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Acute toxicity of triclosan on the native freshwater fish, *Anabas testudineus* (Bloch,1792): behavioral alterations and histopathological lesions

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ABSTRACT

Triclosan is an effective antimicrobial agent commonly used in pharmaceuticals and personal care products. In this study, median lethal concentration i.e., 96h-LC_{50} value, along with behavioural and histopathological modifications of triclosan were investigated in the native fish, *Anabas testudineus*. For determining LC_{50}-96h of triclosan, seven different concentrations of the test chemical at a range from 1 to 2.2 mg/L in a geometric series were selected after a range-finding test. Control group without the test solution and the positive control containing the solubilising agent (DMSO) as a vehicle was also maintained, comprising ten animals per group. The median lethal concentration of triclosan in *Anabas testudineus* estimated by using Probit analysis was 1.767 mg/L. Behavioural modifications noted during the study includes air gulping, surfacing, reduced opercular movement, mucous deposition, bulged and hemorrhagic eyes. Histopathological alterations observed in gill consist of uplifting and hyperplasia of epithelium, aneurysm, lamellar disorganization as shortened primary lamella, fusion or loss of secondary lamella and hyperplasia of gill arches. Hepatocytes showed histological alterations such as irregular or anucleated hepatocytes, cytoplasmic vacuolization, degenerated cytoplasm and aggregation of melanomacrophages. The intensity of morphological lesions increases when the concentration is increased and also substantiates the behavioural alterations exhibited by the fish during triclosan exposure. From the result of probit statistical method, it is evident that the acute toxic effect of triclosan is concentration-dependent, and therefore, triclosan contamination is a threat to aquatic organisms.

Keywords: Triclosan, *Anabas testudineus*, 96h-LC_{50}, Behaviour, Histopathology

INTRODUCTION

Triclosan is an antimicrobial agent widely used in pharmacological and cosmetic products against bacteria and fungi. In India, soaps and handwashes are classified as cosmetic products, where triclosan is used as antibacterial agents and the exposure could finally cause severe health effects, including resistance to bacteria or hormonal imbalance. The concentration of triclosan in personal care products are in the range of 0.1 to 0.3% of the weight of the product (Sabaliunas *et al*., 2003). Recently, it has been documented that majority of triclosan are discharged in municipal wastewater sewage. During wastewater treatment, a considerable amount of triclosan was removed through biodegradation and sorption to biosolids (Bock *et al*., 2010). However, some amounts of triclosan have been detected at high level in the surface waters of aquatic environment through wastewater effluents causing a potential risk to aquatic organisms (Dussault *et al*., 2008). The half-life of triclosan in aqueous media ranged between less than 1h to 10 days in abiotic and freshwater bodies, respectively, and its prolonged exposure could result in bioaccumulation in aquatic organisms (Helbing *et al*., 2011).

The toxicity of triclosan has been characterized in many aquatic organisms including algae, daphnids and fish (Orvos *et al*., 2002). In India, studies on the environmental impact of triclosan were comparatively scanty. One of the studies conducted in three south Indian rivers – Cauvery, Vellar and Tamiraparani - has reported the greater environmental risks for triclosan in the river Cauvery (Ramaswamy *et al*., 2011). Fish are considered as the non-target animal to most of the contaminants, but since it occupy top of the aquatic food web and are highly consumed by humans its health status impose high ecological importance. Thus fish are the good indicators for assessing the characteristics and fate of aquatic environment, especially by monitoring the impact of aquatic contaminants. In this study, one of the native freshwater teleostean fishes, *Anabas testudineus* was selected as a laboratory model for assessing acute toxicity of triclosan. Climbing perch is the common name of the fish; it is highly demanded as protein-rich and nutrient-valued food source having medicinal importance. It is believed that it is the valuable food for the sick as it prevents some disease as male libido and also slows down ageing process in females. Apart from this, several ecotoxicological studies have been conducted in *A. testudineus* owing to its wide distribution, commercial importance, easy availability and effortless acclimatisation in laboratory conditions.

In this context, the present study was designed to determine the acute toxic effects of triclosan on *Anabas testudineus*. Since no regulation has been implemented so far in the use of triclosan containing products in India, the study provides high impact on aquatic ecosystem. Acute toxicity was evaluated by assessing median lethal concentration of triclosan that kills 50% of test animal in a specific time limit. Further, behavioural response and histopathological modifications were also monitored to supplement the toxic effect of triclosan in the fish. Thus, the study provides simple, valuable information about the acute toxic effects of triclosan in fish, thereby providing certain endpoints in ecotoxicological research.

MATERIALS AND METHODS

Fish

Mature freshwater teleostean fish, *Anabas testudineus*, weighing 7 ± 1g and length 7 ± 1.5cm were collected from the fish farm, Pulimukham Hatcheries, Alappuzha District, Kerala. Fishes were acclimatized to the laboratory conditions prior to the experiment in dechlorinated, aerated, static water (40 L capacity) and photoperiod of light and dark (12 h: 12h) was maintained throughout the study.

Preliminary tests

The physicochemical features of the tap water were estimated as per APHA guidelines (1998). Water temperature in the experiment ranged from 26 ± 2°C, oxygen saturation of water maintained between 70 and 100% and pH at 7.4 to 7.6, which were monitored throughout the study using the standardized procedures.

Chemicals

Triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol) of 97% purity, was obtained from HiMedia Laboratories, Mumbai, India. Test chemical was dissolved in 1%DMSO and maintained as positive control (vehicle) along with the blank control. All other chemicals were obtained from local commercial sources.

Acute toxicity test

After acclimatization, fish were exposed to seven different concentrations of triclosan i.e., 1.0, 1.2, 1.4, 1.6,

1.8, 2.0 and 2.2 mg/L for 96 h along with positive and negative controls, maintaining 10 animals in each group. Test concentrations were chosen in geometric range after repeated range-finding experiments. Static test condition was maintained in the experiment for the determination of median lethal concentration. Other disturbances that affect the behaviour of the fish were avoided in order to monitor behavioural changes after triclosan exposure, which were inspected for 2 to 3 h immediately after the toxicant exposure and regularly at 24, 48, 72 and 96 h. Moreover, to record any changes in behaviour, fish were observed for about 1 h once daily. Mortality of fish in each group was recorded throughout the experiment period. Median lethal concentration (LC_{50}-96 h) that kills 50% of animal within 96 h was plotted against different concentrations of triclosan on MS-Excel. The values are then computed with P=0.95 confidence limits by using probit analysis (Finney, 1971).

Collection of tissues

After mortality observed, gill and liver tissues were removed immediately from 1.6 and 1.8 mg/L concentrations along with control groups and used for histological analysis.

Histopathological analysis

Tissues were stored in 10% buffered formalin for 24 h and dehydrated in ascending grades of alcohol and were cleared in xylene. Tissues were embedded in molten paraffin wax and sections of 5-6 µm thickness were prepared using rotary microtome (Roberts and Smail, 2001). Preparations were stained with haematoxylin-eosin and mounted in DPx. The stained sections were observed and photomicrographs were taken using Canon shot camera fitted to the Carl Zeiss Axioscope-2 Plus under trinocular research microscope.

Statistical analyses

Experiments were done in triplicates for the accuracy of the results. Total number of animal used in the experiments, the test concentrations and percentage of mortality observed in each experimental group were fit to a probit analysis using $\log_{10}$ concentration transformation in the statistical package SPSS 17.0.

RESULTS

Median lethal concentration

During the acclimation period, no mortality was observed and the animal was found active and healthy. Similarly, both positive and negative control groups showed no mortality up to 96 h. However, during the exposure period triclosan-treated groups showed different percentage of mortality in the specific time interval (Table 1). Fish are considered dead if there is no movement, especially opercular movement, and no reaction on touching the caudal peduncle. Dead fish are immediately removed and the mortality was recorded for calculating percentage of mortality. It was observed that at 1 mg/L concentration of triclosan, no mortality was observed for 96 h. When the concentrations increased to 1.2, 1.4, 1.6, 1.8 and 2.0 mg/L, showed 10, 30, 40, 60 and 70% mortality, respectively. Triclosan at 2.2 mg/L showed 100% mortality of the animal within 96 h. Concentration-mortality graph plotted showed high degree of positive correlation; r = +0.869 (Figure 1) and the calculated LC_{50} value by using probit analysis was 1.767mg/L at 95% confidence limits.

Table 1 Effect of triclosan on the mortality rate for 96 h in the fish, *Anabas testudineus* (n=10)

Concentration (mg/L)	Mortality (No. of animals)	Mortality (%)	Hour of mortality
DMSO	0	0	96 h
0	0	0	96 h
1.0	0	0	96 h
1.2	1	10	96 h
1.4	3	30	96 h
1.6	4	40	96 h
1.8	6	60	96 h
2.0	7	70	96 h
2.2	10	100	96 h

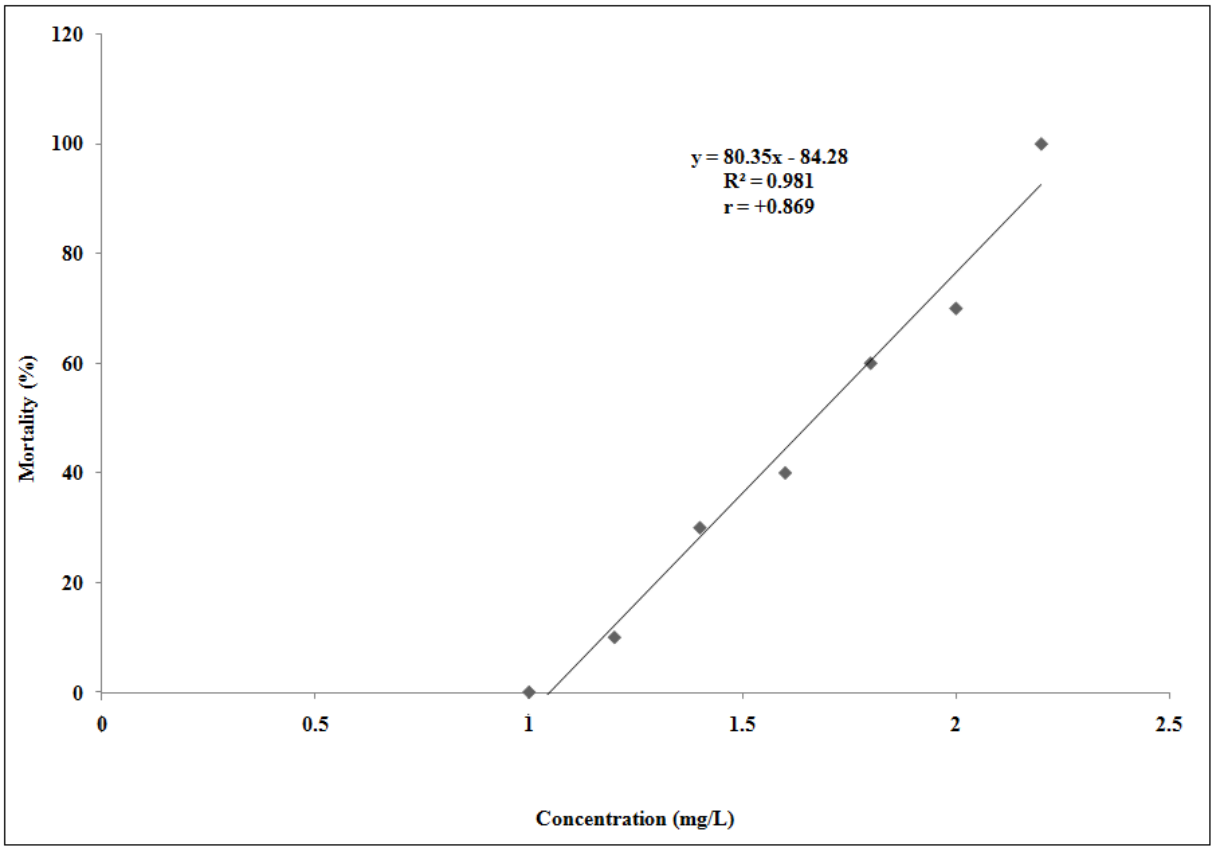

Figure 1. Median lethal concentraction (LC_{50}- 96h) of triclosan in the fish, *Anabus testudineus*

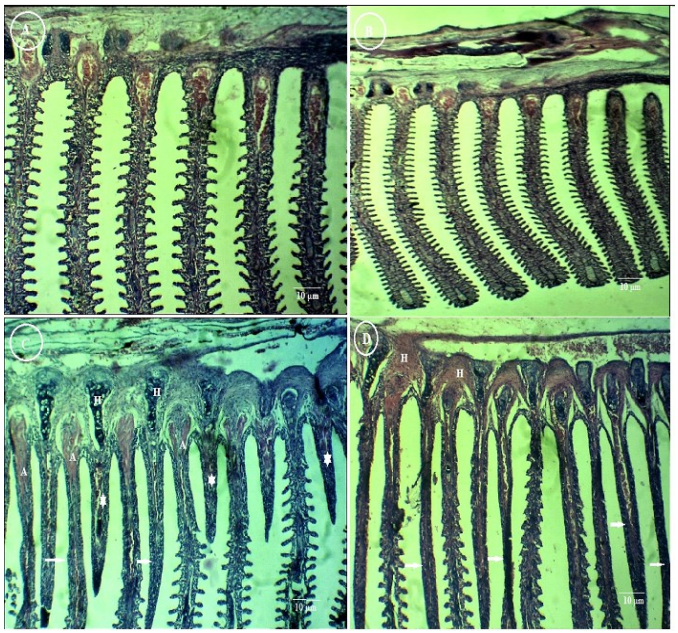

Figure 2: Histopathology of gill tissue. A-Control tissue; B-Vehicle control (DMSO);C-Triclosan-treated gill (1.6 mg/L) showing hyperplasia of gill arches (H), aneurysm (A), lamellar disorganization as shortened primary lamella (*), fusion or loss of secondary lamella (→); D-Triclosan-treated gill (1.8 mg/L) showing uplifting and hyperplasia of gill epithelium (H), fusion or loss of secondary lamella (→).

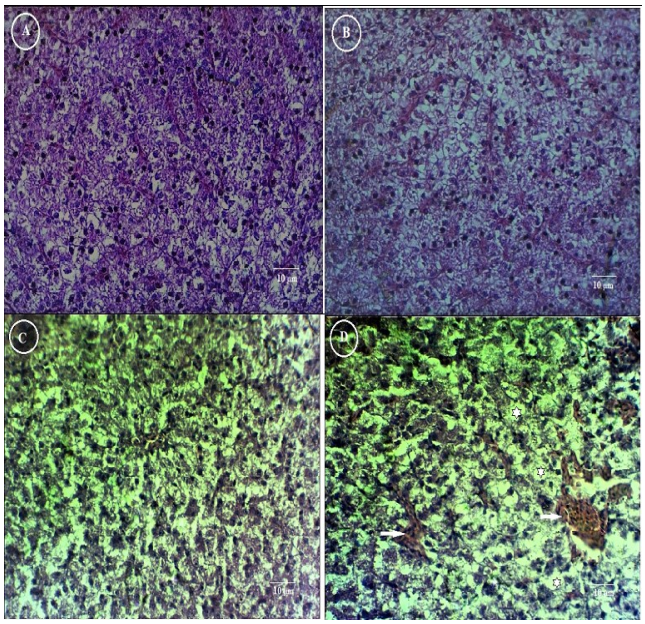

Figure 3: Histopathology of liver tissue. A-Control tissue; B-Vehicle control (DMSO); C-Triclosan-treated liver (1.6 mg/L) showing cytoplasmic vacuolization, degenerated cytoplasm; D-Triclosan-treated liver (1.8 mg/L) showing aggregation of melanomacrophages (→), cytoplasmic vacuolization (*) and degenerated cytoplasm.

Behavioural analysis

In the present study, soon after triclosan exposure, fish showed drastic changes in behaviour at all concentrations when compared to control groups, which includes air gulping, surfacing, reduced opercular movement, mucous deposition, bulged and hemorrhagic eyes.

Histological changes

Microscopic observations of gill tissues of both positive and negative control fish showed normal architecture (Figs. 2A and B; 3A and B), whereas in treated groups extensive tissue damages were recorded. Histopathological alterations observed in gill consist of uplifting and hyperplasia of gill epithelium, aneurysm, lamellar disorganization as shortened primary lamella, fusion or loss of secondary lamella and hyperplasia of gill arches (Figs. 2C and 2D). Hepatocytes showed histological alterations such as irregular or anucleated hepatocytes, cytoplasmic vacuolization, degenerated cytoplasm and aggregation of melanomacrophages (Figs. 3C and 3D).

DISCUSSION

Acute toxicity refers to the adverse toxic effects of exposed test substance on first exposure to a single dose. Acute toxicity of triclosan in different species of fish has been reported extensively. The toxicity of triclosan varies among different species from highly toxic to moderate toxic. In previous literatures, the median lethal concentration of triclosan detailed in various fish are in rainbow trout, *Oncorhynchus mykiss*, which is 4.4 mg/L (Adolfsson-Erici *et al*., 2002), Nile tilapia, *Oreochromis niloticus* is 2.81 mg/L (Vijitha *et al*., 2017), medaka *Oryzias latipes* is 0.37 to 1.7 mg/L (Orvos *et al*., 2002; Nassef *et al*., 2009), zebrafish *Danio rerio* is 0.34 mg/L (Oliveira *et al*., 2009), fathead minnow *Pimephales* promelas is 0.26 mg/L and sunfish *Lepomis macrochirus* is 0.37 mg/L (Orvos *et al*., 2002).

In the present study LC_{50} of triclosan for 96 h in the fish *Anabas testudineus* is 1.767 mg/L. Differences in acute toxicity of triclosan in different fish species may be due to several factors such as variation in species, strain and gender, size of test animal, purity of triclosan used, sensitivity and resistance of the species, mode of entry, adaptation of the animal, detoxifying mechanisms etc.

Behaviour is the adjustment of an organism to external and internal stimuli in order to adapt constantly to the change in physical, chemical, biological and social aspects of the environment (Little and Brewer, 2001). In the present study, as an immediate response to triclosan exposure fish exhibited spontaneous movement with frequent air gulping and surfacing, which gradually decreased until fish became lethargic. Triclosan at lower concentrations i.e., below 1.2 mg/L, showed fish lethargic where it remained static at the bottom of the tank for most of the time during the test period. Reduction in the swimming speed after triclosan exposure has been observed in other fishes as rainbow trout, *Oncorhynchus mykiss* at 0.071 mg/L concentration for 61 days (Orvos *et al*., 2002) and zebrafish, *Danio rerio* at 0.5 and 0.4 mg/L concentrations (Oliveira *et al*., 2009). However, fishes reached to the surface of water once in a while to engulp air. The increase in surfacing and gulping of air from surface water after triclosan exposure could be an attempt of the animal to escape from the toxicant and to avoid breathing in the contaminated water. Similar observations have been reported when the pesticide monocrotophos was exposed to the fish, *Anabas testudineus* (Santhakumar and Balaji, 2000). Opercular movement was also highly reduced in all treatment groups and the dead animal was observed with high mucous deposition all over the body. The dead fish showed wide opened mouth and operculum without movement. High secretion of mucous all over the body and decreased opercular movement during the treatment were a part of first line defensive mechanism of fish to prevent the entry of chemical to the body either through body surface or gill (Asifa and Chitra, 2015; Asifa *et al*., 2016).

At higher concentrations i.e., above 1.4 mg/L of triclosan, fish showed semi-circular swimming behaviour, scoliosis, and loss of balance by vertically hanging, haemorrhagic eyes and body tremors. Before the death, haemorrhaged eyes gradually bulged and become opaque and insensitive. They also exhibited transient erratic movement and hyperactivity by swimming over the surface of water because fish are not able to maintain normal equilibrium, finally loss of opercular movement and death occurs.

Histopathological changes are structural alterations due to direct toxic effects of the toxicant or it may be the part of defensive mechanism to decrease the toxic effects (Bhagwant and Elahee, 2002). The extent of severity of tissue damage may be dependent on the nature of toxicant, concentration exposed and the duration of treatment. Gill and liver are considered as the most

targeted organs of toxicity and hence used as a reliable endpoint in this study to determine the adverse effects of triclosan. Some histological alterations exhibited by gill under triclosan exposure includes epithelial upliftting, hyperplasia of gill arches, fusion or complete absence of secondary lamellae, shortened primary lamellae and aneurysm. The vulnerability of tissue damage is concentration-dependent, which reflected the direct effect of triclosan. Epithelial upliftment and hyperplasia of gill arches are the primary defensive strategy exhibitted by fish to prevent the entry of triclosan into the blood by blocking the spaces towards secondary lamellae (Nowak, 1992). Aneurysm is damages in gill circulation, which was more prominent after triclosan exposure that ultimately resulted in increased blood flow into the gill (Rodriguez *et al*., 2002). Fusion of secondary lamellae and shortening of primary lamallae significantly reduce the gill surface and thus decreases the contact between the pollutant and gill epithelium (Kasherwani *et al*., 2009; Kaur and Dua, 2015). Therefore, triclosan exposure affects osmoregulation and gill respiration which may be correlated to the behavioural changes as noticed by engulping air from surface water.

Liver is the organ associated with detoxification and biotransformation of toxicants and also one of the organs most susceptible to toxicant exposure. In the present study, triclosan related histomorphological changes were observed in liver tissues. It includes irregular or anucleated hepatocytes, cytoplasmic vacuolization, degenerated cytoplasm and aggregation of melanomacrophages. Irregular nucleus in the parenchyma of hepatocytes reflects failure in the synthesis of protein and other substances. The imbalance between the rate of synthesis of substances in the parenchymal cells and its release into the circulatory system leads to vacuolization of hepatocytes. Deficiency of oxygen as a result of degeneration of gill could be the cause for the cellular degeneration of hepatocytes (Gingerich, 1982). In the present study, the observed structural damage of hepatocytes in response to triclosan exposure could eventually result in liver dysfunction.

CONCLUSION

In summary, toxic effects of triclosan on *Anabas testudineus* and its related mortality rate increased in concentration-dependent manner. Widespread use of triclosan as antimicrobial agent pave way to aquatic ecosystem and affect the health status of aquatic life even exposed for short durations. Therefore, regulatory measures should be adopted by the government to reduce the use of triclosan in personal care products so as to save the aquatic organisms.

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