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Suitability of salivary leucocytes to assess DNA repair ability in human biomonitoring studies by the challenge-comet assay

Natalia Fernández-Bertólez [a,b,1], Carlota Lema-Arranz [a,b,c,1], Sónia Fraga [d,e,f], João Paulo Teixeira [d,e,f], Eduardo Pásaro [b,c], Laura Lorenzo-López [g], Vanessa Valdiglesias [a,b,*,2], Blanca Laffon [b,c,2]

[a] Universidade da Coruña, Grupo NanoToxGen, Centro de Investigacións Científicas Avanzadas (CICA), Departamento de Biología, Facultad de Ciencias, Campus A Zapateira s/n, 15071, A Coruña, Spain
[b] Instituto de Investigación Biomédica de A Coruna (INIBIC), Oza, 15071, A Coruna, Spain
[c] Universidade da Coruña, Grupo DICOMOSA, Centro de Investigacións Científicas Avanzadas (CICA), Departamento de Psicología, Facultad de Ciencias de la Educación, Campus Elviña s/n, 15071, A Coruña, Spain
[d] Department of Environmental Health, Portuguese National Institute of Health, Rua Alexandre Herculano, 321, 4000-055, Porto, Portugal
[e] EPIUnit—Instituto de Saúde Pública, Universidade do Porto, Rua das Taipas, nº 135, 4050-600, Porto, Portugal
[f] Laboratório para a Investigação Integrativa e Translacional em Saúde Populacional (ITR), Porto, Portugal
[g] Universidade da Coruña, Gerontology and Geriatrics Research Group, Instituto de Investigación Biomédica de A Coruña (INIBIC), Complexo Hospitalario Universitario de A Coruña (CHUAC), Servizo Galego de Saúde (SERGAS), A Coruña, Spain

HIGHLIGHTS

- Objective: to validate the use of salivary leucocytes in the challenge-comet assay.
- DNA damage of different nature was repaired over the evaluated time points.
- Results demonstrated that salivary leucocytes were as suitable as PBMC for this assay.
- Results were similar in fresh and after 5 months of cryopreservation.
- Recommendations are given depending on the type of DNA repair to be assessed.

GRAPHICAL ABSTRACT

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ABSTRACT

The challenge-comet assay is a simple but effective approach that provides a quantitative and functional determination of DNA repair ability, and allows to monitor the kinetics of repair process. Peripheral blood mononuclear cells (PBMC) are the cells most frequently employed in human biomonitoring studies using the challenge-comet assay, but having a validated alternative of non-invasive biomatrix would be highly convenient for certain population groups and circumstances. The objective of this study was to validate the use of salivary leucocytes in the challenge-comet assay. Leucocytes were isolated from saliva samples and challenged (either in fresh or after cryopreservation) with three genotoxic agents acting by different action mechanisms: bleomycin,

* Corresponding author. Facultad de Ciencias, Campus A Zapateira s/n, 15071, A Coruña, Spain.
E-mail address: vvaldiglesias@udc.es (V. Valdiglesias).
[1] Both should be considered first authors.
[2] Both should be considered senior authors of this manuscript.

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methyl methanesulfonate, and ultraviolet radiation. Comet assay was performed just after treatment and at other three additional time points, in order to study repair kinetics. The results obtained demonstrated that saliva leucocytes were as suitable as PBMC for assessing DNA damage of different nature that was efficiently repaired over the evaluated time points, even after 5 months of cryopreservation (after a 24 h stimulation with PHA). Furthermore, a new parameter to determine the efficacy of the repair process, independent of the initial amount of damage induced, is proposed, and recommendations to perform the challenge-comet assay with salivary leucocytes depending on the type of DNA repair to be assessed are suggested. Validation studies are needed to verify whether the method is reproducible and results reliable and comparable among laboratories and studies.

Abbreviations

%tDNA	percentage of DNA in the comet tail
%RC	percentage of repair capacity
AR	after repair
BER	base excision repair
BLM	bleomycin
BR	before repair
DAPI	4′,6-diamidine-2′-phenylindole
DDR	DNA damage response
DSBR	double strand break repair
dH_2O	distilled water
DSB	double strand breaks
FBS	foetal bovine serum
MMS	methyl methanesulfonate
NER	nucleotide excision repair
net%RC	net percentage of repair capacity
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline solution
PHA	phytohaemagglutinin
PI	propidium iodide
SSB	single strand breaks
SSC	side scattering
$t_{1/2}$	time point at which 50% of the DNA damage induced is repaired
UV	ultraviolet

1. Introduction

Living cells are constantly challenged by exogenous and endogenous agents that can damage DNA and alter its structure, potentially affecting the interpretation and transmission of genetic information. Lesions induced – at a rate of tens of thousands per day in each human cell (Lindahl and Barnes, 2000) – include altered bases, single (SSB) and double (DSB) strand breaks, bulky adducts, inter- or intra-strand DNA-DNA cross-links, and DNA-protein cross-links, in addition to spontaneous loss of bases (Lindahl and Nyberg, 1972) and replication errors. Since preservation of genetic information is crucial, eukaryotes have evolved the DNA Damage Response (DDR), a complex network of mechanisms that sense DNA damage, signal its presence and promote subsequent repair (Ciccia and Elledge, 2010; Huang and Zhou, 2021). As a part of the DDR, DNA repair systems are responsible for amending the different types of DNA lesions before they are fixed and become permanent, so that damage level remains low at the steady state, ensuring an overall survival.

Deregulation or disruption of DDR pathways increases mutagenesis and genomic instability. In fact, mutations affecting DDR machinery components are the cause of several cancer predisposition syndromes, and impaired DNA repair is associated with cancer initiation (Kiwerska and Szyfter, 2019; Vodicka et al., 2019) and immunodeficiency disorders (Tiwari and Wilson, 2019). Likewise, aging is related to failing capacities of a combination of DNA repair pathways (Chatterjee and Walker, 2017). Besides, increasing evidence supports the direct relationship between DNA repair capacity and tumour resistance or hypersensitivity to chemotherapy and radiotherapy (Li et al., 2021), and also that disruptions in repair pathways are likely to contribute to the onset and progression of neurodegenerative disorders, and maybe other neuropsychiatric disorders (major depression, bipolar disorder, schizophrenia) (reviewed in Czarny et al., 2020). Indeed, it has been recommended that molecular epidemiological studies include the assessment of individual's ability to remove DNA damage, in order to complement the interpretation of DNA damage endpoints and the individual's risk to develop pathologies related to genetic damage (Opattova et al., 2022), e.g. that induced by environmental or occupational exposures.

The challenge assay is a simple but effective approach that provides a quantitative and functional determination of individual's DDR capacity. This assay was developed to determine inherent and toxicant-provoked reduction in DNA repair capacity among healthy individuals, individuals exposed to toxicants/mutagens and cancer patients (reviewed in Kaina et al., 2018). The rationale behind this assay is to induce genetic damage in cells with a known genotoxic (challenge) agent and to determine the damage remaining after a certain repair period (Au, 1993). Genetic damage was initially measured by the chromosome aberrations test (reviewed by Au and Salama, 2006), but the assay was later modified to adopt the comet assay (challenge-comet assay), thus providing the additional advantage of allowing to monitor the kinetics of repair activities (by collecting data at multiple time points during the repair period). The outcomes from both assays have been reported to be comparable (Cebulska-Wasilewska et al., 2005). The comet assay (also known as single-cell microgel electrophoresis) is a sensitive, rapid and simple technique for detecting DNA damage at the level of individual cells (Singh et al., 1988). It is extensively used in *in vitro* and *in vivo* studies, and well established as a useful biomonitoring marker of exposure to genotoxic agents. Moreover, evidence has been recently provided that the level of DNA damage in circulating leukocytes of healthy individuals may be predictive of the risk of chronic diseases and mortality, reflecting events such as accelerated aging, telomere capping loss, oxidative stress and more generally genomic instability (Bonassi et al., 2021). Already in the dawn of the comet assay, Ostling and Johanson (1984) and Singh et al. (1988) demonstrated the potential of this technique to assess DNA repair, by following the decrease of DNA damage over time in cells challenged with ionizing radiation. The challenge-comet assay has been proposed as a useful tool to document the DNA repair phenotype in cancer patients, to identify susceptible individuals, e.g. children receiving radiotherapeutic treatment, and to allow a preventive surveillance for radiation-associated tumour development (reviewed in Decordier et al., 2010). Furthermore, it has proven to be useful and sensitive for studying the modulation of DNA repair by environmental exposures, nutritional factors, and disease state (reviewed in Azqueta et al., 2019a).

Peripheral blood mononuclear cells (PBMC, sometimes referred to as lymphocytes, although monocytes are also present up to about 20% of the mononuclear fraction (Kleiveland, 2015) are the cell type most

frequently used in human biomonitoring studies for measuring phenotypic biomarkers of DNA repair, and specifically in the challenge-comet assay. Recently, we optimized the challenge-comet assay protocol for whole blood samples, either fresh or cryopreserved, and demonstrated that their DNA repair abilities were comparable to those found in isolated PBMC (Valdiglesias et al., 2020). Nevertheless, obtaining whole blood samples requires the participation of a healthcare professional and involves a minimally invasive procedure, which may be problematic for certain population sectors, such as individuals with capillary fragility (cancer patients, older adults), children, or dementia patients. Furthermore, the blood sample volume that can be collected in a population study is limited, and usually must be employed for the analysis of a wide range of biomarkers. In those cases, it is of undoubted relevance to have a validated alternative of non-invasive biomatrix available for measuring individual capacity to repair DNA.

Since the suitability of fresh and frozen saliva leucocytes to be employed to detect both primary and oxidative DNA damage was recently demonstrated (Fernández-Bertólez et al., 2021), the main objective of this study was to validate the use of these cells for assessing DNA repair ability by means of the challenge-comet assay. To that aim, leucocytes were isolated from saliva samples taken from ten donors, and they were challenged (either in fresh or after cryopreservation) with three genotoxic agents acting by different action mechanisms: bleomycin (BLM), methyl methanesulfonate (MMS), and ultraviolet (UV) radiation. DNA damage was determined by the alkaline comet assay just after treatment and at other three additional time points, in order to study repair kinetics.

2. Materials and methods

2.1. Chemicals

BLM (CAS No. 11056-06-7), MMS (CAS No. 66-27-3), Histopaque®-1077 sterile-filtered, dimethyl sulfoxide ACS reagent ≥99.9% (DMSO) (CAS No. 67-68-5), propidium iodide (PI) (CAS No. 25535-16-4), and 4′,6-diamidine-2′-phenylindole (DAPI) (CAS No. 28718-90-3) were purchased from Sigma-Aldrich Co. (Madrid, Spain). RPMI 1640, DMEM, heat-inactivated foetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, and phytohaemagglutinin (PHA) were obtained from Gibco (Thermo Fisher Scientific, Madrid, Spain), and Triton X-100 (CAS No. 9036-19-5) from Panreac AppliChem (Barcelona, Spain). BLM and MMS solutions were prepared in sterile distilled water (dH_2O).

2.2. Sample collection and processing

Participants of this study were ten healthy non-smoking volunteers (six women and four men, aged between 18 and 54) with no known recent exposure to genotoxic chemicals or radiation. This study followed ethical criteria established by the Helsinki declaration and was approved by the University of A Coruña Ethics Committee (2021–0027). Each donor signed an informed consent prior to joining the study, and they were asked not to eat or drink anything but water in the hour before sampling. For each experiment, saliva samples were collected from each donor by performing four consecutive mouth rinses for 1 min each with 10 mL of 0.9% NaCl sterile solution.

Oral rinses from each individual (~40 mL) were centrifuged at 1100×*g* and 4 °C for 15 min. After removing the supernatant, 2 mL of phosphate-buffered saline solution (PBS) were added to the cell pellets. Cell suspensions from the ten participants were pooled together and centrifuged again at 1100×*g* and 4 °C for 15 min. Eight mL of RPMI 1640 were added to the cell pellet, and Histopaque®-1077 was used to isolate leucocytes by density gradient centrifugation, according to the manufacturer's instructions. In brief, the cell suspension was carefully layered over 4 mL Histopaque®-1077, and centrifuged with brake turned off at 400×*g* for 30 min. After gently withdrawing the upper layer, leaving the cell pellet (epithelial cells) and the interface (leucocytes) undisturbed, the interface was removed with a sterile Pasteur pipette and transferred to another tube, 5 mL PBS were added, and a new centrifugation was conducted at 1100×*g* for 15 min.

Saliva leucocytes intended to be used after cryopreservation were resuspended at 2.5 × 10^6 cells/mL in freezing medium [50% foetal bovine serum, 40% RPMI 1640, and 10% DMSO] and frozen at −80 °C in cryogenic vials, for five months. At the moment of their use, cells were quickly thawed at 37 °C and the freezing medium was removed by centrifugation at 1100×*g* for 15 min. Procedures carried out with fresh and cryopreserved samples were conducted consecutively in time (total time of analyses 2 months) to minimize the potential inter-assay variability due to seasonal influence.

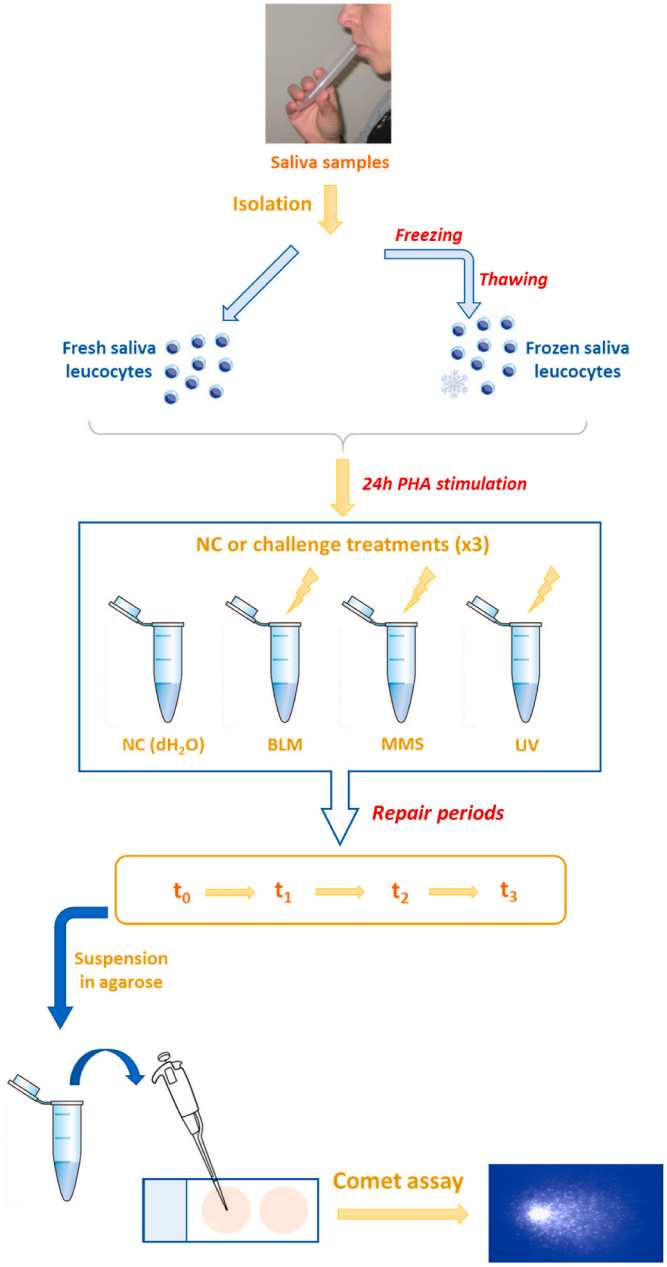

Fig. 1. Scheme of the study design. Leucocytes were isolated from saliva samples and used either in fresh or after cryopreservation. After a 24 h incubation period in the presence of phytohaemagglutinin (PHA), cells were treated with bleomycin (BLM), methyl methanesulfonate (MMS) or ultraviolet (UV) light. Immediately after exposure (t_0) or after three different repair periods (t_1-t_3), leucocytes were suspended in low melting point agarose and standard alkaline comet assay was conducted. NC: negative control.

A complete overview of the study design is depicted in Fig. 1.

2.3. Challenge treatments

In order to assess DDR ability, both fresh and cryopreserved saliva leucocytes were subjected to challenge treatments, and the remaining DNA damage was determined at four different time points by means of the standard alkaline comet assay, as detailed below.

Fresh or thawed saliva leucocytes were resuspended in 0.5 mL culture medium composed of RPMI 1640 containing 15% FBS, 1% penicillin (5000 U/mL)/streptomycin (5000 μg/mL), 1% L-glutamine (200 mM), and 1% PHA, and incubated for 24 h at 37 °C for cell cycle stimulation. Then, cell suspensions were treated at 1% final volume for 30 min at 37 °C with 10 or 20 μg/mL BLM, and 50 or 75 μg/mL MMS. For UV light exposure (254 nm), cells were seeded in 24-well plates placed on ice and irradiated in a CL-1000 Ultraviolet Crosslinker (UVP) with 200 or 300 μJ/cm^2. Negative controls (dH_2O) were included in each experiment.

2.4. Cell viability

Cell viability was checked after treatments and prior challenge-comet assay by flow cytometry using propidium iodide (PI) as vital dye. Briefly, an aliquot of each sample containing 1×10^5 treated cells, was transferred to replicated polystyrene flow cytometry tubes (5×10^4 cells), centrifuged at 1100×*g* for 15 min, and washed in PBS. Cell pellets were then resuspended in 300 μL PBS containing 10 μg/mL PI (final concentration) and incubated 5 min in the dark on ice. Immediately after the incubation period, flow cytometry analysis was conducted in a FACSCalibur flow cytometer (Becton Dickinson, Madrid, Spain). The leucocyte population was gated according to complexity (side scattering, SSC) and size (forward scattering). At least 15,000 events in the leucocyte region were acquired in bidimensional dot plot diagrams, obtaining data from SSC *vs*. FL2 (PI) detectors. The software Cell Quest Pro (Becton Dickinson, Madrid, Spain) was used to analyse the data; total leucocyte population was separated along the PI axis into PI low (viable) and PI high (non-viable) uptake regions. For appropriately gating the viable cells region, untreated control samples were employed. Triton X-100 (1%) was used as positive control. Percent viability was defined as 100 × PI low events/(PI low + PI high events).

2.5. Challenge-comet assay

This manuscript follows the Minimum Information for Reporting Comet Assay procedures and results (MIRCA) recommendations (Møller et al., 2020). At the end of the challenge treatments, cells were centrifuged at 1100×*g* for 15 min and washed with PBS. Comet assay was immediately performed with one fourth of the cells ("repair time 0", t_0) (Fig. 1). The remaining salivary leucocytes were resuspended in fresh culture medium and incubated at 37 °C to allow DNA repair for 3 additional periods (t_1-t_3). Specifically, repair times for BLM were 15, 60 and 120 min, for MMS were 10, 30 and 120 min, and for UV radiation were 60, 90 and 180 min. Treatment doses, exposure times and repair times were selected on the basis of previous studies (Cipollini et al., 2006; Sánchez-Flores et al., 2015; Valdiglesias et al., 2020; Yamauchi et al., 2002). These conditions should not be cytotoxic (at least 80% viability) but able to induce significant increases in the DNA damage, and provide appropriate time-frames to demonstrate DNA repair activity. Moreover, the repair times were chosen considering the type of DNA damage induced by each genotoxic agent, as those previously reported as necessary to complete the repair process of the damage induced.

When each repair time was finished, cells were centrifuged at 8700×*g* for 3 min and washed in PBS. After removing the supernatant, 160 μL of 0.8% low-melting-point agarose (LMA), freshly prepared in PBS (pH 7.4), was added to 20 μL of the remaining cell suspension. This suspension was placed as two drops of 80 μL onto a microscope slide previously pre-coated with a layer of 1% normal melting point agarose. Each drop was covered with a 20 × 20 mm coverslip. Slides were placed on ice for 15 min to allow solidification of the agarose. Coverslips were removed, and slides were immersed overnight at 4 °C in the dark in freshly prepared lysis solution (250 mM NaOH, 100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Tris-HCl, pH 10, with 1% Triton X-100 added just before use).

Slides were immersed in freshly made alkaline electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) on a horizontal electrophoresis tank in an ice bath, and incubated in the dark for 20 min to allow DNA unwinding. Electrophoresis was conducted at 0.83 V/cm for 20 min. Slides were washed (3 × 5 min) with neutralizing solution (0.4 M Tris–HCl, pH 7.5) and air-dried in the dark at room temperature. To prevent drying of the gel, the preparations were kept in a humidified sealed box, and were scored within six days. Staining was performed with 60 μl of 5 μg/mL DAPI, for at least 30 min before analysis. All slides were coded to ensure a blind study.

Image capture and analysis was conducted by a single scorer using the Comet IV Software (Perceptive Instruments). At least 50 cells were scored from each drop (100 cells per slide) using a magnification of 40x. The percentage of DNA in the Comet tail (tail intensity, %tDNA) was considered as DNA damage parameter. Percentage of repair capacity (% RC) at the latter repair period was calculated following Cebulska-Wasilewska (2003), according to the formula:

$$\%RC = (\%tDNA_{X\text{-}BR} - \%tDNA_{X\text{-}AR}) \times 100 / \%tDNA_{X\text{-}BR}$$

where "X" is the genotoxic agent used as challenge, "BR" is before repair (%tDNA at t_0 for BLM and MMS, and at t_1 for UV since UV-induced DNA damage is mostly manifested approximately 1 h after exposure) and "AR" is after repair (%tDNA at t_3). Furthermore, a new formula to calculate net %RC (net%RC) is proposed as follows:

$$net\%RC = (Induced\ damage_{X\text{-}BR} - Induced\ damage_{X\text{-}AR}) \times 100 / Induced\ damage_{X\text{-}BR}$$

where $Induced\ damage_{X\text{-}BR} = \%tDNA_{X\text{-}BR} - \%tDNA_{Control\text{-}BR}$, and $Induced\ damage_{X\text{-}AR} = \%tDNA_{X\text{-}AR} - \%tDNA_{Control\text{-}AR}$.

For each genotoxic agent and dose tested, the time point at which 50% of the DNA damage induced is repaired ($t_{1/2}$) was also calculated.

In order to control inter-experimental variation, a positive reference standard (also called assay control) was included in all experiments, according to recommendations from Azqueta et al. (2019b). It consisted of an aliquot from a single batch of human A172 glioblastoma cells treated with 100 μg/mL MMS for 3 h, suspended in freezing medium composed of 50% FBS, 40% DMEM, and 10% DMSO, aliquoted and cryopreserved at −80 °C. Indications from Collins et al. (2014) were followed to calculate the correction factor for normalization. Correction factor ranged between 0.97 and 1.02 in all cases.

2.6. Statistical analysis

Statistical analyses were conducted using SPSS for Windows statistical package V. 21. For each experimental condition tested, a minimum of three independent experiments with duplicate tests were performed. Mean ± standard error were used to express data from independent experiments. Differences among groups were analysed with Kruskal–Wallis test. Two-by-two comparisons were assessed with Mann–Whitney *U* test. In leucocytes treated with the challenge agents, existence of significant differences was tested as follows: (i) differences between exposed cells and control cells at t_0 (or t_1 in the case of UV radiation), to demonstrate induction of DNA damage; (ii) differences between t_1-t_3 and t0 (or t_2-t_3 and t0 for UV light), to demonstrate reduction in DNA damage during the repair period; and (iii) differences between exposed t_3 and control t_3, to check complete repair of DNA damage induced. Pearson's correlation was employed to determine linear dose-response relationships. Significance was set at *P*-value

<0.05.

3. Results

In this study, suitability of salivary leucocytes, both fresh and after cryopreservation, to be used in the challenge-comet assay for evaluating DNA damage repair capacity was tested, using BLM, MMS and UV radiation as challenge agents, and four different time points to follow-up repair kinetics.

Cell viability was checked after treatments (PI exclusion evaluated by flow cytometry), in order to ensure low cytotoxicity to avoid false positive results in DNA damage evaluation (Fig. S1). No significant differences were observed in any case with regard to the negative control, and percentage of cell viability was in all cases higher than 80%, and in most cases higher than 90%. According to recent recommendations by Azqueta et al. (2022), the threshold of 25% cytotoxicity to avoid false positive results in the comet assay was not reached.

Fig. 2 shows the results obtained with BLM treatments. Both concentrations tested induced a significant increase in the DNA damage over the control at t_0, notably higher in frozen than in fresh leucocytes. The damage induced progressively and significantly decreased with time in a linear way, returning to control values at t_3 (120 min) in all cases ($r = -0.956$, $P < 0.01$ for 10 μg/mL, and $r = -0.973$, $P < 0.01$ for 20 μg/mL in fresh cells, and $r = -0.986$, $P < 0.01$ for 10 μg/mL, and $r = -0.970$, $P < 0.01$ for 20 μg/mL in frozen cells).

Data from experiments with MMS are depicted in Fig. 3. Similar to what occurred with BLM, increases in %tDNA induced at t_0 were more intense in frozen saliva leucocytes than in their fresh counterparts. DNA damage values dropped gradually with time following a linear kinetics ($r = -0.961$, $P < 0.01$ for 50 μg/mL, and $r = -0.964$, $P < 0.01$ for 75 μg/mL in fresh cells, and $r = -0.824$, $P < 0.01$ for 50 μg/mL, and $r = -0.850$, $P < 0.01$ for 75 μg/mL in frozen cells). At the end of the repair period (120 min), no significant differences were detected between the MMS treated leucocytes and control leucocytes, indicating return of the exposed cells to control values (total repair).

Results obtained with UV exposed cells were similar in fresh and frozen salivary leucocytes (Fig. 4). As expected, significantly raised % tDNA values were obtained 1 h after the exposure (Henriksen et al., 1996; Myllyperkiö et al., 2000). Continuous descending DNA damage was then observed over time, according to a time-response linear kinetics ($r = -0.836$, $P < 0.01$ for 200 μJ/cm^2, and $r = -0.839$, $P < 0.01$ for 300 μJ/cm^2 in fresh cells, and $r = -0.934$, $P < 0.01$ for 200 μJ/cm^2, and $r = -0.920$, $P < 0.01$ for 300 μJ/cm^2 in frozen cells). Complete repair for both doses was ascertained at 180 min.

Fig. 5 gathers previous results obtained in cryopreserved PBMC (the gold standard cells for this technique) subjected to the same treatments and experimental conditions used in the current work (Valdiglesias et al., 2020), in order to compare with current results in saliva leucocytes. Clear similarities can be observed between the two types of leucocytes, although PBMC did not repair completely the DNA damage induced by BLM and MMS at the end of the repair period, i.e., %tDNA did not reach the control levels (note that in the case of MMS the final repair time tested was 60 min for PBMC, but it was prolonged up to 120 min in saliva leucocytes to facilitate the total repair). Bivariate correlation analyses between the three types of cell samples (Fig. S2) showed high and significant values of the correlation coefficients ($r = 0.941$ for PBMC *vs.* fresh saliva leucocytes; $r = 0.872$ for PBMC *vs.* frozen saliva leucocytes; and $r = 0.922$ for fresh *vs.* frozen saliva leucocytes; $P < 0.01$ for all correlations).

Basal DNA damage values obtained at all times tested were compared between fresh and frozen saliva leucocytes (Fig. 6). In general, these values were higher in frozen cells, significant for some time points, but the magnitude of the difference was low (less than 1.5 units of %tDNA in all cases).

Repair capacity was calculated according to the traditional formula and a new proposed one (%RC and net%RC) for the longest repair period tested (Table 1). The traditional parameter was significantly higher in frozen than in fresh salivary leucocytes, except in the case of UV-exposures, due to the fact that the DNA damage initially induced was more pronounced in frozen cells. Nevertheless, no differences between fresh and frozen saliva leucocytes were obtained when net%RC was considered, and values for this parameter were close to 100% in most cases, reflecting that the damage induced was almost completely repaired and that both biological samples, fresh and frozen, were equally efficient in repairing this damage, regardless its origin. The time necessary to repair half of the DNA damage induced at t_0 was shorter in frozen than in fresh leucocytes treated with BLM and MMS, and very similar in cells exposed to UV radiation.

4. Discussion

Analysis of DNA repair ability in human populations is an attractive biomarker for clinical investigators, because alterations in several DNA repair pathways are linked to both heritable and sporadically occurring age-associated diseases such as cancer (Trzeciak et al., 2008). Individual differences in DNA repair can be analysed by either determining DNA repair gene polymorphisms (for many of which the functional effect is still unclear) or the transcription level of certain genes from a selected

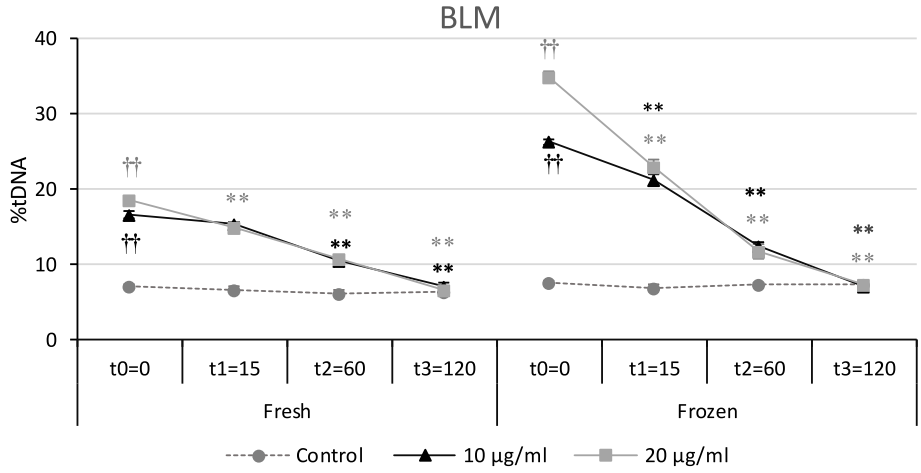

Fig. 2. Results from the challenge-comet assay in fresh and cryopreserved saliva leucocytes treated with bleomycin (BLM). Negative control: dH_2O. $^{**}P < 0.01$, significant differences between t_1–t_3 and the corresponding t_0 condition; $^{\dagger\dagger}P < 0.01$, significant differences between BLM-exposed t_0 and control t_0. X axis indicate incubation time (min).

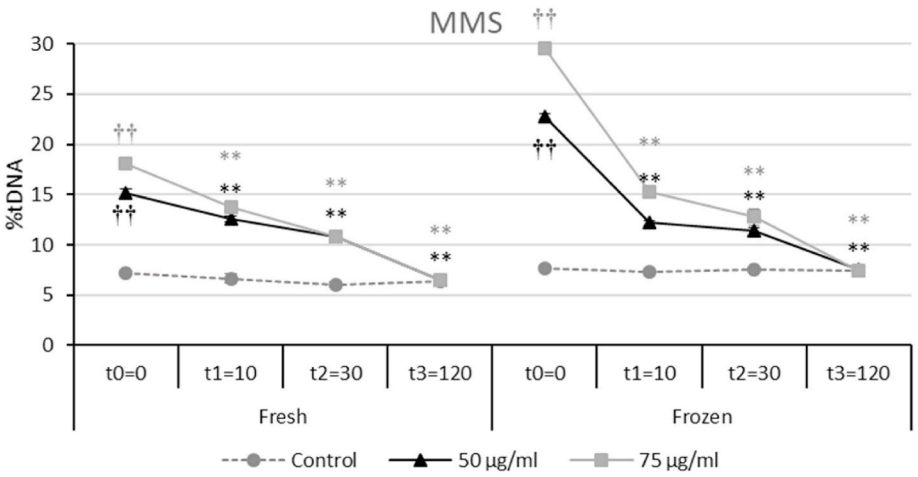

Fig. 3. Results from the challenge-comet assay in fresh and cryopreserved saliva leucocytes treated with methyl methanesulfonate (MMS). Negative control: dH_2O. $^{**}P < 0.01$, significant differences between t_1–t_3 and the corresponding t_0 condition; $^{\dagger\dagger}P < 0.01$, significant differences between MMS-exposed t_0 and control t_0. X axis indicate incubation time (min).

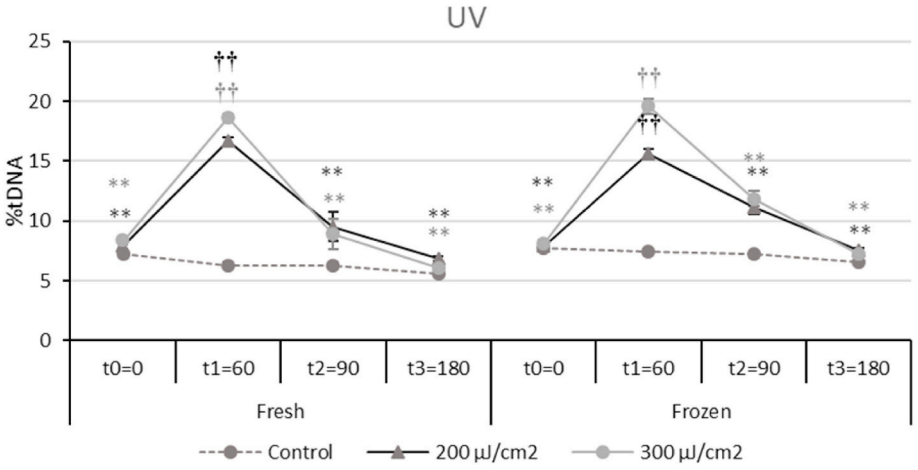

Fig. 4. Results from the challenge-comet assay in fresh and cryopreserved saliva leucocytes treated with ultraviolet (UV) radiation. Negative control: dH_2O. $^{**}P < 0.01$, significant differences between t_0, t_2 and t_3 and the corresponding t_1 condition; $^{\dagger\dagger}P < 0.01$, significant differences between UV-exposed t_1 and control t_1; $^{\#\#}P < 0.01$, significant differences between UV-exposed t_3 and control t_3. X axis indicate incubation time (min).

DNA repair pathway. Still, these approaches do not consider post-transcriptional and/or epigenetic modifications that can modulate DNA repair activity. Moreover, there are numerous genes involved in the different DNA repair routes, which also interact with one another and may have overlapping specificities (Swanson et al., 1999). Thus, phenotypic or functional assays provide a more complete picture of the whole DNA repair process and its consequences, also considering the possible influence of environmental factors (reviewed in Valdiglesias et al., 2011).

Among the different methodologies presently available to assess DNA repair phenotype (reviewed in Decordier et al., 2010 and in Valdiglesias et al., 2011), the challenge-comet assay is a simple approach especially useful to be used in human biomonitoring studies. The challenge-comet assay is described as a functional biomarker of DNA repair that can be used to provide individualized health risk assessment for precision prevention and intervention (reviewed in Xu et al., 2020). In this assay, the entire DNA repair process is assessed, since it depends on the restoration of the normal DNA structure. Essential characteristics of the comet assay [sensitivity, versatility, economy, ease of use, reproducibility, reliability, need of a relatively small number of cells, no requirement of ex vivo culture, and detection of DNA damage at the level of individual cells (Collins, 2004)] are transferred to the challenge-comet assay, resulting more advantageous than the initial versions of the challenge assay using cytogenetic tests (chromosome aberrations or micronucleus tests) to evaluate the remaining genotoxic damage. A further advantage of the challenge-comet assay is that repair kinetics can be followed-up at consecutive time points. The traditional version of this assay was based on the application of X-rays as the challenge agent. Recently, we demonstrated the utility of employing BLM, MMS and UV radiation as challenge agents, thus enabling evaluation of alterations in different DNA repair pathways (Valdiglesias et al., 2020).

As previously mentioned, several reasons support the convenience of having a non-invasive sample type as alternative to PBMC for its use in the challenge-comet assay, particularly when performing human population studies. Oral cavity cells seemed a suitable choice, since they can be obtained easily in reasonable number. Besides, these cells are directly exposed to air passage through the mouth and to drink and food components, representing the target cells for air and oral exposures; hence, they are more appropriate than PBMC for assessing their effects. Exfoliated buccal mucosa and sublingual cells exhibit only a minimal capacity for DNA repair (approximately 0–14% of the level in blood lymphocytes) (Dhillon et al., 2004). Therefore, we chose saliva leucocytes as alternative to PBMC for this study. Results obtained

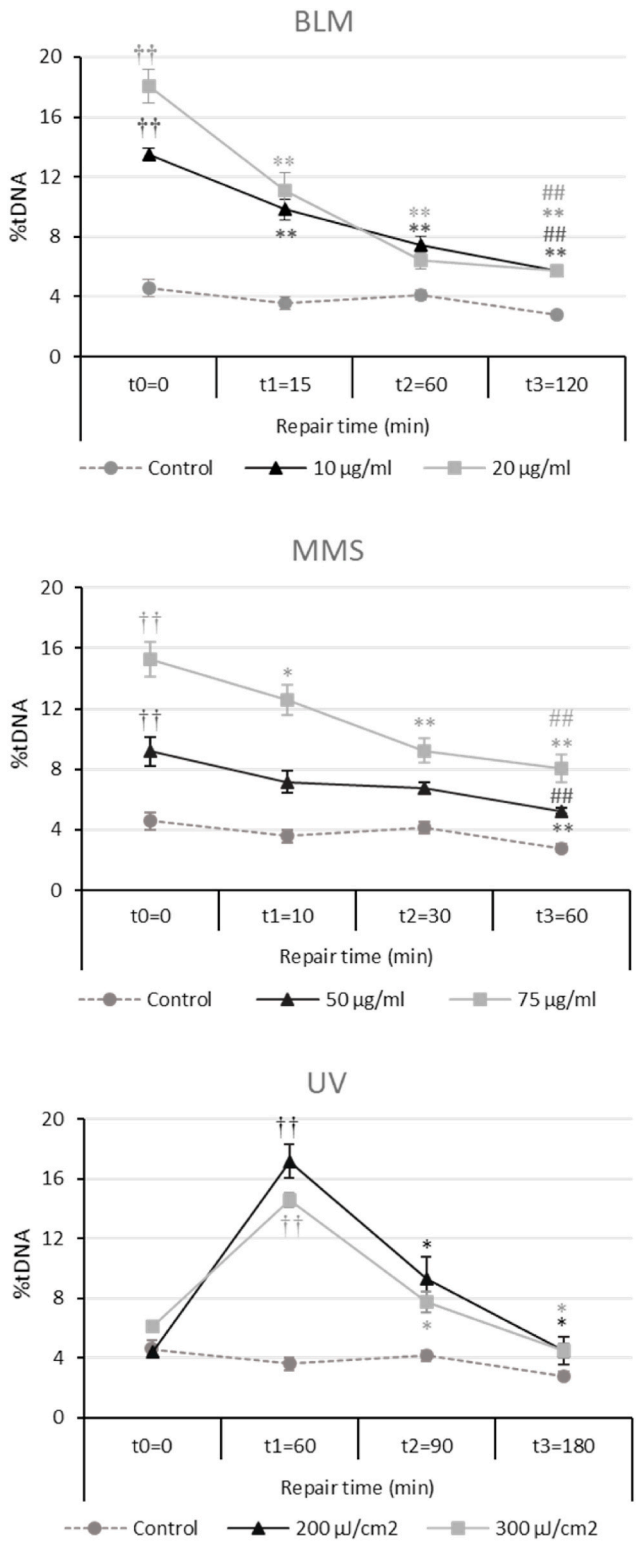

Fig. 5. Results from the challenge-comet assay in peripheral blood mononuclear cells (PBMC) treated with bleomycin, methyl methanesulfonate (MMS) and ultraviolet (UV) radiation. Negative control: dH_2O. $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences between t_1–t_3 and the corresponding t_0 condition; $^{\dagger\dagger}P < 0.01$, significant differences between BLM or MMS-exposed t_0 and control t_0 or between UV-exposed t_1 and control t_1; $^{\#\#}P < 0.01$, significant differences between exposed t_3 and control t_3. Data from Valdiglesias et al. (2020) (reproduced with permission).

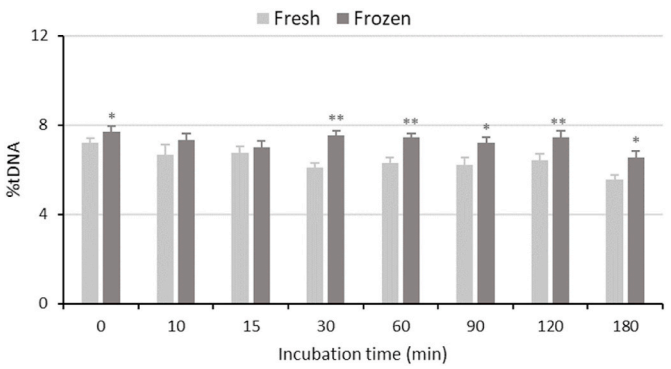

Fig. 6. Comparison of basal DNA damage in negative controls (dH_2O) in fresh and frozen saliva leucocytes at all incubation times tested. $^{*}P < 0.05$, $^{**}P < 0.01$, significant differences with regard to fresh saliva leucocytes.

Table 1
Repair capacity (%RC) at the longest incubation period (t_3), and time necessary to repair 50% of the total DNA damage induced at t_0 ($t_{1/2}$) in fresh and frozen saliva leucocytes treated with bleomycin (BLM), methyl methanesulfonate (MMS) or ultraviolet (UV) light. $^{*}P < 0.05$, significant differences with regard to the same treatment in fresh saliva leucocytes.

		%RC		net%RC		$t_{1/2}$ (min)	
		Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
BLM	10 µg/mL	57.15 ± 2.91	72.95 ± 0.91*	92.85 ± 2.84	101.81 ± 1.36	62.83	49.47
	20 µg/mL	63.84 ± 2.21	78.94 ± 1.12*	97.48 ± 1.36	100.29 ± 0.94	52.97	40.62
MMS	50 µg/mL	57.39 ± 1.67	66.61 ± 1.77*	100.39 ± 5.23	99.07 ± 1.66	51.46	21.99
	75 µg/mL	64.37 ± 1.67	74.81 ± 0.94*	99.62 ± 2.05	100.01 ± 1.57	43.21	23.24
UV	200 µJ/cm^2	58.82 ± 0.58	52.04 ± 0.90*	87.62 ± 2.08	88.45 ± 2.99	106.74	114.86
	300 µJ/cm^2	67.33 ± 0.57	63.07 ± 1.30*	95.98 ± 1.94	94.59 ± 1.45	97.73	106.99

demonstrated that saliva leucocytes were as suitable as PBMC for assessing DNA damage of different nature that was efficiently repaired over the evaluated time points tested (Figs. 2–5). Problems reported related to their use are the limited number of cells obtained per sample, and the microscopy interference with buccal epithelial cells, which prolongs slide scoring time (reviewed in Azqueta et al., 2020). Nevertheless, isolating leucocytes from mouthwashes excludes the presence of buccal epithelial cells in the slides, and the process is sufficiently efficient to render the number of cells necessary to analyse DNA damage at least just after one particular challenge treatment and at three additional time points (preparing four slides with duplicate drops per individual); further experimental conditions could be assessed in higher throughput versions of the comet assay, e.g., GelBond® or 12-Gel Comet Assay Unit. Previous studies have demonstrated that isolation of leucocytes from whole blood by density gradient centrifugation does not lead to increased DNA damage measured by the comet assay (Bausinger and Speit, 2016). The low levels of DNA damage currently observed in control saliva leucocytes (Fig. 6) are comparable to those observed in PBMC and, therefore, allow to also expect no effect of the isolation process in these cells.

In the challenge-comet assay, the residual damage is often assessed at a single time point after the treatment (e.g., 1 h), when most damage has

been repaired, by means of %RC. Although current results show that DNA damage induced by the three challenge agents applied was almost completely repaired in all cases at the longest repair time tested (Figs. 2–4), values of %RC are not close to 100%, providing a confusing idea of the repair efficiency. In contrast, the new formula proposed to calculate this parameter (net%RC) offers results that reflect what can be visually observed in the graphs, i.e. total repair of damage induced. Although limited, this parameter provides relevant information on repair ability, giving a general overview on the repair capacity at the time point selected, useful for the purpose of comparison among individuals. In case it is possible, measuring a full time-course, with short intervals after damage induction, is also a convenient approach since $t_{1/2}$ (i.e. the period of time necessary for repairing half of the DNA damage) provides additional information on the repair velocity. Both $t_{1/2}$ and net %RC are independent of the initial amount of damage induced, and thus are not conditioned by individual susceptibility to the challenge agent applied. In general terms, net%RC reflects the efficacy of the repair process (the fraction of damage induced that could be repaired at the end of the repair period), and $t_{1/2}$ provides information on the speed of the repair procedure.

In the present study, three well-known genotoxic agents were employed to induce several kinds of DNA damage involving different repair pathways. BLM induces a wide range of mutagenic lesions, which eventually result in DNA SSB and DSB (Povirk and Austin, 1991). BLM induced DNA damage is mainly repaired by base excision repair (BER), which takes a short time (usually less than half an hour) (Larsen et al., 2005), and DSB repair (DSBR), which requires a longer period, even hours (Azqueta et al., 2014) (up to 120 min in this study) (Fig. 2). MMS produces alkylation lesions, essentially in guanine and, to a lesser extent, in adenine (Beranek, 1990), that are mainly repaired by BER. Moreover, MMS-induced lesions are considered a source of DSB as a result of collapsed replication forks at the lesions or processed intermediates (Ma et al., 2011), which are repaired by DSBR (Fig. 3). UV light causes covalent linkages between two adjacent pyrimidines. Repair of UV-induced photoproducts [e.g., cyclobutane pyrimidine dimers and 6-4 photoproducts] is conducted by NER and is a relatively slow process (Collins et al., 1997) (up to 180 min in this case) (Fig. 4). Although current results showed that DNA damage induced by the three challenge agents was completely repaired at the end of the repair incubation, it is possible that the loss of heavily damaged cells during this period could contribute to the decreased DNA extent observed. Even though a count of residual cells was not conducted in this work, differences in cell density among slides were not noticed during the blind scoring process. In our previous work, we suggested some recommendations to perform the challenge-comet assay with whole blood samples according to the type of DNA repair to be assessed (Valdiglesias et al., 2020), namely use of 20 μg/mL BLM with evaluations up to 120 min for DSBR, 75 μg/mL MMS with evaluations up to 30 min (for not capturing DSBR) for BER, and 300 μJ/cm^2 UV radiation with evaluations from 60 up to 180 min for NER. Current data support those conditions as suitable to be also used when employing saliva leucocytes as biological sample in the challenge-comet assay.

Molecular epidemiology studies require a quite large number of samples, which are frequently collected at different locations on different days, and must be stored (usually cryopreserved) before use. Hence, stability and suitability of biological samples after cryopreservation-thawing processes is highly convenient for these studies. A recent paper by Møller et al. (2021) reviewed several previous studies reporting similar and comparable results obtained in fresh and cryopreserved PBMC with the challenge-comet assay. Most of them compared fresh and cryopreserved PBMC, and obtained similar results for both conditions either after PHA stimulation (Allione et al., 2013; Bankoglu et al., 2021; Visvardis et al., 1997) or without prior culture in the presence of PHA (Chang et al., 2006; Trzeciak et al., 2008). Recently, Bankoglu et al. (2021) showed that cryopreserved PBMC cannot be used immediately after thawing, but a 16-h recovery with or without mitotic stimulation enabled the application of the repair comet assay. Likewise, analogous results were found in the challenge-comet assay in fresh and cryopreserved whole blood samples pre-incubated for 24 h with PHA, which in addition were equally efficient and comparable in activity to PBMC (Valdiglesias et al., 2020). Results obtained in the present work demonstrate that saliva leucocytes cryopreserved for at least 5 months are also suitable to be used in the challenge-comet assay, after a 24 h incubation with PHA. Although frozen leucocytes presented a higher sensitivity to BLM and MMS treatments, they were as efficient as fresh cells to completely repair the damage induced by all challenge treatments at the end of the established repair period (%tDNA values returned to the control levels), and the basal levels of damage were just slightly higher than those present in fresh leucocytes.

5. Conclusions

Modifications of the comet assay to measure DNA repair activity are increasingly used in human population studies. In this work, it was demonstrated that the challenge-comet assay can be successfully applied in saliva leucocytes and that this methodology is able to detect repair activity of diverse types of DNA damage, i.e., of different DNA repair pathways. This approach is promising for those populations for which collecting whole blood samples is difficult or not possible, and also when blood samples need to be used for the determination of multiple biomarkers. Moreover, the assay can be applied to cryopreserved samples of saliva leucocytes, facilitating the logistics of large human biomonitoring studies. Validation studies are needed to verify whether the method is reproducible and results reliable and comparable among laboratories and studies.

Author contributions statement

Natalia Fernández-Bertólez: Formal analysis, Writing – review & editing, Visualization. **Carlota Lema-Arranz**: Methodology, Writing – review & editing. **Sónia Fraga**: Methodology, Writing – review & editing. **João Paulo Teixeira**: Conceptualization, Formal analysis, Writing – review & editing. **Eduardo Pásaro**: Conceptualization, Formal analysis, Writing – review & editing. **Laura Lorenzo-López**: Supervision, Formal analysis, Writing – review & editing. **Vanessa Valdiglesias**: Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **Blanca Laffon**: Conceptualization, Supervision, Funding acquisition, Writing – original draft preparation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136139.

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