Effect of temperature changes on antioxidant enzymes, and oxidative stress in *Nerita oryzarum* collected along Atomic Power Plant sites

Ajit Ambekar
Smt. Devkiba Mohansinhaji Chauhan College of Commerce and Science Silvassa
Pitchiah Sivaperumal (marinesiva86@gmail.com)
Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences
Priti Kubal
Mangalore University
Kannan Kamala
Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences

Research Article

Keywords: Antioxidant enzyme, Atomic Power station, Nerita oryzarum, Thermal stress

Posted Date: May 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-566305/v1

Abstract

Effect of heated effluent released from the Tarapur Atomic Power Station, India were investigated by analyzing the level of antioxidant enzyme activities on *Nerita oryzarum*. Seasonal variation of antioxidant enzyme (LPO, CAT, SOD, GPx and GST) were determined at six location. CAT increases in the pre-monsoon season (range of 0.12 to 2.1 mM of H_2O_2 consume/min/mg of protein) and LPO activities also increasing trend (range of 0.4 to 2.3 nM of MDA/min/mg) during same pre-monsoon at NIII and SII respectively. Enzyme values encountered high during pre-monsoon, which indicating that increased in temperature is resulting in increased activities. In experimental condition also (30^0C, 35^0C, 40^0C and 25^0C as control), antioxidant enzyme activities were in increasing trend due to raises of water temperatures. Present study was prima facie work related to physiological response of *N. oryzarum* related to heated effluent released from atomic power station and useful as baseline information for future research work.

1. Introduction

To generate the energy in living body, consumption of oxygen through breathing is needed. Most of the oxygen gets incorporated into harmless water molecule except up to 2 percent may be spun off in to our cell as free radical or ROS (Reactive Oxygen Species), which are greatly charged molecules. The production of ROS are dominant in aquatic ecosystem. The oxidative stress are very significant component as stress response, especially in marine environment were intertidal organisms get exposed to assortment of environmental as well as anthropogenic stressors, for example thermal related stress which is very predominant due industrialization, power plants and global climate change.

Oxidative stress can briefly define as, the production of ROS beyond the ability of organism to reduce and be able to cause damage to proteins, lipid and DNA (Halliwell and Gutteridge, 1999). The quenching of $^1O_2$ on site of production and to decrease the fluctuation of other ROS to ultimately inhibit the production of furthermost damaging ROS, *i.e.*, HO. is the primary function of the antioxidant defense system (Cadenas, 1989; Fridovich, 1998). Organisms in marine ecosystem usually get exposed to multiple environmental stressors, such as increase in temperature, reduction in pH, fluctuation in salinity and exposure to metals (Harley et al., 2006; Brierley and Kingsford, 2009; Sparks et al., 2019; Albayrak et al., 2019). Especially the organisms from rocky intertidal and shallow water areas have more negative impact (Millero, 2007) than pelagic zone organisms (Beuchel et al., 2006).

Increase in temperature result in elevated activities of cytosolic and mitochondrial enzyme system that generates ROS in extra mitochondrial compartment which contribute to both increase rate of metabolism and ROS production (Sokolova et al., 2012). High temperature increases mitochondrial ROS production in mollusks (Heise et al., 2003; Abele et al., 2002). A moderate increase in temperature leads to stimulation of the antioxidant protection system and expression of antioxidant enzymes get induced (Ivanina et al., 2009; Gourgou et al., 2010). Since, gastropods are slow moving and inhabitant of intertidal regions, suggesting that they already get exposed to thermal stress due to exposure during tidal rhythm, any additional thermal stress, such as, heated effluent from nuclear power plant, may cross their optimum

tolerance limit. In the present study, the gastropods of *Nerita oryzarum* was selected the study animal due to availability of all seasons around Tarapur Atomic Power Station, India and it could be the greatest demonstrative model for studies of influence of stress on antioxidant defense mechanism.

2. Results

2.1 Measurement of lipid peroxidation level due to temperature changes

The oxidative lipid degradation is known as lipid peroxidation. It is the development in which free radicals take electrons from the lipids in cell membranes, finally cells got damage. These process done by mechanism of free radical chain reaction. During present study the lipid peroxidation in whole body tissue of *N. oryzarom* was analysed spatially at six different locations as well as seasonally during six different seasons in close vicinity of nuclear power plant site, Tarapur (S Table-2). During the monsoon the minimum LPO activity was measured of 0.58 nM of MDA/min/mg at S I and maximum of 2.31nM of MDA/min/mg at SII location with (STDV) ± 0.41. Post-monsoon season the LPO activity measured in *N. oryzarum* got reduced in the range of 0.09 (at NIII) to 1.21 (at SII) nM of MDA/min/mg with (STDV) ± 0.29. During the pre-monsoon it again showed increasing trend and was in the range of 0.4 (at NIII) to 2.3 (at SII) nM of MDA/min/mg with (STDV) ± 0.5 (Fig. 1).

2.2 Catalase analysis

Catalase is present in all aerobic organisms. Within the cell, catalase in mainly located in peroxisomes and mitochondria as both soluble and membrane bound form. Catalase in alternative dismutase enzyme comprises heme moiety at its active position. It changes H_2O_2 in H_2O and molecular oxygen mostly in peroxisomes. It avoids conversion to hydroxyl radical and other toxic ROS. In the present study the CAT was measured in the range of 0.03 (at NI and NII) to 1.1 (at SI) mM of H_2O_2 consume/min/mg of protein with (STDV) ± 0.35 at monsoon season. During the post-monsoon season CAT values does not showed any trend and was in almost similar range as of monsoon season *i.e.*, 0.03 (at SI) to 1.1 (at SII) mM of H_2O_2 consume/min/mg of protein with (STDV) ± 0.34 (S Table-2). The CAT increases in the pre-monsoon season and was in the range of 0.12 (at NIII) to 2.1 (at SII) mM of H_2O_2 consume/min/mg of protein with (STDV) ± 0.42 (Fig. 2).

2.3 Effect of temperature changes on SOD level

Superoxide dismutase catalyzes the dismutation of superoxide (O_2^-) radical into hydrogen peroxide (H_2O_2) or ordinary molecular oxygen (O_2) providing cellular defense against reactive oxygen species. It was observed that the minimum activity of SOD was recorded at NII location during all the season with minimum of 1.8 U/mg of protein during post-monsoon followed by 2.3 U/mg of protein in monsoon and 3.5 U/mg of protein in pre-monsoon season. The maximum values of SOD activity was recorded at SII location throughout study period with similar trend i.e., minimum of 13.4 U/mg of protein in post-monsoon followed by 19.2 U/mg of protein in monsoon and maximum of 27.5 U/mg of protein during pre-monsoon season (Fig. 3). Glutathione peroxidase is selenium comprising antioxidant enzyme that

efficiently reduces H_2O_2 and lipid peroxides to water and lipid alcohol, one-to-one and in turn oxidizes glutathione to glutathione disulfide. The Glutathione peroxidase helps to prevent lipid peroxidation and maintains intracellular homeostasis as well as redox balance. The GPx values were minimum during the post-monsoon season and it was in the range of 0.04 (at NII) to 1.1 (at SII) nmol glutathione oxidase /min/mg protein with (STDV) ± 0.24. During monsoon it was in the range of 0.4 (at NIII) to 2.154 (at SI) nmol glutathione oxidase /min/mg protein with (STDV) ± 0.37 and in pre-monsoon it varied in the range of 0.4 (at NIII and SIII) to 3.04 (at SII) nmol glutathione oxidase /min/mg protein with (STDV) ± 0.58 (Fig. 4).

2.3 Glutathione S-transferases level

Glutathione S-transferases (GST) are a group of enzymes that catalyze conjugation of reduced glutathione to a varied range of substrates, finally resulting in detoxification (Graham et al., 2013). They also function as transport proteins. It catalyses the conjugation of GSH via a sulfhydril group to electrophilic centers on wide variety of substrates to make the compound more water soluble. The GST values showed the increasing trend; minimum during post-monsoon followed by monsoon and pre-monsoon. It was minimum of 8.9 (at SIII) nmoles/min/mg Protein during post-monsoon season. During the monsoon it varied in the range of 10.2 (at NIII and SIII) to 17 (at NI) nmoles/min/mg Protein and it increased in pre-monsoon with the range of 10 (at SIII) to 20.1 (at SII) nmoles/min/mg Protein with (STDV) ± 2.09 (Fig. 5). Similarly, all the antioxidant enzyme activity was studied in experimental organisms and the result showed that, all the activity was in increasing trend and directly proportional to the water temperature. The result of experimental analysis is given S Table no. 2.

In the present study the influence of heated effluent water released from the Atomic plant at Tarapur were investigated by examining the antioxidant enzymes as molecular biomarkers. The five different assay's were analysed such as Lipid peroxidase, Catalase, Superoxide dismutase, Glutathione-S-transferase and Glutathione peroxidase in *N. oryzarum* collected from six different locations during three different seasons. The thermal impact was also studied in similar organism at laboratory condition for similar biomarkers as at environmental conditions by maintaining the organism at three different temperatures (30^0C, 35^0C, 40^0C and 25^0C as control). The correlation of these biomarkers with response to heated effluent was studied (Table-2).

3. Discussion

All aquatic animals, their diversity and community structure are influence by temperature. Various studies have suggested that the temperature bring out considerably the physiological stress and higher metabolic activity which increases oxygen consumption and consequently produces ROS (O_2^-, H_2O_2 and OH^-) (Lushchak and Bagnyukova, 2006; An and Choi, 2010). The temperature limits are different for different species within which they can successfully survive, for some species the range of this limit is very narrow. The metabolic scope and stress response mechanisms are primarily important factors that allow the survival of particular species in that range limit (Somero, 2002 and Portner, 2010). To maintain the

physiological homeostasis within the temperature range various cellular mechanisms plays a vital role. Off this the important are, heat shock proteins, mechanism of general alteration of energetic metabolism and most primitive, antioxidative system (Arad et al., 2010, Pohlmann et al., 2011,Qian et al., 2012 and Mizrahi et al., 2012). According to Handy et al. (2009), marine invertebrate's response to temperature by adjusting mitochondrial electron transport, which in turn produces the superoxide anions as a bi-product (Davidson and Schiestl, 2001). The antioxidant enzyme activity fluctuates as per the production and scavenging of ROS produced. The organism is said to be in oxidative stress when the antioxidant defense mechanisms get oppressed by production of reactive oxygen species (Menghong Hu et al., 2015).

The use of biomarkers in environmental monitoring programs, both in freshwater and marine water, frequently uses molluscs as sentinel species (Marigómez et al., 2006; Laffon et al., 2006; Cajaraville et al., 2006; Lima et al., 2007). The antioxidant defense mechanism of marine mollusks has been the subject of many researchers. Many studies mostly assessed the response of antioxidant defense system of mollusks related to specific pollutants, such as metals (Nikitha Divakaran, 2014), polycyclic aromatic hydrocarbons (Sarkar et al., 2008; Bhagat et al., 2016), oil spill (Moreira et al., 2004; Ana et al., 2009), organic-inorganic pollutants, Industrial and domestic waste disposal etc. In particular most of study focused on bivalve species (for review, Winston and Di Giulio, 1991). But little is known about antioxidant defense of marine gastropods, especially the data related to thermal stress due to heated effluent released from nuclear power plant sites is seldom. Present study investigates the physiological response of *N. oryzarum* in the form of response of antioxidant system towards heated effluent released from nuclear power plant site, Tarapur.

Antioxidant enzymes of SOD, CAT, GPx, GST and GR are significant in coping with oxidative stress produced by metabolism itself or/ and environmental factors also (Tripathi et al., 2006; Pinto et al., 2003). The antioxidant system in mollusks involves some important enzymes which prevent oxidative damage mediated by ROS are Catalase, Superoxide dismutase, Glutathione-S-transferase and Glutathione peroxidase. In the present study most of the enzymatic activity and lipid peroxidation values encountered high during pre-monsoon and at SWIII location, which indicating that increased in temperature is resulting in increased activity. These results are in correlation with experimental condition where increased in temperature also leads to increased in the activity of antioxidant enzymes. Many authors studied the effect of temperature, pH and salinity on antioxidant systems of mollusks. Matozzo et al., 2013, studied the combined effect pH and temperature on bivalve and suggested that exposure of mollusks to the ROS generating stress full condition may either induce or inhibit the activity of antioxidant enzymes.

Lipid peroxidation can generally be described as the process in which the carbon- carbon double bond in lipid especially PUFA (Polyunsaturated fatty acids) get attacked by oxidants such as free radicals (Antonio et al., 2014). In the present study TBARs test was used to represent the level of LPO in *Neritta oryzarum*. The maximum LPO activity (1.88 ± 0.12 nM of MDA/min/mg of protein) was estimated during pre-monsoon 2014, when the maximum water temperature was recorded, indicates that, increased in temperature induces the LPO activity. Production of free radicals increased with increase temperature and

it reacts with polyunsaturated fatty acids of the cell which induces the LPO activity (Jacky Bhagat et al., 2016). Under experimental treatments, the LPO activity was directly proportional to temperature (Table-1) and confirms the present finding. The similar result was also reported by Verlecar et al., 2007, in *Perna viridis* exposed to high temperature.

Superoxide dismutase accumulates in response to oxidative stress and is a main antioxidant defense pathways (Down et al., 2001). Increased SOD level shows that cells are answering to an oxidative stress (Fridovich, 1995). SOD being the primary scavenger of $O_2.^-$ during heat stress indicates that it inhibiting accumulation of oxygen radicals. Verlecar et al., 2007 reported increased activity of SOD in digestive gland and gills of *Perna viridis* at higher temperature (32^0C) than the control condition (26^0C). Increased in temperature substantially increase the oxygen consumption in *Nacella concinna* (Peck, 1989) and also leads to increased SOD activity in digestive gland and gills (Abele et al., 1998). de Oliveira et al., 2015, have also reported the similar trend in *N. concinna* gastropods that increased in temperature leads to increased SOD activity. In the present study maximum SOD activity was detected at SW II location during pre-monsoon 2014 (22.57 ± 0.89 U/mg protein) when the temperature was recorded highest and was in the range of 28.2^0C to 38.5^0C (Avg. 31.20^0C ± 3.78) followed by monsoon 2013 (16.71 ± 0.57 U/mg protein), where temperature was in the range of 28 ^{0}C to 33.75^0C (Avg. 30.71^0C ± 1.99) indicating that increased in temperature leads to increased in SOD activity (although not linear in organism from natural habitat). The result was confirmed in experimental set up where organism was exposed to increased temperature for 8 hrs leads to an increased SOD activity (Table-1). This results is similar with Matozzo et al., 2013, encountered the upward trend of SOD activity with increased in temperature in clams and mussels. Parihar et al. (1997), have also described that, increased in SOD activity in gills of *H. fossilis* on short period temperature improvement. In present study the SOD values shows the seasonal as well as spatial trend. It was minimum during post-monsoon 2013 and maximum in pre-monsoon 2014. Spatially, intake locations i.e. NWI and SWI had less value for SOD activity than the discharge locations. SOD values shows positive correlation with pH (r = 0.439) and water temperature (r = 0.911) and also it is positively correlated with CAT (r = 0.798) and LPO (r = 0.784). M. Hu *et al.*, 2015 also have similar results and stated that SOD activity was high in gills and digestive glands of *M. coruscus* kept at low pH and high temperature.

Increased in SOD activity carry out the procedure of O^{2-} dismutation to H_2O_2 which should ultimately stimulate the activity of Catalase enzyme, which then bring out the reduction of H_2O_2 and protects the tissue from ROS and hydroxyl radicals. Thus catalase act as very important scavenger in the cell. In present study the activity of catalase was recorded high at SWII location (1.52 ± 0.14 mM of H_2O_2 consume/min/mg of protein) during pre-monsoon 2014 (Figure-3). Under the oxidative stress often activity of catalase also increases (Hermes-Lima, 2004), this is in consistent of current finding indicating increased in temperature also indices the activity of SOD and CAT. The result was also similar during the experimental condition where organisms were exposed to thermal stress also have shown similar trend of increased in catalase activity from 0.41 ± 0.2 mM of H_2O_2 consume/min/mg of protein to 1.17 ± 0.3 mM of H_2O_2 consume/min/mg of protein at 30^0C and 40^0C respectively (Table-1). The catalase activity was

higher in experimental thermal set up (i.e. 30^0C< 35^0C< 40^0C) than in control (25^0C, CAT = 0.39 ± 0.1 mM of H_2O_2 consume/min/mg of protein) confirmed that organism exposed to thermal stress induces the higher activity of catalase in order to cope up with oxidative stress. Abele et al., 1998, have demonstrated the thermal stress in *Nacella concinna* by exposing it to increased temperature for 24 hrs and concluded the increased in SOD and CAT activity in the gills. In the present finding CAT activity showed positive correlation with SOD activity (r = 0.798) and with water temperature (r = 0.911) and pH (r = 0.439) indicating both parameters are influencing the enzymatic reactions in *N. oryzarum* (Table-2).

Along with catalase, glutathione peroxidase also acts as important scavenger in the cell. GPx are a large family of selenium dependent and selenium independent enzymes with peroxidase activities. Reduction of organic hydroperoxides to their corresponding alcohol and free H_2O_2 to water are the main biological roles of GPx. In cellular matrix it is located in cytosol, mitochondrial matrix and membrane, thus it removes H_2O_2 from various cellular compartments in combining with catalase (Halliwell and Gutteridge, 2007). During present study, the highest activity of GPx was measured at SWII location (1.98 ± 0.2 nmol glutathione oxidase/min/mg protein) during pre-monsoon 2014, followed by monsoon 2013 at NWII location (1.39 ± 0.1 nmol glutathione oxidase/min/mg protein). Both locations are the discharge point of TAPS 1&2 And TAPS 3&4, respectively and seasons when maximum values for water temperature was reported during study period, indicates that the increased in water temperature influence the activity of GPx. Similarly, when the organism was exposed to thermal stress also shows directly proportional relationship between GPx and water temperature. In the present study GPx activity was positively correlated with water temperature (p = 0.808), SOD activity (p = 0.704) and salinity (p = 0.449) (Table-2).

Glutathione S- transferases (GST), enzymes catalyses the conjugation process of condensed glutathione to extensive range of substrates and ultimately results in detoxification. It catalyses the reaction of glutathione compound with –SH group, thereby neutralizing their electrophilic sites and making the compound more water soluble. Temperature significantly affects the GST activity (Lynn et al., 2000), and it increases with increase in temperature. In present study the GST activity was maximum (16 ± 0.57 nmoles/min/mg protein) was measured during pre-monsoon 2014. At experimental condition the GST activity increased with increase in temperature from 30^0C to 35^0C and it reduces at 40^0C, these could be reason that the organism has already cross the lethal temperature and further response of antioxidant system has stopped. Although, GST is frequently used as biomarker in many experimental studies, the systematic studies of its kinetics, ecologically relevant endpoints and affect of environmental factors are limited. In case of invertebrates, the Glutathione S- transferase activity does not give any predictable trends, because detailed study of its kinetic behavior under stress condition and characterization of several enzymes with in this family is seldom (Domingues et al., 2010).

Biomarkers, when used as diagnostic tool for environmental monitoring, it is important to link the response of biomarker at individual level, potential effect of pollutant on population, ecosystem and on communities of particular organism. However, often it is difficult as meaningful interpretation required deep knowledge of integration of various levels of biological organization and its mechanistic processes

(Forbes et al., 2006). Moreover, one should also consider that spatial and temporal history related to particular contaminants which influence the biochemical changes in organisms, and if these changes are in physiological response and ecologically relevant, then biomarkers are successfully acts as measure of effect (Ankley et al., 2007).

4. Materials And Methods

4.1 Sample collection

The *Nerita oryzarum* (around 20–25 organisms from each site) were collected during three different seasons (monsoon 2013 to pre-monsoon 2014) from six rocky intertidal locations (TAPS1&2, Light house, Varor, TAPS 3&4, Popharan and Nandgaon) around Tarapur nuclear power plant site (S Table-1 & S Fig. 1). The gastropod of similar size (23 to 32mm) and irrespective of their sex was collected for analysis. The organisms were freeze in liquid nitrogen at the site of collection and transported to laboratory. In laboratory, shell of gastropod was gently smashed and entire body tissues were cautiously dissected from other vicera. A soft tissue from 5 to 6 gastropods were pulled together to make 1gm of weight, which was used for preparation of tissue extract for biochemical assay.

4.2. Analaysis of protein and lipid peroxide (LPO):

The estimation of protein in *N. oryzarum*, The Lowry's method (Lowry *et al*., 1955). The standard procedure for estimation of level of lipid peroxide in organism tissue by their reaction with thiobarbituric acid developed by Ohkawa et al., 1979 was used. To estimate lipid peroxidation, 1 g of tissues was homogenized in 9 ml of ice cold 0.25 M sucrose. 0.2 ml of animal tissue homogenate was assorted with 0.2 ml of 8 % SDS followed by 2 ml of 20 % acetic acid and 2 ml of 0.8 % Thiobarbituric Acid (TBA). Further, the mixture was make up to 4 ml by using distilled water and heated for 60 min in boiling water bath. After cooling the mixture was centrifuge at 3000 rpm for 10 min. The absorbance was measured at 532 nm and the results were expressed as nM of MDA $min^{-1}mg^{-1}$ of protein. Lipid peroxide was considered as MDA equivalent.

4.3 Determination of Catalase (CAT):

The catalase assay was measured according to simple calorimetric method for rapid assay of catalase described by Sinha (1972). Tissue homogenate for catalase activity was prepared in 4 ml of phosphate buffer (0.1 M, pH 7.4) by using tissue homogenizer. The total contents were centrifuged at 4^0C, 18,000 rpm for 30 min. The reaction mixture contents 0.5 ml of 0.2 M H_2O_2 (Hydrogen Peroxide), one ml of 0.01 M sodium phosphate buffer (pH 7.0) and 0.4 ml of double distilled water. Further, two ml of dichromate-acetic acid reagent, which contains glacial acetic acid and potassium dichromate and in 3:1 proportions, were added to stop the reaction. The reaction mixture heated for 10 min. in water bath and further kept for cool. After cooling, green color was developed and the absorbance was measured at 583 nm and blank also maintained. The CAT activity was expressed as mM of H_2O_2 consumed/min/mg of protein.

4.4 Determination of Superoxide dismutase (SOD)

The SOD (Superoxide dismutase) activity was measured by the method of Misra and Fridovich (1972) with minor modification. The SOD activity in tissue homogenates were analysed by the level of auto-oxidation of epinephrine to adrenochrome. For SOD determination, 1 g of tissue was homogenized in 0.1M phosphate buffer (pH 7.4), 0.2 ml of chloroform (ice chilled), 0.15 ml of ethanol (ice chilled) followed by 1 ml of distilled water was added in chilled condition. The total contents were centrifuged for 10 min at 4^0C and 3000 rpm in cooling centrifuge machine. The one ml of reaction mixture contained epinephrine (10 mM), sodium carbonate buffer (50 mM with pH 10.2) and EDTA (10 mM). The absorbance was measured for 3 min at 30 sec interval at 320 nm. The SOD activity results were reported as U mg^{-1} (per mg of protein), where 1 U of SOD is distinct as the quantity of sample causing 50 % of inhibition of epinephrine auto-oxidation.

4.5 Determination of glutathione peroxidase (GPx)

The estimation of glutathione peroxidase in tissue homogenate was done by following the method of Paglia and Valentine (1967) with slight modification. The 0.2 ml of tissue homogenate treated with 0.2 ml of phosphate buffer (pH 7.0) followed by 0.2 ml of 0.4 Mm EDTA, 0.1 ml of 10 mM Sodium azide (NaN3) and 0.1 ml of glutathione reductase (2.4 units/ml), mix well. Then add 0.2 ml of Glutathione reduced (GSH, 61.4 mg of GSH in 100 ml of 0.02 M EDTA), 0.2 ml of H_2O_2 (0.01 ml of 30 % solution in 100 ml of phosphate buffer), 0.1 ml of NADPH, incubate the mixture for 10 min. at 37^0C further, add 0.5 ml of TCA (10 %), then centrifuge at 10000 rpm for 5 min. After centrifugation, take 1 ml from supernatant and add 2 ml of 0.4 M Tris buffer (pH 8.9) and 50 µl of 5–5',dithio-bis- (2-nitrobenzoic acid) (DTNB, prepared in methanol). Further, we have read the absorbance at 412 nm for 3 min. The blank also run in same manner by adding 10 % TCA to different concentrations of standard and 2 ml of tris buffer and 50 µl of DTNB and measured the absorbance. The results were expressed as nmol glutathione oxidase /min/mg protein.

4.6 Determination of Glutathione-S-transferase (GSH)

The activity of GSH (Glutathione-S-transferase) measured by the method describe in Habig et al., (1974). The reaction mixture content 0.1 ml of tissue homogenate mixed with 1 ml of phosphate buffer (0.1 M K_2HPO_4, EDTA-Na_2, pH 6.5) followed by 0.1 ml of CDNB (1-Chloro-2,4-Dinitrobenzene). Further, make up the volume of reaction mixture to 2.5 ml by using double distilled water and incubated at 37^0C for 5 min. Then add 0.1 ml of 30 mM reduced glutathione (GSH). The change in absorbance was measured at 340 nm for 3 min at 15 sec interval. The reaction was based on the conjugation of CDNB solution and reduced glutathione reaction in reaction buffer. The GST activity was measured by using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB. The results were stated as µmol/min/mg of protein.

4.7 Experimental set-up for analyzing antioxidant enzymes changes, and oxidative stress in Nerita oryzarum due temperature variation

The gastropod species *Nerita oryzarum* were collected at rocky intertidal locations from Varor which was considered as reference location, as it is situated away from impact of heated effluent. The gastropods of similar size (18 to 20 mm) and irrespective of their sex was collected for our experiment study. The *N. oryzarum* was brought to library in a plastic bucket half filled with sand and seawater from their natural habitat. While collecting the animal, the temperature of sea water was measured (29.7 ± 1 ^{0}C). In laboratory the organisms were transferred carefully to the glass tank of 20 L capacity (S Fig. 2). The sand from natural habitat of organism was spread at the bottom of a tank. The organisms were allowed to acclimatize in aerated seawater for 24 hours. Then the waterproof heater was inserted to each tank after setting it to desire temperature ranges. The 30^{0}C was directly set in heater and for two other temperature ranges it was slowly increased with an interval of 2- 3^{0}C until the temperature of seawater in tank reach 35^{0}C and 40^{0}C. The water temperature, pH, dissolved oxygen and salinity were recorded from 0 hr. time to 8 hr at every 2 hr interval.

The acclimatized *N. oryzarum* samples were divided in three groups and each group had three replicates as tank A, B and C. In each tank, 8–10 animals were placed. Treatment I: Have three tanks labeled as A, B, C (maintained at 30^{0}C temperature). Treatment II: Have three tanks labeled as A, B, C (maintained at 35^{0}C temperature). Treatment III: Have three tanks labeled as A, B, C (maintained at 40^{0}C temperature). Control: Have three tanks labeled as A, B, C (maintained at 25^{0}C temperature) details are mentioned in Table-1. The animals were sacrificed after 8 hrs of experimental duration. The temperature range and duration was fixed based on filed observations. Since, the *N. oryzarum* collected are inhabitant of intertidal region which are exposed to tidal rhythm of around 6 to 7 hrs of diurnal variation.

4.8 Statistical analysis:

The data received from all analytical methods were treated statistically using ORIGIN software. Two-way ANOVA with Duncun's test were run by using SPSS v.20. The graphs were plotted by using SIGMA PLOT software. The correlation analysis between environmental parameters and antioxidant values were analysed on STATISTIKA 7 software. Expressive data analysis were performed, including the calculation of mean, SD, maximum and minimum.

5. Conclusion

The results of present study is prima facie work related to physiological response of gastropod of *N. oryzarum* related to heated effluent released from atomic power station and useful as a baseline information for future research work. Further this study provide a meaningful understanding of how temperature changes affect the antioxidant defense system in marine intertidal organisms. Studies on mechanisms for individual function of enzymes in temperature induced stress finding also required in future.

Declarations

Acknowledgement:

The authors are grateful to The Director, CIFE and Dr. Chandra Prakash, Principal Investigator for BRNS-Tarapur Project, ICAR-CIFE, Deemed University, Mumbai for providing facilities to carry out this study and thankful to Board of Research in Nuclear Science (BRNS), Department of Atomic Energy (DAE), Government of India for providing the financial assistance for this work (CRP - Marine Radioecology Project- Tarapur site). The corresponding author are thankful to Saveetha Dental College and Hospital, SIMATS The authors would like to express their sincere appreciation to the Deanship of Scientific Research, King Saud University, Riyadh, Saudi Arabia, for funding this Research Group Project No. RG-1437-005.

CRediT authorship contribution statement

Ajit ambekar: Investigation, data collection, writing original draft and editing. **P. Sivaperumal:** Conceptualization, Supervision, manuscript review and editing. **Priti kubal:** Data collection, **K. Kamala:** statistical analysis and manuscript editing.

Ethical Approval: Not applicable

Consent to Participate: All authors are consent to participate in this study**.**

Consent to Publish: All authors are agreed to publish the manuscript in this journal

Competing Interests: The authors are confirm that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials: Not applicable

References

Abele, D., Burlando, B., Viarengo, A., Portner, H. O., 1998. Exposure to elevated temperatures and hydrogen peroxide elicits oxidative stress and antioxidant response in the Antarctic intertidal limpet *Nacella concinna*. Comp Biochem and Phys Part B: 120(2): 425-435.

Abele, D., Heise, K., Portner, H., 2002. Temperature dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria.* J. of Exp. Biol. 205:1831-1841.

Albayrak, S., Gülruh Albayrak, Mine Terzi Çelikkol, Aylin Gazdağlı, Emre Yörük, Senem Çağlar, 2019. The effcts of polycyclic aromatic hydrocarbon compounds on antioxidant enzyme activities in *Mytilus galloprovincialis* Lamarck, 1819 from Istanbul Strait, Mar. Poll. Bull. 149: 110662

An, M.I., Choi, C. Y., 2010. Activity of antioxidant enzymes and physiological responses in ark shell *Scapharca broughtonii*, exposed to thermal and osmotic stress: effects on hemolymph and biochemical parameters. Comp. Biochem. Physio. Part B 155: 34–42.

Ana, L.S., Fernando, M., Susana, M., Rui, R., António, J.A.N., Amadeu, M.V.M.S., Lucia, G., 2009. Cholinesterase and glutathione S-transferase activities of three mollusc species from the NW Portuguese coast in relation to the 'Prestige' oil spill. Chemosphere 77:1465–1475.

Ankley, G.T., Brooks, B.W., Huggett, D.B., Sumpter, J.P., 2007. Repeating history: Pharmaceuticals in the environment. Env. Sci. Tech. 41: 8211–8217.

Antonio, A., Mario, F.M., Sandro, A., 2014. Lipid Peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-Hydroxy-2-Nonenal. Oxid. Med. Cell Long. 31. doi.org/10.1155/2014/360438.

Arad, Z., Mizrahi, T., Goldenberg, S., Heller, J., 2010. Natural annual cycle of heat shock protein expression in land snails: desert versus Mediterranean species of *Sphincterochila.* J. Exp. Biol. 213 (20): 3487-3495.

Beuchel, F., Gulliksen, B., Carroll, M.L., 2006. Long-term patterns of rocky bottom macrobenthic community structure in an Arctic fjord (Kongsfjorden, Svalbard) in relation to climate variability. J. Mar. Sys. 63 (1-2): 35-48.

Bhagat, J., Sarkar, A., Deepti, V., Singh, V., Raiker, L., Ingole, B.S., 2016. An integrated approach to study the biomarker responses in marine gastropod *Nerita chamaeleon* environmentally exposed to polycyclic aromatic hydrocarbons. ISJ, Research report 14: 18-31.

Brierley, A.S., Kingsford, M.J., 2009. Impacts of climate change on marine organisms and ecosystems. Cur. Biol. 19(14): R602-R614.

Cadenas, E., 1989. Biochemistry of oxygen toxicity. Ann. Rev. Biochem. 58: 79–110.

Cajaraville, M.P., Garmendia, L., Orbea, A., Werding, R., Gómez-Mendikute, A., Izagirre, U., Soto, M., Marigómez, I., 2006. Signs of recovery of mussels health two years after the Prestige oil spill. Mar. Env. Res. 62: S337-S341.

Davidson, J.F., Schiestl, R.H., 2001. Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in *Saccharomyces cerevisiae*. Mol. cell boil. 21(24): 8483-8489.

de Oliveira, M.F., Junior, E.R., Suda, C.N., Vani, G.S., Donatti, L., Rodrigues, E., Lavrado, H.P., 2015. Interactions of temperature, salinity and diesel oil on antioxidant defense enzymes of the limpet *Nacella concinna*. Mar. Poll. Bull. 97(1-2): 451-459.

Domingues, I., Agra, A.R., Monaghan, K., Soares, A.M., Nogueira, A.J., 2010. Cholinesterase and glutathione-S-transferase activities in freshwater invertebrates as biomarkers to assess pesticide contamination. Env. Tox. Chem. 29(1): 5-18.

Down, C.A., Robert, T., John, E.F., Cheryl, M.W., 2001. A molecular biomarker system for assessing the health of gastropods *Ilyanassa obsoleta* exposed natural and anthropogenic stressors. J. Exp. Mar. Biol.

Eco. 259: 189–214.

Forbes, V.E., Palmqvist, A., Bach, L., 2006. The use and misuse of biomarkers in ecotoxicology. Env. Tox. Chem. 25: 272–280.

Fridovich, I., 1998. Oxygen toxicity: a radical explanation. J. Exp. Biol. 201: 1203–1209.

Gamelin, F.X., Baquet, G., Berthoin, S., Thevenet, D., Nourry, C., Nottin, S., Bosquet, L., 2009. Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur. J. Appl. Physiol. 105: 731-738. https://doi.org/10.1007/s00421-008-0955-8.

Gourgou, E., Aggeli, I.K., Beis, I., Gaitanaki, C., 2010. Hyperthermia-induced Hsp70 and MT20 transcriptional up-regulation are mediated by p38-MAPK and JNKs in *Mytilus galloprovincialis* (Lamarck); a pro-survival response. J. Exp. Biol. 213(2): 347-357.

Graham, S.S., Gail, L.W., Robin, M.W., 2013. Clinical Pathology in Non-Clinical Toxicology Testing. In: Haschek and Rousseaux's Handbook of Toxicologic Pathology, 3rd edn. Volume I. Academic press, pp 565-594.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione Stransferases the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249: 7130-7139.

Halliwell, B., Gutteridge, J., 2007. Free Radicals in Biology and Medicine, 4th edn. Oxford University Press.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. In: Halliwell, B. and Gutteridge, J.M.C., Eds., Free Radicals in Biology and Medicine, 3rd Edition, Oxford University Press, Oxford, 1-25.

Handy, D.E., Lubos, E., Yang, Y., Galbraith, J.D., Kelly, N., Zhang, Y.Y., Leopold, J.A., Loscalzo, J., 2009. Glutathione peroxidase-1 regulates mitochondrial function to modulate redox-dependent cellular responses. J. Biol. Chem. 284(18): 11913-11921.

Harley, C.D.G., Hughes, A.R., Hultgren, K.M., Miner, B.G., Sorte, C.J.B., Thornber, C.S., Rodriguez, L.F., Tomanek, L., Williams, S.L., 2006. The impacts of climate change in coastal marine systems. Ecol. Let. 9: 228–241.

Heise, K., Puntarulo, S., Pörtner, H.O., Abele, D., 2003. Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress. Comp. Biochem. Phys. – C. 134: 79–90.

Hermes-Lima, M., 2004. Oxygen in biology and biochemistry: role of free radicals In: Functional Metabolism: Regulation and Adaptation, K.B. Storey edn., John Wiley & Sons, New York, 319-368.

Ivanina, A., Taylor, C., Sokolova, I., 2009. Effects of elevated temperature and cadmium exposure on stress protein response in eastern oysters *Crassostrea virginica* (Gmelin). Aqua. Tox. 91: 245–254.

Laffon, B., Rabade, T., Pasaro, E., Mendez, J., 2006. Monitoring of the impact of Prestige oil spill on *Mytilus galloprovincialis* from Galician coast. Env. Int. 32(3): 342-348.

Lima, I., Moreira, S.M., Rendon-Von, O.J., Soares, A.M., Guilhermino, L., 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. Chemosphere 66(7): 1230-1242.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lushchak, V.I., Bagnyukova, T. V., 2006. Temperature increase results in oxidative stress in goldfish tissues. 2. Antioxidant and associated enzymes. Comp. Biochem. Physio. Part C: Tox. & Pharm. 143(1): 36-41.

Lynn, H.B., Vanessa, H., Aaron, M., 2000. The effect of environmental parameters on growth, cholinesterase activity and glutathione S-transferase activity in the earthworm (*Apporectodea caliginosa*). Biomarkers 5(1): 46-55.

Marigomez, I., Soto, M., Cancio, I., Orbea, A., Garmendia, L., Cajaraville, M.P., 2006. Cell and tissue biomarkers in mussel, and histopathology in hake and anchovy from Bay of Biscay after the Prestige oil spill (Monitoring Campaign 2003). Mar. Poll. Bull. 53(5-7): 287-304.

Matozzo, V., Chinellato, A., Munari, M., Bressan, M., Marin, M. G., 2013. Can the combination of decreased pH and increased temperature values induce oxidative stress in the clam *Chamelea gallina* and the mussel *Mytilus galloprovincialis*? Mar. Poll. Bull. 72(1): 34-40.

Menghong, Hu., Li, L., Sui, Y., Li, J., Wang, Y., Lu, W., Dupont, S., 2015. Effect of pH and temperature on antioxidant responses of the thick shell mussel *Mytilus coruscus*. Fish & Shellfish Immun. 46(2): 573-583.

Misra, H.P., Fridovich, I., 1977. Superoxide dismutase: “positive” spectrophotometric assays. Anal. Biochem. 79(1-2): 553-560.

Mizrahi, T., Heller, J., Goldenberg, S., Arad, Z., 2012. Heat shock proteins and survival strategies in congeneric land snails (Sphincterochila) from different habitats. Cell Stress Chaperones 17 (5): 523-527.

Moreira, S.M., Moreirados, S.M., Ribeiro, R., Guilhermino, L., 2004. The ‘Coral Bulker’ fuel oil spill on the North coast of Portugal: spatial and temporal biomarker responses in *Mytilus galloprovincialis*. Ecotox. 13: 619–630.

Nikitha, D., 2014. Responses of Antioxidants and Lipid Peroxidation in *Villorita cyprinoides* to Mercury Exposure, Conference proceeding, Semantic Scholar, Chemistry. International journal of innovative

research and development. Corpus ID: 85796576.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95(2): 351-358.

Paglia, D.E., Valentine, W.N., 1967. Studies on the qualitative and quantitative characterisation of erythrocyte glutathione peroxidase. J. Lab Clin. Med. 70: 158–169.

Parihar, M.S.T.J., Hemnani, T., Dubey, A.K., Prakash, P., 1997. Response of superoxide dismutase, glutathione peroxidase and reduce glutathione antioxidant defense in gills of freshwater catfish (*Heteropneustes fossilis*) to short term elevated temperature. J. Ther. Biol. 22: 151-156.

Peck, L.S., Veal, R., 2001. Feeding, metabolism and growth in the Antarctic limpet, *Nacella concinna* (Strebel 1908). Mar. Biol. 138(3): 553-560.

Pinto, E., Sigaud-Kutner, T.C.S., Leitao, M.A.S., Okamoto, O.K., Morse, D., Colepicolo, P., 2003. Heavy metal-induced oxidative stress in algae. J. Phycol. 39: 1008–1018.

Pohlmann, K., Koenigstein, S., Alter, K., Abele, D., Held, C., 2011. Heat-shock response and antioxidant defense during air exposure in Patagonian shallow-water limpets from different climatic habitats. Cell Stress Chaperones 16 (6): 621-632.

Portner, H.O., 2010. Oxygen and capacity limitation of thermal tolerance: a matrix for integrating climate related stressor effects in marine ecosystems. J. Exp. Biol. 213 (6): 881-893.

Qian, Z., Liu, X., Wang, L., Wang, X., Li, Y., Xiang, J., Wang, P., 2012. Gene expression profiles of four heat shock proteins in response to different acute stresses in shrimp *Litopenaeus vannamei.* Comp. Biochem. Phys. - Part C 156 (3): 211-220.

Sarkar, A., Gaitonde, D.C., Sarkar, A., Vashistha, D., D'Silva, C., Dalal, S., 2008. Evaluation of impairment of DNA integrity in marine gastropods (*Cronia contracta*) as a biomarker of genotoxic contaminants in coastal water around Goa, west coast of India. Ecotoxicol. Environ Saf. 71: 473-482.

Sinha, A.K., 1972. Colorimetric assay of catalase. Anal. Biochem. 47(2): 389-394.

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Env. Res. 79: 1-15.

Somero, G.N., 2002. Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. Inte. Comp. Biol. 42 (4): 780-789.

Sparks, C., Jeanine Marnewick, Rashieda Toefy, Reinette Snyman and James Odendaal, 2019. Baseline levels of antioxidant activities in *Mytilus galloprovincialis* along the coast of Cape Town, South Africa,

Mar. Poll. Bull. 140: 287-293.

Tripathi, B.N., Mehta, S.K., Amar, A., Gaur, J.P., 2006. Oxidative stress in *Scenedesmus sp.* during short-and long-term exposure to Cu_2^+ and Zn^{+2}. Chemosphere 62: 538–544.

Verlecar, X.N., Jena, K.B., Chainy, G.B.N., 2007. Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. Chem-biol. Inter. 167(3): 219-226.

Winston, G.W., Di Giulio, R.T., 1991. Pro-oxidant and antioxidant mechanisms in aquatic organisms. Aqu. Tox. 19(2): 137-161.

Tables

Table 1
Effects of different temperatures on enzyme activities in *N. oryzarum*

Parameters / Treatments	LPO	CAT	SOD	GPx	GST
Treatment I (30^0C)	1.16 ± 0.1	0.41 ± 0.1	9.65 ± 0.54	1.28 ± 0.13	13.61 ± 0.48
Treatment II (35^0C)	1.81 ± 0.19	0.49 ± 0.1	17.32 ± 0.84	1.8 ± 0.1	16.27 ± 0.31
Treatment III (40^0C)	2.38 ± 0.32	1.18 ± 0.11	23.56 ± 1.18	2.54 ± 0.93	14.95 ± 0.59
Control (25^0C)	1.03 ± 0.07	0.39 ± 0.04	7.22 ± 0.54	1.09 ± 0.14	9.45 ± 0.36

Table 2
Result of correlation coefficient between physico-chemical parameters and antioxidant enzymes

	SOD	CAT	LPO	GPx	GST	DO	pH	Salinity
CAT	0.798**							
LPO	0.784**	0.407						
GPx	0.704**	0.660**	0.463					
GST	0.461	0.515	0.685**	0.383				
DO	-0.187	-0.192	-0.232	-0.171	-0.171			
pH	0.439**	0.441**	-0.128	-0.137	-0.119	0.228		
Salinity	-0.323	-0.301	0.451**	0.449**	0.296	-0.166	-0.223	
WT	0.911**	0.754**	0.871**	0.808**	0.732**	-0.266	-0.178	0.528**

**. Correlation is significant at the 0.01 level (2-tailed).

WT – Water temperature, DO – Dissolved oxygen

Figures

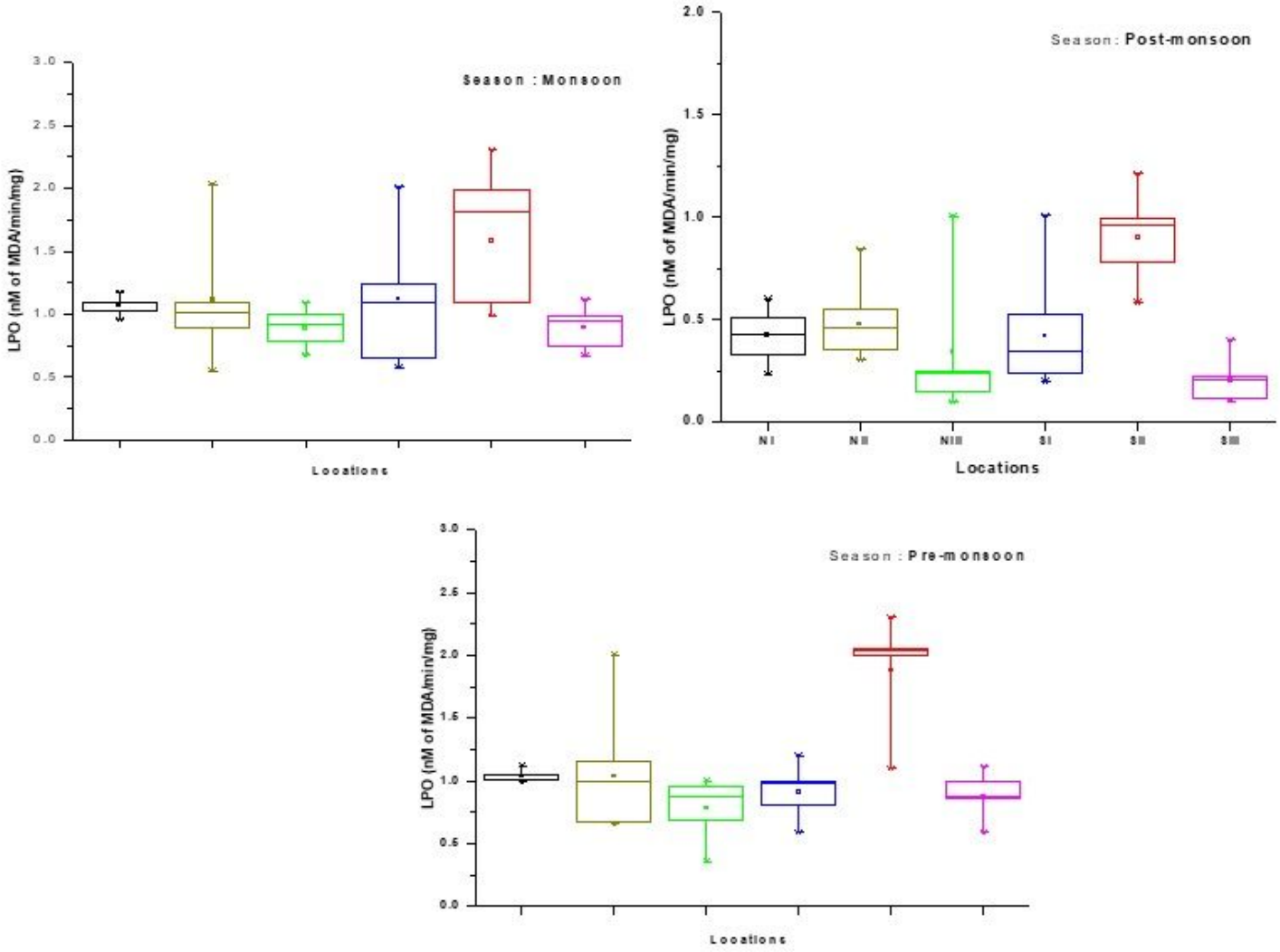

Figure 1

Effect of Lipid peroxidation capacity (Mean±SE) in whole soft tissue of N. oryzarum in different season (2013-14)

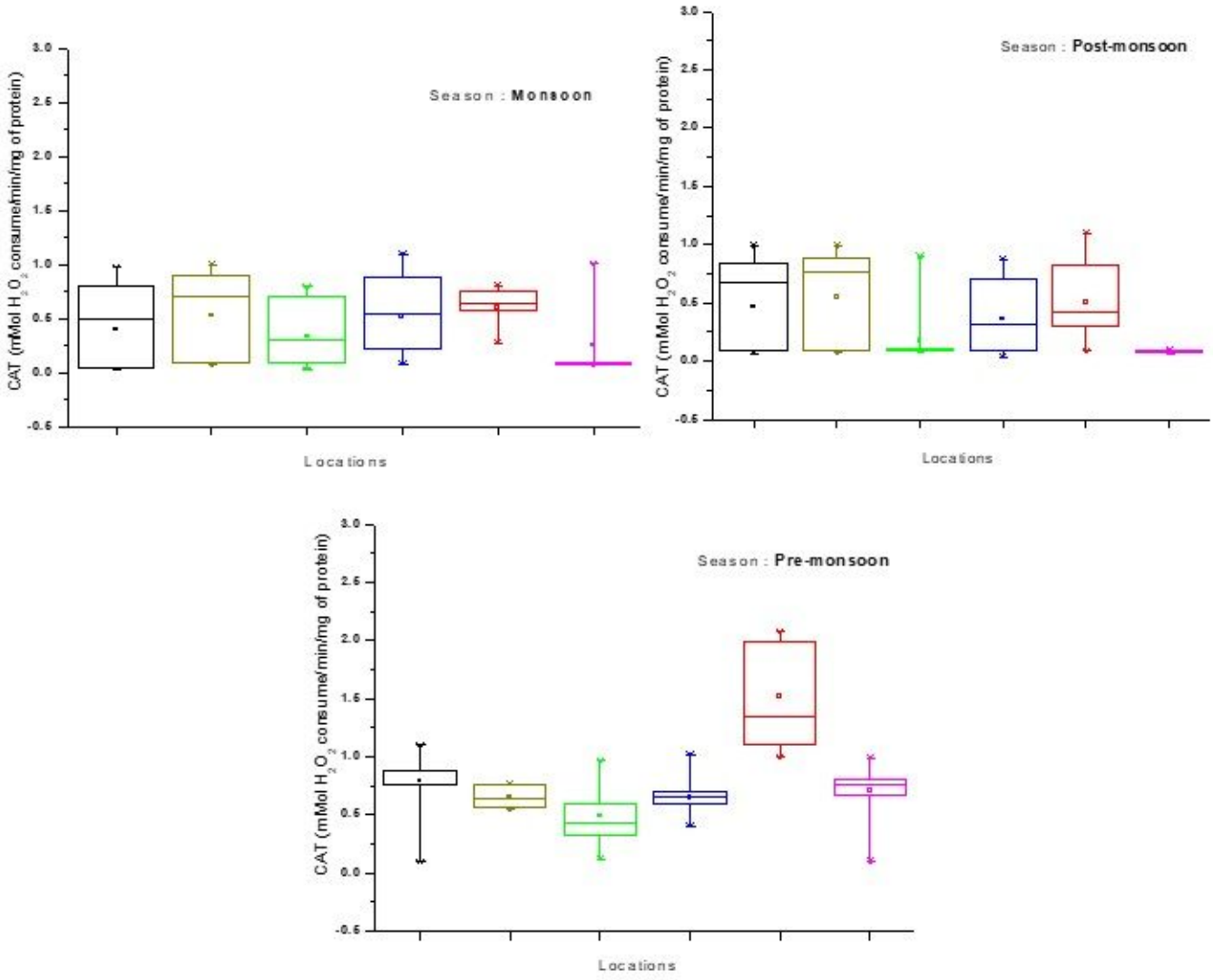

Figure 2

Effect of Catalase capacity (Mean±SE) in whole soft tissue of N. oryzarum in different season (2013-14)

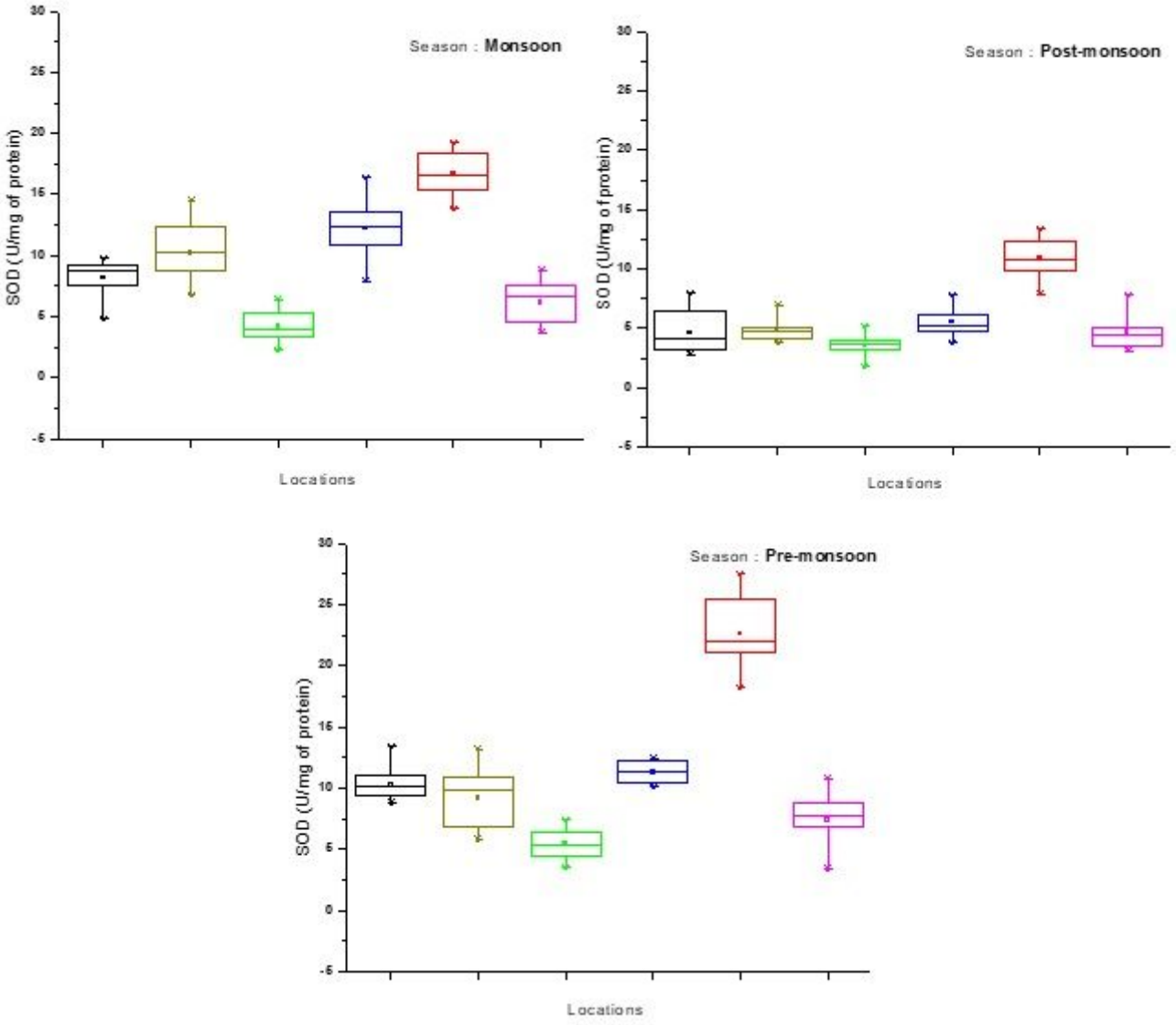

Figure 3

Effect of Superoxide dismutase capacity (Mean±SE) in whole soft tissue of N. oryzarum in different season (2013-14)

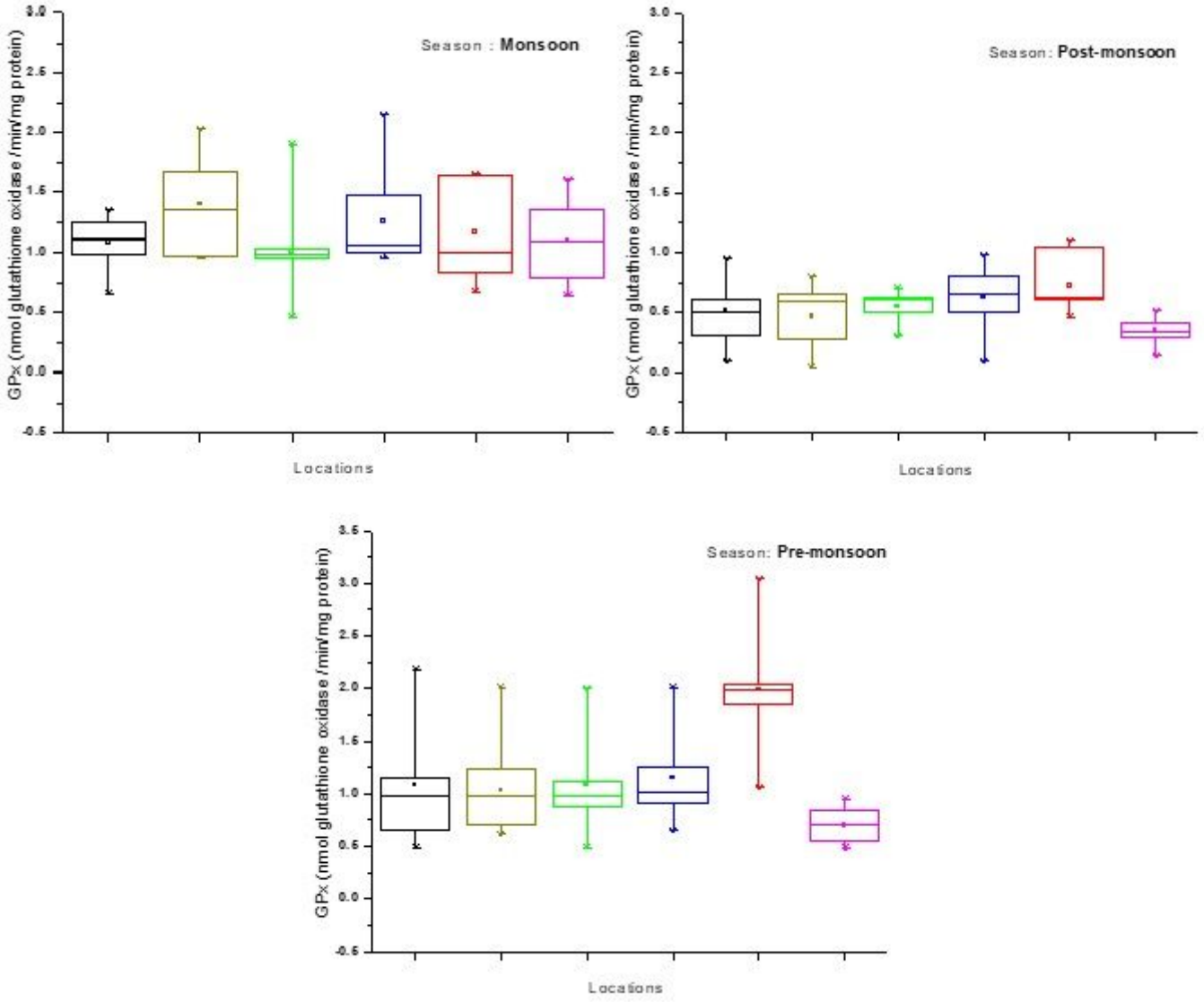

Figure 4

Effect of Glutathione Peroxidase (Mean±SE) in whole soft tissue of N. oryzarum in different season (2013-14)

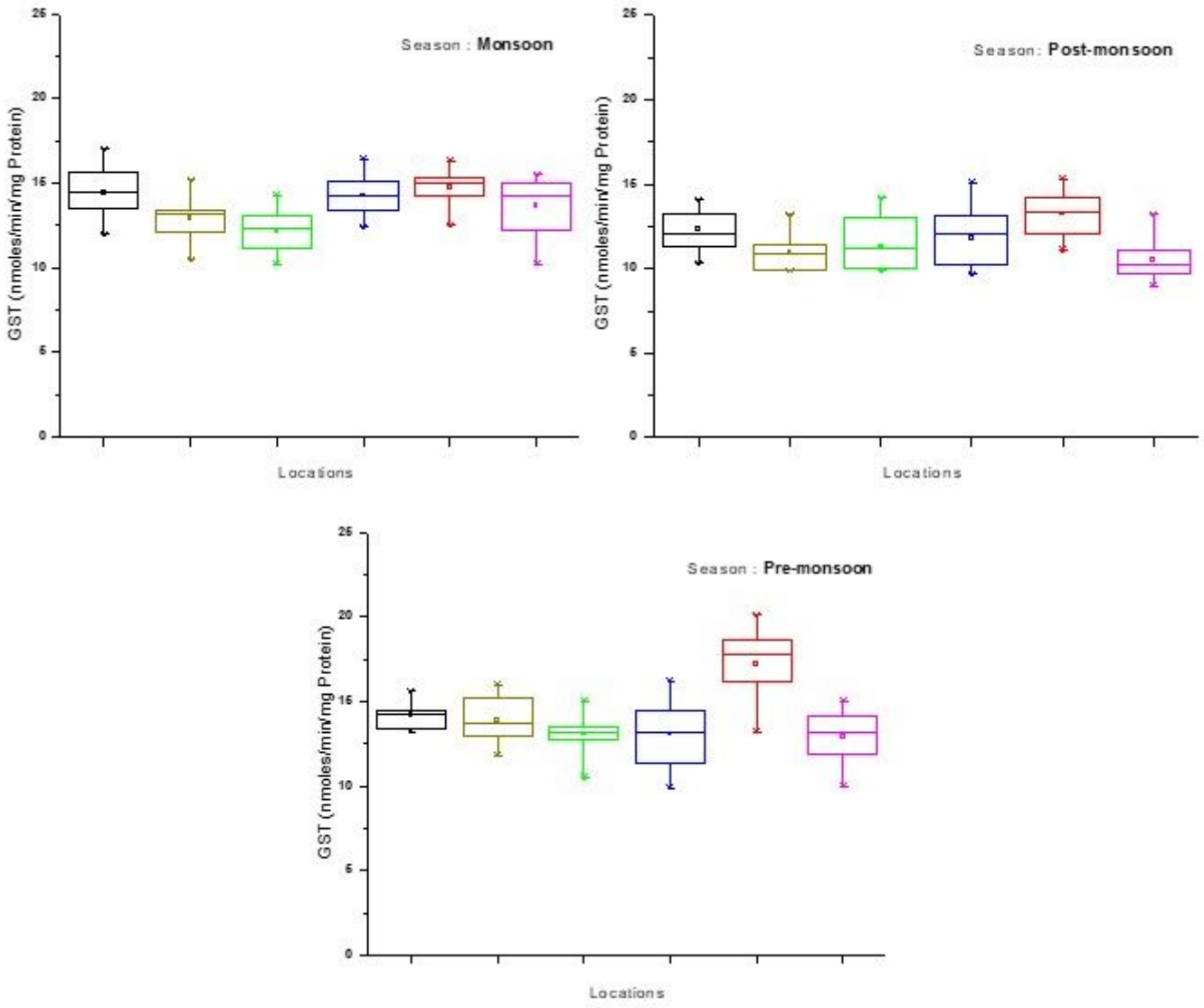

Figure 5

Effect of Glutathione S-transferase (Mean±SE) in whole soft tissue of N. oryzarum in different season (2013-14)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile.docx