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RESEARCH ARTICLE

Response of the freshwater fish, *Oreochromis mossambicus* to the environmental pollutant, nonylphenol

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Abstract

Exposure to nonylphenol on adult freshwater fish, *Oreochromis mossambicus* at sublethal concentration (0.15 mg/ L) for 24 h, 96 h and 7 days did not alter the body weights of the animal when compared with control groups. However, the weight of gill significantly decreased at 7 days of exposure and this was evidenced due to necrosis or atrophy of gill lamellae. Nonylphenol significantly decreased the activities of antioxidant enzymes with concomitant increase in the level of hydrogen peroxide and lipid peroxidation in all treatment groups thereby denoted that nonylphenol upsets the pro-oxidant and antioxidant status in the gills. A significant decrease in the activity of succinate dehydrogenase, a gill marker enzyme, at 96 h could be due to the impairment of aerobic metabolism and stress-related shift towards anaerobiosis at organ level in nonylphenol-treated fishes. Histopathology of gills showed three principal alterations as lamellar fusion, hypertrophy of lamellar epithelium and the mucous deposition over the respiratory epithelium. To summarize, the toxicity of nonylphenol was by the generation of reactive oxygen species in gill of the fresh water fish, *Oreochromis mossambicus*.

Introduction

Environmental pollution is any discharge of material or matter into water, land, or air that cause acute or short-term and sometimes chronic or long-term detriment to the ecosystem and finally that lowers the quality of life. A wide range of man-made chemicals are released either deliberately or unintentionally into the aquatic environment. These chemicals are known as the 'environmental pollutants'. Alkylphenols (AP) are a family of chemicals that are formed when another group of chemicals, the alkylphenol ethoxylates (APEs) partially biodegrade. The most important member of the alkyphenol family is nonylphenol. Approximately 80 percent of the alkylphenols in commerce are available as nonylphenols. APEs are high volume chemicals that have been used for over 50 years as commercial and consumer detergents. Nonylphenol, an important class of non-ionic surfactants is widely used for a variety of industrial, household and commercial applications including plastics, cosmetic products, inks, paints and textiles.

Nonylphenol-surfactants are exposed to both marine and aquatic life when the substantial quantities of the toxicants get discharged into wastewater then it biodegrades into several by-products, including nonylphenol. While a significant source of nonylphenol in the environment, which is unreacted nonylphenol in plastic may result in direct human exposures when the chemical leaches out of plastic in close contact with foods. Nonylphenol is moderately lipophilic and its bioconcentration is approximately 7000 times in the macrophytic algae, leading to levels of nonylphenol of up to 38 mg/ kg (Ahel et al., 1993). In the higher organisms the bioconcentration levels

may include biomagnification through the food chain, and also uptake of sediment containing higher levels than in the water thus accumulate in both freshwater and marine organisms (Ahel et al., 1993).

Nonylphenol is one of the most studied estrogen mimics that appear to interact with development in several organisms (Vazquez-Duhalt et al., 2006). The effects of nonylphenol on different organisms have been reported including developmental abnormalities, changes in the sex ratio towards females, increase in the incidence of hermaphroditism, reduction of fecundity, reduction of gonadal development and reproductive function, decrease in egg production and reduction in egg viability, changes in testosterone conversion rate, vitellogenin induction and many others (Vazquez-Duhalt et al., 2006). When nonylphenol administered orally to male Wistar rats at 1, 10, and 100 mg/kg/day for 45 days significantly decreased the weights of the testes and epididymides and also decreased epididymal sperm counts in a dose-dependent manner while the activities of antioxidant enzymes as superoxide dismutase, catalase and glutathione reductase also has been significantly decreased (Chitra et al., 2002).

Many of nonylphenol's effects on aquatic organisms are attributed to its estrogenic activity, but it also causes toxic effects that are not obviously related to its estrogenic activity, such as effects on growth, behavior, respiration, and osmoregulation. Nonylphenol at sublethal concentration caused genetic damage in freshwater fish, *Oreochromis mossambicus* as evidenced by micronucleus test and Salmonella mutagenicity test (Balakrishnan et al., 2014). There are sufficient qualitative information on acute, chronic, reproductive and developmental toxicity of nonylphenol to aquatic organisms. However, there is no information on the antioxidant status of nonylphenol and its role in the generation of oxygen free radicals after the exposure to the pollutant to fishes. Thus the present study focused on the above issues in the fresh water fish, *Oreochromis mossambicus*.

Materials and Methods:

Fresh water fish, *Oreochromis mossambicus* weighing 6.5 ± 2 g and length 7.5 ± 1 cm were collected from Kaloos fish farm, Malappuram District, Kerala, India. Fishes were acclimatized to the laboratory conditions for four weeks with constant supply of water and good lighting system. During the period of acclimatization, fishes were fed everyday with standard fish pellets. Bath was changed every 24 hour, which was dechlorinated, respectively.

The physico-chemical features of the tap water were estimated as per APHA (1998). Water temperature in the test ranged from 28 ± 2°C during the experiment, oxygen saturation of water ranged between 70 and 100 %, pH is 6.5 to 7.5 which were monitored using a standardized procedures. The LC_{50} values in the respective time intervals were determined by probit analysis, with a confident limit of 5 % level (Finney, 1971), which was 1.5 mg/ L. One-tenth of the dosage (0.15 mg/ L) nonylphenol was chosen to represent sub-lethal concentration.

The specimens were not fed a day prior to and during toxicity tests to reduce faecal and excess food contaminating the test solution. Ten specimens were placed in each tub and were maintained in each test and control groups, they were then aerated using tubed motorized pumps. Monofilament netting was used to cover the tanks to prevent the specimens from jumping out of test solutions. The behaviour of specimens was observed and death was also recorded throughout the study.

Treatments:

There were four groups, three tanks each with toxicant doses maintained for 24 h, 96 h and 7 days, respectively and a tank with control fishes. Single dose with different durations were used in present study. Ten fish specimens were used for every test and also in control groups. The first groups of fishes were maintained in toxicant-free water and were used as control and the second group was treated with nonylphenol at 0.15 mg/ L for 24 h. The third group was treated with nonylphenol at 0.15 mg/ L for 96 h and fourth group was treated with nonylphenol at 0.15 mg/L for 7 days. Biochemical estimation of gill was performed at the end of every treatment and the histopathology was also done at the end of experiments, maintaining the control group.

Tissue processing:

A 1% (w/ v) homogenate of gill 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 8000 ***g*** for 15 min at 4°C to obtain the supernatant, which was then used for the analyses.

Biochemical analysis:

Total protein concentration in the tissue was estimated by the method of Lowry et al. (1951). 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. (1979) and the results were expressed as nmol of MDA produced/ min/ mg protein. Hydrogen peroxide generation was assayed by the method of Pick and Keisari (1981). Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Marklund and Marklund (1974). Catalase (EC. 1.11.1.6) was assayed by the method of Claiborne (1985). The activity of succinate dehydrogenase (EC.1.3.5.1) was assayed by the method of Slater and Bonner, 1952.

Histopathology:

Gill tissue collected by sacrificing the fish was fixed in 10 % buffered formalin for 24 hours. Tissue was dehydrated in ascending grades of alcohol and was cleared in xylene until they became translucent. It was then transferred to molten paraffin wax for 1 hour to remove xylene completely and then impregnated with wax. Then the blocks were cut in a rotary microtome to prepare sections of thickness 4 to 6 microns. The sections were stained with haematoxylin and eosin and mounted in DPx. The structural alteration was observed under light microscope in the sections of gill of fish and was compared with those of control tissues. Photomicrographs were taken using Cannon shot camera fitted to the Carl Zeiss Axioscope 2 Plus Trinocular Research Microscope.

Statistical analyses:

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 17.0. Differences were considered to be significant at $p<0.05$ against control group. Data are presented as mean ± SD for ten animals per group. All biochemical estimations were carried out in duplicate.

Results:

Administration of nonylphenol at the sub lethal concentration of 0.15 mg/ L showed no significant changes in the body weights of fishes after 24 h, 96 h and 7 days of treatment (Table 1). At the end of every treatment, nonylphenol-treated fishes showed a remarkable deposition of mucous all over the body with the percentage increased to 50% when compared with those of control groups (Table 1). Nonylphenol treatment significantly decreased the weights of gill after 7 days of treatment. However no changes were observed at the end of 24 h and 96 h of exposure (Table 1).

Administration of nonylphenol for 24 h and 96 h significantly ($p<0.05$) decreased the activities of superoxide dismutase and catalase in the gills of fishes in concomitant manner as compared with the corresponding group of control animals (Table 2). However after 7 days of treatment the activities of superoxide dismutase and catalase slightly increased than 24 h and 96 h groups but it was significantly decreased when compared with control groups (Table 2). The level of hydrogen peroxide and lipid peroxidation increased significantly ($p<0.05$) at 24 h, 96 h and 7 days of nonylphenol exposure in gills than that of control animals (Table 2).

There was a significant ($p<0.05$) decrease in the activity of succinate dehydrogenase in the gill of nonylphenol-treated fishes after 96 h of exposure. On the other hand, no changes in the activity of succinate dehydrogenase were observed at 24 h and 7 days of nonylphenol treatment as compared with the control group (Table 2).

Histopathological observations showed morphological alterations in the gills when compared with the control groups. The analysis of the control group showed that the gill lamellae are separated from each other (Fig 1A), but in the treated group, after 7 days of nonylphenol exposure, presented three principal alterations: some of the gill lamellae are united by expansions, that is lamellar fusion is observed (Fig 1B); hypertrophy of lamellar epithelium (Fig 1C); and mucous deposition over the respiratory epithelium (Fig 1D).

Table 1 Effect of nonylphenol on the body weight and tissue weights of the fresh water fish, *Oreochromis mossambicus*

Nonylphenol (0.15mg/ L)	Body weight (g)		% of mucous secreted	Weight of gill (g)
	With mucous	Without mucous		
Control	7.82 ± 0.49	7.52 ± 0.52	29.4 ± 0.45	0.75 ± 0.09
24 h	8.21 ± 0.35	7.84 ± 0.33	46.9 ± 0.30*	0.70 ± 0.13
96 h	8.42 ± 1.36	8.14 ± 1.45	47.5 ± 1.30*	0.68 ± 0.13
7 days	8.32 ± 0.77	7.85 ± 0.80	47.1 ± 0.75*	0.62 ± 0.21*

Data are expressed in Mean ± SD for 10 animals per group. Asterisks (*) denote the p value set significant at 0.05 level of significance against the control groups.

Table 2 **Effect of nonylphenol on the biochemical parameters of the fresh water fish, *Oreochromis mossambicus***

Nonylphenol (0.15mg/ L)	Control	24 h	96 h	7 days
Superoxide dismutase [a]	0.226 ± 0.034	0.148 ± 0.072*	0.009 ± 0.007*	0.036 ± 0.022*
Catalase [b]	10.66 ± 2.52	1.30 ± 0.72*	0.25 ± 0.19*	2.94 ± 1.7*
Hydrogen peroxide [c]	2.53 ± 0.47	6.85 ± 2.52*	8.91 ± 3.92*	16.23 ± 3.62*
Lipid peroxidation [d]	8.81 ± 1.13	18.24 ± 8.02*	18.73 ± 6.13*	85.08 ± 13.26*
Succinate dehydrogenase [e]	5.19 ± 0.11	5.20 ± 0.15	3.31 ± 0.26*	4.52 ± 0.32

Data are expressed in Mean ± SD for 10 animals per group. Asterisks (*) denote the p value set significant at 0.05 level of significance against the control groups.

a nmol pyrogallol oxidised/ min/ mg protein
b µmol of hydrogen peroxide consumed/ min/ mg protein
c nmol hydrogen peroxide generated/ min/ mg protein
d nmol of malondialdehyde produced/ min/ mg protein
e mg of formazan formed/ min/ mg protein

Figure 1A-D Effect of nonylphenol on the histology of gill of the fish, *Oreochromis mossambicus* using Hematoxylin and Eosin stains

Figure 1A **Histology of gill from control showing the gill lamellae separated from each other (40x magnification).**

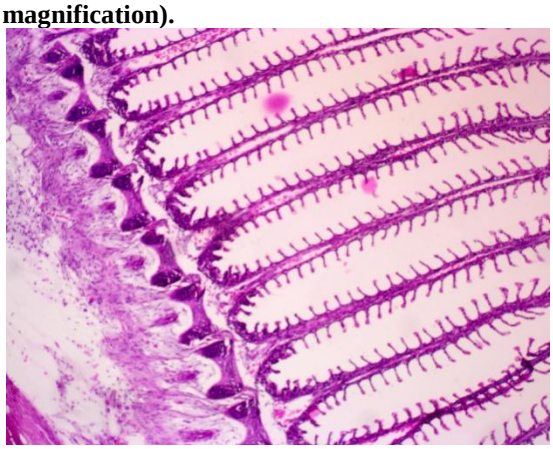


Figure 1B **Histology of gill exposed to nonylphenol for 7 days showing lamellar fusion (40x magnification).**

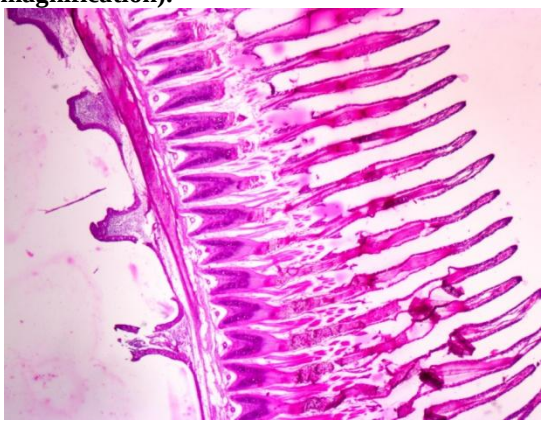


Figure 1C **Histology of gill exposed to nonylphenol for 7 days showing hypertrophy of lamellar epithelium (40x magnification).**

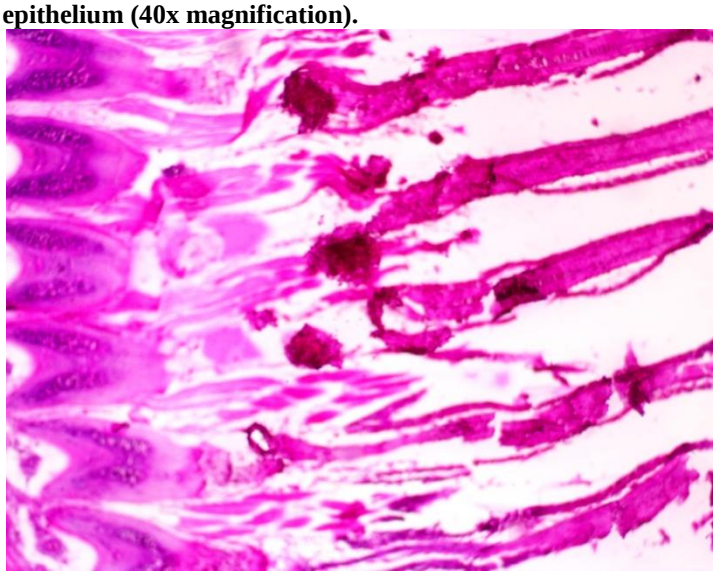
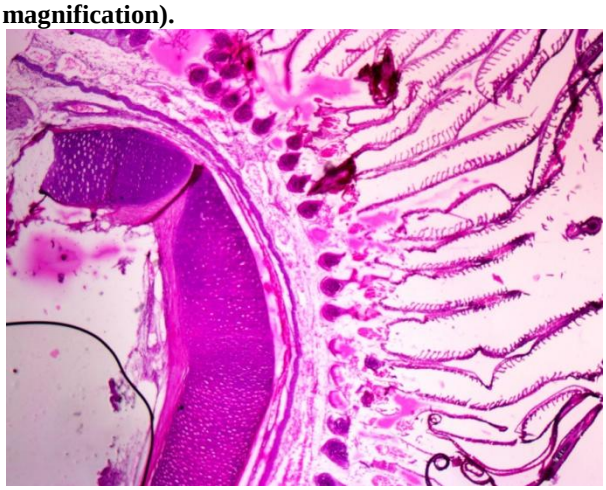


Figure 1D **Histology of gill exposed to nonylphenol for 7 days showing mucous deposition (40x magnification).**



Discussion:

Water is one of the essential factors for the maintenance of the vital functions of the living beings. Unfortunately, the aquatic organisms are greatly exposed to several industrial and domestic wastes and it has increased a great concern in public health. In the present study nonylphenol, one of the environmental pollutants was used and its exposure showed no significant changes in the body weights of fishes. The increase in the mucous production was observed after exposure to nonylphenol in all the treatment groups. Mucous cells are considered efficient in seizing the toxic agents and thus help in the prevention of the entrance of these agents into the gills (Perry and Laurent, 1993). Hypersecretion of mucous may be the consequence of a chronic defensive mechanism of the fish against the exposure to the environmental toxicant nonylphenol. Mucous are normally seen in the filaments, but the mucous can also be found on the respiratory epithelium of fishes only when exposed to stress conditions, which suggest that the mucous layer protects the lamellar surfaces against the toxic agents (Mallat, 1985). Moreover the presence of mucous is an indicative of presence of toxic substances in the water and this could lead to the functional alterations and intrusion in fundamental process such as osmoregulation and antioxidant defense of gills. The presence of mucous in gill was supplemented with histopathological observation of the gill tissue.

In the present study nonylphenol treatment for 7 days significantly decreased the weight of gill, but no such changes were observed at the end of 24 h and 96 h of exposure. This could be possibly due to necrosis or atrophy of gill filament which is evidenced by the histopathological observations. Fish gills are constituted by primary filaments and secondary lamellae, which are formed basically by three different cell types: pillar cells, respiratory cells and erythrocytes that circulate in the lamellae interior. Gills are the first target organ of waterborne pollutants since it is constantly in contact with the water accomplishing gas exchanges; besides this it presents a high adaptation capacity also.

Gills also participate in many important functions in fish such as respiration, osmoregulation and excretion, also remain in close contact with the external environment and particularly sensitive to changes in the quality of the water and thus considered as the primary target of the contaminants (Poleksic and Mitrovic-Tutundzic, 1994). Histological alterations observed in fish gills are acknowledged as a fast and valid method to determine the damages caused by exposure to different pollutants in fishes (Arellano et al., 2001). Alterations like epithelial lifting, hyperplasia and hypertrophy of the epithelial cells, besides partial fusion of some secondary lamellae are examples of defense mechanisms, in general, these results in the increase of the distance between the external environment and the blood and thus serve as a barrier to the entrance of contaminants (Mallatt, 1985).

In the present study exposure to nonylphenol at sub lethal dose for 7 days caused three principal alterations in the gills of fishes as some of the gill lamellae are united by expansions, that is lamellar fusion is observed, hypertrophy of lamellar epithelium and mucous deposition over the respiratory epithelium. Interstitial edema is also one of the more frequent lesions observed in gill epithelium of fish exposed to nonylphenol. Edema with lifting of lamellar epithelium could serve as a mechanism of defense, because separation of epithelial lamellae increases the distance so that the waterborne pollutants must diffuse to reach the bloodstream (Arellano et al., 1999). Lesions in the gill morphology also lead to functional changes and interference in fundamental process such as maintenance of osmoregulation and antioxidant defense of gills.

Exposure to nonylphenol significantly decreased the activities of antioxidant enzymes as superoxide dismutase and catalase, however, it significantly increased the levels of hydrogen peroxide and lipid peroxidation in the gills of fishes in all treatment groups. In general, repeated doses of nonylphenol for a long period of time, may induce a defensive response in *Oreochromis* meanwhile, acute exposure causes inhibition of antioxidant activities.

Superoxide anion ($\bullet O_2^-$), hydroxyl radical ($\bullet OH$) and hydrogen peroxide (H_2O_2) that inevitably form during the metabolism of oxygen, especially in the reduction of oxygen by the electron transfer system of mitochondria are the three species, together with unstable intermediates in the peroxidation of lipids, are referred to as Reactive Oxygen Species (ROS). Damage from ROS as a result of an imbalance between radical-generating and radical-scavenging systems results in a condition called oxidative stress.

Free radicals/ ROS generated in tissues and in sub-cellular compartments are effectively scavenged by the antioxidant defence system, which constitutes antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase. Superoxide dismutase (SOD) catalysis the dismutation of superoxide radical to hydrogen peroxide (H_2O_2) and oxygen (O_2). The conversion of H_2O_2 to $2H_2O$ is by the enzyme glutathione peroxidase and the conversion of H_2O_2 to O_2 and H_2O is by the enzyme catalase. All these enzymes are inevitable for the cell to ROS detoxification (Sikka, 2001).

In the present study a decrease in the activity of superoxide dismutase has been shown to increase the level of superoxide anion, which is known to inactivate catalase activity (Kono and Fridovich, 1982). Similarly, catalase or glutathione peroxidase has been shown to eliminate hydrogen peroxide from the cell leading to the inactivation of superoxide dismutase and generation of lipid peroxides (Bray et al., 1974).

The mitochondrial respiratory stress marker enzyme succinate dehydrogenase is primarily involved in the oxidative catabolism of sugars, which is used as an indicator of osmoregulatory activity. This enzyme is concentrated in the chloride cells within fish gills. In the present study exposure of adult *Oreochromis* at sub lethal concentration of nonylphenol resulted in a significant decrease in succinate dehydrogenase activity in gill after 96 h, which may be due to the impairment of aerobic metabolism in nonylphenol-treated fishes (Rajeswari et al., 1989). Similar observation was noted in the treatment of malathion at 96 h in the freshwater fish, *Oreochromis mossambicus* (Chitra and Mohan, 2013). Inhibition of stress marker enzyme could be considered as an important marker to indicate the state of fish health and their physiological conditions. The result shows that fish exposed to nonylphenol is unable to bear the stress caused due to the pollutant and resulted in a shift towards anaerobiosis at organ level during sub lethal intoxication. It is evident from the present study that the generation of oxygen free radicals induced lipid peroxidation in gill tissues and this could be due to the sub lethal toxicity of nonylphenol in the fresh water fish, *Oreochromis mossambicus*.

It can be, therefore, summarized that the present study provides unequivocal evidence for a highly significant correlation between exposure of *Oreochromis mossambicus* to nonylphenol and a substantial increase in the production of reactive oxygen species in gill tissues. These data do not warrant a causal connection per se, but taken together with reports in the literature, they strongly suggest a consequence of imbalance in pro-oxidant and antioxidant balance in gill was due to the exposure of nonylphenol.

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