

Changes in Oxidative Stress and Antioxidant Status in Stressed Fish Brain

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Abstract: Toxic pollutants entering into aquatic environment exert their effect through altering redox cycling in fish. The antioxidant defence as well as oxidative damage is a common effect in fish exposed to xenobiotics in their water body. Brain requires high and constant oxygen supply to meet its energy needs and generates more free radicals per gram of tissue than does any other organ in the body. The present study evaluates the effects of environmental contaminants on stress biomarkers in brain tissue of *Mugil cephalus*. Effects of xenobiotics on the brain of *M. cephalus* is evaluated in terms of lipid peroxides (LPO), protein carbonyls (PC), nitrite (NO_2^-), 3-Nitrotyrosine (3NT), reduced glutathione (GSH) and total antioxidant capacity (TAC). There was a significant increase in the level of LPO (2 fold), PC (27%), NO_2^- (1 fold), 3-NT ($p < 0.001$) along with significant decrease of GSH (59%) and TAC (75%) in brain of *M. cephalus* inhabiting Ennore estuary than Kovalam estuary. In conclusion, reduction in the level of TAC and GSH indicate that pollutants in water body exert their effect through oxidative/nitrative stress. The damaged products through stress can persist in the organism leading to changes at molecular level in fish which may ultimately affect their survival.

Keywords: Antioxidant, Brain, *Mugil cephalus* (*M.cephalus*), Stress, Xenobiotics

1. Introduction

Aquatic environments are home to a vast diversity of organisms ranging from prokaryotes to higher vertebrates. But these environments also act as sinks for a great variety of anthropogenic contaminants, many of which are toxic (1). It is important to have an understanding of the impact and effects of these toxic chemicals on aquatic life forms such as fish (2). This knowledge apart from helping us in the management of aquatic ecosystems will also essentially help us to understand the fundamental mechanisms that allow the fish to survive in this polluted water.

Xenobiotics entering into the environment exert their effect on redox cycle by formation of reactive oxygen species with the ability to damage cellular molecules. These polluting compounds will often retain their qualities in the aquatic environment with the ability to cause oxidative stress in these organisms (3). The physiological systems of many aquatic organisms, which metabolize these compounds and resulting byproducts, are evolutionarily similar to humans (4). The present study focuses on the effects of xenobiotics on oxidative stress in brain tissue from *M. cephalus*. The grey mullet (*M. cephalus*) has several characteristics required in a sentinel species, such as wide geographic distribution; salinity and temperature tolerance; it is common in coastal waters and enters estuaries, harbors, and rivers that are frequently subjected to pollution. It is capable of concentrating contaminants and is considered suitable for biomarker studies (5). Changes in biomarkers of fish captured from stressed environments may represent a reliable tool in revealing sublethal effects of the pollutants found in aquatic ecosystems (6).

Brain is the master organ of all living organisms as it is the controlling centre for all other receptor and effector organs. Because of its structural complexity and functional diversity, brain performs a number of complex biological functions that are essential for survival. The blood-brain barrier (BBB) is a physical and metabolic barrier between the brain and the systemic circulation, which functions to protect the brain from circulating drugs, toxins, and xenobiotics (7). So the brain serves as an appropriate organ for the study of this type of pollutant effect due to its high metal accumulating capacity, susceptibility to histopathological damage by metals and morphological heterogeneity.

A disturbance in the balance between the prooxidants and antioxidants leading to detrimental biochemical and physiological effects is known as oxidative stress. This is a harmful condition in which increase in free radical production, and/or decreases in antioxidant levels can lead to potential damage of lipids, proteins and DNA (8). Thus changes in antioxidant defenses and oxidative damage are used as biomarkers of oxidative stress (9). Biomarkers enable integration of toxicant interactions in molecular or cellular targets resulting from exposure to complex mixtures of contaminants (10). The enhanced oxidative stress contributes to neuro degeneration (11). Oxidative and nitrative stress results from increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) mediated by pollutants (12).

The lipid peroxidation process affects biomolecules associated with the membrane such as membrane bound proteins or cholesterol, and may be of importance in fish as their membranes contain a higher degree of polyunsaturated fatty acid (PUFA) (13). The brain is more susceptible to free radical attack because of its high oxygen consumption rate,

low level of antioxidant defence system, high PUFA content (14). Malondialdehyde is well characterized oxidation product of PUFAs and thiobarbituric acid reactive substances (TBARS) method of quantifying lipid peroxides in sample measures this end product. The concentration of MDA is the direct evidence of lipid damage caused by free radicals (15).

Oxidation of proteins is of importance in regulating protein function within the cell. It can occur in several manners, where protein carbonylation is the most widely studied and accepted as a marker of oxidative stress. This is due to the fact that carbonylation is not transient, and proteolytic degradation of the protein takes place during these reactions. The formation of carbonyl derivatives is non-reversible and increases the susceptibility of proteins to proteases. Mild oxidation of soluble proteins enhances their susceptibility to proteolytic action while severely oxidized proteins may be stabilized due to aggregation, crosslinking and/or decreased solubility (16).

NO is involved in a wide range of both physiologic and pathologic events. NO can enhance ROS toxicity due to its rapid reaction to form peroxynitrite (ONOO⁻) by the reaction of superoxide with nitric oxide (NO) or by reaction of nitrite and hydrogen peroxide (17). The effect of elevated peroxynitrite and reduced bioavailability of NO, results from enhanced production of free radicals. Peroxynitrite is a potentially harmful reactive oxygen species (ROS) as it exerts cytotoxicity by reacting readily with phenolic and aromatic compounds such as tyrosine to form nitrated product 3-nitrotyrosine (3-NT). Nitrosylation of tyrosine residues, lead to changes in protein conformation and its inactivation (18). The formation of free or protein associated 3-NT serves as a potential biomarker and direct evidence for the generation of reactive nitrogen intermediates *in-vivo* (19).

Reduced glutathione (GSH) is one of the important thiol molecules protecting cells from toxins such as free radicals (20). GSH is the major non-protein thiol in animals, comprising up to 90% of the intracellular non-protein thiol content. It is reported that the levels of glutathione (GSH & GSSG) and glutathione redox ratio (GRR) can be used as an indicator of thiol status during oxidative stress in fish (21). The present study investigates the oxidative stress status and associated antioxidant imbalance in the brain tissue of *M. cephalus* collected from Kovalam (unpolluted site) and Ennore (polluted site) estuaries.

2. Materials and Methods

2.1 Study Site

Two estuaries were chosen as the experimental sites for the present study. Kovalam estuary (12°47'16 N, 80°14'58 E) is situated on the east coast of India and is about 35 km south of Chennai. It runs parallel to the sea coast and extends to a distance of 20 km. It was chosen as the unpolluted site for the present investigation as it is surrounded by high vegetation and it is free from industrial or urban pollution. Ennore estuary (13°14'51 N, 80°19'31 E) also situated on the east coast of India, is about 15 km north of Chennai. It runs parallel to the sea coast and extends over a distance of 36

km. This estuary was chosen as the polluted site as in its immediate coastal neighborhood are situated, a number of industries which include petrochemicals, fertilizers, pesticides, oil refineries, rubber factory and thermal power stations that discharge their effluents directly into this estuary.

2.2 Study animal and sampling

M. cephalus (grey mullet), a natural inhabitant of the estuaries, identified by the use of Food and Agriculture Organization (FAO) species identification sheets (22) was chosen as the experimental animal for the study. Grey mullets with an average length of 30 cm were collected from both Kovalam (n=20) and Ennore (n=20) estuaries using baited minnow traps. Collected fish were placed immediately in insulated containers filled with aerated estuarine water at ambient temperature and salinity. Fish were maintained in the above specified conditions for 4-5 hrs until the start of the experimental procedure for the isolation of brain.

2.3 Protein Preparation

Protein concentration was determined by the classical method (23) with coomassie brilliant blue G-250, using bovine serum albumin as a standard.

2.4 Estimation of lipid peroxides (LPO)

Lipid peroxide content of brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries was analyzed as described by Zhang et al., (24) and measured in terms of malondialdehyde (MDA) equivalents using the thiobarbituric acid (TBA) reaction. The results were expressed in terms of nanomoles/mg protein.

2.5 Estimation of protein carbonyls (PC)

Protein carbonyls content of brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries was estimated according to the method of Baltacioglu et al., (25). The results were expressed in terms of nanomoles/mg protein.

3. Estimation of nitrite (NO₂-)

Estimation of nitric oxide in terms of nitrite was based on the method of Yokoi et al., (26). The results were expressed as nmol/mg protein

3.1 Estimation of reduced glutathione (GSH)

The reduced glutathione (GSH) content of brain homogenate of *M. cephalus* was measured at 412 nm using 5, 5' dithiobis-(2-nitrobenzoic acid) (DTNB) reagent by the method of Moron et al., (27). The result was expressed as micromoles of reduced glutathione formed/min/mg protein.

3.2 Estimation of total antioxidant capacity (TAC)

Total antioxidant capacity of brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries was evaluated by the method described by Prieto et al., (28). The

total antioxidant activity was expressed as Trolox equivalent in mmol /L.

3.3 Immunohistochemical analysis of 3-nitrotyrosine (3-NT) expressions

Immunohistochemical analysis of 3-nitrotyrosine (3-NT) expressions in fish brain was performed by the method of Sternberg et al., (29). Formalin-fixed, paraffin embedded fish brain was processed using an immunohistochemical technique with rabbit polyclonal anti-3-NT (ALX-804-505-C050) antibody. Deparaffinized and rehydrated sections were incubated in 3% hydrogen peroxide (H₂O₂) in absolute methanol for 5 minutes in order to inhibit endogenous peroxidase activity and then rinsed in 0.05 M tris-buffered saline (TBS), pH 7.6, for 5 minutes. Antigen retrieval was performed by heat treating sections in citrate buffer at pH 6 in a microwave oven for 5 minutes (3 cycles). To reduce non-specific binding, slides were incubated in 10% normal goat serum for 10 minutes at room temperature before 1 hour incubation with anti-3-NT (1:2000) antibody in a humidified chamber at 4°C. After rinsing with TBS, biotinylated secondary link antibodies and streptavidin-peroxidase conjugate were added sequentially. The specimen was incubated at room temperature for 30 minutes. Peroxidase activity was detected using 0.1% H₂O₂ in 3, 3'-diaminobenzidine (DAB) solution applied to the tissue sections for 5 minutes, which were then counterstained with hematoxylin for 5 seconds before rinsing, dehydrating and mounting with cover slips using xylene and DPX mountant. The immunohistochemical images were acquired with Olympus (MLXiTR, Olympus). The intensity of the expression was assessed using the Magnus Pro software (CH20i).

3.4 Statistical significance

Data were analyzed using statistical software package version 7.0. Student's t-test was used to ascertain the significance of variations between unpolluted and polluted fish brain homogenate. All data were presented as mean \pm SD of 20 samples. Differences were considered significant at $p < 0.01$ and $p < 0.001$.

4. Results

4.1 Lipid peroxides

The level of lipid peroxide was evaluated in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries (Figure 1). There was a significant increase in the level of LPO ($p < 0.001$) in brain homogenate of *M. cephalus* inhabiting Ennore estuary (2 fold) when compared with brain homogenate of *M. cephalus* inhabiting Kovalam estuary.

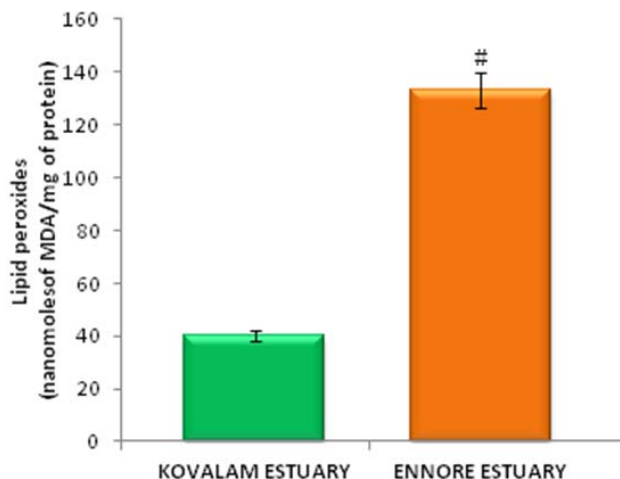


Figure 1: Level of lipid peroxides in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries.

Values are expressed as mean \pm SD (n=20 fish per estuary)
[#] $p < 0.001$ When compared with brain homogenate of *M. cephalus* inhabiting Kovalam estuary

4.2 Protein carbonyls

The level of protein carbonyls content was evaluated in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries (Figure 2). There was a significant increase in the level of PC ($p < 0.01$) in brain homogenate of *M. cephalus* inhabiting Ennore estuary (27%) when compared with brain homogenate of *M. cephalus* inhabiting Kovalam estuary.

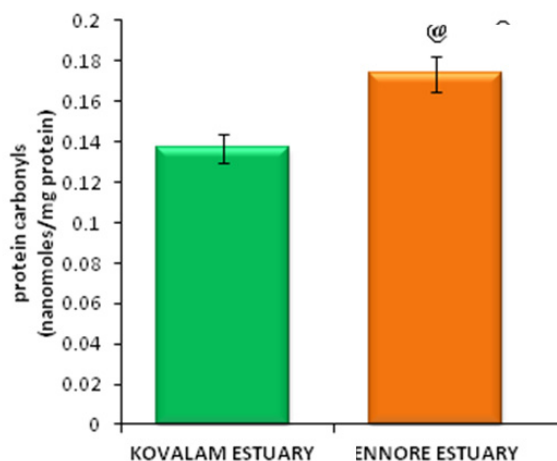


Figure 2: Level of protein carbonyls in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries.

Values are expressed as mean \pm SD (n=20 fish per estuary)
[@] $p < 0.01$ When compared with brain homogenate of *M. cephalus* inhabiting Kovalam estuary

4.3 Nitrite

The level of nitrite was evaluated in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries (Figure 3). There was a significant increase in the level of nitrite ($p < 0.001$) in brain homogenate of *M. cephalus* inhabiting Ennore estuary (1 fold) when compared with brain homogenate of *M. cephalus* inhabiting Kovalam estuary.

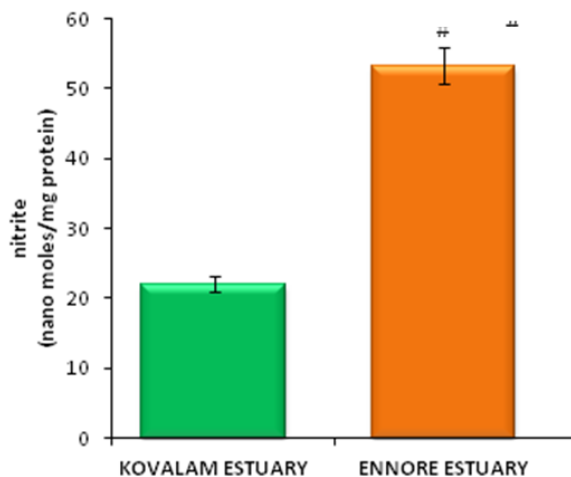


Figure 3: Level of nitrite in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries.

Values are expressed as mean \pm SD (n=20 fish per estuary)
[#]p<0.001 When compared with brain homogenate of *M. cephalus* inhabiting Kovalam estuary

4.4 3-Nitrotyrosine (3-NT)

The immunohistochemical results of 3-NT was evaluated in brain tissue of *M. cephalus* inhabiting Kovalam and Ennore estuaries (**Figure 4**). There was a change in the expression of 3-NT in brain tissue of *M. cephalus* inhabiting Ennore estuary when compared with brain tissue of *M. cephalus* inhabiting Kovalam estuary.

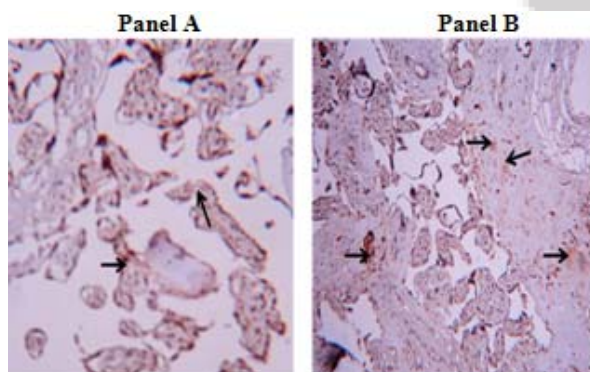


Figure 4: Immunohistochemical analysis of 3-Nitrotyrosine in brain tissue of *M. cephalus* inhabiting Kovalam and Ennore estuaries

Panel A- brain tissue of *M. cephalus* inhabiting Kovalam estuary; Panel B- brain tissue of *M. cephalus* inhabiting Ennore estuary

Arrow heads indicates the expression of 3-Nitrotyrosine. Brain tissue of *M. cephalus* inhabiting Ennore estuary shows more intense staining for 3-NT indicating an increased nitrate stress created by pollutants

5. Reduced glutathione (GSH)

The level of GSH was evaluated in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries (**Figure 5**). A significant decrease in the level of GSH (p<0.001) was observed in brain homogenate of *M. cephalus* inhabiting Ennore estuary (59%) when compared with brain homogenate of *M. cephalus* inhabiting Kovalam estuary.

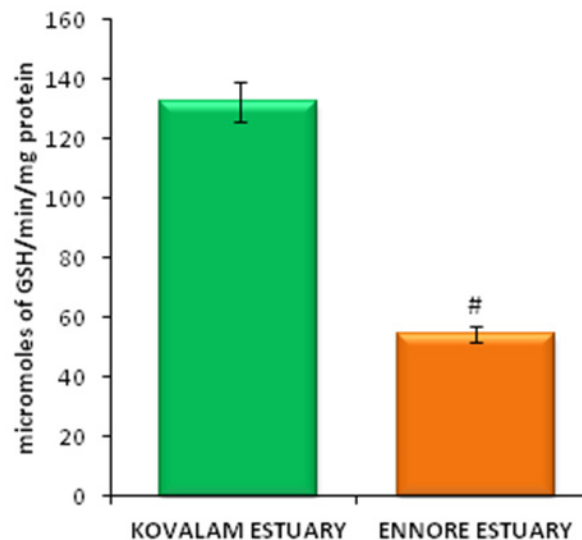


Figure 5: Level of reduced glutathione in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries

Values are expressed as mean \pm SD (n=20 fish per estuary)
[#]p<0.001 When compared with brain homogenate of *M. cephalus* inhabiting Kovalam estuary

5.1. Total antioxidant capacity

The level of total antioxidant capacity was evaluated in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries (**Figure 6**). A significant decrease in the level of TAC (p<0.001) was observed in brain homogenate of *M. cephalus* inhabiting Ennore estuary (75%) when compared with brain homogenate of *M. cephalus* of inhabiting Kovalam estuary.

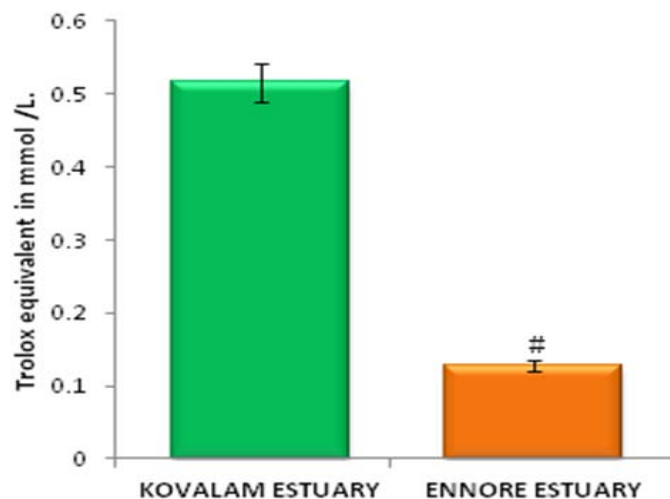


Figure 6: Level of total antioxidant capacity in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries.

Values are expressed as mean \pm SD (n=20 fish per estuary)
[#]p<0.001 When compared with brain homogenate of *M. cephalus* inhabiting Kovalam estuary

6. Discussion

Estuaries are highly sensitive zones subject to pressure from port, industrial, urban and tourist activities. Estuary

contamination with dangerous effect on ecosystems (30) is due to the heavy industrialization and over population along the coastal areas (8). The studies on oxidative stress in fish inhabiting polluted environment have demonstrated significant pollution impact on various organs of fish like gill (31), liver (5) and erythrocytes (32). Accumulation of damaged and oxidized macromolecules like lipid, proteins and DNA in various organs would lead to decrease in reproduction rate, susceptibility to quick infection and sudden death of fish in large numbers (8).

Oxidative stress occur when the production of reactive oxygen species (ROS) overwhelms the endogenous protection afforded by antioxidant enzymes like catalase, superoxide dismutase, glutathione S-transferase and redox sensitive thiol compound, reduced glutathione. Oxygen is an essential element for aerobic metabolism, since it is the terminal acceptor of electrons in oxidative phosphorylation. However, during exposure to pollutants especially heavy metals (33), electron flow may become uncoupled, leading to the production of reactive oxygen species (ROS). Reactive oxygen species, superoxide anion (O_2^-), hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2) can elicit widespread damage to cells, such as lipid peroxidation of polyunsaturated membrane lipids. Lipid peroxidation is a free radical-mediated chain reaction, since it is self-perpetuating. The length of the propagation depends on the chain breaking antioxidant.

The use of pollution biomarkers has been the subject of several studies, especially due to the fact that distinct kinds of pollutants may interfere with animal physiology and behavioral processes, which in turn is of ecological importance (34). Several studies show that toxic agents may affect behavioral parameters (35). Behavioral changes are good indicators of damage to the central nervous system, as a consequence of exposure to toxic agents (36). Toxic effects of cadmium in the brain and nervous system may be associated with aggressive behavior in fish species (37).

In fish, oxidative stress has been documented in both field and laboratory exposure studies. Environmental contaminants present in complex mixtures in sewage treatment effluent and industrial harbor areas contain compounds capable of inducing oxidative stress in exposed fish. This can be manifested in the form of upregulation of antioxidant enzymes as well as increases in oxidative damage, including protein carbonyls, TBARS and DNA damage. Exposures to these various environmental toxicants can often result in cancer, not only in humans but in fish as well (38). However, the relationship between oxidative stress and the pathology of diseases is still unclear in many cases.

The use of TBARS as a biomarker in fish studies is better established than the use of protein carbonylation. Lipid peroxidation can occur both as a result of xenobiotic-related effects and as a result of other cellular damage injuries. Measurements of lipid peroxidation are considered to be of great importance in environmental risk assessment (39). Lipid oxidation products are also essential to address here, as they can affect transcription of antioxidant enzymes. Lipid oxidation is the result of the action of free radicals on lipids that contain PUFA and OS situation is characterized

by increased lipid peroxide formation (40). Lipid peroxides could change the properties of biological membranes, resulting in eventual cell damage (41). Lipid constitutes a major part of brain and plays a main role in membrane integrity. Since polluted fish are subjected to oxidative stress created by pollutants, the lipid constituent is more vulnerable to free radical damage. Hence the level of lipid peroxide was evaluated in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries (**Figure 1**). This indicates the susceptibility of lipid molecules to pollutant induced ROS and the extent of oxidative damage imposed on these molecules. Elevation of MDA concentration is due to the increased peroxidation of lipid membranes and is an indicator of OS (42).

Several other studies have measured protein carbonylation in fish species as protein carbonylation could provide a useful biomarker of oxidative stress resulting from xenobiotic exposure (43). An increase in protein carbonyl levels could indicate that normal protein metabolism is disrupted, resulting in accumulation of damaged molecules. Use of this oxidative damage product is already well established in other species, including humans, especially as a marker of disease pathologies (44). Protein is the most fundamental and abundant constituent present in fish. Increased proteolytic activity to overcome the impeding energy demands created due to pollution induced stress is indicated by the significant increase in the level of PC ($p < 0.001$) in brain homogenate of *M. cephalus* inhabiting Ennore estuary (27%) when compared with brain homogenate of *M. cephalus* inhabiting Kovalam estuary (**Figure 2**).

NO is involved in a variety of physiological events. The short lived NO can be quickly converted to other more stable metabolites such as NO_2^-/NO_3^- with very low bioactivity (45). The increase in nitrite and nitrate levels indirectly reflects the increase in nitrative stress during pollution. The level of nitrite was evaluated in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries (**Figure 3**). NO also exerts its cytotoxicity by nitrosylation to form nitrated product. 3-NT and the altered nitrated protein undergo conformational changes resulting in its decreased function (46). Hence the formation of free or protein associated 3-NT serves as a potential biomarker for the generation of reactive nitrogen intermediates (47). These observations agrees well with the immunohistochemical results of 3-NT which revealed that brain tissue of *M. cephalus* inhabiting Ennore estuary showed a positive, strong and diffuse cytoplasmic immunostaining compared to moderate and light immunostaining observed with brain tissue of *M. cephalus* inhabiting Kovalam estuary (**Figure 4**) confirming the RNS derived nitrative stress under pollutants induced stress condition. GSH can directly scavenge singlet oxygen and hydroxyl radical in cells under oxidative stress (48). The level of reduced glutathione was evaluated in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries (**Figure 5**). This study showed that exposure to contaminants decreased the GSH content in the fish brain. Decreased GSH content may be due to the increase of ROS production which utilized large amount of GSH (49), depressed re-generation of GSH or leakage of GSH from the damaged organ or tissue. Re-generation of GSH depends on Glutathione reductase (GR) which can catalyze the reduction of GSSG back to GSH when it scavenges ROS (50).

Antioxidant responses can be important to include in biomonitoring studies, as many xenobiotics are prooxidants that exert their effects through induction of oxidative stress. However, antioxidant enzymes are often less responsive than other biomarkers (i.e. phase I enzymes) to exposures, may differ greatly in expression levels and inducibility in different species (39). Therefore, antioxidant biomarkers would be most useful as part of a larger battery of biomarkers when investigating effects of xenobiotics on biota. Oxidant radical absorbing capacity (ORAC) is essential to neutralise the prooxidant factor. The level of total antioxidant capacity was evaluated in brain homogenate of *M. cephalus* inhabiting Kovalam and Ennore estuaries (Figure 6). This is indicative of increased free radical generation under conditions of pollution induced stress. From our preliminary investigations, we emphasize this information could provide a knowledge on the toxicological relevance of protein and lipid oxidation. The damaged proteins in particular may be involved in number of cellular processes which could affect organismal health.

7. Conclusion

The relationship between the oxidation of lipid, protein and deficiency of antioxidant defenses suggests that these parameters could also be used as biomarkers for toxicity. Considering the literature and our findings in concert, it appears conceivable to speculate that the pollution induced stress has caused alteration in the redox status and total antioxidant capacity to stabilize the damage to the biomolecules such as lipids and proteins in the brain tissue. However the damaged products through oxidative stress can persist even after the stress has been stabilized by continuous exposure leading to various molecular changes in these fish which may aid in their survival or death process which depends on the intensity of damage caused by the pollutants at one point of time.

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