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Assessment of oxidative stress indices in a marine macro brown alga *Padina tetrastromatica* (Hauck) from comparable polluted coastal regions of the Arabian Sea, west coast of India

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Abstract

Oxidative stress and antioxidant defence systems were assessed in a marine brown alga *Padina tetrastromatica*, commonly occurring from the tropics. Lipid peroxidation (LPX) and H_2O_2 were measured as oxidative stress markers, and antioxidant defences were measured as catalase (CAT), glutathione *S*-transferase (GST) and ascorbic acid (AsA), in order to understand their dissimilarity with respect to pollution levels from selective locations along the central west coast of India. A significant increased levels of LPX, H_2O_2, CAT and GST were observed in samples from relatively polluted localities (Colaba and Karwar) when compared to less polluted locality (Anjuna), while AsA concentration was higher in algal samples from worst polluted region of Colaba. Heavy metals such as Cd and Pb were also higher in the vicinity of polluted areas compared to reference area. Variation of oxidative stress indices in response to accumulation of heavy metals within *P. tetrastromatica* could be used as molecular biomarkers in assessment and monitoring environmental quality of ecologically sensitive marine habitats.

Key words: *Padina tetrastromatica*; lipid peroxidation; antioxidant; biomarker; monitoring

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Introduction

Macro algae are the group of marine plants classified on the basis of pigmentation in to green, brown and red algae and also major primary producers in the marine environment, play an important role in energy transfer. However, lately, various marine habitats are continuously threatened by various stressors such as temperature, ultraviolet radiation, or toxic metals. In biological systems, reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\bullet -}$) and hydroxyl radical ($\cdot OH$) are produced in normal metabolic pathways, as well as due to the exposure of different xenobiotic substances (Halliwell and Gutteridge, 2001). Living beings protect against these oxygen centered molecules, to some extent by developing antioxidant defence systems, which constitute both enzymatic and non enzymatic bimolecules, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione *S*-transferase (GST), reduced glutathione (GSH), and ascorbic acid (AsA) (Halliwell and Gutteridge, 2001).

Marine algae, particularly macro forms, are notable bioindicator species in environmental pollution studies (Amado et al., 1999; Sawidis et al., 2001; Conti and Cecchetti, 2003). Macro algae, especially Phaeophyceae, accumulate more metals due to the presence of negatively charged polysaccharides in physodes (Salgado et al., 2005). In view of this, efforts were made to determine various oxidative stress indices in *Padina tetrastromatica*, from central west coast of India, Arabian Sea. This is the first ever study on application of oxidative stress indices within *P. tetrastromatica*, as flag or key tone species for evaluation of marine environmental qualities.

1 Materials and methods

1.1 Site description

Algal samples (*P. tetrastromatica*) were collected from three sites along central west coast of India (Fig. 1). Anjuna, Goa (15°51′N, 73°53′E), which is cliff-locked beach and away from industrial area with low pollution levels. However, Colaba in Maharashtra (18°52′N, 72°47′E) and Karwar in Karnataka (14°50′N, 74°48′E) receive various effluents from several industrial sources including caustic soda plant, fertilizer plant, iron ore processing plants, dye and pigment processing plants, petroleum refineries, port area, and other sources (CPCB, 1996).

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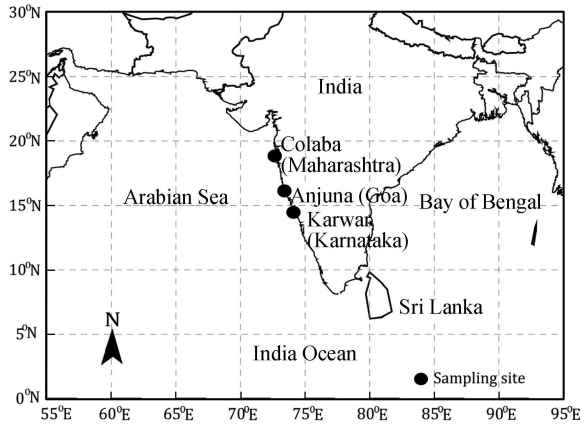

Fig. 1 Sampling sites for *Padina tetrastromatica* collected from central west coast of India, Arabian Sea.

1.2 Sample collection

The brown alga (Phaeophyceae), *P. tetrastromatica* (Family: Dictotaceae) is ubiquitous in the intertidal waters of Indian coasts. Fresh fronds of *P. tetrastromatica* were collected from intertidal zones at three locations during low tides, September 2009. Samples were hand picked along with holdfast, washed with sea water followed by ice cold distilled water, and then immediately frozen in liquid nitrogen.

1.3 Physico-chemical parameters

Physico-chemical parameters at each location were measured during every sampling occasion (Table 1). Surface water salinity was measured with a portable Salinity Refractometer (ATAGO, S/Mill, Japan). The pH of the surface water was measured by a scan microprocessor-based pocket pH tester (YK-35425-10, OAKTON, Malaysia). Surface water temperature was noted using mercury thermometer at the time of collection.

1.4 Metal analysis

Cadmium (Cd) and lead (Pb) contents in algal samples were determined using atomic absorption spectroscopy (A Analyst 300, PerkinElmer, USA) by Toth et al. (1948). Dried algal materials were digested with concentrated HNO_3-$HClO_4$ (3:1, *V/V*).

1.5 Oxidative stress indices

1.5.1 Lipid peroxidation

The level of lipid peroxidation (LPX) was measured in terms of malondialdehyde (MDA), a product of LPX estimated by thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). Fresh algal sample (0.5 g) was homogenized in 5 mL of 10% (*W/V*) trichloro acetic acid (TCA), and the homogenate was centrifuged at 7000 ×*g* for 10 min. One milliliter of the supernatant was mixed with 2 mL of 0.5% TBA solution (in 10% TCA). Then the mixture was heated at 95°C for 45 min and cooled under room temperature. The supernatant was read at 532 nm after removal of any interfering substances by centrifuging at 4000 ×*g* for 10 min. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated by using an extinction coefficient of 1.56×10^5 $(mol/L)^{-1}cm^{-1}$ (Wills, 1969), and expressed as nmol TBARS/g weight (wt) tissue.

Table 1 Physico-chemical parameters of water bodies in different locations during sampling periods

Parameter	Anjuna	Colaba	Karwar
pH	7.71 ± 0.01	8.00 ± 0.04	7.98 ± 0.03
Salinity (‰)	32.1 ± 0.4	32.9 ± 0.6	31.5 ± 0.5
Temperature (°C)	28.8 ± 0.2	28.3 ± 0.2	29.0 ± 0.1

Data are expressed as mean ± SD (n = 3).

1.5.2 Hydrogen peroxide content

The H_2O_2 content was determined according to Sergiev et al. (1997). Algal material (0.5 g) was homogenized with 5 mL of 10% (*W/V*) TCA in an ice bath. The homogenate was centrifuged at 7000 ×*g* for 10 min, and the supernatant (0.5 mL) was added with 1.5 mL of 50 mmol/L potassium phosphate buffer (pH 7.0) and 1 mL of 1 mol/L potassium iodide (KI), and the absorbance was measured at 390 nm (UV 1800, Shimadzu, Japan). H_2O_2 was used as a standard and expressed as nmol H_2O_2/g wt tissue.

1.5.3 Preparation of enzyme extracts

Frozen algal samples (0.5 g) were homogenized in ice cold 50 mmol/L potassium phosphate buffer (pH 7.0) containing 0.1% (*V/V*) Triton X-100 and 1% (*W/V*) polyvinylpyrollidone (PVP). The homogenate was centrifuged at 10,000 ×*g* for 15 min at 4°C, and the supernatants were used for enzyme assays.

1.5.4 Catalase

The activity of CAT was measured by slightly modifying the method of Aebi (1974). The assay mixture contained 2.9 mL of 15 mmol/L H_2O_2 in 50 mmol/L potassium phosphate buffer (pH 7.0) and 0.1 mL of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed as nkat/mg protein (1 katal = 1 mol/sec)

1.5.5 Glutathione *S*-transferase

GST activity was measured by using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, according to the protocol by Habig et al. (1974). The reaction rate was recorded at 340 nm, and enzyme activity was expressed as nmol CDNB conjugate formed/(min·mg protein) using a molar extinction coefficient of 9.6 $(mmol/L)^{-1}cm^{-1}$. Protein content was estimated by the Folin-Phenol reaction as described by Lowry et al. (1951).

1.5.6 Ascorbic acid content

Algal material was homogenized in an ice bath with 5 mL of 10% (*W/V*) TCA and centrifuged at 7000 ×*g* for 10 min. The deproteinised supernatant was used as assay for ascorbic acid following the stoichiometric reduction of phosphomolybdate by ascorbic acid (Mitusi and Ohata, 1961). AsA was used as the standard, and results were expressed as μg AsA/g wt tissue.

1.6 Statistical analysis

Results are expressed as mean ± standard deviation (SD). Differences among the means were analyzed by ANOVA and post hoc tests (Newman-Keuls). Differences were considered statistically significant when $P < 0.05$.

2 Results and discussion

2.1 Metal analysis

In marine environments, the concentration of metals is largely influence by both natural and anthropogenic sources. Metals such as Cu, Mn, Fe, and Zn, are essential micronutrients, but can become toxic at concentrations higher than the amount required for normal growth (Nies, 1999). However, other metals, such as Cd, Hg, and Pb, have so far unknown roles in living organisms, and are toxic even at very low concentrations (Wood, 1974; Nies, 1999). In the present study, Table 2 depicts the Cd and Pb contents in *P. tetrastromatica* from Colaba, Karwar, and Anjuna. The relatively higher values of those metals from samples of Colaba and Karwar as compared to Anjuna could be attributed to high levels of pollution ambience. It has been reported that both of these polluted areas (Colaba and Karwar) receive varieties of pollutants including metals, petrochemicals as well as sewage generated from various anthropogenic activities (NIO, 2001, 2007; CPCB, 1996).

Table 2 Metal accumulation (mg/g dry weight) in tissues of *Padina tetrastromatica* at different sampling sites

Metal	Anjuna	Colaba	Karwar
Cd	0.0022 ± 0.0003	0.006 ± 0.0001	0.004 ± 0.0003
Pb	0.0028 ± 0.0001	0.006 ± 0.0005	0.0034 ± 0.0001

Data are expressed as mean ± SD ($n = 3$).

2.2 Lipid peroxidation

Exposure to environmental stress such as high light intensities, UV radiation, toxicants produces ROS in cells (Aust et al., 1985; Marshall and Newman, 2002), which destabilize the membrane and attributed to LPX (Mead et al., 1982). In the present study, LPX were significantly higher in the samples from Colaba and Karwar than in the samples from Anjuna (Fig. 2A, $P < 0.05$). Similarly, enhanced levels of H_2O_2 were also noticed in the samples from polluted areas (Fig. 2B). Increased levels of LPX and H_2O_2 in *P. tetrastromatica* from different localities were significantly correlated with the heavy metal concentrations (Table 2, $P < 0.05$). It has been reported that, Cd levels in tissue induces a variety of cellular changes, such as damage of membrane integrity (Smeets et al., 2005), reduces photosynthesis (Van Assche and Clijsters, 1990), and impaired CO_2 assimilation (Gouia et al., 2003), which might produce ROS and resulting LPX. Induction of LPX levels were also reported in plants exposed to Cd (Liu et al., 2007) and Pb (Reddy et al., 2005; Dazy et al., 2009).

2.3 Antioxidant defence systems

Plants, as photosynthetic organisms, are continuously producing ROS during photosynthesis and other metabolic processes (Foyer and Noctor, 2000). In addition to this, ozone, salt stress, drought, heat, heavy metals, toxins and organic pollutants also induce the formation of ROS (Pflugmacher, 2004). To protect against the unfavorable ROS environment, cell induces its own conserved antioxidant defences such as SOD, CAT, GPX, GST, GSH, MT, AsA (Halliwell and Gutteridge, 2001). SOD scavenges $O_2^{\bullet -}$ to H_2O_2, and H_2O_2 subsequently scavenged by CAT, GPX and ascorbic acid peroxidase (APX) (Halliwell and Gutteridge, 2001; Pinto et al., 2003). Exposure to stress generates ROS, which serve as signals to activate the

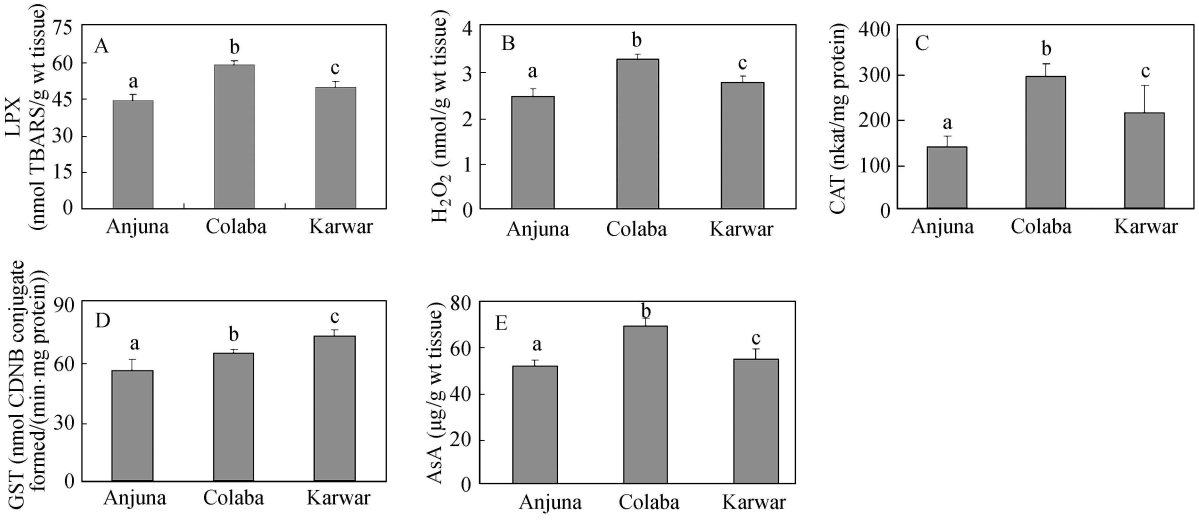

Fig. 2 Biochemical parameters at different sites along west coast of India. (A) lipid peroxidation (LPX); (B) H_2O_2; (C) catalase (CAT); (D) glutathion S-transferase (GST); (E) ascorbic acid content (AsA). Data are expressed as mean ± SD ($n = 5$). Superscripts of different letters are significantly different from each other at $P < 0.05$.

defence system in plants (Mittler, 2002). In the present study, CAT activity was significantly high in samples from Colaba and Karwar, further indicating pollution stress increasing the formation rate of H_2O_2 (Fig. 2C). The importance of CAT as antioxidant lies in its involvement with the Haber-Weiss reaction, responsible for removing H_2O_2 and generating extremely reactive molecule such as OH· (Storey, 1996). Interestingly higher levels of H_2O_2 were measured in samples from respective polluted area as compared to reference sites (Fig. 2B). The induction of CAT activity in *P. tetrastromatica* from polluted region might be to protect against unfavorable ROS environment. A significant correlation was observed between CAT and H_2O_2 scavenging action ($r = 0.99$, $P < 0.01$) to support this statement. Such phenomenon has also been reported in algae treated with Cd and Pb (Dazy et al., 2009).

Macroalgae, like all living organisms are capable of metabolising xenobiotics. Plants and algae have been considered as the "green liver" of ecosystems due to their important role in the biotransformation of xenobiotics (Tang et al., 1998; Pflugmacher et al., 1999; Lei et al., 2003). GST are a family of enzymes with a determinant function in the detoxification processes (Thom et al., 2001) by catalyzing the conjugation of various electrophilic compounds with glutathione, the resulting conjugates being more water soluble and thus easily excretable (Cheung et al., 2001). Increased levels of GST activity in *P. tetrastromatica* samples from Colaba and Karwar, compared to those from Anjuna (Fig. 2D) suggested pollutant stress might have induced GST levels to combat tissues from xenobiotic substances. Elevated GST activity has also been observed in plants treated with Cd (Aravind and Prasad, 2005; Mishra et al., 2009). Similarly to animals, GST activity of some species of algae (eg., *Scenedesmus obliquus*, *Ulva lactuca*, *Laminaria saccharina* and *Cyclotella meneghiniana*) and plants (e.g., *Lemna minor* and *Nuphar lutea*) is increased in the presence of several environmental contaminants such as some herbicides (e.g., atrazine, oxyfluorfen and diuron) and polycyclic aromatic hydrocarbons (PAHs) (Schrenk et al., 1998; Tang et al., 1998; Geoffroy et al., 2002).

AsA and GSH are direct scavengers of ROS, and an additional substrate for various antioxidant enzymes (Halliwell and Gutteridge, 2001). It has been reported that, ascorbate-glutathione cycle (AGC) plays an important role in detoxification of ROS (Kuzniak and Maria, 2001). High concentrations of AsA were also recorded in the samples from Colaba and Karwar as compared to Anjuna (Fig. 2E) could suggest that AsA synthesis might have stimulated to protect against these metal contaminated environment. Although not many studies have reported on AsA levels in response to polluted environmental conditions, interestingly a significant increase in AsA content was observed in response to Cd (Aravind and Prasad, 2005). Therefore, elevated levels of AsA in *P. tetrastromatica* could be attributed to metal ions habitat from those localities. A significant correlation between AsA and Cd levels in the algal tissue also supports this statement (Table 3).

Table 3 Correlation coefficients (r) between accumulation of metals and biochemical parameters

	LPX	H_2O_2	CAT	GST	AsA
Cd	$r = 0.99$	$r = 0.99$	$r = 0.999$	$r = 0.99$	$r = 0.99$
	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
Pb	$r = 0.98$	$r = 0.98$	$r = 0.95$	$r = 0.156$	$r = 0.92$
	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P > 0.05$	$P > 0.05$

3 Conclusions

Padina tetrastromatica displays different biochemical responses to environmental conditions. Elevated levels of LPX and H_2O_2, indicating a state of oxidative stress possibly due to accumulation of metal ions. Elevated antioxidant defences in algae exposed to xenobiotic substances or metals is indicative of required protection against ROS production (Lee and Shin, 2003; Hong et al., 2008), and the evaluation of metal concentrations in *P. tetrastromatica* appears to confirm such a relationship (Table 3). The present data revealed that oxidative stress markers LPX and H_2O_2, non-enzymatic antioxidant such as AsA and antioxidant enzymes (CAT and GST) are useful biomarkers, indicative of this species as key tone or flag species for evaluation and monitoring of marine environment.

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