ZOOLOGIA 27 (6): 956–960, December, 2010
doi: 10.1590/S1984-46702010000600017

Genotoxic effects of the diesel water-soluble fraction on the seahorse *Hippocampus reidi* (Teleostei: Syngnathidae) during acute exposure

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ABSTRACT. The high toxicity of diesel components makes oil spills a threat to the biota in coastal marine environments. The genotoxic effect of the diesel water-soluble fraction (DWSF) on *Hippocampus reidi* (Ginsburg, 1933) was assessed. Fish were exposed to three different DWSF dilutions for up to 96 hours, and genotoxicity was analyzed using the micronuclei test and the comet assay. The micronuclei test revealed no significant differences between any of the DWSF dilutions and the control in the 24-hours period; however, micronuclei increased in fish exposed to 1:500 and 1:100 DWSF dilutions for 96 hours. For all dilutions, there was an increase in micronuclei in fish exposed for 96 hours when compared to those exposed for 24 hours. The tested dilutions increased frequencies of cell classes 2 (medium damage) and 3 (large damage) in the comet assay after 24 and 96 hours. Fish exposed to DWSF 1:100 exhibited a higher frequency of class 4 (apoptosis) cells in the 96-hours period. All dilutions increased the comet score when compared to the control at 24 and 96 hours. The micronuclei and comet tests were efficient in detecting DWSF genotoxic effects in *H. reidi*.

KEY WORDS. Comet assay; diesel; marine fish; micronuclei; toxicity.

Diesel spills are a very important source of marine and coastal pollution (Zhang *et al*. 2004, King *et al*. 2005, Albaigés *et al*. 2006, Simonato *et al*. 2008). They are mainly caused by ships, sailing along the main maritime routes or anchored in harbors or tied up in berths (Gonzáles *et al*. 2006, Silva *et al*. 2009).

The diesel water-soluble fraction (DWSF) contains polar compounds and hydrocarbons of two toxicologically relevant groups: monoaromatic hydrocarbons (benzene, toluene and xylene) and low molecular weight polyaromatic hydrocarbons (PAHs) such as naphthalene, fluorene, and phenanthrene (Simonato *et al*. 2008). Among the diesel oil components, the PAHs raise the greatest environmental concerns due to their biotoxicity (Lee & Anderson 2005, Ferreira *et al*. 2006). Vanzella *et al*. (2007) suggested that the PAHs present in the DWSF form electrophilic compounds that react with DNA molecules and cause serious damage.

The micronuclei test and the comet assay are among the most efficient methods to evaluate DNA damage caused by diesel oil toxicity in fish (Buschini *et al*. 2004, Martínez-Gomez *et al*. 2006, Vanzella *et al*. 2007). These two tests are simple, rapid and highly sensitive to several kinds of DNA damage (Hartman *et al*. 2003).

The seahorse *Hippocampus reidi* (Ginsburg, 1933) is a potential bioindicator of environmental disturbance caused by toxic compounds in coastal waters due to its wide geographic distribution (Foster & Vincent 2004) and sensitivity to contaminants. This study aimed to assess the genotoxic effect of the DWSF on *H. reidi* and to evaluate the efficiency of the micronuclei test and the comet assay to detect genotoxic effects of the DWSF in this species.

MATERIAL AND METHODS

Fifty-six specimens of *H. reidi* (1.94 ± 0.47 g and 8.47 ± 0.68 cm) were obtained from a private breeder in the municipality of Serra, state of Espírito Santo (ES), Brazil, and transported to the laboratory (commute lasting approximately 1 hour) in plastic bags with pressurized oxygen. Acclimation and experiments were conducted using sea water collected at the Itapuã Beach, Vila Velha, state of Espírito Santo, Brazil (20°21'13"S, 40°17'02"W).

In the laboratory, fish were transferred to two 30-L aquaria containing 25 L filtered water, where they were keptfor 30 days. Fish were fed twice a day on live *Artemia* sp. There was no mortality during this period. During acclimation, physicochemical water parameters were measured every three days using a multiparameter YSI 85 instrument and a digital pH meter. The measurements obtained were as follows (mean ± SD): temperature = 22.1 ± 0.4ºC; conductivity = 39.9 ± 0.9 mS/cm; dissolved oxygen = 6.6 ± 0.7 mg/L; pH = 7.5 ± 0.2; salinity = 30.1 ± 0.01.

Commercial diesel oil (type B2) was purchased from a commercial gas station. The density of the oil was 0.849 g/cm^3. The container used to transport the diesel oil was lined with a black plastic bag and sealed to prevent alteration in composition.

The DWSF was prepared in strict accordance with the methodology described by Singer *et al.* (2000). A 5-g aliquot of the oil was weighed and transferred to a 1-L volumetric flask, and sea water was added to bring the final volume to 1 L. The oil + water mixture was placed in an amber container and shaken at 250 rpm for 24 hours without vortex formation. The procedure allowed the DWSF to be obtained. The DWSF was collected using a pipette, and the upper insoluble phase was discarded. The DWSF composition was analyzed by gas chromatography (Perkin-Elmer, Clarus 500*)*. The following parameters were assessed: total PAH (4.8% v/v); benzene (0.11% v/v); toluene (0.59% v/v); m+o+p xylene (2.1% v/v); naphthalene (4.8 mg/L); phenanthrene (0.47 mg/L) and fluorene (0.36 mg/L).

After acclimation, the fish were transferred to 56 aquaria, each containing 1 L of sea water under constant aeration. One animal was placed in each aquarium. Four treatments (seven replicates for each sampling time) were defined according to DWSF dilutions: 1:1000, 1:500, 1:100 and control (without DWSF). These dilutions were based on a previous study by Solé *et al.* (2008). The experiment lasted a total of 96 hours and sampling was conducted in two distinct periods: after 24 hours and after 96 hours. Half of the water volume in each aquarium used to expose fish for 96 hours was replaced after a 48-hours exposure with the same DWSF concentration that had been originally used.

After exposure (24 or 96 hours), fish were anesthetized with benzocaine 1:10 diluted in 95% alcohol. Their blood was collected by caudal vein punction. This procedure was conducted in accordance with the guidelines for the use of fish in research by the American Fisheries Society (Nickum *et al.* 2004).

Physicochemical parameters of the water were measured at the beginning and at the end of the experiment, using a multiparameter YSI 85 instrument. The parameters obtained were (mean ± SD): temperature = 23.2 ± 0.2ºC; conductivity = 41.3 ± 0.5 mS/cm; dissolved oxygen = 6.9 ± 0.5 mg/L; and salinity = 30 ± 0.01.

Peripheral blood samples were smeared on clean slides. Slides were left to dry in the laboratory environment overnight, and then the smears were fixed in methanol PA for 15 minutes and stained for 20 minutes with 5% (w/v) Giemsa, washed with distilled water and left to dry in the laboratory environment. After drying, 2,000 erythrocytes per fish (1,000 from each duplicate slide) were blind analyzed for the presence of micronuclei at 400x magnification under a light microscope (Al-Sabati & Metcalfe 1995).

The blood samples were diluted to 1:120 (v/v) with RPMI 1640 medium and used immediately. The alkaline comet assay was performed as described by Tice *et al.* (2000). Briefly, 5 µL of each diluted blood sample was added to 95 µL of 0.75% (w/v) molten low melting point agarose, and a portion of the mixture was spread on a microscope slide pre-coated with 1.5% (w/v) normal melting point agarose and topped with a coverslip. After the agarose solidified, coverslips were removed, and the slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0-10.5) containing 1% Triton X-100 and 10% DMSO. The slides were kept frozen in lysis solution (4°C) and protected from light for at least 2-3 hours at most subsequently, slides were incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH ⩾ 13, which was experimentally determined) for 20 minutes for DNA unwinding. Electrophoresis (15 minutes at 300 mA and 25 V [0.8 V/cm]) was performed in the same buffer. Every step was carried out under indirect yellow light. After electrophoresis, slides were neutralized in Tris 400 mM (pH 7.5), rinsed three times in distilled water and left to dry overnight at room temperature. Slides were then fixed for 10 minutes in trichloroacetic acid 15% w/v, zinc sulfate 5% w/v, and glycerol 5% v/v, rinsed three times in distilled water, and dried for two hours at 37°C. The dry slides were re-hydrated for five minutes in distilled water and then stained (sodium carbonate 5% w/v, ammonium nitrate 0.1% w/v, silver nitrate 0.1% w/v, tungstosilicic acid 0.25%, formaldehyde 0.15% w/v, freshly prepared in the dark) and constantly shaken for 35 minutes. The stained slides were rinsed twice with distilled water and then submerged in the stop solution (acetic acid 1%), rinsed again, and immediately coded for analysis. To calculate image length, 200 cells from each fish were randomly chosen (100 from each duplicate slide) and analyzed under an optical microscope (400× magnification). Analysis of the slides involved 200 cells/animal, utilizing the visual classification based on the migration of DNA fragments from the nucleus: class 0 (no damage), class 1 (little damage – less than or equal to the diameter of one nucleus), class 2 (medium damage – less than or equal to diameters of two nuclei), class 3 (extensive damage – greater than two times the diameter of one nucleus) and class 4 (apoptosis) (Collins *et al.* 1995, Kobayashi *et al.* 1995). The data are presented as the frequency of cells per class of damage. The comet score (CS) for each fish was calculated as the sum of the number of nucleoids observed in each damage class multiplied by the value of the respective damage class (0, 1, 2, 3 or 4). Results are expressed as the comet score for each treatment group, where 0 represents the absence of damage and 400 indicates the highest damage score.

For all data, normality was tested by the Kolmogorov-Smirnov test. The comet score presented a parametric pattern, and comet assay and micronuclei results did not; therefore, non-parametric tests were used for those results.

For both the micronuclei test and the comet assay, the differences in frequencies obtained for the different dilutions (1:1000, 1:500 and 1:100) in comparison to the respective controls at one of the two measurement times (24 or 96 hours) were analyzed by the Kruskal-Wallis ANOVA and Dunnett's test ($p < 0.05$). The difference between results obtained for the same dilution for the two sampling times was determined using the t test ($p < 0.05$).

For the assessment of CS, the difference in results obtained with each DWSF dilution were compared to the results of respective controls at one of the two sampling times (24 or

96 hours) using the ANOVA and Dunnett's test ($p < 0.05$). The difference between results obtained for same treatment for the two sampling times was determined using the *t* test ($p < 0.05$).

RESULTS

No statistically significant differences were observed in micronuclei frequency with respect to the control in samples collected in the 24-h period for any of the dilutions tested. By contrast, there was a significant increase in the frequency of micronuclei in fish exposed to the 1:500 and 1:1000 DWSF for 96 h Tab. I).

Table I. Micronuclei in the erythrocytes of *H. reidi* exposed to different diesel water soluble fraction dilutions (DWSF: 1:1000, 1:500 and 1:100) for 24 and 96 hours. n = 7 for each combination of dilution and time.

Dilution	Exposure period					
	24 hours			96 hours		
	Median	Min	Max	Median	Min	Max
Control	0.0	0.0	0.5	0.0	0.0	1.0
1:1000	0.5	0.0	1.5	2.0*†	1.0	2.5
1:500	1.0	0.0	2.5	3.5*†	0.5	5.5
1:100	1.5	0.0	2.5	6.0*†	2.0	8.5

* Indicates a significant difference between results obtained for different dilutions at the same exposure period in comparison to the respective controls as indicated by the Kruskal-Wallis test and the Dunnett's test ($p < 0.05$). † Indicates significant difference between samples exposed to the same dilution for the two exposure periods (24 and 96 hours) as indicated by the t test ($p < 0.05$).

No significant difference was observed in the class 1 cells between samples exposed to any of the DWSF dilutions for 24 hours and their controls. However, *H. reidi* exposed to all DWSF dilutions presented significantly greater numbers of damaged cell classes 2 and 3 in comparison with their controls. For class 4 cells, the median was zero for all DWSF dilutions, with no statistically significant difference (Tab. II). As for the fish exposed for 96 h to all DWSF dilutions, the number of class 0 cells was lower than that of the respective control. By contrast, the fish exposed to all DWSF dilutions presented higher numbers of cell classes 2 and 3 when compared with their controls. In addition, the fish exposed to the 1:100 DWSF dilution had more class 4 cells in comparison to the control.

The fish exposed to the 1:1000, 1:500 and 1:100 DWSF dilutions presented higher comet scores when compared with the control groups in the two exposure periods (24 and 96 hours) (Fig. 1). There was no significant difference in comet score between the two exposure periods of fish within the same dilution (Control: $p = 0.368$ and $T = 0.934$; 1:1000 dilution: $p = 0.659$ and $T = 0.452$; 1:500 dilution: $p = 0.722$ and $T = 0.364$; 1:100 dilution: $p = 0.161$ and $T = -1.994$).

Table II. Damage frequency of erythrocytes of *H. reidi* exposed to different diesel water soluble fraction dilutions (DWSF: 1:1000, 1:500 and 1:100) for 24 and 96 hours. n = 7 for each combination of dilution and time. Results are expressed as medians.

Dilution	Comet class				
	0	1	2	3	4
24-h exposure					
Control	60.5	37.5	1.5	0.5	0.0
1:1000	19.5*	48.5	30.5*	1.5*	0.0
1:500	7.0*	46.0	39.0*	8.5*	0.0
1:100	4.5*	25.0	49.5*	25.0*	0.0
96-h exposure					
Control	66.5	31.0	3.5	0.0	0.0
1:1000	21.0*	45.5	32.5*	1.5*	0.0
1:500	7.5*	38.5	42.0*	10.0*	0.0
1:100	2.5*	13.5	47.5*	36.0*	2.5*

* Indicates a significant difference in comparison to control as indicated by the Kruskal-Wallis ANOVA and the Dunnett's test ($p < 0.05$).

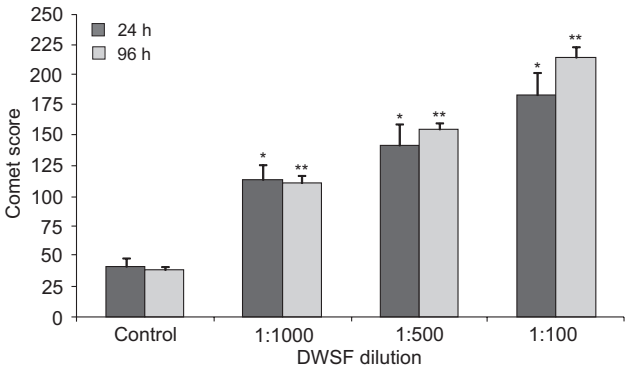

Figure 1. Comet score for *H. reidi* exposed to different diesel water soluble fraction dilutions (DWSF; 1:1000, 1:500 and 1:100) and a control for 24 and 96 hours. Results are expressed as mean and standard deviation. * Indicates a significant difference in comparison to control in the 24-hours exposure period as indicated by an ANOVA and Dunnett's test ($p < 0.05$). ** Indicates a significant difference in comparison to control in the 96-hours exposure period as indicated by an ANOVA and Dunnett's test ($p < 0.05$).

DISCUSSION

The DWSF was a determining factor in the induction of micronuclei in *H. reidi*. From the cellular perspective, micronuclei are formed by chromosome fragments or by chromosomes that, during anaphase, fail to be incorporated into the new nucleus (Fenech 2000). The appearance of micronuclei during cell division results from chromosome breakages, which

in turn result from poor lesion repair or improper chromosome segregation during mitosis (Iarmacovai *et al*. 2008). These errors may be induced by gene defects in the cell cycle, oxidative stress and/or exposure to contaminants (Iarmacovai *et al*. 2008), as observed in the present study.

Hoshina *et al*. (2008) have suggested the tilapia, *Oreochromis niloticus* (Linnaeus, 1758) as an appropriate test organism to assess genetic damage caused by oil because in this organism the exposure to [even low] oil concentrations induces the formation of micronuclei, and triggers cell death. In the tilapia, micronuclei frequency varied from 1 to 3 – values well under those reported here for *H. reidi* in the 96-hours exposure period, which varied between 2 and 6. The micronuclei test proved to be efficient in detecting the magnitude of the genotoxic effects of the DWSF in *H. reidi* because increased micronuclei frequencies were observed for increasing concentrations and longer exposure periods.

Vanzella *et al*. (2007) assessed the effects of acute exposure to DWSF on the fish *Prochilodus lineatus* (Valenciennes, 1837). They observed that relative frequencies of erythrocytes were higher in fish exposed to the xenobiotic for over 96 hours. In the present study, the damage induced to *H. reidi* erythrocytes was also more evident when fish had been exposed to DWSF dilutions for 96-hours. It is possible that the 24-hours exposure period is not long enough to observe erythrocyte damage because by the end of this period the cell division process under the effect of the DWSF is only beginning. The comet assay allows one to distinguish and quantify cells with different DNA damage rates. Therefore, the analysis of the mean DNA damage index for each group is important because it can shed more light on the damage caused by xenobiotics (Vanzella *et al*. 2007). The control fish exhibited a prevalence of class 0 and 1 cells, whereas class 2 and 3 cells were most frequent in samples collected from fish exposed to all dilutions (1:1000, 1:500, and 1:100) over both exposure periods (24 and 96 h) in comparison with the respective controls. Apoptosis, which corresponds to class 4 cells, was observed only in samples collected from individuals of *H. reidi* exposed to 1:100 dilutions of DWSF for 96 hours, though at a rather low frequency (median 2.5%). The results of the comet assay in *H. reidi* exposed to the DWSF appear sufficient to detect environmental genotoxicity. Our results are similar to those reported by Akcha *et al*. (2003), who studied the effects of PAHs in *Limanda limanda* (Linnaeus, 1758) and demonstrated that the comet assay was sensitive enough to detect the effects caused by xenobiotics. An increased CS was observed with the increase in the DWSF concentrations, though this damage was not related to exposure time because no difference was observed in CS in relation to exposure time for any of the dilutions tested.

Hippocampus reidi is sensitive to genotoxic effects of the DWSF. The micronuclei test and the comet assay are efficient biomarkers of contamination by diesel oil in costal environments for this species.

ACKNOWLEDGMENTS

We are indebted to Bruno Ferreira and Frederico Delunardo for their help during fish sampling. This work was supported by a research grant from UVV/FUNDADESP # 09/2009. L.C. Gomes is a research fellowship recipient from CNPq/Brazil.

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Submitted: 03.VIII.2010; Accepted: 21.X.2010.
Editorial responsibility: Antonio Ostrensky Neto