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In-vitro inhalation bioavailability estimation of Metal(oid)s in atmospheric particulate matter ($PM_{2.5}$) using simulated alveolar lysosomal fluid: A dialyzability approach

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ABSTRACT

A novel *in-vitro* method, by using synthetic body fluids, human physiological conditions and a simulated air-blood barrier (by using a dialysis membrane) has been developed and applied to assess *in-vitro* inhalation bioavailability of metal(oid)s associated to particulate matter ($PM_{2,5}$) samples collected from an industrial site of the Northwest of Spain. A validated analytical methodology based on inductively coupled plasma mass spectrometry (ICP-MS) was used to analyse metal(oid)s concentrations in bioavailable fractions. This approach would be a more realistic human health risk assessment since considering processes that occur in human body in contrast the overestimation derived from current models (which consider environmental concentrations). Metal(oid)s such as Cu and Mo seemed to be the most bioavailable (mean *in-vitro* bioavailability ratios higher than 70%); Ba, Cd, Mn, Pb, Rb, Sb, Sn, V and Zn shown mean ratios between 20 and 60%, while low *in-vitro* bioavailability ratios (less than 20%) were observed for metal(oid)s such as Al, Co, Cr, Fe, Ni, Ti, and Tl. Health risk assessment via inhalation based on hazard carcinogenic and non-carcinogenic indexes (HI_c and HI_{nc}, respectively) were performed considering three exposure scenarios using both inhalation bioavailable and total metal(oid)s concentrations in $PM_{2.5}$ samples, suggesting no risk to human health. The influence of chemical composition on *in-vitro* bioavailability ratios was obtained, pointing out that inhalation ratios of Al, Ba, Cr, Cu, Fe, Ni, Pb and V seem to be affected by sea salt and/or crustal and/or biogenic and/or anthropogenic content of $PM_{2.5}$.

1. Introduction

PM toxicity is thought to depend strongly on chemical composition of particles as well as their size (Galvão et al., 2018). As particles are not commonly spherical, they are defined by their aerodynamic diameter (D_{ae}), being particles with $D_{ae} \leq 10$ μm (PM_{10} or coarse fraction) the ones that have potential to penetrate deeper into the respiratory system after inhalation, presenting a major risk. Among them, the most relevant health outcomes have been mainly attributed to particles with $D_{ae} \leq 2.5$ μm ($PM_{2.5}$ or fine fraction) (Tobías et al., 2018), whose exposure was considered responsible for approximately 417,000 premature deaths in 2018 in Europe and UK (EEA, 2020b). Attending to particles' chemical composition, several well-recognised pollutants are associated to PM, being inorganic components (e.g., ions and trace metal(oid)s) mostly associated to natural sources such as marine and crustal. Nevertheless, they could be also emitted to the atmosphere as a result of anthropogenic activity.

As an air pollutant, inhalation is the main exposure pathway to PM, being inhaled billions of particles every day and introduced into the respiratory system. According to deposition studies, particles with D_{ae} 10–100 μm are mostly retained in extrathoracic or nasopharyngeal region (upper airways); particles with $D_{ae} < 10$ μm have the potential to reach tracheobronchial zone (particles with D_{ae} over 4–10 μm mostly retained in this region), and small particles (less than 4 μm of D_{ae}) can reach the lower parts of the respiratory tract and deposit in the pulmonary or alveolar region (Kastury et al., 2017). Consequently, PM-bound pollutants will interact with extracellular pulmonary fluids, being many of them solubilised (bioaccessible fraction) and

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subsequently absorbed to bloodstream (bioavailable fraction). This interaction could be induced by different mechanisms such as paracellular transport (diffusion through tight junctions between alveolar cells) and transcellular transport (transcytosis through alveolar membrane ligands and other non-specific interactions) (Kastury et al., 2017; Knudsen and Ochs, 2018). Within this context, diffusion is considered the main transport mechanism for small (diameter <2 nm) hydrophilic compounds such as metal(oid)s since their ability to pass through the aqueous pores located in the alveolar intercellular junctions (Renkin, 1992). Over the last decades, many studies have focused on the determination of the maximum pollutant fraction that can be solubilised in synthetic body fluids (e.g., lung fluids and gastrointestinal juices) under conditions that simulate the human body processes (i.e., bioaccessible fraction) rather than using total pollutant content, as being considered better PM-associated pollutants' risk predictors and an approximation of how pollutants can behave inside our organisms (Kastury et al., 2017; Innes et al., 2021; Zhao et al., 2021a). Focusing on the inhalation exposure scenario, simulated lung fluids would be employed to obtain PM-bound pollutant's leachates (i.e., inhalation bioaccessible fractions). According to literature, around 100 research papers have been reported using simulated lung fluids (predominantly Gamble's and alveolar lysosomal fluid (ALF) solutions) concerning the study of inhalation bioaccessibility of pollutants (mainly metal(oid)s) in PM and indoor/-outdoor dust during the last four years (Web of Science: accessed 29.06.22), being recently reviewed by Ren et al. (2020). However, the use of bioaccessible concentrations (maximum pollutants' concentrations released in simulated lung fluids) would still provide an overestimation of pollutants risks as not considering their absorption in the air-blood barrier. Thus, assessment of dissolved pollutants in lung fluids that could be also absorbed into the circulation system by crossing the alveolar membrane (inhalation bioavailable fraction), would represent a more realistic evaluation of their potential health risk. Recently, diffusive gradients thin-films water sampler devices (DGT) using 0.40 mm film of Chelex gel has been proposed as an *in-vitro* model to simulate human alveolar sac wall or intestinal wall for bioavailability assays (Luo et al., 2019; Zhao et al., 2021b). Nevertheless, the use of a diffusion gradient could overestimate substances' bioavailable fractions since forcing the membrane crossing. In this framework, dialysis membranes (3.5–10 kDa (KDa) of molecular weight cut-off (MWCO) to mimic cell membranes) combined with physiologically based extraction PBET (to simulate gastrointestinal digestion conditions) have been used by some authors to assess metal(oid)s oral bioavailable concentrations in foods and soil samples (Miller et al., 1981; Haro-Vicente et al., 2006), while it was recently conducted for metal(oid)s (Moreda-Piñeiro et al., 2019) and polycyclic aromatic hydrocarbons (PAHs) (Sánchez-Piñero et al., 2022a) associated to PM_{10} samples. This membrane would simulate the small intestine membrane, whereas piperazinediethanesulfonic acid sodium salt (PIPES) buffer was the recommended acceptor solution (inside the dialysis membranes) because of keeping the pH of small intestine absorption (pH 7.5–8.5). However, to the best of our knowledge, this approach has not been applied to inhalation bioavailability studies to date.

In this research, a novel *in-vitro* PBET methodology to assess metal (oid)s bioavailability in $PM_{2.5}$ samples has been applied. This approach is based on the use of a dialysis membrane of 8 kDa (corresponding with a pore diameter in the range of 1.2–2.0 nm reported for the alveolar epithelium (Barrowcliffe and Jones, 1987)) filled with simulated body fluid (SBF, which simulates human plasma (Marques et al., 2011)), going a step towards realistic lung conditions to obtain more biological relevant data. Furthermore, influence of $PM_{2.5}$ components/optical properties and metal(oid)s *in-vitro* bioavailability ratios will be studied, while a health risk assessment of $PM_{2.5}$-bound metal(oid)s via inhalation will be conducted basing on bioavailable concentrations.

2. Materials and methods

2.1. $PM_{2.5}$ sample collection

$PM_{2.5}$ samples were collected from an industrial site of Vigo city (located on the Northwest coast of Spain, coordinates: 42°12′37.0″N 8°44′11.4″W) over the period of one year (from 10th January to November 6, 2017). Information regarding sampling site has been previously reported by Sánchez-Piñero et al. (2022b). PM collection was performed by using an automatic high-volume sampler DIGITEL DH-77 (Hegnau, Switzerland) at 30 $m^3\ h^{-1}$ for 24 h (00:00–23:59, UTC) and circular quartz filters of 15 cm of diameter (Ahlstrom Munksjö MK360, Falun, Sweden). A total of 52 $p.m._{\cdot 2.5}$ samples (in agreement with the minimum coverage for indicative measurements according to the European Commissions' (EC) Directive 2008/50/EC (EC, 2008)) were selected (1 or 2 samples a week distributed randomly), covering all the sampling campaign period. Sampling and filter treatment were performed according to the European Norm 12,341 (EN 12341:2015) by the European Committee for Standardization (CEN) (CEN, 2015), keeping them at 20 ± 1 °C and relative humidity of 50 ± 5% for 48 h before and during $PM_{2.5}$ mass determination using a microbalance. Also, filters were wrapped in aluminium foil inside envelopes, put inside sealed plastic bags and stored in a freezer (−18 °C) after $PM_{2.5}$ mass determination (Sánchez-Piñero et al., 2022b,c). Field blanks (left inside the sampler, without $PM_{2.5}$ collection) were also collected along with routine samples.

2.2. In-vitro inhalation bioavailability procedure

In-vitro inhalation metal(oid)s bioavailable fractions were assessed using ALF (pH = 4.5 ± 0.1) solution as PM-associated pollutants leaching agent and SBF (pH = 7.4 ± 0.1) as acceptor solution inside the dialysis membrane. ALF (Midander et al., 2007) and SBF (Marques et al., 2011; Leena et al., 2016) compositions are shown in Supplementary Material (Tables S1–2). Both, ALF and SBF were prepared in ultrapure water before extraction by using analytical grade reagents.

Three circular pieces of each $PM_{2.5}$ sample (total area of 9.42 cm^2 and $PM_{2.5}$ mass concentration between 3.0 and 42.0 μg m^{-3}) were placed in 50 mL glass flasks with 20 mL of ALF (pH = 4.5). In this approach, a 100% deposition of $PM_{2.5}$ in the lungs (conservative estimation), an intake air volume of 20 $m^3\ day^{-1}$ (Caboche et al., 2011) and a total volume lining the lung epithelium of 20 mL (Kastury et al., 2017) were assumed to select the most realistic simulated fluid volumes and $PM_{2.5}$ filter amount. Thus, since $PM_{2.5}$ mass concentration varied among the 52 samples studies, solid/liquid ratios (S/L) between 1:10, 000–1:141,000 g mL^{-1} were used, ensuring a full contact between ALF solution and sample, as well as avoiding ALF saturation (Caboche et al., 2011; Kastury et al., 2017). A dialysis membrane of 8 kDa MWCO (Spectra/Por®, Fisher Scientific, Madrid, Spain) filled with 25 mL of SBF (pH = 7.4) as acceptor solution was placed inside the flasks. Flasks were incubated at 37 °C during 24 h (Boxcult incubator, J.P. Selecta, Barcelona, Spain) at 150 rpm orbital shaking (Rotabit orbitalrocking platform shaker, J.P. Selecta, Barcelona, Spain). Temperature, incubation time and agitation rate were selected according to literature (Midander et al., 2007; Kastury et al., 2017; Hernández-Pellón et al., 2018). After incubation and cooling at room temperature for 10 min, bioavailable fractions were collected (using plastic pipettes) and conserved in polyethylene vials at −20 °C. Then, dialysis membranes were removed from flasks, storing non-dialyzable fractions (at −20 °C) until further microwave assisted acid extraction (MAE) procedure. A scheme of the extraction procedure is given in Fig. 1. Two blank filters were also included in each set of samples to control possible contamination.

2.3. Microwave assisted acid extraction

Total metal(oid)s content in $PM_{2.5}$ samples and non-dialyzable

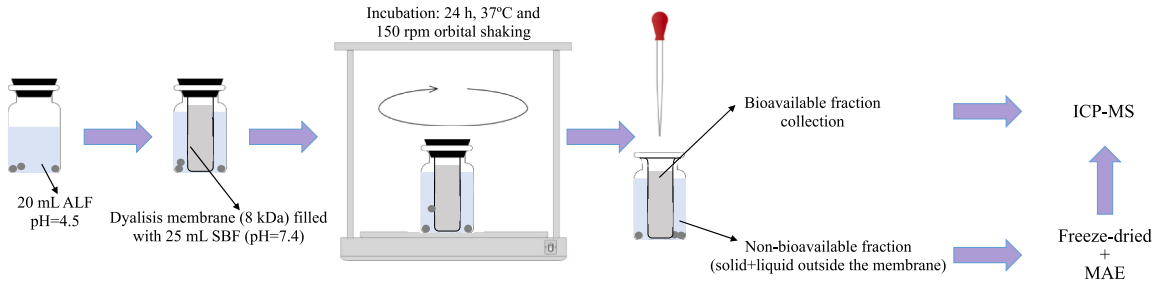

Fig. 1. Scheme of the *in-vitro* inhalation bioavailability procedure.

fractions were achieved by the procedure described in the Supplementary Material.

2.4. Inductively coupled plasma mass spectrometry (ICP-MS) measurements and figures of merit

Procedure for metal(oid)s quantitation in $PM_{2.5}$ samples and in bioavailable and non-dyalizable fractions by ICP-MS are detailed in Supplementary Material. Figures of merit (comprising calibration graphs, linear correlation coefficients, inter-day precision, as well as detection and quantification limits) are shown in Table S3. Accuracy of total metal(oid)s quantification in $PM_{2.5}$ samples was validated by using two certified reference materials (SRM 1648a, urban particulate matter; and CZ120, fine dust, PM_{10}-like). Results were in good agreement with the certified values after statistical evaluation by applying a *t*-test at the 95% confidence level for 7 degrees of freedom (Table S4). Since the absence of reference materials with certified metal(oid)s concentrations, accuracy of the analytical methodology for the analysis of metal(oid)s dissolved in SBF solution was assessed by means of a mass-balance approach considering a $PM_{2.5}$ real sample and two certified reference materials (SRM 1648a and ERM CZ120) (Table S5). Attending to Table S5, accuracy of the analytical methodology used in the present work is therefore demonstrated for all considered metal(oid)s.

2.5. Exposure and health risk assessment

Human health risk assessment was conducted basing on United States Environmental Protection Agency (USEPA) Inhalation Dosimetry Methodology as described in Supplementary Material. Exposure concentrations, carcinogenic risks (CRs) for carcinogenic metal(oid)s, and hazard quotients (HQs) for non-carcinogenic metal(oid)s, were calculated to estimate hazard indexes (HIs), which have been widely used to assess health risks of $PM_{2.5}$-associated metal(loid)s (equations are detailed in Supplementary Material). Furthermore, three scenarios (I) residents (including children and adults); (II) residents working outside the studied area and (III) workers in the area (not residents) were considered (Table S6) (USEPA, 2009, 2014; Hernandez-Pellón et al., 2018).

2.6. Major ions and PM optical properties analysis

Major ions extraction procedure and quantification by zone capillary electrophoresis, together with equivalent black carbon (eBC) and UV-absorbing particulate matter (UVPM) measurement procedures, are detailed in Supplementary Material, while results obtained in analysed $PM_{2.5}$ samples are summarized in Table S7.

2.7. Statistical treatment of data

Metal(oid)s concentration data were studied by Analysis of variance (ANOVA), Principal Component Analysis (PCA) and Univariate Analysis using IBM SPSS® Statistics 28.0.1.0 version Windows software package. Varimax rotation and Kaiser normalization were used for PCA analysis.

3. Results and discussion

3.1. $PM_{2.5}$ mass concentration levels and total metal(oid)s content

The $PM_{2.5}$ mass concentration ranged from 3.0 to 42.0 μg m^{-3}, with an average value of 14.7 μg m^{-3} during the whole sampling period (Table 1). In addition, a statistical summary of metal(oid)s concentrations found in $PM_{2.5}$ samples are also shown in Table 1, whilst concentrations of individual metal(oid)s obtained for each $PM_{2.5}$ sample are detailed in Table S8. Fe (average value of 214 μg m^{-3}), Al (average value of 269 μg m^{-3}) and Zn (average value of 489 μg m^{-3}) were the most profuse elements in $PM_{2.5}$ samples, while Tl, Se, Cd, Bi, Co and As were the less abundant (average values ranged from 0.019 μg m^{-3} (Tl) to 0.36 μg m^{-3} (As)) (Table 1). More than 60% of measured metal(oid)s concentrations were > limit of quantification (LOQ), except for Co, Mo, Bi and Se (quantitation frequencies of 21, 19, 12 and 8%, respectively). Even though some metal(oid)s such as Be, Li, Te and W were also investigated, all concentrations were found <LOQ (being 0.011.1.5.0.011 and 0.027 ng m^{-3}, respectively). Although currently there are no available annual and/or daily limits for metal(oid)s in $PM_{2.5}$, annual-averaged concentrations observed for As, Cd and Ni (0.36 ± 1.2 ng m^{-3}, 0.12 ± 0.30 ng m^{-3} and 1.9 ± 1.9 ng m^{-3} for As, Cd and Ni, respectively) did not exceed annual target values set in PM_{10} (6.0 ng m^{-3}, 5.0 ng m^{-3} and 20 ng m^{-3} for As, Cd and Ni, respectively) (EU, 2004).

3.2. In-vitro inhalation metal(oid)s bioavailable concentrations and inhalation bioavailability ratios in $PM_{2.5}$

A total of 22 metal(oid)s were found in $PM_{2.5}$ bioavailable fractions in concentrations >LOQs, which are summarized in Table 1. Inhalation bioavailable concentrations of individual metal(oid)s found for each $PM_{2.5}$ samples are given in Table S9. The bioavailable (dialyzable) metal (oid) concentrations in $PM_{2.5}$ (N = 3) were within the ranges: <11.0–233, <0.70–4.2, <0.02–0.86, <0.007–0.37, <0.15–1.6, <1.0–32.4, <20–183, <1.2–19.5, <1.5–7.8, <0.10–0.44, <0.70–12.1, <0.20–0.88, 0.85–16.1, <0.20–1.7, <0.05–1.3, <0.003–0.036, 0.11–4.5 and < 15.0–558 ng m^{-3} for Al, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Rb, Sb, Sn, Se, Ti, Tl, V and Zn, respectively (Table 1). Bioavailable concentrations of As, Bi, Se and Sr were <LOQs (0.10, 0.28, 0.10 and 6.6 ng m^{-3} for As, Bi, Se and Sr, respectively) in all dialysates. As expected, metal(oid)s concentrations in bioavailable fractions were lower than total concentrations in $PM_{2.5}$ samples, indicating that metal (oid)s were partially solubilised in ALF solution and partially crossed the dialysis membrane.

Metal(oid)s *in-vitro* bioavailability ratios (B_{av} (%)) were obtained by the following formula:

$$B_{av}\ (\%) = \frac{C_{SBF}}{C_{MAE}}\ x\ 100$$

Table 1
Maximum (Max), minimum (Min), mean, standard deviation (SD) and median for total and in-vitro bioavailable metal(loid)s concentrations expressed as ng m^{-3} of air volume and referred to $PM_{2.5}$ mass (mg kg^{-1} of $PM_{2.5}$, values within brackets), and $PM_{2.5}$ mass concentrations (μg m^{-3}) in samples (N = 52).

	Total concentrations in $PM_{2.5}$					Inhalation bioavailable concentration				
	Mean	SD	Max	Min	Median	Mean	SD	Max	Min	Median
Al	269 (18,900)	343 (27,100)	2070 (172,400)	<1.3 (<86.3)	178 (9750)	29.6 (2520)	37.5 (3830)	233 (22,550)	<11.0 (<370)	21.4 (1500)
As	0.36 (31.4)	1.5 (130)	11.2 (935)	<0.08 (<5.3)	0.13 (8.9)	–[a]	–[a]	–[a]	–[a]	–[a]
Ba	8.0 (970)	15.2 (3590)	81.3 (24,450)	<0.14 (<9.3)	4.5 (263)	1.6 (180)	1.2 (102)	4.2 (504)	<0.7 (<47.0)	1.2 (47)
Bi	0.13 (11.8)	0.41 (34.4)	3.0 (250)	<0.07 (<4.7)	0.07 (4.7)	–[a]	–[a]	–[a]	–[a]	–[a]
Cd	0.12 (6.6)	0.30 (8.8)	2.1 (56.5)	<0.01 (<0.66)	0.065 (4.5)	0.052 (3.0)	0.12 (4.7)	0.86 (22.6)	<0.02 (<1.3)	0.02 (1.3)
Co	0.24 (19.6)	0.54 (50.6)	1.8 (228)	<0.005 (<0.33)	0.005 (0.33)	0.030 (2.1)	0.069 (6.6)	0.37 (45.9)	<0.007 (<0.47)	0.007 (0.47)
Cr	1.8 (168)	2.4 (280)	12.5 (1180)	<0.08 (<5.3)	1.1 (58.5)	0.23 (18.0)	0.25 (24.3)	1.6 (154)	<0.15 (<10.0)	0.15 (10.0)
Cs	2.3 (199)	1.5 (188)	5.3 (802)	<0.33 (<21.9)	2.2 (135)	–[a]	–[a]	–[a]	–[a]	–[a]
Cu	7.0 (490)	10.9 (760)	56.7 (4170)	<0.15 (<10.0)	4.2 (277)	3.3 (230)	5.3 (361)	32.4 (1590)	<1.0 (<67.2)	1.0 (67.2)
Fe	489 (38,700)	529 (45,800)	3860 (321,500)	88.9 (6550)	400 (30,500)	82.9 (6750)	44.9 (5125)	183 (30,680)	<20 (<1340)	80.4 (5715)
Mn	26.3 (1940)	19.8 (1750)	131 (10,900)	1.8 (130)	21.6 (1560)	7.1 (540)	4.0 (375)	19.5 (1510)	<1.2 (<81.0)	6.3 (447)
Mo	1.8 (200)	3.5 (596)	21.3 (3940)	<0.72 (<47.8)	0.72 (47.8)	1.3 (147)	1.0 (136)	7.8 (1710)	<1.5 (<101)	1.5 (101)
Ni	1.9 (147)	1.9 (155)	11.2 (934)	<0.59 (<39.2)	1.2 (88.4)	0.12 (8.7)	0.049 (2.6)	0.44 (21.2)	<0.11 (<7.4)	0.11 (7.4)
Pb	4.7 (318)	5.0 (333)	27.6 (1313)	0.81 (53.9)	2.9 (279)	1.7 (109)	2.2 (99.5)	12.1 (574)	<0.70 (<47.0)	0.76 (47)
Rb	4.6 (390)	1.5 (356)	6.9 (2080)	1.6 (106)	4.9 (346)	0.23 (18.7)	0.12 (1.7)	0.88 (22.5)	<0.20 (<13.4)	0.20 (13.4)
Sb	9.7 (812)	6.9 (781)	28.5 (4040)	2.1 (140)	7.8 (565)	4.5 (394)	4.1 (419)	16.1 (2300)	0.85 (51.2)	3.3 (225)
Se	0.11 (9.8)	0.13 (12.8)	1.0 (84.3)	<0.08 (<5.3)	0.08 (5.3)	–[a]	–[a]	–[a]	–[a]	–[a]
Sn	1.7 (120)	1.3 (111)	8.9 (738)	<0.15 (<10.0)	1.4 (108)	0.35 (23.6)	0.33 (17.5)	1.7 (88.7)	<0.2 (<13.5)	0.2 (13.5)
Ti	13.7 (1100)	19.9 (1810)	128 (10,700)	<0.06 (<4.0)	9.8 (525)	0.17 (10.0)	0.24 (13.3)	1.3 (61.9)	<0.05 (<3.4)	0.05 (3.4)
Tl	0.019 (1.2)	0.019 (1.6)	0.10 (11.6)	<0.002 (<0.13)	0.012 (0.82)	0.004 (0.27)	0.005 (0.29)	0.036 (1.5)	<0.003 (<0.17)	0.003 (0.17)
V	2.2 (179)	1.8 (162)	7.5 (908)	0.25 (16.6)	1.5 (124)	1.2 (117)	1.1 (130)	4.5 (913)	0.11 (8.4)	0.72 (66.4)
Zn	214 (16,300)	212 (17,000)	1070 (90,600)	<2.3 (<153)	166 (10,900)	80.7 (6380)	110 (10,170)	558 (39,900)	<15.0 (<1008)	22.6 (1067)
$PM_{2.5}$ mass	14.7	8.4	42.0	3.0	12.5	–	–	–	–	–

[a] Concentration lower than LOQ.

where C_{SBF} and C_{MAE} are the metal(oid) concentrations in SBF after the *in-vitro* inhalation bioavailability procedure (section 2.2) and acid digestion method (section 2.3), respectively.

Boxplot in Fig. 2 shows inhalation *in-vitro* bioavailability metal(oid)s ratios calculated for studied $PM_{2.5}$ samples. As can be seen from the graph, Cu and Mo were found to be the most bioavailable metal(oid)s (mean ratios higher than 70%); moderate *in-vitro* bioavailabilities (mean ratios between 20 and 60%) were observed for Ba, Cd, Mn, Pb, Rb, Sb, Sn, V and Zn; while metal(oid)s such as Al, Co, Cr, Fe, Ni, Ti, and Tl, were observed to be the less bioavailable (ratios below 20%) (Fig. 2). Based on the results obtained, the wide range of bioavailable metal(oid)s ratios could be derived from different PM sources of $PM_{2.5}$ collected samples.

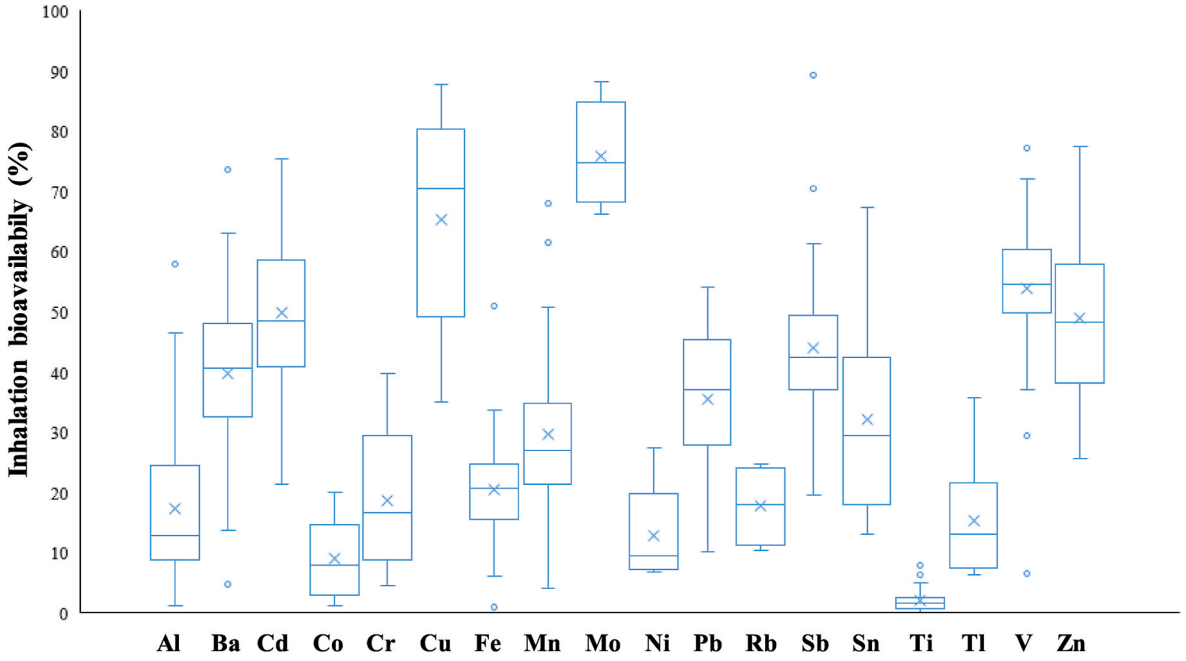

Fig. 2. Box-whisker plot of *in-vitro* inhalation bioavailability ratios (expressed as percentage, %) of metal(loid)s obtained for $PM_{2.5}$ samples. Average values are indicated for each metal(oid) (×).

Also, the low *in-vitro* bioavailability observed for Al, Cr, Fe and Ti might be attributed to their occurrence in insoluble forms (mainly as part of $PM_{2.5}$ crustal material) (Cigánková et al., 2021). Several metal(oid)s bioavailability ratios obtained in the present study were similar to *in-vitro* inhalation bioavailability ratios reported for Cd (~40–45%), Co (~12–15%), Mn (~16–25%) and Pb (~35–40%) in $PM_{2.5}$ samples collected at an urban and industrial area of Nanjing city (eastern China), using ALF solution (pH = 4.5) and DGT devices with Chelex gel film (Zhao et al., 2021b). However, *in-vitro* bioavailability ratios reported for Cr (~3%), Cu (~30–40%) and Fe (~5%) (Zhao et al., 2021b) were lower than those found in this study, which could be attributed to the inclusion of a dialysis membrane in the procedure. Despite the diverse inhalation bioaccessibility protocols found in literature and the different properties/composition of $PM_{2.5}$ samples tested, inhalation bioavailable ratios found in the current study are generally lower than those reported by Ren et al. and Zhao et al. (Ren et al., 2020; Zhao et al., 2021a). On this basis, although most of the metal(oid)s could be highly *in-vitro* bioaccessible (i.e., they can be easily released from $PM_{2.5}$ into body fluids), their ability to cross membranes could be more hindered.

In-vitro inhalation bioavailability ratios of metal(oid)s were also assessed in reference materials SRM 1648a and ERM-CZ120. As is illustrated in Fig. 3, results shown low/intermediate mean *in-vitro* bioavailability ratios, being in the range of 1.8% (Ti) to 55.7% (Zn) and 1.4% (Ti) to 52.7% (Pb) for SRM 1648a and ERM-CZ120, respectively. Se and Sn dialyzability ratios were not assessed in ERM-CZ120 certified reference material since bioavailable concentrations were found <LOQs; as well as for Bi, Sn and Tl, since the absence of certified/indicative values for both reference materials. Moreover, Ba and Mo certified/indicative values in SRM 1648a and Se certified/indicative value in ERM-CZ120 were not available, then their *in-vitro* inhalation bioavailability could not be estimated for such cases. *In-vitro* inhalation bioavailability ratios shown in Fig. 3 are lower than bioaccessibility ratios in SRM 1648a using ALF solution (52.8 ± 1.9%, 65.6 ± 5.5%, 35.0 ± 16.5%, 8.7 ± 0.9%, 55.0 ± 1.1%, 46.8 ± 2.6%, 12.2 ± 4.1%, 75.9 ± 2.2%, 50.9 ± 3.2% and 66.2 ± 2.3% for Ba, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sr and Zn, respectively) reported by Pelfrêne et al., (2017).

3.3. Human health risk assessment of $PM_{2.5}$-bound metal(oid)s

Carcinogenic and non-carcinogenic risks derived from inhalation exposure of $PM_{2.5}$-associated metal(oid)s were assessed in the study area as commented above. In Figs. 4A and 5A are represented mean carcinogenic risks (CRs) and hazard quotients (HQs) estimated for each scenario (±SDs) respectively, basing on USEPA's Inhalation Dosimetry Methodology and considering total metal(oid)s concentrations (USEPA, 2009). Furthermore, mean CRs and HQs were estimated by using bioavailable metal(oid)s concentrations (Figs. 4B and 5B, respectively), which would lead to a more realistic health risk assessment against the overestimation derived from using total concentrations.

Attending to Fig. 4A, CRs for As, Cd and Ni considering total concentrations were less than 1.0×10^{-6} for all scenarios, while CRs higher than 1.0×10^{-6} were found for Co and Cr considering scenarios I (adults and children) and II (Fig. 4A). However, CR for Cr might be overestimated due to the use of an inhalation unit risk value reported for Cr (VI) ($IUR_{Cr(VI)} = 8.4 \times 10^{-2}$ ($\mu g\ m^{-3}$)$^{-1}$, see Supplementary Material), in spite of measuring Cr as Cr(III)+Cr(VI) by ICP-MS. Otherwise, estimated CRs for As, Cd, Co, Cr and Ni were within the safe level when bioavailable concentrations were used for all scenarios (Fig. 4B). Also, carcinogenic hazard index (HI_c), calculated considering total and bioavailable concentrations as the sum of the individual CR_i, did not exceed value of 1.0×10^{-4} for all scenarios (Table 2).

Concerning non-carcinogenic risk assessment, HQ values for Al, As, Ba, Cd, Co, Cr, Mn, Ni, Sb, Se and V considering both total (Fig. 5A) and bioavailable (Fig. 5B) metal(oid)s concentrations were within the acceptable risk level (HQ < 1), indicating non-carcinogenic risks via inhalation for all scenarios. Nevertheless, non-carcinogenic risks from toxic elements by using total metal(oid)s concentrations were higher than those using bioavailable concentrations. Similarly to carcinogenic risk assessment, HQ values for Cr shown in Fig. 5A and B were calculated using Cr total concentration (Cr(III)+Cr(VI)), whereas the reference concentration of chronic inhalation exposure value for Cr(VI) ($RfC_{Cr(VI)} = 1.1\ 10^{-4}$ mg m^{-3}, see Supplementary Information), which could lead to risk overestimations. Moreover, mean non-carcinogenic hazard index (HI_{nc}) calculated as the sum of individual HQ_i for non-carcinogenic metal(oid)s, did not exceed the limit for all scenarios considering bioavailable concentrations (Table 2). Finally, attending to the results obtained for carcinogenic and non-carcinogenic risks, overestimations of 10 and 4 (by average) were accounted for HI_c and HI_{nc} when using bioavailable concentrations (with respect to total concentrations) in $PM_{2.5}$ samples, respectively. Even though the use of *in-vitro* bioavailability concentrations would be a step towards a more realistic human health risk assessment, risks underestimations could be reached since ultrafine particles may enter into the blood stream without previous dissolution of pollutants in lung fluids (i.e., ALF solution) (Oberdörster et al., 2004).

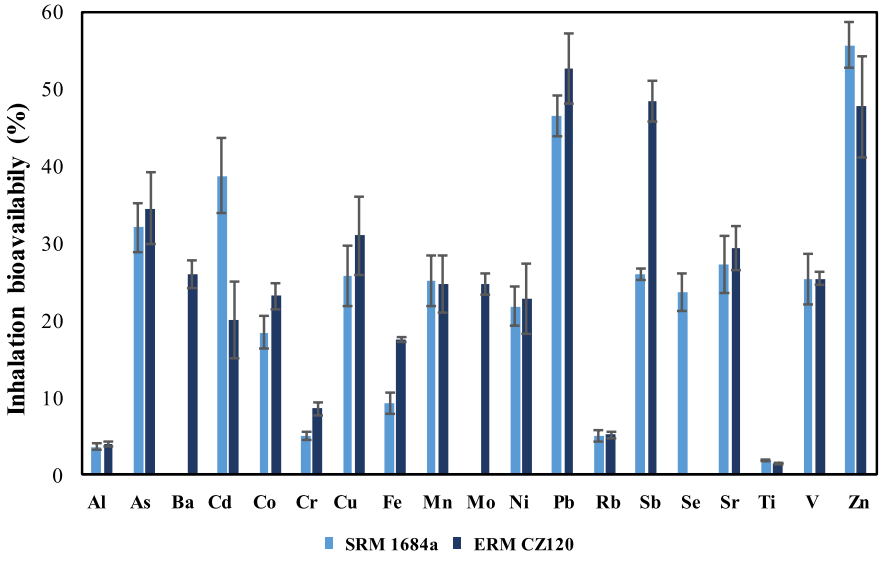

Fig. 3. *In-vitro* inhalation bioavailability expressed as percentage (%) of metal(loid)s obtained for SRM 1648a and ERM CZ120 certified reference materials (N = 3).

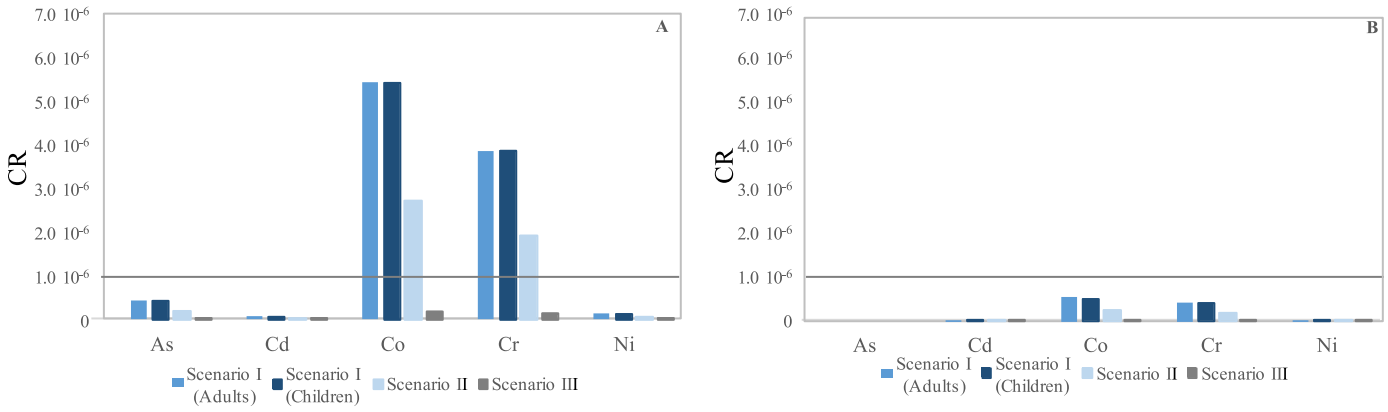

Fig. 4. Carcinogenic risk (CR) values calculated considering (A) total metal(oid)s concentrations in $PM_{2.5}$ samples and (B) *in-vitro* inhalation bioavailable concentrations. The grey line shows the acceptable limit of lifetime cancer risk (i.e., 1.0×10^{-6}) set by the USEPA.

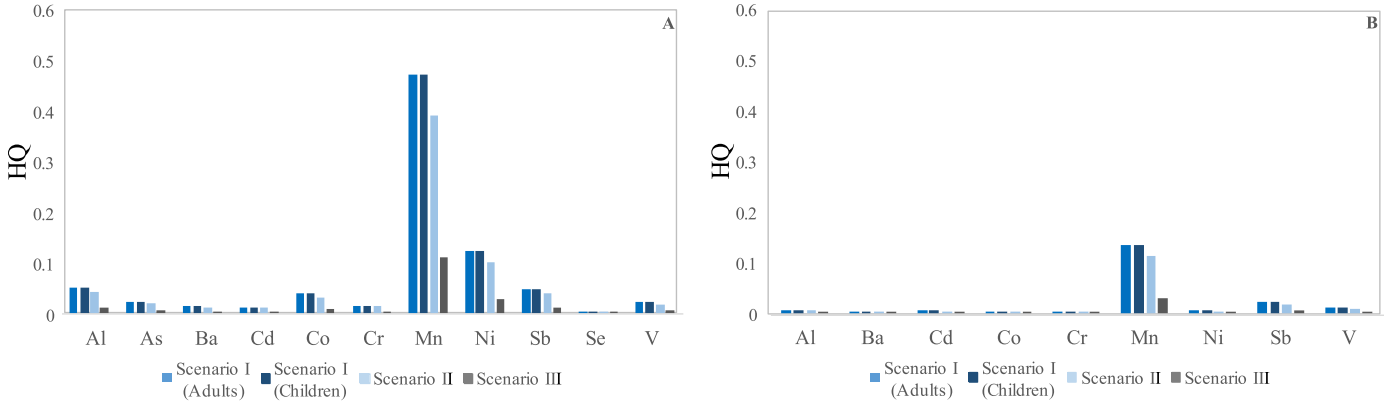

Fig. 5. Hazard quotient (HQ) values calculated using (A) total metal(oid)s concentrations in $PM_{2.5}$ and (B) *in-vitro* inhalation bioavailable concentrations. The acceptable non-carcinogenic risk range set by USEPA is until 1.

Table 2
Mean hazard index for carcinogenic (HI_c) and noncarcenogenic (HI_{nc}) metal(oid)s and standard deviation (SD, within brackets) estimated for each scenario, considering total and *in-vitro* inhalation bioavailable concentrations.

	Scenario I				Scenario II		Scenario III	
	Adults		Children		Total	Bioavailable	Total	Bioavailable
	Total	Bioavailable	Total	Bioavailable				
HI_c	9.9×10^{-6} (1.4×10^{-5})	1.0×10^{-6} (7.4×10^{-7})	9.9×10^{-6} (1.4×10^{-5})	1.0×10^{-6} (7.4×10^{-7})	4.9×10^{-6} (6.5×10^{-6})	5.1×10^{-7} (3.7×10^{-7})	3.2×10^{-7} (4.5×10^{-7})	3.3×10^{-8} (2.3×10^{-8})
HI_{nc}	0.80 (0.49)	0.19 (0.14)	0.80 (0.49)	0.19 (0.14)	0.67 (0.42)	0.16 (0.12)	0.19 (0.16)	0.046 (0.033)

3.4. *Effects of $PM_{2.5}$ composition on in-vitro inhalation metal(oid)s bioavailability*

Several components occurring in PM might greatly influence metal (oid)s solubility/complexation in lung fluids, affecting their *in-vitro* bioavailability ratios. Thus, the study of PM source tracers such as major ions (Cl^-, Na^+, SO_4^{2-}, Mg^{2+}, Ca^{2+}, $C_2O_4^{2-}$, NH_4^+, K^+, NO_3^-) and optical parameters such as eBC (analogous to black carbon present on the filter) and UVPM (an indicator of aromatic organic compounds) would be of interest. $PM_{2.5}$ sources at sampling site during 2017 were previously explored by Sánchez-Piñero et al. (Sánchez-Piñero et al., 2022b,c), indicating that several major ions (SO_4^{2-}, $C_2O_4^{2-}$, NH_4^+, K^+), eBC and UVPM could be used as tracers for anthropogenic source derived from high temperature industrial processes and biomass burning; whereas Cl^-, Na^+, NO_3^- and Mg^{2+} could be associated to sea salt source, and Ca^{2+} could be linked to a crustal source (Sánchez-Piñero et al., 2022b,c). Statistical summary of major ions, eBC and UVPM in $PM_{2.5}$ samples are shown in Table S7. In this framework, a study based on matrix correlation and PCA was performed to investigate the possible effects of $PM_{2.5}$ components/sources on metal(oid)s *in-vitro* inhalation bioavailability ratios. However, some metal(oid)s such as As, Bi, Cd, Co, Mo, Rb, Se, Sn, Sr, Ti and Tl were not enclosed in the study since few bioavailable ratios could be estimated for them.

3.4.1. *Univariate analysis*

Pearson product moment correlations were achieved between the metal(oid) *in-vitro* bioavailability ratios and major constituents of $PM_{2.5}$ (Table S10). A good positive correlation was observed for Ba and Ni *in-vitro* bioavailability ratios and Na^+. Also, Ba and Ni *in-vitro* bioavailability ratios seemed to be positive correlated with $ssSO_4^{2-}$; while Ni *in-vitro* bioavailability ratio was positive correlated with Cl^- (Table S10). Conversely, Cu bioavailable ratio was negative correlated with Cl^-, suggesting that Cu *in-vitro* bioavailability could be decreased in $PM_{2.5}$ samples with a high sea-salt contribution. A similar pattern between

metal(oid)s inhalation bioaccessibility and salt content in PM_{10} samples was observed by da Silva et al. (da Silva et al., 2015) for several metal (oid)s; pointing out that metal(oid)s inhalation bioaccessibility ratios could depend on synthetic fluids selected to perform the *in-vitro* procedure.

Good positive correlations were observed between Cr and Pb *in-vitro* bioavailability ratios and eBC, suggesting that Cr and Pb *in-vitro* bioavailability could be increased in $PM_{2.5}$ samples with a high carbon content (generally, anthropogenic contribution). Also, Cr *in-vitro* bioavailability ratio was observed to be positive correlated with UVPM, which might be attributed to organometallic Cu species in synthetic fluids, as already reported for organometallic Pb species by Fujimori et al. (2018) in comparison to inorganic Pb species (Luo et al., 2019). In addition, Liu et al. (2021) reported that $PM_{2.5}$- and PM_{10}-bound Cr bioaccessibilities increased with traffic PM sources, pointing that metal (oid)s released with soot and ash of vehicular exhaust particles are more soluble in lung fluids than metal(oid)s associated to other sources (Chernyshev et al., 2019). Moreover, a good positive correlation between Pb *in-vitro* bioavailability ratio and K^+ was found, suggesting a great influence of biogenic components. Finally, Al *in-vitro* bioavailability ratio is highly negative correlated with Ca^{2+} content, suggesting that Al *in-vitro* bioavailability could be decreased in $PM_{2.5}$ samples with a high crustal contribution; while no correlation was found for Cd, Co, Mn, Ti, V and Zn *in-vitro* bioavailability ratios and considered $PM_{2.5}$ components/properties.

3.4.2. Principal Component Analysis

PCA results show that 68.12% of the total variance was explained by six principal components (PCs), which eigenvalues higher than 1.0. Bioavailable ratios of several metal(oid)s seemed to be source-dependent, mostly for sea salt and anthropogenic sources (Fig. S1). Cl^-, $ssSO_4^{2-}$, Na^+ and Mg^{2+} content and Ba, Cr and Ni *in-vitro* bioavailability ratios are the highest contributors to PC1 (explaining 19.14% of the total variance). SO_4^{2-}, $nssSO_4^{2-}$, NH_4^+ and oxalate content are the main contributors to PC2 (accounting for 18.30% of the total variance); whereas K^+, eBC and UVPM content and Pb *in-vitro* bioavailability ratio are the main features in PC3, explaining 10.59% of the total variance; while Ca^{2+} content and Al, Fe and V *in-vitro* bioavailability ratios are the main contributors to PC4, explaining 7.75% of the data variance. Finally, PC5 (6.68% of the total variance) is essentially loaded by NO_3^- content and Cr *in-vitro* bioavailability ratio; and PC6 (6.67% of the total variance of the data set) is characterised by Mn, Sb and Zn *in-vitro* bioavailability ratios contribution. Results obtained would suggest that Ba, Cr and Ni *in-vitro* bioavailability ratios are dependent on $PM_{2.5}$ sea salt content; whilst Al, Fe and V *in-vitro* bioavailability ratios could be affected by $PM_{2.5}$ crustal content of samples. Furthermore, Cr and Pb *in-vitro* bioavailability ratios seem to be dependent on biogenic and anthropogenic contents of $PM_{2.5}$ samples.

4. Conclusions

An *in-vitro* methodology for inhalation bioavailable $PM_{2.5}$-bound metal(oid)s estimation, comprising a physiologically-based extraction using simulated biological fluids and a dialysis membrane to simulate alveolar absorption, was applied to a set of 52 p.m.$_{2.5}$ samples collected at an industrial site. The novel inclusion of a dialysis membrane in the test would represent a more realistic evaluation of health risk posed by $PM_{2.5}$-bound metal(oid)s inhalation, which could provide an approximation of how metal(oid)s could interact with organisms after inhalation. Inhalation bioavailable ratios of investigated metal(oid)s were found to vary from 2 to 88%, suggesting that $PM_{2.5}$ contains potentially toxic metal(oid)s that might be absorbed to bloodstream through alveolar absorption, after inhalation. High dialyzability percentages (higher than 70%) were found for several non-carcinogenic metal(oid)s (i.e., Cu and Mo), while *in-vitro* bioavailable ratios between 20 and 60% were observed for Cd and non-carcinogenic metal(oid)s such as Ba, Mn, Pb, Rb, Sb, Sn, V and Zn. Furthermore, Al, Co, Cr, Fe, Ni, Ti, and Tl were found to be the less bioavailable metal(oid)s (*in-vitro* inhalation bioavailability ratios <20%). Averaged CRs and HQs values were assessed using metal(oid)s inhalation bioavailable concentrations, suggesting no carcinogenic and non-carcinogenic risks for all scenarios in the studied area. However, mean CRs estimated for Co and Cr were higher than the acceptable risk limit set by USEPA for scenarios I (adults and children) and II considering total concentrations, while mean HI_c nor HI_{nc} did not exceed the cumulative cancer and non-carcinogenic risks for all scenarios when total and inhalation bioavailable metal (oid)s concentrations were used. Univariate and PCA analysis suggested that *in-vitro* bioavailability ratios of several metal(oid)s depend on $PM_{2.5}$ sources and/or metal(oid)s chemical nature occurring in $PM_{2.5}$ samples. Ba, Cu and Ni *in-vitro* bioavailability ratios were found to be influenced by $PM_{2.5}$ sea salt content; Cr and Pb bioavailabilities seemed to be affected by biogenic and anthropogenic $PM_{2.5}$ components; whereas Al, Fe and V *in-vitro* bioavailability ratios was observed to be influenced by $PM_{2.5}$ crustal content. Although this approach only simulates the diffusion transport across the alveolar barrier, the use of a dialysis membrane during the *in-vitro* PBET procedure might provide a better human health risk assessment of inhaled $PM_{2.5}$-bound pollutants, which could be also applied for the study of further substances such as organic pollutants. Finally, although *in-vitro* methodologies could be promising tools for predicting the human scenario, they would need to be validated against *in-vivo* data, as well as compared to other approaches such as *in-silico* (computer-based) models and permeation and toxicology studies using cultured cells.

Credit author statement

Natalia Novo-Quiza: Investigation, Visualization, Methodology, Formal Analysis. Silvia **Sanromán-Hermida:** Investigation, Methodology. **Joel Sánchez-Pinero:** Visualization, Validation, Formal Analysis, Writing- Original draft preparation. **Jorge Moreda-Piñeiro**: Conceptualization, Validation, Writing- Original draft preparation, Writing-Reviewing and Editing, Project Administration, Funding Adquisition. **Soledad Muniategui-Lorenzo:** Visualization, Resources, Supervision, Project Administration, Funding Adquisition. **Purificación López-Mahía:** Conceptualization, Data Curation, Resources, Project Administration, Funding Adquisition.

Declaration of competing interest

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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.envpol.2022.120761.

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