ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2017; 3(2): 101-109
www.allresearchjournal.com
Received: 20-12-2016
Accepted: 21-01-2017

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Assessment of genotoxic and haematological consequence of triclosan in the fish, *Oreochromis niloticus* (Linnaeus, 1758)

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Abstract
Triclosan, an antiseptic agent, ubiquitous in the pharmaceuticals and personal care products have the ability to enter into the aquatic ecosystems that pose a great threat to the dwelling organisms. The present study was aimed to assess the genotoxic and haematological consequence of triclosan in the fish, *Oreochromis niloticus.* Fish was exposed to triclosan at one-fifth (562 µg/ L) and one-tenth (281 µg/ L) of LC_{50} concentrations for 24, 72 and 96 h maintaining the control group. Triclosan treatment at sublethal concentrations significantly reduced the serum protein and this may be due to increased proteolytic activity and decreased anabolic activity of protein. Exposure to triclosan at both concentrations decreased RBC and haemoglobin and this could be due to haemolysing capacity of triclosan and rapid oxidation of haemoglobin to methaemoglobin. However, WBC content was significantly increased owing to leucocyte mobilization, as the defensive mechanism of fish to overcome treatment-related stress. Genotoxic effects of triclosan are revealed by the nuclear abnormalities as formation of micronucleus, and also lobed, blebbed, notched, irregular and apoptotic nucleus. The present observation was the first study to prove that triclosan induces genotoxicity and also influenced the haematological changes in the fish, *Oreochromis niloticus*.

Keywords: Triclosan, sublethal toxicity, haematology, genotoxicity, *Oreochromis niloticus*

1. Introduction
Triclosan, 5-chloro-2-(2, 4-dichlorophenoxy) phenol, is a synthetic broad spectrum antibacterial or antimicrobial agent which inhibits the activity of bacteria, viruses and fungi. It was initially registered as a pesticide with the Environmental Protection Agency (EPA) in 1969, but since 1990s it is being widely used in household products (US-EPA, 2011) [1]. Food and Drug Administration approved the use of triclosan in Colgate toothpaste in 1997, but the serious effects of the chemical were discovered in the later years (FDA, 2010) [2]. The chemical has been detectable in aquatic organisms ranging from algae, fishes, dolphins as well as in human urine, blood and breast milk (Orvos *et al*., 2002; Adolfsson-Erici *et al*., 2002) [3,4].

Wastewater is the major contributing factor by which considerable amount of triclosan was released into the aquatic environment. Since wastewater treatment plants (WWTPs) fail to remove triclosan from the water and the compound is highly stable for long period of time, a huge amount of triclosan is expected to be emitted into water bodies. Triclosan and its derivatives have been detected in the effluents of waste water across the globe as well as in the surrounding environment. When triclosan is released from the wastewater effluents it reaches the different environmental compartments, where it undergoes various transformation processes. High rates of chemical, photochemical, sonochemical, or biological transformation occurs that leads to the formation of several byproducts.

Triclosan has been detected not only in surface water and estuarine sediment but also in freshwater at concentrations of up to 800 ng/kg. Consequently, researchers have detected triclosan contamination in both aquatic and terrestrial environments and have observed its bioaccumulation in aquatic biota, such as snails, algae, fish, and marine mammals (Chalew *et al*., 2009) [5]. The structure of chlorinated biphenyl ethyl, triclosan is structurally similar to polychlorinated biphenyls, bisphenol A, dioxins, and thyroid hormones (Crofton *et al*., 2007) [6].

Therefore, the receptors of triclosan are structurally similar to the xenoestrogens, including the natural thyroid hormone, which indicates the possibility of interaction of triclosan and possibly its adverse effects on humans.

The accumulation of triclosan has been reported from various studies in the freshwater aquatic organisms mainly from lower trophic level organisms such as algae, crustaceans, and fish. High levels of triclosan exposure at 0.24–4.4 mg/kg were reported in the bile of fish, rainbow trout (*Oncorhynchus mykiss*) when exposed to downstream WWTP discharges (Adolfsson-Erici *et al*., 2002) [4]. The median lethal concentration (LC_{50}) values for *Danio rerio* (zebrafish), *Lepomis macrochirus*, *O. mykiss*, *Oryzias latipes* (medaka), and *Pimephales promelas* ranged from 270-602 µg/L. The sensitivity of each organism on triclosan is dependent on the time of exposure. In addition, it has been reported that triclosan adversely affect the behavior of fish, which includes loss of equilibrium, locking of the jaw, quiescence, and erratic swimming movements (Drummond *et al*., 1990) [7].

Fish live in very intimate contact with their environment, and are therefore very susceptible to physical and chemical changes in the aquatic media, which may be reflected in their blood components (Wilson *et al*., 1993) [8]. Genetic damage that leads to micronucleus formation in periphery of blood is widely accepted bio-indicator tool to screen for toxicant induced chromosomal aberrations. Apart from this, exposure to toxicants could also induce either increase or decrease in haematological parameters. Blood tissue truly reflects physical and chemical changes occurring in the body of the organism and also provides detailed information on the general metabolism and physiological status of the exposed fish. Early diagnosis of the health status of animal is also possible when evaluating haematological data, particularly blood parameters (Luskova, 1997) [9]. Therefore, the present study was designed to assess the genotoxic and haematological consequence of triclosan in the fish, *Oreochromis niloticus.*

2. Materials and Methods

2.1 Laboratory animal

Freshwater fish, *Oreochromis niloticus* weighing 10.5±1.5 g and length 8.5±1 cm were collected from a fish farm, Aqua fish, B.H. Road, Kottakkal, Malappuram District, Kerala, India. Fish were acclimatized to the laboratory conditions for four weeks with constant supply of dechlorinated water in well-aerated tubs (40 L capacity) sustained with good lighting system.

2.2 Preliminary screening test

The physico-chemical features of the tap water were estimated as per APHA [10]. 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 for 96 h (Finney, 1971) [11].

Preliminary tests were conducted to provide guidance on range of concentration of triclosan to use in the bioassay. Ten specimens were placed in each tub and were maintained in each test and control groups, they were then aerated using tubed motorized pumps and tanks are covered with monofilament nets to prevent the specimens from jumping out of test solutions. The behaviour of specimens was observed and death was also recorded throughout the study.

2.3 Chemicals

Triclosan (5-chloro-2-(2, 4-dichlorophenoxy) phenol) of 97% purity was obtained from Hi Media Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals were of analytical grade and obtained from local commercial sources.

2.4 Evaluation of median lethal concentration (LC_{50}-96 h)

The concentration of the toxicant which kills 50 percentage of the test animals during a specific period of time is referred as median lethal concentration (LC_{50}). For determining LC_{50} concentration separate circular plastic tubs of 40 L water capacity were taken and different concentrations of triclosan (1, 1.5, 2, 2.5, 3, 3.5, 4 mg/ L) were added. Then, 10 fishes were introduced into each tub and a control tub with 40 L of water and ten fishes were also maintained without toxicant. The lethal concentration for 50% killing (LC_{50}) values was computed on the basis of probit analysis for 96 h (Finney, 1971) [11], which was 2.81 mg/ L. One-fifth of concentration (0.562 mg/L) and one-tenth of the concentration (0.281 mg/ L) of triclosan were chosen to represent sublethal concentrations.

2.5 Experimental design

There were three groups in the experiment: First group is control group; Second and third are triclosan-exposed groups ie., one-fifth (0.562 mg/ L or 562µg/ L) and one-tenth (0.281 mg/ L or 281 µg/ L) of LC_{50} concentrations, respectively. The toxicant-exposed groups at different concentrations were maintained for 24, 72 and 96 h, respectively. The experiment was designed as follows:

Group 1 – Control group
Group 2 - One-fifth of LC_{50}-96 h of triclosan (562 µg / L)
Group 2a – maintained for 24 h
Group 2b – maintained for 72 h
Group 2c – maintained for 96 h
Group 3 - One-tenth of LC_{50}-96 h of triclosan (281 µg/ L)
Group 3a – maintained for 24 h
Group 3b – maintained for 72 h
Group 3c – maintained for 96 h

2.6 Collection of blood

Each fish was held and wrapped with a clean, dry filter paper and the posterior half of its body was blotted with another clean coarse filter paper. Caudal peduncle of the fish (control and experimental group) was severed with the single stroke using a sharp blade. After discarding the first drop of blood, the freely oozing blood was collected for the biochemical analysis. It was centrifuged at 5000 rpm for 10 minutes to separate the blood serum, which was used for biochemical analysis.

Small quantity of blood was added to anticoagulant (1% ethylenediamine tetraacetic acid - EDTA) for haematological parameters. The blood was thoroughly mixed with the anticoagulant using a thin, blunt glass rod, during the process of collection itself. The whole blood was used for the estimation of erythrocyte, leucocyte counts and haemoglobin in both control and experimental groups.

2.7 Assessment of haematology and genotoxicity

Total protein concentration in the blood serum was estimated by the method of Lowry *et al*. [12]. Erythrocyte (RBC) and leucocytes (WBC) were counted by using Neubauer hemocytometer chamber (Rusia and Sood, 1992) [13] and the number of RBC's and WBC's per cubic millimetre was calculated. Haemoglobin content of the blood was estimated by the method as described by Drabkin [14]. The micronucleus test was performed according to Heddle [15]; and Schmid [16], and nuclear abnormalities were evaluated according to Carrasco *et al*. [17] with slight modification. Cells with nuclear abnormalities were scored among 1000 cells under 100x magnifications.

2.8 Statistical analysis

Statistical analysis 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 (denoted as asterisks in Figures and Table). Data are presented as mean ± SD for ten animals per group. All biochemical estimations were carried out in duplicate.

3. Results

3.1 Effect of triclosan on body weights and mucous deposition

Acute exposure to triclosan at two sublethal concentrations, ie., at one-tenth and one-fifth of LC_{50}-96 h did not showed any significant changes in the weight of fishes in all treatment durations (Figure 1). On the other hand, there was a significant ($P<0.05$) increase in the percentage of mucous deposition after 72 and 96 h of triclosan exposure at 281 µg/ L concentration and a significant ($P<0.05$) time-dependent increase was noted at 562 µg/ L concentration (Figure 2).

3.2 Effect of triclosan on median lethal concentration (LC_{50}-96 h)

In order to determine the median lethal concentration, mortality of the fish was continuously monitored throughout the experiment. Different percentage of mortality at different time interval was observed and recorded for different concentrations of triclosan (Table 1). Fishes undergone mortality was immediately removed after death and their numbers were registered for calculating percentage of mortality. It was observed that at the concentrations 1 and 1.5 mg/ L no mortality was seen after 96 h of triclosan exposure. At 2, 2.5 and 3 mg/ L concentrations 20%, 30% and 50% mortality was observed, respectively, at the end of 96 h treatment. The percentage of mortality was increased to 70% at 72 h when the concentration of triclosan is increased to 3.5 mg/ L. When the concentration is further increased to 4 mg/ L, 100% mortality within 24 h was observed (Table 1). Determination of median lethal concentration by probit analysis showed LC_{50} value as 2.81 mg/ L (Table 2). The values are also plotted as graph and the results of correlation analysis showed that mortality (X-variable) against concentrations of triclosan (Y-variable) was highly positive correlation (r = +0.975). Based on this, the regression line obtained was 3.357X – 4.535, r2=0.952 (Figure 3). Triclosan exposure did not show any drastic behavioural modifications except mucous deposition and lethargy in movement, which was seen immediately after the toxicant exposure.

3.3 Effect of triclosan on total serum protein

Fishes when exposed to triclosan showed significant ($p<0.05$) reduction in the serum level of total protein at both sublethal concentrations in time-dependent manner (Figure 4).

3.4 Effect of triclosan on haematological parameters

3.4.1 RBC count

No significant changes in the count of red blood corpuscles seen after 24 h of triclosan exposure in both concentrations. The significant decrease in the RBC count was observed after 72 and 96 h, in which a time-dependent reduction was noted at one-fifth of LC_{50} concentration when compared to the corresponding control group (Figure 5).

3.4.2 WBC count

Triclosan treatment for 96 h at both sublethal concentrations showed a significant increase in the count of white blood corpuscles in time-dependent manner when compared to the control group (Figure 6).

3.5 Haemoglobin content

Exposure to triclosan showed significant decrease in the gram percentage of haemoglobin at both concentrations in time-dependent manner (Figure 7).

3.6 Effect of triclosan on nuclear abnormalities

Triclosan at sublethal concentrations showed a significant increase in the incidence of nuclear abnormalities in erythrocytes of fish as evidenced by the formation of micronucleus along with lobed, blebbed, notched, irregular and apoptotic nucleus. The frequency of normal nucleus is significantly ($P<0.05$) decreased in time-dependent manner at both concentrations (Table 3; Figures 8).

4. Discussion

Aquatic organisms are more sensitive to the exposure of toxicants and serve as warning signal to environmental damage. Thus the health of the ecosystem is determined by the wellbeing of the organism in which they live. Fishes are economically important source of food and income in many countries and are the good laboratory model for ecotoxicological studies. Fish also serve as a good genetic model for assessing the pollution and threat in the aquatic ecosystems. Triclosan, commonly used as an antimicrobial agent, are found abundant in soaps, toothpastes, hand washes, shampoos, deodorants and other personal care and household products. It enters into aquatic ecosystem readily from the manufacturers or due to its ubiquitous use in the modern society.

Exposure to such contaminants could cause biological changes in organisms and these changes can be measured by using several biomarkers. Among biological changes, hematological parameters are considered as one of the potential biomarkers of exposure to chemical agents, since the latter can induce an increase or decrease in the various hematological components (Heath, 1995) [18]. In the present study the effect of triclosan have been observed in blood parameters and in addition, the genotoxic effects in peripheral blood of fish was also evaluated to understand the toxic effects of the compound in the freshwater fish, *Oreochromis niloticus.*

Acute exposure to triclosan at two sublethal concentrations did not showed any significant changes in the weight of

fishes (Figure 1). But the percentage of mucous deposition was increased (Figure 2) and this could be the first line of defensive mechanism of fish to escape from the toxicant, triclosan. In acute toxicity test or to evaluate the medial lethal concentration fishes were exposed at different concentration of triclosan for 96 h duration. Mortality of the fishes in each group were continuously monitored throughout the experiment and the percentage of mortality was plotted in a graph against different concentrations of triclosan and a high degree of positive correlation (r = +0.975) was observed. Based on this, the regression line obtained was 3.357X – 4.535, r2=0.952 (Figure 3). Higher the concentration of triclosan, higher the mortality rate (Table 1). The median lethal concentration was determined by probit analysis which showed LC_{50} value as 2.81 mg/ L (Table 2). However, triclosan exposure did not show any drastic behavioural modifications except mucous deposition and lethargy in movement, which was seen immediately after the toxicant exposure.

Fishes when exposed to triclosan showed significant (p<0.05) reduction in the serum level of total protein at both sublethal concentrations in time-dependent manner (Figure 4). This may be due to increased proteolytic activity and decreased anabolic activity of protein (Jenkins *et al*., 2003) [19]. Significant reduction in the RBC count was observed after 72 and 96 h, in which a time-dependent reduction was noted at one-fifth of LC_{50} concentration when compared to the corresponding control group (Figure 5). Blood is a vital circulatory fluid composed of cells suspended in intercellular substance called plasma with the major function of maintaining homeostasis (Issac *et al*., 2013) [20]. Haematological components, which consist of red blood cells or erythrocytes, white blood cells or leucocytes, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration are valuable in monitoring the toxicity in blood as well as to assess the health status of animals (Oyawoye and Ogunkunle, 2004) [21]. Red blood cells serve as a carrier of haemoglobin that reacts with oxygen carried in the blood to form oxyhaemoglobin during respiration. It is also involved in the transport of oxygen and carbon dioxide in the body. The reduced RBC due to triclosan exposure may be due to the decrease in the level of oxygen that would be carried to the tissues as well as the level of carbon dioxide returned to the lungs (Soetan *et al*., 2013) [22].

Triclosan treatment showed a significant increase in the count of white blood corpuscles in time-dependent manner when compared to the control group (Figure 6) and this may be due to the triclosan-induced stress stimulus. The increase in WBC count attributed to the stimulation of the immune system in response to tissue damage. White blood cells play a major role in the defense mechanism of fish which may be directly proportional to the severity of the causative stress condition and may be attributed to an increase in leucocytes mobilization. Exposure to triclosan showed significant (P<0.05) decrease in the gram percentage of haemoglobin at both concentrations in time-dependent manner (Figure 7). The decrease in haemoglobin content might be due to rapid oxidation of haemoglobin to methaemoglobin or the release of oxygen radical due to triclosan exposure. Similar observations have been reported in fish, *Cyprinus carpio* exposed to chlorpyrifos (Ramesh and Saravanan, 2008) [23].

Exposure to triclosan at sublethal concentrations showed increase in the incidence of nuclear abnormalities in erythrocytes of fish as evidenced by the formation of micronucleus along with lobed, blebbed, notched, irregular and apoptotic nucleus. The frequency of normal nucleus is significantly (P<0.05) decreased in time-dependent manner at both concentrations (Table 3; Figures 8). Micronucleus are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome acentric fragments or from intact whole chromosomes not included in main nucleus after mitosis (Al-Sabti and Metcalfe, 1995; Ferraro *et al*., 2004) [24,25]. The formation of morphological nuclear alterations was first described in fish erythrocytes. In fishes, micronucleus test is a useful *in vivo* technique for genotoxicity testing to assess the toxic impact of exposed chemicals. The formation of these nuclear abnormalities would represent the way to eliminate toxic chemicals that amplify genetic material from the cell nucleus (Carrasco *et al*., 1990) [17]. The induction of genotoxic damage as revealed by the micronucleus and other nuclear abnormalities reveal the genotoxic effects of triclosan in fish, *Oreochromis niloticus*.

Table 1: Effect of triclosan on the mortality of the fish, *Oreochromis niloticus* when exposed for 96 h

Concentrations (mg/ L)	Mortality (%)	Total (No. of animals)	Hour of mortality
1.0	0	10.00	96 h
1.5	0	10.00	96 h
2.0	20	10.00	96 h
2.5	30	10.00	96 h
3.0	50	10.00	96 h
3.5	70	10.00	72 h
4.0	100	10.00	24 h

Table 2: Probit analysis of 95% confidence limits for effective concentrations of triclosan in the fish, *Oreochromis niloticus*

Prob	Concentration	95% Confidence Limits	
		Lower	Upper
.01	1.46948	.85296	1.83243
.02	1.58586	.97630	1.93638
.03	1.66443	1.06330	2.00604
.04	1.72609	1.13357	2.06054
.05	1.77793	1.19395	2.10631
.06	1.82328	1.24769	2.14636
.07	1.86399	1.29665	2.18236
.08	1.90122	1.34197	2.21534

.09	1.93572	1.38442	2.24600
.10	1.96803	1.42455	2.27479
.15	2.10763	1.60161	2.40081
.20	2.22562	1.75469	2.51049
.25	2.33209	1.89410	2.61344
.30	2.43204	2.02459	2.71499
.35	2.52847	2.14864	2.81901
.40	2.62352	2.26759	2.92883
.45	2.71887	2.38224	3.04771
.50	2.81610	2.49316	3.17903
.55	2.91680	2.60112	3.32638
.60	3.02281	2.70723	3.49388
.65	3.13643	2.81318	3.68668
.70	3.26080	2.92138	3.91199
.75	3.40055	3.03528	4.18098
.80	3.56323	3.16008	4.51268
.85	3.76270	3.30471	4.94360
.90	4.02962	3.48803	5.55763
.91	4.09688	3.53280	5.71867
.92	4.17122	3.58173	5.89950
.93	4.25452	3.63591	6.10561
.94	4.34952	3.69694	6.34510
.95	4.46046	3.76728	6.63063
.96	4.59442	3.85101	6.98370
.97	4.76463	3.95568	7.44514
.98	5.00070	4.09804	8.10847
.99	5.39675	4.33061	9.28086

Table 3: Effect of triclosan on the nuclear abnormalities in the fish, *Oreochromis niloticus*

Nuclear abnormalities (%)	Control	Triclosan (1/10th of LC_{50})			Triclosan (1/5th of LC_{50})		
		24 h	72 h	96 h	24 h	72 h	96 h
Micronucleus	0.1	0.5	1.3	0.3	1	0.8	0.2
Lobed nucleus	1	2.9	3.7	1.2	4.7	2.5	0.5
Blebbed nucleus	1.5	4.8	7.2	7.7	6.4	7.5	3.1
Notched nucleus	0.5	3.3	4.1	1.5	2.6	2	0.6
Irregular nucleus	1.2	4.3	6.5	11.2	3	9	5.2
Apoptotic nucleus	0	0	3	6.9	1	4.4	25.2
Normal nucleus	95.7	84.2	74.2*	71.2*	81.3	73.8*	65.2*

Values are expressed in percentage
Asterisks (*) denotes significance at $P<0.05$ against control group

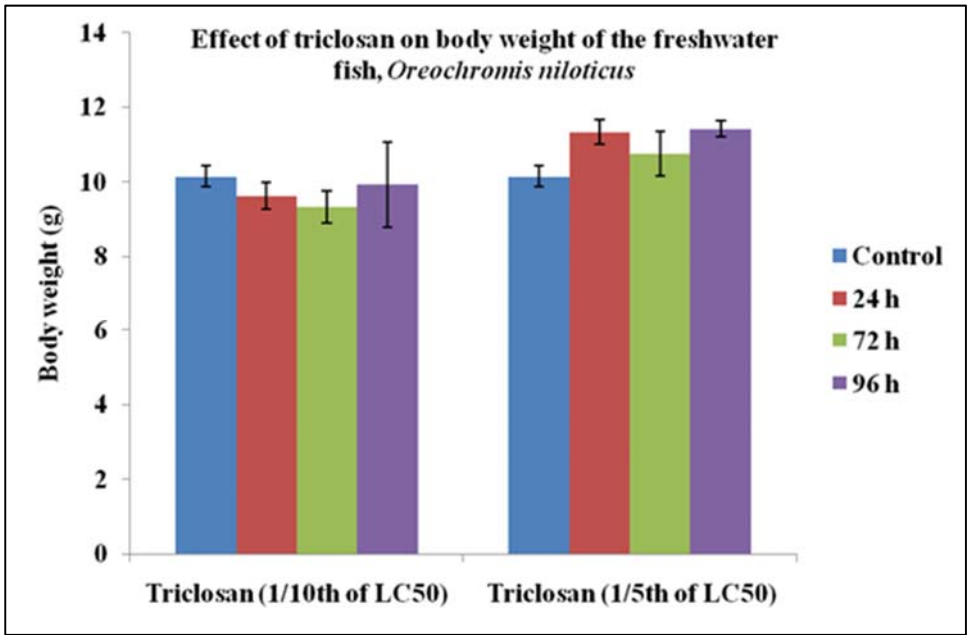
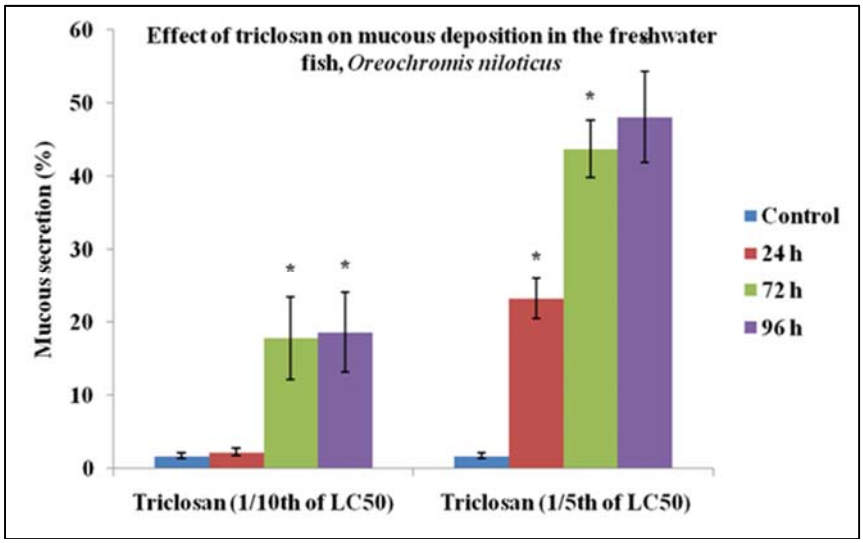


Fig 1



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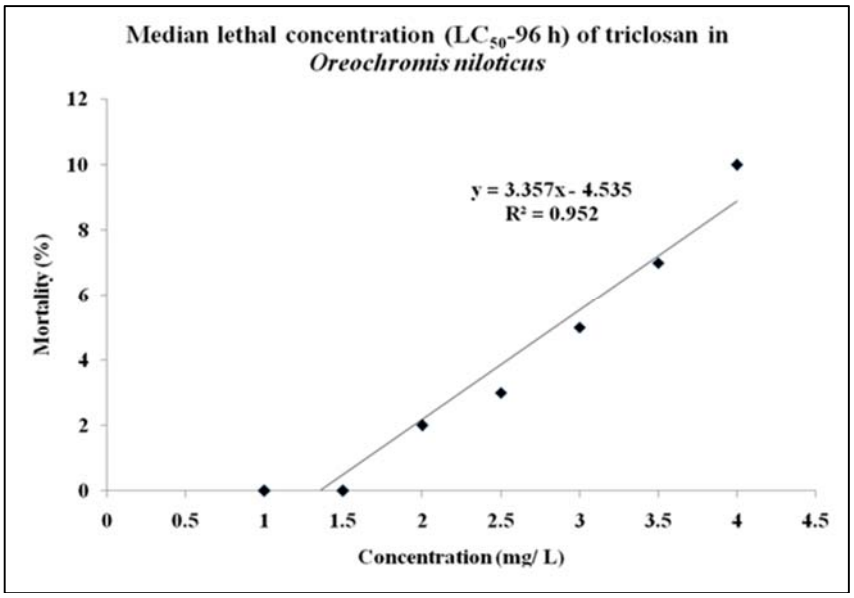

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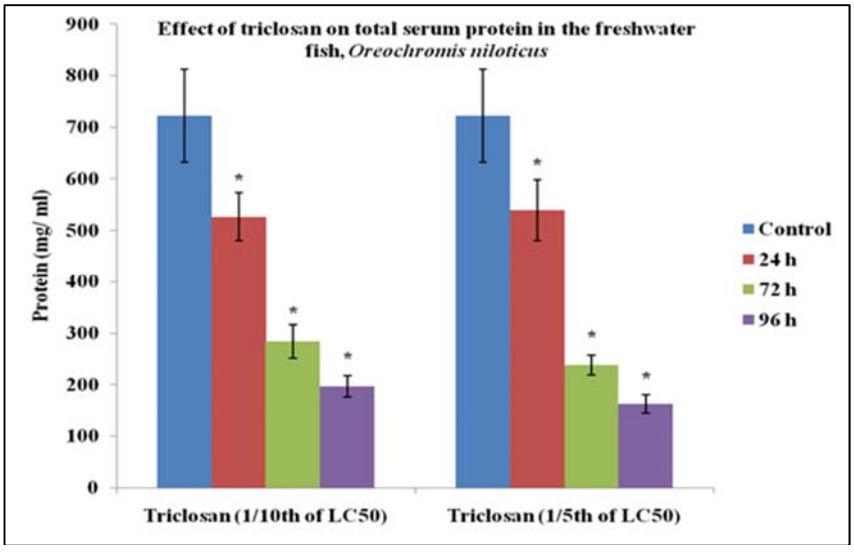

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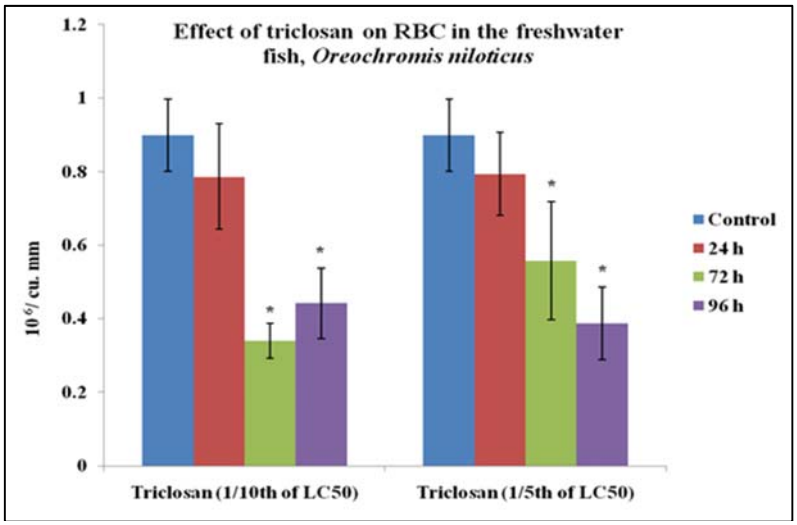

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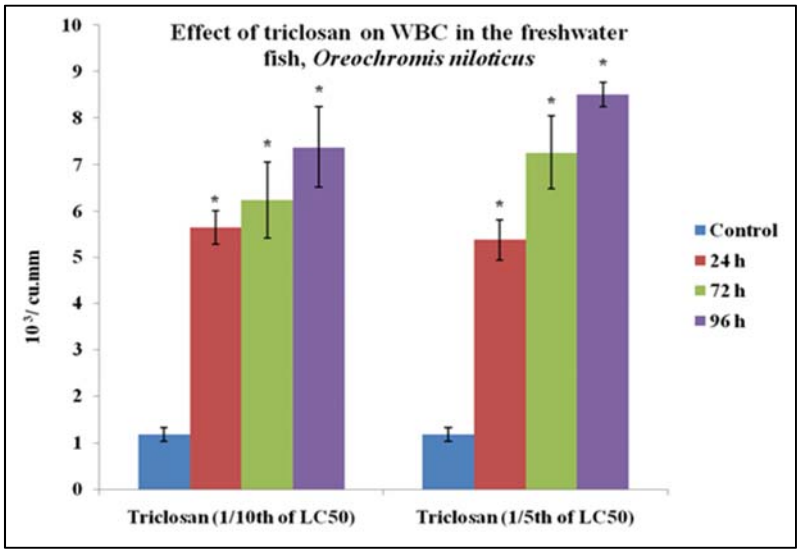

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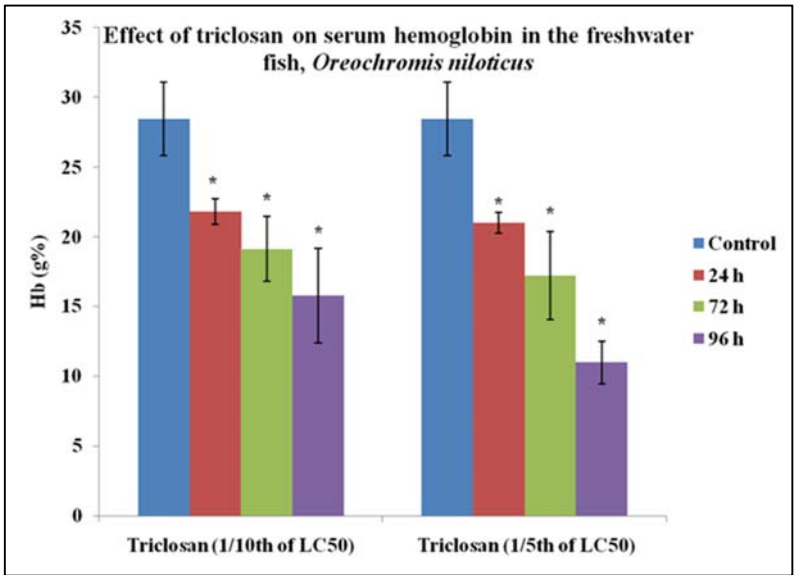

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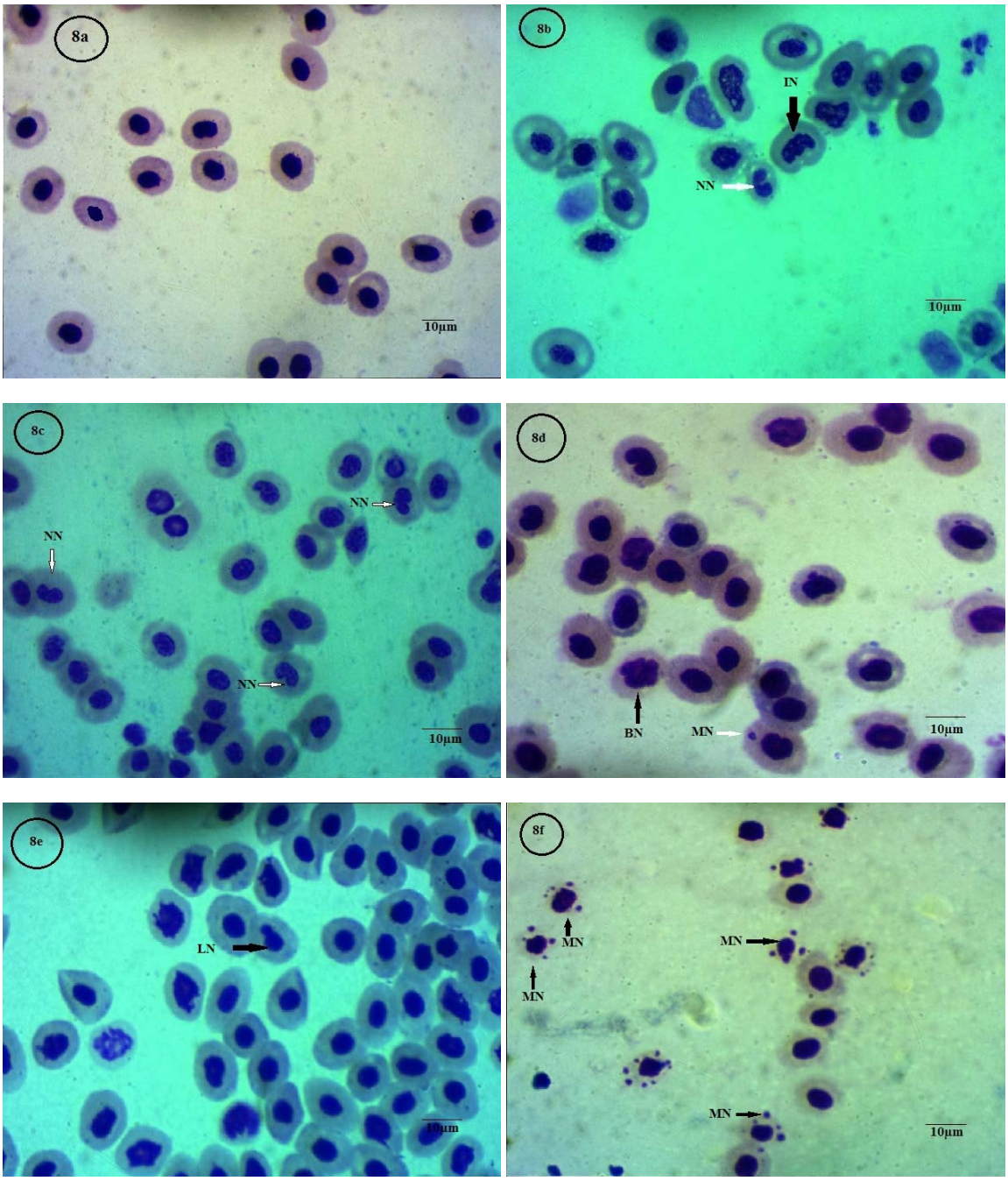

Fig 8

5. Conclusion

On the basis of the foregoing discussion, it can be summarized that exposure to triclosan caused severe genotoxic and haematological changes in the fish, *Oreochromis niloticus*. The consequences of both haematological and genotoxic endpoints observed in the present study are both concentration and time-dependent and can be widely applied to monitor the effects of triclosan in aquatic organism.

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