

## Oxidative stress and antioxidant indices of the marine red alga *Porphyra vietnamensis*

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**Abstract** – Oxidative stress and antioxidant defence systems were assessed in a marine red alga *Porphyra vietnamensis* Tanaka et Pham-Hoang Ho, from India. Lipid peroxidation (LPX) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were measured as oxidative stress markers. Antioxidant defences were measured as catalase (CAT), glutathione *S*-transferase (GST) and ascorbic acid (AsA), in order to understand their dissimilarity with respect to environmental conditions (pollution levels) from selective locations along the central west coast of India. Levels of LPX, H<sub>2</sub>O<sub>2</sub>, CAT and GST were significantly higher in samples collected from Dona Paula than in samples from Malvan and Kunkeshwar, while a lower concentration of AsA was found in samples from Dona Paula. Heavy metals such as Cd, Pb and Hg in higher concentrations in these areas than in other sites were also observed. Variation of oxidative stress indices in response to the accumulation of heavy metals within *P. vietnamensis* could be used as molecular biomarkers for the assessment and monitoring of environmental quality in ecologically sensitive marine habitats.

**Key words:** *Porphyra vietnamensis*, lipid peroxidation, antioxidant, biomarker, oxidative stress

### Introduction

Natural antioxidants are found in some vegetables, fruits and a variety of other foods (MOON and SHIBAMOTO 2009). Many researchers reported the occurrence of various antioxidants in seaweeds, including polysaccharides, dietary fibers, minerals, proteins, amino acids, vitamins, polyphenols and carotenoids (BURTIN 2003). Seaweeds produce antioxidants to counteract environmental stresses (LESSER 2006). In the intertidal zones of rocky beaches around the world, marine algae are exposed to constantly fluctuating and extreme abiotic conditions in, for example, temperature, salinity, pH, and heavy metal pollution,

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resulting in intermittent intracellular oxidative stress conditions developed by the accumulation of reactive oxygen species (ROS) (DAVISON and PEARSON 1996, MITTLER 2002, PINTO et al. 2003). In biological systems, reactive oxygen species (ROS) such as singlet oxygen ( $^1\text{O}_2$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide anion radical ( $\text{O}_2^-$ ) and hydroxyl radical ( $\text{OH}^-$ ) are produced in normal metabolic pathways, as well as from exposure of different xenobiotic substances (HALLIWELL and GUTTERIDGE 2001). Reactive oxygen species are produced directly by excitation of  $\text{O}_2$  and subsequent formation of singlet oxygen, or by the transfer of electrons to  $\text{O}_2$ , which results in the formation of superoxide radicals, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), or hydroxyl radicals, respectively (BAKER and ORLANDI 1995).

Marine algae are a fundamental part of marine food webs, since they are responsible for most of coastal primary production (LOBBAN and HARRISON 1994). Adverse effects on macroalgae caused by natural or anthropogenic phenomena can directly or indirectly affect organisms at higher trophic levels and, finally, the integrity of entire ecosystems (MEDINA et al. 2005). Marine algae protect themselves against such reactive oxygen, to some extent by developing anti-oxidant defence systems, which constitute both enzymatic and non-enzymatic biomolecules, 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 or seaweeds have been found to be notable bioindicator species in environmental pollution studies (SAWIDIS et al. 2001, CONTI and CECCHETTI 2003, MOACIR et al. 2008). Brown algae, for instance, accumulate considerable quantities metals due to the presence of negatively charged polysaccharides (SALGADO et al. 2005). The aim of this study was to examine the various oxidative stress and antioxidant indices in *Porphyra vietnamensis*, from the central west coast of India, Arabian Sea. This is the first known study applying oxidative stress indices to *P. vietnamensis*, a potential model for evaluating marine environmental health.

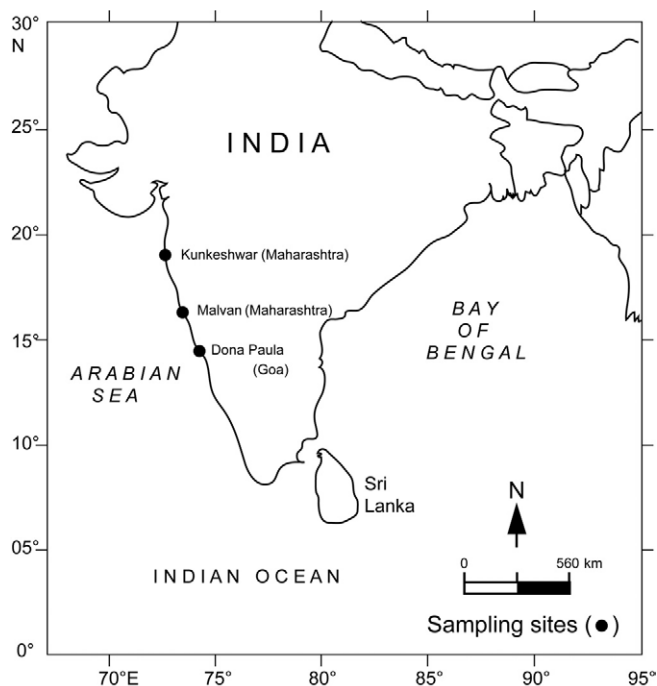
## Materials and methods

### Site description

Algal samples (*P. vietnamensis*) were collected from the central west coast of India (Fig. 1). Two sites were selected from the coast of Maharashtra: Malvan ( $16^\circ 03' 47.5''$  N,  $73^\circ 27' 22.5''$  E) and Kunkeshwar ( $16^\circ 20' 02.4''$  N,  $73^\circ 31.3''$  E), which is a cliff-locked beach. From Goa, one site was selected: Dona Paula ( $15^\circ 51' \text{N}$ ,  $73^\circ 53' \text{E}$ ) which receives various effluents from the Zuari estuary.

### Sample collection

The red alga *P. vietnamensis* (Family Bangiaceae) is common in the intertidal zone of India. Fresh fronds of *P. vietnamensis* were collected from the intertidal zones at the three sites during low tides in April 2011. Whole fronds with holdfast were handpicked, washed in seawater and then in ice cold distilled water, and immediately frozen in liquid nitrogen.



**Fig. 1.** Sampling sites for *Porphyra vietnamensis* collected from the central west coast of India, Arabian Sea.

### Physico-chemical parameters

Physico-chemical parameters at each site were measured during the study period (Tab. 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 a mercury thermometer at the time of collection.

**Tab. 1.** Physico-chemical parameters of water bodies in different locations during sampling periods

Parameter	Malvan	Kunkeshwar	Dona Paula
Water Temp. (°C)	27.5 ± 0.86	26.3 ± 2.20	25.36 ± 0.60
pH	8.06 ± 0.67	7.96 ± 0.73	8.10 ± 0.37
Salinity (PSU)	20.6 ± 1.50	25.3 ± 3.21	22.3 ± 4.93

### Metal analysis

Metal contents were analyzed by digesting 0.5 g of dry algal powder in 20 mL of concentrated HNO<sub>3</sub> in a beaker covered with a watch glass. After complete digestion, the samples were diluted to 100 ml and filtered through Whatman No.1 filter paper (Merck, Mumbai, India). The acid digest was analyzed for heavy metals such as cadmium (Cd), lead

(Pb) and mercury (Hg) using atomic absorption spectrophotometry (Analyst 300 Perkin-Elmer, Waltham, MA, USA; AOAC 2005).

## **Oxidative stress indices**

### ***Lipid peroxidation***

Lipid peroxidation (LPX) was measured according to the malondialdehyde (MDA) content, a product of LPX estimated by the thiobarbituric acid (TBA) reaction (HEATH and PACKER 1968). A 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  $7000 \times g$  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  $95^{\circ}\text{C}$  for 45 min and then cooled at room temperature. The supernatant was read at 532 nm after the removal of interfering substances by centrifuging at  $4000 \times g$  for 10 min. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated by using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  (WILLS 1969), and expressed as nmol TBARS per g of wet tissue.

### ***Hydrogen peroxide content***

The  $\text{H}_2\text{O}_2$  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).  $\text{H}_2\text{O}_2$  was used as a standard and expressed as nmol  $\text{H}_2\text{O}_2$  per g of wet 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  $4^{\circ}\text{C}$ , and the supernatants were used for enzyme assays.

## **Catalase**

The activity of CAT was measured by slightly modifying the method of AEBI (1974). The assay mixture contained 2.9 mL of 15 mM  $\text{H}_2\text{O}_2$  in 50 mM potassium phosphate buffer (pH 7.0) and 0.1 mL of enzyme extract. The decomposition of  $\text{H}_2\text{O}_2$  was followed by a decline in absorbance at 240 nm. The enzyme activity was expressed as nkat per mg of protein (1 katal =  $1 \text{ mol sec}^{-1}$ ).

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

### Ascorbic acid content

Algal material was homogenized in an ice bath with 5 mL of 10 % (W/V) TCA and centrifuged at  $7000 \times g$  for 10 minutes. 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  $\mu\text{g}$  AsA per g of wet tissue.

### Statistical analysis

Microsoft excel (Excel-2007) was used for statistical analyses. ANOVA analysis was used to compare sampling stations and a Tukey test was applied for post-hoc comparisons (ZAR 1996). Differences between means ( $n = 5$ ) were considered significant when  $p < 0.05$ . Pearson correlation analyses ( $p < 0.05$ ) were tested between metal levels in *Porphyra vietnamensis* and oxidative stress responses.

## Results

### Physico-chemical parameter

During the present study, water temperature varied from 25.36 to 27.5 °C; the minimum temperature (25.36 °C) of water was recorded at Dona Paula, and the maximum (27.5 °C) at Malvan. The overall differences in temperatures are consistent with normal variations. The pH varied from 7.96 to 8.10 indicating normal variation. The minimum pH value (7.96) was recorded at Kunkeshwar and maximum (8.10) at Dona Paula during the sampling period (Tab. 1). The salinity of water ranged from 22.3 to 25.3. Kunkeshwar showed the maximum salinity (25.3) and minimum at Malvan (20.6), during the monsoon sampling period.

### Metal analysis

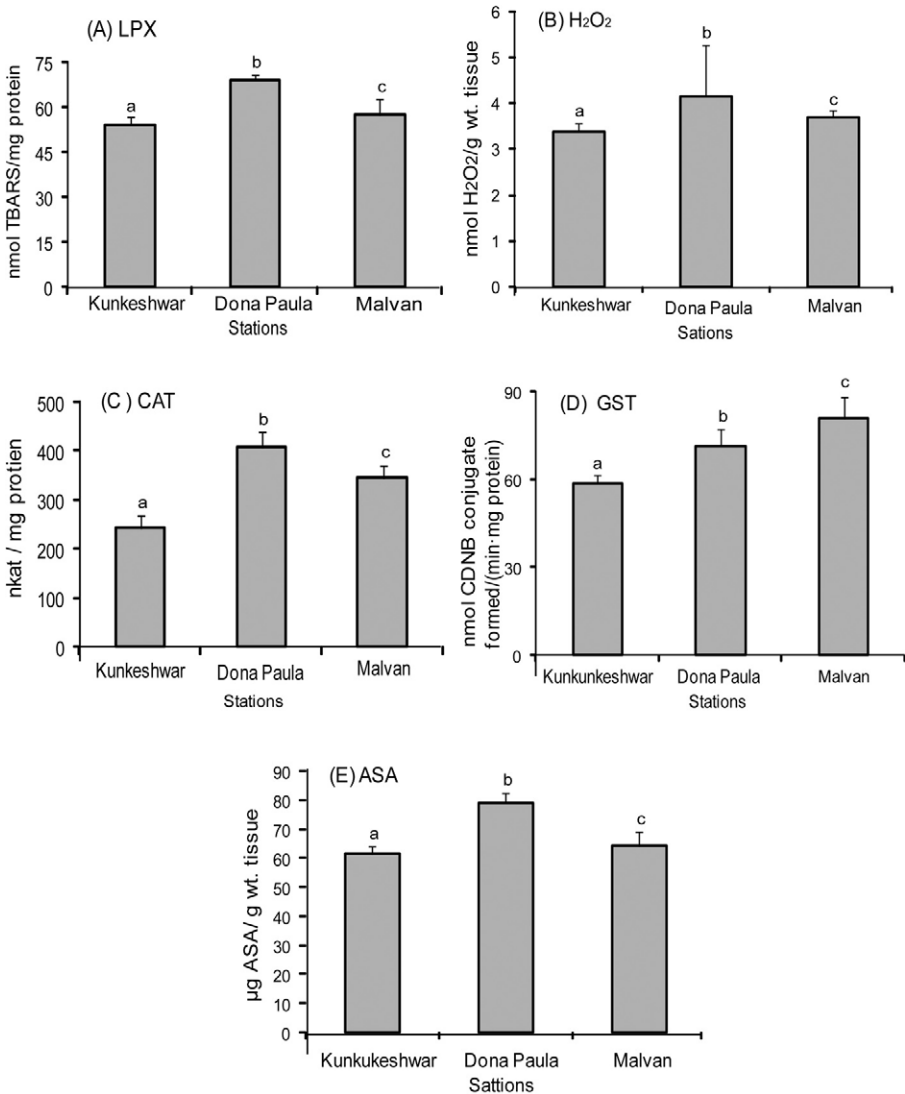
In the present study heavy metal contents in *Porphyra vietnamensis* from Kunkeshwar, Malvan and Dona Paula ranged from 0.47 to 1.98  $\mu\text{g g}^{-1}$  dry weight and occurred in the following rank order of abundance:  $\text{Hg} < \text{Cd} < \text{Pb}$  (Tab. 2). The comparatively higher values of these metals from samples of Dona Paula and Malvan could be attributed to higher levels than the Kunkeshwar. Maximum concentrations of metals in *P. vietnamensis*, from Dona Paula during the monsoon study period, could be due to the disturbances to the substratum, resulting from intensive river runoff and strong currents.

**Tab. 2.** Metal accumulation ( $\text{mg g}^{-1}$  dry weight) in tissues of *Porphyra vietnamensis* at different sampling sites

Metal	Malvan	Kunkeshwar	Dona Paula
Pb ( $\mu\text{g g}^{-1}$ )	$1.77 \pm 0.09$	$1.22 \pm 0.09$	$1.98 \pm 0.17$
Cd ( $\mu\text{g g}^{-1}$ )	$0.68 \pm 0.06$	$0.67 \pm 0.15$	$0.98 \pm 0.18$
Hg ( $\mu\text{g g}^{-1}$ )	$0.58 \pm 0.09$	$0.47 \pm 0.015$	$0.99 \pm 0.19$

**Lipid peroxidation**

In the present study, LPX values were significantly higher in the samples from Dona Paula and Malvan than in the samples from Kunkeshwar (Fig. 2A,  $p < 0.05$ ). Similarly, enhanced levels of  $H_2O_2$  were also noticed in the samples from Dona Paula (Fig. 2B). Increased levels of LPX and  $H_2O_2$  in *P. vietnamensis* from different localities were significantly correlated with the heavy metal concentrations (Tab. 2,  $p < 0.001$ ).



**Fig. 2.** Biochemical measurements at different sites along the central west coast of India. A – Lipid peroxidation (LPX), B – hydrogen peroxide ( $H_2O_2$ ), C – catalase (CAT), D – glutathion S-transferase (GST), E – ascorbic acid content (AsA). The alphabetical letters (a, b, c) mark significant difference at  $p < 0.05$ .

### Antioxidant defence systems

In the present study, CAT activity was significantly higher in the sample from Dona Paula and Malvan, further indicating metal stress increasing the formation rate of  $H_2O_2$  (Fig. 2B). Interestingly higher levels of  $H_2O_2$  were measured in samples from Dona Paula than those from other sites (Fig. 2B). The induction of CAT activity in *P. vietnamensis* from Dona Paula could be attributed to the unfavorable environmental conditions. Also observed were increased levels of GST activity in *P. vietnamensis* samples from Malvan and Dona Paula, compared to those from Kunkeshwar (Fig. 2D). High concentrations of AsA were also recorded in the samples from Dona Paula and Malvan as compared to Kunkeshwar (Fig. 2E) which suggest that AsA synthesis might have been stimulated to protect against a metal-contaminated environment. This response appeared to be particularly associated with physico-chemical parameters (Pb, Cd, Hg, temperature, pH and salinity) and also a significant correlation was found ( $p < 0.001$ , Tab. 3).

**Tab. 3.** Correlation coefficients (r) between physico-chemical and biochemical parameters

Phy.-chem. Parameter	Biochemical Parameters				
	CAT	ASA	GST	LPX	$H_2O_2$
Pb	$r = 0.99^{***}$	$r = 0.82^{***}$	$r = 0.76^{**}$	$r = 0.84^{***}$	$r = 0.94^{***}$
Cd	$r = 0.77^{**}$	$r = 0.98^{***}$	$r = 0.06$	$r = 0.97^{***}$	$r = 0.89^{***}$
Hg	$r = 0.65^*$	$r = 0.93^{***}$	$r = -0.11$	$r = 0.91^{***}$	$r = 0.80^{**}$
Water Temp.(°C)	$r = 0.45$	$r = 0.81^{***}$	$r = -0.35$	$r = 0.78^{**}$	$r = 0.63^*$
pH	$r = 0.99^{***}$	$r = 0.84^{***}$	$r = 0.73^*$	$r = 0.86^{***}$	$r = 0.95^{***}$
Salinity (PSU)	$r = -0.99^{***}$	$r = -0.87^{***}$	$r = -0.64^*$	$r = -0.89^{***}$	$r = -0.96^{***}$

Significance level:  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*)

### Discussion

This study represents the most extensive work on oxidative stress induced by temperature, salinity, pH and metal concentration from selected sites along central west coast of India. Heavy metals are implicated in oxidative injury involved in the formation of ROS and their subsequent attack on proteins, lipids, and nucleic acids, leading to loss of enzyme functions, altered membrane fluidity, and genomic damage (DIETZ et al. 1999).

The physical, chemical and biological features of the coastline and estuarine systems of Goa are adapted to a seasonal rhythm, the monsoon cycle. Increased precipitation and land runoff during the monsoon season causes dynamic changes from typically marine to brackish water conditions (QASIM and SEN GUPTA 1981) resulting in changes in temperature, salinity, pH and metal levels along outer coasts and in estuaries. The water in the Goan estuaries remains well mixed during the pre-monsoon season and gets stratified during the monsoon (QASIM and SEN GUPTA 1981), while diurnal variations in physico-chemical conditions are governed by the tides (SINGBAL 1976). The high precipitation and freshwater influx brings cooler water from the upper reaches of the river, resulting in lower water temperatures during monsoons. It also results in higher pH values due to dilution of

seawater by fresh water inflow (DEHADRAI and BHARGAVA 1972). Solar radiation during the monsoon is reduced because of heavy cloud cover that decreases temperature and the rate of evaporation, thus maintaining lower salinity. However, decreased salinity can be attributed predominantly to heavy precipitation and the influx of fresh water (DEHADRAI and BHARGAVA 1972).

### **Metal analysis**

The concentration of heavy metals, in marine environments is mainly influenced by both natural and anthropogenic sources. In coastal waters, metals can occur at much higher concentrations, probably due to inputs from river systems (MORILLO et al. 2004). However, other metals, such as Cd, Hg, and Pb, are toxic even at very low concentrations (WOOD 1974, NIES 1999). Marine algae are known to concentrate metals, and it is the increased concentration especially of non-essential trace metals such as Cd, Pb, Hg that could induce oxidative stress in their tissues (KUMAR et al. 2010). It has been reported that these polluted areas (along the Goa coastline) receive varieties of pollutants including metals, petrochemicals as well as sewage generated from various anthropogenic activities (CPCB 1996).

### **Lipid peroxidation**

Metal levels in tissue induce a variety of cellular changes, such as damage to membrane integrity (SMEETS et al. 2005), reduction in photosynthesis and impairment of CO<sub>2</sub> assimilation (VAN ASSCHE and CLIJSTERS 1990, GOUIA et al. 2003), which may produce ROS and resulting LPX. Similar results were reported in brown seaweed *Padina tetrastromatica* at polluted locations in Karwar and Colaba (MAHARANA et al. 2010). This suggests that changes in oxidative stress and antioxidant levels might be to the contaminant metals or abiotic factors such as cadmium, mercury, lead, temperature, salinity and pH. 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).

### **Antioxidant defence systems**

Ozone, salt stress, drought, heat, heavy metals, toxins and organic pollutants also induce the formation of reactive oxygen (PFLUGMACHER 2004). In addition, photosynthetic organisms continuously produce reactive oxygen during photosynthesis and other metabolic processes (FOYER and NOCTOR 2000). The defence system against reactive oxygen in plants includes antioxidants such as ascorbate, glutathione,  $\beta$ -carotene and  $\alpha$ -tocopherol and reactive oxygen scavenging enzymes, such as catalase (EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), glutathione reductase (GR, EC 1.6.4.2) and ascorbate peroxidase (APX, EC 1.11.1.11). Higher levels of antioxidants, and increased activities of reactive oxygen scavenging enzymes, correlate with stress tolerance in seaweeds, e.g. brown algae of the genus *Fucus* (COLLEN and DAVISON 1999 a, b).

Catalase is a porphyrin-containing enzyme which catalyses the decomposition of hydrogen peroxide to water and oxygen (ARUOMA 1998). A significant correlation was observed between CAT and H<sub>2</sub>O<sub>2</sub> scavenging action ( $r = 0.97$ ,  $p < 0.001$ ). This suggests either that catalase is the most important enzyme for the breakdown of H<sub>2</sub>O<sub>2</sub>. Increased CAT activity has been described in several aquatic species from impacted areas both by metals



and organic contaminants (ORBEA et al. 2002, NIMPTSCH et al. 2005). Catalase activity increased with treatments with Cd and Pb (DAZY et al. 2009). COLLEN et al. (2003) also reported that the heavy metals also induced the activity of catalase in the *G. tenuistipitata*. Nevertheless, CAT induction in plants has only been reported under laboratory conditions (ROY et al. 1995, RAMA DEVI and PRASAD 1998, COLLÉN et al. 2003).

GST is also capable of detoxifying ROS and its activity increases in cases of oxidative stress in plant cells (PFLUGMACHER et al. 1999, THOM et al. 2001). The observed increase in GST activity at Dona Paula site (Tab. 3), and the positive correlation with Pb ( $p < 0.01$ ;) and pH ( $p < 0.05$ ) suggest that higher metal stress might have induced GST levels to combat tissues from xenobiotic substances. Similar findings were observed in *Padina terastromatica* (MAHARANA et al 2010). GST activity has also been observed in plants treated with Cd (ARAVIND and PRASAD 2005, MISHRA et al. 2009).

Although few 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 (MAHARANA et al. 2010, ARAVIND and PRASAD 2005). A significant correlation between AsA and Cd ( $r = 0.98$ ,  $p < 0.001$ ) levels in the algal tissue also supports this statement (Tab. 3). AsA and GSH are direct scavengers of ROS, and an additional substrate for various antioxidant enzymes (HALLIWELL and GUTTERIDGE 1999). It has been reported that the ascorbate-glutathione cycle (AGC) plays an important role in detoxification of ROS (KUZNIAK and MARIA 2001). Important activities of CAT, GST and ASA consequent to increased LPX and  $H_2O_2$  suggest that *P. vietnamensis* maintains sufficient antioxidant defences to withstand major environmental turbulence related to different stations and abiotic parameters. The evaluation of abiotic parameters and metal concentrations in *P. vietnamensis* appears to confirm such a relationship (Tab. 3).

## Conclusions

*Porphyra vietnamensis* displays different biochemical responses to environmental conditions. Elevated levels of LPX and  $H_2O_2$ , indicate a state of oxidative stress possibly due to accumulation of metal ions. The present data revealed that oxidative stress markers such as LPX and  $H_2O_2$ , and nonenzymatic antioxidant such as AsA and antioxidant enzymes (CAT and GST) may be useful biomarkers, suggesting that this species might be a model species for the evaluation and monitoring of marine environments.

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