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Genetic damage in the bivalve mollusk *Corbicula fluminea* induced by the water-soluble fraction of gasoline

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ABSTRACT

Although gasoline is an important contaminant of aquatic ecosystems, information concerning the potential effects of this petroleum derivative on the DNA of aquatic biota is lacking. The present study aimed to evaluate the genotoxic and mutagenic effects of acute exposures (6, 24, and 96 h) to gasoline water-soluble fraction (GWSF), diluted to 5%, on the Asian clam *Corbicula fluminea*. The comet assay and the micronucleus (MN) test were performed on hemocytes and gill cells of *C. fluminea*. For the three different times tested, the comet assay indicated DNA damage in hemocytes and gill cells of *C. fluminea* exposed to GWSF. The MN test detected significant damage in the genetic material of the hemocytes only after 96 h of exposure to GWSF. The recovery capacity of organisms previously exposed for 6 h to GWSF was also evaluated with the comet assay. The results revealed a great capacity of this species to repair DNA damage; following 6 h of recovery, the comet score returned to that of the control groups. Overall, our findings indicate that GWSF has genotoxic and mutagenic effects on *C. fluminea*. In addition, the present results confirm the sensitivity of *C. fluminea* to damage caused by exposure to environmental contaminants, and, therefore, its suitability for use in environmental monitoring studies.

1. Introduction

Petroleum and its derivatives, such as gasoline, are among the pollutants reaching aquatic ecosystems that have the greatest ecological impact [1]. Although major disasters, such as oil spills, have an important role in the contamination of aquatic environments [2,3], small continuous fuel leaks from gas stations constitute one of the principal sources of soil and water contamination [4–6]. Aquatic recreational activities (with small boats) and widespread use in vehicles and machines make gasoline one of the most commonly spilled petroleum products in the environment [1,7].

Gasoline is a complex mixture of liquid and gaseous organic constituents, including aromatic hydrocarbons [7,8]. In the gasoline water-soluble fraction (GWSF) mono- and polycyclic aromatic compounds are normally present [8]. The monocyclic aromatic hydrocarbons benzene, toluene, and xylene (BTX) are among the most damaging compounds to the environment, due to their high solubility in water (a factor directly related to their pollution potential) and their high chronic toxicity [4–6]. On the other hand, polycyclic aromatic hydrocarbons (PAH) are one of the worst contaminants of aquatic environments, due to their mutagenic and carcinogenic effects on the biota [9,10].

In Brazil, up to 20% ethanol is added to gasoline, making aquatic contamination a particularly serious problem, because ethanol increases the solubility of hydrocarbons in water and facilitates their migration through aquatic environments [5,6]. Although it is recognized that gasoline contaminates aquatic ecosystems and produces genotoxic effects in a range of organisms, including humans [8,11], there are few studies in the literature about the damage it may cause to the genetic material of aquatic organisms.

Bivalves are considered to be good bioindicators of contamination by heavy metals and organic compounds in aquatic environments, because these animals can accumulate a series of contaminants in their tissues, as well as presenting several alterations indicative of damage [12]. Among the bivalves that have been the focus of toxicological studies on contamination by pollutants, *Corbicula fluminea*, popularly known as Asian clam, has been cited as a species-level bioindicator of aquatic contamination, given its high sensitivity to a wide variety of contaminants and the great quantity of water that it filters from its habitat [13,14]. *C. fluminea* is also recognized as a bioaccumulator species [14]. On the other hand, it has been reported in the literature that this species has the capacity to eliminate or degrade xenobiotics [14,15].

In order to understand better the genotoxic and mutagenic effects of gasoline on aquatic biota, we used the comet assay and micronucleus test (MN), two assays recognized for their sensitivity

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in the detection of genetic damage [16,17], to evaluate the acute effects of GWSF on *C. fluminea*. DNA damage was observed after GWSF exposure, and the repair capacity of the organisms was also studied.

2. Materials and methods

2.1. Animals

Freshwater Asian clams (*C. fluminea*), with length 2.5–3.0 cm, were collected from an urban lake in Londrina, Paraná, Brazil and transported to the laboratory, where they were acclimated for 21 days in 20 L glass aquaria containing 10 L of dechlorinated and continually aerated water ($T = 22 \pm 2\,^{\circ}C$), with a 12/12 h light/dark photoperiod. About 40 individuals were maintained per aquarium.

2.2. Gasoline water-soluble fraction (GWSF)

Regular unleaded gasoline without additives was obtained from the same gas station for all the tests. The method used to simulate fuel leakage in tropical conditions followed that of Nicodem et al. [18]. The gasoline, in the form of a film on water (1 part gasoline to 4 parts water), was exposed to weathering under tropical conditions for 24 h, corresponding to 6 h of direct solar radiation. After that, the water phase, corresponding to the gasoline water-soluble fraction (GWSF) was collected. For the toxicity tests, GWSF was diluted to 5% with non-chlorinated water.

Samples of GWSF before dilution (GWSF 100%) were quantitatively analyzed for benzene, toluene, ethylbenzene, and xylene (BTEX), using gas chromatography with a flame ionization detector, and for some polycyclic aromatic hydrocarbons (PAHs), using high performance liquid chromatography with a UV fluorescence detector. GWSF, before and after dilution, was examined qualitatively for the presence of mono- and polycyclic aromatic hydrocarbons by fluorescence emission spectrometry. Synchronous fluorescence spectra were recorded, 250–800 nm.

2.3. Toxicity tests

Animals were exposed to GWSF diluted to 5% (GWSF groups) or to clean water only (negative-control groups or CTR) in static acute toxicity tests of 6, 24, or 96 h duration. The tests for each experimental period and each treatment (CTR and GWSF) were performed in separate glass aquaria of 20 L containing 10 clams each. Thus, there were six aquaria with ten clams each, *i.e.*, 60 animals total. CTR and GWSF groups were sampled simultaneously at the end of each experimental period. For sampling, animals were anesthetized with benzocaine ($0.1\,g\,L^{-1}$) and hemolymph samples were collected from the posterior adductor muscle. Subsequently, animals were sacrificed for gill removal.

In parallel assays, positive control groups were run with the alkylating agent methylmethanesulfonate (MMS). For these tests, clams were exposed to 0.2 L MMS, 0.4 mM, for 6 or 96 h, and the animals sampled as previously described.

2.4. Sampling

Samples of hemolymph for the comet test and micronucleus (MN) assay were obtained following Rigonato et al. [19] and Villela et al. [20]. The samples were centrifuged at $550 \times g$ for 10 min; supernatants were discarded and the precipitated cells were resuspended in saline solution (NaCl 25.22 mM, KCl 1.26 mM, $CaCl_2$ 2.21 mM, $MgCl_2$ 0.59 mM, dextrose 1.0 mM) and maintained under refrigeration for a maximum of 1 h until the assays were carried out. Volumes of saline solution, 50 or 120 μL, were used to resuspend the samples for the comet test and MN assay, respectively.

Gill cell samples for the comet assay were obtained based on the methods of Kilemade et al. [21] and Cavalcante et al. [22], with some modifications. Briefly, gills were removed from the animals and stored for up to 1 h in microtubes with 500 μL Ca^{+2}- and Mg^{+2}-free phosphate-buffered saline (PBS) (KCl 2.68 mM; KH_2PO_4 1.47 mM; NaCl 136.89 mM; Na_2HPO_4 8.1 mM; pH 7.4). Subsequently, gills were sectioned, placed in trypsin solution (0.05%, 2 mL) and gently homogenized in a shaker for approximately 30 min. The gill cell suspension was filtered (30 μm) into fetal bovine serum (FBS, 2 mL) diluted to 10% in PBS, for trypsin inactivation. This suspension was divided into two equal parts and centrifuged at $550 \times g$ for 10 min. Supernatant debris was removed and the precipitate was resuspended in saline solution, 60 μL using this material, three slides per animal were prepared: two for the comet assay and one to verify cellular viability.

2.5. Cell viability assay

Analyses of cellular viability were carried out in parallel to comet and MN assays, using the trypan blue (0.01%) exclusion test. For each animal, 100 hemocytes and 100-gill cells were analyzed; viability was expressed as the percentage of viable cells. Only preparations for which viability were >80% were used.

2.6. Comet assay

Hemolymph and gill cells were submitted to the comet assay according to Villela et al. [20], with some modifications [23]. The steps of the assay were executed as follows for both hemocytes and gill cells: (a) lysis-slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1 mL Triton X-100, pH 10.0) at 4 °C, protected from light, for 1–2 h; (b) DNA unwinding – 30–40 min in the electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13); (c) electrophoresis – 30 min, 300 mA, 25 V, $1\,V\,cm^{-1}$; (d) neutralization – three washes for 5 min each in buffer (0.4 M Tris, pH 7.5). The slides were then fixed with absolute ethanol for 10 min and kept under refrigeration until analysis.

The slides were stained with ethidium bromide ($20\,mg\,mL^{-1}$) and analyzed under Leica fluorescence microscopy with a blue excitation filter (450–490 nm) and a barrier filter of 515 nm, at 1000× magnification. All slides were independently coded and blindly scored. The extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly select and non-overlapping cells per clam according to Villela et al. [24]. DNA damage was classified in four classes (0: no apparent damage; 1: minimum damage; 2: medium damage; 3: maximum damage). The comet score was calculated for each animal with the following formula: $\Sigma(A \times 0) + (B \times 1) + (C \times 2) + (D \times 3)$, where *A*, *B*, *C* and *D* correspond, respectively, to the number of cells in comet classes 0, 1, 2 and 3; the score ranged from 0 to 300 arbitrary units. Results for DNA damage were expressed as the mean number of damaged nucleoids (sum of classes 1, 2 and 3) and the mean comet score for each treatment group, for each exposure period [25].

2.7. Micronucleus test and the occurrence of nuclear abnormalities (NA) in the hemocytes

The micronucleus test was performed with the hemocytes following the methods of Hoftman and de Raat [26]. Hemolymph samples were smeared on glass slides, dried in the dark for 24 h, fixed in absolute methanol for 10 min and stained with Giemsa (5%) for 10 min. A total of 3000 hemocytes per animal were analyzed under an Olympus light microscope (1000× magnification). Intact hemocytes were classified in four categories: normal hemocytes, micronucleated hemocytes, binucleated hemocytes and hemocytes with other nuclear abnormalities. The mean frequencies of each category were estimated and expressed per 1000 cells (‰).

2.8. Recovery test

To verify the recovery capacity of *C. fluminea* from genetic damage to hemocytes caused by 6 h exposure to GWSF, a static toxicity test was carried out with two groups of 18 animals each: a GWSF-recovery group (RecGWSF) with clams exposed to GWSF (5%) for 6 h, and a negative-control recovery group (RecCTR) with animals exposed to clean water, for 6 h. After exposure, the animals from these two groups were submitted to one of three recovery periods (6, 24, or 96 h) in clean dechlorinated water. Clams from the RecGWSF group were transferred to an aquarium containing only clean water (10 L) and animals from the RecCTR group remained in the same aquarium. After each recovery period, hemolymph samples from clams of both groups were collected and processed for the comet assay, as described above.

2.9. Statistical analysis

Data were first tested for normality and homogeneity of variance. The results of the comet score, the number of damaged nucleoids, the frequency of MN and other nuclear abnormalities obtained for CTR and GWSF groups, at each exposure time, were compared using Student's *t*-test or the Mann–Whitney test. The results obtained for positive controls were compared to respective CTR and GWSF groups by ANOVA (one way) followed by the Tukey test when necessary. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Water analysis

The concentrations of BTEX and PAHs in the GWSF are listed in Table 1. The GWSF showed high levels of BTEX, mainly ethylbenzene, xylene and benzene, Among the 12 PAHs analyzed, naphthalene was the most abundant, followed by anthracene and phenanthrene. All the other PAHs analyzed showed concentrations below the detection limit ($0.05\,\mu g\,L^{-1}$). The fluorescence spectrum of GWSF 100% indicated the presence of aromatic hydrocarbons with emission peaks around 300 nm (Fig. 1). The samples of 5% GWSF collected 6, 24, or 96 h after the start of the experiments showed a decrease in the intensities of these compounds when compared with GWSF 100%.

Table 1
Concentrations of monoaromatic hydrocarbons (BTEX) and polycyclic aromatic hydrocarbons (PAH) in the gasoline water-soluble fraction without dilution (100% WSFG).

Hydrocarbons	100% WSFG, μg L^{-1}
Benzene	5700
Toluene	1050
Ethylbenzene	10,500
Xylene	7550
Total BTEX	24,800
Anthracene	53.25
Benzo(a) anthracene	<0.05
Benzo(a) pyrene	<0.05
Benzo(ghi) perylene	<0.05
Benzo(k) fluoranthene	<0.05
Chrysene	<0.05
Dibenzo (a, h) anthracene	<0.05
Phenanthrene	12.2
Fluoranthene	<0.05
Indeno (1, 2, 3) pyrene	<0.05
Naphthalene	2138.25
Pyrene	<0.05
Total PAH	2203.7

Detection limits: BTEX 5 μg L^{-1} and PAH 0.05 μg L^{-1}.

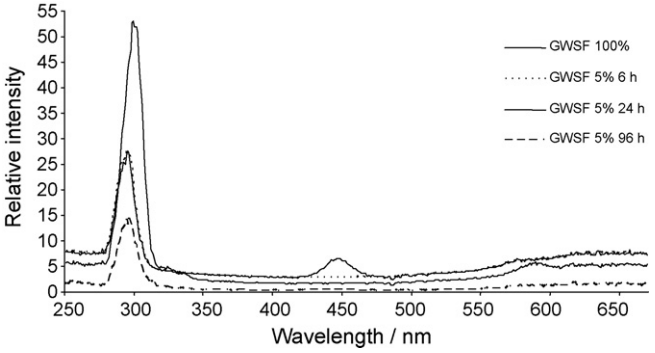

Fig. 1. Synchronous fluorescence spectrum of 100% GWSF and of GWSF diluted to 5% and collected 6, 24 or 96 h from the start of the toxicity tests.

3.2. Animals and cellular viability

During the acute exposures to GWSF, no deaths were observed. Discrepancies in the numbers of individuals analyzed are due to the difficulties either of obtaining samples from some individuals or in their preparation for the experiments. Viability above 95% and 90% was determined for the hemocytes and gill cells, respectively, that were isolated from *C. fluminea*, indicating absence of cytotoxicity for the GWSF group and an absence of damage caused by biological material handling, which could have compromised the analyses.

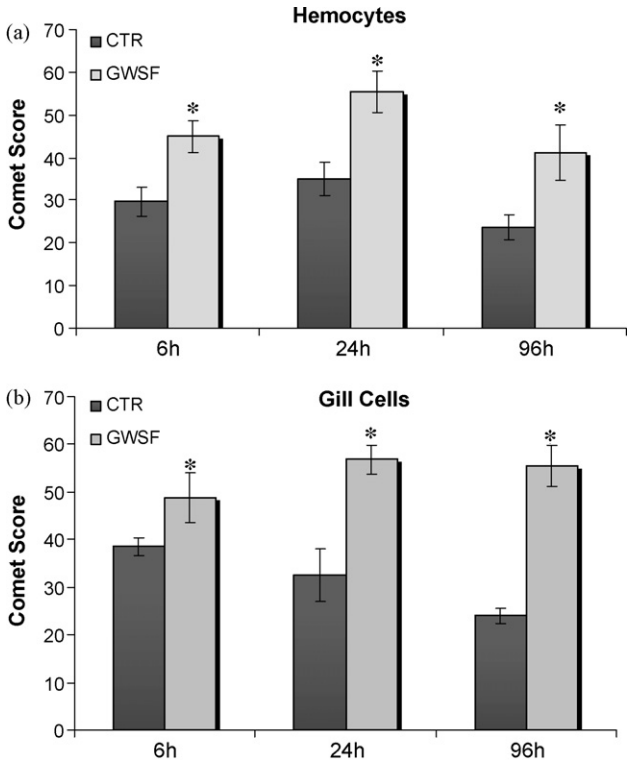

Fig. 2. Comet scores in hemocytes (a) and gill cells (b) of *Corbicula fluminea* exposed to GWSF or to water only (CTR) for each experimental period (6, 24 or 96 h). Bars represent means and vertical lines the standard deviation. *Significantly different from respective CTR group ($p < 0.05$).

3.3. Comet assay

The comet assay performed on hemolymph and gill cells of *C. fluminea* exposed to 0.4 mM MMS indicated a significant increase in the number of damaged nucleoids as well as in the comet score (Table 2). *C. fluminea* exposed to GWSF for 24 or 96 h showed a significant increase in the number of hemocytes with damaged nucleoids (Table 3). With respect to gill cells, the comet assay demonstrated a significant increase in the number of damaged nucleoids in the GWSF group compared to CTR groups, at all exposure times (Table 3). Hemocytes and gill cells of clams exposed to GWSF showed significantly higher comet scores than the respective control groups, at all exposure times (Fig. 2).

3.4. Micronucleus and other nuclear abnormalities in the hemocytes

Clams exposed to MMS showed significantly higher frequencies of micronucleated and binucleated hemocytes, as well as hemocytes with other nuclear abnormalities, in comparison to

Table 2
Relative frequency of nucleoids (%) observed in each comet class (0, 1, 2 and 3), the number of damaged nucleoids and the score of damage in hemocytes and gill cells of *Corbicula fluminea* exposed to methyl methanesulfonate (MMS) or only to water (CTR) for 6 h taking into account the total number of animals (N) analyzed.

	Group	*N*	Comet classes				Damaged nucleoids (mean ± SE)	Comet score (mean ± SE)
			0 (%)	1 (%)	2 (%)	3 (%)		
Hemocytes	MMS	8	18.86	55.86	20.14	5.14	81.14 ± 3.75*	111.57 ± 8.83*
	CTR	7	75.00	20.71	3.86	0.43	25.00 ± 2.16	29.71 ± 3.47
Gill cells	MMS	7	6.00	39.75	51.00	3.25	94.00 ± 1.22*	151.50 ± 4.80*
	CTR	7	69.86	26.29	3.71	0.14	30.14 ± 2.55	38.50 ± 1.82

* Different from respective CTR group ($p < 0.05$).

Table 3
Frequency of nucleoids observed in each comet class (0, 1, 2, 3) and the number of damaged nucleoids in hemocytes and gill cells of *Corbicula fluminea* exposed to gasoline water-soluble fraction (GWSF) or only water (CTR), taking into account the total number of clams (*N*) analyzed for each experimental period (6, 24 and 96 h).

	Time	Group	*N*	Comet classes				Damaged nucleoids (mean ± SE)
				0 (%)	1 (%)	2 (%)	3 (%)	
Hemocytes	6 h	CTR	7	75.00	20.71	3.86	0,43	25.00 ± 2.16
		GWSF	9	67.00	22.66	8.67	1,67	33.00 ± 3.00
	24 h	CTR	9	72.11	21.67	5.22	1,00	27.89 ± 2.75
		GWSF	8	60.13	27.13	10.00	2,75	39.88 ± 3.20*
	96 h	CTR	9	80.00	16.67	3.11	0,22	20.00 ± 2.62
		GWSF	8	68.75	23.38	5.88	2,00	31.25 ± 4.17*
Gill cells	6 h	CTR	7	69.86	26.29	3.71	0.14	30.14 ± 2.55
		GWSF	10	61.20	29.20	8.30	1.30	38.80 ± 2.48*
	24 h	CTR	8	72.88	22.25	4.38	0.50	27.13 ± 4.02
		GWSF	7	56.43	28.14	10.71	1.86	40.71 ± 2.48*
	96 h	CTR	7	78.57	18.86	2.57	0.00	21.43 ± 1.39
		GWSF	6	59.50	28.00	10.00	2.50	40.50 ± 3.96*

* Different from respective CTR group ($p < 0.05$).

the negative-control groups (Table 4). Animals exposed to GWSF demonstrated a trend toward higher MN frequency. However, only the clams exposed to GWSF for 96 h showed a significant increase in the frequency of hemocytes with micronuclei (Table 5).

In relation to the other characteristics considered in this study, binucleated hemocytes were detected with greater frequency, for both the CTR and GWSF groups. Although the frequency of binucleated hemocytes was numerically greater in individuals exposed to GWSF compared to CTR groups, for all experimental times, it did not represent a statistically significant difference (Table 5). A significant increase was also verified for the group categorized as "other nuclear alterations" (MN and binucleated cells excluded), but only in *C. fluminea* exposed for 96 h (Table 5).

Table 4
Frequency of hemocytes micronucleated, binucleated and with other nuclear abnormalities of *Corbicula fluminea* exposed to methyl methanesulfonate (MMS) or only to water (CTR) for 6 h, taking into account the total number of animals (*N*) analyzed.

Hemocytes	Group	*N*	Frequency (‰) (mean ± SE)
Micronucleated	CTR	6	0.00 ± 0.00
	MMS	5	5.10 ± 0.45*
Binucleated	CTR	6	1.22 ± 0.24
	MMS	5	13.40 ± 0.92*
With other nuclear abnormalities	CTR	6	0.00 ± 0.00
	MMS	5	5.67 ± 0.35*

* Different from respective CTR group ($p < 0.05$).

Table 5
Frequency of hemocytes micronucleated, binucleated and with other nuclear abnormalities of *Corbicula fluminea* exposed to gasoline water-soluble fraction (GWSF) or only water (CTR), taking into account the total number of clams (*N*) analyzed for each experimental period (6, 24 and 96 h).

Hemocytes	Time	Group	*N*	Frequency (‰) (mean ± SE)
Micronucleated	6 h	CTR	7	0.10 ± 0.06
		GWSF	8	0.25 ± 0.14
	24 h	CTR	8	0.04 ± 0.04
		GWSF	8	0.21 ± 0.11
	96 h	CTR	6	0.00 ± 0.00
		GWSF	7	0.43 ± 0.10*
Binucleated	6 h	CTR	7	0.62 ± 0.11
		GWSF	8	1.42 ± 0.58
	24 h	CTR	8	0.29 ± 0.08
		GWSF	8	0.67 ± 0.27
	96 h	CTR	6	1.22 ± 0.24
		GWSF	7	1.71 ± 0.37
With other nuclear abnormalities	6 h	CTR	7	0.05 ± 0.05
		GWSF	8	0.13 ± 0.09
	24 h	CTR	8	0.13 ± 0.09
		GWSF	8	0.25 ± 0.14
	96 h	CTR	6	0.00 ± 0.00
		GWSF	7	0.76 ± 0.14*

* Different from respective CTR group ($p < 0.05$).

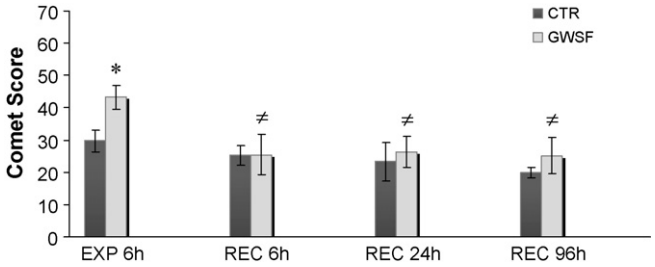

Fig. 3. Comet scores in hemocytes of *Corbicula fluminea* exposed during 6 h (EXP 6 h) to water (CTR) or GWSF and then transferred to clean water for 6 (REC 6 h), 24 (REC 24 h) or 96 h (REC 96 h). *Significantly different from respective CTR group; ≠ significantly different from clams of the group EXP 6 h (GWSF).

3.5. Recovery

The results obtained in the comet assay for the hemocytes of clams exposed to water only or to GWSF for 6 h, and then transferred to clean water for 6, 24 or 96 h, are presented in Table 6 and Fig. 3. The results revealed a significant reduction in the number of damaged nucleoids after the three recovery periods in relation to the results obtained without recovery (Table 6). The comet score also revealed a significant reduction in hemocyte damage after the recovery times (6, 24, and 96 h) in relation to the results obtained without recovery (Fig. 3). Furthermore, no significant difference was detected in the comparisons between the animals of the recovery controls groups and those of the recovery GWSF groups, for the three periods of recovery.

4. Discussion

C. fluminea is sensitive to aquatic pollutants [14,27,28] and is a suitable bioindicator of genotoxic contaminants in aquatic environments [28]. This species has been recommended for biomonitoring of freshwater environments, in conjunction with the comet assay [29]. *C. fluminea,* a sedentary and filter-feeding animal that bioaccumulates xenobiotics, is an interesting model organism for evaluating the action of pollutants in aquatic biota [19,27,28]. In the present study, *C. fluminea* proved sensitive to GWSF diluted to 5%, presenting genetic damage in hemocytes and gill cells after acute exposures.

Table 6
Frequency of nucleoids in each comet class (0, 1, 2 and 3) and the number of damaged nucleoids in hemocytes of *Corbicula fluminea* exposed only to water (CTR) or to gasoline water-soluble fraction (GWSF) for 6 h and then transferred to clean water (recovery) for 6, 24 and 96 h. RecCTR represents clams exposed and transferred to clean water while RecGWSF represents clam exposed to GWSF for 6 h and transferred to clean water. *N* = number of animals analyzed.

Treatment	Time	Group	*N*	Comet classes				Damaged nucleoids (mean ± SE)
				0 (%)	1 (%)	2 (%)	3 (%)	
Exposure	6 h	CTR	7	75.00	20.71	3.86	0.43	25.00 ± 2.16
		GWSF	9	67.00	22.66	8.67	1.67	33.00 ± 3.00*
Recovery	6 h	RecCTR	6	78.33	18.00	3.67	0.00	21.67 ± 2.59
		RecGWSF	6	78.83	18.00	3.00	0.17	21.17 ± 5.44#
	24 h	RecCTR	6	79.33	18.00	2.57	0.00	20.67 ± 4.36
		RecGWSF	5	80.20	14.00	5.20	0.60	19.80 ± 3.53#
	96 h	RecCTR	6	82.67	14.83	2.50	0.00	17.33 ± 1.23
		RecGWSF	6	79.33	16.50	3.83	0.33	20.67 ± 3.71#

* Different from respective CTR control ($p < 0.05$).
\# Different from GWSF group ($p < 0.05$).

Bivalve hemolymph is widely used in toxicological assays because it is easy to collect, hemocytes are already isolated, and it will contain circulating contaminants to which the animal has been exposed [30,31]. Bivalve tissues, such as the gills, have also been used in environment studies [32,33]; gills process the water that enters the animal, so that this tissue is particularly representative for toxicological assays. In the present study, gill cells proved to be more sensitive than hemocytes to the genotoxic effects of GWSF. The gills showed a significant increase in the number of damaged nucleoids compared to control groups, at all exposure periods, while the hemocytes showed such increased damage only after 24 and 96 h exposures. The clams exposed for 6 h to the alkylating agent MMS also showed more genetic damage in gill cells than in hemolymph cells. In another bivalve species, *Mytilus edulis*, exposed to MMS, Rank and Jensen [34] observed that gill cells were more sensitive to DNA damage detected by comet assay than hemolymph cells. This higher gill cell sensitivity to MMS and GWSF could be due to the mode of exposure, with the gills serving as an entrance portal for the contaminant [29]. Rigonato et al. [19] compared the genotoxic sensitivities of hemolymph, gill, and digestive gland cells of *C. fluminea* to MMS and observed that the gill cells were least adequate for genotoxicity studies, not because of their higher sensitivity, but because of their elevated basal levels of DNA damage. Nevertheless, it should be pointed out that these authors exclusively used the technique of mechanical dissociation for obtaining cells from this tissue, a factor that can result in detection of a higher level of basal damage; the isolation of gill cells in the present study was instead accomplished with enzymatic dissociation.

The micronucleus test (MN) is one of the most popular techniques in environmental genotoxicity studies [35] and has, in fact, proved efficient at detecting genetic damage for some species of bivalves exposed to petroleum-derived materials [35,36]. In the present study, the MN test detected significant damage in the genetic material of *C. fluminea* only after 96 h of exposure to GWSF. While the comet assay can detect DNA damage after short time periods, the appearance of MN indicates that the cell has passed through at least one cycle of cellular division. Therefore, in acute assays, the expected number of MN is likely to be low because the cells have passed through few cycles.

As with the pattern observed for MN, other types of hemocyte nuclear abnormalities increased significantly only after 96 h exposure to GWSF. Although the mechanisms involved in the appearance of these abnormalities are not well understood, there are indications that nuclear abnormalities are induced by exposure to genotoxic agents [37,38]. At any rate, such alterations should be considered with great caution because they do not serve as consensus markers among authors.

The results regarding the repair capacity of *C. fluminea* after 6 h exposure to GWSF revealed a great capacity of this species to repair damage to its DNA. The results demonstrated that in just 6 h of recovery, the bivalves were able to reduce scores to basal levels.

The pollution potential of gasoline is directly related to highly water-soluble aromatic hydrocarbons, namely, benzene, toluene, and xylene (BTXs) [5], which are found in the aqueous gasoline fraction [7]. PAH are more toxic than monocyclic aromatic compounds [8]. In the present work, chemical analysis of GWSF showed high concentrations of the monocyclic aromatic hydrocarbons benzene, toluene, ethylbenzene, and xylene (BTEX) and of the PAH naphthalene. Other PAHs, such as anthracene and phenanthrene, were also found, but in lower concentrations. Qualitative analysis indicated the presence of the hydrocarbons in the diluted GWSF after the toxicity tests.

The toxicity of PAH is widely recognized in the literature, as much for its greater persistence in aquatic environments [39] as for the damage that it causes to the biota [1,40,41]. Included among the pollutants with the greatest impact, PAH are considered both mutagenic and carcinogenic agents [42] and have been proven genotoxic to a range of aquatic organisms [3,40]. Studies with other species of bivalve mollusks where there are high concentrations of PAH point to these agents as the most probable cause for the genetic damage found in these areas [43] or in areas affected by oil spills [35,42,44]. Thus, it is possible that PAH are responsible, at least in part, for the DNA damage detected in *C. fluminea* exposed to GWSF in this study. We note also that the interaction of PAH with sunlight can result in the photochemical production of derivatives more genotoxic than the parent compounds [3,43].

PAH are metabolized by cytochrome P450-catalyzed reactions, potentially generating DNA-reactive, adduct-forming metabolites whose repair leads to loss of DNA integrity [3]. Furthermore, PAH biotransformation can generate reactive oxygen species (ROS) that can cause damage to genetic material [3,40]. Silva et al. observed a significant increase in glutathione transferase (GST) activity in *Crassostrea rhizophorae* exposed to diesel oil [45]. The comet assay is an efficient technique for the detection of oxidative damage to DNA, which is often quickly repaired [46]. The rapid repair of DNA damage observed in this study is at least consistent with oxidative damage. The capacity of this species to degrade certain types of petroleum hydrocarbons [15] and for rapid recovery from exposure to some environmental pollutants [14] may be important factors in the recovery observed in this study.

The results of the present study indicate that GWSF has genotoxic and mutagenic actions on *C. fluminea*. The comet assay stood out as a highly sensitive technique for the detection of damage to DNA in this bivalve, corroborating its recognized efficiency for the genotoxic evaluation of aquatic organisms [37,47]. These

findings reinforce recognition of the sensitivity of *C. fluminea* to damage caused by exposure to environmental contaminants, and therefore substantiate its use in environmental monitoring studies [19,28].

Conflict of interest

The authors have no conflict of interest.

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References

[1] M. Pacheco, M.A. Santos, Biotransformation, endocrine, and genetic responses of *Anguilla anguilla* L. to petroleum distillate products and environmentally contaminated waters, Ecotox. Environ. Safe. 49 (2001) 64–75.
[2] C.H. Peterson, S.D. Rice, J.W. Short, D. Esler, J.L. Bodkin, B.E. Ballachey, D.B. Irons, Long-term ecosystem response to the Exxon Valdez oil spill, Science 302 (2003) 2082–2086.
[3] G. Frenzilli, V. Scarcelli, I. Del Barga, M. Nigro, L. Förlin, C. Bolognesi, J. Sturve, DNA damage in eelpout (*Zoarces viviparus*) from Göteborg harbor, Mutat. Res. 552 (2004) 187–195.
[4] R.J. Watts, D.R. Haller, A.P. Jones, A.L. Teel, A foundation for the risk-based treatment of gasoline-contaminated soils using modified Fenton's reactions, J. Hazard. Mater. B76 (2000) 73–89.
[5] E.R.L. Tiburtius, P. Peralta-Zamora, A. Emmel, E.S. Leal, Degradação de BTXs *via* processos oxidativos avançados, Quím. Nova 28 (2005) 61–64.
[6] E.R.L. Tiburtius, P. Peralta-Zamora, A. Emmel, E.S. Leal, Treatment of gasoline-contaminated waters by advanced oxidation processes, J. Hazard. Mater. B126 (2005) 86–90.
[7] C.S. Chen, Y-.W. Lai, C.-J. Tien, Partitioning of aromatic and oxygenated constituents into water from regular and ethanol-blended gasolines, Environ. Pollut. 156 (2008) 988–995.
[8] J.F. Paixão, I.A. Nascimento, S.A. Pereira, M.B.L. Leite, G.C. Carvalho, J.S.C. Silveira Jr., M. Rebouças, G.R.A. Matias, I.L.P. Rodrigues, Estimating the gasoline components and formulations toxicity to microalgae (*Tetraselmis chuii*) and oyster (*Crassostrea rhizophorae*) embryos: an approach to minimize environmental pollution risk, Environ. Res. 103 (2007) 365–374.
[9] M. Poulsen, L. Lemon, J.F. Barker, Dissolution of monoaromatic hydrocarbons into groundwater from gasoline–oxygenate mixtures, Environ. Sci. Technol. 269 (1992) 2483–2489.
[10] R. Van der Oost, F.J. Van Schooten, F. Ariese, H. Heida, N.P.E. Vermeulen, Bioaccumulation, biotransformation and DNA binding of PAHs in feral eel (*Anguilla anguilla*) exposed to polluted sediments: a field survey, Environ. Toxicol. Chem. 13 (1994) 859–870.
[11] F.J. Ahmed, Toxicology and human health effects following exposure to oxygenated or reformulated gasoline, Toxicol. Lett. 123 (2001) 89–113.
[12] T.P. O'Connor, National distribution of chemical concentrations in mussels and oysters in the USA, Mar. Environ. Res. 53 (2002) 117–143.
[13] E.D. Goldberg, The mussel watch: a first step in global marine monitoring, Mar. Pollut. Bull. 69 (1975) 111–132.
[14] S.B. Basack, M.L. Oneto, N.R. Verrengia Guerrero, E.M. Kesten, Accumulation and elimination of pentachlorophenol in the freshwater bivalve *Corbicula fluminea*, Bull. Environ. Contam. Toxicol. 58 (1997) 497–503.
[15] J.C. Colombo, A. Barreda, C. Bilos, N. Cappelletti, S. Demichelis, P. Lombardi, M.C. Migoya, C. Skorupka, G. Suárez, Oil spill in the Río de la Plata estuary, Argentina. 1. Biogeochemical assessment of waters, sediments, soils and biota, Environ. Pollut. 134 (2005) 277–289.
[16] R.R. Tice, M. Furedi-Machacek, D. Satterfield, A. Udumudi, M. Vasquez, J.K. Dunnik, Measurement of micronucleated erythrocytes and DNA damage during chronic ingestion of phenolphthalein in transgenic female mice heterozygous for the *p53* gene, Environ. Mol. Mutagen. 31 (1998) 113–124.
[17] G.I.V. Klobucar, M. Pavlica, R. Erben, D. Papes, Application of the micronucleus and comet assays to mussel *Dreissena polymorpha* haemocytes for genotoxicity monitoring of freshwater environments, Aquat. Toxicol. 64 (2003) 15–23.
[18] D.E. Nicodem, C.L.B. Guedes, R.J. Correa, Photochemistry of petroleum. I. Systematic study of a Brazilian intermediate crude oil, Mar. Chem. 63 (1998) 93–104.
[19] J. Rigonato, M.S. Mantovani, B.Q. Jordão, Comet assay comparison of different *Corbicula fluminea* (Mollusca) tissues for the detection of genotoxicity, Genet. Mol. Biol. 28 (2005) 464–468.
[20] I.V. Villela, I.M. Oliveira, J.C. Silveira, J.F. Dias, J.A.P. Henriques, J. Silva, Assessment of environmental stress by the micronucleus and comet assays on *Limnoperna fortunei* exposed to Guaíba hydrographic region samples (Brazil) under laboratory conditions, Mutat. Res. 628 (2007) 76–86.
[21] M.F. Kilemade, M.G.J. Hartl, D. Sheehan, C. Mothersill, F.N.A.M. Van Pelt, J. O'Halloran, N.M. O'Brien, Genotoxicity of field-collected inter-tidal sediments from Cork Harbour, Ireland, to juvenile turbot (*Scophthalmus maximus* L.) as measured by the comet assay, Environ. Mol. Mutagen. 44 (2004) 56–64.
[22] D.G.S.M. Cavalcante, C.B.R. Martinez, S.H. Sofia, Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*, Mutat. Res. 655 (2008) 41–46.
[23] T.P. Vanzella, C.B.R. Martinez, I.M.S. Colus, Genotoxic and mutagenic effects of diesel oil water-soluble fraction on a neotropical fish species, Mutat. Res. 631 (2007) 36–43.
[24] I.V. Villela, I.M. Oliveira, J. Silva, J.A.P. Henriques, DNA damage and repair in haemolymph cells of golden mussel (*Limnoperna fortune*) exposed to environmental contaminants, Mutat. Res. 605 (2006) 78–86.
[25] T.S. Kumaravel, B. Vilhar, S.P. Faux, A.N. Jha, Comet assay measurements: a perspective, Cell. Biol. Toxicol. 25 (2009) 53–64.
[26] R.N. Hoftman, W.K. de Raat, Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of the eastern mudminnow *Umbra pygmaea* by ethyl methanesulfonate, Mutat. Res. 104 (1982) 147–152.
[27] C. Bilos, J.C. Colombo, M.J.R. Presa, Trace metals in suspended particles, sediments and Asiatic clams (*Corbicula fluminea*) of the Rio de la Plata Estuary, Argentina, Environ. Pollut. 99 (1998) 1–11.
[28] D.H. Cataldo, J.C. Colombo, D. Boltovskoy, C. Bilos, P. Landoni, Environmental toxicity assessment in the Parana river delta (Argentina): simultaneous evaluation of selected pollutants and mortality rates of *Corbicula fluminea* (Bivalvia) early juveniles, Environ. Pollut. 112 (2001) 379–389.
[29] J.D. Caffetti, M.S. Mantovani, M.C. Pastori, S.S. Fenocchio, First genotoxicity study of Paraná river water from Argentina using cells from the clam *Corbicula fluminea* (Veneroida Corbiculidae) and Chinese hamster (*Cricetulus griseus* Rodentia, Cricetidae) K1 cells in the comet assay, Genet. Mol. Biol. 31 (2008) 561–565.
[30] C. Bolognesi, E. Landini, P. Roggieri, R. Fabbri, A. Viarengo, Genotoxicity biomarkers in the assessment of heavy metal effects in mussels: experimental studies, Environ. Mol. Mutagen. 33 (1999) 287–292.
[31] D.R. Dixon, A.M. Pruski, L.R.J. Dixon, A.N. Jha, Marine invertebrate ecogenotoxicology: a methodological overview, Mutagenesis 17 (2002) 495–507.
[32] P. Venier, S. Maron, S. Canova, Detection of micronuclei in gill cells and haemocytes of mussels exposed to benzo[*a*]pyrene, Mutat. Res. 390 (1997) 33–44.
[33] J. Rank, Intersexin *Littorina littorea* and DNA damage in *Mytilus edulis* as indicators of harbour pollution, Ecotox. Environ. Safe. 72 (2009) 1271–1277.
[34] J. Rank, K. Jensen, Comet assay on gill cells and hemocytes from the blue mussel *Mytilus edulis*, Ecotox. Environ. Safe. 54 (2003) 323–329.
[35] J. Baršienė, L. Andreikėnaitė, G. Garnaga, A. Rybakovas, Genotoxic and cytotoxic effects in the bivalve mollusks *Macoma balthica* and *Mytilus edulis* from the Baltic Sea, Ecologija 54 (2008) 44–50.
[36] J. Baršienė, L. Andreikėnaitė, Induction of micronuclei and other nuclear abnormalities in blue mussels exposed to crude oil from the North Sea, Ecologija 53 (2007) 9–15.
[37] M.M. Hoshina, D.F. de Angelis, M.A. Marin-Morales Angelis, Induction of micronucleus and nuclear alterations in fish (*Oreochromis niloticus*) by a petroleum refinery effluent, Mutat. Res. 656 (2008) 44–48.
[38] L. Serrano-Garcia, R. Montero-Montoya, Micronuclei and chromatid buds are the result of related genotoxic events, Environ. Mol. Mutagen. 38 (2001) 38–45.
[39] R. Van Der Oost, J. Beyer, N.P.E. Vermeulen, Fish bioaccumulation and biomarkers in environmental risk assessment: a review, Environ. Toxicol. Pharmacol. 13 (2003) 57–149.
[40] C.L. Mitchelmore, J.K. Chipman, DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring, Mutat. Res. 399 (1998) 135–147.
[41] B. Pérez-Cadahía, B. Laffon, E. Pásaro, J. Méndez, Evaluation of PAH bioaccumulation and DNA damage in mussels (*Mytilus galloprovincialis*) exposed to spilled Prestige crude oil, Comp. Biochem. Physiol. C 138 (2004) 453–460.
[42] S.A. Steinert, R. Streib-Montee, J.M. Leather, D.B. Chadwick, DNA damage in mussels at sites in San Diego Bay, Mutat. Res. 399 (1998) 56–85.
[43] G. Frenzilli, M. Nigro, B.P. Lyons, The comet assay for the evaluation of genotoxic impact in aquatic environments, Mutat. Res. 681 (2009) 80–92.
[44] C. Porte, P. Lemaire, L.D. Peters, D.R. Livingstone, Partial purification and properties of cytochrome P450 from digestive gland microsomes of the common mussel, *Mytilus edulis* L, Mar. Environ. Res. 39 (1995) 27–31.
[45] A.Z. Silva, J. Zanette, J.F. Ferreira, J. Guzenski, M.R.F. Marques, A.C.D. Bainy, Effects of salinity on biomarker responses in *Crassostrea rhizophorae* (Mollusca, Bivalvia) exposed to diesel oil, Ecotox. Environ. Safe. 62 (2005) 376–382.
[46] A.R. Collins, Investigating oxidative DNA damage and its repair using the comet assay, Mutat. Res. 681 (2009) 24–32.
[47] A.N. Jha, Ecotoxicological applications and significance of the comet assay, Mutagenesis 23 (2008) 207–221.