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SEASONAL VARIATION INFLUENCES GLUTATHIONE MEDIATED ANTIOXIDANTS IN *Donax incarnatus* COLLECTED FROM THE TWO SOUTH INDIAN COASTAL REGIONS

R. PARTHIBAN [a] AND P. C. SATHYA NARAYANAN [a++*]

[a] PG and Research Department of Zoology, Pachaiyappa's College for Men, Chennai-600 030, India.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Bivalves comprise the major marine fishery resource; as they are rich in biochemical composition and high nutritional values. The present work deals with studying seasonal variations in the glutathione mediated antioxidant in the edible clam *Donax incarnatus* collected from coastal region of muttukadu-kovalam estuary and Pulicat lake during period of one year, from May' 2021 to April 2022. The changes were assessed in relation to the antioxidant components and the level of heavy metal accumulation. Obtained data indicated seasonal variations in the levels of antioxidant markers (reduced glutathione (GSH) content, glutathione peroxidise and glutathione *s* transferase (GST) activities and the concentrations of heavy metals (Fe, Pb, Cu, Cd and Co) in the bivalves were high during post monsoon season and the lowest values during monsoon. A seasonal variation in the levels of antioxidant components was observed. Among the antioxidant components GST activity was significantly modulated and has significant correlation with the heavy metal accumulation. It therefore appears that bioaccumulation of heavy metals could play a crucial role in regulating the antioxidant components of tissues of Donax species.

Keywords: Glutathione; GPx; GST; heavy metals; seasonal variation; donax.

[++] *Assistant Professor in Zoology;*
**Corresponding author: Email: pcsathyanm@gmail.com, pcsathyazoo@gmail.com;*

1. INTRODUCTION

Bivalves are plentiful around the Indian coastline, and many people rely on them for food [1]. The balance of an ecosystem is crucially maintained by bivalves, which also serve as a significant economic endpoint. They are the significant primary consumer representatives and a crucial link in aquatic food chain [2]. Filter feeders from the Donacidae family, *Donax incarnatus* are frequently seen on India's sandy beaches [3]. These marine molluscs provide a cheap source of high-quality protein, vital minerals, and vitamins [4]. The importance of understanding the nutritional composition of edible organisms is greatly emphasised by the growing need for protein-rich foods in emerging nations [5]. Additionally, it is crucial to comprehend how a mollusc's chemical makeup interacts with its environment in order to sustainably use the resource, including their culture, as well as for conservation and management. This is true both from the perspective of their economic significance and from that of their culture.

Bivalve molluscs are commonly used as an ideal environmental indicator of pollution. According to toxicological research, molluscs serve as bioindicators of metal pollution in the aquatic environment [6]. The present investigation chose clam *Donax incarnatus* and predict the clams are sensitive bioindicators of environmental pollution and the necessity to assess seasonal variation in biomarker responses. The clam belongs to Phylum: Mollusca; Class: Bivalvia; Order: Cardiida; Family: Donacidae; Genus: Donax Species: *Donax incarnatus* has wide geographical distribution in coastal waters of both the east and west coasts of India. It readily accumulates heavy metals and shows various physiological and biochemical responses providing information on the general status of contamination in the coastal environment and health of the animal itself.

The aim of present investigations was to identify the seasonal variations in glutathione-mediated antioxidant and biotransformation enzymes as biomarkers in a local population of bivalve (*D. incarnatus*) from considerably less polluted regions of south India coast. The study period was one year (2021-2022). In order to analyse the principal site of xenobiotic and oxy-radical producing biotransformation enzymes, the hepatopancreas, gill and the mantles were chosen. Activities of GST as a marker for biotransformation enzyme, GSH as a substrate conjugation and GPx as an antioxidant are marker to analyse the impact of seasonal variation in the bioaccumulations of heavy metals on this bivalve. Therefore the current study also determines the bioaccumulation of the heavy metals in *D. incarnatus* whole body tissues.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Specimens of the bivalves *D. incarnatus* were collected from the shore of Muttu kadu backwater and Pulicat lake of east coast of India (Fig. 1). Samples of 20 animals were collected seasonally over one year from May 2021 to April' 2022. Individuals of intermediate size (fully mature) were selected for biochemical and antioxidant analysis. Following collection, the bivalves were left in filtered sea water for three days to allow any sediment to be cleared from the mantle cavity and the gut. The mantle, gills and hepatopancreas of clam was carefully separated using sterile scissors, and put in a sterile tube that was kept at -20°C for future use. Mantles, gills and hepatopancreas tissue samples were homogenised (1:10 w/v) in a pre-chilled 100 mM sodium phosphate buffer, pH 7.0, including 0.5 mM EDTA and a few crystals of phenyl methylsulfonyl fluoride, a protease inhibitor. Centrifuging was done on tissue homogenates at a speed of 10,000 g for 30 minutes at 4°C. The supernatants were collected, aliquoted into different tubes for biomarker analyses, and kept at -20°C until it is used further. Protein estimation in all the tissues was detected following the method of Bradford et al. [7].

2.2 Determination of Antioxidant

The method of Moron et al. [8] was used to determine the glutathione content. By measuring the optical density of the yellow material produced at 412 nm when glutathione reduces 5, 5'-dithio-2-nitrobenzoic acid (DTNB) and the values were expressed as unit per mg protein. The Rotruck et al. [9] method was used to assess glutathione peroxidase (GPx) activity, which required measuring the quantity of reduced glutathione (GSH) consumed in the reaction mixture and enzyme kinetic was used to measure the absorbance at 340 nm (Every 15 seconds). The number of spectrophotometer measurements was six, and the time interval was 15 seconds the values were expressed as unit per mg protein. GST activity of the fraction obtained with the substrate 1-chloro-2,4-dinitrobenzene (CDNB) was measured spectrophotometrically at 37^{o}C by following conjugation of the acceptor substrate with glutathione as described by Habig et al. [10]. Results were expressed as the formed conjugate/min/mg protein.

2.3 Bioaccumulation of Trace Metals in Tissues

For heavy metal analysis in soft tissues of the bivalve, the stored samples were thawed and rinsed with deionized water. Each sample was dried to constant weight in an oven at 95°C and then ground to a fine powder. The 100 µm mesh-sized powder was used for estimation of heavy metals. Briefly, the samples were digested in concentrated nitric acid:perchloric acid (2:1) by placing them in a hot block digester, first at a low temperature (40°C) for one hour and then they were fully digested at a high temperature (180°C) for at least 3 hours. The digested samples were then diluted to a certain volume with double distilled water. After filtration, the digested samples were examined for heavy metals using a Spectra AA-10 Varian atomic absorption spectrophotometer (AAS). Quality control of metal analysis was performed using digestion blanks and reference material (IAEA shrimp MA-A-3/TM and IAEA simulated freshwater W-4). The measured values were in good agreement with the certified values (< 10% deviation).

Mercury: Concentrations of Hg were determined by use of automated cold vapor atomic absorption spectrophotometer (AAS: Spectra AA-10 Varian), according to the methods of Weltz and Schubert-Jacobs (1991). Prior to use of the instrument for measuring the actual concentration of the mercury, the quality control of metal analysis was performed by use of digestion blanks and reference material (Mussel, IAEA-142). All glassware and equipment used were acid washed to avoid contamination and to check for contamination, procedural blanks were analyzed once for every three samples.

2.4 Statistical Analysis

Analysis for obtained results was carried out with the aid of the SPSS computer software programme. Data for antioxidant variables were expressed as mean ± S.E. To evaluate seasonal variations in antioxidant response in *Donax incarnatus* throughout a study year, analysis of variance (One-way ANOVA test, $p < 0.05$) was performed. The differences in body composition between the seasons were also evaluated by comparing the data obtained using Student 't'test.

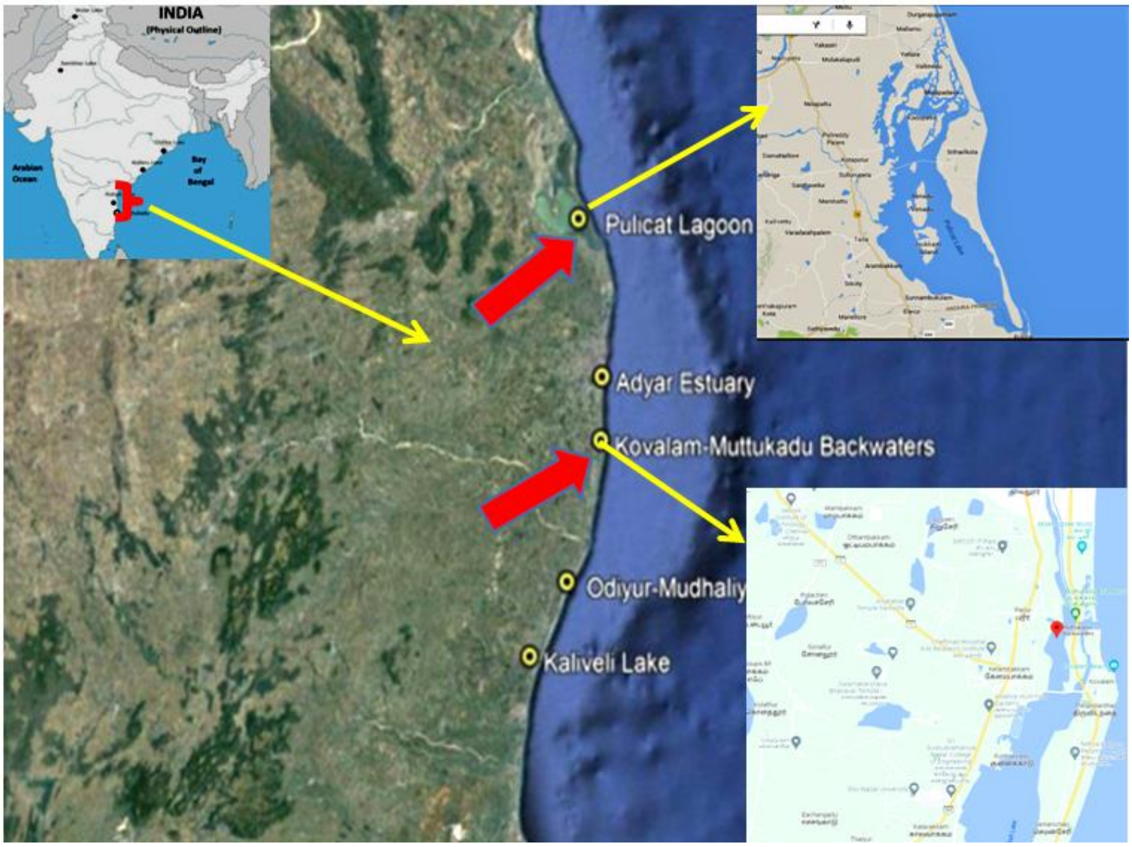

Fig. 1. Map representing two sampling stations along the two South Indian coastal regions where the bivalve molluscs collected

3. RESULTS

3.1 Reduced Glutathione Content

The GSH levels in the mantles gill and hepatopancreas of the *D. incarnatus* collected from Muttu kadu and Pulicat coastal region during all the season shown in Fig. 2A-C. Among the season the GSH contents in the bivalve was comparatively greater during post monsoon period. Between the stations studied bivalve collected at Pulicat coastal region shows higher GSH content. Further among the organs tested gill showed greater GSH than hepatopancreas and mantle (Fig. 2A-C). Clam collected during premonsoon and monsoon period shows a similar GSH level in mantles and such level doesn't show any significant differences. However the samples collected during summer showed significant difference in GSH level in all the organs tested (Fig. 2A-C).

3.2 Glutathione Peroxidise (GPx) Activity

The GPx activity in mantles, gill and hepatopancreas for the bivalve collected from both Muttu kadu and Pulicat coastal region during different season was shown in the Fig. 3A-C. GPx activity in the mantle of bivalve collected at Pulicat showed no significant difference between the four seasons. On contrary the GPx activity in the mantle of the bivalve collected from Muttu kadu showed significant differences between different seasons (Fig. 3A). The highest GPx activity was recorded during the summer followed by the pre-monsoon period (Fig. 3A). GPx activity in the gill and hepatopancreas of the bivalve collected from both Muttu kadu and Pulicat exhibit similar trend, showing greater GPx activity during summer and pre-monsoon periods. The lowest GPx activity recorded in both gill and hepatopancreas in the samples collected during post monsoon season (Fig. 3B-C). Between the stations studied bivalve collected at Muttu kadu coastal region shows greater GPx activities (Fig. 3A-C).

3.3 Glutathione s Transferase (GST) Activity

The GST activity in mantles of the *D. incarnatus* collected from Muttu kadu during summer was greater among all the season. Similarly bivalve collected from the Pulicat coastal region found to be higher among the samples collected during four seasons. Among the organ studied hepatopancreas of the bivalve collected from both Muttu kadu and Pulicat shows greater induction of GST activity (Fig. 4A-C). The gill of *D. incarnatus* collected during post monsoon at Pulicat showed comparatively higher GST activity than Muttu kadu samples (Fig. 4B). The GST activity in all the organ tested shows lesser GST activity during post monsoon season (Fig. 4A-C).

3.4 Bioaccumulation of Heavy Metals in Whole Tissues of *Donax incarnatus*

Figs. 5 and 6 shows accumulation of heavy metals (Cu, Fe, Pb,Co, Cd, Cr, Zn, Hg and Ni) in the whole body tissue of *D. incarnatus* collected from Muttu kadu and Pulicat coastal regions during different seasons. As indicated the levels of metals in whole tissues were generally high during post monsoon and pre-monsoon when compared to monsoon and summer. Among the metals, Iron appeared to be the most accumulated followed by copper and zinc. Not surprisingly Pulicat coastal region showed less bioaccumulation in whole body tissues of *D. incarnatus*. Meanwhile, the results recorded for the Glutathione mediated antioxidant components (GSH and GST) in *D. incarnatus* showed significant variations between seasons at the study year (ANOVA test, $p < 0.05$), with the highest variations of metals accumulation were noticed during post monsoon period and summer.

4. DISCUSSION

"Marine ecosystems are endangered by chemical contaminants associated with urbanization. The biota may be stressed by discharges from specific point sources such as sewage effluents and industrial wastes and from general non-point pollution such as harbour activities, storm drainage, agriculture drainage etc" [11]. "Previous works have shown that heavy metals introduced by domestic, industrial and mining activities can be incorporated into marine sediments by precipitation, flocculation, adsorption and absorption" [12] and "that *D. incarnatus* living in such sediments accumulate, heavy metals to varying degrees" [13]. "Under certain conditions, bivalve *D. incarnatus* are able to absorb metals from ingested sediment as well as from solution in the surrounding water" [14].

"Although published information on metal concentrations in organisms collected from the environment is increasing rapidly, the lack of attention to sampling techniques may invalidate many of the conclusions concerning the relative abundance of metals in different areas. Much attention is correctly paid to inter laboratory comparisons of analytical techniques in order to maximize the analytical accuracy of the results, but unfortunately no such attention is given to biological variables affecting the organism" [15].

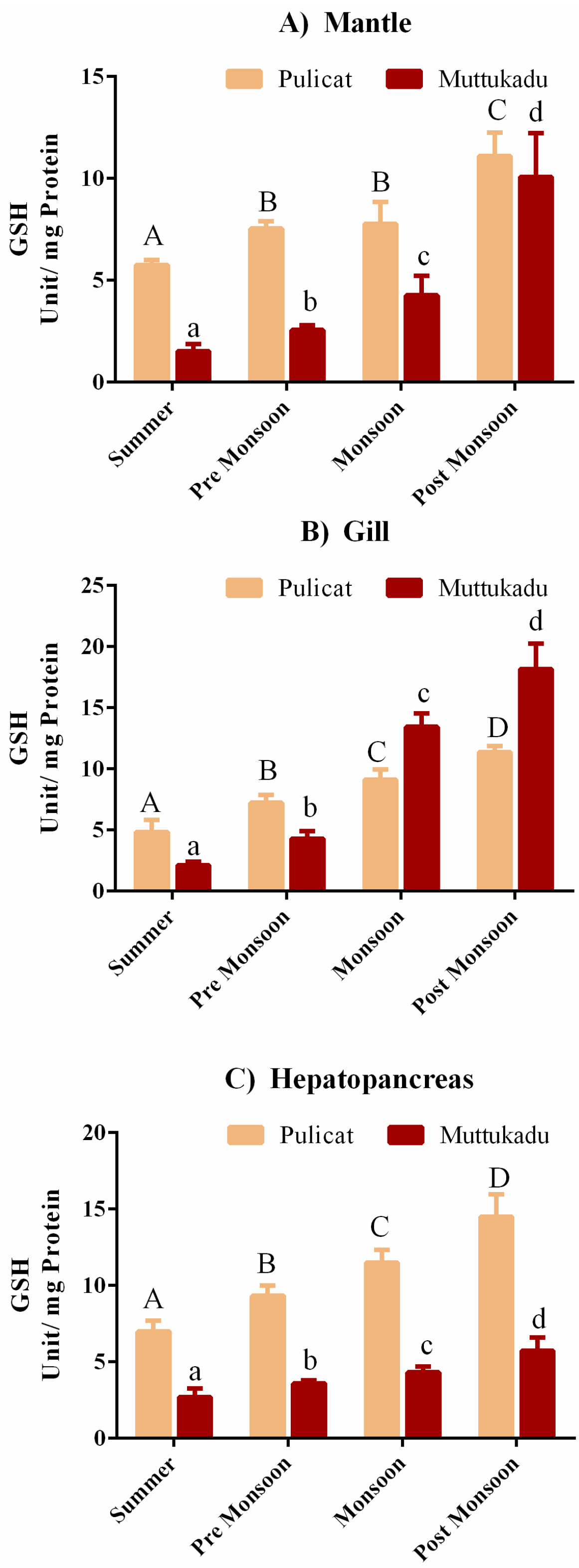

Fig. 2. Reduced glutathione level in different tissues of bivalves collected from Pulicat and Muttukadu coastal regions. Each bar represents mean±standard error of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a, b, c, d) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

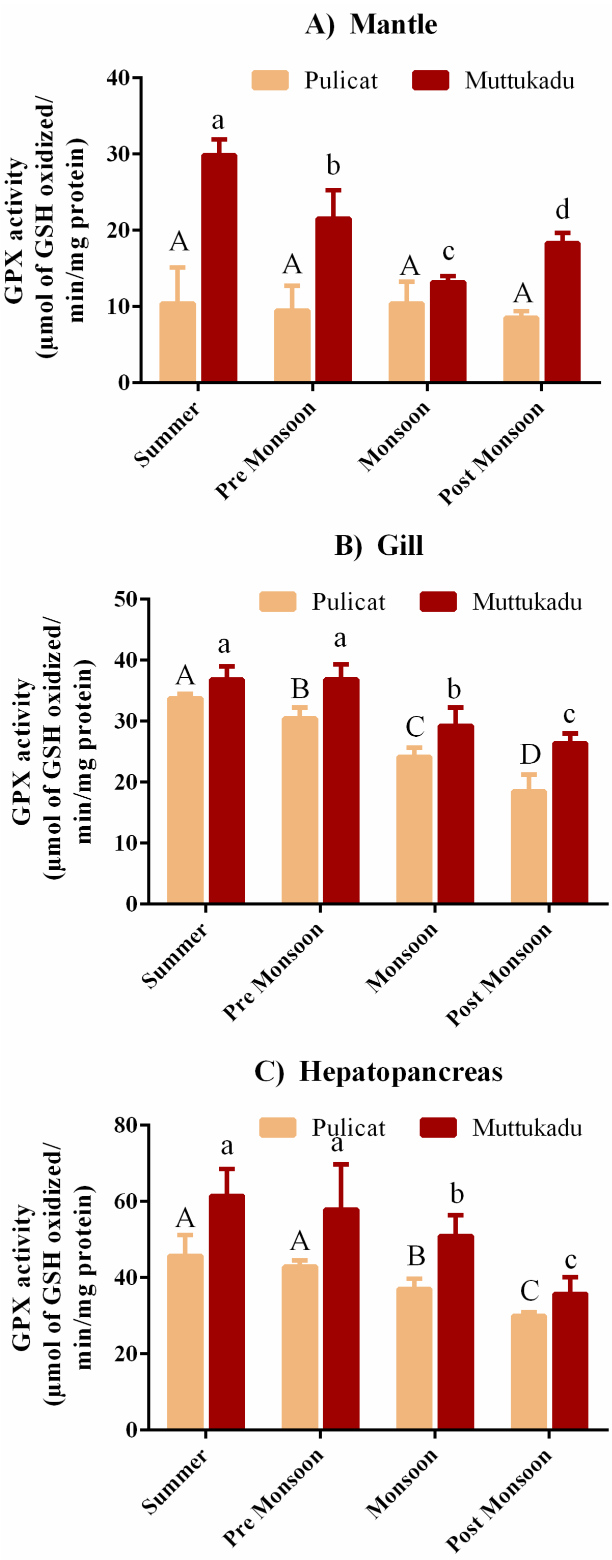

Fig. 3. Glutathione peroxidase activities in different tissues of bivalves collected from Pulicat and Muttukadu coastal regions. Each bar represents mean±standard error of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a, b, c,d) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences (*p* < 0.05) between different groups

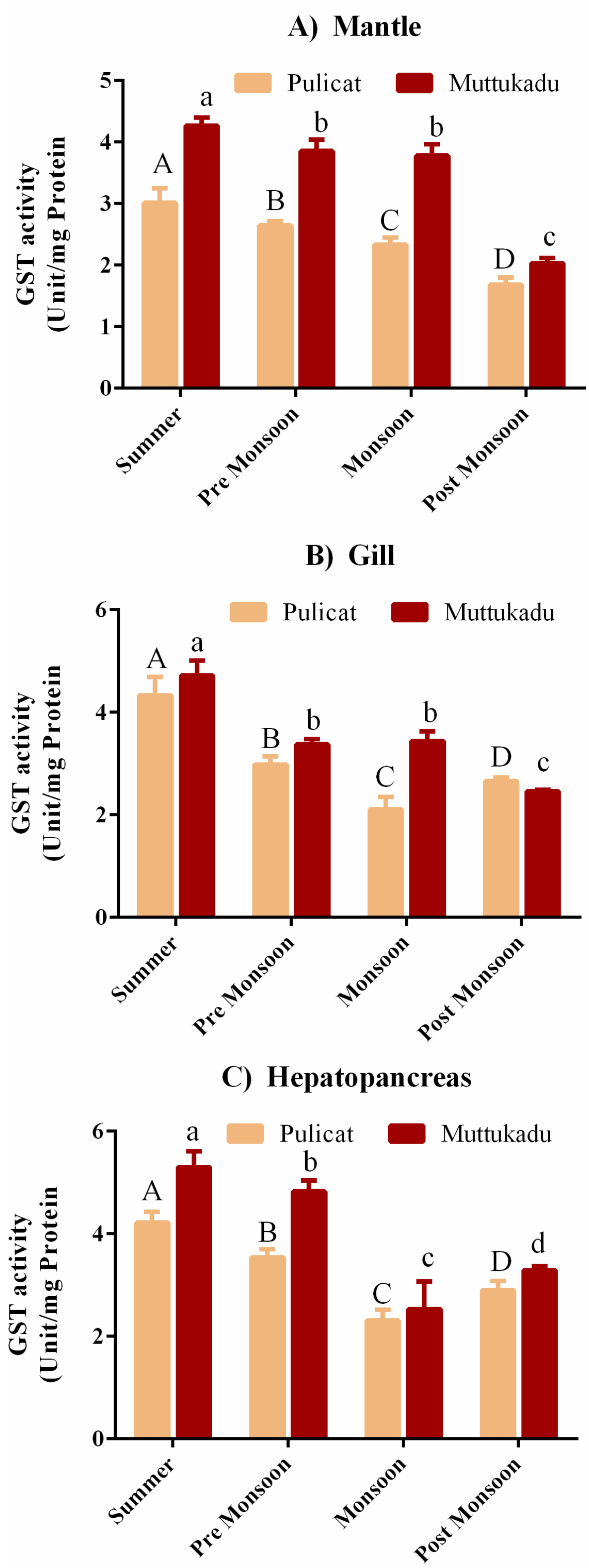

Fig. 4. Glutathione *S* transferase activities in different tissues of bivalves collected from Pulicat and Muttukadu coastal regions. Each bar represents mean± standard error of six determinations using samples from different preparations. Two-way analysis of variance followed by Tukey's post hoc test was used. The same letters (a, b, c,d) indicate no significant difference between the exposure groups whereas different letters indicate statistically significant differences ($p < 0.05$) between different groups

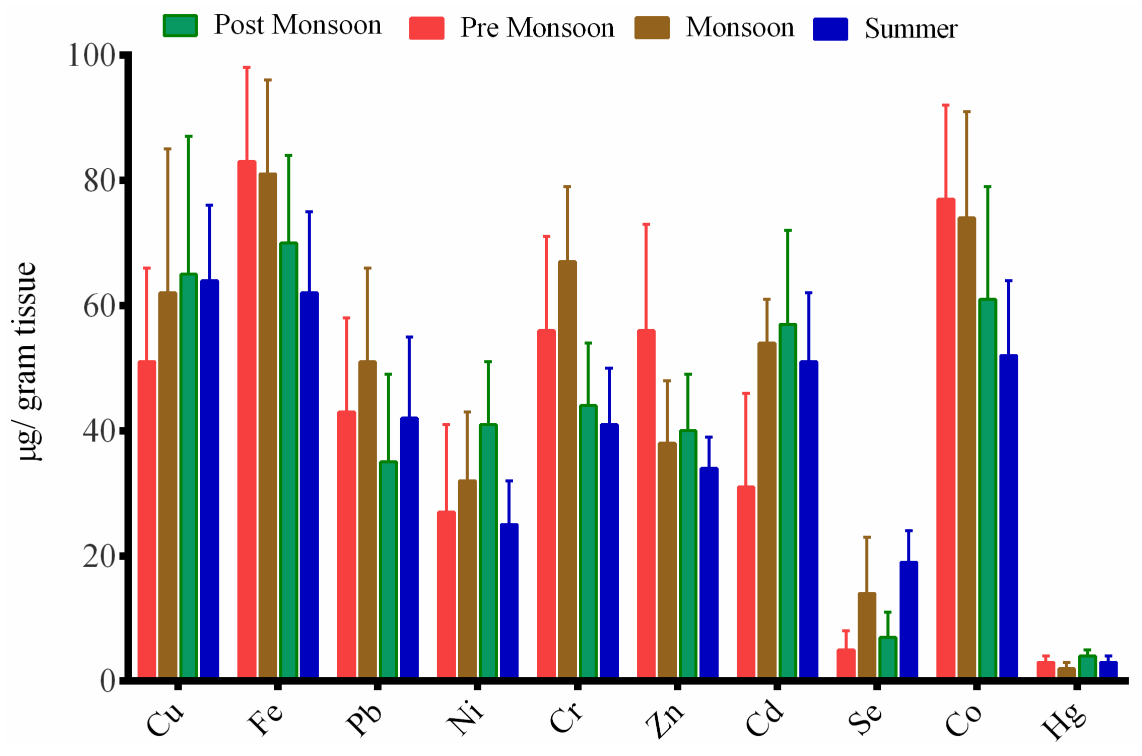

Fig. 5. Bioaccumulation of heavy metals in bivalve collected from Pulicat coastal region during different seasons

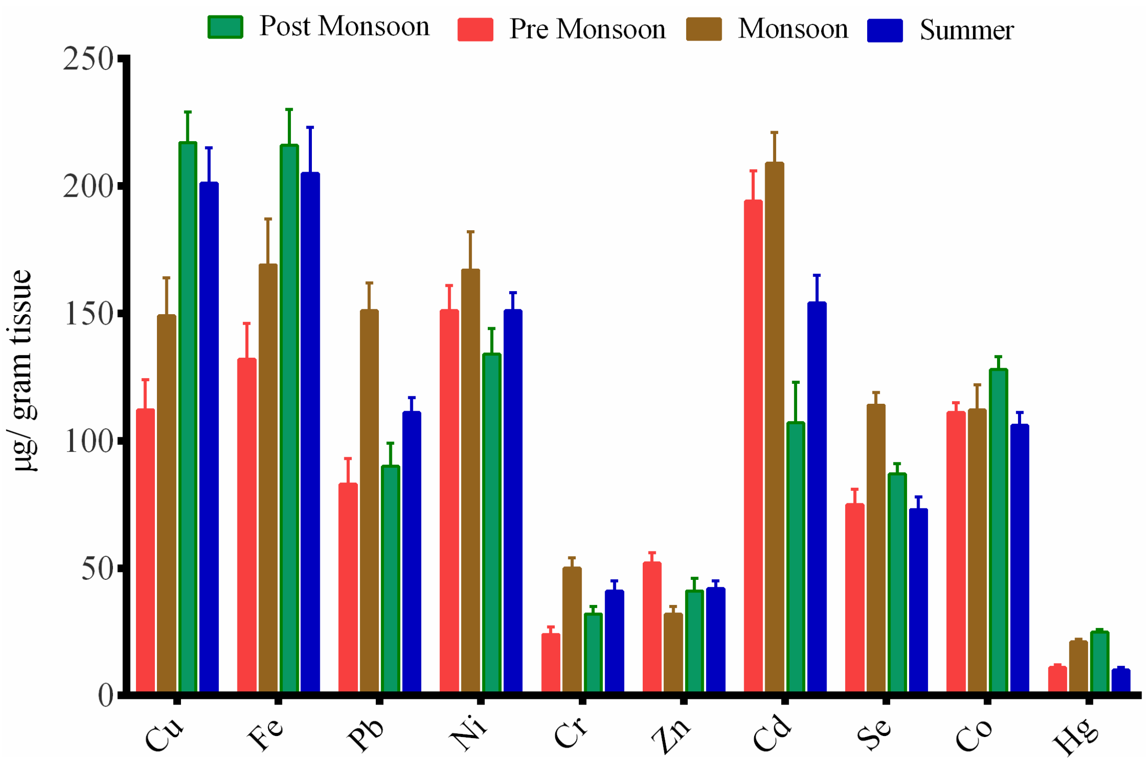

Fig. 6. Bioaccumulation of heavy metals in bivalve collected from Muttu kadu coastal region during different seasons

The effect of season on heavy metal concentrations in *D. incarnatus* showed seasonal patterns with high values recorded during monsoon. However, based on the fact that seasonal trends in Muttu kadu and Pulicat suggests that seasonal changes in heavy metal concentrations in *D. incarnatus* is probably more a result of changes in animal physiology than changes in metal exposure conditions. Improved water quality conditions especially in the coastal region of Pulicate in recent years and resultant remobilization of metals from bottom sediments puts more metals in the water column. Variations in the ratio relating to concentration of metals between suspended particles and dissolved phase has been suggested to be critical [16]. Heavy metal partitioning between particles and dissolved phases can be important for filter feeders such as molluscs and it has been demonstrated that, the relative contribution to total metal body burden from ingested particles increases directly with increasing partitioning coefficient between particles and dissolved metals [17]. It is to be noted that all the glutathione mediated antioxidant enzymes studies failed to show an induction and their levels were drastically reduced in *D. incarnatus* at every season. By contrast numerous studies have reported induction in antioxidant levels as a result of oxidative stress [18].

Earlier studies on biochemical composition of bivalve molluscs defined certain characteristic features that considered of importance in understanding the economic importance of bivalves as food for human. They are: (i) protein represents major organic constituent of bivalves body, compared to the other components, lipids and glycogen [19]. (ii) Bivalve molluscs lack the specific organs for nutrient storage of higher vertebrates and thus body constituents of bivalves represent energy reserves that are changed seasonally, showing cyclic synthesis and utilization [20].

Bivalve molluscs have a variety of antioxidant defence mechanisms, including low molecular weight substances like reduced glutathione (GSH) and enzymes that have been specially adapted [21]. Among these enzymes, GST which catalyses conjugation reactions with GSH and is anticipated to react to modifications in the level of pollution in marine water [22]. The effect of seasonality and site pollution status on the antioxidant components (GSH and GST) in the bivalves *D. incarnatus* was investigated in the current study. The antioxidants that were measured in this study showed a clear seasonal variation, with winter showing very low values while the rest of the year, especially summer, showed elevations in the same parameters.

GST plays an important role in the conjugation of electrophilic compounds with glutathione, and these reactions are of vital importance in the detoxification of heavy metals. GSH offers protection against reactive intermediates, including free radicals and electrophiles [23]. Supportively Lu et al. [24] have suggested that xenobiotic might inhibit antioxidant enzymes. This may explain why no significant positive relationships have been observed between antioxidant enzymes and metal levels.

Some research has looked into the effects of xenobiotic exposure on antioxidant enzyme activity in marine invertebrates [25,26]. These investigations found that antioxidant enzymes can rise in low toxicant concentrations, but can decline or even be inhibited when time is extended or dosage is increased. In our research, we discovered both the tissues, the glutathione mediated antioxidant enzyme activity of *D. incarnatus* studied modulated, indicating an increase and decrease in the formation of oxygen free radicals (O_2^- and H_2O_2) occurred and whether this increase in free radicals by xenobiotics components may leads to affect the physiology of the bivalve needs to elucidate further.

Together with earlier research these data demonstrated a relationship between seasonality and the antioxidant component. The explanation is that in the summer, when it is hotter and there is more food available, the body uses more oxygen and produces more cellular oxygen radicals, which are countered by an increase in antioxidant defences. As a result, the wintertime decline in antioxidant components may point to increased susceptibility of bivalves to oxidative stress [27,28]. The level of aquatic metal pollution, which was shown in the current study by the increased antioxidants (GSH and GST) in *D. incarnatus* collected from Muttu kadu, is the other factor that needs to be studied in relation to the antioxidant defence system.

These results are consistent with those of Dahl et al., [29] who found that sustained exposure to temperature increased the GSH level in the hard clam *Mercenaria mercenaria*. In addition, Le Pennec and Le Pennec [30] showed that the bivalve *Pecten maximus* can increase GST activity in response to exposure to pollution in order to shield cells and the organism from harm. Bioaccumulation of heavy metals in the bivalves' soft tissues *D. semistriatus* and *D. trunculus*. Analyzing water, sediment, and marine organisms can be used to estimate the level of heavy metal pollution in the aquatic environment. The levels of heavy metals in molluscs and other invertebrates are frequently significantly higher than in other marine environment constituents, indicating the capacity of bivalves to bioaccumulate heavy metals.

In this regard, it has been reported that a variety of environmental factors, such as seasonal variations, may have an impact on the degree of metal bioaccumulation in bivalve tissues [31]. This latter finding can be clearly demonstrated by the current study in that the quantified metal levels (Fe, Cu, Pb, and Zn) in the bivalves' entire soft tissues tended to vary from season to season, with the highest values being recorded in the summer and the lowest ones in the winter.

Since there was little evidence of a seasonal pattern in the metal variations in the surface water and sediment in the current study, it is unlikely that the quantified seasonal variations in the metals in the bivalve's tissues are due to changes in the amount of metals reaching the bivalve's environment. Since the peak of the phytoplankton abundance occurred during the summer, this is primarily explained by the fact that increased tissue metal concentrations occurred during this time. As a result, one important factor influencing such seasonal variations may be the availability of food. The results of the current study showed that, in contrast to these seasonal effects, the bivalve tissues from Muttu kadu had higher concentrations of the quantified metals (Fe, Cu, Pb, and Zn) in their whole tissues compared to that of sample collected from the Pulicat coastal region.

This is primarily due to the possibility that the Muttu Kadu backwater could be exposed to higher heavy metal concentrations, particularly heavy metals, which are primarily caused by boat oil spills and any local industrial effluents [32]. The same explanation was established by other researchers, who showed that the concentration of heavy metals in the aquatic environment in which such molluscs are living directly correlated with the bioaccumulation of those metals in the bivalve tissues. Additional field research appeared to confirm the aforementioned findings and revealed that the order in which metals accumulate in bivalve tissues can change depending on the amount of metals present in the environment [33,34].

5. CONCLUSION

The current study clearly demonstrate that the fluctuations of glutathione mediated antioxidant enzymes was comparatively higher in the bivalve collected from Muttu kadu and hepatopancreas showed more variations. The bivalve collected from the Pulicat coastal region showed less metal accumulation in the tissues which could be the one of the reason for difference in the antioxidant system in bivalve collected from the two regions. The information obtained showed that the glutathione-mediated antioxidant activity in *D. incarnatus* varied with the seasons, with summer recording the highest levels and winter recording the lowest. Therefore, the quality of bivalve molluscs consumed by humans as sea food can be largely determined by the season and the level of environmental pollution.

ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s)

DECLARATION

For the care and use of animals, all applicable international, national, and/or institutional guidelines were followed.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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