



Differential accumulation of cadmium and changes in glutathione levels as a function of symbiotic state in the sea anemone *Anthopleura elegantissima*

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Abstract

Coastal ecosystems are increasingly impacted by anthropogenic activities and contaminant inputs, including heavy metals. This paper investigates the responses to laboratory cadmium exposures in the North American Pacific coast temperate sea anemone *Anthopleura elegantissima*. Anemones were exposed to cadmium chloride (control, 20 and 100 $\mu\text{g l}^{-1}$ Cd) for 14 days (sampled at days 0, 2, 7 and 14) and analyzed for Cd content using inductively coupled plasma mass spectroscopy (ICP-MS). Higher levels of Cd were demonstrated in symbiotic anemones compared to symbiont-free (aposymbiotic) anemones (e.g. 100 $\mu\text{g l}^{-1}$ Cd exposure at day 14; 12.4 ± 2.4 and 4.5 ± 1.9 ng Cd mg^{-1} dry weight, respectively; *t*-test; $p < 0.001$). These higher levels in symbiotic anemones were not due to increasing uptake of Cd by the algal symbionts (levels in algal-containing pellet fractions were constant over dose and time; *t*-test; $p > 0.05$) but rather to increased accumulation by both partners. No changes in algal cell density were observed in symbiotic anemone tentacle clips at any dose or time point (ANOVA; $p > 0.05$). Changes in glutathione (GSH) levels have been proposed as a useful biomarker in other marine invertebrates in response to heavy metal stress, but GSH studies have not been reported for this species. We demonstrate the presence of GSH in *A. elegantissima* and its depletion (58.2% of control values) following 1-week exposure to buthionine sulfoximine (BSO), a nontoxic chemical known to deplete GSH. Levels of GSH were higher in symbiotic compared to aposymbiotic anemones (control averages, 563 ± 81 and 485 ± 58 nmol GSH mg^{-1} wet weight, respectively; *t*-test; $p < 0.001$). Following Cd exposure, significant reductions (*t*-test; $p < 0.05$) in GSH levels were observed only in

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the aposymbiotic anemones (exposure to $100 \mu\text{g l}^{-1}$ Cd resulted in a 27% reduction from control levels at day 14). Our results demonstrate that the accumulation of Cd depends upon symbiotic state and provides the first identification of a GSH response in anemones and indeed in any cnidarian in response to heavy metals.

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1. Introduction

Heavy metals emanate from such sources as industrial discharges, sewage treatment discharges and anti-fouling paints, and the toxic effects of heavy metals on a variety of marine organisms have been documented (see [Mason and Jenkins, 1995](#)). Extensive research has been directed towards determining the extent and effects of metal pollution in fish and invertebrate species, particularly in *Mytilus* sp. which have become the species of choice for many biomonitoring programs (e.g. Mussel watch program, [US EPA, 1989](#)). The effects of heavy metals on cnidarians has received relatively little attention, despite the importance of cnidarians in many coastal communities worldwide. In temperate coastal communities, anemones are often conspicuous members of the fauna and 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*. We have used the abundant North American Pacific Coast temperate anemone, *Anthopleura elegantissima*, with its symbiont *Symbiodinium muscatinei*, as a model for the examination of cnidarian–algal symbiosis ([Weis and Reynolds, 1999](#); [Reynolds et al., 2000](#)). This anemone occurs naturally in both the symbiotic and the symbiont-free (aposymbiotic) state, thereby giving us a powerful comparative tool in the study of symbiosis. This allows us to investigate how the symbiotic state affects metal uptake and metal effects without using artificially bleached anemones.

In this study, we examine cadmium accumulation and the effects of cadmium on the *A. elegantissima*–*S. muscatinei* symbiosis. The rationale for choosing this symbiosis for study is three-fold. First, *A. elegantissima* is an important and abundant member of the rocky intertidal community from Alaska to Baja California ([Sebens, 1981](#); [Fitt et al., 1982](#)). Second, understanding the effects of heavy metals on this species will expand our current knowledge beyond a few well-studied organisms such as mussels. Finally, the *A. elegantissima*–*S. muscatinei* symbiosis can serve as a model for the study of the highly threatened coral–algal symbiosis.

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 is known as bleaching. Symbiotic *A. elegantissima*, like corals, has been demonstrated to

bleach under conditions of stress (Dyken and Shick, 1984; Engebretson and Martin, 1994). A few studies to date have examined the biological effects of metal accumulation by cnidarians, which includes a bleaching response (Evans, 1977; Harland et al., 1990; Harland and Nganro, 1990; Miller et al., 1992; Jones, 1997). However, there is little information on the cellular, biochemical and molecular events surrounding these occurrences, especially on the effects of heavy metal toxicity on the underlying regulation of symbiosis and on the responses of well-documented biomarkers of metal stress.

A common biomarker used in marine invertebrates, particularly with respect to metal exposure, is that of changes in total levels of the antioxidant, glutathione (GSH; Regoli and Principato, 1995; Ringwood et al., 1998; Downs et al., 2000). GSH serves many protective functions in cells, including metal-binding and scavenging reactive oxygen species that can be produced by some heavy metals (for a review, see Meister and Anderson, 1983; Ketterer, 1986). To investigate GSH responses, an inhibitor of an enzyme involved in the production of GSH is commonly utilized. This enzyme, γ -glutamyl synthetase, can be inhibited by incubating cells or organisms with buthionine sulfoximine (BSO). Alterations in GSH levels have included decreased, unchanged and even increased levels in metal-exposed organisms compared to controls (Ringwood et al., 1998). To date, no information exists on GSH responses in anemone species. A GSH response has been determined in one coral species in which a statistically significant reduction in GSH levels following exposure to elevated seawater temperatures was demonstrated (Downs et al., 2000).

Cadmium was the metal chosen for this study due to its nonessential function in biological organisms and toxic effects on marine organisms (Goering et al., 1995; Mason and Jenkins, 1995). Levels of cadmium in seawater are generally less than $3 \mu\text{g l}^{-1}$ (Sadiq, 1992). However, levels up to $50 \mu\text{g l}^{-1}$ have been detailed in polluted harbors and ports (Chester, 1990). In this paper, Cd accumulation in *A. elegantissima* and its dinoflagellate symbionts were examined using inductively coupled plasma mass spectroscopy (ICP-MS). In addition, we examined whether Cd accumulation was dependent upon the presence of symbionts, and whether the symbionts preferentially accumulated the metal. The effects of Cd on the loss of algal symbionts from the host, i.e. a bleaching response, were determined by following changes in algal cell density in excised tentacle clips. Finally, the antioxidant biomarker GSH was measured to ascertain whether changes in the levels of this biomarker were apparent following metal exposure, if differences exist in the response of aposymbiotic and symbiotic animals and whether the GSH response could serve as a useful early warning biomarker of stress in the cnidarian symbiosis system.

2. Materials and methods

2.1. Experimental design

Naturally occurring symbiotic and aposymbiotic specimens of *A. elegantissima* were collected from Indian Point, Ecola State Park, Oregon and transferred to a laboratory facility at the Environmental Protection Agency (EPA), Oregon State University's Hatfield

Marine Science Center in Newport, OR. Anemones were placed in 12.5-l plastic tanks in flowing seawater at 14 °C (± 1 °C). Metal halide lamps (5000 W) were used to provide adequate lighting conditions for algal photosynthesis ($200\text{--}250 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, 12:12-h light/dark cycle; Weis and Levine, 1996).

Following acclimatization for 2 weeks, anemones were placed in experimental tanks as follows; control, 20 and 100 $\mu\text{g l}^{-1}$ CdCl₂ exposure concentrations in seawater (10.5-l static fill), with tanks in duplicate (six tanks total). Experimental tanks were placed in a larger tank with flowing ambient seawater to maintain temperature at 14 °C (± 1 °C). Every 2 or 3 days, water was changed and tanks were randomly repositioned to offset any tank position effects. In each tank, 16 symbiotic anemones were placed on the left side of the tank and 16 aposymbiotic anemones were placed on the right. At least three anemones of each type were sampled from each tank on days 0, 2, 7 and 14. To sample, anemones were removed from the tanks, cleaned of mucus (using Kimwipes) 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 prior to analysis. An additional three symbiotic anemones per tank were placed on Plexiglas platforms in the middle of the tanks to monitor algal cell density. Tentacle clips from these animals were taken on each sampling day by anesthetizing the anemones by placing them in 50% 0.37 M MgCl₂ and 50% seawater solution. Three to five tentacles were excised from each anemone, frozen in liquid nitrogen and stored at -80 °C for later cell counts.

An additional experiment was carried out to analyze the glutathione response using symbiotic *A. elegantissima* collected from Seal Rock, Oregon and the mussel *Mytilus californianus*, which was used for comparison (collected from the same site). Two static tanks were set up as previously described. One served as the control and the other contained buthionine sulfoximine (BSO; 20 mg l⁻¹ in ambient seawater), a nontoxic chemical used to deplete glutathione (Ringwood et al., 1998). Four anemones and four mussels were placed in each tank and exposed for 7 days (water changed and re-dosed three times). Anemones were sampled as previously described, and mussels were dissected into digestive gland, gill and mantle tissue, frozen in liquid nitrogen and all tissues stored at -80 °C for later analysis.

2.2. Metal analysis

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), and centrifuged at $2000 \times g$ for 10 min. The resulting animal supernatant was placed in a pre-weighed, acid-washed 10-ml polypropylene tube. Visual inspections of the supernatant demonstrated that no algal cells were present and inspections of the pellet showed intact, unlysed algal cells and contaminating animal material. To address some of the problem of contaminating animal material, the pellet was further processed (see Discussion for further details). The pellet was resuspended with 1 ml of buffer and re-centrifuged at $2000 \times g$ for 10 min. The supernatant was removed and added to the animal supernatant tube and the process was repeated. To remove the top white layer of contaminating animal material (mainly nematocysts), 500 μl of buffer was added over the pellet and gentle pipetting removed the

majority of this white layer. This buffer washing was placed in the animal supernatant tube and the process was repeated. The algal pellet was then resuspended in 4 ml of buffer and placed in a pre-weighed, acid-washed 10-ml polypropylene tube. All samples were first frozen at $-80\text{ }^{\circ}\text{C}$, 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 $\sim 76\%$ water by comparing wet and dry weight values (76.3 ± 1.2 and 76.1 ± 1.2 , respectively, using $n=240$ anemones of each type).

For the digestion of tissues for metal analysis, 2 ml of trace-metal grade nitric acid (Fisher Scientific, USA) was added to all tubes and samples were then incubated overnight at $60\text{ }^{\circ}\text{C}$ in an oven, and the following day heated to $90\text{ }^{\circ}\text{C}$ for 2 h. A 1-ml sample of 30% hydrogen peroxide (Fisher Scientific) was added to each sample and incubations continued for an additional 2 h. Note that this step should be carried out using multiple small volume additions due to excessive bubbling. The tubes were cooled to room temperature and 4 ml of high-purity deionized water was added to all tubes. Analysis of metal content was carried out using a VG Elemental PQ ExCell ICP-MS machine as follows. A 500- μl sample was added to 100 μl of internal standard (containing I and Be) and diluted with 5 ml of 1% ultra-pure double-distilled nitric acid. Metal concentrations were calculated using the ^{111}Cd isotope. A set of calibration standards was added to the analysis together with appropriate blanks. A standard reference (NIST Oyster Tissue, 1566a; expected concentrations; $\text{Cd } 4.15 \pm 0.38\text{ }\mu\text{g g}^{-1}$) was analyzed to verify measurements. The mean measured concentration of standard material was $\text{Cd } 4.07 \pm 0.42\text{ }\mu\text{g g}^{-1}$ dry weight of oyster tissue.

2.3. Algal cell counts/mitotic index

To assess changes in algal cell density and mitotic index, tentacles were thawed, weighed and homogenized in $100 \times$ volume sterile-filtered ($0.2\text{ }\mu\text{m}$) seawater (FSW) and centrifuged at $1500 \times g$ for 10 min to pellet the algae. Algae were resuspended in $100 \times$ volume FSW and counted using a hemocytometer. Algal cell density was determined from hemocytometer counts of algal cells in 20 replicate samples taken directly from each homogenate and expressed as number of algal cells per milligram tentacle wet weight. Concurrently with density estimates, the mitotic index (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.

2.4. Glutathione assay

To investigate changes in levels of total glutathione, samples were analyzed using the 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) enzymatic recycling method as described by [Andersen \(1985\)](#) with modifications. Briefly, samples (anemone quarters or mussel digestive glands) were weighed (wet weight) and immediately homogenized on ice in five volumes of 5% salicylic acid (SSA). Samples were centrifuged at $10,000 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$ and the resultant supernatant kept on ice and used for analysis. Calibration

curves using reduced glutathione (GSH) in 5% SSA were prepared for each experiment. A 975- μl sample of assay buffer (containing 117 mM sodium phosphate, pH 7.8, 5.2 mM disodium EDTA and 1.03 mM DTNB, 0.18 mg ml^{-1} NADPH, at 25 °C) was added to 25 μl of sample in a cuvette and followed by 15 μl of 50 U ml^{-1} GSH reductase in buffer (143 mM sodium phosphate, 6.3 mM disodium EDTA, pH 7.8). The cuvette was inverted several times to initiate the reaction. Final concentrations of reagents in the reaction were; 0.12% SSA, 114.8 mM sodium phosphate, 5.1 mM disodium EDTA, 0.99 mM DTNB, 0.17 mg ml^{-1} NADPH, 0.74 U ml^{-1} GSH reductase. The formation of TNB is followed continuously by following absorbance at 405 nm from 0.25 to 1.75 min. The rate of change of absorbance in the linear portion of the curve from 0.5 to 1.5 min was calculated. The amount of GSH was determined from a standard curve in which GSH (10–180 μM) was plotted against the rate of change of absorbance. Results are expressed as nmol GSH mg^{-1} wet weight of anemone tissue.

2.5. Calculations and statistical design

All data sets were checked for compliance with the normality and variance homogeneity requirements for analysis of variance (ANOVA) testing. Results from replicate tanks in all assays were compared, and significant differences between treatments compared ($n=3-6$) using one-way analysis of variance (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 using ANOVA and subsequent unpaired two-tailed students *t*-tests, which were corrected for multiple comparisons using the Bonferroni correction ($n=6-9$; means \pm S.D.). To determine total Cd accumulation in anemones, both the animal supernatant fraction and the pellet fraction total Cd concentrations were combined and expressed as ng Cd mg^{-1} total dry weight. In the symbiotic anemones, the percentage of total Cd in the pellet fraction was calculated to determine the relative accumulation by the algal-containing pellet fraction. These percentage data sets were log transformed in order to comply with the requirements for ANOVA and subsequent *t*-test analyses.

3. Results

3.1. Metal accumulation and partitioning

Following laboratory exposures to Cd, both symbiotic and aposymbiotic anemones accumulated the metal. Levels increased with both time and concentration of metal over the exposure period. Fig. 1A and B demonstrates this time- and dose-dependent accumulation of Cd in symbiotic and aposymbiotic anemones, respectively. These levels are far higher than expected by passive diffusion between the anemone and seawater. *A. elegantissima* is approximately 76% water (see Methods); hence, it is possible that by passive diffusion, a maximum accumulation of 0.3 ng Cd mg^{-1} dry weight anemone tissue in 100 $\mu\text{g l}^{-1}$ Cd exposures would be expected. The accumulation values measured were an order of magnitude greater than these values. Higher levels of Cd were observed

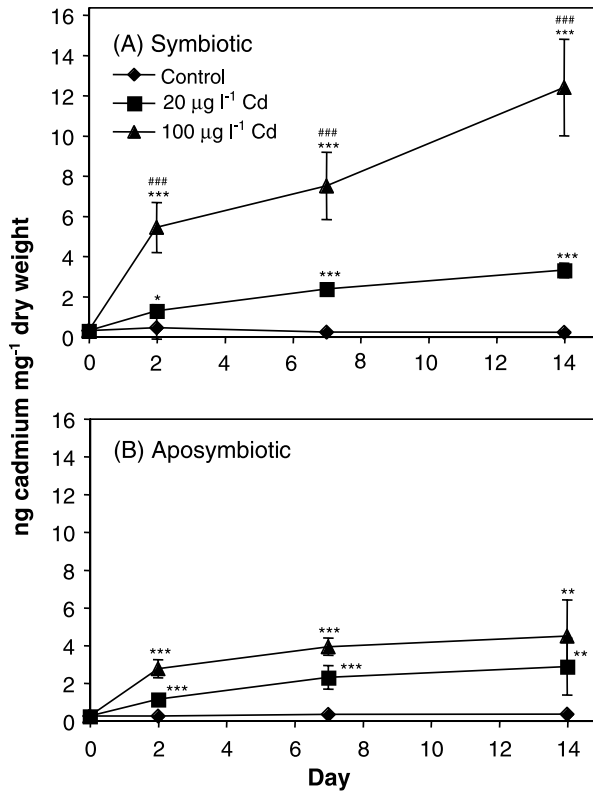


Fig. 1. Extent of Cd accumulation in symbiotic (A) and aposymbiotic (B) *A. elegantissima* following 14-day exposure to 20 and 100 $\mu\text{g l}^{-1}$ added Cd. Data expressed as means (\pm S.D.; $n = 6-9$) and presented as ng Cd mg^{-1} dry weight anemone tissue. No significant differences ($p > 0.05$) were observed between controls over time using unpaired two-tailed Student's *t*-test corrected for multiple comparisons using the Bonferroni adjustment. Statistically significant increases (*t*-test; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) were observed at 20 and 100 $\mu\text{g l}^{-1}$ compared to controls at all time points. Significant differences between symbiotic and aposymbiotic anemones were only observed following exposure to 100 $\mu\text{g l}^{-1}$ Cd (ANOVA; $p < 0.01$), whereby greater levels were accumulated in the symbiotic anemones (*t*-test; #### $p < 0.001$).

in symbiotic compared to aposymbiotic anemones at 100 $\mu\text{g l}^{-1}$ exposure (e.g. day 14; 12.4 ± 2.4 and 4.5 ± 1.9 ng Cd mg^{-1} dry weight, respectively; *t*-test; $p < 0.01$). However, exposure to 20 $\mu\text{g l}^{-1}$ Cd resulted in similar tissue levels in symbiotic and aposymbiotic anemones over the duration of the exposure period (day 14; 3.3 ± 0.4 and 2.9 ± 1.5 ng Cd mg^{-1} dry weight, respectively; *t*-test; $p > 0.05$).

The amount of Cd accumulated in the animal supernatant fraction and in the pellet fraction containing the algae is shown in Fig. 2A and B. Dose- and time-dependent increases were seen in both fractions with similar levels found in each fraction. Statistically significant differences (*t*-test; $p < 0.05$) between these two fractions in symbiotic anemones were only observed in the controls; possibly a result of measured levels of Cd being close to the level of detection for the ICP-MS. Aposymbiotic anemones were

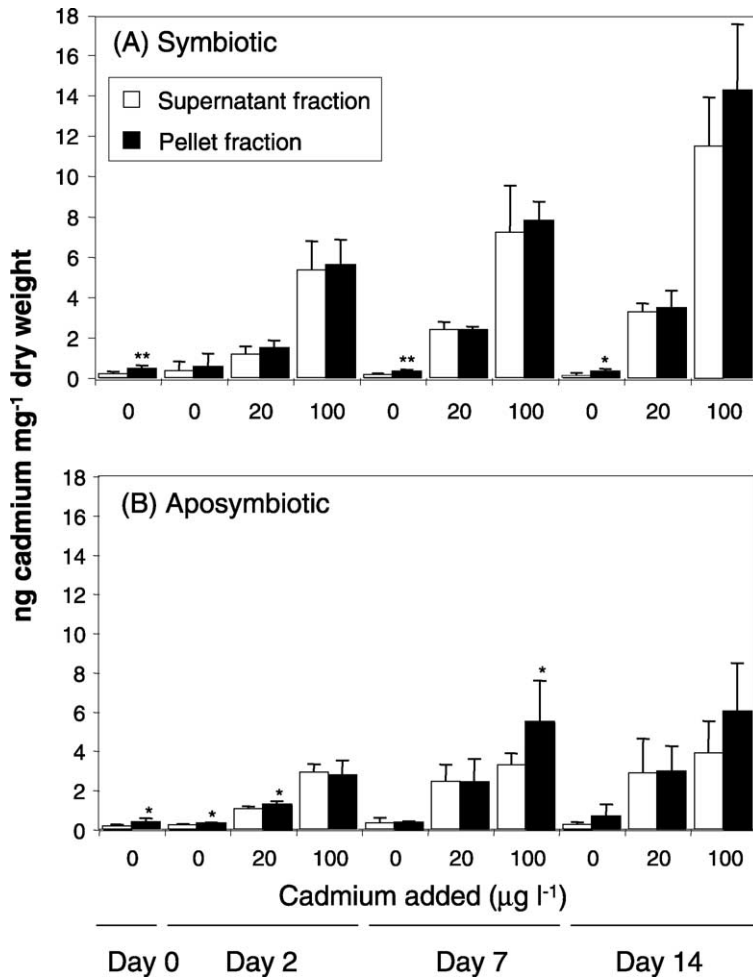


Fig. 2. Extent of Cd accumulation in *A. elegantissima* fractions; the soluble animal supernatant and the pellet fraction in symbiotic (A) and aposymbiotic (B) *A. elegantissima* following 14-day exposure to 20 and 100 $\mu\text{g l}^{-1}$ added Cd. Algae in symbiotic animals were in the pellet fraction. Data expressed as means (\pm S.D.; $n = 6-9$) and presented as ng Cd mg^{-1} dry weight. Statistically significant differences between each fraction pair (supernatant vs. pellet) were determined using unpaired two-tailed Student's *t*-test (* $p < 0.05$; ** $p < 0.01$).

processed in the same way as symbiotic animals and yielded pellet fractions of animal tissue containing accumulated Cd. Very few algae (if at all) were present in this aposymbiotic pellet, as observed by the lack of coloration and visual analysis under the microscope (in a subset of representative samples). This pellet consisted of animal fragments and nematocysts. The similar proportions of Cd accumulation in both fractions for both types of anemones with dose and time indicate that the algae are not preferentially taking up Cd. To investigate this further, the percentage of total Cd observed in the pellet

fraction in the symbiotic anemones was calculated. If algae were preferentially accumulating Cd, we would expect to see an increase in the percentage of total Cd in the pellet fraction with increasing dose and time. However, a similar percentage of the total Cd was continually present in this fraction in the symbiotic anemones irrespective of dose or time point (Fig. 3).

3.2. Algal cell numbers and mitotic index

There was no evidence of metal-induced bleaching in the symbiotic anemones. No statistically significant changes in algal cell density were observed in either control or metal-exposed anemones throughout the duration of the experiment (ANOVA; $p > 0.05$; Fig. 4). In addition, no changes (ANOVA; $p > 0.05$) in mitotic index were observed with an average mitotic index of $1.2 \pm 0.3\%$ observed over the duration of the experiment (data not shown).

3.3. Glutathione

Levels of GSH in control mussel (*M. californianus*) digestive glands were higher than the level of GSH in control symbiotic anemones (t -test; $p < 0.05$; Fig. 5). In both species, however, levels of GSH were depleted to a similar extent (approximately 58% of control values) following 1-week exposure to BSO (20 mg l^{-1}). These reductions were significantly different from the control values (t -test; at least $p < 0.05$). Samples from the metal-exposed anemones were analyzed for total glutathione levels and results are depicted for both symbiotic and aposymbiotic anemones in Fig. 6A and B, respectively.

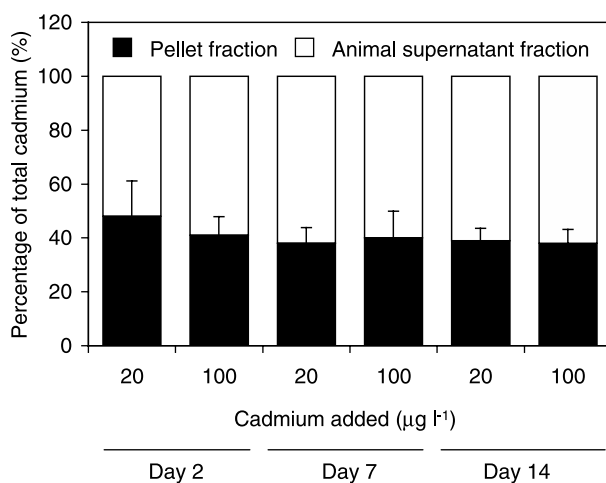


Fig. 3. Percentage of total Cd in the animal supernatant and algal-containing pellet fractions in symbiotic *A. elegantissima*. Data expressed as means (\pm S.D.; $n = 6-9$). Data were log transformed and comparisons were made using unpaired Student's t -test corrected for multiple comparisons using the Bonferroni adjustment. No statistically significant (t -test; $p > 0.05$) changes in levels of accumulated Cd in the algal-containing pellet fraction were observed with increasing dose and experimental duration.

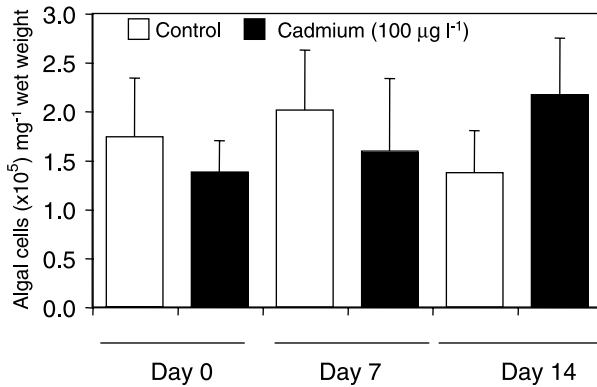


Fig. 4. Numbers of algal cells in excised symbiotic *A. elegantissima* tentacles in control and $100 \mu\text{g l}^{-1}$ added Cd treatments (number of algal cells mg^{-1} wet weight tentacle). Data expressed as means (\pm S.D.; $n=6-9$). No statistically significant (t -test; $p>0.05$) differences between treatments were observed over the duration of the experiment.

Symbiotic anemones contained more GSH compared to aposymbiotic anemones (ANOVA; $p<0.001$). For example, the average of all controls over time gave GSH levels of 563 ± 81 and $485 \pm 58 \text{ nmol GSH mg}^{-1}$ wet weight, respectively (t -test; $p<0.001$). Control values were not significantly different over time for both anemone types (t -test; $p>0.05$). No statistically significant changes (t -test; $p>0.05$) in GSH levels occurred in metal-exposed symbiotic anemones with time. The aposymbiotic anemones, however, did demonstrate a statistically significant (t -test; p at least <0.05) reduction in GSH at both days 7 and 14 when exposed to $100 \mu\text{g l}^{-1}$ Cd. Animals exposed to $100 \mu\text{g l}^{-1}$ Cd

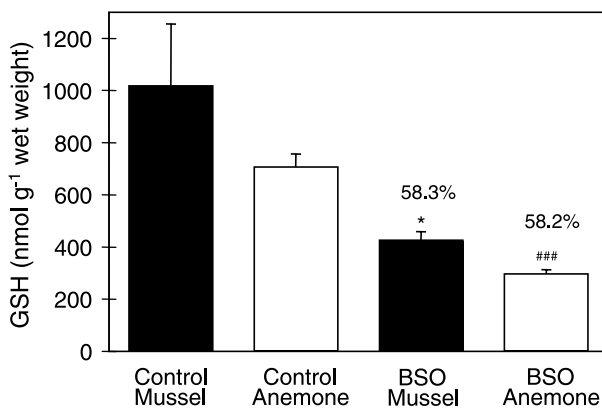


Fig. 5. Reduction of GSH levels in symbiotic anemones (*A. elegantissima*) and mussels (*M. californianis*) digestive gland cells following exposure to BSO (7 days; 20 mg l^{-1}). Data expressed as means (\pm S.D.; $n=4$) and presented as nmol GSH mg^{-1} wet weight tissue. Statistically significant reductions ($*p<0.05$; $###p<0.001$) from respective control values were observed in both species exposed to BSO.

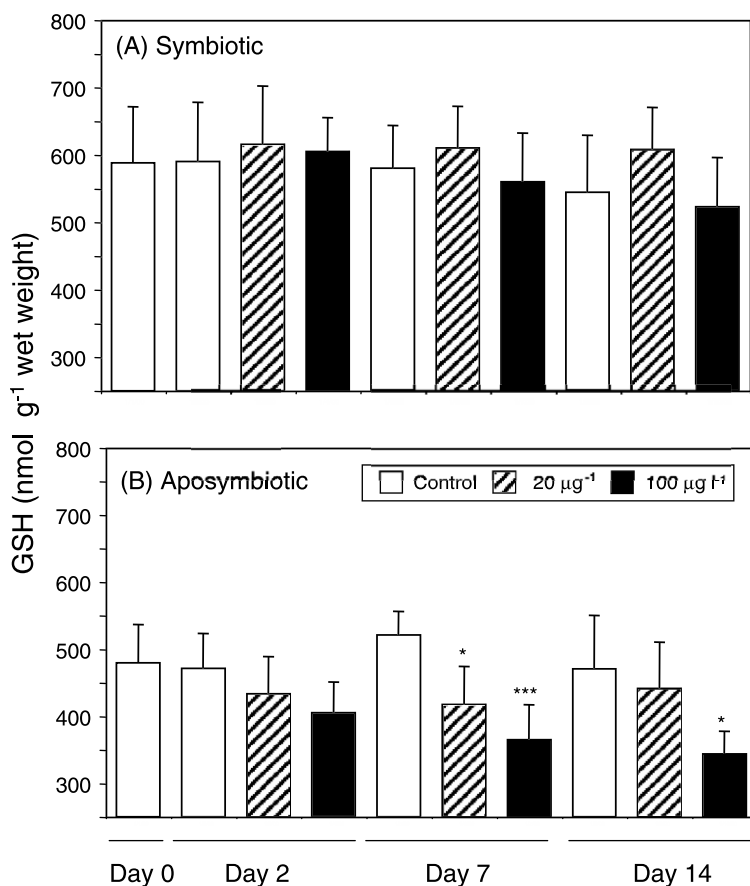


Fig. 6. Levels of GSH in symbiotic (A) and aposymbiotic (B) *A. elegantissima* following a 14-day exposure to 20 and 100 $\mu\text{g l}^{-1}$ added Cd. Data expressed as means (\pm S.D.; $n=6-14$ symbiotic, $n=6$ aposymbiotic) and presented as nmol GSH mg^{-1} wet weight anemone tissue. Higher levels in symbiotic anemones compared to aposymbiotic anemones were observed (ANOVA; $p < 0.05$). Statistically significant decreases (t -test; * $p < 0.05$, *** $p < 0.001$) were observed at 100 $\mu\text{g l}^{-1}$ Cd at days 7 and 14 compared to controls in aposymbiotic anemones, using unpaired Student's t -test corrected for multiple comparisons using the Bonferroni adjustment.

exhibited a 30% and 27% reduction of GSH levels at days 7 and 14, respectively, when compared to control animals at each day.

4. Discussion

We present clear evidence that *A. elegantissima* accumulates Cd in a time- and dose-dependent manner and that differential uptake occurs depending upon symbiotic state. Higher levels of accumulation occurred in symbiotic compared to aposymbiotic anemones. Previous studies exposing anemone species to Cd have demonstrated low levels of Cd

accumulation. For example, less than $0.1 \mu\text{g Cd g}^{-1}$ dry weight was observed in the symbiotic anemone, *Amenonia viridis*, and the non-symbiotic anemone, *Actinia equina*, following 5-day exposures to $5 \mu\text{g l}^{-1}$ Cd (Harland et al., 1990). Low levels of Cd, compared to other marine organisms, were observed in *A. equina* (up to $0.38 \text{ ng Cd mg}^{-1}$ dry weight) collected from Cd-polluted field sites. However, these levels in *A. equina* were nearly 12 times higher than levels of accumulation observed in anemones from control sites (0.032 ng mg^{-1}) suggesting some uptake of Cd in polluted sites (Harland et al., 1990). It is also possible that the higher levels of Cd accumulation in the present study may simply reflect species differences between the Oregon coast anemone *A. elegantissima* and the British anemones *A. viridis* and *A. equina*.

In the present study, symbiotic anemones accumulated Cd more than aposymbiotic anemones (2.8-fold) at the higher concentrations, a pattern that was also shown by the previous Harland et al. (1990) study, in which the symbiotic anemone *A. viridis* accumulated more Cd in comparison to the non-symbiotic *A. equina*. Differences in heavy metal accumulation as a function of symbiotic state have also been previously reported by Harland and Nganro (1990) in *A. viridis* (symbiotic and artificially bleached animals) following laboratory exposures to copper (Cu). However, in contrast to the cadmium studies, greater levels of Cu were observed in the aposymbiotic anemones. Harland and Nganro (1990) proposed that preferential accumulation of Cu by zooxanthellae and their subsequent expulsion led to the observed higher levels of Cu in aposymbiotic cf. symbiotic anemones. However, in the present study, no loss of algal cells were observed in symbiotic *A. elegantissima*, even though this is a common response to heavy metals in other anemone and coral species (Evans, 1977; Esquivel, 1986; Howard et al., 1986; Harland and Nganro, 1990). This lack of algal cell loss in the present study could therefore explain why symbiotic anemones accumulate more Cd.

We aimed to investigate this regulatory mechanism proposed by Harland and Nganro (1990) by determining the levels of Cd accumulation in the algal-containing pellet fraction and animal supernatant fractions. It was evident that our algal pellet fraction was contaminated with animal tissue (microscopic inspection revealed animal fragments and nematocysts). It has been estimated that zooxanthellae represent no more than 10% of total anemone dry tissue weight (Harland et al., 1990). In the present study, the percentage of dry tissue weight in this fraction ranged between 40% and 50% of the total dry weight. Contamination of this fraction was also evident from the results depicted in Fig. 2B, using aposymbiotic (algal-free) anemones which demonstrated similar partitioning results to those observed in the symbiotic anemones (Fig. 2A). Metal accumulation in the pellet could be due to animal membrane fragments, nematocysts, known to accumulate metal cations (Tardent et al., 1990) and/or the presence of metal-rich granules (see review by Brown, 1982), which have been shown to occur in anemones (Van-Praet, 1977). However, despite the contamination of this fraction with animal material, it is still possible to address the question of whether algae preferentially accumulate Cd by calculating the percentage of total Cd in the pellet fraction. If the algae preferentially take up Cd, we would expect to see an increase in the percentage of total Cd in this fraction at higher dose and time points. No such elevation was observed in this study; in contrast, the percentage of Cd in the pellet remained constant through time, even at the highest dose. These results do not negate the involvement of the symbionts, as indeed their presence is correlated with an increase in total

Cd accumulation (at $100 \mu\text{g l}^{-1}$ exposures) over levels in aposymbiotic animals. It is possible that the Cd is taken up by algae and subsequently deposited in host tissues, as was observed with arsenate accumulation in giant clam symbioses (Benson and Simmons, 1981). At present, the mechanisms for increased metal accumulation by symbiotic anemones are unknown. It is likely that the symbiotic partners acting in concert have different mechanisms for handling, detoxifying and eliminating metals than do anemones alone. Further, other physiological differences between the symbiotic and non-symbiotic state, such as differential metabolic rate, could result in differential accumulation.

This is the first paper to report levels of GSH in anemones and the response of GSH levels to heavy metal stress in any cnidarian. Although some data are available on enzymes in the glutathione pathway in cnidarians, including anemones (Gassman and Kennedy, 1992; Hawkridge et al., 2000), no data exist on levels of glutathione in anemones. In marine invertebrates, changes in levels (decreases or increases) of GSH have been implicated as a possible biomarker of stress, including heavy metal stress (e.g. Regoli and Principato, 1995; Ringwood et al., 1998). We employed an experimental regime similar to that used to investigate the GSH response in oysters (Ringwood et al., 1998). Following 7 days of exposure to BSO, we observed statistically significant reductions in levels of GSH to 58.2% of control values in symbiotic anemones. A similar reduction in GSH levels was observed in *M. californianus*, which was used as a positive control. In a preliminary experiment, a low turnover of GSH compared to other marine invertebrates was suggested. No changes in GSH levels were observed in symbiotic anemones following a 48-h exposure to BSO (data not shown) which is in contrast to the 65% GSH reduction observed in oysters using this exposure regime (Ringwood et al., 1998). Despite the wealth of information on the GSH response in other marine invertebrates following a variety of insults, to date, only one study has investigated this response in cnidarians. Downs et al. (2000) demonstrated that levels of GSH decreased in the coral *Montastraea faveolata* following exposure to elevated seawater temperatures ($2.6\text{--}1.3 \mu\text{M GSH } \mu\text{g}^{-1}$ protein) and concluded that this may be a useful biomarker to assess coral health.

In addition to showing the presence of GSH in anemones, this study demonstrates that levels of GSH are higher in symbiotic compared to aposymbiotic anemones. This difference may be attributed to a variety of factors associated with the symbiotic state. First, GSH in symbiotic animals includes any activity from resident symbionts. It has previously been reported that *Symbiodinium* spp. contain various antioxidant enzymes (Shick et al., 1995), although GSH levels have not been measured in zooxanthellae. Symbiotic host cnidarians have also adapted to protect themselves against the high oxygen tension in their tissues originating from photosynthesis by their symbionts (Dykens and Shick, 1982; Dykens et al., 1992; Gassman and Kennedy, 1992). Indeed, there are many reports demonstrating higher levels of antioxidant enzyme activities in symbiotic compared to aposymbiotic host tissues, including those in *A. elegantissima* (Dykens and Shick, 1984). High GSH levels could be among the antioxidant defenses in host tissues.

In the present study, statistically significant reductions in the levels of GSH were seen in metal-exposed aposymbiotic anemones, although a lack of response was observed in symbiotic anemones. Loss of GSH from Cd exposure may be expected due to conjugation reactions of the metal with GSH, thereby reducing the total pool of GSH available (Meister

and Anderson, 1983). The differences in GSH responses to metal exposure in the two types of anemones may reflect the masking of the symbiotic response due to algal GSH presence.

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