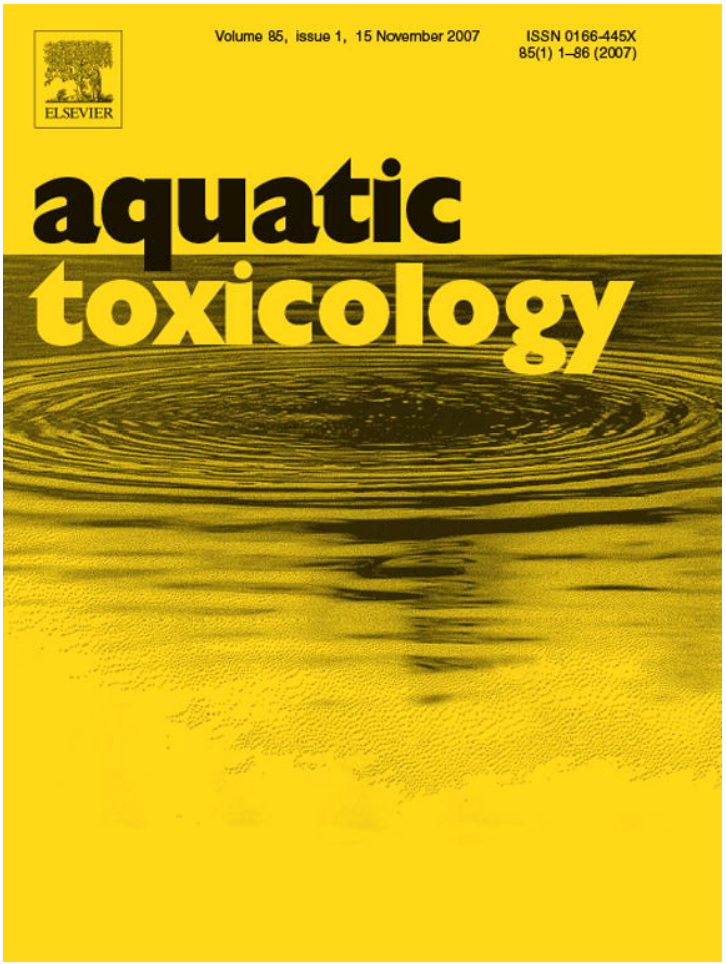
Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article was published in an Elsevier journal. The attached copy is furnished to the author for non-commercial research and education use, including for instruction at the author's institution, sharing with colleagues and providing to institution administration.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

ELSEVIER

Aquatic Toxicology 85 (2007) 48–56

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Uptake and partitioning of copper and cadmium in the coral *Pocillopora damicornis*

Carys L. Mitchelmore [a,*], E. Alan Verde [b], Virginia M. Weis [c]

[a] *University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, 1 Williams Street, P.O. Box 38, Solomons, MD 20688, United States*

[b] *Corning School of Ocean Studies, Maine Maritime Academy, Castine, ME 04420, United States*

[c] *Department of Zoology, Oregon State University, 3029 Cordley Hall, Corvallis, OR 97331, United States*

Received 30 April 2007; received in revised form 26 July 2007; accepted 27 July 2007

Abstract

Coral-reef ecosystems are increasingly being impacted by a wide variety of anthropogenic inputs, including heavy metals, which could be contributing to coral reef stress and bleaching episodes. Fragments of *Pocillopora damicornis* were exposed in the laboratory to cadmium (Cd) or copper (Cu) chlorides (0, 5, 50 $\mu g\,l^{-1}$) for 14 days and analyzed for metal content in the whole association, algal or animal fractions. Various physiological and biochemical parameters were also measured, such as, algal cell counts, mitotic index, chlorophyll content and levels of the antioxidant glutathione (GSH). Cd and Cu accumulation were observed at all time points and doses; there was no evidence of differential metal partitioning between the algal or animal fractions. No changes in algal cell density, mitotic index or chlorophyll content from the controls were observed in any of the metal treatments. GSH levels were significantly higher in the 5 $\mu g\,l^{-1}$ Cd (Day 4) and Cu (Days 4 and 14) treatments compared with controls at the same time point. Although no evidence of a bleaching response occurred, corals in both 50 $\mu g\,l^{-1}$ metal exposures sloughed off tissues and did not survive the duration of the exposure period. Our results demonstrate the accumulation of Cd and Cu in *P. damicornis* and mortality in the absence of a bleaching response.

© 2007 Elsevier B.V. All rights reserved.

Keywords: *Pocillopora damicornis*; Corals; Copper; Cadmium; Glutathione (GSH)

1. Introduction

The mutualistic symbiosis between members of the phylum Cnidaria and dinoflagellate algae in the genus *Symbiodinium* forms both the trophic and structural foundation of coral reef ecosystems (Birkeland, 1996). An increase in the brekdown of this coral–algal association (i.e. coral bleaching) has been occurring globally and has become a common sight in many reefs (Wilkinson, 1999). Coral bleaching can ultimately lead to increased coral mortality, which in turn contributes to various levels of degradation of this important ecosystem. For example, it has been estimated that more than 25% of the coral reefs have been lost worldwide. If this trend continues some have suggested that, in less than 50 years, the majority of the world's reefs will have disappeared (Wilkinson, 1999). Dramatic changes occurring in coral reefs since the 1970s suggest that human activities have had a major impact in these events (Pandolfi and Jackson, 2001). Reefs are experiencing environmental perturbations from a variety of "natural" and anthropogenic sources, which are increasing in frequency and magnitude. These impacts may act in isolation; however, it is more likely that this degradation results from the impact of multiple stressors resulting in synergistic interactions between physical (e.g. temperature, UV light) and chemical factors, including heavy metals (Meehan and Ostrander, 1997; Brown, 2000).

Heavy metals are well known marine pollutants that emanate from such sources as industrial discharges, urban/agricultural run-off, sewage treatment discharges and anti-fouling paints. Many organism's bioconcentrate heavy metals to levels 10 to >10,000 times environmental levels leading to extremely high tissue loads and the toxic effects of heavy metals on a variety of marine organisms have been well documented by Mason and Jenkins (1995). Extensive research has been directed towards determining the extent and effects of metal pollution in fish and bivalve species, however, the effects of heavy metals on corals have received minimal attention despite their ecological

* Corresponding author. Tel.: +1 410 326 7283; fax: +1 410 326 7210.
E-mail address: Mitchelmore@cbl.umces.edu (C.L. Mitchelmore).

0166-445X/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2007.07.015

Author's personal copy

importance and evidence of metal accumulation in reefs and in surrounding sediments (Howard and Brown, 1984; Scott, 1990; Guzman and Jimenez, 1992). A few studies have investigated the use of corals as biomonitors of heavy metal pollution and demonstrated elevated metal content at polluted compared to reference sites (Hanna and Muir, 1990; Guzman and Jimenez, 1992). Additional studies have demonstrated the resulting biological effects of metal exposure including a bleaching response (Jones, 1997), effects on coral or zooxanthellae growth (Jones, 1997; Scott, 1990; Goh and Chou, 1997), metabolism (Nystrom et al., 2001), respiration (Howard et al., 1986), decreased levels of carbonic anhydrase activity (Gilbert and Guzman, 2001), alterations in gene expression levels (Morgan et al., 2001; Mitchelmore et al., 2002), larval mortality and inhibition of reproductive endpoints, such as, fertilization, metamorphosis, settlement success and motility (Esquivel, 1986; Goh, 1991; Reichelt-Brushett and Harrison, 2005).

Ultimately the effects of heavy metals depend upon their uptake and partitioning by the coral. Esslemont et al. (2000) highlighted some similarities and differences in concentrations (partitioning) of various heavy metals (e.g. Cd and Cu) between skeletal and tissue levels in *Pocillopora damicornis*. Preferential partitioning of metals in the algal (compared with animal) components has also been suggested in some symbiotic anemone species (Harland et al., 1990; Harland and Nganro, 1990) but not others (Mitchelmore et al., 2003a,b). Within animal tissues the sequestration of metals into the mucus component has also been shown (Reichelt and Jones, 1994) and excessive mucus production by anemone species exposed to copper appears to be an important response to reduce metal uptake (Mitchelmore et al., 2003a,b).

Copper (Cu) and cadmium (Cd) were the metals chosen for this study because Cu is an essential element required by organisms in trace amounts whereas Cd serves no essential function in biological organisms (Goering et al., 1995); however, at higher concentrations these heavy metals can exert toxic effects on marine organisms. Cu and Cd can affect the function of metal-requiring enzymes and proteins, and cause oxidative stress by generating hydroxyl radicals albeit via different mechanisms depending upon the metal species (Mason and Jenkins, 1995).

Cu concentrations in seawater have been documented at nearly 30 $\mu g\,l^{-1}$ at highly polluted sites (Sadiq, 1992). Data from the Hawaiian Department of Health (DOH) from a variety of coastal sites around Oahu in the mid-1990s demonstrated chronic levels of Cu to be in the range of 0.012–2.331 $\mu g\,l^{-1}$ (Hunter et al., 1995). Levels of Cd in seawater are generally less than 3 $\mu g\,l^{-1}$ (Sadiq, 1992) although levels up to 50 $\mu g\,l^{-1}$ have been detailed in polluted harbors and ports (Chester, 1990). Chronic levels of Cd in seawater from various sites around Oahu in the mid-1990s were demonstrated to be less than 0.14 $\mu g\,l^{-1}$ (Hunter et al., 1995).

In light of recent observations of increases in coral–algal symbioses breakdown or coral bleaching we investigated the extent and partitioning of heavy metal accumulation and the effects of heavy metal exposure on the coral *P. damicornis*, a species stated by Esslemont et al. (2000) to be a good indicator species. We chose concentrations of 5 and 50 $\mu g\,l^{-1}$ metal for our exposures to represent, at the lowest concentration, levels that may be attained in the environment, together with a higher dose, representative of a more acute environmental concentration. We were interested in determining the degree of accumulation of different metals through time in coral tissues, whether accumulation was similar between metal types, whether partitioning of metals occurred between the algal and animal fractions and whether exposure resulted in symbiosis breakdown (bleaching) or alterations in antioxidant capacity (glutathione (GSH) levels) resulting from oxidative stress.

2. Materials and methods

2.1. Animal collection and maintenance

Five colonies of *P. damicornis* were collected during September 2002 on a reef within Kaneohe Bay, Hawaii and transported to the University of Hawaii's Kewalo Marine Laboratory, Honolulu, Hawaii. The coral colonies were sectioned into multiple fragments ($n = 11$ per colony; 55 fragments total) and maintained in ambient light and flowing seawater ($29 \pm 1\,^{\circ}C$) for 14 days prior to the addition of metals in their respective exposure tanks. One day prior to experimental onset, coral fragments were placed in seasoned 12.5 l clear plastic exposure tanks (as detailed below) in the same running seawater. Light intensities impinging on the coral fragments were measured with a quantum meter (QMSW-SS, Apogee Instruments) equipped with a submersible sensor calibrated to solar irradiance by the manufacturer. Daily maximum irradiance levels were observed to be greater than 2000 $\mu mol\ quanta\ m^{-2}\,s^{-1}$ during the late morning (11:00 h) and early afternoon (13:00 h). "Optimal" irradiance regimes ($\sim$800 $\mu mol\ quanta\ m^{-2}\,s^{-1}$) were achieved by screening the static tanks with neutral density black mesh screen to reduce the heating effects (see below).

2.2. Experimental design and metal exposures

Corals were placed in experimental tanks 1 day prior to the start of the exposure experiment as follows. In all five tanks, two fragments of each coral ($n = 5$ colonies for 2 sampling times (Day 4 and Day 14), total $n = 10$ pieces) were placed in specific locations so as to track individual colonies. Five extra pieces were placed in the control tank and represent Day 0 control samples. Metal exposures consisted of three doses: 0, 5 and 50 $\mu g\,l^{-1}$ total Cu or Cd (using chloride salts) in ambient seawater (10.5 l static fill). These static tanks were placed in larger tanks in which circulating ambient seawater was flowing to regulate water temperature. Metal exposure was carried out for 14 days with water changes and metal dosing carried out every day (between 6 and 7 a.m.) with tanks randomly repositioned multiple times to offset any position effects. Complications as to heat exchange between the static tanks and circulating tanks occurred in the first 2 days (0.25–1.0 °C increase in temperature for a few hours around noon). This was solved by adding additional black mesh screens to shade the tanks (reduction in maximal light values from >2500 to $\sim$800 $\mu mol\ quanta\ m^{-2}\,s^{-1}$), so that temperature remained constant throughout the rest of the exposure

Author's personal copy

period. In the late afternoon these screens were removed and subsequently replaced each morning following the metal-water changes.

We investigated the suitability of a variety of buffers to compromise between low salt levels (i.e. to reduce matrix effects in metal ICP-MS analyses) yet still obtaining competent algal cells. Four coral fragments left over after splitting the colonies for metal exposures were water-picked (as detailed below) in either: 50 mM ammonium phosphate (pH 8.0), 10 or 50 mM sodium phosphate buffers (pH 8.0) or filtered artificial seawater (pH 8.2). In both the 50 mM ammonium phosphate and 10 mM sodium phosphate buffers algal cells were compromised (reduced cell counts over time; data not shown) whereas this was not observed with the other two buffers. To reduce interference in metal determinations using the ICP-MS the less complex (compared to artificial seawater) 50 mM sodium phosphate buffer (pH 8.0) was chosen for this study.

At Day 0, one sample from each of the coral colonies ($n = 5$) in the control tank were processed. Corals were removed from the tanks and each coral fragment was exposed to filtered seawater for a minimum of 5 min to rinse off any residual metal-exposure seawater prior to processing. The coral tissue was removed from the skeletal matrix via jets of ice-cold extraction buffer (25 ml of 50 mM sodium phosphate buffer, pH 8.0) by using a commercial oral hygene device. The final homogenate volume was measured and samples kept on ice for processing in various subsequent assays. Subsamples for protein analysis were frozen, thawed, resuspended and processed by the BCA assay (Pierce Chemical) via the 2 h room temperature incubation protocol. Bovine serum albumin was used to construct a standard curve.

2.3. Metal analysis

Coral samples were prepared for metal analysis as follows. A set volume (6–8 ml) of the coral homogenate (total fraction/whole association) was placed in a pre-weighed acid washed 10 ml polypropylene tube and frozen. In addition a second sample of homogenate (6–7 ml) was centrifuged at ~2000 g for 10 min to pellet the algal fraction. The supernatant (animal fraction) was removed and placed in another pre-weighed acid washed 10 ml polypropylene tube. Both the algal pellet and animal supernatant fractions were subsequently frozen at −20 °C. All samples were lyophilized and weighed to determine dry weight of sample (i.e. total, algal or animal fractions). It should be noted that calculations of total fraction weight by combining algal and animal fraction weights were within 5% of the corresponding aliquots of total fraction weight. Corresponding blanks were processed in the same way using the buffer alone for all steps. The average dry weight of the buffer blanks were subtracted from all total and animal fraction samples to adjust for buffer content.

For the digestion of tissues for metal analysis, 2 ml of metal-analysis grade nitric acid was added to all tubes and the samples were incubated overnight at 60 °C, and then at 90 °C for 2 h. One ml of ultra-pure grade hydrogen peroxide was added to each sample (in steps to diminish excessive effervescence) and incubations continued for an additional 2 h at 90 °C. The tubes were cooled to room temperature and 4 ml of ultra high quality water was added to all tubes. Analysis of sample metal content was carried out using ICP-MS as follows. For Cu and Cd analysis, 500 μl of sample was added to 100 μl of internal standard (containing indium (I) and beryllium (Be)) and diluted with 5 ml of 1% ultra-pure double distilled nitric acid. Metal concentrations were calculated using the following isotopes: ^{111}Cd and ^{65}Cu. A set of calibration standards was added to the analysis together with appropriate blanks. Sets of a standard reference material (NIST Oyster Tissue, 1566a; expected concentrations; Cd 4.15 ± 0.38, Cu 66.3 ± 4.3 μg g^{-1}) were analyzed to verify measurements. The mean measured concentration of standard material was 3.99 ± 0.42 for Cd and 66.1 ± 3.3 μg g^{-1} dry weight of oyster tissue for Cu.

2.4. Algal parameters

Density and mitotic index: Algal density was determined from hemocytometer counts of algal cells in 20 replicate samples taken directly from each homogenate. 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.

Chlorophyll: Three replicate 0.5 ml samples of the homogenate were filtered through GF/C filters, each followed by a wash of 1% $MgCO_3$ (0.5 ml) and the filters folded and frozen. Within 12 h of freezing the filters were macerated in 4 ml of 90% acetone (spectrophotometric grade) and the chlorophyll extracted for 12 h in the dark at 4 °C. The chlorophyll preparation was centrifuged for 5 min to pellet the glass fiber fragments and algal cell debris. The absorbance's of the acetone supernatant was determined with a Varian spectrophotometer and were converted to chlorophyll *a* and *c* mass by the equations of Jeffrey and Humphrey (1975) as described by Parsons et al. (1984).

2.5. Measurement of GSH levels

Due to the rapid oxidation and breakdown of GSH and the unsuitability of freezing samples for future processing, samples were immediately processed (<10 min on ice) for levels of glutathione (GSH) using the method described in the commercial GSH-420 glutathione assay kits (Cat. No. 21023; obtained from Oxis International Inc., Portland, OR, USA). Briefly, to 100 μl of samples (in triplicate), 500 μl of reagent 1 (acidic protein precipitant which stabilizes any further GSH degradation) was added and the samples mixed. Subsequent steps were carried out as detailed in the manufacturers protocol. Following incubations in the dark for 30 min, the samples were assayed using a Varian spectrophotometer.

2.6. Calculations and statistical design

All data were checked for normality and homogeneity of variances prior to statistical analysis. In cases where data sets were non-normal or heterogeneous, the data were log transformed prior to analysis. All percentage data (MI and algal–animal metal

Author's personal copy

fraction) were square-root arcsine transformed prior to analysis (Zar, 1999). Using Minitab software metal concentration differences were compared using one-way ANOVA followed by post hoc Tukey (HSD) tests. Statistical significance levels were defined as follows: N.S.: not significant ($P > 0.05$), $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3. Results

3.1. Metal accumulation and partitioning

Following metal exposure, *P. damicornis* accumulated both Cu and Cd in a dose- and time-dependent manner (Fig. 1) in the whole association (total fraction). Severe stress was observed in the high Cu (50 μg l^{-1}) exposure such that the polyps were not fully extended and with increasing exposure time, they retracted further into the skeleton; consequently, all coral fragments exposed to high Cu concentrations were processed at Day 9 (but graphed at Day 14).

Extensive and statistically significant accumulation of Cu was observed in the 50 μg l^{-1} exposure (Fig. 1); after 4 days Cu levels reached 125.9 ± 29.6 ng Cu mg^{-1} dry weight ($P < 0.01$) and continued to increase to 152.9 ± 21.0 ng Cu mg^{-1} dry weight at Day 9 ($P < 0.001$). At the lower exposure levels (5 μg l^{-1}), Cu accumulated to similar extents (~13 ng Cu mg^{-1} dry weight, $P > 0.05$) at both the 4 and 9 Day time points but were significantly different compared with respective controls (both $P < 0.001$).

Compared to controls significant accumulation of Cd was observed at both time points (both $P < 0.001$) in corals exposed to 5 μg l^{-1} (Fig. 1). Accumulated levels were not significantly different ($P > 0.05$) at the different days (Day 4: 7.5 ± 1.0 and Day 14: 8.6 ± 0.7 ng Cd mg^{-1} dry weight). Mortality was observed in the 50 μg l^{-1} Cd exposure between Days 2 and 3; no data are presented for that dose. Coral mortality was also observed in the high Cu exposures, a few days before Day 9.

The amount of metal accumulated in the specific algal and animal fractions of corals exposed to 0 and 5 μg l^{-1} Cu or Cd are shown in Fig. 2. The extent of accumulated Cu and Cd are similar in both of these fractions. To investigate this further, Fig. 3 presents the percentage of the metal of the whole association that was accumulated specifically by the algal or the animal fractions (i.e. percentage of the total metal, which is 100%). No prefer-

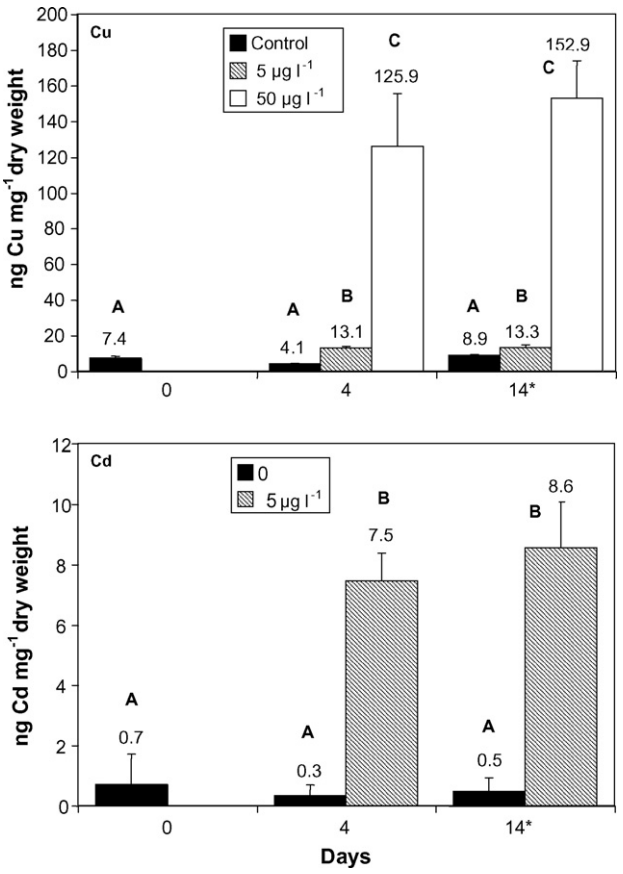


Fig. 1. Metal accumulation in *P. damicornis* exposed to 5 and 50 μg l^{-1} Cu or 5 μg l^{-1} Cd for 14 days. Numerical values represent actual means ± S.E.M. ($n = 5$) and are presented as ng Cu or Cd mg^{-1} dry weight coral tissue. Statistically significant differences ($P < 0.01$) were observed at Days 4 and 14 for both metals (ANOVA, Tukey) compared to respective controls (letters signify significant differences between samples): (*) 50 μg l^{-1} Cu exposed corals were sampled at Day 9 instead of Day 14 due to observations of extreme stress; 50 μg l^{-1} Cd data not presented due to mortality of corals before Day 4.

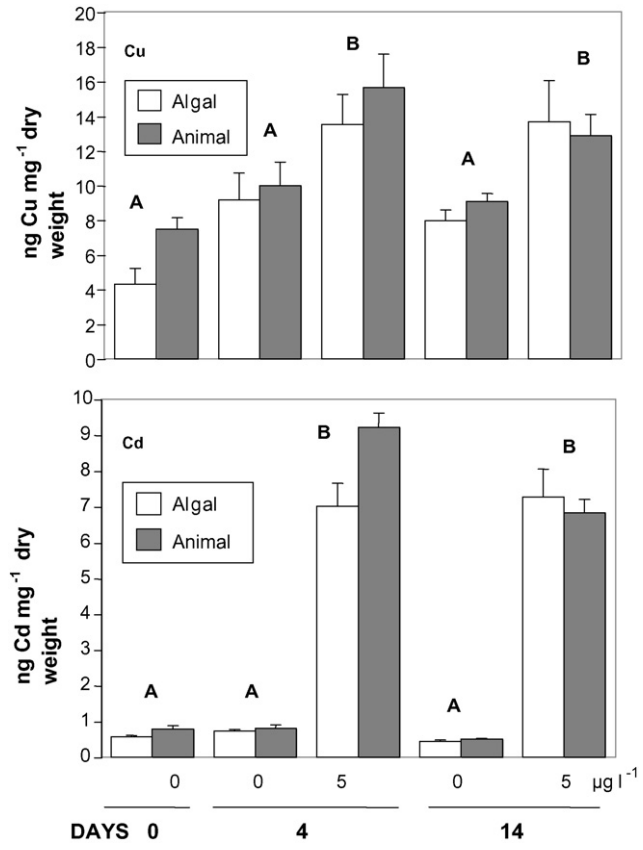


Fig. 2. Metal accumulation in algal and animal fractions of *P. damicornis* exposed for 14 days to 0 or 5 μg l^{-1} Cu or Cd. Data expressed as means ± S.E.M. ($n = 5$) and presented as ng Cu or Cd mg^{-1} dry weight tissue. Statistically significant differences ($P < 0.05$) were observed at Days 4 and 14 for both metals compared with respective controls (ANOVA, Tukey, $P < 0.05$; letters signify significant differences).

Author's personal copy

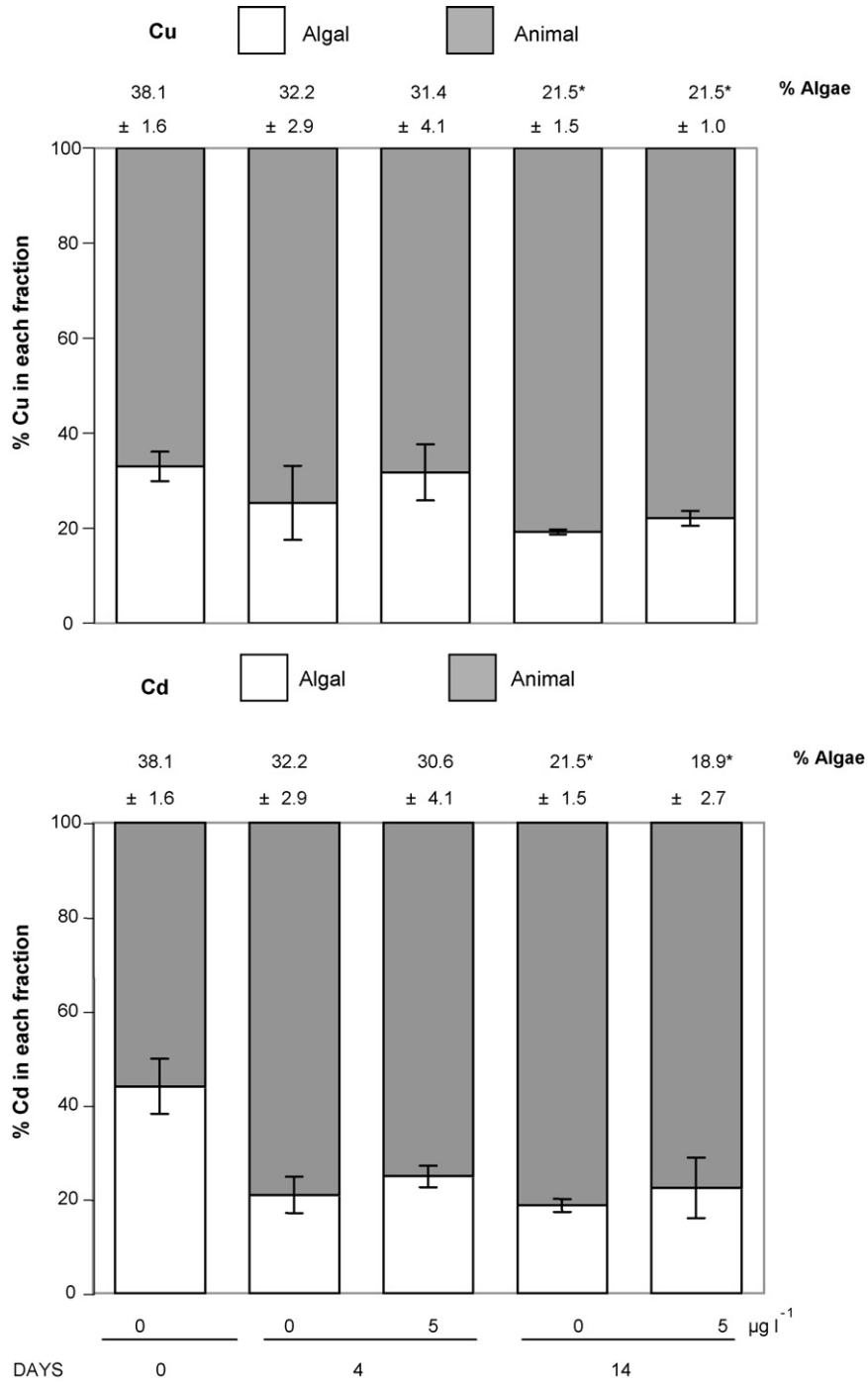


Fig. 3. Average percentage of total Cu and Cd accumulated in the algal pellet fraction and the animal supernatant fraction following exposure to 5 $\mu g\,l^{-1}$ metal over the duration of the experiment. Numerical values listed above the bars report the percentage of the total dry weight (±S.E.M.; $n = 5$) that the algal fraction represents at these specific metal concentrations/time points: (*) statistically significant differences in percentage algal fraction ($P < 0.05$) were observed at Day 14 for controls and both metals compared with Day 0 controls although no differences between treatments at either Day 4 or 14 were observed compared with respective Day controls (ANOVA, Tukey).

ential uptake of metal by any of the algal or animal fractions is suggested since the percentage of metal accumulated by each fraction does not change significantly ($P > 0.05$) over time or dose. For example, if algae were accumulating the metal more so than the animal they would represent a larger percentage of the metal accumulated with exposure (potentially in a time- and dose-dependent manner).

Averaging across all exposures and time points (irrespective of total metal accumulated) the percentage of the total (i.e. whole association) Cu in the algal fraction is 24 ± 6 and $26 \pm 10\%$ for Cd. Coincidently, these values are close to the percentage of the algal dry weight in these samples (i.e. $27.7 \pm 1.3\%$ across all samples). Additionally, at each exposure time and dose, the percentage of the dry weight of the algal fraction (numerically

Author's personal copy

Table 1
Algal cell parameters and GSH levels in control, Cu and Cd treatments

Biomarker	Exposure ($\mu g\,l^{-1}$)	Days of exposure		
		0	4	14[a]
Algal cell density ((cells mg^{-1} protein) $\times 10^5$)	Control	5.5 ± 1.1	4.1 ± 0.8	2.6 ± 0.4[b]
	Cu 5		2.8 ± 0.4	3.0 ± 0.3
	Cu 50		1.6 ± 0.2	3.5 ± 0.7
	Cd 5		3.5 ± 0.4	2.5 ± 0.1
Mitotic index (percentage of cells dividing)	Control	0.08 ± 0.03	0.04 ± 0.02	0.12 ± 0.05
	Cu 5		0.00 ± 0.00	0.08 ± 0.05
	Cu 50		0.05 ± 0.03	0.19 ± 0.06
	Cd 5		0.00 ± 0.00	0.06 ± 0.04
Chlorophyll *a* (pg $cell^{-1}$)	Control	14.0 ± 1.4	13.1 ± 2.3	11.3 ± 1.3
	Cu 5		14.2 ± 1.9	9.9 ± 0.9
	Cu 50		14.4 ± 0.6	13.6 ± 1.9
	Cd 5		10.7 ± 1.3	12.9 ± 0.9
Chlorophyll *c* (pg $cell^{-1}$)	Control	4.4 ± 0.5	6.1 ± 0.9	5.0 ± 1.8
	Cu 5		5.9 ± 1.0	4.1 ± 0.5
	Cu 50		7.4 ± 0.1	5.3 ± 0.9
	Cd 5		4.5 ± 0.7	5.2 ± 0.5
Chlorophyll *a*:*c* ratio	Control	3.2 ± 0.1	2.1 ± 0.1[b]	2.3 ± 0.2[b]
	Cu 5	–	2.5 ± 0.1	2.5 ± 0.1
	Cu 50	–	1.9 ± 0.1	2.6 ± 0.1
	Cd 5	–	2.4 ± 0.1	2.5 ± 0.1
GSH (μM GSH μg^{-1} protein)	Control	6.80 ± 1.13	0.00 ± 0.13[b]	1.02 ± 0.06[b]
	Cu 5		0.00 ± 0.12	1.15 ± 0.11
	Cu 50		1.49 ± 0.07[c]	1.85 ± 0.12[c]
	Cd 5		1.46 ± 0.10[c]	1.01 ± 0.06

Data expressed as means ± S.E.M. ($n = 5$).
[a] Cu 50 $\mu g\,l^{-1}$ sampled at Day 9 and not Day 14. Cd 50 $\mu g\,l^{-1}$ data not presented due to mortality before Day 4.
[b] Statistically significant (ANOVA, Tukey, $P < 0.05$) differences between controls.
[c] Statistically significant (ANOVA, Tukey, $P < 0.05$) differences in exposures compared with respective controls.

detailed in Fig. 3, means ± S.E.M.) was correlated with the percentage of the total metal accumulated by the algal fraction at each dose- and time point. Significant correlations were observed, $r^2 = 0.80$ for Cu and $r^2 = 0.54$ for Cd exposures.

3.2. Algal parameters and additional observations

The algal parameters of cell density, mitotic index (MI) and chlorophylls *a*, *c* and *a*:*c* ratios are listed in Table 1. Most of the statistical comparisons (one-way ANOVA) over the duration of the experiment were not significant (Tukey, $P > 0.05$). There were only significant differences (Tukey, $P < 0.05$) in algal cell density and chlorophyll *a*:*c* ratios over time in control fragment comparisons, no effect of exposure was seen when compared with controls at each respective time point. The reduction in algal cell density in the controls over time (Table 1) is also observed in the percentage of algae (Fig. 3) where Day 14 samples were significantly less ($P < 0.05$) than at Day 0 for control and 5 $\mu g\,l^{-1}$ Cu and Cd exposures.

Following metal exposure, coral skeletons, starting from the tips, were exposed due to the coral tissue sloughing off without prior bleaching of the symbiotic association in both metal treatments (albeit sooner for Cd). In addition, corals in the high Cu dose (and to a lesser extent in the low Cu and Cd doses) released planulae after a few days of metal exposure and many of these planulae settled and adhered to the bottom of the tanks.

3.3. Measurement of glutathione levels

A metal induced alteration in levels of the antioxidant GSH was observed in this study (see Table 1). This was complicated by the brief initial changes in water temperature that occurred during the first 2 days of the static exposure period; consequently, Day 0 control corals displayed higher levels than in subsequent controls and metal exposed corals at subsequent time points. Comparing results at Day 4, statistically significant elevations from controls were observed in the 50 $\mu g\,l^{-1}$ Cu and 5 $\mu g\,l^{-1}$ Cd exposures. At Day 14 elevated levels were only observed in the 50 $\mu g\,l^{-1}$ Cu exposure, although these cannot be directly compared as these corals were actually sampled and processed at Day 9 versus the controls at Day 14.

4. Discussion

The primary objective of this experiment was to investigate the partitioning of the two model heavy metals by the coral association between algal and animal fractions. In this experiment 50 $\mu g\,l^{-1}$ exposures caused severe stress (by Day 9) in the Cu treatments and mortality in the Cd treatments (at 2–3 days).

Author's personal copy

Acute toxicity reports in the literature for corals exposed to these two metals depends on the metal, species and life stage. Howard et al. (1986) reported a 96 h LC50 for *Montipora* sp. exposed to Cu of 48 $\mu g\,l^{-1}$ whereas Evans (1977) reported Cu toxicity to *P. damicornis* at <10 $\mu g\,l^{-1}$. Data for Cd toxicity is limited to the early life stages of corals and demonstrates, in contrast to our study on adult corals, a reduced sensitivity of corals to Cd compared with Cu (Reichelt-Brushett and Harrison, 2005). As noted in the methods and results section a slight thermal/increased UV event occurred across treatments, potentially complicating interpretation of the data, therefore, we discuss the results of the metal treatments with respect to their daily corresponding controls, in addition to changes in controls across time. In two previous preliminary experiments aimed at determining a suitable dose for Cd, similar data in terms of mortality (dose and time to death) and accumulated Cd levels were observed.

Exposure to 50 $\mu g\,l^{-1}$ Cu resulted in extensive accumulation of Cu by the coral tissue. These levels are much higher (over seven times) than we had previously observed with the symbiotic anemone (*Anthopleura elegantissima*) exposed to 100 $\mu g\,l^{-1}$ Cu (i.e. at Day 7, <19 ng Cu mg^{-1} dry weight; Mitchelmore et al., 2003b). However, these higher values in corals are not surprising since many studies have suggested an increased sensitivity of tropical cnidarian species to pollutants compared to their temperate counterparts (Brown, 2000).

Many mechanisms could account for these higher levels in corals. First, corals have a higher surface area to tissue volume ratio compared to *A. elegantissima*. A further mechanism may be due to mucus production. In the anemone exposures, a higher level of Cu (up to 30 ng Cu mg^{-1} dry weight at Day 7) was observed in the aposymbiotic specimens compared with symbiotic anemones. We visually observed extensive production of mucus by the symbiotic anemones, presumably a reflection of food availability (i.e. from symbiont photosynthesis) and hence greater ability to produce copious amounts of mucus. It appears this mucus may act as a barrier to Cu uptake as reported by Reichelt and Jones (1994). The lack of substantial mucus production observed in these corals may explain the extensive accumulation of Cu.

Cu and Cd was significantly accumulated by Day 4 following exposures of 5 $\mu g\,l^{-1}$ (i.e. Cu 3 times control levels, Cd 25 times control levels). Levels were slightly higher at Day 14 but not significantly different ($P > 0.05$) than Day 4. Field and laboratory studies with cnidarians show a variation of response to Cd exposure. For example, in temperate (Atlantic) anemone species exposed for 5 days to 5 $\mu g\,l^{-1}$ Cd, little accumulation was observed (<0.1 μg Cd g^{-1} dry weight; Harland et al., 1990). Low levels of Cd (up to 0.38 ng Cd mg^{-1} dry weight) was observed in temperate anemones collected from a Cd-polluted field site, which was 12 times higher than in specimens from control sites (Harland et al., 1990). Exposure of temperate Pacific anemones (*A. elegantissima*) to Cd for 14 days at 20 $\mu g\,l^{-1}$ resulted in higher accumulations (3.3 ± 0.4 ng Cd mg^{-1} dry weight; Mitchelmore et al., 2003a), similar to those also observed in a further study with this species (using 100 $\mu g\,l^{-1}$ Cd for 7 days; Mitchelmore et al., 2003b). 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). Esslemont (2000) found that metal levels in coral tissue do not always correspond with skeletal or environmental metal availability and concluded that for metals like Cd, evidence of tissue regulation existed in *P. damicornis*.

By separating coral tissues into the algal containing pellet fraction and the animal supernatant fraction, we were able to investigate whether metals were preferentially accumulated by either the algal or animal component of the association. Previous work with the anemone *Anemonia viridis* demonstrated the preferential accumulation of Cu by the algal component such that bleaching represented a potential mechanism of metal detoxification (Harland et al., 1990; Harland and Nganro, 1990). In contrast, this study no evidence of preferential accumulation of Cu or Cd by the zooxanthellae is apparent. If the algae preferentially take up metals we would expect to see an increase in the percentage of total Cu or Cd accumulated in this fraction at higher doses or time points. This elevation of incorporated metal was not observed in this study; only a steady percentage of metal in the algal fraction was observed over the course of the experiment (see Fig. 3). In a previous short-term study of Cd accumulation in the anemone *A. elegantissima*, we also found no evidence for differential accumulation by the algae (Mitchelmore et al., 2003a).

Mechanisms of Cu and Cd toxicity are fairly well documented due to their widespread use and presence in a variety of anthropogenic discharges. These include, effects on redox status (oxidative stress), alteration in calcium homeostasis (de la Torre et al., 2000) and a bleaching response (Evans, 1977; Esquivel, 1986; Howard et al., 1986; Harland and Nganro, 1990; Zamani, 1995; Jones, 1997; Mercier et al., 1997). For example, Jones (1997) demonstrated significant loss of algae from *Acropora formosa* exposed to 10–40 $\mu g\,l^{-1}$ Cu for 48 h. However, metal exposures (when compared to their respective controls at each specific time point) in our study did not cause bleaching of *P. damicornis*. Exposure to levels of 50 $\mu g\,l^{-1}$ Cu resulted in mortality of coral specimens without evidence of prior bleaching. In addition, no changes in algal cell density (or algal pellet percentage of total weight) were observed in both the Cu and Cd exposures at any time point when compared to their respective controls. In some cases coral bleaching is a result not of algal cell loss but in the reduction of algal chlorophyll pigments. In this study no evidence of changes in chlorophyll content in any of the metal exposures over the duration of the experiment was apparent. This lack of visible bleaching and of algal loss with Cu and Cd exposure is similar to other studies. For example, in *A. elegantissima* no bleaching was apparent from exposures to 100 $\mu g\,l^{-1}$ Cu or Cd (Mitchelmore et al., 2003a,b) and even up to 250 $\mu g\,l^{-1}$ Cu (Keel, 1994). However, these data are in contrast to many similar studies in other anemones and in corals as previously discussed.

We also investigated changes in the antioxidant glutathione (GSH) resulting from metal exposure. GSH functions in metal detoxification by various mechanisms. For example, oxidative stress from redox active metals can be alleviated by oxidation of GSH to GSSG. In addition, metals may bind to GSH

Author's personal copy

and be removed from the organism via GSH-conjugation reactions. Within hours of initial exposure to an oxidative stress, intracellular levels of GSH decrease (Sagara et al., 1998). However, as a compensatory action, GSH levels can increase several times over those before the oxidative stress (Sies, 1999). Reduction of GSH levels in metal-exposed organisms, including anemones (Meister and Anderson, 1983; Regoli and Principato, 1995; Mitchelmore et al., 2003a) and corals (Downs et al., 2000) in response to other stressors (e.g. UV and heat) has been described. Downs et al. (2000) found GSH concentrations were significantly reduced (50%) by heat exposure but not light in *Montipora formosa* (2.57–1.30 $\mu M\,mg^{-1}$ total protein). Unfortunately, changes in GSH levels following metal exposure were complicated in this study by the initial heat/UV stress. However, Table 1 clearly demonstrates that UV/heat stress significantly reduces levels of GSH in coral tissues (reduction from $6.75 \pm 2.5\ \mu M\,mg^{-1}$ total protein to levels below the detection of the assay at Day 4 which recovered by Day 14 to $1.02 \pm 0.14\ \mu M\,mg^{-1}$ total protein). Despite these complications, metal induced elevation of GSH compared with controls was observed on both days in the high Cu exposure and at Day 4 in the low Cd exposure.

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 exist, it is important to understand the responses of cnidarian–algal symbiosis to metal exposure. Clearly, the differential response of *P. damicornis* (lack of visible bleaching before mortality) to heavy metal exposure in comparison to other coral species requires further attention and demonstrates that heavy metal accumulation and toxic effects, such as algal cell loss, depend upon metal type and the species. As such, many additional questions need to be addressed regarding the regulatory mechanisms that exist to control or regulate metal accumulation and detoxification.

Acknowledgements

This work was supported by a grant from the Office of Naval Research ONR (N00014-99-1-0564) to V. Weis. Grateful thanks to the faculty, staff and students at the Kewalo Basin Marine Laboratory, Honolulu, HI, especially Drs. M.McFall-Ngai and M. Hadfield for the use of their facilities and equipment. Thanks to Andy Ungerer for assistance with the ICP-MS at OSU's W.M. Keck facility for plasmaspectroscopy and to members of the Weis lab for their comments on the manuscript. This is contribution No. 4116 of the University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory.

References

Birkeland, C., 1996. Life and Death of Coral Reefs. Chapman Hall, New York, p. 536.

Brown, E.B., 2000. The significance of pollution in eliciting the 'bleaching' response in symbiotic cnidarians. Int. J. Env. Pollut. 13 (1–6), 392–415.

Chester, R., 1990. Marine Geochemistry. Allen & Unwin, Australia, p. 698.

de la Torre, F.R., Salibian, A., Ferrari, L., 2000. Biomarkers assessment in juvenile *Cyprinus carpio* exposed to water-borne cadmium. Env. Pollut. 109, 277–282.

Downs, C.A., Mueller, E., Phillips, S., Fauth, J.E., Woodley, C.M., 2000. A molecular biomarker system for assessing the heath of coral (*Montastraea faveolata*) during heat stress. Mar. Biotechnol. 2 (6), 533–544.

Esquivel, I.F., 1986. Short-term bioassay on the planula of the reef coral *Pocillopora damicornis*. In: Jokiel, P.L., Richmond, R.H., Rogers, R.A. (Eds.), Coral Reef Population Biology, vol. 37. Hawaii Institute of Marine Biology Technical Report, pp. 465–472.

Esslemont, G., 2000. Heavy metals in seawater, marine sediments and corals from the Townsville section, Great Barrier Reef Marine Park, Queensland. Mar. Chem. 71 (3/4), 215–231.

Esslemont, G., Harriott, V.S., McConchie, D.M., 2000. Variability of trace-metal concentration within and between colonies of *P. damicornis*. Mar. Pollut. Bull. 40 (7), 637–642.

Evans, E.C., 1977. Microcosm responses to environmental perturbations. Helog Meeresunters 30, 178–191.

Gilbert, A.L., Guzman, H.M., 2001. Bioindication potential of carbonic anhydrase activity in anemones and corals. Mar. Pollut. Bull. 42 (9), 742–744.

Goering, P.L., Waalkes, M.P., Klaassen, C.D., 1995. Toxicology of cadmium. In: Goyer, R.A., Cherian, M.G. (Eds.), Toxicology of Metals. Biochemical Aspects. Springer, Berlin, pp. 189–214.

Goh, B., 1991. Mortality and settlement success of *Pocillopora damicornis* planula larvae during recovery from low levels of nickel. Pacific Sci. 45, 276–286.

Goh, B.P.L., Chou, L.M., 1997. Effects of the heavy metals copper and zinc on zooxanthellae cells in culture. Env. Monit. Assess. 44 (1–3), 11–19.

Guzman, H.M., Jimenez, C.E., 1992. Contamination of coral reefs by heavy metals along the Caribbean coast of central America (Costa Rica and Panama). Mar. Pollut. Bull. 24 (11), 554–561.

Hanna, R.G., Muir, G.L., 1990. Red Sea corals as biomonitors of trace metal pollution. Env. Monit. Assess. 14, 211–222.

Harland, A.D., Nganro, N.R., 1990. Copper uptake by the sea anemone *Anemonia viridis* and the role of zooxanthellae in metal regulation. Mar. Biol. 104, 297–301.

Harland, A.D., Bryan, G.W., Brown, B.E., 1990. Zinc and cadmium absorption in the symbiotic anemone *Anemonia viridis* and the non-symbiotic anemone *Actinia equina*. J. Mar. Biol. Ass. U.K. 70, 789–802.

Howard, L.S., Brown, B.E., 1984. Heavy metals and reef corals. Mar. Biol. Ann. Rev. 22, 195–210.

Howard, L.S., Crosby, D.G., Alino, P., 1986. Evaluation of some methods for quantitatively assessing the toxicity of heavy metals to corals. In: Jokiel, P.L., Richmond, R.H., Rogers, R.A. (Eds.), Coral Reef Population Biology, vol. 37. Hawaii Institute of Marine Biology Technical Report, pp. 452–464.

Hunter, C.L., Stephenson, M.D., Tjeerdema, R.S., Crosby, D.G., Ichikawa, G.S., Goetzl, J.D., Paulson, K.S., Crane, D.B., Martin, M., Newman, J.W., 1995. Contaminants in oysters in Kaneohe Bay, Hawaii. Mar. Pollut. Bull. 30 (10), 646–654.

Jeffrey, S.W., Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls *a*, *b*, *c* and *c*2 in higher plants, algae and natural phytoplankton. Biochem. Physiol. Pflanz. 167, 191–194.

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

Keel, L.W., 1994. Sublethal toxicity of copper sulfate to the intertidal sea anemone, *Anthopleura elegantissima*. M.Sc. Thesis. Western Washington University.

Mason, A.Z., Jenkins, K.D., 1995. Metal detoxification in aquatic organisms. In: Tessier, A., Turner, D.R. (Eds.), Metal Speciation and Bioavailability in Aquatic Systems. John Wiley and Sons, Chichester, p. 679.

Meehan, W.J., Ostrander, G.K., 1997. Coral bleaching: a potential biomarker of environmental stress. J. Toxicol. Env. Health 50, 529–552.

Meister, A., Anderson, M.E., 1983. Glutathione. Ann. Rev. Biochem. 52, 711–760.

Author's personal copy

Mercier, A., Pelletier, E., Hamel, J.-F., 1997. Effects of butyltins on the symbiotic sea anemone *Aiptasia pallida* (Verrill). J. Exp. Mar. Biol. Ecol. 215, 289–304.

Mitchelmore, C.L., Schwarz, J.A., Weis, V.M., 2002. Development of symbiosis-specific genes as biomarkers for the early detection of cnidarian–algal symbiosis breakdown. Mar. Env. Res. 54, 345–349.

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

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

Morgan, M.B., Vogelien, D.L., Snell, T.W., 2001. Assessing coral stress responses using molecular biomarkers of gene transcription. Env. Tox. Chem. 20 (3), 537–543.

Nystrom, M., Nordemar, I., Teengren, M., 2001. Simultaneous and sequential stress from increased temperature and copper on metabolism of the hermatypic coral *Porites cylindrica*. Mar. Biol. 138 (6), 1225–1231.

Pandolfi, J.M., Jackson, J.B.C., 2001. Community structure of Pleistocene coral reefs of Curacao, Netherlands, Antilles. Ecol. Monogr. 71, 49–67.

Parsons, T.R., Maita, Y., Lalli, C.M., 1984. A Manual of Chemical and Biological Methods for Sea Water Analysis. Pergamon Press, New York, pp. 101–104.

Regoli, F., Principato, G., 1995. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomrkers. Aquat. Toxicol. 31, 143–164.

Reichelt, A.J., Jones, G.B., 1994. Trace metal as tracers of dredging activity in Cleveland Bay—field and laboratory studies. Aust. J. Mar. Freswater Res. 45, 1237–1257.

Reichelt-Brushett, A.J., Harrison, P.L., 2005. The effect of selected trace metals on the fertilization success of several scleractinian coral species. Coral Reefs 24, 524–534.

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

Sagara, Y., Dargusch, R., Chambers, D., Davis, J., Schubert, D., Maher, P., 1998. Cellular mechanism of resistance to chronic oxidative stress. Free Radic. Biol. Med. 24, 1375–2189.

Scott, P.J.B., 1990. Chronic pollution recorded in coral skeletons in Hong Kong. J. Exp. Mar. Biol. Ecol. 139 (1/2), 51–64.

Sies, H., 1999. Glutathione and its role in cellular functions. Free Radic. Biol. Med. 27, 916–921.

Wilkerson, F.P., Kobayashi, D., Muscatine, L., 1988. Mitotic index and size of symbiotic algae in Caribbean reef corals. Coral Reefs 7, 29–36.

Wilkinson, C.R., 1999. Global and local threats to coral reef functioning and existence: review and predictions. Mar. Freshwater Res. 50 (8), 867–878.

Zamani, N.P., 1995. Effects of environmental stress on cell division and other cellular parameters on zooxanthellae in the tropical symbiotic anemone *Heteractis malu* Haddon and Shackelton. Ph.D. Thesis. University of Newcastle upon Tyne, UK.

Zar, J.H., 1999. Biostatistical Analysis, 4th ed. Prentice Hall, Englewood Cliffs, New Jersey.