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RESEARCH ARTICLE

Assessment of the Genotoxic Hazard of Estuarine Sediments Using an Integrative Approach With LacZ Plasmid-Based Transgenic Mice

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ABSTRACT

Under the influence of multiple anthropogenic pressures, from industrial to agricultural activities, estuaries have long been regarded as particularly sensitive ecosystems to contamination. The present study aimed at investigating the genotoxic potential of a contaminated sediment sample from an urban and industrial area of the Sado Estuary, by combining the analysis of multiple endpoints in the *LacZ* plasmid-based transgenic mouse model exposed for 28 days to contaminated estuarine sediment extracts through drinking water. The DNA and chromosome damaging effects were monitored in peripheral blood at 7-day intervals using the standard and enzyme-modified Comet assay, as well as the micronucleus assays in peripheral blood cells. After euthanasia, DNA damage was analyzed in several mouse tissues, and *LacZ* mutant frequencies were determined in the liver. Livers were also surveyed for histopathological analysis. A time-dependent increase in micronuclei frequency was seen at all tested doses, in spite of no induction of DNA damage in any organ or mutation induction in the liver of exposed mice. The liver from mice exposed to sediment extracts did not reveal major alterations besides evidence of inflammation. Overall, the integration of the endpoints analyzed in the mice is suggestive of potential chronic, rather than acute, adverse effects in vivo, and points to the need for further research in the resident human population in the area. This experimental design can be used to assess the genotoxicity of complex environmental mixtures, understand how they work, and reduce costs and resources while speeding up data collection and interpretation.

1 | Introduction

Estuarine ecosystems have been recognized as particularly sensitive to anthropogenic pressures, in large part due to limited self-renewal capability. Usually targeted by diverse human industrial activities, estuaries may become reservoirs of a wide variety of pollutants, including mutagenic and carcinogenic substances, particularly when considering

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the interactions with sediments (Chen and White 2004; Ohe et al. 2004). Since exposure to these contaminants occurs for the local biota and human populations, concerns are raised as to their potentially harmful effects to the ecosystem as well as the potential development of environment-associated diseases in humans, either through direct contact with water or sediments (e.g., fishing, agricultural or recreational activities) or through the consumption of local potentially contaminated products. Generally, epidemiological studies in occupational and environmental settings address human exposure and its association to cancer development (Hubaux et al. 2012; Marant Micallef et al. 2018), but these tend to overlook the realistic impact of toxicant mixtures and the mechanisms responsible for their carcinogenicity (Pope 2000). Despite efforts to develop bioassays to determine toxicity and responses to contamination following exposure to sediment-bound aquatic contaminants, including in the study area (e.g., Yang et al. 2010; Costa et al. 2012; Caeiro et al. 2017; Baines et al. 2021), determining cause–effect relationships between aquatic sediment contamination and human health risk remains challenging. In fact, in the context of the assessment and management of estuarine water quality, while several well-established linesofevidence are already being proposed and described (Dagnino et al. 2008; Chapman et al. 2013) there is still a lack of consensus on strategies for determining risk to human health. Hence, a bridge between ecological and human risk assessment is still missing regarding the genotoxic/mutagenic effects of aquatic sediment contaminant mixtures.

Generally, the assessment of the potential hazard of environmental contaminants to humans through the extrapolation of in vitro data in mammalian cells is not straightforward (see for instance Higley et al. 2012; Costa et al. 2014; Pinto et al. 2014; Martins et al. 2024) since cellular systems do not account for the complexity of an organism, including, for example, toxicokinetics and immune responses. In addition, biological effects at the target tissues are known to be greatly influenced by bioavailability and also by the response to genotoxic stress, depending on factors such as cell proliferative rate, metabolic and DNA repair capacities (Louro and Silva 2010). In light of this, in vivo models continue to represent essential tools when investigating the hazardous effects of potentially mutagenic or carcinogenic substances, with studies in mammalian animal models allowing inferences on potential human health risk in the same way as aquatic animal models reflect ecological risk. Thus, to further analyze the potential genotoxic hazard of environmental sediment-bound contaminants in vivo, the present study aimed to investigate the genotoxic potential of one contaminated estuarine sediment sample in a mammalian organism: the *LacZ* plasmid-based transgenic mouse (Gossen et al. 1995; Louro et al. 2014a). This mouse model was selected as it allows assessment of several endpoints in distinct tissues of the same animal, making it a valuable in vivo model for investigating both genotoxic and mutagenic effects and repair mechanisms after exposure to both chemical (Verhofstad et al. 2011; Louro et al. 2002, 2008, 2010) and physical agents (Louro et al. 2014b). Moreover, compared to other transgenic rodent models based on bacteriophage lambda shuttle vectors, this system is the only one that allows the detection and identification of large deletions, providing an accurate quantification of mutant frequency. Furthermore, this model allows the establishment of a multi-endpoint experimental design, reducing the number of animals under experimentation, in compliance with the 3Rs agenda of the EU to Replace, Reduce, and Refine animal experimentation for scientific purposes in the EU recommendations (European Union 2010), and enabling a global analysis of the impact of the exposure on the organism's genetic material.

The Sado Estuary, a large estuarine basin located in SW Portugal, has been reported as an interesting study area where contamination intertwines with ecological and human health. Organic and inorganic pollutants from various anthropogenic or natural sources can become sorbed to particulate matter and sink in sediments, particularly if these have higher loads of finer particles and organic carbon, as is the case of the Northern area of the Estuary, which is also the area where most heavy industry is located (Caeiro et al. 2005; Almeida et al. 2024). Indeed, seemingly impacted by diffuse sources of pollution, high sediment contamination in this area has already been confirmed (Caeiro et al. 2005, 2009, 2017), mostly consisting of complex mixtures of organic and inorganic compounds capable of inducing adverse effects both in local organisms and a human cell line. These studies conducted in the estuary were based on integrated assessment; proven adverse effects on species with commercial value may be used to witness the environmental quality of the estuarine ecosystem and the potential human health risk of the sediments and the development of chronic-degenerative diseases that have a latency period before being symptomatic. In fact, these previous studies showed that sediment samples collected in fishing areas of the Sado Estuary contained a high concentration of metals (namely Cu and Pb) and polycyclic aromatic hydrocarbons (PAHs) with high molecular weight, especially 5- and 6-ring, like benzo[a]pyrene (BaP), benzo[b]fluoranthene, dibenzo[a,h]anthracene, indeno[1,2,3-cd]pyrene and benzo[ghi]perylene (Carreira et al. 2013). The sources of those contaminants are mainly the heavy industry units like Multiuse Terminal and Containership Terminal (naval repairing), pulp and paper industry, chemical and phytopharmaceutical products industry, yeast factory, urban settlements, including urban and fishing ports at the Northern part of the estuary and also the train line (leaks from traction oil used in the brakes) in the southern Alcácer channel (Almeida et al. 2024; Caeiro et al. 2005). These contaminants were shown to induce concentration-dependent cytotoxicity, genotoxic damage and DNA repair inhibition in vitro, when tested in the form of extracts in a human liver cell line (Pinto et al. 2014a, 2015; Costa et al. 2014), and genotoxicity in vivo (although only reported in flatfish) either in laboratory or in situ (Costa et al. 2008, 2011b). Previous work showed that HepG2 cells exposed to crude contaminant extracts obtained from estuarine sediments displayed increased DNA strand breaks and higher micronuclei induction, thus revealing significant clastogenic/aneugenic events (Pinto et al. 2014a), whereas gene mutation was not studied in vitro. Although human cell line-derived studies are crucial to reflect the effects of human exposure, as the observed outcomes are recognized key events in cancer development (Møller 2006; Bonassi et al. 2011) and potential indicators of human health risk, confirmation in complex organisms is necessary. For instance, the toxicity studies performed in local aquatic species,

namely in clam (Carreira et al. 2013; Costa et al. 2013), cuttlefish (Rodrigo et al. 2013) and flatfish (Costa et al. 2009, 2011a, 2012; Gonçalves et al. 2013), revealed that the biomarker responses (examined in biochemical and histopathological endpoints) were consistent with global contamination patterns within the estuary, contributing to both ecological and human health risk assessment. In this context, this study explores the genotoxic effects of contaminated sediments in a mouse model, in order to allow the comparison between in vivo and in vitro data, critical in evaluating the potential for future human health risk assessment of environmentally contaminated areas.

2 | Material and Methods

2.1 | Sediment Sample Contamination and Extract Preparation

The sediment samples were collected from the northern area of the Sado Estuary affected by contaminants from urban and industrial activities. These were overall considered the most contaminated by both metals and organic toxicants such as PAHs and PCBs (Carreira et al. 2013). Sediments were hitherto characterized for contamination and standard physico-chemical parameters (as described in Carreira et al. 2013). Concentrations of toxicants in sediments are detailed in Supplementary Table S1. Total surveyed contaminants concentrations, given *per* gram of sediment dry weight, were 678.83 ± 23.63 μg/g for metallic elements (highlighted by elevated concentrations of Cr, Cu, Pb, and Zn); 1076.98 ± 183.09 ng/g of PAHs (highlighted by elevated concentrations of BaP, fluoranthene, pyrene, phenanthrene, benzo[b]fluoranthene, indeno[1,2,3-cd]pyrene and benzo[ghi]perylene); 2.48 ± 0.42 ng/g of organochlorine pesticides; and 5.37 ± 0.91 ng/g of polychlorinated biphenyls. The extract used for the mice bioassays was prepared from sediment samples that, upon collection, were immediately protected from light and frozen to uphold physico-chemical integrity until toxicant extraction, as described by Pinto et al. (2014a). Briefly, organic and inorganic contaminants were extracted with a mixture of dichloromethane: methanol (2:1) from 1800 g of dry pulverized sediment under agitation. After solvent evaporation, contaminants were resuspended in 180 mL of dimethyl sulfoxide (DMSO), with a final sediment: extract proportion of 10 g sediment dry weight *per* mL of DMSO.

2.2 | Exposure Assay

C57Bl/6 mice harboring the pUR288 plasmid (containing the *LacZ* reporter gene) inserted in head-to-tail sequences homozygously on both chromosomes 3 and 4 were used in this study (Louro et al. 2008, 2010; Gossen et al. 1995). *LacZ* mice were bred, maintained, treated, and euthanized according to European Union directives (European Union 2010) at the National Institute of Health Doutor Ricardo Jorge (INSA), Portugal, during March 2013. All mice were housed in their individual cages until sacrifice, with body weight (bw) measured weekly and any signs of discomfort or disease monitored daily. Individually housed three-month-old *LacZ* mice were randomly assigned to three groups (five or six mice per group): a vehicle control and two treatment groups. The mice bw were determined at the start of the experiment, showing an average of 22.6 ± 1.6 g. Room temperature was kept at 21°C–23°C, light/dark cycle at 12 h/12 h, and lab chow and water were supplied *ad libitum*. Sediment extract and vehicle control were administered orally through drinking water as a surrogate of the expected main route of exposure by the human population in the area, who mostly relies on local produce, well water, and fishery for subsistence (Caeiro et al. 2017). Mice were exposed for a total of 28 days, with drinking water renewed twice weekly (with fresh extract or vehicle control, in water). A daily average of 10 mL water consumption was calculated during the exposure period, leading to doses of 73.7 (Dose 1) and 147.3 g (Dose 2) of sediment equivalent/Kg bw/day (SEQ/Kg bw/day). Doses were selected to ensure sublethal exposure to the toxicants listed in Supplementary Table S1. We set Dose 1 at 1/10 of the lowest effect (oral) dose of B[a]P, the best-studied mutagen and carcinogen in the list, that is, about 100 mg/kg, which at the end of the 21 days of exposure, would add to ≈10 mg internalized B[a]P per kg. Dose 1 is thus the sediment-equivalent dose necessary to expose each mouse to a potential total of 10 mg B[a]P per kg. Dose 2 was set as the double, that is, 1/5 of the lowest effect (oral) dose of B[a]P. The control group was exposed to vehicle only, that is, 3.33% of DMSO in water. No significant differences in bw values, either within or between each tested group, were observed during the exposure period.

2.3 | Tissue Collection and Sample Preparation

Before the exposure and once a week throughout the exposure period (total of five times) 5 μL of peripheral blood were collected from the tail vein for the comet and micronucleus assays in blood cells (the number of sample collections were limited in order to minimize animal stress from repeated manipulations and subsequent potential disturbances of erythropoiesis). Mice were euthanized by direct cervical dislocation (without exsanguination) at the end of the 28-day exposure period, to allow the fixation of mutations in the *LacZ* transgene (Louro et al. 2002, 2008; OECD TG 488, 2011; OECD TG 488 2022). Several tissues were immediately collected for histological analysis, comet and gene mutation assays. Approximately one-third of the liver, spleen, and kidney were washed in phosphate buffer (PBS) and processed immediately for the comet assay. The remaining parts of the liver were washed in cold PBS, flash-frozen, and stored at −80°C until DNA extraction for mutation analysis or immediately fixed before histopathological processing, as described below.

2.4 | Peripheral Blood Micronucleus Assay

Blood smears were freshly prepared on acridine orange (1 mg/mL) coated slides (Hayashi et al. 1990, 2000), two slides *per* animal. Two thousand reticulocytes (RETs) were blindly scored to determine the frequency of micronucleated reticulocytes (MNRET) *per* animal (OECD TG474 2016) under fluorescence microscopy (Axioplan 2 Imaging, Carl Zeiss Microscopy, Göttingen, Germany). The percentage of RETs was also

examined as a measure of toxicity through the analysis of the number of RETs *per* 2000 cells per animal (1000 for each slide).

2.5 | Comet Assay

The Comet assay was performed in blood cells at several timepoints during the exposure and in liver, kidney, and spleen cells collected at the end of the exposure. Cell suspensions were prepared from liver, kidney, and spleen by mincing the tissues in PBS containing 20 mM EDTA and 10% DMSO (pH 7.5). The alkaline Comet assay was carried out essentially according to Azqueta and Collins (2013). In brief, 5–10 μL of the cell suspensions (i.e., from peripheral blood, liver, spleen, and kidney) was embedded in 75 μL of 1% low melting point agarose (Sigma-Aldrich) and three replicate gels were prepared onto three separate precoated microscope slides (1% normal melting point agarose) and allowed to solidify at 4°C. Three slides (using blood or organ cell suspensions from a non-exposed animal) were used as positive controls by submerging them in a H_2O_2 solution at 5 mM for 15 min, followed by PBS washing for 2 min at 4°C. All slides were then immersed in cold lysis solution (2.5 M NaCl, 100 mM $Na_2EDTA.2H_2O$, 10 mM Tris, 10% DMSO and 1% Triton X-100, pH 10) for at least 1 h at 4°C. Slides were then washed (3 × 5 min) with enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) and the gels were treated with 50 μL of the enzyme formamidopyrimidine-DNA glycosylase (FPG) and Endonuclease III (ENDO III) – both diluted in enzyme buffer – or enzyme buffer only for 30 min (FPG) or 45 min (ENDO III) at 37°C. To allow DNA unwinding and to enhance the expression of alkali–labile sites, slides were placed in cold electrophoresis buffer (300 mM NaOH, 1 mM $Na_2EDTA.2H_2O$, pH ≥ 13) for 30 min, and electrophoresis was run at 0.8 V/cm, 300 mA, at 4°C for 40 min. Following neutralization (0.4 M Tris–HCl buffer, pH 7.5), the gels were stained with ethidium bromide (0.125 μg/μL). One hundred randomly selected nucleoids were scored *per* tissue for each mouse under each tested condition, using a Zeiss Axioplan2 Imaging fluorescence microscope equipped with a high-resolution camera (Carl Zeiss, Göttingen, Germany) and the Comet Imager 2.2 software (MetaSystems, Altlussheim, Germany). The median of the percentage of DNA in the nucleoids tail (% DNA in tail) was used as a measure of total DNA strand breakage *per* animal, and the mean value and standard deviation were determined for each group.

2.6 | LacZ Mutant Frequency

Genomic DNA extraction from homogenized livers and *LacZ*-plasmid rescue was conducted at the National Institute of Health Doutor Ricardo Jorge (INSA) as described previously (Louro et al. 2008). Briefly, 50 μg of genomic DNA were incubated with 40 units of the restriction enzyme *HindIII* (New England Biolabs, Ipswich, MA, USA) and magnetic beads (Dynabeads M450 sheep anti-mouse IgG; Invitrogen, Waltham, MA, USA) pre-coated with LacZ/LacI fusion protein (prepared in house as described Louro et al. 2014a). The supernatant fluid was discarded and the plasmid DNA was eluted using isopropyl-β-D-galactopyranoside (Stratagene, La Jolla, CA, USA), followed by heat inactivation of *HindIII* at 65°C. Plasmids were circularized with T4 DNA ligase (Invitrogen) at room temperature and ethanol precipitated. The purified plasmid DNA was electroporated into highly competent *Escherichia coli* C (ΔLacZ, galE-) previously prepared according to Gossen et al. (1992). Electroporation was performed according to the manufacturer (Biorad, Hercules, CA, USA) and cells were immediately allowed to recover in Super Optimal Broth (SOB) medium for 30 min at 37°C. To determine the number of mutant colonies, 99.9% of the transformed *E. coli* were plated on a selective top agar plate containing the lactose analog phenyl-β-D-galactoside (Sigma-Aldrich). The remaining *E. coli* were plated on non-selective medium containing 5-bromo-4-chlor o-3-indolyl-β-D-galactoside (Stratagene) to determine the total number of colonies. The mutant frequency (MF) for each organ was calculated as the ratio of the number of mutant colonies over the total number of colonies (multiplied by the dilution factor). A positive control group was included, consisting of animals exposed to N-ethyl-N-Nitrosurea (ENU in DMSO, 120 mg/Kg bw; CAS# 759–73-9, Sigma-Aldrich, St. Louis, MO, USA) by intraperitoneal (i.p.) injection, performed 2 months before for a previous study (Louro et al. 2014a).

2.7 | Histopathology

Liver portions fixed in Bouin's solution for 24 h at room temperature were washed, dehydrated in a progressive series of ethanol, and embedded in paraplast. Sections (5 μm thick) were obtained with a Jung RM2035 rotary microtome (Leica microsystems) and stained with hematoxylin and eosin, as described in Louro et al. (2014a). The qualitative histopathological assessment was done with a DMLB model microscope equipped with a DFC480 camera (Leica Microsystems, Darmstadt, Germany).

2.8 | Statistical Analysis

All statistics were computed with IBM SPSS Statistics version 20.0, with the threshold of significance set at $p < 0.05$. Comparisons pertaining to Comet assay data were performed using the non-parametric Mann–Whitney *U*-test. The median of the % of DNA in tail was calculated for each animal, and the values obtained for each treatment group were compared to those of the vehicle control group. Comparisons between treatments with and without FPG or ENDO III were also performed. The $\chi 2$ test was used to compare the mean frequency of micronucleated RETs (MNRETs) between each treatment group and the vehicle control. The mean mutant frequencies were compared between the treatment groups and the vehicle control group with Student's *t*-test.

3 | Results

The Comet assay in mouse peripheral blood at either one, two, three, or four weeks of exposure did not show significant induction of DNA damage after the exposure to any dose of the extract (Table 1), as compared to the control group, even when using the endonuclease-modified version of the assay (i.e., with FPG or ENDO III). Still, within each treatment group and timepoint, a slight increase in DNA damage was observed for control and treated animals when using both endonucleases as compared to the conventional Comet assay (Table 1), suggesting residual

oxidative DNA damage. Regarding the Comet assay performed in tissues from mice exposed after 28 days to the sediment extract, no relevant increases in DNA damage were observed either in liver, kidney, or spleen cells, comparatively to the control group (Table 2). Of note, slight increases in the level of DNA damage were detected in the kidney, spleen, and liver of exposed animals following FPG treatment, suggesting the presence of mild oxidative DNA damage.

TABLE 1 | Results of the Comet assay in mouse peripheral blood collected during the 28-day exposure period to the extract sample.

		Median % of DNA in tail (Mean ± SD)		
Timepoint	**Exposure group**	**Buffer**	**FPG**	**ENDO**
t_0	Control	2.05 ± 0.64	7.58 ± 2.40*	4.52 ± 1.35*
	Dose 1	2.68 ± 0.85	7.02 ± 1.26*	5.86 ± 1.37*
	Dose 2	2.33 ± 0.28	8.23 ± 3.08*	5.12 ± 1.06*
t_1	Control	5.36 ± 0.78	5.91 ± 0.29	5.15 ± 2.10
	Dose 1	7.07 ± 4.31	6.62 ± 0.86	5.31 ± 1.65
	Dose 2	5.22 ± 1.31	5.41 ± 2.12	4.68 ± 1.55
t_2	Control	3.82 ± 0.55	6.76 ± 0.84*	5.87 ± 1.56*
	Dose 1	4.27 ± 0.62	6.57 ± 1.38*	6.76 ± 1.86*
	Dose 2	3.19 ± 0.52	5.25 ± 1.50*	7.32 ± 2.06*
t_3	Control	3.52 ± 0.77	5.12 ± 0.92*	4.63 ± 1.29
	Dose 1	2.79 ± 0.85	6.07 ± 2.23*	4.79 ± 1.33*
	Dose 2	3.61 ± 0.93	4.62 ± 0.87	5.28 ± 2.71
t_4	Control	3.40 ± 0.46	4.45 ± 1.40	4.93 ± 1.68
	Dose 1	3.95 ± 0.55	4.69 ± 0.52*	5.98 ± 2.68
	Dose 2	4.15 ± 1.32	3.93 ± 1.32	5.27 ± 1.47
H_2O_2	5 mM	38.07 ± 10.90	49.05 ± 0.44*	46.73 ± 8.86

*Statistically significant differences between modified Comet assay compared to buffer exposed slides, in the same exposure group. SD—standard deviation; t_0—before exposure; t_1–7 days of exposure; t_2–14 days of exposure; t_3–21 days week of exposure; t_4–28 days of the exposure; Dose 1–73.7 g SEQ/Kg bw/day; Dose 2–147.3 g SEQ/Kg bw/day; Control—3.33% DMSO in water. SEQ- sediment equivalent.

TABLE 2 | Results of the Comet assay in mouse tissues collected at the end of the 28-day exposure period to the extract sample.

		Median % of DNA in tail (Mean ± SD)		
Organ	**Exposure group**	**Buffer**	**FPG**	**ENDO**
Kidney	Control	5.95 ± 0.88	5.72 ± 0.83	5.84 ± 1.29
	Dose 1	5.25 ± 0.67	7.28 ± 0.92*	5.42 ± 1.69
	Dose 2	5.40 ± 0.82	6.86 ± 1.15	6.65 ± 0.77*
Spleen	Control	4.70 ± 1.54	6.25 ± 0.59	4.61 ± 0.58
	Dose 1	4.36 ± 1.20	5.42 ± 0.84	4.77 ± 1.19
	Dose 2	3.83 ± 0.90	5.42 ± 0.82*	4.53 ± 1.03
Liver	Control	3.35 ± 0.79	4.85 ± 1.00*	3.69 ± 0.69
	Dose 1	3.03 ± 0.29	5.25 ± 1.22*	3.92 ± 1.00
	Dose 2	3.79 ± 0.64	5.02 ± 0.69	3.83 ± 0.50
Spleen	H_2O_2 5 mM	33.03 ± 17.99	41.60 ± 14.76	49.46 ± 4.64
Liver	H_2O_2 5 mM	6.89 ± 0.60	8.60 ± 1.59	7.06 ± 1.12

*Statistical significant differences between treatment with and without FPG or ENDO III, in the same exposure group. SD—standard deviation; t_0—before exposure; t_1–7 days of exposure; t_2–14 days of exposure; t_3–21 days week of exposure; t_4–28 days of the exposure; Dose 1–73.7 g SEQ/Kg bw/day; Dose 2–147.3 g SEQ/Kg bw/day; Control—3.33% DMSO in water. SEQ- sediment equivalent.

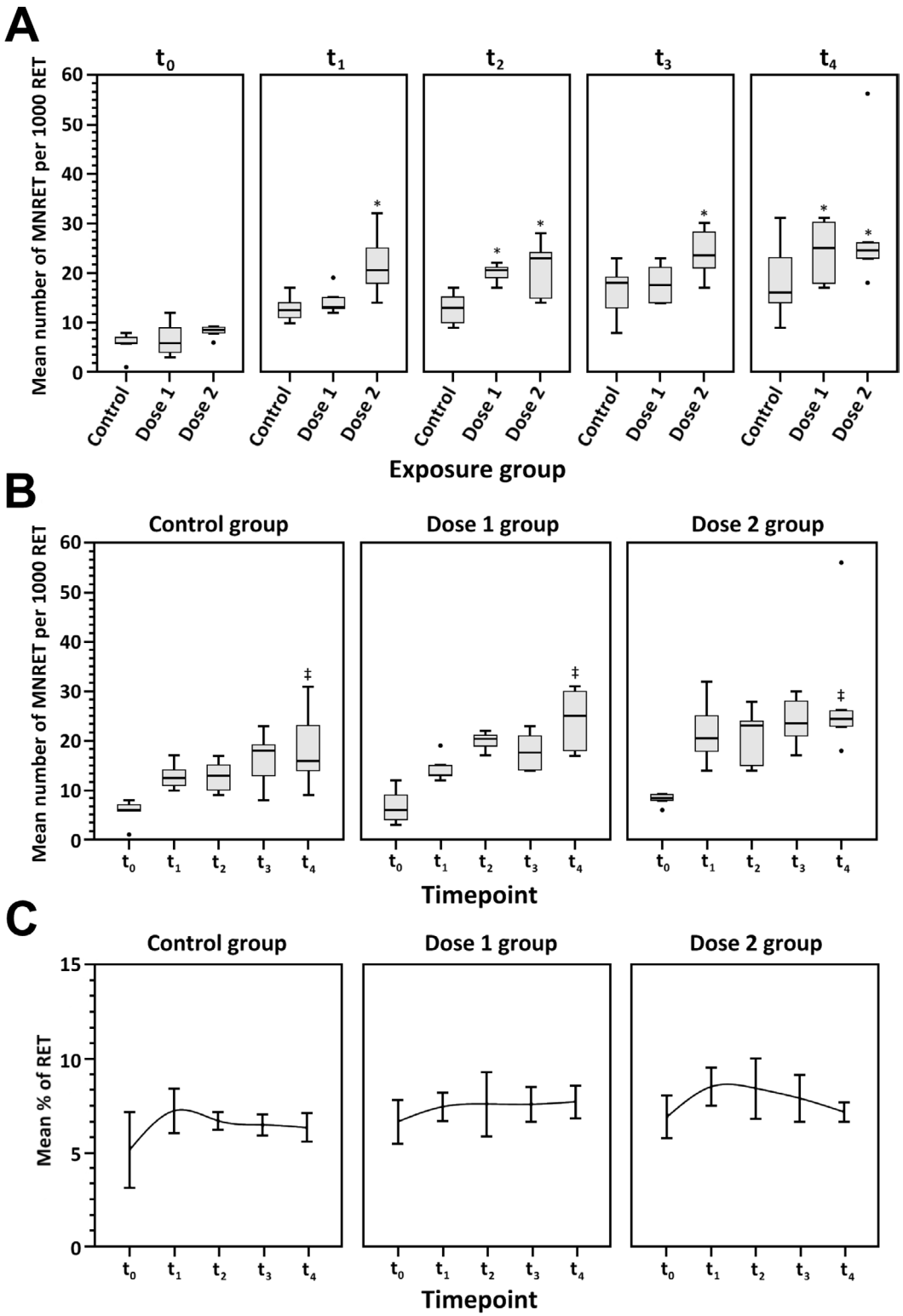

FIGURE 1 | Results for the micronucleus assay in peripheral blood cells from mice exposed to the sediments extract and vehicle controls. (A) Distribution of the mean frequency of MNRET in peripheral blood by tested concentration at each timepoint. *Statistically significant differences over the vehicle control group at the same timepoint. (B) Distribution of the mean frequency of MNRET in peripheral blood for each exposure group along the 28-day exposure period. ‡ Statistically significant difference between timepoints t_1 and t_4 for the same exposure group. (C) Percentage of reticulocytes (RET) in peripheral blood samples collected from mice during the 28-day exposure period to extract sample. t_0—before exposure; t_1–7 days of exposure; t_2–14 days of exposure; t_3–21 days of exposure; t_4–28 days of exposure. Dose 1–73.7 g SEQ/Kg bw/day; Dose 2–147.3 g SEQ/Kg bw/day; Control—3.33% DMSO in water. SEQ —sediment equivalent.

TABLE 3 | *LacZ* mutant frequency in mouse liver collected at the end of the 28-day exposure period to the extract sample.

Exposure group (N)	Total CFU $\times 10^3$ (Mean ± SD)	Mutant CFU (Mean ± SD)	MF $\times 10^{-5}$ (Mean ± SD)
Control (5)	341.40 ± 140.35	34.00 ± 19.33	9.55 ± 2.10
Dose 1 (5)	321.20 ± 59.34	35.20 ± 8.81	10.95 ± 2.10
Dose 2 (6)	296.33 ± 87.49	20.00 ± 6.39	6.92 ± 2.17
Positive control (6)*	317.40 ± 115.29	152.80 ± 87.44	51.02 ± 9.39‡

*Refers to N-ethyl-N-Nitrosurea (ENU), 100 mg/Kg by intraperitoneal injection, previously presented in (Louro et al. 2014a).
‡Significantly different from vehicle controls ($p = 0.012$, *t*-Student's test). CFU—colony forming units; MF—mutant frequency; SD—standard deviation; Dose 1–73.7 g SEQ/Kg bw/day; Dose 2–147.3 g SEQ/Kg bw/day; Control—3.33% DMSO in water. SEQ- sediment equivalent.

In respect to the micronucleus assay in peripheral blood from mice (Figure 1), at each week an increase in MNRET was observed, seemingly dependent on the dose and time of exposure (Figure 1A).

The MNRET frequency from mice exposed to the highest dose was significantly different from that of unexposed mice after one, two, three, and 4 weeks of treatment ($p < 0.001$, $p = 0.001$, $p = 0.005$ and $p < 0.001$, respectively). MNRET frequency also increased with the time of exposure, with the highest value obtained at the end of treatment for all groups (Figure 1B), which was significantly different for all groups from the first week (t_1) of exposure ($p = 0.019$, < 0.001 and $= 0.018$, for control, dose1 and dose 2 groups, respectively). Still, while for dose 1 results suggest that chromosomal damage gradually increased along the exposure period, for dose 2 the observed damage appears more stable in distribution within the exposed group after week one, although increasing in mean along the exposure. Even though a slight but non-significant increase in MNRET frequency was observed for the negative control group at the end of the exposure, all observed values within the control group were still within the range of those obtained in previous studies using the same mouse model (Louro and Silva 2011; Louro et al. 2010). No myelotoxicity was observed following exposure to either dose or any of the timepoints (Figure 1C).

From the *LacZ* mutation assay, it could be inferred that exposure to either dose of extracts did not influence significantly the MF in the liver, as compared to control mice (Table 3).

In fact, the group of mice exposed to the highest dose presented slightly lower MF than both those treated with the lowest dose ($p = 0.020$) and unexposed control mice, although no statistically significant difference was noted as compared to negative controls. Livers of mice exposed to sediment extracts did not reveal major alterations to the expected structure of the liver, compared to controls (Figure 2A). However, in mice exposed to the highest dose, foci of altered hepatocytes characterized by highly vesicular cytoplasms were common (Figure 2B). The foci did not hold necrotic or apoptotic cells. Additionally, foci of inflammatory cells, frequently accompanied by localized hyperemia, were occasionally observed in mice exposed to extracts regardless of dose (Figure 2C).

4 | Discussion

Our preceding work showed that crude extracts of sediment samples collected from fishing areas of the Sado Estuary were able to induce genotoxicity in HepG2 human hepatoma cells () (Pinto et al. 2014a; Costa et al. 2014); therefore, accordant with

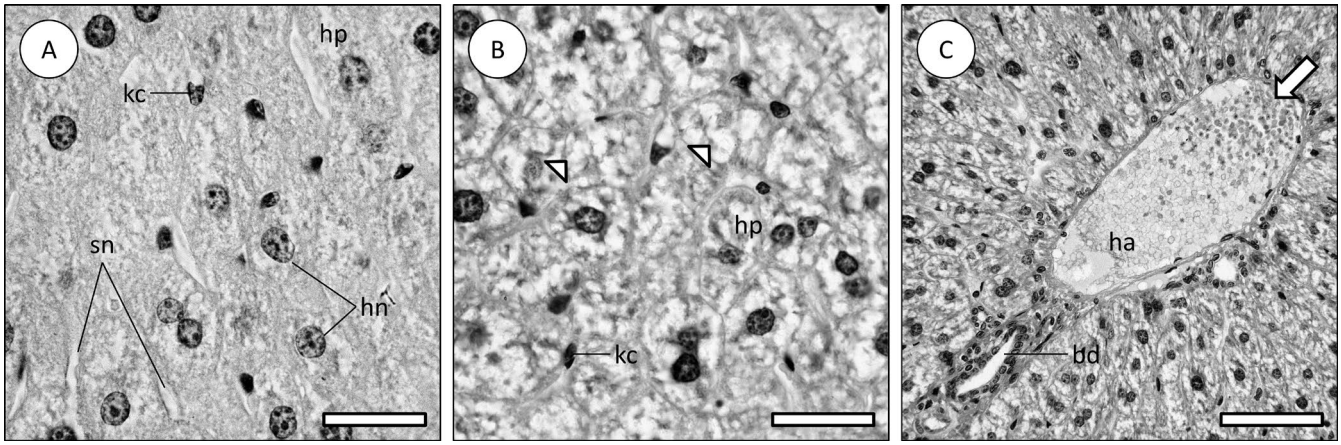

FIGURE 2 | Liver sections of mice (Bouin's, H&E). (A) Section across the liver from a control mouse, exhibiting normal morphology of mammalian hepatocytes (hp) with regular-sized shaped nuclei (hn), arranged in trabeculae lined by many sinusoids (sn) where Kupffer cells (kc) may be found. Scale bar: 25 μm). (B) Same magnification of a section from the liver of a mouse exposed to the highest dose, for comparison. Note the highly vesicular cytoplasm (arrowheads) of hepatocytes (hp). Kupffer cells (kc) can be observed in sinusoids as well, but in this case without evident activation of the reticuloendothelial system. Scale bar: 25 μm). (C) Liver of a mouse subjected to the highest dose of sediment extract, revealing an agglomerate of inflammatory cells (arrow) within a branch of the hepatic portal vein (ha). bd) indicates a bile duct. Scale bar: 70 μm.

earlier findings from a variant of the micronucleus test in fish exposed in situ and in the laboratory to whole-sediment samples (Costa et al. 2008, 2011b). The current study revealed detectable induction of micronuclei in mouse blood as early as 1 week after exposure to the highest dose and 2 weeks after exposure to the lowest dose. These results suggest that contaminants in the extract were reaching the bone marrow, where they induced chromosomal damage during the erythrocyte maturation process. Metallic toxicants present in tested sediments, such as Cd, As, Pb, and Cu, are known to be strong inductors of micronuclei and other nuclear abnormalities (NAs) resulting from chromosomal clastogenic and aneugenic events in fish and other animals (Ayllon and Garcia-Vazquez 2000; Çavaş et al. 2005; Gebel 2001; Palus et al. 2003). Nevertheless, the genotoxic mechanisms associated with the formation of NAs as a result of the exposure to metals and metalloids are still not fully understood and show significant variation. Additionally, past research suggests that potential interactions between organic and metallic contaminants contributing to chromosomal anomalies should not be discarded (Vakharia et al. 2001; Lewińska et al. 2007). Considering the relatively high levels of metals, arsenic, and PAHs in tested sediments and globally in sediments from the Sado estuary ((Caeiro et al. 2005, 2009; Costa et al. 2012; Supplementary Table S1), it can be suggested that the individual and mixed toxicants contribute for ENAs). In fact, among metal concentrations, the concentrations of Cu and the highly toxic metal Pb were ≈2-fold higher in the sample of this study than in other previously surveyed areas of the estuary (Carreira et al. 2013). In addition, higher molecular weight PAHs especially 5- and 6-ring, among which are included known genotoxicants and promutagens like B[a]P, were found in the tested sediment extract. Genotoxicity at the chromosomal level after exposure to metals has already been described in numerous studies, for example, for arsenic (Gebel 2001; Fracasso et al. 2002; Rossman 2003), lead (Bonacker et al. 2005; Celik et al. 2005), cadmium, and copper (Palus et al. 2003; Çavaş et al. 2005). These results are in accordance with those obtained when testing the same extract in HepG2 cells, in which micronuclei induction was found to increase in a dose-responsive manner, which further sustains genotoxic effects (Pinto et al. 2014a).

Since the liver is the main organ for xenobiotic metabolism, ROS or activated metabolites could be produced and disseminated along the blood stream, giving rise to a systemic observable effect. As such, the increase in the micronucleus frequency in contaminant-exposed mice might be indicative of potential adverse effects for human health as well and indicates the need for risk assessment in human populations resident in the affected areas. In fact, the micronucleus test, which is included in the standard battery of genotoxicity assays, is a reliable test for the analysis of chromosome damage [i.e., for the detection of clastogenic and aneugenic events (OECD, 2023)], and a relevant predictor for carcinogenesis, as an association between micronucleus frequencies and cancer risk has already been reported for humans (Bonassi et al. 2011).

Conversely, no induction of significant DNA or oxidative DNA damage occurred, as only residual values were observed either in blood cells or in mouse tissues (kidney, liver and spleen) after exposure to the sediment extract (Tables 1 and 2). The different results between the micronucleus and the Comet assay may rely on the different nature of the lesions detected. The latter allows the identification of DNA lesions, namely single- or double-strand breaks or alkali-labile sites. Such primary lesions usually arise soon after exposure to genotoxic agents and may eventually be repaired by the cells' DNA repair machinery, while micronuclei persist. In fact, other authors have described discrepant results in these two assays (e.g., Hartmann et al. 2001; Valentin-Severin et al. 2003). They suggest that micronuclei may be generated through clastogenic or aneugenic events, and the Comet assay has been shown to produce negative results after exposure to aneugenic agents. Lower sensitivity has indeed been associated with the Comet assay since, for instance, some compounds such as multifunctional alkylating agents induce DNA–DNA and/or DNA–protein crosslinks, which retard DNA electrophoretic migration (Hartmann et al. 2001). Our previous results also suggested that the micronucleus assay might be more sensitive in the detection of genotoxic effects when testing the same complex mixtures in vitro (Pinto et al. 2014a, 2014b). In fact, it was observed that the micronucleus test could be more sensitive than the Comet assay to reveal the genotoxicity of mixtures of toxicants, given that the concentrations of two sediment extracts required to produce a significant increase in the micronucleus frequency over control were lower than those required to significantly raise the level of DNA lesions. The mechanisms to which target cells resort to uphold genome repair, as well as the time elapsed between exposure and analysis, will also contribute to the differences in sensitivity between the micronucleus and Comet assays, due to their particular endpoints. Since it was shown that this extract could in fact induce DNA and oxidative DNA damage in HepG2 after a 48 h exposure (Pinto et al. 2014a), it can now be hypothesized that if DNA damage was occurring, it could have been controlled or even reversed by the first weekly sampling. It must also be noted that although a "long-term" continuous exposure was intended, animals only consume water at intervals, which may have favored the induction of repairable low levels of DNA damage throughout the assay, despite the interference of metals in DNA repair mechanisms, as noted, for example, by (Whiteside et al. 2010).

Although gene mutation tests can be used to confirm and to increase the weight of evidence when inconclusive data has been generated in vitro or in vivo (ICH 2012), in this study mutation analysis in the liver of exposed mice did not exhibit any mutagenic effect under the experimental conditions used (Table 3). Given the probable sensitivity of hepatic cells to the sediment extracts that can be expected based on in vitro analysis (Pinto et al. 2014a), two hypotheses can be foreseen. Firstly, due to the oral administration of the extract, it is possible that the mutagenic agents present reached the liver but were then readily metabolized and eliminated before mutagenic effects could occur. However, this hypothesis is not entirely supported by the findings due to the systemic genotoxic effect of the extract in mouse blood cells. In comparison, the presence of inflammation and lesions in the digestive system of bivalves (Costa et al. 2013) and chromosomal damage in blood cells of fish (Costa et al. 2011b) exposed to similar sediments suggests that the contaminants are able to reach multiple visceral organs while also inducing a systemic effect noticeable in peripheral cells. Even though in the present study histopathological findings are not consistent with acute effects of exposure to sediment extracts and did not reveal alterations that can be specifically pin-pointed to the toxicant

mixture, the changes in hepatocyte ultrastructure (Figure 2), namely vesiculation and microvesiculation, indicate that liver function related to carbohydrate metabolism and storage was compromised, therefore revealing an important chronic effect. Also, no histopathological evidence was found for the occurrence of immune function impairment. Oppositely, the focal occurrence of inflammation indicates that the aforementioned effects may trigger an innate immune response, even though no major lesions were observed. In addition, studies focused on the toxicity of metal mixtures in mice, for instance, have shown that liver alterations can occur after 30 days of exposure to low concentrations of metals (Cobbina et al. 2015a, 2015b). Therefore, an impact on the livers of mice exposed to sediment extract shows an effect in this organ, discouraging this first interpretation of the absence of mutagenic effects in the liver. A second hypothesis is that the mutagenic agents present in the sediment extract reached the liver but the exposure period was not long enough for the fixation of mutations. The observations indicate that mutations tend to accumulate with each treatment. Therefore, a repeated-dose regimen consisting of daily treatments over a 28-day period is generally regarded as sufficient for enabling the accumulation of mutations caused by weak mutagens and for ensuring adequate exposure time to detect mutations in organs and concomitant tissues with a slow cell proliferation rate (OECD 2022). In spite of the null outcomes from the mutation detection assay, one cannot exclude the hypothesis of a secondary mechanism of in vivo mutagenicity, involving inflammation and ROS generation, leading to DNA damage that could become apparent after longer time periods. In fact, residual oxidative DNA damage was observed in the Comet assay, that although too low to be considered significant damage, may suggest induced stress. Nevertheless, the results of the genotoxicity investigation of the sediment extracts in the present study are compatible with an absence of mutagenic effects in mouse concomitant with a clastogenic effect in blood cells. Although the *LacZ* plasmid-based transgenic mouse model has been shown to be sensitive to large deletions (Louro et al. 2002), it is possible that it might not detect large clastogenic or aneugenic events. Furthermore, previous studies in aquatic vertebrates and invertebrates revealed that histopathological alterations in the liver or digestive gland (respectively), although non-specific to contaminants, may occur chronically in animals collected locally or exposed in the laboratory to sediments from the Sado estuary (see for instance Costa et al. 2009, 2011a, 2013; Gonçalves et al. 2013). In accordance with the current work, these findings report, rather than acute effects of exposure (in most cases), chronic alteration to hepatic metabolism including changes in the storage of glycogen and lipids. These effects are less severe than those found in fish exposed to whole sediments (from multiple sites of the estuary) in previous experiments with the same duration (see Costa et al. 2009, 2011a). However, organisms, route of exposure, and dosage are difficult to compare directly.

To summarize, this study highlighted genotoxic damage following direct exposure to bulk contaminants in a crude extract of sediments collected from an impacted urban and industrial estuarine area (specific sources earlier detailed). The integrated approach of this study assembled a battery of genotoxicity tests as well as histopathology, covering a range of endpoints in the *LacZ* plasmid-based transgenic mouse, has the important advantage of allowing a reduction in the number of animals necessary for extensive genotoxicity analysis. For instance, the use of peripheral blood cells avoids the animals sacrifice and enables repeated sampling from the same animal, facilitating its integration in multi-endpoint toxicity studies (Louro et al. 2014a). In addition, the natural environment where organisms are exposed, including humans, the pollutants are present in a mixture where synergistic effects occur, so for risk assessment, direct exposure essays are more appropriate to be used.

5 | Conclusions

The present study aimed at investigating the genotoxic potential of a contaminated sediment sample from a local fishing area of the Sado Estuary, a human-impacted estuary, by combining the analysis of multiple endpoints in the *LacZ* plasmid-based transgenic mouse model. Although several endpoints revealed negative results, the positive induction of micronuclei in blood suggests a systemic genotoxic potential of the present contaminants. Considering the close proximity of the model to humans, along with previous in vitro data, the observations suggest that the exposure to these contaminated estuarine sediments in the environment, or through occupational or recreational activities, might pose a risk to human health. The observation of adverse effects in different research models is now supported by these findings in mice and is indicative of moderate risk, both ecological and to human health, that needs to be further investigated. The experimental design herein presented and the analysis of three distinct and complementary endpoints can successfully be applied to conservatively assess the genotoxicity of complex mixtures of contaminants present in the environment and to provide additional information about their mode of action, while reducing costs and the quantity of test compound needed and expediting data generation and interpretation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.