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RESEARCH ARTICLE

Cadmium and copper toxicity in three marine macroalgae: evaluation of the biochemical responses and DNA damage

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Abstract Marine macroalgae have evolved a different mechanism to maintain physiological concentrations of essential metal ions and non-essential metals. The objective of the present work was to evaluate the antioxidant response and DNA damage of copper and cadmium ions in three halophytes, namely, *Acanthophora spicifera*, *Chaetomorpha antennina*, and *Ulva reticulata*. Accumulation of copper was significantly higher ($P<0.05$) than that of cadmium. Biochemical responses showed that copper was considerably more toxic than cadmium ($P<0.05$). Decreases in glutathione content and fluctuations of super oxide dismutase, catalase, and glutathione peroxidase activities were observed corresponding to time and concentration of exposure. Interestingly, it was also observed that antioxidant levels decreased as a result of metal accumulation, which may be due to free radicals generated by copper and cadmium in seaweeds. The present study also showed that copper and cadmium increased oxidative stress and induced antioxidant defense systems against reactive oxygen species. The order of toxicity for metals in the studied seaweeds was *U. reticulata* > *A. spicifera* > *C. antennina*. DNA damage index analysis supported that copper was significantly ($P<0.05$) more toxic than cadmium. Bioaccumulation, biochemical responses, and DNA damage observed in the here analyzed marine macroalgae after exposure to selected metals indicate that these marine organisms represent useful bioindicators of marine pollution.

Keywords Cadmium · Copper · Seaweeds · Antioxidants · DNA damage · Bioaccumulation

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M.Yokesh Babu and L. Palanikumar equally contributed to this paper.

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Introduction

Marine florae are sustainable resources in coastal ecosystem, and have been used as a source of food, feed, and medicine. It was estimated that about 90 % of all marine plants are algae and about 50 % of the global photosynthesis is contributed by algae (Dhargalkar and Neelam 2005). Seaweeds are traditionally consumed in the orient as part of the daily diet. Currently, human consumption of green algae (5 %), brown algae (66.5 %), and red algae (33 %) is the greatest in Asia, compared to the rest of the world (Dawes 1998).

The bioaccumulation of metals is a useful indicator since metals are not metabolized (Luoma and Rainbow 2005). Metals can be accumulated by marine organisms either directly (e.g., macroalgae) or through food chain, eventually posing a significant risk to inhabitants of an ecosystem, including humans (Chan et al. 2003; Luoma and Rainbow 2005; Stengel et al. 2005; Rodríguez-Figueroa et al. 2009; Simon et al. 2011; Saez et al. 2012). As suggested by Luoma and Rainbow (2005), bioaccumulation of metals can be complex, and it can be influenced by (i) diet and solution and (ii) geochemical impacts on bioavailability. The evaluation of the impact of pollutants on environmental quality has become a priority globally, and requires rapid assessment of their potential impacts on an ecosystem (Wells et al. 2001; Palanikumar et al. 2012a, b; Janaki Devi et al. 2012). Among pollutants, metals

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such as cadmium (Cd), copper (Cu), lead (Pb), and mercury (Hg) are of particular concern as they are non-biodegradable and strongly phytotoxic, partly because of their tendency to generate reactive oxygen species (ROS), which react with lipids, proteins, photosynthetic pigments, and nucleic acids, causing lipid peroxidation, membrane damage, metabolite degradation, inactivation of enzymes, and cell death (Hegedus et al. 2001; Nagarani et al. 2012). Metal toxicity in macroalgae is often linked to the formation of free reactive oxygen radicals causing the inhibition of development (Collén et al. 2003; Pinto et al. 2003). Molecular oxygen is unreactive with organic molecules because it has two unpaired valence shell electrons in outer shell. However, when activated through reduction, it forms reactive oxygen species (ROS) such as superoxide radical (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and finally water (H_2O) (Collén et al. 2003). ROS are toxic because they have the ability to interact rapidly with biological molecules (proteins, lipids, DNA) causing oxidative stress which can result into cell death via apoptosis or necrosis (Palanikumar et al. 2013a).

Cu and Cd have received much attention because of their great toxicity to plants and other organisms. The concentrations Cu and Cd in marine and estuarine systems are highly variable due to freshwater runoff, intermittent discharge of domestic sewage and industrial effluents, tides and currents, hydrological parameters, and ecological factors (Wang et al. 2009). Copper can accumulate in plants and it induces toxicity and oxidative damage by generating ROS such as $^1O_2$, H_2O_2 and OH (Halliwell and Gutteridge 1999; Iseri et al. 2011). The lack of angiosperms in macrophytic algae can uptake metals only from the solution, which can be a excellent biomonitor for metal availability in water columns (Rainbow 2005), since the compound leaves of macrophytic algae are not in direct contact with the sediment. The mechanisms by which Cu and Cd are toxic have been studied extensively (Mehta et al. 2006; Achard-Joris et al. 2007; Wang et al. 2008).

Cd has no known metabolic function in macroalgae; however, it has been shown to be a cofactor in a carbonic anhydrase of the diatom *Thalassiosira weissflogii* (Lane and Morel 2000). In higher plants, Cd causes disturbances in growth, photosynthesis, ion, and water transport, as well as decreases in enzyme activities through reactions between Cd and thiol groups (Prasad 1995; Archana Devi and Kumaraguru 2008). In contrast to Cu, Cd cannot contribute to·OH formation in the Fenton reaction (Halliwell and Gutteridge 1999).

Limited studies on the bioassays were conducted with macroalgae of the Indian coasts (Murthy and Umamaheswara Roa 2003; Archana Devi and Kumaraguru 2008), and several reports have signified the importance of macroalgal community in evaluating the toxicity of pollutants (Han and Choi 2005; Baumann et al. 2009; Kumar et al. 2010, 2012). The aim of this study was to characterize the toxicities of metal concentration that were representative of the highly contaminated regions to three macroalgae (Luoma and Rainbow 2008; Stauber et al. 2005). Several studies reported concentrations of Cu in *Padina durvillaei* that ranged between 1.9 and 61 $mg.kg^{-1}$ under reference conditions at El Monteón Beach at Mexico (Páez-Osuna et al. 2000) and a highly contaminated region in Baja California Sur, Mexico, respectively (Carrillo Domínguez et al. 2002). In seawater, Cu concentrations were reported as high as 10 $\mu g.l^{-1}$ at Caleta Pinto and 37 $\mu g.l^{-1}$ in Caleta La Lancha, Chile (Stauber et al. 2005). Accumulation of Cd by *P. durvillaei* was between 5 and 6 and 8 mg kg^{-1} in algae from El Monteón Beach, Mexico (Páez-Osuna et al. 2000) and a mining region in Santa Rosalia, Mexico, respectively (Rodríguez Meza 2005).

In view of this, the present study was designed to investigate (i) the extent of Cd and Cu-induced oxidative stress in three marine algae species (*U. reticulata*, *A. spicifera,* and *C. antennina)*, and (ii) whether antioxidant responses differ in algal species for each metal. The first objective was analyzed by determining lipid peroxidation and the second one was to examine changes in antioxidant enzyme activities such as superoxide dismutase, catalase, reduced glutathione, and glutathione peroxidase in the tissue of three seaweeds upon exposure to Cu and Cd. The present study has focused on understanding the effect of lethal doses of Cu and cadmium in relation to biochemical enzyme markers and DNA damage analysis in three species of seaweeds.

Material and methods

Seaweed collection

The Gulf of Mannar represents a rich source of different seaweed species. It is located in the Indo-Pacific region, and it is considered to be one of world's richest marine biological resource regions (Palanikumar et al. 2011). Three seaweed species were collected from the intertidal rocky shore region of the Pudumadam coast, Gulf of Mannar, Southeast coast of India at a latitude 09° 16.26′ N and longitude 94° 12.88′ E during low tide from a site rich in different seaweed species.

Toxicity bioassay

Collected seaweed samples were washed with sand-filtered seawater to remove epiphytes and other marine organisms. They were transported to the laboratory in sand-filtered seawater and under continuous aeration in a plastic trough. The experiments were conducted in the tanks with 16:8-h day and night, respectively. Prior to receiving test specimen, all test chambers used during the experiments were washed and soaked overnight in 10 % nitric acid, then soaked overnight in the de-ionized water. Seaweeds were acclimatized in 500-l

fiberglass tanks containing well-aerated seawater for a period of 10 days. The test concentrations for Cu ($Cucl_2$. $2H_2O$; ml wt. 170.48; Merck India Ltd, Mumbai) and cadmium ($CdCl_2.5H_2O$; ml.wt. 228.40; Merck India Ltd, Mumbai) were 0.25, 0.50, 0.75, and 1.00 $mg.l^{-1}$ (Archana Devi and Kumaraguru 2008). Cu (1000 $mg.l^{-1}$) and Cd (1000 $mg.l^{-1}$) stock solutions were prepared in Milli-Q water.

Seawater was collected in the Gulf of Mannar off the Pudhumadam coast in an area of 10 m depth. Water samples were filtered using 10 μm and 1 μm filters, passed through an UV sterilizer, and stored at 27±4 °C as described in detail by Palanikumar et al. (2013b). Water quality characteristics of filtered seawater was calcium hardness 355 mg l^{-1}, magnesium hardness 1,381 mg l^{-1}, dissolved oxygen 6.32 mg l^{-1}, silicates 6.35 μg l^{-1}, inorganic phosphate 3.28 μg l^{-1}, nitrite-nitrogen 1.59 μmol l^{-1}, nitrate-nitrogen 4.35 mmol l^{-1}, and ammonia 0.10 μg l^{-1}. This filtered seawater was used as test medium. The experimental setup using static renewal test systems was conducted in accordance with the methods of Sprague (1973) and USEPA (2002). Each test medium and control seawater was replaced at an interval of 24 h. The test chambers (60 cm × 30 cm × 30 cm) contained 10 g (wet weight) of each alga with 10 l of test medium. Test chambers were maintained in quadruplicate for each test concentration (total 40 g fresh seaweed) and control group. Illumination was maintained at ratio of 16:8-h day and night period. The tests were conducted over a period of 15 days. At the end of the exposure periods, algae were collected, washed with filtered seawater, and deionized water and stored at 4 °C, and the wet samples in treatment chambers were used for biochemical, comet assay, and bioaccumulation pattern in period intervals.

For metal analysis, algae were pooled and 1 g of wet algae was washed, dried, and digested with 10 % nitric and 10 % perchloric acid at room temperature for 12 h. The digested sample was heated to 180 °C until the sample volume was reduced to 2–3 ml (Grasshoff et al. 1999). The extract was analyzed for the occurrence of Cu and Cd using atomic absorption spectroscopy (AAS, model: PerkinElmer Flame AAnalyst 800, MA, USA). The detection levels for Cu and Cd by AAS were 1 and 5 μg, respectively.

Estimation of thiobarbituric acid reactive substances

The levels of lipid peroxidation (LPO) were determined from the thiobarbituric acid-reacting substance (TBARS) contents resulting from the thiobarbituric acid (TBA) reaction as described by Health and Packer (1968). From the pooled samples, seaweeds of 0.2–1.0 g were homogenized in 10 ml 5 % trichloroacetic acid (TCA) with acid washed sand. The extract was centrifuged at 10,000 rpm for 20 min. One milliliter of the supernatant was mixed with 4 ml of TCA-TBA reagent (20 % containing 0.5 % TCA) and kept in a water bath at 95 ° C for 30 min. It was then cooled down quickly in an ice bath; then centrifuged at 10,000 rpm for 30 min. The absorbance of the supernatant was measured at 532 nm using UV-visible spectrophotometer (Cecil, CE, 1021). The TBARS content was calculated based on $A_{532}–A_{600}$ with the extinction coefficient of 155 mM^{-1} cm^{-1}.

Reduced glutathione

The amount of reduced glutathione (GSH) of a sample was estimated by the method of Boyne and Ellman (1972). One milliliter of the sample extract was treated with 4.0 ml of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA, and 30 g NaCl dissolved in 100 ml water). After centrifugation, 2.0 ml of the protein-free supernatant was mixed with 0.2 ml of 0.4 M Na_2HPO_4 and 1.0 ml of DTNB reagent (40 mg DTNB in 100 ml of aqueous 1 % trisodium citrate). Absorbance was measured at 412 nm within 2 min using UV-visible spectrophotometer (Cecil, CE, 1021). GSH concentration was expressed as nmol/mg protein.

Catalase

Catalase (CAT) activity was assayed by the method of Sinha (1972). The enzyme extract (0.5 ml) was added to the reaction mixture containing 1 ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H_2O_2, 0.4 ml H_2O, and incubated for different time periods. The reaction was terminated by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture), which was prepared by mixing 5 % potassium dichromate with glacial acetic acid (1:3 by volume). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10 min and the absorbance was measured at 610 nm using UV-visible spectrophotometer (Cecil, CE, 1021). CAT activity was expressed in terms of micromoles of H_2O_2 consumed/min/mg protein.

Superoxide dismutase

The assay of superoxide dismutase (SOD) was performed using the procedure of Das et al. (2000). In this method, 1.4 ml aliquots of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer of pH 7.4, 0.075 ml of 20 mM L-methionine, 0.04 ml of 1 % (*v*/*v*) triton X-100, 0.075 ml of 10 mM hydroxylamine hydrochloride, and 0.1 ml of 50 mM EDTA) was added to 100 μl of the sample extract and incubated at 30 °C for 5 min. Eighty microliters of 50 mM riboflavin was then added, and the tubes,were exposed for 10 min to 200 W Philips lamps. After the exposure time, 1 ml of Greiss reagent (mixture of equal volume of 1 % sulphanilamide in 5 % phosphoric acid) was added, and the absorbance of the color formed was measured at 543 nm using UV-visible

spectrophotometer (Cecil, CE, 1021). One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50 % of nitrite formation under assay conditions.

Glutathione peroxidase

Glutathione peroxidase (GPx) was assayed using the procedure of Rotruck et al. (1973) with some modifications. The reaction mixture, consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10 mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, and 0.2 ml of water, was added to 0.5 ml of enzyme and incubated at 90 s. The reaction was terminated with 0.5 ml of 10 % TCA, and after centrifugation, 2 ml of the supernatant was added to 3 ml of phosphate buffer and 1 ml of DTNB reagent (0.04 % DTNB in 1 % sodium citrate). The color developed was read at 412 nm using UV-visible spectrophotometer (Cecil, CE, 1021); the enzyme activity was expressed in terms of microgram of glutathione utilized/min/mg protein.

Protoplast isolation and comet assay

Protoplast isolation and purification were performed according to Gupta et al. (2011). Briefly, following Cu and Cd treatment (after 15 days of exposure) and washing, the algal fragments (300 mg FW) were chopped into small pieces of tissue (1 mm thin) in natural sterile seawater (NSW) in two replicates, each with 150 mg of tissue. The chopped tissues were then rinsed several times with NSW to remove debris. The cleaned tissues were incubated in an enzyme solution consisting of 0.5 % dextron sulfate, 2 % cellulase, 2 % NaCl, and 0.8 % mannitol in 50 mM MES buffer (pH 6.0) for 4 h on a rotary shaker (50 rpm) in the dark at 25 °C. The protoplasts were passed through a muslin cloth and centrifuged at 300*g* for 10 min. The precipitates were suspended in saline phosphate buffer (PBS), at pH 7.4, and kept on ice at 4 °C. The protoplast yields were estimated by counting the cells using a hemocytometer under a light microscope.

For the comet assay, the protoplast solution (50 μl) containing approximately 1×10^3 protoplasts was mixed with 50 μl of 1 % low-melting temperature agarose (LMPA) dissolved in phosphate-buffered saline. An 80 μl aliquot of the solution was layered onto a base slide, which was precoated with 1 % agarose, and then covered with a cover slip. When the agarose gel solidified, the cover slip was gently slid off, and another agarose layer (90 μl, 0.5 % LMPA) was layered while covering it with a new cover slip and then left for 10 min on a chilled metal plate in order for solidification of the agarose layer to occur. Following this, the cover slip was removed, and the slides were submerged in alkali lysis solution (2.5 M NaCl, 10 mM Trizma, and 100 mM EDTA) at pH>13 overnight at 4 °C. Thereafter, the glass slide was incubated in a fresh, cold electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13) in a horizontal electrophoresis tank for 30 min at room temperature to allow for DNA unwinding. Electrophoresis was performed for 10 min at 25 V and 300 mA in a chamber cooled in an ice bath. After electrophoresis, the glass slides were neutralized in 0.4 M Tris HCl (pH 7.5) buffer, washed twice in distilled water, and left overnight for drying at room temperature. All preparation steps were performed under dim light to prevent additional DNA damage. The slides were analyzed using a Carl Zeiss HB50 fluorescence microscope (magnification at×40) with an excitation filter of 510–560 nm. Images of 100 randomly selected cells (50 counts on each duplicate slide) were analyzed for each sample. Mean score and standard deviation were calculated for the comet classes.

Statistical analyses

Experiments were performed in triplicates and the results are expressed in terms of mean values ± SD. The data were initially analyzed for homogeneity of variance and normal distribution. One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests was applied to compare the significant difference between control and different concentrations of metal-exposed seaweeds. $P<0.05$ was considered as statistically significant.

Result

Water quality of test medium

The water quality parameters of toxicant and control medium were relatively constant during the experimental period. The pH value ranged between 7.4 and 7.6. Dissolved oxygen concentration ranged between 4.2 and 6.4 $mg.l^{-1}$; salinity ranged between 30 and 33 ppt; and temperature of the test solutions varied between 28 and 31 °C (Table S1).

Cu and Cd analysis in seaweeds

Cu and Cd accumulation in seaweeds were analyzed at the end of 15 days (Figs. 1 and 2). Cu and Cd concentrations in control seaweeds were negligible or below the detection limit. Cu was found to be highly accumulated, and tissue concentrations were 7.83 $mg.g^{-1}$ wet weights and 7.25 $mg.g^{-1}$ wet weights in *U. reticulata* and *A. spicifera*, respectively (Table 1). Maximum Cd accumulation was 1.30 $mg.g^{-1}$ wet weights after 15 days in *U. reticulata*. In Cu, the order of the accumulation was *U. reticulata* > *A. spicifera* > *C. antennina*.

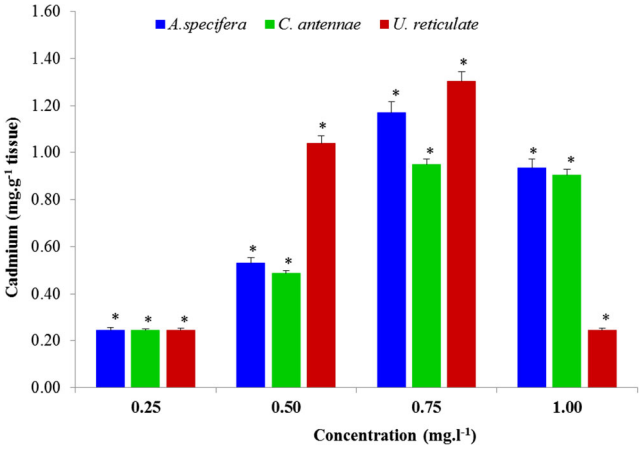

Fig. 1 Bioaccumulation pattern of cadmium in seaweeds after 15 days of exposure. *P<0.01 statistically significant when compared to control (*error bars* represent mean ± SD, n=3)

Lipid peroxidation

Lipid peroxidation was measured and expressed in terms of MDA. The MDA increased gradually with increasing concentrations of Cu and Cd. Maximum MDA levels (a significant 10-fold increase compared to controls) were recorded for 1 mg l^{-1} of Cd on 15 days in *U. reticulata* and *A. spicifera* species (Table 2). At 1 mg l^{-1}, increases in MDA levels were slightly less in Cu when compared to Cd at 15 days of exposure (Table 1). The increase in MDA content was significant (P<0.05) for all the concentrations of Cu- and Cd-treated algae, when compared to their respective controls. Among the seaweeds, *U. reticulata* showed maximum increase in MDA content when treated with the metals. In Cd exposure, MDA levels elevated up to 101.49 and 144.77 % (R^2=0.9308 decreases to R^2=0.80) when compared to control, while treatment with Cu had shown 10 and 24 % (R^2=0.80) increase than control groups, based on time and dose. Increases in MDA levels were greatest in *U. reticulata,* followed by *A. spicifera* and *C. antennina*.

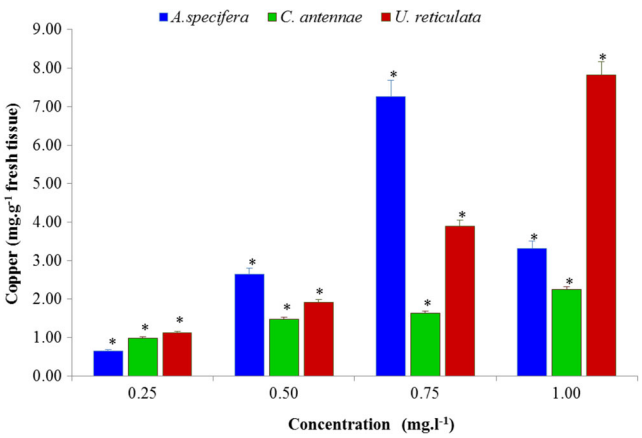

Fig. 2 Bioaccumulation pattern of copper in seaweeds after 15 days of exposure. *P<0.01 statistically significant when compared to control (*error bars* represent mean ± SD, n=3)

Antioxidant biomarkers

Cd and Cu had significant effects on the activities of antioxidant enzymes at most of the experimental doses in comparison with the controls (Tables 1 and 2).

Reduced glutathione

Irrespective of their concentrations, the levels of GSH decreased in *U. reticulata* when exposed to Cu and Cd at 15 days of exposure. GSH levels in *C. antennina* and *A. spicifera* were decreased twofold times, at higher concentrations, while little differences were observed at lower concentrations of Cd treatment. In Cu-treated seaweeds, the GSH levels of *A. spicifera* increased onefold time at the end of treatment, while GSH levels in *C. antennina* were gradually decreased twofold times at the end of 15 days treatment. The responses of GSH levels in *A. spicifera* and *C. antennina* were opposite.

Catalase

CAT activities increased in a concentration-dependent manner with increasing concentrations of Cu and Cd (Tables 1 and 2). In *C. antennina*, the increase of CAT was more than the other tissues (to the maximum of 50.39 and 64.72 %; R^2=0.9796) in Cd, while Cu treatment had decreased the CAT activity. Twofold increases in CAT activity were observed at the end of 15 days for both *A. spicifera* and *U. reticulata* for Cu treatment. Overall, significant difference in CAT activity was noted for both the metals, when compared to their control.

Superoxide dismutase

Among the antioxidant enzymes, SOD was found to increase in all algae on Cd and Cu treatment (Tables 1 and 2). In Cd-exposed algae, *A. spicifera* was observed to induce 82.78 % (R^2=0.9837) and *C. antennina* (twofold increase; R^2= 0.8366). In *U. reticulata*, we observed 2.75-fold increase in SOD synthesis (R^2=0.9884). In Cu-treated algae, the order of increase in SOD formation was observed to be high in *U. reticulata* (twofold; R^2=0.964) followed by *C. antennina* and *A. spicifera* (with R^2=0.8463 and R^2=0.9672, respectively).

Glutathione peroxidase

In the present study, GPx was observed to the overall increase on both Cu and cadmium exposure. Maximum rise was observed in Cd-treated alga *A. spicifera*, followed by *U. reticulata* and *C. antennina* (10-fold, 8-fold, and 2-fold; R^2=0.8396; R^2=0.7117, and R^2=0.9032, respectively). In Cu treatment, GPx were observed to be increased in the same

Table 1 Changes in biochemical endpoints in *A. spicifera*, *C. antennina*, and *U. reticulata* exposed to copper at different time intervals (mean ± SD; n=3)

	MDA			GSH			CAT	
	0 day	7th day	15th day	0 day	7th day	15th day	0 day	7th day
A. spicifera								
Control	0.18±0.02	0.21±0.81	0.24±0.62	1790±2.81[a]	1803±1.47[a]	1956±0.81[a]	2.85±0.12	2.62±0.81
0.25	0.43±0.23	0.90±0.11*	1.45±0.02*	1700±0.81[a]	2000±2.81*[a]	2453±1.81*[a]	2.00±0.02	3.21±0.81*
0.50	0.5±0.81	1.22±0.81*	1.68±0.47*	1690±1.81[a]	2127±0.02*[a]	2502±1.22*[a]	2.07±0.81	3.99±0.02*
0.75	0.6±0.13	1.37±0.62*	1.83±0.02*	1643±0.98 [a]	2354±2.11*[a]	2710±0.81*[a]	1.97±0.81	4.45±0.04*
1.00	0.93±0.01	1.53±0.81*	2.17±0.81*	1590±0.81 [a]	2588±1.81*[a]	2842±0.62*[a]	1.61±0.02	5.17±0.81*
C. antennina								
Control	0.12±0.02	0.12±0.81	0.19±0.02	687±0.54[a]	700±0.81*[a]	717±0.81[a]	4.96±0.01	3.03±0.10
0.25	0.31±0.01	0.40±0.81	0.52±0.81*	628±3.81[a]	654±0.02*[a]	542±1.10*[a]	4.72±0.02	3.89±0.81*
0.50	0.38±0.02	0.48±0.81	0.61±0.81*	542±0.23[a]	478±0.1*[a]	368±0.10*[a]	4.57±0.01	3.63±0.81*
0.75	0.49±0.81	0.79±0.01*	0.84±0.06*	444±0.82[a]	220±0.06*[a]	200±0.23*[a]	4.21±0.81	3.46±0.01*
1.00	0.61±0.1	0.92±0.90*	1.12±0.09*	345±0.33[a]	192±0.81*[a]	180±0.01*[a]	3.98±0.02	3.21±0.10*
U. reticulata								
Control	0.5±0.02	0.52±0.02	0.62±0.1	1500±0.02[a]	1046±0.81*[a]	923±0.82[a]	1.3±0.10	1.35±0.70
0.25	0.55±0.81	1.87±0.02*	2.21±0.02*	1630±0.81[a]	50±0.02*	17.9±0.02*	0.86±0.01	1.82±0.10
0.50	0.62±0.10	2.11±0.81*	2.78±0.02*	1589±0.02[a]	10±0.81*	4.6±0.02*	1.02±0.01	2.41±0.33
0.75	1.60±0.05	3.60±0.81*	3.92±0.01*	1400±0.81[a]	9.3±0.45*	2.27±0.05*	1.14±0.01	2.89±0.81
1.00	1.60±0.81	3.51±0.01*	4.07±0.01*	1227±0.01[a]	7.25±0.5*	0.00±0.01*	1.61±0.81	3.57±0.04

	CAT	SOD			GPx		
	15th day	0 day	7th day	15th day	0 day	7th day	15th day
A. spicifera							
Control	2.71±0.33	267±0.81	280±1.32	280±0.81	0.24±0.93	0.24±0.63	0.23±0.61
0.25	3.72±0.33*	290±0.81*	367±0.93	423±1.34*	0.24±0.81	0.29±0.47*	0.5±0.33*
0.50	4.19±0.41*	356±0.23*	435±1.33	535±0.02*	0.37±0.81	0.47±0.81*	0.71±0.81*
0.75	4.73±0.81*	422±1.22*	512±0.81	602±0.79*	0.65±0.01	0.88±0.23*	1.42±0.01*
1.00	5.31±0.81	500±0.23*	535±2.81	666±1.44*	0.7±0.81	1.02±0.81*	1.6±0.81*
C. antennina							
Control	3.00±0.12	142.0±0.23	147±0.81	150±0.82	0.12±0.02	0.17±0.02	0.23±0.81
0.25	3.61±0.01*	165.5±0.02	190±0.47	234±0.02*	0.10±0.02	0.20±0.02*	0.40±0.81*
0.50	3.45±0.02*	182.3±0.63	222±0.81	300±0.02*	0.20±0.02	0.30±0.02*	0.44±0.10*
0.75	3.28±0.02*	210.0±0.81	289±0.01	318±0.81*	0.30±0.02	0.44±0.02*	0.50±0.90*
1.00	3.125±0.01*	225.5±0.01	264.5±0.81	320±0.81*	0.45±0.1	0.80±0.20*	0.71±0.45*
U. reticulata							
Control	1.36±0.22	142±0.23	147±0.1	150±0.9	0.4±0.01	0.44±0.02	0.49±0.01

Table 1 (continued)

	CAT		SOD		GPx		
	15th day	0 day	7th day	15th day	0 day	7th day	15th day
0.25	3.98±0.03*	182±0.87*	190±0.91*	200±0.01*	0.56±0.01	1.26±0.02*	1.73±0.01*
0.50	4.00±0.01*	205±0.99*	222±0.81*	240±0.50*	0.58±0.01	1.69±0.05*	2.01±0.01*
0.75	4.22±0.01*	210±0.80*	248±0.50*	267±0.20*	0.73±0.01	1.83±0.30*	2.34±0.08*
1.00	4.73±0.81*	215±0.033*	231±0.81*	285±0.81*	0.96±0.1	2.09±0.81*	2.69±0.01*

MDA malondialdehyde (lipid peroxidation) in mM MDA formed/min/mg protein; *CAT* catalase H_2O_2 consumed/min/mg protein; *GSH* reduced glutathione in nmol/mg protein; *SOD* superoxide dismutase in mg/protein; *GPx* glutathione peroxidase—microgram of glutathione utilized/min/mg protein

*$P<0.05$, when compared to their respective controls and previous exposure periods

[a] Standard error expressed in terms of percentage

order as 7-fold, 5.5-fold, and 6.75-fold ($R^2=0.9534$, $R^2=0.8984$, and $R^2=0.9292$, respectively).

DNA damage

From the comet assay, it was clearly evident that the ion-induced DNA damage responded in a dose-dependent manner (Figs. 3 and 4). The DNA damage measured as percentage of tail DNA in whole tissues of the control and treatment groups indicated that the alga exposed to different concentrations of Cu and cadmium ions exhibited significantly higher DNA damage in their tissues than the control sample. Analysis of comet data indicated significant differences between the control and all concentrations for both toxicants (significance $P<0.05$ for Cu and $P<0.005$ Cd). Increasing concentrations of metals increases the percentage of DNA damage. *U. reticulata* shows maximum DNA damage followed by *A. spicifera* and *C. antennina*. Compared to Cu, the cadmium showed significantly higher genotoxicity ($P<0.005$) in the selected macroalgae.

Discussion

Marine macroalgae have been shown to be good bioindicators of metal contamination in seawater, which is a reason why macroalgae are used in phytoremediation (Akcali and Kucuksezgin 2011). Accumulation of metals in marine environments has been extensively studied using macroalgae due to their ability to concentrate and tolerate high metal levels (Bermúdez et al. 2011; Huang et al. 2010). Their upper tiers of the aquatic food chain make them important in energy transformation and nutrient cycling, leading them as vital organisms to be monitored (Mendes et al. 2013).

Accumulation and exposure of metals to seaweeds may increase oxidative stress, that can mitigate ion disequilibrium, cellular osmolarity, and ROS detoxification due to non-enzymatic and complex enzymatic pathway (Kumar et al. 2010; Wu and Lee 2008). From the present investigation, it can be deliberated that the absorption of metals by plants and algae can be a good indicator of metal accumulation, which can be accompanied by an induction of a variety of cellular changes. The total dissolved metal concentrations tested in the present investigation were only the representatives of highly contaminated marine environments. The present results can be a reference to assess the metal contamination using seaweeds in the coastal regions, as reported in other aquatic plants, such as *Hydrodictyon reticulatum* and *Chara globularis*, *Chlorella vulgaris*, *Nasturtium officinale*, *Mentha aquatica*, and *Bacopa monnieri* (Aslan et al. 2003; Bajguz and Godlewska-Żyłkiewicz 2004; Singh et al. 2006; Johnson et al. 2007).

One of the mechanisms that was involved in the prevention of metal-induced cell destruction has been the synthesis of

Table 2 Changes in biochemical endpoints in *A. spicifera*, *C. antennina*, and *U. reticulata* exposed to cadmium at different time intervals (mean ± SD; n=3)

	MDA			GSH			CAT	
	0 day	7th day	15th day	0 day	7th day	15th day	0 day	7th day
A. spicifera								
Control	0.16±0.01	0.19±0.02	0.19±0.10	2920±0.01[a]	2883±0.10[a]	2812±0.01[a]	2.78±0.1	2.62±0.10
0.25	0.98±0.23	1.66±0.01*	3.43±0.10*	2898±0.23[a]	2367±0.50*[a]	1802±0.50*[a]	3.46±0.23	5.03±0.10*
0.50	1.23±0.01	1.94±0.01*	3.76±0.10*	2812±0.90[a]	2168±0.50*[a]	1674±0.50*[a]	3.87±0.05	5.82±0.10*
0.75	2.41±0.01	3.98±0.01*	4.12±0.10*	2745±0.50[a]	2102±0.77*[a]	1428±0.50*[a]	4.12±0.42	6.07±0.10*
1.00	2.86±0.01	3.18±0.01*	4.60±0.10*	2710±0.50[a]	1943±0.50*[a]	867±0.86*[a]	4.57±0.5	6.36±0.50*
C. antennina								
Control	0.12±0.5	0.18±0.41	0.13±0.5	627±0.50[a]	1100±0.50[a]	1127±0.95[a]	5.07±0.02	3.96±0.50
0.25	0.76±0.1	1.46±0.5*	2.63±0.01*	925±0.50[a]	862±0.80*[a]	900±0.50*[a]	2.60±0.50	4.12±0.05*
0.50	0.93±0.1	2.43±0.1*	2.9±0.23*	628±0.01[a]	536±0.50*[a]	698±0.50*[a]	3.46±0.30	4.67±0.23*
0.75	1.38±0.5	2.43±0.1*	3.33±0.01*	515±0.01[a]	678±0.01*[a]	1060±0.50*[a]	3.67±0.02	7.98±0.50*
1.00	1.41±0.3	2.99±0.5*	3.89±0.5*	500±0.50[a]	715±0.50*[a]	1120±0.50*[a]	4.01±0.50	8.12±0.50*
U. reticulata								
Control	0.67±0.01	0.7±0.02	0.72±0.02	1490±0.1[a]	1108±0.1*[a]	700±0.23[a]	0.785±0.5	1.02±0.5
0.25	0.92±0.02	2.17±0.01*	3.64±0.01*	2050±0.44[a]	1490±0.98* [a]	682±0.50*[a]	1.53±0.23	2.17±0.01*
0.50	0.92±0.02	3.43±0.01*	4.23±0.01*	1530±0.23[a]	1466±0.81*[a]	590±0.23*[a]	1.48±0.1	2.23±0.01*
0.75	1.35±0.81	3.92±0.01*	4.67±0.01*	1344±1.10[a]	1420±0.50*[a]	515±0.10*[a]	1.32±0.50	2.61±0.23*
1.00	1.64±0.81	4.61±0.01*	5.10±0.02*	1212±0.50[a]	1372±0.10*[a]	458±0.50*[a]	1.12±0.23	2.74±0.81*

	CAT	SOD			GPx		
	15th day	0 day	7th day	15th day	0 day	7th day	15th day
A. spicifera							
Control	2.53±0.01	273±0.10	273±0.50	280±0.50	0.24±0.02	0.24±0.01	0.27±0.01
0.25	3.96±0.50*	278±0.1	280±0.70*	316±0.50*	0.44±0.02	1.27±0.01*	1.53±0.10*
0.50	4.23±0.50*	291±0.23	300±0.63*	390±0.05*	0.72±0.02	0.91±0.50*	1.83±0.23*
0.75	4.65±0.50*	302±0.50	305±0.62*	423±0.50*	0.53±0.02	1.41±0.02*	2.01±0.10*
1.00	5.20±0.50*	302±0.01	312±0.50*	499±0.50*	0.90±0.02	1.72±0.02*	2.34±0.10*
C. antennina							
Control	3.77±0.50	140±0.23	142±0.50	156±0.50	0.11±0.05	0.17±0.50	0.19±0.02
0.25	5.67±0.50*	195±0.50	213±0.50*	237±0.33*	0.2±0.50	0.40±0.50*	0.48±0.40*
0.50	6.21±0.01*	198±0.50	247±0.50*	243±0.50*	0.2±0.81	0.44±0.01*	0.56±0.50*
0.75	8.01±0.01*	210±0.50	284±0.50*	282±0.50*	0.24±0.50	0.56±0.81*	0.40±0.50*
1.00	9.64±0.23*	230±0.23	312±0.50*	283±0.50*	0.34±0.81	0.72±0.81*	0.40±0.50*
U. reticulata							
Control	1.14±0.5	140±0.5	142±0.50	156±0.10	0.4±0.02	0.48±0.01	0.51±0.02

Table 2 (continued)

	CAT		SOD		GPx		
	15th day	0 day	7th day	15th day	0 day	7th day	15th day
0.25	3.13±0.01*	195±0.23	213±0.50*	237±0.50*	0.62±0.01	0.96±0.10*	2.59±0.02*
0.50	3.42±0.01*	198±0.50	247±0.50*	275±0.50*	0.86±0.01	2.13±0.01*	2.79±0.81*
0.75	3.66±0.01*	210±0.23	284±0.50*	330±0.50*	0.98±0.02	2.53±0.50*	2.91±0.81*
1.00	3.81±0.50*	230±0.50	312±0.50*	385±0.50*	1.06±0.02	2.91±0.02*	3.26±0.02*

LPO lipid peroxidation in mM MDA formed/min/mg protein; *CAT* catalase H_2O_2 consumed/min/mg protein; *GSH* reduced glutathione in nmol/mg protein; *SOD* superoxide dismutase in mg/protein; *GPx* glutathione peroxidase—µg of glutathione utilized/min/mg protein

*$P<0.05$, when compared to their respective controls and previous exposure periods

[a] Standard error expressed in terms of percentage

antioxidative enzymes (Collin et al. 2003; Wu and Lee 2008). Elevated levels of antioxidant enzymes predominantly SOD, CAT, and GPX in algae following the Cd and Cu treatment in this study indicated that these enzymes could act in combination to reduce the impact of metal toxicity.

Cu is a transition metal involved in the Fenton reaction and was among the source of hydroxyl radicals; however, Cd cannot participate in redox reaction (Huang et al. 2010), while Cd can promote the pro-oxidant status via reduction of the GSH pool, and bind the sites of essential elements, thus leading to the induction of hydroperoxides and superoxide anions (Huang et al. 2010). Accordingly, increase in lipid peroxidation (TBARS content) content following the exposure to Cd and Cu in the present study clearly indicates the ROS generation confirming the state of oxidative stress (Palanikumar et al. 2012a).

Yan et al. (2010) reported an increase of MDA content in *Avicennia marina* when exposed to lead. Kumar et al. (2010) reported that TBARS increased twofold times when *Ulva lactuca* exposed to higher concentrations of Cd. The increases in LPO (TBARS content) were high as when the accumulation of metal increase as supported by other researchers (Burrit et al. 2002; Contreras et al. 2005). Collén et al. (2003) reported that Cu exposure was able to induce a greater extent of LPO than Cd exposure. However, at the intial time point, Cd had shown greater extent than Cu, and prolonged Cu treatment influenced elevated LPO, that might be due to varying metal accumulation in the chosen seaweeds and the results are in agreement with other reports (Baumann et al. 2009; Kumar et al. 2010).

Control cultures of marine algae possessed the minimum value of antioxidant enzymes. A concentration-dependent increase in antioxidant activity was observed in the present work, similar to the reported results in *C. vulgaris* (Bajguz 2010). Increased glutathione levels had been shown to correlate with plant adaptation to extreme metal stress, and decreased glutathione pool shows marked alterations in response to metal stress (Jin et al. 2008; Masood et al. 2012). Moreover, glutathione is also a precursor of phytochelatins, which are low molecular mass peptides produced by plants to immobilize toxic metals (Tsuji et al. 2005; Groppa et al. 2007). Therefore, the increased glutathione level noted in *A. spicifera* when treated with Cd and *C. antennina* when treated with Cu at a range of concentrations, that may precede phytochelatin accumulation by intracellular sequestration of metal ions (De Vos et al. 1992).

Induction of SOD activity in plant cells had been correlated with increased tolerance to a variety of chemical compounds and physical stresses (Mittler 2002). Induced SOD activity can either be due to the increased production of ROS or the protective measure adopted by macroalgae against oxidative damage. CAT is one of the key enzymes involved in the

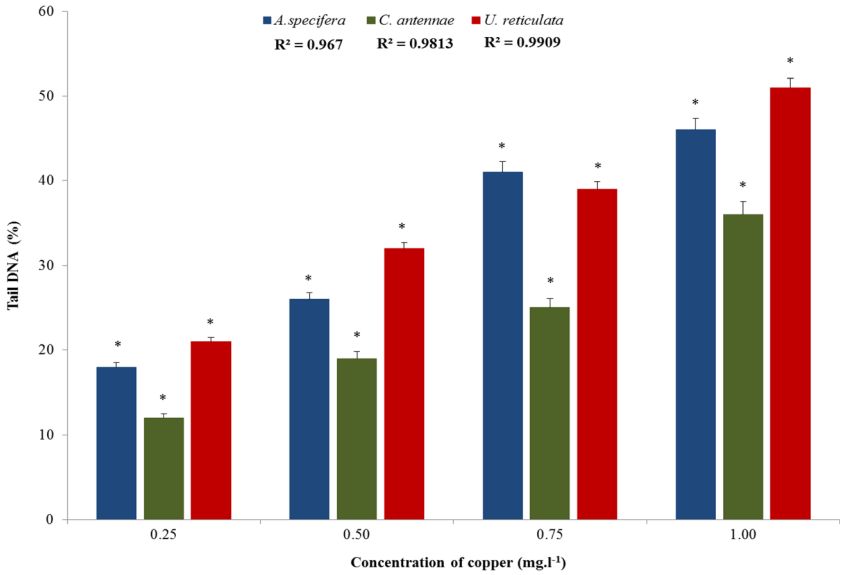

Fig. 3 Tail DNA damage measurement in cadmium treated seaweeds. *P<0.01 statistically significant when compared to control (*error bars* represent mean ± SD, n=3)

removal of toxic peroxides as it quenches H_2O_2 to water and molecular oxygen. In the present study, the increase in CAT activity can be considered as an adaptive mechanism developed by plants (Reddy et al. 2005). Reduction of CAT activity observed at the higher concentration of metals might be attributed to inactivation of enzyme by ROS, decrease in synthesis of enzyme, or change in the assembly of its subunits (Verma and Dubey 2003).

In this work, SCGE turned out as a useful and efficient tool to evaluate the genotoxic effect of increasing Cd and Cu contamination. Very distinct DNA alteration in three macroalgae was observed when exposed to higher concentrations of Cd and Cu. But the effect of Cu was slightly less than that of Cd at higher metal concentrations. Exposure to Cd and Cu generates ROS in plant cells, including H_2O_2, usually metabolized into water and oxygen in the presence of catalase (Olmos et al. 2003). Consequently, the high content of catalases and other enzymes inactivating ROS in algae may prevent their reaction with DNA. In conclusion, in the marine algae, the short-term exposure to metals seriously impairs cell DNA stability, as evidenced by the alkaline SCGE (comet) method, as well as plant growth. The increase in SOD, CAT, and GPx activity together with a higher redox level of non-enzymatic antioxidants could prevent the increase of metal ion induced ROS level and consequently limit DNA damage in macroalgae exposed to low concentration.

In summary, Cu and Cd induce the formation of harmful ROS, which causes irreversible damage to macromolecules such as lipid and proteins. To overcome this, cells have evolved enzymatic mechanisms such as SOD, CAT, GPx,

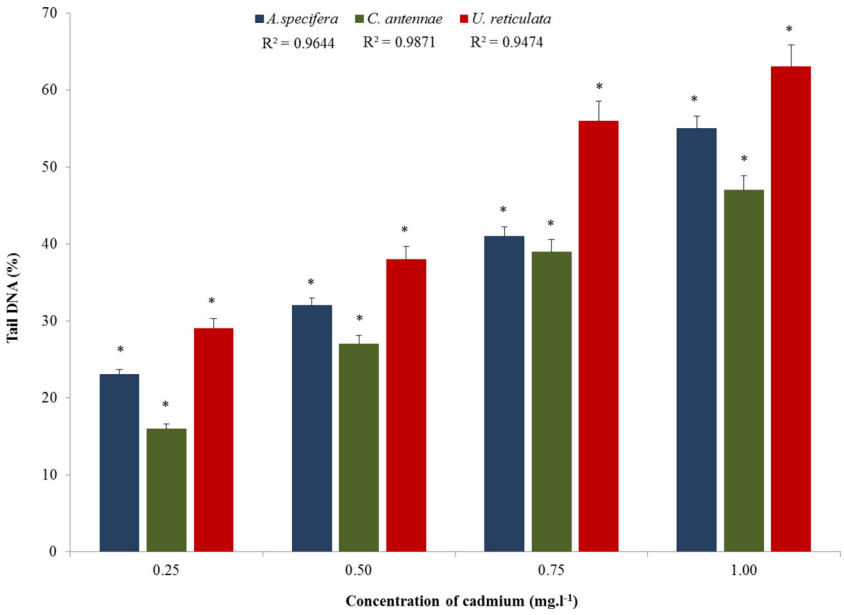

Fig. 4 Tail DNA damage measurement in copper treated seaweeds. *P<0.01 statistically significant when compared to control (*error bars* represent mean ± SD, n=3)

and GSH to eliminate or reduce their damaging effects. The results indicate that the early oxidative stress induced was more pronounced with Cd than with Cu; however, after prolonged metal exposure, oxidative stress was mainly enhanced under Cu exposure. This makes these species potentially interesting for metal absorption/filtration application in wastewater treatment plants for the future. The variations in bioaccumulation levels of metals were observed in the present study when compared to the results reported by several authors may be due to various environmental factors including the physico-chemical parameters and regional influence (Palanikumar et al. 2012a).

Conclusion

Pollution in aquatic environments by metals has received considerable attention. The majority of the organisms taken from aquatic environments for human consumption come from marine ecosystems. For this reason, pollution impact studies that focus on the marine environment are receiving more attention. In general, excess of metals has a negative effect on plant growth and metabolism. Furthermore, redox-active metals such as Cu stimulate formation of ROS by the Fenton reaction, which may result in oxidative stress leading to damage of biomolecules such as lipid and proteins. Hence, regulatory measures have to be taken by the authorities to limit the concentration of metal pollutants in the marine environment. This study also shows a range of biochemical responses measured in macroalgae that can be used as biomarkers or bioindicators of Cd or Cu contaminations in highly contaminated marine ecosystems.

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