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Methodological approaches for fractionation and speciation to estimate trace element bioavailability in engineered anaerobic digestion ecosystems: an overview

Eric D. van Hullebusch^{1,*}, Gilles Guibaud², Stéphane Simon², Markus Lenz^{3,4}, Sepehr Shakeri Yekta⁵, Fernando G. Feroso⁶, Rohan Jain⁷, Lars Duester⁸, Jimmy Roussel⁹, Emmanuel Guillon¹⁰, Ulf Skjellberg¹¹, C. Marisa R. Almeida¹², Yoan Pechaud¹, Mirco Garuti¹³, Luigi Frunzo¹⁴, Giovanni Esposito¹⁵, Cynthia Carliell-Marquet⁹, Markus Ortner^{16,17}, Gavin Collins¹⁸

¹Université Paris-Est, Laboratoire Géomatériaux et Environnement (LGE), EA 4508, UPEM, 77454 Marne-la-Vallée, France

²Université de Limoges, Groupement de Recherche Eau Sol Environnement, Faculté des Sciences et Techniques, EA 4330, 123 Av. Albert Thomas, 87 060 Limoges, France

³University of Applied Sciences and Arts Northwestern Switzerland (FHNW), School of Life Sciences, Institute for Ecopreneurship, CH-4132 Muttenz, Switzerland

⁴Sub-Department of Environmental Technology, Wageningen University, 6700 EV Wageningen, The Netherlands

⁵Department of Thematic Studies-Environmental Change, Linköping University, SE-581 83 Linköping, Sweden

⁶Instituto de la Grasa (C.S.I.C.), Campus Universidad Pablo de Olavide. Edificio 46. Ctra. de Utrera km. 1, 41013-Sevilla, Spain

⁷Tampere University of Technology, Department of Chemistry and Bioengineering, Korkeakoulunkatu 10, FI-33720 Tampere, Finland

⁸Federal Institute of Hydrology, Department G2 - Aquatic Chemistry, Am Mainzer Tor 1, 56068 Koblenz, Germany

⁹School of Civil Engineering, University of Birmingham, Edgbaston, Birmingham, B15 2TT, United Kingdom

¹⁰Université Reims Champagne Ardenne, Institut de Chimie Moléculaire de Reims (ICMR), UMR 7312 CNRS-URCA, Moulin de la Housse, BP 1039, 51687 Reims Cedex 2, France

¹¹Swedish University of Agricultural Sciences, Dept. of Forest Ecology and Management, SE-90183 Umeå, Sweden

¹²Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal

¹³Centro Ricerche Produzioni Animali (CRPA), Viale Timavo 43/2, 42121 Reggio Emilia, Italy

¹⁴Department of Mathematics and Applications Renato Caccioppoli, University of Naples Federico II, via Cintia, Monte S. Angelo, I-80126 Naples, Italy

¹⁵Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, via Di Biasio 43, 03043 Cassino, FR, Italy

¹⁶University of Natural Resources and Life Sciences Vienna, Institute for Environmental Biotechnology, Konrad Lorenz-Str. 20, A-3430 Tulln, Austria

¹⁷BIOENERGY 2020⁺ GmbH, Konrad Lorenz-Str. 20, A-3430 Tulln, Austria

¹⁸Microbiology, School of Natural Sciences, National University of Ireland Galway, University Road, Galway, H91 TK33, Ireland

*Corresponding author: Eric.vanHullebusch@univ-paris-est.fr, Tel : +33 1 49 32 90 42 ;
Fax +33 1 49 32 91 37

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Abstract

Optimal supply of trace elements (TE) is a prerequisite for microbial growth and activity in anaerobic digestion (AD) bioprocesses. However, the required concentrations and ratios of essential TE for AD biotechnologies strongly depend on prevailing operating conditions as well as feedstock composition. Furthermore, TE in AD bioreactors undergo complex physico-chemical reactions and may be present as free ions, complex bound or as precipitates depending on pH, or on the presence of sulfur compounds or organic macromolecules. To overcome TE deficiency various commercial mineral products are typically applied to AD processes. The addition of heavy metals poses the risk of overdosing operating systems, which may be toxic to microbial consortia and ultimately the environment. Adequate supplementation, therefore, requires not only appropriate knowledge about the composition, but also on the speciation and bioavailability of TE. However, very little is yet fully understood on this specific issue. Evaluations of TE typically only include the measurement of total TE concentrations but do not consider the chemical forms in which TE exist. Thus detailed information on bioavailability and potential toxicity cannot be provided. This review provides an overview of the state-of-the-art in approaches to determine bioavailable TE in anaerobic bioprocesses, including sequential fractionation and speciation techniques. Critical

aspects and considerations, including with respect to sampling and analytical procedures, as well as mathematical modelling, are examined. The approaches discussed in this review are based on our experiences and on previously published studies in the context of the 'COST Action 1302: European Network on Ecological Roles of Trace Metals in Anaerobic Biotechnologies'.

Keywords

anaerobic digestion; trace elements; analytical methods; fractionation; speciation; bioavailability

1. INTRODUCTION

Anaerobic environments prevail in water-logged soils (e.g., rice fields, flood plains and artificial wetlands) and “anoxic” sediments (e.g., salt marshes) where the physico-chemical conditions are characterised by the absence of dissolved oxygen as well as reduced redox potential (Kirk 2004; Borch et al. 2010; ref). Such environments provide ecologically important ecosystems (e.g. as biodiversity support, water quality improvement, flood abatement and carbon sequestration), and are underpinned by various environmental redox processes involved in the formation and dissolution of mineral phases controlling the release or immobilization of inorganic compounds (Borch et al. 2009). Redox processes control the chemical speciation, bioavailability, toxicity and mobility of many trace elements (TE). For instance the formation of low solubility metal sulfide minerals may constrain the mobility of trace elements and their availability to microorganisms (ref Capone et al. 1983; Gambrell 1994; Du laing et al. 2009). Similar phenomena may also occur in engineered anaerobic digestion bioprocesses applied for the bioconversion of organic compounds into biofuel (i.e. methane) (Callander and Barford 1983a,b; Paulo et al. 2015).

1.1. Trace elements supply in anaerobic digestion (AD) processes

Optimal supply of TE is a prerequisite for microbial growth and metabolism in AD processes. Consequently, deficiency results in significant limitation of microbial activity that can result in the failure of AD processes. This is, in part, because methanogenesis, which is the final metabolic pathway during AD, involves the participation of various metal-rich enzymes, such as carbon monoxide dehydrogenase/acetyl-CoA synthase

(Cdh) and methyl coenzyme M reductase (Mcr). Those enzymes catalyse key metabolic steps and require sufficient supply of iron (Fe), nickel (Ni) and cobalt (Co) (Choong et al. 2016; Glass and Orphan 2012; Zerkle et al. 2005).

Typically, it has been observed that the deficient supply of TE can significantly limit AD processes resulting in accumulation of metabolic intermediates, such as volatile fatty acids (VFA), leading to bioreactor acidification and reduced methane yield (Demirel and Scherer 2011; Ortner et al. 2015; Pobeheim et al. 2010; Schattauer et al. 2011).

However, excess supply of essential TE may also inhibit AD processes. Some studies have found that exceeding threshold concentrations of certain TE resulted in significant loss of methane yield (for Se see Lenz et al. (2008b); for Ni see Bartacek et al. (2010); and for Co see Bartacek et al. (2008)). In Figure 1, an overview of TE supply and process performance is illustrated.

The determination of total TE concentration is a starting point for the evaluation of the effect of deficiency or excess of TE on AD processes. However, it is commonly accepted that total TE concentration is a poor indicator of the elemental fraction available to microorganisms (Smith et al. 2015). This is underlined by a review reporting the TE concentrations required for optimal operating conditions in anaerobic bioreactors (Table 1) (Schattauer et al. 2011). Optimal concentrations differed by as much as four orders of magnitude based on total TE content (Schattauer et al. 2011), but the chemical form of the elements was not considered, which obviously has an important impact on TE bioavailability.

1.2. Trace elements bioavailability

By definition of ISO 11074 (2005), bioavailability is the degree to which elements are available for interaction with biological systems (Marcato et al. 2009). As reported by Harmsen (2007) bioavailability may be assessed by applying chemical sequential extraction techniques, in which the pool of bioavailable elements decreases after each extraction step. The distribution of elements (chemical speciation), in quantity and quality, across different fractions (available, adsorbed, precipitated and unavailable in soil, sediment and sludge matrices) indicates their availability for metabolic activity (Filgueiras et al. 2002; Osuna et al. 2003). Fuentes et al. (2008) concluded that the exchangeable fraction – and not only the water-soluble fraction – is highly bioavailable .

These bioavailable TE can be divided into two categories by their uptake mechanisms: those with (i) active uptake i.e. internalisation processes requiring direct metabolic activity from microorganisms to transfer TE through the plasma membrane; and (ii) passive uptake, i.e. uptake based only on concentration gradients across the cell membrane. In addition to these processes, TE bioavailability is controlled by TE partitioning between the liquid and solid phases, and the diffusion of TE towards the microbial membrane surface. Once internalised, TE can impact the methane production yield *via* the intracellular, bioavailable fraction (Figure 2).

Figure 2. Simplified, conceptual representation of TE bioavailability in anaerobic digesters (adapted from NRC (2003)). A, B and C are related to bioavailability processes: TE interactions between phases, transport of TE to microorganisms and bio-uptake of TE through the biological membrane, respectively. D represents the biological

response (i.e. methane production yield) as a function of the bioavailable TE intracellular concentration.

Dissolved TE are present in AD as free ions and as soluble complexes (Jansen 2004). Commonly used models to predict TE bioavailability consider the free ion as the major bioavailable species (Worms et al. 2006). However, increases in TE bioavailability in the presence of TE organic or inorganic complexes have frequently been found even if the initial free TE concentration is the same (Zhao et al. 2016). For instance intact metal TE complexes may be internalised, *via* uptake of hydrophilic complexes through a ligand transporter system (e.g. metal citrate complexes or neutral metal phosphate complexes (MeHPO_4)) or passive diffusion of lipophilic complexes (e.g. metal complexes with dissolved organic matter), enhancing the bioavailability of TE in complex media, as in AD bioreactors (Zhao et al. 2016). Furthermore, such observations have, on several occasions, been linked to the variable degree of lability of TE complexes species (step A, Fig. 2). In the case of fast-dissociating (i.e. labile) complexes, the bio-uptake might be significantly enhanced compared to slow-dissociating (i.e. non-labile) complexes (Van Leeuwen 1999). The free-ion concept should, therefore, be considered carefully when transport limitations of the free metal ion to the uptake site predominate (step B, Fig. 2). Finally, TE equilibration (adsorption–desorption) with the cell surface of the microorganism occurs, followed by the facilitated transport of the TE into the microorganism (uptake) (Step C – Figure 2).

1.3. Definition of fractionation and speciation of TE

TE chemical species may strongly differ in their solubility and partitioning, and, eventually, their biological action i.e. in their 'behaviour' (referred to as 'fate'). In this sense, a chemical specie is defined as being specific in its isotopic composition, oxidation state, and/or complex or molecular structure (Templeton et al. 2000). For instance, though both are As species, the arsenite anion is acutely toxic to humans, whereas arsenobetaine (Leermakers et al. 2006) is hardly toxic. Therefore, it is of utmost importance to perform TE speciation analysis, which is the analytical activity of identifying and/or measuring the quantities of individual chemical species.

In contrast, fractionation analysis refers to separation procedures with insufficient separation power to differentiate between individual chemical species, classifying a group of analytes according to their physical (e.g. size, solubility) or chemical (e.g. bonding, reactivity) properties.

1.4. Bottlenecks and challenges, and the aim of this review

In heterogeneous biological systems, such as engineered AD ecosystems, TE distribution and partitioning is involved in the control of a complex network of physical, chemical, and biological reactions (Fermoso et al. 2009). The effect of these complex processes is a dynamic TE partitioning among different fractions: free TE ions, soluble organic and inorganic TE complexes, TE bound to colloidal and biotic (microorganisms) particulate materials. Figure 3 shows the chemical reactions occurring both in liquid (i.e. TE reduction, precipitation or complexation) and solid phases (i.e. TE sorption) that play key roles in the chemical speciation of TE in AD bioreactors in relation to S. In addition

to the precipitation of metals by sulfide (S^{2-}), carbonates (CO_3^{2-}) and phosphates (PO_4^{3-}) – and their deposition in bioreactor sludges and biofilms – also play a pivotal role in nutrients and TE turnover (Fig. 3) (Fermoso et al. 2015; Thanh et al. 2016).

A direct determination of the entire speciation patterns fractions of TE in AD ecosystems is often not attainable. TE fractionation can provide valuable information on TE pools useful to assess bioavailability (Ortner et al. 2014; Ortner et al. 2015).

Regarding TE fractionation, operationally defined sequential and kinetic extractions are the most widely used methods. A multitude of variations has been developed and is frequently applied for different solid phases of anaerobic materials, such as sediments and sludges (Filgueiras et al. 2002). In contrast, only a few studies using sequential extraction methods were carried out on anaerobic sludges to study TE fractionation (van Hullebusch et al. 2005a; van Hullebusch et al. 2005b; Zandvoort et al. 2005b). Though straightforward, these methods are limited by only providing information on operationally defined TE fractions in the solid phase and do not provide information on speciation.

In the last three decades many methods for fractionation and speciation have been applied to study anoxic sediments and their interstitial waters (Filgueiras et al. 2002); however, information on other anaerobic ecosystems, such as in anaerobic digesters, is still lacking. Recently, Thanh et al. (2016) provided a detailed overview of what is known regarding TE speciation and bioavailability in anaerobic digesters.

The present overview paper describes the approaches suitable to monitor the fate of TE in the specific context of complex and dynamic AD ecosystems (considering

both liquid and solid phases). To assess the true speciation in these fragile systems, recommendations on sampling and preservation strategies are included. Further, the most suitable modelling approaches to gain information on TE fate and bioavailability in AD are also discussed.

2. SAMPLING AND LIQUID/SOLID SAMPLE PREPARATION AND PRESERVATION

TE are directly or indirectly impacted by redox changes, affecting their speciation and fractionation (Borch et al. 2010). As a consequence, the mobility and bioavailability is changed across redox gradients.

In AD ecosystems, basically, two different incipient stages can be addressed for liquid/solid separation - *in situ* and *ex situ* of the anaerobic bioreactor. Both approaches deliver advantages and disadvantages and may help to avoid, or may lead to, various methodological artefacts with respect to the change of TE speciation or fractionation. For sample preservation – even more than for sampling – it holds true that the choice of the best practice depends mainly on the respective scientific question(s) and the chosen analytical tools. The concepts of sampling, and sample preparation and preservation, are detailed in this section as well as the respective challenges connected with the methods. This is supported by examples from literature and the authors' experiences.

A typical example, which may help to raise awareness for challenges in AD speciation / fractionation is the redox speciation of iron. Whereas the dissolved divalent Fe(II) species is only stable at near-neutral pH under low redox conditions, contact with traces of oxygen leads to rapid oxidation and induces formation of Fe (hydr)oxides. Such Fe (hydr)oxides are well known for sorbing / co-precipitating several TE. Thus, formation of

Fe(III)-containing colloids must be suppressed during sampling in low redox environments, including anaerobic digesters, if more information than just the total elemental content is required (Tack et al. 1996; Zehl and Einax 2005). Preservation by acid addition –commonly applied to avoid those effects – may change chemical speciation and is, thus, not appropriate.

In addition to demands derived from assessing unaltered speciation, samples collected from, e.g., full-scale biogas reactors, should ideally be representative of the whole system. Hence, precautions should be taken to ensure homogeneity of reactors material in the case of fully-mixed systems. Sampling is often a major source of uncertainty and may have a high impact on analytical results.

Ortner et al. (2014) demonstrated that for evaluation and interpretation of the results of TE analysis, a deviation of 6-12% (at minimum) derived from the sampling should be considered in addition to the analytical error. It is supposed that the variation of the results is mainly attributed to heterogeneities originating from low mixing efficiency in digesters, which is a typical phenomenon of biogas plants.

Important parameters directly affecting the speciation of TE must be controlled along with sample collection, handling, transport, preservation and preparation. As an example, the overpressure due to biogas production during sample transport should be avoided. Low preservation temperatures also alter gas-liquid solubility and chemical equilibrium (e.g. carbon dioxide in solution affects pH, (Angelidaki et al. 1998; Rozzi and Remigi 2004)) and related TE speciation. Sample preparation methods should be carefully evaluated to minimise the influence on original TE speciation and fractionation.

For example, the results of fractionation analysis may be affected by drying (humidity level, formation of metal oxides) and grinding (high surface to volume ratio, therefore enhanced metal oxide formation) the solid phase prior to extractions of different TE fractions (Baeyens et al. 2003).

2.1. *In situ* sampling

Speciation analysis commonly requires sampling of representative fractions of AD reactors. Whereas direct *in situ* speciation analyses can be done without sampling (e.g. electrochemical methods such as voltammetry or by ion selective electrodes; detailed in section 3), indirect methods depend on sampling devices. Examples are diffusion-driven samplers (e.g., DGT (Williams et al. 2014) or 2D peeper (Lewandowski et al. 2002)), which are based on separating the bulk and parts of the liquid matrix from the analytes of interest by diffusion across a membrane. Quantitative information is achieved by removing the respective sampler from the studied environment prior to analysing the receiving matrix (e.g., ultrapure water exposed in peeper experiments or re-elution of analytes from ion exchanger in DGT experiments). The advantages of diffusion-based sampling in anaerobic environments are: (i) a simple installation, (ii) a multitude of available methods and device designs, and (iii) simple sampling mechanisms. Drawbacks are the time-integrating character of the methods, whereas it is not possible to run experiments at high time resolutions (hours). Further, disturbances of the experiment may occur during removal and installation of the diffusion-driven samplers. More details are provided in the section 3.3.

Suction-based samplers with hollow fibres (Vink 2002) or porous ceramic or glass probe heads (Hofacker et al. 2013) can be installed directly in a bioreactor (Fig. 4). The samplers deliver a filtered sample (different cut-offs can be used for size fractionation questions), which is obtained by pumping (Duester et al. 2008) or by applying a vacuum (Seeberg-Elverfeldt et al. 2005). The general advantage of *in situ* bulk/liquid separation is that it is less prone to artefacts caused by changes in TE partitioning during *ex-situ* separation in conditions different to those in the reactor. Using gas-tight (e.g., Luer-Lock) connectors embedded in the reactor walls and flushing of the porous materials and tubing with inert gas prior to installation limits such disturbances. In addition, since the filtration step is included online, the sample preparation is straightforward. The drawbacks include chemical fouling (in particular under strongly varying redox conditions) and biofouling (in particular with high biological activity and long experimental times).

2.2. *Ex situ* sampling

With *ex situ* sampling from anaerobic environments (e.g., by pumping slurry samples from a bioreactor) contamination with oxygen must be avoided. The best practice is probably to directly sample into a glove box with the respective oxygen-free conditions of the reactor. Sample preparation transfers the respective sample to a form applicable to a specific analytical method. After preparation, the sample preservation aims at stopping the microbial processes and at preserving the speciation or fractionation of TE of interest, though the latter is not addressed in total TE content analyses. Using a glove box, the standard procedures for sample preparation can be carried out in an inert

atmosphere. Common examples are: 1. Squeezing, 2. Centrifugation, 3. Sedimentation, 4. Filtration 5. Freeze-Drying (details on the different methods are available in, e.g., Bufflap and Allen (1995a) and Bufflap and Allen (1995b)). Squeezing is more useful for work with core sections and less interesting for reactor studies. Filtration is comparably time consuming in comparison to centrifugation, but may suffer from shifts in apparent cut-off due to filter-cake formation. Drying under an inert gas at room temperature or shock freezing by liquid nitrogen with subsequent lyophilisation are additional options if analysis of the bulk phase requires dried materials (e.g. Scanning Electron Microscope) (Bordas and Bourg 1998; Rapin et al. 1986).

If a glove box is unavailable, one can try to limit potential sampling artefacts by use of inert gas flushed tubes or vessels. Centrifugation is more rapid than sedimentation and it seems easier to avoid oxygen contamination if no glove box is available.

2.3. Sample preparation and preservation

The main objective of a sample preparation method is to convert a real matrix into a sample with a format that is suitable for analysis (Cornelis et al. 2003; Mitra 2004). For common analytical techniques, such as optical or mass spectrometry, the analyte has to be transferred into a single liquid phase. After preparation, the sample preservation aims at stopping the microbial processes and preserving the speciation or fractionation of TE of interest.

2.3.1 Total TE content analyses

Bulk phase

For total TE concentration either destructive or non-destructive methods can be applied. In destructive methods, the TE are solubilised prior to elemental analysis through an acid digestion procedure, such as *Aqua Regia* digestion. A microwave oven may be used to accelerate the dissolution process (Niemelä et al. 2005). Non-destructive methods commonly refer to X ray fluorescence, whereas Neutron Activation Analysis remains a niche (for low concentrated TE). In both cases, it is strongly recommended to make use of certified standard materials to ensure accuracy / precision of the applied methods.

Liquid phase

In liquid phase, the total TE content can be determined on the whole sample or for different size fractions. If the total TE content of the whole liquid sample is addressed and if a cut-off $> 0.45 \mu\text{m}$ is chosen, samples have to be digested prior e.g., ICP based analyses. However, direct slurry applications without digestion steps by graphite furnace atomic absorption spectroscopy (Ferreira et al. 2010) or graphite furnace coupled to ICP-MS are available (Duester et al. 2011). If a cut-off of $< 0.45 \mu\text{m}$ is chosen, usually the samples are acidified with high purity nitric acid (below pH 2) and can be stored for a certain time without additional treatment. Despite the "conserving" effect of acid addition, one should consider potential problems such as precipitation and interferences with respect to the respective matrix beforehand. Common examples include e.g. silver chloride precipitation or formation of $^{40}\text{Ar}^{35}\text{Cl}^+$ interference on $^{75}\text{As}^+$ upon hydrochloric

acidification. To determine the total content in different colloidal fractions several different methods are available. Some of these methods are straightforward (*e.g.*, sequential filtration (Droppo et al. 1995; Logan 1995) or cloud point extraction (Hartmann and Schuster 2013)), whereas others are more sophisticated, like field flow fractionation (Meermann et al. 2014) or single particle ICP-MS (Tuoriniemi et al. 2015). The preservation of the size distribution is certainly challenging and often discussed in the frame of engineered nanoparticles fate.

2.3.2. Evolution of TE fractionation and speciation during sample preparation

In anoxic samples, such as sediments, sulfide-based phases can play a significant role in the retention of cationic TE due to their high affinity (low solubility products) for sulfide. When such anoxic samples are brought into contact with air, these sulfide phases can be oxidized, eventually yielding sulfate. At the same time, TE incorporated or sorbed to these phases will be released into solution. Among the released metals, Fe and Mn can further generate new solid phases (Fe oxyhydroxides, Mn oxides, etc) (Figure 5).

These phenomena can induce a significant change of the TE partitioning. Indeed, applying sequential extractions in the presence or absence of air led to completely different conclusions regarding TE fractionation (Buykx et al. 2000). Similar effects have been described for sequential extraction of anaerobic granular sludge (Lenz et al. (2008c), underlining the importance of careful sampling / sample preparation when studying anaerobic systems.

In this respect, the dissolution of solid phases upon oxygenation firstly enriches the liquid phase in TE (Fig. 5). However, several studies (Almeida et al. 2008; Caetano et al. 2003; Caille et al. 2003) reported that TE initially present in the liquid phase, as well as released during the dissolution of solid phases, could sorb onto the newly-formed Fe and Mn phases. As a result, the concentrations of TE of the original liquid phase can increase or decrease (even below their initial value) according to these antagonistic phenomena (Fig. 5). Such evolution can occur within a few hours, as shown by Caetano et al. (2003) who observed a significant increase of Fe, Mn, Pb, Cu and Cd between 20 and 40 min after exposing an anoxic sediment to an aerated estuarine water. Following this rapid release, metal concentrations underwent a decrease due to sorption and/or co-precipitation phenomenon.

3. TE ANALYSIS IN LIQUID MATRICES

3.1. Determination of total dissolved TE

Whereas atomic spectroscopy (AAS, OES) may be used at times (typically $\mu\text{g/L}$ to mg/L level), ICP-MS has certainly become the method of choice for TE quantification in many fields, including natural and in engineered systems. This is due to unique capability of rapid multi-element detection of all essential TE at outstandingly low limits of detection / limits of quantification (typically ng/L to $\mu\text{g/L}$ levels). Briefly, ICP-MS uses an argon plasma to dry the sample aerosol, dissociate molecules, atomize and ionize the analyte making it available to mass spectrometric detection. The main challenge of using ICP-MS for total TE determination in AD is not the limit of detection (LOD) / limit of quantification (LOQ) itself, but the typically high content of total dissolved solids (TDS).

These cause several problems, including interferences, ionization suppression and material deposition on the interface cones. To achieve acceptable TDS levels, often a dilution step prior analysis is required, increasing the LOD / LOQ for the elements of interest. Further, AD digestates are rich in major elements, such as Na, K, Ca, Cl, and S. These elements result in the formation of polyatomic ions that have a very similar mass-to-charge ratio as the TE of interest (for instance, $^{43}\text{Ca}^{16}\text{O}^+$ on $^{59}\text{Co}^+$; $^{40}\text{Ar}^{23}\text{Na}^+$ on $^{63}\text{Cu}^+$; $^{23}\text{Na}^{35}\text{Cl}^+$ on $^{58}\text{Ni}^+$, to list just a few). If no alternative interference-free isotope is available for measurement, [costly] high-resolving MS and / or [challenging] interference corrections can overcome these problems. Above all, the recent advances in collision / reaction cell technologies (e.g. Bishop et al. 2015; D'Illio et al. 2011) nowadays increasingly establish economical (*i.e.* non-high resolution) ICP-MS as a routine method for TE determination in such challenging matrixes.

3.2. Determination of dissolved TE speciation

Individual species present in the liquid phase of AD can be determined by different means. The discussion of this section will be limited to truly species-specific techniques – referred to as "hyphenated methods" – which make use of coupling a separation technique (typically high performance liquid chromatography, HPLC; capillary electrophoresis, CE) with spectroscopic / spectrometric detection (e.g. Evans et al. 2015; Harvanová and Bloom 2015). By matching sample analyte retention times with those of known standards, single species can be identified and quantified. The element-specific detection of spectroscopic and spectrometric instruments then allows quantifying unknown species even though no specific compound standard is available.

In recent years the number of speciation applications using ICP-MS as detector has increased considerably (Evans et al. 2015). For advances in the field of speciation, readers are referred to the comprehensive "atomic spectrometry update" series (Evans et al. 2015). Speciation methods based on classical normal- and reversed-phase, and ion-exchange, chromatography are becoming 'routine' and are reviewed elsewhere (Evans et al. 2015; Montes-Bayón et al. 2003; Popp et al. 2010). Besides the investigation of metals and metalloids, and their species (*e.g.* Sn, Hg, As, Cr or Se), representing 'classical' elements in environmental science, other elements (*e.g.* P, S, Br, I) amenable to ICP-MS determination were more recently addressed (Popp et al. 2010). It is generally accepted that TE undergo redox reactions in biologically-active reducing environments (such as AD, the gut, sediments, water logged soils, wetlands) (Gadd 2010; Lovley 1993; Lloyd et al. 2003) with innumerable examples for speciation measurements. In contrast, speciation analysis in AD – in particular for essential TE – is poorly developed.

General challenges for hyphenated techniques to address TE liquid speciation in anaerobic environments are related to the fragility of analyte, the mismatch of the original aqueous sample chemistry and the eluent composition, the concentration of the analytes, the tendency of many elements to biotransform into volatile (alkylated) forms, and at times, the lack of standards for identification. In addition, AD liquids represent a particularly challenging matrix due to high TDS, both of inorganic and organic nature.

The fragility of the analyte relates to both complex and redox stability of the analyte. For instance, Sb/Cr complexes with organic acids are weaker in contrast to the

corresponding EDTA complex. EDTA can be used on the one hand to preserve the original oxidation state of the element (Hansen et al. 2011; Markiewicz et al. 2015). On the other hand, however, the true original speciation information may be lost, since more labile complexes (*e.g.* Sb(III)-citrate, tartrate (Hansen et al. 2011)) dissociate in favour of the EDTA complex.

In most cases, at least several minutes are required to achieve chromatographic separation of TE species. Whereas sampling and sample preparation can be done in anaerobic conditions (see section 2), analyte oxidation by oxygen dissolved in the eluent requires further efforts to be prevented. Eluents that allow for separation of single species may not have the same aqueous composition than the original samples. This is the cause for two problems. Firstly, upon mixing analyte and eluent, spontaneous precipitation may occur, resulting in bias by co-precipitation and sorption of the analyte on the newly formed phases. The latter can be predicted using thermodynamical equilibrium modelling and prevented by choosing appropriate eluents (Floor et al. 2011). Secondly, the eluent may represent a more diluted system in contrast to AD digestate, and contain complexing molecules (*e.g.* EDTA) and salts. Thus, labile complexes may disintegrate and TE partitioning may change (Wrobel et al. 2003). For instance, size-exclusion chromatography (SEC) is generally well suited for TE speciation in organic-rich environments (such as AD), allowing a size-separation of metal complexes (Bolea et al. 2006; Laborda et al. 2008; Sadi et al. 2002; Vogl and Heumann 1997; Wu et al. 2004). However, to prevent undesired electrostatic interactions of stationary phase and analyte, salts, buffers or ligands have to be added to the mobile phase. In this context

they have to be compatible with ICP-MS detection. It should be noted that added compounds might induce a modification of the original TE partitioning, as demonstrated for Zn and Fe (Jahromi et al. 2010).

Several distinct problems (plasma instability, deposition on cones, interferences) associated with the introduction of organic HPLC-effluents into the ICP have been also reported, limiting the use of classical reverse-phase separation for hyphenation. Samples with high salt contents may require matrix separation prior to analysis to avoid polyatomic interferences (see section 3.1). For instance, the use of a sulfonic-acid-based cation-exchange resin allowed to assess Hg speciation even in highly saline seawater (Jia et al. 2012).

A whole range of TE is known to be readily biotransformed into alkylated forms (Thayer 2002). Some (yet not all) of these alkylation products are even volatile, which poses the risk of partial, or complete, loss during sample preparation and analysis. Though little is understood on the underlying mechanisms, it appears that – particularly when essential TE are dosed in excess to AD – alkylation may be favoured (Lenz et al. 2011). For volatile derivatives, release with the biogas represents a loss of essential TE, potentially leading to deprivation of the biomass. Alkylated non-volatile derivatives can be analysed by LC-ICP-MS coupling, whereas volatile derivatives can be rather analysed using GC-ICP-MS coupling (Michalke et al. 2000; Peitzsch et al. 2010; Vriens et al. 2014).

One of the challenges that is difficult to overcome regarding liquid phase speciation in AD is the lack of defined standards needed for identification. This appears

to be particularly true for elements that are known to readily form soluble species with S, such as Se and As (Lenz et al. 2008a; Petrov et al. 2012; Planer-Friedrich et al. 2010). Identification of unknown species following fraction collection by high-resolution MS techniques is not straightforward due to low concentrations of the single species.

Certainly, discussing the most appropriate techniques for each TE in AD in detail is beyond the scope of this review. Instead, the reader should carefully examine current literature while keeping the mentioned general limitations / challenges in mind to identify a truly species-specific, speciation-preserving approach for sample matrices.

3.3. *In situ* methodological applications for free / labile metal ion determination

Toxicity models (e.g. Biotic Ligand Model, BLM) assume free metal ion concentrations to be the most relevant species crossing biological membranes, ultimately triggering physiological reactions (incl. toxicity) (Smith et al. 2015), despite on-going discussions on the role of neutral complexes and nanomaterials (An et al. 2015; Beddoes et al. 2015; Brandt et al. 2008; Sanchez-Marin et al. 2007) (see section 1.2). To determine the free metal ion concentrations in aqueous solutions, several methodologies are available (Pesavento et al. 2009; Weng et al. 2011).

The Donnan Membrane Technique (DMT) is based on semipermeable membranes separating the solution to be analysed (the donor, *i.e.* the anaerobic digester) and an acceptor solution collecting the free metal ions after membrane passage (Temminghoff et al. 2000; Weng et al. 2011). A main advantage with respect to AD is its applicability to anoxic systems, the avoidance of sample matrix effects, the possibility to conduct *in situ* measurements and – if ICP-MS is used for detection – the

multi-analyte capability. DMT was used to determine Co and Ni speciation in anaerobic media containing varying concentrations of complexing ligands [carbonates, phosphates and EDTA] (Bartacek et al. 2008; Bartacek et al. 2010). The average TE concentration causing 50% inhibition of methanogenic activities were as little as 13 and 15 $\mu\text{mol/L}$ for free Co^{2+} and free Ni^{2+} , respectively. The applicability of DMT, however, was clearly limited to TE excess (i.e. toxicity by addition of 0.1 to 2 mmol Ni / L and 0.1 to 5 mmol Co / L). In case where the free ion concentration in the acceptor side does not exceed the quantification limit of the analytical technique applied, accumulation / ligand addition are needed (Weng et al. 2011). In these cases, DMT requires long equilibration times, incompatible with highly dynamic systems such as AD. Whereas DMT is frequently used to determine free cation species, only a limited number of anions has been studied (e.g. Se (Vega et al. 2010)).

The diffusive gradients in thin films (DGT) technique are passive samplers utilizing the flux of metal ions through a diffusive layer (porous gel matrix) into an underlying layer of binding phase irreversibly sorbing ions (strong cation/anion exchangers) (Pesavento et al. 2009). DGT has been frequently used for freshwater, wastewater, sediments and soils. DGT devices accumulating free and labile metal species were originally not designed to determine the actual chemical species present in the solution, but the amount measured is considered as a proxy of (bio)available TE (Davison and Zhang 1994). During the deployment, a TE concentration gradient is established in the diffusive layer between the exposition media and the binding phase, whereas after the deployment, the TE is eluted from the binding phase to determine the

total amount of TE sorbed. Then its original concentration in the exposition media is back-calculated (Davison and Zhang 2012). By using different binding phases, different target species can be assessed. For example, ZrO_2 or AgI can be used to sample free S^{2-} , as demonstrated for anoxic sediments. Phases with Chelex-100 resin or TiO_2 are widely used to determine free trace metallic cations and free oxyanions, respectively (reviewed in Zhang et al. 2014).

DGT can be combined with further methods to investigate TE speciation of redox sensitive elements. For example, a combination of HPLC–ICP-MS and DGT techniques were suitable to investigate Hg species availability in the soil- plant-root-system (Cattani et al. 2008). In another study, As speciation was investigated in anoxic sediment columns, using both DGT and DET (Diffusive Equilibrium in Thin films) (Bennett et al. 2012). The DGT technique has not been applied yet to AD; nevertheless, it can be assumed that such applications would be similar to applications on anoxic sediments or soils (Hong et al. 2014; Williams et al. 2014).

In addition to the above, electrochemical methods, such as voltammetry, stripping chronopotentiometry, and Nernstian equilibrium stripping are widely used for determination of particular metal forms and free ions in dissolved phase. However, there are several major limitations in the application of the latter for TE speciation in AD processes. Most of them are related to the low concentration of metal species (particular free metal ions), the presence of interfering compounds in complex biological matrices (*i.e.* the anaerobic digestate) and disturbances of chemical equilibrium by the electrodes applied [reviewed in Pesavento et al. 2009]. Application of ion-selective

electrodes overcomes some of the latter disadvantage, yet electrodes are only available for a limited number of TE, e.g. Cu, and still has to face to artefacts due to the matrix complexity.

In conclusion, mainly DMT and DGT appear promising to study the complex interaction of essential TE free ion concentrations in AD, if methodological limitations from above mentioned can be overcome.

4. TE FRACTIONATION AND SPECIATION IN SOLID MATRICES

4.1. Sequential extraction and metal fractionation

To obtain information on TE fractionation in AD ecosystems different chemical extraction approaches might be used to provide information regarding the potential mobility and bioavailability of these TE. However one should be aware the results obtained by sequential extraction are operationally defined, i.e. the ‘forms’ of TE are a direct result of the extraction procedure used (Quevauviller et al. 1997).

For instance, the sequential extraction (SE) approach aims at treating the samples with a series of reagents selected for their ability to react with different, major solid components of the matrix, releasing associated TE upon dissolution of the latter. This method has been popularized by Tessier et al. (1979), who developed an analytical procedure for determining the partitioning of particulate TE (Cd, Co, Cu, Ni, Pb, Zn, Fe, and Mn) into five fractions: exchangeable, bound to carbonates, bound to Fe-Mn oxides, bound to organic matter, and residual. The contribution of the sulfide fraction was not mentioned, as the samples treated in this study were oxic fluvial bottom sediments. Later on, in 1989, the Tessier procedure was implemented on anaerobically treated

sludge, revealing that organic matter and sulfide fractions are the most important carriers of metals in these matrixes (Angelidis and Gibbs 1989). Readers should note that TE bound to this fraction are simultaneously extracted and, therefore, no information regarding the contribution of each phase in TE binding is provided. However, before the publication of the Tessier procedure, Stover et al. (1976) developed an SE scheme for determining the forms and amounts of Pb, Zn, Cu, and Ni in anaerobically digested wastewater sludges obtained from twelve municipal treatment plants. The Stover SE scheme provides information regarding the TE present in exchangeable, adsorbed, organic matter-bound, carbonate and sulfide forms. It therefore allows discriminating metal distribution between the organic matter and sulfide fractions. However, the higher number of extraction steps compared to the Tessier scheme results in poor recovery of the TE extracted compared to the initial TE budget (van Hullebusch et al. 2005b). Many other SE procedures were later developed, or adapted, including the Bureau Communautaire de Reference (BCR) SE scheme, which has been developed to harmonize all SE studies and certified SE through inter-laboratory trials (Rauret et al. 2000), as well as the accelerated BCR SE scheme (Pérez-Cid et al. 1999), which significantly reduced the extraction time: from 2.5 days for the Tessier SE scheme to half a day.

A constant debate regarding SE schemes concerns the most suitable sample pre-treatment, namely the discussion around wet *versus* dried samples. Lately, Ortner et al. (2014) tested the modified Tessier scheme (A) (TE extraction from dried and milled samples) and an adapted version (B) (TE extraction from centrifuged wet

samples) on applicability for TE fractionation in different biogas slurries. Both methods provided reproducible results and fractionation features for the TE studied. Method A showed better reproducibility and lower coefficients of variation than method B due to the homogenization in pretreatment. However, sample pre-treatment by drying and milling was not only time-consuming, but also led to artificial shifting of TE towards insoluble fractions and thus did not reflect the native TE fractionation of the sample in method A. The so-called “bioavailable” fractions were, in particular, far better represented by method B, which was considered the more appropriate approach (Ortner et al. 2015).

Even though several SE schemes have been proposed, it must be mentioned that no single fractionation scheme achieves to dissolve distinct TE bearing phases exclusively and exhaustively (Figueiras et al. 2002). Nevertheless, despite uncertainties in the selectivity of the various extractants and possible problems due to re-adsorption and partial oxidation of oxygen sensitive elements (e.g. Fe and S), SE procedures are a well established, justified mean to study metal partitioning among the various solid phases of soils and sludges (see Figueiras et al. 2002 for overview).

Most of the TE analysed are cations, which are able to precipitate with anionic compounds such as carbonates, phosphates or sulfides. Apart from precipitation, there is evidence that carbonates and sulfides are also able to form metal complexes (i.e. $[\text{MeHS}]^+$ or $[\text{Me}(\text{HS})_2]$) and may serve in this form as a source of metal for cell uptake (Jansen et al. 2007; Rickard and Luther 2006). Due to the positive charge of some cationic TE (e.g. Ni, Co) they are also able to adsorb onto microbial cell or particle

surfaces. Consequently, these elements can be found in all fractions depending on concentration and physico-chemical conditions. In contrast Mo is mainly present as (molybdate, MoO_4^{2-}), a highly soluble anion which neither form any precipitates nor adsorb onto surfaces. However, under low redox (anaerobic) conditions Mo (VI) might be reduced to Mo (IV) and precipitation in form of MoS_2 is very likely in presence of sulfidic compounds (Mendel 2005).

There are also approaches to adapt existing SE procedures to better study bioavailability of anions, such as Se or Mo (Wright et al. 2003). However, Lenz et al. (2008c) showed that the interpretation can be biased by unselective extraction of targeted species and artefacts introduced during the extraction as subsequently discussed by Huang and Kretzschmar (2010) for As chemical species.

Other methodologies selected to obtain information on TE fractionation involved the use of SE combined with other approaches, such as sorption isotherm analysis.

It has been shown that combining SE with isotherm analysis is very useful when comparing TE fractionation in similar sample types prone to different physico-chemical conditions, either in static or dynamic systems. In fact, van Hullebusch et al. (2005a) investigated the mechanisms of Co and Ni sorption by anaerobic granular sludges under different experimental conditions (mono-metal and competitive conditions) by coupling SE with sorption isotherm analysis. The retention of TE in methanogenic (non-fed) sludges could be assessed by the latter, whereas the isotherms provided information on the retention capacity and the strength by which the sorbate is retained onto the sludge. However, the information gained through adsorption isotherms is

limited because the immobilization mechanisms of the metals by the solid phases could not be determined and the actual coordination chemistry of metals in various chemical phases could not be identified (van Hullebusch et al. 2004). In fact, due to the complexity of the metal retention processes in anaerobic sludges and the limitations associated with only considering isotherms in understanding sorption mechanisms, the aforementioned combination is highly recommended.

The combination of SE with further methodology, such as pH titration, can be used to gain more information on the species extracted in one specific fraction. Roussel (2013) found that most of manganese extracted in the exchangeable fraction of SE was bound with carbonate by pH titration. However, the pH of the first extracting solution dedicated to the exchangeable fraction is already around 3, which reduce the pH selectivity of the SE (Dodd et al. 2000). Dissolution of TE with slow drop of pH from 8 to 3 allows an increase of information on some chemical species of TE extracted in the SE fractions.

The use of SE was also shown to be useful to monitor the time evolution of TE fractionation during the operation of AD. For instance Zandvoort et al. (2005a) studied the effect of a sulfur source, as well as the type of sulfur source (L-cysteine or sulfate), on the performance and metal retention dynamics in an upflow anaerobic sludge bed (UASB) reactors using modified Tessier SE scheme (Osuna et al. 2004). Methanol-fed UASB reactors inoculated with Co preloaded granular sludge were used as a model system. Results showed that Co washed out from the sludge at similar rates in both reactors. The leaching of Co occurred at two distinct rates: first at a high rate due to

release mainly from the exchangeable and carbonate fraction; later at a relatively low rate due to release from the organic/sulfide fraction (Zandvoort et al. 2005a).

Another methodology to obtain information on TM fractionation is through simultaneously extracted metals / acid-volatile sulfides (SEM/AVS). In sediment geochemistry, the ratio between HCl-extractable metals (SEM) and AVS is used to assess toxic effects of TE in anaerobic systems (Di Toro et al. 1992; Di Toro et al. 1990). A ratio below 1 indicates the presence of sufficient sulfide in the system to bind TE as insoluble TE sulfides. This reduces TE toxic potential compared to situations with a lower sulfide content (in which $SEM/AVS > 1$). Although this theory does consider the toxicity of non-complexed sulfide, it can also be used to describe the potential (bio)availability of TM in anaerobic wastewater reactors (Jong and Parry 2004; Shakeri Yekta et al. 2012b; Van der Veen et al. 2007). In this respect, van der Veen et al. (2007) showed that sulfides are the dominating bonding form of essential TE Co and Ni in anaerobic methanogenic granular sludge, and, therefore, have a strong influence on TE bioavailability. Nonetheless, this methodology also presents flaws. As previously mentioned here, the oxidisable or so-called organic/sulfide fraction of the modified Tessier sequential extraction scheme is not a good indicator for TE sulfides. However, a high portion of Fe was extracted in the residual fraction with unknown composition, although an excess of sulfide (AVS) was present in the sludge that would allow to precipitate the iron content with sulfide (Van der Veen et al. 2007).

4.2. Solid TE speciation

In this sub-section, species-specific methods are covered. These methods, in principal, take advantage of the interplay between chemical forms and electromagnetic radiation i.e. spectroscopy. To be able to conduct species-specific spectroscopic measures in non-crystalline, complex matrices with low concentration of analyte, the high brilliance of synchrotron radiation (SR) is a prerequisite for success. There are currently about 50 SR lightsources around the world (www.lightsources.org seen on September 2016), but so far there are relatively few reports on the use of SR to characterize the chemical speciation of samples in AD (compare Table 2) mainly because of the relatively low TE concentrations that hamper the acquisition of good quality data.

Similar to organic soils and sediment, the matrix of AD samples is dominated by the low-mass elements H, C, N, O and S. Biological and physical structures built by these elements can be visualized in high detail by, for instance, confocal microscopy, and transmission and scanning electron microscopy (TEM and SEM, respectively). Coupling the latter with energy-dispersive X-ray absorption (EDX), TEM/SEM provides useful tools for elemental mapping linking structure to the occurrence of TE. To go one step further i.e. to provide species-specific information, ^{13}C -NMR and ^{15}N -NMR have been extensively used to characterize and quantify organic carbon functionalities (Kögel-Knabner 1997) in bulk. The recent development of SR techniques is opening up the possibility for species-specific identification of most elements in the periodic table. For the low-mass elements, C, N, S and Fe, K-edge XANES (X-ray absorption near-edge spectroscopy) is a useful technique. Using XANES data libraries of reference

compounds, the relative contribution of different classes of aliphatic and aromatic C compounds (carboxyls, alcohols, carbonyls, phenols) can be quantified in an unknown sample (Lehmann et al. 2005; Schumacher et al. 2005). Sulfur K-edge XANES provides a powerful tool to separate distinct reduced forms (e.g. FeS and FeS₂) from zero-valent S and polysulfides, which are difficult to distinguish using more conventional techniques (Mossbauer spectroscopy or XPS, resp.). Further, S K-edge XANES can also assess sulfoxides, sulfonates and sulfate and sulfate esters (e.g. Manceau and Nagy 2012; Pickering et al. 1998; Shakeri Yekta et al. 2012b; Xia et al. 1998), allowing to study S cycling on a molecular scale. Because reduced S species form stable complexes and solid phases with TE, the information provided by S XANES is essential for chemical speciation modelling of AD reactors (Shakeri Yekta et al. 2014a; Shakeri Yekta et al. 2014b). For Fe, species-specific information can, in principal, be provided by K-edge XANES (O'Day et al. 2004). Because S and Fe react with each other and form metastable and stable chemical compounds, such as FeS and FeS₂, a combination of S and Fe XANES is highly beneficial for the characterization of anoxic environments. In this way the uncertainty, which is in the order of 15% in the quantification of S and Fe species by fitting model compounds to their respective XANES spectra, could be lowered substantially. At SR beamlines with focusing lenses, XANES spectra for C, N, S and Fe can be provided with high spatial resolution (μ -XANES).

Still, due to the low abundance of TE in AD (and many natural systems), which is often below $\mu\text{g kg}^{-1}$, speciation remains inaccessible for spectroscopic methods. In this case, speciation of TE still needs to be calculated by thermodynamic or kinetic

modelling (e.g. Shakeri Yekta et al. 2014b). However, the on-going development of SR sources with increasing light brilliance keeps lowering the detection limits. As a drawback of the high photon flux, samples need to be protected from radiation damage. Today, Fe is the only metal that, at least in some AD samples, reaches concentrations that can be directly studied by use of EXAFS (extended X-ray absorption fine structure) spectroscopy in bulk samples. All other TE need to be added to samples to reach at least concentrations in the range 1-10 $\mu\text{g g}^{-1}$ to yield reasonable signal-to-noise ratios for XANES analysis. The drawback with this addition is obvious, and the chemical speciation in many cases would be expected to change from the situation in the AD reactor.

A technique that partly avoids the problem with low TE concentration samples is scanning transmission X-ray microscopy (STXM). This technique is primarily oriented towards the low mass elements, in particular C, and provides high-quality electron density microscope images in essence similar to SEM/TEM (Hitchcock et al. 2005; Kinyangi et al. 2006). On top of that high resolution, data of C XANES are extracted in each pixel. Thus, different types of organic compounds may be quantified down to a sub-nm spatial scale. Because of the high resolution of this technique, localised high concentrations of also heavier elements (e.g. TE) can be detected and specified. Thus, nano-particles of TE (e.g. metal sulfides) or local concentrations of TE at, e.g., cell surfaces or in cell vacuoles, can be identified by STXM (Behrens et al. 2012). One problem is that samples need to be very thin to enable transmission detection.

Therefore specific sample holder cells and detectors sensitive to TE fluorescence currently are under development at beamlines devoted to STXM.

The use of more conventional and more accessible spectroscopic techniques, other than synchrotron-based techniques in the characterization of TE speciation also offers important information. For example Electron Paramagnetic Resonance (EPR) is very sensitive with fluorescence allowing analysis of very low TE concentrations, yielding information on TE oxidation state, geometry, and chemical environment of, e.g., for Cu, Fe(II) and Fe(III). Mössbauer Spectroscopy (for Fe) can provide information on the nature of major solid phases, but also about the oxidation state of TE. Although these techniques are only useful for certain types of TE (see Table 2), they can contribute significantly to mechanistic knowledge on the fate of TE.

4.3. Combination of the TE fractionation and speciation approaches

Recently Shakeri Yekta et al. (2012b) investigated the effect of SE of TE on sulfur speciation in anoxic sludge samples from two lab-scale biogas reactors augmented with Fe. Analyses of S K-edge XANES spectroscopy and AVS were conducted on the residues from each SE step. The S speciation in sludge samples after AVS analysis was also determined by S XANES. Sulfur was mainly present as FeS (~60% of total S) and reduced organic S (~30% of total S), such as organic sulfide and thiol groups, in the anoxic solid phase. Sulfur XANES and AVS analyses showed that during first step of the extraction procedure (the removal of exchangeable cations), a part of the FeS fraction corresponding to 20% of total S was transformed to zero-valent S. Fe was not released into the solution during this extraction step. After the last extraction step

(organic/sulfide fraction) a secondary Fe phase was formed. The change in chemical speciation of S and Fe occurring during SE procedure suggests indirect effects on TE associated to the FeS fraction that may lead to incorrect results. Furthermore, by S XANES it was verified that AVS extraction quantitatively dissolved the FeS fraction. The present results identified critical limitations for the application of SE for trace metal speciation analysis outside the framework for which the methods were developed.

Table 3 summarizes the main drawbacks and advantages of each chemical extraction method described in this section.

5. MODELLING OF TE SPECIATION

Analytical techniques for TE speciation studies often do not provide information on the overall TE speciation. Furthermore, their application in AD practices encounters some limitations with respect to, e.g., sample disturbances due to sample collection and preparation, potential analytical interferences caused by complex chemical matrices, and detection limit of the instruments. Accordingly, modelling of TE speciation is widely used as a theoretical approach to either compile or verify the analytical results. Chemical speciation modelling approaches are based on the generic assumption of 'local equilibrium', which implies that metal forms are predicted at their thermodynamic equilibrium state, while kinetic factors and intermediate species involved are not taken into account. In this section, an overview of the applications of TE speciation modelling in the context of AD and reflections upon important considerations for future development of TE speciation models are provided.

5.1. Chemical speciation modelling of TE in AD context

So far, relatively few studies have explored application of chemical equilibrium modelling approaches for assessment of TE speciation and bioavailability in AD processes (e.g. Aquino and Stuckey 2007; Callander and Barford 1983a; Callander and Barford 1983b; Jansen et al. 2007; Shakeri Yekta et al. 2014b). An early TE speciation modelling approach was developed and implemented for the assessment of TE ion availability in anaerobic digesters (Callander and Barford 1983a; Callander and Barford 1983b). By evaluating the discrepancy between the model-predicted and measured TE solubility, these researchers studied the importance of precipitation and chelation reactions on the overall solubility of TE (Callander and Barford 1983b). Aquino and Stuckey (2007) implemented the chemical speciation modelling to assess TE speciation in the absence of biomass during AD. By comparing the modelling results with TE fractions measured using sequential extraction methods, the authors evaluated the effects of “biomass and microbial chelators” on TE speciation.

Later on, Jansen (2004) implemented a combined TE bio-uptake and speciation modelling approach to quantify TE uptake under well-defined experimental conditions in relation to the microbial activities. The modelling results were used to assess the response of the microbial community to TE deficiency and excess in terms of the regulation of TE uptake. In a later study, Jansen et al. (2007) provided evidence that the discrepancy between model-predicted and measured TE solubility may not entirely be related to the effect of biological processes. Based on the chemical speciation modeling results, it was argued that the solubility of TE in their system may instead be related to

dissolution kinetics and formation of soluble metal-sulfide species. Furthermore, chemical speciation models may be combined with TE fractionation techniques to determine the nature of the extracted compounds in each fraction. Roussel (2013) used the chemical speciation modelling to confirm the extraction of vivianite and iron sulfide precipitates in the exchangeable and residual fractions of the BCR sequential extraction, respectively. In a study carried out by Shakeri Yekta et al. (2014a), the interrelationship between overall chemical speciation of S, Fe, and TE was assessed by comparison of the model-predicted and measured solubility of metals under different S and Fe concentrations, as the most important chemical factors regulating the TE speciation during AD.

5.2. Considerations for developing TE speciation models in AD context

Development of thermodynamic models for prediction of TE speciation during AD requires information on the nature of metal-binding compounds present and the stability constants of their reactions with TE. Three main metal-binding groups in the AD processes include: (i) inorganic and organic ligands which control soluble TE complex formation and precipitation-dissolution reactions; (ii) inorganic and organic surface active sites which promote adsorption-desorption reactions of TE; and (iii) microbial biomass whose affinity and capacity for binding TE determine the interaction of TE with microorganisms (Callander and Barford 1983a; Callander and Barford 1983b; Hughes and Poole 1991; Nolan et al. 2003; Jansen 2004; Worms et al. 2006; Reeder et al. 2006; Fernández-Martínez and Charlet 2009; Hooda, 2010). The microbial response to metal deficiency and excess, such as variation in bio-uptake affinity and/or metal efflux

out of the cells are also important components for development of TE speciation models (see Jansen (2004) and Jiang (2006)).

A comprehensive selection of stability constants for various organic and inorganic ligands can be found in databases, such as from the International Union of Pure and Analytical Chemistry Critical Database (Pettit and Pettit 2009), National Institute of Standards and Technology (www.nist.gov), and Joint Expert Speciation System (http://jess.murdoch.edu.au/jess_home.htm, seen on September 2016). A suite of chemical speciation modelling software is also available which are supplied with thermodynamic databases (e.g. MINEQL (Westall et al. 1976); MINTEQA2 (Allison et al. 1991); The Geochemist's Workbench (Bethke 1992); WHAM (Tipping 1994); PHREEQC (Parkhurst and Appelo 1999); Visual MINTEQ (<http://vminteq.lwr.kth.se/>, seen on September 2016) (Gustavsson et al. 2013)). Common multicomponent thermodynamic equilibrium modelling approaches are used by these software (e.g. (Lichtner 1996; Morel and Morgan 1972; Yeh and Tripathi 1989). Major input data required for application of chemical speciation modelling software and expected output information are summarized in Figure 6.

5.2.1. Aqueous complex formation and precipitation-dissolution

Modelling of TE complex formation and precipitation-dissolution reactions requires quantitative information on major inorganic ligands present in the anaerobic digesters such as carbonate, phosphate, sulfide, and polysulfides (Callander and Barford 1983a; Shakeri Yekta et al. 2014b). In addition, information regarding the major cations present in the digester is also needed as model input in order to account for their ionic exchange

and competition with TE for binding to ligands and negative surface sites. This information is also essential for a proper estimation of the ionic strength in the system (Figure 6).

Due to compositional complexity and variety of organic substrates used in AD processes, a multitude of organic ligands emerge as a result of chemical and microbial processes. Shakeri Yekta et al. (2012a) demonstrated that composition of dissolved organic matter in a number of stirred tank biogas reactors in a relatively narrow mass window of 150 to 700 Da includes 900 to 1900 individual molecules. Organic matter characterization in AD processes mainly aimed for assessing the transformation and structural change of different classes of organic compounds (e.g. Greenwood et al. 2012; Tambone et al. 2013) and information on their TE-binding properties is scarce. Organic functional groups with high affinity for TE are mainly phosphoryl, carboxyl, amino, alcohol, phenol, and thiol groups (Smith et al. 2002). Among those, thiols are suggested to be substantially influential on TE speciation in AD processes (Shakeri Yekta et al. 2014a). Accordingly, complexity of the pool of organic matter in AD and lack of information on their metal-binding properties may impede development of reaction networks for TE speciation modelling based on interaction of individual organic molecules with TE.

Several generic approaches are presented for modelling of TE binding to natural organic matter such as Gaussian Dissolved Organic Matter, Non-Ideal Competitive Adsorption, and Stockholm Humic Models (Gustafsson 2001; Kinniburgh et al. 1996; Perdue et al. 1984) which may be used in the context of AD. Dudal and Gérard (2004)

discussed the ways that interactions of TE and organic matter have been integrated in different chemical speciation models, emphasizing the need for fundamental knowledge regarding characteristics and TE-binding properties of the pool of organic compounds to improve current approaches. Thus, identification, characterization, and quantification of major TE-binding organic functional groups with high affinity towards TE (e.g. carboxyl, amino, and thiol groups) may be a first step for inclusion of the effects of organic compounds into TE speciation models.

5.2.2. Sorption

The surface active sites contributing to TE sorption during AD processes originate from influent and endogenous inorganic minerals, solid organic matter, and microbial biomass. For example, it has been demonstrated that extracellular polymeric substances and FeS(s) particles, which are extensively formed during AD, have high TE adsorbing affinity (van Hullebusch et al. 2006; Watson et al. 1995). Furthermore, uptake of TE by microorganisms initially involves TE adsorption to membrane-bound active sites and association to cell surface (Mason 2013). The adsorption isotherms are widely used for analysis of TE sorption (e.g. Langmuir, Freundlich, Dubinin Raduskevich, Redlich–Peterson, etc.; Fomina and Gadd 2014; Volesky 1999). The models are inherently empirical and provide numerical relationships between concentration of sorbed and soluble TE under defined experimental conditions. Sorption models cannot be utilized to predict metal adsorption behaviors under different conditions (i.e. pH, ionic strengths and TE concentrations, (Goldberg and Criscenti 2007)) due to their empirical nature. Furthermore, empirical adsorption models do not take the sorption mechanisms

into account and their applications for adsorption studies in complex systems are limited (Fomina and Gadd 2014). Alternatively, multi-component models (e.g. Brunauer-Emmett-Teller) may be used to express multilayer adsorption in complex biological systems (Morley and Gadd 1995).

Surface complexation models provide a deeper description of TE sorption by calculating adsorption equilibrium through mole balance and mass action equations. The main advantage of these pseudo-mechanistic equilibrium models is that they are able to describe the effects of the operating conditions, such as pH, in terms of supposed reactions among active sites and metal species in solution (Guibaud et al. 2008; Stumm 1987). However, their applications in describing adsorption by biological materials are rather limited. In order to use these models, the adsorption mechanisms and types of surface complexes must be specified for all adsorbing metal ions. These models can be characterized by different degrees of complexity or accuracy to account for surface heterogeneity and other factors contributing to non-ideal sorption phenomena in a system (Stumm 1987). In addition, microbial uptake of TE may be explained by surface complexation reactions between metal ions and membrane-bound organic acids (Flynn et al. 2014). Thus, modelling the adsorption-desorption reactions between TE and biomass may be used to link the chemical speciation to bio-uptake of TE.

5.2.3. Modelling uncertainties

The results of TE speciation modelling using main-stream databases and software need to be critically evaluated considering potential errors arising from human intervention

during data compilation and possible use of thermodynamically inconsistent set of data (May and Filella 2011). Furthermore, thermodynamic uncertainties can be related to application of stability constants for TE speciation analysis in a system whose chemical condition is considerably different from the laboratory conditions under which the constants were obtained (e.g. different ionic strengths and pH, (VanBriesen et al. 2010)). Stochastic approaches can be utilized for correction of stability constants when the chemical environment of a system substantially deviates from original laboratory conditions (e.g. Weber et al. 2006).

The level of uncertainty in the results of TE speciation modelling is also linked to the “system’s competitiveness” (VanBriesen et al. 2010). Under the chemical environments where one TE specie dominates, the uncertainty is mainly related to its stability constant. However, the uncertainty increases when more and more species with similar stability constants contribute to speciation of TE. Accordingly, the stability constants of TE reactions with dominant organic and inorganic ligands in AD systems need to be specifically evaluated. A number of computer codes are developed which allow for assessment of uncertainties in speciation modelling results associated with stability constants and environmental factors such as ionic strength changes and elemental composition (e.g. Ekberg and Ödegaard-Jensen (2011); Pettit and Pettit (2009)). To the best of our knowledge, assessment of the uncertainties in application of stability constants for TE speciation analysis in the context of AD is scant.

5.3. Inclusion of biological processes in TE speciation models

Biological activities can influence TE speciation through a range of possible mechanisms including precipitation, dissolution, sorption, chelation, and redox transformation (Lemire et al. 2013; Sunda 1988; Worms et al. 2006). The availability and speciation of TE can, therefore, influence, and be influenced by, microbial ecology (Bennett et al. 2001; Morel and Price 2003; Uroz et al. 2009; Ünal et al. 2012). Numerous studies have focused on microbial utilization of TE (particularly iron) in well-oxygenated ocean surface waters (e.g., Hutchins et al. 1999; Morel and Price 2003; Vraspir and Butler 2009). For instance, Hutchins et al. (1999) investigated the competition between prokaryotes and eukaryotes for organically-bound iron. The chemical nature (i.e. speciation) of available iron and biogenic organic complexes was shown to be able to steer the growth of eukaryotic phytoplankton or prokaryotic picoplankton (cyanobacteria) indicating its influence on microbial ecology (Hutchins et al. 1999). However, far fewer studies have investigated the role of other TE in marine microorganisms living in low-oxygen environments (Glass et al. 2015). Indeed, under such conditions, microorganisms may need to adapt for TE acquisition and utilization due to lower bioavailability as a result of metal sulfide precipitation. These survival strategies can generally be classified into four categories: scavenging ; shifting speciation ; storage ; and substitution (see Glass et al. 2015 for further details). Glass et al. (2014) showed that microbial consortia catalyzing anaerobic oxidation of methane (AOM) were able to utilize scarce micronutrients (cobalt and tungsten) in addition to nickel and molybdenum. They suggested that AOM consortia use specialized

biochemical strategies to overcome the challenges of TE availability in sulfidic environments.

Although the relationships between specific microorganisms, or consortia, and TE has been documented (as in Duxbury 1985; Hughes and Poole 1991; Plugge et al. 2009; Thauer et al. 2010; Worm et al. 2011). It is unlikely that even near-complete information has been uncovered on the TE requirements of uncultured microorganisms in complex, multi-species consortia underpinning AD ecosystems (Feng et al. 2010; Ünal et al. 2012; Munk and Lebuhn 2014; Westerholm et al. 2015). Therefore, new experimental and investigative approaches to identify, and quantify, the impact of TE on AD ecosystems are now required. Examples include tests to measure the influence of TE on rates of biomethane production and hydrolytic activity; as well as protein production assays employing radiolabeled metals to study incorporation into, and direct influence on the activity of, metalloproteins (e.g., *Clostridium thermoaceticum* + ^{75}Se (Andreesen and Ljungdahl 1973) and *Helicobacter pylori* + ^{63}Ni (Eitinger and Mandrand-Berthelot 2000)).

A modelling approach aiming to deal with chemical and biological interactions in aqueous systems was proposed by VanBriesen and Rittmann (1999) *i.e.* Co-Contaminants in a BATCH reactor (CCBATCH). In CCBATCH, the aqueous speciation affects the biodegradation reactions, where the effect of biological reactions on the concentration of chemical species (e.g. H_2CO_3 , NH_4^+ , O_2) are explicitly taken into account. Bulk-phase chemical speciation reactions including acid-base and complexation reactions are thermodynamically controlled, while biological processes

are modeled as kinetically-controlled reactions. A key feature of CCBATCH is its use of the mass balance on proton condition (*i.e.* H^+) as the keystone for linking processes that involve production of acid or base. The main CCBATCH modelling limitations are the following: (i) CCBATCH employs the Newton-Raphson method as numerical solution technique which requires user-input starting values “in the neighborhood” of the solution; (ii) Uncertainty due to the complexation constants, (iii) Precipitation and sorption processes are neglected, (iv) only reactions in batch (non-transport) systems can be modeled.

Following the first CCBATCH model, several extensions have been introduced. Rittmann et al. (2002) developed the biogeochemical framework in CCBATCH in order to include precipitation and dissolution phenomena. Willet and Rittmann (2003) included a new sub-model for slow aqueous complexation reactions. Finally, Schwarz and Rittmann (2007) expanded the CCBATCH model in order to take into account surface complexation and the formation of soluble and solid products. This latest model version has been applied to understand the relative importance of the various key ligands of sulfidic systems in Zn detoxification. Based on CCBATCH, a new model made for modelling the biofilm anode *i.e.* PCBIOFILM (Marcus et al. 2011). The model is founded on the framework of CCBATCH, but PCBIOFILM tracks the analytical concentrations of chemical species in the aqueous phase of the biofilm (Marcus et al. 2011). Recently, chemical speciation software included the models for bio-uptake of TE in order to link chemical speciation of TE to metal toxicity (*e.g.* inclusion of Biotic Ligand Model in Visual Minteq 3.1, <http://vminteq.lwr.kth.se/>, seen on September 2016). Up to this point,

the database available for this combined model is limited to a few numbers of TE and microorganisms.

6. CONCLUSIONS AND RESEARCH NEEDS

When one wants to determine total TE content as well as true TE fractionation and / or speciation, dedicated liquid and solid phase sampling and preservation methodology should be implemented.

TE content in liquid phase is rather low and in addition the free TE (*i.e.* bioavailable species) usually display lower concentration of several order of magnitude compared to total dissolved TE. Direct TE speciation information is therefore very difficult to capture due to sampling artefacts as well as analytical limitations.

TE fractionation or speciation in solid phase can be assessed by chemical extraction methods or spectroscopic methods, respectively. The former one might be easily implemented, yet is limited in the depth of information; the later one provides a molecular understanding, yet requires the use of advanced, laborious techniques.

In contrast, modelling approaches may allow to process simultaneously TE speciation and to combine such information to methane production which is the most interesting information for process engineer. However, the complexity of the system on the one hand, and insufficient / inaccurate knowledge on thermodynamic constants may be limits to this approach.

From the literature and the discussion presented in this review, further research is particularly required in the following areas to better assess the role of TE in anaerobic digesters performance:

1. Standard sampling procedures should be developed for the collection of liquid and solid samples from anaerobic digesters to allow TE preservation, or at least to minimise TE speciation changes,
2. For a deeper understanding of the dynamic behaviour of TE speciation and bioavailability in anaerobic digesters, advanced analytical methods are required to determine, directly or indirectly, the concentration of bioavailable TE and to identify pitfalls that should be avoided.
3. Simple analytical procedures should be developed for the use of AD operators, to allow assessment of TE “bioavailability”
4. One should be able to link TE speciation and bioavailability with microbial growth and anaerobic digester performance by monitoring the dynamics and activity of microbial populations; much greater insight, and more data, on the microbial ecophysiology of TE will be needed to support this goal.
5. One should be able to integrate TE speciation and bioavailability information into anaerobic digestion models (e.g. ADM1) allowing the prediction of AD performance in terms of biogas production. The information required should be sufficiently easy to obtain to be used by operators on site to optimise the biogas production in the case of TE limitation.

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Table 1. TE concentration measured or calculated in AD (modified from Schattauer et al. (2011)).

	References								
	h	d	c	e	f	b	a	g	i
	μM [fresh matter]								
B							0.092 - 1.02 × 10 ³		79.5 - 442.1
Ca						>13.5 - 998.1			
Co	1.02	0.0 5 - 1.0 2	8.5 - 339	0.05 - 1.0	0.05 - 1.0	>0.01 - 2.0	1.0	0.4 - 169. 7	0.5 - 27.8
Cu							0.9 - 1007.2		0.4 - 25.8
Fe		17. 9 - 179	179 - 3.6 10 ³	17.9 × - 179	17.9 - 170	>5 - 902.6			859 - 1.09 × 10 ⁵
Mg						14.8 × 10 ³ - 1.97 × 10 ⁵			2.7 × 10 ³ - 1.65 × 10 ⁵
Mn	0.09 - 910						0.09 - 1001		103.2 - 1354
Mo	0.52	0.0 5 - 0.5 2	1.04 - 3.64	0.05 - 0.52	0.05 - 0.52	>0.01 - 0.5	0.52	1.7 - 521	1.4 - 4.8
Ni	0.1	0.0 85 - 8.5	8.5 - 511	0.08 5 - 8.5	0.085 - 8.5	0.1 - 85.1	0.1	0.4 - 10.6	3.9 - 61.2
S						9.98 - 5.1 × 10 ⁵			1.1 × 10 ³ - 7.2 × 10 ³
Se (IV)	0.1		1.3 - 4.4	0.1		1 - 10	0.1		0.13 - 5.7
W			0.5 - 1.9	0.5 - 2.2		0.09 - 99.5			
Zn									131 - 1044

^aSahm (1981)

^bTakashima & Speece (1990)

^cKloss (1986)

^dWeiland (2006)

^eSeyfried et al. (1990)

^fMudrack and Kunst (2003)

^gPobeheim et al. (2010)

^hBischofsberger (2005)

^lSchattauer et al. (2011)

Table 2. Methods available for TE and major elements speciation in samples from AD reactors.

Methods	Elements	Advantages	Drawbacks
TEM/SEM-EDX	C, N, S, Fe, TE, others	Microscopic technique providing contrasting images of electron density to identify biological and physical structures coupled to element mapping. Complementary to techniques below.	No species-specific information.
XANES (NEXAFS)	C, N, S, Fe, TE, others	Can be used to separate classes of mainly low mass elements.	Character more of fingerprinting than strict species-specificity.
EXAFS	Fe, (TE)	A non-destructive method in complex matrices that provides species-specific information.	The sensitivity is restricted to about 1-10 $\mu\text{g g}^{-1}$ for the most brilliant 4 th generation SR sources. Radiation damage is a concern.
μ -XANES, μ -EXAFS	C, N, S, Fe, (TE)	Provides XANES/EXAFS species-specific information at micro-scale.	Similar to conventional XANES/EXAFS but higher energy flux required
STXM	C, S, TE	Localized (nm scale) species-specific (XANES) information in contrasting images	Sub nm thickness of samples required making the method less sensitive to dispersed elements. For TE high local concentrations is required (e.g. nano-materials)
XPS	C, S, Fe, TE	Surface sensitive method for chemical speciation within the nm scale of surfaces	TE addition is required due to insensitivity
EPR	Paramagnetic elements (Cu, Fe, Mn, Cr ...)	Non-destructive method Very sensitive Easy and fast data acquisition	Data sometimes difficult to analyze
Mössbauer	Fe	Non-destructive method Best technique for quantitative determination of	Restricted to Fe

		the valence state of iron and identification of various iron oxides.	
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Table 3 : Advantages and drawbacks of different chemical extraction methods.

Method	Advantages	Drawbacks	Reference
Tessier Sequential extraction	Easy to implement TE fractionation	Not designed for anoxic samples fractionation	(Tessier et al. 1979)
Modified Tessier sequential extraction	Easy to implement TE fractionation	Designed for anoxic samples fractionation	(Osuna et al. 2004; van Hullebusch et al. 2005b)
Accelerated BCR extraction scheme	Standard procedure, Certified reference materials available	Only few fractions, not designed for anoxic samples fractionation	(Pérez-Cid et al. 1999)
SEM-AVS	Easy to implement	Fail to determine TE bioavailable concentration	(Di Toro et al. 1992; Di Toro et al. 1990)
SEM-AVS + sequential extraction	Determination of sulfide contribution to TE binding	Both procedure induces artefacts e.g. sulfide oxidation into elemental sulfur, laborious, no routine method	(Van der Veen et al. 2007)
SEM-AVS + sequential extraction + XAS S K-edge	Determination of sulfide contribution to TE binding		(Shakeri Yekta et al. 2012b)

SE + XAS	Extraction easy to implement, species specific information by XAS	Laborious, no routine method	(Lenz et al. 2008c)
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Table 4 : Advantages and drawbacks of different modelling methods

Methods	Advantages	Drawbacks	Reference
Modelling precipitation-dissolution and dissolved complex formation (organic and inorganic ligands)	Availability of stability constants and modelling software	Need for quality assessment of stability constants Lack of information on the TE interactions with organic compounds	(Pettit and Pettti 2009) (Dudal and Gérard 2004)
Modelling adsorption-desorption reaction	Isotherm: Simple expressions, interpretable parameters Surface complexation: Including effects of the operating conditions, describing non-ideal adsorption	Isotherm: adsorption parameters are case specific due to empirical nature of the models Surface complexation: need for information on types of surface complexes	(Fomina and Gadd 2014) (Guibaud et al. 2008)
Modelling microbial regulation of TM uptake	Potential application for process operation and control	Complexity of microbial dynamics in AD processes is not captured	(Willet and Rittmann 2003) (Schwarz and Rittmann 2007)

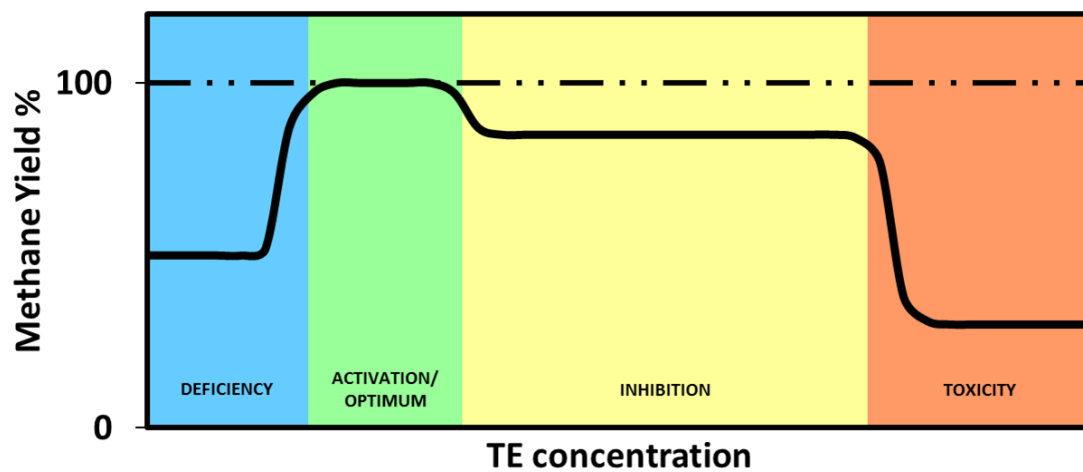


Figure 1. Effect of TE concentration in AD showing deficiency, optimal, inhibitory and toxic effect on methane production yield.

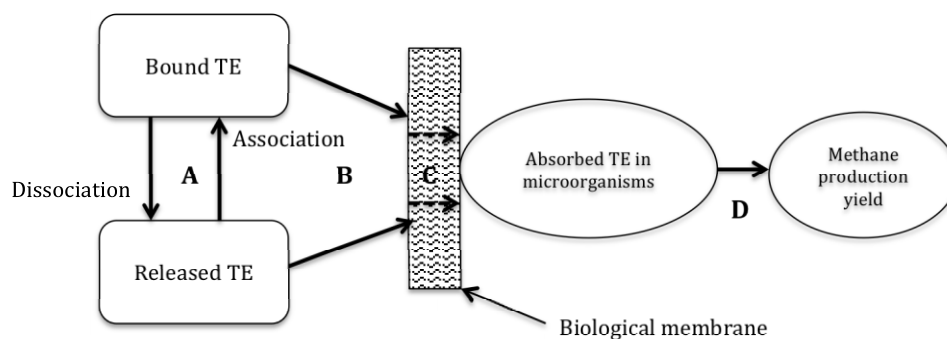


Figure 2. Conceptual simplified representation of TE bioavailability in anaerobic digesters (adapted from NRC (2003)). A, B and C are related to bioavailability processes : TE interactions between phases, transport of TE to microorganisms and bio-uptake of TE through the biological membrane, respectively. D represents the biological response (i.e. methane production yield) as a function of the bioavailable TE intracellular concentration.

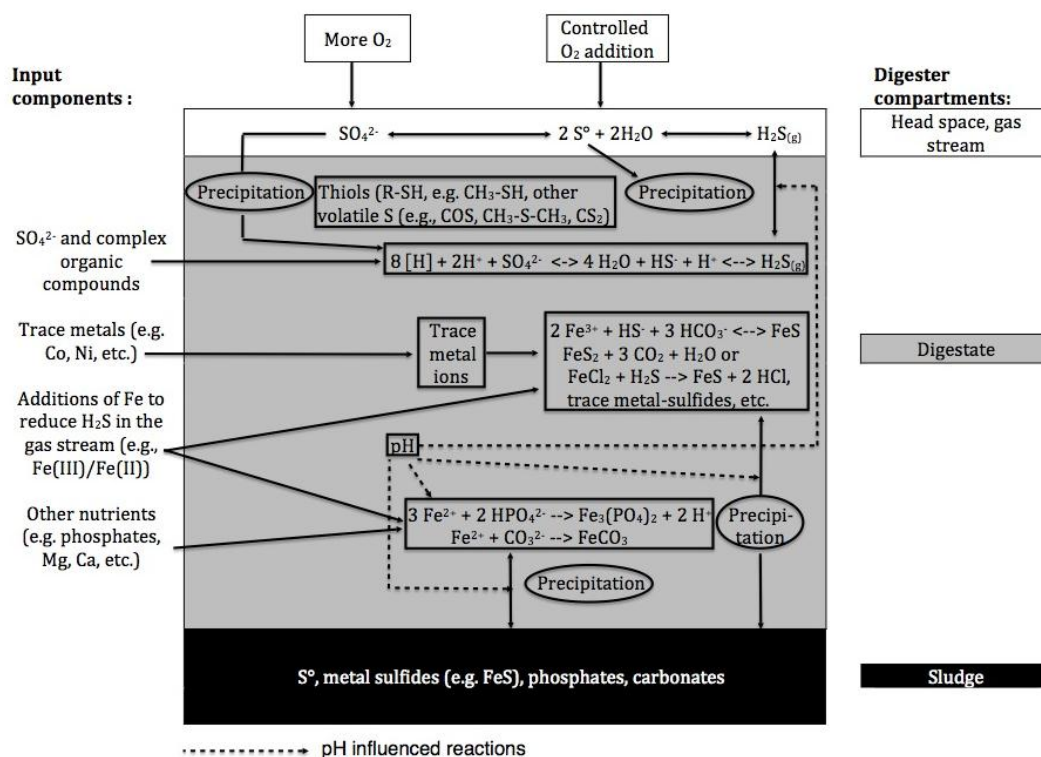


Figure 3. Sulfur turnover in biogas bioreactors and its influence on TE and nutrients speciation (modified from Möller and Müller (2012)).

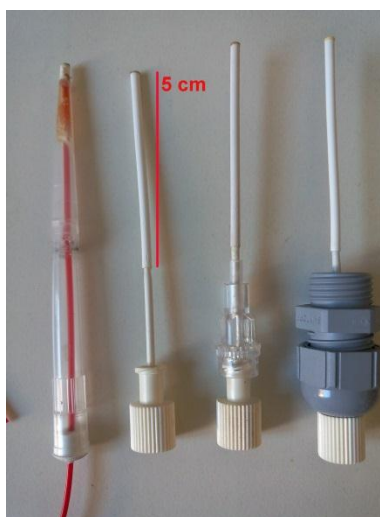


Figure 4. The figure shows from left to right a self-manufactured probe for microprofiling with 3 mm hollow fibre (Fabricius et al. 2014), a 5 cm hollow fibre probe with Luer connector (PIJPKER Laboratorium Technik, NL), the same probe type with a Luer connector which can be glued into a reactor walls (BBraun, Germany) and a Skintop PG connector (LAPPKABEL, Germany) that can be screwed into a reactor wall.

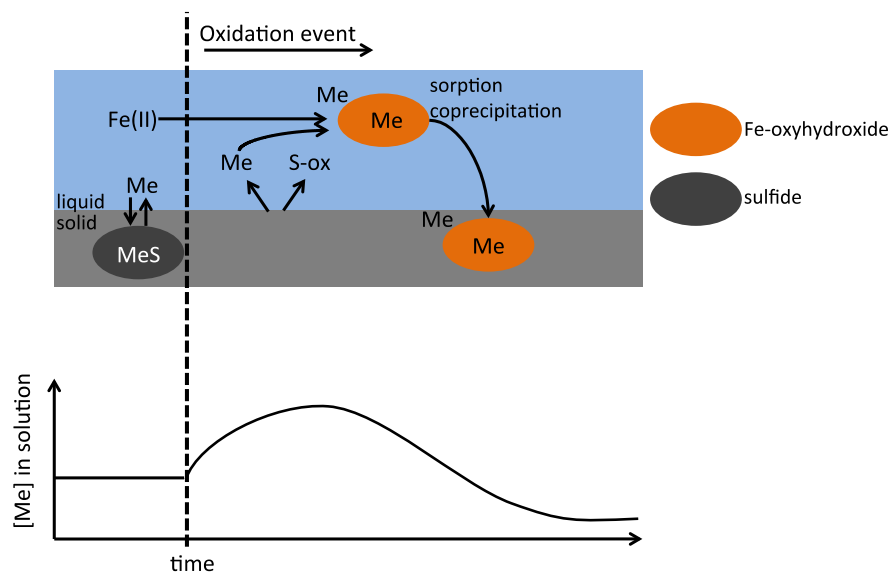


Figure 5. Conceptual scheme showing the impact of anoxic samples oxygenation on the TE solubility.

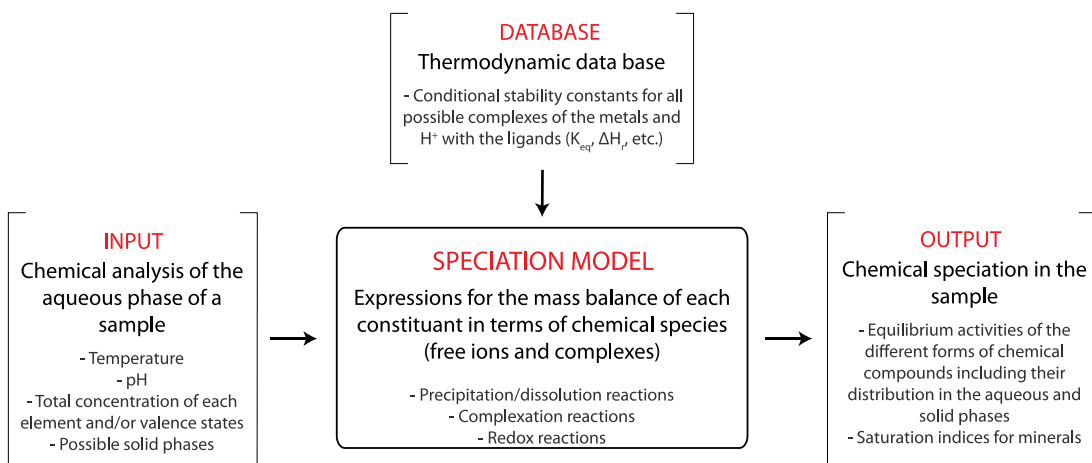


Figure 6. A summary of major input data for application of chemical speciation modelling software as well as output information.