



Impact of nonylphenol on oxygen consumption, haematological and histological parameters in the cichlid fish, *Pseudotropheus maculatus* (Bloch, 1795)

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Abstract

The present study was designed to investigate the acute sublethal toxic effects of nonylphenol in the south Indian cichlid fish, *Pseudotropheus maculatus*. The fish was exposed to sublethal concentrations of nonylphenol (89 and 178 $\mu\text{g/L}$) for 24, 72 and 96 h durations. The effects of nonylphenol on the rate of oxygen consumption, haematological and histopathological parameters were evaluated. Nonylphenol treatment at both sublethal concentrations caused significant increase in the rate of oxygen consumption up to 48 h, followed by a decrease after 72 h when compared to the control groups. Haematological parameters such as erythrocyte count were decreased whereas total leukocyte count increased significantly in concentration and time-dependant manner. Nonylphenol exposure altered normal architecture of gill as revealed by curling and destruction of secondary lamellae, aneurysm and hyperplasia in gill arches. Hepatic cells showed cytoplasmic vacuolization, degeneration of hepatocytes and severe necrosis. The present study revealed that nonylphenol caused alterations in oxygen consumption and haematological parameters along with degenerative changes in various vital organs thereby proving non-estrogenic adverse effects, which possibly disturb general health condition of fish in natural population.

Keywords: nonylphenol, *pseudotropheus maculatus*, oxygen consumption, haematology, histology, gill, liver

Introduction

Nonylphenol is an intermediate in the manufacture of commercially important non-ionic surfactant nonylphenol ethoxylate, which is extensively used in detergents, paints, lubricating oils, pesticides, emulsifiers, plastics and personal care products [1]. Nonylphenol is grouped under 'high production volume chemicals' because of the exponential increase in the manufacture and it has been reported that the global production exceeds 100 to 500 million pounds by every year [2]. Due to its widespread domestic and industrial applications, alkylphenol ethoxylates, especially nonylphenol ethoxylates are frequently found in wastewater discharges, sewage treatment plant effluents or in the environment, from where it is degraded into more persistent metabolites such as alkylphenols including nonylphenol and octylphenol. Toxicological studies have reported the existence of metabolites of alkylphenol ethoxylates in various environmental compartments such as air, water, soil, sediments and surface waters. Most of the metabolites are more toxic than the parent compound and have the potential to mimic natural hormones by interfering with the endocrine system [3].

Nonylphenol enters into aquatic ecosystems from variety of sources such as industrial wastes, domestic wastewater discharges, effluents from wastewater treatment plants, agricultural activities, transportation systems and through atmospheric deposition [4]. The consequence of widespread use and production of nonylphenol increase possible exposure pathways in aquatic environment from the upper rural portions of the river to the urbanized and industrialized lower segments [5]. Moreover, the persistence nature of nonylphenol and its

resistant to biodegradation take several months or years to degrade in surface waters, soils, and sediments making it more susceptible to aquatic species. Nonylphenol is less soluble in water but are highly lipophilic that enhance the tendency to bioaccumulate and partition to organic-rich sediments [6]. The capacity of bioaccumulation and body burdens of nonylphenol has been well documented in different fish species and other aquatic animals [7, 8]. Owing to its persistence and bioaccumulation, it has been recommended that nonylphenol could be transported over long distances and has a global reach that draw out far from the site of contamination.

Several studies have investigated the toxicity of nonylphenol on wide variety of animals including fish. The toxicological effects of nonylphenol are mediated through multiple pathways in different fish species. Nonylphenol is an endocrine disruptor having estrogenic properties that disrupt the natural balance of hormones in affected organisms. Evidence of estrogenic effect of nonylphenol in fish has been evidenced by interaction with estrogen receptors and competitively displaces estrogen from its receptor site [9]. Nonylphenol has been known to stimulate the expression of vitellogenin-making gene in male fish for the production of female specific egg yolk precursor, vitellogenin thereby altering gonadal development, steroidogenesis and formation of intersex causing more specific impacts on male reproduction [10]. Besides the estrogenic effects, nonylphenol has been shown to alter growth, ovosomatic index, behavioural modifications and caused cellular apoptosis in rainbow trout and medaka [11-13]. Nonylphenol at sublethal concentration has been also known to alter the antioxidant status and induced oxidative stress in various tissues of

zebrafish, *Oreochromis mossambicus* and *Etroplus maculatus* [14-16]. Nonylphenol exposure induced genotoxicity in *Oreochromis mossambicus* as evidenced by micronucleus test and Salmonella mutagenicity test [17]. In order to investigate the potency of nonylphenol with respect to lethality other than estrogenicity, the present study evaluates the acute sublethal toxic effects on oxygen consumption, haematological as well as histological parameters in the cichlid fish, *Pseudotroplus maculatus*.

Materials and methods

Animal

The cichlid fish, *Pseudotroplus maculatus* (body weight: 7 ± 1 g; length: 6.5 ± 1 cm) were collected from local fish farm near Parappanangadi, Malappuram district, Kerala, India. Fish were acclimatized to laboratory conditions for two weeks prior to the experiments in aquarium tank (40 L capacity) with dechlorinated water and good lighting system (12 h light: 12 h dark).

Preliminary tests

The physico-chemical characteristics of the tap water were estimated as per APHA guidelines [18]. Water temperature ($28 \pm 2^\circ\text{C}$), oxygen saturation of water (70 to 100 %), and pH (6.5 to 7.5) were continuously monitored throughout the experiment in both control and treatment groups.

Chemicals

Technical grade nonylphenol, 4-(2, 4-dimethylheptan-3-yl) phenol, of 97% purity was purchased from SISCO Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals were of analytical grade and obtained from local commercial sources.

Treatment and analyses

After two weeks of acclimatization, fishes were kept in different tanks for the experiment maintaining ten animals per group. Since nonylphenol is insoluble in water it was first dissolved in 1% DMSO and therefore used as a solvent (vehicle) control in the experiment. Earlier studies from our laboratory reported the median lethal concentration (96 h LC_{50}) of nonylphenol in *P. maculatus* as $890 \mu\text{g/L}$ [19]. One-tenth ($89 \mu\text{g/L}$) and one-fifth ($178 \mu\text{g/L}$) of LC_{50} were selected as sublethal concentrations in the present study and exposed for short-term (24 h, 72 h and 96 h) durations along with negative control and vehicle control groups.

Oxygen consumption of fish was measured by Winkler's method [20]. Before starting the experiment, initial water sample was collected immediately from each group without causing any damage to the animal. Subsequently, at every 24 h intervals water sample was collected up to 96 h and the rate of oxygen consumption in each samples were calculated considering net weight of fish by using formula given in Winkler's method. The rate of oxygen consumption was expressed as ml of O_2 / litre/ g/ h in control and treated samples. Experiments were done in triplicate and the statistical data are presented as mean \pm SD for ten animals per group.

At the end of every treatment period, fishes were caught very gently using a small dip net, one at a time with least

disturbance. Blood collected by cardiac puncture using 2 ml glass syringe was transferred into a vial containing 1% ethylenediaminetetraacetic acid - EDTA as anticoagulant. The whole blood was then immediately used for the estimation of erythrocyte and leukocyte counts [21] in both control and experimental groups.

After collection of blood sample, gill and liver tissues were extirpated from one-tenth sublethal concentration group after 96 h of exposure. Tissues are then stored in 10% buffered formalin for 24 h for histological examination. Tissues were dehydrated in ascending grades of alcohol, cleared in xylene and embedded in molten paraffin wax for 1 h. Sections were cut at $6 \mu\text{m}$ and stained with haematoxylin and eosin, and mounted in DPX. The structural alterations in the tissues of fish from 96 h exposure groups were then compared with both control groups by observing under light microscope. Photomicrographs were taken using Canon 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) and the means were compared by Duncan's Multiple Range test using statistical package (SPSS17.0). Differences were considered to be significant at $p < 0.05$ against the control groups. Data are presented as mean \pm SD for ten animals per group. All haematological examinations were performed in duplicate.

Results

Effect of nonylphenol on oxygen consumption

The rate of oxygen consumption was found to be increased significantly ($P < 0.05$) after 24 h when compared to the corresponding control groups, and then decreased in time-dependent manner showing significant ($P < 0.05$) decrease at the end of 96 h of nonylphenol treatment at both sublethal concentrations (Fig. 1).

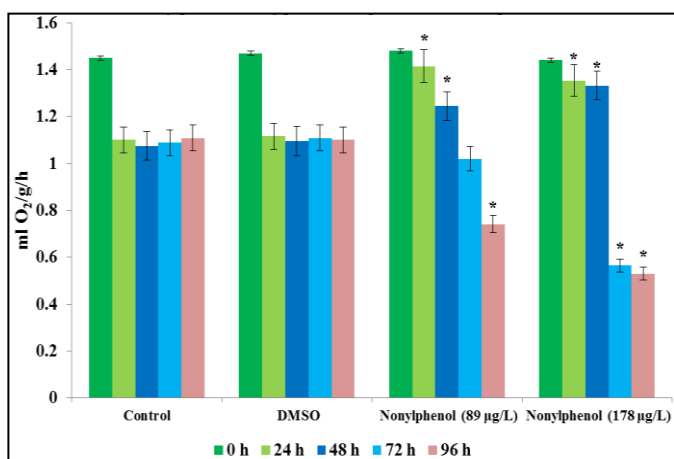


Fig 1: Effect of nonylphenol on oxygen consumption in *Pseudotroplus maculatus*

Effect of nonylphenol on haematological parameters

Nonylphenol exposure at two sublethal concentrations showed significant ($P < 0.05$) decrease in erythrocyte count whereas leukocyte count increased significantly ($P < 0.05$) in

concentration and time-dependent manner when compared to the control groups (Figs. 2 and 3).

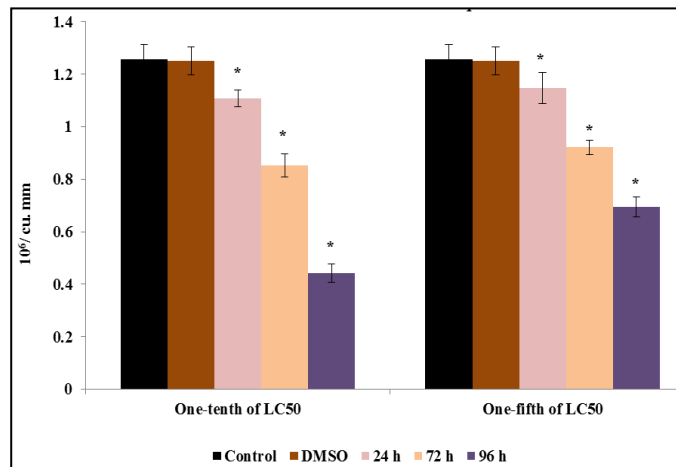


Fig 2: Effect of nonylphenol on erythrocyte count in the fish, *Pseudetroplus maculatus*

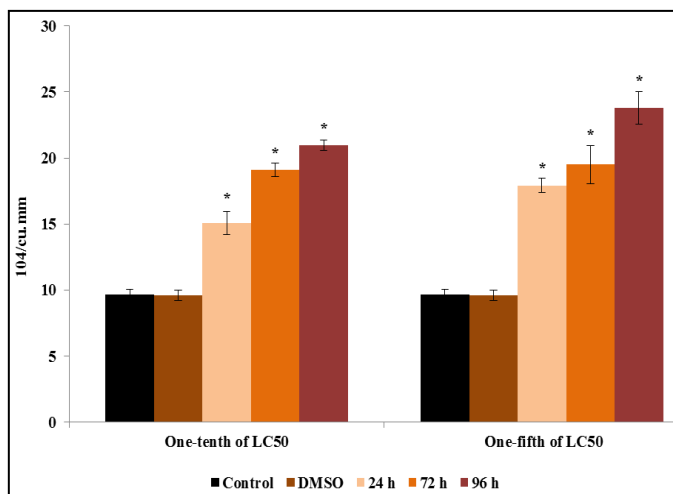


Fig 3: Effect of nonylphenol on leukocyte count in the fish, *Pseudetroplus maculatus*

Effect of nonylphenol on histology of tissues

DMSO-treated fishes showed no significant variation in both gill and liver tissues and it was found similar to negative control tissues (Figs. 4 and 5). Nonylphenol treatment altered normal architecture of gill as revealed by curling and destruction of secondary lamellae, aneurysm and hyperplasia in gill arches (Fig. 4). Hepatic cells showed cytoplasmic vacuolization, degeneration of hepatocytes and severe necrosis (Fig. 5).

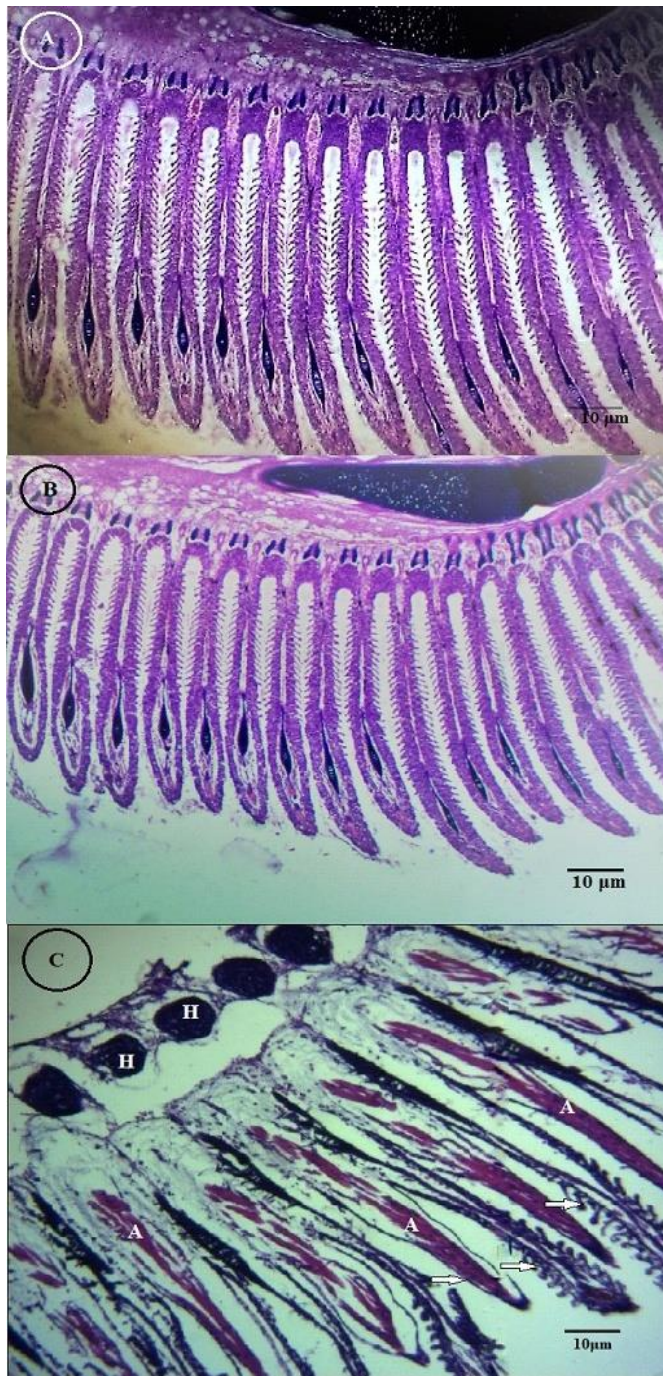


Fig 4: Histology of gill tissue exposed to nonylphenol in *Pseudetroplus maculatus*. A-Gill Control; B-DMSO exposed; C-1b: Nonylphenol at 89 µg/ L concentration exposed for 96 h showing hyperplasia of gill arches (H), aneurysm (A), curling and absence of secondary lamellae (→)

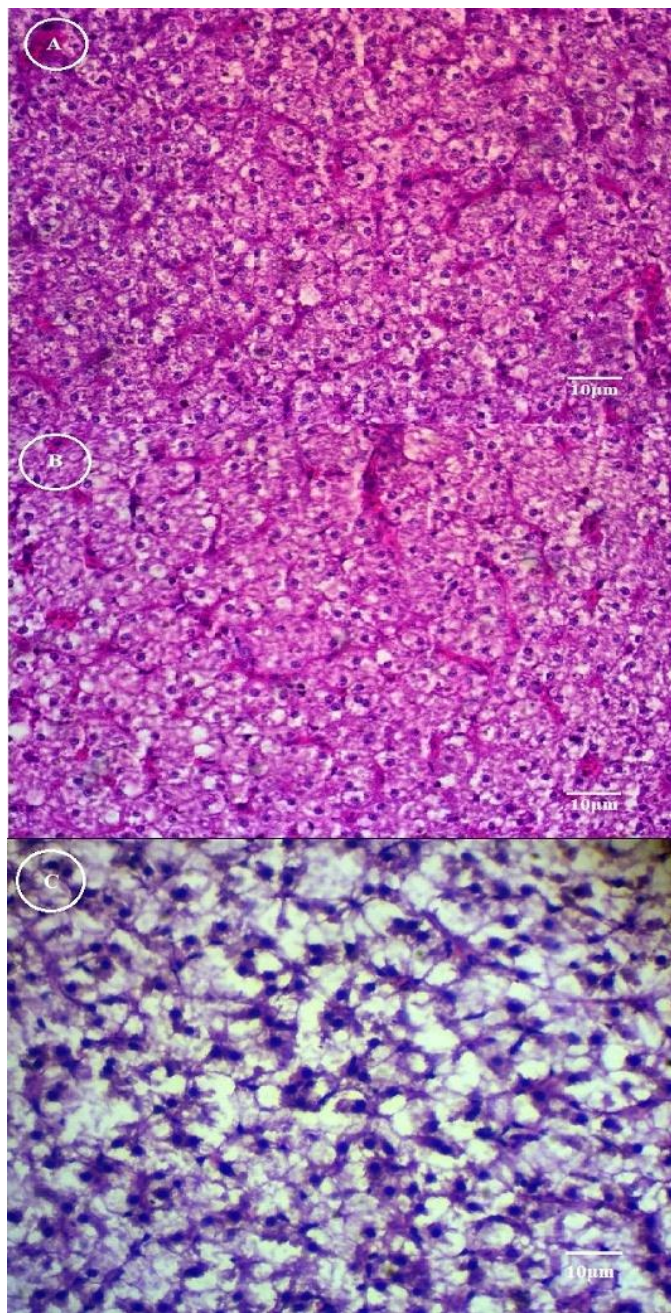


Fig 5: Histology of liver tissue exposed to nonylphenol in *Pseudotropheus maculatus*. A-Liver Control; B-DMSO exposed; C-1b: Nonylphenol at 89 µg/ L concentration exposed for 96 h showing cytoplasmic vacuolization, degeneration of hepatocytes and severe necrosis

Discussion

One of the key motivations of conducting laboratory experiments on environmental contaminants in fish is directly related to assessing the undeviating effects of pollutants in controlled conditions. Thus the interpretation of nonylphenol-exposed laboratory fish gives exact details on the toxic effects of pollutants and can be easily correlated to the natural population. Therefore, apart from recognizing and managing the source of pollution, it is essential to scrutinize the adverse effects of the pollutants in organisms inhabiting in it, which in turn reflects the health status of aquatic ecosystem. The

present study exploits the cichlid fish, *Pseudotropheus maculatus* as piscine model for the assessment of nonylphenol toxicity. The fish is very easy to maintain in the laboratory and are best to lodge in both freshwater and brackish waters. Moreover, it is very sensitive to minute environmental variations including chemical contamination.

Generally, fish gills comprise over 50% of the surface area of fish and are equipped for aquatic respiration [22]. During the process of respiration, fish engulf water along with air that supplies oxygen to the tissues and takes away carbon dioxide through diffusion. Thus, oxygen uptake is a vital physiological phenomenon and is considered as an important biological indicator of general metabolic activity of the animal [23]. Therefore, any variation in the rate of oxygen consumption is a sign of changes in the internal metabolic activities. In the present study, nonylphenol at both sublethal concentrations increased the rate of oxygen consumption after 24 h and subsequently decreased after 72 h onwards. Exposure to environmental contaminants has been known to stimulate the peripheral nervous system that results in increased activity of fish, which requires more oxygen to fulfill the energy demand [24]. This could be one of the reasons for initial elevation in the rate of oxygen consumption. Thus the fish made an attempt to escape from the pollutant by increasing the rate of oxygen consumption, however, failed due to the continuous exposure of nonylphenol for 96 h, which is evident by the decrease in oxygen consumption after 72 h that reflects respiratory stress and impaired oxidative metabolism in fish. Similar results of elevated oxygen uptake have been reported during first 24 h of dimethoate exposure followed by decrease in subsequent hours in *Channa punctatus* [25]. Decreased rate of oxygen consumption has been documented in *Pseudotropheus maculatus* when exposed to sublethal concentrations of the nanoparticles fullerene C₆₀ and bisphenol A [26, 27]. One of our previous studies reported that immediately after nonylphenol exposure, fish showed aggressive swimming behaviour for few hours followed by slow and restricted swimming activity with decreased rate of opercular movement, which also correlates the altered rate of oxygen consumption observed in the present study [19].

Responses of blood cells are one of most significant marker for changes in the internal and external environment of the animal. Usually, the blood cell count is a stable index and fishes try to maintain the index between certain limits. But presence of toxicants in surrounding environment or attack of disease causing agents may eventually change normal blood cell count [28]. Therefore in presence of toxicant or other stressors, blood parameters can be used as typical laboratory parameter to determine diseased conditions and metabolic disturbances in fish. The exposure of *P. maculatus* to sublethal concentrations of nonylphenol caused a significant decrease in erythrocyte count while leukocyte count increased in concentration and time-dependent manner. Similar observations have been noted in *Clarias gariepinus* when exposed to nonylphenol for 7 days [29]. Reduction in erythrocyte count indicates the condition of anemia caused by disruptive effects of nonylphenol on erythropoietic tissues and cell viability [30]. The reduction in the number of erythrocytes was also reported in *Oreochromis mossambicus* when exposed to sublethal concentration of quinalphos [31]. Leukocytes play

vital role in nonspecific or innate immunity, and thus the leukocyte count indicates the health status of fish [32]. The increase in leukocyte count in treated fish could be due to the direct stimulation of defense system induced by nonylphenol toxicity. It also reflects increased demand for leukocyte for removal of cellular debris at a faster rate or puts a greater demand for phagocytic cells [33]. Progressive increased levels of total WBC count have also been reported in *Cyprinus carpio* and *Channa punctatus* when exposed to chlorpyrifos and copper, respectively [34, 35]. Similarly, bisphenol A at sublethal concentration has been shown to cause reduction in RBC and induction of WBC count in *P.maculatus* [27].

In toxicology studies, histological analysis have been proved as a responsive tool to detect the direct cytotoxic effects of chemical compound on target organs such as gill, liver, brain, muscle and gonads of fish in laboratory and field conditions [36]. Moreover, it is considered as one of the cost-effective tools to detect the nature and health status of fish populations, thereby reflecting the well-being of the entire aquatic ecosystem. In all organisms, the cellular damages are contributed due to different mechanisms and factors that influence various cell types. Reactive oxygen species associated damages to cellular components are usually considered as important mechanism involved in the histopathological alterations [37]. Nonylphenol induced alteration in antioxidant defense system and reactive oxygen species generation in gill and liver tissues of *Pseudotroplus maculatus* were previously reported in our laboratory [38, 39]. In the present study, nonylphenol induced gill and liver tissue damages in the fish after 96 h of nonylphenol exposure was studied in detail. The major deformalities found in the gill tissue are curling and destruction of secondary lamellae, hyperplasia of gill arches and aneurysm. Hepatic cells showed cytoplasmic vacuolization, degeneration of hepatocytes, and severe necrosis after exposure to nonylphenol when compared to control tissues. Hyperplasia of epithelium and fusion of secondary lamellae in gills and disappearance of the cell membrane with necrosis in the liver tissue has been observed in rosy barb after nonylphenol treatment at 0.17, 0.34 and 0.68 μM concentrations [40]. Similarly, lamellar fusion, hypertrophy of lamellar epithelium and mucous deposition over the respiratory epithelium has been observed in the gill of *Oreochromis mossambicus* after nonylphenol exposure at 0.15 mg/ L concentration [15]. Thus, the histopathological alterations in gill and liver tissues of *Pseudotroplus maculatus* indicate that nonylphenol exposure at sublethal concentrations could cause morphological alterations that widely reflect the health status of exposed fish in laboratory condition.

Conclusion

The investigation, however, yielded reliable outcome on the toxic effects of nonylphenol in optimal laboratory condition and further research on field studies could contribute for the better understanding on the adverse effects of the pollutant in natural environment. Once the risk assessment in natural ecosystem is established may possibly provide the best risk management practices to minimize or prevent the release of such contaminants into the environment.

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