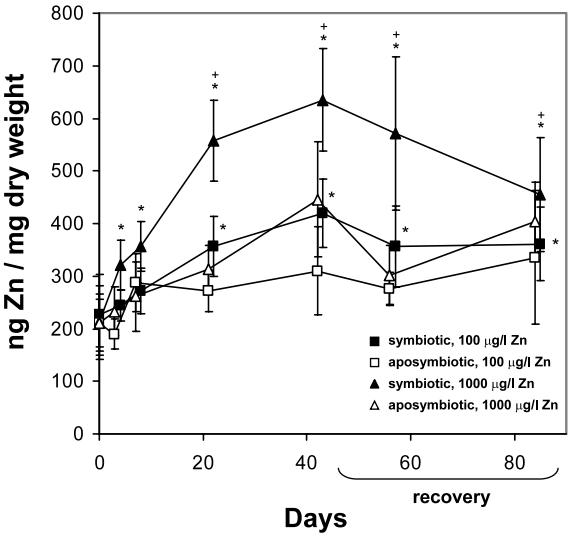


Fig. 5. Zn accumulation in symbiotic and aposymbiotic anemones exposed to 100 and 1000 μg $l^{-1}$ Zn. Anemones were exposed for 42 days followed by a 42-day recovery. Data are presented as means (± S.D.; $n = 6–8$) and expressed as ng Cu $mg^{-1}$ dry weight anemone tissue. Statistically significant differences for symbiotic (*) and aposymbiotic (+) anemones were observed within treatments over time when compared to their respective Day 0 values ($P$ at least $< 0.05$). For clarity purposes significance symbols (* and +) are grouped together by dose with the higher dose above the lower dose. Significantly greater levels of Zn were accumulated in the symbiotic compared to aposymbiotic anemones following exposure to 1000 μg $l^{-1}$ Zn except at Day 0 and Day 42 recovery ($P < 0.01$).

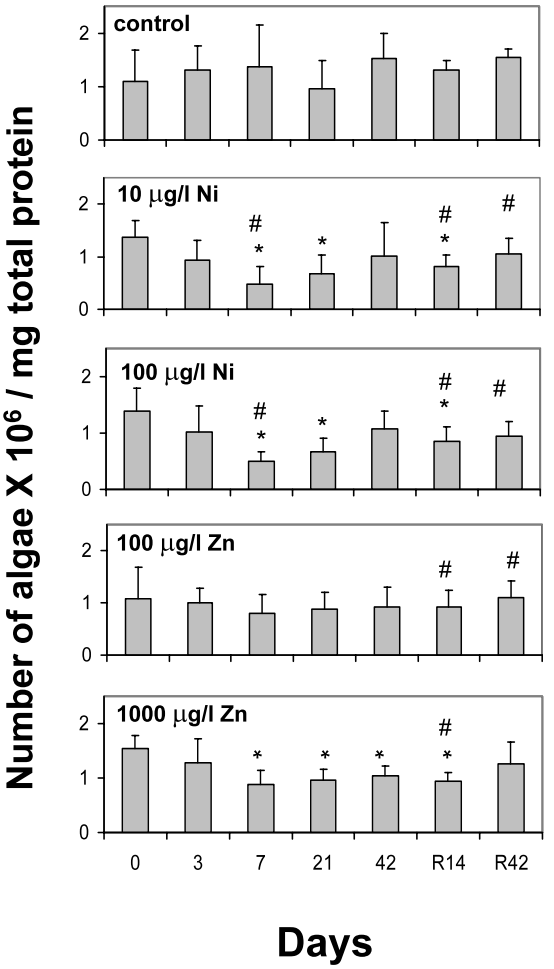


Fig. 6. Algal cell density in excised symbiotic anemone tentacles in control, Ni and Zn treatments (number of algal cells × $10^6$ $mg^{-1}$ total protein). Data are expressed as means ($n = 8$) ± S.D. An asterisk (*) indicates treatments that were significantly different ($P < 0.05$) from Day 0 values. A # indicates significant differences between treatments at each time point ($P < 0.05$).

($P < 0.05$) were observed in the Ni exposures at Days 7, 21, and recovery 14. At both doses this was significant compared with the respective controls on that day only on Days 7, recovery 14 and recovery 42. There was a trend of lower algal densities in the 1000 μg $l^{-1}$ Zn exposures from Day 7; although this is only significantly different from control values at recovery Day 14 ($P < 0.05$). No changes in MI were observed and an average MI of 0.2 ± 0.1% was observed over the duration of the experiment at all exposures.

## 4. Discussion

The results of these studies provide evidence that *A. elegantissima* can accumulate heavy metals and that differential uptake occurs depending upon metal type and symbiotic state. For Cd, Ni and Zn, higher levels of accumulation occurred in symbiotic compared with aposymbiotic anemones. However, the response to Cu exposure was strikingly different, with more Cu accumulation in aposymbiotic than in symbiotic anemones. Following recovery in ambient seawater, the tissue metal levels in anemones were reduced, often to near pre-exposure levels. Metal exposures in this study did not cause dramatic bleaching of symbio-

tic *A. elegantissima*. No significant changes in algal cell density were observed during the Cu and Cd exposures, although there was a tendency for reduced levels at the higher doses. Reduced algal cell densities were observed at some time points following Ni and high Zn exposures. Furthermore, algal cell densities returned to pre-incubation levels during the recovery period. This lack of dramatic algal cell loss is in contrast to accumulation studies performed on other anemones and corals (Evans, 1977; Esquivel, 1986; Howard et al., 1986; Harland and Nganro, 1990; Zamani, 1995; Jones, 1997; Mercier et al., 1997). Temperature stress could exacerbate the toxic effects of metals, and may be an important consideration when comparing temperate to tropical cnidarians. Brown (2000) made similar conclusions based on studies in European temperate anemones.

The extent of Cu accumulation and the higher levels accumulated by the aposymbiotic compared with symbiotic anemones is similar to results from a previous study on the European temperate anemone *A. viridis* (Harland and Nganro, 1990). Artificially bleached specimens of *A. viridis* accumulated more Cu than symbiotic ones and both anemone types accumulated Cu to a similar level at a comparable exposure time to anemones in this study. Harland and Nganro (1990) concluded that mucus production by the host could explain some of the differences observed between the two anemone types, although this protective mechanism has recently been questioned by Marshall (2002). Harland and Nganro (1990) also proposed a novel metal regulatory mechanism. They hypothesized that Cu was accumulated by the symbionts, which were preferentially expelled from the host, which would then result in lower levels of Cu accumulation in symbiotic compared with aposymbiotic animals. This theory does not explain the results in the present study as no significant loss of algal cells was observed in symbiotic *A. elegantissima* exposed to Cu. It is noteworthy that recent attempts to detect Cd and Cu in zooxanthellae by X-ray microanalysis in corals and anemones have been unsuccessful (see Marshall, 2002). The lack of visible bleaching and of algal loss with Cu exposure in our studies is similar to another study on *A. elegantissima* which showed no bleaching from exposures to 250 $\mu g\ l^{-1}$ Cu (Keel, 1994), but is in contrast to many other similar studies reported for other anemones and corals (Evans, 1977; Esquivel, 1986; Howard et al., 1986; Harland and Nganro, 1990; Zamani, 1995; Jones, 1997; Mercier et al., 1997). At the higher Cu dosage in this study, anemones remained contracted for the duration of the exposure and did not expand their tentacles until the recovery period. These observations were also made in other studies of anemones (Harland and Nganro, 1990; Keel, 1994; Mercier et al., 1997).

The response by *A. elegantissima* to Cd exposure was particularly interesting in that the anemones demonstrated signs of recovery from the metal before removal from the stress. First, anemones in high Cd exposures up to Day 7 appeared swollen and enlarged, suggesting an osmotic imbalance. Cd has been shown to disrupt cellular calcium homeostasis and osmotic balance in other organisms (see de la Torre et al., 2000). After Day 7, however, all specimens appeared normal and seemed to recover from the putative osmotic imbalance. Second, Cd levels in animals exposed to high Cd peaked at Day 28, only midway through the Cd exposure. By Day 42, levels in both anemone types were lower than those at Day 28. This suggests that, after an initial lag period, the anemones may have up-regulated metal efflux mechanisms or some mechanism for clearing the metal.

Symbiotic anemones accumulated Cd to a much greater extent than aposymbiotic anemones in contrast to the findings with Cu. These data agree with a Cd-exposure study comparing symbiotic *A. viridis* with a non-symbiotic species *A. equina*, although accumulation was much lower than in *A. elegantissima* (Harland et al., 1990). Field studies of cnidarians show a variation of responses to Cd accumulation. There was no difference in Cd accumulation in Red Sea corals collected from pristine and polluted sites in contrast to the responses of other metals, such as Cu, Zn and Ni (Hanna and Muir, 1990). However, Romeo et al. (1992) found increased levels of Cd in the pelagic hydroid *Velella velella* in polluted coastal compared with pristine oceanic sites.

Ni exposures had the greatest effect on algal cell density of any of the metals tested, although it was accumulated to the least degree. In addition, although levels were reduced following recovery, they did not return to pre-exposure conditions. Despite studies demonstrating Ni accumulation in corals and coral skeletons (e.g., Hanna and Muir, 1990), to date only one study on Ni effects in cnidarians has been described in which Ni exposures reduced survival of coral planulae in *Pocillopora damicornis* (Goh, 1991). No studies have been performed on adults.

The levels of Zn accumulation in the present study were similar to levels in European symbiotic and non-symbiotic anemones (Brown and Howard, 1985; Harland et al., 1990). Following recovery, a reduction in levels of Zn were observed in the present study although levels of Zn were significantly higher than Day 0 levels. Similarly, Harland et al. (1990) demonstrated a 50% reduction in Zn levels after 5 days recovery from exposure to dietary Zn in *A. viridis* in the laboratory. Studies on field-collected anemones also provide evidence of Zn accumulation in anemones. There was an almost twofold difference in Zn levels between *A. equina* samples collected from polluted versus pristine sites in the United Kingdom (Harland et al., 1990).

Our data show clear differences in metal accumulation between symbiotic and aposymbiotic anemones. It is possible that differential metal accumulation by the algal symbionts could account for some of the observed differences in accumulation. In the present study, we did not examine the partitioning of metals between the algal and host components, but this was examined for Cd in another study where we found no evidence for differential accumulation in the algae (see Mitchelmore et al., 2003). Although symbiotic *A. elegantissima* accumulated more Cd compared with aposymbiotic anemones (as in the present study), the algae did not function as sinks for Cd. At present, the mechanisms for increased, or in the case of Cu, decreased metal accumulation as a function of symbiotic state are unknown. Examination of metal partitioning in algal versus animal tissues in symbiotic anemones for other metals besides Cd is a topic for future studies.

The observations of (1) differential metal accumulation depending on metal type, (2) the maximal accumulation of Cd at Day 28 before the end of the stress, (3) the removal of accumulated metals during recovery, and (4) differential accumulation as a function of symbiotic state, all suggest that mechanisms exist to exclude, detoxify and clear accumulated metals in anemones. A common mechanism employed to exclude and control metal entry is the binding of metals by external mucus coatings. Indeed increased mucus production had been documented in cnidarians as a response to heavy metal exposure (Harland and Nganro, 1990; Mercier et al., 1997). Anemones in this study produced excessive amounts of mucus, particularly in the Cu exposures. Symbiotic anemones appeared to produce more mucus than aposymbiotic ones. Mucus production in other anemone species (Stambler and Dubinsky, 1987; Davy et al., 1996) and in corals (Muscatine and Weis, 1992) has been directly tied to the productivity of symbiotic algae. This may explain why aposymbiotic anemones, presumably producing less mucus than symbiotic animals, had higher levels of Cu accumulation than symbiotic anemones.

The sequestration of metals into granules has been demonstrated in cnidarians (Van-Praet, 1977; Gupta and Hall, 1984; Hyne et al., 1993) and in other marine invertebrates (review; Brown, 1982; Klerks and Bartholomew, 1991; Pullen and Rainbow, 1991; Mason and Jenkins, 1995) as a mechanism for regulating metals. This process may act in conjunction with mucus production such that metal granules are concentrated in mucus and sloughed off. Nematocyst discharge has also been shown to play a role in removing sequestered metal granules (Gupta and Hall, 1984; Tardent et al., 1990). Metal-rich granules have been demonstrated in both symbiotic partners in *Hydra* (Hyne et al., 1993) and could explain why symbiotic anemones contain more accumulated metal. It is also possible that metals are taken up by algae and subsequently deposited in host tissues, as was observed with arsenate accumulation in giant clam dinoflagellate symbioses (Benson and Simmons, 1981). This could explain why Marshall (2002) demonstrated a lack of metals

found in zooxanthellae from metal-exposed cnidarians.

Metal detoxification by binding to peptides or proteins is another metal regulatory mechanism that could be active in anemones. In a short-term study of Cd effects on *A. elegantissima* we have found higher levels of the metal-binding antioxidant glutathione (GSH) in symbiotic compared with aposymbiotic anemones (Mitchelmore et al., 2003). This may reflect a GSH contribution from the symbiont GSH and/or increased host GSH induced to counteract the oxygen radicals generated by symbiont photosynthesis (Dykens and Shick, 1982; Dykens et al., 1992). Reduction of GSH levels in metal-exposed organisms, including anemones (Meister and Anderson, 1983; Regoli and Principato 1995; Mitchelmore et al., 2003) and corals (Downs et al., 2000) in response to other stressors has been described. Regulation of metal levels by metallothionein (MT) in the host and/or phytochelatins in the symbiont is also possible. Although these two common metal detoxification pathways have been described in many species (Roesijadi, 1992; Torres et al., 1997; Poonguzhali and Rao, 1998) to date no evidence of these regulatory mechanisms has been shown in any cnidarian or dinoflagellate. Indeed, the only study to date on cnidarians suggests the absence of MT regulation in *Hydra* (Andersen et al., 1988), although only short-term (2–4 days) Cd and Zn exposures were investigated. Whatever the mechanisms for metal accumulation, differential uptake and removal, it is clear that metal regulation mechanisms exist in *A. elegantissima* and that this regulation is different depending on symbiotic state.

## 5. Conclusion

Considering the increasing threat of heavy metal impact to cnidarians in coastal environments, particularly in tropical regions where synergistic responses to elevated temperatures may exist, it is important to understand the responses to metal exposures in cnidarian-algal symbioses. Clearly, the differential responses of *A. elegantissima* to heavy metal exposure requires further attention and demonstrates that heavy metal accumulation and toxic effects depend upon metal type and the symbiotic state. Many questions need to be addressed regarding the regulatory mechanisms that must exist to control metal accumulation and detoxification in this invertebrate group.

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