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Effects of octylphenol on oxidative stress-mediated neurotoxicity in brain of the fish, *Oreochromis niloticus* (Linnaeus, 1758)

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Abstract
The neurotoxic effect of octylphenol was determined on the brain tissue of the tilapian fish, *Oreochromis niloticus*. Fishes were exposed to octylphenol at sublethal concentration, i.e., 50.6 µg/L for 24, 48, 72 and 96 h duration along with control groups. Octylphenol treatment showed no significant changes in the body weight and the weight of brain tissues. Exposure to octylphenol showed significant ($P<0.05$) increase in the activity of superoxide dismutase after 48 h. However, the activities of catalase and glutathione reductase showed significant ($P<0.05$) reduction in all treatment groups in time-dependent manner. The biochemical estimation of the levels of lipid peroxidation and hydrogen peroxide generation showed a significant increase in all treatment groups and this could be due to generation of reactive oxygen species in brain tissues. There was a significant decrease in the activity of acetylcholinesterase in the brain and blood serum after 48 h indicating neurotoxicity due to octylphenol treatment. Histological changes like atrophy, infiltration and neuronal cell degeneration were also observed in the brain tissues of the treated groups. Therefore, the present study suggests that octylphenol induced neurotoxicity by impairing the antioxidant status and also affects the histomorphology of brain tissues in the freshwater fish, *Oreochromis niloticus*.

Keywords: octylphenol, brain, neurotoxicity, oxidative stress, histopathology, *Oreochromis niloticus*

1. Introduction
Agricultural contaminants namely pesticides, fertilizers, manure, wastes from farms, slaughterhouse and poultry farms are drained from agricultural lands into water bodies through rain water run-off or by accidental spillage during transport. India is the largest manufacturer of basic pesticides in Asia and fourth position globally producing about 226 products including 75% pesticides, 12% fungicides and 10% herbicides [1]. Pesticides not only kill insect and pest, but it also enters the human body through drinking water or through food chain by biomagnifications. Most of the pesticides are less soluble in water, but are highly soluble in fats. Therefore, such contaminants are not only restricted to aquatic organisms but have been observed often in human food chain. The other major contaminants that pollutes natural aquatic ecosystem are man-made chemicals or xenobiotics possessing endocrine disrupting properties. It includes many pollutants, among which the most popular is alkylphenol ethoxylates. They are synthetic surfactants found in detergents, cleaning products, lubricants, pesticides, hair dyes and other hair care products, and even spermicides. Alkylphenols and their ethoxylates has been the subject of considerable regulatory attention due to its concern about aquatic toxicity and weak estrogenic activity.

Ethoxylates of alkylphenol namely nonylphenol ethoxylates and octylphenol ethoxylates are the most abundantly found contaminants. Although nonylphenol and octylphenol ethoxylates entering in sewage treatment are non-toxic and hydrophilic, degradation of the ethoxylates into the metabolites such as 4-nonylphenol and 4-*tert*-octylphenol during sewage treatment process are highly toxic [2]. Alkylphenols are known to bioaccumulate in body fluids and fats of animal tissues and have the ability to bind to estrogen receptors that interfere to the actions of endogenous estrogens [3]. The metabolite, nonylphenol is produced at 80% while the production of octylphenol is only at 15-20% of the total production, which is proved to contain estrogenic activity *in vitro* [4].

Octylphenol released into the environment from any sources are likely to be degraded rapidly by reacting with hydroxyl radicals.

However, in water and soil, octylphenol is known to persist for a long time and tend to bioaccumulate through the food chains. Majority (80%) of octylphenol is used in the production of *para-tert*-octylphenol based resins, which are used as tackifiers in tyre manufacture and also used for metals to rubber bonding applications in the technical rubber goods manufacture [5]. It is also reported that octylphenol is present as an impurity in commercial grade nonylphenol and that this way account for its detection in the environment. Several studies suggest that octylphenol is very toxic to aquatic organisms, as it is not easily degraded in the aquatic ecosystem.

Normal cellular function depends on a balance between the reactive oxygen species (ROS) produced and the antioxidant defense mechanisms available for the cell. Thus pro-oxidant and antioxidant balance is vital for normal biological functioning of the cells. If any of the complex components such as, environmental contaminants affecting this balance can provoke excessive production of reactive oxygen species that can be effectively scavenged by endogenous antioxidant defence system to prevent from internal cellular damage [6]. These electrophilic metabolites or radicals can readily interact with essential biomolecules, including DNA, proteins and lipids, leading to oxidative modification that finally leads to structural and functional alterations of the cells [7].

There are several evidences reporting the toxic effects of octylphenol on freshwater fishes, but the information remains scanty on the effect of this environmental contaminant on the brain antioxidant system in *Oreochromis niloticus*. Based on the above context the present work has been designed to carry out to study the neurotoxicity effect of octylphenol by evaluating the biomarkers of oxidative stress in the brain tissue of *O. niloticus* at sublethal concentration.

2. Materials and methods

2.1 Animal

Freshwater fish, *Oreochromis niloticus* weighing 14 ± 1 g and length 8 ± 1.5 cm were collected during June-October month, from the fish farm, Aquafish Aquarium, B.H. Road, Kottakal, Malappuram District. Fishes were acclimatized to the laboratory conditions for four weeks with a constant supply of water and good lighting system. They were maintained in well-aerated tubs (40 L capacity), which was dechlorinated and sustained with fresh water maintaining light and dark at 12: 12 h. The physico-chemical features of the tap water were estimated as per APHA [8]. Water temperature (28 ± 2°C), oxygen saturation of water (70 and 100 %), pH (6.5 to 7.5) were maintained and monitored using a standardized procedures.

2.2 Evaluation of median lethal concentration

The LC_{50} values in the respective time intervals were determined by probit analysis, with a confident limit of 5 % level for 96 h [9]. In the experiment, the concentration of octylphenol at which 50 percentage of the exposed fish undergo mortality at a specific period, i.e., for 96 h is LC_{50}-96 h is median lethal concentration of octylphenol. Fishes were not fed a day prior to and during the test to reduce fecal and excess food contaminating the test solution. For determining LC_{50} concentration, separate tanks of tap water (40 L capacity) were taken, which was dechlorinated and aerated using tubed motorized pumps. Monofilament netting was used to cover the tanks so as to prevent the specimens from jumping out of test solutions. Seven different concentrations (350, 400, 450, 500, 550, 600 and 650 µg/ L) of octylphenol were added in each separate tank. Octylphenol was dissolved in 1% DMSO (dimethyl sulfoxide) and was used as the solvent (vehicle) control in the experiment. Control tank, without toxicant, and vehicle control (DMSO) were also maintained along with the treatment groups. In both controls and experimental tanks, 10 fishes were introduced and mortality as well as the behaviour of fishes was recorded throughout the study. The lethal concentration for 50% killing (LC_{50}) values was computed on the basis of probit analysis for 96 h with a confident limit of 5 % level [9].

The above acute toxicity tests were repeated three times in order to confirm the mortality and to reduce the statistical errors. Data were analyzed by Probit of regression analysis as statistical method using SPSS 19.0 statistical analysis software. The LC_{50} value (with 95% confidence limits) was calculated, and then the correlation between mortality against concentrations and the best-fit line were also obtained.

2.3 Chemicals

Octylphenol (4-(1,1,3,3-tetramethylbutyl)phenol) of 90% purity was obtained from SISCO Research Laboratories Pvt. Ltd., Mumbai, India. Malondialdehyde, NADPH, glutathione oxidized, thiobarbituric acid, pyrogallol and dithiobisnitrobenzoic acid were obtained from Himedia Laboratories, Mumbai, India. Acetylthiocholine iodide was obtained from Alfa Aesar, England. All other chemicals were of analytical grade and obtained from local commercial sources.

2.4 Experimental design

One-tenth of median lethal concentration of octylphenol was selected as sublethal concentration i.e., 50.6 µg/L. The test concentration was maintained for four durations i.e., 24, 48, 72 and 96 h, respectively along with control fishes, positive and negative controls. Single concentration with different durations was used in the present study and ten fish specimens were used for every test and also in control groups. The first group of fishes was maintained in toxicant-free water and was used as control and the second group was treated with vehicle (1% DMSO) and served as positive control. The third group was treatment groups exposed to octylphenol at 50.6 µg/L concentration for 24, 48, 72 and 96 h, respectively.

2.5 Killing of animals

The fish was caught very gently using a small dip net, one at a time with least disturbance. At the end of each exposure time, fishes were weighed and decapitated. Brain of both control and all treated groups were dissected and stored at 4ºC until the biochemical analyses were performed.

2.6 Preparation of tissue samples for biochemical analysis

A 1% (w/ v) homogenate of brain was prepared in ice-cold normal saline with the help of a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenate was centrifuged at 800 *g* for 15 min at 4 °C to obtain the supernatant, which was then used for the analyses. Total protein concentration in the tissue was estimated by the method of Lowry *et al*. [10]. Hydrogen peroxide generation was assayed by the method of Pick and Keisari [11]. The levels of lipid peroxidation were measured via the thiobarbituric acid color reaction for malondialdehyde (MDA) at 535 nm, according to the method of Ohkawa *et al*. [12]. Activities of antioxidant enzymes such as superoxide dismutase [13],

catalase [14] and glutathione reductase [15] were assayed. The activity of acetylcholinesterase [16] was assayed in both tissue and blood serum.

2.7 Histopathology

At the end of treatment, brain tissue from control and treatment groups were dissected, rinsed in physiological saline to remove blood and debris and fixed in 10% buffered formalin for 24h. Tissues were dehydrated in ascending grades of alcohol and were cleared in xylene till the tissues become translucent. Tissues were then transferred to molten paraffin wax for an hour for complete impregnating with wax. The tissue blocks were made then tissues were cut in sections of thickness 4 to 6 microns using rotary microtome. The sections were double stained with haematoxylin and eosin and mounted in DPX [17]. The slides were carefully examined and photographs were taken using Cannon shot camera fitted to the Carl Zeiss Axioscope-2 plus Trinocular Research Microscope.

2.8 Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 19.0. Differences were considered to be significant at $p<0.05$ against control groups. Data are presented as mean ± SD for ten animals per group. All biochemical estimations were carried out in duplicate.

3. Results

The median lethal concentration for 96 h (LC_{50}-96 h) in the fish, *Oreochromis niloticus* observed by probit analysis was 506.17µg/L (Figure 1). Octylphenol exposure at sublethal concentration (50.6µg/L) showed slight reduction in the animal weight and brain tissue weights only after 96 h (Figure 2 and 3).

Sublethal exposure of octylphenol showed significant ($p<0.05$) increase in the activity of superoxide dismutase after 48 h of exposure (Figure 4). Meanwhile, the activities of catalase and glutathione reductase showed significant reduction in all treatment durations in time-dependent manner (Figures 5 and 6). The levels of hydrogen peroxide generation and lipid peroxidation showed a significant ($p<0.05$) increase in all treatment groups in time-dependent manner when compared to the corresponding control groups (Figures 7 and 8). There was a significant ($p<0.05$) decrease in the activity of acetylcholinesterase in the brain and blood serum after 48 h of octylphenol treatment in time-dependent manner than that of the corresponding control groups (Figure 9 and 10).

Control and vehicle tissues showed no histopathological alterations (Figures 11A and 11B). Octylphenol treatment showed several pathological lesions as atrophy, infiltration, degeneration and vacuolization in brain tissues (Figures 11C-11F). The severity of these modifications was observed in a time-dependent manner.

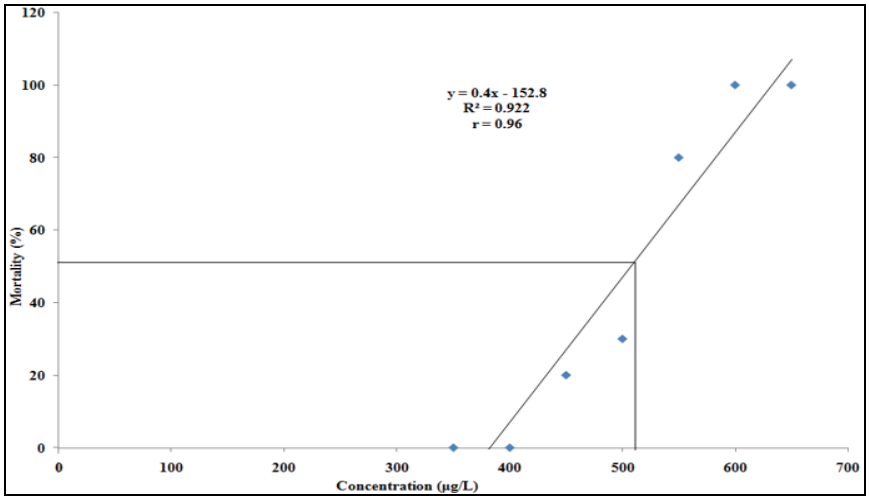

Fig 1: Effect of octylphenol on the median lethal concentration (LC_{50}-96 h) in the fish, *Oreochromis niloticus*

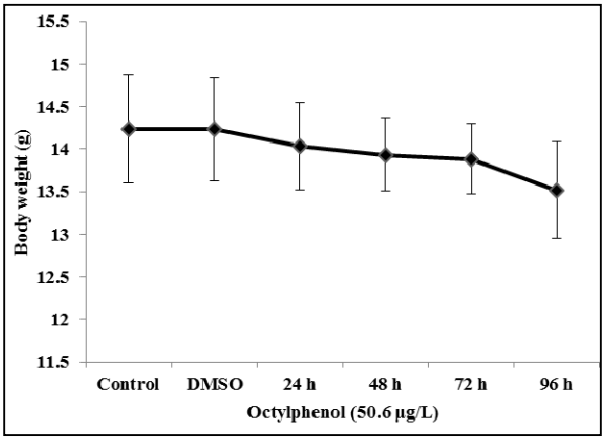

Fig 2: Effect of octylphenol on the body weight of the fish, *Oreochromis niloticus*

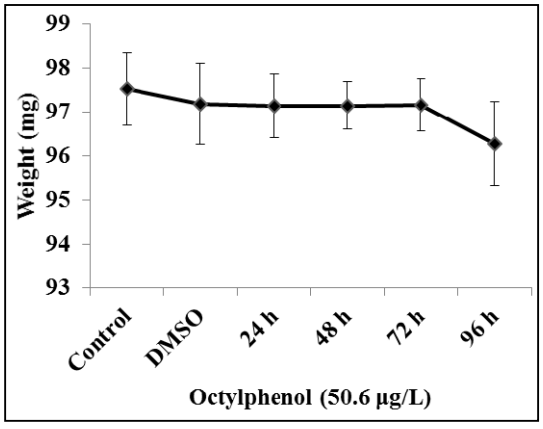

Fig 3: Effect of octylphenol on the weights of brain of the fish, *Oreochromis niloticus*

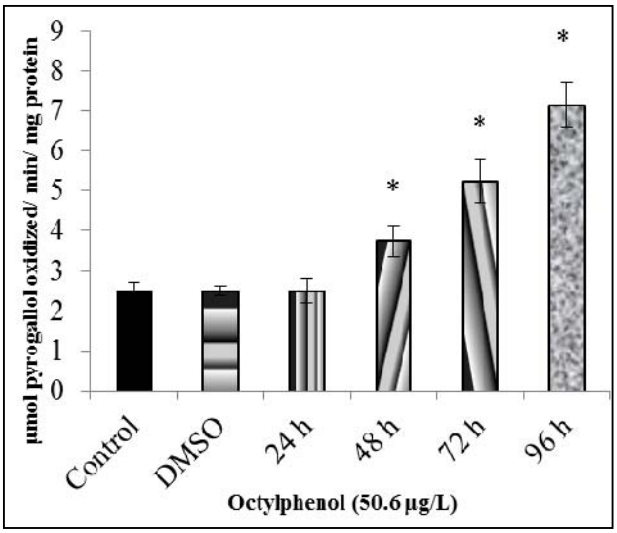

Fig 4: Effect of octylphenol on the activity of superoxide dismutase in the brain of the fish, *Oreochromis niloticus*

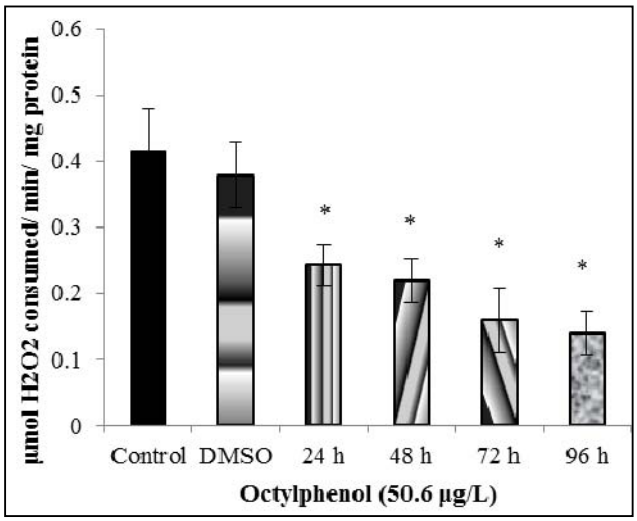

Fig 5: Effect of octylphenol on the activity of catalase in the brain of the fish, *Oreochromis niloticus*

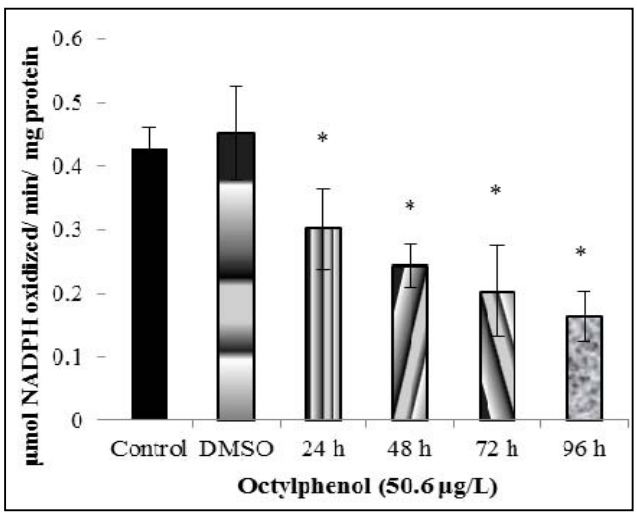

Fig 6: Effect of octylphenol on the activity of glutathione reductase in the brain of the fish, *Oreochromis niloticus*

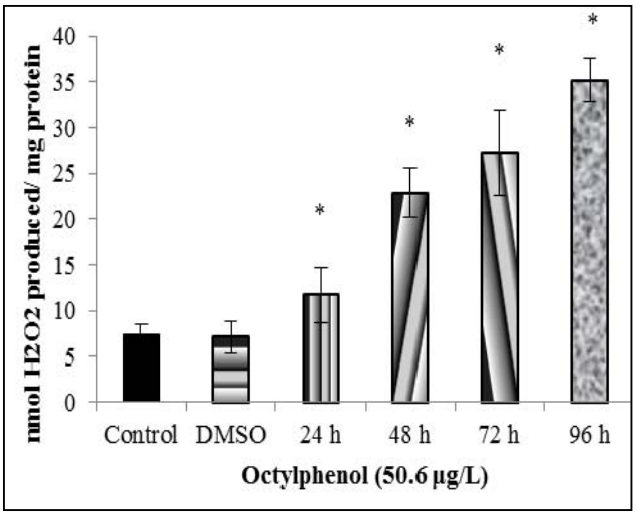

Fig 7: Effect of octylphenol on the level of hydrogen peroxide generation in the brain of the fish, *Oreochromis niloticus*

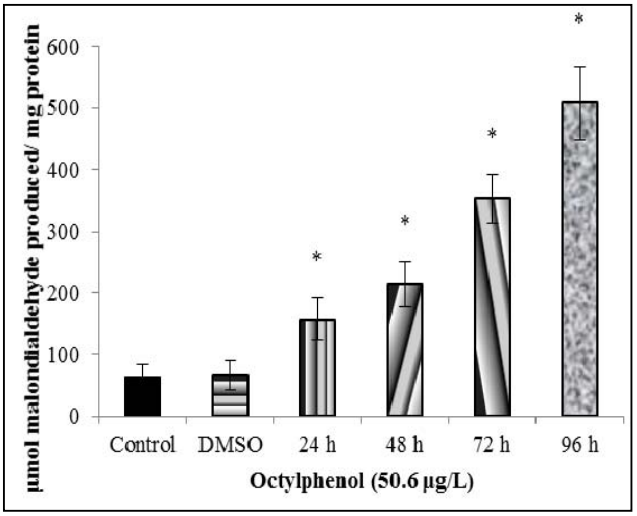

Fig 8: Effect of octylphenol on the level of lipid peroxidation in the brain of the fish, *Oreochromis niloticus*

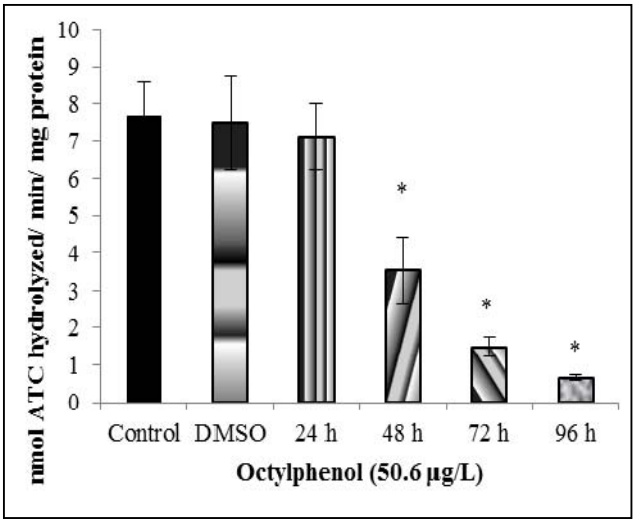

Fig 9: Effect of octylphenol on the activity of acetylcholinesterase in the brain of the fish, *Oreochromis niloticus*

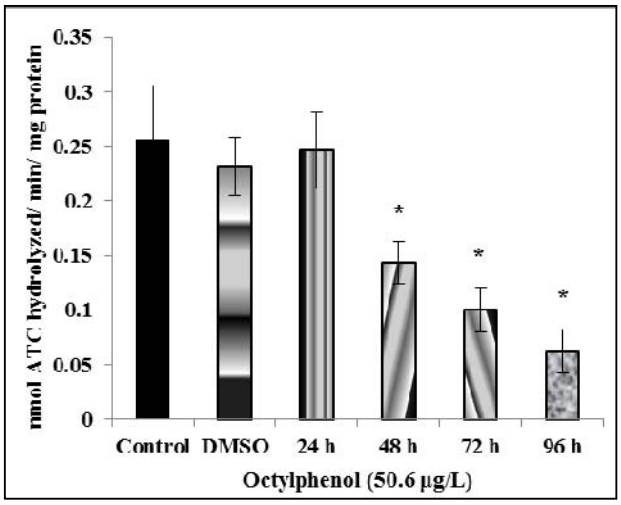

Fig 10: Effect of octylphenol on the activity of acetylcholinesterase in the serum of the fish, *Oreochromis niloticus*

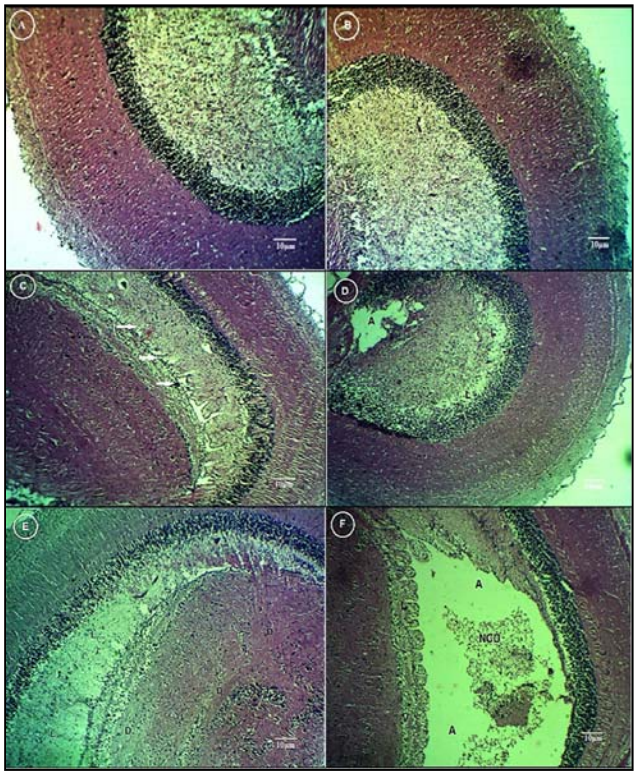

Fig 11: Photomicrographs of histological sections, stained with H&E in the control brain (A); Vehicle (B); C- showing infiltration (arrow) after 24 h of exposure; D- showing L-infiltration; A-atrophy after 48 h of exposure; E- L-infiltration; D-degeneration after 72 h exposure; F- A-atrophy; NCD-neuronal cell degeneration after 96 h of exposure in *Oreochromis niloticus*

4. Discussion

Release of large quantities of pollutants in the aquatic environment possibly results in large-scale sudden mortality of fish population. However, lower discharge of toxicants results in bioaccumulation in the aquatic organisms. Fish resources are natural assets which strengthens the economic status of the country. Recently there are many literatures reported the adverse effects on health status of aquatic organisms, particularly fish, due to the long-term exposure to certain pollutants. Octylphenol is one of the environmental toxicants widely used in household, industrial and agricultural products that enter the aquatic environment through wastewater. Octylphenol is frequently detected at low levels in aquatic ecosystems and have negative impact on fish population.

The purpose of the present study was to evaluate the oxidative stress responses and neurotoxicity potential of octylphenol in the fish, *Oreochromis niloticus*. The endpoint of octylphenol toxicity in brain tissue was evaluated by assessing the activity of antioxidant defensive enzymes. A wide range of differences in the oxidative damage has been reported in several aquatic animals from polluted region compared to non-polluted area. Brain is relatively more sensitive tissue to free radicals generated as a result of toxicant exposure. It is well known fact that any modifications in biochemical, cellular or tissue level induces a number of modifications in different vital organs such as, gill, liver, kidney, brain and reproductive organs. Accordingly, histomorphological changes induced by octylphenol in brain tissues were also evaluated to establish a relation of the toxicity of the compound.

Acute toxicity of octylphenol was performed based on the standard method prescribed in OECD guidelines. The median lethal concentration (LC_{50}-96 h) was investigated for 96 h by adopting Probit analysis [9]. Fish were exposed to the different concentrations of test solution ranging from 350 to 650 µg/L octylphenol and mortality was observed and recorded for 96 h. By using the basic program of probit analysis 95% confidence limit were calculated and it was observed as 506.17µg/L. Fish exposed to different concentrations of octylphenol showed abnormal behaviour. At the start of exposure, fish were aware to the change in the environment owing to the exposure of toxicant. It was noted by the sudden stopping of swimming where it remained static on the addition of octylphenol. After some time, fish tried to avoid the toxic water with fast swimming and jumping in order to engulp more air from the surface. Faster opercular movement was also observed at the time of surfacing to gulp more air. In tanks with higher concentrations of octylphenol, the fish showed more irregular and unsteady swimming with jerky movements and hyperexcitability. It resulted in imbalance of swimming which was observed by regular tapping on the sidewalls of the tank, energy became exhausted, lost consciousness and became lethargic. The fins became hard and stretched at the time due to stretching of body muscles. Finally, fish remained in vertical position without any movement for a few minutes with the anterior side or terminal mouth up near the surface of water, trying to gulp the air and the tail of fish faced in downward direction. Soon the dead fish settled towards the bottom of the tank showing bulged bellies with severe mucous deposition throughout the body and no movement confirmed the mortality of the fish. The death was regularly monitored and recorded, which showed high degree of positive correlation (r = +0.96) against the various concentrations. It was clear from the observation that higher the concentration, higher the mortality of the animal.

The body weight recorded after octylphenol exposure showed no significant changes when compared to the control groups, but a slight decrease was observed at the end of 96 h and it could be due to the toxic effects of octylphenol treatment. Similarly, the weight of brain showed a slight reduction only after 96 h of exposure while other treatment groups showed no significant changes than that of control groups. This could be due to the brain tissue damage as atrophy, degeneration

and vacuolization as observed in histological analysis. Also, it is evident that octylphenol can cross blood-brain barrier in fish to induce damage to brain tissues.

Brain is the master controlling centre of all organisms, and its structural complexity and functional diversity performs various biological functions. The barrier that exists between brain and blood is blood-brain barrier that prevents the entry of circulating toxins, drugs and xenobiotics from the systemic circulation into the brain tissue [18]. The present study focused on the effects of octylphenol on oxidative stress and neurotoxicity in the brain of the fish. Brain tissues are characterized by high levels of polyunsaturated fatty acids, and, therefore, more susceptible to toxicant-induced oxidative stress [19, 20]. The enhanced oxidative stress possibly leads to neurodegeneration and tissue damage [21]. It is evident from the present study that octylphenol induced oxidative stress by altering the activities of antioxidant enzymes and increase in the levels of lipid peroxidation and hydrogen peroxide. Lipid peroxidation is widely used as a biomarker of environmental stress, reflecting damage to cell membranes and tissue injury from free radicals [22]. The extent of damage caused by free radical production is dependent on the activities of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione reductase and peroxidase [23]. Therefore, antioxidant enzymes are the most commonly used toxicant-induced stress enzymes in ecotoxicological studies. Sublethal exposure to octylphenol significantly increased the activity of superoxide dismutase after 48 h of exposure whereas the activities of catalase and glutathione reductase showed significant reduction in all treatment durations in time-dependent manner. Superoxide dismutase is an enzyme that converts the highly reactive superoxide molecule to hydrogen peroxide. The increase in the production of more superoxide dismutase enzyme reflects the defensive mechanism of the fish to get rid of free radicals generated by the exposure to octylphenol. However, the activities of catalase and glutathione reductase decreased after octylphenol treatment and this indicates the failure of enzymes to eliminate free radicals generated and was confirmed by the increase in the level of hydrogen peroxide in all groups of octylphenol treatment in a time-dependent manner. Although hydrogen peroxide is not a free radical, but a reactive oxygen species, they possess higher activity than molecular oxygen [24]. The generation of more hydrogen peroxide has been shown to involve in the oxidation of cellular components such as proteins, lipids and DNA [25].

Octylphenol exposure at sublethal concentration increased the level of lipid peroxidation in time-dependent manner. The most widely used method to examine the level of lipid peroxidation is the production of malondialdehyde and it is measured with thiobarbituric acid reactive substances [26]. Though there are other new specific measures to examine the concentration of lipid peroxidation, in the present study malondialdehyde was measured as the end product. It was clearly obvious from the observation that exposure to octylphenol at sublethal concentration induced oxidative stress in the brain tissues of fish.

Acetylcholinesterase is the neurotransmitter enzyme found in neuromuscular junction and cholinergic nervous system that are capable of splitting the neurotransmitter acetylcholine to choline and acetate in order to terminate the synaptic transmission [27]. Acetylcholine plays an important role in sending the signal from one neuron to the other across the synapse. Therefore, acetylcholinesterase is an important biomarker to diagnose neurotoxicity induced by toxicant stress. The activity of acetylcholinesterase in the brain tissue and blood serum decreased significantly after 48 h of octylphenol treatment in time-dependent manner when compared to corresponding control groups. Octylphenol-induced inhibition of acetylcholinesterase summarizes neurotoxicity and alteration in behaviour of the exposed fish. Similar observations have been reported when one of the organochlorine pesticides, chlordecone, exposed to the freshwater cichlid fish, *Pseudetroplus maculatus* [21]. Further analysis of the results showed that the concentration of the enzyme is 20-fold higher in brain tissue when compared to the circulatory level of blood serum and therefore, it is clear that the toxicant, octylphenol induced neurotoxicity in brain tissue.

Neurotoxic effect of octylphenol was further confirmed by histological analysis in brain tissue of fish. Histopathological investigation is the sensitive tool to detect the direct effects of any toxicants within target organs of fish in laboratory condition. Histological studies are the proved document to establish correlation between tissue lesions in response to toxicant exposure. The present study showed no histological lesions in the brain of control and DMSO-treated groups. The major histological changes observed after 24 h of octylphenol exposure includes degeneration of granular layer and dilation of meningeal vessels that resulted in infiltration of mononuclear cells. After 48 h of octylphenol treatment, the cellular damage in the interior region of the brain was noted with severe atrophy of cells in cerebrum. Other pathological changes observed in the brain of 72 h treatment group includes more severity as shown by infiltration and complete degeneration of the nerve cells. After 96 h of octylphenol exposure, the lesion of brain was more prominent with vacuolization that leads to atrophy of the brain cells and neuronal cell degeneration that proves the neurotoxic effect of the toxicant. These observations were supported by the brain tissue damage in the fish due to exposure of chlordecone [21].

5. Conclusion

Based on the above discussed observations of the study, it can be summarised that exposure to octylphenol induced oxidative stress in the brain of the fish, *Oreochromis niloticus.* The neurotoxic effect of octylphenol at sublethal concentration was further proved by histopathological lesions which also lead to impaired behaviour in the stressed animal.

6. References

1. Dubey B, Kumar A. Agrochemical challenges and outlook for India. Chemical News. 2011; 14.
2. Ahel M, Giger W, Koch M. Behavior of alkylphenol polyethoxylate surfactants in the aquatic environment—I. Occurrence and transformation in sewage treatment. Water Research. 1994; 28:1131-1142.
3. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM *et al*. Endocrine-disrupting chemicals: an endocrine society scientific statement. Endocrine Reviews. 2009; 30:293-342.
4. Routledge EJ, Sumpter JP. Structural features of alkylphenolic chemicals associated with estrogenic activity. The Journal of Biological Chemistry. 1997; 272:3280-3288.
5. European Tyre, Rubber Manufacturers' Association (ETRMA). EU Risk Reduction Strategy proposed for octylphenol. 2007; 4.
6. Krieger TR, Loch-Caruso R. Antioxidants prevent

gamma-hexachlorocyclohexane-induced inhibition of rat myometrial gap junctions and contractions. Biology of Reproduction. 2001; 64:537-547.
7. Fernandez V, Massa L, Quinones L, Simon-Giavarotti KA, Giavarotti L, D'Almeida V *et al*. Effects of gamma-hexachlorocyclohexane and L-3,3›,5-triiodothyronine on rat liver cytochrome P4502E1-dependent activity and content in relation to microsomal superoxide radical generation. Biological Research. 2003; 36:359-365.
8. APHA. Standard methods for the examination of water and waste water, 20th Edition, Washington, DC, 1998.
9. Finney DJ. Probit analysis, 3rd (Ed.), Cambridge University Press, London. 1971; 333.
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry. 1951; 193:265-275.
11. Pick E, Keisari Y. Superoxide anion and H_2O_2 production by chemically elicited peritoneal macrophages-induced by multiple nonphagocytic stimuli. Cell Immunology. 1981; 59:301-318.
12. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 1979; 95:351-358.
13. Marklund S, Marklund G. Involvement of superoxide anion radical in antioxidation of pyrogallol and a constituent assay for superoxide dismutase. European Journal of Biochemistry. 1974; 47:469-474.
14. Claiborne A. Catalase activity. In: CRC Handbook of methods for oxygen radical research. R Greenwald (ed.), CRC Press, Boca Raton, Florida. 1985; 283-284.
15. Carlberg I, Mannervik BJ. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. The Journal of Biological Chemistry. 1985; 250:5474-5480.
16. Ellman GL, Courtney KD, Anders V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology. 1961; 7:88-90.
17. Roberts RJ, Smail DA. Laboratory methods In: Ronald J. Roberts, Fish pathology. Edn 3, Harcourt publishers limited. 2001, 380-390.
18. Eliceiri BP, Gonzalez AM, Baird A. Zebrafish model of the blood-brain barrier: morphological and permeability studies. Methods in Molecular Biology. 2011; 686:371-378.
19. Rejitha R, Asifa KP, Chitra KC. Induction of reactive oxygen species in brain of *Etroplus maculatus* after exposure to bisphenol A. Journal of Applied Natural Science. 2016; 8:386-391.
20. Sumi N, Chitra KC. Fullerene (C_{60}) induced alteration in the brain antioxidant system of the cichlid fish, *Pseudetroplus maculatus* (Bloch, 1795). Journal of Global Biosciences. 2017; 6:4908-4917.
21. Asifa KP, Chitra KC. Sublethal effects of chlordecone on histomorphology of the freshwater cichlid fish, *Pseudetroplus maculatus* (Bloch, 1795). Global Journal for Research Analysis. 2017; 6:497-499.
22. Ringwood AH, Conners DE, Keppler CJ, Dinovo A. Biomarker studies with juvenile oysters (*Crassostrea virginica*) deployed *in situ*. Biomarkers. 1999; 4:400-414.
23. Doyotte A, Cossu C, Jacquin M, Babut M, Vasseur P. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve, *Unio tumidis.* Aquatic Toxicology. 1997; 39:93-110.
24. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine, second ed. Clarendon Press, Oxford, UK, 1989.
25. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochemical Journal. 1997; 324:1-18.
26. Draper HH, Squires EJ, Mahmooch H, Wu J, Agarwal S, Handley M. A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. Free Radical Biology and Medicine. 1993; 15:353-363.
27. Tripathi A, Srivastava UC. Acetylcholinesterase: A versatile enzyme of nervous system. Annals of Neurosciences. 2008; 15:106-110.