

Genotoxic and mutagenic effects of diesel oil water soluble fraction on a neotropical fish species

T.P. Vanzella^a, C.B.R. Martinez^b, I.M.S. Cólus^{a,*}

^a Departamento de Biologia Geral, Universidade Estadual de Londrina (UEL), Paraná, Brazil

^b Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina (UEL), Paraná, Brazil

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Abstract

Numerous spills and leakages involving petroleum and its derivatives have recently occurred in Brazilian rivers. Considering the lack of information regarding the genotoxic response of neotropical fish to these events and the predominance of information regarding saltwater fish, which offers no genuine comparisons, the present work aimed to evaluate the genotoxicity and mutagenicity of the diesel water soluble fraction (DWSF) on the neotropical fish *Prochilodus lineatus* under acute (6, 24 and 96 h) and subchronic (15 days) exposures, using the comet (SCGE) and micronucleus assays. The results indicated genotoxic and mutagenic damage in erythrocytes of *P. lineatus* exposed to DWSF. Comet scores for fish exposed to DWSF in all experimental periods were significantly higher than the respective negative control groups (fish exposed to clean water for the same period). The relative frequencies of micronucleated erythrocytes for *P. lineatus* exposed to DWSF under acute and subchronic treatment were also significantly higher than their respective negative controls. Taken together these results showed that acute and subchronic exposures to DWSF produce mutagenic and genotoxic effects on the blood cells of *P. lineatus* and that the combination of comet and micronucleus assays proved to be both suitable and useful in the evaluation of the genotoxicity of diesel oil due to their complementary action.

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1. Introduction

The deliberate discharge and accidental release of harmful chemical compounds into the aquatic environment has the potential to disturb the structure and functioning of natural ecosystems [1,2]. The major contributors to the high release of foreign substances in aquatic environment are industrial, urban and agricultural activities and the discharges from waste water

treatment plants and oil refineries [3–5]. A large proportion of aquatic contaminants of anthropogenic origin are constituted of potentially genotoxic and carcinogenic substances [2].

Petroleum and its refined products are found all over the planet [6], which justifies the recent global concern with pollution originated from oil spills that can result in the deposition of large quantities of aromatic hydrocarbons in the aquatic ecosystem [7]. In Brazil, accidents involving oil spills occur frequently, arising from oil tanker spills and transportation duct ruptures [8]. Therefore, oil spills are a source of water pollution and oil slicks can spread and reach rivers and other aquatic ecosystems, causing serious pollution problems

* Corresponding author. Tel.: +55 43 3371 4417;
fax: +55 43 3371 4527.

E-mail address: colus@sercomtel.com.br (I.M.S. Cólus).

[9]. Thus, the evaluation and prediction of the damage caused by these petroleum products on the aquatic environment have become very important and urgent issues [10].

Petroleum can be distilled into several products, like engine fuels such as diesel oil and gasoline. The toxicity of petroleum and its derivatives is mostly attributed to its soluble part [11], which contains low weight molecular polar compounds, such as monoaromatic hydrocarbons (benzene, toluene and xylene) and small polyaromatic hydrocarbons (PAHs) [9].

Diesel oil is a complex chemical mixture of hundreds of chemical substances and its main components can be divided in two classes: aliphatic and aromatic hydrocarbons. In diesel oil, naphthalene (the simplest PAH) and its compounds are the most frequently found (0.4%) [9].

Currently, there are several works that have evaluated the genotoxicity of petroleum hydrocarbons and their derivatives in aquatic organisms, and most of these used bivalve and fish species [12–16]. The biotransformation of PAHs by fish provides the appearance of intermediary compounds potentially genotoxic, capable of inducing acute toxicity and genetic alterations and their presence in water sediments are correlated with liver neoplasm in benthic fish species [2,4]. Most of the research concerning the toxicity of petroleum and its products is related to saltwater organisms, while petroleum hydrocarbons toxicity to freshwater organisms is poorly researched [17]. Particularly, some reports have been published concerning the effects of petroleum hydrocarbons on freshwater fish such as detection of DNA strand breaks and adducts [18], increases of micronuclei [19], absence of micronuclei in erythrocytes [20] and induction of erythrocytic nuclear abnormalities [21]. The use of neotropical fish in this sort of research is even scarcer [22,23] and these studies did not apply methods to study DNA damage.

The fish species *Prochilodus lineatus* (Valenciennes, 1847) is native to the south and southeast regions of Brazil and represents a well suited species to environmental monitoring as it is a bottom feeder fish which is in contact with xenobiotics in water and in sediment and also has been shown to be sensitive to petroleum derivatives [23,24].

Numerous spills and/or leakages involving petroleum and its by-products have recently occurred in Brazilian rivers. In 2001, the most important oil spills in a freshwater ecosystem ever reported were when Petrobrás Co. accidentally discharged 50,000 L of crude oil in the Barigui River, in southern Brazil [22]. Although these kind of large oil spills are widely covered in the media, it is believed that the principal source of inland waters contamination from petroleum and its derivatives is due

to small and continuous leakages from underground bulk storage tanks of fuel oils, such as diesel oil, thereby reaching groundwater and later rivers [25]. Thus, considering the lack of information regarding the genotoxic response of neotropical fish to these events and the predominance of information regarding saltwater fish which offers no genuine comparisons, the present work aimed to evaluate the genotoxicity and mutagenicity of the diesel water soluble fraction (DWSF) on a neotropical fish species from Brazilian rivers under laboratory conditions, using the comet (SCGE) and micronucleus assays.

2. Material and methods

2.1. Animals

Juvenile specimens of *P. lineatus* (Valenciennes, 1847), weighting 32.15 ± 14.97 g (mean \pm S.D., $n = 117$), were supplied by the Universidade Estadual de Londrina Hatchery Station. Prior to the toxicity tests fish were acclimated to laboratory conditions for a minimum of 7 days in a 600-L tank with aerated water ($T \cong 21.3$ °C; $pH \cong 7.35$; $OD \cong 7.79$ mgO₂ L⁻¹; conductivity $\cong 110$ μ S cm⁻¹; hardness $\cong 80$ mg L⁻¹ CaCO₃). During this period, fish were fed with pellet food each 48 h. Fish were not fed during and on the day preceding the experiments.

2.2. Preparation of diesel water soluble fraction (DWSF)

To obtain the DWSF one part of commercial diesel oil was added to four parts water in a glass container and placed in darkness for 16 h prior to exposure to intense solar light for 6 h, simulating a diesel spill in tropical conditions [26]. After that the upper insoluble phase was discharged and the remaining water phase was collected and diluted to 50% DWSF with dechlorinated water. DWSF (before and after dilution) was examined spectrofluorimetrically for the presence of mono- and polyaromatic hydrocarbons.

2.3. Toxicity tests

Animals were exposed to a 50% dilution of DWSF in water in acute (6, 24 and 96 h) and subchronic (15 days) static toxicity tests, performed in glass aquaria of 100 L containing eight fish each. One negative control group, consisting of eight animals exposed only to clean water (the same as that used for acclimation) was terminally sampled at each experimental interval along with the experimental groups exposed to water plus DWSF. Positive control groups, consisting of fish injected with the clastogenic agent cyclophosphamide (20 mg kg⁻¹, Sigma—CAS no. 64-86-8) were terminally sampled 6 and 24 h after treatment. Replicates were carried out for each acute experimental interval. During the subchronic exposure water (in control group) or water plus DWSF (in experimental group) was renewed after 7 days. During the tests water was contin-

uously monitored for temperature, dissolved oxygen, pH and conductivity.

At each exposure period, animals were removed from the aquaria, immediately anesthetized with benzocaine (0.1 g L^{-1}) and blood samples were taken from the caudal vein by means of heparinized plastic syringes. Subsequently fish were killed by cervical section.

2.4. Comet assay

Alkaline comet assay was performed as described by Singh et al. [27] and Speit and Hartmann [28]. At first microscope slides were coated with 1.5% normal melting point agarose (NMP) at 60°C , dried at room temperature for 24 h and stored at 4°C . Blood samples were diluted (1:100 v/v) in saline solution (17 mM NaCl, 4.8 mM KCl, 1.5 mM CaCl_2 , 1.2 mM NaHCO_3 , 4.5 mM Na_2HPO_4 , 2.9 mM NaH_2PO_4) and 15 μL of cell suspension were mixed with 120 μL of 0.5% low melting point agarose (LMP) at 37°C . These suspensions were spread on the slides previously coated with NMP which were then covered with a cover slip and maintained for 20 min at 4°C to allow the agarose to solidify.

The cover slips were then removed and slides were immersed in freshly made lysing solution, for 1 h, at 4°C and protected from light. The slides were then placed in an electrophoresis buffer (pH > 13.0), in the dark for 20 min, to allow the DNA to unwind, and a current of 25 V (1.0 V/cm; 300 mA) was applied for another 20 min. Following electrophoresis, slides were washed three times with neutralization buffer, fixed with 100% ethanol for 10 min, dried at room temperature and kept under refrigeration until cytological analyses. Slides stained with ethidium bromide ($20 \mu\text{g mL}^{-1}$) were analyzed using a Nikon fluorescence microscope (100 \times objectives) fitted with a 515–560 nm excitation filter and a 590 nm barrier filter. Slides were coded independently and scored blindly. DNA migration was visually determined in 100 randomly select and non-overlapping cells per fish. Fifty cells were analyzed per slide, with two slides per animal. DNA damage was classified in four classes, according to the tail length of the comet: 0, undamaged; 1, minimum damage; 2, medium damage; 3, maximum damage, according to Kobayashi et al. [29]. The mean number of damaged nucleoids (classes 1–3) was calculated per specimen exposed to DWSF and their respective controls, for each exposure period, as the total number of damaged nucleoids divided by the number of fish in each group. The score of damage for each fish was calculated as the number of nucleoids observed in each damage class multiplied by the value of its respective damage class (0, 1, 2 or 3). Results were expressed as the mean score of damage, for each treatment group, where 0 represents absence of damage and 300 indicates the highest damage score.

2.5. Micronucleus test

The micronucleus test was performed according to the methodology of Hoofman and de Raat [30]. Immediately after

sampling, a drop blood was smeared on clean slides which were dried at room temperature and after 24 h were fixed in 100% methanol for 10 min. Afterwards, they were stained with 5% Giemsa solution for 20 min, air-dried and then prepared for permanent use.

Cytological analysis was done under a Nikon optical microscope (100 \times objective). A total of 3000 erythrocyte cells were examined per fish in coded slides. The mean micronucleus frequencies found in each experimental group were calculated.

2.6. Statistical analysis

Differences in comet score and micronucleus frequency between the groups of fish exposed to DWSF and their respective controls, for the same experimental interval, were determined using the Mann–Whitney non-parametric statistical test. Positive controls were compared to its respective negative controls using Student's *t*-test. Comparison among the same experimental treatment (DWSF or Control), in different exposure periods (6, 24, 96 h and 15 days) were done using the Kruskal–Wallis analysis of variance followed by Dunn's Multiple Comparison test. Statistical analyses were done according to Zar [31] and values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Water analysis

Qualification of the DWSF showed the presence of monoaromatic and polyaromatic hydrocarbons at all exposure times. The fluorescence emissions showed similar profiles along the period of 15 days and indicated a predominance of compounds that are normally found in DWSF, like benzene, toluene, xylene, naphthalene, fluorine and phenanthrene. The physical–chemical characteristics of the water during the experiment and in the control groups, for all the experimental periods, remained stable and the values obtained were (mean \pm S.D.): temperature $22.63 \pm 0.10^\circ\text{C}$; pH 6.97 ± 0.03 ; dissolved oxygen $6.59 \pm 0.08 \text{ mg O}_2 \text{ L}^{-1}$; conductivity $111.89 \pm 2.36 \mu\text{S cm}^{-1}$.

3.2. Comet assay

The results obtained using the comet assay in erythrocytes of *P. lineatus* exposed to the DWSF and its respective negative and positive controls are presented in Table 1.

Animals subjected to DWSF in all exposure periods showed a significant increase in the number of damaged nucleoids in relation to their respective negative control. The mean number of damaged nucleoids did not differ

Table 1

Number of nucleoids observed in each comet class (0–3) and mean number of damaged nucleoids per fish in erythrocytes of *Prochilodus lineatus* exposed to diesel water soluble fraction (DWSF), taking into account the total number of fish (*N*) analyzed for each experimental treatment (acute and subchronic) and their negative and positive controls (100 cells per specimen were analyzed)

Period of exposure	Treatments	<i>N</i>	Comet classes				Number of damaged nucleoids per fish (mean \pm S.D.)
			0	1	2	3	
6 h	DWSF	16	57	637	788	118	96.438 \pm 4.381a*
	Negative control	16	1299	249	50	2	18.813 \pm 12.150
	Positive control	6	2	208	337	53	99.667 \pm 0.516*
24 h	DWSF	14	141	752	428	79	89.929 \pm 4.411b**
	Negative control	14	1110	282	8	0	20.714 \pm 3.384
	Positive control	7	4	310	340	46	99.429 \pm 0.535*
96 h	DWSF	16	161	349	490	600	89.938 \pm 8.250bc*
	Negative control	12	950	196	54	0	20.833 \pm 11.052
15 days	DWSF	6	13	134	278	175	97.833 \pm 2.137ac*
	Negative control	7	535	146	19	0	23.571 \pm 7.345

Different letters indicate significant differences ($p < 0.05$) among exposure periods.

* Significant difference from respective negative control ($p < 0.05$).

** Significant difference from respective negative and positive controls ($p < 0.05$).

between fish exposed to DWSF for 6 h and 15 days, for 24 and 96 h and for 96 h and 15 days (Table 1).

Comet scores for fish exposed to DWSF in all experimental periods were significantly higher than the respective negative control groups (Fig. 1). Fish exposed to DWSF for longer periods (96 h and 15 days) presented comet scores significantly higher than those exposed to DWSF for 24 h (Fig. 1).

3.3. Micronucleus assay

The relative frequencies of micronucleated erythrocytes for *P. lineatus* exposed to DWSF under acute (6,

24 and 96 h) and subchronic treatment (15 days) were significantly higher than their respective negative controls (Table 2). Comparison of micronuclei frequencies among different exposure time to DWSF, indicated that the highest frequency occurred at 24 h, which was similar to that obtained at 96 h and different from the others (6 h and 15 days). The lowest value observed for this parameter occurred after 6 h of exposure to DWSF and showed no statistical difference from the results obtained after the subchronic treatment (15 days).

4. Discussion and conclusion

The present study demonstrates genotoxic and mutagenic damage in erythrocytes of the neotropical fish *P. lineatus* exposed to DWSF. In the comet assay the high number of damaged cells (Table 1) in fish exposed to diesel oil indicates an elevated level of DWSF genotoxicity, similar to that seen after 6 and 24 h treatment with cyclophosphamide (positive control). Studies of aquatic organisms exposed to crude oil [13] or monitoring environments contaminated with industrial petroleum effluents [4,7,15,32] regarding marine aquatic fish or mussels demonstrated that the compounds derived from petroleum induced high indices of DNA damage by the comet assay.

Studies with freshwater species exposed to the PHAs, polychlorinated biphenyls (PCBs) or petroleum by-products, as reported by Winter et al., Pandrangi et al., and Klobucar et al. [18,33,34] also confirm the genotoxicity of these pollutants in freshwater organisms. The

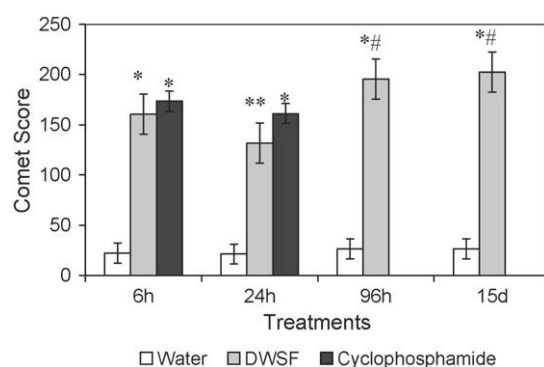


Fig. 1. Comet scores for erythrocytes of *Prochilodus lineatus* exposed to diesel water soluble fraction (DWSF) under acute and subchronic treatments and their respective negative (water) and positive controls (cyclophosphamide). *Significant difference from respective negative control ($p < 0.05$). **Significant difference from respective negative and positive controls ($p < 0.05$). #Significant difference from fish exposed to DWSF for 24 h ($p < 0.05$).

Table 2

Frequencies of micronucleated erythrocytes in *P. lineatus* after acute exposures (6, 24, 96 h) and subchronic exposure (15 days) to diesel water soluble fraction (DWSF) and the respective negative and positive controls (3000 erythrocytes per specimen were analyzed)

Period of exposure	Treatment	N	Total number of micronuclei observed	Frequency of micronucleated erythrocytes (mean \pm S.D.)
6 h	DWSF	16	52	3.25 \pm 2.27a*
	Negative control	15	13	0.87 \pm 1.13
	Positive control	6	28	4.00 \pm 0.82*
24 h	DWSF	16	233	16.64 \pm 4.78b*
	Negative control	15	27	1.80 \pm 0.77
	Positive control	7	34	4.86 \pm 1.07*
96 h	DWSF	16	163	10.19 \pm 2.95bc*
	Negative control	12	14	1.17 \pm 1.19
15 days	DWSF	6	36	6.00 \pm 1.67ac*
	Negative control	7	13	1.86 \pm 1.07

Different letters indicate significant differences ($p < 0.05$) among exposure periods. N = total number of sampled specimens.

* Significant difference from respective negative control ($p < 0.05$).

effects of diesel oil to *P. lineatus* observed in the present study can result in different forms of biological damage. Since this fish species is of great commercial value and represents an important food item for human consumption [35], it could act as vectors of these contaminants from the environment to humans and others vertebrates. PAH availability and the occurrence of genotoxic damage in mammals after consuming mussels contaminated with these pollutants were reported by Lemiere et al. [36].

The process of PAHs biotransformation in fish, in contrast to that which occurs with the most of chemical compounds, frequently converts these xenobiotics into intermediary highly toxic reactive substances [37] that may cause oxidative damage to DNA. The genetic effects, such as DNA strand breakage and micronuclei presence may cause mutagenicity, carcinogenicity, teratogenicity and subsequent population alterations [20,38].

The cell damage observed in *P. lineatus* exposed to DWSF in the present work might have resulted from these oxidative processes, where the monoaromatic and polyaromatic hydrocarbons (PAHs) present in the DWSF tested may have generated electrophilic compounds, which in association with DNA molecule sites caused several lesions.

In the present work the high frequency of damaged cells found in *P. lineatus* after 6 h of exposure to DWSF shows that the exposure time was probably not sufficient for a significant increase in the expression of the enzymes responsible for the detoxification of the hydrocarbons present in DWSF. Simonato et al. [23,24] have already demonstrated that GST liver activity of *P. lineatus* exposed to DWSF increases after 96 h of exposure.

This enhanced GST activity may have contributed to the decrease in the number of damaged nucleoids observed after 96 h of exposure to DWSF. These results agree with those found by Maria et al. [15] which showed that the fish *Anguilla anguilla* exposed for 8 h to contaminated waters with PAHs and PCBs also presented DNA damage in blood cells and these authors observed an increase in cytochrome P450 and GST enzymes followed by the reduction of DNA damage after 48 h of exposure.

In the comet assay is possible to quantify and to distinguish cells with different rates of DNA damage, thus the analysis of the mean values of the scores for each group becomes very important. In the present work, a predominance of comet classes 0 and 1 was observed in all negative control groups (Table 1).

In the acute exposures to diesel oil of 6 and 24 h, a predominance of comet classes 1 and 2 occurred, distinguishing minor and medium damage to fish DNA. Regarding the acute exposure of 96 h and subchronic exposure (15 days) to DWSF, comet classes 2 and 3 predominated, with these latter results showing maximum DNA damage. The mean score values for exposure to DWSF confirms the results obtained for the damaged cell scores (Table 1). The results of this work corroborate those found by Nigro et al. [39], who described high indices of DNA damage in erythrocytes of *A. anguilla* treated in laboratory with different hydrocarbons. High damage indices were also reported in erythrocyte DNA of the fish *Zoarces viviparus* collected in an estuary in Switzerland, where the sediments were contaminated with several compounds, including PAHs and PCBs [4].

The consequences of disturbances in the DNA molecule, such as adducts, single and double-strand breaks, might cause lesions that become permanent. The

use of cytogenetic assays, like micronucleus assay, is an excellent means to evaluate any permanent damage in the genetic material. According to Klobucar et al. [34], this assay has been successfully used since the 1980s to evaluate environmental pollutants and different chemical compounds. In a freshwater environment, the principal objects of study are vertebrates [40,41] and their MN frequencies are strongly related to the water quality under analysis [3,42–44].

In the present study, evaluation of the induction of micronuclei in blood cells of *P. lineatus* blood cells exposed to DWSF under acute and subchronic conditions, demonstrated that this test is efficient in detecting mutagenic compounds present in the fraction tested.

The induction of micronuclei occurred at all the exposure periods tested and the lowest frequencies of micronuclei were obtained after 6 h and 15 days of exposure. One hypothesis that could explain this observation is that 6 h of exposure were not sufficient to promote such damage in the cells, which were in interphase or at the beginning of the cell cycle, which reflects the kinetics of the cell cycle in the majority of fish. The explanation for the low frequency of micronucleated cells observed after exposure to DWSF for 15 days is related to erythrocytes renewal. A high percentage of apoptotic cells were reported in marine fish exposed to oil refinery effluents [4]. This phenomenon occurs when cell damage is very intense and the defense mechanisms are unable to repair the problem. Consequently, the number of available micronucleated cells for analysis decreases. During blood cell kinetics, erythrocytes are continually renewed and damaged erythrocytes tend to be eliminated from the organism quicker than those which are not damaged [45]. Thus, although the micronuclei are often reported as genotoxic biomarkers in fish, several studies with fish subchronically or chronically exposed to pollutants demonstrated that the frequency of micronucleated erythrocytes tended to diminish after the 15th or 21st day of exposure [46–48].

In the present work, the greatest frequencies of micronuclei were observed after the exposure of fish to DWSF for 24 and 96 h. It seems that 24 h is a more adequate period of time for the evaluation of mutations in *P. lineatus*, because after this interval the cell lesions that occurred were probably not repaired and the cells presented micronuclei.

The frequency of micronuclei in *Cyprinos carpio* [19] and *Oreochromis niloticus* [49] blood cells collected at three sites contaminated with PAHs, showed a significant increase when compared with the frequencies obtained from fish from non-polluted sites. Significant increases in micronuclei frequency in mussel (*Perna viridis*) and

fish (*Dicentrarchus labrax*) blood cells exposed for different times to benzo[a]pyrene were observed by Siu et al. [12] and Gravato and Santos [50], respectively.

The assessment of DNA damage when using the comet assay results from the complex interaction between two processes: DNA damage and repair (activation or inhibition); the damage in this case indicates a recent response to the pollutant. On the other hand, when micronuclei are formed, they continue present throughout the lifetime of the cell [34]. Therefore, the combination of these two assays in the present work proved to be both adequate and useful in the evaluation of the genotoxicity of the diesel water soluble fraction due to their complementary action. The results obtained will hopefully stimulate other experiments on this subject and allow for a clearer understanding of the genetic effects of diesel oil spills on neotropical fish.

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