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Genotoxicity assessment in estuarine environments using oyster hemocytes: a comparative comet assay study

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ABSTRACT

The comet assay with sentinel organisms has been employed to determine the genotoxicity of water samples for environmental risk or hazard assessment. Methodologies with diverse sensitivities have been proposed to elucidate the sources of genetic damage, thereby enhancing environmental assessment and informing decision-making processes. The conventional method and the modified conventional method with the DNA excision enzyme formamidopyrimidine-DNA glycosylase (FPG) were used to evaluate DNA damage in oyster (*Crassostrea rhizophorae*) hemocytes. The oysters were exposed for seven days to water samples from three estuarine regions with different degrees of impact on the Brazilian equatorial margin: (1) Carimã Island, which is distant from the main industrial activities of Maranhão Island (reference area), (2) Itaqui Port, and (3) Bacanga Lagoon. Genetic damage tests were performed in parallel with an early-stage assay using oyster embryos. The results revealed that the degree of genetic damage varied depending on the water sampling location and genetic damage method used. Regarding sampling sites, the frequency of anomalies in embryos/larvae and genotoxic damage increased as follows: Carimã Island<Itaqui Port<Bacanga Lagoon. Regarding the method, the total genetic damage index was 58%, 69%, and 41% greater when the FPG comet assay was used than when the conventional comet assay was used for samples from Carimã Island, Itaqui Port, and Bacanga Lagoon, respectively. However, it was only 13% in samples of the positive control group (Carimã Island + H_2O_2). Furthermore, a stronger correlation (R = 0.8) was observed between the frequency of anomalies in embryos/larvae and the genotoxic damage measured via the FPG comet assay than via the conventional comet assay (R = 0.4). Therefore, the FPG comet assay was a more sensitive method, which enabled a better classification of environmental risks because of the teratogenic and genetic effects observed in oysters.

Keywords: Biomonitoring, Estuarine pollution, Mangrove oyster

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INTRODUCTION

Urban centers tend to be concentrated near water bodies, especially estuaries, which are a decisive factor in increasing aquatic pollution

(De Seixas Filho et al., 2020; Freeman et al., 2019; Vlahogianni and Valavanidis, 1990). Estuaries are particularly influenced by coastal drainage basins and the substantial input of contaminants via surface runoff. Indeed, port activities and the discharge of urban and industrial effluents may have biological and ecological impacts on estuarine areas (Cuong et al., 2005; Freeman et al. 2019; Geret et al., 2011; Nigro et al., 2006). Therefore, different approaches and techniques are required for the detection, assessment, and monitoring of these impacts.

Early-stage assays have been used to assess the acute toxicity of marine and estuarine environmental samples, enabling the detection of harmful pollutants even at low concentrations (Geffard et al, 2001; His et al., 1999; Picone et al., 2016). In addition, molecular biomarkers have assisted in diagnostic studies of impacts on estuarine environments and are valuable tools in the scope of environmental quality monitoring. Biomarkers are adaptive molecular responses to known, but unspecified, stressors (Walker et al., 1996). These changes start as molecular-level responses and are characterized by the induction of defense mechanisms, enabling the evaluation of interactions between xenobiotics and the organisms exposed to them (Jesus and Carvalho, 2008). These initial changes are warnings for potential long-term alterations at the ecosystem level (Milinkovitch et al., 2019), so a combination of early-stage assays and biomarkers are useful for assessing environmental status (Edge et al., 2012).

Filter-feeding mollusks, especially oysters, are useful sentinel organisms for determining the environmental quality of coastal ecosystems (Aguirre-Rubí et al., 2018; Moreau et al., 2014; Moreau et al., 2015; Rizo et al., 2010; Vaisman et al., 2005; Vázquez-Boucarda et al., 2014). Oysters of the species *Crassostrea rhizophorae* (Guilding, 1828) are sessile, intertidal, euryhaline, widely distributed along the Brazilian coast, and easy to acclimate to test conditions. These characteristics make them ideal candidates to be used as model organisms in toxicological assessments of coastal aquatic environments (Zagatto and Bertoletti, 2008). Indeed, several oyster tissues have been employed in bioaccumulation and biomarker studies, e.g., hemolymph hemocytes have been reported to be useful for investigating the stress associated with environmental contamination (Moreau et al., 2014, 2015).

Hemocytes produce excessive amounts of reactive oxygen and nitrogen species (RONS) when in contact with contaminants (Ribeiro et al., 2005; Vasconcelos et al., 2007). RONS can cause oxidative stress and subsequent DNA damage when produced in high quantities and not neutralized by the antioxidant defense system, which can result in apoptosis, mutation, or carcinogenesis if not corrected by the repair system (Dizdaroglu, 2012). The redox balance in the cells of aquatic organisms is altered in the presence of certain xenobiotics, making genotoxic evaluation relevant in chronic exposures and their potential consequences for populations (Devaux et al., 2011). In this context, damage to nuclear chromatin represents a genotoxic effect and can be quantified by the "comet assay"—single-cell gel electrophoresis—SCGE (Christl et al., 2004).

The comet assay applied to mollusks involves assessing DNA fragmentation to identify molecular damage caused by environmental factors (Dhawan et al., 2009; Gagnaire et al., 2006; Gajski et al., 2019), which is observed via cell lysis followed by gel electrophoresis under alkaline conditions to visualize chromatin fragmentation by the migration of DNA fragments away from the nucleoid center (Gielazyn et al., 2003). Thus, the comet tail is observed under a fluorescence microscope, and its size correlates with the amount of damaged DNA. Generally, the damage quantified by the comet assay includes single-strand breaks (SSBs), double-strand breaks (DSBs), and unstable alkaline sites (ALS) (Emmanouil et al., 2007; Gielazyn et al., 2003). Notably, a positive and significant correlation was demonstrated between genotoxicity, measured via the comet assay, and embryotoxicity in oysters (Wessel et al., 2007). However, this assay enables a modification—using the DNA-formamidopyrimidine glycosylase (FPG) enzyme—that gives information on the involvement of oxidative stress (oxidized purines). Additionally, the conventional and modified methods show different sensitivities to the mechanism of action of environmental contaminants. Therefore, this study assessed the genotoxicity of water samples from different estuarine environments located on Maranhão Island (Brazilian equatorial margin) toward hemocytes of the oyster *C. rhizophorae* by comparing the results of two comet assay methods (conventional and modified). We hypothesize that the modified comet assay will show greater sensitivity in response to the environmental contaminants in the tested water samples than the conventional method. The results can contribute to the selection of relevant, reliable, and more accurate biomarkers to be employed in future programs aimed at monitoring environmental quality and health.

METHODS

Sampling sites

Carimã is located on Curupú Island (02°24'39"S, 44°05'12"W) in Raposa, which is one of the four municipalities on Maranhão Island (Maranhão state, Northeastern Brazil), and is characterized by few potentially polluting activities (Figure 1). Situated far from metropolitan centers, it is highly suitable for fishing (Diniz et al. 2020) and has been considered a natural environment with minimal alterations (Corrêa et al., 2021; Lima, 2018; Piorski et al., 2009; Rêgo et al., 2018). Carimã is a nesting area and feeding ground for many wild and endemic species (Costa-Lotufo et al., 2006; Miranda et al., 2009; Rodrigues et al., 2010; Ribeiro et al. 2014) and hosts a bank of oysters (*C. rhizophorae*), which were previously identified via morphological and genetic analyses (Rocha Jr. et al., 2018). Therefore, Carimã was chosen as a reference site for the collection of the oysters employed in the assays. Additionally, seawater from this site was collected for oyster acclimation in the laboratory, as well as for the assays performed as "negative control."

The São Marcos Estuarine Complex (CESM) is in the Maranhense Gulf and receives water from important rivers such as Pindaré, Mearim, and Bacanga, which are mixed with northern coastal waters. Within the CESM lies Bacanga Lagoon and Itaqui Port (Figure 1), which have experienced various impacts, including contamination from various points and diffuse sources such as effluents (domestic, industrial, and hospital), metals, metalloids, nutrients, and organic compounds (Cabral et al., 2020; Carvalho-Neta and Abreu-Silva, 2010; Castro et al., 2019; Lima et al., 2021; Righi et al., 2022; Silva et al., 2023).

The Bacanga River basin is considered the most important of São Luís, capital of the state of Maranhão. The basin area accounts for 12.3% of São Luís and is composed of 10 subbasins in which 64,000 households are distributed across 60 neighborhoods, housing estates, and urban clusters with approximately 256,000 inhabitants (Soares et al., 2021). This is also characterized by residential clusters along water bodies, accommodating vulnerable populations (Lopes, 2017). The Bacanga River is influenced by saltwater due to the region's macrotidal regime and since the 1960s, significant changes have occurred with the construction of the Bacanga dam, which has led an artificial lagoon system controlled by gates and substantially increased the water residence time (Silva et al., 2014). The dam was planned to shorten access to Itaqui Port, generate electricity (from tidal power), and create an artificial lake (lagoon) to facilitate the city's urbanization process (Barros et al., 2009; Pereira et al., 2018). However, this intense urbanization has led to numerous issues, including siltation, mangrove infilling, water contamination from raw sewage discharge, and waste accumulation (Duarte dos Santos et al., 2016; Lopes, 2017; Martins, 2005; Martins, 2008; Morais et al., 2021).

The Itaqui region, which is an industrial area of São Luís, has a port infrastructure to meet the demand for imports and exports and the intense movement of large ships. The following port facilities are in the Itaqui Port complex: the Organized Port of Itaqui, Ponta da Madeira Maritime Terminal (TMPM), Alumar Private Use Terminal (TUP) (Alcoa), and terminals that are in the project/implementation phase—the São Luís Port Terminal, Mearim Port Terminal, and Alcântara Port Terminal (Cassia, 2023; MTPA, 2018). Near the Itaqui Port complex, the Itaqui Thermal Power Plant (Eneva S.A.) was inaugurated in 2013 with the capacity to produce 360 KW, with its cooling system using and returning water from São Marcos Bay (Branco et al., 2013).

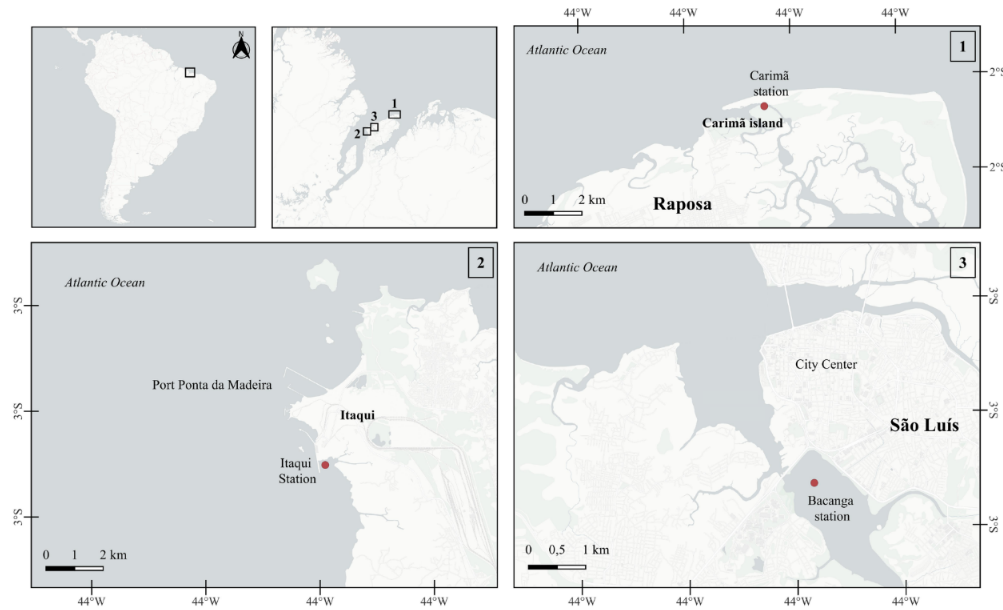

Figure 1. The estuarine environments of Maranhão Island and the locations of the sampling sites: 1- Carimã, 2- Itaqui Port, and 3- Bacanga Lagoon.

Oyster Embryotoxicity Assays

The acute toxicity of the water samples was determined via the embryo–larval assay with oocytes from adult oysters collected at Carimã following the procedures described by Cruz et al. (2007), Magris et al. (2006) and Nascimento et al. (2002). The collection, storage, and processing of water samples followed the recommendations of standard NBR 15469 (ABNT, 2021). A total of 25 L of water was collected at each sampling station at a depth of approximately 1.0 m via a Van Dorn-type oceanographic bottle, and in the laboratory, the samples underwent the following treatments: (1) settling for 48 h, (2) pH and salinity adjustment, (3) filtration through a Whatman® GF/C 1.2 µm filter, and (4) autoclaving (120 kgf/cm^2 for 20 minutes). After treatment, the cells were observed for at least 24 hours before the assays were performed.

Twenty females and four males were used for in vitro fertilization. Gametes were obtained by incising the gonads with the aid of a Pasteur pipette, were observed under a microscope to confirm sex, and were counted using a Neubauer chamber. The female and male gametes were gently mixed at a ratio of 50:1 in a Petri dish (Velasco et al., 2010), which was chosen to prevent polyspermy and/or low fertilization rates (Beiras and His, 1995; Rampersad et al., 1994). After 1 h of rest, the gametes were once again observed in a Sedgwick-Rafter chamber to confirm fertilization (approximately 90% of the oocytes), which resulted in the formation of the polar body and first cleavage.

The tissue remnants were removed via a 150 µm sieve, and the oocytes were then transferred to a 45 L aquarium with treated seawater (from Carimã) containing a mixture of antibiotics (Rocha Jr. et al., 2018) under constant aeration. After reaching the gastrula phase, approximately 24 h after fertilization, the embryos were removed from the aquarium, and the density was adjusted to 20 µm mesh for 500 to 1,500 embryos/mL. The embryo suspension was used for the assays. For acute tests, glass containers (~300 mL; three replicates per treatment) containing the environmental samples (undiluted) were used, in addition to the positive control consisting of a 1.0 mg/L solution of sodium dodecyl sulfate (SDS).

The test vials were filled with 100 mL of treated seawater (from Carimã) and 1 mL of embryo suspension. After 24 h, the embryos were fixed with 4% formalin and left for an additional 24 h to settle, then the supernatant material was removed, and a 10-mL aliquot of the settled material was transferred to test tubes for subsequent examination in a Sedgwick-Rafter chamber.

Embryos were considered normal if they showed a D-shaped shell, empty D-shaped shells, or smaller embryos with a D-shaped shell. Embryos were considered abnormal if they showed anomalies on the shell margins or hinge, lacked shells, showed multiple combined anomalies, or did not reach the larval stage before the veliger stage.

The results were calculated as the net percentage of abnormality via the Abbott formula, with anomalies calculated based on the net rate of normal development adjusted by the controls (Finney, 1971; Mottier et al., 2013). Group comparisons were conducted via the Kruskal–Wallis test ($p<0.05$) and Statistical analyses were performed via R software (v.4.4.3).

Genotoxicity assays with oyster hemocytes

Oyster immersion was performed according to Christl et al. (2004) with modifications. Briefly, *C. rhizophorae* were collected from Carimã and transferred to the laboratory in a moist container, there they were cleaned of encrustations with a brush and tap water. They underwent a three-day period of acclimation in a 60-L aquarium containing filtered water from Carimã under constant aeration.

Ninety-six oysters of similar size (6.46 ± 0.36 cm in length and 4.11 ± 0.26 cm in width) were selected and distributed in 20-L tanks (n =12 per tank) containing water from human-impacted environments (Bacanga and Itaqui), in addition to the group kept in Carimã water (control). A positive control group of oysters was maintained in Carimã water that received hydrogen peroxide during the hemocyte extraction and treatment stages. The oysters remained in the tanks for seven days under controlled conditions of salinity (25 g/kg), temperature (26 ± 1°C), pH (8.0-8.5), dissolved oxygen (> 4.1 mg/L), and a photoperiod cycle of 12 h light/12 h dark.

In the comet assay, cell lysis followed by gel electrophoresis under alkaline conditions enables the visualization of chromatin fragmentation via the migration of DNA fragments away from the nucleoid center (Gielazyn et al., 2003). The comet tail is observed under a fluorescence microscope, and its size correlates with the amount of damaged DNA. Generally, the damage quantified by the comet assay includes single-strand breaks (SSBs), double-strand breaks (DSBs), and unstable alkaline sites (ALS) (Emmanouil et al., 2007; Gielazyn et al., 2003). However, the conventional comet assay can be modified via the DNA-formamidopyrimidine glycosylase (FPG) enzyme, which removes damaged DNA by hydrolyzing the N-glycosidic bond, creating abasic sites. Therefore, the results of the modified comet assay mainly represent single-strand breaks associated with repair incision sites (SSBe) (Kienzler et al., 2012). In this study, both conventional and modified versions of the comet assay were employed. The single-cell gel electrophoresis (SCGE) comet assay (conventional method) was adapted from Machella et al. (2006), Ribeiro et al. (2003), and Rigonato et al. (2005). All the steps described below follow the recommendations of Møller et al. (2020) regarding the minimum information for reporting on the Comet Assay (MIRCA):

Isolation of cells: All the procedures were performed at a laboratory at 20–25°C. Oyster hemolymph containing hemocytes was collected by puncturing the adductor muscle using a syringe (needle gauge: 13×3.8 mm);

Embedding the cells in agarose. The low-melting point agarose was melted in a microwave for 45 sec and kept in a water bath at 36°C. Immediately after the hemolymph extraction of each individual, 20 µL aliquots were transferred into two 1.5 mL microtubes containing 100 µL of LMA each (final concentration of 0.052%). For the positive control (assay control), 10 µL of hydrogen peroxide was added to the hemolymph suspension and left to stand for 10 min. The hemolymph+LMA mixture was homogenized very gently. The material was pipetted (100 µL) onto frosted microscope slides (26×76 mm) with three replicates (1-gel/slide format) per oyster and cooled to 4°C for 5 min. These slides were previously covered with normal melting point agarose (NMA) (1.5 g of agarose NMA + 10 mL of PBS, 1%).

Lysis: Slides were placed in lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 0.01 M Tris, pH 10 + 1% Triton X-100, and 10% DMSO) for 12 h in the dark at 4°C. For the modified comet assay (FPG), after the cell lysis step, the slides were immersed in 1X FLARE TM buffer (Trevigen) for 30 min.

Enzyme treatment: Each slide was treated with 150 µL of a solution containing the FPG enzyme

(8 u/mL, DNA-formamidopyrimidine glycosylase, Trevigen's Comet Assay® analysis kit, BioTechne), covered with coverslips, and incubated in a humid chamber (water bath) for 45 min at 37°C.

Alkaline treatment: The slides were incubated for 20 min in an alkaline solution (10 M NaOH, 0.2 M EDTA, distilled water, pH 13) at 4°C, placed in an electrophoresis tank, and covered with the same buffer.

Alkaline electrophoresis: The electrophoresis run lasted 20 min at a voltage of 25 V (0.72 V/cm) and 300 mA at room temperature.

Neutralization: Slides were placed in a neutralization buffer solution (0.4 M Tris, pH 7.5) at room temperature for 15 min and left to dry for 12 h.

Staining and visualization: The slides were immersed in ethanol (99.3%) for 4 min for fixation. After 20 min of drying, the slides were stained with ethidium bromide (20 µg/mL) and immediately analyzed under a fluorescence microscope (BX51-Olympus, filter 516-560 nm, 40x objective), with 100 hemocyte nucleoids being analyzed per slide.

Scoring and data analysis: The effects were classified according to Speit and Hartmann (1995) into five damage classes regarding the size and quantity of DNA in the nucleoid's tail: Class 0: no damage (<5%); Class 1: low level of damage (5–20%); Class 2: medium level of damage (21–40%); Class 3: high level of damage (41–94%); and Class 4: total damage (>94%). The total damage index (TDI) was calculated considering the sum of comets in each class multiplied by the number of their respective class, with the final total divided by 100, as per the formula below:

$$TDI = \frac{0 \times (nClass0) + 1 \times (nClass1) + 2 \times (nClass2) + 3 \times (nClass3) + 4 \times (nClass4)}{100}$$

Statistical analysis: The results did not show a parametric distribution according to the Shapiro–Wilk and Levene tests ($p > 0.05$), so they were subjected to Yeo–Johnson transformation. After normalization, the TDI values were compared between the different treatments (conventional and modified comet assay methods [FPG] and positive control [Carimã + H_2O_2]) via one-way ANOVA, followed by the Tukey ($p < 0.05$) post hoc test to identify significant differences between groups. Statistical analyses were performed via R software (v.4.4.3).

RESULTS

The water samples from the Bacanga site showed greater toxicity than those from the other sampling sites did ($p<0.05$). Indeed, the effects observed with water samples from the Bacanga site surpassed the effect observed in the treatment with the reference substance (DSS; 1.0 mg/L) by approximately 37% (Table 1). Compared with those from the Carimã site, the water samples from the Itaqui site also showed toxicity to oyster embryos ($p<0.05$). The effect was like the one observed in the positive control.

Table 1. Percentage (average ± standard deviation) of anomalies in embryos/larvae of the mangrove oyster *Crassostrea rhizophorae* exposed to different water samples from estuaries of Maranhão Island. NC = negative control; DSS = sodium dodecyl sulfate; PC = positive control. Different small letters indicate significant difference among the experimental groups (Kruskal–Wallis, $p<0.05$).

Carimã (NC)	DSS (PC)	Itaqui	Bacanga
18,63±4,98 [a]	68,77±9,26 [b]	69,00±11,39 [b]	94,47±1,30 [c]

The degree of genetic damage to oyster hemocytes also varied depending on the water collection site. For both comet assay methods employed, the samples from the Bacanga site showed the greatest genotoxicity, which confirmed the results of the acute tests with embryos (Figure 2). However, they indicated a significant difference in the genetic damage induced by water samples from the Itaqui site only when the modified FPG test was used ($p<0.05$). The observed effect was like the one observed in the positive control (PC). When the conventional test was used, the genetic damage induced by the water samples from the Itaqui site was like the one observed with the water samples from the Carimã site ($p>0.05$).

The FPG comet assay with samples from the Itaqui site showed an increased response (~220%) compared with the conventional test. Despite the lack of difference between the two positive controls, there was a difference in the responses of the two methods for the water samples from the Carimã site (121%) and the Bacanga site (72%). Thus, water samples from the studied environments affected the mangrove oysters in the following order: Carimã < Itaqui < Bacanga.

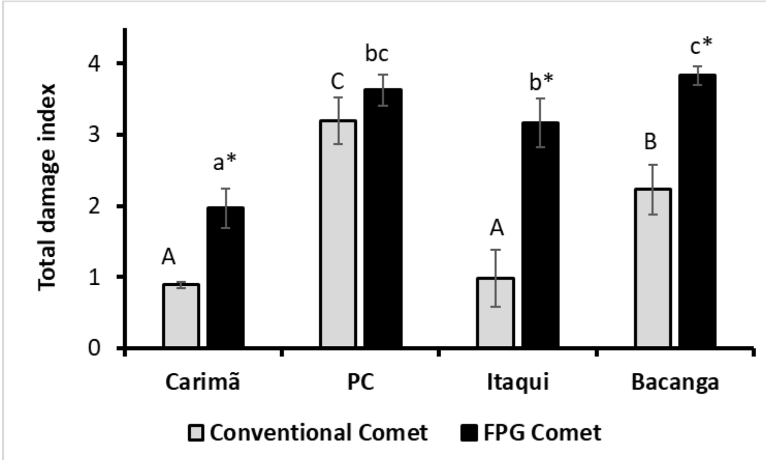

Figure 2. Genetic damage index (average ± standard deviation) in *Crassostrea rhizophorae* hemocytes exposed for seven days to surface water samples from three estuaries of Maranhão Island determined by the conventional and modified (FPG) comet assay methods. PC = positive control (Carimã + H_2O_2). Different capital and small letters indicate significant differences between the experimental groups (ANOVA with Tukey's test, $p<0.05$) via the conventional and modified comet assay methods, respectively. * Indicates a significant difference between the comet assay methods (ANOVA, $p<0.05$).

DISCUSSION

Genotoxicity spatial variation

The acute toxicity test with oyster embryos showed an ideally low frequency (<20%) of anomalies in the negative control test with water samples from the reference site (Carimã). Indeed, an anomaly frequency >25% in the negative control would invalidate the test (Nascimento, 1991). This ideal result is associated with the adequate conditions adopted to obtain the tested embryos (Butler et al., 1992). The results of the acute toxicity test with oyster embryos/larvae indicated greater teratogenicity in the water samples from Bacanga Lagoon and Itaqui Port than in those from the Carimã site (Figure 2). This result was also observed when the results of the DNA damage assay with oyster hemocytes via the modified FPG comet assay were considered. These findings agree with the impacts that these two estuarine environments have been subjected to in recent decades. Indeed, several studies performed in these areas have shown the presence of different classes of contaminants and the occurrence of various effects on environmental biomarkers in several bioindicators (Table 2).

Additionally, our results indicated greater genotoxicity in the water samples from Bacanga Lagoon than in the water samples from Itaqui Port. This result was observed in both teratogenic (anomalies in embryos/larvae) and DNA damage (conventional and modified FPG comet assays) tests and can be explained, at least in part, by the different contamination profiles of these two sites. Notably, despite being geographically close to the Carimã site and subject to some common sources of pollution (such as domestic and industrial effluents), the environmental dynamics between the Bacanga Lagoon and Itaqui Port sites are quite distinct from each other. The high mixing rate in the estuarine complex of São Marcos, associated with its macrotidal regime, can contribute to the dispersion of pollutants, thus reducing their concentration in certain areas (Czizeweski et al., 2020; Dos Santos et al., 2020; Dos Santos et al., 2023; Lima et al., 2021). In contrast, Bacanga Lagoon shows a different scenario. The primary function of the Bacanga dam, which is controlled by gates, is to regulate the volume of the reservoir, thus limiting variations in its water level, which would prevent excessive levels that could lead to flooding while providing the minimal water levels necessary for sustaining fishing activities (Ferreira and Estefen, 2009; Leite Neto et al., 2017). These characteristics result in a significant increase in the water residence time, thus leading to a decrease in contaminant dispersion and a consequent increase in contaminant concentration in Bacanga Lagoon, which could explain the greater genotoxic response in the water samples from this site than with those from Itaqui Port.

Table 2. Contaminants in different environmental compartments (water, porewater, sediment, and biota) and some effects observed in bioindicator species in two impacted estuaries (Itaqui and Bacanga) of Maranhão Island (Maranhão state, Northeastern Brazil).

Estuary	Matrix	Contaminant/biomarker	Reference
Itaqui (São Marcos)	Water	Biocides (irgarol and diuron)	Diniz et al. (2014)
		Coliforms	Costa et al. (2014)
		Metal (Al, Cd, Pb, Cr, Fe, and Hg), phenolics, tributyltins, and PCB	Carvalho Neta et al. (2017)
		Metal (B and Zn), total phosphorus, and residual chlorine	Almeida et al. (2021)
		Metal (Mn and Zn)	Sousa et al. (2023)
	Water and sediment	Metal (Al and Fe)	Sousa (2009)
		Biocides (irgarol and diuron)	Viana et al. (2020)
		Metal (Al, Cd, Pb, Cr, Fe, and Hg), benzene, total phenols, tributyltin and PCB	Carvalho Neta et al. (2013)
	Porewater and sediment	Biocides (irgarol and diuron) biocide stable degradation products (DMSA and DCPMU)	Viana et al. (2019)
	Sediment	Metal (Al, Fe, Cu, and Ni)	Nunes et al. (2020)
		Metal (As and Ni)	Ribeiro et al. (2023)
	Sediment and macroalgae	Metal (Cr, Cu, Fe, Mn, Pb, and Zn)	Corrêa et al. (2023)
	Gastropods	Imposex	Viana et al. (2021)
	Crab	Biometric alterations, histological lesions, and GST	De Oliveira et al. (2019)
	Fish	Gonadosomatic index and GST	Carvalho Neta et al. (2013; 2014)
		Histopathological lesions	Castro et al. (2018; 2019); Sousa et al. (2013)
		Gonadosomatic index, GST, and reproduction	Carvalho Neta et al. (2017)
		Metal (Al, Fe, Cd, Cu, Hg, and Ni), CAT and GST	Nunes et al. (2020)
		DNA damage (Micronuclei and Comet Assay)	Almeida et al. (2021)
		Condition factor, gonadosomatic index, and histological alterations	Ribeiro et al. (2023)
Bacanga	Water	Coliforms	Liao et al. (1984)
		Aeromonas	Martins et al. (2009)
		Eutrophication	Sá et al. (2021); Silva et al. (2014)
		Eutrophication, surfactants, and phenolics	Duarte dos Santos et al. (2016)
		Metal (Hg and Pb)	Cantanhêde et al. (2016)
		PPCPs (albendazole and ketoconazole) and caffeine	Chaves et al. (2020)
		Metal (Cd, Pb e Zn)	Cabral et al. (2020)
		Estrogens	De Sousa et al. (2020)
	Sediment	Metal (Cd, Cr, Cu, Ni, Pb, and Zn)	Da Silva et al. (2015)
		PPCPs (albendazol and cetoconazol)	Chaves et al. (2020)
	Mussel and oyster	Metal (Cu and Zn)	De Carvalho et al. (2020); Ibañes Rojas et al. (2007)
	Mussel and fish	Coliforms	Nascimento et al. (2001)
	Fish	Micronuclei and gills lesions	Cantanhêde et al. (2016)
		Coliforms	Marreira et al. (2017)
		Micronuclei	De Sousa et al. (2020); Tchaicka et al. (2018)

PCB = Polychlorinated biphenyl. PPCPs = Pharmaceuticals and personal care products. DMSA = N′-dimethyl-N-phenyl-sulfamide. DCPMU = 1-(3,4-dichlorophenyl)-3-methylurea. CAT = Catalase activity. GST = Glutathione S-transferase activity.

Interestingly, a large industrial plant for Al production is operated by the Aluminum Company of America (ALCOA) in the influence area of the Itaqui Port complex. Spatial differences in genotoxic effects were also reported for hemocytes of the *C. virginica* oyster in Lavaca Bay (Texas, USA) and, coincidently, the more intense toxic effects observed in hemocytes of *C. virginica* were also found to be near an industrial plant (Bissett Jr. et al., 2009). In this case, a chlorine–alkali processing unit also operated by ALCOA from the late 1960s to the early 1970s and discharged Hg into Lavaca Bay. Additionally, coal tar processing has contaminated other areas around facilities with polycyclic aromatic hydrocarbons (PAHs). Lavaca Bay is an estuary of the Matagorda Bay System and is located along the central Gulf Coast of Texas, which has been under a long process of environmental remediation since 1998. In fact, it has been considered a severe threat to the marine ecosystem (USEPA, 2005). Although there are some similarities between the Itaqui port complex and the Lavaca Bay complex, comparisons must be done with precaution, considering the different contamination histories and types of contaminants, as well as the potential seasonal and hydrodynamic effects on the dispersion and fate of chemical contaminants in these two areas (USEPA, 2025).

Oxidative stress-induced genotoxicity

Our results revealed significant differences in the degree of genotoxicity among the sampling sites according to the comet assay method (Figure 2). As previously mentioned, the difference between the total DNA damage quantified by the conventional and modified FPG comet assays represents the amount of damage caused by oxidative stress, since the conventional assay detects strand breaks and alkali-labile sites but does not distinguish the underlying mechanisms responsible for such damage (Azqueta et al., 2013; Evans et al., 1995; Gielazyn et al., 2003). Therefore, the results indicate that the water samples from the Itaqui Port area have a significantly greater contribution of oxidative stress-inducing contaminants than those from the Bacanga Lagoon. These findings agree with previous reports on studies using biomarkers of oxidative stress (GST activity and DNA damage) in the catfish *Sciades herzbergii* from the Itaqui Port area (Almeida et al. 2021; Carvalho-Neta and Abreu-Silva, 2013). In this context, there is a marked difference in the chemical contamination profiles of water samples from Itaqui Port and Bacanga Lagoon, in which a wider variety of both inorganic and organic contaminants is observed in water samples from Itaqui Port than in those from Bacanga Lagoon. This could be explained not only by the different hydrodynamics described above for these two sampling sites but also by the different point sources associated with the chemical contamination reported for the Bacanga Lagoon and the Itaqui Port area. These environmental factors lead not only to differences in the quantity of contaminants accumulated but also to differences in the classes of contaminants at the two sampling sites.

The chemical contamination reported in the Itaqui Port area is more representative of industrial activities, whereas the Bacanga Lagoon seems to be more associated with domestic and urban sewage (Table 2). Indeed, chemical contamination of water samples from the Itaqui Port area is reportedly associated with the presence of organic compounds (benzene, diuron, irgarol, phenol, tributyltin, and PCB), as well as metals (Al, B, Cd, Cr, Fe, Hg, Pb, and Zn), and nonmetal (Cl and P) elements. In turn, water samples from the Bacanga Lagoon are reported to also be contaminated with some metals (Cd, Hg, Pb, and Zn) but also with other different types of organic contaminants (albendazole, ketoconazole, phenolics, caffeine, estrogens, and surfactants). Therefore, the different profiles of the classes of contaminants at the two sampling sites could explain the differential responses observed with the two comet assay methods (conventional and FPG modified).

Regarding the organic contaminants in water samples from the Itaqui Port area, several studies have demonstrated the association of oxidative stress with exposure to the biocides irgarol (Downs and Downs, 2007; Mohr et al., 2008), diuron (Felício et al., 2018), phenolic compounds (Hiraku and Kawanishi, 1996; Roche and Bogé, 2000; Varadarajan and Philip, 2016), tributyltins (Dash and Rahman, 2023; Tang et al., 2021; Zhang et al., 2017), and PCBs (Ferreira et al., 2005; Liu et al., 2020; Valavanidis et al., 2006; Winston, 1991). Additionally, metallic ions, such as Al, Cd, Cr, Cu, Hg, Ni, Pb, and Zn, which have already been detected and quantified in the Itaqui Port area, are known to induce oxidative stress in aquatic animals (Hellou et al., 2012; Lee et al. 2019; Lushchak, 2011; Lynn et al., 1997; Rahman and Singh, 2019; Sevcikova et al., 2018; Valavanidis et al., 2006), including hemocytes of the mussel Mytilus edulis (Emmanouil et al., 2007).

As reported for Lavaca Bay (Bissett Jr. et al., 2009), our results show that biomarkers of genotoxicity via oyster hemocytes are reliable tools for evaluating spatial differences in the quality of water samples from the São Marcos Estuarine Complex (Maranhão Gulf), where Itaqui Port and Bacanga Lagoon are located. However, using the modified FPG comet assay, we could better characterize the genotoxic effects of oxidative stress-inducing contaminants among the sampling sites (Figure 2). The addition of FPG to the alkaline comet assay protocol enables the detection of oxidative damage that is not identified in the conventional version, as lesions in nitrogenous bases, such as 8-oxoguanine and other oxidized purines, are converted into single-strand breaks (Azqueta et al., 2013; Gajski et al., 2019). Oxidative stress associated with inorganic and organic contaminants is induced by the depletion of key cellular antioxidants and increased generation of reactive oxygen species (ROS) (Ercal et al., 2001). In this case, free radicals, such as hydroxyl (HO^-), superoxide anion (O_2^-), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2), are oxidants of macromolecules, including DNA (Manduzio et al. 2005, Phaniendra et al., 2015). DNA oxidation also occurs through reactions with singlet oxygen ($^1O_2$) and hydrated electrons [e-(aq)] in smaller proportions (Berra et al. 2006). Therefore, the oxidative stress induced by the exposure of aquatic animals to chemical contaminants can lead to changes in cell morphology and, depending on the exposure conditions, can cause damage that may trigger cell cycle modulation, carcinogenesis, or apoptosis (Manduzio et al., 2005; Reyes-Becerril et al., 2019). Notably, such effects can be detected with greater sensitivity with the modified FPG comet assay than with the conventional assay (Cant et al., 2023; Kienzler et al., 2012). Indeed, DNA adducts caused by oxidative stress are evidenced by the application of the FPG enzyme, which results in an increase in comet tail length in nucleoids subjected to electrophoresis (Dušinská and Collins, 1996). Therefore, the total damage index (TDI) observed in the hemocyte's DNA mainly represents single-strand breaks caused by repair incisions (SSBe) (Kienzler et al., 2012). At this point, note that the greater frequency of anomalies in embryos/larvae and DNA damage in hemocytes of the oyster *C. rhizophorae* are in complete agreement with the fact that free radicals that are harmful to cellular chromatin can form guanine adducts that can block DNA transcription, which indicates a strong relationship with mutagenesis (Berra et al., 2006; Dizdaroglu, 2012).

CONCLUSIONS

Our results confirm that the mangrove oyster *C. rhizophorae* is an adequate and suitable test organism for use in teratogenic and genotoxicity assays. In fact, it is sensitive to contaminants in water samples from different estuaries of Maranhão Island on the Brazilian equatorial margin. Importantly, water samples from the metropolitan estuary of the Bacanga River showed greater acute toxicity to oyster embryos and genetic damage, as assessed by the conventional comet assay, which is likely associated with the hydrological characteristics and the significant contributions of several pollution sources reported in literature. For the Itaqui Port area, genotoxicity was confirmed by the modified FPG comet assay, which better highlighted the effects related to oxidative stress-inducing contaminants. Overall, the modified comet assay in hemocytes of the mangrove oyster *C. rhizophorae* was more sensitive for detecting the genotoxic effects of pollutants in the estuarine environments of Maranhão Island than the conventional method.

Although some potential limitations or factors could influence the results (e.g., genetic variability of oysters, extreme environmental conditions, and seasonal variations in hydrodynamics and pollutant dispersion), our study highlights the greater reliability and robustness of the modified FPG comet assay for use in future studies evaluating and monitoring water quality in estuarine environments using the oyster *C. rhizophorae* as a promising bioindicator. Furthermore, the genotoxic effects, which were measured via embryo/larva anomalies and DNA damage to the hemocytes of adult oysters, highlight the need for continuous evaluation and monitoring of the spatial and temporal fluctuations in the level of contamination of water samples from the different estuarine environments of the Maranhão Gulf. Future monitoring and restoration studies should focus on the major inorganic and organic contaminants in these environments, especially those leading to oxidative stress-induced genotoxic and mutagenic effects. Additionally, our results indicate the urgent need to implement efficient effluent control strategies. Mitigation and restoration of the quality of estuarine environments in the Maranhão Gulf would certainly benefit biodiversity conservation, fisheries, and human health at the Brazilian equatorial margin.

Data availability statement

Data will be available upon request.

Supplementary materials

There is no supplementary material available for this article.

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Author Contributions

C.L.R.J.: Investigation, Methodology, Software, Formal Analysis, Writing – original draft.

L.F.C.C.: Supervision, Investigation, Writing – review & editing.

M.B.M.: Software, Writing – original draft.

A.B.: Project Administration, Funding Acquisition, Writing – review & editing.

R.L.S.: Conceptualization, Supervision, Resources, Project Administration, Funding Acquisition, Writing – review & editing.

Conflicts of interest

The authors declare no conflicts of interest.

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