Chapter 2

Biogeochemistry of trace elements in anaerobic digesters

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ABSTRACT

Essential trace elements (TE) are a prerequisite that ensures optimal performance of the anaerobic digestion (AD) process. However, finding the proper way to deliver these micronutrients to microbial communities is not an easy task. The chemical speciation of TE and the complex environment characterizing AD play a critical role in their mobility, bioavailability, and toxicity. These aspects are particularly
critical when establishing the total versus bioavailable concentration of TE, by properly balancing the two sides of the same coin, namely essentiality and toxicity. Both non-redox sensitive (e.g. Co, Cu, Ni, Zn) and redox-sensitive (e.g. Fe, Mn, Mo, Se, W) elements engage in a complex interplay with the mineral and organic phases present in AD. In addition, TE can also interact with each other, thus further complicating our current understanding. All these ‘parasitic’ reactions may render a large fraction of supplemented TE non-bioavailable for the efficient degradation of organic matter by microbial consortia, therefore limiting the biomethane yield. Current analytical limitations related to sampling and assessing the speciation, bioavailability, and matrix (liquid/solid) analysis add to the difficulty of understanding the bigger picture. This chapter reviews and discusses at length all these aspects, providing an up-to-date presentation of the biogeochemistry of TE in AD.

**KEYWORDS**: anaerobic digestion, biogas, biogeochemistry, resource recovery, trace elements

### 2.1 INTRODUCTION

Anaerobic digestion (AD) is a biological process commonly used for the treatment of organic waste streams and production of methane as a renewable energy carrier. The effluents from anaerobic digesters, so called digestate, contain high levels of nutrients such as nitrogen (N) and phosphorous (P) and can be applied as a sustainable alternative to conventional mineral fertilizers in agricultural practices (Monlau *et al.*, 2015). Efficient AD of organic substrates requires a balanced amount of trace elements (TE) for growth and activities of the diverse microbial consortia involved in AD of organic matter (Schnürer, 2016). Since the feedstock of AD processes may lack sufficient amounts of TE, a constant TE supplementation by operators is often required to assure the stability and efficiency of the AD processes (Demirel & Scherer, 2011). It has been well documented that the absence of key TE results in a serious underperformance of the AD processes (e.g. Banks *et al.*, 2012; Gustavsson *et al.*, 2011; Jarvis *et al.*, 1997; Moestedt *et al.*, 2015; Molaey *et al.*, 2018a; Schmidt *et al.*, 2014). Several TE such as iron (Fe), nickel (Ni), cobalt (Co), molybdenum (Mo), selenium (Se), tungsten (W), or zinc (Zn) are identified as essential additives to the AD processes in which TE deficient feedstock is treated (Glass & Orphan, 2012).

A number of TE, including Fe, Mn, Mo, Se, or W, may exist under several valence states, implying that redox conditions play an important role in the regulation of their chemical speciation. Furthermore, non-redox sensitive TE, including Co, Ni, or Zn, often demonstrate a restricted mobility under anaerobic conditions as a result of their limited valence-state interchange (Shakeri Yekta *et al.*, 2014). Once introduced in the anaerobic system, redox-sensitive and non-redox sensitive TE are involved in a complex network of reactions, such as oxidation-reduction, precipitation,
co-precipitation, adsorption, and ion exchange, which may limit their availability for microorganisms (Fermoso et al., 2015). Accordingly, TE concentrations supplemented to AD processes often exceed the ‘trace levels’ required by microorganisms (Pinto-Ibieta et al., 2016). This raises the challenging question of TE addition optimization, which requires an understanding of the biochemical processes regulating the chemical speciation and bioavailability of TE in AD processes. Over-supplementation of TE is counterproductive as it: (i) entails additional process costs; (ii) elicits toxicity of TE with low toxicity threshold concentrations (e.g. Se); and (iii) poses restriction for down-stream application of digestate as fertilizer/soil conditioner due to its TE contents.

The purpose of this chapter is to review and discuss the current state of knowledge on the biogeochemistry of redox sensitive and non-redox sensitive TE with relevance in AD. In addition, important aspects related to total versus bioavailable TE concentration and the challenge to assess speciation and bioavailability of TE for optimum dosing to anaerobic digesters will be discussed.

2.2 TOTAL VERSUS BIOAVAILABLE TRACE ELEMENTS

The determination of total TE concentration is important when one is seeking to evaluate the potential effect of the deficiency or excess of TE on AD processes. However, it has been reported that the total TE concentration is a poor indicator of the elemental fraction available to microorganisms (Thanh et al., 2016). As shown in Table 2.1, the optimal TE concentrations required for optimal operating conditions in anaerobic bioreactors, based on total TE content, differ by as much as four orders of magnitude (Schattauer et al., 2011). However, the chemical form of the reported elements is not considered, which obviously has an important impact on TE bioavailability as discussed by Thanh et al. (2016).

In order to illustrate the importance of TE speciation on methanogens activity, Bartacek et al. (2008) investigated the influence of Co speciation on its toxicity to methylotrophic methanogenesis in anaerobic granular sludge. The Co speciation in three different media that contained varying concentrations of complexing ligands (carbonates (CO$_3^{2-}$), phosphates (PO$_4^{3-}$) and ethylenediaminetetraacetic acid (EDTA)) was studied. Three Co fractions (total Co added, dissolved Co and free Co ion) were measured in the liquid media and were correlated with data from batch toxicity experiments. The average concentration of Co that was required for 50% inhibition of methanogenic activity (IC$_{50}$) for free Co$^{2+}$ in the three sets of measurements was about $1.3 \times 10^{-5}$ mol L$^{-1}$ in the three different media. Complexation (and/or precipitation) with EDTA, PO$_4^{3-}$ and CO$_3^{2-}$ was shown to decrease the toxicity of Co on methylotrophic methanogenesis. However, the free Co concentration is considered as the key parameter to correlate with Co toxicity onto methanogens, as this metal species is defined as being fully bioavailable. Such a conclusion
Table 2.1 TE concentration measured or calculated for optimal operating conditions in AD (modified from Schattauer et al. 2011).

<table>
<thead>
<tr>
<th>Element</th>
<th>μM (Fresh Matter)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>0.092–1.02 × 10³&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.5–442.1&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ca</td>
<td>&gt;13.5–998.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>1.0</td>
<td>&gt;0.01–2.0</td>
</tr>
<tr>
<td>Cu</td>
<td>0.9–1007.2</td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>&gt;5–902.6</td>
<td>179–3.6 × 10³</td>
</tr>
<tr>
<td>Mg</td>
<td>14.8 × 10³–1.97 × 10⁵</td>
<td>2.7 × 10⁵–1.65 × 10⁵</td>
</tr>
<tr>
<td>Mn</td>
<td>0.09–1001</td>
<td>0.09–910</td>
</tr>
<tr>
<td>Mo</td>
<td>0.52</td>
<td>&gt;0.01–0.5</td>
</tr>
<tr>
<td>Ni</td>
<td>0.1</td>
<td>0.1–85.1</td>
</tr>
<tr>
<td>S</td>
<td>9.98–5.1 × 10³</td>
<td>1.1 × 10³–7.2 × 10³</td>
</tr>
<tr>
<td>Se(IV)</td>
<td>0.1</td>
<td>1–10</td>
</tr>
<tr>
<td>W</td>
<td>0.09–99.5</td>
<td>0.5–1.9</td>
</tr>
<tr>
<td>Zn</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Sahm (1981).  
<sup>b</sup>Takashima <i>et al.</i> (1990).  
<sup>c</sup>Kloss (1986).  
<sup>d</sup>Weiland (2006).  
<sup>e</sup>Seyfried <i>et al.</i> (1990).  
<sup>f</sup>Mudrack & Kunst (2003).  
<sup>g</sup>Pobeheim <i>et al.</i> (2010).  
<sup>h</sup>Bischofsberger <i>et al.</i> (2005).  
<sup>i</sup>Schattauer <i>et al.</i> (2011).
could be extrapolated to cases where concentrations of Co are added to stimulate the methylotrophic methanogens with a free Co ion concentration being optimal for a concentration in the range of $10^{-12}$–$10^{-13}$ mol L$^{-1}$ (van Hullebusch et al., personal communication).

The bioavailability is the degree to which elements are available for interaction with biological systems (Marcato et al., 2009). These bioavailable TE can be divided into two categories by their uptake mechanisms: those with (i) active uptake i.e. internalization processes requiring direct metabolic activity from microorganisms to transfer TE through the plasma membrane; and (ii) passive uptake, i.e. uptake based only on a concentration gradient 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 internalized, TE can impact the methane production yield via the intracellular, bioavailable fraction (Figure 2.1).

![Figure 2.1 Simplified, conceptual representation of TE bioavailability in anaerobic digesters (adapted from NRC (2003) and reprinted from van Hullebusch et al., 2016). 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.](image)

**2.3 BIOGEOCHEMICAL PROCESSES**

**2.3.1 Introduction**

In heterogeneous biological systems, such as engineered AD ecosystems, the distribution and fate of TE are controlled by a complex network of physical, chemical, and biological reactions (Fermoso et al., 2009). The effect of these processes is a dynamic TE partitioning among different fractions: free TE ions, soluble organic and inorganic TE complexes, and TE bound to colloidal and biotic (microorganisms) particulate materials. Figure 2.2 shows the chemical reactions occurring both in liquid (i.e. TE reduction, precipitation or
complexation) and solid phases (i.e. TE sorption in sludge) that play key roles in the chemical speciation of TE in AD bioreactors in relation to sulfur (S). In addition, the precipitation of metals by sulfide ($S^{2-}$), $CO_3^{2-}$ and $PO_4^{3-}$ may also play a pivotal role in nutrients and TE turnover (Figure 2.2) (Fermoso et al., 2015; Maharaj et al., 2018; Thanh et al., 2016).

2.3.2 Non-redox sensitive elements

Non-redox sensitive metals such as Ni, Co, Zn or copper (Cu) occur in natural and engineered environments such as sediments or bioreactors (in vast majority) only in one valence state, most often $+\text{II}$. Even though other valences are known (e.g. Co(III) or Ni(I)), these forms are rare and their transformations (i.e. oxidation and reduction) do not occur under conditions prevailing in most natural environments.

Indirectly, redox potential influences the chemistry of non-redox sensitive TE by the occurrence of various redox sensitive anions. Specifically, $S^{2-}$ dominates the chemistry of non-redox sensitive TE in anaerobic environments as it forms extremely strong precipitates. Under aerobic conditions, sulfide occurs only in trace amounts and sulfate does not form precipitates with TE.

Figure 2.2 Sulfur turnover in biogas bioreactors and its influence on TE and nutrients speciation (modified from Möller and Müller (2012) and reprint from van Hullebusch et al., 2016).
All most important non-redox sensitive TE, i.e. Co, Ni, Zn and Cu, can form a great number of organic and inorganic complexes ranging from simple [NiCl]$^{+}$ to extremely complex cobalamins. This increases the bioavailability of non-redox sensitive TE in case of TE limitation, because complexes are much more mobile than precipitates. Even though the equilibrium concentrations of free ions are very low in the presence of these complexes, strong complexes such as EDTA have been shown to alleviate TE limitation (Fermoso et al., 2008).

Immobilization (or biosorption) of non-redox sensitive elements by anaerobic microorganisms, usually clustered in biofilms, includes a number of processes, most important of which are adsorption, ion exchange, absorption in extracellular polymers (EPS), precipitation and internalization (van Hullebusch et al., 2003). In real systems, it is difficult to distinguish individual processes and therefore the summary term biosorption is often used. Out of all processes included in the biosorption term, precipitation, absorption in EPS and internalization are probably the most significant.

An accurate description of TE precipitation in anaerobic biofilms is extremely challenging, and an experimental description of actual distribution is almost impossible. In most environments, S$^{2-}$, CO$_3^{2-}$ and PO$_4^{3-}$ anions are the most important precipitation agents for non-redox sensitive TE. In particular, S$^{2-}$ complexes are extremely poorly soluble. On the contrary, CO$_3^{2-}$ complexes are relatively soluble and therefore more bioavailable. TE are not completely biologically inert when precipitated with S$^{2-}$. Gustavsson et al. (2013a) observed an increase in methanogenic activity as the result of CoS dosing. Thus, S$^{2-}$ precipitates with TE can, in some cases, create a pool of micronutrients which will be very slowly washed out from anaerobic bioreactors or sediments.

EPS play a crucial role in biosorption because they mostly contain anionic groups which form complexes with TE. The retention of TE in these complexes can then cause concentration gradient formation and, thus, regulate the exposure of bacteria to TE. TE bound in EPS can also play the role of a stock in the event of micronutrient deficiency in the environment (Fermoso et al., 2009). The strength of the bond between TE and the EPS depends on the conditions prevailing in the environment (e.g. pH, temperature) and the concentration of other TE present in the environment. At the same time, the properties of the given TE (e.g. electrical charge, radius) are very important.

### 2.3.2.1 Cobalt

Free cobalt ion (Co$^{2+}$) is present in natural and engineered environments in extremely low concentrations, i.e. at the level of pmol L$^{-1}$ (Yekta et al., 2017). The vast majority of Co is present as inorganic complexes (e.g. [Co(OH)]$^{+}$ to [Co(OH)$_4$]$^{2-}$, [CoCl]$^{+}$, [CoCO$_3$]$^{0}$, [CoHS]$^{+}$ or [CoSO$_4$]$^{0}$), organic complexes (e.g. vitamin B$_{12}$ or complexes with humic acids) and precipitates (mostly CoS(s), CoCO$_3$(s) and Co(OH)$_2$; Pitter, 2009).
Specifically in anaerobic environments, $S^{2-}$ concentration controls the speciation of Co (Gustavsson et al., 2013b) and only very strong complexing agents such as EDTA can keep Co in solution (Bartacek et al., 2008).

As Co is an extremely important TE, especially for methanogenic organisms (Fermoso et al., 2009), both bacteria and archaea (Marie Sych et al., 2016; Rempel et al., 2018) developed a way of Co extraction even from the environments where free Co ions are extremely rare, i.e. complexation of cobalt in cobalamins (or vitamin B$_{12}$). Besides their vital role in methyl transfer and radical generation (Matthews, 2001), they have been reported to play a crucial role in an active transport of cobalt across cell membranes (Rempel et al., 2018; Zhang & Gladyshev, 2009). It has been shown by Fermoso et al. (2010) that supplementation of Co in the form of vitamin B$_{12}$ is much more efficient than in any other form ([CoH$_2$EDTA], CoCl$_2$ etc.). It should be noted as well that improving bioavailability of Co might also lead to Co toxic concentrations (Pinto-Ibieta et al., 2016).

### 2.3.2.2 Nickel

Nickel, similarly to cobalt, can form many different complexes such as $[\text{Ni(OH)}]^{2+}$ to $[\text{Ni(OH)}_3]^{2-}$, $[\text{NiCl}]^{+}$, $[\text{NiCO}_3]^0$, $[\text{NiHS}]^{+}$ or $[\text{NiSO}_4]^0$. In anaerobic digesters, ammonia complexes are also important (from $[\text{Ni(NH}_3)]^{2+}$ to $[\text{Ni(NH}_3)_6]^{2+}$). Precipitates formed in most anaerobic environments are similar to Co precipitates, i.e. mainly NiS(s), NiCO$_3$(s) and Ni(OH)$_2$ (Pitter, 2009), with NiS(s) usually being vastly dominating.

### 2.3.2.3 Zinc

Zinc is also, most often, present in $^{+II}$ valence and its physical-chemical speciation is similar to Co and Ni. It forms similar complexes (with CO$_3^{2-}$, ammonia or hydroxyl ligands), but its complexing abilities are much weaker than for Co, Ni and Cu (Pitter, 2009). It also forms precipitates, again mainly with S$^{2-}$, CO$_3^{2-}$ and hydroxyl ions (Legros et al., 2017; Le Bars et al., 2018).

### 2.3.2.4 Copper

Copper prevails in anaerobic environments as Cu($^{II}$), but it can be also present in small amounts as Cu($^{I}$). Its complexation ability is the strongest of all non-redox sensitive TE (Pitter, 2009). Again, Cu forms strong hydroxo-complexes and complexes with CO$_3^{2-}$, PO$_4^{3-}$ and S$^{2-}$. It also forms extremely strong organic complexes. Similar to other non-redox sensitive TE, Cu precipitates with S$^{2-}$, CO$_3^{2-}$, and hydroxyl ions (Legros et al., 2017).

### 2.3.3 Redox-sensitive elements

In contrast to non-redox sensitive elements, those displaying several valence states (i.e. redox sensitive) have higher mobility and display complex behavior
with changing environmental conditions. Table 2.2 below compiles several redox-sensitive elements used in AD (Fe, Mn, Mo, Se, and W), together with their valence states and commonly reported form (compound). Of the five elements mentioned, Fe and Mn are commonly present in aquatic solutions as positively charged metal cations, whereas Mo, Se, and W form negatively charged oxyanions. When present in the same system, these elements often interact with each other in a complex interplay.

### Table 2.2 Redox-sensitive elements commonly used in anaerobic digestion.

<table>
<thead>
<tr>
<th>Chemical Element</th>
<th>Valence States*</th>
<th>AD Supplemented Valence State**</th>
<th>Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>+VI, +IV, +III, +II</td>
<td>+II</td>
<td>FeCl₂</td>
</tr>
<tr>
<td>Mn</td>
<td>+VII, +VI, +IV, +III, +II</td>
<td>+II</td>
<td>MnCl₂</td>
</tr>
<tr>
<td>Mo</td>
<td>+VI, +III, +II</td>
<td>+VI</td>
<td>MoO₂⁻***</td>
</tr>
<tr>
<td>Se</td>
<td>+VI, +IV, 0, −II</td>
<td>+VI, +IV</td>
<td>SeO₄²⁻, SeO₃²⁻</td>
</tr>
<tr>
<td>W</td>
<td>+VI, +V, +IV, +III, +II</td>
<td>+VI</td>
<td>WO₄²⁻</td>
</tr>
</tbody>
</table>

*From Lide (2004); **Commonly reported in literature; ***Many articles report the addition of molybdenum as ammonium heptamolybdate: (NH₄)₆Mo₇O₂₄ • 4H₂O.

### 2.3.3.1 Iron

Iron is the fourth most abundant element on Earth and is usually found combined with oxygen as iron oxide minerals: hematite (Fe₂O₃), magnetite (Fe₃O₄), and siderite (FeCO₃) (Lide, 2004). Iron forms compounds of relevance in biology mainly in the +II (ferrous) and +III (ferric) oxidation states. The mineralogy of iron is complicated by its propensity to form non-stoichiometric compounds whose composition may vary. Complicating matters further, many coordination compounds of iron are known. Iron sulfide (FeS₂, pyrite) is an important compound in AD systems, especially in sulfidogenic environments where SO₄²⁻ is reduced to highly reactive S²⁻, by sulfate-reducing bacteria (SRB). Sulfide is particularly important since it binds readily with Fe and other metallic cations yielding insoluble precipitates, thus rendering essential micronutrients biologically unavailable. Both FeO and FeS minerals play a major role in adsorption and precipitation phenomena involving other metals and metalloids present in AD. Iron is regularly added to AD systems as Fe(II) (Table 2.2), although the use of zero valent iron (ZVI) has also been documented (Zhang et al., 2011). ZVI may also act as an additional electron donor and buffer for the undissociated H₂S. Iron is
involved in numerous biological processes (e.g. oxidation, transport, respiration) being an essential TE. It is noteworthy that iron-binding proteins are found in all living organisms.

In spite of its environmental abundance, Fe is a difficult element to exploit by living organisms as a consequence of its presence in insoluble states. Due to its importance for microbial metabolism, bacteria have evolved high-affinity sequestering molecules (siderophores) involved in extracellular Fe uptake (Neilands, 1995). Interestingly, certain siderophores are broad-range metal chelators (Braud et al., 2009). For instance, azotobactin, a fluorescent pyoverdine-like siderophore produced by *Azotobacter vinelandii*, binds molybdenum and vanadium (V) (Wichard et al., 2009).

### 2.3.3.2 Manganese

Manganese (Mn) minerals are widely distributed in nature: oxides, silicates, and carbonates being the most common. Pyrolusite (MnO₂) and rhodochrosite (MnCO₃) are commonly encountered Mn minerals (Lide, 2004). Manganese reacts with S²⁻, forming insoluble MnS. As in the case of the other redox sensitive elements described in this chapter, the formation of MnS renders the micronutrients biologically unavailable. Interestingly, the addition of Mn oxides to AD systems can help mitigate ammonia presence. Ammonia is known to be an undesirable compound for methanogenic microbial consortia (Romero-Guiza et al., 2016). Various classes of enzymes have Mn cofactors, thus acting as an element of biological importance.

### 2.3.3.3 Molybdenum

Molybdenum (Mo) is a transition metal found in various valence states in minerals such as molybdenite (MoS₂), wulfenite (PbMoO₄), and powellite [Ca(MoW)O₄]. Industrially, Mo is a by-product of Cu and tungsten (W) smelting operations (Lide, 2004). Most molybdenum compounds have low water solubility, with the exception of molybdate oxyanion, MoO₄²⁻.

Xu et al. (2005) investigated the adsorption of two major Mo species, molybdate (MoO₄²⁻) and tetrathiomolybdate (MoS₄²⁻), onto iron minerals pyrite (FeS₂) and goethite (FeOOH) and found the maximum sorption capacity in the acidic pH range (pH < 5). In addition, they observed a powerful competitive effect of phosphate for the adsorption sites of Fe minerals, while silicate and sulfate exhibited only a minor influence. MoS₄²⁻ showed the greatest propensity for Fe minerals, thus suggesting a possible mechanism of Mo immobilization under sulfidogenic and iron-rich conditions. In addition, MoS₄²⁻ was shown to strongly interact with organically modified montmorillonite (organo-smectite) at low pH (<5), displaying a maximum sorption capacity of 705 mmol kg⁻¹ (Muir et al., 2017). The main sorption mechanisms proposed are chemisorption and ion exchange.
2.3.3.4 Selenium

Selenium (Se) occurs rarely as a pure element, being commonly associated with metal-sulfide minerals (e.g. pyrite and chalcopyrite) and bionitites (e.g. coal, crude oil, bituminous shales). As such, Se is typically found together with Cu, Zn, lead (Pb), gold (Au), and Ni ores (Kyle et al., 2011). Apart from the abiotic release of Se through rock weathering, volcanism and wildfires, the biotic component plays a major role in the mobilization of this element. Bacteria, possessing a versatile metabolism, have been documented to participate in almost all known valence-state transformations of Se (Staicu & Barton, 2017). In aquatic solutions, Se forms high valence state oxyanions: selenate, Se(VI), SeO_4^{2-}, and selenite, Se(IV), SeO_3^{2-}. These two Se oxyanions have a high toxicological potential in aquatic ecosystems, but under anaerobic conditions various bacteria can respire them to generate cellular energy (Staicu et al., 2017). Unlike S, a closely related element, Se can be biologically reduced to elemental Se, Se⁰, directly, a solid product, which is biologically unavailable (Figure 2.3a and Figure 2.4). It is noteworthy that Se can be incorporated into selenocysteine (Sec), the 21st proteinogenic amino acid, from its most reduced valence state, selenide, Se(-II). For a fuller discussion on the biosynthesis of Se, the reader is referred to Staicu et al. (2017). Selenocysteine exists naturally in all three domains of life and, in selenoproteins, serves oxidoreductase functions against reactive oxygen species (Labunskyy et al., 2014). Formate dehydrogenase (FDH), which plays an important role in syntrophic acetate oxidation in AD, is dependent on Se as well as Mo and W (Molaey et al., 2018b). When Se is not supplemented to AD in its selenide form (Figure 2.3b), there is a significant risk of losing part of it as the biologically unavailable Se⁰. Furthermore, biogenic Se⁰ exhibits colloidal properties that make it environmentally persistent and prone to long distance transport in aquatic ecosystems when escaping the bioreactor setting, leading to secondary pollution (Cordoba & Staicu, 2018).

Selenium was shown to be complexed to organic matter through the formation of Se-metal–humic ternary complexes, although selenate appears to be considerably less reactive than selenite (Bruggeman et al., 2007). On the other hand, at the mineral/water interface different processes involving Se can be taken into account such as adsorption, co-precipitation and surface precipitation processes (Fernandez-Martinez & Charlet, 2009). Adsorption of Se species at the mineral/water interface has been described to occur through both outer-sphere and inner-sphere complexation mechanisms. Selenium association with Fe, aluminum (Al) and Mn oxides and hydroxides has been reported in numerous articles (reviewed by Fernandez-Martinez & Charlet, 2009). In general, selenite is more reactive with various mineral surfaces and a possible explanation may be the inner-sphere complexation characterizing this interaction. In the case of selenate, due to the ionic strength dependence of its sorption capacity,
the outer-sphere (electrostatic) adsorption mechanism is thought to be the main factor. In complex environments, co-precipitation and surface precipitation are sometimes intermingled with adsorption phenomena.

Figure 2.3 Selenium transformations in bacteria related to the supplementation of different forms. a) Assimilatory and dissimilatory metabolism of selenium: (A) Import of various forms (selenate/selenide) of Se inside the bacterial cell; Inset 1 (B): the assimilation of selenide into selenoproteins; Inset 2 (C) respiration of selenate to selenite in G- bacteria; Inset 2 (D) reduction of selenite to red Se⁰ by glutathione reductase (GSH). b) Bacterial synthesis of selenoproteins from selenides. (Sec – selenocysteine; SelA – selenocysteine synthase; SelB – SECIS-binding protein; SelC – Sec-specific tRNA (tRNASec); SelD – selenophosphate synthetase; Ser – serine; SerRS – seryl-tRNA synthetase; GTP – guanosine-5′-triphosphate; SECIS – selenocysteine insertion sequence) (modified from Staicu et al., 2017).

2.3.3.5 Tungsten

Tungsten (W) is a rare metal that occurs in minerals such as wolframite, (Fe, Mn) WO₄, scheelite, CaWO₄, huebnerite, MnWO₄, and ferberite, FeWO₄ (Lide, 2004). The element reacts with oxygen by forming tungstic oxide, WO₃, which further solubilize in aqueous alkaline solutions and form tungstate ions, WO₄²⁻ (Wiberg & Holleman, 2001). Tungsten supplements are provided to AD reactors in the form of tungstate. From the biological perspective, tungsten is the heaviest
element displaying known biological functions. Several oxidoreductase enzymes containing tungsten were characterized and some require selenium for proper functioning (Stiefel, 1998).

Adsorption of tungsten and tetrathiotungstate (\(\text{WS}_2\text{O}_4\)) onto pyrite was investigated, showing an increased adsorption capacity with decreasing pH and a higher adsorption of \(\text{WO}_2\text{O}_4\) over \(\text{WS}_2\text{O}_4\) (Cui & Johannesson, 2016). The authors correlate the adsorption capacity of W forms with different inner-sphere complexation on the pyrite surface. Another study investigated the interaction of tungsten with hematite showing strong adsorption and, similarly to pyrite, increased adsorption capacity with decreasing pH (Rakshit et al., 2017). Tungstate was also shown to adsorb onto organically modified montmorillonite and the main sorption mechanisms involve chemisorption and ion exchange (Muir et al., 2017). The sorption capacity was high at acidic pH (pH < 5), while at pH > 5 sorption was limited or completely inhibited.

2.4 HOW TO ASSESS SPECIATION AND BIOAVAILABILITY OF TE IN ANAEROBIC DIGESTERS

TE can be present under different chemical species (speciation), which can be free or associated to various constituents of the matrix (fractionation). Their determination
may be performed using *in situ* or *ex situ* analysis, both approaches having limitations. For example, in the *ex situ* methodologies, inappropriate sampling and/or storage may result in TE sorption/release or redox state modifications. In contrast, *in situ* methodologies are less prone to alteration of the initial speciation and fractionation, but may have lower analytical performances than laboratory techniques.

### 2.4.1 Sampling

One of the main challenges in TE sampling in anaerobic digesters is the ability to maintain anoxic conditions. Indeed, in anoxic samples, sulfide-based phases may play a significant role in the retention of TE. Upon oxygenation of the sample, these phases can be oxidized, resulting in the release of the associated TE into solution. Among the released metals, Fe and Mn can further generate new solid phases (Fe oxyhydroxides, Mn oxides, etc.), which could sorb the TE initially present in the liquid phase or released during the dissolution of solid phases (Almeida *et al.*, 2008; Caetano *et al.*, 2003; Caille *et al.*, 2003). 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. Such evolution can occur within a few hours (Caetano *et al.* 2003). These phenomena may, thus, induce a significant change of the TE partitioning, as highlighted in comparative sequential extractions performed in the presence or absence of air (Buykx *et al.*, 2000; Lenz *et al.*, 2008a). Special care should, thus, be taken during sampling and sample preparation when studying anaerobic systems.

A second challenge lies in the necessity that samples collected from a full-scale biogas reactor should be representative of the whole system. Hence, precautions have to be taken to ensure homogeneity of the reactor material in case of fully mixed system. Sampling may, thus, be the weak point in the analytical procedure as it may represent a major source of uncertainty and bias. Ortner *et al.* (2014) demonstrated that the sampling itself may induce an additional deviation of 6%–12% at minimum on TE analysis repeatability. It is supposed that this is mainly attributed to inhomogeneities originated from low mixing efficiency in the digesters, which is a typical phenomenon of biogas plants.

#### 2.4.1.1 In-situ sampling

*In-situ* sampling can be performed through the installation of porous liquid-phase sampling probes in the bioreactor or through the deployment of semipermeable membranes or diffusion-based passive samplers.

Suction-based samplers with hollow fibers (Vink, 2002) or porous ceramic or glass probe heads (Hofacker *et al.*, 2013) can be installed directly in a bioreactor. The samplers deliver a filtered sample by pumping (Duester *et al.*, 2008) or by applying a vacuum (Seeberg-Elverfeldt *et al.*, 2005). The *in situ* bulk/liquid separation is less prone to induce changes in TE partitioning and more
straightforward than *ex-situ* separation. The drawbacks are chemical fouling, in particular under strongly varying redox conditions, and biofouling, in particular with high biological activity and long experimental times.

The Donnan Membrane Technique (DMT) is based on semipermeable membranes separating the donor solution (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). Once the Donnan equilibrium is reach between both solutions, the TE present in the acceptor solution are quantified, generally using a spectrometric technique (e.g. ICP-MS). A main advantage with regard to AD is its applicability to anoxic systems and the avoidance of sample matrix effects. A major limitation arises in the case of low TE presence in the bioreactor, where the concentration in the acceptor solution may be too low to be determined. This technique has already been used to determine Co speciation in anaerobic media (Bartacek *et al.*, 2008).

The diffusive gradients in thin films (DGT) passive sampling is based on the diffusion flux of TE through a diffusive layer (porous gel matrix) followed by its irreversible sorption onto an underlying layer of binding phase (Davison & Zhang, 1994). During the deployment, a TE concentration gradient is established in the diffusive layer between the exposition media and the binding phase. The amount of accumulated TE is thus correlated to its concentration in the exposition media and the duration of deployment. After the deployment, the TE is eluted from the binding phase and quantified, usually by atomic spectrometry, then its original concentration in the exposition media is back-calculated (Davison & Zhang, 1994). Initially developed to assess the sum of free and labile metal concentration in water, the DGT technique has also been used for wastewater, sediments, and soils. In the latter two situations, the accumulation of TE in the DGT device induces a decrease in the TE concentration in the interstitial solution at the vicinity of the device, resulting in some cases in a release of TE from the solid phase. Such behavior could mimic the uptake of TE by plants, making the DGT technique able to estimate the bioavailable TE (Hooda & Zhang, 2008; Sun *et al.*, 2014). In recent studies, the speciation of redox-sensitive TE can also be addressed either by using a binding phase that retains a specific TE species (e.g. a thiolated binding phase will accumulate As (III) only; Bennett *et al.*, 2011) or by analyzing the eluted species with a hyphenated technique (e.g. Hg speciation could be determined by analyzing the DGT eluate by LC-ICP-MS; Cattani *et al.*, 2008). DGT devices main advantages are their ease of use and their ability to limit the risk of modification of the sample by introduction of oxygen. Their major drawback lies in the fact the DGT measurements give only an integrated concentration over the whole deployment duration, and not a dynamic evolution of TE concentration over time. The DGT technique has recently been tested to determine Cd fractionation in biological methane potential tests during anaerobic digestion of dairy waste (Bourven *et al.*, 2017).
2.4.1.2 Ex-situ sampling

To avoid oxygenation during ex-situ sampling from anaerobic environments (e.g. by pumping slurry samples from a bioreactor), the best practice is probably to directly sample into a glove box with the respective oxygen-free conditions of the reactor. After collection, the sample must be prepared to be compatible with the analytical method. For example, TE should be transferred to a liquid phase to perform atomic spectrometry analysis. In the meantime, sample preservation may also be necessary to limit TE species alteration during transport and storage of the sample, through (de)sorption, chemical or biological processes. Once again, oxygenation should be avoided during all these steps.

One main step of the sample preparation procedure is the separation of solid and liquid phase, which can be carried out by squeezing, centrifugation, sedimentation, filtration or freeze-drying (details on the different methods are available in Bufflap & Allen, 1995a, b). Briefly, squeezing is more useful for work with core sections and less interesting for reactor studies. Filtration is 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 (e.g. N2) at room temperature or shock freezing by liquid nitrogen with subsequent lyophilization are additional options if analysis of the bulk phase requires dried materials (e.g. Scanning Electron Microscope) (Bordas & Bourg, 1998; Rapin et al., 1986). Centrifugation is more rapid than sedimentation and it seems easier to avoid oxygen contamination if no glove box is available.

Before performing direct solid analysis or extraction of TE, the solid phase needs to be homogenized, which may generally include drying and grinding steps. Such sample preparation methods should be carefully evaluated in order to minimize their influence on original TE speciation and fractionation. Indeed, it has been reported that drying and grinding procedures may affect the results of TE fractionation in anaerobic sediments or biogas slurries (Baeyens et al., 2003; Ortner et al., 2014; Zehl & Einax, 2005). The drying of samples may allow better homogenization compared with wet samples, thus improving the repeatability of sequential extractions (Baeyens et al., 2003). But, if not performed under oxygen-free conditions, it may also result in drastic modification of TE fractionation, either by oxidation of the TE itself or by oxidation of the associated binding phases (Ortner et al., 2014; Zehl & Einax, 2005).

2.4.2 TE speciation in liquid samples

Various analytical techniques can be used to determine the individual species of a given TE in the liquid phase of AD. Most of these species-specific analyses are performed with ‘hyphenated techniques’, which corresponds to the coupling of a separation technique (typically liquid chromatography, LC, or capillary electrophoresis, CE) with spectrometric detection. Ion exchange chromatography is the most common separation mechanism, followed by reverse phase (usually
using an ion pairing agent). Both allow the separation of the main species of, for example, As, Cr, Hg, Sb or Se. For the study of complexes involving TE and organic matter, size exclusion chromatography (SEC) is more suitable (Laborda et al., 2008; Sadi et al., 2002; Vogl & Heumann, 1997). Inductively coupled plasma mass spectrometry (ICP-MS) has become the detector of choice, due to its low limits of detection (a few to sub-µg L\(^{-1}\)) for a large range of elements. Several reviews dealing with examples and advances in TE speciation by LC-ICP-MS or CE-ICP-MS can be found in the literature (Harrington et al., 2010, 2015; Harvanova & Bloom, 2015; Michalski et al., 2011; Popp et al., 2010).

The major challenges for TE speciation in AD samples by hyphenated techniques are related to the stability of analytes during the separation step, the complexity of the sample matrix, and the lack of standards for species identification. The fragility of the analyte relates to both complexes and redox stability of the analyte. During the chromatographic separation (at least several minutes), analytes can be oxidized by oxygen dissolved in the eluent. Furthermore, as the eluent required for the separation may not have the same aqueous composition than the original samples, spontaneous precipitation or complexation may occur upon mixing analyte and eluent. This would result in bias due, respectively, to co-precipitation and/or sorption of the analyte on the newly formed phases or to modification of the original complexation equilibrium involving the studied TE. For example, the addition of EDTA in the mobile phase may induce a dissociation of weak complexes of antimony (Sb) and organic acids (Hansen et al., 2011). Mobile-phase composition and pH should thus be compatible with the AD sample. Once the different species are correctly separated, they can be individually identified by matching retention times with known standards and then quantified. Unfortunately, such standards are not always available, in particular for elements that are known to readily form soluble species with S, such as Se and As (Lenz et al., 2008b; Petrov et al., 2012; Planer-Friedrich et al., 2010). Due to the low concentration of the analytes, the identification by collection of chromatographic fractions and subsequent analysis using mass spectrometry is not a straightforward option.

### 2.4.3 TE analysis in solid samples

#### 2.4.3.1 TE speciation

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 (Table 2.3). There are currently about 50 SR light sources around the world (www.lightsources.org), but so far there are relatively few reports on the use of SR to characterize the chemical speciation of samples in AD. Due to the low abundance of TE in AD, often below µg kg\(^{-1}\), speciation remains generally inaccessible for spectroscopic methods. However, the on-going development of SR sources with increasing
light brilliance is continually lowering the detection limits. Today, Fe is the only metal that, at least in some AD samples, reaches concentrations that can be directly studied by use of Extended X-ray Absorption Fine Structure (EXAFS) spectroscopy in bulk samples.

**Table 2.3** Synchrotron-radiation spectroscopic methods available for TE and major elements speciation in samples from AD reactors.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Advantages</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>XANES (NEXAFS)</td>
<td>Can be used to separate classes of mainly low mass elements</td>
<td>Provide more fingerprinting information (mostly redox state) than strict species-specificity</td>
</tr>
<tr>
<td>EXAFS</td>
<td>A non-destructive method in complex matrices that provides species-specific information</td>
<td>The sensitivity is restricted to about 1–10 µg g⁻¹ for the most brilliant 4th generation SR sources. Radiation damage is a concern</td>
</tr>
<tr>
<td>µ-XANES, µ-EXAFS</td>
<td>Provides XANES/EXAFS species-specific information at micro-scale</td>
<td>Similar to conventional XANES/EXAFS, but higher energy flux required</td>
</tr>
<tr>
<td>STXM</td>
<td>Localized (nm scale) species-specific (XANES) information in contrasting images</td>
<td>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)</td>
</tr>
<tr>
<td>XPS</td>
<td>Surface-sensitive method for chemical speciation within the nm scale of surfaces</td>
<td>TE addition is required due to insensitivity</td>
</tr>
</tbody>
</table>

Similar to organic soils and sediments, the matrix of AD samples is dominated by low mass elements such as 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. The recent development of SR techniques opens up the possibility for species-specific identification of most elements in the periodic table.

For Fe, in principal species-specific information can be provided by K-edge X-ray Absorption Near-Edge Spectroscopy (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).

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). Because of the high resolution of this technique, localized high concentrations of heavier elements (e.g. TE) can be detected and its coordination chemistry unraveled. 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 about 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, they can contribute significantly to mechanistic knowledge on TE fate.

2.4.3.2 TE fractionation using sequential extraction

To assess TE fractionation, which can provide information regarding their potential mobility and bioavailability in AD ecosystems, different chemical extraction approaches might be used. However, the results obtained are operationally defined, i.e. the ‘forms’ of TE are a direct result of the used extraction procedure (Quevauviller et al., 1997).

The sequential extraction (SE) approach lies on successive extractions, involving a series of reagents selected for their ability to react with different major solid components of the sample, to release the associated TE. This method has been popularized by Tessier et al. (1979) who developed an analytical procedure to differentiate particulate cationic TE (Cd, Co, Cu, Ni, Pb, Zn, Fe, and Mn) into five main fractions: exchangeable, bound to carbonates, bound to Fe–Mn oxides, bound to organic matter, and residual. Its application to anaerobically treated sludge revealed that organic matter and sulfide fractions are the most important carriers of metals in these matrices (Angelidis & Gibbs, 1989), but both fractions
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 a SE scheme for determining the fractionation of Pb, Zn, Cu, and Ni in anaerobically digested wastewater sludges from municipal treatment plants. The Stover scheme discriminates the TE present in exchangeable, adsorbed, organic matter-bound, carbonate, and sulfide forms. This, therefore, allows discriminating metal distribution between the organic matter and sulfide fractions. However, the higher number of extraction steps compared with the Tessier scheme results in a poor recovery of the TE extracted compared with the initial TE budget (van Hullebusch et al., 2005). The Bureau Communautaire de Reference (BCR) developed a SE scheme to harmonize all SE studies and certified SE through inter-laboratory trials (Quevauviller, 1994). An accelerated BCR scheme, based on ultrasound-assisted extractions, was also proposed to significantly reduce the extraction time: from 2.5 days for Tessier scheme to half a day (Perez-Cid et al., 1999).

Even though several SE schemes have been proposed, it must be mentioned that no single fractionation scheme solves for distinct TE bearing phases exclusively and exhaustively (Filgueiras 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 means to study metal partitioning among the various solid phases of soils and sludges (see Filgueiras et al., 2002 for overview).

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

Shakeri Yekta et al. (2012) investigated the effect of SE of TE on S speciation in anoxic sludge samples from two lab-scale biogas reactors augmented with Fe. Analyses of S K-edge XANES spectroscopy and Acid Volatile Sulfides (AVS) were conducted on the residues from each SE step. The S speciation in sludge samples after AVS analysis was also determined. In the anoxic solid phase, S was mainly present as FeS (~60% of total S) and reduced organic S (~30% of total S), such as organic sulfide and thiol groups. During the 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, the FeS fraction was quantitatively dissolved by AVS extraction. These results identify critical limitations for the application of SE for TE speciation analysis outside the framework for which the methods were developed.
2.5 CONCLUDING REMARKS

Trace elements are often supplemented in high amounts. This is mainly due to the lack of clear understanding of TE speciation and bioavailability. Initially, the amount of added TE was generally determined based on the total rather than ‘free’ or bioavailable TE concentrations. However, it is now clear that chemical speciation is critical when predicting bioavailability. Although TE chemical fractionation has been studied extensively over the last two decades to determine the fate of TE in anaerobic environments, its relationship with TE bioavailability is still not clearly understood. Such studies are further complicated as non-redox sensitive (e.g. Co, Cu, Ni, Zn) and redox-sensitive (e.g. Fe, Mn, Mo, Se, W) TE are involved in different biogeochemical reactions with mineral and organic phases present in AD.

The well known chemical sequential extraction methods and, more recently, some more advanced analytical techniques such as DMT or XAS have been applied to determine the speciation of TE in liquid and solid samples, respectively; however, the application of the advanced analytical techniques to anaerobic digesters is still in development.

In conclusion, a fundamental approach, which includes the basic biogeochemical processes of TE and the available analytical techniques, is highly needed. From a practical point of view, a deeper understanding of TE bioavailability in anaerobic digestion will help to minimize TE supplementation costs, while also maximizing the performance of anaerobic treatment processes operated at full scale.

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