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Research Paper

EFFECT OF POLLUTION ON ANTIOXIDANT DEFENSE IN VARIOUS TISSUES OF SCALE CARP, CYPRINUS CARPIO COMMUNIS (LIN.)

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This study was carried out to assess the status of oxidative stress and antioxidant defense in various tissues of fresh water scale carp, *Cyprinus carpio communis* (Lin.) living in a hyper-eutrophic urban Edulabad water reservoir system, which is being polluted from industrial, domestic and sewage effluents. The seasonal effects of environmental pollution on antioxidant responses in liver, kidney and gills of the fish were studied. The results obtained in present study indicated the increase in oxidative stress in gills, liver and kidney when compared to that of Bibi Nagar water reservoir, which is fresh water reservoir system with lesser contaminants. Further, increased reactive oxygen species and lipid peroxidation with sequence of liver gill kidney was observed. However, the decreased GSH and GSH/GSSG with sequence of gill liver kidney. The decreased GSH/GSSG in all tissues indicated the oxidized thiol status in fish living in polluted water. All these changes were in the order of pre-monsoon monsoon post-monsoon season. The present study indicates that the presence of pollutants in Edulabad water reservoir might be affecting aquatic life through enhancing the oxidative stress in the fish. Further, the tissue specific responses in fish adaptation are seasonally variable by environmental pollution.

Keywords: Edulabad water reservoir, Oxidative stress, Glutathione, Lipid peroxidation, Cyprinus carpio

INTRODUCTION

Today different highway agencies, institutions, The aquatic environment surrounding the urban areas is being subjected to various contaminants resulted from industrial, agricultural and domestic effluents. Due to these pollutants, water quality become deteriorated through eutrophication, acidification and ultimately causes a serious risk for the aquatic organisms (Nayaka *et al*., 2009; Upadhyay *et al*., 2006). The contaminants such as heavy metals organic waste can be deposited in aquatic organisms through food chain process (Bagnyukova *et al*., 2006; Bacanskas *et al*., 2004; Sherry, 2003) leading to altered cellular homeo

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stasis by interaction at molecular level in a variety of ways. Since, fish species are strongly respond to stress conditions, they can be considered as bioindicators of aquatic pollution for environmental monitoring (Almroth *et al*., 2008; Schmitt, 2004; Vander Oost *et al*., 2003). Further, the contaminants uptake by fishes may induce tissue-specific responses through metabolism and their elimination process in fish (Gravato *et al*., 2010; Giari *et al*. 2007; Geist *et al*. 2007; Zhou *et al*. 2008). Those responses during exposure to environmental toxins include extensive generation of deleterious free radicals via Fenton type reactions. These free radicals cause covalent modification of biological macromolecules and alter cellular redox balance (Livingstone, 2001). In addition, antioxidant defense systems might be stimulated in organisms exposed to pollutants such as heavy metals, pesticides etc (Almroth *et al*., 2008; Almedia *et al*., 2002; Berntssen *et al*., 2003). Such defense mechanism includes antioxidant enzymes such as superoxide dismutase (SOD) and catalase were altered in mullet and flounder by exposure to pollutants (Ferreira *et al*., 2005). Several studies have been reported the enhanced oxidation of polyunsaturated fatty acids has been observed in various aquatic systems which were grown in polluted water (Stegeman *et al*., 1992). Further, the disturbances in glutathione metabolism occur by various toxicants in fishes which can be considered as biomarker of oxidative stress (Almar *et al*., 1998). In this perspective, various tissues of fish responses may provide valuable information concerning environmental pollution (Ozmen *et al*., 2006). In addition to various natural biological factors, seasonal variations also have been proposed to have a significant influence on aquatic environment because the activity of antioxidant defense enzymes and other biomarkers also depends on availability of nutrients, reproductive status, and season related growth throughout the year (Padmini *et al*., 2008; Verlecar *et al*., 2008). Various reports revealed the existence of seasonal variations on biological parameters in red mullet (Pavlovic *et al*., 2010), horse mussels (Lesser and Kruse, 2004), blue mussels (Manduzio *et al*., 2004) and in the digestive gland of brown mussels (Filho *et al*., 2001). Evaluation of seasonal variations in oxidative stress biomarkers in model organisms constitute a research strategy that is widely recommended today. Hence, the present study was ascertained to elucidate the seasonal variations in lipid oxidation, glutathione redox ratio and antioxidant enzyme activities in liver, kidney and gills of Scale carp, *Cyprinus carpio communis* living in highly eutrophic and polluted Edulabad water reservoir (EDWR) located in urban area of Andhra Pradesh. Hence, EDWR was chosen for studying the seasonal variation as well as intend of pollution on oxidative damage and endogenous antioxidants in fish, C. Carpio, a cheap food item with good source of protein for the human diet. The results obtained in present study indicated more oxidative damage and decreased antioxidant defense in the various tissues of the fish living in EDWR when compared to that of Bibi Nagar water reservoir (BNWR), which is in oligotrophic nature, located in Andhra Pradesh. These changes in oxidative stress were more significant in pre-monsoon season than monsoon season.

MATERIALS AND METHODS

Study Area

The present study was conducted in two medium reservoirs for two years during 2005-2007. Edulabad water reservoir, located at the intersection of latitude 17° 24' North and longitude 78° 45' East (at an elevation of 350 m above mean sea level) in Ranga Reddy district of Andhra Pradesh, India selected as a polluted reservoir, which is in highly eutrophic in nature and is used

as experimental for the study and geographical representation was shown in our previous publication (Sagar *et al*., 2013). The Common Effluent Treatment Plants located near urban areas reached the water reservoir through Musi river. Unpolluted Bibi Nagar water reservoir is located at the intersection of latitude 17° 28' and longitude 78° 47 in Nalgonda district of Andhra Pradesh, which is oligotrophic in nature, is used as control for the study.

Sampling

The common fresh water fish, scale carp, *Cyprinus carpio communis* were collected thrice in each season in three stations (n=3 at each station) of the each reservoir for a period of two years from the both experimental and control reservoirs. The seasons were categorized into three types such as pre-monsoon (February-May), monsoon (June- September) and post-monsoon (October - January) in each year. Adult individuals of similar size (length, 11.8 ± 1.9 cm; weight, 140 ± 10.21 g) were used for the study for each experiment; 54 fishes were sacrificed, out of which 27 for control and remaining 27 for experimental during each season. The liver, kidney and gill were dissected out, washed with ice cold distilled water and processed immediately or frozen in liquid nitrogen till further use.

Measurement of Reactive Oxygen Species

Determination of reactive oxygen species (ROS) in fish liver, kidney and gills using a cell permeable non-fluorescent probe, 2', 7'-dichlorodihydro-fluorescein diacetate (2', 7'-DCFDA) as per the method reported by Driver et al. (2000), with minor modifications. Briefly, 10% homogenates of the fish tissues was prepared in ice cold PBS (50 mM, pH 7.4) using glass-Teflon homogenizer at 4ºC and homogenates were centrifuged at 1000 ×g for 10 min at 4°C to obtain clear supernatants. The reaction mixture (1 ml) containing PBS buffer (50 mM, pH 7.4), 0.2 ml homogenate (100 µg protein) and 10 µl of 2', 7'-DCFDA (10 µM) (Molecular Probes, Eugene, USA) was incubated for 30 min at 37°C ± 1, in the dark. The fluorescence intensity of formed 2', 7'-dichlorofluorescein upon reaction with ROS was measured at λ_{Ex}= 490 nm and λ_{Em} = 520 nm in steady state spectroflourometer (Perkin Elmer LS-50B, Boston, USA). The background fluorescence was corrected by using the reaction mixture without homogenates as blank. Protein content in the homogenates was determined by Bradford's method (1976). The ROS accumulation in each sample was calculated for 100 g protein and expressed as Relative Fluorescence Intensity/100 g protein.

Measurement of Lipid Peroxidation

Products of lipid peroxidation were measured as thiobarbituric acid reactive substances (TBARS) in cytosolic extracts (Ernster and Nordenbrand, 1967) isolated from 10% homogenates of fish tissues containing 50 g protein by reacting with 0.67% thiobarbituric acid after 20% trichloroacetic acid precipitation and absorbance was measured in clear supernatants at 533 nm UV Vis spectrophotometer (Shimadzu, Japan) employing malondialdehyde as the reference standard.

Determination of Intracellular Glutathione Levels

Reduced (GSH) and oxidized (GSSG) forms of glutathione concentrations were measured as per Hissin and Hilf [1976]. Cytosolic extracts were isolated from homogenates of fish tissues (50 mg/ 500 l) by employing centrifugation at 15000 ×g for 30 min at 4°C in 0.2 M Tris-0.02 M EDTA, pH 8.2. The cell free extract (0.3 ml containing 1.2 mg protein) prepared in above buffer was treated with 60 l of 25% phosphoric acid, centrifuged at 10,000 g for 30 min at 4 C to collect clear supernatant. Different aliquots of the protein free filtrate were made up to 0.1 ml with cold distilled water followed by addition of 1.8 ml of 0.1 M sodium

phosphate buffer (pH 8.0) containing 5 mM EDTA for GSH or 1.8 ml of 0.1 N NaOH for GSSG. To this, 0.1 ml of 1 mg/ml o-phthalaldehyde was added, mixed and incubated at room temperature for 15 min. Standards ranging from 0-2 g GSSG and 0-10 g for GSH were evaluated simultaneously. GSH and GSSG react with o-phthalaldehyde to yield a fluorescent complex and the fluorescence intensity was measured at λ_{Ex}=350 nm and λ_{Em}= 420 nm using Fluoromax-3 spectrofluorimeter (Horiba Jobin yvon Inc., USA.

Statistics

Data were expressed as Mean ± SD and the mean values of various groups were compared by one way ANOVA followed by Post hoc Duncan's test for multiple comparisons using Sigma Plot Ver 11.0 software. The criteria for statistical significance were p ? 0.05.

RESULTS

Based on comparison of results, the oxidative stress was significantly ($p < 0.05$) higher in the fish living in Edulabad water reservoir (EBWR) with that of Bibi Nagar water reservoir (BNWR), evidenced by increase in ROS, lipid peroxidation, decrease in total glutathione levels and GSH/GSSG. However, the oxidative stress was slightly increased in 2006-07 when compared to 2005-06. Further, all these parameters were higher in pre monsoon (PM) than monsoon (M) and post monsoon (MP) seasons throughout two years (PM MP M).

Enhanced ROS Observed in Various Tissues of Fish Living in EBWR

In 2005-06, ROS in liver of the fish living in EBWR was 2.4, 1.9 and 2.3 fold significantly higher in PM, M and MP seasons respectively when compared with that of BNWR (p 0.05) (Table 1). ROS in gill was 2.1, 1.8 and 2.0 fold significantly higher in PM, M and MP seasons respectively (p 0.05). ROS in kidney was 1.9, 1.8 and 1.9 fold higher in PM, M and MP seasons respectively. Mean ROS was 220 ± 51, 183 ± 34 and 151 ± 18 RFI/100 µg protein in liver, gill and kidney of EBWR fish respectively throughout year 2005-06. It was slightly increased in 2006-07. Further, increased ROS was observed in the order of liver gill kidney.

Enhanced Lipid Peroxidation Observed in Various Tissues of Fish Living in EDWR

Liver lipid peroxidation of the fish living in EBWR was 94, 78 and 87% increased significantly in PM, M and MP seasons respectively when compared with that of BNWR In 2005-06 (p 0.05) (Table 1). Further, lipid peroxidation in gill was enhanced significantly up to 69, 42 and 57% in

Table 1: Lipid Peroxidation

Tissue	Year	Thiobarbituric Acid Reactive Substances (µg/min/mg protein)					
		Pre-monsoon		Monsoon		Post monsoon	
		BNWR	EDWR	BNWR	EDWR	BNWR	EDWR
Liver	2005-06	62.2 ± 5.9	121.2 ± 6.1*	66.3 ± 4.1	118.6 ± 9.7*	66.3 ± 5.6	124.5 ± 8.6*
	2006-07	55.3 ± 2.0	124.4 ± 9.3*	58.9 ± 3.9	111.9 ± 8.3*	64.2 ± 4.9	124.1 ± 10.4*
Gill	2005-06	62.5 ± 3.6	106.2 ± 8.9*	69.3 ± 7.0	98.8 ± 5.4*	69.9 ± 2.8	109.9 ± 3.3*
	2006-07	63.3 ± 2.7	128.1 ± 2.9*	72.9 ± 3.5	115.4 ± 6.4*	70.3 ± 3.5	130.8 ± 8.6*
Kidney	2005-06	52.2 ± 4.7	80.9 ± 4.4*	62.9 ± 3.0	79.2 ± 4.7*	62.3 ± 3.5	92.8 ± 4.1*
	2006-07	63.6 ± 3.4	114.2 ± 3.8*	73.2 ± 3.6	103.9 ± 6.7*	71.2 ± 4.7	105.8 ± 5.3*

*Lipid peroxidation in tissues of C. carpio living in Bibinagar water reservoir (BNWR) and Edulabad water reservoir (EDWR) during two consecutive years was measured as thiobarbituric acid reactive substances and represented as µg/min/mg protein. All values are represented as Mean ± SD obtained from three independent experiments. *p<0.05 indicates statistically significant between fishes of EDWR in comparison to that of BNWR.*

Table 2: Reduced Glutathione Levels

Tissue	Year	Reduced glutathione levels (µg/mg protein)					
		Pre-monsoon		Monsoon		Post monsoon	
		BNWR	EDWR	BNWR	EDWR	BNWR	EDWR
Liver	2005-06	48.7 ± 1.6	24.0 ± 2.2	37.3 ± 2.1	25.6 ± 3.9*	45.6 ± 2.9	26.0 ± 1.7*
	2006-07	50.6 ± 2.2	27.3 ± 2.3*	42.9 ± 1.2	25.2 ± 0.8*	46.8 ± 1.9	25.6 ± 0.9*
Gill	2005-06	38.9 ± 1.8	15.3 ± 1.5*	27.2 ± 2.3	15.7 ± 2.5*	36.1 ± 1.9	17.3 ± 1.5*
	2006-07	41.6 ± 2.0	17.7 ± 1.3*	29.1 ± 4.9	16.0 ± 1.3*	37.0 ± 1.3	18.6 ± 2.2*
Kidney	2005-06	41.4 ± 1.7	21.2 ± 1.6*	28.8 ± 1.8	19.9 ± 1.8*	40.2 ± 2.6	20.9 ± 1.1*
	2006-07	45.9 ± 1.5	25.3 ± 1.1*	31.6 ± 1.9	22.8 ± 1.9*	48.8 ±1.0	27.5 ± 0.9*

*Reduced form of glutathione (GSH) levels in tissues of C. carpio living in Bibinagar water reservoir (BNWR) and Edulabad water reservoir (EDWR) during two consecutive years represented as µg/mg protein. All values are represented as Mean ± SD obtained from three independent experiments. *p<0.05 indicates statistically significant between fishes of EDWR in comparison to that of BNWR.*

PM, M and MP seasons respectively ($p \leq 0.05$). Similarly, lipid peroxidation in kidney increased up to 55, 25 and 48% in PM, M and MP seasons respectively ($p \leq 0.05$). Mean lipid peroxidation 121 ± 3, 105 ± 6 and 88 ± 5 nmoles/min/mg protein in liver, gill and kidney of EBWR fish respectively throughout year 2005-06. It was slightly increased in next year. Further, lipid peroxidation was in the order of liver gill kidney.

Decreased glutathione levels in various tissues of fish living in Edulabad water reservoir

Liver GSH levels of the fish in living in EBWR was 51, 31 and 43% significantly decreased in PM, M and MP seasons respectively during 2005-06, when compared with that of BNWR ($p < 0.05$) (Table 1). Further, GSH levels significantly decreased up to 61, 42 and 52% in PM, M and MP seasons respectively ($p < 0.05$). In addition, kidney GSH was decreased significantly ($p \leq 0.05$) up to 49, 31 and 40% in PM, M and MP seasons respectively. Further, mean GSH level observed in liver, gill and kidney of EBWR fish were 25 ± 1, 16 ± 1 and 22 ± 3 µg/mg protein respectively throughout year 2005-06. It was slightly increased in 2006-07. However, decrease in GSH was in the order of gill liver kidney

Similarly, GSH/GSSG was 36, 24 and 29% significantly higher in PM, M and MP seasons respectively in the liver of the fish in living in EBWR during 2005-06 when compared with that of BNWR ($p \leq 0.05$) (Table 1). In 2005-06, GSH/GSSG in gill was 39, 27 and 31% significantly higher in PM, M and MP seasons respectively (p 0.05). Further, kidney GSH/GSSG observed in PM, M and MP seasons was 27, 17 and 19% higher when compared with that of BNWR. In addition, mean GSH/GSSG in liver, gill and kidney of EBWR fish was 1.34 ± 0.09, 1.43 ± 0.15 and 1.16 ± 07 respectively throughout year 2005-06. It was slightly increased in consecutive year.

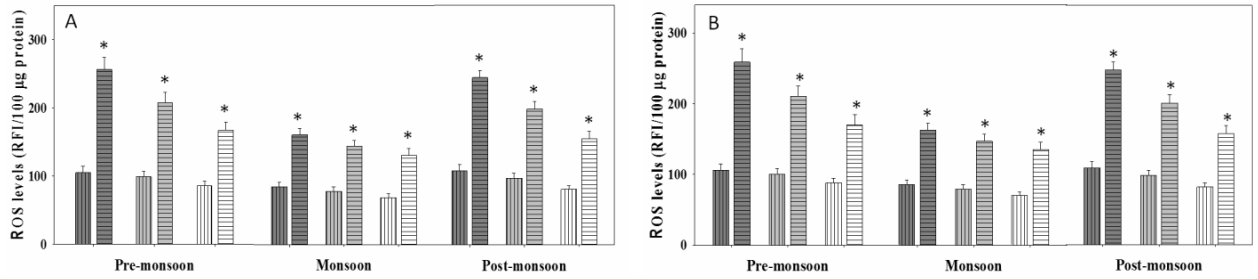
DISCUSSION

The basic purpose of the present investigation is to evaluate the status of antioxidant defense elicit by the *Cyprinus carpio* inhabiting polluted EDWR, which exhibited higher in biological oxygen demand and chlorides, nitrates (Gummadavelli *et al*., 2011) when compared with BNWR. EDWR receives a source canal from the river musi, a highly polluted due to intake of drains, toxic substances released from industries, agricultural wastes and domestic wastes. Such industrial wastes influence the aquatic life and use of biomarkers for environmental monitoring has been frequently applied to assess the integrated response to pollutants (Hanson and Larsson, 2009, Nedjoud *et al*., 2009; Vinodhini and Narayanan, 2008). Recent report evidenced the higher bioaccumulations of metals in gills, liver and kidney with decrease in total protein, glycogen and lipid levels in the fish living in EDWR (Gummadavelli *et al*., 2013). These reservoirs are useful to supply drinking water and majorly for fish production. Hence, the reservoir was chosen to study the antioxidant defense in C. carpio living in EDWR. The results indicated the higher ROS, lipid peroxidation, decreased total glutathione levels and GSH/GSSG in all the examined tissues of fish living in EDWR when compared to BNWR.

Several studies has been reported that fish living in aquatic bodies polluted with heavy metals and other toxic substances induces number of subtle biological changes depends on their capacity to enhance highly deleterious ROS (Marijic and Raspor, 2003; Viarengo *et al*., 2007) that damages the crucial cellular components including membrane lipids, intracellular proteins and nucleic acids due to the imbalance in oxidation-reduction reactions, leads oxidative stress in various fish species either short term or longer periods (Kopecka-Pilarczyk and Correia, 2009; Jung *et al*., 2009; Narghang *et al*., 2009; Hannam *et al*., 2010). In this perspective, the analysis of antioxidant defenses as well as lipid peroxidation in fish species becomes highly relevant in biomonitoring studies (Almar *et al*., 1998; Valavanidis *et al*., 2006; Ahmad *et al*., 2000; Whyte *et al*., 2000; Schmitt, 2004).

In present study, the increased oxidative damage in fish of EDWR was evidenced by increased ROS in liver (2.4 fold), gill (2.1 fold) and kidney (1.9 fold) as well as lipid peroxidation in liver (94%), gill (69%) and kidney (74%) in PM season when compared to that of BNWR (Figure 1) Some previous reports have been demonstrated the increase in lipid peroxidation

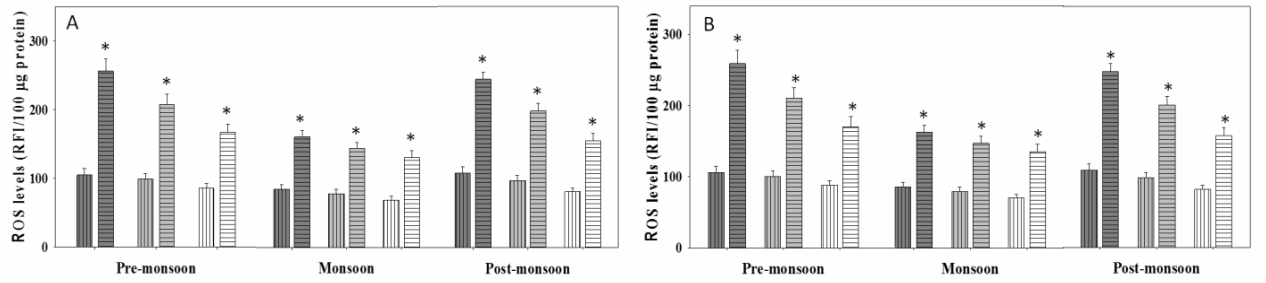
Figure 1: ROS Accumulation



A. During 2005-06. B. During 2006-07. ROS accumulation in tissues of C. carpio living in Bibinagar water reservoir (BNWR) and Edulabad water reservoir (EDWR) was measured using 2', 7'-dichlorodihydrofluorescein diacetate and expressed as Relative Fluorescence Intensity/100 g protein. The vertical bars representing liver (dark grey), gill (grey) and kidney (light grey) with grid lines of vertical for BNWR and horizontal for EDWR depicted in figure. Vertical bars and error bars are

Mean and standard deviation obtained from three independent experiments. *p ≤ 0.05 indicates statistically significant between the lipid peroxidation in fishes of EDWR in comparison to that of BNWR.

Figure 2: GSH/GSSG: A. During 2005-06. B. During 2006-07



in fish species living in metal contaminated river in both spring and autumn (Ruas *et al*., 2007). Similarly, some studies have shown individual variations in glutathione redox status and lipid peroxidation in wide range of fish species (Bagnyukova *et al*., 2007; Oruc and Usta, 2007; Oakes *et al*., 2004). Further, increased ROS and lipid peroxidation was denoted with sequence of liver gill kidney. Even though the more intake of toxic substances present in water by the gill, the liver is more prone to injury since it involves in metabolizing the toxic substances through various mechanisms. Further, the toxic substances formed all over the body can be passed to kidney for elimination (Oliveira *et al*., 2010; Singh *et al*., 2001). In present study, the decreased levels of GSH in all examined tissues implying the increased levels of ROS and lipid peroxidation may cause loss in cellular membrane damage, physiological integrity and ultimately reduce cell survival. The increased lipid peroxidation and decrease in SOD and catalase activities suggesting that lack of antioxidant defenses could result in oxidative damage in C. Carpio captured in the Yellow River, China, a river contaminated by phenols, oils, PAHs and ammonia (Huang *et al*., 2007).

However, the decreased GSH and GSH/GSSG with sequence of gill liver kidney. Our results were similar to earlier reports where contaminants caused decrease in GSH contents of gill in golden grey mullet (Milinkovitch *et al*., 2011), in killifish (Bacanskas *et al*., 2004) and in Carassius auratus (Yin *et al*., 2007) and GSH synthesis in contaminant exposure (Canesi *et al*., 1999). Even though, the fish is adapted to waterborne contaminants due to presence of higher amounts of non enzymatic antioxidants, the GSH depletion might be occur during detoxification process (Parvez *et al*. 2006; Hamed *et al*., 2003; Rao, 2006) reported GSH content was depleted in liver, brain and gill tissues in Tilapia (oriochromis mosambicus) fish exposed to RPR-II an organo phosphorus insecticide. Seasonal changes in GSH level has also been reported by (Power and Sheehan, 1996, and Filho *et al*., 2001). GSH/GSSG ratio is decreased, this is the indication of the fish is under the stress.

Further, all oxidative stress indicators studied throughout two years were in the order of pre-monsoon monsoon post-monsoon season. Since, there is continuous release of industrial and agricultural wastes and lack of adequate amount of rain fall, the concentration of toxic substances increases during PM season. In addition, the increased atmospheric temperature also enhances the solubility of those pollutants in water might be enhanced the oxidative stress

in fish organs during PM season. It can possibly attribute to varied water temperature as well as its physiological characteristics. Similar with earlier reports (Giari *et al*., 2007; Geist *et al*., 2007; Mohamed, 2008; Saravanan *et al*,. 2011) the present study also revealed that the tissue specific responses have been observed under environmental pollution.

The results obtained in present study shown an increased oxidative stress in the fish living in EDWR when compared to that of BNWR predominantly in PM season. The combined results indicated that seasonal variations of antioxidant defense enzyme activities and lipid peroxidation might be used for biomonitoring studies in fish species.

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