

Assessing genotoxic potential of petroleum refinery wastewater using biomarkers of laboratory exposed and field captured fishes

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Received: 05.05.2021 Revised: 13.06.2021 Accepted: 17.06.2021 Online: 15.09.2021

Abstract Petroleum refinery wastes contain mixtures of toxic compounds including Polycyclic Aromatic Hydrocarbons (PAH) which may pose genotoxic threats to the biota. The objective of the present study was to assess the genotoxic potential of wastewaters of the petroleum refinery at Sapugaskanda, Sri Lanka using erythrocytic micronuclei and nuclear abnormalities as genotoxic biomarkers in the fish *Oreochromis niloticus* exposed to the refinery wastewater under controlled laboratory conditions and in the field captured fishes from the canals close to the refinery. Erythrocytic micronuclei and nuclear abnormalities in the peripheral blood were evaluated using cytogenetic tests. Patterns of fluorescent aromatic compounds in the fish bile were also examined using fixed fluorescence spectrometry to assess potential PAH exposure. Erythrocytic micronuclei, nuclear buds and notched nuclei in the peripheral blood were highly induced ($P < 0.05$) in *O. niloticus* exposed to the wastewater for 7 days under laboratory conditions in comparison to the control fish. The field-captured fishes, *Trichogaster pectoralis* and *Dawkinsia singhala* inhabiting water canals near the petroleum refinery also demonstrated significant induction ($P < 0.05$) of erythrocytic micronuclei and other nuclear abnormalities. PAH exposure indicative bile fluorescence patterns (naphthalene, phenanthrene, pyrene, and benzo(a)pyrene types) were detected in the fish exposed to the wastewater under laboratory conditions and in the field captured fishes. The results revealed that the wastewater of petroleum refinery contains genotoxic chemicals including PAHs. Hence, incorporation of genotoxicity tests as bioanalytical tools for regulating the discharge of final refinery wastewater to the aquatic ecosystems would be prudent in consideration of sustainable development goals focusing on the ecosystem and human safety.

Keywords: Bile fluorescent aromatic compounds; genotoxicity; petroleum refinery wastewater; polycyclic aromatic hydrocarbons; fish micronuclei; nuclear abnormality

INTRODUCTION

The petroleum industry generates large volumes of wastewater as petroleum refining is a water-intensive practice. Petroleum refinery waste may contain a great variety of organic and inorganic contaminants such as hydrocarbons, phenols, heavy metals, sulfides, ammonia, nitrogen compounds, oil, and grease as well as suspended solids (Daflon et al. 2017; Varjani et al. 2017; Saien and Shahrezaei 2012). Although most of these compounds/ substances are treated and recovered in the refinery before they enter the final effluent, a

significant amount of toxic contaminants can enter the wastewater. Hence concern on the toxicity of final wastewater generated by the petroleum refining industry has been increased in recent years due to the potential health threats associated with their release into the waterways (Almasi et al. 2014; Daflon et al. 2017).

Bioassays and biomarkers are considered modern bio-analytic tools for assessing environmental quality (Wieczerszak et al. 2016). In recent years, genotoxic and mutagenic potential of petroleum refinery effluents has been assessed using various bioassays (Gupta et al. 2015; Hara



and Marine-Morales 2017; Iqbal et al. 2017). Induction of micronuclei and other nuclear abnormalities in the cells are considered as biomarkers of the genotoxic damage (Heddle et al. 1991; Van der Oost et al. 2003; Arslan et al. 2015; Hemachandra and Pathiratne 2016; Hemachandra and Pathiratne 2017). Fish are excellent animal models for assessing the potential genotoxicity of refinery effluents discharged to aquatic ecosystems. Previous studies have reported induction of micronuclei in the fish *Oreochromis niloticus* upon exposure to the effluents from several petroleum refineries (Cavas and Ergene-Gozukara 2005; Hoshina et al. 2008; Hoshina & Marin-Morales 2010) and river water affected by a refinery effluent (Souza and Fontanetti 2006, Hara et al. 2017; Radelyuk et al. 2021).

Polycyclic Aromatic Hydrocarbons (PAHs) are considered the most likely cause for genotoxicity associated with petroleum refinery effluents (Rodrigues et al. 2010; Vargini et al. 2017). PAH parent molecules and highly reactive intermediates produced during biotransformation of PAH within the body may induce deleterious effects on the genetic material leading to mutations and cancers (Shafy and Mansour 2016). As bile represents the major excretory route for the biotransformed PAH metabolites in the fish, biliary fluorescence patterns relevant to specific PAH metabolites can be used as biomarkers for assessing the exposure of fish populations to PAHs (Van der Oost et al. 2003; Johnson-Restrepo 2008; Beyer et al. 2010, Bali et al. 2016). PAH contamination in some inland water bodies in Sri Lanka had been assessed based on bile fluorescence patterns as biomarkers of exposure in resident fish species (Pathiratne et al. 2010; Hemachandra and Pathiratne 2011; Ranasinghe and Pathiratne 2015).

The petroleum refinery located at Sapugaskanda in Gampaha District is the single largest oil refinery of Sri Lanka. It plays a major role in the Sri Lankan economy and petroleum has been identified as a nonindigenous energy form that can supplement the local resources in fulfilling the energy demand of the country. Physico-chemical characteristics of wastewater discharged from the petroleum refinery are expected to comply with the tolerance limits of the general standards of industrial effluents discharged into inland surface waters in Sri Lanka (Anonymous 2008). However, no scientific studies had been conducted to assess

the potential toxicity of wastewater generated from this petroleum refinery and the effects associated with petrochemical pollution on surrounding ecosystems. For the first time, the present study was aimed at assessing potential genotoxicity associated with wastewater of petroleum refinery in Sri Lanka using biomarkers of laboratory exposed and field captured fishes. The genotoxic potential was assessed using erythrocytic micronuclei and nuclear abnormalities of the test fish, *Oreochromis niloticus* under laboratory bioassays and field captured fishes, *Trichogaster pectoralis* and *Dawkinsia singhala* inhabiting water canals near the petroleum refinery. In addition, bile fluorescence patterns in these fishes were also examined as biomarkers for potential PAH exposure.

MATERIALS AND METHODS

Test fish for bioassay

Fingerlings of Nile Tilapia, *O. niloticus* were obtained from the Udawalawe freshwater fish breeding station of the National Aquaculture Development Authority of Sri Lanka. Fish were kept in large circular tanks filled with aerated aged tap water for two weeks for acclimation to laboratory conditions. Half of the water in the tanks was renewed every 2 days. The fish were daily fed with commercial fish food pellets. Fish were not fed 24 hours before the commencement of the bioassay. During the acclimation period, temperature, pH and dissolved oxygen (DO) concentration in the water tanks (range: minimum to maximum) were 26-30°C, 6.8-7.1, and 4-5 mg/L respectively.

Petroleum refinery wastewater

In the present study, samples of petroleum refinery final wastewater were collected into double-walled polythene bags from wastewater pond of Petroleum refinery Division of Ceylon Petroleum Corporation (6°58'45"N; 79°55'40"E) in June 2011 and transported within 30 minutes to the laboratory for the bioassay. Temperature, pH, Total Dissolved Solids (TDS) and DO levels in the composite wastewater samples were measured in situ using calibrated multiprobe water quality checker (YSI/Model: 556 MPS, USA). For the determination of selected chemical parameters, wastewater samples were brought to the laboratory

in glass bottles. Biochemical Oxygen Demand (BOD₅), Chemical Oxygen Demand (COD), oil and grease levels in the wastewater were measured following APHA (1998).

Fish bioassay: Laboratory exposure to refinery wastewater

O. niloticus (8.9±0.2 cm in total length, 10.3±0.8 g in body weight) which had been acclimated to the laboratory conditions were exposed separately to 50% and 100% of petroleum refinery wastewater samples in glass tanks (40 L) in triplicates (n=10 fish per tank) for 7 days under static conditions. Concurrently the fishes in another three sets of tanks (n=10 fish per tank) were maintained in the aged tap water used for dilution of the wastewater samples (dilution water control). The exposure media were moderately aerated. General water quality parameters in the fish tanks with the dilution water and 50% and 100% of the wastewater used for exposure studies were measured daily using the water quality checker (YSI/Model: 556 MPS, USA) and found to be within the desirable limits for fish (temperature: 27-28°C, pH:7-7.4, and DO levels: 4.9-5.3 mg/L). The Oil and grease content of the exposure media was measured on day one of the bioassays as described by APHA (1998). Oil and grease content in the 50% and 100% of wastewater were 3.4 and 7.2 mg/L respectively. Oil and grease were not detected in the dilution water. Fish were not fed during the seven days exposure period. After 2 days of exposure, ten fish representing each exposed water type were sampled randomly from each replicate tank (3-4 fish per tank) for biomarker studies. The remaining fish were maintained under static exposure conditions and sampled again on the 7th day for biomarker studies. The remaining fish were sacrificed individually under an overdose of the anaesthesia (70 mg/L Benzocaine). Blood was drawn from the caudal vein of each fish onto a glass slide and a thin smear of blood was prepared, air-dried, and processed further as described below for assessment of erythrocytic micronuclei and nuclear abnormalities. Bile samples were taken from each fish to Eppendorf tubes and frozen at -80°C until bile fluorescence analysis. All applicable international guidelines for the care and use of animals were followed in this study concerning the laboratory maintenance and bioassessment with the fish.

Field captured fish in the canals near Petroleum Refinery

The Pattiwila canal and Heiyanthuduwa canal flows through the area under the influence of the Petroleum Refinery at Sapugaskanda (Figure 1). In the present study, three sites of the Pattiwila canal (Sites A, B and C) and one site of the Heiyanthuduwa canal (Site D) were selected for capturing wild fish species for biomarker assessments. Site A which is situated behind the Petroleum Refinery, Sapugaskanda, is the source point (6°57'24.37"N; 79°57'28.66"E) from which the Pattiwila canal commences. Site B (6°57'06.24"N; 79°57'03.58"E) is the middle stream of the Pattiwila canal which is situated about 1.2 km away from the site A. Site C (6°56'17.91"N; 79°57'31.51"E) is downstream of the Pattiwila canal which is about 0.8 km away from site B. Site D (6°58'08.52"N; 79°57'28.30"E) is located in the Heiyanthuduwa canal which is located in the vicinity of the petroleum refinery. In addition, a relatively less polluted water body, Bathalagoda reservoir (7°45'N; 80°37'E) was used as the reference site (Site R) as no other suitable reference site could be found in the canals near the area. Temperature, pH, TDS, and DO levels of surface water of each site were measured onsite in July 2011 using the water quality checker. BOD₅, COD, and oil and grease contents in the surface water samples were determined as described by APHA (1998). Snakeskin gourami, *Trichogaster pectoralis* (7.7±0.3 cm in total length, 6.8±0.4 g body weight) were collected from the three sites in Pattiwila canal whereas blackspot barb, *Dawkinsia singhala* (6.8 ±0.3 cm in total length, 6.1 ±0.2 g body weight) were collected from Heiyanthuduwa canal using cast nets. They were commonly available fish species in these study sites. In addition, samples of *T. pectoralis* (7.9±0.3cm in total length, 6.9±0.3 g body weight) and *D. singhala* (6.4 ±0.2cm in total length, 5.9±0.3 g body weight) were collected from the reference site (Bathalagoda reservoir) using cast nets for comparison. These fish were collected into polythene bags filled with water samples collected from the same habitat and the water in the bags was well oxygenated. The fish samples were transported to the laboratory and peripheral blood samples and bile samples were taken from each fish for biomarker studies as mentioned previously.

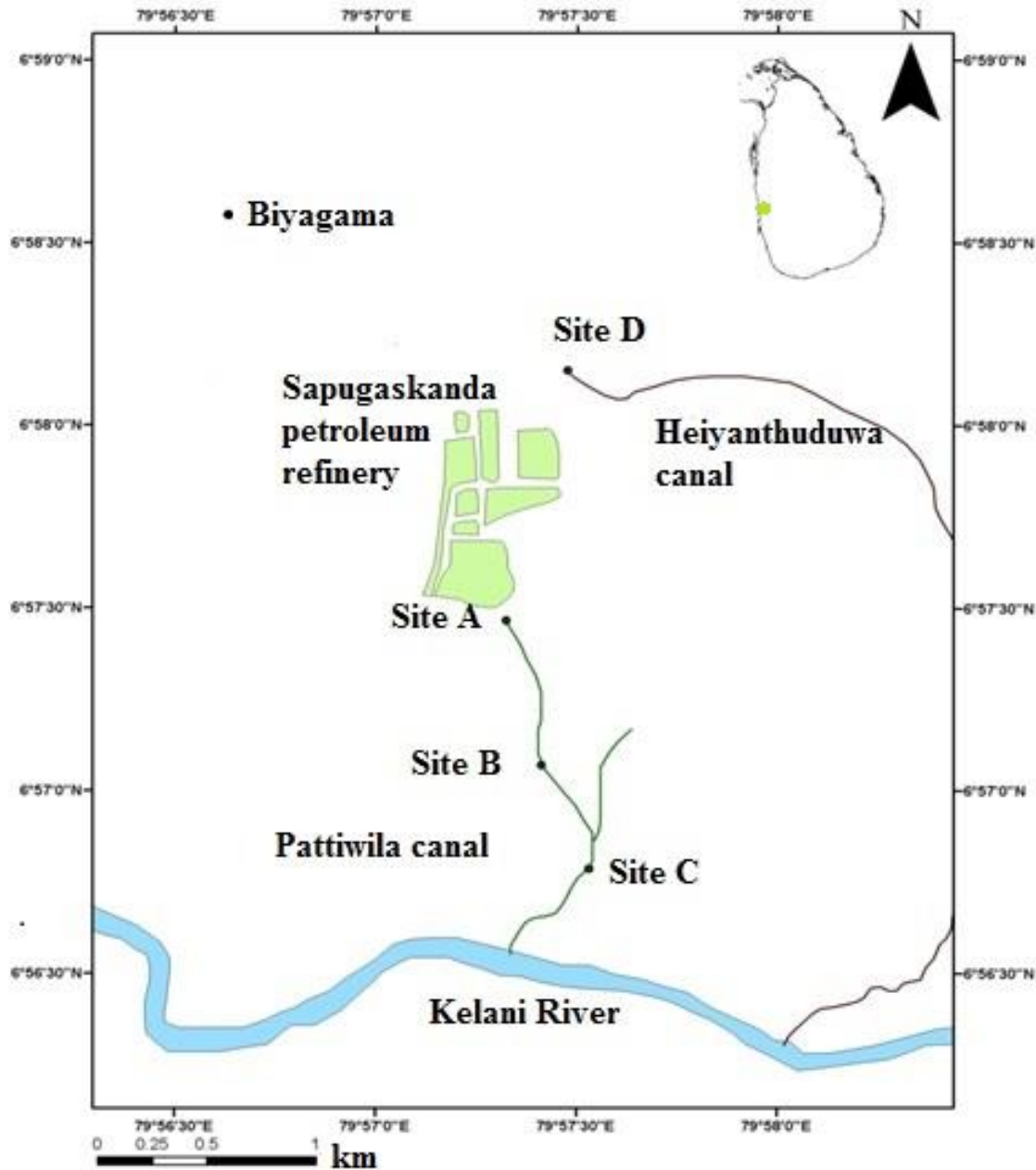


Fig 1 Location of the fish captured from the canals near petroleum refinery at Sapugaskanda for biomarker studies: Site A: upstream of the Pattiwila canal, Site B: middle stream of the Pattiwila canal, Site C: downstream of the Pattiwila Canal, Site D: the Heiyanthuduwa canal

Genotoxicity assessment

Peripheral blood samples were smeared onto glass microscope slides and were air-dried. The slides were fixed in methanol for 20 minutes and stained for 7 minutes with 5% Giemsa solution (Cavas and

Ergene-Gozukara 2005). The slides were rinsed with distilled water, air-dried, and examined under oil immersion ($\times 1000$ magnification) of a binocular bright light microscope. At least 1000 erythrocytes were examined for each fish on coded slides. Small, non-refractive, circular, or ovoid chromatin bodies

in the cytoplasm, displaying the same staining and focusing pattern as the main nucleus, were scored as micronuclei (Al-Sabti and Metcalfe 1995). Nuclear abnormalities in the erythrocytes other than micronuclei were classified as nuclear buds, binuclei, and notched nuclei (Cavas and Ergene-Gozukara 2005). Nuclear buds were characterized as extruded nuclear material that appears like a micronucleus with a narrow bridge to the main nucleus. The erythrocytes with two nuclei were considered binucleated cells. Nuclei with voids with appreciable depth into the nucleus were recorded as notched nuclei. The frequencies of micronuclei, nuclear buds, bi-nuclei, and notched nuclei in the erythrocytes were expressed separately as numbers per 1000 erythrocytes examined (%).

Detection of fluorescent aromatic compounds in fish bile

PAH metabolites in fish bile were screened by detecting fluorescent aromatic compounds with fixed wavelength fluorescence technique using computer-controlled Varian Cary Eclipse fluorescence spectrophotometer. Two μL of bile diluted in 4 mL of 48% ethanol were used to decrease the absorption and quenching of the fluorescence signal. Fluorescence measurements were performed in quartz cuvettes. Fixed wavelength fluorescence at the excitation/emission wavelength pairs 290/335 nm and 341/383 nm and 380/430 nm were used for detection of naphthalene type, pyrene type, and benzo(a)pyrene type metabolites respectively as described by Aas et al. (2000). Fixed wavelength fluorescence at the wavelength pair 260/380 nm was used for the detection of phenanthrene type-metabolites (Krahn et al. 1993). The slit width was set at 2.5 nm for the detection of PAH metabolites. The fixed fluorescence at the respective wavelength pair was expressed as arbitrary fluorescence units (A.F.U.).

Statistical analysis

Micronuclei and nuclear abnormality data were subjected to ArcSin transformation and other data were transformed to $\text{Log}_{10}(x+1)$ scale where appropriate before statistical analysis (Zar 1998). The transformed data on frequencies of micronuclei and other nuclear abnormalities, and bile fluorescence measurements of the fishes, *O.*

niloticus and *T. pectoralis*, were analyzed separately using one-way analysis of variance test. If there were significant differences, Tukey's test was used for the comparison of means. The accepted level of significance was $P < 0.05$. Student's t-test was used for comparison of the data sets on the fish, *D. singhala*.

RESULTS

Physico-chemical characteristics of refinery wastewater and surface water of canals near the refinery

Measured physico-chemical parameters of the composite sample of petroleum refinery wastewater were within the acceptable limits for discharge of industrial effluents into inland surface waters in Sri Lanka (Table 1). Physico-chemical parameters of the water in the Pattiwila and Heiyanthuduwa canals near petroleum refinery and the reference site (Bathalagoda reservoir) indicate that the TDS, BOD₅, COD, and oil and grease levels in the Site A of Pattiwila canal and Site D of Heiyanthuduwa canal were comparatively higher than those of the other sites (Table 1).

Genotoxicity assessment of O. niloticus exposed to the refinery wastewater under laboratory conditions

All *O. niloticus* could survive until the end of the exposure period. However, the fish exposed to 50% and 100% of the wastewater were lethargic and displayed sluggish behavior at 5 days of exposure onwards compared to the control fish. Erythrocytes with micronucleus and different types of nuclear abnormalities (nuclear bud, binuclei, and notched nucleus) seen in the examined blood smears of *O. niloticus* are shown in Figures 2(a) to 2(d). The fish exposed to 100% wastewater showed significant induction of micronuclei after 2 days and 7 days exposure (Table 2). Concentration-dependent significant increase in the occurrence of erythrocytes with nuclear buds or notched nuclei were seen in the fish exposed to the refinery wastewater at 2 days and 7 days of exposure. Binuclei were not seen in the control fish, but a significant increase in the occurrence of binucleated erythrocytes was observed in the fish exposed to 100% wastewater (Table 2).

Table 1 Measured physico-chemical parameters of the petroleum refinery wastewater and the surface water in the canals near Petroleum Refinery and reference site

Parameter	National tolerance limits*	Refinery wastewater	Pattiwila Canal			Heiyanthuduwa Canal	Reference site (Bathalagoda reservoir)
			Site A	Site B	Site C	Site D	
Temperature(°C)	< 40	31	30	30	31	32	30
pH	6-8.5	7.47	7.4	7.4	7.3	7.3	7.1
Total dissolved solids (mg/L)	-	100	108	88	49	308	26
Dissolved Oxygen (mg/L)	-	4.7	4.3	4.2	4.6	4.1	5.2
Biochemical oxygen demand 5 days (mg/L)	30	20	12	5	3	22	2
Chemical oxygen demand (mg/L)	250	110	57	22	26	75	10
Oil and grease (mg/L)	10	7.2	1.6	0.4	0.1	1.8	0

*Tolerance limits for discharge of industrial effluents into inland surface waters in Sri Lanka assuming at least 8 times dilution with clean receiving water (Anon. 2008)

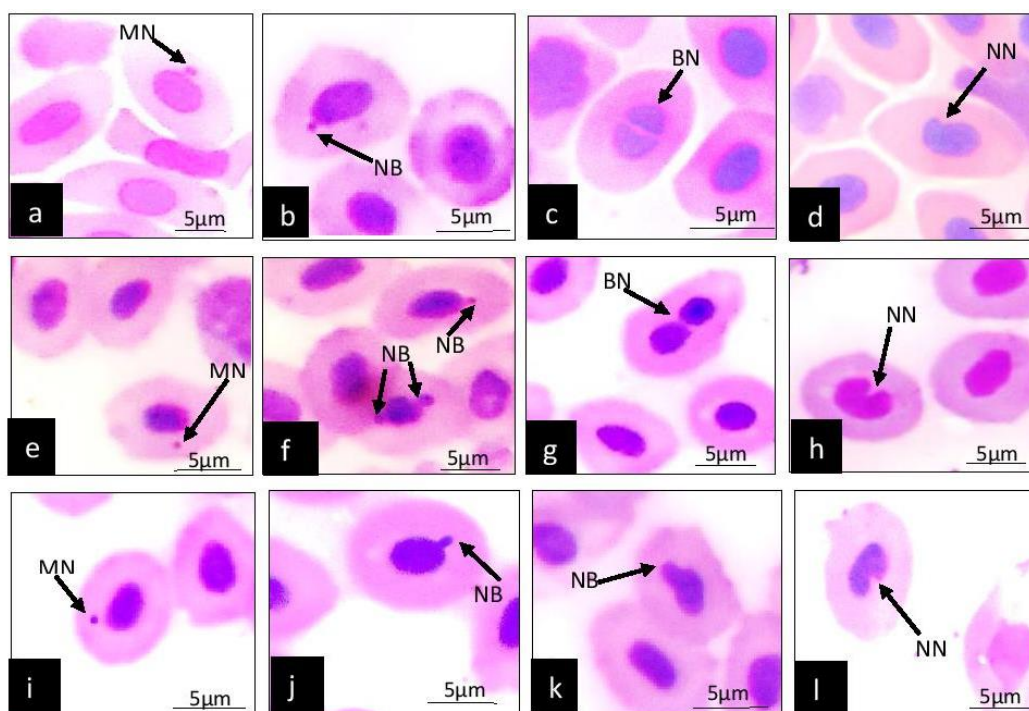


Fig 2 Erythrocytic micronuclei and nuclear abnormalities found in the blood of test fishes: *O. niloticus* (a, b, c, d) following exposure to petroleum refinery wastewater in the laboratory; field captured *T. pectoralis* (e, f, g, h) and *D. singhala* (i, j, k, l) residing canals near the petroleum refinery. MN: Micronucleus; NB: Nuclear bud; BN: Binuclei; NN: Notched nucleus

Table 2 Occurrence of erythrocytic micronuclei, nuclear buds, binuclei and notched nuclei in the peripheral blood of *O. niloticus* after two days and seven days exposure in the laboratory to petroleum refinery wastewater

Exposure	Micronuclei	Occurrence of nuclear abnormality (%)		
		Nuclear buds	Binuclei	Notched nuclei
2 days				
Control (dilution water)	0.1±0.1 ^a (0-1)	0.5±0.3 ^a (0-2)	0± 0 ^a	3.5±0.7 ^a (1-6)
50% wastewater	1.5±0.5 ^a (0-4)	6.6±1.8 ^b (0-21)	0.5±0.3 ^a (0-2)	19.3±4.6 ^b (12-25)
100% wastewater	5.5 ±0.9 ^b (1-10)	17.7±1.6 ^c (11-29)	3.9±0.6 ^b (1-7)	31.3±6.5 ^c (26-56)
7 days				
Control (dilution water)	0.1±0.1 ^a (0-1)	0.7±0.4 ^a (0-3)	0± 0 ^a	4.3±0.6 ^a (1-7)
50% wastewater	2.5± 0.5 ^a (1-5)	12.3±2.7 ^b (1-24)	1.4± 0.5 ^a (0-4)	18.8±5.4 ^b (13-27)
100% wastewater	9.0±1.6 ^b (1-17)	31.6±2.9 ^c (12-44)	5.5± 0.5 ^b (2-8)	42.8±7.9 ^c (32-61)

* Data are presented as Mean ± SEM (Minimum and maximum range), n=10 fish per group. In a column, for a specific exposure duration, data indicated with different superscripts are significantly different from each other. (P <0.05).

Figure 3 shows the fixed fluorescence intensities of bile of *O. niloticus* exposed to 50% and 100% of petroleum refinery wastewater and the dilution water (control) after 2 days and 7 days exposure. Bile fluorescence intensities corresponding to naphthalene-type and phenanthrene-type metabolites were significantly higher in the fish exposed to the 50% and 100% wastewater in comparison to the respective controls (Figure 3). However, no significant differences were observed either with the duration of exposure or strength of the wastewater to these metabolite

types. Bile fluorescence intensity corresponding to phenanthrene-type metabolites was decreased at 7 days exposure to 100% wastewater in comparison to 2 days exposure. Bile fluorescence intensity corresponding to pyrene-type metabolites was significantly higher than controls only in the bile of fish exposed to 50% wastewater for 7 days. The fluorescence intensity of benzo(a)pyrene-type metabolites was significantly higher only in the fish exposed to 50% wastewater after 2 days and 7 days of exposure in comparison to the control fish (Figure 3).

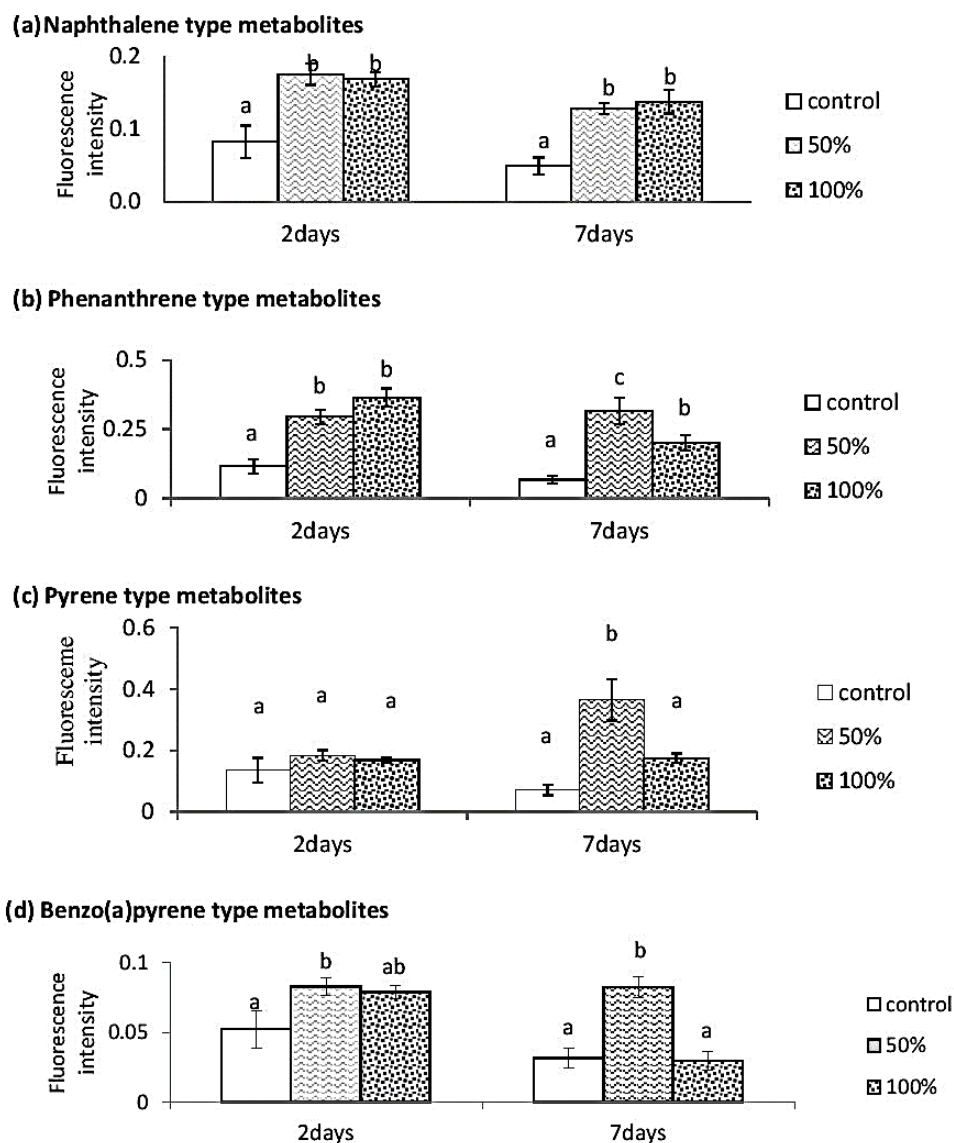


Fig 3 Fixed fluorescence intensities (arbitrary fluorescent units) of bile of *O. niloticus* exposed to 50% and 100% of petroleum refinery wastewater and the dilution water (control) after 2 days and 7 days exposure. Results are presented as mean \pm SEM, n=10 fish per group. For each category, bars indicated with different letters are significantly different from each other ($P < 0.05$).

Genotoxicity assessment of *T. pectoralis* and *D. singhala* collected from canals near the petroleum refinery

Erythrocytes with micronuclei and nuclear buds, binuclei, and notched nuclei seen in the examined blood smears of field captured *T. pectoralis* are presented in Figure 2 (e) to 2 (h). Binuclei were seen only in the erythrocytes collected from *T. pectoralis* collected from Site A. Significantly higher occurrence of micronuclei, and nuclear buds were observed in the erythrocytes of *T. pectoralis* (Table 3) collected from site A of Pattiwila canal in comparison to the fish from the control site and other sites (B and C). The frequency of erythrocytes with notched nuclei was significantly higher in the

blood of *T. pectoralis* captured from sites A and B in comparison to those from the reference site and site C. In the blood smears of *D. singhala* only erythrocytes with micronuclei, nuclear buds, and notched nuclei were seen (Figure 2 (i) to 2 (l)) in addition to the normal erythrocytes. Micronuclei were not seen in *D. singhala* captured from the reference site whereas induction of micronuclei up to 21‰ was observed in some individuals of *D. singhala* captured from Site D of the Heiyanthuduwa canal. Erythrocytes with nuclear buds and notched nuclei were significantly higher in *D. singhala* captured from the Heiyanthuduwa canal in comparison to those from the reference site (Table 3).

Table 3 Occurrence of erythrocytic micronuclei, nuclear buds, binuclei and notched nuclei in the peripheral blood of field captured *T. pectoralis* and *D. singhala* inhabiting the canals near petroleum refinery (Sites A, B, C of the Pattiwila canal and Site D of Heiyanthuduwa canal)

Fish and sampling site	Occurrence of nuclear abnormality (‰)			
	Micronuclei	Nuclear buds	Binuclei	Notched nuclei
<i>T. pectoralis</i>				
Site A	17.8±1.7 ^b (14-22)	14.8±2.2 ^b (10-23)	0.4± 0.4 ^a	45.2±5.5 ^b (32-60)
Site B	0.8±0.5 ^a (0-2)	5.2±0.7 ^a (3-7)	0± 0 ^a	32.4±3.6 ^b (22-44)
Site C	0.4±0.4 ^a (0-2)	1.2±0.8 ^a (0-4)	0± 0 ^a	15.4±2.1 ^a (11-23)
Reference site	0.2±0.2 ^a (0-1)	1.0±0.4 ^a (0-3)	0± 0 ^a	11.8±1.1 ^a (9-15)
<i>D. singhala</i>				
Site D	14.4±2.4 ^b (7-21)	17.0±1.9 ^b (12-22)	0± 0 ^a	42.2±5.4 ^b (23-53)
Reference site	0±0 ^a	1.8±0.7 ^a (0-3)	0± 0 ^a	10.2±1.3 ^a (7-14)

*Data are presented as Mean±SEM (Minimum and maximum range), n=5 fish per group. In a column, for a particular species, data indicated with different superscripts are significantly different from each other. (P <0.05). Reference site (Site R) is the Bathalagoda reservoir

Bile fluorescence (fixed fluorescence) intensities of field captured *T. pectoralis* and *D. singhala* are presented in Figure 4. *T. pectoralis* collected from site A of Pattiwila canal demonstrated significantly high fluorescent intensities corresponding to Naphthalene-type, metabolites compared to the fish from other sites of the canal and the reference site (Figure 4a). Fluorescent intensities relevant to phenanthrene-type and benzo(a)pyrene-type metabolites were significantly higher in the fish collected from Sites

A and B of the canal compared to the fish from the other sites. Pyrene-type metabolites in the bile were significantly higher in *T. pectoralis* collected from the three sites of the Pattiwila canal compared to the fish from the reference site. *D. singhala* captured from the Heiyanthuduwa canal (Figure 4b) demonstrated significantly higher fluorescence intensities corresponding to Naphthalene-type, Phenanthrene-type, Pyrene-type, and Benzo(a)pyrene-type metabolites in comparison to the fish collected from the site R (reference site).

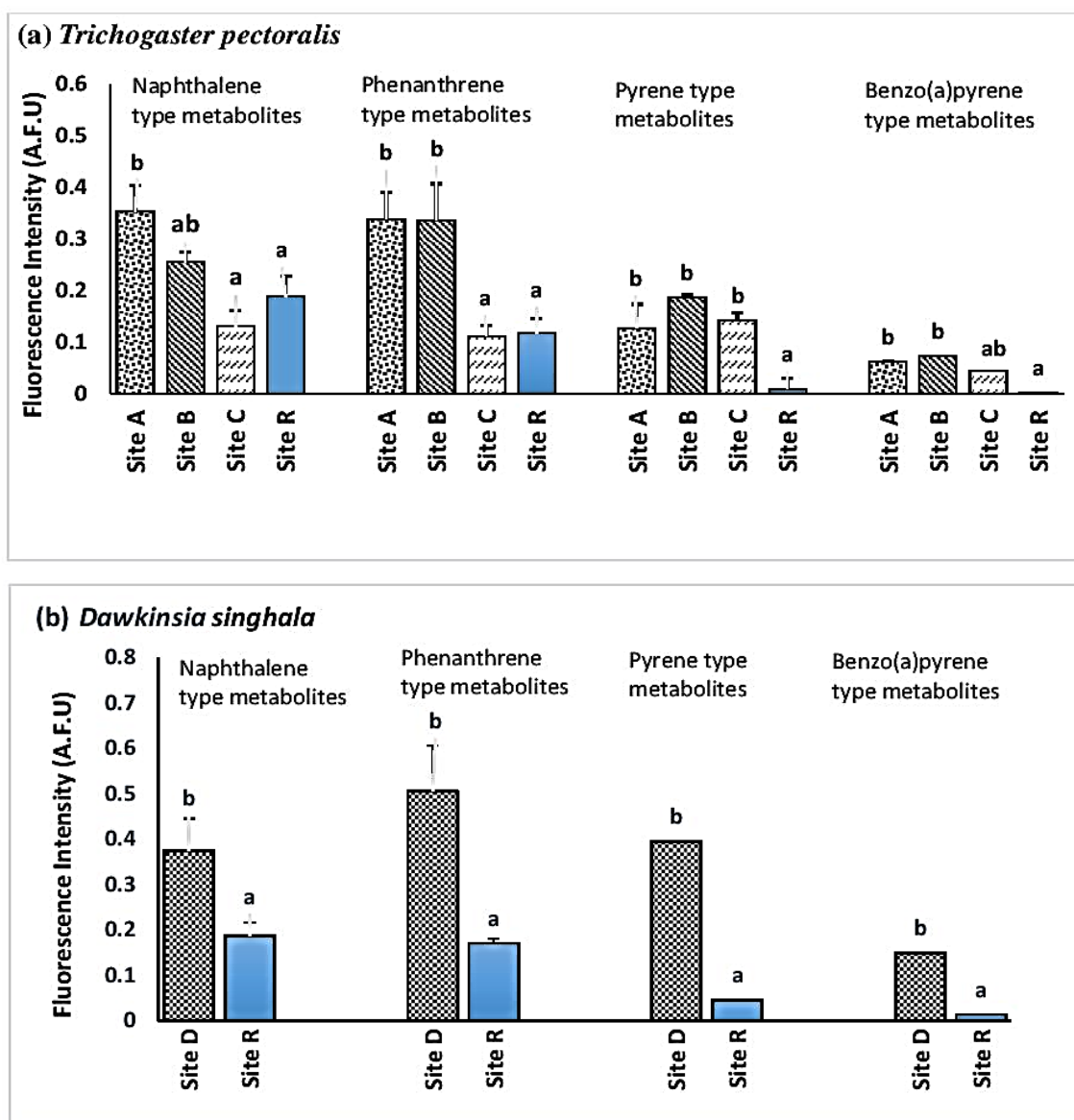


Figure 4 Fixed fluorescence intensities (arbitrary fluorescent units) of bile of *T. pectoralis* and *D. singhala* captured from canals near the petroleum refinery (Sites A, B, C of the Pattiwila canal and Site D of the Heiyanthuduwa canal) and Bathalagoda reservoir, the reference site (Site R). Results are presented as mean \pm SEM, n=5 fish per group. For each type of metabolite, bars indicated with different letters are significantly different from each other ($P < 0.05$).

DISCUSSION

The impact of wastewater generated by the petroleum refining industry on aquatic ecosystems have been increasingly concerned in recent years as the refinery waste contains genotoxic PAHs, phenols, and heavy metals which can directly or indirectly induce DNA damage (Almasi et al. 2014;

Gupta et al. 2015; Daflon et al. 2017). Testing wastewaters of several petroleum refineries by a variety of bioassays revealed that the refinery wastewaters are genotoxic and mutagenic which would pose health risks to the exposed biota (Cavas and Ergene-Gozukara 2005; Hoshina et al. 2008; Gupta et al. 2015; Iqbal et al. 2017). Genotoxic effects associated with wastewater can be assessed using fish biomarkers as early warning signals. For

the first time, the present study provided scientific evidence for genotoxic potential associated with the petroleum refinery wastewater generated by the oil refinery of Sri Lanka. Even though measured physico-chemical parameters of the tested petroleum refinery wastewater at Sapugaskanda were within the acceptable limits for discharge of industrial effluents into inland surface waters in Sri Lanka (Anonymous 2008), the wastewater exhibited genotoxic properties as revealed by induction of erythrocytic micronuclei and nuclear abnormalities in the fish *O. niloticus* exposed to the wastewater under laboratory conditions.

Among the genotoxic tests that can be conducted routinely for the genotoxic evaluation, the micronucleus test with fish is a simple, reliable, and economical test that can be used in refinery wastewater quality assessments (Al-Sabti and Metcalfe 1995; Cavas and Ergene-Gozukara 2005; Hoshina et al. 2008). Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments after clastogenic action or intact whole chromosomes lagging in the anaphase stage of cell division as a result of aneugenic effects. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Heddle et al. 1991; Udroui 2006). In the present study, *O. niloticus* exposed to the wastewater showed significant induction ($P < 0.05$) of micronuclei after 2 days and 7 days exposure indicating genotoxicity. In the field captured fishes, significantly higher frequencies of erythrocytic micronuclei were observed in *T. pectoralis* and *D. singhala* captured from the sites close to the petroleum refinery (site A of Pattiwila canal and Site D of Heiyanthuduwa canal respectively) in comparison to those from the other sites indicating the genotoxic impact associated with the refinery. Physico-chemical analysis of the surface waters of these sites revealed high TDS, COD, oil, and grease levels compared to the other sites indicating that Sites A and D of the nearby canals are more polluted than the other sites. Field studies have shown that oil refinery effluents often have an impact on the fauna, which is usually restricted to the area close to the outfall (Wake 2005). Our study with field-captured fishes also showed that sites subject to petrochemical influence were under higher genotoxic impact. Micronuclei induction in fish indicates genotoxic and mutagenic effects (Heddle

et al. 1991) which may eventually lead to malignancy (Hintzsche et al. 2017).

In addition to micronuclei, nuclear abnormalities have been detected in fish erythrocytes exposed to various industrial effluents indicating genotoxic impacts (Hemachandra and Pathiratne 2016; 2017; Weldetinsae et al. 2017). Although the processes such as problems in segregating tangled and attached chromosomes, aneuploidy, and cellular degradation are speculated, the mechanisms underlying the formation of such nuclear abnormalities are not fully explained (Weldetinsae et al. 2017). Concentration-dependent increase ($P < 0.05$) in the frequencies of erythrocytic nuclear buds and notched nuclei were seen in *O. niloticus* exposed to the refinery wastewater at 2 days and 7 days. In the field captured fishes, significantly higher occurrence of nuclear buds and notched nuclei were observed in the erythrocytes of *T. pectoralis* and *D. singhala* captured from the sites close to the refinery in comparison to those from the other sites. Even though binuclei were not seen in *O. niloticus* exposed to the dilution water (controls), a significant increase in the frequency of binucleated erythrocytes was observed in the fish exposed to 100% wastewater. Binuclei were seen only in the erythrocytes of *T. pectoralis* captured from the Site close to the refinery (Site A). The occurrence of binucleated cells is an indicator of abnormal cell division due to the blocking of cytokinesis which would result in a genetic imbalance in the cells (Weldetinsae et al. 2017).

PAHs can contribute to the genotoxicity associated with petroleum refinery effluents (Wake 2005; Vargini et al. 2017). Analysis of bile fluorescence using fixed wavelength fluorescence spectrometry is a simple method that can be used to screen semi-quantitatively the recent exposure of fish to PAHs (Aas et al. 2000; Beyer et al. 2010). In the present study, a significant increase in bile fluorescence patterns corresponding to naphthalene-type, phenanthrene-type, pyrene-type, and benzo(a)pyrene-type metabolites were detected in the fish *O. niloticus* exposed to the wastewater under laboratory conditions. However, a consistent pattern was not observed either with the duration of exposure or strength of the wastewater to these PAH metabolite types. This may be associated with the differences in the absorption and metabolism of each type of PAH in the fish body as well as the degradation of PAHs in the exposed water over

time. Naphthalene-type metabolites were significantly higher in the field captured *T. pectoralis* collected from the site closest to the refinery (Site A) compared to the other sites of the Pattiwila canal. Phenanthrene-type, pyrene-type, and benzo(a)pyrene-type metabolites in *T. pectoralis* captured from other sites of the Pattiwila canal were also significantly higher than those from the reference site in most cases. Bile fluorescence patterns corresponding to naphthalene-type, phenanthrene-type, pyrene-type, and benzo(a)pyrene-type metabolites were significantly higher in the fish *D. singhala* captured from the Heiyanthuduwa canal compared to the reference site. Bile fluorescence patterns of laboratory exposed and field captured fishes indicate bioavailability of PAHs through exposure to petroleum refinery wastewater. As aromatic hydrocarbons produced by the refineries are difficult to remove from the final effluent by conventional treatment systems, a combination of additional techniques is required before it is released into the environment (Almasi et al. 2014). If the waste treatment process at the refinery is not improved, continuous genotoxic stress posed by the refinery wastewater may ultimately lead to an increase of mutations and ineffective adaptation to changing environmental conditions affecting the health of native fauna especially the fish populations in the receiving waterways.

In conclusion, analysis of biomarker responses in the laboratory exposed and field captured fishes in the present study revealed that the wastewater of petroleum refinery of Sri Lanka contains genotoxic chemicals including PAHs. Hence, the release of inadequately treated refinery wastewater to the aquatic environment may pose genotoxic impacts to the biota especially the native fish populations in the receiving ecosystems. This study presents the scientific evidence for the necessity of improving current wastewater treatment technologies for reducing biological impacts. In view of human and ecosystem health under chronic exposure, genotoxicity tests may serve as powerful bioanalytical tools when regulating refinery wastewater discharges into inland waterways as they can detect potential toxicity due to interactive effects of all substances present in the wastewaters. Hence, incorporation of genotoxicity tests for final refinery wastewater quality assessments would be

prudent considering sustainable development goals focusing on the ecosystem and human safety.

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