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Oxidative stress and antioxidant indices of the estuarine alga *Gayralia oxysperma*

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Abstract
Green alga *Gayralia oxysperma* sampled from three estuaries in central west cost of India i.e., Ratnagiri (RA), Achara (AC) and Terekhol (TE). In addition lipid peroxidation and hydrogen peroxide were analysed as oxidative stress markers, and activities of antioxidants i.e. enzymatic CAT, GST and non-enzymatic GSH and ASA were investigated for the understand their pollution levels and environmental condition. We observed a significance increase in lipid peroxidation, hydrogen peroxide and antioxidant enzymes of the RA site, suggesting that the seaweed at RA site are maximum as compared to remaining both the sites AC and TE. However, non- enzymatic ASA and GSH antioxidant was also observed high level in the RA site indicate (GSH and ASA) deu to the protection against stress metals. Study shows that changes in antioxidants at different sites in the *G. oxysperma*.

Keywords: *Gayralia oxysperma*, lipid peroxidation, antioxidants, biomarker, oxidative stress

Introduction
Gayralia oxysperma is a light green, monolayer and floating macro-algal species is found in estuarine zone. G. *oxysperma* higher concentrations of dietary fibers, proteins and minerals as well as different functional properties are reported by Pise *et al*., (2012). Marine macro-algae are exploited from past few decades due to major or potential biological compounds are present in higher concentration (Ellouali *et al*., 1993; Bergé *et al*., 2002). Along the central west coast of Maharashtra and Goa increased industrial sectors such as pharmaceuticals, fertilizers, shipping and mining units and also tourism activities. Estuarine marine algae continuously connected to tidal activities along with those abiotic components which including salinity, temperature, pH and trace metals pollution (Pinto *et al*., 2003**)**. Overall seaweeds in the estuarine areas are regularly influenced due to prominent changes in their environment that's why may be present defense mechanisms for the protection from different stressors. Dring *et al*., (2006) is reported that the rate of reactive oxygen species production can be increased by exposure of different stressors like higher temperature, UV radiation, toxic chemicals and desiccation and also Foyer and Noctor (2003) noticed that ROS produced by the metabolic processes as a byproduct in the mitochondrial respiration and photosynthesis in the plants. The ROS like $^1O_2$, H_2O_2, O_2^- and OH^- is evolved in the normal metabolic pathways and due to unusual xenobiotic substances reported by Halliwell and Gutteridge (2000). ROS is formed due to excitation of O_2 and produce singlet oxygen, which creats superoxide radicals, hydrogen peroxide or hydroxyl radicals reported by Baker and Orlandi (1995). ROS impacted to damage membrane lipids, proteins and nucleic acid, is caused when higher rate of production exceeds the rate of scavenging of ROS.
Estuarine marine algae are developing antioxidant enzymatic and non enzymatic defense system due to protection from reactive substances bimolecules. Two enzymes, superoxide dismutase and catalase are also involved in ROS-scavenging, removing superoxide anion and hydrogen peroxide by converting them to hydrogen peroxide and water, respectively. The protection plant cells from ROS, glutathione plays important role in the ascorbate glutathione pathway reported by Pastori (2003). Ascorbic acid is a used for the scavenger of reactive substances (Halliwell and Gutteridge, 2001). Antioxidant enzymes are the prime subject of the redox regulation and demonstrate adaptive response to the oxidative stress. The antioxidant system must maintain just enough ROS to ensure they play their essential roles; however, it should also remove harmful ROS to avoid damage. In the present study, represents changes in the oxidative stress and antioxidant responses in *Gayralia oxysperma.* Comprehensive investigation of antioxidant variation with respect pollution levels along the selected sites. This study may be useful for the biomonitoring of the ecologically sensitive marine habitats.

Site selection
Alga *Gayralia oxysperma* was collected from estuaries along central west coast of India, during (Pre-Monsoon, March - April) 2019 to 2021. In Maharashtra two sites were selected (Fig.1) i.e. Achara, (16°11'57.71"N,

73°26'38.99"E) and Shirgao creek at Ratnagiri, (17°01'36.29"N, 73°17'03.58"E). However, only one site Goa (Fig.1) was selected i.e. Terekhol, (15°45'04.15"N, 73°41'33.73"E).

Materials and methods

Chemicals

Thiobarbituric acid, trichloro acetic acid, bovin serum albumin, glutathione reductase, 1-chloro-2, 4-dinitrobenzene, 5, 5'-dithio-2-nitro benzoic acid were purchased from Sigma Chemicals Co., USA. Methanol (HPLC) grade, ethanol (AR) grade were obtained from Mark Chemicals Co., India.

Sample collection

The green alga *G. oxysperma* (Monostromataceae, Ulvales) is found floating in shallow water or attached to pneumatophores of the Mangrove. Fresh algal material were collected during low tide and frozen in liquid nitrogen.

Physico-chemical parameters

For the Physico-chemical parameters salinity, pH and temperature was measured at sample collection sites.

Metal analysis

For the trace metal analysis acid digestion and atomic spectrophotometeric method used.

Oxidative stress indices

Oxidative stress indices were estimated with different methods i.e lipid peroxidation were estimated as per Heath and Packer, (1968) reported method. Along with that hydrogen peroxide content was determined according to Sergiev *et al*., (1997). For the enzymatic essay algal material homogenized in phosphate buffer with Trion X-100 and PVP. The extract supernatants were used for measured catalase activity with the method of Aebi (1974) and Glutathion S transferase activity measured with Habig *et al*., (1974) [21] method.

Non-enzymatic antioxidants

Estimation of glutathione (GSH)

GSH is measured according to the protocol by Moron *et al*., (1979) [34] and ascorbic acid content was measured by (Mitusi and Ohata, 1961) [31]

Statistical analysis

Statistical data analyzed with standard deviation, one way ANOVA and Turkey test was used to analysed post hoc comparison.

Results and discussion

This study represents the most extensive work on oxidative stress induced by heavy metals, temperature, salinity and pH 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) [12].

Physico-Chemical Parameters

Measurable differences in physico-chemical parameters were marked among the three sites investigated during 2019 - 2021 in the present study. Table 1 shows the Physico-chemical parameters of water bodies. Temperature of water varied from 25.37^{0}C to 29.83^{0}C, higher water temperature was reported (25.37±0.6^{0}C) in 2019, and the maximum (29.83±1.66^{0}C) in 2021 at Ratnagiri. The overall differences in temperatures are consistent with normal variations. The pH varied from 7.22 to 8.16, indicating normal variation. The minimum pH value (7.22±0.2, 2019) was recorded at Terekhol in 2020 and maximum (8.16±0.32, 2021) at Ratnagiri in 2021 (Table 1). Salinity ranges recorded in between 10.8 - 23.16 psu (Table 1). Terekhol showed the maximum salinity (23.16± 1.89 psu, 2020) and minimum was seen at Achara (10.8±0.36 psu, 2019), during the sampling period (Pre-Monsoon).

Table 1: pH, Salinity and temperature measured at different sampling site for three year 2019 to 2021

Parameter	Terekhol			Achara			Ratnagiri		
	2019	2020	2021	2019	2020	2021	2019	2020	2021
Temp. (°C)	27.17±0.15	27.67±2.6	26.73±1.91	27.13±0.98	27.97±2.44	27.33±1.52	25.37±0.6	26.00±2.7	29.83±1.66
pH	7.45±0.03	7.22±0.2	7.81±0.99	7.33±0.15	8.04±0.18	7.94±0.32	7.54±0.08	8.09±0.47	8.16±0.32
Salinity (%)	19.43±0.4	23.16±1.89	24.5±1.17	10.8±0.36	12.3±2.51	12.33±1.52	18.00±0.6	21.36±0.81	21.66±1.52

Estuarine ecosystem is a unique assemblage of different environmental factors. It is a result of tidal action together with freshwater inflow and geomorphology of the region. Increased precipitation and land runoffs cause dynamic changes from typically marine to brackish water conditions (Qasim and Sen Gupta, 1981) [42] resulting

in changes in salinity, pH, metal levels and temperature in the coastline and estuaries. Qasim and Gupta (1981) reported that the estuary water remains well mixed at the pre monsoon season, while diurnal variations in physico-chemical conditions are governed by the tides (Singbal, 1976) [46]. The high precipitation and freshwater influx from upper reaches of the river and it results in increased pH values (Dehadrai and Bhargava, 1972) [11]. The solar radiations during pre-monsoon are high, which increases the rate of evaporation due to incresd temperature and resulting in to increased salinity.

Trace metal analysis

Due to the anthropogenic activities and natural sources are impacted or influenced trace metals in the marine environment. In the present study metal concentrations (Cd, Hg and Pb) ranged from 0.19 to 1.92 µg g^{-1} DW from all selected sites. The relative quantity of metals were observed in the order of Cd > Hg > Pb in all three sampling sites. *Gayralia oxysperma* contained the highest Pb content at Ratnagiri in 2021 (1.92±0.36 µg g^{-1} DW) while low value (0.89±0.036 µg g^{-1} DW) were recorded in Terekhol. Mercury (Hg) content was found high in Ratnagiri during, 2020 (0.95±0.01 µg g^{-1} DW) and a low value was recorded in 2019 at Achara (0.42±0.04 µg g^{-1} DW). Cadmium (Cd) showed a high value in 2021 at Ratnagiri site (0.34±0.02 µg g^{-1} DW) and low value was recorded at Achara (0.19±0.041 µg g^{-1} DW, 2019), respectively (Table 2). Maximum concentrations of metals in *G. oxysperma* from Ratnagiri could be due to the received from sewage and other various anthropogenic activities. Nies (1999) is reported that the heavy metal like Cd, Hg and Pb are toxic even its very low content. In the marine or esturine algae are known to concentrate heavy metal and it is increased content particularly non essential trace metals like Cd, Pb and Hg that could increases oxidative stress reported by the Kumar *et al*., (2010).

Table 2: Trace metals present in tissue of G. oxysperma at different sampling locations

Metal	Terekhol			Achara			Ratnagiri		
	2019	2020	2021	2019	2020	2021	2019	2020	2021
Pb (µg g-1)	0.90±0.03	0.91±0.03	0.89±0.036	0.032	0.94±0.047	1.01±0.03	1.76±0.39	1.61±0.045	1.92±0.36
Cd (µg g-1)	0.25±0.01	0.25±0.015	0.26±0.025	0.19±0.02	0.19±0.041	0.2±0.02	0.29±0.015	0.33±0.01	0.34±0.02
Hg (µg g-1)	0.59±0.04	0.71±0.025	0.55±0.035	0.42±0.041	0.61±0.05	0.69±0.02	0.95±0.036	0.95±0.01	0.93±0.02

Lipid peroxidation

LPX was measured higher at Ratnagiri site as comparison with Achara and Terekhol sites (Fig.2A, P<0.05).

In the same way, higher content of hydrogen peroxide were recorded in the algal sample from Ratnagiri (Fig. 2B) and significantly correlated with H_2O_2 (Fig.4G). Higher levels of lipid peroxidation in *G. oxysperma* from different sites were positively correlated with metals (Table 3), Cd (r = 0.92, P < 0.000), Pb (r = 0.98, P < 0.000) and Hg (*r* = 0.73, *P* < 0.023). Similar findings also reported by Maharana *et al*., (2010) in the brown alga P. tetrastromatica at polluted site i.e. Karwar and Colaba akong with that Liu *et al* (2007 reported Cd metal also influenced and Reddy *et al*., (2005) Pb metal influenced LPX values in the higher plant tissue. Moacir *et al* (2008) also stated that the changes in antioxidants and oxidative stress might be due to the metal content or biotic factors.

Table 3: The correlation coefficient in between antioxidant and physic-chemical parameters.

Parameters	CAT	ASA	GSH	GST	LPX	H_2O_2
Pb	*r* =.950	*r* =.925	*r* =.888	*r* =.862	*r* =.981	*r* =.727
	p=.000	p=.000	p=.001	p=.003	p=.000	p=.026
Cd	*r* =.872	*r* =.913	*r* =.896	*r* =.785	*r* =.928	*r* =.759
	p=.002	p=.001	p=.001	p=.012	p=.000	p=.018
Hg	*r* =.760	*r* =.920	*r* =.875	*r* =.758	*r* =.738	*r* =.865
	p=.017	p=.000	p=.002	p=.018	p=.023	p=.003
Temp.	*r* = -.144	*r* = -.165	*r* =.034	*r* = -.057	*r* = -.005	*r* = -.368
	p=.711	p=.672	p=.930	p=.883	p=.990	p=.331
pH	*r* =.163	*r* =.418	*r* =.590	*r* =.268	*r* =.303	*r* =.481
	p=.676	p=.262	p=.095	p=.485	p=.429	p=.190
Salinity	*r* =.534	*r* =.453	*r* =.578	*r* =.734	*r* =.262	*r* = -.036
	p=.139	p=.221	p=.103	p=.024	p=.496	p=.927

Correlations (new. sta) significant at p <.05000 N=9

Antioxidant defense systems

G. oxysperma was found to increase catalase activity, suggesting that the scavenging of ROS may be related to the increase in antioxidant enzyme activity. In the present study, Catalase (CAT) activity observed in *G. oxysperma*, among the three sites during 2019-2021 and in between (226.66±1.5 to 390.33±1.5 nkat/mg protein), indicated higher activity observed in Ratnagiri site, compared to that from Achara and Terekhol (Fig. 2C) sites.

The higher CAT activity indicates metal stress on *G. Oxysperma*, which results in an increase in H_2O_2 formation. Catalase significant correlated with GSH (Fig. 3A), ASA (Fig. 3B), H_2O_2 (Fig. 3C) and LPX (Fig. 3D), which supports the above statement. This indicates that Catalase plays important role for the breakdown of hydrogen peroxide. Such phenomenon has also been reported by Dazy *et al*., 2009 in algae treated with Cd and Pb. A positive correlation was also observed (Table 3) with Cd ($r = 0.87$, $P < 0.002$), Pb ($r = 0.94$, $P < 0.0001$) and Hg ($r = 0.76$, $P < 0.02$). Nimptsch *et al*., (2005) [35] and Orbea *et al*., (2002) [37] also reported increased CAT activity in several aquatic species from areas impacted both by metals and organic contaminants. That catalase activity increased due to higher heavy metals in the *G. tenuistipitata*. However, it is also reported in the lab experimentcatalase increased in the G. tenuistipiatata reported by Collen *et al*.,(2003). Catalase induction *G. oxysperma* from Ratnagiri may be against ROS environment. Higher GST activity in *G. oxysperma* algal material at Ratnagiri, with compared Achara and Terekhol (Fig. 1E.). However, a positive correlation was also observed (Table 3) with Cd ($r = 0.78$, $P < 0.012$), Pb ($r = 0.86$, $P < 0.003$) and Hg ($r = 0.75$, $P < 0.018$). Similar findings were observed in *Padina terastromastica* (Maharana *et al*., 2010) [29]. Present study GST positive correlated CAT (Fig.3E), H_2O_2 (Fig. 3F) and LPX (Fig.3G). Glutathione S- transfarase have been also influenced due Cd treatment (Mishra *et al*., 2009) [30]. Glutathione makes an ideal biochemical role to protect plants against stresses including oxidative stress, heavy metals and certain exogenous and endogenous organic chemicals (Foyer and Noctor, 2005) [15]. In the present study, High concentration of GSH was observed at the Ratnagiri site (Fig. 2F), compared with other two sites. Increased levels of GSH with the increasing concentrations of trace metals were observed, indicating that the pollutant stress might have induced GSH levels. However, a positive correlation was also observed with GST (Fig. 3H), H_2O_2 (Fig. 4A), LPX (Fig. 4B) and trace metals (Table 3) i.e. Cd ($r = 0.89$, $P < 0.001$), Pb ($r = 0.88$, $P < 0.001$) and Hg ($r = 0.87$, $P < 0.002$), which signify that exposure of heavy metals induces ROS, and also directly or indirectly influences the level of GSH. Several studies reported an increase in GSH content with exposure of metal stress in plants (Tukendorf and Rauser, 1990) [48], including *Ulva* sp. (Pawlik-Skowrońska *et al*., 2007) [38], that can be associated to the activation of GSH synthesizing enzymes (Tukendorf and Rauser, 1990) [48]. However, under field conditions an opposite pattern has also been observed in *Ulva* sp. with higher metal accumulation being accompanied by lower GSH levels (Pawlik-Skowrońska *et al*., 2007) [38]. Ratnagiri site found high concentrations of metals and H_2O_2 compared with the other two sites, indicating increase in activity of GSH to control reactive oxygen formation. It is also reported that GSH participates in the control of H_2O_2 level of plant cells (Foyer and Noctor, 2005) [15]. Change in the ratio of its reduced (GSH) to oxidized (GSSG) form during degradation of H_2O_2 is important in certain redox signaling pathway. It has been suggested that the GSH /GSSG ratio, an indicative of the cellular redox balance, may be involved in ROS perception. Reduced glutathione (GSH) acts as an antioxidant and is involved directly in the reduction of most ROS generated during stress (Foyer and Noctor, 2005) [15]. As described above, one protective role of GSH in plants during exposure to heavy metal stress is the quenching of ROS. Secondly, GSH acts as a precursor for the synthesis of phytochelatins (PCs). Phytochelatins (PCs) are a set of novel heavy metal-binding peptides (Fig. 4). These were first isolated from cell suspension cultures of a higher plant after exposure to Cd. Since then, PCs have been found in some eukaryotes, including higher plants (Gekeler *et al*., 1989) [17]. PCs are synthesized inductively by exposure to not only Cd, but also to other heavy metals such as Hg, Cu, Zn, Pb and Ni. During the exposure of plants to such metals, PCs are synthesized from GHS by phytochelatin synthase (PCS) activity. Thereafter, numerous physiological studies have indicated their role in heavy metal detoxification as well as in the maintenance of ionic homeostasis (Hirata *et al*., 2005) [24].

Ascorbic acid content is noticed in higher concentrations at the Ratnagiri as match up to Achara and Terekhol (Fig. 2D). This response was found to be particularly associated with metal concentration (Pb, Cd and Hg). The AsA content in *G. oxysperma* among the three sites varied from (60.61 ± 3.67 to 79.78 ± 4.1 µg g^{-1} FW) and was maximum in Ratnagiri (Fig.3), which suggests that ascorbic acid acted to protect against trace metal stress. Higher content of antioxidants in Ratnagiri site compared to Achara and Terekhol sites suggest their higher resistance power against the oxidative stress, as antioxidant AsA plays a crucial role in the detoxification of ROS, which can be generated as a by-product during the biotransformation reaction of toxins or xenobiotics (Pastore *et al*., 2003). Therefore, elevated levels of AsA in *G. oxysperma* could be attributed to metal ions from those sites. A significant correlation observed between AsA and metal levels; Cd ($r = 0.91$, $P < 0.001$), Pb ($r = 0.92$, $P < 0.001$) and Hg ($r = 0.91$, $P < 0.001$), (Table 3). Ascorbic acid also significantly correlated with LPX (Fig. 4C), GST (Fig. 4D), GSH (Fig. 4E) and H_2O_2 (Fig.4F). Maharana *et al*. (2010) and Halliwell and Gutteridge, (2000) also reported enhanced level of enzymes in response to metals.

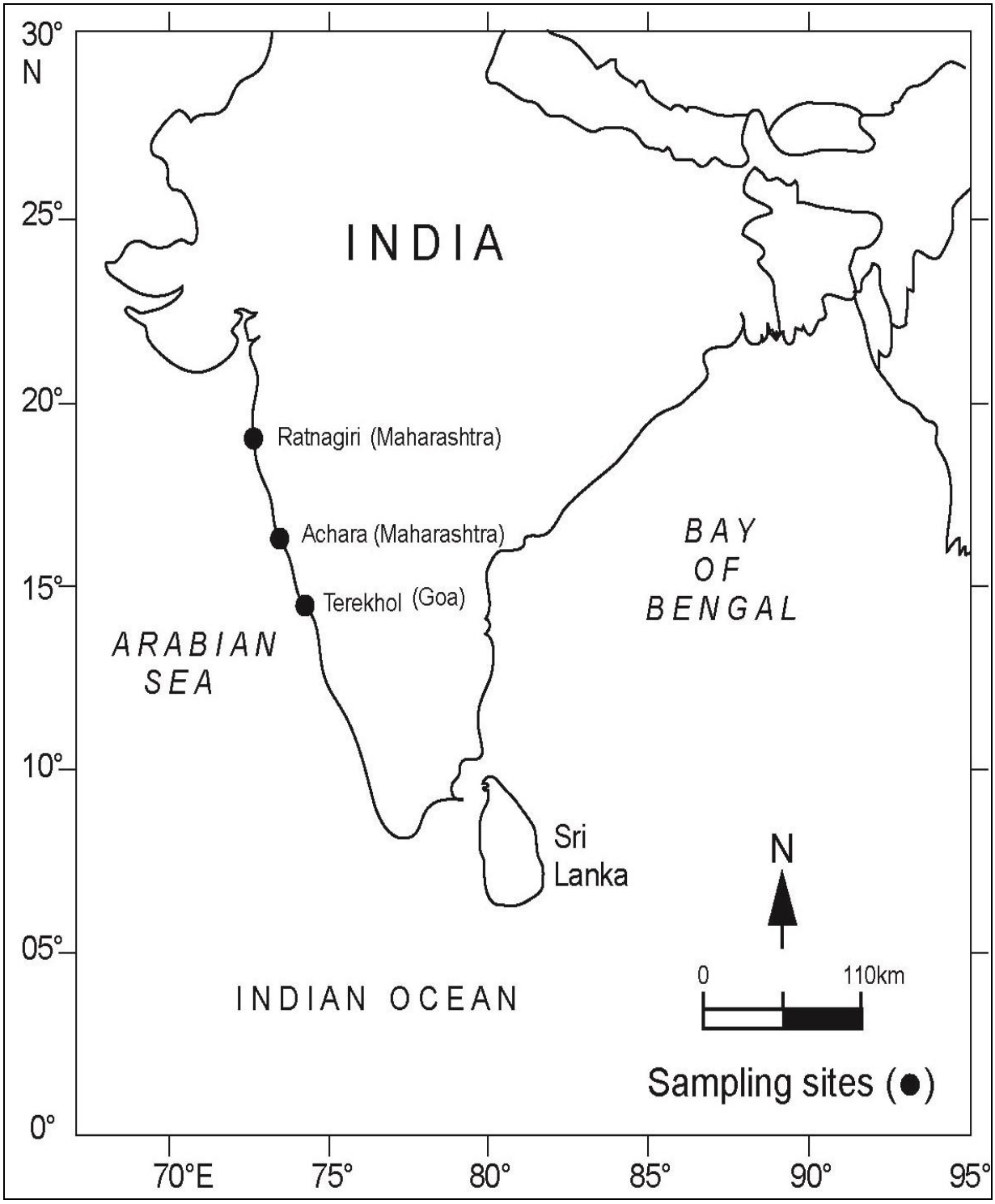


Fig 1: Sampling sites for *G. oxysperma* collection estuarine water of Maharashtra and Goa coast.

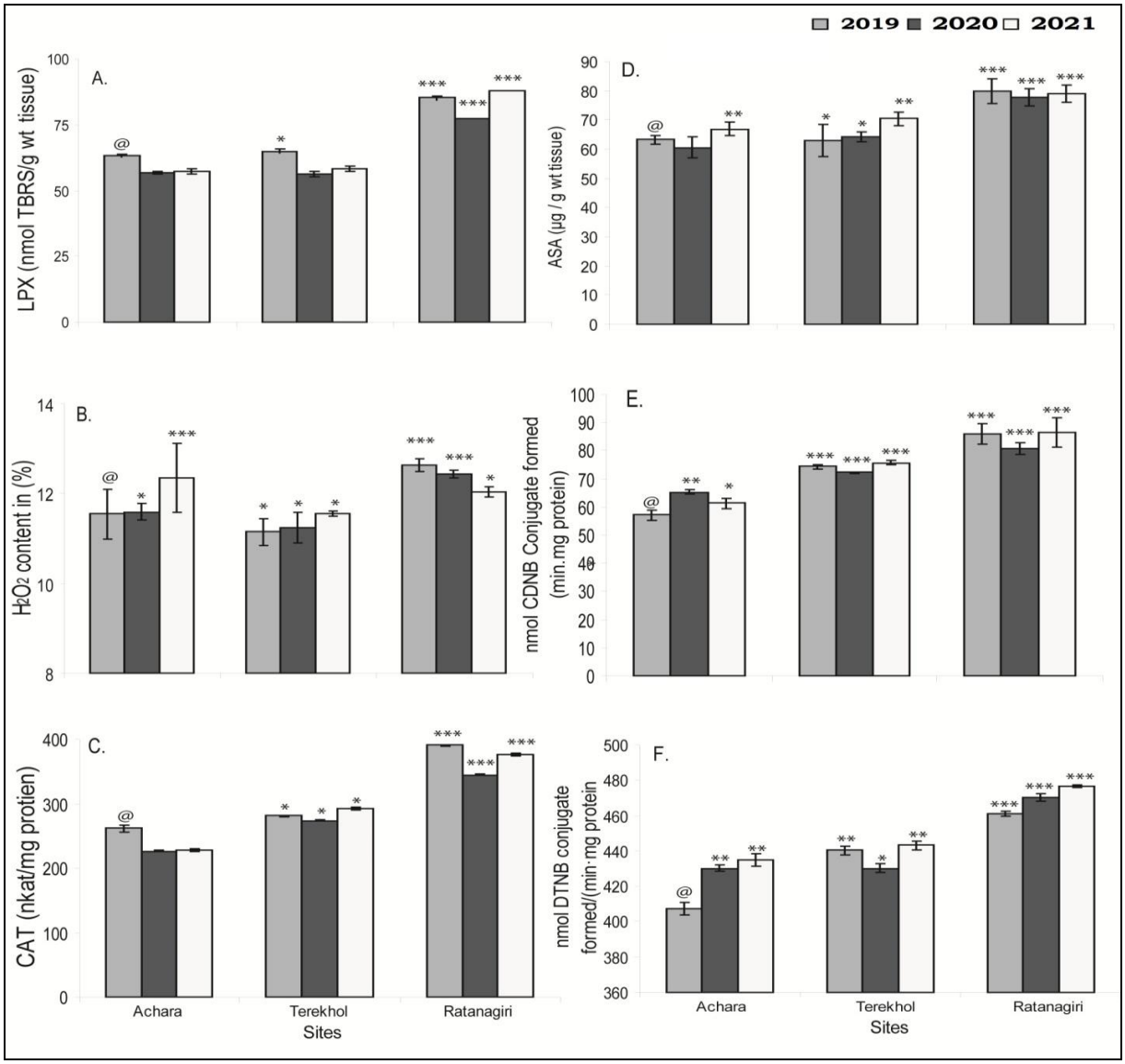
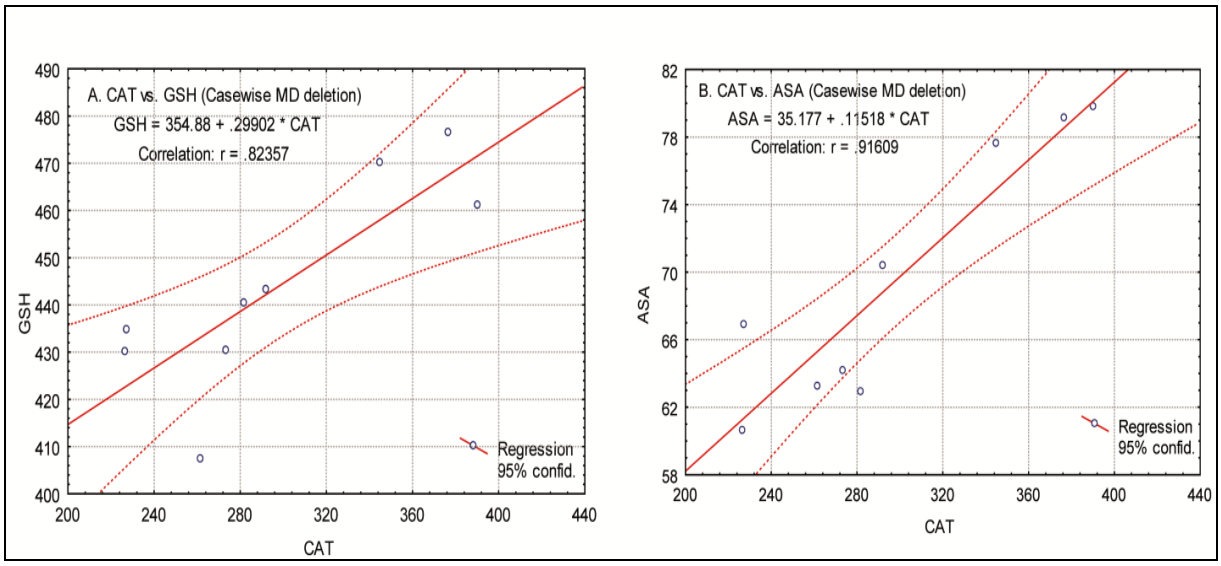


Fig 2: A. Lipid peroxidase (LPX), B. Hydrogen peroxide (H2O2), C. Catalase (CAT), D. Ascorbic acid (ASA), E. Glutathione s-transfrase (GST) and F. Glutathione Reductase (GSH) activities in tissue of G. oxysperma from control sites (AC - Achara, TE - Terekhol) and polluted site (RA- Ratnagiri). Data are expressed as mean + SD (n=3). Superscripts of different letters are significantly different from each other at P< 0.05



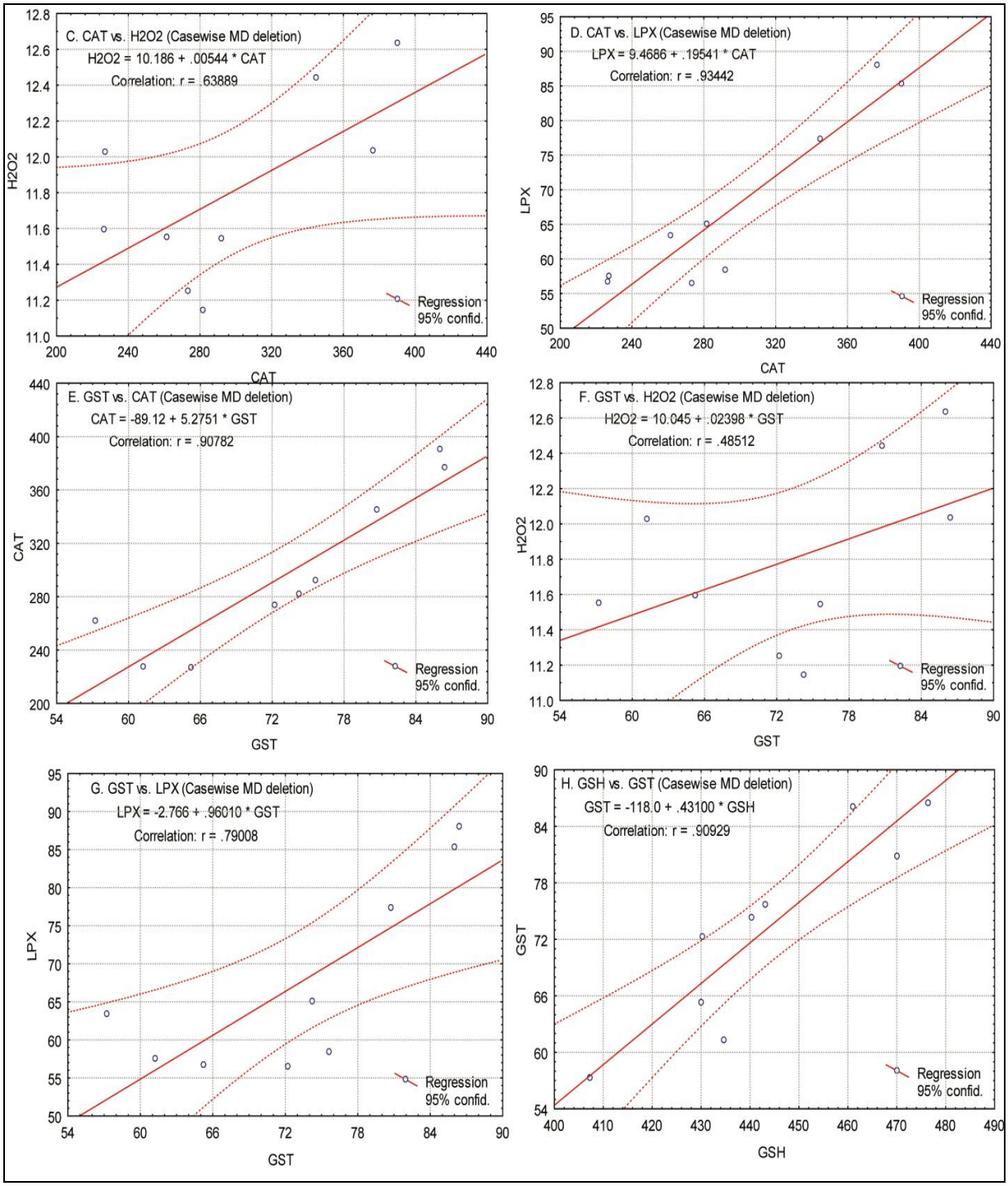
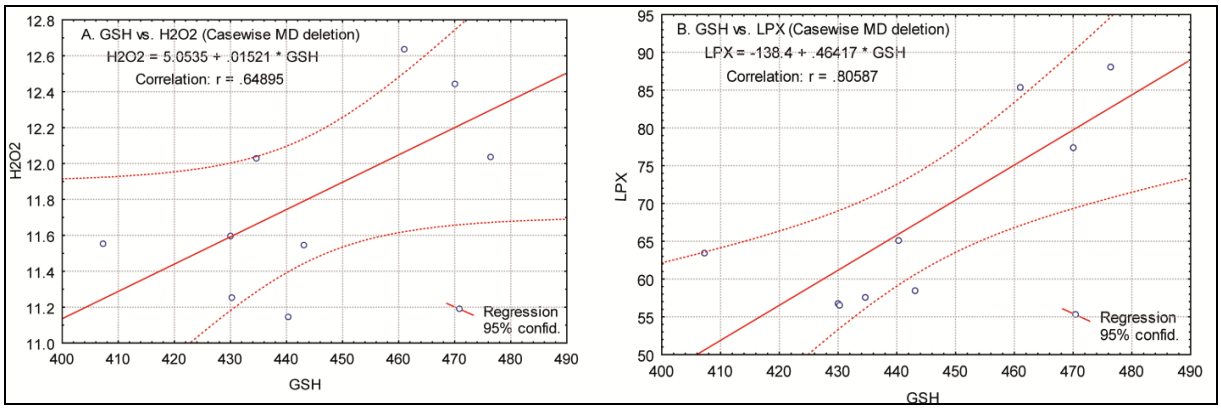


Fig 3: Correlation coefficients (r) between antioxidant parameters i.e. (A) CAT vs GSH, (B), CAT vs ASA, (C) CAT vs H_2O_2, (D) CAT vs LPX, (E) GST vs CAT, (F) GST vs H_2O_2, (G) GST vs LPX, (H) GSH vs GST.



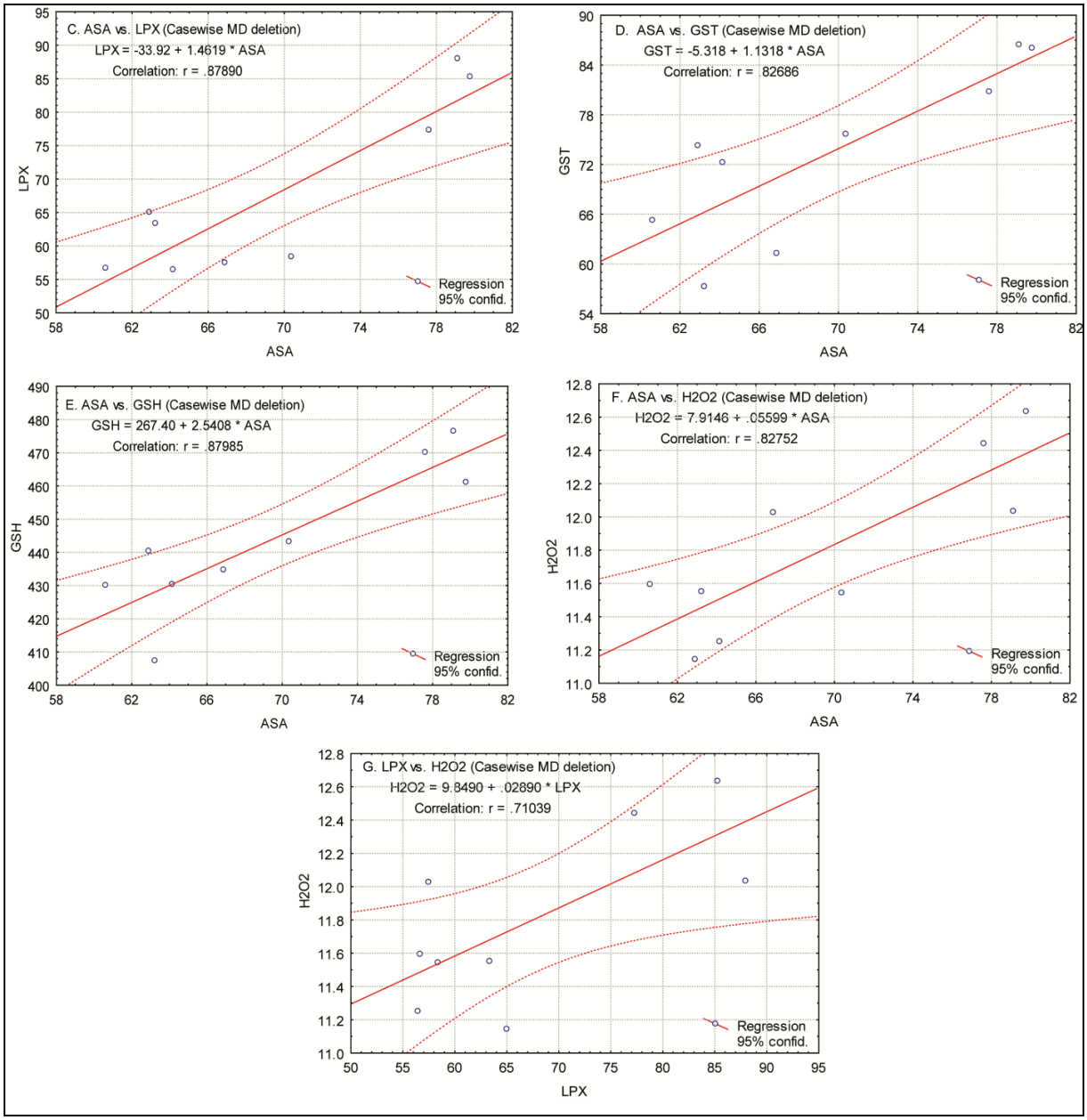


Fig 4: Correlation coefficients (r) between antioxidant parameters i.e. (A) GSH vs H_2O_2, (B) GSH vs LPX, (C) ASA vs LPX, (D) ASA vs GST, (E) ASA vs GSH (F) ASA vs H_2O_2 and (G) LPX vs H_2O_2.

Conclusions

Gayralia oxysperma displays different biochemical responses to environmental conditions. We observed evaluated TBARS and antioxidants (non-enzymatic, enzymatic) at Ratnagiri, Achara and Terekhol sites. Elevated high levels of (LPX, H_2O_2) at Ratnagiri site point out a state of oxidative stress possibly due to amassing of stress metals. Study revealed of volatility in antioxidative responses of marine algae sampled from different three sites with various water quality. This study helps to understand the whether antioxidant parameter serve as bio-indicator for biomonitoring studies.

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