

Oxidative stress tolerance in intertidal red seaweed *Hypnea musciformis* (Wulfen) in relation to environmental components

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Abstract

Oxidative stress parameters in relation to temperature and other factors have been analysed in *Hypnea musciformis*, the red seaweed from Anjuna beach, Goa with an aim to understand its susceptibility to changing seasons. The results indicate that elevated temperature, sunshine and desiccation during peak summer in May enhanced activity of LPX, H₂O₂ and antioxidants such as catalase, GSH and ASA. Statistical tests using MANOVA and correlation analysis showed that oxidative stress and antioxidants maintain significant relation with temperature, salinity, sunshine and pH at an individual or interactive level. The dissolved nitrates, phosphates and BOD in ambient waters and the trace metals in seaweeds maintained sufficiently low values to provide any indication that could exert contaminant oxidative stress responses. The present field studies suggest that elevated antioxidant content in *H. musciformis* offer sufficient relief to sustain against harsh environmental stresses for its colonization in the rocky intertidal zone.

Keywords: *Hypnea musciformis* · Lipid peroxidation · Catalase · Temperature · Metal

Introduction

Hypnea musciformis is a red algae prevailing throughout Indian coast and most parts of the world (Khan and Satam 2003). This species is an important carrageenan resource and often used as raw material in phycocolloid gelling industry. The presence of kappa carrageenan similar to *Kappaphycus* in *Hypnea* has prompted several countries to initiate cultivation of this species (Humm and Kreuzer 1975; Mshigeni 1976). Commercial exploitation of seaweed resources in India began with the establishment of a number of small and medium-scale seaweed phycocolloid industries during late 1970s. This led to the depletion of natural stocks and triggered attempts to cultivate seaweeds locally for commercial use. In this process, *Kappaphycus alvarezii*, an alien species favoured commercial cultivation in south India (Eswaran et al. 2002). *H. musciformis* although an indigenous species, having large demand, was not seriously attempted for cultivation (Ganesan et al. 2006). Probably, information on its physiological behaviour with respect to seasons and environmental factors has been inadequate to decide its standing stock assessment and cultivation strategies.

Marine seaweeds being predominant in intertidal zones, they are periodically subjected to direct exposure to solar radiation, aberrant temperatures and emersion due to tidal fluctuations. Similarly, annual monsoon precipitation also brings about seasonal changes in salinity, ambient water quality and mineral composition. These changes are likely to influence the normal metabolic functions in algae creating oxidative stress through increased production of reactive oxygen species (ROS). ROS are highly reactive species of molecular oxygen such as singlet oxygen ($^1\text{O}_2$), the superoxide anion radical ($\text{O}_2^{\bullet-}$), the hydroxyl radical ($\bullet\text{OH}$) and hydrogen peroxide (H_2O_2). Overproduced ROS cause lipid peroxidation and consequent membrane damage, protein and nucleic acid degradation and pigment bleaching (Hendry and Crawford 1994). Plants are well-equipped with antioxidant defences to provide effective protection against the oxidative stress produced. Intertidal seaweeds have also developed mechanisms to prevent lethal physiological damages incurred to sustain environmental changes and maintain their physiological integrity. This could be in the form of enzymatic antioxidants such as SOD, CAT and GPX. SOD dismutates reactive $\text{O}_2^{\bullet-}$ to H_2O_2 which is reduced to water and molecular oxygen by CAT. Similarly, GPX catalyzes the reduction of H_2O_2 to water and organic peroxide to alcohols using GSH as a source of reducing equivalent. GR regenerates GSH from oxidized glutathione (GSSG), which is a scavenger of ROS as well as a substrate for the other enzymes. GST is involved in metabolism of many endogenous hydroperoxides and prevents any hydrogen peroxide formation (Arvind and Prasad 2005). It also conjugates xenobiotics with GSH for excretion. The non-enzymatic

component consists of small organic molecules such as β -carotene, GSH, vitamin E and vitamin C. These ROS scavenging enzymes and antioxidants are present within algal cells also as protective agents against the damages commonly associated with ROS. Since ROS are highly reactive with most cellular components damaging proteins, lipids and nucleic acids they must be neutralized quickly and efficiently at once formed.

To date there has been not much information on field evaluation of antioxidant properties of *H. musciformis* with respect to seasons and environmental stresses. Determination and evaluation of antioxidant status is important to decide about the seasonal growth and behavior of the plants with respect to the fluctuating biotic and abiotic conditions in marine habitat (Choo et al. 2004). The attempt of the present studies is to examine the influence of antioxidants in protecting the cellular components to sustain growth of *H. musciformis* against harsh environmental conditions arising from changing seasons. The observations are therefore designed to indicate how the temperature, salinity, pH and sun exposure would affect the cellular damage and antioxidant responses in *H. musciformis* inhabiting the intertidal zone from the Anjuna Beach, Goa (West coast of India).

Materials and Methods

Sampling site

Hypnea musciformis collected from the Anjuna coast, Goa (Lat 15°51' N, Long 73°53'E; Fig. 1), along central west coast of India covering the three annual periods of post-monsoon i.e. November 2008, pre-monsoon i.e. February 2009 and May 2009 and monsoon i.e. August 2009. Anjuna is a cliff-locked beach, away from any polluting industrial site. Algal samples from the intertidal region, exposed during low tide were hand-picked along with holdfast and washed with sea water followed by ice-cold distilled water, then immediately frozen in liquid nitrogen for further analysis of antioxidant parameters. Seaweed sample collected during November, May and August was dried, powdered, acid-digested and analysed for trace metals such as Fe, Mn, Cu, Cd, Pb, Hg and As using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Perkin-Elmer, and Optima 2000).

Analytical methods

Metal analysis in seaweed samples

The whole seaweed sample collected during November, May and August was dried and powdered for metal analysis. For this purpose, 0.5g of dried samples were digested with 20ml concentrated HNO₃ in a

beaker covered with a watch glass. After complete digestion, the samples diluted to 100 ml and filtered through Whatman No.1 filter paper. The acid digest was analysed for trace metals such as Fe, Mn, Cu, Cd, Pb, Hg and As using atomic absorption spectrophotometer (A Analyst 300, Perkin Elmer, USA; Toth et al. 1948).

Water analysis

On the spot measurement of aquatic parameters during 4 occasions included water temperature using a hand-held thermometer, salinity with a portable Salinity Refractometer (Atago, S/Mill, Japan) and pH by waterproof pH scan microprocessor-based pocket pH tester (YK-35425-10, Oakton, Malaysia). Surface water samples were collected from intertidal region for analysis of nutrients, BOD and metal content once during each season of post-monsoon (November), pre-monsoon (May), and monsoon (August). Nutrients such as nitrate, nitrite, phosphate and silicate in water were measured in the laboratory by following procedures described by Grasshoff et al. (1983), while BOD was measured using Winkler method. Dissolved metals in water such Fe, Mn and Pb were analysed by pre-concentrating the metals in filtered water samples by chelating the metals with ammonium pyrrolidine dithiocarbamate (APDC), followed by extraction in an organic solvent- methyl iso-butyl ketone (MIBK). The organic extract was back extracted in ultra-pure HNO₃. The metals Fe, Mn, Cd, and Pb were measured in the inorganic extract using a graphic furnace atomic absorption spectrophotometer (A Analyst 300, Perkin Elmer). Standards of metals were run in similar manner for calibration purpose.

Oxidative stress indices

Seaweed samples for lipid peroxidation, hydrogen peroxide and other antioxidant parameters were analyzed during 4 occasions in November (autumn/post-monsoon), February (early summer/pre-monsoon) and May (peak summer/pre-monsoon) and August (rainy/monsoon).

Lipid peroxidation

The level of lipid peroxidation (LPX) was measured as 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 ml 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 then cooled under room temperature. The supernatant was read at 532 nm after removal of any interfering substances by

centrifuging at $4000 \times g$ for 10 min. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Wills, 1969), and expressed as nmol TBARS/g wt. tissue.

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 $7000 \times g$ for 10 min, and the supernatant (0.5 mL) was added with 1.5 mL of 50 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M 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.

Preparation of enzyme extracts

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

Catalase (CAT)

The activity of CAT was measured by slightly modifying the method of Aebi (1974). The assay mixture contained 2.9 mL of 15 mM H_2O_2 in 50 mM 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}).

Glutathione S-transferase (GST)

GST activity was measured 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 $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$. Protein content was estimated by the Folin-Phenol reaction as described by Lowry et al. (1951).

Reduced Glutathion and Ascorbic acid content (GSH and AsA)

Algal material was homogenized in an ice bath with 5 mL of 10 % (W/V) TCA and centrifuged at $7000 \times g$ for 10 min. The deproteinised supernatant was used as assay for GSH using DTNB (Ellman 1959) and expressed as nmol/g wt. tissue. Ascorbic acid (AsA) was analysed following the stoichiometric

reduction of phosphomolybdate by AsA (Mitusi and Ohata 1961). AsA was used as the standard and results were expressed as $\mu\text{g AsA/g wt. tissue}$.

Statistical analysis

Results of biochemical analysis are expressed as mean \pm standard deviation (SD). Differences among the means were analyzed by ANOVA and post hoc tests (Newman-Keuls). Differences were considered statistically significant at $P < 0.05$. A MANOVA test was applied to oxidative stress and antioxidant parameters in relation to salinity, temperature, pH and solar radiations and a simple regression analysis was also worked out with the environmental data.

Results

Biochemical observations in *Hypnea musciformis*

LPX content in the tissue samples of *Hypnea musciformis* ranged from 33 to 60 nmol TBARS/mg protein (Fig. 2A) during 4 sampling occasions being highest in May followed by November and with lower values in February and August. H_2O_2 levels ranged from 2.34 to 3.28 nmol/g wt. tissue (Fig. 2B) fluctuating in almost a similar trend of LPX remaining high in November and May and low during other two sampling occasions. CAT activity varied from 116.58 to 233 nkat/mg protein (Fig. 2C) and almost consistent with LPX and H_2O_2 with high values in May and November and low in February and August. GST however showed independent fluctuations ranging from 45.42 to 74.99 nmol CDNB conjugate formed/min/mg protein (Fig. 2D) with higher values in February and August and lowest in May. GSH which is involved in detoxification of electrophilic xenobiotics and reducing ROS ranged from 283.28 to 347.06 nmol/g wt. tissues with elevated values in November and May (Fig. 2E) and fluctuated similarly as CAT, LPX and H_2O_2 . Highest AsA values of 48 $\mu\text{g/g wt.}$ were recorded in May and the lowest of 39 $\mu\text{g/g wt. tissue}$ in August while the November and February values remained almost similar (Fig. 2F).

Water characteristics

Surface water salinity exhibited marked seasonal variations attaining highest of 34.2 psu in May (peak summer/pre-monsoon, Fig. 3C) and lowest of 30 psu in August (rainy/monsoon, Fig. 3B). Surface water temperature also remains high in May with a maximum of 30.2°C and the lowest of 24°C in February (early summer/pre-monsoon, Fig. 3C). Elevated pH of 8.12 in surface waters was seen in May and the lowest of 7.76 in August (Fig. 3C, B). Higher salinity and pH in May were consistent with increased temperature and solar radiations in peak summer (23.87 mega J/m^2). Higher salinity is because of higher

evaporation rate and negligible river discharges in May, while lower salinity and pH in August are the result of monsoon precipitation and land runoff. Water quality parameters analyzed were nutrients, biological oxygen demand (BOD) and dissolved metal content. Along with this, the fluctuations in biomass and tissue metal content was also measured to determine the interference of any contaminants in ROS production. BOD was moderately low ranging from 1.01 to 2.32 mg/g with higher values in November (autumn/post-monsoon, Fig. 3A). Nutrients such as nitrite, nitrate, phosphate and silicates ranged from 0.22 to 0.89, 4.23 to 6.95, 0.48 to 6.96 and 0.41 to 0.75 $\mu\text{mol/l}$, respectively with highest concentrations in May (Fig. 3C). This indicates that the nutrients adequately support the *H. musciformis* growth. The biomass values ranged from 27 to 98 g/m^2 being highest in May followed by November and August. Dissolved metals in intertidal waters such as Mn, Fe and Pb ranged from 0.12 to 0.4 mg/l, 2.02 to 10.9 mg/l and BDL (below detection limit) to 14 $\mu\text{g/l}$, respectively, remaining high in monsoon (Fig. 3B).

Tissue metal content

Trace metal content of Mn, Fe, Cu, Cd and Pb ranged from 102.2 to 144.2, 377 to 443.6, 0.94 to 1.58, 1.56 to 3.04 and 2.22 to 3.44 mg/100g dry tissue respectively. Similarly, Hg and As content ranged from 1.16 to 1.72 and 0.78 to 0.86 $\mu\text{g/g}$ dry tissue, respectively. Higher values of tissue metals content prevailed in monsoon followed by post-monsoon and pre-monsoon (Fig. 3A, B, C).

Solar radiations

Data on total daily solar radiation for Goa region during the three seasons were obtained from Metrological Laboratory, Pune indicated a highest average of 22.88 ± 1.09 mega j/m^2 for pre-monsoon (February to May 2009, Fig. 3C) and lowest of 16.74 ± 2.23 mega j/m^2 for monsoon (June to September 2009, Fig. 3B).

Discussion

Under field conditions, the seaweeds may be experiencing combined effects of several changes in environmental parameters. Hence quantification of the influence of single environmental factor may be less useful when the effects are related to more than one such factor. As shown under results oxidative changes in seaweeds appear to have strong relation to seasonal changes in environmental features. The statistical significance of this concept was carried out / done using MANOVA test of antioxidants with temperature, salinity, pH and sunshine in direct and interactive way. The results suggest direct as well as

combined influence of each of these factors on ROS and most antioxidants as evidenced by highly significant values in MANOVA test (Table 1, Table 2).

As per our investigations the rise in temperatures in November and May are accompanied with the elevated LPX and H₂O₂ content in *H. musciformis* while the reverse is true for the decreased temperatures in February and August (Fig. 2, Fig. 3). Increased reactive oxygen production at higher temperatures of 20°C compared to 0°C has been reported for intertidal brown macroalga *Fucus vesiculosus* under laboratory conditions (Collen and Davison 2001). An enhanced level of lipid peroxidation with increasing temperatures has been observed in the root and leaf segments of *Phalaenopsis* exposed to temperatures from 25 to 40°C (Ali et al. 2005). ROS generation due to temperature stress is stated to be main reason for increasing the LPX content in these species. This is mediated through OH[•] which is the most reactive oxygen radicals and has sufficient reactivity to remove a hydrogen atom from a methylene group of lipids and thus to initiate membrane peroxidation (Halliwell and Gutteridge 1989). MANOVA tests have also shown highly significant relation of LPX and H₂O₂ with changing temperatures in present observations. It appears therefore that heat stress in November and May could be one of the reasons for the increased values of lipid peroxidation and H₂O₂ in *H. musciformis*.

Among the antioxidant enzymes CAT fluctuated as per LPX and H₂O₂ indicating its direct involvement in scavenging hydroperoxides. CAT is activated in peroxisomes to scavenge H₂O₂ created by photorespiration (Asada 1992). Collen and Davison (1999) observed increased catalase activity associated with higher *in vivo* scavenging of H₂O₂ in red seaweed *Mastocarpus stellatus* suggesting it to be an important enzyme for H₂O₂ breakdown. Increase in SOD and CAT activities have also been recorded in *P. gatunense* under high irradiance and high pO₂/pCO₂ (Butow et al. 1994; 1997). Sampath-Wiley et al (2008) while studying the combined effects of heat, sunshine and dehydration reported elevated activities of CAT and other antioxidants during tidal emersion of *Porphyra umbilicalis* from intertidal zone. Thus, the present results give the indication that *H. musciformis* maintains sufficient antioxidant tolerance to sustain the elevated reactive oxygen metabolism of harsh environmental challenges especially during summer.

Physiological stress arising from high temperature and emersion has well been documented in red algae *Mastocarpus stellatus* (Smirnoff 1995; Collen and Davison 1999). Salinity and pH also show significant statistical relation with ROS as observed with temperature and solar radiations in present

studies (Table 1, Table 2). Increased oxidative stress in terms of elevated TBARS and antioxidants enzymes resulting from combined effects of high irradiance (photo-oxidative stress) and/or carbon depletion has been reported by Choo et al. (2004) for filamentous green algae *Cladophora glomerata* (L.) and *Enteromorpha ahlnneriana*. Elevated pH associated with decreased carbon availability is reported to be responsible to stimulate ROS production (Vardi et al. 1999) and catalase activity in dinoflagellate *Peridinium gatunense* (Butow et al. 1994). Increase in H₂O₂ production arising from CO₂ limitation during carbon fixation in *Ulva rigida* has also been reported by Collen et al. (1995). Significant relation seen in MANOVA test of LPX and H₂O₂ with abiotic factors both individually and interactive way indicates that temperature in association with salinity, sunlight and pH are together involved in ROS generation in *H. musciformis* observed at present (Table 1, Table 2).

Semidiurnal nature of the tidal system prevailing along the western Indian coast exposes our intertidal species *Hypnea musciformis*, to emersion stress twice during the 24-h period. Of this day time emersion during which samples were collected, is more crucial for ROS production because of excess desiccation arising from the combined effects of sunshine, temperature and salinity. Changing seasons could also affect ROS generation because of alterations in abiotic features. Accordingly, overcast sky and precipitation in monsoon (August) reduces solar radiations and salinity and ultimately the pH due to decreased photosynthesis. As skies get clearer and rainfall reduced from November to May, progressive increase in these abiotic parameters is evident (Fig. 3). But the temperature trend differs a bit with a significant drop in August influenced by monsoon precipitation and February by cooler northerly winds of fading winter season in between the high values in November and May. As indicated above, ROS production and CAT content tend to follow the temperature profile (Fig. 2, Fig. 3). It appears therefore that influence of environmental parameters such as solar radiations, salinity and pH in ROS production, revolve around the temperature fluctuations. Observations on *P. umbilicalis* are in support of the present studies where greatest increase in CAT and other antioxidants were reported during summer when temperature and sunshine were at its peak (Sampath-Wiley et al. 2008).

Although GSH is consistent with CAT values, the changes GST shows a reverse trend with a decline in November and May and increase in February and August. GST involves in catalyzing nucleophilic addition of glutathione to electrophilic compounds. Consumption of GSH further occurs due to its direct interaction with electrophilic compounds to replace hydrogen, chlorine, nitro-groups and all kinds of other so-called leaving groups. This is reflected in elevated GSH values during stressful periods of November and May. Replenishment of depleted GSH reserves is brought about by glutathione reductase

(GR) via oxidation of NADPH to NADP⁺. Restoration of the GSH is essential to bring about the recycling of reduced ascorbate via dehydroascorbate reductase. In present studies, AsA varied from lowest monsoon (August) to highest values in May. Ascorbate is an excellent electron donor and an important redox molecule of the complex ascorbate-glutathione cycle (Zhang and Kirkham 1996). This is the main H₂O₂ scavenging pathway operating in chloroplast as well as the cytosol. Similarly, GSH is a valuable component of thiol pool responding independently or in combination with other antioxidants like GST or GR to protect the cells from oxidative stress (May et al. 1998). MANOVA and correlation analysis also showed significant relation of GST with environmental variables as depicted by LPX, H₂O₂ and CAT. In case of GSH, MANOVA test was valid only at interactive level while correlation is significant for environmental variables both for GSH as well as ASA. Thus, the observed changes such as GST consumption and excess reserves of GSH and ASA during stressful periods of May and November reflect important role of these antioxidants to alleviate the algae from ROS stress.

Influence of salinity in inducing remarkable stress in algae has been shown for freshwater macrophytes *Pistia stratiotes* and *Salvinia molesta*. These algae indicated higher responses of H₂O₂, LPX, catalase, glutathione, ascorbate and others with salinity increase (Upadhyay and Panda 2005). It has been reported that the growth of the red algae *Gracilaria corticata* is favoured within a salinity range of 25-34 psu and hypo- or hyper-saline conditions create stressful situation increasing antioxidant content (Kumar et al. 2010). Influence of salinity on ROS and antioxidants is clear from their valid statistical relation in present studies. However, fluctuations in salinity are in lower range of 30 to 34.2 psu. It appears therefore that increased salinity in summer could act in synergism with other abiotic parameters in inducing the observed changes in antioxidant in these field samples. The function of AsA is vital to keep itself recycling continuously without which all of the available ascorbate would be oxidized in a matter of seconds during photosynthesis. Ultimately, this would cause a decline in APX activity to bring about lethal levels of oxidative damage (Asada and Takahashi 1987; Smirnoff 1995). Obviously, pre-monsoon/peak summer (May) appears to be a crucial period when most of the antioxidants and H₂O₂ remain elevated with highest rise in temperature, salinity, pH and solar radiations. Kumar et al. (2011) have reported that desiccation in *Gracilaria corticata* during the low tide was responsible for the loss of water accompanied by increase in ROS and antioxidants including GSH and ASA, during first 2-3h indicating stress conditions. Sampath-Wiley et al. (2008) have similarly observed the greatest antioxidant increase in *Porphyra umbilicalis* during summer months when temperatures followed by sun exposure and emersion attained their peak.

Nutrient contents in waters such as nitrates and phosphates determine the growth while the vitamin and minerals in edible seaweeds make them nutritionally valuable. Photosynthetic responses of *H. musciformis* from Florida coast were reported to be temperature dependent being highest in summer between a temperature range of 24 to 32⁰C (Durako and Dawes 1980). Sunlight being one of the prime factors for photosynthetic production the increased nitrates, phosphates and silicates (Fig. 3) associated with extended sunshine hours during peak summer was probably responsible for higher biomass production of *H. musciformis*. Fe, Mn and Cu are the bio-essential elements associated with a catalytic enzyme superoxide dismutase (SOD) and involved in photosynthetic activity of eukaryotic algae (Asada and Takahashi 1987). Hence uptake of these elements under the influence of solar radiations may be obvious.

Although the algal biomass remain high in pre-monsoon (May) and low in monsoon (August), the metal content in seaweeds showed inverse relation with low and high values in respective seasons. It appears therefore that the metal content in seaweed not only depends on photosynthetic activity but also on their bioavailability in surrounding waters (Jothinayagi and Anbazhagan 2009). This observation is supported by the analysis of some of the dissolved metals such as Mn, Fe and Pb in intertidal waters which varied from ND to 0.4, 203 to 1.089 and 0 to 0.0013 mg 100 ml⁻¹ respectively during the 3 seasons indicating elevated values in monsoon because of land drainage.

It has been reported that the concentration levels of essential and non-essential metals i.e. Mn, Pb, Zn, Cd, Cu and Cr in various seaweed samples from Wandoor, south Andaman Island, however, *A. calyculus* contained the highest concentration of heavy metals as compared to other algal species (Karthicka et al. 2012). Basically, seaweeds are known to concentrate metals and it is the increased concentration especially of non-essential trace metals such as Cd, Pb, Hg and As that could induce the oxidative stress in their tissues (Kumar et al. 2010; Collen et al. 2003). Our study reveals the Fe, Mn, Cu, Cd and Pb content in the seaweeds were much lower than those reported from contaminant free areas of Mandapam Coastal waters (Karthikai et al. 2010) and the marine biosphere reserve at Gulf Mannar in south India (Karthikai et al. 2009). Other toxic metals such as Pb and As are also lower than the seaweeds in moderately contaminated lake Venice in Italy (Caliceti et al. 2002). Similarly the Hg in seaweeds and BOD content in ambient waters were sufficiently low (Fig. 3). Also as per our previous report the present seaweed collection site (Anjuna beach) is not much affected by contaminant sources as reported for *Padina tetrastromatica* having low Cd and Pb content in relation to much higher values observed at pollution prone areas of Karwar and Colaba (Maharana et al. 2010). Under these conditions

it is not likely that BOD or contaminant metals could have much influence on the oxidative stress and antioxidants levels in *H. musciformis*. But the fact remains that the high biomass production is supported during the peak summer (May) period when environmental conditions such as temperature, sunshine, desiccation are at its peak to induce increased oxidative stress. It is obvious therefore that *H. musciformis* sustains physiological advantages to facilitate colonization in the rocky intertidal zone which is largely supported by elevated antioxidant contents. Thus the present field studies suggest that these seaweeds are sufficiently photo protected and it is the elevated antioxidant metabolism that are involved in providing them adequate relief to encounter acute environmental stresses in the intertidal zone.

Acknowledgements

Authors are thankful to the Director, CSIR-National Institute of Oceanography, Goa, India for providing the research facilities and encouragement. We are also thankful to Ms. Lakshmi Priya, Researcher, CSIR-NIO, Goa for grammatical corrections in the manuscript. This work was carried under the funds from CSIR-Network Project 0018. This document forms NIO contribution no-----.

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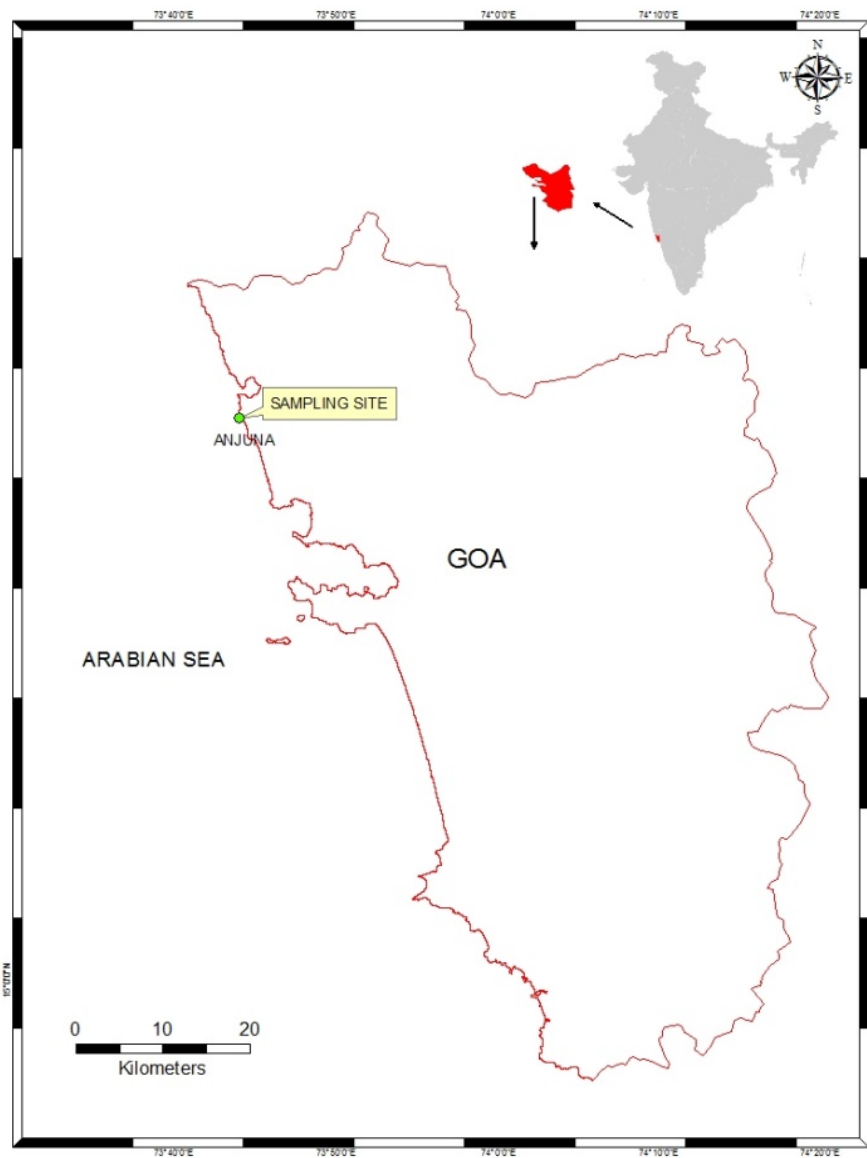


Fig.1 Represents sampling station at Anjuna, Goa (Lat15° 51' N, Long 73° 53' E)

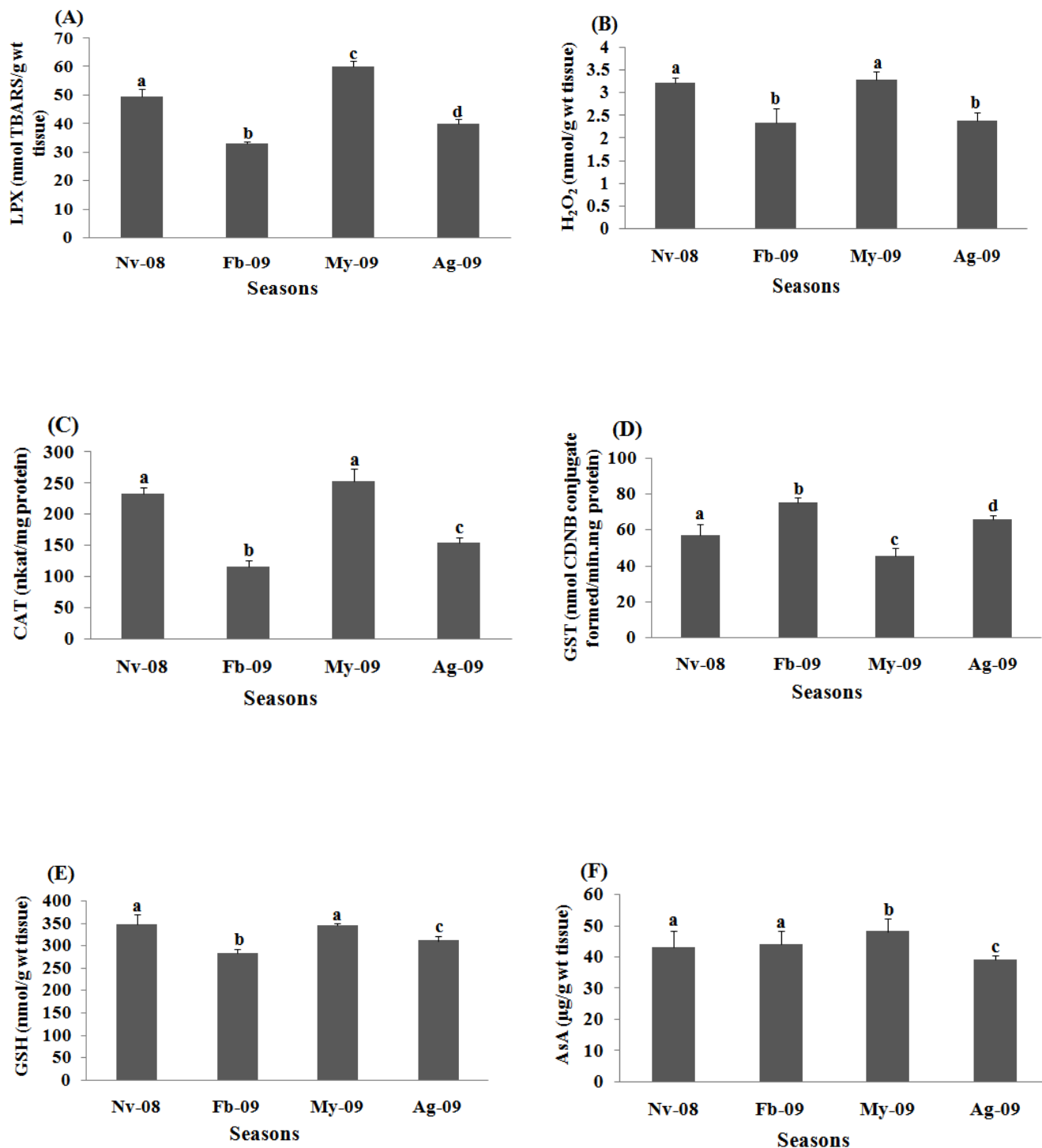


Fig. 2 Biochemical parameters i.e. Lipid peroxidation (LPX) (A); hydrogen peroxide (H₂O₂) (B); catalase (CAT) (C); glutathion *S*-transferase (GST) (D); reduced glutathion (GSH) (E); ascorbic acid content (AsA) (F) at different seasons along west coast of India. Data are expressed as mean \pm SD ($n = 5$). Superscripts of different letters are significantly different from each other at $P < 0.05$

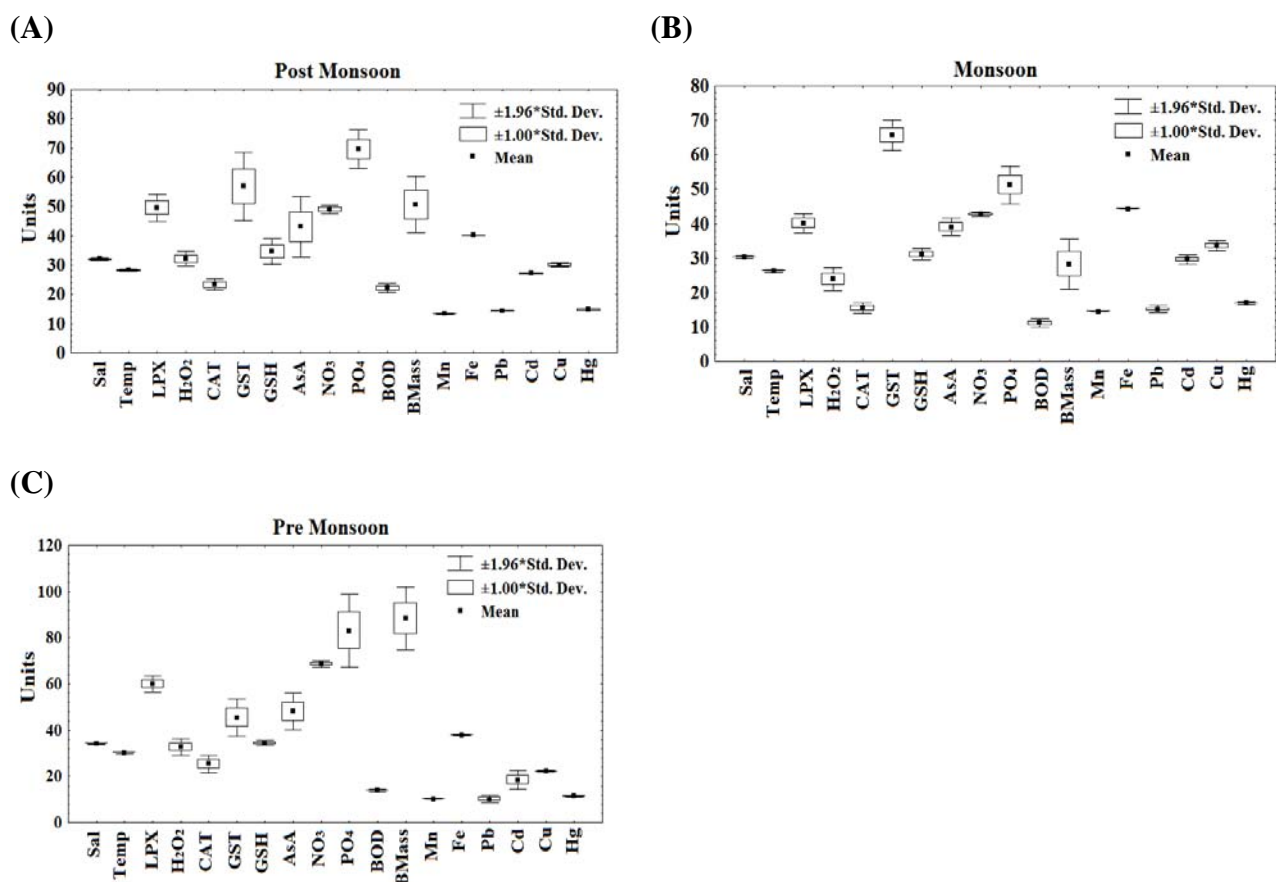


Fig. 3 Box and Whisker Plot: Mean and \pm SD values of different antioxidant and environmental parameters in tissue and water column during post-monsoon (A), monsoon (B) and pre-Monsoon (C). Units on Y axis constitute, Salinity (psu), Temp ($^{\circ}$ C), LPX (nmol TBARS/g wt. tissue), H₂O₂ (nmol/g wt. tissue), CAT (nkat/mg protein), GST (nmol CDNB conjugate formed/min·mg protein), GSH (nmol/g wt. tissue), AsA (μ g/g wt. tissue), NO₃ and PO₄ (nmol/l), BOD (mg/l), Biomass (g/m²), Mn, Fe, Pb, Cd and Cu (mg/100g dry wt.), Hg (μ g/100g dry wt.).

Table 1 Stepwise multiple regression analysis of antioxidant and environmental parameters with salinity (psu) in three different seasons of Anjuna coast during Nov-2008, Aug-2009 and May-2009.

Multiple Regression				
With salinity (psu)	Post-monsoon (Nov-2008)	Monsoon (Aug-2009)	Pre-monsoon (May-2009)	Total
LPX	0	0	0	0
H ₂ O ₂	0	0	0.206131	0
CAT	0	0	0.027121	0
GST	0	0	0	-0.0087
GSH	0	0	0	0
ASA	0	0	-0.07929	0
Temp	0	0	0	0.405525
NO ₃	-1.75383	-9.19169	0	0
PO ₄	0	0	0	0
BOD	0	-2.24211	0	-0.94263
Biomass	0	0	0	0
Mn	0	0	0	0
Fe	0	-0.11759	0	-0.0752
Pb	-13.1536	0	0	-0.69473
Cd	0	0	0	0
Cu	8.954381	0	0	0
Hg	0	0	0	5.393862
Intercept	52.177	14.74	41.05	46.43
R ²	0.999	0.999	0.999	0.995
Std. Error	1.25	0.146	0.089	15.69

Table 2 Stepwise multiple regression analysis of antioxidant and environmental parameters with temperature (°C) in three different seasons of Anjuna coast during Nov-2008, Aug-2009 and May-2009.

Multiple Regression				
With temperature (°C)	Post-monsoon (Nov-2008)	Monsoon (Aug-2009)	Pre-monsoon (May-2009)	Total
LPX	0	0	0	0.126557
H ₂ O ₂	-1.91072	0	0	0
CAT	0	-0.00679	0	0
GST	-0.00825	0	0	0
GSH	0	0	0	0
ASA	0	0	0	0.021473
Salinity	0	0	0	0.921503
NO ₃	0	0	-0.95193	2.97719
PO ₄	0	0	0	-4.17204
BOD	0	0	0	3.334444
Biomass	0	-0.00859	0	-0.01337
Mn	-0.10036	0	0	-0.00267
Fe	0	0	0	0.136371
Pb	0	0	-1.13248	0
Cd	0	0	0	0
Cu	0	0	0	0
Hg	0	9.929105	0.641037	0
Intercept	28.87	120.176	26.49	-81.4
R ²	0.999	0.999	0.999	0.989
Std. Error	0.44	8.326	0.06	32.65