# A simultaneous assessment of organic matter and trace elements bio-accessibility in substrate and digestate from an anaerobic digestion plant

## Authors and affiliation

Andreina Laera[a,d,*], Sepehr Shakeri Yekta[b], Mattias Hedenström[c], Rémy Buzier[d], Gilles Guibaud[d], Mårten Dario[b], Giovanni Esposito[e], Eric D. van Hullebusch[f]

[a] University of Paris-Est, Laboratoire Géomatériaux et Environnement (EA 4508), UPEM, 77454 Marne-la-Vallée, France, andreina.laera@u-pem.fr or piedelibero89@gmail.com

[b] Department of Thematic Studies-Environmental Change and Biogas Research Center, Linköping University, 581 83 Linköping, Sweden

[c] Department of Chemistry, Umeå University, 901 87 Umeå, Sweden

[d] University of Limoges, PEIRENE, Equipe Développement d'indicateurs ou prévision de la qualité des eaux, URA IRSTEA, 123 Avenue Albert Thomas, 87060 Limoges Cedex, France

[e] University of Napoli "Federico II", Department of Civil, Architectural and Environmental Engineering, via Claudio 21, 80125 Napoli, Italy

[f] Université de Paris, Institut de Physique du Globe de Paris, CNRS, UMR 7154, F-75238 Paris, France

*Corresponding author: andreina.laera@u-pem.fr

## Abstract

This study evaluates a simultaneous assessment of organic matter (OM) and trace elements (TE) bio-accessibility in substrate and digestate from a full-scale anaerobic digester by a sequential OM extraction method. Simultaneous release of TE was determined along with the extraction of different OM fractions and the effects of extracting reagents on characteristics of OM were evaluated by nuclear magnetic resonance (NMR) spectroscopy. The reagents used for sequential extraction of OM were not enough selective. However, proteins were particularly removed by 0.1 M NaOH, while 72% $H_2SO_4$ mainly extracted hemicellulose and cellulose. The OM fractionation allowed for simultaneous extraction of >60% of total As, Cd, Co, Fe, Mn, Ni and Zn, while the extraction efficiency was limited for Al, Cr, Cu, Mo, and Pb. In substrate, >50% of total As, Co, Mn and Ni and <40% of total Fe, Zn and Mo were identified in bio-accessible fractions. In digestate, all elements demonstrated poor bio-accessibility except for As.

## Keywords

Sequential chemical extraction

Organic matter fractionation

Trace elements fractionation

NMR spectroscopy

Anaerobic co-digestion

# 1. Introduction

Availability of trace elements (TE) as micronutrients is essential for stable and efficient performance of anaerobic digestion processes (Feng et al., 2010; Karlsson et al., 2012; Lindorfer et al., 2012). Due to a complex network of reactions, controlling the chemical speciation of TE, a small fraction of TE in the digesters environment is often available for microbial activities, while the majority of TE occur as precipitates, adsorbed or complexed species (Aquino and Stuckey, 2007; Maharaj et al., 2019, 2018). Several studies attempted to determine the fraction of TE available for uptake by microorganisms with sequential extraction procedures, such as the modified Tessier method (van Hullebusch et al., 2005) and the Community Bureau of Reference (BCR) method (Rauret et al., 1991) to guarantee an adequate supply of TE in digesters (Braga et al., 2017; Cao et al., 2018; Ortner et al., 2014). The procedure involves sequential treatment of the samples by chemical reagents such as neutral salts, weak and strong acids or reducing and oxidizing agents to selectively extract TE. Despite the limitations associated to the sequential extraction procedures including the poor selectivity of chemical reagents (Bacon and Davidson, 2008; Filgueiras et al., 2002), the lack of uniformity in the procedure and the complexity of chemical composition of anaerobic samples (Thanh et al., 2016), there is a growing interest in using sequential extraction procedures to fractionate TE (Ortner et al., 2014; Zhu et al., 2014). Indeed, results obtained from sequential extraction methods are valuable for anaerobic digestion processes and for environmental risk assessments of digestate utilization as a soil fertilizer (Ortner et al., 2014; Zhu et al., 2014), as it allows to quantitatively determine the TE fractions with different degree of solubility and reactivity. Moreover, sequential extraction methods are useful tools to assess mobility, accessibility, and potential bio-availability of TE (Harmsen, 2007). Braga et al. (2017) observed that micronutrient TE, including Se, Zn, Ni and Fe were

mainly found in the organic matter (OM)/sulfide fraction of the modified Tessier method applied on sewage sludge samples. Similar results were also found by Zufiaurre et al. (1998) in sewage sludge samples. Such fraction is considered less mobile than other TE fractions extracted by the sequential extraction procedure and less bio-accessible for microbial uptake (Filgueiras et al., 2002).

Different chemical interactions between TE and organic/inorganic compounds originating from the substrate and generated during the anaerobic digestion process will determine the chemical speciation of TE and consequently their bio-accessibility and bioavailability for anaerobic microorganisms in digesters and for plants and soil microorganisms when digestate is spread on lands (Fermoso et al., 2015; Thanh et al., 2016). Inorganic compounds such as sulfide ($S^{2-}$), phosphate ($PO_4^{3-}$) and carbonate ($CO_3^{2-}$) may precipitate TE in anaerobic digestion systems as simulated by Maharaj et al. (2018) with a dynamic mathematical model based on anaerobic digestion model no.1 (ADM1). Such inorganic compounds would compromise the availability of TE for microbial uptake in anaerobic digesters (Callander and Barford, 1983). In addition, TE could be complexed with organic chelators becoming either more or less available for microbial uptake depending on the binding strength of metal-organic complexes. Organic compounds contain functional groups such as carboxyl, hydroxyl or amino groups with high affinity to complex with TE (Callander and Barford, 1983). Gonzalez-Gil et al. (2003) observed that amino acids in yeast extracts form soluble complexes with Ni and Co which prevent their precipitation with sulfide and consequently increased their availability for microorganisms. Moreover, the degradation process of bio-accessible OM deriving from substrate or digestate, can release TE in solution that becomes potential bio-accessible for up-take by anaerobic digester microorganisms or soil-dwelling organisms (Knoop et al., 2018). Accordingly, association of TE with OM play an important role in bioavailability of TE.

Jimenez et al. (2017, 2014) assessed the bio-degradability and the bio-accessibility of OM in organic wastes using a physical-chemical sequential extraction procedure for a large number of samples, including municipal sludge samples, municipal solid wastes, digestate and compost. The sequential extraction procedure includes the following fractions: i) Extractable Soluble from Particulate Organic Matter (SPOM), which contains water-soluble proteins and sugars; ii) Readily Extractable Organic Matter (REOM), representing easily accessible proteins and lipids; iii) Slowly Extractable Organic Matter (SEOM), containing humic-like and fulvic acid-like structures as well as complex proteins and certain lignocellulosic compounds and iv) Poorly Extractable Organic Matter (PEOM), targeting hemicellulose and cellulose (Jimenez et al., 2017). The development of OM fractionation methods allows assessing the accessibility of OM as carbon and energy sources for microorganisms in anaerobic digesters. However, to the best of our knowledge, no research work assessed simultaneous OM and TE extraction for assessment of the bio-accessibility of a combined source of carbon, energy and micronutrient TE.

Thus, we aim to evaluate the application of a sequential extraction method adapted for OM fractionation by Jimenez et al. (2017, 2014) to determine simultaneously the accessibility of TE in substrate and digestate samples. For this purpose, substrate and digestate samples from a full-scale anaerobic digester, regularly supplied with TE supplements, were used to determine the TE concentrations in different OM fractions. The TE investigated in this study are Co, Fe, Ni, Mn, Mo, and Zn, which are important micronutrients for microbial activities (Gustavsson et al., 2013, 2011) and Al, As, Cd, Cr, Cu and Pb, which could be harmful to soil microorganisms and plant growth once digestate is applied as soil amendment (Bajgiran, 2013; Kupper et al., 2014; Nkoa, 2014). Moreover, changes in structural characteristics of OM after each step of the extraction procedure were studied by nuclear magnetic resonance (NMR) spectroscopy in order to assess

different organic groups in the samples, which were removed by extracting reagents during the sequential extraction procedure.

## 2. Material and methods

### 2.1. Samples

Substrate and digestate were collected from a full-scale anaerobic co-digestion plant located in Linköping, Sweden. The anaerobic digestion plant has a capacity of 125 000 tons of waste per year and treats the organic fraction in household waste (50%), slaughterhouse waste (25%) and industrial waste (25%) at 42°C with an organic loading rate of 4,5 kgVS/L·d and a hydraulic retention time of 37 days. The substrate was collected from a tank after 1-hour pasteurization at 70°C and TE addition, whereas the digestate was collected from the main anaerobic digester sampling port. About 1 liter of each sample was collected in acid washed polypropylene (PP) bottles. The bottles were flushed with nitrogen ($N_2$) prior to sampling and closed with a lid after collection to reduce sample exposure to air during sampling and transportation from the plant to the laboratory. Once in the laboratory, the samples were immediately treated in accordance to the sequential extraction procedure.

### 2.2. Sequential extractions procedure

The sequential extractions of dissolved organic matter (DOM), REOM and SEOM were carried out as described by Jimenez et al. (2014), while SPOM and PEOM fractions were extracted according to Jimenez et al. (2017). The latter modified protocol includes calcium chloride ($CaCl_2$) reagent for SPOM extraction and sulfuric acid ($H_2SO_4$) for PEOM extraction compared to the procedure proposed by Jimenez et al. (2014). Moreover, we slightly modified the protocols to adapt the method for simultaneous extraction of OM and TE (Table 1). The main modifications involve the use of raw sample, rather than freeze dried sample, $N_2$ flushing during operations to reduce sample oxidation and changes in TE speciation (*e.g.* formation of metal

95 oxides (Ortner et al., 2014)) which would determine a change in the bio-accessibility pattern of
96 trace elements. Moreover, the mass of the sample and the volume of reagents were decreased
97 compared to the original procedures to adapt the method to facilities available in the laboratory
98 such as the high-speed centrifuge (Beckman J2-21M, USA), which was used to separate the
99 supernatants from the solids. In short, the first step of the procedure separates DOM from the
100 solid residue. Approximately 300-600 ml of sample, with a total solids content of 4.8±0.2 wt%,
101 and 14.6±0.1 wt% for digestate and substrate, respectively, was centrifuged at 18600×g for 30
102 min at 10°C. Then, the supernatant, containing DOM, was filtered through 0.45 µm
103 polyethersulfone (PES) syringe filters (Pall Laboratory). The solid residue was flushed with $N_2$,
104 sealed and stored at 4°C in PP centrifuge tubes (Sarstedt) before performing the next extraction
105 step. In the second step, SPOM was extracted according to the procedure by Jimenez et al.
106 (2017). Approximately 3 g of pellet were shaken in polypropylene copolymer tubes (Termo
107 Scientific Nalgene) with 24 ml (mass ratio 1:8) of 10 mM $CaCl_2$ (pH 8) at 200 rpm and at 30°C
108 for 15 min. The suspension was then centrifuged at 18600×g for 30 min at 4°C and the
109 supernatant containing SPOM was recovered and filtered through 0.45 µm PES syringe filters.
110 The residual solid was treated with the same reagent three more times. During extraction of
111 SPOM, $N_2$ was flushed in the tubes. Subsequently, the solid residue was rinsed four times with 24
112 ml of 10 mM NaCl and 10 mM NaOH (pH 11) (Jimenez et al., 2014). The suspension was
113 shaken, centrifuged and filtered to recover REOM fraction. Thereafter, the residual pellet was
114 used to extract carbonate, sulfides and hydroxides (CSH) fraction by adding 24 ml of 0.1 M HCl
115 for 1 h at 30°C and 200 rpm (Jimenez et al., 2014). Unlike the original procedure (Jimenez et al.,
116 2014), this fraction was recovered for further analyses. The resulting solid residue was washed
117 with ultrapure water and neutralized to pH 7 with 0.1 M NaOH. Subsequently, the solid residue
118 was suspended in 24 ml of 0.1 M NaOH (pH 12) and shaken at 200 rpm and at 30°C for 1 h to

recover the SEOM fraction (Jimenez et al., 2014). This step was repeated three more times. Finally, the residual pellet was shaken two times with 24 ml of 72% (w:w) $H_2SO_4$ for 3 h at 30°C and 200 rpm for extraction of PEOM (Jimenez et al., 2017). The residual solid, which is the Non-Extractable Organic Matter (NEOM), was recovered and freeze-dried for further analyses. The sequential extraction was performed on triplicate samples. All reagents were prepared in acid washed glassware and with ultrapure deaerated water.

Table 1 shows the sequential extraction steps of the procedure.

(Table 1 here)

## 2.3. Analytical procedures

### 2.3.1. Chemical analysis

The pH of the samples was measured with a pH meter (InoLab 7310, WTW, Weilheim, Germany). Total solids (TS) and volatile solids (VS) content in the raw samples and pellet collected after DOM extraction were quantified in triplicates according to the Swedish Standard method (SS-028113; 25). Thus, an aliquot of sample was dried in porcelain crucibles at 105 °C for 20 h to measure TS content. Then, the dried samples were heated up to 550°C for 2 h in a muffle furnace to determine VS content. Dissolved organic carbon (C) was measured in the filtered supernatants by total organic carbon analyzer (TOC-VCHS, Shimadzu, Japan). During analysis, ultrapure water was analyzed after each set of triplicates to avoid cross contamination.

Total C and N content in the solid residue collected at each extraction step was determined by CHNS/O elemental analyzer (EA2400, Perkin Elmer, USA). Prior to analysis, the samples were freeze-dried and finely ground with a mortar and pestle.

140 The concentration of TE for each extracted faction was quantified in the filtered supernatants,
141 whereas the total TE' content (*i.e.* Al, As, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb and Zn) was
142 extracted after digestion of substrate and digestate samples according to the Swedish standard
143 method (SS028311) using 7 M $HNO_3$ in an autoclave at 120 °C for 30 min as described by
144 Shakeri Yekta et al. (2014a). Trace element concentrations in the samples collected both after the
145 acid digestion method and the sequential extraction procedure were quantified by inductively
146 coupled plasma mass spectrometry (ICP-MS, Nexion 300D, Perkin Elmer, USA). Before ICP-
147 MS analysis, if required, samples were acidified with concentrated $HNO_3$ (1% of the sample
148 volume) and stored at 4°C until analysis. To reduce possible chemical interference, some of the
149 TE were analyzed by using kinetic energy discrimination (KED) or dynamic reaction cell (DRC)
150 mode during ICP-MS analysis. A graphical scheme of the analyses performed on the collected
151 samples is showed in Figure 1.

152 (Figure 1 here)

153 2.3.2. Nuclear magnetic resonance spectroscopy

154 NMR analysis was performed on the solid residues recovered at each step of the extraction
155 procedure. Solid residues were preferred to the liquid fractions to reduce possible interferences,
156 generated by the chemical reagents, with the sample NMR signals.

157 About 0.4 g dry mass of sample was pre-treated with 2 M HCl for 1 h to remove the
158 paramagnetic TE that would be detrimental to the quality of the NMR spectra according to
159 Shakeri Yekta et al. (2018). The suspension was centrifuged and the supernatant was discarded,
160 while the solid residue was recovered and freeze-dried. Approximately 80 mg of each sample was
161 transferred to 4 mm $ZrO_2$ rotors for solid state cross polarization magic angle spinning (CPMAS)
162 $^{13}C$ NMR analysis and approximately 100 mg of sample was milled using a Fritsch Pulverisette 7

planetary ball-mill to prepare it for solution-state 1D $^{1}H$ and 2D $^{1}H$-$^{13}C$ heteronuclear single quantum coherence (HSQC) NMR analysis. The protocol used for grinding consisted of 5 × 10 min milling with 5 min pauses in between to prevent overheating of the samples. 20 mg of milled sample were transferred to 5 mm NMR tubes and 600 µl of deuterated dimethyl sulfoxide (DMSO-$d_6$) was added. The CPMAS $^{13}C$ analysis was performed in triplicate and these were later pooled to get sufficient material for liquid state NMR analysis.

Solid state CPMAS $^{13}C$ NMR spectra and liquid state 1D $^{1}H$ and 2D HSQC $^{1}H$-$^{13}C$ NMR spectra were acquired using a Bruker 500 MHz AVANCE III spectrometer equipped with a 4 mm MAS probe and a Bruker 600 MHz AVANCE III HD spectrometer equipped with a 5 mm cryoprobe, respectively. CPMAS $^{13}C$ NMR spectra were recorded using cp pulse sequence and 3500 scans. The relaxation delay was 1 s and spin-rate was 10 kHz. 1D $^{1}H$ and 2D HSQC $^{1}H$-$^{13}C$ NMR spectra were recorded using zg30 and hsqcetgpsisp2.2 pulse sequences and 8 and 16 scans, respectively. The relaxation delay was 1.5 s and 2 s for 1D $^{1}H$ and 2D HSQC $^{1}H$-$^{13}C$ NMR, respectively. The spectra processing was performed in Topspin 3.5 (Bruker Biospin, Germany) and spectra were calibrated using adamantane as an external reference for CPMAS spectra or the residual DMSO peak ($\delta_{H/C}$: 2.49/39.5 ppm) in the case of 2D $^{1}H$-$^{13}C$ HSQC spectra.

## 2.4. Analytical quality control

The methodological limit of detection (MLD) and quantification (MLQ) were calculated using the conservative formula as the average plus three or ten times the standard deviation of the blanks, respectively, to consider contamination from elements in the reagents. During the sequential extraction procedure, we used 36 and 96 procedural blanks for TE and dissolved organic C analysis, respectively. Quality controls at 20 and 50 mg/L dissolved organic C were analyzed for every 10 samples during dissolved organic C analysis. The recovery was equal or

above 94% among all analyses. A reference material Sewage sludge CRM029-050 was acid digested in triplicate to evaluate the performance of the adopted total acid digestion method for TE' analysis. The accuracy of the method, calculated by dividing the mean observed concentration to the certified value (van Reeuwijk and Houba, 1998), was equal or higher than 90% for all analyzed elements.

In addition, spiked samples were analyzed to check possible matrix interferences on the measured concentrations of analytes due to the diversity of reagents used to perform the sequential extraction procedure. The method adopted to evaluate the matrix effect is standard addition method. We observed no significant matrix effect for dissolved organic C analysis ($p \geq 0.04$) in all OM fractions, whereas we observed matrix effect for TE analysis in PEOM fraction extraction and therefore results for this fraction are not presented.

# 3. Results and discussion

## 3.1. Organic matter composition of substrate and digestate

The TS content of substrate and digestate samples were 14.6±0.1 and 4.8±0.2 wt%, respectively, with the VS contents of 91.4±0.4 and 76.2±0.6 % of TS. The TS content after centrifugation and separation of the liquid fraction (*i.e.* solid pellets used for the sequential extraction procedure) were 33.1±0.4 wt% for substrate and 19.6±0.5 wt% for digestate samples. The pH of substrate and digestate was 4.9 and 8.1, respectively. We observed different distribution of C among the operationally defined organic fractions (*i.e.* DOM, SPOM, REOM, SEOM and PEOM) for substrate and digestate samples (Figures 2a and 2b). A large proportion of organic C in the substrate was present as DOM (76% of extracted organic C), whereas PEOM had the highest proportion among the OM fractions of the digestate (47% of extracted organic C). The DOM fraction mainly contains water-soluble organic substances, whereas PEOM contains recalcitrant and insoluble organic compounds according to Jimenez at al. (2017, 2014). In substrate, only 9% of extracted organic C was contained in PEOM and 15% of extracted organic C was present in SPOM, REOM and SEOM, which mainly contain proteins and sugars, lipids, humic-like and fulvic acid-like structures based on fluorescence spectroscopic characterization of the OM extracted in the supernatant after each step by Jimenez et al. (2017). In digestate, 28% of extracted organic C was as DOM, while 25% of extracted organic C was present in SPOM, REOM and SEOM.

To support the OM characterization of the extracted fractions provided by Jimenez et al. (2017, 2014), NMR spectroscopy was applied to look into the structural compositions of the organic molecules in the solid residues recovered after each extraction step of the sequential extraction

219 procedure. Moreover, NMR spectroscopy has shown to yield good resolutions of spectra in
220 complex organic matrices such as substrate and digestate (Shakeri Yekta et al., 2018).

221 Based on Kögel-Knabner (1997) and on Tambone et al. (2009), $^{13}C$ CPMAS NMR spectra,
222 presented in Figures 2c and 2d , were divided in five regions corresponding to different organic
223 structures: aliphatic chain C ($\delta_C$ 0-47 ppm), carbohydrates ($\delta_C$ 47-90 ppm), anomeric C ($\delta_C$ 90-
224 110 ppm), aromatic C ($\delta_C$ 110-160 ppm) and carbonyl C ($\delta_C$ 160-187 ppm). Figures 2c and 2d
225 show a higher contribution of aliphatic, aromatic and carbonyl C resonances in spectra of the
226 digestate compared to the substrate, while carbohydrates signals had a higher contribution in the
227 substrate spectra. Accordingly, aliphatic, aromatic and carbonyl C, mainly attributed to lipid-
228 and/or protein-like structures (Keeler et al., 2006; Kögel-Knabner, 1997; Simpson et al., 2011),
229 were enriched in the solid phase of the digestate upon anaerobic digestion. These observations are
230 in agreement with previously reported $^{13}C$ CPMAS NMR results by Tambone et al. (2013), who
231 showed a decrease of O–alkyl carbon signals ($\delta_C$ 47-113 ppm) attributed to carbohydrates in
232 substrates, whereas aromatic-C ($\delta_C$ 113–160 ppm) and aliphatic chain C ($\delta_C$ 0– 47 ppm)
233 accumulated in digestate samples.

234 Additionally, 2D $^{1}H$-$^{13}C$ HSQC NMR spectra of solid residue from substrate and digestate show
235 that anomeric signals at $\delta_H$ 4.45-5.2 ppm and $\delta_C$ 99-102 ppm (Simpson et al., 2011) as well as O-
236 alkyl signals at $\delta_H$ 3.4-3.8 ppm and $\delta_C$ 68-79 ppm (Soucémarianadin et al., 2017) from
237 hemicellulose and starch were more readily degraded during the anaerobic digestion process
238 compared to non-anomeric ($\delta_H$ 3.24-3.63 ppm and $\delta_C$ 60-72 ppm) (Soucémarianadin et al., 2017)
239 and anomeric C signals ($\delta_{H/C}$: 4.32/102.4 ppm) (Soucémarianadin et al., 2017) from cellulose,
240 which was left in the digestate. Interestingly, the peak from unsaturated double bonds of aliphatic
241 structures such as fatty acids ($\delta_{H/C}$: 5.3/130.1 ppm) is not visible in the digestate sample,

242 indicating that aliphatic double bonds in substrate OM were susceptible to degradation during the
243 anaerobic digestion.

244 (Figure 2 here)

245 3.2. Structural characteristics of sequentially extracted organic matter fractions

246 NMR spectroscopy analyses of the solid pellets after each step of the sequential extraction
247 allowed to differentiate the major structural groups, which remain after application of chemical
248 reagents used for fractionation of particulate OM (Table 1).

249 Additional results display that the solid residues recovered after DOM, SPOM, REOM and
250 SEOM extractions have a comparable distribution of C among different organic groups in both
251 substrate and digestate samples. Only a slight reduction of carbohydrates was observed in the
252 SEOM solid residue in substrate. On the other hand, we observed a reduction of C in anomeric
253 and carbohydrate organic groups in solid residues of substrate and digestate after the PEOM
254 extraction, implying that application of 72% $H_2SO_4$ resulted in partial dissolution of cellulosic
255 structures.

256 The $^{1}H$-$^{13}C$ HSQC NMR spectra of digestate solid residues collected after extraction of DOM,
257 SPOM, and REOM fractions were qualitatively similar. Thus, OM extraction by 10 mM $CaCl_2$
258 and a mixture of 10 mM NaCl and 10 mM NaOH, used for extraction of SPOM and REOM, did
259 not selectively remove OM groups from the particulate OM. However, the $^{1}H$-$^{13}C$ HSQC NMR
260 spectrum of pellet collected after extraction of SEOM fraction contained fewer cross peaks within
261 the chemical shift regions assigned to CH(α) groups of amino acids in peptide chains and proteins
262 ($\delta_{H/C}$: 4.0-4.7/45-62 ppm) (Simpson et al., 2011). Furthermore, several peaks in the aliphatic
263 region where signals from amino acid side-chains appear, e.g. the peak at $\delta_{H/C}$: 2.0/15.1 ppm

assigned to methionine $CH_3$-groups (Shakeri Yekta et al., 2018), were absent or had reduced intensities in the spectra of pellets after SEOM extraction. The major peaks in the aromatic region, $\delta_{H/C}$: 6.5-7.4/113-134 ppm, assigned to aromatic side-chains of amino acids (based on comparisons with reference spectra) also experienced a reduction in signal intensities after SEOM extraction. Accordingly, 0.1M NaOH reagent mainly extracted proteins in SEOM fraction. It is notable that the presence of protein-derived resonances in the spectra even after SEOM extraction indicates that the proteins were only partially extracted during this step.

The $^{1}H$-$^{13}C$ HSQC NMR spectra of digestate proved that carbohydrate signals mainly from cellulose, $\delta_{H/C}$: 4.1-5.2/94-106 ppm (anomeric) and $\delta_{H/C}$: 2.9-4.11/59-84 ppm (O-alkyl), and signals related to amino acids were removed from the solid residue after the PEOM extraction. Thus, the OM extracted by 72% $H_2SO_4$ mainly originate from carbohydrate and partially from protein contents of the samples. These results support the findings in Jimenez et al. (2015), where the authors identified the biochemical nature of each extracted fractions by testing the sequential extraction protocol on several representative samples (e.g. lipid-rich agri-food waste, cardboard and crispbread). Based on percentage of chemical oxygen demand (COD) extracted in each fraction from the representative samples, the authors found that protein-like and lipid-like compounds were mainly extracted in SEOM fraction, whereas carbohydrates and holocelluloses in the PEOM fraction.

Similarly, $^{1}H$-$^{13}C$ HSQC NMR spectra of substrate solid residues, collected after extraction of DOM, SPOM, and REOM were qualitatively similar, whereas peaks from CH(α) groups of the amino acids and peaks from aromatic amino acid side-chains were significantly reduced in solid residue collected after SEOM fraction. Moreover, signals related to cellulose and amino-acids were removed from the solid residue of substrate after the PEOM extraction.

287 Furthermore, comparison of the amount of C extracted during the sequential extraction procedure
288 and total C content of the samples demonstrated that more than 60% of the total initial C was still
289 retained in both the substrate and digestate samples after sequential extraction. Nevertheless, the
290 structural characterization of OM in our study revealed that NEOM, representing the fraction
291 with low degree of bio-accessibility (Jimenez et al., 2015), comprised mainly of aliphatic and
292 aromatic CH groups of the protein/biomass fraction of the OM.

293 The major organic molecules targeted by the chemical reagents used for fractionation of
294 particulate OM according to Jimenez et al. (2017, 2015, 2014) and the findings of this study are
295 summarized in Table 2. It should be highlighted that in this study, NMR spectroscopy was
296 performed on the solid residues recovered at each step of the extraction procedure, whereas
297 Jimenez et al. (2017, 2015, 2014) identified the nature of the organic molecules in the liquid
298 fractions extracted by the fractionation procedure. Similarities are only found in SEOM and
299 PEOM fractions, whereas the organic molecules found in NEOM fraction reflects the nature of
300 substrate analyzed.

301 (Table 2 here)

302 ## 3.3. Simultaneous extraction of trace elements with organic matter fractions

303 Concentrations of TE extracted at each step during sequential extraction of OM are reported in
304 Table 3. Element contents in CSH fraction are also reported as this fraction may include TE
305 likely bound to inorganic ligands. Quantification of TE concentrations in PEOM fraction had a
306 high degree of uncertainty due to analytical interferences, caused by reagent matrix and are
307 omitted from Table 3.

308 Overall, we observed a higher concentration of total TE in digestate than substrate (on TS basis),
309 which is also a result of OM content reduction in digestate compared to substrate. Among

310 elements, total concentrations of Fe, Al, Mn and Zn prevailed in digestate and substrate, whereas
311 total concentration of As, Cd, Co, Cr, Mo, and Pb were lower than 11 µg/$gTS_{in}$ in both samples.

312 (Table 3 here)

313 More than 60% of total Cd, Co, Fe, Mn, Ni and Zn were extracted together with DOM, SPOM,
314 REOM, CSH and SEOM organic fractions of the digestate and substrate samples, whereas it is
315 assumed that the remaining concentrations were in the residual pellet after SEOM fraction
316 extraction. Additionally, sum of As concentrations in the fractions extracted along with
317 sequential extraction of OM from substrate was higher than the As concentrations measured after
318 total digestion of the samples by 7M $HNO_3$ (Table 3). Molybdenum concentration in the samples
319 was relatively low (2.3±0.1 µg/gTSin in digestate, 0.68±0.02 µg/gTSin in substrate) and only
320 13% of total Mo was recovered during the sequential extraction procedure, primarily in DOM
321 and SPOM fraction. The recovery of Cr was also low, *i.e.* 18% and 29% for digestate and
322 substrate, respectively, mainly found in DOM and CSH fractions, whereas only 4% of Cu was
323 recovered in DOM fraction in digestate. In general, the highest concentration of all TE was found
324 in DOM and CSH fractions of digestate and substrate. Low concentrations of Al, Fe, Mn, Mo and
325 Ni were found in SPOM fraction of substrate and digestate, additionally Co and Zn were
326 extracted from substrate in SPOM fraction. Notably, the concentration of five elements was
327 below MLD and MLQ in digestate and substrate. Among quantified elements, Co, Fe, Mn and
328 Mo were found in REOM fraction of both samples, additionally Al and Ni were extracted from
329 digestate in this fraction. Finally, SEOM contained Al, Cr, Fe, Mn, Ni and Zn with relatively low
330 concentrations extracted from both samples.

331 It should be emphasized that, except for the DOM fraction obtained by centrifugation, the other
332 extraction steps involve reagents that may interact with TE species in the sample and promote the

dissolution/precipitation of elements together with OM extraction. However, we do not exclude that TE, which were extracted together with the operationally defined fractions of OM, may originate from organically-bound and/or inorganic TE compounds (*e.g.* CSH fraction) in the samples. To further assess the origin of TE in OM fractions and assess the contribution of TE containing minerals compounds during the sequential extraction, we performed the CSH fraction extraction step between DOM and SPOM extraction steps. Addition of 0.1M HCl during CSH extraction results in dissolution of metals bound to minerals under acidic conditions *e.g.* metals bound to carbonate, phosphate and amorphous metal sulfide (Albacete et al., 2015; Filgueiras et al., 2002; Rickard and Morse, 2005). Thus, shifting the extraction of CSH fraction prior to sequential extraction of SPOM, REOM, and SEOM allows the removal of metals bound to minerals, whereas TE simultaneously extracted during the subsequent extraction steps represent the fractions most likely bound to OM. Thus, concentrations of TE in each fraction provide information on potential association of elements with operationally defined OM fractions. Indeed, we noticed that the concentration of elements found in the "shifted" CSH fraction is similar to the concentration of elements found in CSH fraction of the original fractionation procedure. Accordingly, the assessment of simultaneous extraction of OM and TE suggested that 31% to 98% and from 61% to 94% of total elements' content, depending on the specific element, are associated with the mineral fraction (*i.e.* CSH fraction) (or strongly bound to organic compounds) in substrate and digestate, respectively, whereas the remaining portion is likely associated with the extracted OM fractions.

### 3.4. Implications for simultaneous assessment of trace elements and organic matter bio-accessibility

Comparison of the OM fractionation of digestate and substrate demonstrated that the anaerobic digestion process resulted in a decrease of dissolved organic C in DOM fraction of the substrate, while the PEOM fraction was enriched in the digestate (Figure 2Figure a, b). The DOM fraction contains more bio-accessible organic substances compared to the other fractions, whereas PEOM represents the least bio-accessible fraction of the OM, which is mainly composed of hemicellulose, cellulose and starch based on $^{13}C$ CPMAS and $^{1}H$-$^{13}C$ HSQC NMR spectroscopy in this study (Table 2).

A distribution of TE based on different degree of bio-accessibility is proposed in Figure 3Figure. The proposed distribution is based on the knowledge of the leaching strength of the reagents used during the extraction procedure and the results obtained from the modified sequential extraction procedure (*i.e*. mineral fraction extracted at the beginning of the extraction procedure). Therefore, we suggest that TE found in DOM fraction are mobile in the digester environment and thus, more bio-accessible. The TE found in SPOM fraction are potentially bio-accessible, whereas TE found in REOM, CSH and SEOM fractions are considered poorly-bio-accessible. It is noteworthy that the CSH fraction contains metals bound to minerals with different solubility (e.g. metals bound to phosphate and carbonate minerals encompass a higher solubility and potential bioavailability in the digester environment than amorphous metal-sulfides). Moreover, part of TE extracted in CSH fraction could be associated to mineral particles present in extracellular polymeric substances (EPS) as observed by D'Abzac et al. (2010) using scanning electron microscopic analysis in anaerobic granular sludge. Therefore, association of metals in CSH to poorly-accessible fraction should be considered relative to the operationally-defined fractions of metals in this study, which

376 is based on leaching strength of the reagent used. Finally, the elements not extracted by the
377 sequential extraction procedure are likely not immediately bio-accessible, but they may be
378 mobilized on the long term after degradation of the OM present in PEOM and NEOM fractions.

379 The high concentration of TE found in DOM fraction is related to the presence of dissolved metal
380 species (free ions and complexes with inorganic and organic metal-binding ligands) as well as
381 metal-containing colloids and particles (<0.45 μm). Organic macromolecules such as proteins
382 may as well contain metals (e.g. Co-containing vitamin B12), which contribute to the pool of
383 metals associated with DOM (Shakeri Yekta et al., 2014a; Zhu et al., 2014). Therefore, we
384 hypothesize that TE in DOM fraction are accessible for interaction with the biological interface.
385 Regarding TE found in SPOM fraction, obtained by washing the sample pellets with $CaCl_2$
386 reagent, we assume that elements are potentially mobile and bio-accessible since they were likely
387 released in solution by ion exchange mechanisms with $Ca^{2+}$ or $Cl^-$, therefore TE bio-accessibility
388 is related to availability of this fraction for taking part in ion-exchange reactions. Indeed, $CaCl_2$
389 reagent is commonly used in soil analysis to extract the exchangeable fraction of TE which is
390 also the most available fraction for plant uptake (Filgueiras et al., 2002; Houba et al., 1996). The
391 TE associated with CSH fraction is likely related to the TE as minerals, such as amorphous metal
392 sulfide, metal carbonate and metal phosphate precipitates, which are dissolved under acidic
393 conditions upon addition of HCl. Accordingly, the accessibility of TE in the form of inorganic
394 precipitates in solid phase is probably limited, and the availability of TE bound to this fraction is
395 largely dependent on the solubility of the metal-containing minerals. Chemical speciation
396 analysis of TE in different anaerobic digesters suggested that sulfide is likely the major inorganic
397 ligand, scavenging TE from aqueous phase in co-digesters and the TE in solid phase is dominated
398 as TE-sulfide (Shakeri Yekta et al., 2014b). Due to poor solubility of TE-sulfide minerals as
399 potential dominant species in the CSH fraction, the accessibility of metals in this fraction for

400 microorganisms is likely constrained. REOM and SEOM fractions include elements extracted
401 from the samples under alkaline condition (pH 11-12). Prevalence of such high pH is uncommon
402 in anaerobic digesters and in environment. Furthermore, dissolution of metal species, which
403 commonly occur at low pH, is unlikely to occur during extraction of REOM and SEOM
404 fractions, implying that TE extracted might potentially originate from the simultaneously
405 extracted OM. As this fraction of OM is considered less accessible and could be solubilized only
406 after a pH increase, the bio-accessibility of TE associated with REOM and SEOM fractions may
407 be limited.

408 In substrate, more than 50% of total As, Co, Mn and Ni are bio-accessible or potentially bio-
409 accessible, whereas less than 40% of total Fe, Zn and Mo are bio-accessible or potentially bio-
410 accessible. It is well reported that Co, Fe, Mn, Mo, Ni and Zn are important TE for optimal
411 performance of the anaerobic digestion processes as demonstrated by Gustavsson et al.
412 (Gustavsson et al., 2013, 2011) and reviewed by Zandvoort et al. (2006) and by Demirel and
413 Scherer (2011). Therefore, their bio-accessibility for anaerobic microorganisms is relevant. In
414 digestate, except for As, all elements have poor or limited bio-accessibility, suggesting the
415 formation of more stable forms of trace elements during anaerobic digestion process. This
416 information is relevant to fulfill national or European requirements for application of digestate as
417 soil amendment. Indeed, the current results show that less than 20% of total Cd, Cr, Cu, Ni, Pb
418 and Zn, considered harmful elements for plant uptake when present at high concentrations
419 (Saveyn and Eder, 2014), are immediately bio-accessible or potentially bio-accessible. However,
420 the other elements which serve as nutrient for plants also have poor or no bio-accessibility.

421 (Figure 3 here)

## 4. Conclusions

More than 60% of total As, Cd, Co, Fe, Mn, Ni and Zn were extracted during the fractionation procedure mainly in DOM and CSH fractions, which were defined as the immediately and poorly bio-accessible fractions, respectively. Between 31% and 98% of total elements was likely associated to minerals (CSH fraction) in both substrate and digestate, whereas the remaining elements were associated with OM. We observed that SEOM fraction mainly contains proteins, whereas PEOM contains hemicellulose, cellulose, starch and certain proteins. However, no specific organic molecules were extracted along with SPOM and REOM fractions.

## Appendix A. Supplementary data

E-supplementary data for this work can be found in e-version of this paper online.

## Conflict of interest

The authors declare no conflict of interest.


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## Figure Captions

**Figure 1.** Analyses performed on the supernatants and solid residues collected during the sequential extraction procedure. DOC: dissolved organic carbon; TE: trace elements analysis; NMR: nuclear magnetic resonance spectroscopy; C/N: total carbon and nitrogen content; TS-VS: total and volatile solids content.

**Figure 2**. Organic matter characterization of substrate and digestate according to the sequential extraction procedure and NMR spectra. a-b) Relative distribution of organic C extracted after each step of the sequential extraction procedure form substrate and digestate samples. c-d)$^{13}$C CPMAS NMR spectra of DOM solid residues (n:3) from substrate and digestate. The integrated peak areas are expressed in % of total integral.

**Figure 3.** Interpretation of metals fractionation in terms of potential bio accessibility in substrate and digestate.

## Table Captions

**Table 1.** Sequential extraction procedure adapted from Jimenez et al. (2017, 2014) with some modifications highlighted in italic font. The extracted fractions are listed in order of decreasing bio-accessibility.

**Table 2.** Target molecules extracted by the sequential extraction procedure adopted by Jimenez et al. (2017, 2015, 2014) and the one performed in this study. The nature of the organic molecules was identified by using reference samples and/or 3D fluorescence spectroscopy in the work of Jimenez et al. (2017, 2015, 2014), whereas NMR spectroscopy was used in this study.

465 **Table 3**. Trace elements concentration found in each extracted fraction and total elements
466 concentration in digestate (grey rows) and substrate (white rows). Except of DOM fraction
467 extraction, results are mean of triplicate ± standard deviation.

468

469

## Tables and Figures

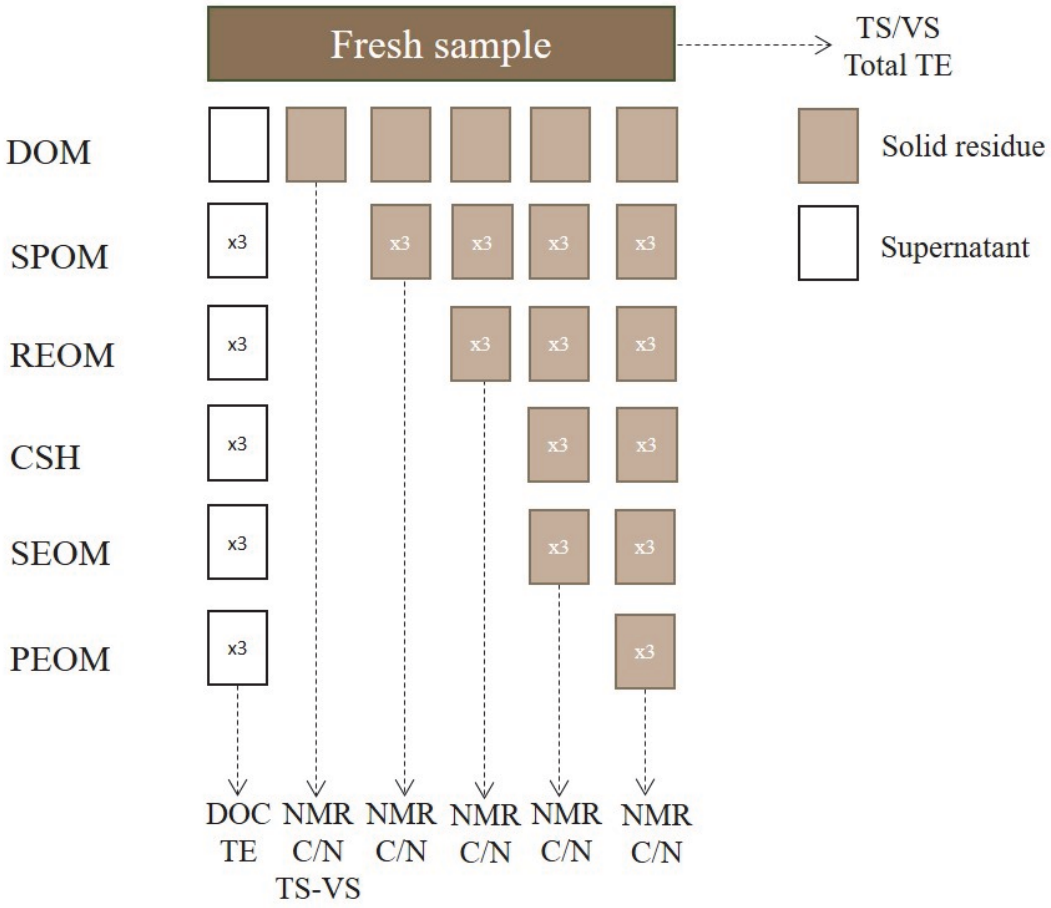
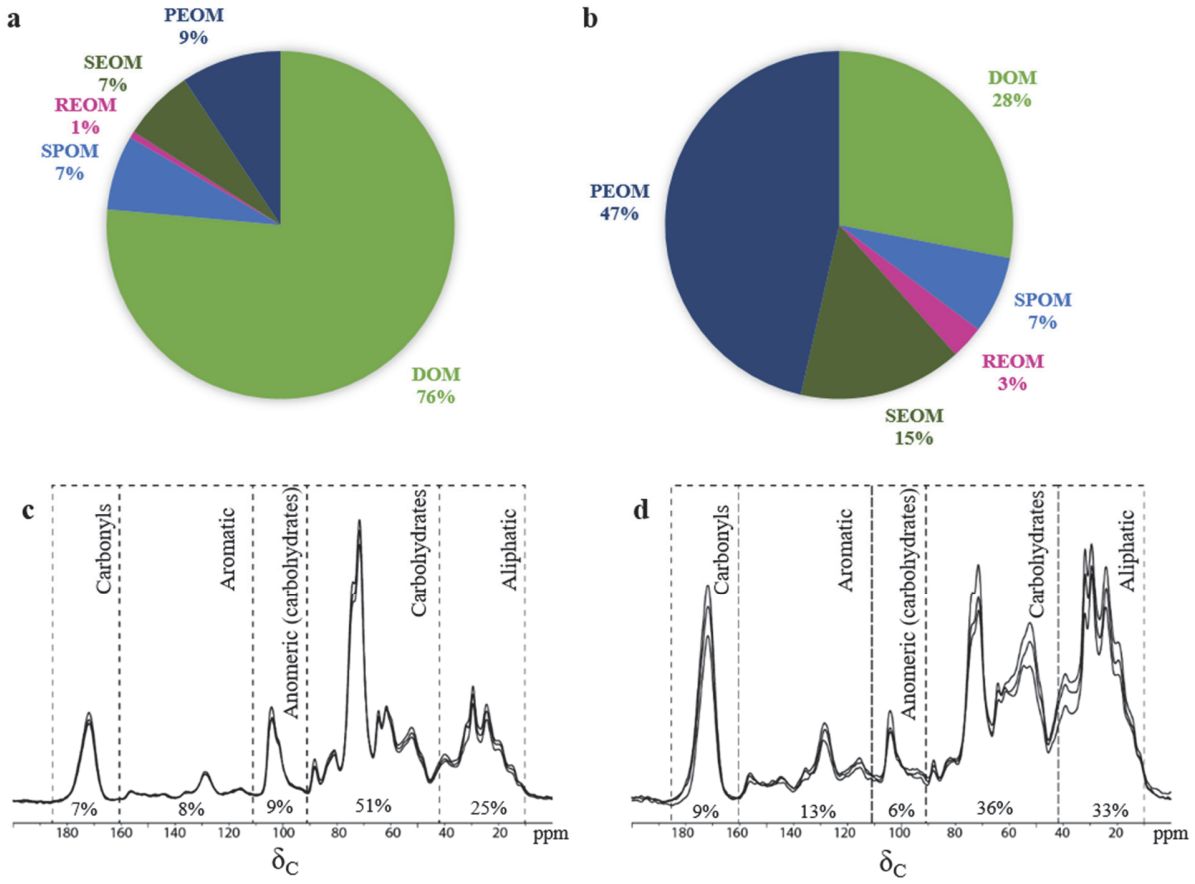
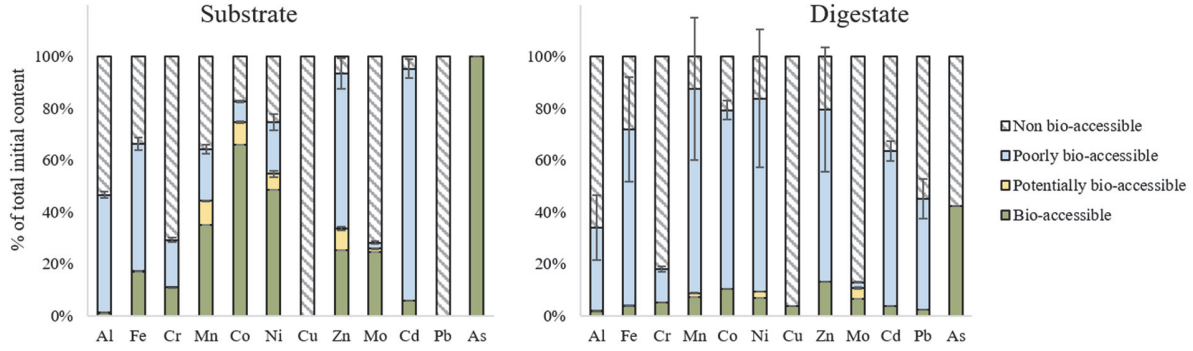



**Figure 1.**




473

474 **Figure 2.**




475

476 **Figure 3.**

477 **Table 1.**

| OM Fraction | Reagent | Extraction Method | Bio-accessibility Degree |
|---|---|---|---|
| DOM | - | Centrifugation (18600g, 30 min, *10°C*), filtration 0.45 µm, $N_2$ *flushing* | High |
| SPOM | *24 ml* of 10 mM $CaCl_2$ | 4 × shaking (*200 rpm*, 30°C, 15 min), centrifugation, filtration, $N_2$ *flushing* | |
| REOM | *24 ml* of 10 mM NaCl + 10 mM NaOH | 4 × shaking (200 rpm, 30°C, 15 min), centrifugation, filtration, $N_2$ *flushing* | |
| CSH | *24 ml* of 0.1 M HCl + ultrapure water rinsing | 1 × shaking (200 rpm, 30°C, 60 min), centrifugation, filtration, $N_2$ *flushing* | |
| SEOM | *24 ml* of 0.1 M NaOH | 4 × shaking (200 rpm, 30°C, 60 min), centrifugation, filtration, $N_2$ flushing | |
| PEOM | *24 ml* of 72% $H_2SO_4$ | 2 × shaking (*200 rpm*, 30°C, 3 h), centrifugation, filtration, $N_2$ *flushing* | Low |

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479

480 **Table 2.**

| Fractions | Target molecules by Jimenez et al. (2017, 2015, 2014) (Reference samples + 3D fluorescence spectroscopy) | Target molecules according to this study (NMR spectroscopy) |
|---|---|---|
| SPOM | Water-soluble proteins and sugars | *The reagent did not selectively remove OM* |
| REOM | Proteins and lipids | *The reagent did not selectively remove OM* |
| SEOM | Humic-like and fulvic like acids, complex proteins (i.e. glucolated proteins) and certain lignocellulosic compounds | Certain proteins (CH(α) groups of the amino acids; methionine $CH_3$-groups; aromatic side-chains of amino acids) |
| PEOM | Hemicellulose and cellulose | Carbohydrate (e.g. hemicellulose, cellulose and starch) and certain proteins |
| NEOM | Lignin-like compounds and non-extractable humic-like acids (i.e. humin) | Protein/biomass (aliphatic and aromatic CH groups) |

481

482 **Table 3.**

| | DOM ($\mu g/gTS_{in}$) | SPOM ($\mu g/gTS_{in}$) | REOM ($\mu g/gTS_{in}$) | CSH ($\mu g/gTS_{in}$) | SEOM ($\mu g/gTS_{in}$) | Total ($\mu g/gTS_{in}$) | % of total content |
|---|---|---|---|---|---|---|---|
| Al | 23.2 | 1.4±0.1 | 7.4±0.8 | 386.6±147.1 | 44.2±23.6 | 1359.4±33.7 | 34% |
| | 7.7 | 0.9±0.2 | <1.0# | 318.0±8.6 | 10.3±1.0 | 722.1±59.7 | 47% |
| As | 0.6 | <0.1* | <0.02# | <0.4* | <0.1# | 1.5±0.1 | 42% |
| | 0.4 | <0.1* | <0.01# | <0.2# | <0.1# | 0.22±0.02 | 180% |
| Cd | 0.01 | <0.1# | <0.0003# | 0.13±0.01 | <0.004* | 0.22±0.01 | 63% |
| | 0.01 | <0.04# | <0.01# | 0.09±0.00 | <0.001# | 0.10±0.00 | 95% |
| Co | 1.1 | <0.2* | 0.05±0.00 | 6.9±0.4 | 0.28±0.02 | 10.5±0.1 | 79% |
| | 2.7 | 0.35±0.01 | 0.01±0.00 | 0.32±0.02 | <0.01* | 4.04±0.04 | 83% |
| Cr | 0.3 | <0.1# | <0.01# | 0.7±0.1 | 0.08±0.00 | 5.9±0.2 | 18% |
| | 0.2 | <0.1# | <0.01* | 0.32±0.01 | 0.05±0.00 | 2.0±0.1 | 29% |
| Cu | 1.4 | <0.9# | <0.8# | <3.4* | <0.1* | 40.4±6.4 | 4% |
| | 0.2 | <0.5# | <0.5# | 6.2±0.1 | <0.4# | <35.8§ | - |
| Fe | 471.8 | 12.8±1.0 | 9.1±0.7 | 8502.1±2535.0 | 68.7±9.0 | 12623.1±222.8 | 72% |
| | 749.3 | 6.8±2.1 | 2.9±0.3 | 2119.9±108.3 | 36.1±1.9 | 4393.0±71.0 | 66% |
| Mn | 8.7 | 1.9±0.1 | 0.18±0.02 | 95.0±33.3 | 0.4±0.1 | 121.1±5.1 | 88% |
| | 16.2 | 4.2±0.1 | 0.15±0.02 | 9.0±0.8 | 0.07±0.00 | 46.1±1.5 | 64% |
| Mo | 0.2 | 0.09±0.01 | 0.04±0.00 | 0.01±0.00 | <0.1# | 2.3±0.1 | 13% |
| | 0.2 | 0.01±0.00 | 0.02±0.00 | <0.02* | <0.1# | 0.68±0.02 | 28% |
| Ni | 1.6 | 0.56±0.01 | 0.17±0.02 | 17.1±6.2 | 0.22±0.02 | 23.5±0.2 | 84% |
| | 1.3 | 0.17±0.03 | <0.01* | 0.5±0.1 | 0.03±0.01 | 2.7±0.1 | 75% |
| Pb | 0.1 | <0.1# | <0.04# | 1.6±0.3 | <0.1* | 3.8±0.6 | 45% |
| | 0.01 | <0.05# | <0.02# | 0.6±0.1 | <0.1* | <1.8§ | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Zn | 22.3 | <0.7# | <1.1# | 109.8±40.3 | 2.0±0.1 | 168.4±6.8 | 80% |
| | 17.2 | 5.6±0.5 | <0.7# | 39.3±3.7 | 1.5±0.3 | 68.1±0.6 | 93% |

The percentage of total extracted is the ratio of the sum of element's concentration in DOM, SPOM, REOM, CSH and SEOM over the total element' concentration.

*MLQ=average blanks ± 10*standard deviation blanks (n=36) expressed on the same concentration basis (µg/$gTS_{in}$) as those for the samples using 25.6 $gTS_{in}$/l and 41.6 $gTS_{in}$/l as conversion factor for digestate and substrate respectively.

#MLD=average blanks ± 3*standard deviation blanks (n=36)

§MLQ=average blanks ± 10*standard deviation blanks (n=3), using 10.5 $gTS_{in}$/l as conversion factor.

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# Supplementary data for

# A simultaneous assessment of organic matter and trace elements bio-accessibility in substrate and digestate from an anaerobic digestion plant

## Authors and affiliation

Andreina Laera[a,d,*], Sepehr Shakeri Yekta[b], Mattias Hedenström[c], Rémy Buzier[d], Gilles Guibaud[d], Mårten Dario[b], Giovanni Esposito[e], Eric D. van Hullebusch[f]

[a] University of Paris-Est, Laboratoire Géomatériaux et Environnement (EA 4508), UPEM, 77454 Marne-la-Vallée, France, andreina.laera@u-pem.fr or piedelibero89@gmail.com

[b] Department of Thematic Studies-Environmental Change and Biogas Research Center, Linköping University, 581 83 Linköping, Sweden

[c] Department of Chemistry, Umeå University, 901 87 Umeå, Sweden

[d] University of Limoges, PEIRENE, Equipe Développement d'indicateurs ou prévision de la qualité des eaux, URA IRSTEA, 123 Avenue Albert Thomas, 87060 Limoges Cedex, France

[e] University of Napoli "Federico II", Department of Civil, Architectural and Environmental Engineering, via Claudio 21, 80125 Napoli, Italy

[f] Université de Paris, Institut de Physique du Globe de Paris, CNRS, UMR 7154, F-75238 Paris, France

*Corresponding author: andreina.laera@u-pem.fr

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## Matrix effect

The method adopted to evaluate matrix effect consists of calculating the slope of a standard addition curve where the y-axis is the observed concentration of trace elements or organic carbon and the x-axis is the theoretical added concentration. The absence of matrix effect is identified when the slope of the curve is equal to 1. The standard addition curve was built for each reagent used during the sequential extraction procedure.

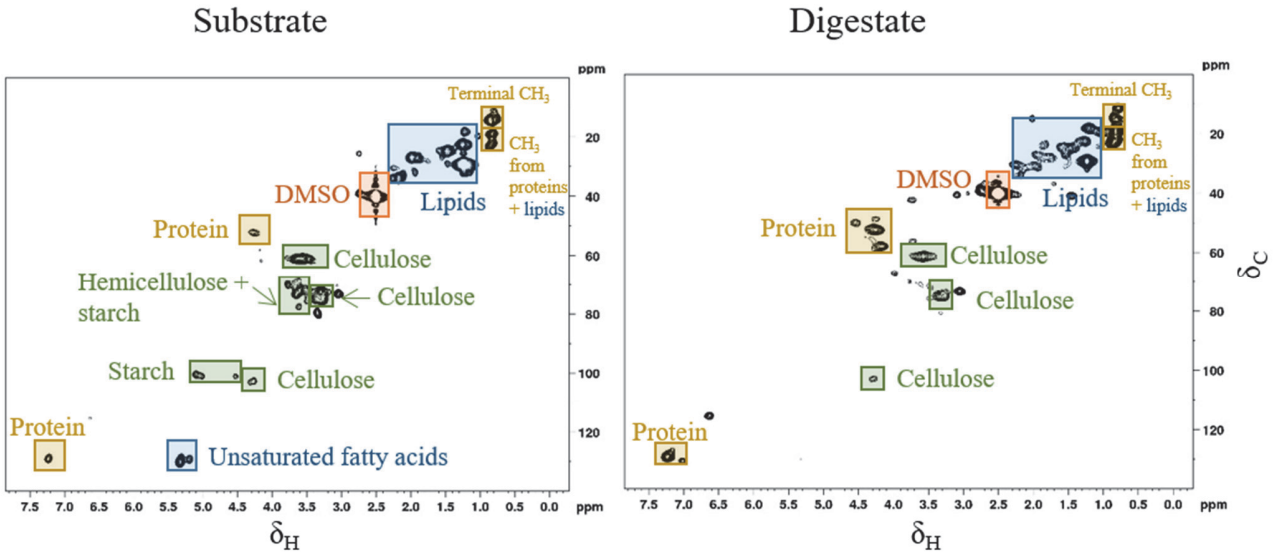



**Figure 1.** 2D $^{1}H$-$^{13}C$ HSQC NMR spectrum of DOM solid residue from substrate and digestate. The x and y-axis reports the chemical shift of $^{1}H$ and $^{13}C$ respectively in part per million (ppm) and peak originating from proteins, lipids and carbohydrates are marked in orange, blue and green, respectively.

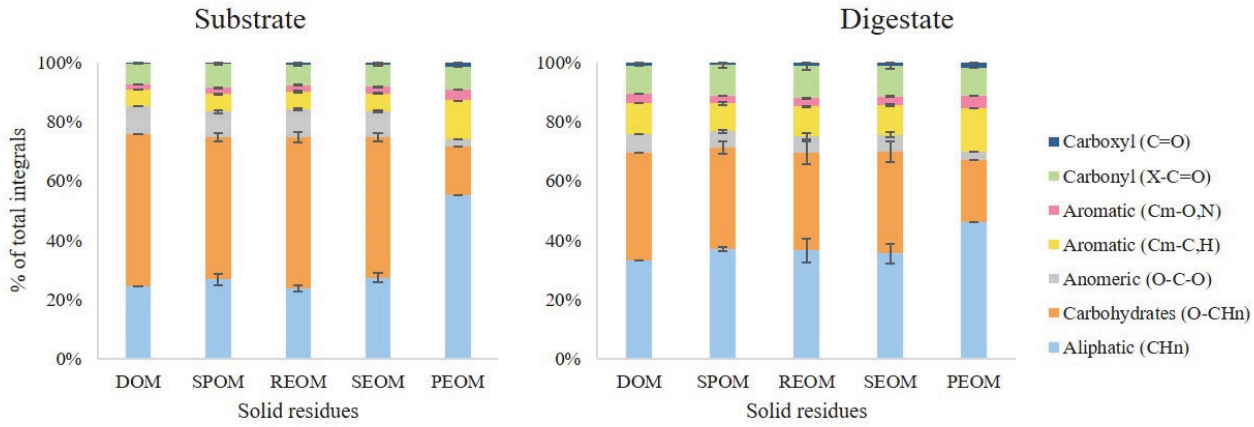
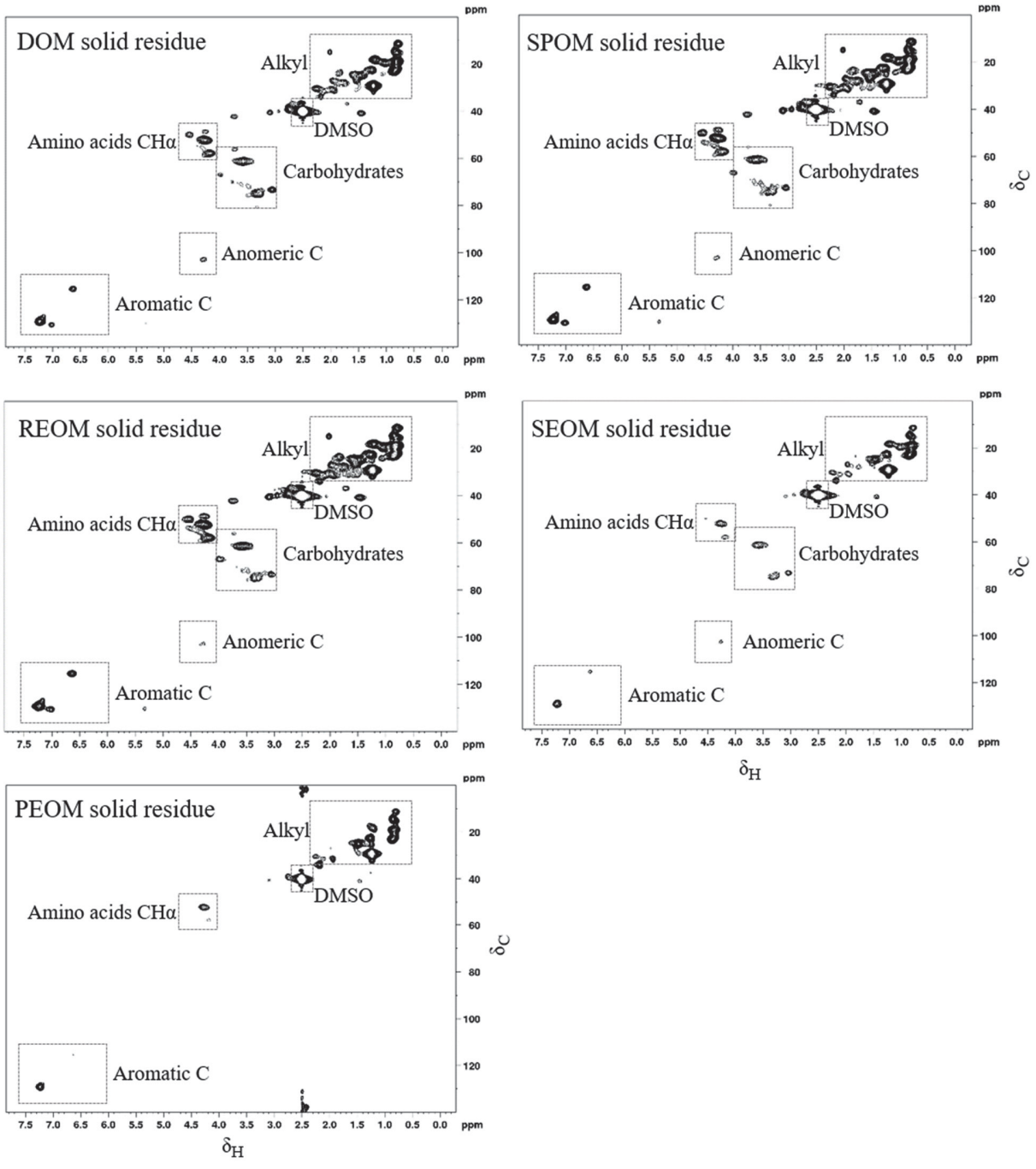



**Figure 2.** Distribution of C among different organic groups for the substrate and digestate solid residues. Results are expressed as % of total integrals of $^{13}C$ CPMAS NMR spectra.

Digestate


DOM solid residue
Alkyl
DMSO
Amino acids CHα
Carbohydrates
Anomeric C
Aromatic C
SPOM solid residue
REOM solid residue
SEOM solid residue
PEOM solid residue
ppm
δC
δH

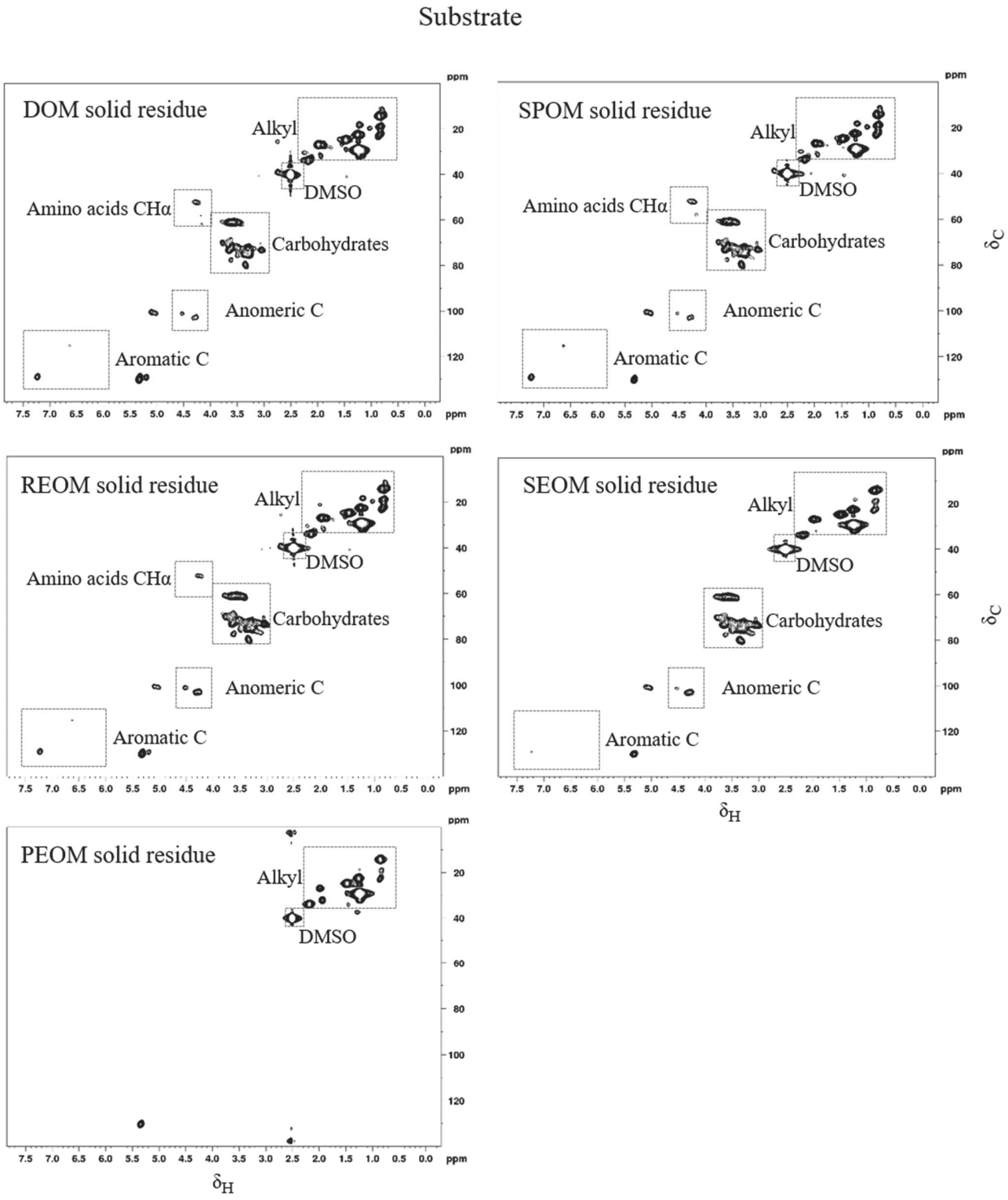



**Figure 3.** 2D $^{1}H$-$^{13}C$ HSQC NMR spectra of digestate and substrate solid residues collected during the sequential extraction procedure. Spectral regions representative for different chemical structures or functionalities are indicated by rectangles (Simpson et al., 2011; Soucémarianadin et al., 2017).

**Table 1.** Mass balance of C. Extracted dissolved organic C and initial C content are compared to estimate the percentage of non- and extracted C.

| | Substrate | Digestate |
|---|---|---|
| Extracted dissolved organic C* (mg) | 213.6 | 121.4 |
| Initial C content[#] (mg) | 546.0 | 316.9 |
| Extracted C (%) | 39.1 | 38.3 |
| Non-extracted C (%) | 60.9 | 61.7 |

*It is the sum of dissolved organic C extracted in SPOM, REOM, SEOM and PEOM fractions

[#] It is quantified in the solid residue after DOM extraction

**Table 2.** Trace elements concentration found in each extracted fraction by the modified sequential extraction procedure and total elements concentration in digestate (grey rows) and substrate (white rows). Except of DOM fraction extraction, results are mean of triplicate ± standard deviation

| | DOM ($\mu g/gTS_{in}$) | **CSH** ($\mu g/gTS_{in}$) | SPOM ($\mu g/gTS_{in}$) | REOM ($\mu g/gTS_{in}$) | SEOM ($\mu g/gTS_{in}$) | Total ($\mu g/gTS_{in}$) | % of total content |
|---|---|---|---|---|---|---|---|
| Al | 23.2 | 503.2±47.9 | 8.5±1.8 | <1.7# | 27.2±4.9 | 1359.4±33.7 | 41% |
| | 7.7 | 403.5±33.9 | 7.9±1.8 | <1.0# | 10.3±0.1 | 722.1±59.7 | 59% |
| As | 0.6 | <0.3# | 0.3±0.1 | <0.02# | <0.1# | 1.5±0.1 | 64% |
| | 0.4 | <0.2# | 0.09±0.02 | <0.01# | <0.1# | 0.22±0.02 | 220% |
| Cd | 0.01 | 0.14±0.02 | <0.1# | <0.0003# | <0.001# | 0.22±0.01 | 64% |
| | 0.01 | 0.13±0.01 | <0.04# | 0.01±0.00 | <0.001# | 0.10±0.01 | 138% |
| Co | 1.1 | 7.6±0.9 | 0.4±0.1 | 0.05±0.01 | 0.23±0.03 | 10.5±0.1 | 89% |
| | 2.7 | 1.4±0.1 | 0.5±0.1 | <0.01* | <0.01* | 4.04±0.04 | 111% |
| Cr | 0.3 | 0.67±0.05 | 0.04±0.00 | <0.01# | 0.10±0.00 | 5.9±0.2 | 19% |
| | 0.2 | 0.36±0.03 | <0.1# | <0.01* | 0.06±0.00 | 2.0±0.1 | 32% |
| Cu | 1.4 | <1.5# | <3.2# | <0.8# | <1.8* | 40.4±6.4 | 4% |
| | 0.2 | 6.4±0.3 | <0.5# | <0.5# | <0.4# | <35.8§ | - |
| Fe | 471.8 | 11322.4±2116.7 | 234.4±59.8 | 21.0±10.2 | 50.3±4.8 | 12623.1±222.8 | 96% |
| | 749.3 | 2390.1±133.3 | 44.2±4.8 | 4.7±1.6 | 47.0±1.4 | 4393.0±71.0 | 74% |
| Mn | 8.7 | 124.6±10.4 | 2.3±0.8 | 0.1±0.1 | <0.1* | 121.1±5.1 | 112% |
| | 16.2 | 20.3±2.1 | 5.4±1.5 | 0.2±0.1 | <0.04* | 46.1±1.5 | 91% |
| Mo | 0.2 | <0.04* | <0.1# | <0.01* | <0.1# | 2.3±0.1 | 7% |
| | 0.2 | <0.02* | <0.01# | 0.01±0.00 | <0.1# | 0.68±0.02 | 26% |
| Ni | 1.6 | 22.1±2.7 | 2.9±0.5 | 0.08±0.04 | 0.15±0.03 | 23.5±0.2 | 114% |
| | 1.3 | 0.9±0.1 | 0.3±0.1 | <0.01# | <0.02* | 2.7±0.1 | 95% |
| Pb | 0.1 | 1.3±0.5 | <0.1# | <0.03# | <0.1* | 3.8±0.6 | 37% |
| | 0.0 | 0.46±0.03 | <0.04# | <0.02# | <0.1* | <1.8§ | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Zn | 22.3 | 140.2±10.3 | 4.6±1.7 | <1.1# | <1.4* | 168.4±6.8 | 99% |
| | 17.2 | 51.6±2.8 | 4.0±1.2 | <0.7# | 1.3±0.5 | 68.1±0.6 | 109% |

*MLQ=average blanks ± 10*standard deviation blanks (n=36) expressed on the same concentration basis (µg/$gTS_{in}$) as those for the samples using 25.6 $gTS_{in}$/l and 41.6 $gTS_{in}$/l as conversion factor for digestate and substrate respectively.

#MLD=average blanks ± 3*standard deviation blanks (n=36)

§MLQ=average blanks ± 10*standard deviation blanks (n=3), using 10.5 $gTS_{in}$/l as conversion factor.

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