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Potentially harmful elements in house dust from Estarreja, Portugal: characterization and genotoxicity of the bioaccessible fraction

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Abstract:	Due to their behavioral characteristics, young children are vulnerable to the ingestion of indoor dust, often contaminated with chemicals that are potentially harmful. Exposure to potentially harmful elements (PHEs) is currently exacerbated by their widespread use in several industrial, agricultural, domestic and technological applications. PHEs cause adverse health effects on immune and nervous systems, and can lead to cancer development via genotoxic mechanisms. The present study is an integrated approach that aims at assessing the genotoxicity of bioaccessible PHEs following ingestion of contaminated house dust. A multidisciplinary methodology associating chemical characterization of five house dust samples, extraction of the bioaccessible PHEs in gastric extracts by the Unified BARGE Method, determination of the bioaccessible fraction and in vitro genotoxicity of gastric extracts in adenocarcinoma gastric human (AGS) cells was developed. The five gastric extracts induced dose-dependent genotoxicity in AGS cells. Copper (bioaccessible concentration up to 111 mg/kg) was probably the prevalent PHE inducing primary DNA damage (up to 5.1-fold increase in tail DNA at 0.53 g/L of gastric extract). Lead (bioaccessible concentration up to 245 mg/kg) was the most prevalent

	PHE inducing chromosome-damaging effects (r = 0.55; p < 0.001 for micronucleated cells induction). The association of Principal Component Analysis and Spearman's correlations were decisive to understand the chromosome damaging properties of the bioaccessible PHEs in AGS cells. This methodology could be used on a larger scale study to provide useful information for science-based decision-making in regulatory policies, and a better estimation of human exposure and associated health risks.
Response to Reviewers:	Reviewer #2: This is a very interesting contribution combining geochemistry and genotoxicology and brings some new indications of effects caused by potential harmful elements (PHE) in urban/industrial dust. Given the fact that I am not a biologist, I admit that I am not able to fully evaluate sections devoted to genotoxicity. I have a number of comments specified below that need to be addressed in the revision. I think that the paper can be published after moderate revision. Specific comments: Abstract does not contain any results/data. Number of dust samples studied should be mentioned (in the third paragraph of the abstract authors say that "The five gastric extracts..."; thus numbers are important, they probably correspond to 5 samples of total set of 19 samples previously studied). According to the reviewer, major data were added to the abstract. In order to be more comprehensible, we specified the number of house dust samples (page 2, line 9). In order to provide an abstract of less than 250 words, numerous minor modifications were made in the abstract. Concentrations of PHEs should be mentioned. The main goal of our work was to develop an interdisciplinary study involving chemical and in vitro biological characterization of PHEs contained in house dust. By performing all the methods required for this purpose on only 5 house dust samples, PHEs concentrations cannot be considered as representative of PHEs content in house dust of Estarreja. Therefore, we did not add PHEs concentrations in the abstract. The major result, i.e. the role of Pb, which is highly developed in the paper (direct relationship to chromosome damage) is even not mentioned in the abstract. Although lead was already mentioned in the abstract, we added data related to the relationship between chromosome damage and lead as follow: “Lead (bioaccessible concentration up to 245 mg/kg) was the most prevalent PHE inducing chromosome-damaging effects (r = 0.55; p < 0.001 for micronucleated cells induction).” (page 2, lines 16-17). P3, para 2, L15: Aung et al. (2004) not given in the reference list. According to the reviewer remark, we added the missing reference in the reference list: “Aung, N.N., Yoshinaga, J., Takahashi, J., 2004. Exposure assessment of lead among Japanese children. Environmental Health and Preventive Medicine 9, 257–261. doi:10.1007/BF02898139”. (page 20, lines 1-2). P4, paras 3 & 4: I understand that some data on these dusts have already been published elsewhere, but at least some major results should be given here. Basic statistics of total and bioaccessible fractions in 19 studied dust samples could be reported in a table (min, max, median, mean, percentiles) or briefly mentioned in the text. The publication of Reis et al (2015) mainly focused on only 2 PHEs: copper and manganese that were quantified in house dust, in gastric extracts and in toenail clippings. Although a quantification of 53 chemicals contained in house dust was performed for this study, the publication of Reis et al (2015) did not report the corresponding data. We published for the first time these data for PHEs only. In order to avoid any confusion, we modified the manuscript as follow: “The relationships between biomarkers of exposure, levels of two PHEs (copper and manganese) in house dust and their bioaccessible fractions (BAF) were investigated in the Aveiro district (North of Portugal) (Reis et al., 2015).” (page 4, lines 19-21) As suggested by the reviewer, data on PHEs concentrations measured in the 19 houses are now provided as Table S2 in the Supplementary Material. Moreover, data on the oral bioaccessibility of the PHEs are now provided as Figure S2

	in the Supplementary Material. The manuscript was modified as follow: “The arithmetic means, geometric means and medians of all analyzed PHEs in the 5 studied dust samples were calculated. Table 1 shows values for the 16 PHEs under study and compares the PHEs concentrations with those reported in the literature (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001). Further data on PHEs concentrations in the full set of indoor dust samples collected from 19 private households in the city of Estarreja are provided in the form of supplementary material (Table S2).” (page 10, lines 14-19). “Oral bioaccessibility data for the set of 19 samples are presented in supplementary material (Figure S1), while Table 2 shows the results obtained for the subset of 5 samples used in this study.” (page 11, lines 11-13). Finally, we added a sentence to mention that the bioaccessible fractions data obtained in the 5 houses selected in this study and in the 19 houses studied in Reis et al. (2015) were very closed: “The overall assessment of the results (Table 2 and Fig. S2) shows that the trends in the oral bioaccessibility of the PHEs are the same in the selected subset and in the broader set of samples subjected to the UBM protocol.” (page 11, lines 20-22). Saying that "PHEs were characterized using ICP-MS..." should be deleted from this introduction part as the details are given later in the text. In the end of the Introduction section, we have just mentioned the name of each method used as our goal was to propose a new methodology linking chemical characterization, bioaccessibility and genotoxicity. Therefore, the name of the methods was not deleted. P5, chapter 2.2: The rationale for selecting 5 dust samples studied here should be better explained!!! According to the reviewer comment, the manuscript was modified as follow: “To carry out the present study on the genotoxic potential of the house dust bioaccessible fractions, 5 out of 19 houses dust sampled by Reis et al. (2015) were selected to be representative of the various sources of PHEs on Estarreja. Indeed, the 5 selected sites were far from each other (Fig.1) compared to the geographical distribution of the sampled sites of Reis et al. (2015).” (page 5, lines 20-23). P6, chapter 2.3.1: Give more details for certified reference materials. What are these GRX-1, GRX-6 and SAR-M materials? Give QC/QA data in the Supplement to help the reader to understand the accuracy of the analytical procedures. The United States Geological Survey (USGS) Geochemical Exploration Reference Materials chose GXR-1 to GXR-4 and GXR-6 as different soil and/or materials from various US areas, while SAR-M is a composite of contaminated sediment from the Animas River watershed in Colorado. These were considered to represent a wide range of total elemental concentrations in relevant matrices. Further QA/QC data are provided in the form of Table 1 of the supplementary material. The manuscript was modified as follow: “The Certified Reference Materials GRX-1, GRX-4, GRX-6 and SAR-M (United States Geological Survey) were selected to represent a wide range of total elemental concentrations. The blanks results were always below detection limits. In this study, we focused on the 16 following PHEs: Al, Zn, Cu, Pb, Mn, Ba, Ni, Cr, Sn, V, As, Co, Sb, Mo, Ga, and Cd. Values for precision (expressed as RSD %) are typically < 15 % for all elements. Further information on QA/QC is provided as supplementary material (Table S1).” (page 6, lines 8-13). P11 and 12: Why simple calculations of exposures were not performed for the major PHEs (e.g. calculating exposures to 60 mg dust per day and comparisons with tolerable daily intake limits) to have an idea about the extent of potential risk. As already mentioned, we consider that house dust PHEs concentrations generated from 5 sites cannot be used for assessing exposure levels in Estarreja. As indicated at

	the end of the Abstract, “This methodology could be used on a larger scale study to provide useful information for science-based decision-making in regulatory policies, and a better estimation of human exposure and associated health risks.” I am also convinced that generalities about "priority pollutants" (P12, para 1 on cadmium) have nothing to do in the Results section and should be moved to Discussion. According to the reviewer comment, the results section relative to cadmium was modified as follow: “The biogeochemistry of Cd, which is currently ranked seventh on the Priority List of Hazardous Substances by the Agency for Toxic Substances and Diseases Registry (ATSDR 2013), was also noteworthy.” (page 12, lines 13-15). We did not mentioned Cadmium in the discussion section as no significant effect was associated with this PHE in the present study. P17, para 3: I cannot judge, if these results aren't only speculations: there is a lot of "probably", based only on the statistical data treatment. Maybe reformulation of these major results could help. In the paragraph 3 on page 17, according to the reviewer suggestion, we have modified the sentences containing “probably” as follow: “Although gastric extracts containing numerous elements were assessed, Cu is thought to be the prevalent PHE inducing primary DNA damage, while Pb was the most prevalent PHE inducing chromosome-damaging effects.” (page 18, lines 10-12) P18, last para: It is great that these genotoxicological methods have finally been combined with geochemical in vitro testing. However, I think that the results cannot "provide information on PHEs sources" as the authors claim. According to the reviewer comment, we suppressed "provide information on PHEs sources". Further, the corresponding paragraph was extensively modified according to the following comment. Maybe also a financial outlook could be given here to have an idea, how accessible this method could be for researchers, who have been using only leaching in simulated body fluids so far. According to the reviewer comment, the accessibility of the methods has been mentioned as follow: “Further, the use of the UBM and of these two in vitro genotoxicity assays can be performed in few weeks and are rather cheap (about 1,000€ per site), compared to in vivo assays.” (page 19, lines 3-4). P19, para 2. This paragraph is containing repetitive information. Maybe rewriting could be suggested here. According to the reviewer comment, we modified the two last paragraphs of the Conclusion section in order to delete repetitive information, as follow: “The option of selecting house dust samples with PHEs concentrations in the range of the current EU soil screening values for potentially unacceptable risk, unraveled the potential of this methodology to provide further information that can be used for science-based decision-making in regulatory policies, such as deriving soil screening values that are currently lacking in Portugal. By developing this methodology in broader studies, encompassing larger areas and comprehensive datasets, we should be able to correlate house dust PHEs concentrations with the physical environment of the house, as well as with exterior anthropogenic contributions. Establishing links between site characteristics, PHEs concentrations and their speciation, bioaccessibility and genotoxicity is likely to provide an accurate characterization of sources, pathways and fate of environmental PHEs, enabling more effective assessment of human exposure and associated health risks.” (page 19, lines 8-24). Tables: Give numbers to 3 digits, i.e. 2755 mg/kg should be given as 2780 mg/kg. According to the reviewer comment, tables 1 and 2 were corrected and the numbers

	given with 3 digits. Corrections appear in bold in the tables. Reviewer #3: The paper is well written with thorough coverage of the background material the methods, statistical analysis and interpretation. I cannot comment on the genotoxicity study as it is outside of my area of expertise. Specific comments: Page 12 line 8 should be "Regardless of" According to the reviewer comment, we replaced “Regardless” by “Regardless of”. (page 12, line 21). Bottom of page 14 the opposite projection of Al-Cr relative to Cd-Cu is likely to be a consequence of the closed nature of elemental compositions (add up to 100%), i.e. the more Al there is the less Cd-Cu can be present. I would be careful about saying that Al has an inverse relationship with chromosome damage. According to the reviewer comment, the corresponding sentence was modified as follow: “However, the diametric opposite projection of Al-Cr relative to Cd-Cu; and Ni-Mo-Sn relative to Pb suggested that these elements were less abundant and, therefore, not involved in chromosome damage induction.” (page 15, lines 9-12). Page 15 third paragraph line 4 "Pb Thus" appears twice According to the reviewer comment, we removed “Thus Pb”, that was appearing twice. (page 15, line 28). Page 15 end of third paragraph should be "extent" not "extend" We agree with the reviewer, and replace “extend” by “extent” twice. (page 16, lines 4-5).

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Title

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The authors declare that they have no conflict of interest.

Abstract

Due to their behavioral characteristics, young children are ~~more~~ vulnerable to the ingestion of indoor dust, often contaminated with chemicals that are potentially harmful. Exposure to ~~these~~ potentially harmful elements (PHEs) is currently exacerbated by their widespread use in several industrial, agricultural, domestic and technological applications. PHEs cause adverse health effects on immune and nervous systems, and can lead to cancer development via genotoxic mechanisms.

~~Hence, t~~**T**he present ~~pilot~~ study is an integrated approach that aims at assessing the genotoxicity of bioaccessible PHEs following ~~incidental~~ ingestion of contaminated house dust. ~~We developed a~~**A** multidisciplinary methodology associating ~~the~~ chemical characterization of five house dust **samples**, ~~the~~ extraction of the bioaccessible PHEs in gastric extracts by the Unified BARGE Method, ~~the~~ determination of the bioaccessible fraction and ~~the~~ *in vitro* genotoxicity of gastric extracts in adenocarcinoma gastric human (AGS) cells **was developed**.

~~A cytotoxicity assay was performed to determined non-cytotoxic concentrations.~~ The five gastric extracts induced dose-dependent genotoxicity in AGS cells. Copper **(bioaccessible concentration up to 111 mg/kg)** was probably the prevalent PHE inducing primary DNA damage (**up to 5.1-fold increase in tail DNA at 0.53 g/l of gastric extract). L~~, while l~~ead **(bioaccessible concentration up to 245 mg/kg)** was the most prevalent PHE inducing chromosome-damaging effects **($r = 0.55$; $p < 0.001$ for micronucleated cells induction)**. The association of ~~two statistical methods (~~Principal Component Analysis and Spearman's correlations~~)~~ were decisive to understand the chromosome damaging properties of the bioaccessible PHEs in AGS cells. This methodology could be used on a larger scale study to provide **useful** information ~~that could be used~~ for science-based decision-making in regulatory policies, ~~as well as~~ **and** a better estimation of human exposure and associated health risks.

Keywords

House dust; Unified BARGE method; Bioaccessibility; Genotoxicity; Alkaline comet assay; Cytokinesis-block micronucleus assay.

1. Introduction

It has been reported that trace elements such as cobalt (Co), copper (Cu), chromium (Cr), molybdenum (Mo), manganese (Mn) and zinc (Zn) are essential nutrients required for various biochemical and physiological functions in the human body (WHO, 1996). For some, including Cr, Cu and Zn, there is a narrow range of concentrations between beneficial and adverse effects (Chang et al., 1996; Lee et al., 2012). Other trace elements such as aluminium (Al), antimony (Sb), arsenic (As), barium (Ba), cadmium (Cd), gallium (Ga), lead (Pb), nickel (Ni), thallium (Tl), tin (Sn) and vanadium (V) have no established biological functions and are considered as potentially harmful to humans. These potentially harmful elements (PHEs) contribute to genotoxicity involving reactive oxygen species and/or DNA repair inhibition, apoptosis, and cancer development in mammals (Anetor et al., 2007; De Boeck et al., 2003; Denys et al., 2012; Gebel, 1997; Lee et al., 2012).

Human exposure to environmental PHEs has risen dramatically as a result of an exponential increase of their use in several industrial, agricultural, domestic and technological applications (Tchounwou et al., 2012). Ingestion, inhalation and dermal absorption are the likely routes of exposure. Experimental lines of evidence suggest that, soil/dust ingestion is the major source of human exposure to environmental PHEs contaminants (Davis and Mirick, 2006; Reis et al., 2014). Furthermore, given the large amount of time people spend indoors, either at home, school or workplace, the potential health risks posed by contaminants in house dust raises additional concerns (Lin et al., 2015). Indeed, recent studies suggested that exposure to contaminated house dust may represent an increased human health risk compared to contaminated exterior soil (Ibanez et al., 2010; Niu et al., 2010). Ingestion of dust generally occurs through deliberate hand-to-mouth movements, or unintentionally by eating food that has dropped on the floor and particles that have adhered to skin (U.S. Environmental Protection Agency, 2008). Young children (< 6 years old), while exploring their environment, are particularly vulnerable to this exposure pathway due to frequent hand-to-mouth and object-to-mouth habits (Mohmand et al., 2015; Reis et al., 2015; Tulve et al., 2002). Characterization of PHEs house dust by several measures (concentration, load and loading rate) has been used to document the presence and influence of metal sources (Rasmussen et al., 2013). Further, in the particular case of lead, Aung et al (2004) have shown that house dust and soil ingestion can be the predominant routes of exposure among children.

Whilst the intake dose (the amount of a chemical ingested by an individual) is the most commonly used measure of exposure in toxicity studies, the absorbed dose is the one causing the most, if not all, adverse effects on human health. In addition to the determination of PHEs concentrations in dust, data relative to the PHEs bioavailability (fraction of an ingested chemical that is absorbed by the body) and/or bioaccessibility (fraction of the

contaminant that is released from its solid matrix in the gastrointestinal tract and is therefore available for absorption by the body) are key information in human health risk assessment. Several protocols have been developed for the ingestion pathway to assess the bioavailability of a PHE from its bioaccessibility (Oomen et al., 2002). The Bioaccessibility Research Group of Europe (BARGE) has developed an *in vitro* test, the Unified BARGE Method (UBM), which mimics the solubilisation process during digestion and thus allows estimating the bioaccessibility of PHEs in the solid-phase (Denys et al., 2009; Wragg et al., 2011). The UBM is considered to be representative of the physiological conditions in the human gastrointestinal (GI) tract (Wragg et al., 2011).

Previous studies investigating oral bioaccessibility of house dust mainly focused on organic compounds (i.e. polycyclic aromatic carbons, phthalates esters) obtained after organic extraction (Kang et al., 2013, 2011; Maertens et al., 2008; Pohren et al., 2012; Wang et al., 2013a, 2013b). They quantified the toxicity by evaluating the excess lifetime cancer risk and some assessed the *in vitro* cytotoxic and genotoxic potentials of organic extracts by using bacterial mutagenic assays such as the Ames test, SOS chromotest and Salmonella assay (Kang et al., 2013, 2011; Maertens et al., 2008; Pohren et al., 2012; Wang et al., 2013a, 2013b). Likewise, the toxicity of PHEs from house dust has been investigated using either the evaluation of excess lifetime cancer risk or cytotoxicity tests (Chen et al., 2014; Deschamps et al., 2013; Granero and Domingo, 2002; Kurt-Karakus, 2012). However, the ingested dose was used as the relevant measure to assess the health risk and the oral bioavailability of the PHEs was not estimated. Notably, as far as we know, no study was published focusing on the genotoxicity of PHEs in house dust taking into account their oral bioavailability.

The relationships between biomarkers of exposure, ~~**PHEs**~~ levels **of two PHEs (copper and manganese)** in house dust and their bioaccessible fractions (BAF) were investigated in the Aveiro district (North of Portugal) (Reis et al., 2015). Reis et al. (2015) evidenced that manganese levels in toenails was associated with house dust manganese contents and this association was supported by the bioaccessibility estimates. In this study, dust containing PHEs were characterized in 19 houses located at various distance from the chemical complex of Estarreja (Reis et al., 2015). Large variations in PHEs levels were found in the different sampled house dust.

The present pilot study is about an integrated approach that aims at assessing the genotoxicity of the BAF of PHEs considering that, the ingested dose following incidental ingestion of contaminated house dust, usually overestimates the absorbed dose. PHEs were characterized using ICP-MS on dust samples and on their BAF obtained by the UBM. The cytogenotoxic potentials of these extracts were evaluated on adherent human adenocarcinoma gastric stomach (AGS) cells using the WST-1 assay for cytotoxicity, the alkaline comet assay

for primary DNA lesions quantification, and the centromeric micronucleus assay for chromosome breakages and losses assessment. AGS cells were chosen as a relevant model to assess the toxicity of PHEs contained in gastric extracts. AGS cells were previously used to assess *in vitro* cytotoxicity and genotoxicity of drugs and nanoparticles that may be ingested by humans (Alam-Escamilla et al., 2015; Botelho et al., 2014).

It is a detailed interdisciplinary study, performed on 5 houses dust sampled in the study of Reis et al. (2015) and carried out for purposes of methodology development. Our study does not envisage achieving further information on the diagnosis, spreading or prevention of disease in the studied area.

2. Material and methods

2.1 Characterization of the study area

Estarreja is a small coastal city in Portugal close to the broad "Ria de Aveiro" lagoon, which is a protected "Natura 2000 net" area (Fig.1). The coastal plain around the lagoon supports an intensive and diversified agriculture but, since 1950 the region is also known for the large industrial chemical complex of Estarreja (CCE). For many years, several of these industries dumped their solid residues (including ashes and dust with As, Pb, Cu, Zn) in an improvised park inside the CCE, albeit the proximity of the urban center of Estarreja is only 1 km away. Awareness on the pollution of the "Ria de Aveiro" and on its ecosystems started in the 80s (Patinha et al., 2014). Since then, technological upgrades associated to remediation measures implemented by the industries have strongly reduced the environmental burden of the area. However, by its dimension and configuration, the CCE is still regarded as the major pollutant of the Aveiro district.

2.2 Dust sampling

To carry out the present study on the genotoxic potential of the house dust bioaccessible fractions, 5 out of 19 houses dust sampled by Reis et al. (2015) were selected **to be representative of the various sources of PHEs on Estarreja. Indeed, the 5 selected sites were far from each other (Fig.1) compared to the geographical distribution of the sampled sites of Reis et al. (2015).** ~~**based on the PHEs levels heterogeneity (Fig. 1).**~~

The floor surfaces were not cleaned for a period of 7 days before the scheduled house dust sampling. Composite house dust samples were collected from the floor and carpets using the high-volume small surface (HVS3) vacuum sampler, which is designed to capture 99% of the particles ≥ 0.5 μm (Reis et al., 2015). Collected dust samples were oven-dried at 40°C for 24 h, sieved through a 150 μm nylon mesh and stored in polyethylene containers at ambient temperature. The particle size < 150 μm was chosen to the exploratory study because it

represents the particle size range likely to adhere to hands and thus, to be ingested following hand-to-mouth movements (Gron, C. and Andersen, L., 2003; U.S. Environmental Protection Agency, 2000).

2.3 Chemical analysis

2.3.1 House dust PHEs total concentrations

Dust samples were digested in Aqua Regia at 90 °C in a microprocessor controlled digestion block for 2 h, and the analysis of 16 PHEs was carried out by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) at the ACTLABS Analytical Laboratory, Canada. Each sample batch prepared for ICP-MS analysis included dust samples, duplicates and blanks. The Certified Reference Materials GRX-1, **GRX-4,** GRX-6 and SAR-M (United States Geological Survey) were selected to represent a wide range of total elemental concentrations. The blanks results were always below detection limits. In this study, we focused on the 16 following PHEs: Al, Zn, Cu, Pb, Mn, Ba, Ni, Cr, Sn, V, As, Co, Sb, Mo, Ga, and Cd. Values for precision (expressed as RSD %) are **typically** < ~~10~~**15** % for all elements**. Further information on QA/QC is provided as supplementary material (Table S1)**.

2.3.2 Bioaccessibility of the PHEs in gastric extracts

The bioaccessibility of the PHEs was determined by subjecting a small quantity of sample to the UBM. The UBM protocol consists of two parallel sequential extractions simulating the chemical processes occurring in the mouth, stomach and intestine compartments using synthetic digestive solutions according to physiological transit times, which allow us to obtain both gastric and gastrointestinal extractions (Denys et al., 2009). The methodology has been validated against a juvenile swine model for As, Cd and Pb in soils, but not yet for less common PHEs (Denys et al., 2012). Several studies comparing *in vivo* bioavailability and *in vitro* bioaccessibility have shown that the *in vitro* gastric phase was generally closer to *in vivo* PHEs concentrations (Wragg et al., 2011). Indeed, likely precipitation reactions attributed to basic pH solutions used in the gastrointestinal phase of the UBM protocol usually decrease the bioaccessibility estimates, which may underestimate the bioavailability of the PHEs.

The chemicals used in this experiment were provided by Sigma-Aldrich (Sintra, Portugal). For the gastric extraction, an aliquot of 0.6 g dust was extracted by 9 ml of simulated saliva fluid (manually shaken) and 13.5 ml of simulated gastric fluid. After adjusting the pH to 1.2 ± 0.05, the mixture was rotated at 37°C for 1 h on an end-over-end shaker. After checking that the pH value was between 1.2 et 1.5, the solution was centrifuged at

4500 rpm for 15 min, then the supernatant was removed and stored at < 4°C for further analysis (Wragg et al., 2011).

The BAF corresponds to the ratio of PHE concentration in the gastric extract to total PHE concentration in dust, and is expressed as percentage (Oomen et al., 2002):

$$\text{BAF } \% = \frac{\text{PHE concentration in gastric extract}}{\text{PHE concentration in house dust}} \times 100$$

The gastric extracts were analyzed by ICP-MS at CEREGE, France. Duplicate samples, the BGS 102 bioaccessibility reference material and blanks were extracted with every batch of UBM bioaccessibility extractions, for quality control purposes. The blanks always returned results that were below the detection limit.

2.4 Genotoxicity assays

2.4.1 Reagents

4'-6-diamidino-2-phenylindole (DAPI), Goat anti-human Alexa Fluor® 488, Trypsin 0.25% EDTA, Fetal Bovine Serum (FBS), and PBS Dulbecco's were obtained from Life technologies (Saint Aubin, France). Paraformaldehyde (PAF) 4% PBS was from EMS (Hatfield, PA, USA). Bovine Serum Albumin (BSA) fraction V was purchased from Eurobio (Courtaboeuf, France). Vectashield was provided from Vector Laboratories (CA, USA). Human anti-kinetochore antibodies (CREST) were obtained from Laboratory of Immunology at the Hôpital de la Conception (Marseille, France). The other reagents were from Sigma-Aldrich (Lyon, France).

2.4.2 Cells culture and cell viability assay

Adherent human adenocarcinoma gastric stomach (AGS) cells were maintained in DMEM supplemented with 4.5 g/l glucose, 2 mM L-glutamine, 10% FBS, and 1% penicillin and streptomycin, at 37°C under 5% of CO_2. Cell Line Service (Eppelheim, Germany) provided all these reagents.

Cytotoxicity testing was performed by a colorimetric test, the WST-1 assay, to analyze the number of viable AGS cells by the cleavage of tetrazolium salts added to the culture medium. The choice of the concentration range to be tested was defined to approach the physiological gastric concentration of PHEs following dust ingestion by young children. As the ingestion rate of house dust by young children has been set by the INVS at 60 mg/d and the gastric volume of young children is about 500 ml, the PHEs physiological gastric concentration is estimated at 0.12 g/l (INVS, Institut de Veille Sanitaire, 2012).

AGS cells, grown in a 96-well plate at a density of 10^5 cells/well, were incubated with gastric extracts at 0.067 – 0.13 – 0.27 – 0.53 g/l, and with the relative controls for 2 h and 24 h before adding the WST-1 reagent. After 2 h incubation period, the formazan dye was quantified with a scanning multi-well spectrophotometer microplate reader (Multiskan, Thermo Scientific; Waltham, MA, USA) using a 450 nm emission filter. Three independent experiments were performed for each extract.

2.4.2 Alkaline comet assay

The alkaline comet assay was performed to evaluate primary DNA damage (Collins et al., 1993; Tice et al., 2000). Cells were seeded at a concentration of 2.5 x 10^4 cells/well in a 6-well plate. 24 h after the seeding, cells were exposed for 2 h to increasing concentrations of gastric extracts (0.067 – 0.13 – 0.27 – 0.53 g/l) and vehicle control (2% gastric blank solution in DMEM), then trypsinized and pelleted. After dropping a layer of 0.8% NMP agarose in PBS onto SuperFrost® Microscope slides (Thermo Scientific; Braunschweig, Germany) pre-coated with 1.6% NMP agarose, the cellular pellets were resuspended in 1% LMP agarose and spotted onto the slides. Positive control was performed at this step by exposing AGS cells to 125 µM H_2O_2 for 5 min at 4°C. After cellular lysis (90 min at 4°C, in the dark) by using a freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 300 mM NaOH, 10 mM Tris, pH 10 supplemented with 10% DMSO and 1% Triton X-100), slides were immersed, for 20 min at 4°C in the dark, in a denaturation solution (300 mM NaOH and 1 mM EDTA). Electrophoresis was carried out at 27 V and 300 mA for 20 min at 4°C. Then the slides were rinsed with neutralization buffer (4 mM Tris, pH 7.5) and finally dehydrated few seconds in methanol.

To evaluate DNA damage (% tail DNA), the slides were stained with propidium iodide and analyzed using a fluorescence microscope BX 60 (Olympus; Rungis, France) equipped with an appropriate filter combination and a black-and-white camera (Andor Luca S) and driven by Komet 6.0 software (Andor™ Technology; Belfast, UK). For each experimental condition, 100 cells were analyzed, and the experiments were repeated tree times. Two slides were prepared per concentration and 50 cells per slide were analyzed. Results were expressed as mean of n = 2 slides × 3 independent experiments.

2.4.3 Cytokinesis-block micronucleus (CBMN) assay in combination with centromere labeling

The CBMN assay was performed according to the original method described by Fenech (Fenech, 2007), with minor modifications for AGS cells, while centromere labeling was performed as described by González (González et al., 2011). Cells were seeded at a concentration of 2.5 x 10^4 cells/chamber in LabTek™ 4-Chamber Slide™ (Nuc International; Villebon-sur-Yvette, France), and cultured under standard conditions (37°C, 5%

CO_2). After 24 h culture, slides were treated with increasing concentrations of gastric extracts (0.067 – 0.13 – 0.27 – 0.53 g/l), vehicle control (2% gastric blank solution in DMEM), and appropriate clastogenic and aneugenic positive controls (10 ng/ml mitomycin C (MMC) and 25 nM colchicine, respectively). At the end of the treatment (24 h), the medium was removed and replaced by fresh medium containing 3 µg/ml cytochalasin B to inhibit cell division after mitosis. Following 24 h incubation, the culture medium was further changed and, after 2 h incubation, cells were washed in PBS and fixed (4% PAF). To discriminate chromosome losses and breakages, cells were incubated with CREST serum (1:1000 in 1% BSA/PBS) for 30 min. Cells were washed in 0.5% Triton X-100/PBS and incubated with secondary antibody Alexa Fluor® 488 goat anti-human (1:200 in 1% BSA/PBS) for 1 h. Cells were then washed in 0.5% Triton X-100/PBS and incubated with a 0.06 µg/ml solution of phalloidin-TRITC (tetramethylrhodamine B isothiocyanate) for 30 min to stain the cytoplasm. After two washes in 0.5% Triton X-100/PBS, cells were incubated with a solution of 4'-6-diamidino-2-phenylindole (DAPI, 1:50000) for 10 min to stain the nuclei. Finally, the slides were mounted in Vectashield and stored in dark at 4°C until analysis.

Micronuclei (MN) contained in binucleated cells were scored using a fluorescence microscope BX 60 (Olympus; Rungis, France) equipped with a black-and-white camera (Andor Luca S), and appropriate filters for DAPI, phalloidin-TRITC, and Alexa Fluor® 488.

Before the analysis, we calculated the cytokinesis block proliferation index on 500 cells to provide the average number of cell divisions completed by the cells, as previously described (Benameur et al., 2011; Kirsch-Volders et al., 2003). Micronucleus analysis data are expressed as a frequency of binucleated micronucleated (BNMN) cells per 1000 binucleated cells; scoring criteria were in accordance with those previously described (Fenech, 2007). Taking advantage of the centromeric labeling, micronuclei containing whole chromosome(s) positively labeled (centromeric micronuclei cell, C+MN), and acentric chromosome fragments that are not stained due to the absence of centromere (acentromeric micronuclei, C−MN) were discriminated (González et al., 2011; Iarmarcovai et al., 2006; Mateuca et al., 2006; Natarajan et al., 1996). Two chambers were prepared per concentration, and micronuclei were counted for 1000 binucleated cells on each chamber. Results were expressed as mean of n = 2 slides × 3 independent experiments.

2.5 Statistical analysis

Results of cytotoxicity and genotoxicity were expressed as the mean value of three independent experiments ± standard deviation (SD). Data were submitted to statistical evaluation using one-way ANOVA. GraphPad Prism

6 (GraphPad Software, Inc., La Jolla, USA) software was used to perform statistical analysis and to draw the graphics. The significance was compared to negative control (vehicle: 2% gastric blank solution in DMEM). Values were considered statistically significant starting from $p < 0.05$.

Relationships between bioaccessible PHEs and chromosome damage were investigated using two complementary methods, principal component analysis (PCA) and Spearman's correlation. Principal component analysis (PCA) is a mathematical technique adapted to quantitative variables that transforms *n* possibly correlated variables into a (smaller) number of uncorrelated variables referred to as principal component (Jolliffe, 2014; Reis et al., 2015). Moreover, Spearman's correlation is adapted to analyze non-linear monotonic relationships (increasing or decreasing) between the observation's ranks of two defined characteristics (chemical composition and chromosome damage).

3. Results

3.1 House dust PHEs concentrations

House dust samples collected from five selected houses in Estarreja were analyzed by ICP-MS to determine elemental concentrations (Table 1). The arithmetic means, geometric means and medians of all analyzed PHEs in the **5** studied dust samples were calculated. Table 1 shows values for the 16 PHEs under study and compares the PHEs concentrations with those reported in the literature (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001). **Further data on PHEs concentrations in the full set of indoor dust samples collected from 19 private households in the city of Estarreja are provided in the form of supplementary material (Table S2).**

In all sites, the major element was Al, whose median concentration was 11100 mg/kg, followed by trace elements Zn, Cu, Pb, Mn, Ba, Ni, Cr, Sn, V, and As. Co, Sb, Mo, Ga, and Cd were the less abundant PHEs (median concentrations ≤ 5 mg/kg). Notably, Pb (78-1180 mg/kg; RSD = 119%), Zn (991-5210 mg/kg; RSD = 85%), Ga (0.9 – 4.0 mg/kg; RSD = 56%), Mo (1.2-4.7 mg/kg; RSD = 43%), Mn (98-296 mg/kg; RSD = 42%) and Cd (0.8-2.2 mg/kg; RSD = 42%) showed a wide range of values, reflecting the heterogeneity of house dust chemistry among the five sites under investigation.

The Estarreja median levels for Zn (1520 mg/kg) and Cu (339 mg/kg) were higher than the ones reported for other cities (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001). Pb median concentration in house dust from Estarreja was

equivalent to that reported for Canadian house dust (Rasmussen et al., 2001), while it was nearly ten times higher than the ones from Turkey and Brazil (Deschamps et al., 2013; Kurt-Karakus, 2012). Median levels of Ni, Cr, As and Co were similar to the majority of those reported in the literature (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001), whereas Ba and V were lower than the ones from China (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001)).

3.2 PHEs bioaccessibility in gastric extracts

In the present study, the PHE concentrations extracted in the gastric phase were considered to be the relevant measure of exposure. Since our study focuses on the fate of the PHEs in the gastric compartment following incidental ingestion of house dust, bioaccessible PHEs were determined by using the UBM to mimick *in vitro* interactions between the dust and the gastric solutions (Table 2). **Oral bioaccessibility data for the set of 19 samples are presented in supplementary material (Figure S1), while Table 2 shows the results obtained for the subset of 5 samples used in this study.**

The BAF of the 16 studied PHEs ranged from 4 to 85% (Table 2), showing that some PHEs were highly bioaccessible, and others almost not. PHEs can be classified into three groups according to their bioaccessibility: first, Zn and Cd were highly bioaccessible (85% and 80%, respectively); second, Pb, Mn, As, Ba, Co, Ni, Ga, and V were moderately bioaccessible (from 62% to 41%); and third Cu, Al, Cr, Sb, Sn, and Mo were almost not available to humans (less than 27%). Otherwise, Sn, Al, Ba, and Ga bioaccessibility were highly variable (RSD ranged from 64% to 54%) from one house to another, suggesting variations in physicochemical forms, thus different PHE states of speciation. **The overall assessment of the results (Table 2 and Fig. S2) shows that the trends in the oral bioaccessibility of the PHEs are the same in the selected subset and in the broader set of samples subjected to the UBM protocol.**

In the present study, the bioaccessibility of the 16 PHEs was considerably lower than the one reported for house dust in Turner and Simmonds (2006), which employed a simple surrogate (pepsin in 0.075 M HCl) for the human stomach. They found that the bioaccessibility of Al, Cu, Ni and Pb averaged between 60 and 100% (Turner and Simmonds, 2006), whereas our results were in accordance with those of Rasmussen (2004) reporting BAF of 30−40% for Ni and of 55−75% for Pb using 0.07 M HCl as extraction medium (Rasmussen, 2004). These discrepancies between the reported results are likely related to the different protocols used. Differences in

fluid formulation, pH values, residence time or type of shaking within the various *in vitro* tests can significantly affect the degree to which the PHEs are extracted from the soil/dust matrix (Oomen et al., 2002).

Results of the oral bioaccessibility testing indicate that a large proportion of the total Cu, Al, Cr, Sb, Sn, and Mo content in the house dust were not in bioaccessible forms, and are therefore unavailable to the residents via ingestion. Moreover, such low BAF values (< 30%) suggested that, in the dust, these PHEs were associated to resistant mineral phases that were hardly dissolved by the acidic solutions used in the gastric phase of the UBM protocol. In a previous study on these house dust that aimed at identifying likely environmental sources, Reis et al. (2015) suggested that Cu was probably related to waste materials of the CCE (Reis et al., 2015).

An overall analysis of the results obtained so far showed that the studied PHEs concentrations were not elevated, although Zn, Pb and Cu dust contents were above the ones reported in the literature (Table 1) (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001). While major fractions of Cu dust content were not available for absorption in the digestive system, Zn was quite bioaccessible (Table 2). The biogeochemistry of Cd, **which is currently ranked seventh on the Priority List of Hazardous Substances by the Agency for Toxic Substances and Diseases Registry (ATSDR 2013),** was also noteworthy. Despite the low Cd concentrations in the house dust samples (Table 1), the averaged Cd BAF was 76%, showing that Cd was greatly available for absorption to the body (Table 2).~~**Cd is currently ranked seventh on the Priority List of Hazardous Substances by the Agency for Toxic Substances and Diseases Registry (ATSDR 2013), who has derived a Minimum Risk Level (MRL) of 0.1 µg/kg/day for chronic duration oral exposure (≥ 1 year). Cd is stored in the body and oral exposure to low levels of Cd over a long period of time can damage the kidneys.**~~

Regardless **of** the average PHEs levels in the house dust samples, investigation on the genotoxic potential of their BAF is a new way to determine hazards closely associated with health risks

3.3 *In vitro* cytotoxicity

The *in vitro* cytotoxicity of bioaccessible PHEs was assessed in AGS cells by using concentrations ranging from 0.067 g/l to 0.53 g/l, surrounding the estimated physiological gastric concentration of 0.12 g/l (Fig.2).

Except for the highest tested concentration (0.53 g/l), the percentage of AGS cells viability was not statistically different from the negative control after 2 and 24 h exposure to the gastric extracts, and it always remained above 80%.

These results showed that the chosen concentrations were not cytotoxic. These concentrations were thus used for the analysis of bioaccessible PHEs genotoxic potentials by the alkaline comet and the cytokinesis-block micronucleus assay (CBMN) in combination with centromere labeling.

3.4 *In vitro* genotoxicity of gastric extracts from house dust

The *in vitro* genotoxicity of the gastric extracts was assessed in AGS cells using two complementary tests, namely the alkaline comet assay, in order to assess the primary DNA lesions and the cytokinesis-block micronucleus (CBMN) in combination with centromere labeling assay in order to quantify chromosome breakages and losses induction.

3.4.1 Alkaline comet assay

DNA integrity of AGS cells exposed to the gastric extracts (0.067 – 0.13 – 0.27 – 0.53 g/l) was evaluated at molecular level by the alkaline comet assay (Fig. 3), which allowed us to perform a quantification of single- and double- strand breaks, as well as alkali-labile and abasic sites formation (Collins et al., 2008).

A dose-dependent increase in the tail DNA content (% tail DNA) was observed after 2 h exposure to the gastric extracts for all sites although no significant induction of primary DNA lesions was noted at 0.13 g/l for the site 4. Gastric extracts obtained from house dust of sites 1, 2 and 3 induced more severe primary DNA damage: following exposure at 0.53 g/l, a 4.7-fold, 4.8-fold and 5.1-fold increase in DNA damage were observed, respectively. When AGS cells were exposed to the same concentration of gastric extracts obtained from house dust of sites 4 and 5, lower (3.0-fold and 3.3-fold, respectively) but still highly significant ($p < 0.001$) increases in primary DNA damage were noted.

The lowest tested concentration (0.067 g/l) induced a highly significantly ($p < 0.001$) increase in primary DNA damage compared to negative control for sites 1 and 3, a significant increase for sites 2 and 4 ($p < 0.01$ and $p < 0.05$, respectively), while not-significant DNA lesions were quantified for site 5 at this concentration. A significant increase ($p < 0.01$) relative to the negative control was observed for 0.27 g/l for site 4 and, for the same site, the only highly significant ($p < 0.001$) induction of primary DNA lesions was measured at 0.53 g/l.

Hereafter, the experimental data show that all gastric extracts induced primary DNA lesions although the induction observed for sites 1, 2 and 3 were higher than those noted for sites 4 and 5.

3.4.2 Cytokinesis-block micronucleus assay (CBMN) in combination with centromere labeling

Genome integrity was evaluated *in vitro* at chromosomal level by the CBMN assay (Fig. 4), a test that determines the frequency of micronuclei formation in exposed AGS cells and the CBMN assay was performed in combination with centromere labeling, allowing the discrimination between breakages (C-MN) and losses (C+MN).

The frequency of binucleated micronucleated cells (BNMN) per 1000 binucleated AGS cells increased in a dose-dependent manner ($p < 0.001$) after 24 h of exposure to the gastric extracts from the 5 houses at all the tested concentrations (0.067 – 0.13 – 0.27 – 0.53 g/l). Gastric extracts corresponding to sites 3 and 4 induced more chromosome damage than those corresponding to sites 1, 2 and 5. At the highest tested concentration (0.53 g/l), for sites 3 and 4, a 4.3-fold increase in chromosome damage was observed after 24 h exposure, while the frequency of BNMN increased up to 3.5-fold, 2.6-fold and 3.0-fold for sites 1, 2, and 5, respectively.

We further analyzed whether the BAF of the studied PHEs induced the formation of centromere positive (C+MN) or centromere negative micronuclei (C-MN) in AGS cells. Results showed that all gastric extracts induced dose-dependent increases of C+MN in binucleated AGS cells after 24 h, indicating an induction of aneugenic events such as chromosome migration abnormalities leading to chromosome losses. The highest *in vitro* aneugenic effect was noted for the gastric extract obtained from site 4 ($p < 0.001$).

Gastric extracts obtained from house dust induced dose-dependent C-MN increases in binucleated AGS cells after 24 h exposure, indicating that clastogenic events (chromosome breakage, inducing a partial chromosome loss) occurred. For sites 1, except for the highest concentration (0.53 g/l), and 2 no significant induction of C-MN was observed. The lowest concentration (0.067 g/l) was significantly different from the negative control for sites 3 and 4 ($p < 0.05$), as well as for site 5 ($p < 0.01$). A statistically significant ($p < 0.01$) induction of C-MN compared to the negative control was detected at 0.13 g/l for site 5, whereas this increase was highly significant ($p < 0.001$) for sites 3 and 4. The highest *in vitro* clastogenic effect was noted for the gastric extract obtained from site 3.

The highest *in vitro* chromosome damage were noted for the gastric extracts obtained from sites 3 and 4. The extracts from sites 3 and 4 were responsible for the most severe clastogenic and aneugenic effects, respectively. Finally we observed that the gastric extracts from sites 3, 4 and 5 were efficient to induce chromosome breakages (C-MN) as well as chromosome losses (C+MN); conversely the gastric extracts from sites 1 and 2 induced mostly chromosome losses.

3.4.3 Multivariate analysis

Principal components analysis (PCA) was performed to unravel possible relationships between bioaccessible PHEs in gastric extracts and their genotoxicity, in terms of chromosome damage (Fig.5). The data matrix used to carry out the PCA was composed by the BAF of the 16 PHEs under study, in all sampled sites; in addition, C+MN, C-MN and BNMN were projected as supplementary variables. The two first components, PC1 and PC2, accounted for approximately 72% of the total variance of the dataset and were therefore selected to investigate geometrical relationships between the variables of interest.

The results showed that the chromosome damage (BNMN, C+MN and C-MN) were associated to a PHEs cluster composed by Cd-Cu-Pb, and Zn to a certain extent (Fig.5). Other clusters in the factorial plane were composed by Ga-Sb, and Zr-Co-As-V-Ba-Mn, which were not related with chromosome damage. However, the diametric opposite projection of Al-Cr relative to Cd-Cu; and Ni-Mo-Sn relative to Pb suggested that these elements were ~~**negatively correlated and, therefore, had a likely inverse relationship with chromosome damage**~~ **less abundant and, therefore, not involved in chromosome damage induction**.

Spearman's correlation coefficients (*r*) were calculated between chromosome damage (C+MN, C-MN and BNMN) and PHEs concentrations in the gastric extracts to better support the interpretation of PCA results (Table 3).

Pb showed significant correlations with chromosome damage (BNMN: $p < 0.001$; $r = 0.55$), chromosome losses (C+MN: $p < 0.01$; $r = 0.32$) and chromosome breakage (C-MN: $p < 0.001$; $r = 0.72$) induced by gastric extracts in AGS cells. The induction of aneugenic events (C+MN) was also significantly correlated with V ($p < 0.05$; $r = 0.28$). Finally, Zn also induced significant clastogenic events ($p < 0.01$; $r = 0.34$) in AGS cells. In addition significant negative correlations were highlighted between PHEs and chromosome damage: (1) BNMN induction with Ba ($p < 0.05$; $r = -0.27$), Ni ($p < 0.05$; $r = -0.28$), Cr ($p < 0.001$; $r = -0.50$) and Co ($p < 0.01$; $r = -0.35$); (2) C+MN induction with Ni, Cr and Mo ($r = -0.28$, $r = -0.31$ and $r = -0.33$, respectively with $p < 0.05$); and (3) C-MN induction with Cu ($p < 0.05$; $r = -0.26$), Mn ($p < 0.01$; $r = -0.35$), Ba ($p < 0.001$; $r = -0.49$), Cr ($p < 0.001$; $r = -0.62$), Co ($p < 0.001$; $r = -0.56$), Sb ($p < 0.01$; $r = -0.38$) and Mo ($p < 0.01$; $r = -0.46$).

The joint interpretation of the results produced by the statistical tools used to investigate relationships between PHEs and genotoxic effects allows further detailing the effects detected in AGS cells exposed to the gastric extracts. Whilst, in Figure 5 Cu and Cd were projected associated with BNMN, C+MN and C-MN inductions, the correlation coefficients were not statistically significant (Table 3). Thus, Pb ~~**Thus, Pb**~~ appeared to be the PHE directly related with the observed chromosome damage. Pb-induced clastogenic events seem to be

prevalent over aneugenic events such as chromosome migration abnormalities leading to chromosome losses. Both PCA and Spearman's correlation highlighted a weak positive influence of V on C+MN induction and of Zn on C-MN induction in AGS cells. Finally the combination of the two methods shows few negative correlations: (1) BNMN induction with Ni and Cr; (2) C+MN induction with Ni, Mo, and Cr to a certain **~~extend~~ extent**; and (3) C-MN induction with Cr, and Sb to a certain **~~extend~~ extent**.

4. Discussion

House settled dust is a mixture of displaced soil particles, outside airborne particles transferred either by wind, pets or shoes, and particles produced directly in the indoor environment (Glorennec et al., 2012). Hence, the chemical composition of house dust particles is influenced by both indoor and outdoor sources, which may partially explain the higher PHE contents usually found in indoor dust relative to exterior soil (Rasmussen et al 2001; Reis et al., 2015). Thereupon, improvement of residential exposure assessments is achieved by dividing soil ingestion into separate categories for indoor house dust and exterior soil. In this study, exposure of a human adenocarcinoma gastric stomach cell line to gastric extracts obtained by unified BARGE method from house dust and assessment of the *in vitro* genotoxic potentials of these extracts at physiological concentrations aimed at establishing a novel methodology for human exposure assessment studies. The combination of bioaccessibility and genotoxicity evaluated by *in vitro* methods sequentially performed at physiological concentrations enables to assess the hazards relative to PHEs as close as possible to human exposure. The use of this methodology may be of uppermost importance, especially when the total PHEs contents in the environmental media are close to the established regulatory screening values.

The cytotoxicity and genotoxicity assays of the gastric fractions extracted from house dust were carried out in AGS cells, a cell line representative of the cells exposed to the gastric liquid in humans. To avoid non-specific DNA fragmentation by necrosis and/or apoptosis under cytotoxic conditions, AGS cells were treated with gastric extracts concentrations inducing less than 20% cellular death. The genotoxicity was assessed with two complementary tests. The alkaline comet assay enabled the detection of single- and double-strand breaks directly produced or associated with incomplete excision repair processes, as well as alkali-labile sites (Collins et al., 2008; Karlsson et al., 2015). The CBMN assay with centromeric labeling (CREST antibodies) was suitable to determine *in vitro* chromosomal damage and to discriminate between clastogenic (chromosome breakage consecutive to protein DNA crosslinks, interstrand crosslinks and various DNA lesions occurring during the

DNA replication) and aneugenic (chromosome loss consecutive to disruption of the mitotic apparatus) effects. Our data showed that the five tested extracts had dose-dependent genotoxic properties *in vitro*.

In the five samples selected in our exploratory study, PHEs dust contents were not elevated, although Zn, Pb and Cu concentrations were above the ones reported in some recent studies (Table 1). However, the average BAF of Cu in the house dust samples under study was 27% (Table 2), which decreased the potential health risk posed by the element to the residents. Notably, the geometrical relationships between the PHEs of concern and the damages induced in AGS cells displayed in the PCA loadings plot suggested that chromosome damage (BNMN, C+MN and C-MN) were largely associated to Cd, Cu, Pb, and Zn to a certain extent (Fig.5). Whilst Cu was primarily not in bioaccessible forms and Cd contents were weak, Pb came forth as potential inducer of clastogenic effects (C-MN), aneugenic effects (C+MN) and global chromosome damage (BNMN). Also, to a certain extent, chromosome damage seemed to be influenced by V and Zn concentrations in the extracts for aneugenic and clastogenic effects, respectively.

The alkaline comet assay results indicated that the gastric extracts obtained from sites 1, 2 and 3 induced the most severe primary DNA damage, meaning that these samples affected more severely the DNA integrity of AGS cells. Sites 1, 2 and 3 presented the highest Cu concentrations in the gastric extracts, while no other PHE showed the highest concentrations in these three extracts. Whilst dust Cu concentrations were elevated (Table 1) only a 27% fraction was on average solubilized by the gastric fluids, the results achieved indicated that the bioaccessible Cu concentrations (Table 2) were likely related to primary DNA damage. It is well established in the literature that some PHEs, namely Cu, may directly damage lipids, proteins, and DNA due to their capacity to generate hydroxyl radicals, ROS production and oxidative DNA damage (Delfino et al., 2011). Further, lipid peroxidation products consecutive to the action of reactive oxygen species are well known to contribute to the formation of interstrand DNA crosslinks and DNA-protein conjugates (Ayala et al., 2014). In these three sites (1, 2 and 3) the range of total Cu concentrations (303-407 mg/kg) was close or below the soil screening values for potentially unacceptable risk (residential soil use) provided by some EU countries (Carlon 2007).

The chromosome damaging properties of the gastric extract were assessed by the use of the CBMN assay and our results showed that the gastric extracts obtained from sites 3 and 4 induced the highest global chromosome damage (aneugenic and clastogenic effects) in AGS cells. Besides sites 3 and 4, the gastric extract from site 5 was also efficient to induce chromosome breakages (C-MN). These three house dust samples were the ones having the highest total and bioaccessible concentrations of Pb. According to the literature, Pb is able to induce

either clastogenic or aneugenic effects, suggesting a potential health risk in populations exposed to this PHE (Agency for Toxic Substances and Disease Registry (ATSDR), 2007; García-Lestón et al., 2010). Gastric extract from site 3, with the highest Zn concentrations, showed the most severe clastogenic effects, while the extracts from site 4, with the highest V concentrations, presented the most severe aneugenic effects. These results were also in accordance with the literature and the multivariate analysis results. V is found in different oxidation states, the most common being vanadium pentoxide is known to induce aneugenic events (Zhong et al., 1994). Concerning Zn exposure, even if at low concentrations, this PHE acts as a protector of genome stability, at concentrations higher than physiologic ones, weak clastogenic effects were reported (Roney et al., 2006).

Finally, and as a first attempt to untangle the effects of exposure to a chemical mixture (represented by the UBM gastric extracts) in AGS cells, several interrelations can be highlighted. **Although gastric extracts containing numerous elements were assessed,** Cu **is thought to be ~~was probably~~** the prevalent PHE inducing primary DNA damage, while Pb was the most prevalent PHE inducing chromosome-damaging effects. Whilst methodology development is the main purpose of this study, our results are in accordance with several recommendations (i.e. Pb) and thus seem relevant to human health risk assessment considerations. Additionally, as clatogenic compounds are closely associated with an increased risk of carcinogenesis and as no threshold value can be established for direct DNA damaging agents (a single mutation is still considered to be able to give rise to a clonal expansion and to a tumor), further investigations (*in vivo* studies) and preventive strategies and should be considered.

5. Conclusions

The pilot study described here proposes an interdisciplinary approach integrating environmental and health data derived from house dust samples collected from households of Estarreja. No similar study was found in the relevant literature, and the development of an integrated approach combining environmental and toxicity data was the primary endpoint of the present study. Thereby, our study did not envisaged achieving further information on the diagnosis, spreading or prevention of disease in the studied area.

The association of alkaline comet and CBMN assays proved to be effective tools to investigate both primary DNA lesions and chromosome damage induced by bioaccessible PHEs in environmental samples. In fact, our study demonstrates that a combination of *in vitro* methods representative of the bioaccessibility and the genotoxicity of environmental contaminants could be useful to document hazards relative to human exposure to complex mixture, taking into account the representativeness of the cellular model used and the physiological

concentration to be tested. Moreover, the method associating CBMN assay and CREST anti-kinetochore antibody labeling for centromere detection allowed us to reveal the mechanism of action (clastogenic or aneugenic) of bioaccessible PHEs**. Further, the use of the UBM and of these two *in vitro* genotoxicity assays can be performed in few weeks and are rather cheap (about 1,000€ per site), compared to *in vivo* assays.** The combined interpretation of the bioaccessible data and the genotoxicity results is well complemented by the association of two statistical methods (geometric: PCA and analytic: Spearman's correlations) that turned out to be decisive to understand the role and action mechanisms of the bioaccessible PHEs in a human cell model.

The option of selecting house dust samples with PHEs concentrations in the range of the current EU soil screening values for potentially unacceptable risk, unraveled the potential of this methodology to provide further information that can be used for science-based decision-making in regulatory policies, such as deriving soil screening values that are currently lacking in Portugal. ~~**This methodology could be used for a further study on a larger scale with numerous sampling sites that might enable to correlate house dust PHEs concentrations with household attributes and exterior contribution. The statistically significant responses between sites characteristics, PHEs concentrations, bioaccessibility and genotoxicity could provide information on PHEs sources, as well as a better estimation of human exposure and associated health risks. Also, concerning the chemical characterization, nitro and oxidized PAHs concentrations should be determined as well as metals' speciation to obtain a finer characterization.**~~ **By developing this methodology in broader studies, encompassing larger areas and comprehensive datasets, we should be able to** ~~**It is our aim to further develop this methodology to be used in broader studies, encompassing larger areas and comprehensive datasets, to**~~ correlate house dust PHEs concentrations with the physical environment of the house, as well as with exterior anthropogenic contributions. Establishing links between site characteristics, PHEs concentrations and their speciation, bioaccessibility and genotoxicity is likely to provide an accurate characterization of sources, pathways and fate of environmental PHEs, enabling more effective assessment of human exposure and associated health risks.

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Figures captions

Fig. 1 Map of Estarreja showing the sampling sites, the CCE, and the location of Estarreja within the Aveiro district and Portugal

Fig. 2 Viability of AGS cells exposed for 2 h and 24 h to the gastric extracts

Mean ± SD values % cell viability of three independent experiments were determined. Statistical analysis *versus* the negative control was performed by one-way ANOVA: ¤ $p < 0.05$.

Fig. 3 Primary DNA damage investigated by alkaline comet assay in AGS cells exposed for 2 h to gastric extracts

Mean ± SD % tail DNA of three independent experiments were determined. Statistical analysis *versus* the negative control was performed by one-way ANOVA: ¤ $p < 0.05$, ¤¤ $p < 0.01$, ¤¤¤ $p < 0.001$.

Fig. 4 Chromosome losses and breakages evidenced with CBMN assay in combination with centromere labeling in AGS cells exposed for 24 h to the gastric extracts

Frequency of BNMN, mean ± SD of C-MN and C+MN of three independent experiments were determined. Statistical analysis *versus* the negative control was performed by one-way ANOVA: ¤¤¤ $p < 0.001$ for BNMN, and by two-way ANOVA: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for C-MN and C+MN.

Fig. 5 Projections of the variables (PHEs) in the first factorial plane (PC1/PC2) of PCA analysis carried out for bioaccessible PHEs in gastric extracts of the five sites

Fig. S1 Box and whisker plots of the bioaccessible fraction (BAF) estimated for 16 PHEs using the UBM protocol in the 19 houses sampled by Reis et al. (2015)

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Title

Potentially harmful elements in house dust from Estarreja, Portugal: characterization and genotoxicity of the bioaccessible fraction

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Abstract

Due to their behavioral characteristics, young children are vulnerable to the ingestion of indoor dust, often contaminated with chemicals that are potentially harmful. Exposure to potentially harmful elements (PHEs) is currently exacerbated by their widespread use in several industrial, agricultural, domestic and technological applications. PHEs cause adverse health effects on immune and nervous systems, and can lead to cancer development via genotoxic mechanisms.

The present study is an integrated approach that aims at assessing the genotoxicity of bioaccessible PHEs following ingestion of contaminated house dust. A multidisciplinary methodology associating chemical characterization of five house dust samples, extraction of the bioaccessible PHEs in gastric extracts by the Unified BARGE Method, determination of the bioaccessible fraction and *in vitro* genotoxicity of gastric extracts in adenocarcinoma gastric human (AGS) cells was developed.

The five gastric extracts induced dose-dependent genotoxicity in AGS cells. Copper (bioaccessible concentration up to 111 mg/kg) was probably the prevalent PHE inducing primary DNA damage (up to 5.1-fold increase in tail DNA at 0.53 g/L of gastric extract). Lead **(**bioaccessible concentration up to 245 mg/kg) was the most prevalent PHE inducing chromosome-damaging effects ($r = 0.55$; $p < 0.001$ for micronucleated cells induction). The association of Principal Component Analysis and Spearman's correlations were decisive to understand the chromosome damaging properties of the bioaccessible PHEs in AGS cells. This methodology could be used on a larger scale study to provide useful information for science-based decision-making in regulatory policies, and a better estimation of human exposure and associated health risks.

Keywords

House dust; Unified BARGE method; Bioaccessibility; Genotoxicity; Alkaline comet assay; Cytokinesis-block micronucleus assay.

1. Introduction

It has been reported that trace elements such as cobalt (Co), copper (Cu), chromium (Cr), molybdenum (Mo), manganese (Mn) and zinc (Zn) are essential nutrients required for various biochemical and physiological functions in the human body (WHO, 1996). For some, including Cr, Cu and Zn, there is a narrow range of concentrations between beneficial and adverse effects (Chang et al., 1996; Lee et al., 2012). Other trace elements such as aluminium (Al), antimony (Sb), arsenic (As), barium (Ba), cadmium (Cd), gallium (Ga), lead (Pb), nickel (Ni), thallium (Tl), tin (Sn) and vanadium (V) have no established biological functions and are considered as potentially harmful to humans. These potentially harmful elements (PHEs) contribute to genotoxicity involving reactive oxygen species and/or DNA repair inhibition, apoptosis, and cancer development in mammals (Anetor et al., 2007; De Boeck et al., 2003; Denys et al., 2012; Gebel, 1997; Lee et al., 2012).

Human exposure to environmental PHEs has risen dramatically as a result of an exponential increase of their use in several industrial, agricultural, domestic and technological applications (Tchounwou et al., 2012). Ingestion, inhalation and dermal absorption are the likely routes of exposure. Experimental lines of evidence suggest that, soil/dust ingestion is the major source of human exposure to environmental PHEs contaminants (Davis and Mirick, 2006; Reis et al., 2014). Furthermore, given the large amount of time people spend indoors, either at home, school or workplace, the potential health risks posed by contaminants in house dust raises additional concerns (Lin et al., 2015). Indeed, recent studies suggested that exposure to contaminated house dust may represent an increased human health risk compared to contaminated exterior soil (Ibanez et al., 2010; Niu et al., 2010). Ingestion of dust generally occurs through deliberate hand-to-mouth movements, or unintentionally by eating food that has dropped on the floor and particles that have adhered to skin (U.S. Environmental Protection Agency, 2008). Young children (< 6 years old), while exploring their environment, are particularly vulnerable to this exposure pathway due to frequent hand-to-mouth and object-to-mouth habits (Mohmand et al., 2015; Reis et al., 2015; Tulve et al., 2002). Characterization of PHEs house dust by several measures (concentration, load and loading rate) has been used to document the presence and influence of metal sources (Rasmussen et al., 2013). Further, in the particular case of lead, Aung et al (2004) have shown that house dust and soil ingestion can be the predominant routes of exposure among children.

Whilst the intake dose (the amount of a chemical ingested by an individual) is the most commonly used measure of exposure in toxicity studies, the absorbed dose is the one causing the most, if not all, adverse effects on human health. In addition to the determination of PHEs concentrations in dust, data relative to the PHEs bioavailability (fraction of an ingested chemical that is absorbed by the body) and/or bioaccessibility (fraction of the

contaminant that is released from its solid matrix in the gastrointestinal tract and is therefore available for absorption by the body) are key information in human health risk assessment. Several protocols have been developed for the ingestion pathway to assess the bioavailability of a PHE from its bioaccessibility (Oomen et al., 2002). The Bioaccessibility Research Group of Europe (BARGE) has developed an *in vitro* test, the Unified BARGE Method (UBM), which mimics the solubilisation process during digestion and thus allows estimating the bioaccessibility of PHEs in the solid-phase (Denys et al., 2009; Wragg et al., 2011). The UBM is considered to be representative of the physiological conditions in the human gastrointestinal (GI) tract (Wragg et al., 2011).

Previous studies investigating oral bioaccessibility of house dust mainly focused on organic compounds (i.e. polycyclic aromatic carbons, phthalates esters) obtained after organic extraction (Kang et al., 2013, 2011; Maertens et al., 2008; Pohren et al., 2012; Wang et al., 2013a, 2013b). They quantified the toxicity by evaluating the excess lifetime cancer risk and some assessed the *in vitro* cytotoxic and genotoxic potentials of organic extracts by using bacterial mutagenic assays such as the Ames test, SOS chromotest and Salmonella assay (Kang et al., 2013, 2011; Maertens et al., 2008; Pohren et al., 2012; Wang et al., 2013a, 2013b). Likewise, the toxicity of PHEs from house dust has been investigated using either the evaluation of excess lifetime cancer risk or cytotoxicity tests (Chen et al., 2014; Deschamps et al., 2013; Granero and Domingo, 2002; Kurt-Karakus, 2012). However, the ingested dose was used as the relevant measure to assess the health risk and the oral bioavailability of the PHEs was not estimated. Notably, as far as we know, no study was published focusing on the genotoxicity of PHEs in house dust taking into account their oral bioavailability.

The relationships between biomarkers of exposure, levels of two PHEs (copper and manganese) in house dust and their bioaccessible fractions (BAF) were investigated in the Aveiro district (North of Portugal) (Reis et al., 2015). Reis et al. (2015) evidenced that manganese levels in toenails was associated with house dust manganese contents and this association was supported by the bioaccessibility estimates. In this study, dust containing PHEs were characterized in 19 houses located at various distance from the chemical complex of Estarreja (Reis et al., 2015). Large variations in PHEs levels were found in the different sampled house dust.

The present pilot study is about an integrated approach that aims at assessing the genotoxicity of the BAF of PHEs considering that, the ingested dose following incidental ingestion of contaminated house dust, usually overestimates the absorbed dose. PHEs were characterized using ICP-MS on dust samples and on their BAF obtained by the UBM. The cytogenotoxic potentials of these extracts were evaluated on adherent human adenocarcinoma gastric stomach (AGS) cells using the WST-1 assay for cytotoxicity, the alkaline comet assay

for primary DNA lesions quantification, and the centromeric micronucleus assay for chromosome breakages and losses assessment. AGS cells were chosen as a relevant model to assess the toxicity of PHEs contained in gastric extracts. AGS cells were previously used to assess *in vitro* cytotoxicity and genotoxicity of drugs and nanoparticles that may be ingested by humans (Alam-Escamilla et al., 2015; Botelho et al., 2014).

It is a detailed interdisciplinary study, performed on 5 houses dust sampled in the study of Reis et al. (2015) and carried out for purposes of methodology development. Our study does not envisage achieving further information on the diagnosis, spreading or prevention of disease in the studied area.

2. Material and methods

2.1 Characterization of the study area

Estarreja is a small coastal city in Portugal close to the broad "Ria de Aveiro" lagoon, which is a protected "Natura 2000 net" area (Fig.1). The coastal plain around the lagoon supports an intensive and diversified agriculture but, since 1950 the region is also known for the large industrial chemical complex of Estarreja (CCE). For many years, several of these industries dumped their solid residues (including ashes and dust with As, Pb, Cu, Zn) in an improvised park inside the CCE, albeit the proximity of the urban center of Estarreja is only 1 km away. Awareness on the pollution of the "Ria de Aveiro" and on its ecosystems started in the 80s (Patinha et al., 2014). Since then, technological upgrades associated to remediation measures implemented by the industries have strongly reduced the environmental burden of the area. However, by its dimension and configuration, the CCE is still regarded as the major pollutant of the Aveiro district.

2.2 Dust sampling

To carry out the present study on the genotoxic potential of the house dust bioaccessible fractions, 5 out of 19 houses dust sampled by Reis et al. (2015) were selected to be representative of the various sources of PHEs on Estarreja. Indeed, the 5 selected sites were far from each other (Fig.1) compared to the geographical distribution of the sampled sites of Reis et al. (2015).

The floor surfaces were not cleaned for a period of 7 days before the scheduled house dust sampling. Composite house dust samples were collected from the floor and carpets using the high-volume small surface (HVS3) vacuum sampler, which is designed to capture 99% of the particles ≥ 0.5 µm (Reis et al., 2015). Collected dust samples were oven-dried at 40°C for 24 h, sieved through a 150 µm nylon mesh and stored in polyethylene containers at ambient temperature. The particle size < 150 µm was chosen to the exploratory study because it

represents the particle size range likely to adhere to hands and thus, to be ingested following hand-to-mouth movements (Gron, C. and Andersen, L., 2003; U.S. Environmental Protection Agency, 2000).

2.3 Chemical analysis

2.3.1 House dust PHEs total concentrations

Dust samples were digested in Aqua Regia at 90 °C in a microprocessor controlled digestion block for 2 h, and the analysis of 16 PHEs was carried out by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) at the ACTLABS Analytical Laboratory, Canada. Each sample batch prepared for ICP-MS analysis included dust samples, duplicates and blanks. The Certified Reference Materials GRX-1, GRX-4, GRX-6 and SAR-M (United States Geological Survey) were selected to represent a wide range of total elemental concentrations. The blanks results were always below detection limits. In this study, we focused on the 16 following PHEs: Al, Zn, Cu, Pb, Mn, Ba, Ni, Cr, Sn, V, As, Co, Sb, Mo, Ga, and Cd. Values for precision (expressed as RSD %) are typically < 15 % for all elements. Further information on QA/QC is provided as supplementary material (Table S1).

2.3.2 Bioaccessibility of the PHEs in gastric extracts

The bioaccessibility of the PHEs was determined by subjecting a small quantity of sample to the UBM. The UBM protocol consists of two parallel sequential extractions simulating the chemical processes occurring in the mouth, stomach and intestine compartments using synthetic digestive solutions according to physiological transit times, which allow us to obtain both gastric and gastrointestinal extractions (Denys et al., 2009). The methodology has been validated against a juvenile swine model for As, Cd and Pb in soils, but not yet for less common PHEs (Denys et al., 2012). Several studies comparing *in vivo* bioavailability and *in vitro* bioaccessibility have shown that the *in vitro* gastric phase was generally closer to *in vivo* PHEs concentrations (Wragg et al., 2011). Indeed, likely precipitation reactions attributed to basic pH solutions used in the gastrointestinal phase of the UBM protocol usually decrease the bioaccessibility estimates, which may underestimate the bioavailability of the PHEs.

The chemicals used in this experiment were provided by Sigma-Aldrich (Sintra, Portugal). For the gastric extraction, an aliquot of 0.6 g dust was extracted by 9 ml of simulated saliva fluid (manually shaken) and 13.5 ml of simulated gastric fluid. After adjusting the pH to 1.2 ± 0.05, the mixture was rotated at 37°C for 1 h on an end-over-end shaker. After checking that the pH value was between 1.2 et 1.5, the solution was centrifuged at

4500 rpm for 15 min, then the supernatant was removed and stored at < 4°C for further analysis (Wragg et al., 2011).

The BAF corresponds to the ratio of PHE concentration in the gastric extract to total PHE concentration in dust, and is expressed as percentage (Oomen et al., 2002):

$$\text{BAF } \% = \frac{\text{PHE concentration in gastric extract}}{\text{PHE concentration in house dust}} \times 100$$

The gastric extracts were analyzed by ICP-MS at CEREGE, France. Duplicate samples, the BGS 102 bioaccessibility reference material and blanks were extracted with every batch of UBM bioaccessibility extractions, for quality control purposes. The blanks always returned results that were below the detection limit.

2.4 Genotoxicity assays

2.4.1 Reagents

4'-6-diamidino-2-phenylindole (DAPI), Goat anti-human Alexa Fluor® 488, Trypsin 0.25% EDTA, Fetal Bovine Serum (FBS), and PBS Dulbecco's were obtained from Life technologies (Saint Aubin, France). Paraformaldehyde (PAF) 4% PBS was from EMS (Hatfield, PA, USA). Bovine Serum Albumin (BSA) fraction V was purchased from Eurobio (Courtaboeuf, France). Vectashield was provided from Vector Laboratories (CA, USA). Human anti-kinetochore antibodies (CREST) were obtained from Laboratory of Immunology at the Hôpital de la Conception (Marseille, France). The other reagents were from Sigma-Aldrich (Lyon, France).

2.4.2 Cells culture and cell viability assay

Adherent human adenocarcinoma gastric stomach (AGS) cells were maintained in DMEM supplemented with 4.5 g/l glucose, 2 mM L-glutamine, 10% FBS, and 1% penicillin and streptomycin, at 37°C under 5% of CO_2. Cell Line Service (Eppelheim, Germany) provided all these reagents.

Cytotoxicity testing was performed by a colorimetric test, the WST-1 assay, to analyze the number of viable AGS cells by the cleavage of tetrazolium salts added to the culture medium. The choice of the concentration range to be tested was defined to approach the physiological gastric concentration of PHEs following dust ingestion by young children. As the ingestion rate of house dust by young children has been set by the INVS at 60 mg/d and the gastric volume of young children is about 500 ml, the PHEs physiological gastric concentration is estimated at 0.12 g/l (INVS, Institut de Veille Sanitaire, 2012).

AGS cells, grown in a 96-well plate at a density of 10^5 cells/well, were incubated with gastric extracts at 0.067 – 0.13 – 0.27 – 0.53 g/l, and with the relative controls for 2 h and 24 h before adding the WST-1 reagent. After 2 h incubation period, the formazan dye was quantified with a scanning multi-well spectrophotometer microplate reader (Multiskan, Thermo Scientific; Waltham, MA, USA) using a 450 nm emission filter. Three independent experiments were performed for each extract.

2.4.2 Alkaline comet assay

The alkaline comet assay was performed to evaluate primary DNA damage (Collins et al., 1993; Tice et al., 2000). Cells were seeded at a concentration of 2.5 x 10^4 cells/well in a 6-well plate. 24 h after the seeding, cells were exposed for 2 h to increasing concentrations of gastric extracts (0.067 – 0.13 – 0.27 – 0.53 g/l) and vehicle control (2% gastric blank solution in DMEM), then trypsinized and pelleted. After dropping a layer of 0.8% NMP agarose in PBS onto SuperFrost® Microscope slides (Thermo Scientific; Braunschweig, Germany) pre-coated with 1.6% NMP agarose, the cellular pellets were resuspended in 1% LMP agarose and spotted onto the slides. Positive control was performed at this step by exposing AGS cells to 125 µM H_2O_2 for 5 min at 4°C. After cellular lysis (90 min at 4°C, in the dark) by using a freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 300 mM NaOH, 10 mM Tris, pH 10 supplemented with 10% DMSO and 1% Triton X-100), slides were immersed, for 20 min at 4°C in the dark, in a denaturation solution (300 mM NaOH and 1 mM EDTA). Electrophoresis was carried out at 27 V and 300 mA for 20 min at 4°C. Then the slides were rinsed with neutralization buffer (4 mM Tris, pH 7.5) and finally dehydrated few seconds in methanol.

To evaluate DNA damage (% tail DNA), the slides were stained with propidium iodide and analyzed using a fluorescence microscope BX 60 (Olympus; Rungis, France) equipped with an appropriate filter combination and a black-and-white camera (Andor Luca S) and driven by Komet 6.0 software (Andor™ Technology; Belfast, UK). For each experimental condition, 100 cells were analyzed, and the experiments were repeated tree times. Two slides were prepared per concentration and 50 cells per slide were analyzed. Results were expressed as mean of n = 2 slides × 3 independent experiments.

2.4.3 Cytokinesis-block micronucleus (CBMN) assay in combination with centromere labeling

The CBMN assay was performed according to the original method described by Fenech (Fenech, 2007), with minor modifications for AGS cells, while centromere labeling was performed as described by González (González et al., 2011). Cells were seeded at a concentration of 2.5 x 10^4 cells/chamber in LabTek™ 4-Chamber Slide™ (Nuc International; Villebon-sur-Yvette, France), and cultured under standard conditions (37°C, 5%

CO_2). After 24 h culture, slides were treated with increasing concentrations of gastric extracts (0.067 – 0.13 – 0.27 – 0.53 g/l), vehicle control (2% gastric blank solution in DMEM), and appropriate clastogenic and aneugenic positive controls (10 ng/ml mitomycin C (MMC) and 25 nM colchicine, respectively). At the end of the treatment (24 h), the medium was removed and replaced by fresh medium containing 3 µg/ml cytochalasin B to inhibit cell division after mitosis. Following 24 h incubation, the culture medium was further changed and, after 2 h incubation, cells were washed in PBS and fixed (4% PAF). To discriminate chromosome losses and breakages, cells were incubated with CREST serum (1:1000 in 1% BSA/PBS) for 30 min. Cells were washed in 0.5% Triton X-100/PBS and incubated with secondary antibody Alexa Fluor® 488 goat anti-human (1:200 in 1% BSA/PBS) for 1 h. Cells were then washed in 0.5% Triton X-100/PBS and incubated with a 0.06 µg/ml solution of phalloidin-TRITC (tetramethylrhodamine B isothiocyanate) for 30 min to stain the cytoplasm. After two washes in 0.5% Triton X-100/PBS, cells were incubated with a solution of 4'-6-diamidino-2-phenylindole (DAPI, 1:50000) for 10 min to stain the nuclei. Finally, the slides were mounted in Vectashield and stored in dark at 4°C until analysis.

Micronuclei (MN) contained in binucleated cells were scored using a fluorescence microscope BX 60 (Olympus; Rungis, France) equipped with a black-and-white camera (Andor Luca S), and appropriate filters for DAPI, phalloidin-TRITC, and Alexa Fluor® 488.

Before the analysis, we calculated the cytokinesis block proliferation index on 500 cells to provide the average number of cell divisions completed by the cells, as previously described (Benameur et al., 2011; Kirsch-Volders et al., 2003). Micronucleus analysis data are expressed as a frequency of binucleated micronucleated (BNMN) cells per 1000 binucleated cells; scoring criteria were in accordance with those previously described (Fenech, 2007). Taking advantage of the centromeric labeling, micronuclei containing whole chromosome(s) positively labeled (centromeric micronuclei cell, C+MN), and acentric chromosome fragments that are not stained due to the absence of centromere (acentromeric micronuclei, C−MN) were discriminated (González et al., 2011; Iarmarcovai et al., 2006; Mateuca et al., 2006; Natarajan et al., 1996). Two chambers were prepared per concentration, and micronuclei were counted for 1000 binucleated cells on each chamber. Results were expressed as mean of n = 2 slides × 3 independent experiments.

2.5 Statistical analysis

Results of cytotoxicity and genotoxicity were expressed as the mean value of three independent experiments ± standard deviation (SD). Data were submitted to statistical evaluation using one-way ANOVA. GraphPad Prism

6 (GraphPad Software, Inc., La Jolla, USA) software was used to perform statistical analysis and to draw the graphics. The significance was compared to negative control (vehicle: 2% gastric blank solution in DMEM). Values were considered statistically significant starting from $p < 0.05$.

Relationships between bioaccessible PHEs and chromosome damage were investigated using two complementary methods, principal component analysis (PCA) and Spearman's correlation. Principal component analysis (PCA) is a mathematical technique adapted to quantitative variables that transforms *n* possibly correlated variables into a (smaller) number of uncorrelated variables referred to as principal component (Jolliffe, 2014; Reis et al., 2015). Moreover, Spearman's correlation is adapted to analyze non-linear monotonic relationships (increasing or decreasing) between the observation's ranks of two defined characteristics (chemical composition and chromosome damage).

3. Results

3.1 House dust PHEs concentrations

House dust samples collected from five selected houses in Estarreja were analyzed by ICP-MS to determine elemental concentrations (Table 1). The arithmetic means, geometric means and medians of all analyzed PHEs in the **5** studied dust samples were calculated. Table 1 shows values for the 16 PHEs under study and compares the PHEs concentrations with those reported in the literature (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001). Further data on PHEs concentrations in the full set of indoor dust samples collected from 19 private households in the city of Estarreja are provided in the form of supplementary material (Table S2).

In all sites, the major element was Al, whose median concentration was 11100 mg/kg, followed by trace elements Zn, Cu, Pb, Mn, Ba, Ni, Cr, Sn, V, and As. Co, Sb, Mo, Ga, and Cd were the less abundant PHEs (median concentrations $\leq$ 5 mg/kg). Notably, Pb (78-1180 mg/kg; RSD = 119%), Zn (991-5210 mg/kg; RSD = 85%), Ga (0.9 – 4.0 mg/kg; RSD = 56%), Mo (1.2-4.7 mg/kg; RSD = 43%), Mn (98-296 mg/kg; RSD = 42%) and Cd (0.8-2.2 mg/kg; RSD = 42%) showed a wide range of values, reflecting the heterogeneity of house dust chemistry among the five sites under investigation.

The Estarreja median levels for Zn (1520 mg/kg) and Cu (339 mg/kg) were higher than the ones reported for other cities (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001). Pb median concentration in house dust from Estarreja was

equivalent to that reported for Canadian house dust (Rasmussen et al., 2001), while it was nearly ten times higher than the ones from Turkey and Brazil (Deschamps et al., 2013; Kurt-Karakus, 2012). Median levels of Ni, Cr, As and Co were similar to the majority of those reported in the literature (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001), whereas Ba and V were lower than the ones from China (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001)).

3.2 PHEs bioaccessibility in gastric extracts

In the present study, the PHE concentrations extracted in the gastric phase were considered to be the relevant measure of exposure. Since our study focuses on the fate of the PHEs in the gastric compartment following incidental ingestion of house dust, bioaccessible PHEs were determined by using the UBM to mimick *in vitro* interactions between the dust and the gastric solutions (Table 2). Oral bioaccessibility data for the set of 19 samples are presented in supplementary material (Figure S1), while Table 2 shows the results obtained for the subset of 5 samples used in this study.

The BAF of the 16 studied PHEs ranged from 4 to 85% (Table 2), showing that some PHEs were highly bioaccessible, and others almost not. PHEs can be classified into three groups according to their bioaccessibility: first, Zn and Cd were highly bioaccessible (85% and 80%, respectively); second, Pb, Mn, As, Ba, Co, Ni, Ga, and V were moderately bioaccessible (from 62% to 41%); and third Cu, Al, Cr, Sb, Sn, and Mo were almost not available to humans (less than 27%). Otherwise, Sn, Al, Ba, and Ga bioaccessibility were highly variable (RSD ranged from 64% to 54%) from one house to another, suggesting variations in physicochemical forms, thus different PHE states of speciation. The overall assessment of the results (Table 2 and Fig. S2) shows that the trends in the oral bioaccessibility of the PHEs are the same in the selected subset and in the broader set of samples subjected to the UBM protocol.

In the present study, the bioaccessibility of the 16 PHEs was considerably lower than the one reported for house dust in Turner and Simmonds (2006), which employed a simple surrogate (pepsin in 0.075 M HCl) for the human stomach. They found that the bioaccessibility of Al, Cu, Ni and Pb averaged between 60 and 100% (Turner and Simmonds, 2006), whereas our results were in accordance with those of Rasmussen (2004) reporting BAF of 30−40% for Ni and of 55−75% for Pb using 0.07 M HCl as extraction medium (Rasmussen, 2004). These discrepancies between the reported results are likely related to the different protocols used. Differences in

fluid formulation, pH values, residence time or type of shaking within the various *in vitro* tests can significantly affect the degree to which the PHEs are extracted from the soil/dust matrix (Oomen et al., 2002).

Results of the oral bioaccessibility testing indicate that a large proportion of the total Cu, Al, Cr, Sb, Sn, and Mo content in the house dust were not in bioaccessible forms, and are therefore unavailable to the residents via ingestion. Moreover, such low BAF values (< 30%) suggested that, in the dust, these PHEs were associated to resistant mineral phases that were hardly dissolved by the acidic solutions used in the gastric phase of the UBM protocol. In a previous study on these house dust that aimed at identifying likely environmental sources, Reis et al. (2015) suggested that Cu was probably related to waste materials of the CCE (Reis et al., 2015).

An overall analysis of the results obtained so far showed that the studied PHEs concentrations were not elevated, although Zn, Pb and Cu dust contents were above the ones reported in the literature (Table 1) (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001). While major fractions of Cu dust content were not available for absorption in the digestive system, Zn was quite bioaccessible (Table 2). The biogeochemistry of Cd, which is currently ranked seventh on the Priority List of Hazardous Substances by the Agency for Toxic Substances and Diseases Registry (ATSDR 2013), was also noteworthy. Despite the low Cd concentrations in the house dust samples (Table 1), the averaged Cd BAF was 76%, showing that Cd was greatly available for absorption to the body (Table 2).

Regardless of the average PHEs levels in the house dust samples, investigation on the genotoxic potential of their BAF is a new way to determine hazards closely associated with health risks

3.3 *In vitro* cytotoxicity

The *in vitro* cytotoxicity of bioaccessible PHEs was assessed in AGS cells by using concentrations ranging from 0.067 g/l to 0.53 g/l, surrounding the estimated physiological gastric concentration of 0.12 g/l (Fig.2).

Except for the highest tested concentration (0.53 g/l), the percentage of AGS cells viability was not statistically different from the negative control after 2 and 24 h exposure to the gastric extracts, and it always remained above 80%.

These results showed that the chosen concentrations were not cytotoxic. These concentrations were thus used for the analysis of bioaccessible PHEs genotoxic potentials by the alkaline comet and the cytokinesis-block micronucleus assay (CBMN) in combination with centromere labeling.

3.4 *In vitro* genotoxicity of gastric extracts from house dust

The *in vitro* genotoxicity of the gastric extracts was assessed in AGS cells using two complementary tests, namely the alkaline comet assay, in order to assess the primary DNA lesions and the cytokinesis-block micronucleus (CBMN) in combination with centromere labeling assay in order to quantify chromosome breakages and losses induction.

3.4.1 Alkaline comet assay

DNA integrity of AGS cells exposed to the gastric extracts (0.067 – 0.13 – 0.27 – 0.53 g/l) was evaluated at molecular level by the alkaline comet assay (Fig. 3), which allowed us to perform a quantification of single- and double- strand breaks, as well as alkali-labile and abasic sites formation (Collins et al., 2008).

A dose-dependent increase in the tail DNA content (% tail DNA) was observed after 2 h exposure to the gastric extracts for all sites although no significant induction of primary DNA lesions was noted at 0.13 g/l for the site 4. Gastric extracts obtained from house dust of sites 1, 2 and 3 induced more severe primary DNA damage: following exposure at 0.53 g/l, a 4.7-fold, 4.8-fold and 5.1-fold increase in DNA damage were observed, respectively. When AGS cells were exposed to the same concentration of gastric extracts obtained from house dust of sites 4 and 5, lower (3.0-fold and 3.3-fold, respectively) but still highly significant ($p < 0.001$) increases in primary DNA damage were noted.

The lowest tested concentration (0.067 g/l) induced a highly significantly ($p < 0.001$) increase in primary DNA damage compared to negative control for sites 1 and 3, a significant increase for sites 2 and 4 ($p < 0.01$ and $p < 0.05$, respectively), while not-significant DNA lesions were quantified for site 5 at this concentration. A significant increase ($p < 0.01$) relative to the negative control was observed for 0.27 g/l for site 4 and, for the same site, the only highly significant ($p < 0.001$) induction of primary DNA lesions was measured at 0.53 g/l.

Hereafter, the experimental data show that all gastric extracts induced primary DNA lesions although the induction observed for sites 1, 2 and 3 were higher than those noted for sites 4 and 5.

3.4.2 Cytokinesis-block micronucleus assay (CBMN) in combination with centromere labeling

Genome integrity was evaluated *in vitro* at chromosomal level by the CBMN assay (Fig. 4), a test that determines the frequency of micronuclei formation in exposed AGS cells and the CBMN assay was performed in combination with centromere labeling, allowing the discrimination between breakages (C-MN) and losses (C+MN).

The frequency of binucleated micronucleated cells (BNMN) per 1000 binucleated AGS cells increased in a dose-dependent manner ($p < 0.001$) after 24 h of exposure to the gastric extracts from the 5 houses at all the tested concentrations (0.067 – 0.13 – 0.27 – 0.53 g/l). Gastric extracts corresponding to sites 3 and 4 induced more chromosome damage than those corresponding to sites 1, 2 and 5. At the highest tested concentration (0.53 g/l), for sites 3 and 4, a 4.3-fold increase in chromosome damage was observed after 24 h exposure, while the frequency of BNMN increased up to 3.5-fold, 2.6-fold and 3.0-fold for sites 1, 2, and 5, respectively.

We further analyzed whether the BAF of the studied PHEs induced the formation of centromere positive (C+MN) or centromere negative micronuclei (C-MN) in AGS cells. Results showed that all gastric extracts induced dose-dependent increases of C+MN in binucleated AGS cells after 24 h, indicating an induction of aneugenic events such as chromosome migration abnormalities leading to chromosome losses. The highest *in vitro* aneugenic effect was noted for the gastric extract obtained from site 4 ($p < 0.001$).

Gastric extracts obtained from house dust induced dose-dependent C-MN increases in binucleated AGS cells after 24 h exposure, indicating that clastogenic events (chromosome breakage, inducing a partial chromosome loss) occurred. For sites 1, except for the highest concentration (0.53 g/l), and 2 no significant induction of C-MN was observed. The lowest concentration (0.067 g/l) was significantly different from the negative control for sites 3 and 4 ($p < 0.05$), as well as for site 5 ($p < 0.01$). A statistically significant ($p < 0.01$) induction of C-MN compared to the negative control was detected at 0.13 g/l for site 5, whereas this increase was highly significant ($p < 0.001$) for sites 3 and 4. The highest *in vitro* clastogenic effect was noted for the gastric extract obtained from site 3.

The highest *in vitro* chromosome damage were noted for the gastric extracts obtained from sites 3 and 4. The extracts from sites 3 and 4 were responsible for the most severe clastogenic and aneugenic effects, respectively. Finally we observed that the gastric extracts from sites 3, 4 and 5 were efficient to induce chromosome breakages (C-MN) as well as chromosome losses (C+MN); conversely the gastric extracts from sites 1 and 2 induced mostly chromosome losses.

3.4.3 Multivariate analysis

Principal components analysis (PCA) was performed to unravel possible relationships between bioaccessible PHEs in gastric extracts and their genotoxicity, in terms of chromosome damage (Fig.5). The data matrix used to carry out the PCA was composed by the BAF of the 16 PHEs under study, in all sampled sites; in addition, C+MN, C-MN and BNMN were projected as supplementary variables. The two first components, PC1 and PC2,

accounted for approximately 72% of the total variance of the dataset and were therefore selected to investigate geometrical relationships between the variables of interest.

The results showed that the chromosome damage (BNMN, C+MN and C-MN) were associated to a PHEs cluster composed by Cd-Cu-Pb, and Zn to a certain extent (Fig.5). Other clusters in the factorial plane were composed by Ga-Sb, and Zr-Co-As-V-Ba-Mn, which were not related with chromosome damage. However, the diametric opposite projection of Al-Cr relative to Cd-Cu; and Ni-Mo-Sn relative to Pb suggested that these elements were less abundant and, therefore, not involved in chromosome damage induction.

Spearman's correlation coefficients (r) were calculated between chromosome damage (C+MN, C-MN and BNMN) and PHEs concentrations in the gastric extracts to better support the interpretation of PCA results (Table 3).

Pb showed significant correlations with chromosome damage (BNMN: $p < 0.001$; $r = 0.55$), chromosome losses (C+MN: $p < 0.01$; $r = 0.32$) and chromosome breakage (C-MN: $p < 0.001$; $r = 0.72$) induced by gastric extracts in AGS cells. The induction of aneugenic events (C+MN) was also significantly correlated with V ($p < 0.05$; $r = 0.28$). Finally, Zn also induced significant clastogenic events ($p < 0.01$; $r = 0.34$) in AGS cells. In addition significant negative correlations were highlighted between PHEs and chromosome damage: (1) BNMN induction with Ba ($p < 0.05$; $r = -0.27$), Ni ($p < 0.05$; $r = -0.28$), Cr ($p < 0.001$; $r = -0.50$) and Co ($p < 0.01$; $r = -0.35$); (2) C+MN induction with Ni, Cr and Mo ($r = -0.28$, $r = -0.31$ and $r = -0.33$, respectively with $p < 0.05$); and (3) C-MN induction with Cu ($p < 0.05$; $r = -0.26$), Mn ($p < 0.01$; $r = -0.35$), Ba ($p < 0.001$; $r = -0.49$), Cr ($p < 0.001$; $r = -0.62$), Co ($p < 0.001$; $r = -0.56$), Sb ($p < 0.01$; $r = -0.38$) and Mo ($p < 0.01$; $r = -0.46$).

The joint interpretation of the results produced by the statistical tools used to investigate relationships between PHEs and genotoxic effects allows further detailing the effects detected in AGS cells exposed to the gastric extracts. Whilst, in Figure 5 Cu and Cd were projected associated with BNMN, C+MN and C-MN inductions, the correlation coefficients were not statistically significant (Table 3). Thus, Pb appeared to be the PHE directly related with the observed chromosome damage. Pb-induced clastogenic events seem to be prevalent over aneugenic events such as chromosome migration abnormalities leading to chromosome losses. Both PCA and Spearman's correlation highlighted a weak positive influence of V on C+MN induction and of Zn on C-MN induction in AGS cells. Finally the combination of the two methods shows few negative correlations: (1) BNMN induction with Ni and Cr; (2) C+MN induction with Ni, Mo, and Cr to a certain extent; and (3) C-MN induction with Cr, and Sb to a certain extent.

4. Discussion

House settled dust is a mixture of displaced soil particles, outside airborne particles transferred either by wind, pets or shoes, and particles produced directly in the indoor environment (Glorennec et al., 2012). Hence, the chemical composition of house dust particles is influenced by both indoor and outdoor sources, which may partially explain the higher PHE contents usually found in indoor dust relative to exterior soil (Rasmussen et al 2001; Reis et al., 2015). Thereupon, improvement of residential exposure assessments is achieved by dividing soil ingestion into separate categories for indoor house dust and exterior soil. In this study, exposure of a human adenocarcinoma gastric stomach cell line to gastric extracts obtained by unified BARGE method from house dust and assessment of the *in vitro* genotoxic potentials of these extracts at physiological concentrations aimed at establishing a novel methodology for human exposure assessment studies. The combination of bioaccessibility and genotoxicity evaluated by *in vitro* methods sequentially performed at physiological concentrations enables to assess the hazards relative to PHEs as close as possible to human exposure. The use of this methodology may be of uppermost importance, especially when the total PHEs contents in the environmental media are close to the established regulatory screening values.

The cytotoxicity and genotoxicity assays of the gastric fractions extracted from house dust were carried out in AGS cells, a cell line representative of the cells exposed to the gastric liquid in humans. To avoid non-specific DNA fragmentation by necrosis and/or apoptosis under cytotoxic conditions, AGS cells were treated with gastric extracts concentrations inducing less than 20% cellular death. The genotoxicity was assessed with two complementary tests. The alkaline comet assay enabled the detection of single- and double-strand breaks directly produced or associated with incomplete excision repair processes, as well as alkali-labile sites (Collins et al., 2008; Karlsson et al., 2015). The CBMN assay with centromeric labeling (CREST antibodies) was suitable to determine *in vitro* chromosomal damage and to discriminate between clastogenic (chromosome breakage consecutive to protein DNA crosslinks, interstrand crosslinks and various DNA lesions occurring during the DNA replication) and aneugenic (chromosome loss consecutive to disruption of the mitotic apparatus) effects. Our data showed that the five tested extracts had dose-dependent genotoxic properties *in vitro*.

In the five samples selected in our exploratory study, PHEs dust contents were not elevated, although Zn, Pb and Cu concentrations were above the ones reported in some recent studies (Table 1). However, the average BAF of Cu in the house dust samples under study was 27% (Table 2), which decreased the potential health risk posed by the element to the residents. Notably, the geometrical relationships between the PHEs of concern and the

damages induced in AGS cells displayed in the PCA loadings plot suggested that chromosome damage (BNMN, C+MN and C-MN) were largely associated to Cd, Cu, Pb, and Zn to a certain extent (Fig.5). Whilst Cu was primarily not in bioaccessible forms and Cd contents were weak, Pb came forth as potential inducer of clastogenic effects (C-MN), aneugenic effects (C+MN) and global chromosome damage (BNMN). Also, to a certain extent, chromosome damage seemed to be influenced by V and Zn concentrations in the extracts for aneugenic and clastogenic effects, respectively.

The alkaline comet assay results indicated that the gastric extracts obtained from sites 1, 2 and 3 induced the most severe primary DNA damage, meaning that these samples affected more severely the DNA integrity of AGS cells. Sites 1, 2 and 3 presented the highest Cu concentrations in the gastric extracts, while no other PHE showed the highest concentrations in these three extracts. Whilst dust Cu concentrations were elevated (Table 1) only a 27% fraction was on average solubilized by the gastric fluids, the results achieved indicated that the bioaccessible Cu concentrations (Table 2) were likely related to primary DNA damage. It is well established in the literature that some PHEs, namely Cu, may directly damage lipids, proteins, and DNA due to their capacity to generate hydroxyl radicals, ROS production and oxidative DNA damage (Delfino et al., 2011). Further, lipid peroxidation products consecutive to the action of reactive oxygen species are well known to contribute to the formation of interstrand DNA crosslinks and DNA-protein conjugates (Ayala et al., 2014). In these three sites (1, 2 and 3) the range of total Cu concentrations (303-407 mg/kg) was close or below the soil screening values for potentially unacceptable risk (residential soil use) provided by some EU countries (Carlon 2007).

The chromosome damaging properties of the gastric extract were assessed by the use of the CBMN assay and our results showed that the gastric extracts obtained from sites 3 and 4 induced the highest global chromosome damage (aneugenic and clastogenic effects) in AGS cells. Besides sites 3 and 4, the gastric extract from site 5 was also efficient to induce chromosome breakages (C-MN). These three house dust samples were the ones having the highest total and bioaccessible concentrations of Pb. According to the literature, Pb is able to induce either clastogenic or aneugenic effects, suggesting a potential health risk in populations exposed to this PHE (Agency for Toxic Substances and Disease Registry (ATSDR), 2007; García-Lestón et al., 2010). Gastric extract from site 3, with the highest Zn concentrations, showed the most severe clastogenic effects, while the extracts from site 4, with the highest V concentrations, presented the most severe aneugenic effects. These results were also in accordance with the literature and the multivariate analysis results. V is found in different oxidation states, the most common being vanadium pentoxide is known to induce aneugenic events (Zhong et al., 1994).

Concerning Zn exposure, even if at low concentrations, this PHE acts as a protector of genome stability, at concentrations higher than physiologic ones, weak clastogenic effects were reported (Roney et al., 2006).

Finally, and as a first attempt to untangle the effects of exposure to a chemical mixture (represented by the UBM gastric extracts) in AGS cells, several interrelations can be highlighted. Although gastric extracts containing numerous elements were assessed, Cu is thought to be the prevalent PHE inducing primary DNA damage, while Pb was the most prevalent PHE inducing chromosome-damaging effects. Whilst methodology development is the main purpose of this study, our results are in accordance with several recommendations (i.e. Pb) and thus seem relevant to human health risk assessment considerations. Additionally, as clatogenic compounds are closely associated with an increased risk of carcinogenesis and as no threshold value can be established for direct DNA damaging agents (a single mutation is still considered to be able to give rise to a clonal expansion and to a tumor), further investigations (*in vivo* studies) and preventive strategies and should be considered.

5. Conclusions

The pilot study described here proposes an interdisciplinary approach integrating environmental and health data derived from house dust samples collected from households of Estarreja. No similar study was found in the relevant literature, and the development of an integrated approach combining environmental and toxicity data was the primary endpoint of the present study. Thereby, our study did not envisaged achieving further information on the diagnosis, spreading or prevention of disease in the studied area.

The association of alkaline comet and CBMN assays proved to be effective tools to investigate both primary DNA lesions and chromosome damage induced by bioaccessible PHEs in environmental samples. In fact, our study demonstrates that a combination of *in vitro* methods representative of the bioaccessibility and the genotoxicity of environmental contaminants could be useful to document hazards relative to human exposure to complex mixture, taking into account the representativeness of the cellular model used and the physiological concentration to be tested. Moreover, the method associating CBMN assay and CREST anti-kinetochore antibody labeling for centromere detection allowed us to reveal the mechanism of action (clastogenic or aneugenic) of bioaccessible PHEs. Further, the use of the UBM and of these two *in vitro* genotoxicity assays can be performed in few weeks and are rather cheap (about 1,000€ per site), compared to *in vivo* assays. The combined interpretation of the bioaccessible data and the genotoxicity results is well complemented by the association of two statistical methods (geometric: PCA and analytic: Spearman's correlations) that turned out to be decisive to understand the role and action mechanisms of the bioaccessible PHEs in a human cell model.

The option of selecting house dust samples with PHEs concentrations in the range of the current EU soil screening values for potentially unacceptable risk, unraveled the potential of this methodology to provide further information that can be used for science-based decision-making in regulatory policies, such as deriving soil screening values that are currently lacking in Portugal. By developing this methodology in broader studies, encompassing larger areas and comprehensive datasets, we should be able to correlate house dust PHEs concentrations with the physical environment of the house, as well as with exterior anthropogenic contributions. Establishing links between site characteristics, PHEs concentrations and their speciation, bioaccessibility and genotoxicity is likely to provide an accurate characterization of sources, pathways and fate of environmental PHEs, enabling more effective assessment of human exposure and associated health risks.

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Figures captions

Fig. 1 Map of Estarreja showing the sampling sites, the CCE, and the location of Estarreja within the Aveiro district and Portugal

Fig. 2 Viability of AGS cells exposed for 2 h and 24 h to the gastric extracts

Mean ± SD values % cell viability of three independent experiments were determined. Statistical analysis *versus* the negative control was performed by one-way ANOVA: ¤ $p < 0.05$.

Fig. 3 Primary DNA damage investigated by alkaline comet assay in AGS cells exposed for 2 h to gastric extracts

Mean ± SD % tail DNA of three independent experiments were determined. Statistical analysis *versus* the negative control was performed by one-way ANOVA: ¤ $p < 0.05$, ¤¤ $p < 0.01$, ¤¤¤ $p < 0.001$.

Fig. 4 Chromosome losses and breakages evidenced with CBMN assay in combination with centromere labeling in AGS cells exposed for 24 h to the gastric extracts

Frequency of BNMN, mean ± SD of C-MN and C+MN of three independent experiments were determined. Statistical analysis *versus* the negative control was performed by one-way ANOVA: ¤¤¤ $p < 0.001$ for BNMN, and by two-way ANOVA: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for C-MN and C+MN.

Fig. 5 Projections of the variables (PHEs) in the first factorial plane (PC1/PC2) of PCA analysis carried out for bioaccessible PHEs in gastric extracts of the five sites

Fig. S1 Box and whisker plots of the bioaccessible fraction (BAF) estimated for 16 PHEs using the UBM protocol in the 19 houses sampled by Reis et al. (2015)

Figure

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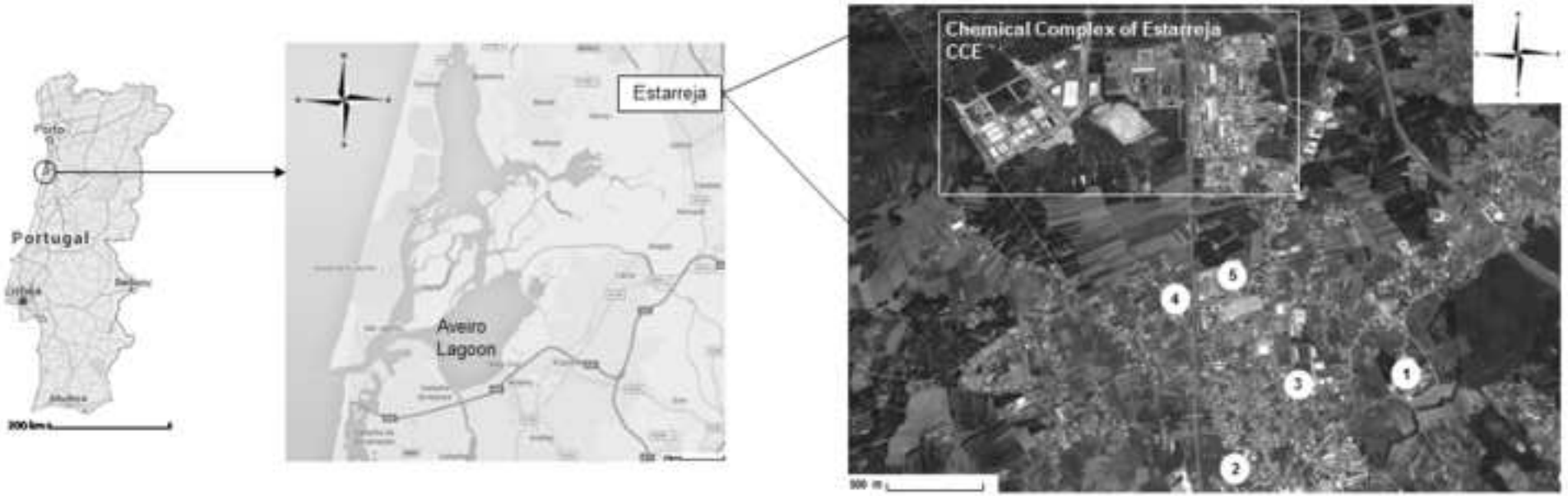

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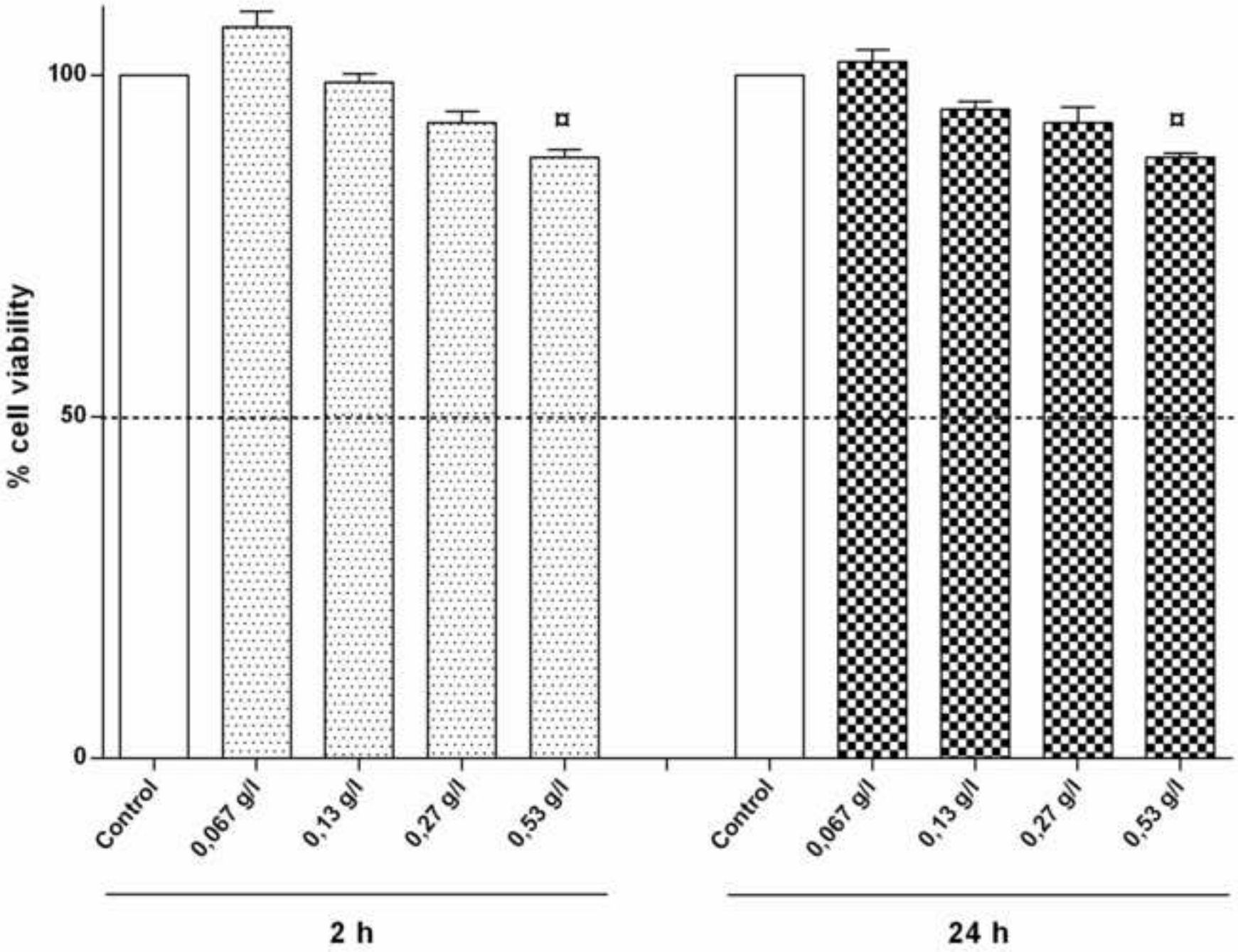

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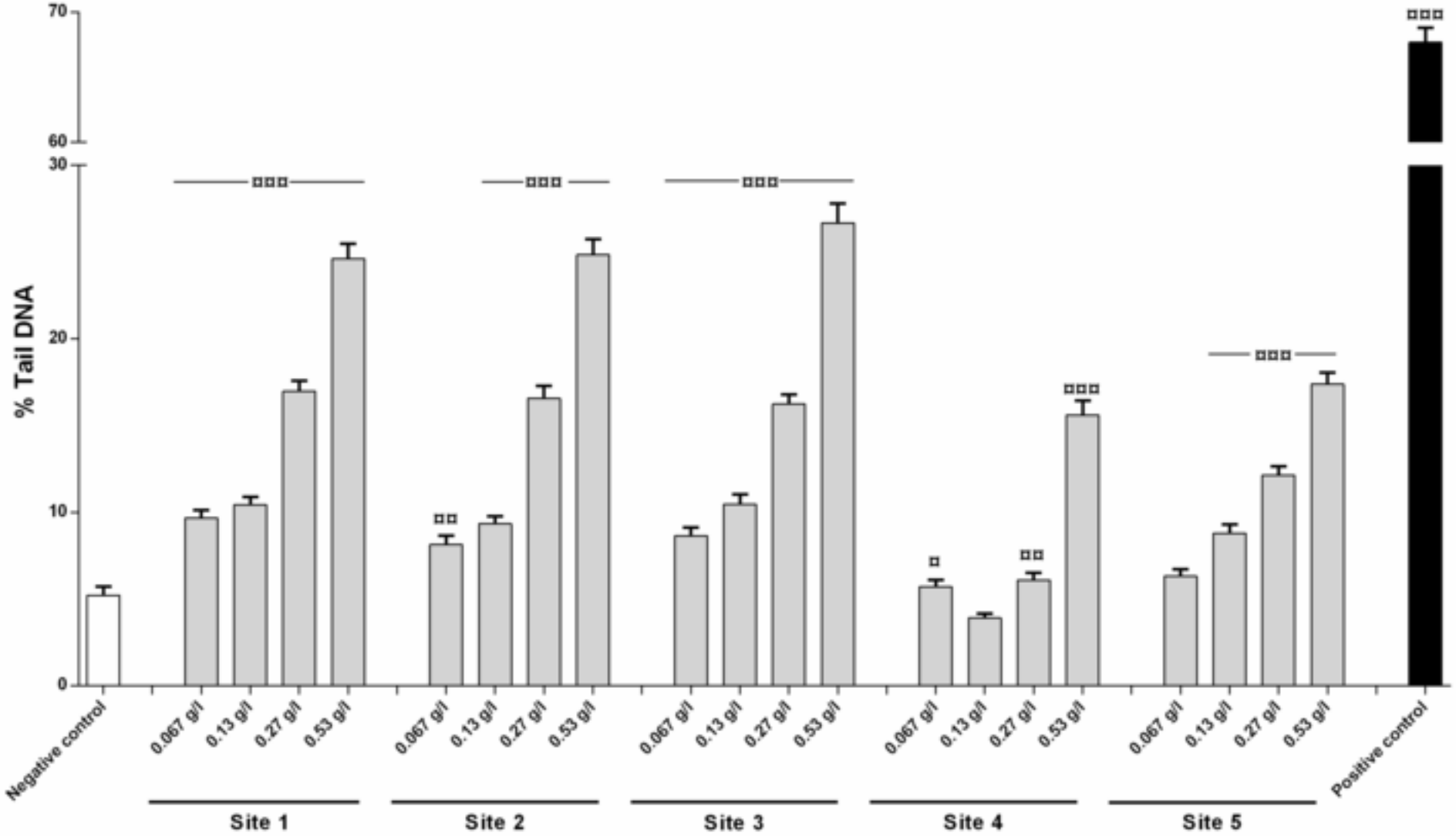

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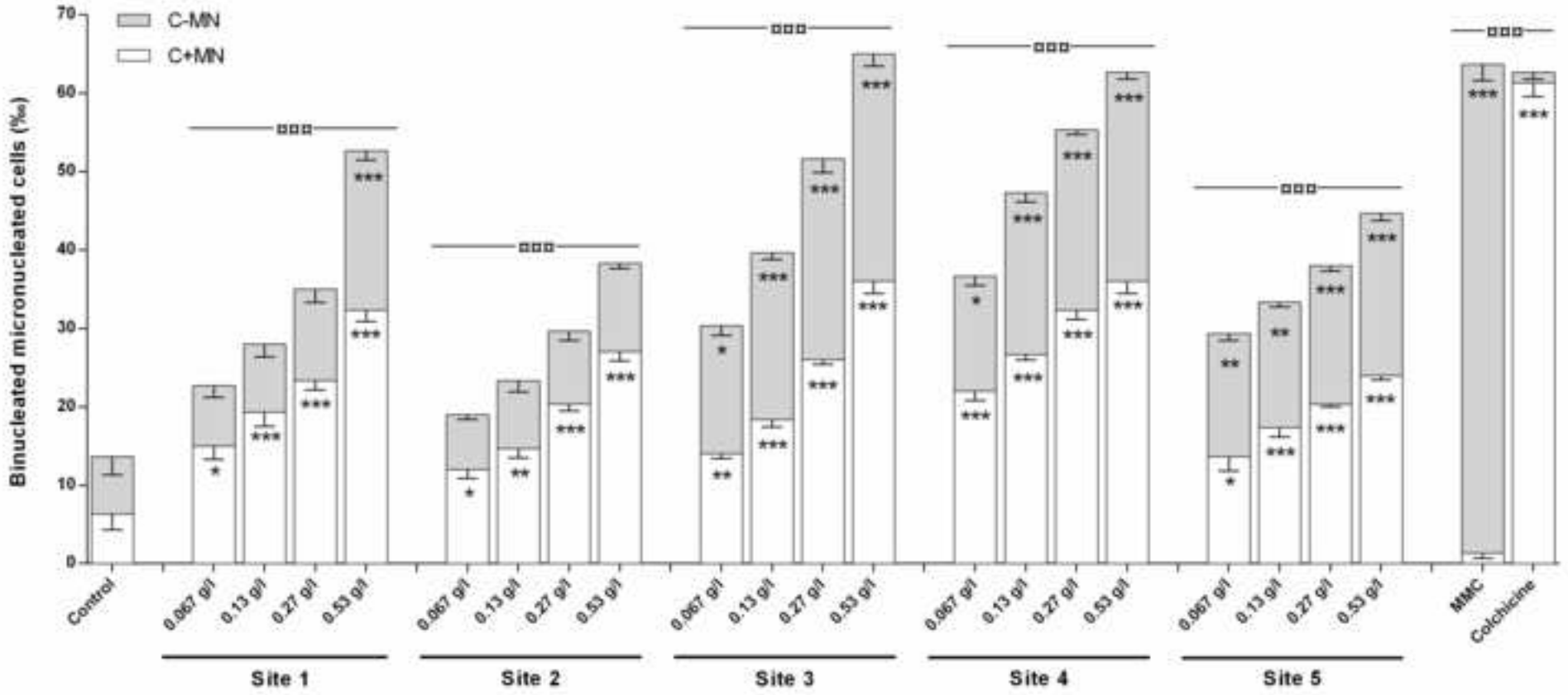

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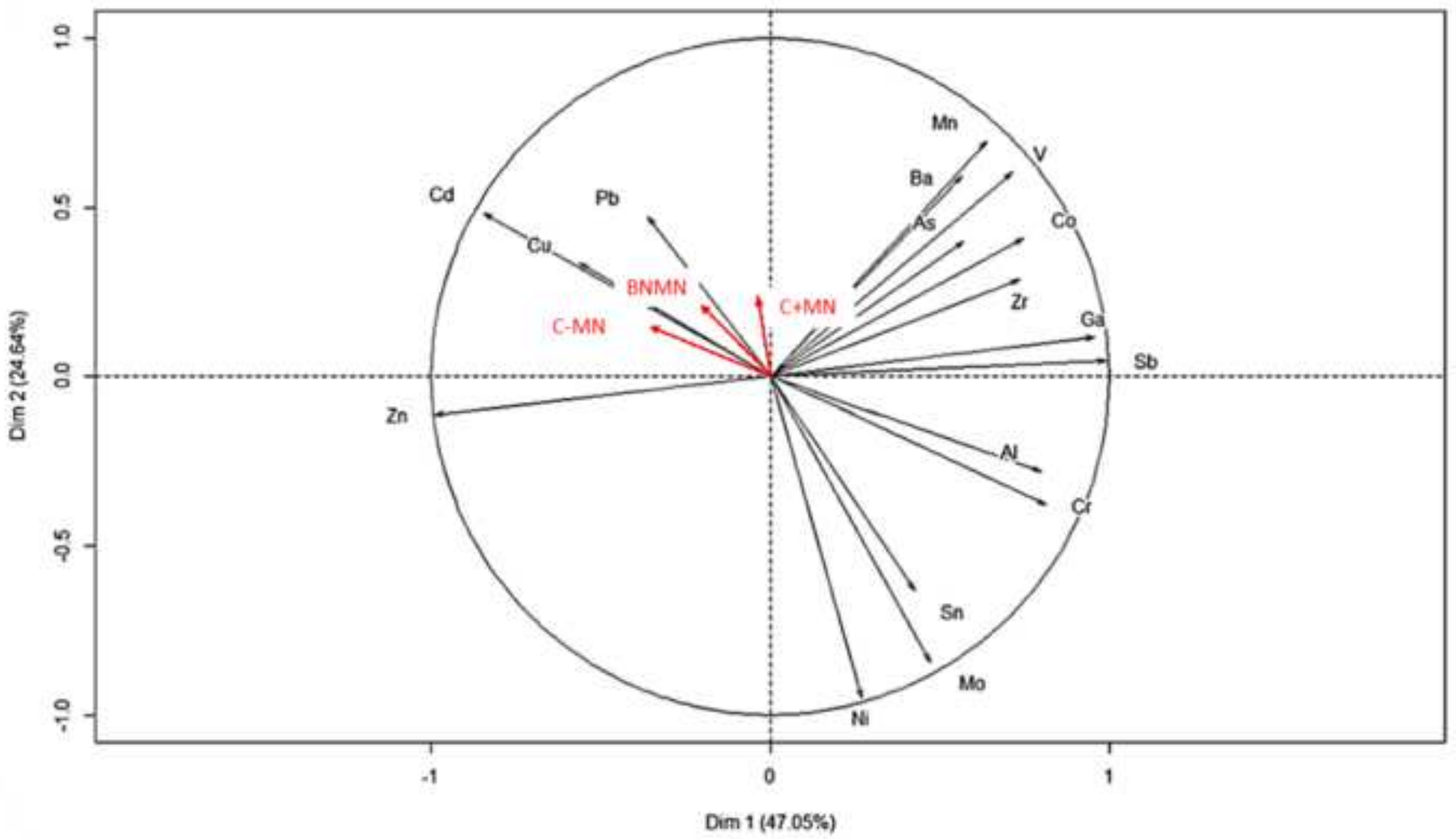

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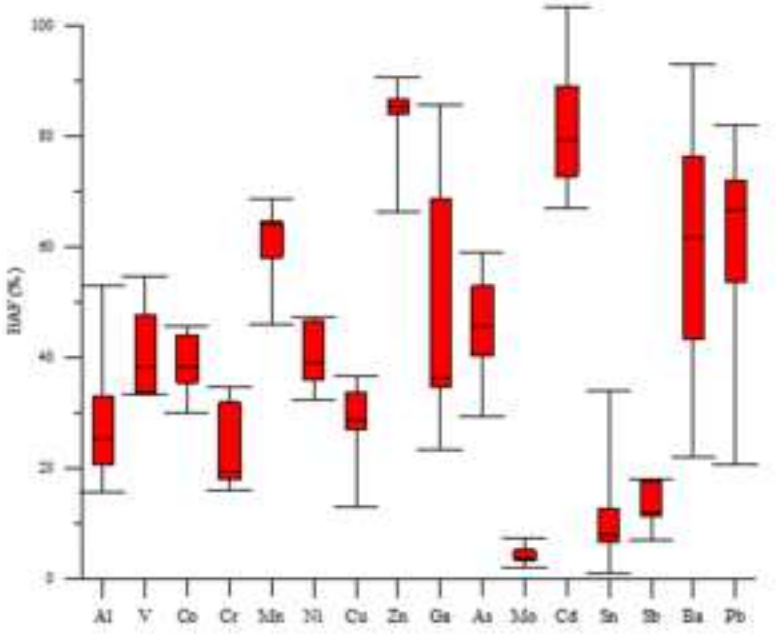

Table 1 PHEs concentrations (mg/kg) in house dust samples and comparison with other studies also focusing on house dust

Samples	Location	n	Al	Zn	Cu	Pb	Mn	Ba	Ni	Cr	Sn	V	As	Co	Sb	Mo	Ga	Cd
Site 1	Estarreja, Portugal		11100	1520	303	78	193	103	81	67	33	19	9	5.4	4.2	4.0	2.3	1.0
Site 2			16200	1110	339	147	296	71	70	77	19	23	19	6.6	7.1	4.7	4.0	1.8
Site 3			6800	5210	407	266	98	90	64	25	25	12	10	3.4	2.0	1.2	0.9	2.2
Site 4			15100	991	158	1180	228	71	65	61	13	23	14	5.0	4.6	2.7	3.1	1.2
Site 5			8300	1590	350	229	126	134	85	88	18	11	16	5.3	4.4	4.4	1.2	0.8
Mean (*)	Estarreja. Portugal	5	11500	20**90**	311	380	188	94	73	63	21	18	13	5.1	4.4	3.4	2.3	1.4
SD			41**10**	17**70**	94	453	79	26	10	24	8	6	4	1.2	1.8	1.5	1.3	0.6
% RSD			36	85	30	119	42	28	13	38	36	33	31	22	41	43	56	42
Median (**)			11100	1520	339	229	193	90	70	67	19	19	14	5.3	4.4	4.0	2.3	1.2
Min			6800	991	158	78	98	71	64	25	13	11	9	3.4	2.0	1.2	0.9	0.8
Max			16200	5210	407	1180	296	134	85	88	33	23	19	6.6	7.1	4.7	4.0	2.2
(Kurt-Karakus. 2012) **	Istanbul. Turkey	31		832	156	28	136		263	55				5.0				0.8
(Rasmussen et al.. 2001) **	Ottawa. Canada	50		633	157	222	267		52	69				8.8				4.3
(Chattopadhyay et al.. 2003) **	Sydney. Australia	82		372	93	76	48		15	65								1.6
(Lisiewicz et al.. 2000) **	Warsaw. Poland	27		1070	109	124			30	90								
(Chen et al.. 2014)**	Zi'an. China			400	67	145	547	865	34	129		68	12	40				
(Deschamps et al.. 2013)*	Brazil	50		242	64	29			108	546			45					

SD: standard deviation; RSD: relative standard deviation (= SD/mean*100); Min: minimum; Max: maximum; n: number of samples
* Value refers to the mean; ** Value refers to the median

Table 2 Bioaccessible PHEs concentrations (mg/kg) and PHEs bioaccessible fraction (BAF in %) determined in the gastric extracts

Samples	Al	Zn	Cu	Pb	Mn	Ba	Ni	Cr	Sn	V	As	Co	Sb	Mo	Ga	Cd
Site 1	25**60**	1260	82	52	98	45	32	16	3.8	6.4	3.6	2.0	0.5	0.2	0.8	0.7
	23	***83***	***27***	***67***	***51***	***43***	***39***	***23***	***12***	***34***	***40***	***37***	***12***	***4***	***35***	***69***
Site 2	27**60**	951	111	100	192	66	23	16	0.6	7.8	7.4	2.7	0.5	0.1	0.9	1.2
	17	***86***	***33***	***68***	***65***	***93***	***33***	***21***	***3***	***34***	***40***	***40***	***7***	***3***	***23***	***67***
Site 3	107**0**	44**50**	111	218	57	28	23	4.7	0.3	4.1	4.3	1.2	0.4	0.1	0.4	1.8
	16	***85***	***27***	***82***	***58***	***31***	***36***	***19***	***1***	***34***	***44***	***36***	***18***	***7***	***47***	***81***
Site 4	31**10**	658	58	245	157	49	21	9.8	1.2	11	8.3	2.1	0.5	0.1	1.1	0.9
	21	***66***	***37***	***21***	***69***	***70***	***33***	***16***	***9***	***48***	***59***	***41***	***11***	***3***	***35***	***80***
Site 5	43**90**	14**40**	46	165	81	29	35	15	1. 5	6.0	8.2	1.7	0.5	0.2	1.0	0.6
	53	***91***	***13***	***72***	***64***	***22***	***41***	***18***	***8***	***55***	***52***	***31***	***11***	***4***	***86***	***83***
Mean	27**80**	17**50**	82	156	117	44	27	12	1.5	7.1	6.3	1.9	0.5	0.1	0.9	1.1
	26	***82***	***27***	***62***	***61***	***52***	***36***	***19***	***7***	***41***	***47***	***37***	***12***	***4***	***45***	***76***
SD	11**90**	15**40**	30	80	56	16	6.3	5.0	1.6	2.6	2.2	0.6	0.0	0.1	0.3	0.5
	15	***10***	***9.1***	***24***	***7.0***	***29***	***3.6***	***2.7***	***4.5***	***9.9***	***8.3***	***3.9***	***4.0***	***1.6***	***24.3***	***7.4***
% RSD	43	88	36	51	48	36	23	41	106	36	36	29	8.9	55	30	44
	59	***12***	***34***	***38***	***12***	***56***	***10***	***14***	***64***	***24***	***18***	***11***	***33***	***41***	***54***	***10***
Median	27**60**	1260	82	165	98.	45	23	16	1.2	6.4	7.4	2.0	0.5	0.1	0.9	1.0
	21	***85***	***27***	***68***	***64***	***43***	***36***	***19***	***8***	***34***	***44***	***37***	***11***	***4***	***35***	***80***
Min	107**0**	658	46	52	57	28	21	4.7	0.3	4.1	3.6	1.2	0.4	0.1	0.4	0.6
	16	***66***	***13***	***21***	***51***	***22***	***33***	***16***	***1***	***34***	***40***	***31***	***7***	***3***	***23***	***67***
Max	43**90**	44**50**	111	245	192	66	35	16	3.8	11	8.3	2.7	0.5	0.2	1.1	1.8
	53	***91***	***37***	***82***	***69***	***93***	***41***	***23***	***12***	***55***	***59***	***41***	***18***	***7***	***86***	***83***

Bold and italic: bioaccessible fraction (BAF)

SD: standard deviation; RSD: relative standard deviation (= SD/mean*100); Min: minimum; Max: maximum

Table 3 Spearman's correlation coefficient and p values between chromosome damage and bioaccessible PHEs concentrations

	BNMN		C+MN		C-MN	
	r	**p-value**	**r**	**p-value**	**r**	**p-value**
Al	-0.14	2.71E-01	-0.14	2.99E-01	-0.13	3.16E-01
Zn	0.18	1.77E-01	0.00	9.84E-01	***0.34***	***7.07E-03***
Cu	-0.20	1.27E-01	-0.12	3.73E-01	-0.26	4.63E-02
Pb	***0.55***	***4.67E-06***	***0.32***	***1.23E-02***	***0.72***	***8.63E-11***
Mn	-0.17	1.98E-01	0.02	8.70E-01	-0.35	6.33E-03
Ba	-0.27	3.72E-02	-0.03	8.27E-01	-0.49	7.15E-05
Ni	-0.28	3.29E-02	-0.28	2.91E-02	-0.23	7.93E-02
Cr	-0.50	5.01E-05	-0.31	1.47E-02	-0.62	9.77E-08
Sn	-0.15	2.64E-01	0.01	9.54E-01	-0.29	2.41E-02
V	0.18	1.73E-01	***0.28***	***3.01E-02***	0.04	7.50E-01
As	0.05	7.31E-01	-1.74E-03	9.89E-01	0.09	4.97E-01
Co	-0.35	6.20E-03	-0.10	4.25E-01	-0.56	3.05E-06
Sb	-0.20	1.28E-01	-0.01	9.56E-01	-0.38	3.06E-03
Mo	-0.42	8.36E-04	-0.33	1.08E-02	-0.46	2.35E-04
Ga	-0.09	5.07E-01	0.01	9.33E-01	-0.18	1.68E-01
Cd	0.14	2.85E-01	0.05	6.98E-01	0.22	9.78E-02

Bold and italic: positive and significant (from $p < 0.05$) correlation

r: Spearman's correlation coefficient

Table S1 Values of precision (repeatability) for the 16 PHEs in the 19 houses sampled by Reis et al. (2015).

PHE	Precision (%)
Al	3.5
Zn	5.8
Cu	1.7
Pb	4.0
Mn	5.5
Ba	12.7
Ni	3.8
Cr	4.4
Sn	11.9
V	8.6
As	8.6
Co	2.8
Sb	7.0
Mo	10.7
Ga	7.2
Cd	8.2

Table S2 Summary statistics for concentrations of 16 PHEs in the indoor dust sampled in the 19 houses studied by Reis et al. (2015). All elements are expressed as mg/kg, with the exception of Al that is expressed as %.

	Minimum	Median	Mean ± Standard Deviation	Maximum
Al	0.7	1.0	1.0 ± 0.3	1.6
Zn	582.0	1110.0	1348.7 ± 1020.4	5210.0
Cu	148.0	210.0	260.5 ± 118.4	585.0
Pb	53.2	118.0	173.8 ± 250.2	1180.0
Mn	98.0	173.0	178.4 ± 58.5	304.0
Ba	51.0	76.0	81.0 ± 24.2	134.0
Ni	37.4	63.1	67.0 ± 21.2	117.0
Cr	24.5	72.3	70.6 ± 20.8	102.0
Sn	10.7	18.3	20.5 ± 7.9	36.6
V	10.0	15.0	15.4 ± 3.8	23.0
As	4.1	9.6	11.1 ± 4.1	18.7
Co	2.8	5.0	5.5 ± 1.9	12.0
Sb	2.0	4.9	6.9 ± 5.9	25.2
Mo	1.2	2.7	3.2 ± 1.7	8.1
Ga	0.9	2.3	2.2 ± 0.8	4.0
Cd	0.6	0.9	1.0 ± 0.4	2.2

IMBE – MEDITERRANEAN INSTITUTE OF MARINE AND TERRESTRIAL BIODIVERSITY AND ECOLOGY

DEPARTMENT OF BIOGENETOXICOLOGY, HUMAN HEALTH AND ENVIRONMENT

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Marseille, 26th September 2016

To the Editors of *Environmental Geochemistry and Health Journal*
Ref : EGAH-D-16-00043

Dear Editors,

Thank you for the opportunity to revise our manuscript entitled: **"Potentially harmful elements in house dust from Estarreja, Portugal: characterization and genotoxicity of the bioaccessible fraction"** (Ref: EGAH-D-16-00043).

The point-by-point answer to reviewers is given in a dedicated file. According to their requests, we modified the abstract, the Introduction, Materials and Methods, Results and Discussion sections, as well as the Tables 1 and 2. We also added supplementary data provided in tables S1 and S2, and in the figure S1.

We thank you for your consideration of this report, and look forward to your editorial decision.

On behalf of the coauthors, sincerely yours,

Best regards,

Thierry Orsière

Reviewer #2: This is a very interesting contribution combining geochemistry and genotoxicology and brings some new indications of effects caused by potential harmful elements (PHE) in urban/industrial dust. Given the fact that I am not a biologist, I admit that I am not able to fully evaluate sections devoted to genotoxicity. I have a number of comments specified below that need to be addressed in the revision. I think that the paper can be published after moderate revision.

Specific comments:

Abstract does not contain any results/data. Number of dust samples studied should be mentioned (in the third paragraph of the abstract authors say that "The five gastric extracts..."; thus numbers are important, they probably correspond to 5 samples of total set of 19 samples previously studied).
According to the reviewer, major data were added to the abstract. In order to be more comprehensible, we specified the number of house dust samples (page 2, line 9). In order to provide an abstract of less than 250 words, numerous minor modifications were made in the abstract.

Concentrations of PHEs should be mentioned.
The main goal of our work was to develop an interdisciplinary study involving chemical and in vitro biological characterization of PHEs contained in house dust. By performing all the methods required for this purpose on only 5 house dust samples, PHEs concentrations cannot be considered as representative of PHEs content in house dust of Estarreja. Therefore, we did not add PHEs concentrations in the abstract.

The major result, i.e. the role of Pb, which is highly developed in the paper (direct relationship to chromosome damage) is even not mentioned in the abstract.
Although lead was already mentioned in the abstract, we added data related to the relationship between chromosome damage and lead as follow:
“Lead (bioaccessible concentration up to 245 mg/kg) was the most prevalent PHE inducing chromosome-damaging effects (r = 0.55; $p < 0.001$ for micronucleated cells induction).”
(page 2, lines 16-17).

P3, para 2, L15: Aung et al. (2004) not given in the reference list.
According to the reviewer remark, we added the missing reference in the reference list:
“Aung, N.N., Yoshinaga, J., Takahashi, J., 2004. Exposure assessment of lead among Japanese children. Environmental Health and Preventive Medicine 9, 257–261. doi:10.1007/BF02898139”.
(page 20, lines 1-2).

P4, paras 3 & 4: I understand that some data on these dusts have already been published elsewhere, but at least some major results should be given here. Basic statistics of total and bioaccessible fractions in 19 studied dust samples could be reported in a table (min, max, median, mean, percentiles) or briefly mentioned in the text.

The publication of Reis et al (2015) mainly focused on only 2 PHEs: copper and manganese that were quantified in house dust, in gastric extracts and in toenail clippings. Although a

quantification of 53 chemicals contained in house dust was performed for this study, the publication of Reis et al (2015) did not report the corresponding data. We published for the first time these data for PHEs only.
In order to avoid any confusion, we modified the manuscript as follow:
“The relationships between biomarkers of exposure, levels of two PHEs (copper and manganese) in house dust and their bioaccessible fractions (BAF) were investigated in the Aveiro district (North of Portugal) (Reis et al., 2015).”
(page 4, lines 19-21)
As suggested by the reviewer, data on PHEs concentrations measured in the 19 houses are now provided as Table S2 in the Supplementary Material.
Moreover, data on the oral bioaccessibility of the PHEs are now provided as Figure S2 in the Supplementary Material.
The manuscript was modified as follow:
“The arithmetic means, geometric means and medians of all analyzed PHEs in the 5 studied dust samples were calculated. Table 1 shows values for the 16 PHEs under study and compares the PHEs concentrations with those reported in the literature (Chattopadhyay et al., 2003; Chen et al., 2014; Deschamps et al., 2013; Kurt-Karakus, 2012; Lisiewicz et al., 2000; Rasmussen et al., 2001). Further data on PHEs concentrations in the full set of indoor dust samples collected from 19 private households in the city of Estarreja are provided in the form of supplementary material (Table S2).”
(page 10, lines 14-19).
“Oral bioaccessibility data for the set of 19 samples are presented in supplementary material (Figure S1), while Table 2 shows the results obtained for the subset of 5 samples used in this study.”
(page 11, lines 11-13).
Finally, we added a sentence to mention that the bioaccessible fractions data obtained in the 5 houses selected in this study and in the 19 houses studied in Reis et al. (2015) were very closed:
“The overall assessment of the results (Table 2 and Fig. S2) shows that the trends in the oral bioaccessibility of the PHEs are the same in the selected subset and in the broader set of samples subjected to the UBM protocol.”
(page 11, lines 20-22).

Saying that "PHEs were characterized using ICP-MS..." should be deleted from this introduction part as the details are given later in the text.
In the end of the Introduction section, we have just mentioned the name of each method used as our goal was to propose a new methodology linking chemical characterization, bioaccessibility and genotoxicity. Therefore, the name of the methods was not deleted.

P5, chapter 2.2: The rationale for selecting 5 dust samples studied here should be better explained!!!
According to the reviewer comment, the manuscript was modified as follow:
“To carry out the present study on the genotoxic potential of the house dust bioaccessible fractions, 5 out of 19 houses dust sampled by Reis et al. (2015) were selected to be representative of the various sources of PHEs on Estarreja. Indeed, the 5 selected sites were far from each other (Fig.1) compared to the geographical distribution of the sampled sites of Reis et al. (2015).”

(page 5, lines 20-23).

P6, chapter 2.3.1: Give more details for certified reference materials. What are these GRX-1, GRX-6 and SAR-M materials? Give QC/QA data in the Supplement to help the reader to understand the accuracy of the analytical procedures.

The United States Geological Survey (USGS) Geochemical Exploration Reference Materials chose GXR-1 to GXR-4 and GXR-6 as different soil and/or materials from various US areas, while SAR-M is a composite of contaminated sediment from the Animas River watershed in Colorado. These were considered to represent a wide range of total elemental concentrations in relevant matrices. Further QA/QC data are provided in the form of Table 1 of the supplementary material.

The manuscript was modified as follow:

"The Certified Reference Materials GRX-1, GRX-4, GRX-6 and SAR-M (United States Geological Survey) were selected to represent a wide range of total elemental concentrations. The blanks results were always below detection limits. In this study, we focused on the 16 following PHEs: Al, Zn, Cu, Pb, Mn, Ba, Ni, Cr, Sn, V, As, Co, Sb, Mo, Ga, and Cd. Values for precision (expressed as RSD %) are typically < 15 % for all elements. Further information on QA/QC is provided as supplementary material (Table S1)."

(page 6, lines 8-13).

P11 and 12: Why simple calculations of exposures were not performed for the major PHEs (e.g. calculating exposures to 60 mg dust per day and comparisons with tolerable daily intake limits) to have an idea about the extent of potential risk.

As already mentioned, we consider that house dust PHEs concentrations generated from 5 sites cannot be used for assessing exposure levels in Estarreja. As indicated at the end of the Abstract, "This methodology could be used on a larger scale study to provide useful information for science-based decision-making in regulatory policies, and a better estimation of human exposure and associated health risks."

I am also convinced that generalities about "priority pollutants" (P12, para 1 on cadmium) have nothing to do in the Results section and should be moved to Discussion.

According to the reviewer comment, the results section relative to cadmium was modified as follow:

"The biogeochemistry of Cd, which is currently ranked seventh on the Priority List of Hazardous Substances by the Agency for Toxic Substances and Diseases Registry (ATSDR 2013), was also noteworthy."

(page 12, lines 13-15).

We did not mentioned Cadmium in the discussion section as no significant effect was associated with this PHE in the present study.

P17, para 3: I cannot judge, if these results aren't only speculations: there is a lot of "probably", based only on the statistical data treatment. Maybe reformulation of these major results could help.

In the paragraph 3 on page 17, according to the reviewer suggestion, we have modified the sentences containing "probably" as follow:

“Although gastric extracts containing numerous elements were assessed, Cu is thought to be the prevalent PHE inducing primary DNA damage, while Pb was the most prevalent PHE inducing chromosome-damaging effects.”
(page 18, lines 10-12)

P18, last para: It is great that these genotoxicological methods have finally been combined with geochemical in vitro testing. However, I think that the results cannot "provide information on PHEs sources" as the authors claim.
According to the reviewer comment, we suppressed "provide information on PHEs sources". Further, the corresponding paragraph was extensively modified according to the following comment.

Maybe also a financial outlook could be given here to have an idea, how accessible this method could be for researchers, who have been using only leaching in simulated body fluids so far.
According to the reviewer comment, the accessibility of the methods has been mentioned as follow:
“Further, the use of the UBM and of these two in vitro genotoxicity assays can be performed in few weeks and are rather cheap (about 1,000€ per site), compared to in vivo assays.”
(page 19, lines 3-4).

P19, para 2. This paragraph is containing repetitive information. Maybe rewriting could be suggested here.
According to the reviewer comment, we modified the two last paragraphs of the Conclusion section in order to delete repetitive information, as follow:
“The option of selecting house dust samples with PHEs concentrations in the range of the current EU soil screening values for potentially unacceptable risk, unraveled the potential of this methodology to provide further information that can be used for science-based decision-making in regulatory policies, such as deriving soil screening values that are currently lacking in Portugal. By developing this methodology in broader studies, encompassing larger areas and comprehensive datasets, we should be able to correlate house dust PHEs concentrations with the physical environment of the house, as well as with exterior anthropogenic contributions. Establishing links between site characteristics, PHEs concentrations and their speciation, bioaccessibility and genotoxicity is likely to provide an accurate characterization of sources, pathways and fate of environmental PHEs, enabling more effective assessment of human exposure and associated health risks.”
(page 19, lines 8-24).

Tables: Give numbers to 3 digits, i.e. 2755 mg/kg should be given as 2780 mg/kg.
According to the reviewer comment, tables 1 and 2 were corrected and the numbers given with 3 digits. Corrections appear in bold in the tables.

Reviewer #3: The paper is well written with thorough coverage of the background material the methods, statistical analysis and interpretation. I cannot comment on the genotoxicity study as it is outside of my area of expertise.

Specific comments:

Page 12 line 8 should be "Regardless of"
According to the reviewer comment, we replaced “Regardless” by “Regardless of”.
(page 12, line 21).

Bottom of page 14 the opposite projection of Al-Cr relative to Cd-Cu is likely to be a consequence of the closed nature of elemental compositions (add up to 100%), i.e. the more Al there is the less Cd-Cu can be present. I would be careful about saying that Al has an inverse relationship with chromosome damage.
According to the reviewer comment, the corresponding sentence was modified as follow:
“However, the diametric opposite projection of Al-Cr relative to Cd-Cu; and Ni-Mo-Sn relative to Pb suggested that these elements were less abundant and, therefore, not involved in chromosome damage induction.”
(page 15, lines 9-12).

Page 15 third paragraph line 4 "Pb Thus" appears twice
According to the reviewer comment, we removed “Thus Pb”, that was appearing twice.
(page 15, line 28).

Page 15 end of third paragraph should be "extent" not "extend"
We agree with the reviewer, and replace “extend” by “extent” twice.
(page 16, lines 4-5).