

Multiple biomarker responses of Nile tilapia (*Oreochromis niloticus*) exposed to textile industry effluents reaching Bolgoda North Lake, Sri Lanka

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Abstract

Textile industry effluents contain a complex mixture of chemicals which may have potential threats to biota. The present study was carried out to assess the potential impacts of selected textile industry effluents entering Bolgoda North Lake, Sri Lanka using multiple biomarkers responses of Nile tilapia viz. brain acetylcholinesterase (AChE), hepatic ethoxyresorufin *O*-deethylase (EROD) and erythrocytic micronuclei/nuclear alterations. The biomarker responses were determined in the fish upon exposure to the undiluted and diluted effluents along with the respective controls. Brain AChE activities of the fish exposed to the textile industry effluents were depressed (up to 40%) and frequencies of erythrocytic micronuclei and nuclear alterations were increased (up to 9 folds) significantly indicating the availability of neurotoxic and genotoxic substances in the effluents. Strong induction of hepatic EROD activities (up to 23 folds) in the exposed fish revealed the availability of CYP1A inducing pollutants which may have contributed to enhance the genotoxic potential of the effluents. The results revealed the sensitivity of these biomarkers of Nile tilapia to assess the biological effects of textile industry effluents. The depression of AChE activities and induction of EROD levels along with enhanced micronuclei and nuclear alterations in the fish exposed to the selected effluents can be considered as early warning signs for possible impacts pose by the textile industry effluents on fish populations inhabiting the effluent receiving water bodies.

Introduction

The use of biomarkers in fish for biomonitoring contaminants in aquatic environments has become popular in recent years. Cholinesterase especially acetylcholinesterase (AChE) in fish is regarded as an effective biomarker of neurotoxic chemical exposure (van der Oost et al. 2003). The measurement of a phase I biotransformation enzyme, Cytochrome P4501A (CYP1A) dependent ethoxyresorufin *O*-deethylase (EROD) in fish has become a promising biomarker for detecting aquatic contaminations of a variety of highly toxic organic pollutants especially aryl hydrocarbon receptor agonists (Whyte et al. 2000; van der Oost et al.

2003). Many genotoxic organic compounds only become harmful after biotransformation to active metabolites by the CYP1A system. Among the cytogenetic tests currently available for genotoxicity assessments of environmental pollutants, simultaneous expression of morphological erythrocytic nuclear alterations together with micronuclei in fish exposed to genotoxic substances has received considerable attention (Cavas and Ergene-Gözükara 2003; Lemos et al. 2008).

With the rapid industrialization, the discharge of industrial effluents containing undesirable toxic wastes into nearby surface waters has become a major source of water pollution especially in developing countries. Textile industrial sector is one of the largest industrial sectors of Sri Lanka. Disposal of textile industry effluents into nearby surface waters may pose severe environmental problems due to the generation of large volumes of waste water and the production of effluents with complex compositions including dyes, heavy metals and surfactants during various operations of textile processing. Aquatic contamination derived from industry effluents with complex chemical mixtures has raised concerns about safety of the environment (Bandara 2003). Several studies have reported mutagenic and genotoxic potential of textile dyes (Rajaguru et al. 1999; Oliveira et al. 2007). However no information is available on the biological effects of the effluents entering Sri Lankan waters. Objective of the present study was to assess the neurotoxic and genotoxic potential of several textile industry effluents discharged to Bolgoda North Lake, Sri Lanka using brain AChE activities and erythrocytic micronuclei and nuclear alterations in *Oreochromis niloticus*, a potential fish species for biomonitoring aquatic pollution under tropical conditions. In addition, hepatic EROD activities of the fish were determined to evaluate the influence of textile industry effluents on CYP1A system.

Materials and Methods

Study sites

The Bolgoda North Lake (6°44'16" – 6°49'52"N; 79°53'10" – 79°55'18"E) is an urban water body situated in the Western province of Sri Lanka which directly receives inputs from multiple pollution sources including the effluents from nearby textile industries. In the present study, three points which receive several textile industry effluents to the lake (Figure. 1) were selected as sampling sites (Site 1, 2 and 3). The Site 1 which is located in the extreme north part of the lake (Aththidiya area) received blackish colour effluents from a nearby textile industry which directly discharge the effluent to the site through pipes. The Site 2 (Borupana area) received bluish colour effluent from nearby two textile industries which discharge their effluents to the lake water through a ditch. The Site 3 (Thelawala) received light bluish colour effluent from another textile industry. The three sites were visited in January and February 2007 and temperature and pH of the effluents were measured in situ using portable water quality monitors. The temperature and pH of the effluents were respectively 27° C, pH 6.5-6.6 in Site 1; 27° C, pH 6.1-6.4 in Site 2; 27-28° C, pH 6.4-6.7 in Site 3. The effluents (nearly 100 L) were collected

separately from each site into transparent polythene bags and were brought to the laboratory within an hour for experimental exposure studies with Nile tilapia.

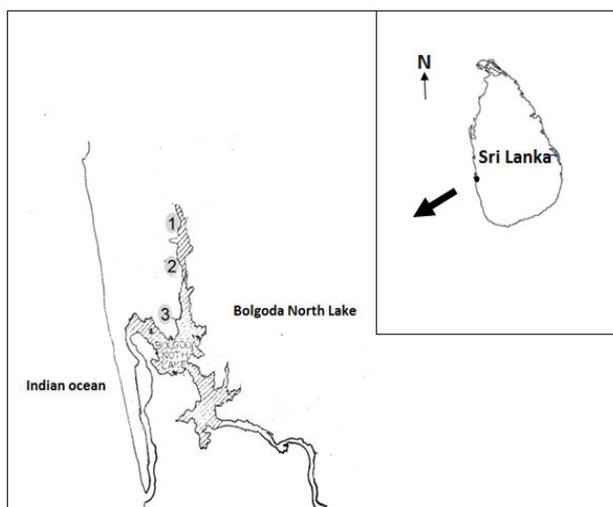


Figure 1. A map showing selected textile industry effluent receiving sites (Site 1- Aththidiya), Site 2 -Borupana and Site 3- Thelawala) in the Bolgoda North Lake, Sri Lanka

Effluent exposure

Nile tilapia (15- 28 g in body weight and 9-13 cm in total length) used in the effluent exposure studies had been obtained from Udawalawe freshwater fish breeding station, National Aquaculture Development Authority, Sri Lanka and acclimated to the laboratory conditions (water temperature 27-28 °C; pH 7.1-7.4; Dissolved oxygen 4.9-5.1 mg/L) for two weeks under the natural photoperiod. Fish were placed in aquaria (34 L, 4 fish per aquarium) with aged tap water (negative control) and two different concentrations (50% or 100%) of the textile industry effluent collected from each site. Cadmium was used as the positive control for genotoxicity testing (Cavas et al. 2004). Exposure aquaria were set up in duplicates. Due to the limited number of aquaria, exposure studies with the cadmium could not be carried out concurrently. Hence, exposure of fish to water borne cadmium (3 mg/L in aged tap water) was carried out separately along with another set of negative control (aged tap water only). After three days of exposure to the textile industry effluents, fish were anesthetized using benzocaine and blood samples were collected by severing the caudal vein for micronuclei and nuclear abnormality tests. Fish were dissected and brain and liver tissues were removed and frozen at -80° C in an ultra low temperature freezer until processing for enzyme assays.

Erythrocytic micronuclei and nuclear abnormalities testing

Blood smears of each fish were prepared and fixed in absolute ethanol for 20 minutes and air dried. The dried smears were flooded with 10% Giemsa solution for 25 minutes. Excess stain was rinsed off with running deionized water and slides

were blot dried before examination. Small non-refractive, circular or ovoid chromatin bodies lying in the cytoplasm showing the same staining pattern as the nucleus were considered as micronuclei. Cells with abnormal nuclei were classified as notched, blebbed and lobbed nuclei and binuclei according to Carrasco et al (1990). Thousand erythrocytes from each slide were scored under 10 X 100 magnifications using a bright field light microscope and the number of micronuclei and abnormal nuclei per 1000 cells ($^0/_{00}$) were determined.

Biomarker enzyme assays

The enzyme source for AChE assay was prepared by homogenizing the brain tissue of individual fish separately in ice-cold 0.1 M pH 8 phosphate buffer (20 mg tissue in 1 mL buffer). AChE activities in the tissue homogenates were determined at 28° C colourimetrically, following the method of Ellman et al. (1961) as a kinetic assay as described previously (Pathiratne et al. 2009). Microsomal fraction of liver tissues was prepared by differential centrifugation (Pathiratne et al. 2009). EROD activity in the liver microsomes was determined following the spectrophotometric method of Klotz et al. (1984). The enzyme activity was measured at 572 nm as a kinetic assay at 28 °C using a recording spectrophotometer (GBC Cintra 10e) fitted with a thermostated cuvette holder. Proteins present in the liver microsomes and brain homogenate were determined according to the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Data analysis

Data are presented as Mean \pm SEM, for 8 fish. Frequency of micronuclei and nuclear abnormalities in peripheral erythrocytes of the control and treated fish were transformed to $\ln(x + 1)$ before analysis. As no statistical differences in the data were found initially between the duplicate aquaria, the data were pooled and compared using One-way analysis of variance (ANOVA). The accepted level of significance was $p < 0.05$; where difference were significant, differences among the means were compared using Tukey's pair wise comparison test (Zar 1999).

Results

Brain AChE and hepatic EROD enzyme activities

Brain AChE and hepatic EROD activities of the fish exposed to the effluents are presented in Table 1. Brain AChE activity of the fish exposed to the diluted and undiluted effluents (except the diluted effluent from site 1 in the first sampling occasion) depressed to 24-40 % of the enzyme levels of the control fish. Hepatic EROD activity was significantly higher in the fish exposed to the diluted (2-19 folds) and undiluted (7-23 folds) effluents compared with the respective controls (Table 1). Greater EROD induction (12-23 folds) was observed in the fish exposed to the effluents from the Site 2 in both sampling occasions.

Erythrocytic micronuclei and nuclear abnormalities

Peripheral blood smears of Nile tilapia contained erythrocytes with micronuclei (Figure 2) and abnormal shaped nuclei in addition to the majority of

erythrocytes with normal elliptical or oval shape nuclei. Small non-refractive, circular or ovoid chromatin bodies lying in the cytoplasm showing the same staining pattern as the nucleus were considered as micronuclei (Figure 2a). Among the nuclei that were clearly deviated from their normal shape (Figure 2b) the nuclei with depth into a nucleus were recorded as notched nuclei. The nuclei with relatively small evaginations of the nuclear membrane which contain euchromatin were recorded as blebbed nuclei whereas evaginations larger than the blebbed nuclei which could have several lobes were considered as lobbed nuclei. The erythrocytes which were recorded as binucleated cells contained approximately equal size two nuclei with same staining intensity.

Table 1. Brain AChE and hepatic EROD activities in Nile tilapia exposed to selected textile industry effluents entering Bolgoda North Lake, Sri Lanka.

Exposure	Effluent concentration	AChE activity nmol/min/mg protein	EROD activity pmol/min/mg protein
January, 2007			
Aged tap water	-	429±5 ^a	57±8 ^a
Site 1 Effluent	50%	402±8 ^a	117±12 ^b
	100%	327±3 ^b	544±21 ^{cd}
Site 2 Effluent	50%	306±9 ^b	557±18 ^{cd}
	100%	301±11 ^b	697±8 ^d
Site 3 Effluent	50%	324±13 ^b	401±14 ^c
	100%	292±15 ^b	535±26 ^{cd}
February, 2007			
Aged tap water	-	425±8 ^a	48±3 ^a
Site 1 Effluent	50%	323±5 ^b	298±28 ^{bc}
	100%	302±11 ^b	703±17 ^d
Site 2 Effluent	50%	307±13 ^b	898±18 ^d
	100%	266±12 ^b	1087±31 ^e
Site 3 Effluent	50%	283±11 ^b	289±13 ^c
	100%	253±9 ^b	320±16 ^c

Data are presented as Mean ± SEM, n=8. In each column data indicated with similar superscript letters are not significantly different from each other (ANOVA, Tukey's Test, $p > 0.05$). Data were transformed to $\log(x+1)$ before statistical analysis. Aged tap water was used as the control.

Frequency of occurrence of micronuclei, binuclei and abnormally shaped nuclei (notched, blebbed and lobbed nuclei) in the peripheral erythrocytes of Nile tilapia exposed to cadmium (a genotoxin) and two different concentrations of textile industry effluents are presented in Table 2 along with the respective controls. Nile tilapia exposed to cadmium displayed significantly increased levels of micronuclei (4 fold), binuclei (6 fold) and nuclear abnormalities (3 fold) in comparison to the controls. Exposure of fish to the undiluted textile industry effluents collected from the three sites on both sampling dates caused significant increases (3-9 folds) in

frequency of micronuclei in the erythrocytes. Most prominent increases (9 fold) in micronuclei frequencies were observed in the fish exposed to the effluents collected from the Site 2. Exposure of fish to the diluted effluent (50% effluent) from the Site 2 also caused significant increases (4-5 folds) in erythrocytic micronuclei in comparison to the respective controls in both sampling occasions. Occurrence of binucleated erythrocytes was significantly higher in the fish exposed to undiluted effluents collected from the Sites 2 and 3 in both occasions. Frequency of the abnormally shaped nuclei (notched, blebbed and lobbed nuclei) was significantly higher in the fish exposed to the diluted and undiluted effluents from the Sites 2 and 3 in both occasions.

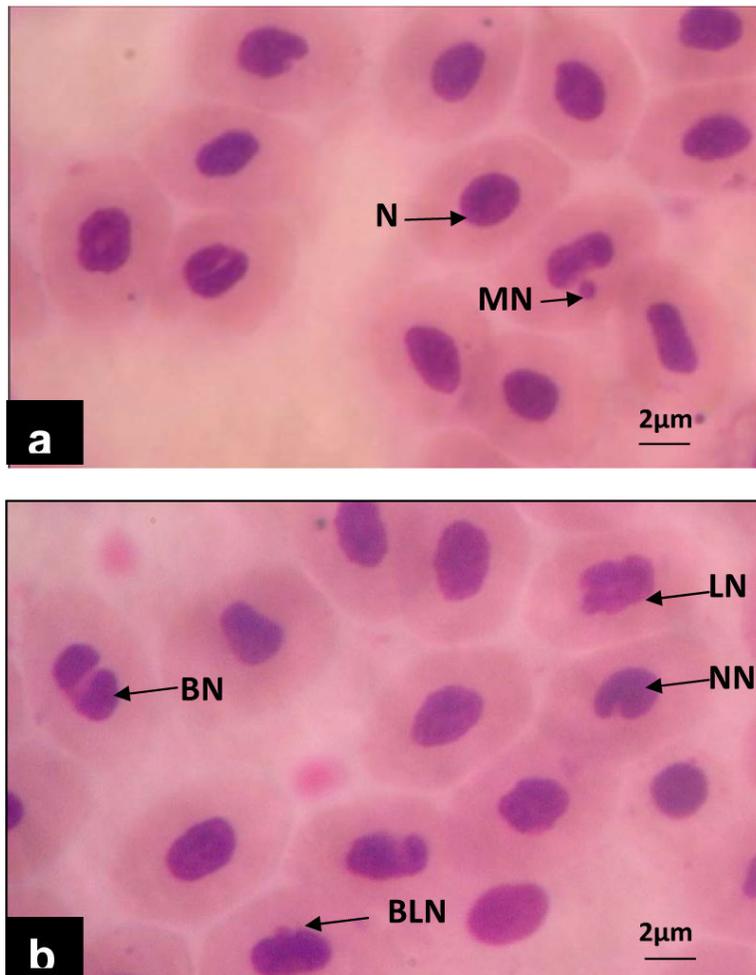


Figure 2. Peripheral blood smear of Nile tilapia showing normal nucleus (N), micronucleus (MN), noched nucleus (NN), blebbed nucleus (BLN), lobbed nucleus (LN) and binuclei (BN) in erythrocytes.

Table 2. Occurrence of erythrocytic micronuclei/nuclear alterations in Nile tilapia exposed to selected textile industry effluents entering Bolgoda North Lake.

Exposure	Effluent concentration	Micronuclei ($^0/_{00}$)	Binuclei ($^0/_{00}$)	Abnormal shaped nuclei ($^0/_{00}$)
Aged tap water	-	1.9±0.1 ^a	0.9±0.5 ^a	14.6±2.8 ^a
Cadmium(3 mg/L)	-	8.3±0.2 ^b	5.4±0.4 ^b	47.9±1.5 ^b
January, 2007				
Aged tap water	-	1.7±0.2 ^a	1.2±0.6 ^a	11.2±1.5 ^a
Site 1 Effluent	50%	3.6±0.6 ^{ab}	2.7±0.3 ^a	11.3±1.3 ^a
	100%	8.3±0.4 ^b	3.1±0.7 ^{ab}	44.3±2.8 ^b
Site 2 Effluent	50%	8.9±0.7 ^b	5.2±0.5 ^b	49.4±1.3 ^b
	100%	15.3±1.6 ^c	6.3±0.1 ^{bc}	105.6±4.5 ^d
Site 3 Effluent	50%	2.3±0.5 ^a	2.1±0.3 ^a	40.3±1.3 ^b
	100%	9.4±0.8 ^b	5.2±0.8 ^b	68.9±4.3 ^c
February, 2007				
Aged tap water	-	2.1±0.4 ^a	1.4±0.3 ^a	12.3±0.5 ^a
Site 1 Effluent	50%	2.2±0.3 ^a	1.7±0.3 ^a	10.2±0.6 ^a
	100%	7.3±0.4 ^b	3.2±0.5 ^{ab}	26.1±1.9 ^{ab}
Site 2 Effluent	50%	9.1±0.5 ^b	1.0±0.3 ^a	41.1±0.7 ^b
	100%	18.3±1.7 ^c	8.0±0.8 ^c	89.9±3.8 ^{cd}
Site 3 Effluent	50%	10.3±0.7 ^b	3.3±0.6 ^{ab}	36.9±2.5 ^b
	100%	11.9±1.1 ^b	7.0±1.1 ^{bc}	79.1±3.8 ^c

Data are presented as Mean ± SEM, n=8. In each column data indicated with similar superscript letters are not significantly different from each other (ANOVA, Tukey's Test, $p > 0.05$). Data were transformed to $\ln(x+1)$ before statistical analysis. Aged tap water was used as the control. Cadmium was used as the positive control.

Discussion

Depending on the nature of the raw material and product, textile processing industry employs a variety of chemicals which are very diverse in chemical composition ranging from the inorganic compound to polymers and organic products. Textile industrial processes generate waste water with contaminations of various heavy metals and textile dyes. Other toxic wastes in textile processing industries include materials such as toxic chlorinated organic solvents (e.g. perchloroethylene, trichlorobenzene, methylene chlorides and chloroform), non degradable surfactants such as ethoxylated phenols, biocides such as pentachlorophenol and toxic anions including sulfides (De Lima et al. 2005). The intense black/blue colours of the tested effluents tested in this study showed that they had been contaminated with different dyes used in the dyeing process. The pH of the effluents collected from the Site 2 of the lake were slightly acidic and not

within the national tolerance limits specified for waste from textile industry being discharged into inland waters of Sri Lanka (Anonymous 2008).

In the present study, significant inhibition (24-40%) of brain AChE activity was observed in Nile tilapia exposed to different textile industry effluents collected from the study sites. AChE in fish has been recognized as a biomarker for neurotoxic contaminants in aquatic environments especially organophosphate and carbamate pesticides. Some studies have suggested inhibition of cholinesterase activities with other types of pollutants including heavy metals, detergents and complex mixture of pollutants (van der Oost et al. 2003; Silva and Pathiratne 2008). Anticholinesterase chemicals including heavy metals present in the effluents tested in this study may have contributed to depression of brain AChE activities in the experimentally exposed fish. High levels of heavy metals in effluent receiving surface water (in $\mu\text{g/L}$: lead-28.6-32.4; cadmium-6.3-8.1, chromium 6.7-9.6, copper 9.1-40.3; zinc 26.3-53.8) and sediments (in $\mu\text{g/g}$ dry weight; lead-26.8-68.7; cadmium-2.4-4.8; chromium- 23.4-65.8; copper-45.2-56.8; zinc- 87-298) have been reported in this area of the Bolgoda Lake recently (Pathiratne et al. 2009). Depression of AChE activities by the textile industry effluents indicates their neurotoxic potential on aquatic fauna especially on native fish populations in the receiving water bodies. Effluent induced inhibition of brain AChE activities(24-40%) may not directly induce fish mortalities. Nevertheless, it could be an additional physiological stress for the fish populations inhabiting the lake.

The measurement of CYP1A dependent EROD in fish has become a promising biomarker for detecting aquatic contaminations of a variety of highly toxic organic pollutants especially aryl hydrocarbon receptor agonists (Whyte et al. 2000; van der Oost et al. 2003). In the present study, EROD activity was significantly higher in the fish exposed to the diluted (2-19 folds) and undiluted (7-23 folds) effluents compared with the respective controls. Greater EROD induction (12-23 folds) was observed in the fish exposed to the effluents from the Site 2. In a recent study, induction of CYP1A dependent EROD activity in gill filaments of rainbow trout upon water borne exposure to the textile dye, indigo has been observed (Jönsson et al. 2006). It has been reported that the natural and synthetic dye indigo has potency similar to that of dioxin to activate human AhR (Adachi et al. 2001). In the present study, strong induction of hepatic EROD activities in Nile tilapia upon 3 days exposure to diluted/undiluted textile industry effluents revealed the existence of CYP1A inducing toxic organic pollutants in these textile industry effluents. Effluent induced elevated EROD activities in the fish may be attributed to the presence of CYP1A inducing textile dyes such as indigo in the effluents which warrants further studies.

Azo dyes are the class most widely used industrially, having a world market share of 60-70%, and they have been found to be genotoxic and mutagenic in various test systems (Rajaguru et al. 1999; Oliveira et al. 2007; Carita and Marin-Morales 2008). In typical dyeing and printing processes, 50 -100% of the colour is fixed on the fiber and the remainder is discarded in the form of spent dye baths or in waste water from subsequent textile washing operations. Genotoxic potential of textile industry effluents on fish cells has been shown by evaluation of micronuclei frequencies (Cavas and Ergene-Gözükara 2003). Micronuclei are chromosome

fragments or whole chromosomes that lag at cell division due to the lack of centromere, damage, or a defect in cytokinesis. Micronuclei are formed by both clastogenic substances which induce breaks and produce alterations in the chromosome structure and aneugenic substances which induce alterations in chromosome distribution during the cell division process giving rise to aneuploidies (Heddle et al. 1991). The formation of morphological alterations in the nuclear envelope described by Carrasco et al. (1990) as blebbed, lobbed and notched nuclei have also been reported in erythrocytes of fish, as a consequence of exposure to environmental and chemical contaminants of genotoxic action (Cavas and Ergene-Gözükara 2003). Although the mechanisms underlying the formation of nuclear abnormalities have not been fully explained, these abnormalities are considered to be indicators of genotoxic damage and, therefore, they may complement the scoring of micronuclei in routine genotoxicity surveys. Cadmium is a known genotoxicant to fish (Cavas et al. 2004). In the present study, as expected cadmium significantly increased the formation of micronuclei (4 fold), binuclei (6 fold) and nuclear (notched, blebbed and lobbed nuclei) abnormalities (3 fold) in Nile tilapia. Significant increases in the frequency of micronuclei (up to 9 folds) were seen in the peripheral erythrocytes of Nile tilapia exposed to textile effluents collected from the three sites on two sampling stages compared to the respective controls. In general undiluted effluents caused the highest increases in the occurrence of micronuclei/binuclei and other nuclear abnormalities. The highest induction in the frequency of micronuclei was recorded from the effluents entering the Site 2 (up to 9 folds) indicating the high genotoxic potential. The results indicate that the effluents reaching the lake contain genotoxic substances (clastogenic and/or aneugenic) which can induce DNA damage and micronuclei formation in the erythrocytes. Consequences of continuous genotoxic stress on native fish populations can be initiation of mutations, accelerated aging of cells, ineffective adaptation to changing environmental conditions or as worst case, carcinogenesis.

In conclusion, the present study demonstrates the sensitivity of tested biomarkers of Nile tilapia to assess the toxic effects of textile industry effluents. Depression of brain AChE activities of the fish exposed to the textile industry effluents entering the study sites of the lake indicates the availability of neurotoxic substances in the effluents. The results also indicate that the textile industry effluents contain genotoxic agents that can induce DNA damage in the erythrocytes of Nile tilapia. Strong induction of hepatic EROD activities in the fish revealed the availability of CYP1A inducing pollutants in the textile industry effluents which may have contributed to enhance the genotoxic potential. The results demonstrate that the textile industry effluents entering the Site 2 may pose more hazards compared to the other sites. The depression of AChE activities and induction of EROD levels along with enhanced micronuclei and nuclear alterations in the fish exposed to the selected effluents can be considered as early warning signs for possible ecological impacts pose by the textile industry effluents on fish populations residing in effluent receiving water bodies. This was the first study which used the biomarkers to assess toxicity of textile industry effluents entering Sri Lankan waters.

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