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Di(2-ethylhexyl)phthalate-induced Lipid Peroxidation and Associated Oxidative stress in Gill, Liver and Muscle Tissues of the Fish, *Oreochromis mossambicus* (Peters, 1852)

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ABSTRACT: The present study was focused to investigate the consequences of one of the phthalate plasticizers, di(2-ethylhexyl)phthalate (DEHP) on the induction of lipid peroxidation and its associated oxidative stress in gill, liver and muscle tissues of the freshwater fish, *Oreochromis mossambicus*. DEHP at 60 ppm concentration was exposed to fish for short-term (24, 48, 72 and 96 h) and long-term (7, 14, 30, 60days) durations along with control groups. Oxidative stress markers such as the activities of antioxidant enzymes superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase, and the level of lipid peroxidation were evaluated in gill, liver and muscle tissues. All antioxidant enzymes showed significant changes when compared to the control groups with significant (P<0.05) induction in the level of lipid peroxidation in all tissues. Activity of acid phosphatase increased significantly (P<0.05) after 96 h of treatment in both gill and muscle tissues. However, the activity of alkaline phosphatase showed significant (P<0.05) increase after 72 h onwards in gill and muscle tissues. In liver tissue, alanine and aspartate aminotransferase enzyme activities increased significantly (P<0.05) after the phthalate exposure. The present findings concludes that the toxic effect of DEHP is mediated through the induction of lipid peroxidation thereby causing oxidative stress to the vital tissues, which might be one of the reasons behind the tissue damage as evidenced by the alteration in tissue marker enzymes. Thus the chronic release of phthalate plasticizers even at low concentration can be correlated to tissue injury and eventually may pose risks to the entire aquatic life in the natural environment.

Keywords: Oxidative stress, *Oreochromis mossambicus*, antioxidant enzymes, gill, liver, muscle.

I. INTRODUCTION

Phthalates are added to the vinyl polymers in order to improve the features of plastic products and it is susceptible to leach from dumped plastic wastes, teethers, food wraps and medical devices into the natural ecosystems. The ability of phthalates for long range transport from the production site to enter into the environment eventually invades human through the food chain. Phthalates was first introduced in 1940s and currently the annual production exceeds about 18 million pounds, out of which the major portion is used in the synthesis of many commercial goods including medical devices and remaining is used in solvents, sprays and emulsions. Di(2-ethylhexyl) phthalate (DEHP), one of the low molecular weight phthalates, was reported as carcinogenic, mutagenic or reproductive toxicant by several toxicology agencies as National Toxicology Program [1], Agency for Toxic Substances and Disease's Registry [2] and Environmental Protection Agency [3]. Regulation for the use of DEHP on medical devices is strictly followed in the European Union; however, its widespread application is not only restricted in the medical equipments but also found extensively in other products as toys, cosmetics, rubber clogs and some consumer foodstuffs that contribute to the route of exposure to humans [4].

Enteric coating of oral medications and various dietary supplements ranging from fish oils to probiotics plays major possible entry for DEHP. Thus all organisms, including human is exposed to DEHP on a daily basis by multiple routes of exposure. After ingestion, phthalates are cleaved into corresponding hydrolytic monoesters where 6-12% is absorbed by the action of esterase and lipases, and approximately 65% of parental alkyl chain undergoes oxidative metabolism and ends in glucouridination depending on the species [5]. The primary and secondary metabolites of DEHP have been identified in several body fluids as urine, breast milk, blood serum and saliva [6]. DEHP was also found in the drinking water at levels ranging from 0.04 to 30 ppb [2] thus alarming the release of phthalates in the aquatic ecosystems.

DEHP act as endocrine disruptors causing negative impacts on reproduction and also in the antioxidant defense system, metabolism, electron transfer and cell signaling [7].

There are few reports showing toxic effects of DEHP on rainbow trout *Onchorhynchus mykiss* [8], Japanese medaka, *Oryzias latipes* [9] and in guppy fish, *Poecilia reticulata* [10]. The differential involvement of peroxisome proliferator activated receptors (PPARs) binding and activation of phthalates has been identified as the common pathway for the induction of oxidative stress [11]. One of the phthalates, diisononyl phthalate has been shown to induce oxidative stress in gill, liver and muscle tissues of the fish, *Oreochromis mossambicus* [12]. In ecotoxicology, oxidative stress in aquatic organisms is considered as suitable biomarkers to detect the toxic effects of environmental pollutants. Under normal physiological conditions, all tissues in the biological systems maintain redox homeostasis through the generation and elimination of both reactive oxygen and nitrogen species [13]. Redox state is essential for proper functioning of the cell and it differ among various cells and tissues. Imbalance in redox homeostasis results in the generation of free radicals, which shows potent oxidative activity towards cellular components such as, biomolecules, membranes, enzyme and DNA [14]. Oxidative stress represents an increase in the level of reactive oxygen species generation, which is harmful to the tissue and thereby leading to several chronic diseases and other degenerative ailments such as damages in DNA, protein and other macromolecules, neurodegeneration, development of tumor etc. [15]. The present study was undertaken to examine the toxic effects of DEHP by the formation of free radicals and induction of lipid peroxidation in gill, liver and muscle tissues of the freshwater fish, *Oreochromis mossambicus*.

II. MATERIALS AND METHODS

A. Test animal

Freshwater fish, *Oreochromis mossambicus* of 5.5±1.5cm length and 3.5±0.75g weight were collected from Safa aquarium, Kozhikkode, Kerala. Fish were brought to laboratory without stress to the animal and acclimatized in dechlorinated and well-aerated water (40 L capacity) for two weeks prior to experiment. The physico-chemical characteristics of water such as pH (7.5±1), temperature (28±2^{o}C), oxygen saturation (70-100%) were maintained throughout the study according to APHA guidelines [16].

B. Chemicals

Di(2-ethylhexyl)phthalate (DEHP; CAS No. 117817) of 99.7% purity was obtained from Sigma Aldrich chemical Co., USA. Malondialdehyde, NADPH, glutathione oxidized, thiobarbituric acid, pyrogallol, horseradish peroxidase, sodium pyruvate, 2,4-dinitrophenyl hydrazine, L-aspartate, 2-oxoglutarate, DL-α-alanine were obtained from Himedia Laboratories, Mumbai, India. All other chemicals were of analytical grade and obtained from local commercial sources.

C. Experimental design

DEHP (60 ppm) is dissolved in propylene glycol (1 M) and hence used as vehicle control. In each experimental group 10 animals were maintained, which was further grouped as follows:

Group 1: Control group (vehicle and toxicant-free)
Group 2: Vehicle control group (propylene glycol)
Group 3: Short-term treatment group (DEHP-60 ppm for 24, 48, 72 and 96 h)
Group 4: Long-term treatment group (DEHP-60 ppm for 7, 14, 30 and 60 days)

D. Processing of tissues

At the end of every treatment period, fish were captured using a dip net with least disturbance, weighed and decapitated. Weights of gill and liver were recorded, and 1% (w/v) of gill, liver and muscle tissue homogenates were prepared in ice-cold saline using a motor driven tissue homogenizer. The homogenates were centrifuged at 3000 rpm for 15min at 4^{o}C and the supernatants collected were then used for the biochemical analysis.

E. Biochemical assays

Total protein concentration in the tissues was estimated as described by Lowry *et al*. [17]. Activities of antioxidant enzymes such as superoxide dismutase [18], catalase [19], glutathione reductase [20] and glutathione peroxidase [21] and the level of lipid peroxidation [22] were assayed in the supernatants of gill, liver and muscle tissues. The activities of acid phosphatase [23] and alkaline phosphatase [24] were assayed in gill and muscle tissues. The activities of alanine and aspartate aminotransferase were measured by the method of Bergmeyer [25] in liver tissue.

F. Statistical analysis

One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed in order to find the significant ($P<0.05$) difference against the control groups by using the statistical package SPSS 17.0. Data are presented as mean±SD for ten animals per group and all biochemical estimations were carried out in triplicate.

III. RESULTS

A. Effect of DINP on body weights and tissue weights

No treatment related changes in the body weight of the animal was noticed throughout the treatment period when compared to the control groups (Table 1). The weights of gill and liver tissues showed significant ($P<0.05$) decrease only after 30 and 60 days of DEHP exposure (Table 1).

Table 1: Effect of DEHP on the body weight and tissue weights of the fish, *Oreochromis mossambicus.*

Parameters	Control	Vehicle control	DEHP (60ppm)							
			Short-term exposure					Long-term exposure		
			24 h	48 h	72 h	96 h	7 days	14 days	30 days	60 days
Body weight (g)	3.47±0.5	3.47±0.6	3.46±0.14	3.44±0.51	3.50±0.03	3.49±0.29	3.45±0.43	3.40±0.06	3.43±0.57	3.39±0.05
Gill weight (mg)	122±1.15	122±1.24	121±1.22	122±0.96	123±0.81	122±0.51	120.6±0.84	119.4±1.26	117±1.63*	113±1.20*
Liver weight (mg)	51.9±0.82	51.3±1.72	52±0.67	51±1.26	51±0.78	50±0.70	49.7±1.49	49.1±1.37	48.5±1.71*	47±1.64*

Values expressed in Mean±SD; n=10/group; *P<0.05 against the control groups.

B. Effect of DEHP on gill antioxidant system

The activity of superoxide dismutase enzyme showed slight increase after 24 h of DEHP exposure followed by significant (P<0.05) reduction from 96 h of treatment onwards when compared to the control groups (Fig. 1A). The activities of catalase and glutathione reductase showed significant (P<0.05) increase in both short-term and long-term exposure groups (Fig. 1B and C). While the activity of glutathione peroxidase showed significant (P<0.05) decrease only after 14 days of DEHP exposure and the activity remained unchanged in short-term exposure group (Fig. 1D).

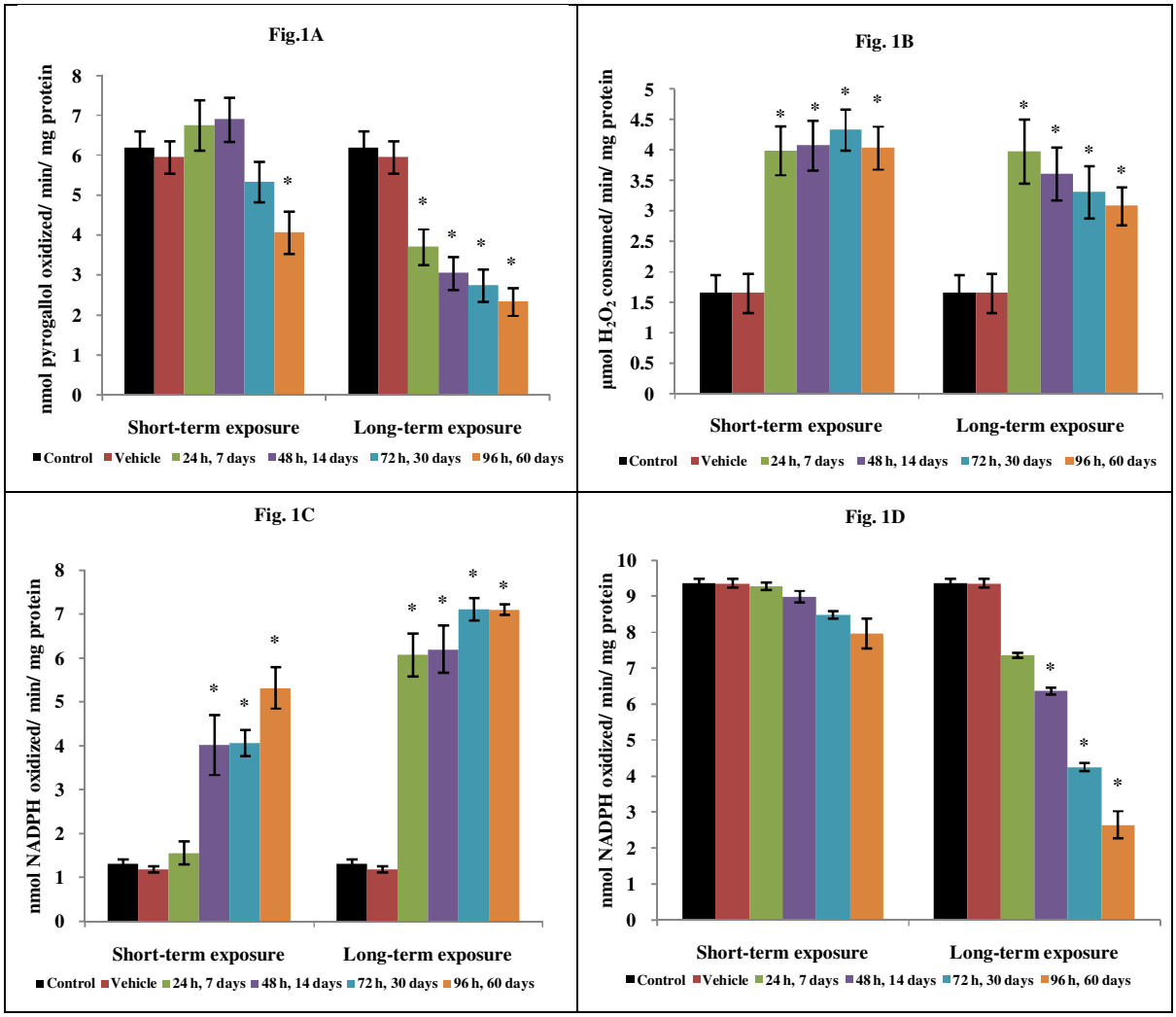

Fig. 1. A. Effect of DEHP on the activity of superoxide dismutase in the gill of the fish, *Oreochromis mossambicus.* **B.** Effect of DEHP on the activity of catalase in the gill of the fish, *Oreochromis mossambicus.* **C.** Effect of DEHP on the activity of glutathione reductase in the gill of the fish, *Oreochromis mossambicus.* **D.** Effect of DEHP on the activity of glutathione peroxidase in the gill of the fish, *Oreochromis mossambicus.*

Fig. 1E

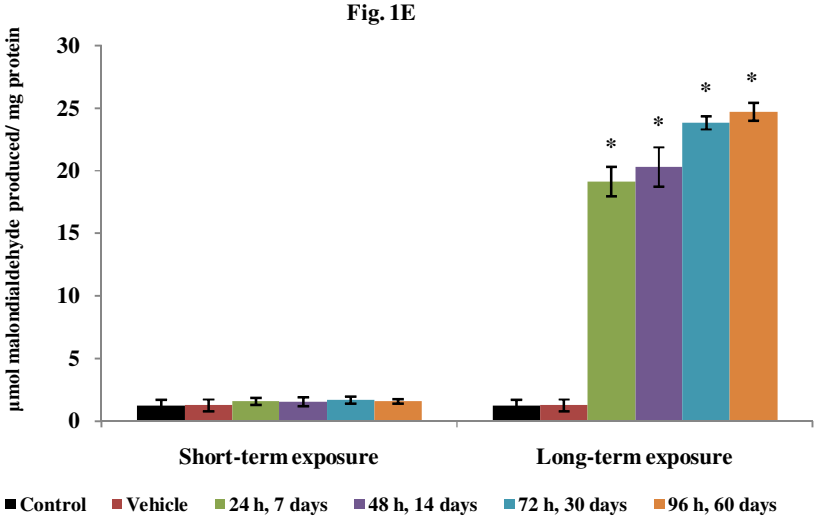

Fig. 1E. Effect of DEHP on the level of lipid peroxidation in the gill of the fish, *Oreochromis mossambicus.*

The level of lipid peroxidation increased to 20-25 folds when compared to the corresponding control groups in long-term exposure without any changes in the short-term treatment (Fig. 1E). The activities of gill tissue marker enzymes, acid and alkaline phosphatase showed significant (P<0.05) increase after DEHP exposure (Figs. 4A and B).

C. Effect of DEHP on liver antioxidant system

Superoxide dismutase enzyme activity showed significant (P<0.05) decrease right from 24 h of DEHP exposure in time-dependent manner throughout the study than that of the control groups (Fig. 2A).

Activity of catalase was found to decrease significantly after 72 h, which is duration dependent (Fig. 2B). However, the activity of glutathione reductase increased significantly (P<0.05) immediately after 24 h of phathalate exposure and ultimately significant (P<0.05) reduction was observed after 60 days of treatment (Fig. 2C). Glutathione peroxidase enzyme decreased significantly (P<0.05) from 96 h onwards when compared to the control groups (Fig. 2D). The level of lipid peroxidation was found increased significantly (P<0.05) in both short-term and long-term exposure groups (Fig. 2E).

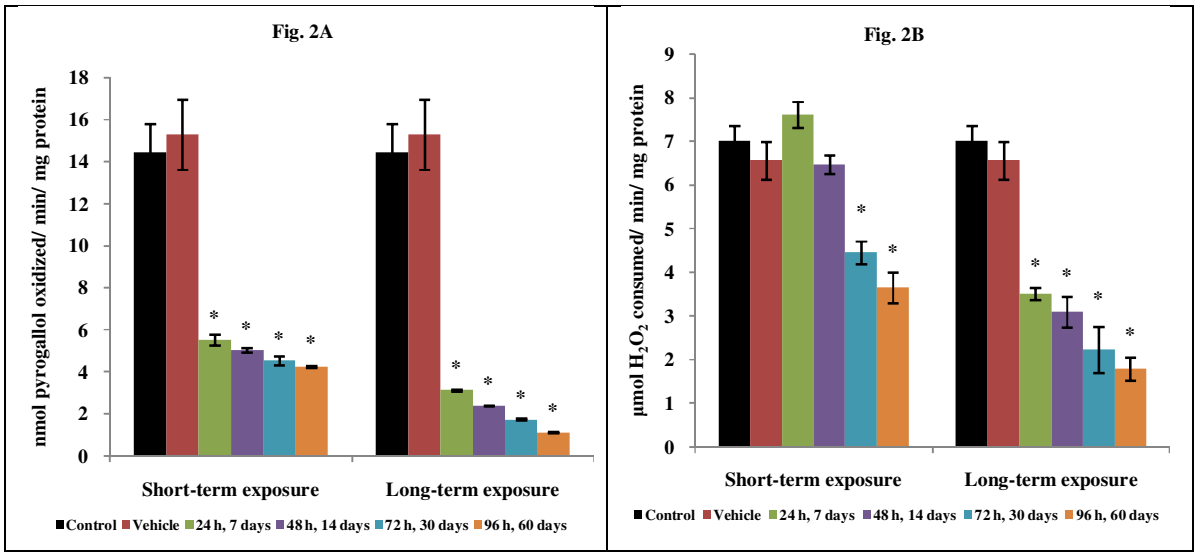

Fig. 2 A. Effect of DEHP on the activity of superoxide dismutase in the liver of the fish, *Oreochromis mossambicus.*
B. Effect of DEHP on the activity of catalase in the liver of the fish, *Oreochromis mossambicus.*

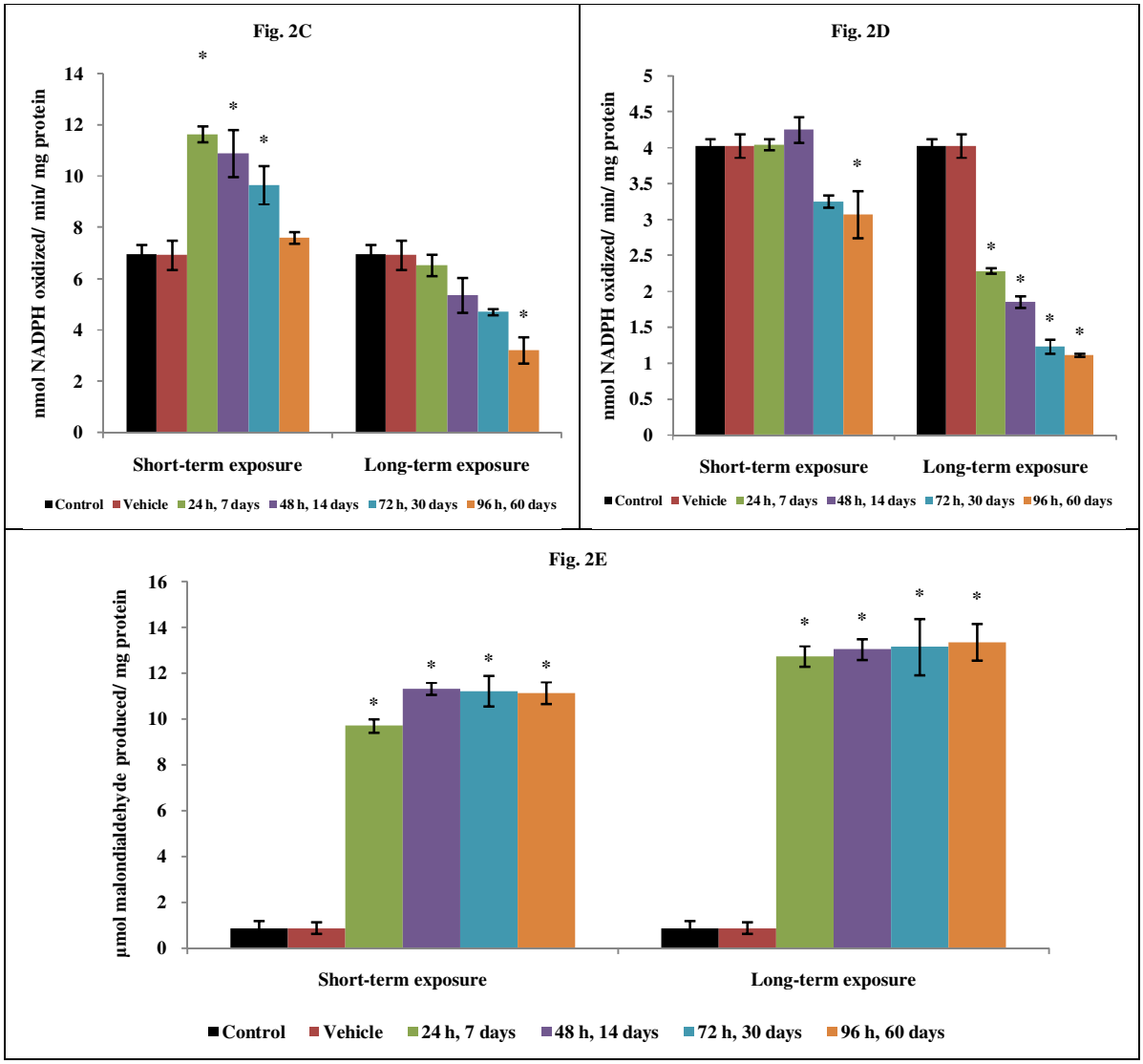

Fig. 2C. Effect of DEHP on the activity of glutathione reductase in the liver of the fish, *Oreochromis mossambicus.* **D.** Effect of DEHP on the activity of glutathione peroxidase in the liver of the fish, *Oreochromis mossambicus.* **E.** Effect of DEHP on the level of lipid peroxidation in the liver of the fish, *Oreochromis mossambicus.*

The activity of liver marker enzyme, alanine aminotransferase increased significantly ($P<0.05$) in time-dependent manner from 72 h (Fig. 4C) whereas aspartate aminotransferase activity showed significant ($P<0.05$) increase only in the long-term exposure group (Fig. 4D).

D. Effect of DEHP on muscle antioxidant system

The activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase showed significant ($P<0.05$) time-dependent reduction in both short-term and long-term exposure groups when compared to the control groups (Figs. 3A-D). Meanwhile, the level of lipid peroxidation increased significantly ($P<0.05$) in all exposure groups (Fig. 3E). However, the activities of acid and alkaline phosphatase, tissue specific markers showed significant ($P<0.05$) increase after DEPH exposure in time-dependent manner (Figs. 4E and F).

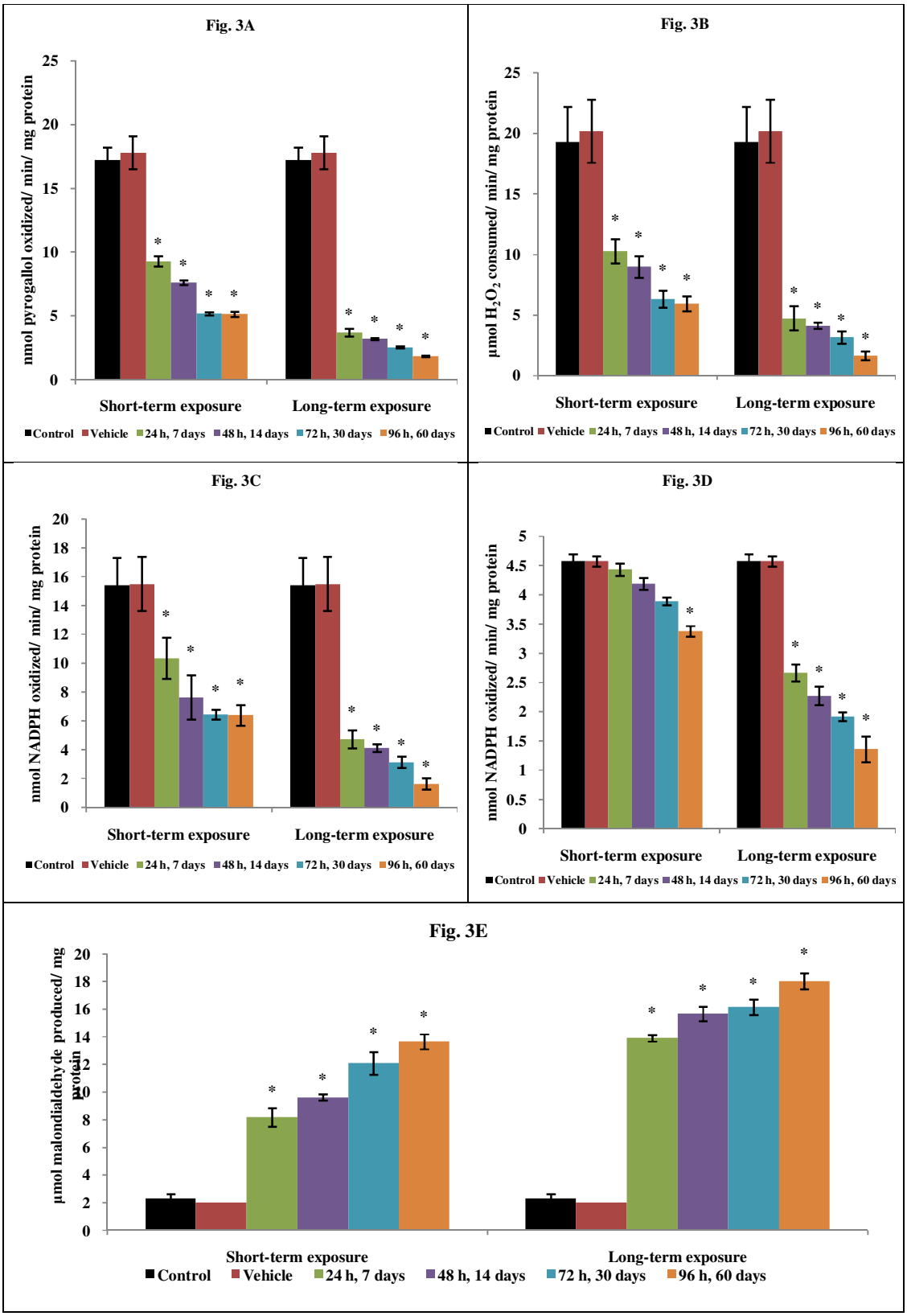

Fig. 3 A. Effect of DEHP on the activity of superoxide dismutase in the muscle of the fish, *Oreochromis mossambicus.* **B.** Effect of DEHP on the activity of catalase in the muscle of the fish, *Oreochromis mossambicus.* **C.** Effect of DEHP on the activity of glutathione reductase in the muscle of the fish, *Oreochromis mossambicus.* **D.** Effect of DEHP on the activity of glutathione peroxidase in the muscle of the fish, *Oreochromis mossambicus.* **E.** Effect of DEHP on the level of lipid peroxidation in the muscle of the fish, *Oreochromis mossambicus.*

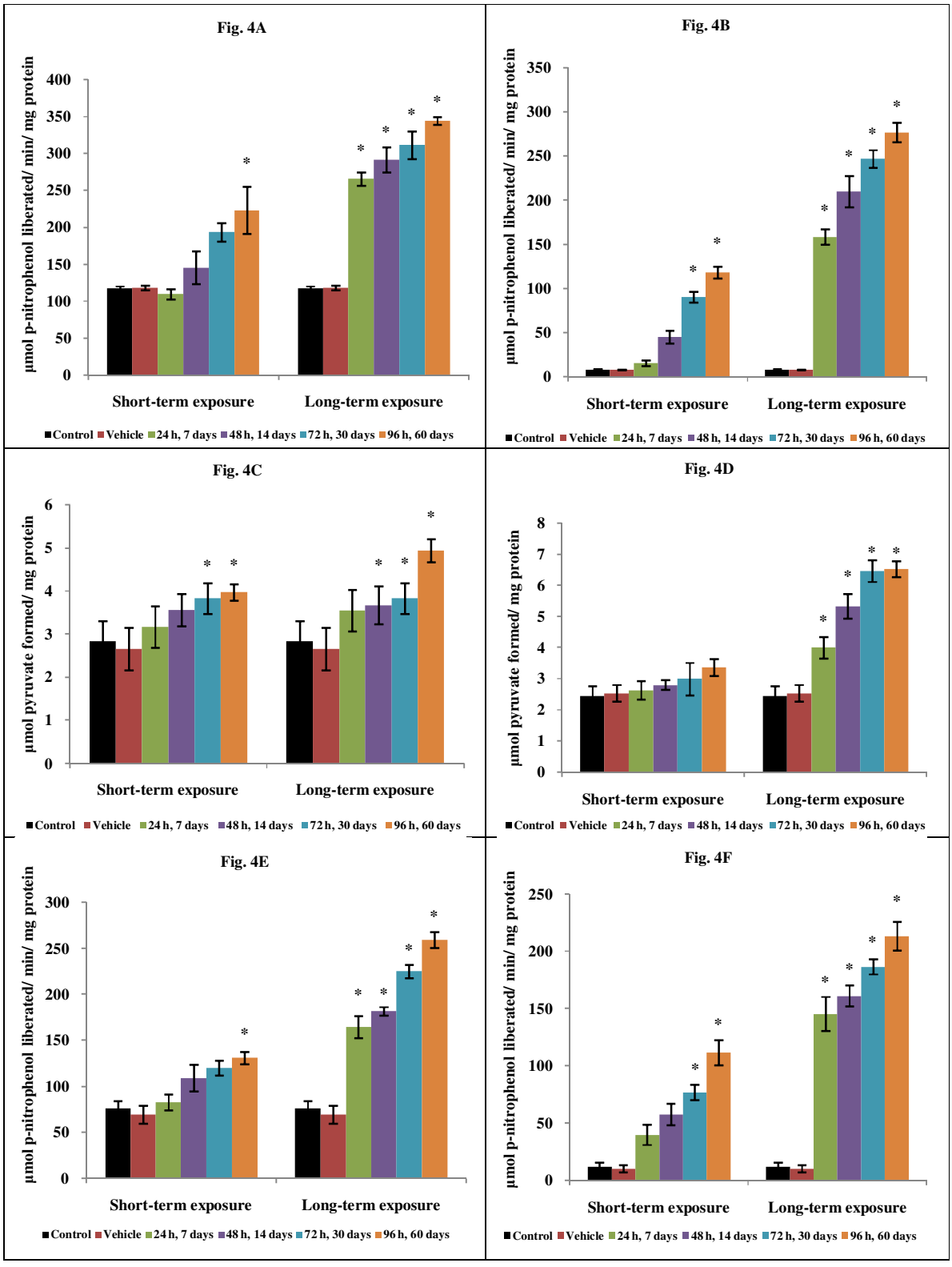

Fig. 4. A Effect of DEHP on the activity of acid phosphatase in the gill of the fish, O*reochromis mossambicus.* **B.** Effect of DEHP on the activity of alkaline phosphatase in the gill of the fish, *Oreochromis mossambicus*. **C.** Effect of DEHP on the activity of alanine aminotransferase in the liver of the fish, *Oreochromis mossambicus*. **D.** Effect of DEHP on the activity of aspartate aminotransferase in the liver of the fish, *Oreochromis mossambicus*. **E.** Effect of DEHP on the activity of acid phosphatase in the muscle of the fish, *Oreochromis mossambicus.* **F**. Effect of DEHP on the activity of alkaline phosphatase in the muscle of the fish, *Oreochromis mossambicus*.

IV. DISCUSSION

Phthalates are esters of phthalic acid widely used as plasticizers to enhance the flexibility and durability of polyvinyl chloride (PVC) products. Phthalates are added during the manufacture of plastics, cosmetics, printing ink, paper and selected types of packaging [26]. Phthalates has been shown to migrate easily to water, air, soil and food from the production site [27]. DEHP, one of the most popular phthalate plasticizers are ubiquitous in the environment and are identified as a source of phthalate pollution in the environment. DEHP are lipophilic thereby known to readily dissolved and accumulated in lipids of tissues. The evaluation of toxic effects of phthalates in aquatic organisms, especially on fish has been well documented in the form of embryonic deformalities [28], reproductive failure [29] and oxidative stress [30]. Fish is a notable model for assessing aquatic contamination and used as an environmental sentinel for water pollutants. Pollutants elicit toxicity through the generation of free radicals as a result of imbalance between the pro-oxidants and antioxidant defense system. Naturally all tissues are equipped with endogenous and exogenous antioxidants that act as a free radical scavengers. Free radicals are highly unstable molecules that have electrons to react with various macromolecules like proteins, lipids and nucleic acids [31]. Therefore, exposure to pollutants in the form of phthalates may modify the normal activities of antioxidant enzymes, undergo oxidation of proteins, lipids and nucleic acids and also disturb redox status of the cells or tissues. Therefore, in the present study DEHP induced toxicity was evaluated by assessing the antioxidant status in the vital tissues as gill, liver and muscles of the fish, *Oreochromis mossambicus*.

The body weight of fish remained unchanged throughout DEHP treatment, however, the weights of gill and liver tissues declined after 30 and 60 days indicating pollutant related necrosis or tissue damage. Exposure of DEHP caused alteration in the antioxidant defense system in gill tissue of the fish, which was evident by alteration in the activities of antioxidant enzymes. Superoxide dismutase is the first line of defensive enzyme against the generation of free radicals. The enzyme catalyzes the dismutation of superoxide anion radical ($O2^{\bullet -}$) into hydrogen peroxide by reduction. The activity of superoxide dismutase slightly increased after 24 h of DEHP exposure followed by significant decrease after 96 h onwards, which indicate the failure of the enzyme to remove the free radicals formed due to the toxicant exposure. Short-term and long-term exposure to DEHP increased the activities of catalase and glutathione reductase which indicates an attempt made by the enzymes to transform the hydrogen peroxide formed into water and oxygen. The present results showed reduction in the activity of glutathione peroxidase after 14 days of DEHP exposure and this could be due to the failure of the enzyme to eliminate the generated hydrogen peroxide in gill tissue though an effort was produced by catalase and glutathione reductase enzymes. Besides hydrogen peroxide, glutathione peroxidase has been shown to reduce lipid or non-lipid hydroperoxides [32]. It is a well-known fact that generation of free radicals severely damages the membrane lipids and thereby leads to the induction of lipid peroxidation. This was further proved by 20-25 fold increase in the level of lipid peroxidation after DEHP exposure. Thus the modification in the antioxidant enzymes in gill tissue of fish could be the cause of induction in the level of lipid peroxidation, one of the deteriorative reactions in cellular mechanism of toxicant induced oxidative stress. The activities of gill marker enzymes, acid and alkaline phosphatases increased after DEHP exposure owing to the disturbances of membrane transport and severe tissue damage attributed to toxicant related stress in gill tissue.

Liver is an important tissue associated to biotransformation and detoxification of pollutants and therefore they are highly equipped with antioxidant defense system to eradicate the free radicals formed in relation to the exposure of pollutants. In the present study DEHP exposure decreased the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase by concomitant increase in the level of lipid peroxidation. The results suggest the adaptive response of liver tissue against the reactive oxygen species formed. The impairment of antioxidant enzyme system may accumulate the free radicals formed in the liver tissue and that could be responsible for the induction of lipid peroxidation on DEHP exposure. Further, hepatic damages were evident by the increase in the activities of liver marker enzymes, alanine and aspartate aminotransferase. The rise in the activity of aminotransferases indicates the high energy demand of the tissue as it is strongly implicated in the production of energy and also considered as stress indicator [33].

Fish muscle is equipped with natural antioxidant defense system, which not only neutralizes the excessive reactive oxygen species, but also involved in myogenic regeneration, induce angiogenesis and reduce fibrosis. The present findings suggest that DEHP treatment decreased the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in time-dependent manner in both short-term and long-term exposure groups. Usually during muscular contraction, an increase in oxygen consumption occurs which through electron transport chain is reduced to water. However, about 5% of oxygen formed has been converted to superoxide and therefore, mitochondria in muscle tissue is regarded as the primary source for the formation of free radicals [32]. The reactive oxygen species formed are effectively scavenged by antioxidant defense enzyme system.

Oxidative stress in muscle tissue was evident by the failure of antioxidant status which was observed by the reduction in enzyme activities. The reduction in antioxidant enzyme activities plays a significant role in the induction of lipid peroxidation. Lipids are most susceptible to free radicals by attacking polyunsaturated fatty acids thereby leading to change in the properties of cellular membranes [34]. Further, increase in the activities of acid and alkaline phosphatase enzymes was noticed after DEHP exposure, which could be an adaptive mechanism of the fish to meet the energy demand. Similar results have been reported previously from our laboratory when Di-isononyl phthalate (DINP) was exposed to the fish, *Oreochromis mossambicus* [12]. The modification in the activities of these enzymes has been also associated to the disturbance in the structure and integrity of cell organelles, like endoplasmic reticulum and membrane transport system [35].

The short-term and long-term toxicity study concludes that DEHP exposure induced lipid peroxidation in gill, liver and muscle tissues leading to the formation of oxidative stress in the fish, *Oreochromis mossambicus*. Production, release and application of DEHP create major risk of exposure to aquatic ecosystems and thereby susceptible to adverse effects on the fish population, in addition to humans through the food chain.

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