University of São Paulo
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Human bioaccessibility and absorption by intestinal cells of potentially harmful elements from urban environmental matrices

Alexys Giorgia Friol Boim

Thesis presented to obtain the degree of Doctor in Science. Area: Soil and Plant Nutrition

Piracicaba
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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Purificar o Subaé
Mandar os malditos embora
Dona d'água doce quem é?
Dourada rainha senhora
Amparo do Sergimirim
Rosário dos filtros da aquária
Dos rios que deságuam em mim
Nascente primária
Os riscos que corre essa gente morena
O horror de um progresso vazio
Matando os mariscos e os peixes do rio
Enchendo o meu canto
De raiva e de pena
(Purificar o Subaé – Caetano Veloso)

"The scientific man does not aim at an immediate result. He does not expect that his advanced ideas will be readily taken up. His work is like that of the planter—for the future. His duty is to lay the foundation for those who are to come and point the way."
(The Problem of Increasing Human Energy - Nikola Tesla)

CONTENTS

RESUMO

Bioacessibilidade humana e absorção por células intestinais de elementos potencialmente nocivos em matrizes ambientais urbanas

Elementos potencialmente nocivos (EPN), dentre eles os metais pesados, são encontrados naturalmente nos solos, geralmente em baixas concentrações. Porém, devido à intensidade das atividades antrópicas, as concentrações destes elementos podem aumentar e ocasionar efeitos negativos ao meio ambiente e à saúde humana. Métodos para a avaliação de risco podem prever ou indicar o nível da exposição de uma área à contaminação. Além do teor total ou pseudototal de EPN, geralmente extraídos com soluções ácidas, pode-se determinar os teores nas frações reativa, biodisponível, bioacessível destes elementos para avaliação do grau de contaminação do solo. Por sua vez, métodos *in vitro* têm sido utilizados em vários países para avaliar a bioacessibilidade de PHE em seres humanos. Neste trabalho foram utilizadas amostras de solo urbano coletadas em Piracicaba e solos coletados em áreas de uma antiga usina de metalurgia de chumbo (Usina do Calabouço/IPT) na cidade de Apiaí, ambas localizadas no Estado de São Paulo; e na cidade de Santo Amaro, Bahia, onde foram coletadas amostras de solos urbanos localizados em áreas residenciais, principalmente próximas a uma antiga área de refino de chumbo, onde foram detectados níveis elevados de EPN. Foram avaliados procedimentos baseados na ingestão e na inalação de solos coletados por meio dos métodos *Unified BARGE Method* (UBM) e do *Artificial Lysosomal Fluid* (ALF) para obtenção do teor bioacessível nos fluidos gastrointestinais e nos fluidos pulmonares, respectivamente. Como a fração bioacessível não estima a concentração absorvida e transportada para a corrente sanguínea, o método *in vitro*, utilizando células Caco-2, que são extraídas de adenocarcinoma do cólon humano, foi usado para avaliar a quantidade de EPN que as células intestinais podem absorver. As características mineralógicas das amostras e a extração sequencial de As, Cd, Cu, Mn, Pb e Zn foram obtidas para avaliar a interação com fluido pulmonar e do o suco gástrico e gastrointestinal. Como esperado, as amostras de rejeito de mineração apresentaram as maiores concentrações pseudototais de EPN em comparação com as amostras de solo e sedimento, tanto nas amostras < 2mm, como nas amostras de tamanho < 250 µm e < 10 µm. A bioacessibilidade respiratória e oral dos EPN variou amplamente entre as matrizes, indicando que foram influenciadas por características químicas, físicas e mineralógicas das matrizes. A fração bioacessível respiratória, calculada como porcentagem das concentrações de EPN pseudototal, variou de 13 a 109% para As; 14 - 98% para Cd; 21 - 89% para Cu; 46 - 140% para Pb, 35 - 88% para Mn e; 21 - 154% para Zn. A bioacessibilidade gástrica foi maior que a bioacessibilidade gastrointestinal, variando de 0 a 33% e 0 a 26% para As; 0-69% e 0-40% para Cd; 18-75% e 12-89% para Cu; 24-83% e 7-50% para Pb; 43-105% e 27-97% para o Mn; 14-88% e 6-46% para Zn. A concentração pseudototal forneceu uma boa estimativa para bioacessibilidade respiratória e oral, mas os métodos *in vitro* fornecem resultados mais precisos. A linhagem celular Caco-2 foi um bom modelo para avaliar o efeito da exposição ao PHE, mas são necessários mais estudos sobre o transporte e biodisponibilidade de PHE em células intestinais.

Palavras-chave: Bioacessibilidade oral e respiratória; Avaliação de risco; Células Caco-2; Saúde humana; Poluição do solo

ABSTRACT

Human bioaccessibility and absorption by intestinal cells of potentially harmful elements from urban environmental matrices

Potentially harmful elements (PHE) are found naturally in soils, usually in low concentrations. However, due to the intensity of the anthropic activities, the concentrations of these elements may increase and have negative effects on the environment and human health. Methods for risk assessment may predict or indicate the level of exposure to contamination of an area. In addition to the total or pseudo-total concentration of PHE, generally extracted with acidic solutions, it is possible to determine the reactive, bioavailable and bioaccessible levels of these elements in order to evaluate the degree of soil contamination. Urban soil samples located in residential areas were collected in Piracicaba, State of São Paulo (SP) and in Santo Amaro, State of Bahia, including soils collected near a primary lead smelter area (COBRAC/Plumbum), where researchers detected elevated levels of PHE. Soils samples in an old lead metallurgy plant (Usina do Calabouço / IPT), which today belongs to the Centro Integrado de Ensino Multidisciplinar (CIEM/ Companhia de Pesquisa de Recursos Minerais (CPRM) - Geological Survey of Brazil) in Apiaí, located in the Upper Ribeira Valley (SP) were also collected. *In vitro* methods have been used in several countries to assess the bioaccessibility of PHE in humans. In this study, procedures based on ingestion and inhalation of soils using the Unified BARGE Method (UBM) and Artificial Lysosomal Fluid (ALF) methods were used to obtain the bioaccessible concentration in the gastrointestinal and pulmonary tract, respectively. As the bioaccessible fraction does not estimate the concentration absorbed and transported into the bloodstream, the *in vitro* method using Caco-2 cells, which are derived from human colon adenocarcinoma, was used to assess the amount of PHE that intestinal cells can absorb. The mineralogical data was obtained, and the sequential extraction of As, Cd, Cu, Mn, Pb and Zn was carried out to evaluate their interaction with lung fluid and gastric/gastrointestinal fluids. As expected, mine tailing samples had the highest pseudo-total concentrations of PHE in comparison to soil and sediment samples, both in the bulk soil (2 mm) and in the 250 µm and 10 µm sizes. Both respiratory and oral bioaccessibility of PHE varied widely among matrices, indicating that they were influenced by matrices´ chemistry, physical and mineralogical characteristics. The respiratory bioaccessible fraction, calculated as a percentage of the PHE pseudo-total concentrations, ranged from 13 - 109% for As; 14 - 98% for Cd; 21 - 89% for Cu; 46 - 140% for Pb, 35 - 88% for Mn and; 21 - 154% for Zn. Gastric bioaccessibility was greater than gastrointestinal bioaccessibility, ranging from 0-33% and 0-26% for As; 0-69% and 0-40% for Cd; 18-75% and 12-89% for Cu; 24-83% and 7-50% for Pb; 43-105% and 27-97% for Mn; 14-88% and 6-46% for Zn. Pseudo-total concentration provided a good estimate of respiratory and oral bioaccessibility, but the *in-vitro* methods provided more accurate results. Caco-2 cell line (*in vitro* test) was a good model for evaluating the effect of PHE exposure, but further studies on the transport and bioavailability of PHE in intestinal cells are needed.

Keywords: Oral and respiratory bioaccessibility; Risk assessment; Caco-2 cells; Human health; Soil pollution

LIST OF ABBREVIATIONS AND ACRONYMS

ALF	Artificial lysosomal fluid
ANOVA	Analysis of variance
AP	Apiaí city
BA	Bahia state
BAF	Bioaccessible fraction
BARGE	Bioaccessibility Research Group of Europe
BCR	Bureau Communautaire de Reference
BGS	British Geological Survey
BS	Bulk sample
CEC	Cation-exchange capacity
CIEM	Centro Integrado de Estudos Multidisciplinares de Apiaí
CONAMA	Conselho Nacional do Meio Ambiente
CPRM	Companhia de Pesquisa de Recursos Minerais
CRM	Certified reference material
DCB	Dithionite-citrate-bicarbonate
DMA	Dimethylarsinate
DMEM	Dulbecco's Modified Essential Medium
DRS	Diffuse reflectance spectroscopy
EDS	Energy dispersive spectrometry
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
FF	Fine fraction
FTIR	Fourier transform infrared
G	Gastric fluid
GFAAS	Graphite furnace atomic absorption spectrometry
GI	Gastrointestinal fluid
HHRA	Human health risk assessment
HSD	Honestly significant difference
IC	Inorganic carbon
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
ISO	International Organization for Standardization
LD	Limit of detection
LQ	Limit of quantification
MMA	Monomethylarsonate
MT	Metallothionein
NDIR	Non-dispersive infrared sensor
NEAA	Non-essential amino acids
NIST	National Institute of Standards and Technology
O.M	Organic matter
OC	Organic carbon
PBS	Phosphate buffered saline
PC	Piracicaba city
PHE	Potentially harmful elements
PM 10	Particulate material less than 10 μm
PM 2.5	Particulate material less than 2.5 μm
rpm	Rotations per minute
RSD	Relative standard deviation
SA	Santo Amaro city

SD	Standard Deviation
SEM	Scanning electron microscopy
SHIME	Simulator of Human Intestinal Microbial Ecology
SP	São Paulo state
SRM	Standard Reference Materials
TC	Total carbon
TEET	Transepithelial electrical resistance
UBM	Unified BARGE Method
USEPA	United States Environmental Protection Agency
VI	Value of research
VP	Value of prevention
XRD	X-Ray Diffraction

1. INTRODUCTION

The development of cities is proportional to the needs and to the size of the population. Unfortunately, the same thing occurs with pollution levels in these places, mainly because of the high quantity of solid waste and atmospheric pollutants released into the environment. The quality of the urban environment and places like, gardens and leisure areas are usually diminished by the presence of traffic, industries, building material, urban waste and so forth. Atmospheric pollution, for example, can provide the deposition of particulate material onto the soil which, in turn, can release substances or elements contaminating the environment that could be exposed to humans, either by inhalation, by ingestion or by dermal contact.

Soils from urban areas are generally modified by human activity and can present coarse or fine textured materials of anthropic origin (Morel *et al.*, 2014) that alters their pedogeochemical characteristics (Angelone & Udovic, 2014). Soil studies in urban areas are necessary, since humans have direct contact especially in recreation areas where there is great exposure, especially by children through hand-to-mouth contact or pica behaviour during outdoor activity (Luo *et al.*, 2012). The outdoor activities of children generally occur under good climatic conditions, low relative humidity, when their immune system is more fragile than those of adults and are more susceptible to the development of diseases.

Potentially harmful elements (PHE) are defined as elements that can be either contaminants or nutrients to humans, depending on their concentration and can be released by different sources into the environment, such as nature or human activities (Bini & Wahsha, 2014). The levels of toxic elements such as As, Cd, Pb and Hg or nutrients, such as Cu, Zn and Mn, have increased in the environment and the soil is one of the main sinks of large amounts of these elements that can affect human health. In consequence, many studies have been carried out mainly in relation to soil quality and the availability or bioaccessibility of PHE to human. At the same time, guidelines and regulations have been established to assist with risk assessment of potentially contaminated soils.

In a risk assessment, the investigation and exploration link the source of a contaminated substance and the exposed population (Elom *et al.*, 2013). The human health is the primary concern and the policy decisions are based on the concentration of a particular contaminating substance combined with exposure modelling (Casarini *et al.*, 2001; Hansen *et al.*, 2007; CONAMA, 2009).

In Brazil, the value of research (VI) is the concentration of an element or substance in soil or groundwater that may present some potential direct or indirect risks to human health,

considering a standardized exposure scenario. The VI were derived initially by Environmental Agency of São Paulo (CETESB) through the CETESB Worksheet for Human Health Risk Assessment (CETESB, 2013) from equations developed by USEPA (1989a), with parameters derived from Sao Paulo conditions.

The National Environment Council (CONAMA) Resolution nº 420, 2009 recommends the extraction of pseudo-total concentration of PHE for assessment of their VI (National Environment Council - CONAMA, 2012). The PHE concentrations are determined by extraction with an acid extractor, such as *aqua Regia* (HCl: HNO_3, 3:1, v/v) or US. EPA 3051A (HNO_3: HCl, 3:1, v/v). However, pseudo-total concentration is not a good indicator of the bioavailability of PHE as it may overestimate the concentration that actually comes into contact with humans and in risk assessment this can result in unnecessary and expensive remediation and avoiding heightened public concern about the potentially contaminated site (Cave *et al.*, 2013; Ng *et al.*, 2015; Yan *et al.*, 2017).

The determination of the bioaccessible concentration is used to assess health risks. Bioaccessibility is defined as amount of PHE in soils or other environmental matrices, such as sediments, tailing, dust, etc., that may be soluble in fluids in the lungs or the gastric/gastrointestinal tract, but are not necessarily absorbed into the circulatory system and can be a surrogate for bioavailability measurements (Intawongse & Dean, 2006; Turner, 2011; Deshommes *et al.*, 2012; Ianni *et al.*, 2014).

Generally, studies with experimental animals, using rodents or swine methods, may determine an enable estimative of available levels to humans, but these tests are expensive and time-consuming. Therefore, researchers have developed *in vitro* tests to evaluate the bioaccessibility of contaminants to humans (Ruby *et al.*, 1993, 1999, Oomen *et al.*, 2002, 2003a; Schroder *et al.*, 2004; Van de Wiele *et al.*, 2007; USEPA, 2008; Wragg *et al.*, 2009, 2011; Zia *et al.*, 2011)

In relation to human bioaccessibility of contaminants, the risk occurs when there is a likelihood of adverse effect to contaminants and will depend on the soluble fraction in the gastrointestinal or respiratory system.

The contaminant is bioavailable when the bioaccessible PHE crosses the intestinal epithelium tissue or is solubilized by macrophages in alveolar system and passes into the bloodstream (Cave *et al.*, 2011; Rogers, 2011; Pelfrêne *et al.*, 2017). Cells derived from a human colon adenocarcinoma (Caco-2) are widely used to predict the absorption of medicines and drugs or food, but they are still little used in soil science. The absorption of PHE by Caco-2 cells can

estimate the concentration that may be bioavailable to humans and this is an alternative to *in vivo* tests (Vasiluk *et al.*, 2011) and this method mimics the process of intestine PHE retention (Fu & Cui, 2013). The Caco-2 cells play an important role to estimate the bioavailable levels of PHE to humans, although cautious is necessary when using caco-2 cells because of their peculiarities in relation to the normal intestinal epithelium (Lea, 2015).

In Brazil, there are few studies that focus on the relationship between urban soils and human health, giving rise to the need for more detailed investigations on the interaction between the transfer of PHE to the soil solution and the gastrointestinal and respiratory systems.

A bibliometric survey using Scopus database showed a total of 13 articles and proceedings published by Brazilian researchers, some with foreign partnerships. The key words used were "bioaccessibility", “Brazil”, "soil" and "sediments". Of these 13 papers, one was carried out with soils from state of São Paulo (Rodrigues et al., 2018, Souza et al., 2018), one with soils from Amazon forest (Moreira et al., 2018), one with soils of the city of Três Marias, Minas Gerais state (MG) (Ono et al., 2016), two articles and three procedures with soils of Paracatu, MG (Ono et al., 2012a, b; Guilherme et al., 2014; Ng et al., 2014a, b, Ciminelli et al., 2018), two articles with soils from Adrianópolis, State of Paraná (Bosso & Enzweiler, 2008a; Bosso et al., 2008) and a review (Bosso & Enzweiler, 2008b). While around 350 articles and 13 international reviews were found at the Scopus research base, most of them were published by Chinese, American and British researchers.

In this context, the hypothesis was that the gastric and/or gastrointestinal fluids alter the speciation of PHE in the solid fraction of the sample and that the properties of each type of material can reflect the bioaccessibility behaviour of a PHE in the any matrices.

The general objective was to evaluate the efficiency of *in vitro* bioaccessibility and the absorption by Caco-2 cells assays for PHE in urban environmental matrices (soil, sediments and Pb tailing) with different levels of contamination as well as to evaluate the influence of mineralogy and geochemistry on the oral and respiratory bioaccessibility. Caco-2 cells method was used to estimate the absorption of PHE in some matrices samples and likely bioavailability of PHE by the intestine cells. It is intended that this study can serves as a reference for further studies focusing on the urban environment and its relationship with human health. Therefore, bioaccessibility methods in humans could become an integral part of the risk assessment procedure for potentially contaminated areas.

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2. RESPIRATORY BIOACCESSIBILITY OF POTENTIALLY HARMFUL ELEMENTS IN BRAZILIAN URBAN ENVIRONMENTAL MATRICES

Abstract

Humans living in large urban centres with heavy traffic and industrial activities, such as steel, metallurgy, mining etc. can be exposed to potentially harmful elements (PHE) daily. Atmospheric pollution, for example, can deposit particulate material containing anthropogenic and geogenic contaminants onto soils, to which humans are exposed via inhalation or ingestion routes during normal, everyday activities. There are few documented studies focusing on the relationship between urban soils and human health in Brazil, giving rise to the need for more detailed investigations regarding this interaction. In this study, the respiratory bioaccessibility fraction of urban environmental matrices contaminated with As, Cd, Cu, Pb, Mn and Zn were assessed in samples collected from parks, gardens and recreational areas in residential districts, near metallurgic industrial activities and in an area known to contain Pb tailings from previous mining activity. The matrices were classified into soil, sediment and tailing. Samples were collected from a depth of 0 to 5 cm and sieved to <10 µm. To simulate lung fluid an artificial lysosomal fluid (ALF) solution was employed to evaluate the bioaccessible concentrations of PHE, and the BCR (*Bureau Communautaire de Reference*) sequential extraction was performed to evaluate how the bioaccessibility of the PHE is related to the solid phase partitioning in the different matrices. The PHE pseudo-total (extracted with HCl: HNO_3, v/v 1:3) and the bioaccessible concentration in the samples covered a wide range. The bioaccessible fraction, calculated as a percentage of the PHE pseudo-total concentrations ranged from 13 - 109% for As; 14 - 98% for Cd; 21 - 89% for Cu; 46 - 140% for Pb, 35 - 88% for Mn and; 21 - 154% for Zn. The bioaccessibility of the PHE was high for some samples and related to particle size, mineralogy and to different soil phases from the BCR sequential extraction. The average bioaccessible fraction of the elements decreased in the following order: (i) Soil: Cd> Pb> Mn> As> Zn> Cu; (ii) Tailing: As> Pb> Cd> Zn> Cu> Mn; (iii) Sediments: As> Pb> Mn> Cd> Zn> Cu. ALF solubilized more PHE than the pseudo-total metal digestion in some samples, and this was attributed to the aggressive nature of ALF and to the presence of organic compounds that are capable of forming complexes with PHE. A high positive correlation was observed between the pseudo-total and the bioaccessible concentrations indicating that, for these matrices, the pseudo-total concentration can simply estimate the respiratory bioaccessibility of As, Cd, Cu, Pb, Mn and Zn.

Keywords: Artificial lysosome fluids; Human inhalation exposure; Particulate material; Soil pollution

2.1. Introduction

In the urban environment, potentially harmful elements (PHE) have several sources, mainly vehicular traffic and industries. Cities grow to meet population's needs, and the potential for urban pollution increases accordingly. Urban soils can be a sink of a substantial amount of waste products, such as industrial and mining waste, particulate matter emitted by motor vehicles or industrial chimneys whose contaminated particles can be deposited onto the most superficial layers of urban soils. Such particulate materials (PM), as well as the soil fine fraction, can be carried by the wind and reach human respiratory airways.

One of the main routes of exposure to PHE is the inhalation of particulate material or the fine fraction of the soils (PM10 or PM2.5, aerodynamic diameters < 10 µm and < 2.5 µm, respectively). The risk represented by inhalation of PHE from soils depends on the soluble fraction in the respiratory system. PM10 and PM2.5 can be suspended in the air for long periods of time, e.g. the PM10 can be suspended for a few hours, while PM2.5 can remain suspended in the atmosphere for several days to weeks (Leelasakultum & Kim Oanh, 2017) and when inhaled are deposited onto the surfaces of the respiratory system (Figure 1).

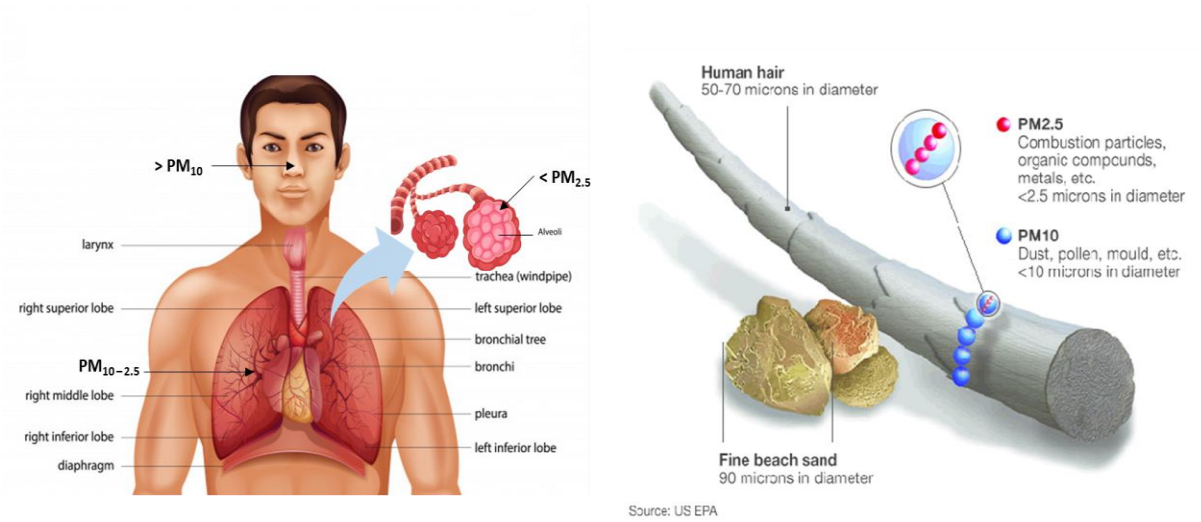

Figure 1. Figure (A) Respiratory system, inhalable particles (PM10 and PM2.5) can penetrate in the lungs and (B) comparison of particle size with human hair and sand grain (Source: (USEPA (United States Environmental Protection Agency), 2017)).

In humans, PM, when inhaled, pass through the tracheobronchial system and can arrive at the cellular system in the form of soluble particles which are transported into the circulatory system (Lehnert, 1990). Galle *et al.* (1992) demonstrated, however, that the opposite might occur, since lung macrophages are able to concentrate and precipitate various inhaled elements preventing the diffusion of toxic substances through the circulatory system. When a particle is not solubilized by the epithelial lining fluids (extracellular environment), it can be phagocytized by alveolar macrophages (intracellular environment), that allows the dissolution of a variety of substances (Figure 2) to a higher degree than under extracellular conditions (Kreyling, 1992).

In the context of human health risk assessment, the bioavailability of PHE or organic substances has been estimated using *in vivo* tests with animal models, such as rodents (for example, Moreda-Piñeiro *et al.*, 2011). Molina *et al.* (2013) evaluated bioaccessibility of Zn

by simulating phagolysosomal fluid (pH 4.5) and bioavailability assisted by intranasal administration of Zn (from Zn mine waste and minerals) in rodents and observed a positive relationship between the *in vivo* and *in vitro* tests (R^2 = 0.86). However, there is no standardised procedure (Calas *et al.*, 2017; Pelfrêne *et al.*, 2017) where *in-vitro* methods that have been developed can be compared with *in-vivo* studies.

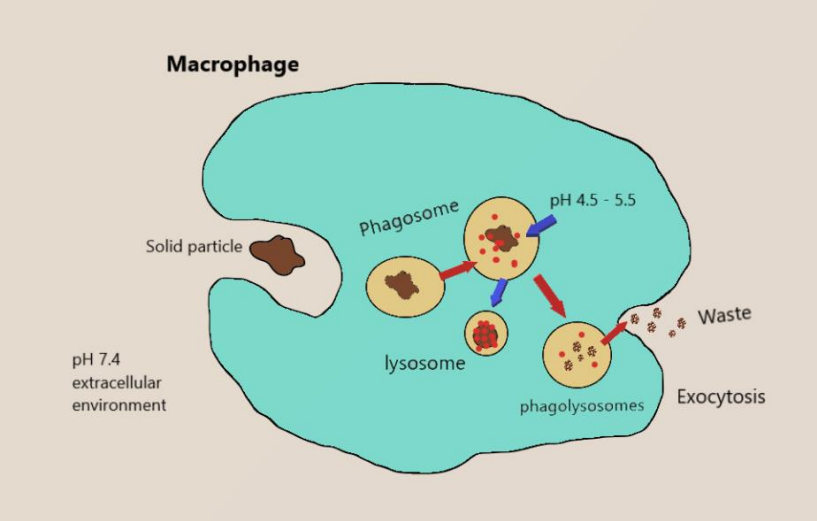

Figure 2. Dissolution of a particle by phagocytosis processes on a macrophage alveolar

In vivo tests in risk assessment are generally difficult to reproduce, expensive, time-consuming and have ethical constraints (Broadway *et al.*, 2010; Guney *et al.*, 2017). The *in vitro* method, as a simulator of PHE dissolution by pulmonary (extra/intra cellular) fluid can be used as a surrogate for *in vivo* tests, by estimating their dissolution by pulmonary (extra/intra cellular) fluid (Taunton *et al.*, 2010).

In vitro methods use simulated lung fluids to solubilize and quantify the elemental composition of substances or elements adsorbed to particulate materials, for example soil or dust, and considered bioaccessible to humans. Bioaccessibility is defined as a concentration of a substance or a PHE potentially available for uptake in the respiratory (or other) system and that could be transported into the bloodstream (Ruby *et al.*, 1996; National Research Council, 2003). Niu *et al.* (2010); Julien *et al.* (2011) and Huang *et al.*, (2014) evaluated the respiratory bioaccessibility of metals from atmospheric particles; similarly Wragg & Klinck (2007); Drysdale *et al.* (2012); Boisa *et al.* (2014); Pelfrêne *et al.* (2017) and Guney *et al.* (2017) have looked at bioaccessibility of metals from soil; Colombo *et al.* (2008); Hu *et al.* (2012) and Witt III *et al.* (2014) have assessed road dust; oxide nanoparticles have been investigated by

Cruz *et al.* (2015) and Zhong *et al.* (2017); and pharmaceutical materials have been looked at by Tronde, (2002) and Marques *et al.* (2011).

Gamble's solution is widely used to simulate the extracellular lung fluids and to estimate the quantity of PHE potentially available for absorption via respiratory tract (Wragg & Klinck, 2007; Drysdale, 2008). Whereas artificial lysosomal fluid (ALF), mimics the intracellular conditions in the lungs, i.e., the particle comes into contact with the lung fluids after being phagocytosed by alveolar and interstitial macrophages, and represents a more acidic environment (pH value of 4.5, against 7.3 for Gamble solution) (Colombo *et al.*, 2008; Calas *et al.*, 2017). The presence of complexing ligands (organic acids) and the low pH in the intracellular environment indicates that PHE-related solubilization may occur where they are adsorbed on oxides matrices, since the complexation of polyvalent cations with chelating agents (e.g. citrate acid) increase the mobilisation of PHE in the lung (Wiseman, 2015; Calas *et al.*, 2017). Organic acids can solubilize Fe from soil and form strong complexes due to dissociation from hydroxyl groups resulting in the multi-dentate and multi-nuclear complex species formation (Miller *et al.*, 1986). This has led to the hypothesis that PHE associated with more labile solid phases (soluble and exchangeable) in the soil and those phases associated with Fe oxides or/and organic matter in fine soil particle (< 10 μm) can be released into the pulmonary tract.

Pelfrêne *et al.* (2017) applied four pulmonary fluids (phosphate buffered saline solution (PBS at pH 7.3), Gamble's solution (pH 7.3), modified Gamble's solution (pH 7.3) and ALF (pH 4.5)) to three samples of standard reference material (BCR-723, NIST 2710a and NIST 1648a) to evaluate the lung bioaccessibility of a set of PHE (Ba, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sr and Zn). Pelfrêne *et al.* (2017) observed that the bioaccessibility of the elements was higher using the ALF compared to the other fluids, which was expected, since ALF is a more aggressive solution because of its low pH. The authors also conclude that ALF solution could be used to assess the lung bioaccessibility of PHE because it provides a more conservative estimation of the bioaccessible PHE. In this study respiratory bioaccessibility in the PM10 size fraction from a set of soils, sediments and mine tailings with chemical, physical and mineralogical measurements was compared to identify and understand the solid phase geochemical sources of bioaccessible PHE.

2.2. Material and Methods

2.2.1. Collection and sampling areas

The samples were collected in the cities of Piracicaba (n = 3), the eastern region of São Paulo State (SP); Apiaí (n = 5), Upper Ribeira Valley (SP); and Santo Amaro, in the region of Recôncavo Baiano, Bahia State (BA) (Table 1). In Piracicaba, the samples were collected from Mario Telles Square, José Bonifácio Downtown Square and at the athletics track of the Luiz de Queiroz College of Agriculture, University of São Paulo, near Independência Avenue, one of the busiest in the city (Appendix A, Figure 1). These are recreational locations of residential areas, frequented by children and have a high traffic density. These samples were classified as soils because of their chemical and mineralogical characteristics as will be seen in the next items.

The Apiaí samples were collected at the Centre for Integrated and Multidisciplinary Studies of Apiaí (CIEM), a unit of the Mineral Resources Research Company (CPRM) of Geological Survey of Brazil, located in the Upper Ribeira Valley Region (Appendix A, Figure 2). A lead (Pb) and silver (Ag) foundry and an old slag deposit operated in this area (Calabouço Mill) from 1940 to 1956. The main environmental issues in this area are mineral dust, metallic waste and Pb slag deposits (Martins & Figueiredo, 2014). The samples were collected from five sites: four in the plant area (classified as mining tailing) and one in the native forest near the unit (classified as soil). The collections were based on isolines map of Martins and Figueredo (2014).

In Santo Amaro, the samples were collected from within the urban perimeter and in an old Brazilian Pb company. The city is known for the intense galena (PbS) mining between the years of 1958 to 1993 and is considered to be one of the most Pb polluted cities in the world (CETEM/MCTI, 2012). During the operation of the company, 490,000 tons of accumulated slag were deposited around the factory, besides that used during the paving of the public and private areas (Andrade & Moraes, 2013). The soil samples were collected based on "Map of soil contamination by chemical elements in Santo Amaro da Purificação" carried out by Carvalho et al. (2010). Residents who worked or lived during the operational period of the company were interviewed and indicated where the slag was deposited. A total of nine samples were collected: one tailing (collected in the deactivated plant area), four soils (samples collected in residential areas, mainly frequented by children) and four sediments (two samples collected on the Subaé banks river and the other two samples collected into a street gutter and in an unpaved road close to the plant area). (Appendix A, Figure 3).

Table 1. Coordinates and sites characteristics of the local collection of soil, sediments and tailing matrices

Matrix	#ID	City	Coordinate		Site
Soil 1	35PC	Piracicaba	22°42.054'S	47°40.072'W	Mario Telles Square and Playground
Soil 2	47PC	Piracicaba	22°43'27"S	47°38'52.94"W	José Bonifácio Downtown Square
Soil 3	58PC	Piracicaba	22°42.850'S	47°37.975'W	Athletics track of the Luiz de Queiroz College of Agriculture campus
Soil 4	3SA	Santo Amaro	12°33.111'S	38°42.514'W	Saudade Cemetery Garden
Soil 5	5SA	Santo Amaro	12°33.287'S	38°41.675'W	Neighbourhood Derba, residential area
Soil 6	6SA	Santo Amaro	12°32.406'S	38°43.637'W	Soccer field for children near the old company facilities
Soil 7	9SA	Santo Amaro	12°33.651'S	38°42.073'W	Garden of Municipal School Maria dos Anjos Salles Brasil
Soil 8	5AP	Apiaí	24°32.443'S	48°49.851'W	Native forest, next to the old company facilities
Sediment 1	2SA	Santo Amaro	12°32.804'S	38°42.491'W	Subaé riverbank, next to Pedro Lago School
Sediment 2	4SA	Santo Amaro	12°33.019'S	38°42.385'W	Subaé riverbank, near the Forum
Sediment 3	7SA	Santo Amaro	12°32.427'S	38°43.632'W	Unpaved road connecting Rui Barbosa Avenue and the old facilities
Sediment 4	8SA	Santo Amaro	12°32.415'S	38°43.602'W	Street gutter of Rui Barbosa Avenue
Tailing 1	1AP	Apiaí	24°32.315'S	48°49.991'W	Soil with slag from the landfill at old company facilities
Tailing 2	2AP	Apiaí	24°32.323'S	48°49.909'W	Soil with slag from the landfill at old company facilities
Tailing 3	3AP	Apiaí	24°32.356'S	48°49.955'W	Soil with slag from the landfill at old company facilities
Tailing 4	4AP	Apiaí	24°32.349'S	48°49.899'W	Soil with slag from the landfill at old company facilities
Tailing 5	1SA	Santo Amaro	12°32.371'S	38°43.855'W	Soil with slag from the landfill at old company facilities

Each sample was composed of five subsamples collected from the 0 to 5 cm layer in an area of approximately 4 m² using a stainless-steel shovel and thoroughly mixed to obtain approximately 5 kg of soil. The samples were then placed in plastic bags and transported to the laboratory. The depth of 0-5 cm was chosen because it is assumed that this soil layer can be carried by the wind and exposed to human activities (Drysdale *et al.*, 2012). The samples were air dried, sieved in a 2 mm and quartered for further chemical, physical and mineralogical analyses. Each sample was stored in plastic pots and classified according to the type of matrices: soil, sediment and tailings. This classification was necessary to allow the evaluation of the effect of ALF solution on different matrices.

2.2.2. Preparation of the samples for the *in-vitro* test

The main interest was focused on particles with aerodynamic diameter of < 10 µm because these particles affect the tracheobronchial system and can reach the alveolar region (Boisa *et al.*, 2014; Guney *et al.*, 2017). Samples for the respiratory bioaccessibility test were prepared by mechanically shaking the 2 mm size fraction through a series of 1 mm, 500 µm, 250 µm, 125 µm and 63 µm (18, 35, 60, 120 and 230 Mesh) sieves for 30 min, retained in a collector and stored in plastic pots prior to further separation to obtain the 10 µm fraction. All materials used were plastic, except for sieves, which were made of stainless steel, to avoid contamination.

The separation method was adapted from Ljung *et al.* (2008, 2011). Approximately 40 g of the < 63 µm sub-sample was transferred to a 600 mL beaker containing 500 mL of ultrapure water (18 ΩM cm^{-1}). The suspension was shaken by hand, followed by ultrasonic dispersion for 5 min (three times), allowed to stand for 10 min and filtered using a 10 µm aperture nylon filter with the aid of a vacuum pump (Figure 3). The supernatant was transferred to a beaker and dried at 60 °C for approximately three days. After drying, samples were homogenized using an agate pestle and mortar, stored in plastic pots previously washed with 10% HCl and rinsed with ultrapure water.

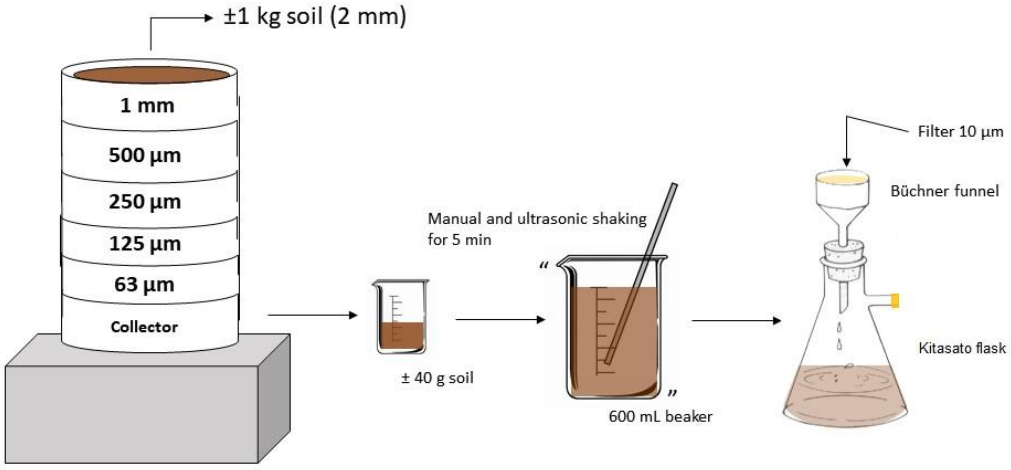

Figure 3. Preparation of the samples for the *in vitro* bioaccessibility test.

Samples classified as sediments commonly had high sand contents (Table 3) and contained some pieces of very small materials of dark colour, likely materials derived from the old metallurgical (Sediments 3 and 4).

The amount of material filtrated at 10 µm varied between the samples ranged from 1 to 12 g (data not shown). This fraction was nominated as "fine fraction" or "FF" (diameter < 10 µm) to differentiate it from bulk samples, that is, the sample as a whole (< 2 mm).

2.2.3. Chemical characterization and PHE pseudo-total concentrations

The pH was determined by potentiometry in 0.01M $CaCl_2$ (1:2.5 soil: solution ratio) according to the method suggested by Anderson & Ingram (1993). Sodium dithionite-citrate-bicarbonate solution method (DCB) (Mehra & Jackson, 1960) extracted the contents of Al and Fe crystalline oxides, while the acid ammonium oxalate solution method (Loeppert & Inskeep, 1996) extracted levels of amorphous Al, Fe, and Mn oxides.

Pseudo-total concentrations of PHE were obtained by the EPA 3051A method (1:3 HCl: HNO_3, v/v) according to USEPA (2007). For this, 0.25 g of fine soil (10 µm) was weighed in Teflon tubes, and then, 1.5 mL HCl (37%) and 4.5 mL HNO_3 (69%) were added. The samples were microwaved for 10 min at 175°C and then heated in a block for one or two days at 50ºC until complete evaporation of the acid solution. After evaporation, 2% HNO_3 solution was added to decant the residue. The extracts were diluted 600-fold for determination by mass spectrometry with coupled plasma source (ICP-MS). A blank and the standard reference material BCR 723 (Road Dust) sample were used for quality control (Table 2).

Table 2. Pseudo-total concentration of potentially harmful elements (PHE) of the SRM extracted by the EPA 3051a method (mean ± standard deviation)

SRM	As	Cd	Cu	Pb	Zn
	----------	----------	mg kg^{-1}	----------	----------
BCR 723 (measured)	12.8 ±0.01	1.8 ±0.001	221.0±0.8	833.2 ±2.1	1326.9 ±11.3
BCR 723 (certified)	NA	2.5 ±0.4	226 ±3.0[a]	866.0 ±16.0	1660 ±100.0

SRM = standard reference material; Value from Pelfrêne et al. (2017) [a]; NA = concentration value not available on certificate

2.2.4. X-ray diffraction

All samples (2 mm) were ground in a tungsten mill and sieved to 100 µm for the identification of the crystalline materials in the urban matrices. The mineralogical identification was performed by X-ray diffraction (XRD) using a Philips PW 1877 diffractometer operated at a potential of 40 kV, 40 mA currents, CuKα (k = 1.54186 Å), with a monochromator for the elimination of Kβ radiation, and step increment of one second for

each 0.02º (2θ). The clay fractions, free from iron oxides and treated with Mg^{2+} and K^{+}, were deposited on glass slides and analysed in a scanning range of 3º to 65º (2θ).

2.2.5. Determination of respiratory bioaccessibility of PHE in the soil, sediment and tailings matrices

To simulate the intracellular conditions in lung fluids, an ALF solution was prepared as described in Pelfrêne *et al.* (2017). Details of the procedure are described in ANNEX A. 0.05g (± 0.0001) of the fine soil fraction (10 µm) was weighed in 85 mL polycarbonate centrifuge tubes and 50 mL of the simulated fluid (ratio - 1:1000) was added. Samples were shaken at 37 ºC on an end-over-end shaker for 24 h. This extraction time was chosen because various authors have shown (Cruz *et al.*, 2015; Pelfrêne *et al.*, 2017; Guney *et al.* ,2017) that it is sufficient for maximum dissolution of the soil PHE For each element, the bioaccessible fraction (BAF%) for the lung compartment was calculated according to the following equation: BAF (%) = ALF concentration * 100 / Pseudo-total concentration

2.2.6. Sequential Extraction

The BCR-modified method (Rauret *et al.*, 1999) has been successfully applied in studies with several environmental matrices such as soils, sediments and dust (Davidson *et al.*, 2006; Pascaud *et al.*, 2014; Pueyo *et al.*, 2008; Unda-Calvo *et al.*, 2017; Zhang & Wang, 2009).

The sequential extraction was performed according to Rauret *et al.* (1999) in three steps: (i) F1: extraction with 0.11 mol L^{-1} CH_3COOH, the soluble and exchangeable phase (non-specifically adsorbed species); (ii) F2: extraction with 0.5 mol L^{-1} $NH_2OH.HCl$, reducible phase, i.e., bound to the Fe and Mn oxides and oxyhydroxides; and (iii) F3: extraction with hydrogen peroxide followed by extraction with 1 mol L^{-1} ammonium acetate (pH 2), the oxidizable fraction, i.e., bound to organic matter and sulphides. Details of this procedure are described in ANNEX C. The residual fraction was calculated as the difference between the pseudo-total concentration extracted by the 3051A method (USEPA. 2007) and the sum of the F1, F2 and F3 fractions (Abbad & Torres 2002; Abdu *et al.* 2011, 2012; Puga *et al.* 2016). The residual fraction is associated with the elements strongly adsorbed to the crystalline matrix, mainly by specific adsorption, suggesting predominantly a geogenic origin

(Patinha *et al.*, 2015b). The elements were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES, Thermo Scientific iCAP 6300 Duo).

2.2.7. Statistical Analyses

The software SPSS Statistics 20 (SPSS Inc.) was used for descriptive statistics and for the parametric and non-parametric tests. The non-parametric tests Kruskal-Wallis and Wilcoxon-Mann-Whitney were chosen rather than ANOVA, due to their greater power when small samples with different dimensions are analysed (Marôco, 2011). Data were plotted by Origin 8 (Origin Lab® Corporation). Simple linear regression was used to evaluate the relative impact of PHE pseudo-total levels as a predictor of PHE bioaccessible concentration. The data were log-transformed to normalize their distribution.

2.3. Results and Discussion

2.3.1. Chemical characterization, PHE pseudo-total concentration and particle size

The results of the chemical characterization and the comparison between the PHE pseudo-total concentration found in bulk samples (BS), that is, sieved to 2 mm; and in the fine fraction samples <10 µm (FF) are reported in Table 3.

Pseudo-total concentration of both fractions varied greatly among samples, with the highest levels found in the samples collected in the mine tailings areas in the city of Apiaí, whose contents in the FF were: 6.2% Pb, 2.2% Cu, 1.5% Zn and, 1.3% As (Tailing 1 sample). The Wilcoxon test shows that the concentrations in BS and FF differ for Cu ($Z = -2.58$; $p = 0.01$) and Cd ($Z = -2.63$ $p = 0.009$), with Cu presenting higher median in FF than in BS. and Cd presenting higher median in BS than in FF (Figure 4). The statistical output report containing the summary of the nonparametric analysis (Wilcoxon test) is in Appendix B. The higher reactivity of the elements in the FF compared to BS (Table 3) was probably due to the greater specific surface area of the different particle sizes. Moreover, materials with particle size <10 µm are classified as medium silt and clay (ISO 14688-1, 2017); thus the influence of mineralogical composition (e.g., phyllosilicates and Al, Fe and Mn oxides) and the organic

matter can increase the negatively charged sites that will provide higher cation adsorption (Luo *et al.*, 2011).

Even though there was no difference from the Wilcoxon test between the BS and FF fraction, the highest concentrations of PHE in the tailings samples were present in the BS fraction (Table 3). Heavy metals can be associated with Fe and Mn oxides and aluminosilicate minerals and incorporated into the structure of minerals (Batista *et al.*, 2018). Batista *et al.* (2018) found Pb associated with Fe and Mn, such as plumboferrite [$Pb_2Mn^{2+}_{0.2}Mg_{0.1}Fe^{3+}_{10.6}O_{18.4}$], and primary mineral, such as trioctahedral mica and also found Pb minerals, cerussite ($PbCO_3$), magnetoplumbite [$Pb_{1.1}Fe^{3+}_{7.7}Mn^{3+}_{2.6}Mn^{2+}_{0.6}Ti_{0.6}Al_{0.4}Ca_{0.1}O_{19}$], humboldtine [$Fe^{2+}(C_2O_4)\cdot 2(H_2O)$] and plumbogummite [$PbAl_3(PO_4)_2(OH)_5\cdot H_2O$] in sand and silt fractions. High levels of crystalline and amorphous oxides of Fe, Al and Mn were found in tailing samples (Table 3) that the PHE could be associated in the coarser fractions. The main minerals identified by XRD in all BS were quartz and kaolinite (APPENDIX D), with illite-montmorillonite, gibbsite and calcite being found in smaller amounts in the clay fraction. In the tailing samples, it was possible to observe peaks corresponding to cerussite ($PbCO_3$). XRD analyses were not performed on the FF samples due to small amount of material available. The concentrations of As, Cu, and Zn are relatively high in tailing samples, however no crystalline phases associated with these elements were found, but it can be inferred that these elements are in the form of amorphous oxides or insoluble precipitates, probably due to the pH value of the samples collected in the areas (5.2 to 6.6). In the soil samples, the peaks of quartz with high crystallinity interfere with those minor mineral containing heavy metals (Batista *et al.*, 2018)

Although the concentrations in BS and FF were not different ($p > 0.05$) for the most elements, the bioaccessibility and fractionation analyses were performed on FF, since this fraction has a greater potential risk to humans, mainly when inhaled (Witt III *et al.*, 2014).

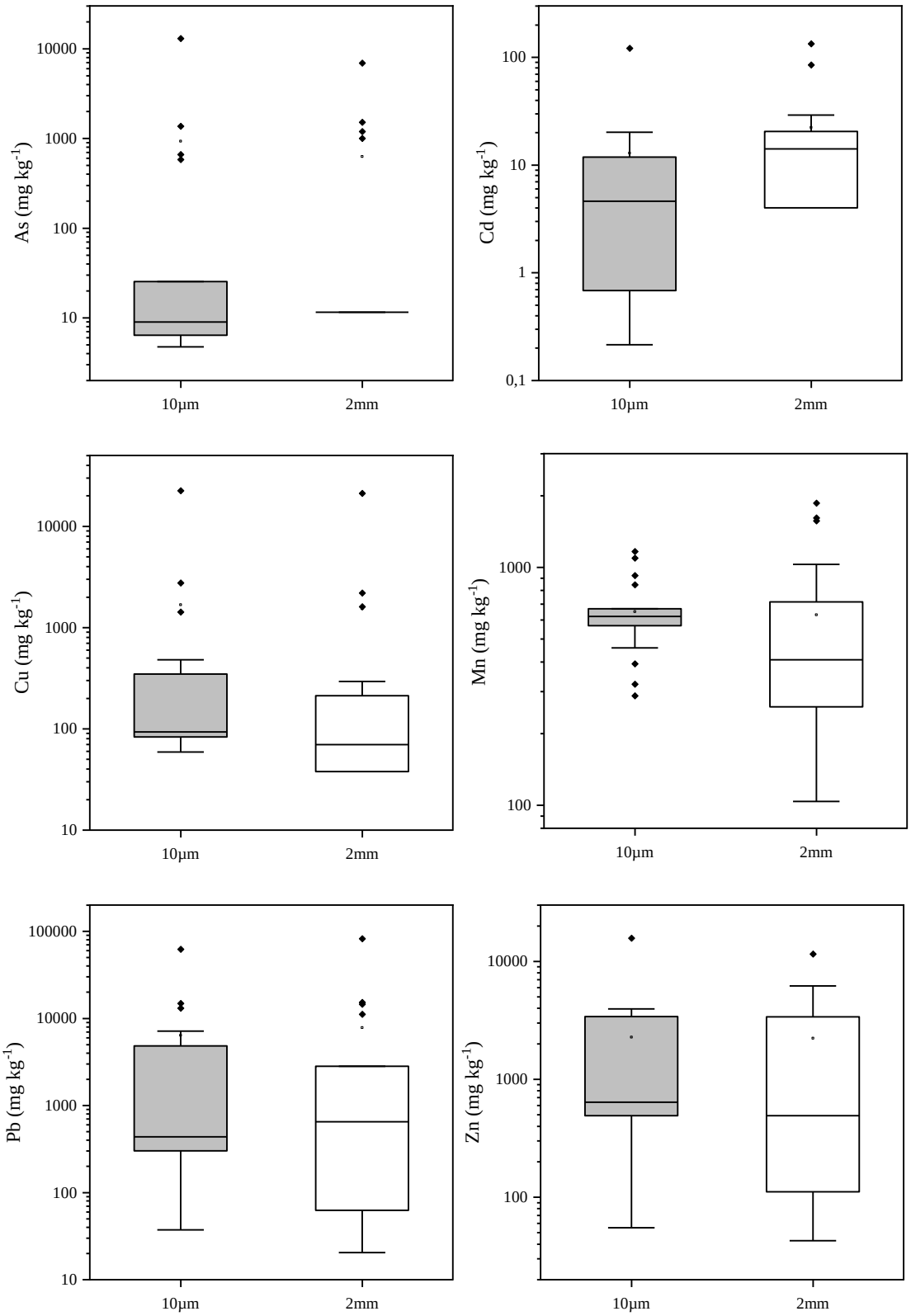

Figure 4. Distribution of potentially harmful elements in the bulk sample (2mm) and fine fraction (10µm) of the urban environmental matrices. Data has been log-transformed for better visualization.

Table 3. Chemical and physical characterization and potentially harmful elements (PHE) pseudo-total concentrations of samples collected in urban regions in Brazil.

Matrix	pH	Al_{DCB}	Fe_{DCB}	Al_{OX}	Fe_{OX}	Mn_{OX}	Sand	Silt	Clay	C_{org}	As	Cd	Cu	Mn	Pb	Zn	As	Cd	Cu	Mn	Pb	Zn
	---------- Bulk sample (BS) ----------																---------- Fine fraction (FF) ----------					
		---------- g kg^{-1} ----------					----------%----------				---------- mg kg^{-1} ----------											
Sediment 1	6.6	32.0	22.2	2.1	3.7	0.3	89.4	4.3	6.2	0.5	0.0	0.0	0.0	103.5	32.0	76.3	7.3	4.6	87.9	922.4	336.7	529.5
Sediment 2	6.9	33.4	47.3	2.8	4.3	0.2	85.9	6.6	7.5	0.9	0.0	2.9	0.0	135.8	62.5	154.2	6.4	3.8	86.4	658.1	317.0	637.7
Sediment 3	7.1	23.9	20.3	2.3	12.6	0.3	23.7	28.9	47.4	1.1	0.0	16.4	69.7	714.3	1048.9	968.9	5.5	8.6	66.4	585.3	1055.9	638.0
Sediment 4	7.1	17.5	55.4	3.6	31.3	0.9	55.5	17.6	27.5	1.3	0.0	14.4	93.0	407.9	2011.3	3171.8	8.1	11.8	100.0	567.1	2209.4	2609.9
Soil 1	6.2	18.8	44.1	2.7	11.8	1.4	56.1	18.7	25.1	2.1	0.0	0.0	21.1	352.1	20.4	48.3	9.2	0.2	58.5	627.3	41.8	127.8
Soil 2	5.3	21.2	61.4	3.5	8.3	1.4	48.6	16.3	35.1	3.2	11.5	4.2	30.0	459.0	23.7	56.9	17.9	0.4	127.2	610.1	37.8	94.5
Soil 3	5.6	10.0	24.8	2.4	10.2	0.5	76.0	9.0	15.1	0.8	4.5	2.1	211.7	412.6	131.1	110.9	11.5	0.5	623.4	845.9	321.2	351.8
Soil 4	4.2	40.0	100.4	7.8	7.0	0.2	70.0	12.6	17.5	3.7	0.0	20.5	70.4	258.7	47.4	42.7	25.4	0.4	111.5	288.0	59.9	55.0
Soil 5	6.8	19.0	12.3	2.4	5.1	0.4	67.6	15.0	17.5	1.9	0.0	4.0	46.7	180.7	646.8	491.4	5.5	2.7	93.2	458.0	434.6	579.2
Soil 6	7.1	12.6	3.6	0.6	1.8	0.1	68.6	11.5	20.0	1.8	0.0	4.3	39.8	343.5	163.4	322.9	4.7	2.0	83.8	393.0	368.3	857.6
Soil 7	6.6	8.1	6.7	1.2	2.7	0.2	63.4	10.4	26.2	1.7	0.0	14.1	64.4	387.7	2813.8	1779.6	23.0	20.1	128.0	656.0	4812.1	3953.4
Soil 8	7.1	13.2	4.8	0.7	2.1	0.1	17.8	27.2	55.0	6.3	0.0	5.6	37.7	190.0	83.8	234.1	8.6	0.7	68.2	322.3	145.0	490.3
Tailing 1	6.0	11.9	11.7	1.4	6.0	0.1	52.2	27.3	20.5	3.2	6906.8	133.5	2191.4	1611.2	82476.1	11569.4	13856.5	119.1	2751.8	665.4	67673.0	15667.7
Tailing 2	6.0	9.5	14.1	1.3	9.4	0.3	38.3	26.7	35.0	3.4	1194.6	85.0	1603.4	1861.8	15270.2	5613.9	670.7	8.6	1417.5	626.7	7154.6	3378.5
Tailing 3	5.2	11.2	20.0	2.2	5.0	0.5	23.0	27.1	49.9	2.3	999.2	29.1	293.5	1567.4	14545.8	3531.5	574.9	12.7	341.3	1154.2	12995.3	3768.6
Tailing 4	6.6	11.5	17.2	2.2	9.3	0.3	61.3	23.7	15.0	3.2	1515.7	23.1	21112.9	1026.0	11132.4	3387.4	1368.9	14.8	22513.5	1093.4	14889.4	3621.8
Tailing 5	6.6	10.2	11.1	1.7	3.2	0.1	28.3	26.1	45.7	0.6	0.0	20.3	125.2	695.4	2626.5	6187.5	5.7	5.4	82.8	575.3	858.7	1025.4

DCB = Al and Fe crystalline oxides extracted by sodium dithionite-citrate-bicarbonate method; OX = amorphous Al, Fe, and Mn oxides extracted by acid ammonium oxalate solution.

2.3.2. Respiratory bioaccessibility of PHE in different environmental matrices

The PHE bioaccessibility fraction varied widely among matrices, indicating that they were influenced by their chemistry, physical and mineralogical characteristics, as well as the land uses of the sampled areas. The median bioaccessible concentration were 4.1 (As); 0.5 (Cd); 31.1 (Cu); 192.7 (Pb); 325.4 (Mn) and 238.8 (Zn) mg kg^{-1} for soil matrix, 5.7 (As); 5.0 (Cd); 49.2 (Cu); 638.5 (Pb); 512.0 (Mn); and 481.5 (Zn) mg kg^{-1} for sediment matrix, and 714.9 (As); 11.4 (Cd); 949.7 (Cu); 10,219.8 (Pb); 416.4 (Mn); and 2,842.1 (Zn) mg kg^{-1} for tailing matrix (data not shown).

Table 4. Mean, standard deviation, median, and minimum and maximum values of the potentially harmful elements in urban matrices (soil, n = 8); (sediment, n = 4); (mining tailings, n = 5).

Element	Fraction	Mean	Standard Deviation	Median	Min.	Max.
		---------- mg kg^{-1} ----------				
As	Pseudo-total	977.0	3239.7	9.2	4.7	13856.5
Cd		13.5	27.1	4.6	0.2	119.1
Cu		1690.7	5249.4	100.0	58.5	22513.5
Pb		6688.9	15888.1	434.6	37.8	67673.0
Mn		649.9	233.2	626.7	288.0	1154.2
Zn		2258.0	3626.6	638.0	55.0	15667.7
As	ALF	396.5	1.011.9	5.7	3.1	4154.0
Cd		10.7	22.5	3.9	0.2	98.5
Cu		1380.4	4669.5	20.9	18.2	19986.5
Pb		5903.4	14212.6	437.0	19.4	60668.0
Mn		453.4	179.0	428.3	172.3	876.9
Zn		1555.4	2353.0	458.2	25.8	9918.4

ALF = Artificial lysosomal fluid

The tailing matrix, as expected, had the highest bioaccessible concentrations, but some soils samples (sample 7) and sediment (sample 4) collected in Santo Amaro had high levels of bioaccessible Pb (4,812.1 and 2,014.7 mg kg^{-1}, respectively) and Zn (2,516.9 and 1,849.0 mg kg^{-1}, respectively). These samples were collected from a soccer field and the sidewalk, both located on an avenue near an old of Pb metallurgical facility. The presence of these elements at these sites is probably associated with a flood that occurred during 2015, prior to soil collection, when several particulates and coarse materials, derived from the plant, were dragged to the sites. The sample sites are less than 1 km from the old company and these results might indicate serious contamination due to the particulate material in this region.

These concentrations are above the Brazilian regulatory guidance values (Resolution # 420, 28/12/2009, CONAMA, 2012) established for soils in both residential and industrial areas (300 and 900 mg kg $^{-1}$, respectively). Concentrations of PHE in soils above the values established by CONAMA #420 may present direct or indirect risks to human health.

The bioaccessible Mn concentration did not vary among matrices ($p > 0.05$). Mn is one of the elements that was not included in the table of background guiding values for Brazilian soils (CONAMA, 2012), although it is essential for humans, in the synthesis and metabolism of neurotransmitters (Dieter *et al.*, 2005). Prolonged exposure to fumes and dust containing high concentrations of Mn represents a risk factor for diseases such as Parkinson's (Kwakye *et al.*, 2015) or Alzheimer's (Tong *et al.*, 2014), as well as diseases associated with pulmonary inflammation (Santamaria & Sulsky, 2010). Research on the contamination of soils or dust by Mn and its effects on human health is little studied in Brazil, and more studies focused on Mn exposure are necessary, especially on the dose-effect relationship (Santamaria & Sulsky, 2010). Despite being a nutrient for humans, Mn needs to be better evaluated and should be considered during risk assessment because its high capacity to cause harm to human health under prolonged exposure. According to USEPA (1995) the reference dose for Mn is 10 mg day^{-1} (or 0.14 mg kg^{-1} day^{-1} for 70 kg adults) for chronic ingestion and the inhalation lowest-observed-adverse-effect level (LOAEL) is 0.793 mg m^{-3}. Neurotoxicity has been reported in environments where there is chronic exposure containing > 1 mg m^{-3} of Mn (Santamaria & Sulsky, 2010).

The sediment samples have a different median distribution as compared to soil and tailings samples (Figure 5), and it seems to be related to mineralogy of sediment samples and the location of collection. The BAF variation was higher in soils than in sediments and tailings, but the BAF median was above 50% in all matrices, except for Cu in the soil samples.

The sediments 1 and 2 were collected in Subaé bank river, where the deposition and sedimentation of coarser materials such as quartz are likely to be greater, while sediments 3 and 4 were collected close to the old metallurgical facility and had high concentrations of Pb and Zn. These contamination levels, as well as in the soil 6 sample, can also be justified by the flood action that occurred at the site.

Sediment samples contained 24 to 90 % sand (data not shown) and low-Mn amorphous oxide contents (30 to 90 mg kg^{-1}) in the BS fraction compared to soils and tailings (Table 3). This is likely because of the higher contribution of quartz and lower presence of

clay minerals (Appendix D) and Fe and Mn oxides that could increase the sample reactivity (Ciszewski, 1997). .

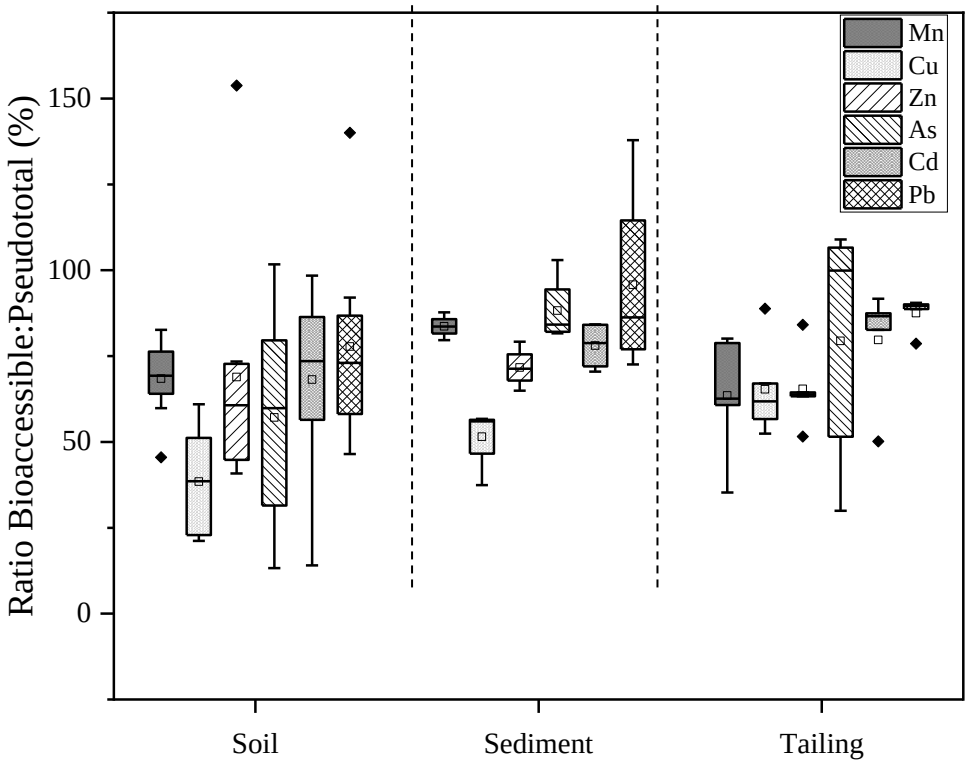

Figure 5. Box and whisker plot of the respiratory bioaccessible fraction (BAFp (%)) of As, Cd, Cu, Mn, Pb and Zn separated by type of matrix (Sediments (n = 4). Soil (n = 8) and Tailings (n = 5))

2.3.3. Influence of solid phase matrices in the respiratory bioaccessibility of potentially harmful elements

A high variability of the geochemical phases in the soil, sediment and tailing matrices was observed in the sequential extraction (BCR) results (Figure 6). In tailing areas, the F1 fraction, i.e., the elements associated with the mobile phase of the soil, contained a larger proportion of Cd and Zn (median 44 and 51%, respectively) compared to other elements. High concentrations of carbonates are generally found in soils with pH > 6.0 such as the samples collected in the urban and mining areas. In sediments, the elements with higher amount in F1 were Cd and Mn with median 43 and 48%, respectively. The F1 fraction, exchangeable + carbonate Cd fraction, varied from 0 to 46% in the soil samples, 41 to 49% in the tailing matrix, and 38 to 48% in sediments. In soils, Mn had the higher amount in F1 (average = 40%), though a soil sample (58PC) had almost 85% of the total content in the soluble or exchangeable fraction (Figure 6). Three soil samples, two collected in the city of

Piracicaba, and one collected in a native forest in the city of Apiaí, did not contain Cd extracted by the 0.11 mol L^{-1} acetic acid (Rauret *et al.*, 1999). However, Cd was associated with either the reducible fraction (F2) or the residual fraction (F4) in these samples. Pb and Cu had lower amounts in F1 than the other elements, most like due to the pH > 6 that favoured the formation of precipitates or insoluble substances and also to the presence of organic matter, as C-organic ranged from 0.5 to 6.5% (Table 3), thus forming stable complex with heavy metals.

In the F2 fraction or reducible phase, the elements had the same behaviour in each sample, i.e. soil samples 2, 4 and 8 had available amounts of Cd, Cu, Pb, Mn and Zn in this fraction, while the other samples did not have. The same behaviour was also observed in sediment 4 (9SA) and tailings samples 2, 3 and 4. Tailing samples had higher contents in F2 than the sediment and soil samples in all elements, indicating that these elements occur bound to Fe/Mn oxides. The hydroxylamine reagent used in F2 extraction can solubilize Fe and Mn oxides/hydroxides. The high median content was observed for Cu and Pb (> 30%) in tailing samples 2, 3 and 4. Samples 1 and 5 did not have levels available in this fraction.

F3 is related to oxidizable fraction, that is, the fraction of the elements that may be linked to organic matter or to sulphides (Rauret *et al.*, 1999). There was little expression of this fraction, with relative amounts <16%. The Kruskal-Wallis test showed that there was no difference in the distribution of Cu ($p = 0.128$) and Mn ($p = 0.390$) values in the F3 and that for Cd ($p = 0.043$), Pb ($p = 0.025$) and Zn ($p = 0.002$) there were differences in the distribution of data. The high amounts of PHE in the F3 were found for Cd (median = 13%) and Zn (median = 12%).

When the two first fractions were examined together (sum of F1 + F2), it seems that probably the most mobilizable elements were Cd (44%) and Mn (40%) in soils, Cd (43%) and Mn (50%) in sediments, and Zn (82%) > Cd (58%) > Cu (54%) > Mn (47%) > Pb (45%). The groups of samples containing considerable proportions of PHE in the mobile and reducible phases were likely to be derived from anthropogenic sources, while the less mobile (oxidizable and residual) were linked to geogenic sources (Patinha *et al.*, 2015).

High proportions in the residual fraction (F4) were observed for all matrices and indicate that these elements may be either related to the silicate matrix of the samples or present in several metal alloys not dissolved by the reagent solutions used in the previous fractions. On the other hand, low proportion of Cd (7%) and Cu (2%) were found in the soil matrix 2 (47PC) and 3 (58PC), respectively. The Zn proportion was low in matrix of sediment 4 (8SA) and tailing 3 (3AP) and 4 (4AP) - Figure 6.

The relationship between As bioaccessible and the solid fractionation of each matrices was not evaluated because the fractions containing As could not be fully resolved when a high concentration of crystalline oxides was present. This highlights the need for alternative reagents (e.g. more aggressive conditions for solubilizing As bound to the crystalline oxides matrices) and alternative approaches to the operationally defined methods such as the BCR (Cave *et al.*, 2004).

The use of the BCR methodology indicated that Pb was associated with all the fractions across the range of soil, sediment and tailings sampled (Figure 6). This is not surprising given the varied potential sources of Pb and its varying solubility under different geochemical conditions/extraction scenarios. In general, soil Pb is immobile at high pH and when associated with silicate fraction (Shotyk & Le Roux, 2005). All samples contained Pb associated with F1 (pore-water, exchangeable, carbonate phases) (Figure 6). Witt III *et al.* (2014) evaluated Pb dust collected in a mining area and observed that the high proportion of Pb in the mobile fraction was associated with the presence of cerussite ($PbCO_3$), litharge (PbO) and anglesite ($PbSO_4$).

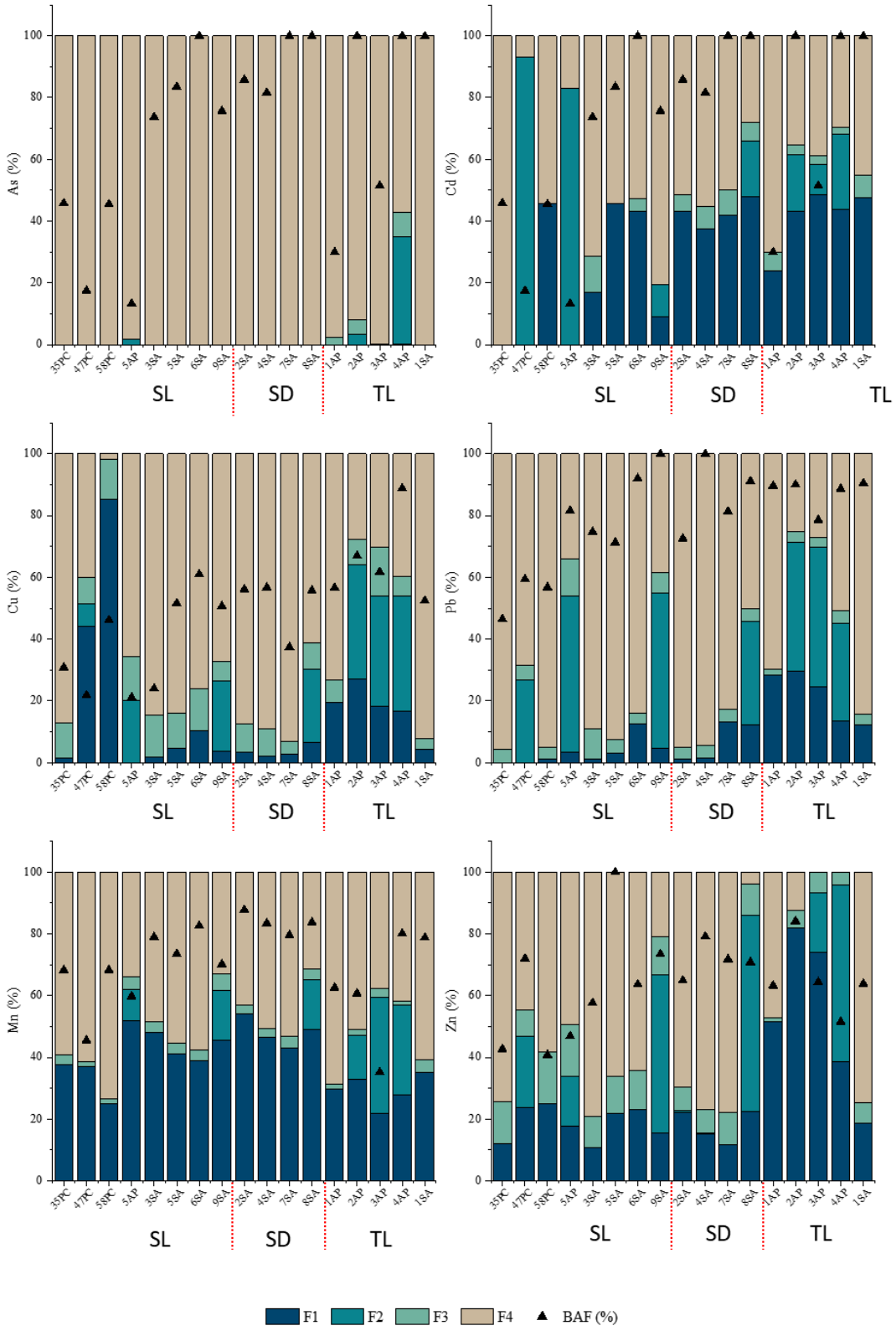

Figure 6. Solid-phase distribution and bioaccessible fraction of potentially harmful elements in samples of fine urban soil fraction from urban matrices. BAF—bioaccessible fraction (%). F1: Exchangeable and acid soluble carbonate fraction; F2: hydroxides and mixed oxy-hydroxide phases (reducible fraction); F3: Organic substance and sulphide fraction (oxidizable fraction) and; F4: the residual non-silicate bound trace metal concentration. The red dashed lines separate the groups SL (soil), SD (sediment) and TL (tail).

2.3.4. Pseudo-total concentration as a predictor of respiratory bioaccessibility potentially harmful elements

The extraction with simulated lung fluid resulted in PHE proportions (bioaccessible: pseudo-total) equal to or greater than 100% in some samples (Table 4 and Figure 6). This is probably because the ALF solution is a complex medium with a high concentration of organic complexes, low pH (pH 4.5) and is considered to be able to solubilize high concentrations of these elements (Pelfrêne *et al.,* 2017). Moreover, the high solid: solution ratio (1:1000) used in the procedure, unlike the solid: solution ratio in the 3051A method (1:24), can contribute to a greater dissolution of the <10 µm material (Guney *et al.,* 2017). It suggests that the pseudo-total concentration in these cases should be interpreted with caution during risk assessment, because ALF solution could be more aggressive than acid solutions (i.e. 3051A method) due to the higher complexity of the solution.

Comparing results of the respiratory bioaccessibility extraction with the sequential extraction shows that the simulated fluid could extract all the solid phases (Figure 6), including the residual phase, i.e. it was able to extract those elements that are strongly adsorbed to the crystalline phase of the matrix. The high release of bioaccessible PHE in the different matrices is due to the high complex forming capacity of the solution that contains six types of organic chemical substances (0.077 g L^{-1} trisodium citrate dihydrate, 0.059 g L^{-1} Glycine, 20.8 g L^{-1} Citric acid, 0.090 g L^{-1} disodium tartrate, 0.085 g L^{-1} sodium lactate and 0.172 g L^{-1} sodium pyruvate).

The release of PHE may increase with increasing complex forming capacity and concentration of binders in the solution (Hedberg *et al.,* 2011). The effect of pH alone is not as significant as the action of organic complexing agents and the number of available functional groups (Hedberg *et al.,* 2011). The presence of citric acid and other organic substances makes the simulated lung fluid able to form complexes with the metals, resulting in increased solubility, thus replacing the adsorbed metals on the surface of the particulate matter and forming organometallic complexes in the ALF solution (Kim *et al.,* 2013; Henderson *et al.,* 2014).

Particulate matter may pass through two compartments in the lung: extracellular environment in the deep lung tissue where the pH is 7.4 (often simulated with Gamble's solution) and intracellular environment with a pH of 4.5, where the particles are phagocytized by alveolar and interstitial macrophages in the lung (Guney *et al.,* 2016). The ALF method mimics a more acidic environment in the lungs and can solubilize the maximum concentration

of PHE that have the potential to reach the circulatory system (Colombo *et al.*, 2008; Calas *et al.*, 2017). Respiratory bioaccessibility in this study varied among the elements and solid matrices, following the descending order, Soil: Cd> Pb> Mn> As> Zn> Cu; Tailing: As> Pb> Cd> Zn> As> Mn; Sediments: As> Pb> Mn> Cd> Zn> Cu. Mineralogy can be a major influence on the solubility of PHE in the lung fluid, since the presence of metal oxides, carbonates and chlorides in the matrices can be easily dissolved in the lung fluids while phosphates and silicates are not (Pelfrêne *et al.*, 2017).

A relationship was observed between pseudo-total concentrations and the bioaccessible fraction (Figure 7). The relationship presented $R^2 > 0.9$, except for Mn $R^2 = 0.7$; standard error (s.e.) < 0.5 and indicates that extraction using method 3051A (HCl: HNO3, 1: 3, USEPA 2007) could be a good predictor of respiratory bioaccessibility for intracellular conditions in all environmental matrices. Witt III *et al.* (2014) measured the Pb-pseudo-total with Pb-bioaccessible extracted by ALF from dust samples at two particle sizes (<1 µm and > 1 µm). The authors observed that linear regression analysis explained 82% (s.e. = 18%) of the variability (at both particle sizes > 2mm and < 10um) and suggested that the model is a good predictor for Pb in the bioaccessible phase. Pelfrêne & Douay (2018) also obtained a good relationship between the pseudo-total and bioaccessible respiratory contents for Cd and Pb (R^2 = 0.965 and 0.980, respectively) in samples of sidewalk dust, indicating that a large amount of the pseudo-total content is present in the mobilizable fractions of the lungs.

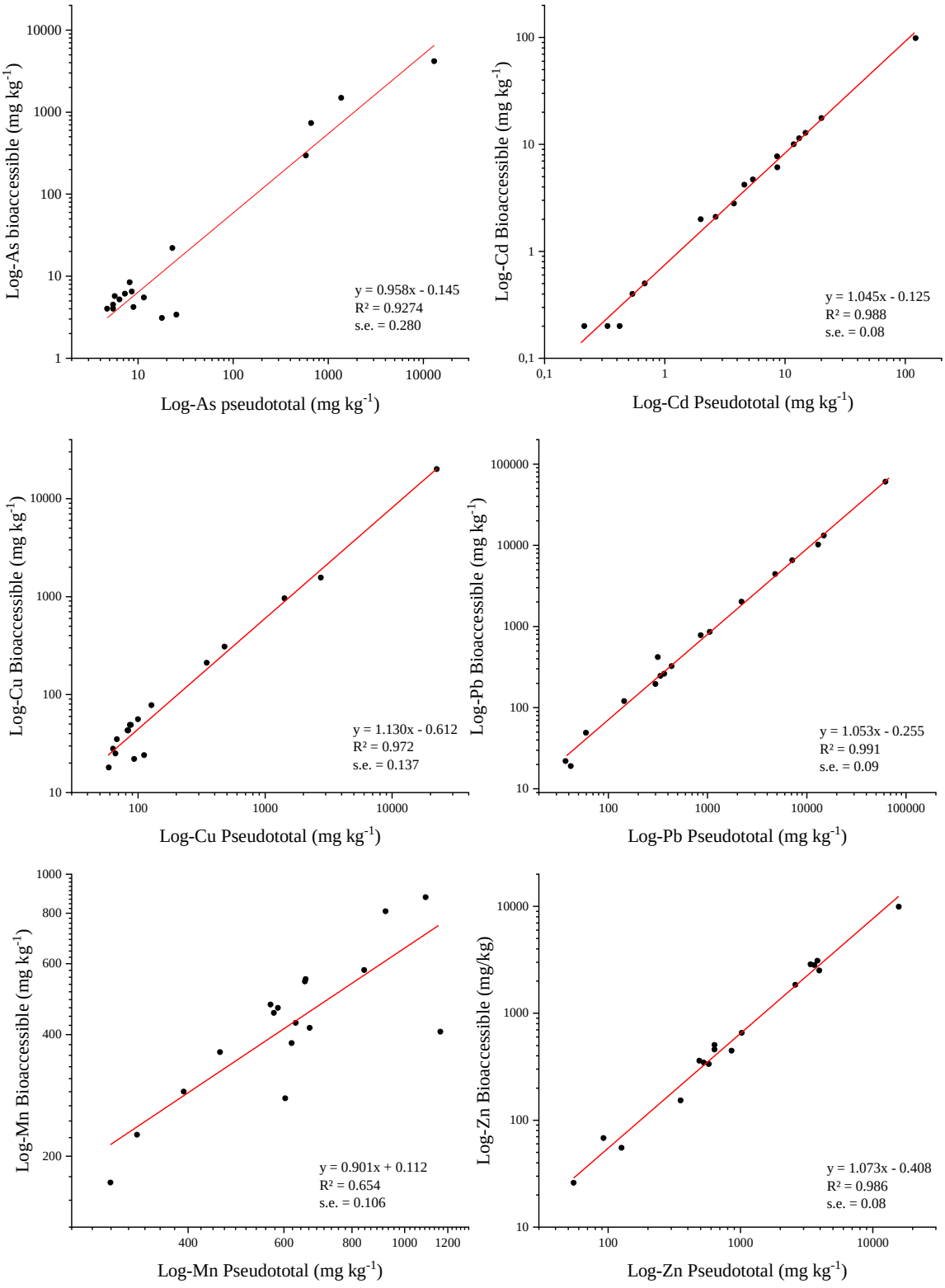

Figure 7. Correlation between the concentrations of As, Cd, Cu, Pb, Mn and Zn extracted by the USEPA 3051A method and lung bioaccessible extracted by the ALF method from soils, sediments and tailing (n = 17, data were log-transformed).

2.4. Conclusions

The pseudo-total PHE concentrations of the test materials provided a good estimate of the respiratory bioaccessibility and their laboratorial achievement was simpler to undertake than the *in vitro* method used. The organic acid in the ALF solution (intracellular condition) solubilized the labile and non-labile forms of PHE in solid fraction.

The application of the BCR sequential extraction procedure to urban environmental matrices samples indicated that PHE mobility are followed by the sequence: Cd > Mn > Zn > Cu > Pb in soils; Mn > Cd > Zn > Pb > Cu in sediments and Zn > Cd > Cu > Mn > Pb. The As did not present relative amount to the mobile fraction (F1+F2) and was present only in the tailings matrix.

Mine tailing samples had the highest pseudo-total concentrations of PHE in comparison to the soil and sediment samples, both in the bulk soil and fine fraction. However, the all three matrices presented high proportion of bioaccessible PHE (> 60%) in most samples, which highlights the potential risk from inhaled PHE. Mn is not included in the table of guideline values for Brazilian soil quality, but the data showed that 35 to 88% of the pseudo-total Mn concentration is bioaccessible.

The CONAMA Brazilian Resolution #420 recommends the use of strong acid solutions in the determination of inorganic substances, except Hg, in soils sieved with a 2mm mesh, but the use of these procedures to determine the contents of PHE and the particle size do not necessarily estimate the bioaccessible concentration to humans. Therefore, a more detailed analysis of each contaminated site and adequate sample preparation is required.

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3. HUMAN ORAL BIOACCESSIBILITY AND DISTRIBUTION OF POTENTIALLY HARMFUL ELEMENTS IN URBAN ENVIRONMENTAL MATRICES

Abstract

Potentially harmful elements (PHE) are found in soils, generally at low levels in nature, but increases through input from anthropic sources, such as mining activities and industrial or vehicle emissions, can cause harmful effects to human health by either dermal contact, inhalation or ingestion of the contaminated material. The input of PHE into soils is of global concern, as they are potential bioaccumulators and can cause various health problems. Oral bioaccessibility of As, Cd, Cu, Mn, Pb, and Zn was evaluated in different environmental samples (urban soils, sediments and mine tailings), from the cities Piracicaba and Apiaí, both in São Paulo state, and Santo Amaro, Bahia State in Brazil. The in-vitro method used to obtain bioaccessibility data based around human physico-chemical parameters e.g. body temperature, fluid pH, soil residence time etc. The method used to determine the concentration of bioaccessible PHE in the gastric (G) and gastrointestinal tract (GI) was UBM (Unified BARGE Method). 0.43 mol L^{-1} HNO_3 was also used to determine whether this simple extraction could be used as a proxy for the UBM gastric phase or not. In addition, the BCR (*Bureau Communautaire de Référence*) sequential extraction was performed to assess the distribution of PHE throughout soil components/fractions (solid phases e.g. carbonates) to understand their influence on oral bioaccessibility. The highest concentrations of PHE were found in the tailing samples. G bioaccessibility was greater than GI bioaccessibility, with values of 0-33% and 0-26% for As; 0-69% and 0-40% for Cd; 18-75% and 12-89% for Cu; 24-83% and 7-50% for Pb; 43-105% and 27-97% for Mn; 14-88% and 6-46% for Zn, respectively. Cd, Mn and Zn were associated with the exchangeable and carbonate phases, while Pb and Cu were associated with the reducible phase. A high positive correlation was observed between the acid extraction using 0.43mol L^{-1} HNO_3 and the UBM gastric phase for all the elements tested, except As.

Keywords: UBM method; Gastric fluids; Gastrointestinal fluids; 0.43M HNO_3; Solid phase

3.1. Introduction

Soils in urban areas undergo a degree of modification because of human activities, either in terms of function or composition. The environment around these areas (automotive traffic, industries, buildings, municipal waste etc.) affects soil quality.

Smelting activities are a type of source of urban contamination. In Brazil, these companies are placed in areas close to urban centres. During ore processing of mine tailings, waste water and particulate material are produced (Li *et al.*, 2006) and affect many people living in the surrounding areas. Atmospheric pollution, for example, can contribute to the contamination of soil particulate materials.

Humans regularly come into contact with contaminated soil in the urban environment, particularly in areas dedicated to recreation and leisure, where there is great

potential for exposure through oral ingestion, inhalation and particulate dermal contact. This is particularly true for children, who are more vulnerable to effects of exposure (Luo *et al.*, 2012). Their curiosity or physiological needs can result in direct ingestion of soil, with indirect ingestion resulting from hand-to-mouth behaviour. Because of their weak immune system compared to adults, children are more likely to be susceptible to the detrimental effects of soil ingestion.

In Brazil, guideline values for soil and groundwater quality (GVSQ) have been established by the National Environment Council (CONAMA) under resolution #420 of December 28, 2009 (National Environment Council - CONAMA, 2012). These GVSQ values are divided into three categories: (i) Reference Value of Quality (VRQ), that is the concentration of a substance that defines the natural quality of the soil (Background); (ii) Prevention Value (VP), the concentration threshold value of organic or inorganic substances in the soil in order to ensure its main functions; and (iii) Investigation Value (VI), the concentration at which a given soil contaminant is a direct or indirect potential risk to human health, considering three exposure scenarios: rural, housing and industrial (CONAMA, 2012). For analysis, Resolution 420 recommends acid digestion methods 3050B or 3051A (USEPA, 1996, 2007) to extract total (or in this case acid extractable) concentration of inorganic elements in sediments, sludges and soils etc. However, neither of these acid digestion methods consider the bioaccessibility of inorganic elements and may overestimate the concentration available to humans via the three exposure scenarios mentioned above.

The bioaccessible concentration of PHE is defined as the concentration in the soil that may be soluble in the gastrointestinal (GI) tract but are not necessarily absorbed into the circulatory system (Van de Wiele *et al.*, 2007; Deshommes *et al.*, 2012). The contaminant is bioavailable when the bioaccessible element crosses the intestinal epithelial tissue lining, the inner wall of the small intestine that absorbs nutrients and allows the passage into the bloodstreams (Rogers, 2011), i.e., bioavailability is the maximum amount of contaminant that the intestine can absorb (Oomen *et al.*, 2002). The risk posed by ingesting PHE present in the soil depends on the soluble fraction in the GI system and the behaviour of the element. This fraction is important to quantify the risk associated with oral exposure to soil PHE and can be measured by *in vitro* tests that quantify the bioaccessible concentration to humans.

The Bioaccessibility Research Group of Europe (BARGE) has been involved in comparing and evaluating many models to measures the bioaccessibility of contaminants to *in vivo* models (Oomen *et al.*, 2002; Wragg *et al.*, 2009, 2011; Pelfrêne *et al.*, 2012; Denys *et al.*,

2012; Cave *et al.*, 2016). The BARGE group developed a method to standardize the operational variables to avoid the differences in *in vitro* methods and the resulting difference in bioaccessible concentrations that could be found in the same soil sample using several different methods, thus hindering the actual risk assessment (Wragg *et al.*, 2011). This test is aimed to simulate the process of digestion in the mouth, the stomach and the intestines of the human digestive system (Wragg *et al.*, 2011).

Bioaccessibility methods are less costly, faster than *in vivo* methods and do not have problems with ethical constraints (Van de Wiele *et al.*, 2007). Furthermore, the bioaccessible concentration provides more conservative values for a risk assessment than bioavailable concentrations. The UBM method was assessed by an inter-laboratory trial (Wragg *et al.*, 2011) and subsequently validated for As, Cd and Pb using an *in vivo* juvenile swine model (Denys *et al.*, 2012). The validation used 16 soils contaminated by either smelting or mining activities resulting in a slope 0.8 to 1.2 and $R^2 > 0.6$, meeting the requirements set out by Wragg *et al.* (2011). The authors of the study to validate the UBM considered that the UBM was robust for the assessment of As, Cd and Pb, but they recommended further studies on a variety of samples with distinct attributes and characteristics with the UBM method before it can be used as an appropriate tool for assessing the risk of PHE in humans.

Studies that focus on the relationship between urban soils and human health need for more detailed investigations. In this context, the hypothesis that the gastric and/or gastrointestinal fluids alter the speciation of PHE in the solid fraction of each sample type and that the properties of each type of material can reflect the bioaccessibility of a determined soil PHE. For this, the UBM *in vitro* method was tested on three urban sample types: soil, sediment and tailing, slightly and highly contaminated collected from different Brazilian regions. The influence of the distribution of As, Cd, Cu, Mn, Pb and Zn in solid fractions using BCR sequential extraction and mineralogy information on these materials was also evaluated.

3.2. Material and Methods

3.2.1. Collection and sampling areas

Soil samples were collected in the cities of Piracicaba (n = 3), the eastern region of the State of São Paulo (SP), Apiaí (n = 4), Upper Ribeira Valley (SP) and Santo Amaro, in the

region of Recôncavo Baiano, State of Bahia (BA) (Figures 1, 2 and 3 in Appendix A). A brief description of the areas has already been given in Chapter 2, section 2.2.1.

Four of the 16 samples were classified as sediment, eight as soils and four as tailing. These classifications were based on the collection site, i.e. the samples classified as sediment were those collected on the Subaé riverbank, and sediments accumulated in street gutter of paved and unpaved streets. Samples classified as soil were collected in urban gardens and native forest, while samples classified as tailing were collected in the areas of lead tailings in Apiaí and Santo Amaro.

3.2.2. Characterization chemical and physical of soils samples

The extraction of the pseudo-total concentration was carried out using the EPA method 3051A (1:3 HCl:HNO3, v/v) according to USEPA (2007) in matrices samples sieved to 2mm and 250 µm. For this purpose, 0.5 g of sample was weighed in a Teflon tube, 3 ± 0.1 mL of 37% HCl and 9 ± 0.1 mL of 69% HNO_3 were added and allowed to stand for at least 16 h for pre-digestion at room temperature. The tubes were then closed and microwaved in a Mars Xpress (CEM Corporation) for 10 min at 175 °C. After cooling, the extracts were filtered, and the volume made up to 50 mL with ultra-pure water (18.25 MΩ cm) from a Milli-Q system (Millipore, Milford Corp., USA). The extracts were diluted (1:4, v/v) for determination by inductively coupled plasma optical emission spectroscopy (ICP-OES, Thermo Scientific iCAP 6300 Duo). Three certified standard materials, NIST CRM 2711a (Montana soil), BGS 102 (Wragg, 2009) and Embrapa MR-06/13 (Nogueira *et al.*, 2016) were used for quality control (Table 1).

A solution of 0.43 mol L^{-1} HNO_3 was used to extract the reactive concentration of PHE (Rodrigues *et al.*, 2012; Groenenberg *et al.*, 2017; Boim *et al.*, 2018). For this purpose, 5.0 g of soil was weighed in polypropylene flasks, and then 50 mL of 0.43 mol L^{-1} HNO_3 were added. The procedure was performed in triplicate. The samples were mechanically shaken for 2 h at room temperature and filtered. The reactive concentrations were measured by ICP-OES.

Table 1. Pseudo-total concentration of the PHE of the Reference Materials extracted by the EPA method 3051a (mean ± standard deviation)

Reference Material	As	Cd	Cu	Mn	Pb	Zn
	---------- mg kg^{-1} ----------					
BGS (measured)	104.6 ±8.3	NM	25.2 ±4.3	6347.6 ± 482	73.4 ± 4.5	174.1 ±11.5
[a] BGS (certificate)	104 ± 1	0.3 ± 0.2	26 ±1.5	7330 ± 49	79.4 ± 1.4	191 ±2
NIST 2711a (measured)	105.2 ± 0.4	49.4 ± 0.6	143.3 ±2.6	580.2 ± 0.9	1444.5 ± 14.2	381.3 ± 9.6
[b] NIST 2711a (certificate)	81 - 110	43 - 56	120-160	450 - 580	1100-1400	310 - 380
Embrapa (measured)	58.5 ± 4.7	83.31 ±2.0	9.7 ± 1.1	95.6 ± 6.6	174.5 ± 5.3	13.6 ± 2.4
Embrapa (certificate)	59.3 ±7.2	94 ±11.4	8.8 ±4	130±20	173.8 ±18.8	ND

[a] Total concentration was carried out by X-Ray Fluorescence (XRF) analysis (Wragg, 2009); [b] Concentration range; ND = concentration value not available on certificate; <LQ = below the limit of quantification; NM = not measured

3.2.3. Sample preparation for in-vitro bioaccessibility tests

To evaluate the oral bioaccessibility, matrices samples were sieved to < 250μm representing the fraction of soil that could adhere to children's hands and be inadvertently ingested (Rodrigues *et al.*, 2014).

All materials used were made of plastic, except the sieves, which were made of stainless steel, to avoid any type of contamination. For the extraction of the < 250 μm solid fraction (bioaccessible oral fraction), approximately 1 kg of air-dried fine soil, < 2 mm, was used. Samples were passed through 1 mm, 500 μm and 250 μm mesh sieves by mechanically shaking for 30 min. The sample retained in the collector was stored and weighed, and the material retained in the previous sieves was sent to the Laboratory of Chemical Wastes at the University of São Paulo for proper disposal.

3.2.4. Determination of oral bioaccessibility of potentially harmful elements in soils

UBM was performed in two stages: (i) gastric stage (G), pH 1.2 ± 0.05, which consisted of the extraction of the PHE after simulation of the saliva and stomach compartments; (ii) and the gastrointestinal (GI) step, pH 6.3 ± 0.5, consisted of the extraction

of the PHE after simulation of the saliva + stomach + small intestine compartments, as described in Denys *et al*. (2012) and Wragg *et al*. (2011).

The fluids consisted of saliva, gastric fluid, duodenal fluid, and bile. The simulated organic and inorganic fluids were prepared separately (500 ml each) and combined to a final volume of 1000 ml. Enzymes were added after the reagents were completely dissolved and mixed (Wragg *et al.*, 2009; BARGE - INERIS, 2011). These solutions were prepared one day prior to the extractions to ensure complete dissolution of the reagents. For details of the procedure, see ANNEX B

The samples were weighed in duplicate (where each sample consisted of one for the G-phase and one for the G plus GI-phase). 0.6 g of sample was weighed into an 80 mL centrifuge tubes. The extraction fluids solutions were pre-heated to 37ºC and the pH adjusted where necessary. 9 mL of saliva was added to the centrifuge tubes, the samples were shaken for 5 min at 37ºC (body temperature), then 13.5 mL of the gastric fluid was added. The pH of the samples was adjusted to 1.2 ± 0.5 with 37% HCl. After the pH was adjusted, the samples were stirred for 1 h at 37ºC at 30 rpm (end-over-end rotation). After stirring the pH was adjusted again to ensure it was within the recommended range (BARGE - INERIS, 2011; Wragg *et al.*, 2009).

Samples from the gastric step (G) were centrifuged at 3000 × g for 5 min. The extracts were diluted 1:10 with 2% HNO_3 solution. Subsequently, they were transferred to *Falcon*® tubes and kept under refrigeration. Once the pH was measured, the GI digestion was continued by adding 27 ml of the duodenal fluid and 9 ml of bile. The final pH was adjusted to 5.8-6.8 with 1 mol L^{-1} NaOH. The samples were then shaken at 30 rpm (end-over-end) for 4 h at 37 °C followed by centrifugation at 3000 × g for 5 min. The extracts were diluted in a 1:10 ratio with 2% HNO_3 and kept under refrigeration until analysis. For quality control, the reference material BGS 102 (Ironstone Soil) from the British Geological Survey (Wragg, 2009) and duplicate blank samples were included. The analysis was carried out by inductively coupled plasma mass spectrometry (ICP-MS). The bioaccessible fraction (BAF, %) of PHE was calculated as follows:

$$BAF = \frac{Bioaccessible\ concentration\ (G\ or\ GI)}{Pseudototal\ concentration} \times 100$$

3.2.5. Sequential Extraction

The sequential extraction was performed according to Rauret et al. (1999) in three steps: (i) extraction with CH_3COOH (0.11 mol L^{-1}), the soluble and exchangeable phase (non-specifically adsorbed species); (ii) extraction with $NH_2OH.HCl$ (0.5 mol L^{-1}), the reducible phase, i.e., bound to the Fe and Mn oxides and oxyhydroxides; and (iii) extraction with hydrogen peroxide followed by extraction with 1 mol L^{-1} ammonium acetate (pH 2), this phase contained the oxidizable fraction, i.e., bound to organic matter and sulphides. The PHE concentration in the residual material was obtained by the difference between the pseudo-total concentration extracted by the 3051A method and the sum of the other fractions (Luo *et al.*, 2003; Szolnoki & Farsang, 2013; Mendoza *et al.*, 2017). The elements were determined by ICP-OES.

3.2.6. Mineralogical characterization

3.2.6.1. Identification of clay fraction

The powder X-ray diffraction (XRD) method was employed to characterize the minerals present in the samples sieved to <250 µm. Samples were ground in a tungsten carbide mill, passed through 106 µm sieve and were housed in an appropriate sample holder to favour or not the preferential orientation of the particles.

The clay fraction was analysed after treatment with sodium dithionite-citrate-bicarbonate solution (DCB) to eliminate the iron oxides and consequently concentrated the silicates in the sample soils. The clay samples were then treated as follows: (i) magnesium (Mg) saturation and air drying; (ii) Mg saturation and glycerol solvation; (iii) saturation with potassium (K) and air drying; (iv) saturation with K and heating at 110, 350 and 550 °C in a muffle.

The analysis was performed using a Philips PW 1877 diffractometer operated at a potential of 40 kV, current 40 mA, using a monochromator to eliminate Kα radiation, and Cu source (Kα1 = λ = 1.54606 Å).

3.2.6.2. Identification of iron oxides and oxyhydroxides by diffuse reflectance spectroscopy

The identification of Fe oxides and oxyhydroxides was carried out by diffuse reflectance spectroscopy (DRS) in samples (without organic matter), ground in a tungsten carbide mill and passed through 106 μm sieve. The samples were pelletized and placed in a sample holder with a quartz window. The measurements were performed on a Varian, Cary 5 (UV-VIS-NIR) spectrometer equipped with a 110 mm integrating sphere.

Iron oxides and oxyhydroxides were identified by the position of the most intense absorption bands. The identification of goethite occurred by absorption bands between 475 and 481nm and the hematite was identified by absorption bands between 526 and 553 nm (Scheinost *et al.*, 1998).

3.2.7. Statistical Analyses

Statistical analyses were performed using the SPSS 20 for Windows statistical program, in order to analyse the relationship between geochemical and bioaccessible forms. The Shapiro-Wilk normality test was used to assess the normality of both the original and the processed data.

Non-parametric methods were used to evaluate the sample data because most of the results were not normally distributed and not easily transformed into a normal distribution. Descriptive analyses were performed by calculating the Spearman correlation coefficients. A non-parametric ANOVA was performed to assess differences and similarities between the urban environmental sample types. The Wilcoxon-Mann-Whitney and Kruskal-Wallis rank sum tests were used to compare the PHE concentrations between the sediments, soils and tailings.

Geochemical groups were defined for each element using the results from the K-Means cluster analyses. K-means analysis identifies groups of homogeneous cases of a data set from a defined number of clusters (Raghuwanshi & Arya, 2012). The distribution of solid-phases identified three groups. Distances were computed using the Euclidean distance. The similarities among the samples based on their geochemical phases were evaluated: Group I = Very Mobile; Group II = Potentially Mobile; and Group III = Immobile (Witt III *et al.*, 2014).

3.3. Results and Discussion

3.3.1. Pseudo-total concentration of PHE and particle size in different environmental sample types

The highest concentrations of PHE were found in the tailing areas (Table 2) in comparison with soils and sediments. The PHE concentrations in some of the soils, sediments indicated higher PHE concentrations than those concentrations established as industrial investigation values (IV) by the National Environmental Council, Resolution # 420, December 28, 2009 (As = 150, Cd = 20, Cu = 600, Pb = 900 and Zn = 2000 mg kg^{-1}) (CONAMA, 2012)

Based on Kruskal-Wallis test the distribution of these material types indicates that statistical differences did not occur for Cd and Cu ($p > 0.05$), but there were differences between the distributions of As, Pb, Mn and Zn ($p < 0.05$). The differences for As occurred between sediments and tailing ($p = 0.019$), while for Mn, Pb and Zn the nonparametric test shows that their distribution in soils and tailings were different ($p = 0.020$; 0.026; 0.044, respectively). These differences among the tailings samples were already expected since they presented high concentrations of PHE as compared to the prior concentration in the ore processing.

In the tailing areas, both in the city of Apiaí and in the city of Santo Amaro, there are high quantities of slag resulting from Pb ore, which also contained Cu, Zn and As. In Apiaí, the area is used for teaching purposes and is mainly attended by children and adolescents, while the area of Santo Amaro (1SA) has been interdicted by the Brazilian courts since the 1990s.

Some of the soils collected from Santo Amaro contained PHE concentrations higher than the industrial VI limits according to CONAMA Resolution # 240/2009 (CONAMA, 2012), in particular Pb (2,814 and 10,198 mg kg^{-1}, in sieved soil at 2 mm and < 250 µm, respectively). These samples were collected from a soccer field (6SA), frequented mainly by children and another two sediment samples (7SA and 8SA) from the same city were also found to have high Pb concentrations (1,049 and 2,011 mg kg^{-1} at 2mm). These three samples were collected in the same region, less than 1 km from the old lead foundry.

Table 2. Descriptive statistic of pseudo-total concentration of potentially harmful elements of sediments (n = 4), soils (n = 8) and tailing (n = 4)

Element	mean	stnd dev	median	min	max
	---------- mg kg^{-1} ----------				
			Sediments		
As	1.9	3.36	-	-	7.8
Cd	9.6	11.59	4.9	-	28.4
Cu	83.4	77.92	55.6	14.4	207.9
Mn	360.9	292.42	275.6	80.4	812.2
Pb	1,627.7	2,036.54	713.2	47.6	5,036.9
Zn	1,747.7	2,501.23	429.1	76.5	6,055.9
			Soils		
As	8.7	8.99	5.0	-	28.5
Cd	6.8	12.68	2.3	-	40.1
Cu	100.9	116.93	45.4	17.4	362.4
Mn	290.8	106.16	271.3	154.4	535.7
Pb	1,393.9	3,328.80	173.3	19.0	10,197.6
Zn	973.0	2,038.95	269.4	35.2	6.356.0
			Tailing		
As	1,392.1	1,116.2	1,219.1	6.6	3,123.5
Cd	20.7	12.3	15.7	9.7	41.5
Cu	13,582.8	21,979.4	1,300.1	106.8	51,624.2
Mn	1,141.9	293.0	1,197.6	737.9	1,434.5
Pb	17,759.3	10,995.6	18,280.9	1,803.5	32,671.6
Zn	5,696.9	3,250.7	5,023.1	2,217.1	10,524.3

Arsenic was another element of interest with median concentrations of 1,097 and 1,219 mg kg^{-1}, in < 2 mm and < 250 µm, respectively. This PHE is often associated with Pb deposits and released into the soil as a mining by-product, but can also occur naturally as arsenopyrite (FeAsS) (Figueiredo *et al.*, 2007). In the soils and sediments, the median concentration was lower than the prevention value recommended by CONAMA #420, 2009 (15 mg kg^{-1}). Exceptions to this were soil samples 5AP (native area from Apiaí, 17 mg kg^{-1}) and 6SA (soccer field in Santo Amaro, 28.5 mg kg^{-1}). Although both the sampled areas are close to sources of contamination, this could not confirm that the presence of this element was due to previous mining related activities.

Cadmium, Cu and Zn were also present at high concentrations in the tailings areas, in both fraction (2mm and 250µm), as expected. These metals are present in mining and smelting activities, with the concentrations dependent on the type of mineralization, the composition of ore minerals, geology, topography, method of mining, and smelting etc. (Jung, 2008). In the soils and sediments, Cd was above the Brazilian guideline values, residential VI 8 mg kg^{-1} (CONAMA, 2012), indicating that more attention is required as these areas are

frequented by the local population, which could increase the risk of accidental ingestion of this element.

3.3.2. Oral bioaccessibility of potentially harmful elements in environmental samples

Figure 1 shows the box and whiskers plot of pseudo-total concentration in both the < 2 mm and < 250 µm size fractions (Table 1 and 2, from Appendix C), the oral bioaccessible fraction, gastric (G) and gastrointestinal (GI). The distribution between the elements in the fractions 2mm and < 250 µm did not differ for Cd, Mn and Zn by Wilcoxon test ($p > 0.05$). The median pseudo-total concentration of the Cu and As was higher in the fine fraction (250µm) than in the bulk sample (2mm) material, indicating that element reactivity is more pronounced in the <250µm first fraction than in the <2mm. This is probably due to the larger surface area of the particle that increases the cation exchange capacity and the number of charged sites that strongly influence cation adsorption. This was in agreement with Pelfrêne & Douay (2018) who found increasing PHE bioaccessibility with decreasing size particle. Elements normally are concentrated in the clay and silt fractions, although depending on the type of material they can also be found at high concentrations in the sand fraction (Otero *et al.*, 2013). The median concentration for Pb was higher in the 2 mm (405 mg kg^{-1}) than in 250 µm (257 mg kg^{-1}). Several Pb-bearing minerals can be found in the coarsest fractions of the soil (Batista *et al.*, 2018). A greater distribution of the data, however, was observed by the interquartile range in fraction 250 µm than in the fraction 2 mm (Figure 1a). The data distribution can be quite sensitive to the presence of outliers, damaging the values of the medians.

The UBM method extracted large quantities of PHE in both compartments G and GI (e.g. 7 - 16,700 and 2 - 5,835 mg kg^{-1} of Pb in the G and GI phases, respectively), mainly in the tailings samples (Table 4 and 5 from APPENDIX C). However, the G bioaccessible concentration was greater than GI bioaccessible concentration because of the lower G-fluid pH and the complexity of the GI fluid composition. The presence of complexing agents as phosphate, carbonate and enzymes, in the fluid composition can favour the formation of metallic precipitates. These results agree with those found by Wragg *et al*. (2011) in an inter-laboratory test on the bioaccessibility of As, Cd and Pb. The authors used several different types of sample, data from in vivo testing methods and two standard reference materials NIST 2710 and NIST 2711 and a soil prepared by the British Geological Survey (BGS), BGS 102.

Table 3 shows the bioaccessible fraction (BAF%) separated by material type and the differences in the relative solubility in the GI fluids. The BAF values were calculated based on the pseudo-total concentration extracted in the fraction of 250 μm, according to the equation described in item 3.2.4. In general, As, Cd and Cu had lower gastric BAF% as compared to Mn, Pb and Zn which may indicate a competition amongst these elements for adsorption sites. This may occur because the pH had a greater influence on the solubility of Mn, Pb and Zn than the other PHE. According to Smith *et al.* (2011) at pH 1.5 the competition for surface adsorption sites is higher mainly due to the presence of H_3O^+ that could dissociate other cations. Moreover, the higher concentration of ions such as Ca^{+2}, Mg^{2+} and K^+ in the gastric solution may also have contributed to the cation competition for the sorption sites on the particles, although the relationship between these divalent metals is currently not well understood.

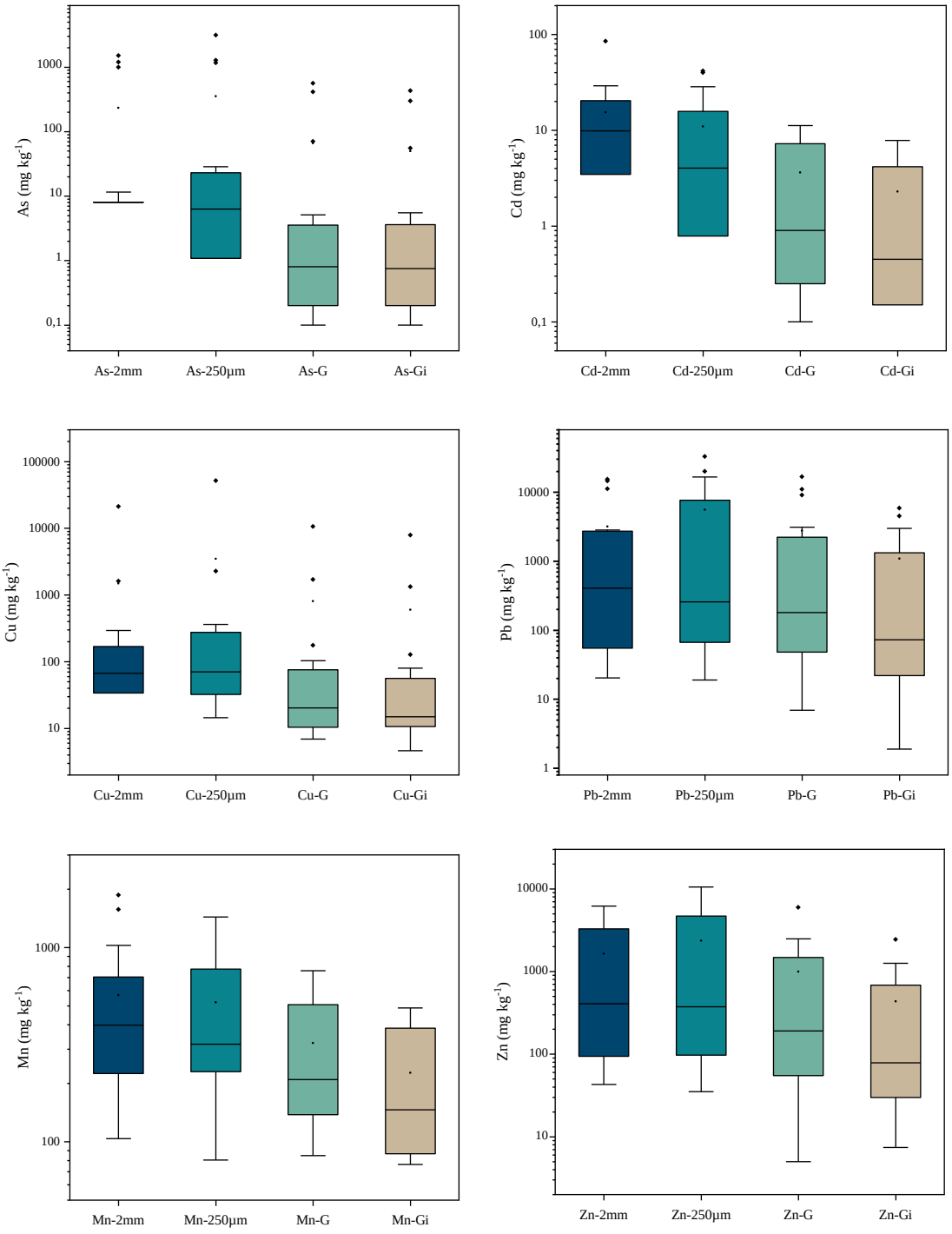

Figure 1. Comparison between PHE pseudo-total concentration (<2 mm and <250 µm) and bioaccessible concentration in gastric and gastrointestinal phases in all material types. The results were log-transformed to allow the visualization of the data distribution. The diamonds represent the outliers.

Table 3. Descriptive statistics of gastric (%BAF-G) and gastrointestinal (%BAF-GI) bioaccessible fraction in sediments (n = 4), soils (n = 8) and tailing (n = 4) samples

Element	median	min	max	median	min	max
	----------%BAF-G ----------			----------%BAF-GI ----------		
			Sediments			
As	0.0	0.0	19.2	0.0	0.0	18.7
Cd	10.5	0.0	61.8	7.6	0.0	39.8
Cu	32.2	17.6	56.2	22.2	12.3	89.1
Mn	89.7	63.5	105.2	67.1	46.4	97.7
Pb	72.7	26.1	75.6	45.2	17.1	50.2
Zn	55.1	20.2	66.4	27.6	10.8	42.9
			Soils			
As	6.5	0.0	34.0	0.0	0.0	27.4
Cd	3.8	0.0	45.8	1.9	0.0	26.2
Cu	36.9	21.5	45.0	25.6	14.6	40.1
Mn	71.5	43.5	92.3	44.8	31.8	58.6
Pb	56.9	24.0	82.7	19.5	7.4	40.2
Zn	51.9	14.2	66.3	21.3	6.7	46.0
			Tailing			
As	21.7	6.0	32.4	18.6	4.8	25.9
Cd	52.6	19.3	69.0	29.2	13.3	39.7
Cu	46.0	20.6	75.0	32.1	15.3	58.2
Mn	52.2	46.4	75.1	38.3	27.3	60.0
Pb	70.6	27.7	83.4	20.2	17.9	31.8
Zn	73.4	20.4	88.2	27.5	11.9	35.9

Arsenic has a similar behaviour to phosphorous in the soil, being highly adsorbed onto oxides at low pH. Ruby *et al*. (1996) observed that the pH in the stomach phase was the parameter that had lowest contribution to As bioaccessibility. Among oxides, hydrous ferric oxide, goethite and hematite are considered to be the most important sinks for As and may play an important role in regulating As retention in soils (Kim *et al.*, 2014). Goethite and Hematite were detected by diffuse reflectance spectroscopy (DRS) in all samples studied. The low As bioaccessibility in gastric phase was because As adsorption occurs in form of As_2S_3 (Brookins, 1988) because of the reduction environment and the presence of sulphur in G fluid. However, increasing pH in the gastrointestinal phase promotes a decrease of positive charges on the oxides surfaces promoting the desorption of As, as well as the formation of $HAsO_4^{-}$. (Brookins, 1998; Masscheleyn *et al.*, 1991). Hamilton *et al.* (2015) also observed the same results for As in gastric and gastrointestinal phases (3.9 and 3.3 mg kg^{-1}, respectively), stating that the As anionic species are formed in solution with increasing pH. On the other hand, the metallic cations in the gastrointestinal fluid are less bioaccessible than in the gastric solution (Table 3 and Figure 1). Hamilton *et al*. (2015) showed a decrease in this phase due their lower solubility at higher pH (6.3 ± 0.5) and by the formation of insoluble precipitates such as sulphates and phosphates in the gastrointestinal compartment.

The use of gastric compartment in a risk assessment has been discussed by several authors (Roussel *et al.*, 2010; Reis *et al.*, 2013; Rosende *et al.*, 2013; Wragg *et al.*, 2014; Patinha *et al.*, 2015a). In general, the higher bioaccessible concentrations will equate to a more conservative measurement. Figure 2 is a box and whisker plot summarizing the BAF% of gastric phase separated by the sample types: sediment, soil and tailing. Tailings samples had a higher BAF% than both the soils and sediment, except Mn, which was higher in the sediment samples.

No differences were observed between soils, sediments and tailing ($p > 0.05$). The BAF% values for the elements by sample type was in the order: Sediment = Mn > Pb > Zn > Cu > Cd > As; Soil = Mn > Pb > Zn > Cu > As > Cd; Tailing = Zn > Pb > Cd ≈ Mn > Cu > As.

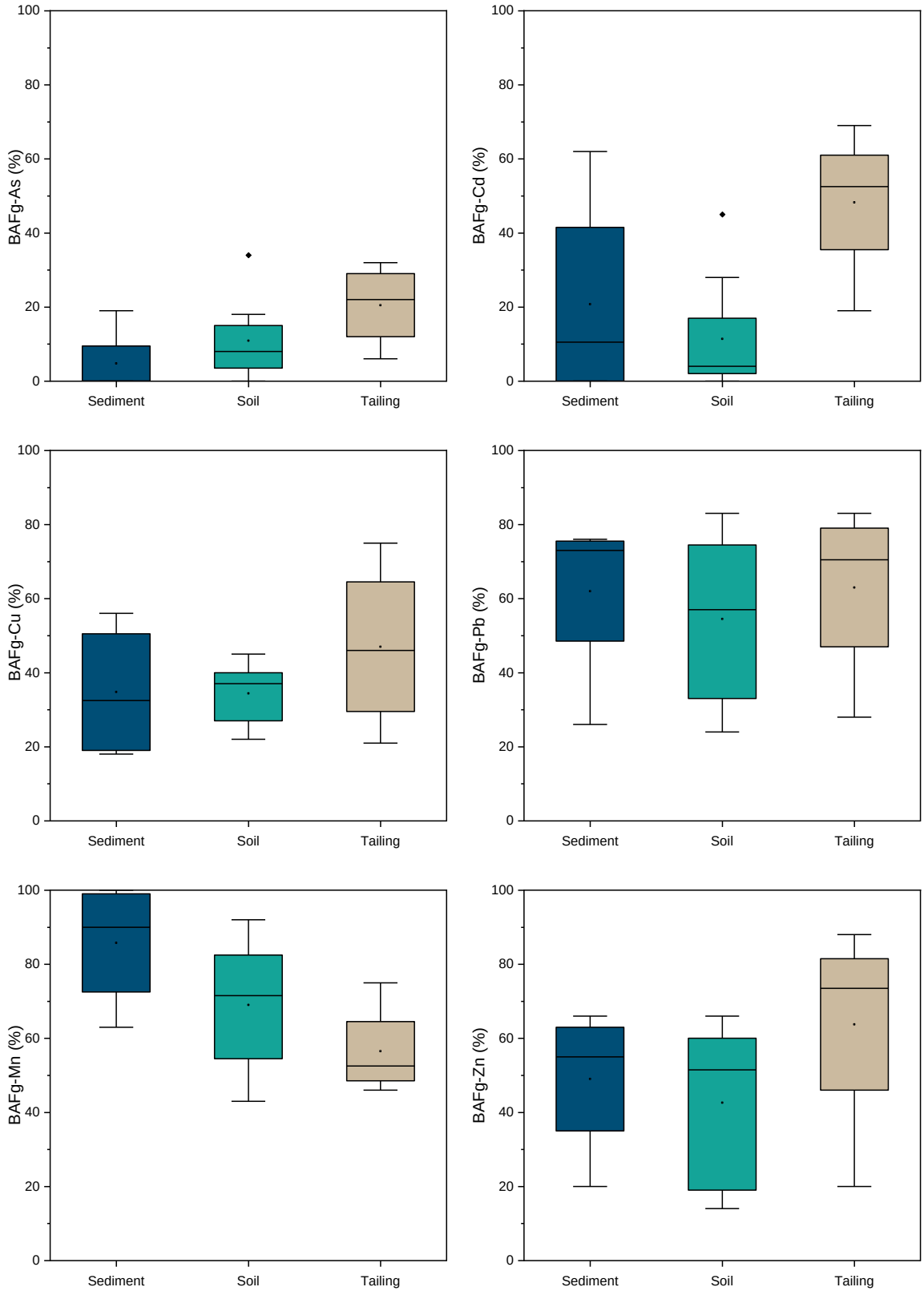

Figure 2. Box and whisker plot showing the difference between the bioaccessible gastric fraction (BAFg) of PHE by sample type

3.3.3. Influence of the distribution of potentially harmful elements between solid fractions (phases) on oral bioaccessibility

3.3.3.1. Sequential extraction

Figure 3 shows the proportion of i) the four chemical fractions (exchangeable, reducible, oxidizable and residual) and ii) gastric and gastrointestinal PHE bioaccessibility (%BAF) in each sample. The PHE had different distribution patterns between solid fractions. The exchangeable fraction (F1) corresponds to the most easily solubilized fraction, extracted by 0.11 mol L^{-1} CH_3COOH and represented 0 – 46% of Cd pseudo-total; 1 – 16% of Cu; 0 – 34% of Pb; 18 – 69% of Mn; and 2 – 78% of Zn, with low/negligible % of As concentration in F1. Fraction 2 is the reducible fraction, where the reducing agent, hydroxylammonium chloride ($NH_2OH.HCl$) can solubilize iron and manganese oxides (poorly crystallized). Percentages of Cd extracted in this fraction varied from 0 – 34% of pseudo-total; 8 – 42% of Cu; 14 – 84% of Pb; 12 – 47% of Mn; 6 – 50% of Zn and 4 – 26% of As, which was found only in three samples of tailing matrices (2AP, 3AP and 4AP). In fraction 3, the oxidizable fraction that represents the elements associated with organic matter and sulphides are extracted by H_2O_2 and ammonium acetate. Cd ranged from 0 – 8%; 3 – 40% of Cu; 1 – 10% of Pb; 1 – 7% of Mn; and 0 – 10% of Zn. The As concentration in F3 ranged from 2 to 8% in tailing samples from Apiaí city.

Fraction 4 represents residual fraction and varied from 0 – 100% of Cd; 34 – 86% of Cu; 8 – 80% of Pb; 0 – 52% of Mn; and 0 – 91% of Zn. The As residual fraction was 100% for most samples, except 2AP (66%), 3AP (93%) and 4AP (85%).

The BCR was unable to extract As from most of samples in this study, although the BCR sequential extraction method has been successful in the extraction of As by previous workers (Baig *et al*., 2009; Sahuquillo *et al.*, 2003), Larios et al. (2012), when evaluated against other methods of sequential extraction for As in highly polluted mining sediments, the authors found that the BCR method was not suitable because some reagents, e.g. $NH_2OH.HCl$ and H_2O_2 were unable to completely extract the element from fraction 2 and 3. For this study extractable As (from any phase) was not observed in four of the samples (concentration < limit quantification).

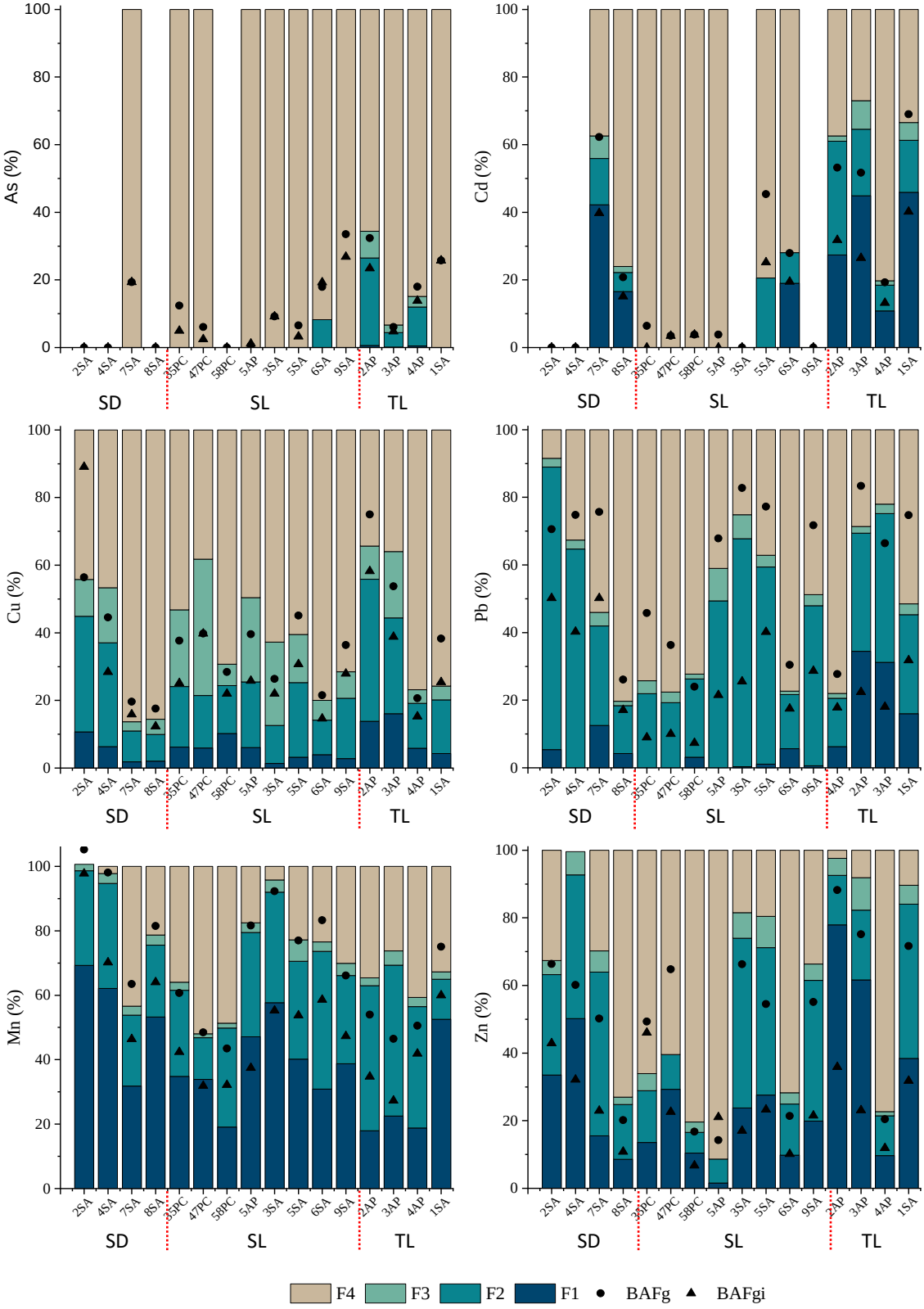

Figure 3. Distribution of As, Cd, Cu, Pb, Mn and Zn in the solid phases from each sample type in relation to the simulated gastric (BAFg) and gastrointestinal(BAFgi) fluid extraction. F1: Exchangeable and acid soluble carbonate fraction; F2: hydroxides and mixed oxy-hydroxide phases (reducible fraction); F3: Organic substance and sulphide fraction (oxidizable fraction) and; F4: the residual non-silicate bound trace metal concentration. The red dashed lines separate the groups SL (soil), SD (sediment) and TL (tail).

Cd, Pb, Mn and Zn present in the tailing samples could be associated with carbonates (Figure 3), exemplified by the presence of calcite, ($CaCO_3$), by intensity(I/Io): 0.303 nm (1) and cerussite ($PbCO_3$), by intensity(I/Io): 3.593(1), 3.498(0.5), 2.487(0.32) (Mineralogical Society of America, 2001) identified by the XRD analysis of the bulk samples and the clay fraction (APPENDIX D). The presence of calcite and other carbonates (i.e. cerussite $Pb(CO_3)$ and siderite ($FeCO_3$)) can increase soil pH and other environmental matrices due to release of OH^- and HCO_3^-.

Significant proportions of Mn associated with fraction 1 (water + acid-soluble + carbonates fraction) were observed in sediments (58%) and soils (37%) and this result agrees with the those found by Wali *et al.* (2015) in soils contaminated with phosphorgypsum. Mn may be as $MnCO_3$ and/or MnO in the sample types, and the Mn^{2+} ion can be released into solution when extracted by 0.11 mol L^{-1} acetic acid (Abali *et al.*, 2007). Mn in tailings samples, however, was associated with F2 and F4. Although Mn minerals were not identified in XRD in this study, they can be found as nodules and concretions with iron oxides which may be reduced and dissolved by the $NH_2OH.HCl$ solution or found as unweathered primary minerals (Chao, 1972).

Copper was observed in association with fraction 1, however, to a lesser extent. These results are in contrast to Perlatti *et al.* (2015) where high Cu concentration were observed in the carbonate phase and this was attributed to the presence of malachite and pseudo-malachite minerals in the tailings samples. In this study, Cu was mainly associated with the reducible (17%) and residual (62%) fractions. Generally, the mobility of Cu was similar in the three matrices. In soils, the metal association with reducible and oxidizable fractions was similar (17 and 18%). The oxidizable fraction level was not pronounced, with less than 10% Cd, Pb Mn and Zn. Nevertheless, Cu was present in the highest proportion, with values ranging from 3 to 40% due to the high affinity of Cu for organic matter.

Cadmium had a different distribution pattern and was present in the more easily mobilized fractions of sediments and tailings. In contrast, soils had a higher proportion in the residual fraction. The Cd concentration in 2SA, 4SA (sediments) and 6SA and 9SA (soils) was lower than the LQ (Figure 3).

The F2 fraction, extracted by 0.5 mol L^{-1} $NH_2OH.HCl$ at pH 2.0, was associated with Mn and Fe, identifying PHE associated with crystalline oxyhydroxides and amorphous forms (Rauret *et al.*, 1999). Generally, heavy metals have a high affinity for manganese oxides. According to McKenzie (1980), Pb can bind in Mn oxides either by strong specific adsorption or the formation of some specific Pb-Mn minerals such as coronadite. Adsorption on Mn and

Fe oxides decreased in order Pb > Zn > Mn > Cu > Cd (sediment); Pb > Mn > Cu > Zn > Cd (soil); Mn > Pb > Cu > Zn = Cd (tailing).

In some soil and sediments samples, 2:1 minerals such as smectites, vermiculite and illite were found as well as the 1:1 minerals such as kaolinite in clay fraction. These minerals may contribute to cation adsorption, explaining the high proportion of metals in the residual phase. 2:1 minerals have an adsorption capacity greater than 1:1 minerals (predominant in tropical soils), because of the higher total surface area that may contribute to metal immobilization (Dube *et al.*, 2001). On the other hand, kaolinite has a pH-dependent variable charge and in samples with high pH (> 6.6) the high affinity charge sites may increase with cation adsorption on hydroxylated sites on edges of silicate layers (Rieuwerts, 2007).

3.3.3.2. Solid fraction and bioaccessibility of PHE

Soil mineralogy can influence PHE bioaccessibility and bioavailability. According to Ollson *et al.* (2016), As dissolution in an acidic medium, similar to gastric conditions, depends on mineralogy. Ollson *et al.* (2016) found 30%, 49% and 82% for As bioaccessibility for tailings, calcinated and grey slimes, respectively. Arsenic sulphides and iron arsenate are less bioaccessible than arsenic sulphates and calcium-iron arsenates (Meunier *et al.*, 2010). Molina *et al.* (2013) observed a higher Zn bioavailability and bioaccessibility in mine waste at pH 1.5 and 4.5 than at pH 7.4 and *in vitro* bioaccessibility decreased as follows: mine waste > hydrozincite > hemimorphyte > zincite ≈ smithsonite >> sphalerite.

Comparing the results of the UBM method with the BCR method, the gastric fluid solubilized all the fractions in most samples (n = 16), including F4 (residual), for Cd, Cu and Pb. On the other hand, Mn and Zn only in fractions 2 or 3 were solubilized by gastric fluid (Figure 3). The mix between saliva and gastric fluid was extremely aggressive (pH 1.2 ± 0.05) and could dissolve even the PHE strongly adsorbed onto the solid matrix. There is a reduction in the negatively charged sites with decreasing of pH and can increase the desorption of PHE by the gastric juice. Besides the influence of pH, the presence of glucuronic acid ($C_6H_{10}O_7$), which has a carboxylic group in its structure, chlorides, phosphates groups and enzymes have high capacity to complex heavy metals. Ruby et al. (1992) evaluated the effect of particle size and pH using kinetic models for mechanisms for Pb dissolution in both mine-waste-impacted soil and pure anglesite ($PbSO_4$) through stomach phase. The authors observed that the

dissolution rate and equilibrium concentration of Pb might be dependent on $PbSO_4$ dissolution and the concentration of Cl^- due to the formation of $PbCl^+$.

The chemical and physical properties of the samples, as well as the pseudo-total concentration of PHE, can also give a preliminary estimate of human bioaccessibility. Figure 4 shows a linear positive relationship between the pseudo-total and gastric phase for all elements ($R^2 > 0.7$). Using linear regression models, Poggio *et al.* (2009) derived the bioaccessible content of soils from the pseudo-total PHE concentration, pH, organic matter and clay content and obtained significant models for Cu, Pb and Zn. Barsby *et al.* (2012) observed a positive correlation between the bioaccessible concentration in the gastric phase and the pseudo-total concentration of the PHE. Barsby *et al.* (2012) also associated the bioaccessibility of PHE to the presence of lithological material in the region studied.

An approach that has been well studied to estimate the bioaccessibility of PHE is the extraction with 0.43 mol L^{-1} HNO_3 (Cruz *et al.*, 2015; Li *et al.*, 2015; Rodrigues *et al.*, 2018). This method extracts the 'geochemically reactive fraction' (Rodrigues *et al.*, 2018), which is represented by the adsorbed elements in the organic matter, poorly crystallized oxides and silicate surface mineral that is easily desorbed into the soil solution, increasing PHE mobility and/or availability (Römkens *et al.*, 2004; Spijker *et al.*, 2011).

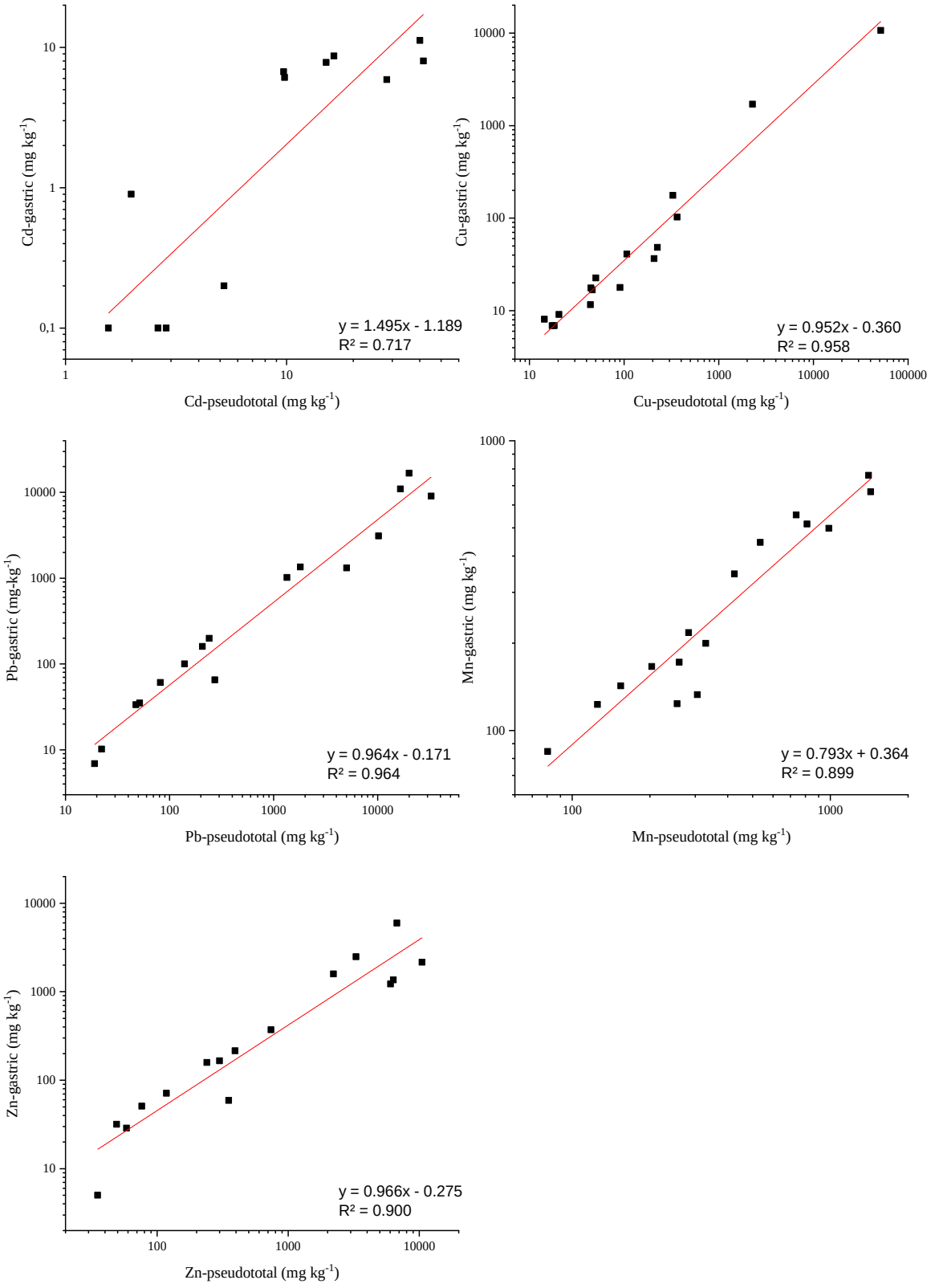

Figure 4. Relationship between the pseudo-total concentration extracted by 3051A (USEPA, 2007) and the gastric bioaccessibility. The data were log-transformed.

Table 4. Reactive concentration extracted by 0.43 mol L^{-1} HNO_3 solution and the relative proportion of PHE

Matrix	Sample	Cd		Cu		Mn		Pb		Zn	
		mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}	%
Sediment	SA2	0.6	0	6.4	45	117.7	146	30.0	94	46.3	97
Sediment	SA4	0.7	0	7.4	36	235.2	188	54.0	86	100.7	124
Sediment	SA7	2.3	23	7.0	8	426.0	52	350.0	33	164.2	12
Sediment	SA8	3.5	12	17.7	9	356.4	84	782.0	39	427.1	8
Soil	PC35	0.2	13	3.6	20	72.4	22	3.7	18	11.2	50
Soil	PC47	0.2	7	4.6	27	97.5	38	3.5	15	17.8	94
Soil	PC58	0.2	8	139.4	38	252.4	83	92.2	70	33.6	12
Soil	AP5	0.3	6	11.7	26	95.5	47	19.9	42	5.4	10
Soil	SA3	1.0	0	12.9	29	145.1	94	270.0	42	219.9	91
Soil	SA5	1.0	50	14.6	29	115.9	41	119.0	73	158.2	76
Soil	SA6	6.6	16	26.6	12	367.0	69	1674.3	60	432.3	4
Soil	SA9	0.5	0	10.0	22	233.9	90	74.0	88	162.9	117
Tailing	AP2	5.6	34	671.0	30	220.2	16	7704.6	50	644.7	3
Tailing	AP3	6.2	41	107.5	33	108.3	8	7343.0	50	642.7	4
Tailing	AP4	3.7	9	4017.6	8	105.1	11	3933.6	35	462.0	1
Tailing	SA1	2.8	29	10.9	10	144.1	20	464.2	18	377.2	21

PC = Piracicaba city; AP Apiaí city; SA = Santo Amaro city

The 0.43 mol L^{-1} HNO_3 extractable PHE varied from 0.2 to 6.6 mg kg^{-1} (median 1.0 mg kg^{-1}) for Cd; 3.6 to 4,018 mg kg^{-1} (median 12.3 mg kg^{-1}) for Cu; 3.5 to 7,705 mg kg^{-1} (median 194.5 mg kg^{-1}) for Pb; 72.6 to 426 mg kg^{-1} (median 72.4 mg kg^{-1}) for Mn; 14.7 to 93.9 mg kg^{-1} (median 163.6 mg kg^{-1}) for Zn. The As concentration for the soil and sediment samples and one tailing sample (1SA) were below the LQ. The tailing samples from Apiaí city had As concentrations ranging from 31.4 to 176 mg kg^{-1}. The linear correlations between 0.43 mol L^{-1} HNO_3 and UBM gastric (UBM-G) and UBM gastrointestinal (UBM-GI) phases were carried out in order to verify the efficiency of the diluted acid solution in simulating the PHE bioaccessibility (Figure 5). A good relationship between these methods was observed for all elements, $R^2 > 0.9$ (UBM-G), except for As.

Rodrigues et al. (2018) evaluated the bioaccessibility of Ba, Cd, Cu, Ni, Pb and Zn in soil samples from Portugal, Brazil and Netherlands and compared the results from the UBM method with SBET (Simplified Bioaccessibility Extraction Test) and 0.43 mol L^{-1} HNO_3. The authors observed that there was no difference ($p > 0.05$) for Cd, Cr, Cu, Ni, Pb and Zn values extracted by these methods and demonstrating a good linear relationship ($R^2 = 0.8 – 0.99$). In this study, for As, however, there was a difference ($p < 0.05$) between 0.43 mol L^{-1} HNO_3 and UBM-G, but this difference was limited because the As pseudo-total concentration was below the LQ (0.01 mg L^{-1}) in most of the samples. Conversely, Li *et al.* (2015) reported a linear relationship between UMB-G and 0.43 mol L^{-1} HNO_3 for arsenic, $R^2 > 0.7$ (mean = 29.4 and

27.2%, respectively), in soils from farming, mining, and smelting with high As concentration (22.2 to 4,172 mg kg^{-1}). Li *et al.* (2015) concluded that 0.43 mol L^{-1} HNO_3 has potential for determination of bioaccessible As in soils.

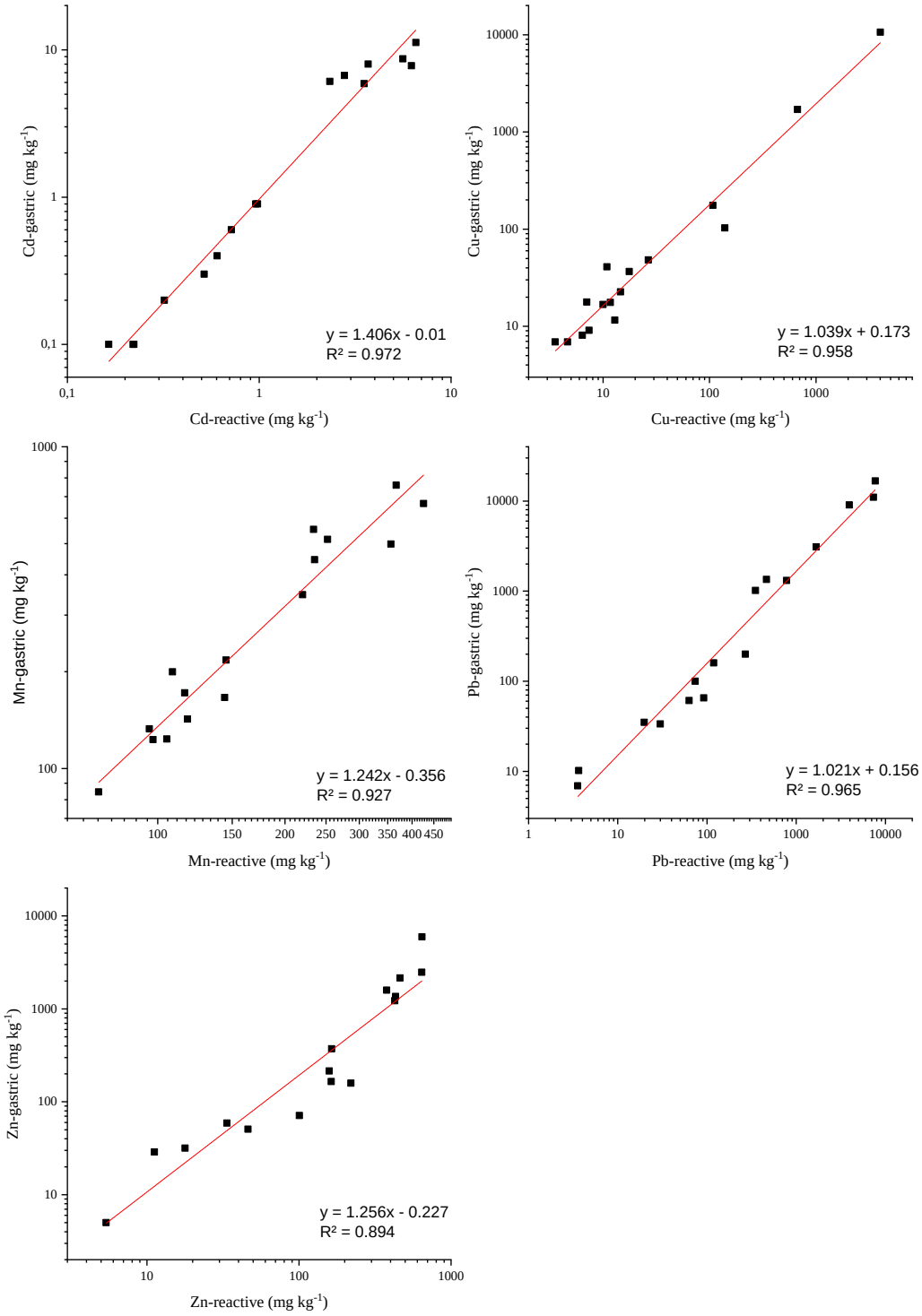

Figure 5. Relationship between reactive concentrations extracted with 0.43 mol L^{-1} HNO_3 and gastric bioaccessibility. The data were log-transformed.

3.3.4. Relevance of bioaccessibility studies in risk assessment

Ingestion of soil is one of the most important pathways of PHE exposure to humans, and some researchers recommend that *in vitro* methods should be included in the health risk assessment to estimate the PHE concentration that may release into the gastric system (Luo *et al.*, 2012; Li *et al.*, 2015; Rodrigues *et al.*, 2018). Ng *et al.* (2015) reported that in Australia, the National Environmental Protection Measures (NEPM) recommends that the bioaccessibility and bioavailability approaches should be incorporated into health risk assessments.

Usually, the neutralization of contaminated areas by liming or other techniques can reduce the solubility of PHE metals in soils by adsorption and ion precipitation. However, these procedures do not guarantee that the soil immobilized elements are not solubilized by the gastric tract or changing environmental conditions at the sampling location. This can be observed with the samples utilized in this study (Figures 6). Witt III *et al.* (2014) classified the solid-phase distribution into three groups: (i) very mobile (group I), PHE associated with exchangeable and carbonates phases, (ii) potentially mobile (group II), those PHE associated with reducible phase and (iii) immobile (group III), associated with oxidizable and residual phases. K-means clustering analysis allows identification of the three groups of solid-phase distribution based on the sequential extraction results (Figure 6). The three matrices presented different behaviour amongst themselves, that is, material from the same sample type could be classified into any of the three groups I, II and III (Figure 6).

The immobile phase had a low contribution to the oxidable fraction, and these results agree with Witt III *et al.* (2015) who found low organic matter content in road dust from mining areas, since these areas are often disturbed by anthropic actions, and the organic matter does not tend to accumulate on the superficial layers of unsurfaced roads. In this study, the sampling areas suffered diverse interferences by humans and nature such as movement of people, deposition of particulate materials from the atmosphere, dragging coarse particulate material from unpaved streets, flooding and other factors.

The pH of gastric and gastrointestinal simulated fluids was the main factor that contributed to PHE dissolution in both compartments. Usually, when an individual is fasted, the gastric pH is generally acidic (< 2.0) and suggesting that metal solubility is greater than in an individual that is fed (Cave *et al.*, 2011). However, Patinha *et al.* (2012) tested two pH values to simulate stomach condition, pH 2 (fasting) and pH 4 (fed) using the SHIME (Simulator of Human Intestinal Microbial Ecology) method and observed that Pb

bioaccessibility under fasted conditions was lower than under fed conditions. This fact could be due to the increase in enzyme and bile concentrations under fed conditions. According to Van de Wiele *et al.* (2007), in the SHIME method, 25 mL of a solution of pancreatic enzymes and bile salts are added under fasted conditions, while 100 mL of the same solution is used under fed condition. This corresponds to the *in vivo* tests where the rate of enzymes and bile addition increases during feeding. Bile is a route for the excretion of some divalent metals, Pb, Cu, Mn, Cd and Zn, but the mechanisms involved in the excretion of metals by bile are not clear (Boyer, 2013). There are many advantages and disadvantages associated with the bioaccessible extraction method, and this will depend on the methodology applied and its validation (Wragg & Cave, 2002).

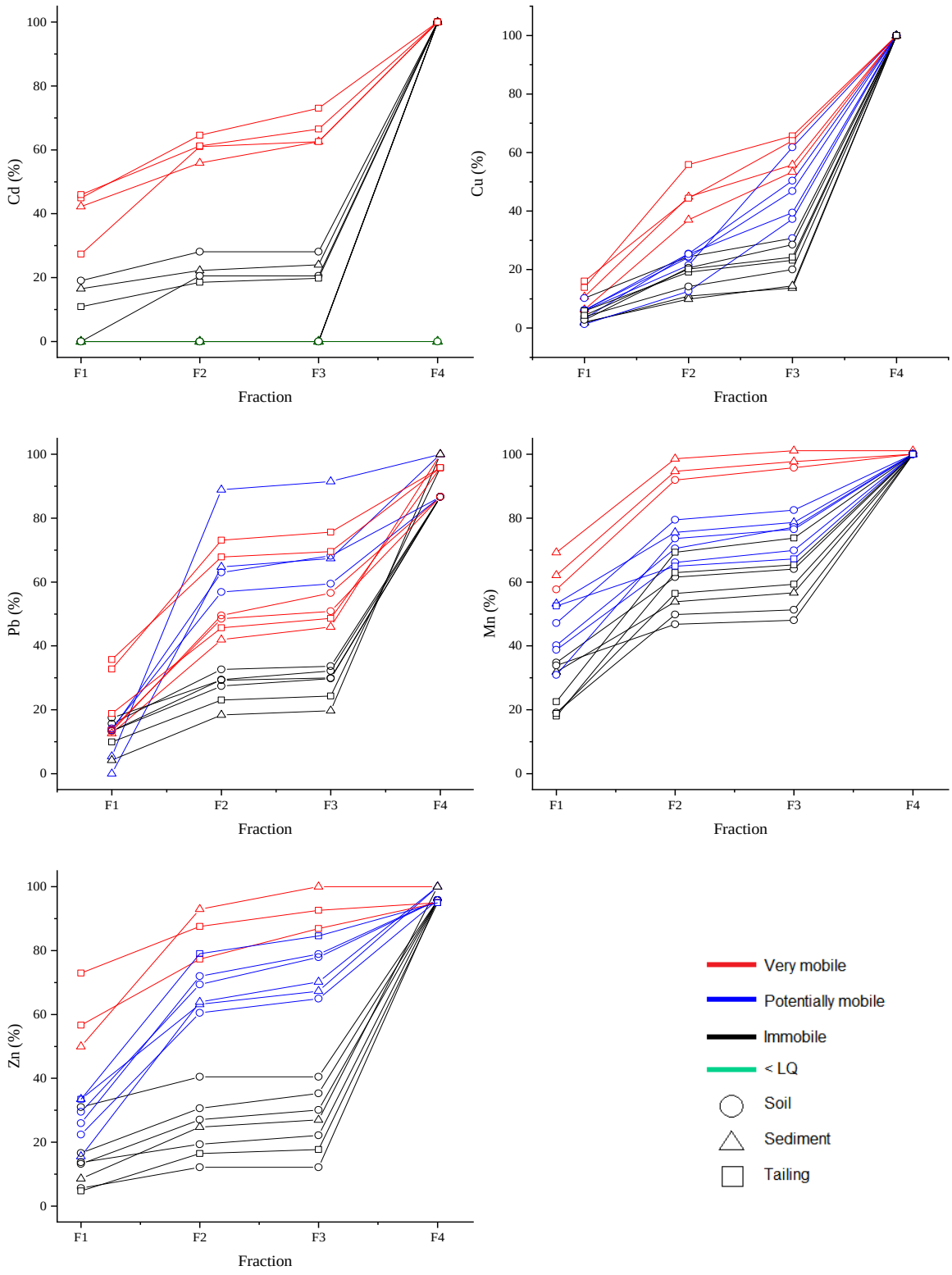

Figure 6. Cumulative extraction curve of Cd, Cu, Pb, Mn and Zn from soil (circle), sediment (triangle) and tailing (square) based on k-means cluster analysis separated by very mobile (red); potentially mobile (blue) and immobile (black). Green indicates that the concentration was low than the limit of quantification. F1: Exchangeable and acid soluble carbonate fraction; F2: hydroxides and mixed oxy-hydroxide phases (reducible fraction); F3: Organic substance and sulphide fraction (oxidizable fraction) and; F4: the residual non-silicate bound trace metal concentration

3.4. Conclusions

Samples from different urban sites differ in their PHE distribution pattern. Despite the high proportion of PHE associated with the residual phase, Cd, Mn and Zn were more associated with the available phase, mainly in the presence of minerals such as of calcite, cerussite and siderites. Lead and Cu were associated with the reducible phase, and the presence of high contents of iron oxides, such as hematite and goethite and amorphous iron oxides, may have favour their adsorption.

The BCR sequential extraction was not an efficient method to evaluate the fractionation of As in the samples, because the reagents were unable to completely extract the element, mainly in the reducible and oxidizable fractions.

PHE desorption/dissolution was more effective in the gastric simulation fluid than in gastrointestinal fluid, because of the low value of pH, the presence of organic substances (enzymes) and PHE complexing, with the gastric fluid results being more conservative.

Dilute nitric acid had similar results for Cd, Cu, Pb, Mn and Zn when compared to the UBM method. It is interesting to use this extractor for risk assessment, mainly due to its low analytical cost and time-consuming, but the *in vitro* method that mimics the human body provides results that are more accurate.

The Brazilin Resolution 420/2009 does not present guideline values for soil quality with respect to Mn. However, Mn has similar behaviour to the other elements studied, with high levels available in the environment and potentially bioaccessible to humans. There are several studies regarding the influence of manganese on human health, mainly related to Alzheimer and Parkinson's diseases. Therefore, there is a need for further studies on manganese in soils focused on exposure to Mn, especially on the dose-effect relationship.

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4. HUMAN INTESTINAL CACO-2 CELL LINE IN-VITRO ASSAY TO EVALUATE THE ABSORPTION OF Cd, Cu, Mn AND Zn FROM ENVIRONMENTAL URBAN MATRICES

Abstract

The Caco-2 cell line is derived from a human colon adenocarcinoma and is generally used in toxicity assays. The ingestion of soil or dust is a significant route of human exposure to potential harmful elements (PHE) and assays of bioaccessibility or bioavailability assay can be used to measure the potential hazard posed by exposure to toxic substances. The *in-vitro* digestion (*UBM* method) and Caco-2 cell model were used to investigate the bioaccessibility and absorption by intestinal cells of the PHE in four soil samples (two urban soils and 2 soils with lead mining tailings) along with the guidance material for bioaccessibility measurements, BGS 102. The gastrointestinal (GI) compartment was simulated and the resulting material added to Caco-2 cells. In the GI, the average bioaccessibility was 24% for Cd, 17% for Cu, 0.2% for Pb, 44% for Mn and 6% for Zn. The poor reproducibility of GI test was attributed to the pH (6.3) and the highly complex GI fluid that formed PHE precipitates and complexes. In 2h, Caco-2 cells absorbed 0.2 ng mg^{-1} of cellular protein for Cd, 13.4 ng mg^{-1} for Cu, 5.4 ng mg^{-1} for Mn and 31.7 ng mg^{-1} for Zn. Pb absorption was lower than the limit of quantification (< 2 µg L^{-1}). Cd was present in the cell monolayer and could interfere in the intracellular accumulation of Cu, Mn and Zn. The use of *in vitro* assays allowed an estimate of the absorption of Cd, Cu, Mn and Zn from environmental matrices and, except for Mn, it had a good correlation with bioaccessible concentration, suggesting a common association of this elements in cellular environment.

Keywords: Potentially harmful elements; Intestinal epithelium; Bioaccessibility; Bioavailability; Toxicity; Caco-2 cell line

4.1. Introduction

The ingestion of soil or dust is the main exposure route of potentially harmful elements (PHE) with their cellular absorption endangering human health. When soil particles are ingested, the bioaccessible PHE contents are solubilized into the gastrointestinal system, but not necessarily absorbed by the organism (Intawongse & Dean, 2006). The contaminant is absorbed or bioavailable when the bioaccessible element crosses the intestinal epithelium tissue lining the inner wall of small intestine that absorbs nutrients and allows their passage thereof into the bloodstreams (Rogers, 2011).

Exposure to contaminated soils is greater in children because of their pica behaviour or the simple fact of putting a dirty hand into their mouth. Because of their weaker immune system, as compared to that of adults, children are more susceptible to disease and illnesses associated with exposure to soil PHE. Pica behaviour is the recurrent ingestion of high amounts of soil (i.e., clay, yard soil and flower-pot soil) from 1 to 5 g day^{-1} and varies with

age. Where exposure is a result of non-pica behaviour, soil + dust ingestion is estimated at 0.1 g day^{-1} (US EPA, 2011).

The study of oral bioaccessibility of PHE in contaminated soil can be used in a human health risk assessment (HHRA) to evaluate the hazardous exposure of a contaminant through the soil ingestion. Oral exposure to PHE and organic substances (such as persistent organic pollutants (POPs) and aromatic hydrocarbons (PAHs)) have been studied with *in vivo* experiments using juvenile swine or adult rats (Juhasz *et al.*, 2007; Smith *et al.*, 2011), because this assay can determine the bioavailability of chemicals to organisms, however, these methods are costly, time-consuming, and there are ethical constraints (Cui *et al.*, 2016). This has resulted in the development of *in-vitro* models that simulate human gastrointestinal digestion.

In vitro methods can evaluate the bioaccessible concentration of soil contaminants and a more suitable approach in a HHRA than the use of pseudo-total PHE concentration (Pelfrêne *et al*, 2012). The oral bioaccessible concentration of contaminants in soils is normally greater than the concentration actually bioaccumulated into the body (Vasiluk *et al.*, 2007). Normally, the gastric compartment provides most conservative potentially available concentrations for human absorption than gastrointestinal compartment (Farmer *et al.*, 2011). Because of acidic conditions (pH 1.5) the simulated gastric fluid is able to extract a large amount of PHE of soils (Reis *et al.*, 2014). However, the gastric measurement does not estimate the concentration that could be absorbed by intestinal walls and which proportion can be transported into the bloodstream (Laparra *et al.*, 2007).

Oomen *et al.* (2003) defined four steps to evaluate element or substance bioavailability to a human: (i) the daily amount of soil ingested; (ii) bioaccessibility concentration of the material; (iii) how much is absorbed by intestinal epithelium; and (iv) quantification of first-pass effect (biotransformation of the contaminant in the intestine or liver followed by excretion). According to Oomen *et al.* (2003), metallic PHE, such as Pb are not biotransformed in the intestines or liver, consequently in this particular case, the first-pass effect was not considered.

To simulate absorption by human intestinal epithelium, cells derived from a human colon adenocarcinoma (Caco-2) are widely used to predict the potential absorption of medicine and drugs human intestinal mucosa (Hilgers *et al.*, 1990; Souza *et al.*, 2007; Artursson *et al.*, 2012), and in the availability of toxic substances in food (Laparra *et al.*, 2007; Fu & Cui, 2013; Aziz *et al.*, 2015; Cai *et al.*, 2017). However, few researches have

looked at their use to determine the bioavailability of PHE in soils (Oomen *et al.*, 2003b; Vasiluk *et al.*, 2011; Yin *et al.*, 2013). The Caco-2 cell line is similar to normal enterocytes with typical and organized brush border microvilli, grow in monolayer, present polarized cells and are maintained by the tight junctions complex (Delie & Rubas, 1997).

In this study the bioaccessibility and absorption of PHE by human intestinal cells (Caco-2 cells) were evaluated for contaminated soils and Pb mining tailings. The samples were selected considering the contrasting chemical, physical and mineralogical characteristics. The intracellular accumulation of cadmium (Cd), copper (Cu), manganese (Mn), lead (Pb) and zinc (Zn) using the Caco-2 cells assay and the likely competition between the elements for entry into the cells and thereby providing insight into the associated risk was investigated.

4.2. Materials and methods

4.2.1. Sampling location and preparation of samples

Two urban soils were selected, one from Santo Amaro (6SA), state of Bahia, Brazil (12°32.406'S, 38°43.637'W), a soccer field were children normally play and that presented high PHE concentrations, and the other from athletic track of University of São Paulo in Piracicaba city (58PC), state of São Paulo (SP), Brazil (22°42.850'S, 47°37.975'W) with lower concentrations of PHE relative to the first sample. The athletic track is next to Independência Avenue, one of the busiest avenues in the city of Piracicaba. The other two Pb mine tailing samples were also selected from Santo Amaro, 1SA (12°32.371'S, 38°43.855'), at the old lead ore beneficiation plant (Plumbum Mining and Metallurgy Ltd., initially COBRAC – Brazilian Lead Company) and Apiaí (SP) localized at the Upper Ribeira Valley, southeaster, Brazil (24°32.349'S, 48°49.899'W) at Lead and Silver Experimental Plant (4AP), a lead beneficiation plant deactivated in 1951, whose tailings were deposited in the soil around the facilities. These activities left a great environmental legacy for both cities.

All coarse material and covering grass was removed on the surface before the collection. Samples were collected from the 0 to 5 cm layer using a stainless-steel shovel or a plastic spade and stored in plastic bags. Each sample was derived from five sub-samples to obtain a bulk mixed. Then they were air-dried at room temperature, sieved into <2 mm fractions and quartered for further chemical, physical and mineralogical analysis. For the

determination of pseudo-total and bioaccessible elements concentration the samples were sieved to < 250 µm because it is assumed that this is the fraction of soil which sticks to children's hands that they then might put in their mouths (Rodrigues et al. 2014).

4.2.2. Chemical and physical characterization of soil samples

The pH was measured by potentiometry in water suspension using a liquid to solid (L/S) ratio of 2.5:1 according to (Anderson & Ingram, 1993). Total organic carbon was determined using a non-dispersive infrared sensor (NDIR). The densimeter method described by Gee & Or (2002) was used to obtain the clay content.

Mineralogical identification was made in bulk samples (2 mm) and clay samples, that were performed by X-ray diffraction (XRD) using a Philips PW 1877 diffractometer operated at a potential of 40 kV, 40 mA currents, CuKα (k = 1.54186 Å). The clay fraction was analysed after treatments with sodium dithionite-citrate-bicarbonate solution (DCB) to eliminate iron oxides and to concentrate the silicates. Clay samples received the following treatments: (i) magnesium (Mg) saturation and air drying; (ii) Mg saturation and glycerol solvation; (iii) saturation with potassium (K) and air drying; (iv) saturation with K and heating at 110, 350 and 550 °C in a muffle.

Pseudo-total concentrations were obtained by an acid extraction using (1:3 HCl: HNO3, v/v) according to the US. EPA 3051A method (USEPA 2007). A standard sample certified by the National Institute of Standards and Technology (NIST), SRM 2711a (Montana Soil), BGS 102 and blanks were used for quality control. The extracts were diluted four times for determination by inductively coupled plasma optical emission spectroscopy (ICP-OES).

Except for BGS 102, all the other samples were sieved to 250 µm to prior analysis. Pseudo-total concentrations (Table 2) were obtained by an acid extraction using (1:3 HCl: HNO_3, v/v) according to the US. EPA 3051A method (USEPA, 2007).

4.2.3. Oral bioaccessibility

The simulated gastrointestinal digestion was performed using the Unified BARGE Method (UBM) where the gastric stage (G) was conducted at pH 1.2 ± 0.05 and the

gastrointestinal (GI) stage at pH 6.3 ± 0.5 (Wragg *et al.*, 2011 and Denys *et al.*, 2012). The digestion has been described in full by many authors (Broadway et al. 2010; Denys et al. 2012; Wragg et al. 2009; 2011), but in brief each soil was exposed to a sequential extraction using saliva, gastric the gastrointestinal (duodenal and bile) fluids at physiologically relevant pH, and extraction time, temperature and soil:solution ratio (1:100). In this study no sample was collected at the end of the gastric phase. For more details, see Annex B. The extracts (chyme) from the gastrointestinal phase were filtered through syringe filter with 0.22 µm cellulose membrane into sterile Falcon® tubes and frozen at -80 ° C prior to analysis. All extractions were performed in quadruplicate. Four blanks and the guidance material BGS 102 prepared at the British Geological Survey (Wragg, 2009) were included for the analysis control. The BGS 102 is derived from an ironstone soil and presented bioaccessible reference values for 57 elements (Hamilton et al. 2015) however this study provides the first estimates of PHE absorption by Caco-2 cells for this material. The percentage bioaccessible fraction (%BAF) was also calculated as:

$$BAF\ (\%) = \frac{Element_{bioaccessible}}{Element_{pseudototal}} \times 100$$

4.2.4. Caco-2 cell line culture

The human intestinal epithelial cells, Caco-2, were obtained from American Type Culture Collection (ATCC® HTB37™). Monolayers of Caco-2 cells from 28th and 31st passages were seeded at 1 – 2 × 10^6 cell mL^{-1} into T75 flasks (75 cm^2) following the procedure described by Vázquez *et al.* (2014) and Wise (2002). The culture medium was Dulbecco's Modified Eagle's Medium (DMEM) with a high glucose content supplemented with 1% L-glutamine, 1% non-essential amino acid solution (NEAA), 1% antibiotic PEN-STREP (Penicillin: 10,000 U.I. mL^{-1} - Streptomycin: 10 mg mL^{-1}), 0.8% Amphotericin B and 20% heat inactivated foetal bovine serum (FBS). The pH of the medium was adjusted to 7.2 ± 0.1 with 1 mol L^{-1} HCl. In the post-confluence period, the culture medium FBS was reduced to between 8 and 10%.

The assays were undertaken in a biological safety cabinet (BSC) in aseptic conditions. The cells were maintained in an incubator at 37ºC with the internal atmosphere regulated to 95% relative humidity and 5% CO_2. The culture medium in the flask was changed every two or three days. When cells achieved 60% of confluency, the medium was

removed by vacuum aspiration and discarded. The cells were removed from the T75 flask surface with 10 mL of 0.25% Trypsin-EDTA for 5 min at 37 ºC. The trypsinized cells were transferred into Falcon® tubes and centrifuged at 373 × *g* by 10 min at 26ºC. The Caco-2 cells were transferred to T25 flasks (25 cm^2) at a density of approximately 2.5×10^5 cells mL^{-1} with supplemented DMEM. The medium was changed every 2 days until 80% confluence was reached. An inverted microscope (Figure 1) was used to assess cell growth and monolayer integrity, confluence and the post-confluence period.

4.2.1. Exposure

After the 15th day post-confluence, the experiment was undertaken with four replicates of each of the five matrices: two soils samples and two Pb mining tailings samples and BGS 102 guidance material, one blank positive and one negative control were also included.

To initiate the experiments, the GI fluid with soils (chyme) were diluted 1:3, chyme: DMEM (pH 6.5) to avoid cell lysis (Yin *et al.*, 2017). The old medium was removed by vacuum aspirate and discarded; 5 mL of chyme + DMEM (pH 6.0 ± 0.2) was added to the cells and incubated for 2 h based on that proposed by Aziz *et al.* (2014, 2015) and Ferruzza *et al.* (2000). For the negative control, the cell line was treated with DMEM (pH 6.5) used to dilute the chyme and for the positive control the cells line were treated with DMEM (pH 6.5) and GI fluid. The integrity of the cell layer was maintained, no displacement or cell death was observed by inverted microscope (Figure 1).

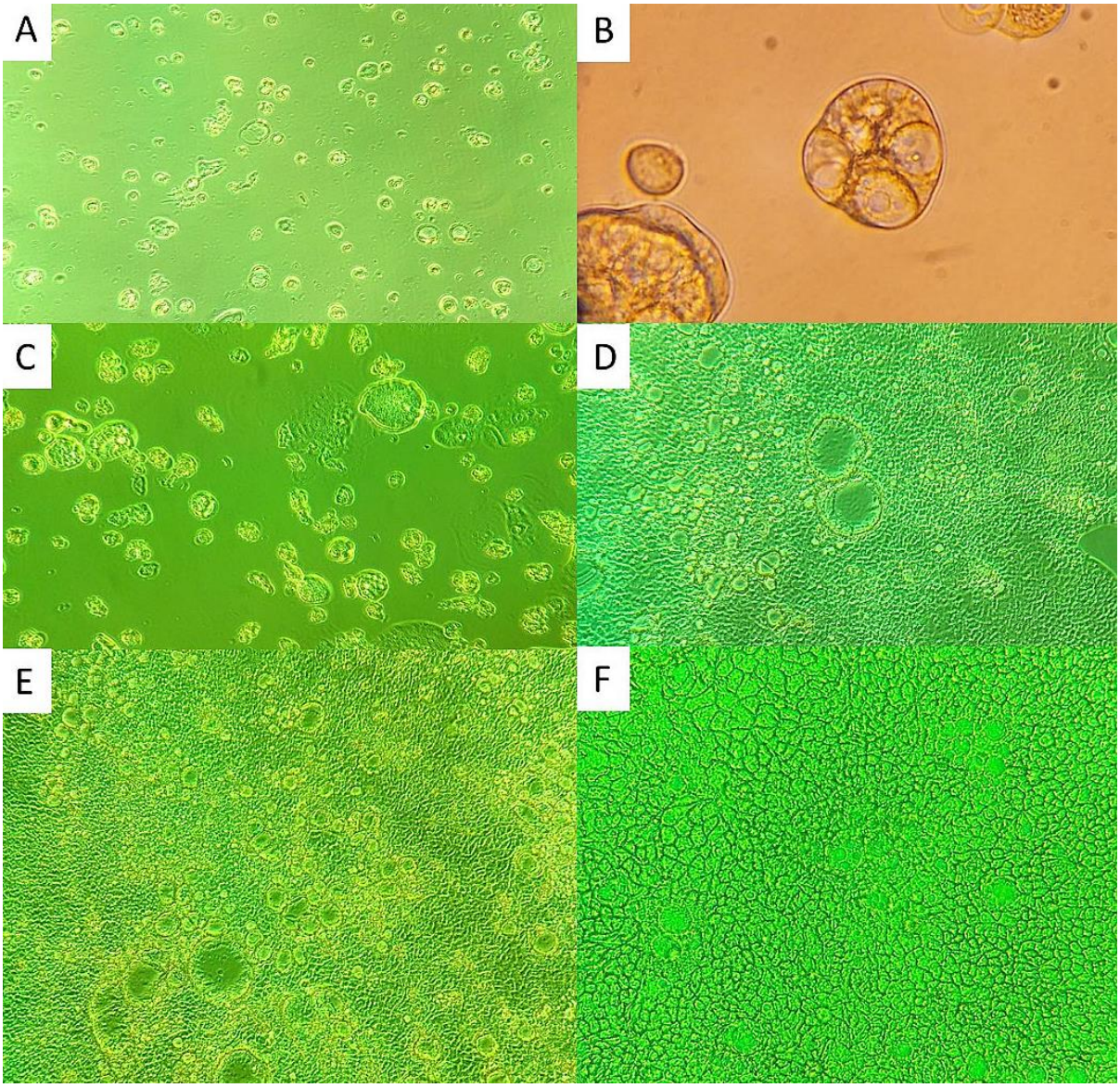

Figure 1. Inverted microscopy photographs of the grow Caco-2 cell over the time-course. (A) 1st day, Caco-2 in T25 flasks; (B) 3rd day, first change of medium, the image shows the moment of the cell division; (C) seventh day of experiment; (D) twelfth day of experiment (± 80% of confluence); (E) Post-confluence; (F) Experiment Caco-2 with chyme (sample Soil 1) after 2h of exposure.

The cells were rinsed with 3 mL of phosphate buffered saline (PBS) to remove the extracellular PHE and discarded. Then cells were washed three times with 2 mL of PBS and the cells scraped off transferred to centrifuge tubes (cells + PBS) and centrifuged (400 × *g* by 5 min) at 4 ºC and the supernatant discarded. The cell "pallets" were covered with N_2, sealed and stored at -80ºC until the extraction.

To extract the PHE, 1 mL 69% HNO_3 was added to the cell "pallets" and left overnight for pre-digestion. After 24 h, the tubes were placed in a water bath at 85ºC to complete digestion. Samples were diluted with 4 mL of ultrapure water (18.*2* MΩ) and frozen

at -20 °C prior to analysis. The concentration of Cu, Pb, Mn and Zn in all of the following extractions were measured using ICO-OES and for Cd graphite furnace absorption spectroscopy (GFAAS).

The absorption of PHE by the Caco-2 cells (%ABS) was expressed as a percentage of the measured bioaccessible concentration that was added and calculated with follow equation:

$$\%ABS = \frac{(M\_Abs - M\_NegContr)}{M\_Bio} \times 100$$

in which M_Abs is the PHE's concentration absorbed by Caco-2 cell (µg L^{-1}); M_NegContr is the negative control, that is, the PHE's concentration in Caco-2 cell without GI fluid (µg L^{-1}); and M_Bio is the bioaccessible concentration of PHE in the gastrointestinal phase (µg L^{-1}).

4.2.1. Protein determination

The levels of PHE accumulated in the cells were expressed as ng mg^{-1} cell protein, according to the international units (Aziz *et al.*, 2014). For that, aliquots of 20µL were withdrawn from each lysed cell sample to determine by the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) as the calibration standard. The protein concentration was estimated using the absorbance intensity at 595 nm.

4.2.2. Statistical Analyses

Data were analysed using SPSS 23 (SPSS Inc., Chicago, USA). Differences between the groups of samples by bioaccessibility and Caco-2 cell absorption were evaluated by the one-way ANOVA test followed by Tukey or Games-Howell *post hoc* tests with the differences considered significant at $p \leq 0.05$. The normality assumptions were evaluated by the Shapiro-Willks test since the sample size was less than 50 ($p \geq 0.05$) and the assumptions of variance homogeneity were evaluated by the Levene test based on the mean ($p \geq 0.05$) (Marôco, 2011). Non-normalized data were transformed by the Two-Steps approach, according to Templeton (2011). Relationships between the proportion of PHE absorbed and PHE concentration by cellular protein were analysed by Pearson's correlation analysis.

4.3. Results

4.3.1. Sample characterization and pseudo-total concentrations of Cd, Cu, Pb, Mn and Zn

The samples are slightly acidic to slightly alkaline (pH 6.6 – 7.3), presented sandy loam to clay texture according to USDA soil texture classification and have total carbon varying from 1 to 7% (Table 1). The Cd, Cu, Pb, Mn and Zn pseudo-total concentration varied between samples with high concentration (Table 1) in relation to the prevention value (PV) established by Brazilian Resolution CONAMA #420, December 28, 2009 (National Environment Council - CONAMA, 2012), except Mn that does not have a value established by Brazilian Resolution. High concentration of Cu, Pb and Zn were found in the samples 4AP, 1SA (soil + mine tailing) and 6SA (soil) compared to 58PC. The sample 6SA was collected in an area less than 1km from the site where 1SA sample was collected.

Table 1. Chemical and physical characterization of soil sample

Parameters	Particle-size	58PC	4AP	1SA	6SA	BGS 102
pH-H_2O	< 2mm	6.6	7.2	7.3	7.2	7.4[ac]
C-total[b] (%)	< 2mm	1	7	2	2	2[ac]
Clay (%)	< 2mm	15	15	46	20	-
Silt (%)	< 2mm	9	24	26	11	-
Sandy (%)	< 2mm	76	61	28	69	-
Cd (mg kg^{-1})	< 250 µm	3	42	10	40	13[c]
Cu (mg kg^{-1})	< 250 µm	362	51,624	107	224	25 [c]
Pb (mg kg^{-1})	< 250 µm	273	32,672	1,804	10,198	73 [c]
Mn (mg kg^{-1})	< 250 µm	255	988	738	536	6,348 [c]
Zn (mg kg^{-1})	< 250 µm	352	10,524	2,217	6,356	174 [c]
BS mineralogy	< 2 mm	Qz, K, Hm	Qz, Ca, Cu, K, Hm	Qz, Ca, Sm, Ti	Qz, R, Or, K	
Clay mineralogy	< 2 mm	K, Gb, Vm, Il, Br	K, Gb, Ca	Il, Sm, K	K, Sm, Il, Fe	

[a] Results from Wragg (2009); [b] Total carbon; [c] the particle size of BGS 102 guidance material 102 is < 40µm. BS = Bulk sample sieved to 2 mm. Br = Brucite; Ca = Calcite; Cu = Cuprite; Fe = Siderite; Gb = Gibbsite; Hm = Hematite; Il = illite; K = Kaolinite; Mg = Magnetite; Mv = Muscovite; Or = Orthoclase; Qz = Quartz; R = Rutile; Sm = Smectite; Ti = Anatase; Vm = Vermiculite.

4.3.2. Oral bioaccessibility

The values of bioaccessible fraction (BAF) ranged between 0 to 50% (Cd), 13 to 23% (Cu), 0 to 0.4% (Pb), 32 to 59% (Mn) and 3 to 21% (Zn) (Table 2, Figure 2). Cd concentration in 58PC sample was lower than the LQ (10 µg L^{-1}), while the Cd concentration of 4AP sample was higher than in the other samples, however 6SA sample did not differ (Tukey *post-hoc* test, $p > 0.05$). Sample 1SA differed from all samples (Table 2). Although, the 1SA had a lower bioaccessible concentration than samples 4AP and 6SA, its BAF was higher, with 37% relative to the pseudo-total, while the 4AP tailing sample was 24% bioaccessible and the 6SA soil sample was 17% bioaccessible (Figure 2 and Table 2).

Bioaccessible Cu concentration varied between all samples ($p > 0.05$). Sample 4AP had the highest concentration of bioaccessible Cu (8,164.1 mg kg^{-1}) accounting for 16% of pseudo-total Cu, lower than 58PC (23%) while, the 1SA sample also derived from mining tailings presented 21% of BAF.

Table 2. Oral bioaccessibility of PHE in soils extracted by gastrointestinal fluids and the bioaccessible factor (%BAF)

Sample	Matrix	Cd		Cu		Mn		Pb		Zn	
		mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}	%	mg kg^{-1}	%
1SA	Tailing	3.6 ±2.4 b	37	22.3 ± 3.0 a	21	436.8 ± 29.8 c	59	5.3 ± 6.1 a	0.3	467.9 ± 37.5 c	21
4AP	Tailing	10.1 ± 1.1 c	24	8164.1 ± 257.5 b	16	338.6 ± 51.0 bc	34	130.0 ± 113.6 b	0.4	337.9 ± 99.8 bc	3
58PC	Soil	<LQ a	-	84.7 ± 4.4 c	23	97.5 ± 9.3 a	38	<LQ a	-	<LQ a	-
6SA	Soil	7.0 ± 1.6 c	17	29.3 ± 0.9 d	13	293.0 ± 17.5 b	55	31.2 ± 10.0 ab	0.3	307.7 ± 16.0 b	5
BGS 102	Soil	5.2 ± 1.0	42	< LQ	13	2219.1 ± 122.2	35	<LQ	-	<LQ	-

< LQ = concentration below the limit of quantification (Cd, Cu and Pb = 10 µg L^{-1}; Zn = 50 µg L^{-1}). Means followed by the same letters in the column do not differ (Tukey or Games-Howell *post hoc* test, 5 %).

Pb and Zn had the lowest BAF (%), probably because of they can form stable or precipitated complexes at pH > 6.5, making them unavailable in the GI fluids. Sample 4AP had the highest bioaccessible Pb concentration (130 mg kg^{-1}) compared to the other samples, however, it did not differ from sample 6SA (31 mg kg^{-1}) (*post hoc* Tukey HSD test, $p > 0.05$). Sample 6SA had a Pb pseudo-total concentration around 1800 mg kg^{-1}, and 0.4% of Pb concentration in this sample was bioaccessible. Even though the 4AP and 6SA samples had pseudo-total concentration higher than 1SA sample (Table 1), the BAF were similar, (0.3-

0.4%) (Figure 2 and Table 2). The Pb bioaccessible concentration in sample 58PC was not possible to determine because it was lower than the LQ (10 µg L^{-1}).

Bioaccessible Zn contents in samples 6SA and 4AP were not different, and the 4AP sample also did not differ from the 1SA sample ($p > 0.05$). In 58PC sample did not have Zn bioaccessible concentration (concentration smaller than the LQ of 10 µg L^{-1}). In addition, the BAF of 1SA sample had the highest BAF (21%) as compared to the other samples that varied between (3 – 5%).

Similar results to Zn were observed in the bioaccessible concentration of Mn, in which the 6SA and 4AP samples did not differ ($p > 0.05$), but the 4AP also did not differ from the 1SA sample ($p > 0.05$), and the 58PC had the lowest Mn bioaccessible concentration (97. Mg kg^{-1}). The samples derived from the same city (1SA and 6SA) had the highest BAF (59 and 55%, respectively) compared to the other samples.

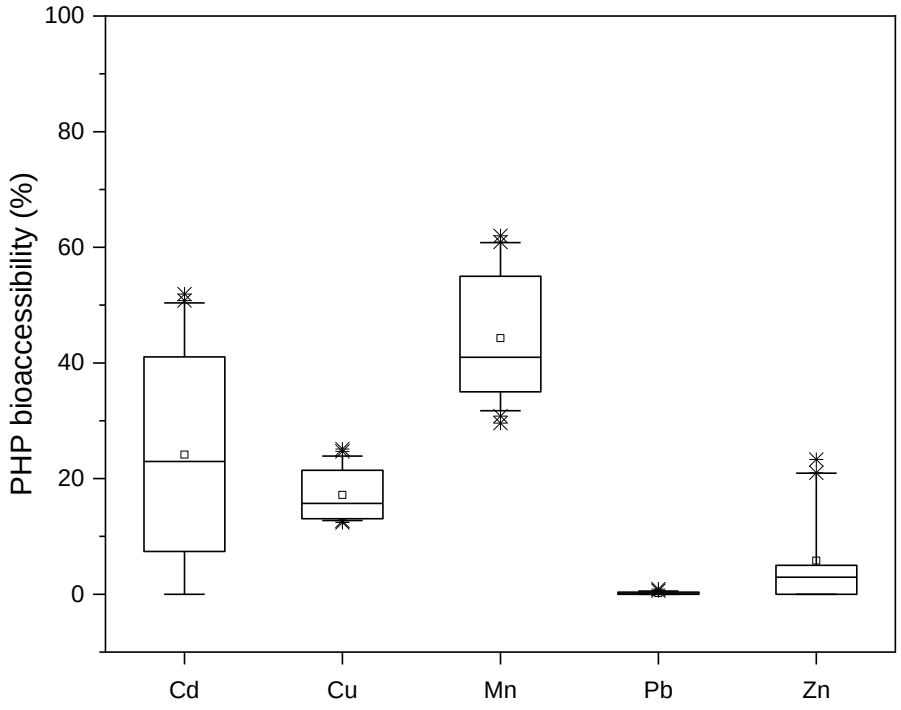

Figure2. Box-plot for oral bioaccessibility of Cd, Cu, Pb, Mn and Zn in the matrices (asterisk - extremes; degree sign - outliers).

4.3.3. Cellular uptake of potentially harmful elements

The Pb concentration determined using Caco-2 cell was lower than the LQ (< 2 μg L^{-1}), most likely because of the presence of amino acids and high concentrations of salts in the media (chyme + DMEM) where the Caco-2 cells were exposed to the PHE. The formation of complexes with Pb may have occurred and impaired their absorption (Oomen *et al.*, 2003b). Thus, only the results for Cd, Cu, Mn and Zn are discussed (Table 3).

Sample 4AP had a higher concentration of Cu and BGS a higher Mn concentration was absorbed by Caco-2 cells than all the other samples after 2 h of exposure, as shown by the outliers (asterisks) in Figure 3. This probably occurred because of the higher bioaccessible concentration of these elements in these samples.

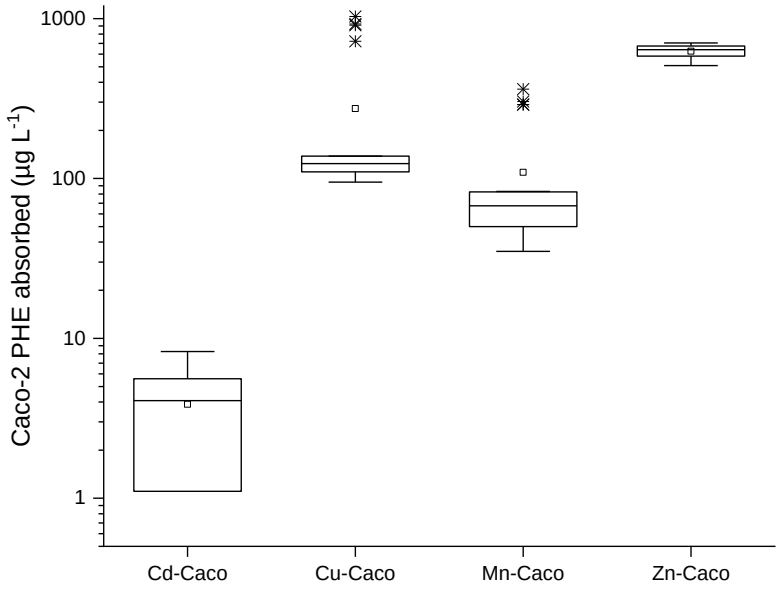

Figure 3. Box-plot of absorption of PHE by Caco-2 cell after 2h of exposure (mg L^{-1}). The values were log-transformed

There were differences in the accumulation of Cd, Cu and Mn ($p < 0.05$) between the samples (Table 3). Conversely, there was no significant difference in Zn between samples and the positive control ($p > 0.05$), that is, there was probably no absorption by Caco-2 cells of bioaccessible Zn from samples (Figure 1, Appendix E). The mean of the PHE accumulated by Caco-2 cells ranged from 0.2 ng mg^{-1} for Cd; 12 ng mg^{-1} for Cu; 5 ng mg^{-1} for Mn and 31 ng mg^{-1} for Zn of cellular protein. The Cd absorption varied between 0 to 0.4 ng mg^{-1} of cellular protein, but samples BGS102 did not differ from the positive control, which was not possible

to quantify (< LQ = 5 µg L^{-1}). The bioaccessible Cd concentration (17 to 37%) did not necessarily correlated to the absorption by the Caco-2 cells. The proportion absorbed (ABS%) in relation to the bioaccessible ranged from 0 to 44% (Figure 3) after 2 h of exposure.

Table 3. Absorption of PHE by Caco-2 cells after 2h of exposure (mean ± standard deviation, n = 4)

Sample	Matrix	Cd / cell protein	Cu / cell protein	Mn / cell protein	Zn / cell protein
		---------- ng mg^{-1} ----------			
1SA	Tailing	0.3 ± 0.06 bc	5.3 ± 1.0 a	3.5 ± 0.8 b	32.7 ± 5.3 ns
4AP	Tailing	0.2 ± 0.03 b	43.7 ± 12.2 b	3.1 ± 1.3 ab	30.1 ± 6.8 ns
58PC	Soil	ND a	6.1 ± 1.6 a	2.2 ± 0.7 ab	28.0 ± 6.6 ns
6SA	Soil	0.4 ± 0.01 c	6.2 ± 0.8 a	2.9 ± 0.8 ab	35.1 ± 3.6 ns
BGS 102	Soil	0.3 ± 0.3 abc	ND	15.2 ± 1.2 c	32.5 ± 1.9 ns
Positive Control	Blank	ND a	4.5 ± 0.8 a	ND a	25.5 ± 3.0 ns

Different letters in the column indicate a significant difference ($p < 0.05$, Tukey test; ns = not significant). ND = not detected

The samples did not differ in the accumulation of Cu by Caco-2 cells, except for sample 4AP (43.7 ng mg^{-1} of cellular protein); however, the ABS (%) in relation to bioaccessible concentration was only 4%, less than samples 6SA (82%), 1SA (68%) and 58PC (23%). Though the bioaccessible concentration of Mn in samples ranged from 97.5 to 2,219.1 mg kg^{-1} there was low absorption of the element (6 to 16%) compared to the other essential elements (for humans), Cu and Zn (Figure 3).

The highest Mn accumulation (15.2 ng mg^{-1} cell protein) was from sample BGS 102, however, the absorbed proportion (6%) was similar to samples 4AP (7%) and 1SA (6%), while with 58PC 16% of the bioaccessible content was absorbed by the cells (Figure 3).

Table 4 presents the Pearson correlation coefficients obtained for the relations of the PHE (Cd, Cu, Mn, Zn) bioaccessible and absorbed by Caco-2 cells, expressed in mg L^{-1}. A good correlation was observed between bioaccessibility and absorption of Cd, Cu and Mn ($p > 0.01$) shown in bold, indicating that the Caco-2 cell model was a good assay for estimate accumulation for some elements in the intestinal cells. These results might imply the existence of significant relations.

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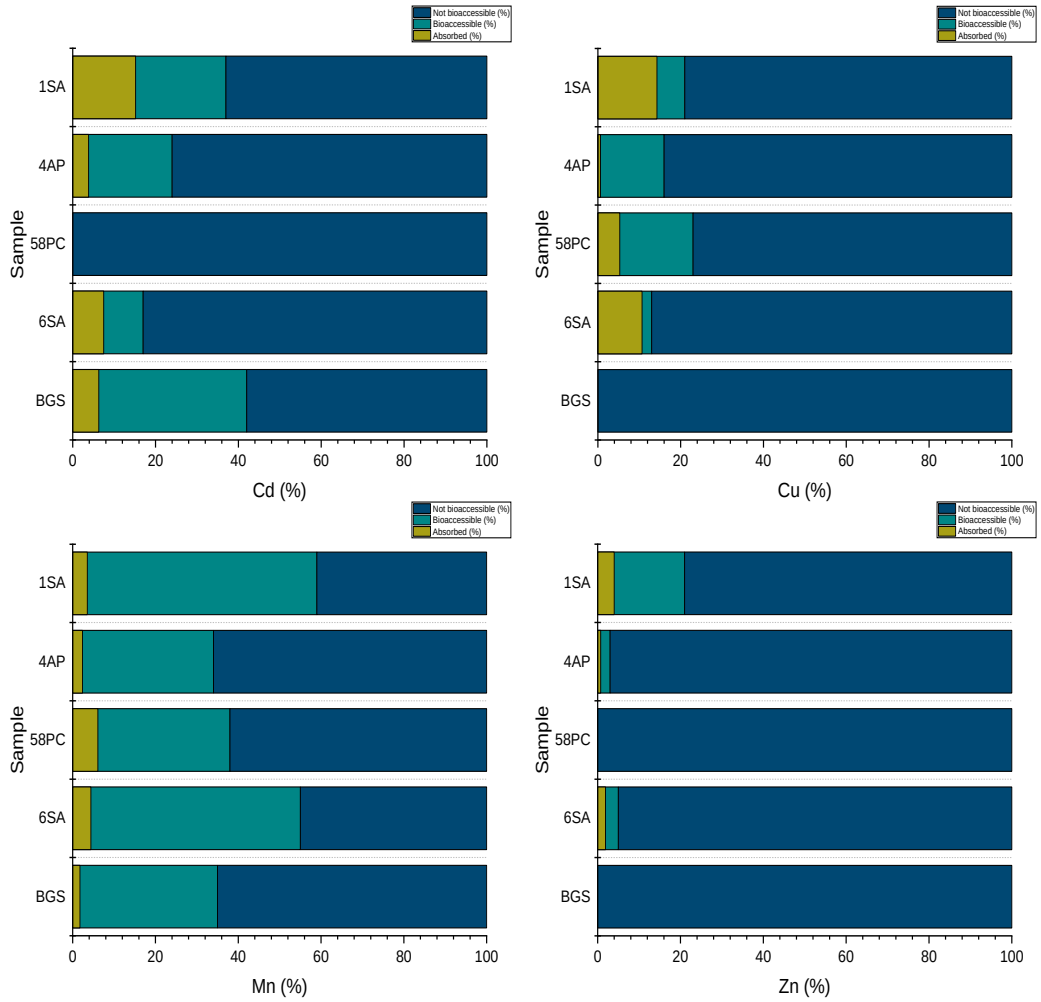

Figure 3. Relative amount of Cd, Cu, Mn and Zn not-bioaccessible (blue), bioaccessible (green) and absorbed by Caco-2 cell after 2h of exposition (yellow).

Table 4. Pearson's correlation coefficients (r) for absorbed and bioaccessible Cd, Cu, Mn and Zn (mg L^{-1}) in urban environment matrices (n = 20)

Element	Cd-Absorbed	Cu-Absorbed	Mn-Absorbed	Zn-Absorbed
Cd-Bioaccessible	**0.609****	0.677**	0.085	0.537*
Cu-Bioaccessible	0.043	**0.988****	-0.232	-0.125
Mn-Bioaccessible	-0.175	-0.209	**0.980****	0.223
Zn-Bioaccessible	0.708**	0.295	-0.492*	0.416

Correlation is significant at the ***p* < *0.01*, **p* < *0.05* (two-tailed)

Good positive correlation was also observed between Cd bioaccessible and Cu and Zn absorbed; Zn bioaccessible and Mn absorbed (Table 4). The close correlations (r > 60%)

established between the PHE bioaccessible and PHE absorbed by cells suggests a common association of this elements in cellular metabolism.

4.4. Discussion

4.4.1. In-*vitro* bioaccessibility studies

The GI concentrations of PHE were different than those obtained in Chapter 3 (APPENDIX C, Table 4). In the previous chapter the analyses of oral bioaccessibility were carried out in the geochemistry laboratory at the Geosciences Department of the University of Aveiro in Portugal, while the analyses carried out in this chapter were carried out at the laboratory of environmental analyses of the Department of Soil Science of the University of São Paulo (ESALQ / USP) in Brazil.

The relationship between PHE bioaccessibility in Brazil and in Portugal can be seen in Figure 4. A linear regression demonstrated a good relationship with Cu, Mn and Zn data (data not shown), with $R^2 > 0.9$ (Figure 4), however, only Cu and Mn showed relative standard deviation (RSD) lower than 10%, suggesting good reproducibility (data not shown). Wragg *et al.* (2011) found poor reproducibility in the gastrointestinal phase in an inter-laboratory trial of the UBM method. Koch *et al.* (2013) also found high values of relative standard deviation (RSD) for Pb (83%) and Zn (56%) in the gastrointestinal phase compared to the gastric phase (45% and 23% respectively), indicating poor reproducibility. A framework of reproducible (RSD ≤ 10%) bioaccessibility guidance values was established for 27 elements in the gastric phase and for only two elements in the gastrointestinal phase (Mg and Rb) in the BGS 102 sample, although Cd, Cu, Mn and Pb presented RSDs of 26, 22, 18 and 23%, respectively (Hamilton *et al.*, 2015).

The gastrointestinal compartment has a pH above 6.3 and includes the presence of enzymes, such pancreatin or bile that may form precipitates or complexes with PHE such as Cd, Pb and Zn. Specific adsorption sites could also form at high pH values due to the presence of organic matter, Fe and Al oxides and clays (Ellickson *et al*., 2001; Wragg *et al*., 2011).

Mica and Smectite group (2:1 minerals) peaks are more pronounced in samples 6SA and 1SA (APPENDIX D, Figures 11 and 16, respectively), different from 58PC and 4AP sample (APPENDIX D, Figures 3 and 15, respectively), that had predominance of Kaolinite (1:1 mineral). 2:1 minerals have a large surface area and significant cation exchange capacity able to adsorb PHE to their retention surface, even in soils with high pH value (Lamb *et al.*,

2009). Based on this, the GI fluids compounds, such as, bile and albumin, that are in contact with soil can remove the element bound to the minerals surface forming organic complexes and the likely PHE bioaccessibility increase (Wapnir, 1998).

The likely low BAF(%) in sample 4AP occurred because of the presence of sulphides minerals that are generally found in mining tailings and may form insoluble sulphides complex with chalcophile elements, such as Cd, Cu, Pb and Zn (Mehta *et al.*, 2019). However, these minerals were not detected by XRD and this means that they can be in the form of amorphous minerals. On the other hand, Mn proved to be the most bioaccessible in the samples studied (47% BAF, in averaging). Factors such as pH and redox reaction affects the dynamic of Mn in soils (Abreu *et al.*, 2004; Porter *et al.*, 2004; Suda & Makino, 2016). Furthermore, the proteins readily bind to element, mainly pepsin, present in gastric fluid, because of acidic character in its structure (Lassalle *et al.*, 2011). The pepsin activity decrease and may occurs denaturation of enzyme with increase of pH (Piper & Fenton, 1965) in GI fluid, based on this the elements previous binding to pepsin can be released to medium.

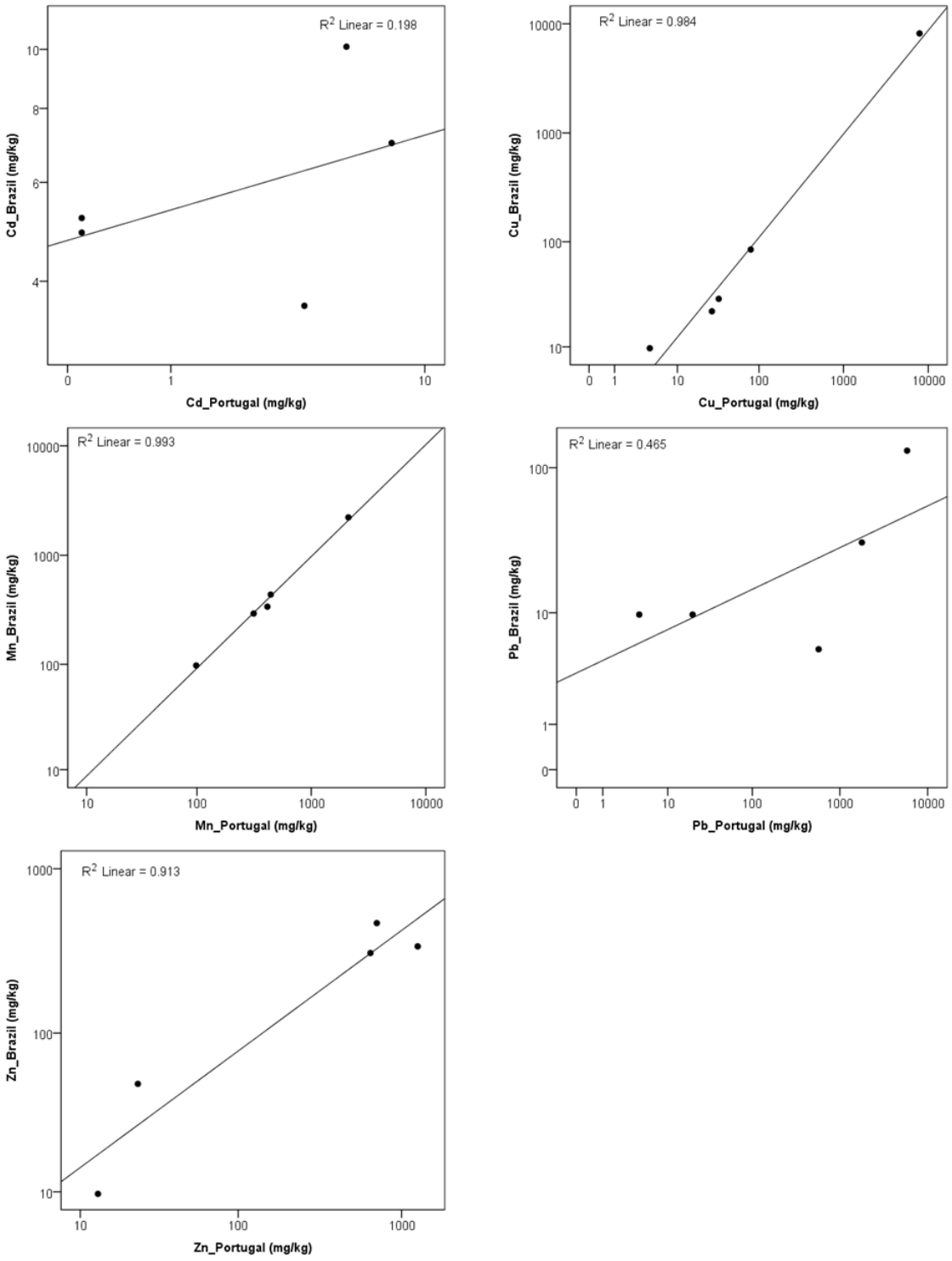

Figure 4. Comparison of PHE bioaccessibility determined in Brazil at University of São Paulo and in Portugal at University of Aveiro using the UBM-GI method. Data were log-transformed.

4.4.2. The PHE absorption by Caco-2 cell line

Caco-2 cell assay is a good predictor of nutrients or harmful elements that are bioavailable to humans (Hilgers *et al.*, 1990; Jumarie, 1997; Tallkvist *et al.*, 2000; Reboul *et*

al., 2005; Kang *et al.*, 2013; Fu & Cui, 2013). After absorption, the elemental species can form complexes with proteins, including enzymes (Apostoli *et al.*, 2006). The level of Cd absorption in Caco-2 cells varied between the two soils and two tailing samples and this is probably due to the difference in bioaccessible concentration where bioaccessibility is influenced by the chemical and mineralogical characteristics of the matrices studied.

Several authors have reported the transfer of PHE into cells after the extraction of bio-accessible PHE using soils samples. Oomen *et al.* (2003) evaluated the transport of bioaccessible Pb employing Caco-2 cell line and observed that not all the ingested Pb was bioaccessible in the GI tract and that 27% of bioaccessible Pb was present in the apical side of Caco-2 cells after 24h of exposure. Vasiluk *et al.* (2011) evaluated the bioaccessibility of Ni in contaminated soils using PBET (Physiologically Based Extraction Test) method for gastric and gastrointestinal digestion and assessed Ni absorption using Caco-2 cells and observed that bioaccessibility and bioavailability did not exceed 30%. Yin *et al.* (2017) observed that the species As(V), As(III), MMA^V, DMA^V were metabolized by human gut microbiota and absorbed by Caco-2 cells, with the intestinal absorption of As(III) was greater than the other As species. On the other hand, no studies were found in the literature with more than one chemical element in the same soil or other environmental matrices samples and likely competition.

Pearson correlation analysis showed a negative correlation between Cd and Mn absorbed. (r = -0.567; $p = 0.009$, 2-tailed) and a positive correlation between Cd and Zn (r = 0,.671; $p = 0.001$), Cd and Cu (r = 0.756; $p = 0.000$) and, Zn and Cu (0.645; $p = 0.002$)indicating that presence of Cd negatively influenced the absorption of Mn and it is proportion to the Zn and Cu absorption.

Cadmium is classified as a group B1, probably carcinogenic to humans by USEPA (United Stated Environmental Protection Agency) because of its high acute toxicity and the RfD for dietary exposure to cadmium is 0.001 mg kg^{-1} d^{-1} (USEPA, 1989b). When absorbed Cd is transported to the intestine through metallothionein (MT) complexes previously produced at the liver (Aziz *et al.*, 2014). Cadmium has a high affinity with MT may forming the Cd-MT complex in the intestinal mucosa besides competing with Ca^{2+} into cytoplasm even low concentrations (Rossi *et al.*, 1996). Zn, Cu and Mn may affect Cd absorption. After addition of 30 µmol L^{-1} of Cu, Mn and Zn, Noël *et al.* (2006) observed that Mn had the highest inhibitory potential for Cd absorption, while Cu was the lowest. Cu, Mn and Zn are

essential for humans and are present in the metabolic activities (Arredondo *et al.*, 2006; Leblondel & Allain., 1999).

Samples 1SA and 6SA had the largest amounts of Cd absorbed by Caco-2, 41 and 46%, respectively, compared to the other samples. This element can interact with Cu and Zn which had high amounts absorbed by cells (Figure 3). High Zn concentrations in the medium of Caco-2 cell can affect the Cu transport across the monolayer. This is probably because high concentration of Zn absorbed by cells can induce MT which are able to sequester Cu and reduce its absorption (Reeves *et al.*, 1996, 1998). MT are low molecular weight proteins found in mammalian cells, are related to the control and transport of PHE to the other proteins and have an important role against oxidative stress condition (Petering *et al.*, 2009). Reeves *et al.* (1998) incubated Caco-2 cells with doses of 5, 35 and 140 μmol L^{-1} of Zn ($ZnSO_4.7H_2O$ solution) and observed that the amount of Zn absorbed increased proportionately with the concentration of Zn in the media and that the last dose decreased the cellular absorption of Cu by 20%.

The intracellular concentration of Zn did not increase with the addition of chyme, remaining around the control level (25.5 ng mg^{-1} cellular protein). These results agree with those reported by Rossi *et al.* (1996) in the apical compartment. However, sample 1SA (tailing) showed the lowest proportion of absorbed Zn and the highest absorption of Cd (41%) and Cu (68%) (Figure 3 and 4). The interaction between this elements may be explained by competitive binding to MT, which participates in divalent cations transport (Arredondo *et al.*, 2006).

4.5. Conclusions

The application of an *in vitro* assay in a HHRA allowed an estimate of the bioaccessibility of Cd, Cu, Mn, Pb and Zn from environmental matrices. However, the UBM method for the gastrointestinal compartment had low reproducibility when compared with results obtained in the Chapter 3, suggesting that the complexity of the chemical and mineralogical composition of the samples can cause problems in the reproducibility of this method. Thus, *in vivo* tests are required to validate *in vitro* estimates of the elements evaluated in this study.

Cadmium may interfere in the intracellular accumulation of Cu, Mn and Zn, but these elements behave differently between soil and tailing samples because of the varying bioaccessible amount of each element. This supports the assertion by Pearson's correlation

analysis and suggests a common association of these elements in bioaccessible forms in cellular metabolism.

Caco-2 cell assay should be validated for soils using *in vivo* methods because it provides relevant information and was a useful tool for estimating the absorption of PHE by human rather than using *in vivo* tests which would require a high investment and time consumption.

This study highlights the importance for more bioaccessible studies in HHRA, but more studies are necessary, mainly with respect to the transport of the elements by the cells. Apical and basolateral compartments could be suitable for assessing PHE in both directions across the gastrointestinal epithelium.

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5. FINAL REMARKS

PHE can enter the body through ingestion or inhalation. In this study, the importance of bioaccessibility studies in a human health risk assessment in contaminated areas was highlighted.

Bioaccessibility methods can estimates the soluble amount of PHE in the digestive and respiratory systems in human. The Brazilian Resolution (CONAMA 420) recommends the use of strong acid solutions to extract inorganic substances from soils to establish prevention or investigation values for agriculture, residential and industrial scenarios. These values, however, cannot be assumed to be available or potentially available to humans, because such solutions extract total or pseudo-total concentrations of PHE that may include inert or precipitate elements in soils.

In vitro tests in a risk assessment may be used to estimate the amount of a determined element that can be harmful to human health and the capacity of extraction varies with the pedogeochemical characteristics of each matrix. The PHE behaved differently in soils, sediments and tailings, and the amount of PHE solubilized in simulated fluids depended on the Fe, Al and Mn oxides contents, organic matter, and type and content of clay in the matrix.

Artificial lysosomal fluid (ALF) was able to dissolve a large amount of PHE. In some samples, it extracted more than the concentration extracted by the US.EPA 3051A solution (labile and non-labile forms of PHE). This probably occurred because of the presence of organic acids that can dissolve the matrix and released PHE into the fluids. These elements extracted by ALF are not necessarily available to the lungs and reach blood streams, and for this, *in vivo* methods are needed.

The extraction of pseudo-total PHE concentration of the test matrices provided a good estimate of the respiratory bioaccessibility, but it is recommended that the extraction be performed on particles with less than 10 μm to simulate the size fraction that can reach the tracheobronchial region and gain access to the alveolar system.

The Unified BARGE method (UBM) provided a reliable estimate of bioaccessibility of PHE in gastric and gastrointestinal compartments. The oral bioaccessibility of PHE also varied among soils, sediments and tailings. The bioaccessibility in the gastric compartment was more conservative that in gastrointestinal compartments and should be used as a tool in risk assessment. The 0.43 mol L^{-1} HNO_3 has good potential to extract the bioaccessible fraction of PHE, however *in vitro* methods provide results that are more accurate.

Caco-2 cells were able to absorb Cd, Cu, Mn and Zn after exposure for 2 h. The use of this procedure is a useful tool in a risk assessment to investigate the toxicity and the accumulation of elements in human intestines, but more research is necessary to validate this method with *in vitro* and *in vivo* models. This study showed the probable competition between Cd, Cu, Mn and Zn, but further studies are needed to understand the behaviour of these elements in intestinal cells.

It can be concluded that this study serves as a basis for the development of new strategies for evaluating sites considered contaminated and as a basis for toxicological studies. The PHE do not necessarily have the same behaviour in different matrices; therefore, each case must be carefully investigated. An accurate assessment is important for a correct management or a cost-effective site remediation. The exposure to a chemical substance will depend on the type of the matrix, the amount in which it is ingested, as well as the group of which the individual belongs: child or adult. Children are more vulnerable to exposure to PHE than adults, because they are more vulnerable to effects on your body.

This study indicates the necessity for a more detailed investigation of the soil-solution interaction, the gastric/gastrointestinal and respiratory system, and proposes that studies on the bioaccessibility of PHE in humans should become part of risk assessment procedures to evaluate potentially contaminated areas.

This thesis allows a discussion of the incorporation of *in vitro* oral and respiratory bioaccessibility methods for inorganic elements into a human health risk assessment, thus helping to ensure an adequate management of contaminated land relative to the risk that may pose the population. The development of public policies by stakeholders may use bioaccessibility results as one of the evaluation criteria in a determinate site.

APPENDICES

APPENDIX A

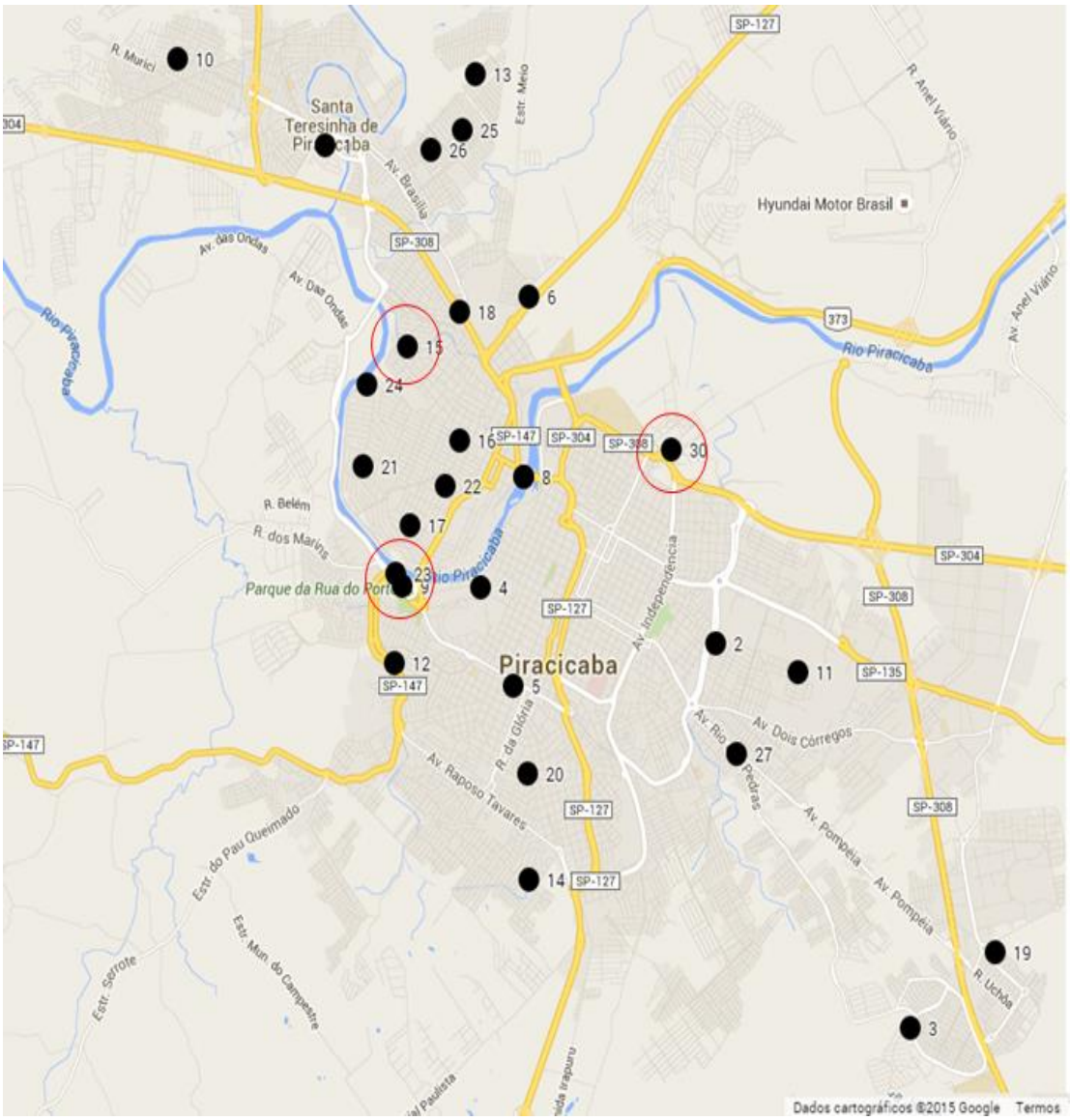


Figure 1 - Collection sites of set samples soils in Piracicaba. Samples selected for this study are marked with a red circle

Figure 2 - Collection sites in the old lead smelting plant (CIEM/CPRM), located in the neighbourhood Palmital in Apiaí, SP

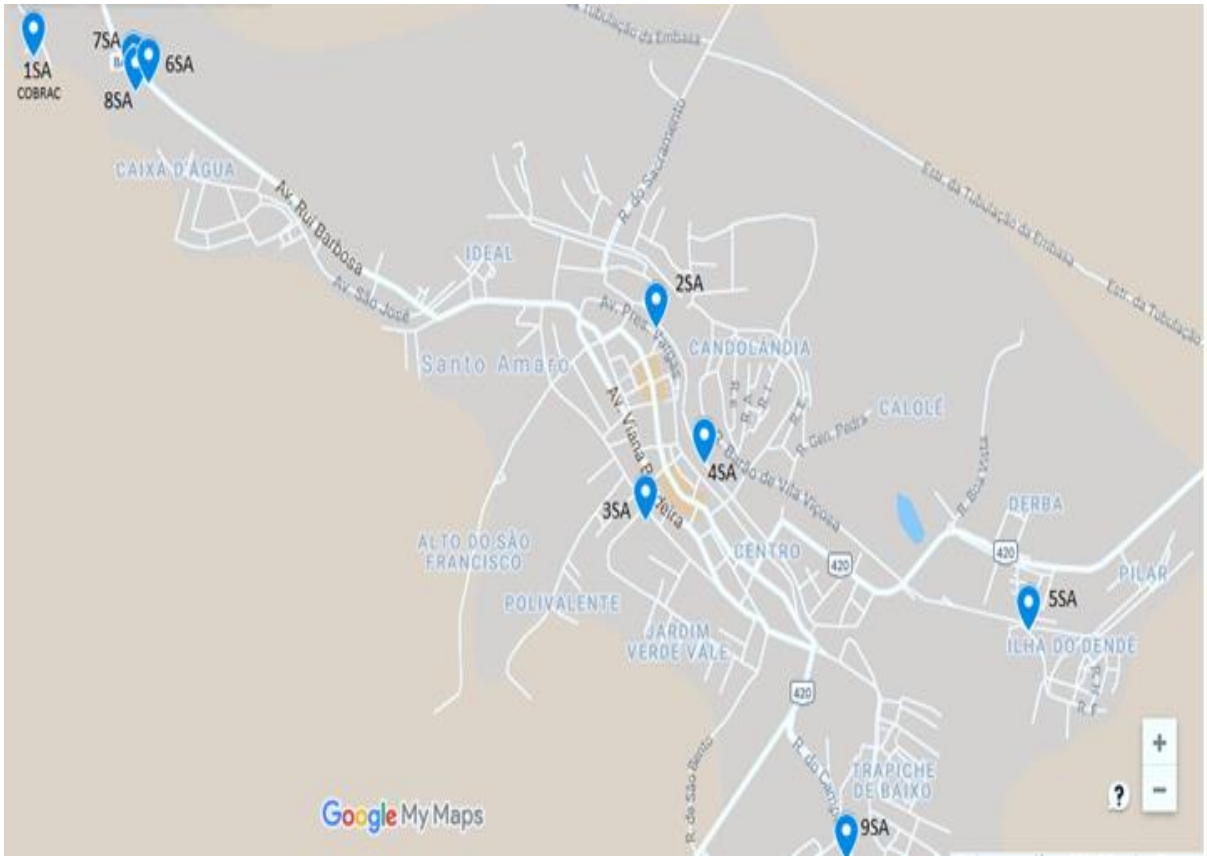


Figure 3 - Soil collection sites in the city of Santo Amaro, BA

APPENDIX B

Wilcoxon Signed Ranks Test

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum	Percentiles		
						25th	50th (Median)	75th
as	17	976,941	3339,3557	5,0	13856,0	6,000	9,000	300,000
cu	17	1678,588	5414,1055	58,0	22514,0	75,500	93,000	410,000
zn	17	2258,000	3738,2506	55,0	15668,0	421,000	638,000	3500,000
cd	17	12,729	28,0388	,2	119,1	,600	4,600	12,250
pb	17	6688,882	16377,0789	38,0	67673,0	231,000	435,000	5983,500
mn	17	649,765	240,3028	288,0	1154,0	512,500	627,000	755,500
as2mm	17	625,432	1691,4609	,0	6906,8	,000	,000	505,345
cu2mm	17	1530,052	5083,6970	,0	21112,9	33,865	69,670	252,625
zn2mm	17	2220,445	3146,3907	42,7	11569,4	93,605	491,410	3459,445
cd2mm	17	22,324	34,9635	,0	133,5	3,450	14,080	21,810
pb2mm	17	7831,535	19919,8010	20,4	82476,1	54,950	646,800	6973,100
mn2mm	17	629,856	554,8484	103,5	1861,8	224,350	407,940	870,180

Ranks

		N	Mean Rank	Sum of Ranks
as2mm - as	Negative Ranks	14[a]	7,71	108,00
	Positive Ranks	3[b]	15,00	45,00
	Ties	0[c]		
	Total	17		
cu2mm - cu	Negative Ranks	14[d]	9,36	131,00
	Positive Ranks	3[e]	7,33	22,00
	Ties	0[f]		
	Total	17		
zn2mm - zn	Negative Ranks	13[g]	7,62	99,00
	Positive Ranks	4[h]	13,50	54,00
	Ties	0[i]		
	Total	17		
cd2mm - cd	Negative Ranks	4[j]	5,25	21,00
	Positive Ranks	13[k]	10,15	132,00
	Ties	0[l]		
	Total	17		
pb2mm - pb	Negative Ranks	12[m]	7,17	86,00
	Positive Ranks	5[n]	13,40	67,00
	Ties	0[o]		
	Total	17		
mn2mm - mn	Negative Ranks	12[p]	8,25	99,00
	Positive Ranks	5[q]	10,80	54,00
	Ties	0[r]		
	Total	17		

a. as2mm < as
b. as2mm > as
c. as2mm = as
d. cu2mm < cu
e. cu2mm > cu
f. cu2mm = cu
g. zn2mm < zn
h. zn2mm > zn
i. zn2mm = zn
j. cd2mm < cd
k. cd2mm > cd
l. cd2mm = cd
m. pb2mm < pb
n. pb2mm > pb
o. pb2mm = pb
p. mn2mm < mn
q. mn2mm > mn
r. mn2mm = mn

Test Statistics[a]

	as2mm - as	cu2mm - cu	zn2mm - zn	cd2mm - cd	pb2mm - pb	mn2mm - mn
Z	-1,492[b]	-2,580[b]	-1,065[b]	-2,627[c]	-,450[b]	-1,065[b]
Asymp. Sig. (2-tailed)	,136	,010	,287	,009	,653	,287

a. Wilcoxon Signed Ranks Test
b. Based on positive ranks.
c. Based on negative ranks.

Output of Wilcoxon test

APPENDIX C

Table 1. Pseudo-total content of PHE in the sample soils sieved at 2 mm mesh

Matrix	Sample	As	Ba	Cd	Co	Cr	Cu	Mn	Ni	Pb	Zn
		---------- mg kg^{-1} ----------									
Sediment	SA2	<LQ	31.4	<LQ	2.7	3.5	<LQ	103.5	2.9	32.0	76.3
Sediment	SA4	<LQ	35.6	2.9	3.3	4.3	<LQ	135.8	4.1	62.5	154.2
Sediment	SA7	<LQ	188.7	16.4	19.6	43.5	69.7	714.3	35.8	1,048.9	968.9
Sediment	SA8	<LQ	88.6	14.4	10.1	20.6	93.0	407.9	16.5	2,011.3	3,171.8
Soil	PC35	<LQ	68.9	<LQ	3.7	21.2	21.1	352.1	6.0	20.4	48.3
Soil	PC47	11.5	63.7	4.2	6.6	44.5	30.0	459.0	7.7	23.7	56.9
Soil	PC58	4.5	46.2	2.1	3.8	16.1	211.7	412.6	7.0	131.1	110.9
Soil	AP5	<LQ	12.8	20.5	4.1	86.7	70.4	258.7	8.1	47.4	42.7
Soil	SA3	<LQ	54.7	4.0	8.1	12.1	46.7	180.7	8.8	646.8	491.4
Soil6	SA5	<LQ	70.6	4.3	4.9	18.9	39.8	343.5	9.8	163.4	322.9
Soil	SA6	<LQ	84.4	14.1	12.5	18.1	64.4	387.7	12.1	2,813.8	1,779.6
Soil	SA9	<LQ	57.1	5.6	6.3	35.6	37.7	190.0	12.7	83.8	234.1
Tailing	AP1	6,906.8	101.3	133.5	18.6	37.9	2,191.4	1,611.2	33.5	82,476.1	11,569.4
Tailing	AP2	1,194.6	90.7	85.0	21.7	49.6	1,603.4	1,861.8	16.0	15,270.2	5,613.9
Tailing	AP3	999.2	30.4	29.1	15.0	65.7	293.5	1,567.4	13.3	14,545.8	3,531.5
Tailing	AP4	1,515.7	160.9	23.1	54.6	40.9	21,112.9	1,026.0	35.8	11,132.4	3,387.4
Tailing	SA1	<LQ	179.4	20.3	25.5	32.1	125.2	695.4	34.1	2,626.5	6,187.5
VP		15	150	1.3	25	75	60	-	30	72	300
VIA		35	300	3	35	150	200	-	70	180	450
VIR		55	500	8	65	300	400	-	100	300	1000
VII		150	750	20	90	400	600.0	-	130	900	2000

<LQ = value less than the limit of quantification; VP = Prevention Value; VIA = Agriculture Research: Value; VIR = Residential Research: Value; VII = Industrial Research: Value (National Environment Council – CONAMA, 2009)

Table 2. Pseudo-total content of PHE in the sample soils sieved at 250 µm mesh

Matrix	Sample	As	Ba	Cd	Co	Cr	Cu	Mn	Mo	Ni	Pb	Zn
		----------mg kg^{-1} ----------										
Sediment	2SA	<LQ	38.5	<LQ	2.5	<LQ	14.4	80.4	2.4	4.3	47.6	76.5
Sediment	4SA	<LQ	45.3	<LQ	3.5	<LQ	20.4	125.4	2.8	6.6	81.4	118.1
Sediment	7SA	7.8	216.5	9.8	21.8	44.9	90.7	812.2	3.2	43.7	1.345.0	740.2
Sediment	8SA	<LQ	329.5	28.4	37.7	95.6	207.9	425.8	3.9	60.0	5.036.9	6.055.9
Soil	35PC	4.0	75.3	1.6	3.9	<LQ	18.3	329.1	3.8	6.5	22.3	58.4
Soil	47PC	8.3	44.3	2.9	3.5	29.8	17.4	255.0	4.5	5.7	19.0	49.1
Soil	58PC	<LQ	114.5	2.6	9.2	29.7	362.4	305.6	4.4	12.5	272.6	352.3
Soil	5AP	17.3	14.5	5.2	2.7	89.0	44.5	203.7	2.3	9.0	51.6	35.2
Soil	3SA	2.2	67.0	<LQ	4.4	<LQ	44.0	154.4	3.0	9.5	240.7	239.3
Soil	5SA	3.1	86.4	2.0	5.7	38.3	50.2	282.4	3.4	14.9	207.1	394.1
Soil	6SA	28.5	293.4	40.1	44.9	80.7	224.3	535.7	2.7	47.0	10.197.6	6.356.0
Soil	9SA	6.0	85.5	<LQ	8.9	86.2	46.2	260.1	3.8	22.3	139.4	299.4
Tailing	2AP	1.274.4	88.2	16.4	19.7	62.1	2.273.1	1.407.7	2.5	22.7	20.027.8	6.747.3
Tailing	3AP	1.163.8	35.1	15.1	12.1	58.9	327.1	1.434.5	2.2	14.7	16.534.1	3.298.9
Tailing	4AP	3.123.5	457.6	41.5	141.5	127.0	51.624.2	987.5	2.8	122.2	32.671.6	10.524.3
Tailing	1SA	6.6	222.9	9.7	24.7	49.4	106.8	737.9	2.5	42.5	1.803.5	2.217.1

<LQ = value less than the limit of quantification

Table 3. Pseudo-total content of PHE in the sample soils sieved at 10 µm mesh

Matrix	Sample	As	Ba	Cd	Co	Cr	Cu	Mn	Mo	Ni	Pb	Zn
		----------mg kg^{-1} ----------										
Sediment	2SA	7.3	283.3	4.6	23.3	76.3	87.9	922.4	1.1	39.5	336.7	529.5
Sediment	4SA	6.4	256.4	3.8	18.5	78.1	86.4	658.1	1.0	39.7	317.0	637.7
Sediment	7SA	5.5	235.9	8.6	20.0	69.5	66.4	585.3	0.1	50.0	1,055.9	638.0
Sediment	8SA	8.1	230.1	11.8	20.7	76.7	100.0	567.1	0.7	45.3	2,209.4	2,609.9
Soil	35PC	9.2	204.7	0.2	8.9	45.7	58.5	627.3	2.0	17.4	41.8	127.8
Soil	47PC	17.9	107.7	0.4	6.8	49.7	127.2	610.1	2.8	18.6	37.8	94.5
Soil	58PC	11.5	167.4	0.5	10.0	43.7	623.4	845.9	1.7	21.4	321.2	351.8
Soil	5AP	25.4	30.1	0.4	4.5	132.6	111.5	288.0	0.9	29.0	59.9	55.0
Soil	3SA	5.5	204.5	2.7	12.9	70.1	93.2	458.0	1.6	35.6	434.6	579.2
Soil	5SA	4.7	201.4	2.0	14.4	82.7	83.8	393.0	0.5	36.1	368.3	857.6
Soil	6SA	23.0	281.9	20.1	26.2	84.4	128.0	656.0	0.8	46.9	4,812.1	3,953.4
Soil	9SA	8.6	164.9	0.7	13.6	89.8	68.2	322.3	0.8	39.4	145.0	490.3
Tailing	1AP	13,856.5	8.7	119.1	10.3	38.5	2,751.8	665.4	1.5	32.8	67,673.0	15,667.7
Tailing	2AP	670.7	138.4	8.6	10.5	66.1	1,417.5	626.7	0.2	35.0	7,154.6	3,378.5
Tailing	3AP	574.9	47.1	12.7	9.6	67.9	341.3	1,154.2	0.4	35.1	12,995.3	3,768.6
Tailing	4AP	1,368.9	265.1	14.8	42.6	68.7	22,513.5	1,093.4	1.5	59.7	14,889.4	3,621.8
Tailing	1SA	5.7	265.9	5.4	21.2	88.3	82.8	575.3	1.2	48.3	858.7	1,025.4

Table 4. Gastric phase-UMB method

Matrix	Sample	Li	Be	Al	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Mo	Ag	Cd	Sn	Sb	Ba	W	Tl	Pb	Th
		mg kg^{-1}																					
Sediment	2SA	<LQ	0.1	347.4	1.1	0.3	84.6	300.0	1.1	0.4	8.1	50.7	<LQ	0.1	0.1	0.4	<LQ	0.1	21.7	0.1	0.0	33.6	0.2
Sediment	4SA	<LQ	0.1	523.2	2.4	0.7	122.9	437.5	1.5	1.0	9.1	71.0	<LQ	0.0	0.0	0.6	<LQ	0.1	30.4	0.0	<LQ	60.9	0.1
Sediment	7SA	<LQ	0.3	1664.7	3.8	1.0	515.5	981.1	7.9	5.1	17.8	371.4	1.5	<LQ	0.3	6.1	<LQ	0.2	149.0	0.0	<LQ	1017.5	0.1
Sediment	8SA	<LQ	0.3	1629.4	5.8	1.9	347.0	1540.8	4.8	3.3	36.6	1222.2	1.1	0.5	1.1	5.9	0.3	0.4	68.0	0.7	0.0	1313.4	1.9
Soil	35PC	<LQ	0.1	790.2	5.8	0.5	199.7	403.5	1.6	0.9	6.9	28.8	0.5	0.0	0.0	0.1	0.0	0.0	35.6	0.0	0.0	10.2	0.1
Soil	47PC	<LQ	0.1	928.9	6.6	1.2	123.6	393.6	0.9	0.9	6.9	31.8	0.5	<LQ	0.1	0.1	0.0	0.0	25.0	0.0	0.0	6.9	0.1
Soil	58PC	<LQ	0.1	774.3	2.7	0.3	132.8	330.8	1.2	0.8	102.8	59.0	0.4	<LQ	0.0	0.1	0.1	0.1	31.8	0.0	0.0	65.4	0.1
Soil	5AP	<LQ	0.1	3333.6	4.2	1.1	166.2	869.7	0.3	0.9	17.6	5.0	0.1	0.1	0.3	0.2	0.1	0.1	8.4	0.2	0.1	35.0	0.2
Soil	3SA	<LQ	0.1	820.6	3.2	0.7	142.5	475.7	1.9	2.1	11.6	158.6	<LQ	<LQ	0.0	0.9	<LQ	0.1	43.5	0.0	<LQ	199.1	0.2
Soil	5SA	<LQ	0.2	980.7	5.2	1.9	217.5	935.1	2.0	2.1	22.6	214.6	0.2	<LQ	0.0	0.9	<LQ	0.2	60.3	0.1	<LQ	160.0	0.1
Soil	6SA	<LQ	0.2	843.6	5.1	1.8	445.9	2211.4	8.2	3.9	48.3	1358.7	5.1	0.0	1.2	11.2	0.2	1.5	63.6	0.0	0.0	3102.7	0.1
Soil	9SA	<LQ	0.2	1538.2	5.5	3.0	171.9	741.4	3.2	2.2	16.8	165.0	2.0	0.2	0.2	0.3	0.1	0.2	57.2	0.2	<LQ	100.0	0.7
Tailing	2AP	<LQ	0.2	1266.7	9.9	0.4	759.3	1097.5	9.6	2.3	1704.3	5950.3	412.5	0.1	21.7	8.7	0.9	3.3	46.6	0.2	0.1	16695.6	0.3
Tailing	3AP	<LQ	0.1	1384.5	11.1	0.4	666.3	869.7	5.5	1.5	175.8	2477.4	70.3	<LQ	6.4	7.8	0.2	1.4	13.3	0.2	0.1	10983.9	0.3
Tailing	4AP	<LQ	0.3	2201.5	13.4	2.7	498.8	1999.0	14.5	9.0	10646.0	2148.3	561.9	0.2	17.7	8.0	0.8	3.6	85.6	0.3	0.0	9058.0	0.2
Tailing	1SA	<LQ	0.2	1939.0	4.0	1.4	554.0	2644.1	13.1	6.3	40.9	1588.2	1.7	0.0	0.2	6.7	0.1	0.2	176.6	0.2	0.1	1347.0	0.2

<LQ = value less than the limit of quantification

Table 5. Gastrointestinal phase-UMB method

Sample	Matrix	Li	Be	Al	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Mo	Ag	Cd	Sn	Sb	Ba	V	Tl	Pb	Th
		mg kg^{-1}																					
2SA	Sediment	0.4	<LQ	167.2	1.2	0.4	78.6	187.0	1.0	0.9	12.8	32.8	<LQ	0.2	0.02	0.3	0.04	0.2	11.8	0.05	<LQ	23.9	0.1
4SA	Sediment	0.1	<LQ	237.0	1.1	0.4	88.0	186.0	1.1	0.5	5.8	38.0	<LQ	0.2	0.01	0.4	0.05	0.1	17.0	0.04	<LQ	32.8	0.04
7SA	Sediment	0.7	0.2	1157.8	2.3	1.5	376.5	547.5	4.9	4.5	14.4	169.8	1.4	0.2	0.04	3.9	0.1	0.4	104.9	0.2	0.04	674.8	0.2
8SA	Sediment	0.6	0.2	1191.8	3.5	1.6	272.8	1200.1	3.3	3.4	25.6	655.9	1.3	0.4	0.2	4.3	0.1	0.9	55.4	0.09	0.01	860.3	0.1
35PC	Soil	0.3	<LQ	397.1	2.5	0.3	139.4	212.0	0.7	0.7	4.6	26.9	<LQ	0.2	0.01	0.03	0.04	0.06	20.1	0.05	<LQ	2.0	0.1
47PC	Soil	0.2	<LQ	368.9	1.8	0.6	81.2	163.4	0.2	0.7	6.9	11.1	<LQ	0.2	0.01	0.06	0.03	0.1	13.5	0.08	<LQ	1.9	0.1
58PC	Soil	0.1	<LQ	312.1	1.2	0.2	98.3	140.2	0.8	1.0	79.6	23.7	<LQ	0.1	0.01	0.05	0.1	0.3	17.7	0.2	<LQ	20.1	0.1
5AP	Soil	0.2	<LQ	1236.7	0.9	1.0	76.3	337.6	0.1	0.8	11.5	7.4	<LQ	0.1	0.02	0.04	0.02	0.09	3.8	0.05	<LQ	11.0	0.2
3SA	Soil	0.6	<LQ	328.9	1.0	0.5	85.3	197.4	0.5	0.9	9.7	40.7	<LQ	0.2	0.01	0.3	0.03	0.2	22.8	0.04	<LQ	61.5	0.06
5SA	Soil	0.6	<LQ	729.2	3.0	1.1	151.9	663.7	1.1	1.8	15.4	91.9	0.1	0.3	0.2	0.5	0.1	0.2	42.9	0.2	0.05	83.3	0.3
6SA	Soil	0.5	0.1	654.3	2.7	1.7	314.0	1639.5	5.2	3.8	32.8	645.0	5.5	0.3	0.6	7.8	0.1	2.8	45.9	0.4	0.1	1783.2	0.2
9SA	Soil	1.0	<LQ	901.0	2.9	1.7	123.0	447.5	1.7	1.9	12.9	64.5	1.6	0.4	0.03	0.2	0.05	0.3	36.4	0.1	<LQ	40.0	0.2
1AP	Tailing	1.8	<LQ	1411.3	4.5	1.4	629.9	3568.4	5.8	10.8	1066.5	8032.1	298.3	0.2	2.9	5.2	0.7	6.6	23.6	0.2	<LQ	4488.5	0.2
2AP	Tailing	0.6	<LQ	689.2	4.7	0.6	488.8	688.7	6.3	2.4	1323.9	2423.3	55.5	0.1	0.4	4.0	0.1	3.7	5.9	0.1	<LQ	2978.5	0.1
3AP	Tailing	0.9	<LQ	576.5	3.7	0.3	391.4	400.3	3.0	1.6	126.9	761.0	431.1	1.3	3.8	5.5	0.8	4.1	64.4	1.5	<LQ	5835.3	0.4
4AP	Tailing	3.4	0.2	1455.1	9.4	3.5	413.9	1466.5	10.2	9.1	7881.4	1254.0	1.7	0.3	0.03	3.9	0.07	0.5	77.9	0.1	<LQ	573.7	0.09

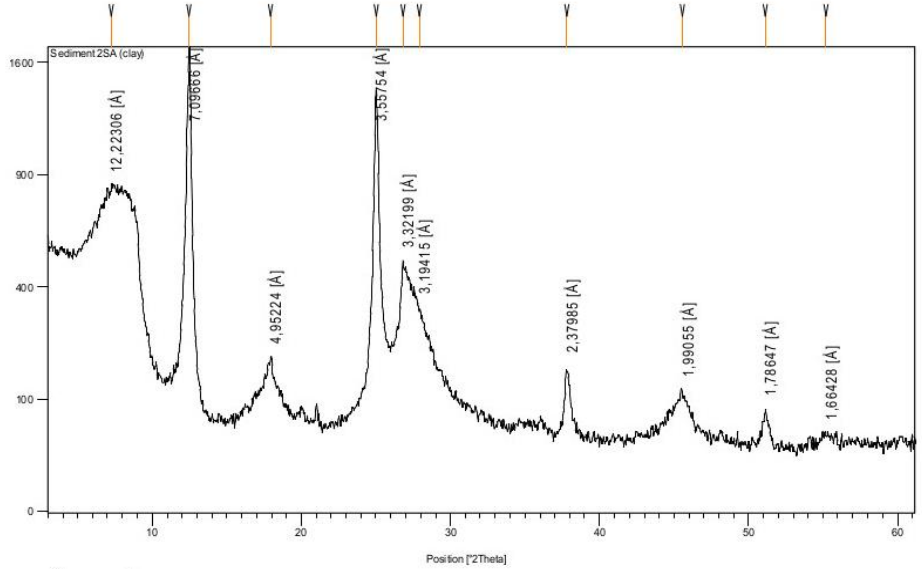
<LQ = value less than the limit of quantification

Table 6. Respiratory bioaccessibility results - ALF method

Sample	Matrix	Al	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Mo	Ag	Cd	Ba	Pb
		mg kg^{-1}													
35PC	Soil	2072.0	5.9	428.0	3278.0	3.8	13.4	18.0	55.0	4.2	4.9	0.4	0.2	91.3	19.0
47PC	Soil	2179.0	8.4	278.0	4069.0	2.1	0.0	28.0	68.0	3.1	3.7	0.2	0.2	79.0	22.0
58PC	Soil	2804.0	4.9	578.0	3218.0	5.0	14.0	309.0	153.0	5.5	3.9	0.1	0.4	132.2	196.0
1AP	Tailing	2778.0	7.0	416.0	11664.0	6.0	23.4	1559.0	9918.0	4154.0	3.2	6.8	98.5	79.3	60668.0
2AP	Tailing	1876.0	5.6	381.0	2878.0	5.4	12.9	961.0	2865.0	732.0	2.8	0.9	7.7	60.9	6537.0
3AP	Tailing	1811.0	4.6	407.0	2343.0	3.9	13.6	211.0	3094.0	296.0	3.3	0.4	11.4	37.0	10220.0
4AP	Tailing	4536.0	16.0	876.0	6884.0	27.2	33.5	19990.0	2828.0	1492.0	5.2	9.1	12.8	242.5	13204.0
5AP	Soil	4764.0	10.3	172.0	5915.0	0.5	0.0	24.0	26.0	3.4	2.7	0.1	0.2	26.0	49.0
1SA	Tailing	2720.0	6.2	453.0	2889.0	10.2	17.5	43.0	655.0	5.7	2.8	0.1	4.7	264.6	777.0
2SA	Sediment	3581.0	15.2	809.0	9285.0	12.0	18.1	49.0	345.0	6.1	3.2	0.1	4.2	255.3	245.0
3SA	Soil	2368.0	8.2	362.0	3822.0	4.5	17.9	22.0	334.0	4.0	4.4	0.1	2.1	169.7	325.0
4SA	Sediment	3452.0	10.9	549.0	6647.0	8.6	0.0	49.0	505.0	5.2	3.4	0.1	2.8	225.0	418.0
5SA	Soil	4141.0	13.1	289.0	6229.0	3.9	15.1	43.0	447.0	4.0	2.8	0.1	2.0	192.6	262.0
6SA	Soil	3245.0	12.1	542.0	9867.0	11.9	19.1	78.0	2503.0	22.0	3.6	0.1	17.6	251.6	4419.0
7SA	Sediment	2755.0	7.3	466.0	3282.0	8.0	0.0	25.0	458.0	4.5	3.2	0.1	6.1	255.0	859.0
8SA	Sediment	4464.0	12.9	475.0	7158.0	7.8	18.0	56.0	1849.0	8.4	3.7	0.1	10.0	233.7	2015.0
9SA	Soil	3143.0	14.8	226.0	3425.0	4.5	0.0	35.0	360.0	6.5	3.3	0.0	0.5	158.0	120.0

APPENDIX D

Figure 1 - X-ray diffractogram of bulk sample (BS) and clay fraction of sediment 1 sample (2SA).



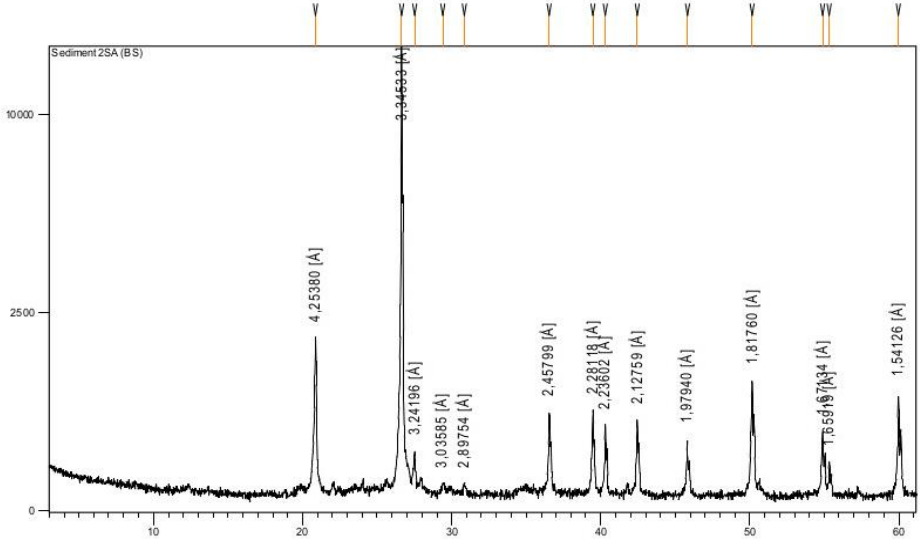


Figure 2 - X-ray diffractogram of bulk sample and clay fraction of sediment 2 sample (4SA).

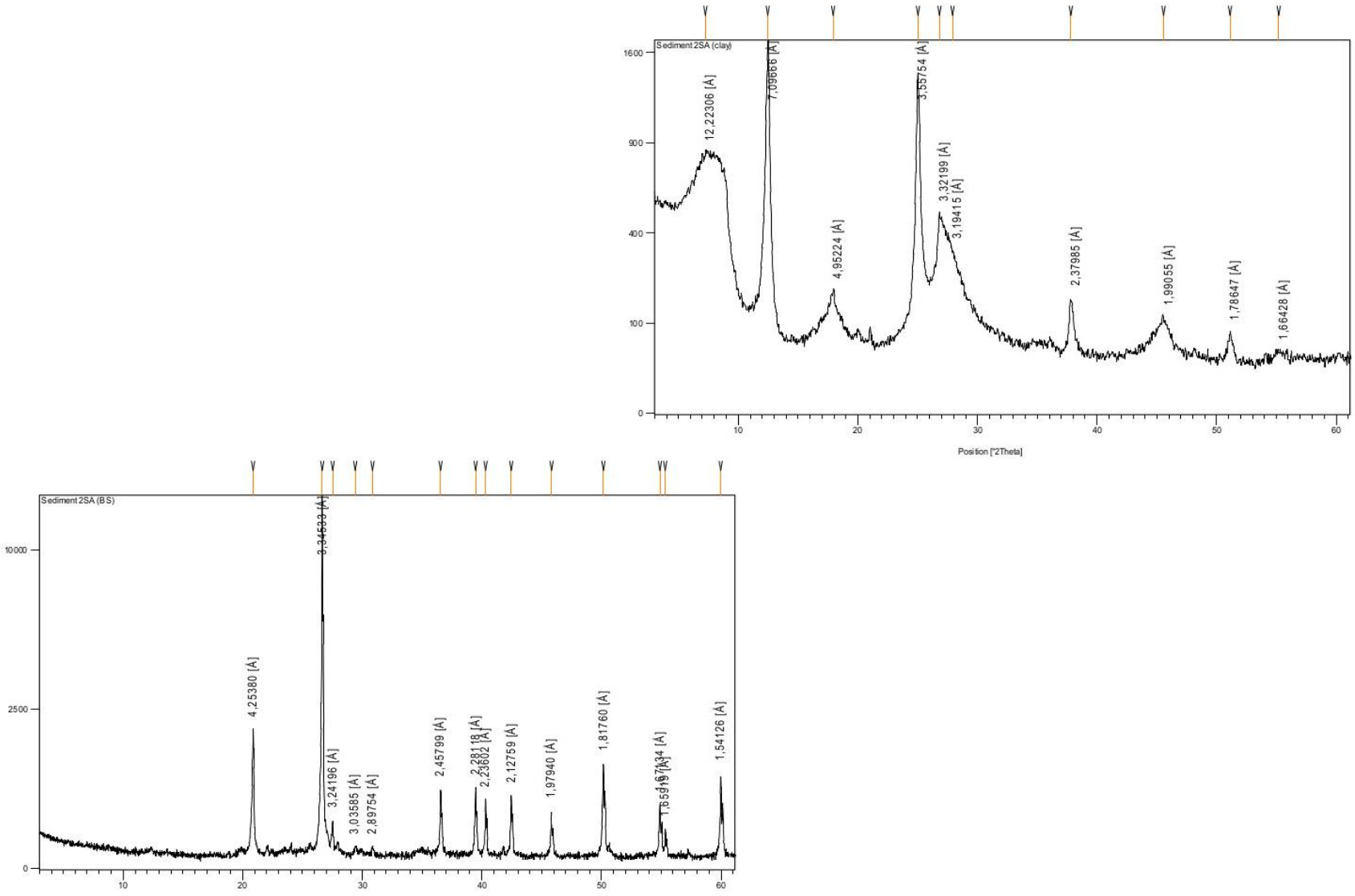


Figure 3 - X-ray diffractogram of bulk sample and clay fraction of sediment 3 sample (7SA)

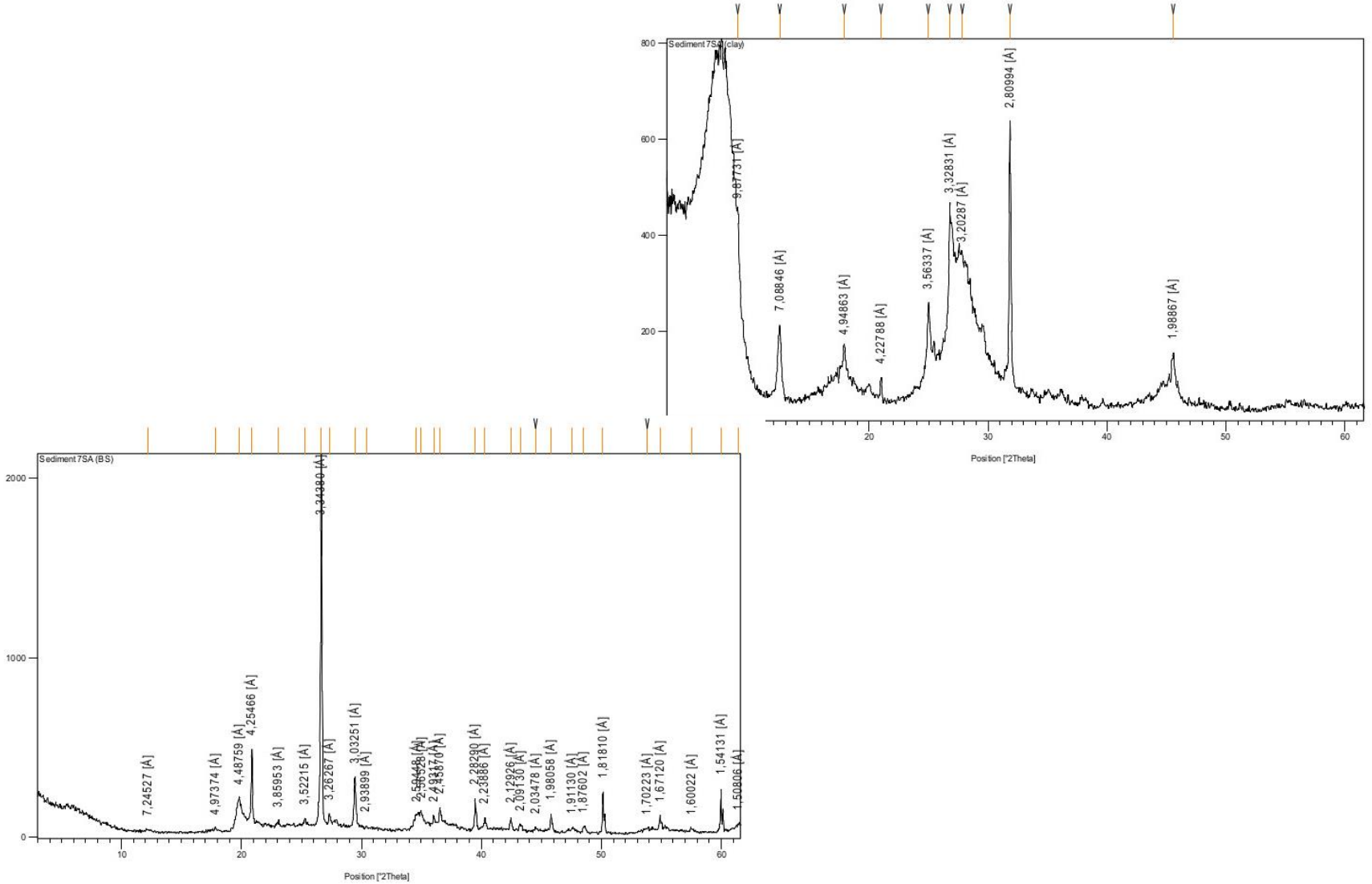


Figure 4 - X-ray diffractogram of bulk sample and clay fraction of sediment 4 sample (8SA)

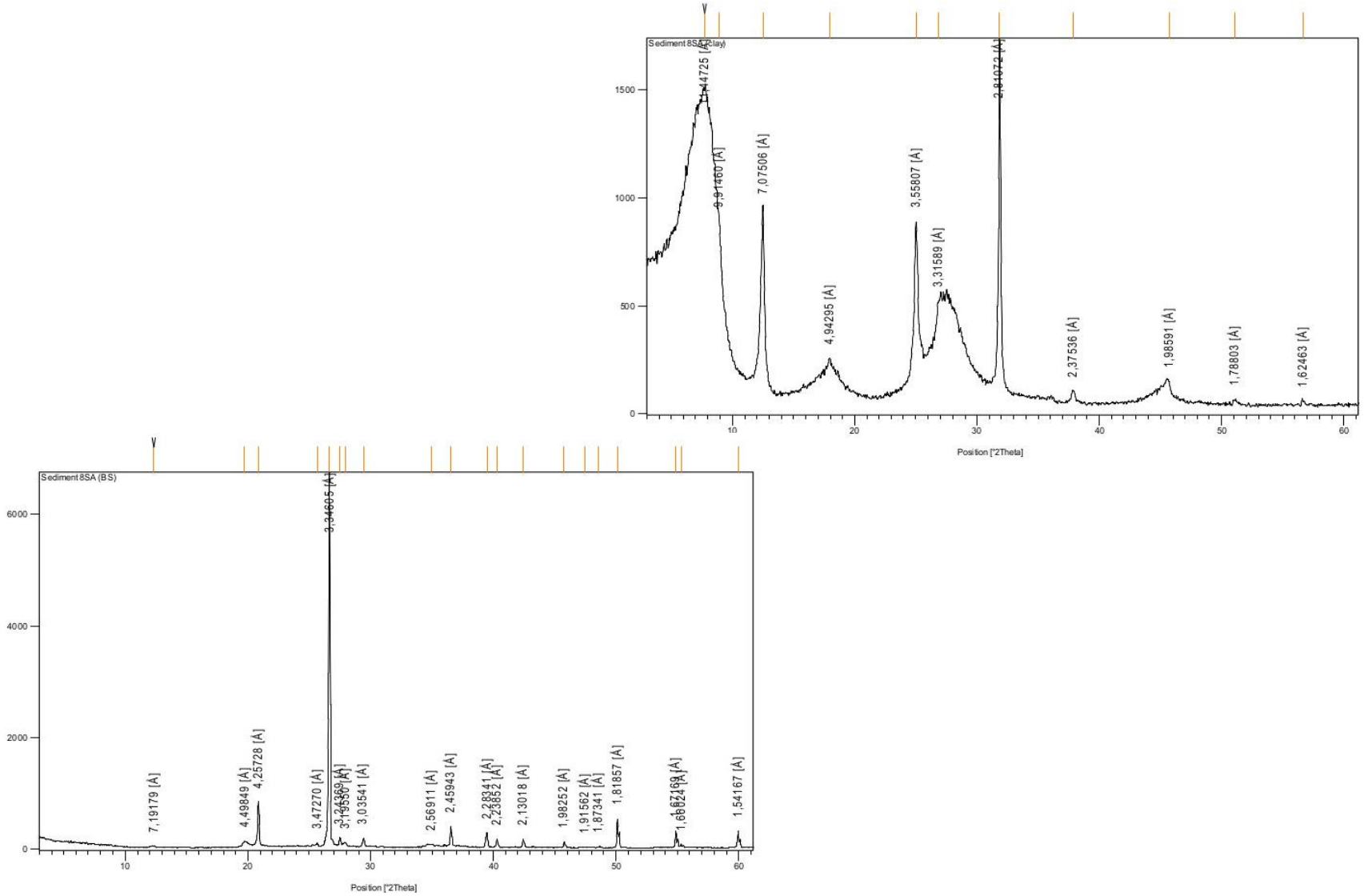


Figure 5 - X-ray diffractogram of bulk sample and clay fraction of soil 1 sample (35PC).

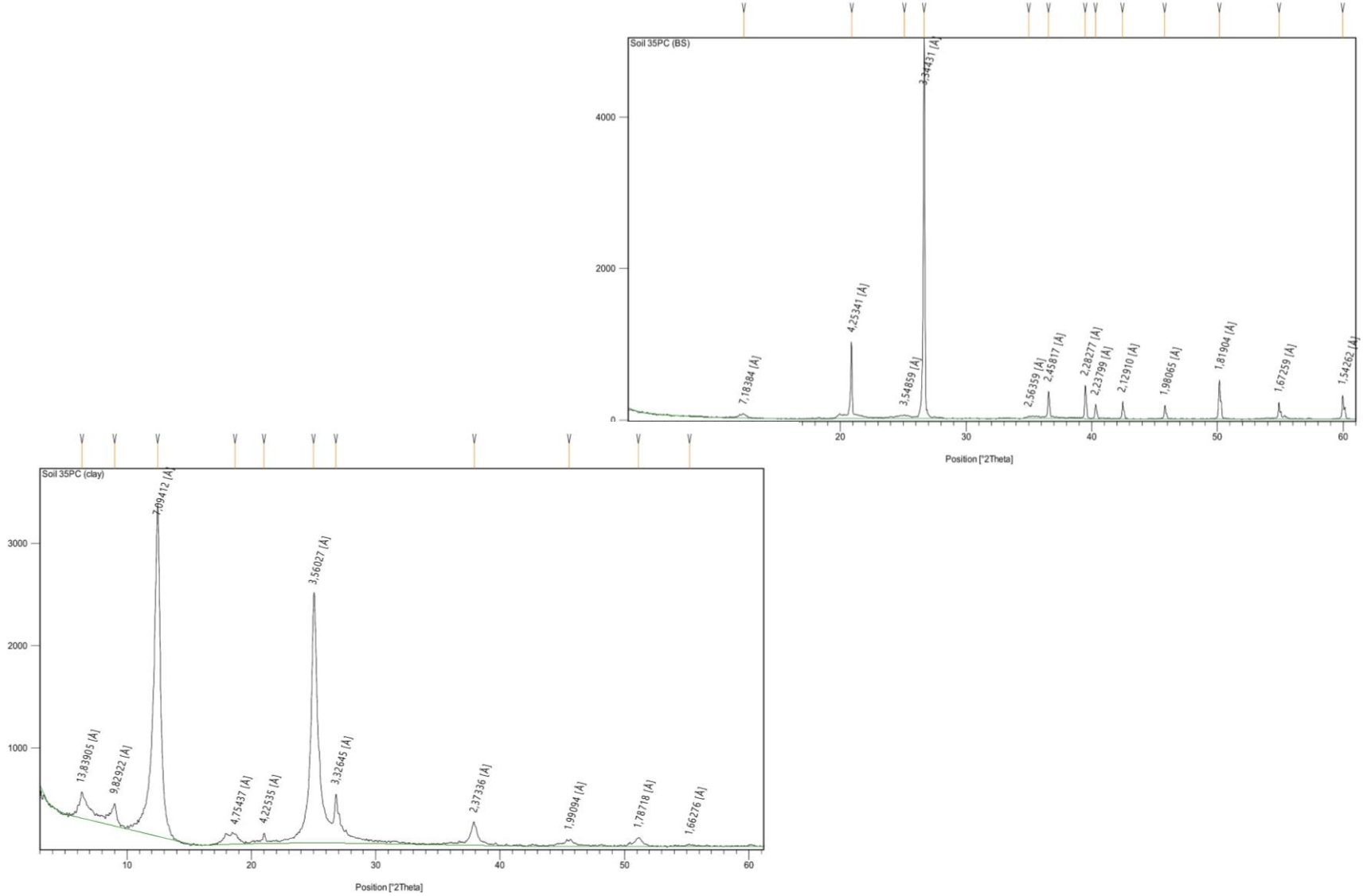


Figure 6 - X-ray diffractogram of bulk sample and clay fraction of soil 2 sample (47PC).

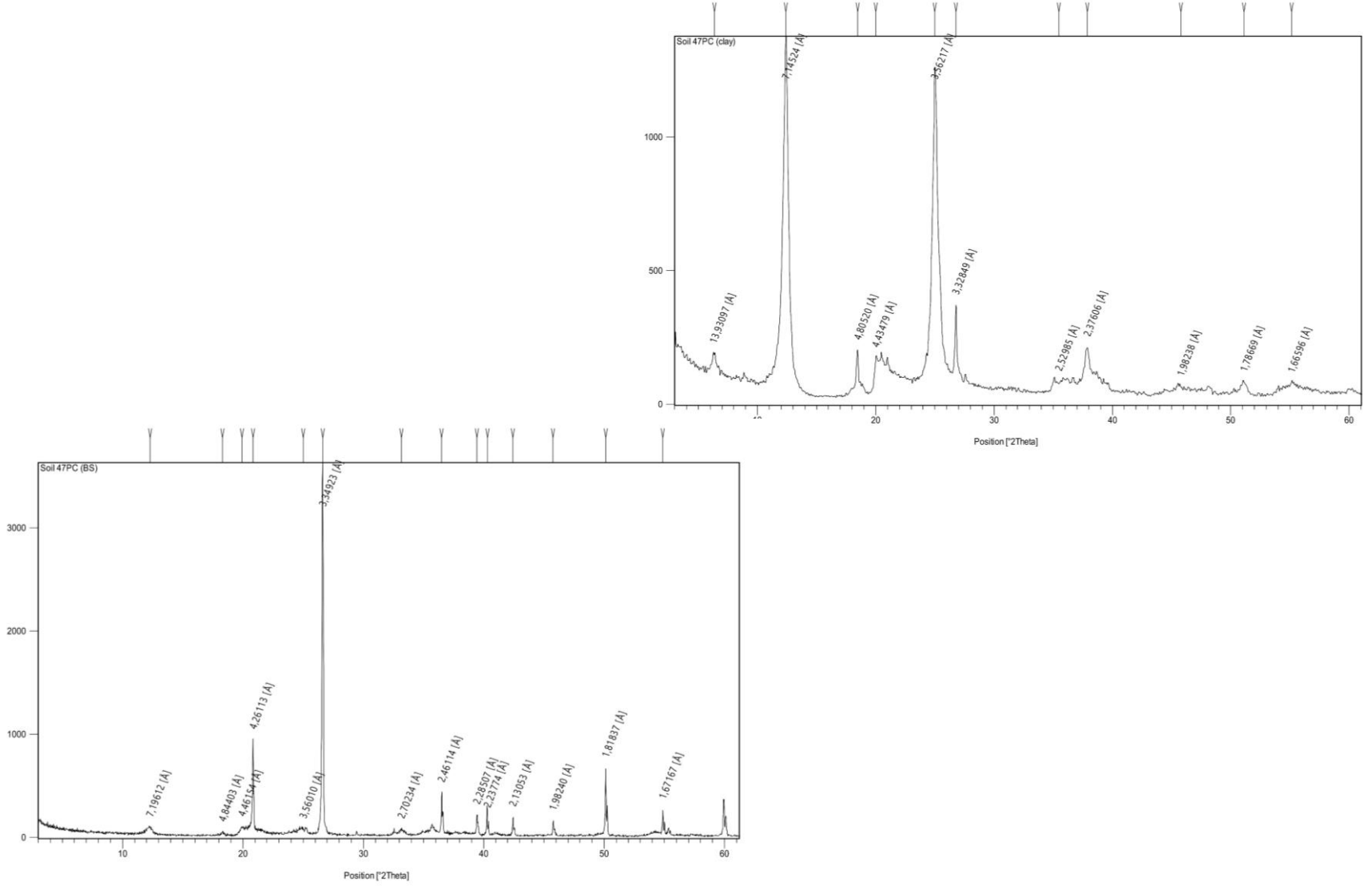


Figure 7 - X-ray diffractogram of bulk sample and clay fraction of soil 3 sample (58PC).

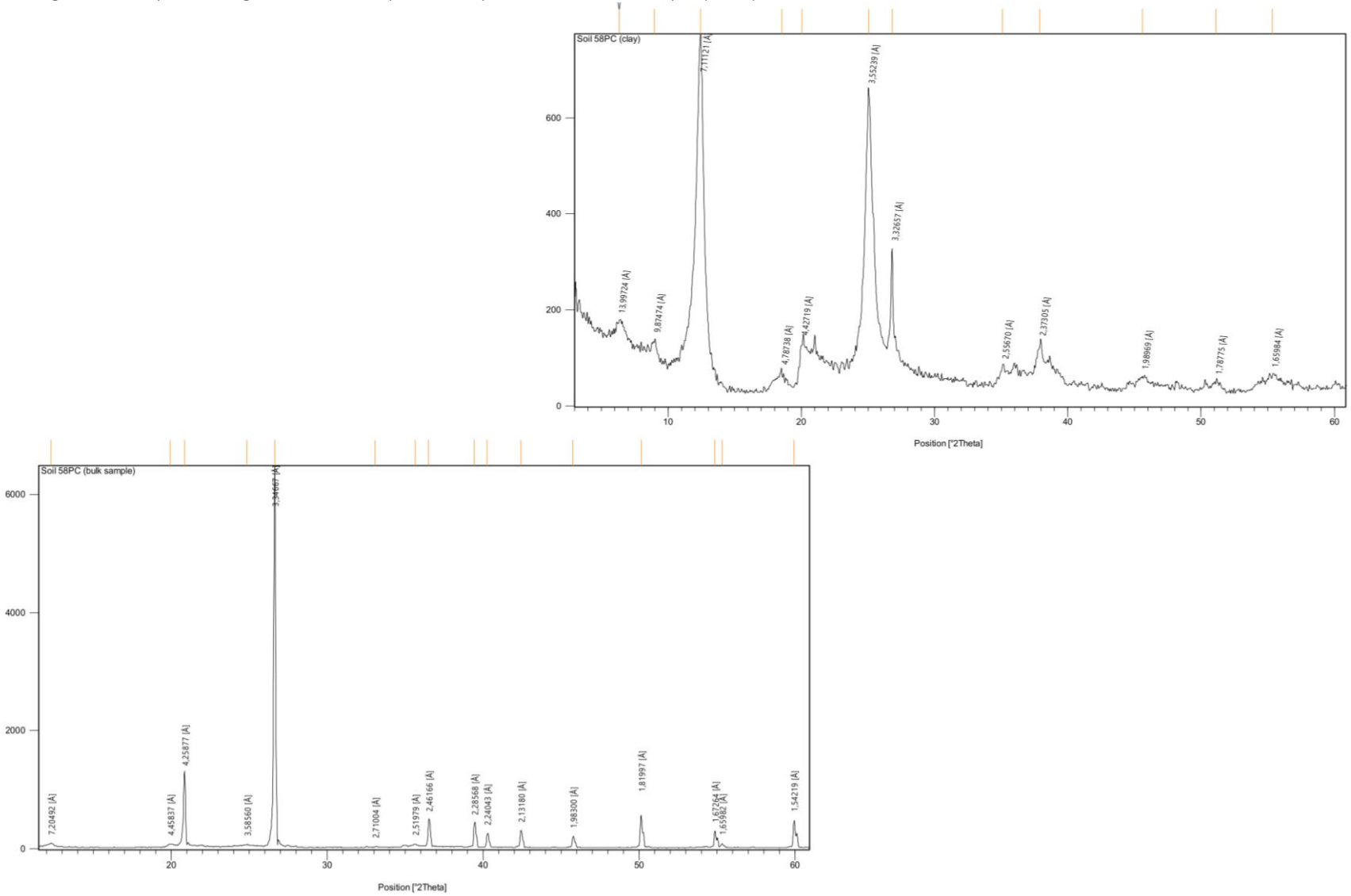


Figure 8 - X-ray diffractogram of bulk sample and clay fraction of soil 4 sample (3SA).

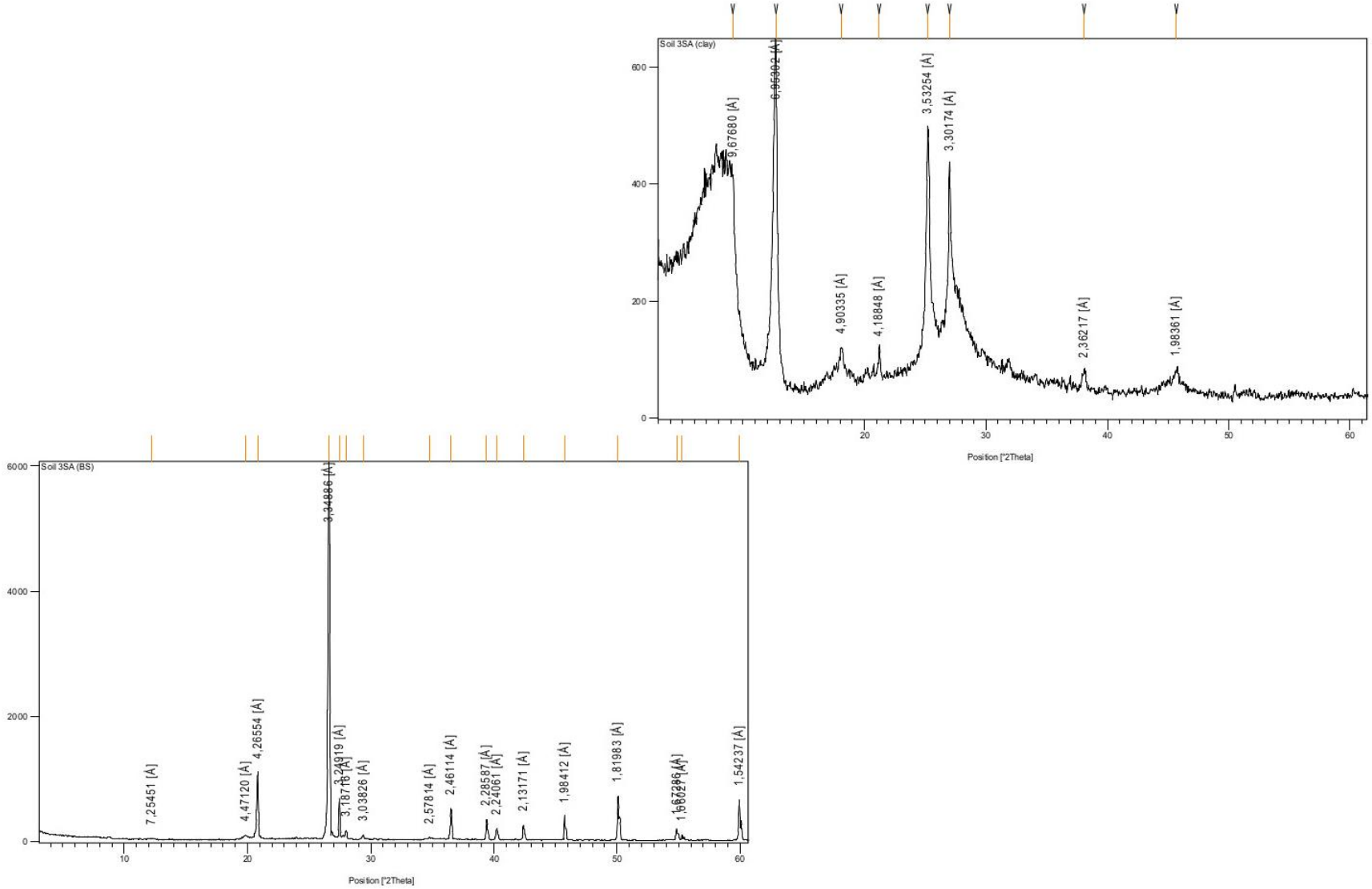


Figure 9 - X-ray diffractogram of bulk sample and clay fraction of soil 5 sample (5SA).

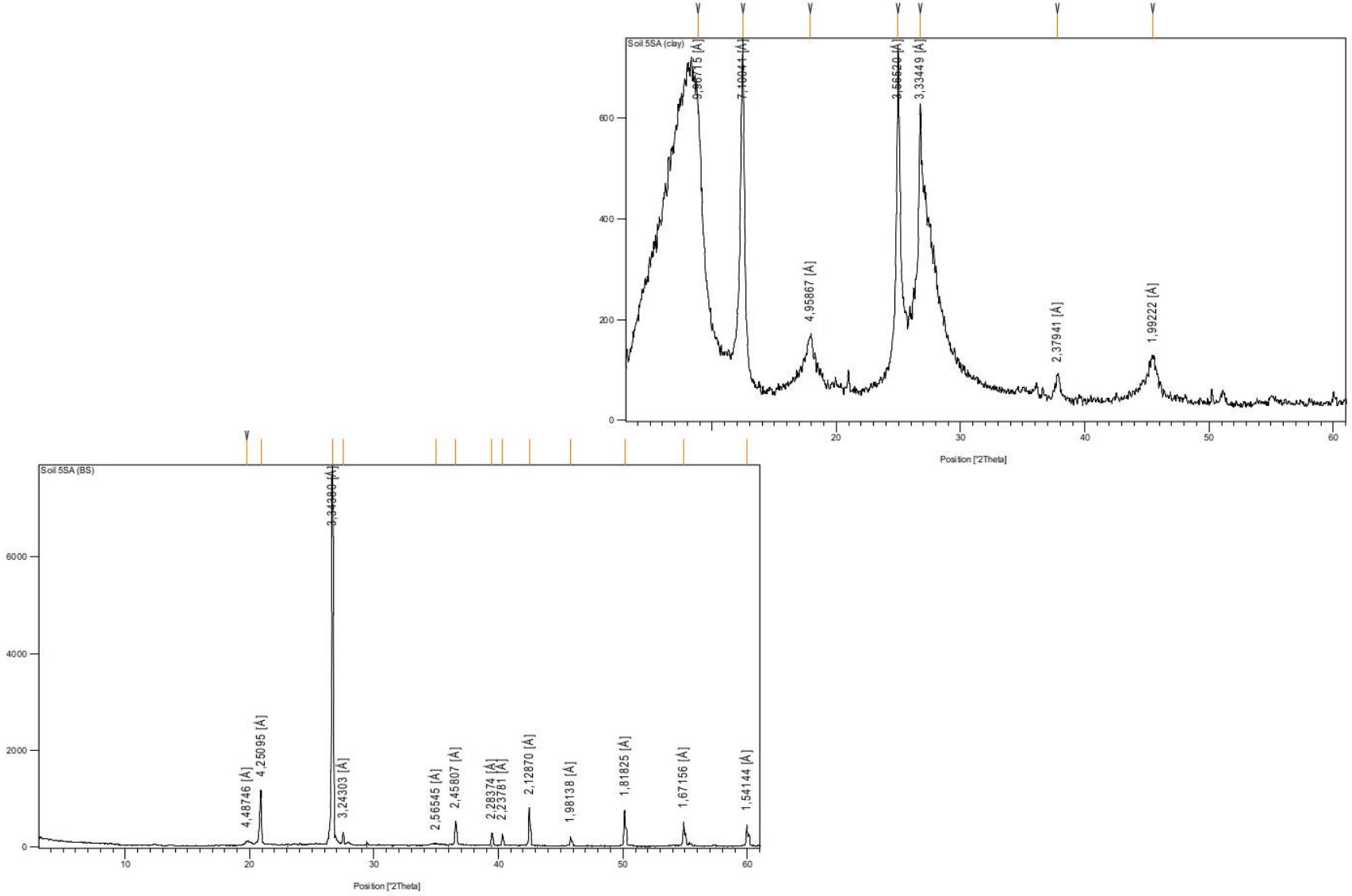


Figure 10 - X-ray diffractogram of bulk sample and clay fraction of soil 6 sample (6SA).

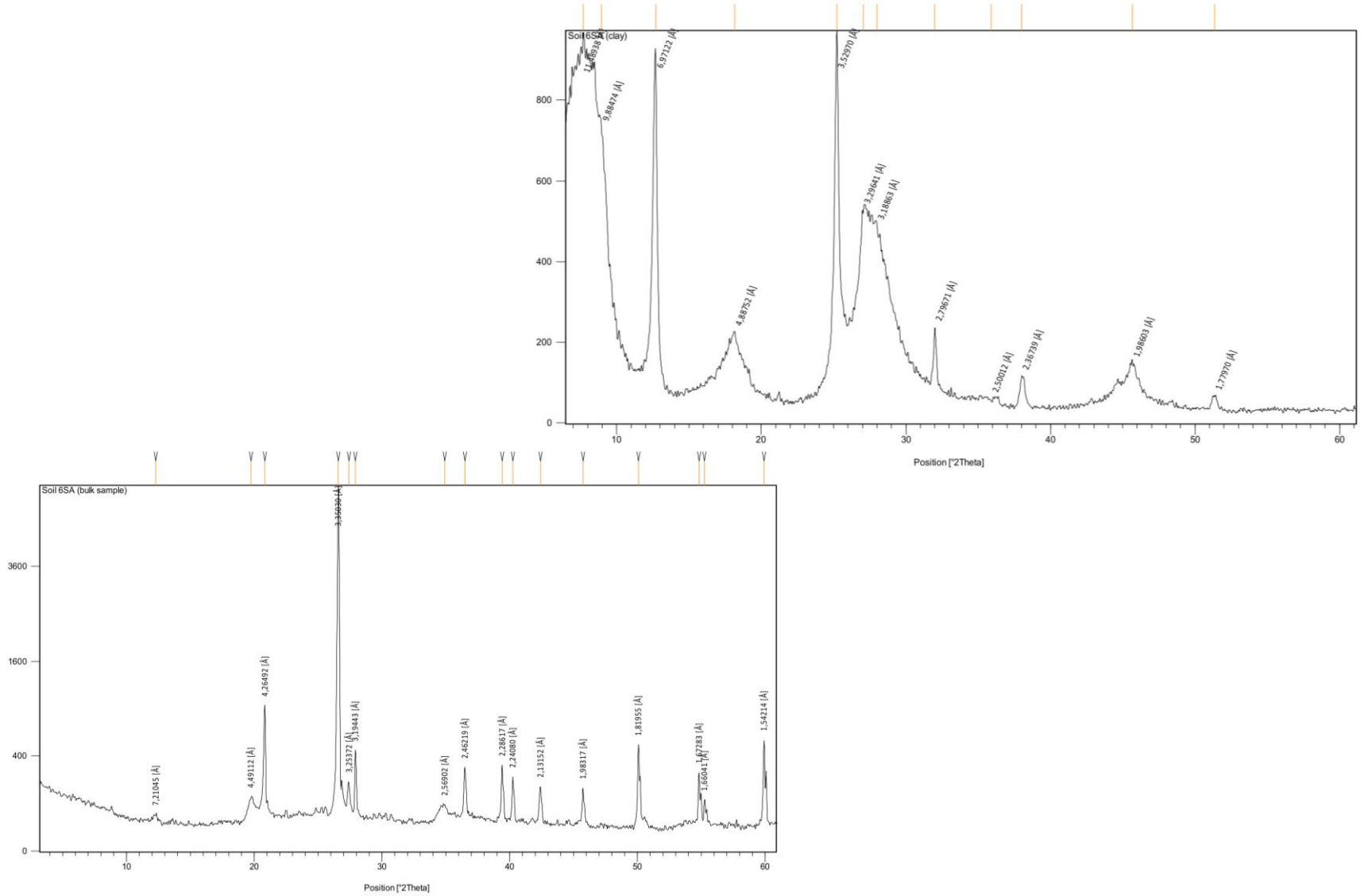


Figure 11 - X-ray diffractogram of bulk sample and clay fraction of soil 7 sample (9SA).

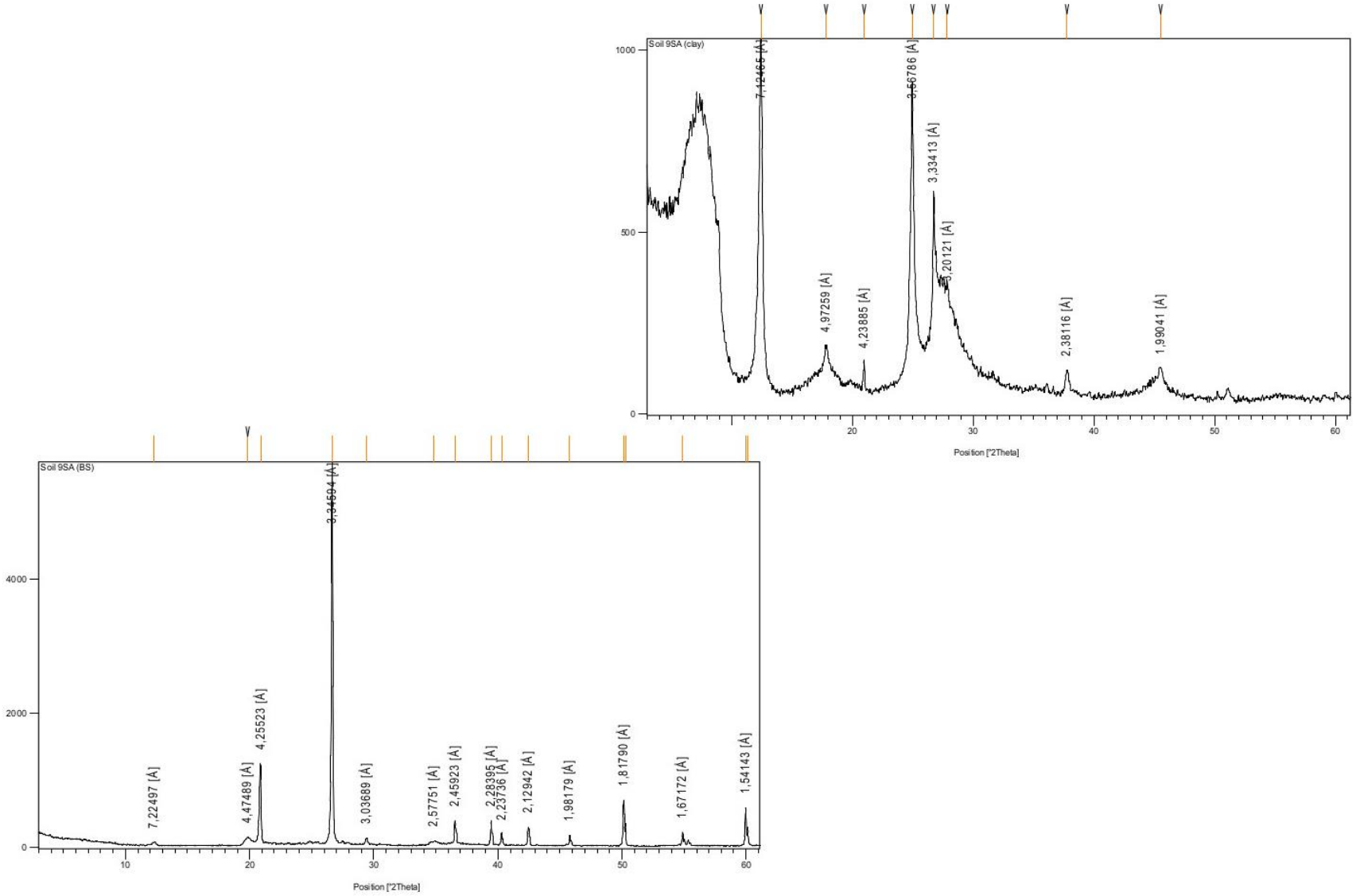


Figure 12 - X-ray diffractogram of bulk sample and clay fraction of soil 8 sample (5AP).

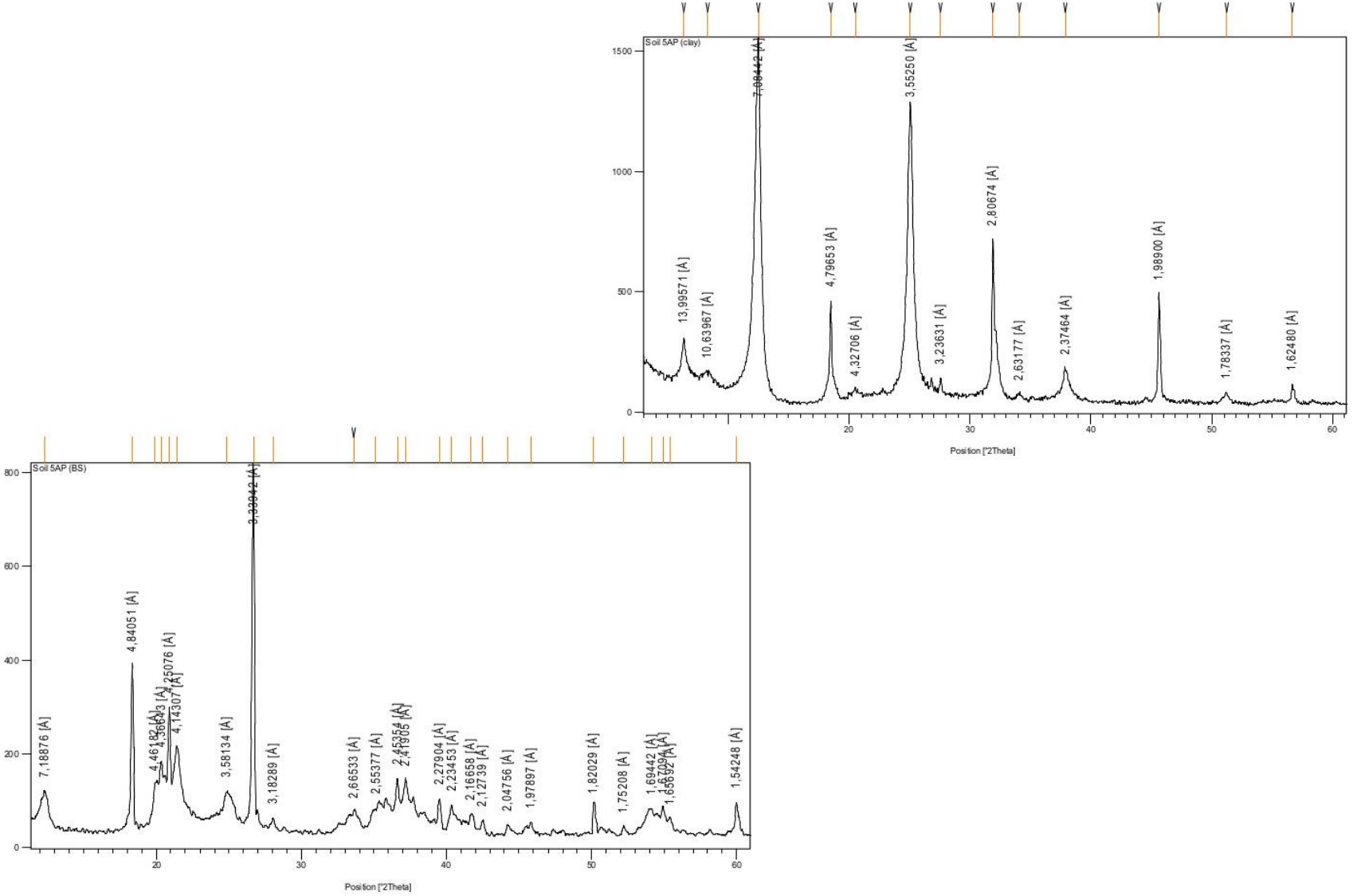


Figure 13 - X-ray diffractogram of bulk sample and clay fraction of tailing 1 sample (1AP).

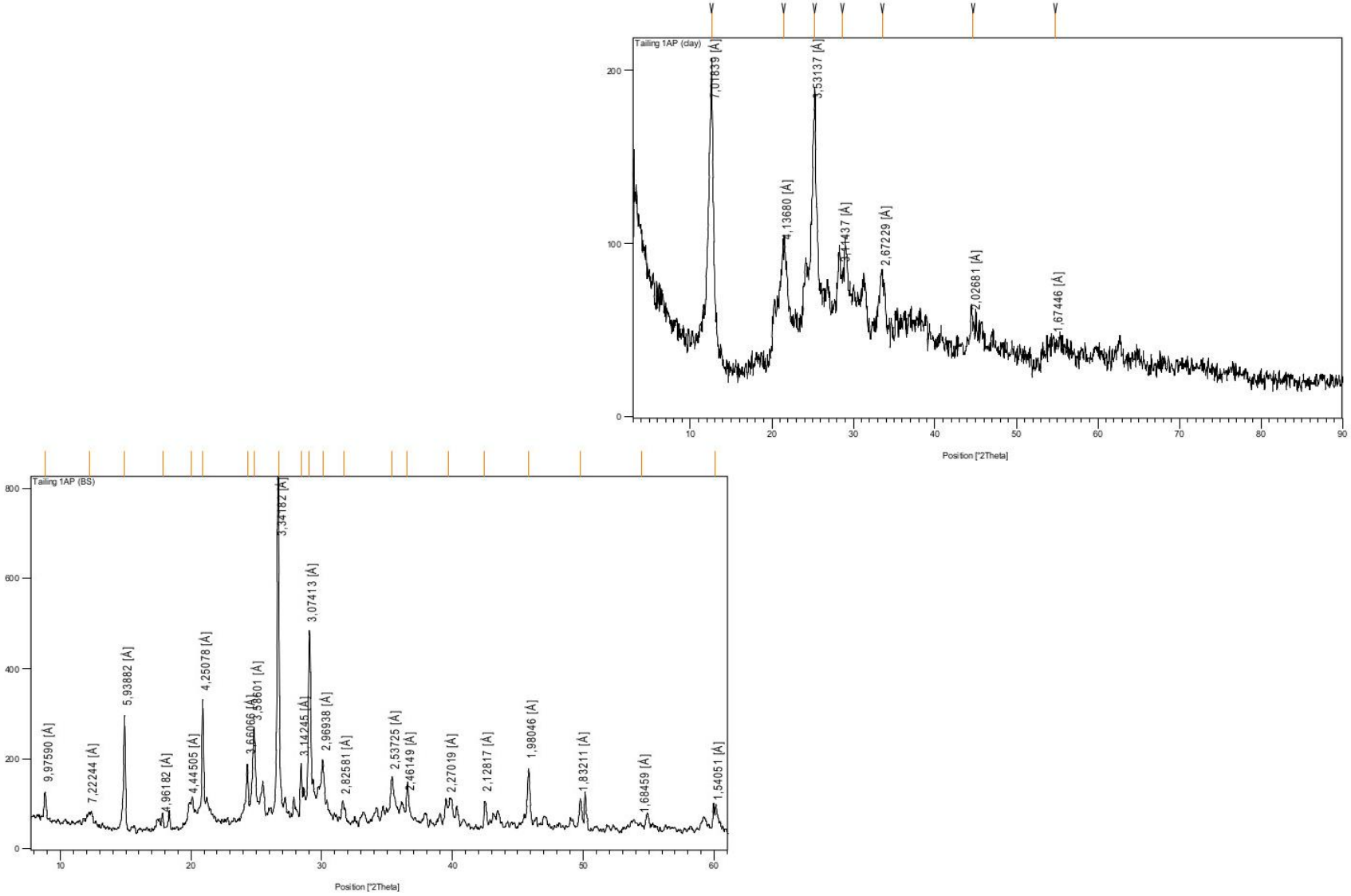


Figure 14 - X-ray diffractogram of bulk sample and clay fraction of tailing 2 sample (2AP).

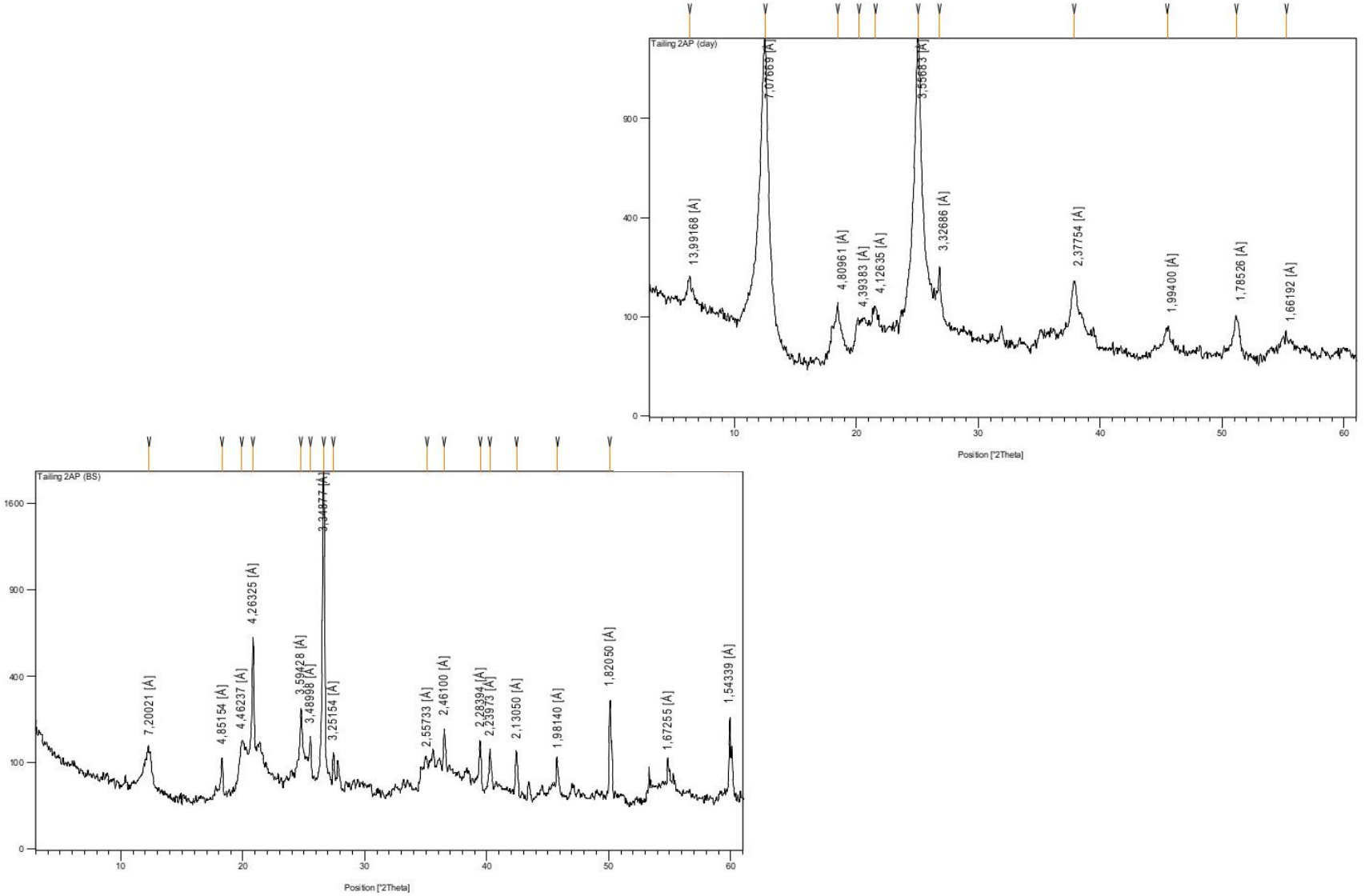


Figure 15 - X-ray diffractogram of bulk sample and clay fraction of tailing 3 sample (3AP).

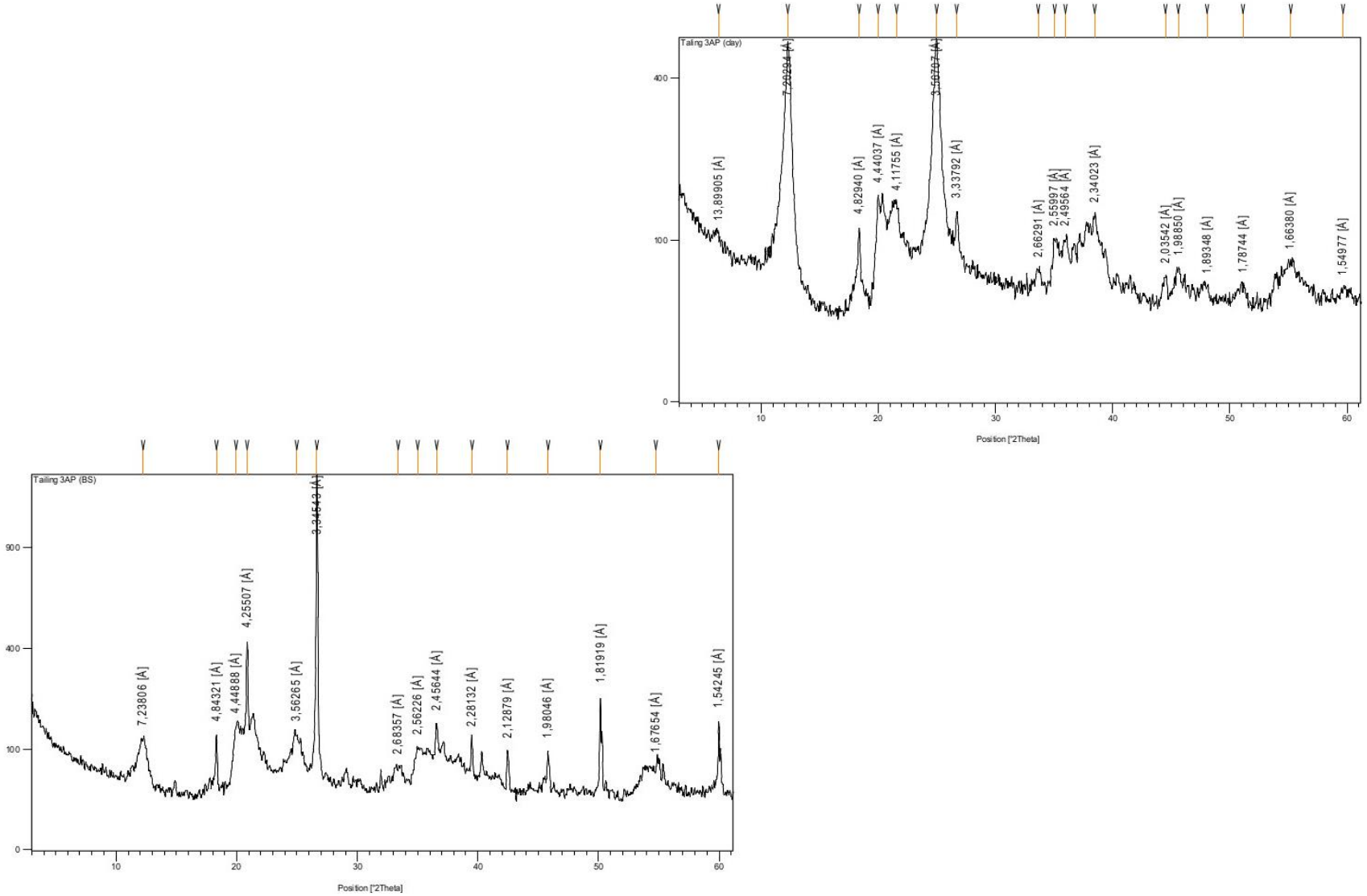


Figure 16 - X-ray diffractogram of bulk sample and clay fraction of tailing 4 sample (4AP).

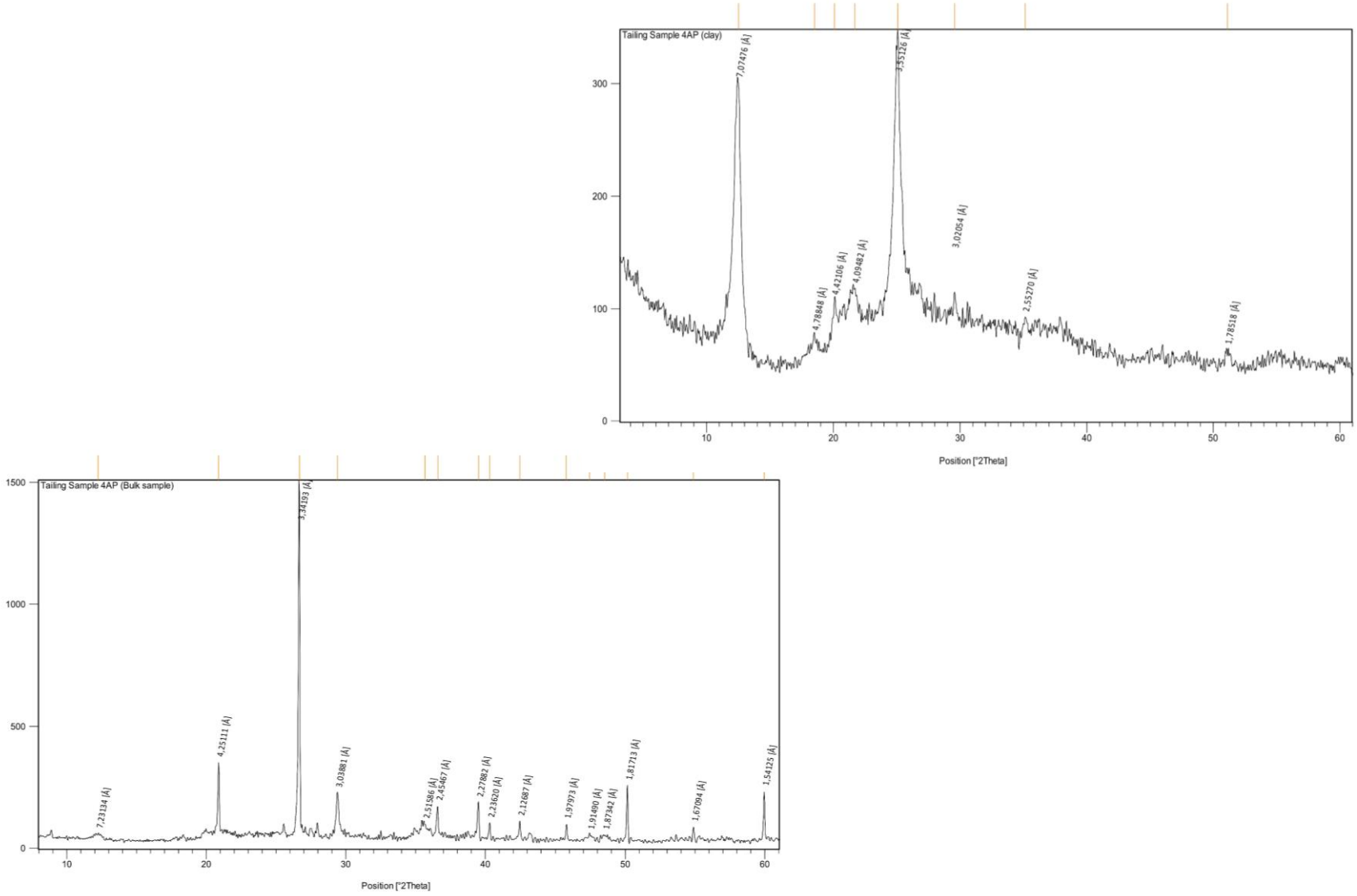
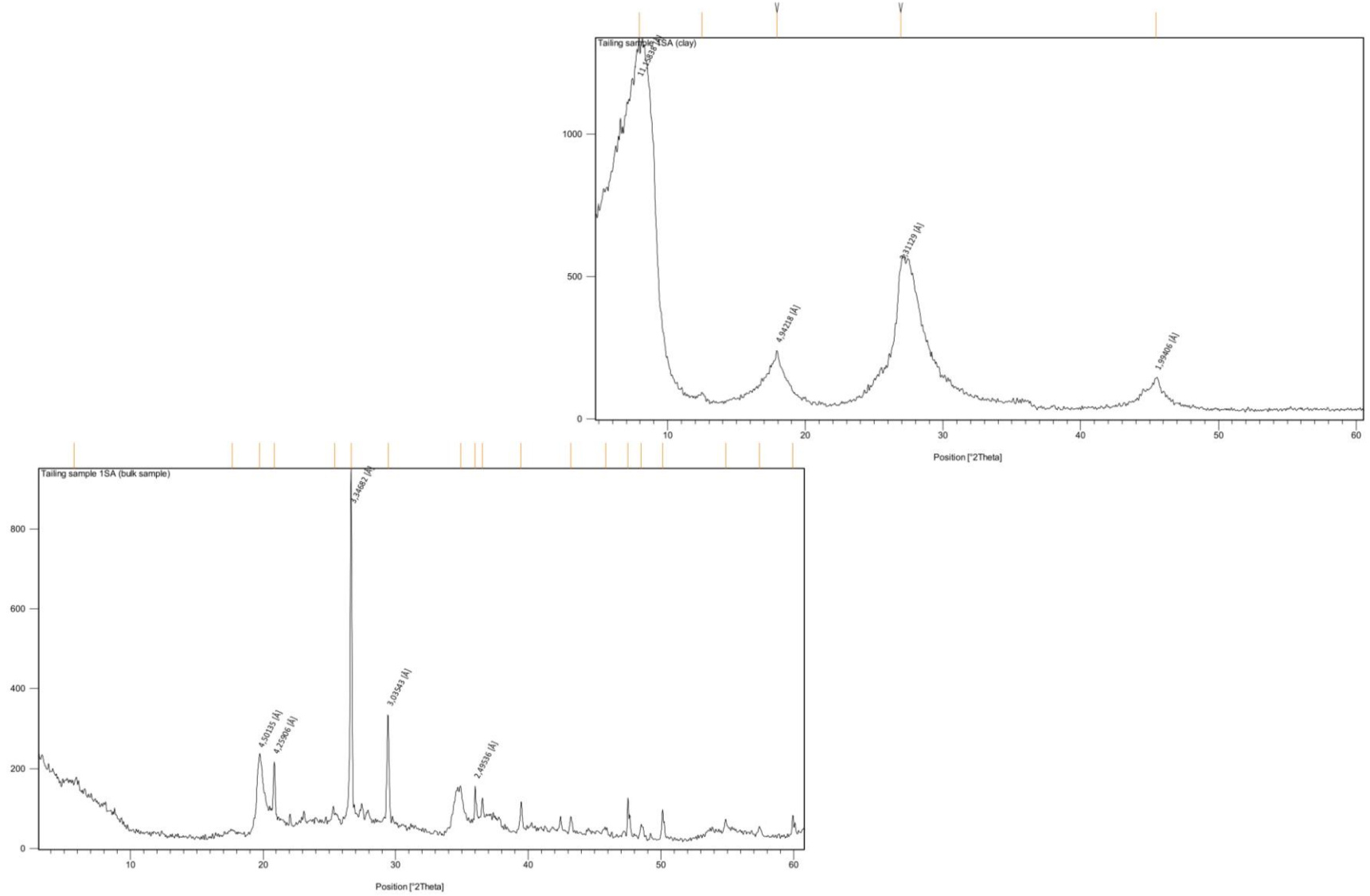


Figure 17 - X-ray diffractogram of bulk sample and clay fraction of tailing 4 sample (1SA).



APPENDIX E

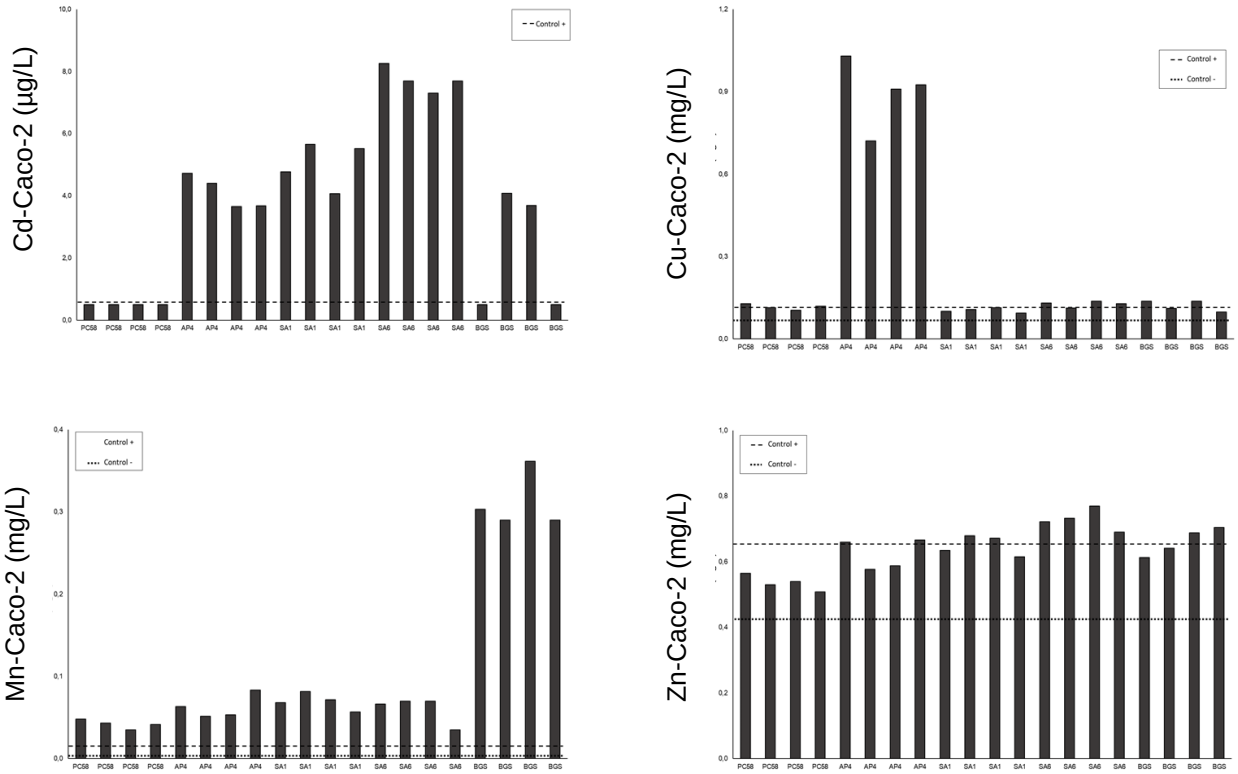


Figure 1 - Absorption of PHE Caco- 2 cells after 2 h. Values are presented by repetition (n = 4). Dashed line is the mean of positive control and dotted line is the mean of negative control.

ANNEXES

ANNEX A

Respiratory *in vitro* method: *Artificial Lysosomal Fluid* (ALF)

(Pelfrêne *et al.*, 2017).

Table 1 shows the composition of the artificial lysosomal fluid.

Table 1. Composition and concentration of reagents for preparation of Artificial Lysosomal Fluid (ALF)

Reagents	Lung fluid
	Concentration (g L^{-1})
NaCl	3.210
Na_2HPO_4	0.071
Trisodium citrate dihydrate	0.077
Glycine	0.059
NaOH	6.000
Citric acid	20.800
$CaCl_2.2H_2O$	0.128
Na_2SO_4	0.039
$MgCl_2$	0.050
Disodium Tartrate	0.090
Sodium Lactate	0.085
Sodium Pyruvate	0.172
pH	4.5 ± 0.1

Procedure

1. Weigh 0.05 g of sample sieved at 10 μm into centrifuge tube of 50 or 100mL.
2. Add 50 mL of the ALF solution.
3. Check the pH 4.5 ± 0.1. Adjust with 37% HCl or NaOH 1 mol L-1 if necessary.
4. Shake for 24 h at 37 ° in an end-over-end rotator at 50 rpm.
5. Centrifuge the samples at 4500 g for 15 min
6. Collect 1mL of the supernatant and transfer for Falcon® tubes and dilute with 9mL of 2% HNO_3. Store at 4 °C for further analysis.

Reference

Pelfrêne, A. Cave, M. Wragg, J. & Douay, F. 2017. In Vitro Investigations of Human Bioaccessibility from Reference Materials Using Simulated Lung Fluids. *International Journal of Environmental Research and Public Health*. **14(2)**. 112. doi: 10.3390/ijerph14020112

ANNEX B

Gastric and gastrointestinal i*n vitro* method. Unified BARG Method (UBM)

(BARGE - INERIS. 2011; Denys *et al.*. 2012; Hamilton *et al.*. 2015)

The method is divided into three stages of simulation of the gastrointestinal system: (i) saliva; (ii) stomach and (iii) intestinal compartments. The four simulated fluids (saliva. stomach. duodenum and bile) should be prepared individually one day prior to the extraction to ensure that all reagents are completely dissolved.

The inorganic (I) and organic (O) components of the solutions should be prepared separately in a 250 mL flask for a total amount of 500 mL of each fluid. as shown in Table 1 of Annex A. Dissolve Reagents I and O well and then transfer both solutions to the same vial and add the enzymes. Shake each solution at least 3 hours.

Table 1. Composition and quantity of reagents for preparation of saliva. gastric. duodenal and bile solutions

Solutions	Reagents		Saliva (S)	Gastric (G)	Duodenum (D)	Bile (B)
		Volume (mL)	Weight (g)			
Inorganic (I)	KCl	250	0.448	0.412	0.282	0.188
	NaH_2PO_4		0.444	0.133	-	-
	KSCN		0.100	-	-	-
	Na_2SO_4		0.285	-	-	-
	NaCl		0.149	1.376	3.506	2.63
	$CaCl_2$			0.2	-	-
	NH_4Cl			0.153	-	-
	$NaHCO_3$				2.804	2.893
	KH_2PO_4				0.040	
	$MgCl_2$				0.025	
	NaOH (1 M)		0.9		-	
	HCl (37 %)		-	4.15 mL	90 µL	90 µL
Organic (O)	Urea	250	0.1	0.0425	0.050	0.125
	Glucose		-	0.325	-	-
	Glucuronic Acid		-	0.010	-	-
	Glucosamine Hydrochloride		-	0.165	-	-
Enzymes	Alpha Amylase	500	0.0725	-	-	-
	Mucin		0.0250	1.5	-	-
	Uric acid		0.0075	-	-	-
	Bovine Albumin		-	0.500	0.500	0.900
	Pepsin		-	0.500	-	-
	$CaCl_2$		-	-	0.100	0.111
	Pancreatin		-	-	1.500	-
	Lipase		-	-	0.250	-
	Bile		-	-	-	3.000

Preparation of solutions

1. Inorganic Solutions: Dissolve all inorganic reagents in 200 mL of ultrapure water and make up to 250 mL in a volumetric flask.
2. Organic solutions: Dissolve all orangish reagents in 200 mL of ultrapure water and make up to 250 mL in a volumetric flask.
3. Final Solution: Mix the inorganic and organic solutions in a 500 ml flask and add the enzymes. Mix thoroughly and check the pH of each fluid: 6.5 ± 0.5 (saliva); 1.1 ± 0.1 (Gastric); 7.4 ± 0.2 (Duodenum); 8.0 ± 0.2 (Bile). If necessary. adjust pH to the correct tolerance with NaOH 1M and/or HCl 37%.

Procedure

The next day. check the final pH of the Saliva + Gastric (Mixture A) and Saliva + Gastric + Duodenal + Bile (Mixture B). The solutions should be heated at 37ºC. If the pH is not in indicated range adjust the pH of the fluids with 37% HCl or 1 M NaOH.

Mixture A: 2 mL Saliva + 3 mL Gastric fluid - pH = 1.2 - 1.4

Mixture B: 1 mL Saliva + 1.5 mL Gastric fluid + 3 mL Duodenal fluid + 1 mL Bile - pH = 6.5 ± 0.5

- weigh 0.6 g of soil in centrifuge tubes of 80 or 100 mL

<u>Gastric phase extraction</u>

1. add 9 mL of Saliva.
2. Shake for 5 min at 37 ° C. 50 rpm in the end-over-end (360 °) rotator.
3. add 13.5 mL of Gastric Juice.
4. Shake by hand and check the pH 1.2 ± 0.05. Adjust with 37% HCl or NaOH 1 mol L-1 if necessary.
5. Shake for 1 h at 37 ° C. 50 rpm in an end-over-end (360 °) rotator.
6. Check the final pH <1.5. if it is not within the tolerable range. the sample should be discarded and repeat the procedure.
7. Centrifuge the samples at 4500 × *g* for 15 min.

8. Collect 1mL of the supernatant and transfer to Falcon® tubes and dilute in 9mL of 2% of HNO_3. Store at 4 °C for further analysis.

To simulate the intestinal phase. the above procedure must be repeated until the step 6.

1. Add 27 mL of duodenal fluid and 9 mL of Bile.
2. Check the pH 6.3 ± 0.5. Adjust with 37% HCl or NaOH 1 mol L-1 if necessary.
3. Shake for 4 h at 37 ° C. 50 rpm in an end-over-end rotator (360 °).
4. Record the final pH value.
5. Centrifuge the samples at 4500 × *g* for 15 min
6. Collect 1mL of the supernatant and transfer to Falcon® tubes and dilute in 9mL of 2% of HNO_3. Store at 4 °C for further analysis.

References

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ANNEX C

Extraction Sequence by BCR-modified method

(Rauret *et al.* 1999)

Reagents:

Solution A (0.11 M Acetic Acid): Add 6.3 mL of Acetic Acid to a beaker containing 900 mL of ultrapure water. Complete volume for 1L in volumetric flask.

Solution B (0.5 M Hydroxylamine hydrochloride): Dissolve 34.75 g of hydroxylamine hydrochloride in 400 ml of ultrapure water. Transfer to a 1 L volumetric flask. Add 25 mL of 2 M HNO_3 solution. Complete the volume. Prepare the solution on the day of use

C (8 M Hydrogen peroxide. 300 mg g^{-1}): Use hydrogen peroxide supplied by the manufacturer. With pH 2-3. stabilized with HNO_3.

Solution D (1 M Ammonium acetate): Dissolve 77.08 g of ammonium acetate in 900 ml of ultrapure water. Adjust the pH to 2 ± 0.1 with concentrated HNO_3. Complete the volume in 1L volumetric flask.

Procedure

Fraction 1

1. Weigh 1g of soil in a 100 mL centrifuge tube with round bottom
2. Add 40 mL of solution A.
3. Shake at 30 rpm on end-over-end rotator for 16 h at room temperature.
4. Centrifuge at 3000 g for 20 min
5. Filter and store the supernatant under refrigeration <4 ° C
6. Wash residual soil with 20 mL of ultrapure water
7. Shake at 30 rpm on end-over-end rotator for 15 min at room temperature
8. Centrifuge at 3000 g for 20 min and discard the supernatant

Fraction 2

1. Add 40 mL of solution B in the centrifuge tubes to the residual soil of the 1st fraction
2. Repeat steps 3 to 8 of Fraction 1

Fraction 3

1. Add 10 mL of solution C in the centrifuge tubes to the residual soil of the 2nd fraction (A careful attention to the probable violent reaction)
2. Cover the tubes and let them react for 1 h at room temperature. Shake occasionally.
3. Continue digestion for 1h at 85ºC in a water bath. and then let the volume reduce to ± 3 mL
4. Add 10 mL of solution C and continue digestion for another 1 hour.
5. Reduce the volume of solution C. but do not allow it to dry completely.
6. Add 50 mL of solution D
7. Repeat steps 3 to 8 of Fraction 1
8. Remove the residual sample from the centrifuge tube and let dry in an oven at 60ºC

Residual fraction: (3051A. USEPA. 2007)

1. Weigh 0.5g of dry residual sample in a Teflon microwave tube
2. Add 3 mL HCl and 9 mL HNO_3.
3. Take to the microwave (closed system) and heat to 175ºC.
4. Filter the supernatant on blue filter paper in 50 mL volumetric flasks and make up to volume with ultrapure water.
5. Transfer to Falcon® tubes and store for further analysis.

Alternatively. residual fraction can be obtained from the difference between the pseudo-total PHE extract by 3051A and the sum of the three fractions above.

References

Rauret, G. López-Sánchez, J.F. Sahuquillo, A., Rubio, R. Davidson, C. Ure, A. & Quevauviller, P. 1999. Improvement of the BCR three step sequential extraction procedure prior to the certification of new sediment and soil reference materials. *Journal of environmental monitoring: JEM*. **1**. 57–61.

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