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Development and application of an inhalation bioaccessibility method (IBM) for lead in the PM_{10} size fraction of soil

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ABSTRACT

An approach for assessing the inhalation bioaccessibility of Pb in the PM_{10} size fraction is presented, using an *in vitro* simulated epithelial lung fluid to represent the extracellular environment of the lung. The developed inhalation bioaccessibility method (IBM) is applied to a range of urban surface soils and mining wastes obtained from Mitrovica, Kosovo, a site where impacts upon human health following exposure to Pb have been internationally publicised. All Pb determinations were undertaken by inductively coupled plasma mass spectrometry (ICP-MS). The pseudo-total concentration of Pb (microwave acid digestion using aqua-regia) varied between matrices: smelter (20,900–72,800 mg kg^{-1}), topsoil (274–13,700 mg kg^{-1}), and tailings (2990 mg kg^{-1}–25,300 mg kg^{-1}). The *in vitro* inhalation bioaccessibility was typically several orders of magnitude lower: smelter (7.0–965 mg kg^{-1}), topsoil (9.8–1060 mg kg^{-1}), and tailings (0.7 mg kg^{-1}–49.2 mg kg^{-1}). The % inhalation bioaccessibility ranged from 0.02 to 11.0%, with the higher inhalation bioaccessible Pb concentrations being observed for samples from the Bosniak Mahalla area of Mitrovica (an area proposed for the relocation of internally displaced peoples). The estimated inhalation dose (for adults) calculated from the PM_{10} pseudo-total Pb concentration ranged from 0.369 to 1.284 μg kg^{-1}_{BW} day^{-1} (smelter), 0.005–0.242 μg kg^{-1}_{BW} day^{-1} (topsoil), and 0.053–0.446 μg kg^{-1}_{BW} day^{-1} (tailings). When daily inhalation doses were calculated using the bioaccessible Pb concentration the modelled exposure doses were much lower: smelter (0.0001–0.0170 μg kg^{-1}_{BW} day^{-1}), topsoil (0.0002–0.0187 μg kg^{-1}_{BW} day^{-1}) and tailings (0.0001–0.0009 μg kg^{-1}_{BW} day^{-1}). Modelled for the neutral pH conditions of the interstitial lung environment, the results indicate a low potential inhalation bioaccessibility for Pb in these samples. Given the already elevated environmental Pb burden experienced by the local population, where significant prolonged dust or particulate generating activities are taking place, or where the inhaled particles are phagocytized, then inhalation exposure has the potential to significantly add to the overall Pb burden. Such data are important for local policy makers to better enable them to assess risk, especially in areas where soils/dusts have elevated levels of contamination.

1. Introduction

Potentially harmful elements (PHEs) released into the environment either through natural or anthropogenic activities may cause adverse health effects to humans. The three main exposure pathways to humans are via ingestion, inhalation and topical absorption (typically referred to as dermal as usually across the skin) (Environment Agency, 2009a). For public health safety it is essential that acceptable methods are developed for estimating realistic human exposure concentrations resulting from exposure to PHEs in the environment. Several protocols have been developed for the ingestion pathway including physiologically-based *in vitro* bioaccessibility methods. Previous bioaccessibility work has predominantly focused on As, Cd and Pb where the ingestion pathway is considered to be the main exposure route to humans. Indeed there are several *in vitro* oral models currently in use for contaminants in food and environmental matrices, and reviews of this work are numerous (e.g. Abrahams, 2012; Hur et al., 2009; Intawongse and Dean, 2006; Oomen et al., 2002; Ruby et al., 1999; Wragg and Cave, 2003). In certain contexts and for certain elements however, inhalation, and specifically the inhalation of suspended particles in air, can pose an increased human health risk via this exposure pathway (Colombo et al., 2008; Harrad et al., 2004; Jones-Otazo et al., 2005; Voutsa and Samara, 2002). Indeed for Ni, the inhalation pathway is considered to be the most significant exposure pathway (Environment Agency, 2009b). Epidemiological, animal toxicological and *in vitro* studies have indicated that it is the chemical composition, as well as the physical presence, of the inhaled particles that plays a major role in associated

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Table 1
The composition and concentrations of *in vitro* lung bioaccessibility fluids.

Composition	Original Gamble solution	Pseudo alveolar fluid	Simulated lung fluid	Artificial interstitial fluid	Gamble solution	Modified Gamble solution	Synthetic serum	Gamble solution	SELF
$MgCl_2 \cdot 6H_2O$ (mg L^{-1})	203	212	212	203					200
NH_4Cl (mg L^{-1})					535	5300	535	118	
NaCl (mg L^{-1})	6019	6415	6400	6193	6786	6800	6786	6400	6020
$CaCl_2$ (mg L^{-1})					22		22		
$CaCl_2 \cdot 2H_2O$ (mg L^{-1})	368	255	255	368		290		225	256
Na_2SO_4 (mg L^{-1})	71	79		71					72
H_2SO_4 (mg L^{-1})					45	510	45		
$Na_2SO_4 \cdot 10H_2O$ (mg L^{-1})			179						
Na_2HPO_4 (mg L^{-1})		148	148	142				150	150
NaH_2PO_4 (mg L^{-1})	142				144		144		
$NaH_2PO_4 \cdot H_2O$ (mg L^{-1})						1700			
H_3PO_4 (mg L^{-1})						1200			
$NaHCO_3$ (mg L^{-1})	2604	2703	2700	2604	2268	2300	2268	2700	2700
Na_2CO_3 (mg L^{-1})						630			
$NaHC_4H_4O_6 \cdot 2H_2O$ (sodium hydrogen tartrate dihydrate) (mg L^{-1})		180	180						
$H_2C_6H_5O_7Na \cdot 2H_2O$ (sodium dihydrogen citrate dihydrate) (mg L^{-1})	97	153	153						
$CH_3CHOHCOONa$ (sodium citrate) (mg L^{-1})		175			52		52	160	
Citric acid $\cdot H_2O$ (mg L^{-1})						420			
$NaOCOCOCH_3$ (sodium pyruvate) (mg L^{-1})		0.72	172						
NH_2CH_2COOH (glycine) (Gly) (mg L^{-1})		118	118		375	450	450	190	376
L-Cysteine ($C_3H_7NO_2S$) (mg L^{-1})					121				122
DPPC (dipalmitoyl phosphatidyl choline) ($C_{40}H_{80}NO_8P$) (mg L^{-1})								200	100
$CH_3COONa \cdot 3H_2O$ (sodium acetate trihydrate) (mg L^{-1})	953			952					
Sodium acetate (CH_3COONa) (mg L^{-1})						580			
HOC (COONa) $(CH_2COONa)_2 \cdot 2H_2O$ (sodium citrate dihydrate) (mg L^{-1})				97		590			
$C_3H_5NaO_3$ (sodium lactate) (mg L^{-1})			290						
KCl (mg L^{-1})	298			298					298
Potassium hydrogen phthalate ($C_8H_5KO_4$) (mg L^{-1})						200			
$C_{14}H_{23}N_3O_{10}$ (DTPA) (pentetic acid) (mg L^{-1})					79				
$C_{21}H_{38}NCl$ (ABDAC) (mg L^{-1}) (benzalkonium chloride)					50				
Ascorbic acid (mg L^{-1})									18
Uric acid (mg L^{-1})									16
Glutathione (mg L^{-1})									30
Albumin (mg L^{-1})									260
Mucin (mg L^{-1})									500
pH (adjustment with HCl)		7.6		7.4	7.3	7.4	7.3		7.4
Reference	Moss, 1979	Takaya et al., 2006,	Taunton et al., 2010	Stopford et al., 2003	Wragg and Klinck, 2007	Gray et al., 2010;	Kanapilly et al., 1973	Caboche et al., 2011	This study

toxic, carcinogenic and other health effects (e.g. Adamson et al., 2000; Ghio and Devlin, 2001; Godleski et al., 2002; Hunt et al., 2003).

With respect to the inhalation route, a particle may reside in one of at least two "compartments": the extracellular environment typified by lung fluid of neutral pH and the more acidic environment within macrophages (Zoitos et al., 1997). The pioneer synthetic lung fluid (SLF), commonly referred to as Gamble's solution, has been widely used for the exposure assessment of humans to inhalable pollutants. Gamble's solution attempts to simulate the extracellular environment of the deep lung (Holliday, 2000; Margues et al., 2011). This initial formula has been modified by several researchers (Caboche et al., 2011; Wragg and Klinck, 2007) but the name "Gamble solution" is often retained. Names like "modified Gamble's solution" (Gray et al., 2010), "Gamble serum simulant" (Ansoborlo et al., 1990), "simulated lung fluid" (Taunton et al., 2010), "synthetic serum" (Kanapilly et al., 1973), "artificial interstitial fluid" (Stebounova et al., 2011; Stopford et al., 2003), and "pseudo alveolar fluid" (Takaya et al., 2006) have also been employed. The formulae for these different versions of Gamble's solution are summarised in Table 1 and indicate both similarities and differences in their constituent compounds. Several other substitute fluids such as deionised water, dilute acids, polydentate chelants (i.e. ethylene diaminetetraacetic acid), acetate buffers, phosphate buffers and physiological sodium chloride solutions have also been used to estimate inhalation bioaccessibility (e.g. Artelt et al., 1998; Birmili et al., 2006; Canepari et al., 2006, 2010; Harrington et al., 2012; Santos et al., 2009), as have fluids that represent different conditions within the lung, such as artificial lysosomal fluid representative of the more acidic fluid with which particles come into contact after phagocytosis (Colombo et al., 2008). Whilst the Bioaccessibility Research Group of Europe (BARGE) has formulated fluids for the different compartments of the digestive system (mouth, stomach and intestine) that attempt to mimic a full complement of chemical groups identified in the digestive fluids of healthy humans i.e. the Unified Bioaccessibility Method (UBM) (Wragg et al., 2009), there is presently no consensus on the fluid composition for the inhalation pathway and many existing fluids appear to be limited when their formulae at the molecular level are compared with the UBM for oral bioaccessibility studies. At the molecular level the UBM contains minerals (salts), water, proteins (e.g. albumin, mucin, uric acid) and lipids (bile salt); thus all four molecular components of human body fluids are represented. Typical recipes (Table 1) for SLFs indicate sufficient salt (mineral) content but appear to be deficient in representing the mix of organic molecules in the native respiratory tract environment (Margues et al., 2011). Though some authors have substituted proteins with citrates/citric acid and organic acids with acetates (Gray et al., 2010; Stopford et al., 2003; Takaya et al., 2006; Wragg and Klinck, 2007), it may be difficult to justify these substitutions especially when the same organic molecules have been used in other aqueous *in vitro* protocols (e.g. UBM) and are readily available on the market. Lipids, known to be present in SLFs (Hamm et al., 1996; Kendall, 2007; Lohninger et al., 1983), have also been excluded in many lung bioaccessibility models with minimal to no justification in the literature.

The primary aims of this study were the development of i) a robust *in vitro* simulated epithelial lung fluid (SELF), to represent the extracellular environment of the lung, and ii) an inhalation bioaccessibility extraction (IBM) protocol. The protocol was then used, iii) to assess the inhalation bioaccessibility of Pb in the PM_{10} size fraction that originate from surface soils, tailings and smelter wastes from across Mitrovica, Kosovo. A mounting body of literature underlines the growing concern for the impact of PM, especially the $<PM_{10}$ fraction on human health. The inhalation of dust derived from soils is generally an underestimated exposure to date. Mass contributions to ambient PM_{10} in several cities of the world have been traced to several sources including soil materials (Almeida et al., 2005; Bi et al., 2007; Lim et al., 2010; Marcazzan et al., 2003). Significant human health risks at Mitrovica have been identified originating from the inhalation of ambient dust aerosols containing PHEs (UNEP, 2010). Ardtisoglou and Samara (2005) in their investigation of total suspended particulate matter and associated trace elements in Mitrovica identified the resuspension of soil dust as the most significant contributor of PHEs into the local atmosphere. Our focus was on the PM_{10} fraction. Following inhalation, particles with aerodynamic diameters of >10 μm are mainly deposited in the upper respiratory tract, whilst particles with aerodynamic diameters of less than or equal to 10 μm are likely (>50%) to reach the tracheobronchial region (Merget and Rosner, 2001). The larger inhaled particles are known to become trapped in the mucus that lines the airways and are transported by cilia away from the lungs (the so called mucociliary escalator) and subsequently expectorated or swallowed (gastro-intestinal pathway). Particles <4 μm are mainly respirable (i.e. deposit in the alveolar region or deep lung).

2. Experimental

2.1. Simulated epithelial lung fluid (SELF) formulation

A critical review of the published literature indicates that inorganic salts, surfactant lipids, large molecular-mass proteins, low molecular-mass antioxidant proteins and organic acids are all native to the epithelial lung fluids of healthy non-smoking humans (Cantin et al., 1990; Hamm et al., 1996; Kendall, 2007; Kirkham and Rahman, 2006; Lohninger et al., 1983; Vanbever et al., 1999; Vliet et al., 1999). Generally the range of inorganic salts (sodium chloride, calcium chloride, sodium dihydrogen phosphate, sodium bicarbonate, potassium chloride, magnesium chloride hexahydrate, sodium sulphate) and their concentrations used in existing models (Table 1) closely approximate published *in vivo* data for healthy humans (Cadwell et al., 2002; Hull et al., 1998; Jayaraman et al., 2001; Joris et al., 1993; Knowles et al., 1997; Vanthanouvong and Roomans, 2004; Zhang and Engelhardt, 1999). For our SELF formulation the same salts and similar concentrations were maintained (Table 2A). The *in vivo* concentrations of ascorbic acid, uric acid and glutathione in the respiratory tract fluids of healthy human subjects have been documented as 17.6 mg L^{-1}, 15.1 mg L^{-1}, 30.7 mg L^{-1}, respectively (Cross et al., 1994), and approximately the same concentrations were chosen for this formulation (Table 2B). Similarly, dipalmitoyl phosphatidyl choline (DPPC), a major lipid in human respiratory tract fluids (Gregory et al., 1991; Griese, 1999; Johansson et al., 1994; Pison et al., 1986), is included. A concentration of 100 mg L^{-1} DPPC is suitable for mimicking the airway's fluid surfactant properties (Davies and Feddah, 2003) and was adopted as part of the SELF formulation (Table 2C).

Table 2
Simulated epithelial lung fluid formulation.

Reagent	Weight of reagent (mg) made up to 500 mL
(A) Inorganic phase reagent	
NaCl	6020
$CaCl_2$	256
Na_2HPO_4	150
$NaHCO_3$	2700
KCl	298
$MgCl_2$	200
Na_2SO_4	72
(B) Organic phase reagent	
Ascorbic acid	18
Uric acid	16
Glutathione	30
(C) Additional reagents	
Albumin	260
Cysteine	122
DPPC	100
Glycine	376
Mucin	500

Studies investigating the composition of human SLFs have also identified albumin and mucin as the most abundant proteins (Bakker, 1988; Bredberg et al., 2012; Su et al., 1998). The airway mucus gel protects the respiratory tract against environmental challenges (Cooper et al., 1985; Khanvilkar et al., 2001; Thornton et al., 2008) and it has been suggested that glycoproteins such as albumin and mucin bind with metals in solution (Cross et al., 2001; Duranti et al., 2001) thereby influencing metal solubility. Twining et al. (2005) adopted an albumin concentration of 200 mg L^{-1} in their inhalation bioaccessibility model. This reflects the lower end of the reported *in vivo* concentration range of 50–5500 mg L^{-1} (Fick et al., 1984; Lamer et al., 1993). For our SELF formulation an albumin concentration of 260 mg L^{-1} was adopted (Table 2C); beyond this concentration albumin may bind to polystyrene and glass surfaces (Bakker, 1988; Su et al., 1998). Mucin also exhibits extensive aggregations and forms gel-like solids at physiologic concentrations of 20,000–50,000 mg L^{-1} (Bansil and Turner, 2006; Bansil et al., 1995; Bromberg and Barr, 2000; Cao et al., 1999; Lee et al., 2005). However, at dilute concentrations of <1500 mg L^{-1} such aggregations are limited but the characteristic lubricating properties of mucin are retained (Lee et al., 2005). A concentration of 500 mg L^{-1} mucin (0.5 wt.%), sufficient for the attainment of surface active properties (Griffiths et al., 2010), was adopted in our SELF (Table 2C).

The interaction of metal ions with proteins is well known and can lead to aggregation and precipitation reactions (Hughes and Klotz, 1956). Glycoproteins, such as mucin for example included in our SELF, have been shown to interact with Pb^{2+} at neutral pH values reducing the solubility of Pb (Duranti et al., 2001). However the role of proteins and antioxidant reagents warrants further investigation as Harris and Silberman (1988) compared the leaching efficiency of normal canine serum with that of a synthetic serum and concluded the opposite that the lower leaching efficiency of their synthetic serum was due to the absence of proteins in the synthetic serum. Voutsa and Samara (2002) also attributed the very low inhalation bioaccessibility (<1%) for Pb from urban and industrial airborne particulate matter in their synthetic serum to the neutral pH and/or the absence of strong Pb complexing agents.

Organic acid components of human SLFs have previously been represented by glycine (Ansoborlo et al., 1990; Caboche et al., 2011; Drysdale et al., 2012; Gray et al., 2010; Kanapilly et al., 1973; Takaya et al., 2006; Wragg and Klinck, 2007) and cysteine (Ansoborlo et al., 1990; Harris and Silberman, 1983; Vousta and Samara, 2002; Wragg and Klinck, 2007). Both acids help maintain the glutathione balance (Kelly, 1999) and exhibit anti-inflammatory effects during endothelial inflammation (Hasegawa et al., 2012). In the SELF formulation the concentrations of glycine and cysteine were set at 376 mg L^{-1} and 122 mg L^{-1}, respectively (Table 2C), and are based on the formulation by Wragg and Klinck (2007).

2.2. Reagents

Chemicals used in fluid formulation and analyses were of certified analytical grade. Concentrated nitric acid (HNO_3) and concentrated hydrochloric acid (HCl) were supplied by Fisher Scientific Ltd. (Loughborough, Leicestershire, UK). Sodium hydrogen phosphate (NaH_2PO_4) and potassium hydrogen phosphate ($NaHPO_4$) were purchased from Sigma Aldrich Ltd. (Gillingham, Dorset, UK). Sodium chloride (NaCl), anhydrous sodium sulphate (Na_2SO_4), potassium chloride (KCl), calcium chloride ($CaCl_2{\cdot}2H_2O$), sodium bicarbonate ($NaHCO_3$), magnesium chloride ($MgCl_2{\cdot}6H_2O$), sodium hydroxide (NaOH), uric acid, bovine serum albumin (BSA) and concentrated nitric acid (69% HNO_3) were all obtained from Merck Ltd. (Poole, UK). Mucin (pig) was obtained from Carl Roth Ltd., Germany. Ascorbic acid, glutathione, cysteine, dipalmitoyl phosphatidyl choline (DPPC), and glycine were obtained from Sigma Aldrich Ltd. (Gillingham, Dorset, UK). A Pb standard and internal standard solutions (indium and terbium) were obtained from SPEXCertPrep (Middlesex, UK). Ultra-pure water of conductivity 18.2 MΩ-cm was produced by a direct QTM Millipore system (Molsheim, France). Certified reference materials: BCR 038 (fly ash from pulverised coal with a particle size fraction of <10 μm), BCR 143R (sewage sludge amended soil with a particle size fraction of <90 μm), BCR 176R (fly ash with a particle size fraction of <105 μm), BCR 723 (road dust with a particle size fraction of <105 μm) as well as SRM 2783 (air particulate on filter media) were obtained from LGC-Promochem (London, UK); a Guidance Material 102 (naturally contaminated soil, from North Lincolnshire, with a particle size of <40 μm) was obtained from the British Geological Survey (Keyworth, UK).

2.3. Preparation of simulated epithelial lung fluid (SELF)

The list of the chemicals required to prepare 1000 mL of SELF are given in Table 2. To prepare 500 mL of the inorganic and organic phases, the chemicals specified in Tables 2A and 2B were dissolved in deionised water in separate 1 L high density polyethylene (HDPE) screw top bottles and made up to 500 mL. The 500 mL inorganic and the 500 mL organic phases were then combined to form 1000 mL of reagent in a 1 L bottle which contained the solids listed in Table 2C and the resulting fluid was mixed thoroughly. About 0.4 mL of HCl was added to attain the desired pH of 7.4 ± 0.2.

2.4. Procedure for inhalation bioaccessibility extraction

A static dissolution technique was chosen. Such techniques are amongst the easiest and most widely implemented *in vitro* protocols and avoid the production of large quantities of SLF associated with flow-through systems (e.g. Zoiltos et al., 1997). An in-house test soil was utilised for method development work. This soil sample was collected from a former industrial site known for its Pb works (St. Antony's, Newcastle upon Tyne, UK); the soil sample was sieved with a nylon sieve of particle size fraction 250 μm and then ball milled to reduce the particle size to a fine powder.

A respiratory tract fluid volume of 0.3 mL kg^{-1} body mass has been recorded for healthy non-smoking human subjects by both Miserocchi (1997) and Noppen et al. (2000). For a 70 kg adult this would correspond to 21 mL. For this study the value was rounded down to 20 mL and set as the experimental volume of the simulated epithelial lung fluid. A similar volume has also been used in previous related studies (Gray et al., 2010; Twining et al., 2005; Wragg and Klinck, 2007).

The basal human body temperature (37 °C) was chosen for the experimental protocol for this study. Based on McShane et al. (2003), where a pH of 7.1 ± 0.1 was reported, and an upper pH value of 7.5 reported by Fischer and Widdecombe (2006), the pH for this inhalation bioaccessibility study was set at 7.4 ± 0.2.

In terms of a suitable sample mass a range of 250–550 mg has been applied across a range of published studies (e.g. Drysdale et al., 2012; Wragg and Klinck, 2007). A conservative upper annual inhalation dose of 300 mg (PM_{10}) was calculated as a reasonable upper-bound value and is consistent with the published range. This was calculated for adults according to Sexton et al. (1995) as follows:

$$\text{Annual}\,PM_{10}\,\text{inhalation} = PM_{\text{limit}} \times V_{\text{resp}} \times \text{EF} \quad (1)$$

where PM_{limit} is the exposure limit of PM_{10} (40 μg/m^3; this represents the upper daily PM_{10} concentration not to be exceeded in a calendar year, European Environment Agency, 2011), V_{resp} is the inhalation rate (20 m^3 day^{-1}) (U.S. EPA, 1991) and EF is the exposure frequency (365 days).

The SELF was heated to 37 ± 2 °C in a thermostatically controlled water bath before use. Approximately 0.3 g of test sample was accurately weighed into a centrifuge tube to which 20 mL of the prepared SELF was added. The resulting suspensions were rotated for a range of fixed times at a speed of 30 RPM, then centrifuged at 3000 RPM for 10 min

and the clear supernatant was transferred into fresh tubes, diluted and stored in the fridge (4 °C) for subsequent analysis. The resultant residual fraction was microwave acid digested (temperature, 160 °C; power, 750 watts; extraction time, 40 min; and cooling time, 30 min) using aqua-regia (HCl:HNO_3 in the ratio 3:1 v/v) to allow both a total and a mass balance for the system to be established. Analysis was performed by inductively coupled plasma mass spectrometry (ICP-MS) X Series II from Thermo Electron Corporation (Winsford, UK).

The percentage inhalation bioaccessible fraction (%IBAF) was calculated as follows:

$$\%\text{IBAF} = (C_{\text{ibio}}/C_{\text{total}} \times 100) \quad (2)$$

where C_{ibio} is the inhalation bioaccessible concentration of Pb and C_{total} is the total concentration of Pb determined by aqua regia digestion. For each digestion, reagent blanks were also prepared. The filtrate obtained from the digestion was refrigerated (<4 °C) prior to analysis.

2.5. Quality control procedures

Procedural blanks were included within the inhalation bioaccessibility extraction procedure and to determine reproducibility all samples were extracted in triplicate. For the microwave acid digestion 0.5 g of a certified reference material (BCR 143R) was accurately weighed and digested to assess the efficiency of the digestion procedure. For the ICP-MS analysis a blank solution and two quality control standards (low and high) were analysed after no more than 10 unknown samples. Instrumental detection limit for Pb was 0.1 μg L^{-1}, with the method detection limit two orders of magnitude greater at approximately 10 μg L^{-1}.

All reported data are based on the average of three replicate. Also to assess the quality of data generated by the ICP-MS standard solutions (0, 20, 40, 60, 80, 100, 200, and 400 ng mL^{-1}) were prepared and analysed prior to the test samples.

3. Study site

Mitrovica, Northern Kosovo contains a number of former and current metallurgical industries including the now inactive Zvecan Pb/Zn smelter, located approximately 5 km to the north of the city (Fig. 1). Internationally publicised concerns around the uptake of Pb by internally displaced populations (IDPs) within Mitrovica since the Kosovan War (1998–1999) have been highlighted (CDC, 2007; Human Rights Watch, 2009). Our previous work has assessed the potential daily intake of soil-bound PHEs in surface soils and metallurgical waste in the area using physiologically-based *in vitro* analyses (Boisa et al., 2013). The samples analysed in this study ($n = 33$) included 4 smelter waste samples (from Gornje Polje waste dumps), 24 topsoil samples (1–10 cm depth from Bosniak Mahalla, Roma Mahalla and Cesmin Lug) and 5 mine tailing samples. The pH of the topsoils was determined using a Hanna pH209 electrode in a slurry containing sample and deionised water in a 1:2.5 (w:v) ratio. The pH of the topsoils ranged from slightly acidic to moderately alkaline (6.08 to 8.5) with a neutral-mildly alkaline median of 7.3. Organic matter content was quantified gravimetrically by loss-on-ignition (LOI) following the ignition of sample material at 375 °C for 16 hours (Ball, 1964). The samples indicated a very low–

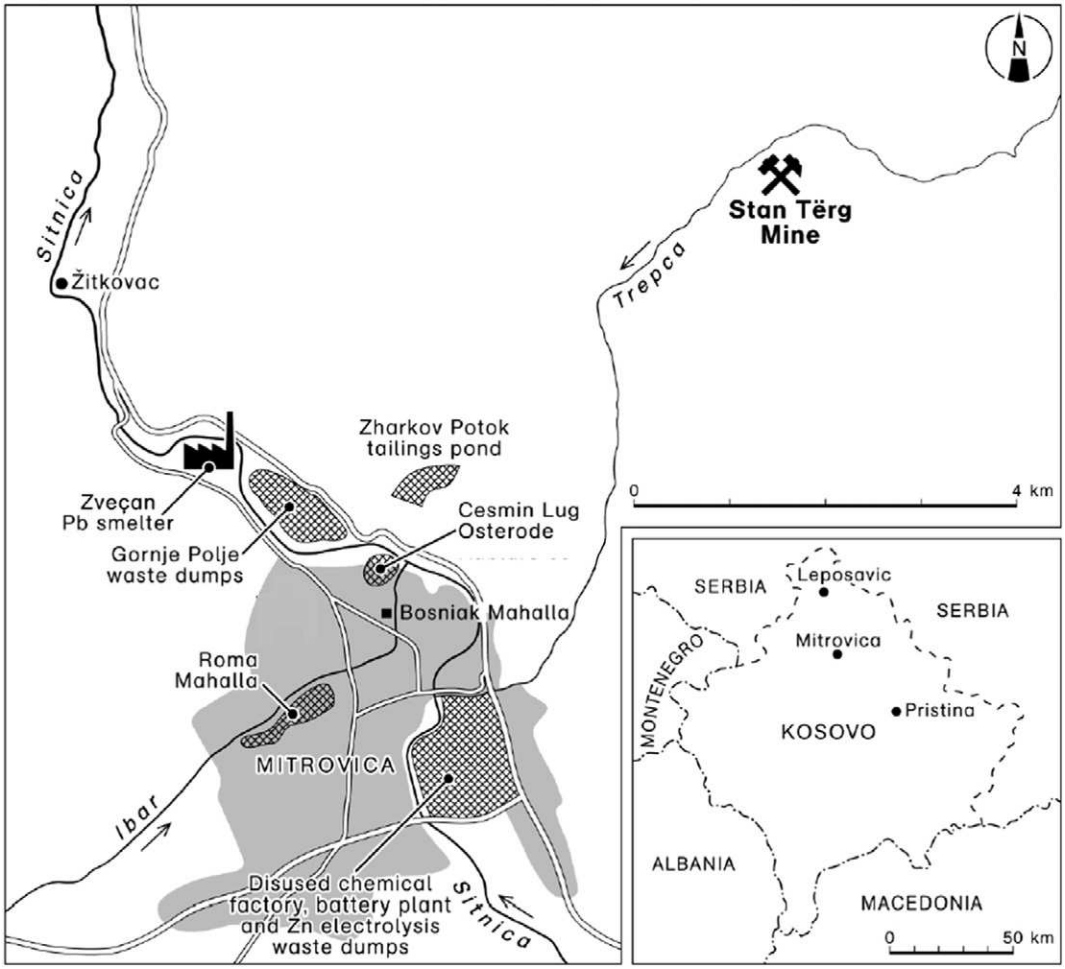

Fig. 1. Sample locations in Mitrovica, Kosovo (from Boisa et al., 2013).

moderate organic matter (% LOI) status (0.2–3.3%) with a median of 0.7%. Low organic status soils are typically more friable, especially when dry, compared to those with higher organic matter status with a higher aggregate binding potential.

Samples (<250 μm) were dry sieved to obtain the <63 μm size fraction and fugitive dusts (<10 μm) were extracted from the <63 μm fraction by wet extraction (e.g. Ljung et al., 2011; Luo et al., 2011) with the method optimised and adjusted to the laboratory scale in this study. The low %LOI content of these soils reduced the need for pretreatment with hydrogen peroxide to remove the organic matter. The water soluble fractions of PHEs can be a source of error for water-based PM_{10} sampling methods. To assess the loss of Pb during our wet extraction procedure the supernatant for each sample (the visually clear liquid obtained after centrifugation) was analysed for its Pb content by ICP-MS. The % loss of Pb during the PM_{10} extraction (expressed as a % of the pseudo-total Pb concentration) was consistently low, and ranged from 0.4–0.94%. However, when expressed as a % of the lung bioaccessible Pb concentration, the loss of Pb during the PM_{10} extraction ranged between 1.1 and 7.6% for the majority of the topsoils but the tailing and smelter samples indicated a far higher % loss of Pb (some samples in excess of 80%). Given the extremely high Pb concentration in these wastes, the Pb lost during the water separation procedure still only represents <1% of the pseudo-total concentration; so in percentage terms minimal Pb is extracted from the tailing and smelter samples either at the water separation stage or by the lung bioaccessibility procedure. Given the low % loss of Pb from the topsoils we proceeded to extract PM_{10} from these samples using the wet extraction technique. All PM_{10} samples were subsequently subjected, in triplicate, to our developed IBM.

4. Results and Discussion

4.1. Method development: experimental time

The residence time of inhaled particulate matter in the respiratory tract is known to vary depending on the characteristics of the material, the health state of the individual concerned (Caboche et al., 2011), and the region of the lung being modelled. The lungs act as a continuous sampler of inhaled particulate matter some of which is deposited in the lung and of these a proportion is retained (Abraham et al., 1991). Indeed clearance time of particles can actually be in the order of months or even years. Typically, PM deposited in the tracheobronchial region are cleared by two main routes: (1) via mucociliary transport (such as to the gastrointestinal tract) or (2) to regional lymph nodes or blood capillaries (Sturm, 2007). Mathematical approaches for modelling pulmonary clearance are numerous (Stöber and McClellan, 1997), and it is clear that deposited/deposited in the lung are subject to short-term to long-term retention processes and require complex multi-compartment models for simulating tracheobronchial clearance (Sturm, 2007). No consistent experimental extraction time has been applied in the scientific literature, with time variations of between 5 min and 26 days reported, with most studies using ≤24 hours (e.g. Caboche et al., 2011). An investigation to determine a suitable extraction time for the IBM (i.e. optimal or maximum bioaccessibility) was undertaken using the abundant ball-milled in-house test soil. The extraction profile indicated that extraction of the bioaccessible Pb was time dependent, with rapid initial dissolution and a maximum bioaccessible Pb concentration determined at 96 hours and then an asymptotic response up to the experimental maximum time of 170 hours (Fig. 2). It has been suggested that about 10–35% of particles initially deposited in the human bronchial tree are still retained beyond 24 hours (Hoffman and Asgharian, 2003). It has also been suggested that materials deposited in the alveolar zone (i.e. deep lung) by sedimentation and diffusion are removed slowly, with a clearance time amounting to days (Lippman et al, 1980). We concluded that 96 hours (4 days) provides a suitable approximation of the steady state of inhalation bioaccessible Pb. This equates well with other authors (e.g. Wragg and Klinck, 2007) who have noted that an extraction time of 100 hours provides a conservative (maximum) estimate of Pb inhalation bioaccessibility.

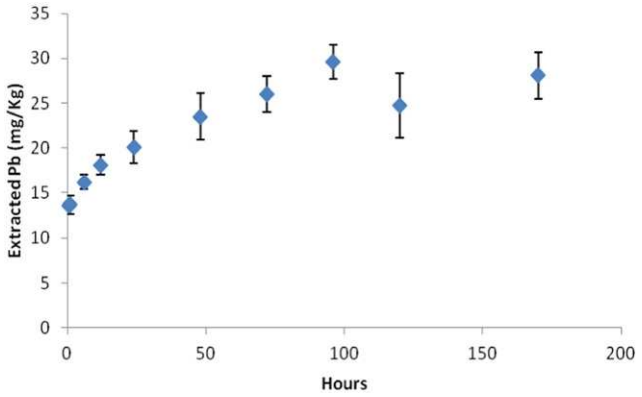

Fig. 2. Time-dependent extraction of Pb in the simulated epithelial lung fluid (SELF). The mean ($n = 9$) with a 1 standard deviation error bar is displayed.

4.2. Quality control

Excellent agreement is shown between the measured total Pb concentrations and their certificate/guidance values (Table 3). There are no certified reference materials for assessing *in vitro* inhalation bioaccessibility methods. The developed IBM was thus applied to a range of certified reference materials (BCR 038, BCR 143R, BCR 176R, BCR 723), and a guidance material (BGS 102) to investigate the precision and suitability of the approach as assessed using a mass balance approach: the sum of the stage 1 (inhalation bioaccessible) data and stage 2 (residual fraction) producing % total recoveries ranging from 100% to 103% (Table 3). The mass balance results show that the loss (or gain) of Pb in all the experimental stages were insignificant indicating that the analytical protocols employed are robust. For the *in vitro* inhalation extraction methodology the precision, expressed as relative standard deviation (% RSD), is also presented in Table 3. In this study the *in vitro* IBM extraction exhibited precision in the range of 5.7 to 16.3%.

The % inhalation bioaccessible fraction (% IBAF) ranged between 0.3% (BCR 038) and 8.4% (BCR 143R) illustrating the low extraction from a range of environmental matrices with particle sizes ranging from <10 μm to <105 μm.

4.3. Total and inhalation bioaccessible Pb concentration in PM_{10} fraction

The range of total and inhalation bioaccessible concentrations in the smelter, topsoils and tailings from Mitrovica (<10 μm fraction) is shown in Table 4. The total mean concentration of Pb varied between matrices: smelter (20,900–72,800 mg kg^{-1}), topsoil (274–13,700 mg kg^{-1}), and tailings (2990 mg kg^{-1}–25,300 mg kg^{-1}), and also by location (Fig. 3). The *in vitro* inhalation bioaccessible fraction (% IBAF) ranged between 0.02 and 11.0%, with high inhalation bioaccessible concentrations being observed for samples from the Bosniak Mahalla area of Mitrovica (Fig. 3). Samples without Pb-bearing mineral phases indicated the highest mean % IBAF (for indicative XRD-derived mineralogy of these samples the reader is referred to Boisa et al., 2013). The observed range (0.02 to 11.0% bioaccessibility) closely approximates the range 0.17% to 10.7% previously reported by Harris and Silberman (1988) for Pb in inhalable particles of fly ash (<22 μm fraction) extracted with canine serum (a biological fluid selected to mimic the fluid lining human airways). The mean Pb bioaccessibility obtained for the 5 mine tailings from Mitrovica (0.29%; % IBAF range: 0.02–1.26) is well below the values (14.4%, 18.0% and 25.3%) recorded for tailing samples from a Welsh mine site (Wragg and Klinck, 2007). The large difference observed in inhalation bioaccessibility between these two different studies may be due to different Pb mineral forms in the samples or differences

Table 3
Determination of lead in certified reference and guidance materials: total and inhalation bioaccessible fractions.

Certified reference or guidance material	Certified value (mg kg^{-1})	Determined concentration (mg kg^{-1})	*In vitro* inhalation bioaccessibility method (IBM) (mg kg^{-1} Pb)					
			Stage I (inhalation bioaccessible fraction)			Stage II (residual digest)	Total content (Stage I + II)	
		Mean ± SD (n = 3)	Mean ± SD (n = 3)	% IBAF[a]	% RSD[b]	Mean ± SD (n = 3)	Mean (n = 3)	% Total recovery[c]
BCR 038	262 ± 11	252 ± 3.0	0.8 ± 0.1	0.3	7.50	252 ± 20	253	100
BCR 143R	174 ± 5	172 ± 3.0	14.4 ± 2.2	8.4	15.3	160 ± 2.0	174	101
BCR 176R	5000 ± 500	5024 ± 51	190 ± 31	3.8	16.3	4880 ± 311	5070	101
BCR 723	866 ± 16	851 ± 21	33.8 ± 3.0	4.0	8.90	832 ± 18	866	102
BGS 102	79.4 ± 1.4	70.2 ± 3.4	3.5 ± 0.2	5.0	5.70	68.8 ± 0.6	72.3	103

BCR 038 (fly ash from pulverised coal);
BCR 143R (sewage sludge amended soil);
BCR 176R (fly ash);
BCR 723 (road dust);
BGS 102 (naturally contaminated soil, from North Lincolnshire).
[a] % IBAF: stage related inhalation bioaccessible fraction, calculated as a fraction of the total concentration.
[b] % RSD (Relative standard deviation): calculated as (standard deviation / mean)*100.
[c] % Total recovery: sum of residual and bioaccessible Pb calculated as a fraction of the total concentration.

in chemical composition of the leaching liquids employed in the studies. The four smelter samples from Kosovo also exhibited a very low mean % IBAF (1.20%; range: 0.03–4.60%). Given the variety of composition of *in vitro* simulated lung fluids currently in the literature, the fact that the inhalation bioaccessibility values obtained in this study differ to ranges previously reported for Pb on similar materials is to be expected. As previously highlighted, the SELF developed in this study includes antioxidants, large molecular mass proteins (mucin and albumin) and a surfactant lipid that have the potential to influence the dissolution kinetics of Pb (Cross et al., 2001; Duranti et al., 2001; Gehr et al., 1994; Thornton et al., 2008). The extent to which this occurs clearly warrants further investigation; however this was beyond the scope of the current project.

4.4. The relevance of inhalation bioaccessibility in estimating potential human health impacts

Human exposure to airborne contaminants can be estimated by calculation of respiratory intake or inhalable dose. Exposure to Pb in the resuspendable (PM_{10}) fraction of surface soils and metallurgic waste was estimated for adult receptors in the study area. Children are typically considered the most sensitive receptors, being more at risk following exposure to Pb because their growing bodies more readily absorb the Pb and because their tissues are especially sensitive to damage (Chandramouli et al., 2009). However, as the developed IBM is based on information available for adults and as children are not "mini-adults," either biologically or physically, we modelled our inhalable dose for an adult receptor.

In order to consider the relevance of the lung bioaccessibility results in terms of potential human health impacts we need to calculate a daily exposure to Pb (daily intake (DI) μg kg$^{-1}_{BW}$ day^{-1}). This was done using a modified version of the literature approach detailed in Chen et al. (2011):

$$DI = \left(F_{Pb} \times TR \times PM_{10} \times V_{resp}\right)/BW \quad (3)$$

In Eq. (3), PM_{10} is the concentration of particles with a diameter less than 10 μm (μg m^{-3}); F_{Pb} is the mass fraction (total or bioaccessible) of Pb in the PM_{10}; TR is the tracheobronchial retention, expressed as a fraction (i.e. the fraction of original intake retained in the respiratory tract once some of the deposited particles have been cleared by the bodies various clearance mechanisms); V_{resp} is the inhalation rate (m^3 day^{-1}); and BW is the body weight (kg). The advantage of using a mass fraction for the Pb content and a fraction for the tracheobronchial retention is that the units generate output from the equation directly as μg kg$^{-1}_{BW}$ day^{-1}.

The parameters used to evaluate the inhalation exposure to Pb in the Mitrovica samples are summarised in Table 5. Environmental monitoring in the Mitrovica region by Arditsoglou and Samara (2005) reports a mean (annual) TSP in the local atmosphere of 181 μg m^{-3}. Published ratios of PM_{10}:TSP range from 0.59 to 0.8 in studies where traffic is likely to be the main source and from 0.37 to 0.53 where mining and industry are predominant sources (e.g. Berico et al., 1997; Roosli et al., 2000; Triantafyllou et al., 2006), whilst the U.S. EPA (1995) uses a ratio of 0.5 (PM_{10}:TSP) for windblown dust from aggregate storage areas. In a recent study measuring PM_{10} concentrations at locations within the same study area as ours, a maximum observed concentration of 83 μg m^{-3} was observed (Shala et al., 2010), consistent with the TSP value of 181 μg m^{-3} reported by Arditsoglou and Samara (2005) when using a PM_{10}:TSP ratio of 0.5. This maximum observed concentration of 83 μg m^{-3} PM_{10} was used to model the inhalation dose.

Another factor that influences the modelled inhalation dose is the fraction of particulate matter retained in the respiratory tract following inhalation. As noted earlier, particles inhaled/deposited in the lung are subject to short-term to long-term retention processes and require complex multi-compartment models for simulating tracheobronchial clearance (Sturm, 2007). To apply a tracheobronchial retention factor (TR) is clearly an oversimplification of the system, but at this stage of procedural development was considered a pragmatic approach. Chen et al. (2011) applied 75% for lung retention (based on SFT, 1999) whilst 50% retention is reported after about 5 days according to Sturm (2007). Again taking a conservative approach, the former retention estimate used by Chen et al. (2011) is applied in this study.

The estimated inhalation dose (for adults) calculated from the total Pb concentration in PM_{10} ranged from 0.005 to 0.242 μg kg$^{-1}_{BW}$ day^{-1} from the topsoils, 0.053–0.446 μg kg$^{-1}_{BW}$ day^{-1} from the tailings, and 0.369–1.284 μg kg$^{-1}_{BW}$ day^{-1} from the smelter samples. JECFA (2012) report that an intake of 1.9 μg kg$^{-1}_{BW}$ day^{-1} is associated with a population decrease of 3 IQ points, whilst a dietary intake of 0.50 μg kg$^{-1}_{BW}$ day^{-1}, derived using the Integrated Exposure Uptake Biokinetic (IEUBK) model, has been linked to neurodevelopmental effects in children (ESFA, 2010). Evidence for the impact of even low-level environmental Pb exposure on human health, behaviour and cognition is increasing, along with a growing consensus that there is likely to be no clear threshold, nor linear dose–response relationship, between Pb exposures in children and neurodevelopmental impacts (Bellinger, 2008; Budtz-Jørgensen et al., 2013; Chandramouli et al., 2009; Grandjean, 2010; Jakubowski, 2011; Lanphear, et al., 2005). A number of recent studies suggest that the threshold for clinical concern should be reduced to a blood Pb level of 5 μg dL^{-1} (e.g. Chandramouli et al., 2009) whilst the European Food Safety Authority (2010) concluded that there is no known safe exposure to Pb. Significantly, in the USA, the California Office of Environmental Health Hazard Assessment has

Table 4
Descriptive statistics for total and inhalation bioaccessible Pb from the Mitrovica samples. Smelter ($n = 4$); topsoil ($n = 24$); tailings ($n = 5$).

Matrix	Mean	Minimum	Maximum	Median
Total concentration (mg kg^{-1})				
Smelter	43,000	20,900	72,800	39,200
Topsoil	3240	274	13,700	2280
Tailings	8450	2990	25,300	4720
Inhalation bioaccessible concentration (mg kg^{-1})				
Smelter	272	7.00	965	58.0
Topsoil	202	9.80	1060	57.4
Tailings	15.0	0.70	49.2	10.2
Inhalation bioaccessible fraction (IBAF) (%)				
Smelter	1.20	0.03	4.60	0.08
Topsoil	5.34	0.50	11.00	5.70
Tailings	0.29	0.02	1.26	0.05
Total mass recovery (%)				
Smelter	101	99.3	104	100
Topsoil	99.9	96.2	104	99.5
Tailings	101	98.3	102	101

developed a 1 μg dL^{-1} benchmark for source-specific incremental change in blood lead levels for protection of school children and foetuses (OEHHA, 2009). A similar downward revision of the UK guideline values for Pb would greatly increase the urban land area with soil considered as hazardous in terms of its Pb content. The recently released provisional category 4 screening levels (pC4SL) for Pb base their upper modelled pC4SLs on a blood Pb concentration of 5 μg dL^{-1} (DEFRA, 2013). The resultant UK residential (with home grown produce) pC4SLs for Pb in soil range from 200 to 210 mg kg^{-1}. This is lower than even the minimum total Pb concentration observed in the topsoils across Mitrovica (Table 4). Our findings, however, suggest that Pb within the extracellular lung environment is not readily bioaccessible in any of the three sample groups (topsoils; smelter waste; tailings). When daily inhalation doses were calculated using the bioaccessible Pb concentration the exposure doses are generally lower, by about two orders of magnitude, than the doses derived from total Pb. The bioaccessible inhalation dose from topsoil extracted PM_{10} ranged from 0.0002 to 0.0187 μg kg$^{-1}_{BW}$ day^{-1}, 0.0001–0.0170 μg kg$^{-1}_{BW}$ day^{-1} from smelter samples, and 0.0001–0.0009 μg kg$^{-1}_{BW}$ day^{-1} in the tailing samples. For the general European population, human exposure to Pb is mainly via food and water with an average adult Pb dietary exposure ranging from 0.36 to 2.43 μg kg$^{-1}_{BW}$ day^{-1} in high consumers in Europe (EFSA, 2010). Clearly in contexts such as Mitrovica where non-dietary Pb exposure from soil and dust may not be negligible and may add to the exposure burden then the inhalation pathway needs to be considered as part of the overall risk assessment. This may be particularly relevant as previous work on oral (ingestion) bioaccessiblity in Northern Kosovo has indicated the high gastric bioaccessibility of Pb present within these same topsoils (13.40–92.20%; Boisa et al., 2013). Clearly any inhalation exposure will add to an already elevated Pb burden to which the local population are exposed.

A recent study using Pb isotopes to investigate the key sources of Pb in these topsoils from Mitrovica indicated ratios of $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ that fell between those for smelter waste stored at Gornje Polje, bedrock from the Mitrovica area and those for Eastern European petrol combustion residues, with localised inputs from metallurgical slag resulting from secondary vehicle battery processing. Wind-blown dispersal of Pb-rich waste from the Zvecan smelter (whilst it was active) and the Gornje Polje tailings dump are the principal sources (FLUVIO, 2010). The social and economic consequences of limited primary prevention of exposure to Pb have been reported in the literature, highlighting the benefits of removing sources of lead exposure (Gould et al., 2009; Grosse et al., 2002; Pichery et al., 2011; Schwartz, 1994).

5. Conclusion

There is growing concern for the impact of PM on human health. Studies such as ours which seek to develop robust methods to enhance the reliability and practicality of assessing release from inhaled particles are needed if we are to adequately assess the potential health risks associated with exposure to metals in the inhaled fraction. We propose a new *in vitro* simulated epithelial lung fluid (SELF) that, in addition to the full complement of chemical groups present in other formulae, also contains high molecular mass proteins, antioxidants and a

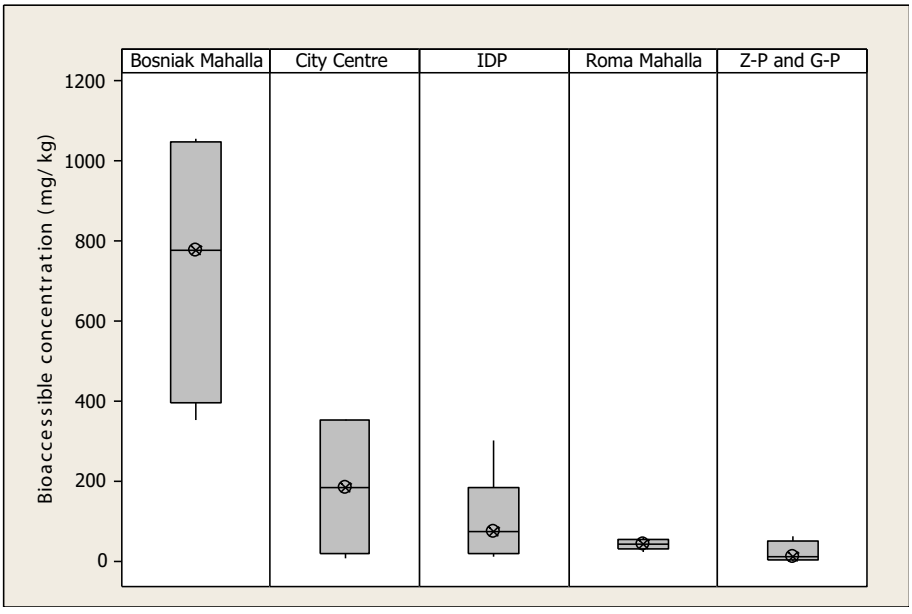

Fig. 3. Inhalation bioaccessible Pb concentrations at the different sample locations across Mitrovica. Bosniak Mahalla ($n = 4$); Mitrovica City Centre ($n = 7$); internally displaced peoples camps (IDPs) ($n = 6$); Roma Mahalla ($n = 8$); Zharkov Potok (Z-P) and Gornje Polje (G-P) ($n = 9$). The mean is represented by a circle and the median a solid line through the box.

Table 5
Variables used in calculating the exposure dose of Pb in PM_{10}.

Parameter	Description	Adult
F_{Pb}	Total or bioaccessible Pb concentration range in PM_{10} (dimensionless)	Total range[a]: 0.0003–0.0728 Bioaccessible range[a]: 0.000007 to 0.00106
TR	Tracheobronchial retention	0.75[b]
PM_{10}	Concentration of particles with an aerodynamic diameter less than 10 μm (μg m^{-3})	83[c]
V_{resp}	Inhalation rate expressed (m^3 day^{-1})	17[d]
BW	Body weight expressed (kg)	60[d]

[a] Reported as mass fraction (mg kg^{-1} divided by 10^{-6}).
[b] SFT (1999).
[c] Shala et al. (2010).
[d] ESTA (2013).

surfactant. The proposed IBM is designed to mimic the tracheobronchial tract of the human respiratory system. Given the seemingly contradictory research findings in the literature on the role of proteins (such as mucin) and antioxidant reagents with respect to the bioaccessibility of PHEs, further work is needed in this area. Literature data on the respiratory uptake of PHEs are, in general, limited and difficult to compare due to the lack of a standard procedure to simulate the *in vivo* respiratory uptake. Presently there is no human or animal *in vivo* data to validate lung bioaccessibility protocols, although the observed range using our IBM closely approximates the range of 0.17 to 10.7% reported by Harris and Silberman (1988) for Pb in inhalable particulates of fly ash (<22 μm fraction) in canine serum. The development of specialised fluids to mimic other parts of the human respiratory system may also be required, possibly as a two, or more, phase process such as the UBM for gastric and intestinal phases (Wragg et al., 2009). Particles that get into the distal lung are likely to be phagocytosed by macrophages and then undergo dissolution in the more acidic phagolysosomal environment within the cell (Stefaniak, 2005). Work undertaken on roadside dusts (63–125 μm fraction) by Potgieter-Vermaak et al. (2012) concluded that the Pb concentration released in their artificial lysosomal fluid (pH 4.55) was substantially higher than that found for their Gamble's solution (pH 7.35) and concluded that this was linked to the reduced extractant pH. A recent study on nanoparticles (<3 μm) of galena (PbS) also indicated high bioaccessibility in the modelled human lung environment as a result of phagocytosis (Beeston et al., 2010). The current literature therefore implies that inhaled particles would have to be phagocytized before significant Pb dissolution occurs (in the more acidic environment). As such, our developed IBM, based on SELF with a neutral pH (pH 7.4), may be an underestimate of the overall lung bioaccessibility, and this must be considered in any estimates of exposure, particularly where the PM_{10} fraction is dominated by the smaller "respirable" fraction as it is the smaller particles that are more likely to be phagocytized.

This paper also reports on the application of our IBM method to determine the Pb inhalation bioaccessibility of urban topsoil (and related samples) from Mitrovica, Kosovo. Although determined inhalation bioaccessibilities are generally lower, by about two orders of magnitude, than those modelled using total Pb concentration, it is widely accepted that airborne dust from tailings and other urban and industrial processes may represent an important exposure pathway and subsequent risk to human health. So, whilst the results from our Kosovo study indicate a low potential inhalation bioaccessibility for Pb (modelled for the neutral pH conditions of the interstitial lung environment), it is not unreasonable to speculate that, under changing climatic conditions (e.g. dry and arid), or where significant prolonged dust or particulate generating activities are taking place (e.g. unregulated battery reprocessing, demolition and construction sites) or where the inhaled particles are phagocytized, inhalation exposure has a significant potential to add to an already elevated Pb burden experienced by the local population. Such data are important for local policy makers to better enable them to assess risk, especially for those soils/dusts which have elevated levels of contamination, providing additional information on potential sources of Pb exposure.

This paper has focussed solely on the direct inhalation pathway and consideration of the human health risk via exposure to PHEs requires assessment of multiple routes (i.e. ingestion, inhalation and dermal absorption) to allow an overall systemic loading to be derived and evaluated. Given the significance of the inhalation route for certain PHEs, such as Ni (Environment Agency, 2009b), and in certain contexts (e.g. dust generating contexts) it is timely that work developing robust inhalation bioaccessibility protocols receives wider attention by the bioaccessibility research community. One can only truly define the most appropriate *in vitro* model by comparison with *in vivo* data. In the absence of *in vivo* data our approach was to critically appraise the literature to contribute to an understanding of the factors pertinent to the design of simulants of the lung environment and the subsequent experimental protocol for the study of particle dissolution using a static dissolution technique. Whilst bioaccessiblity in soils is often highly site-specific, the results of this study are also of potential wider value, allowing for the cross-comparisons between studies focusing on the same element, method and/or media of interest.

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