Chemical Speciation of Sulfur and Metals in Biogas Reactors

Implications for Cobalt and Nickel Bio-uptake Processes

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Biogas Reactors

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Abstract

A balanced supply of micronutrients, including metals such as iron (Fe), cobalt (Co), and nickel (Ni), is required for the efficient and stable production of biogas. During biogas formation, the uptake of micronutrient metals by microorganisms is controlled by a complex network of biological and chemical reactions, in which reduced sulfur (S) compounds play a central role. This thesis addresses the interrelationship between the overall chemical speciation of S, Fe, Co, and Ni in relation to the metals bio-uptake processes. Laboratory continuous stirred tank biogas reactors (CSTBR) treating S-rich grain stillage, as well as a number full-scale CSTBRs treating sewage sludge and various combinations of organic wastes, termed co-digestion, were considered. Sulfur speciation was evaluated using acid volatile sulfide (AVS) extraction and S X-ray absorption near edge structure (XANES). The chemical speciation of Fe, Co, and Ni was evaluated through the determination of aqueous metals and metal fractions pertaining to solid phases, as well as kinetic and thermodynamic analyses (chemical speciation modelling).

The relative Fe to S content in biogas reactors, which in practice is regulated through the addition of Fe for the purpose of sulfide removal or prior to the anaerobic digestion of sewage sludge, is identified as a critical factor for the chemical speciation and bio-uptake of metals. In the reactors treating sewage sludge, the quantity of Fe exceeds that of S, inducing Fe(II)-dominated conditions under anaerobic conditions, while sulfide dominates in the co-digestion and laboratory reactors due to an excess of S over Fe. Under sulfide-dominated conditions, chemical speciation of the metals is regulated by hydrogen sulfide and the formation of metal sulfide precipitates, which in turn restrict the availability of metals for microorganisms. However, despite the limitations set by sulfide, aqueous concentrations of different Co and Ni species were shown to be sufficient to support metal acquisition by the microorganisms under sulfidic conditions. Comparatively, the concentrations of free metal ions and labile metal-phosphate and -carbonate complexes in aqueous phase, which directly participate in bio-uptake processes, are higher under Fe-dominated conditions. This results in an enhanced metal adsorption on cell surfaces and faster bio-uptake rates. It is therefore suggested that the chemical speciation and potential bioavailability of metals may be controlled through adjustments of the influent Fe concentration in relation to S content. The results also indicated that the pool of metal sulfides in the biogas reactors could be regarded as a source of metals for microbial activities. Thus, the recovery and utilisation of this fraction of metals may be considered as a measure with which to minimise the metal dosing concentrations to CSTBRs.

Keywords: Biogas, Anaerobic digestion, Chemical speciation, bio-uptake, Sulfur, Iron, Cobalt, Nickel

Sammanfattning

För att en effektiv och stabil biogasproduktion från organiskt avfall skall uppnås, behöver mikroorganismer i biogasreaktorer ha tillgång till näringsämnen inklusive spårmetaller såsom järn (Fe), kobolt (Co), och nickel (Ni). Mikroorganismernas upptag av spårmetaller styrs av biologiska och kemiska reaktioner som påverkar metallernas tillgänglighet, där framför allt interaktioner mellan metaller och reducerat svavel (S) spelar en viktig roll. Avhandlingen analyserar sambandet mellan kemisk speciering av S, Fe, Co, och Ni i relation till metallernas biologiska upptagsprocesser. Omrörda tankreaktorer (CSTBR) i lab.- och fullskala för produktion av biogas från spannmålsdrank, avloppsslam, och olika kombinationer av organiska avfall (samrötning) har utgjort basen för studierna. Svavelspeciering analyserades med hjälp av AVS (acid volatile sulfide) extraktion och S XANES (sulfur X-ray absorption near edge structure). Speciering av Fe, Co, och Ni utvärderades med hjälp av sekventiell extraktion, mätning av metall koncentrationer i löst och fast faser samt genom kinetiska och termodynamiska analyser (kemisk specieringsmodellering).

Biogasreaktorers relativa mängder av Fe och S, identifierades som en central faktor för kemisk speciering och bio-upptag av metaller. Järn-mängden regleras bl a genom tillsats av Fe för att rena biogasen från vätesulfid eller vid diverse fällningsreaktioner i reningsverk före rötningssorgen av avloppsslam. Därför är järnhalterna högre än S-halterna i reaktorer, som behandlar avloppsslam. Detta leder till en Fe(II)-dominerande miljö. Däremot dominerade vätesulfid i de samröttnings- och laboratoriereaktorer, som ingick i studien. Under dessa förhållande styrs den kemiska metallspecieringen av sulfid och fr a genom fällning av metallsulfider, som då begränsar tillgängligheten av metaller för mikroorganismerna. Trots begränsningarna via sulfidfällningen var koncentrationen av de lösta Co och Ni formerna tillräckliga för bio-upptag av dessa metaller. Vid de Fe-dominerade förhållandena var koncentrationer av fria metalljoner och labila komplex (t.ex. med fosfat och karbonat), som direkt deltar i bio-upptagsprocesser, relativt höga, vilket medför relativt goda möjligheter för metalladsorption till cellytor och bio-upptag. Resultaten visar att den kemiska specieringen och därmed biotillgängligheten av metaller skulle kunna regleras genom justering av inflödet Fe i förhållande till S. Resultaten visade också att metallsulfider i fast fas sannolikt utnyttjas av mikroorganismer som en källa till metaller. Det innebär att en återanvändning av denna metallfraktion skulle kunna utnyttjas som en del i att minimera metalldoseringskoncentrationer.

Nyckelord: Biogas, Anaerob nedbrytning, Kemisk speciering, Bio-upptag, Svavel, Järn, Kobolt, Nickel

List of papers

The thesis is based on the following papers, which will be referred to in the text by the corresponding Roman numerals:

- I. Shakeri Yekta S, Gustavsson J, Svensson BH, Skyllberg U (2012). Sulfur K-edge XANES and acid volatile sulfide analyses of changes in chemical speciation of S and Fe during sequential extraction of trace metals in anoxic sludge from biogas reactors. *Talanta*. 89: 470-477
- II. Gustavsson J, Shakeri Yekta S, Karlsson A, Skyllberg U, Svensson BH (2013). Potential bioavailability and chemical forms of Co and Ni in biogas process – an evaluation based on sequential and acid volatile sulfide extractions. *Engineering in Life Sciences*. 13: 572–579.
- III. Shakeri Yekta S, Lindmark A, Skyllberg U, Danielsson Å, Svensson BH (2014). Importance of reduced sulfur for equilibrium chemistry and kinetics of Fe(II), Ni(II) and Co(II) supplemented to semi-continuous stirred tank biogas reactors fed with stillage. *Journal of Hazardous Material*, 269: 84-89.
- IV. Shakeri Yekta S, Svensson BH, Björn A, Skyllberg U (2014). Thermodynamic modeling of iron and trace metal solubility and speciation under sulfidic and ferruginous conditions in full scale continuous stirred tank biogas reactors. *Applied Geochemistry*, 47: 61–73.
- V. Shakeri Yekta S, Matsson L, Svensson BH, Danielsson Å, Skyllberg U. Effects of sulfide removal by Fe addition on chemical speciation of Co(II) and Ni(II) during anaerobic digestion of stillage – Implications for microbial metal uptake (manuscript).

List of abbreviations

AVS	Acid volatile sulfide
AVS-M	Simultaneously extracted metals
BL	Biotic ligand
CD	Co-digester
CSTBR	Continuous stirred tank biogas reactor
FIW	Food industry waste
HRT	Hydraulic retention time
ML	Metal-ligand complexes
OFMSW	Organic fraction of municipal solid waste
OLR	Organic loading rate
PBSS	Primary and biological sewage sludge
SE	Sequential extraction
ShW	Slaughterhouse waste
SS	Sewage sludge digester
TS	Total solid
VCA	Volatile carboxylic acid
VS	Volatile solid
XANES	X-ray absorption near edge structure

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1. Introduction

Anaerobic digestion is a widely applied process for the management of organic waste and the production of biogas, a gas mixture of methane and carbon dioxide (Krishania et al. 2013). The process depends on the growth of anaerobic microorganisms, and involves a sequence of microbial pathways initiated by hydrolysis of organic composites i.e. carbohydrates, proteins, and lipids into soluble mono- and oligomers such as sugars, amino acids, and fatty acids (Ahring 2003). The hydrolytic products are subsequently degraded into intermediate fermentation products such as acetate, carbon dioxide, hydrogen, and a number of organic acids. The final steps include the conversion of organic acids into acetate and additional hydrogen, and the production of methane via methanogenic pathways. The methane content of the biogas is used as a renewable energy carrier. Concerted efforts are at present focused on improving the production of biogas in order to expand the economic viability and application of anaerobic digestion technologies (Krishania et al. 2013).

Biogas formation using organic materials depends on the supply of macronutrients such as carbon (C), phosphorus (P), nitrogen (N), and sulfur (S), as well as micronutrients including several metal ions, both of which are required for the growth of the microorganisms involved (Kim et al. 2002). Among the micronutrients, iron (Fe), cobalt (Co), and nickel (Ni) are particularly important for anaerobic microorganisms. The availability of these metals during anaerobic digestion has a significant effect on microbial growth and activities and, thus, the performance efficiency of the process (Demirel and Scherer 2011, Vintiloiu et al. 2012). Iron, Co, and Ni are, therefore, commonly added to biogas reactors to ensure that there is a sufficient supply for the desired microbial processes (Demirel and Scherer 2011). Iron addition is also an important measure for controlling hydrogen sulfide concentrations in reactors through FeS(s) precipitation, in order to attenuate disturbances associated with the presence of sulfide (Lens et al. 1998). Hydrogen sulfide is formed as a result of the decomposition of S-containing organic compounds and microbial sulfate reduction under anaerobic conditions (Hulshoff et al. 1998, Muyzer and Stams 2008).

Several studies have shown that the addition of Fe, Co, and Ni increases anaerobic digestion efficiency, primarily through increasing organic matter decomposition, intermediate fermentation product conversion, and biogas production (Gustavsson et al. 2011, Pobeheim et

al. 2011, Lindorfer et al. 2012, Takashima et al. 2011, Karlsson et al. 2012, Facchin et al. 2013, Schmidt et al. 2014). The availability of metals for microorganisms is essentially controlled by the environmental conditions regulating the chemical properties of the metal species in contact with the biological interface (Bell et al. 2002, Erickson 2013). Complex and heterogenic biochemical environments emerge in biogas reactors as a result of the differing chemical composition of the substrates and the operational conditions implemented (Möller and Müller 2012). Thus, the critical biological and chemical processes which govern metal availability for microbial uptake are difficult to assess. It is widely accepted that the chemical speciation of metals in anaerobic environments is determined by reactions with reduced S compounds due to their high affinity for metals (Rickard and Luther 2006). Similarly, the importance of sulfide as a controlling factor for chemical speciation and the bioavailability of metals, including Co and Ni, during the anaerobic digestion processes has been emphasised (e.g. Gonzalez-Gil et al. 1999, Barber and Stuckey 2000, Jansen et al. 2007, Gustavsson et al. 2011).

The beneficial effects of Fe, Co, and Ni on process performance, along with their relationships with microbial processes, have been widely addressed. However, information regarding important chemical factors, particularly the role of reduced S, which is involved in the regulation of the metal-microbe interactions during anaerobic digestion, remains scarce. Accordingly, the addition of metals to the biogas reactors generally relies on trial and error or case-specific experiments. Thus, in-depth knowledge of the role of reduced S species, due to their inherent occurrence in biogas reactors, is required to identify the critical biochemical processes which control the biological uptake of the supplemented metals. This information is important with regard to metal dosage strategies for biogas reactors, and may have implications related to both improvements in biogas production and positive downstream effects for recipients.

In this thesis, the chemical speciation of S, Fe, Co, and Ni in biogas reactors, along with the implications for Co and Ni bio-uptake processes, are assessed. In the following background chapter, the importance of Fe, Co, and Ni for biogas formation is underlined, and an overview of the interrelationships between chemical speciation and the bio-uptake processes of metals is presented. A summary of the major chemical reactions involved in the speciation of Fe, Co, and Ni in biogas reactors, with a focus on the contribution of reduced S compounds, is also provided. The short background motivates the main research objective and questions of the thesis, which

are specified in Chapter Three. The biogas reactor systems studied and the analytical approaches implemented to provide answers for the specific research questions of the thesis are briefly presented in Chapter Four. In Chapter Five, the main outcomes of the research are discussed in the context of the knowledge status of the research area. Concluding remarks are offered in Chapter Six, which present possible practical measures related to the supply of trace elements in biogas reactors.

Comprehensive descriptions of the material and methods, along with the outcomes are presented in the five papers appended to this thesis. The methods for the chemical extraction of different S, Fe, Co, and Ni chemical fractions were assessed in Paper I. The chemical extraction methods were further implemented to study the chemical forms of Fe, Co, and Ni, and their potential bioavailability, in Paper II. The kinetics and thermodynamics of the supplemented metals were evaluated in Paper III. In Papers IV and V, the chemical speciation of S, Fe, Co, and Ni under different sulfide concentrations, and the consequent effects on their bio-uptake processes, were assessed.

2. Background

2.1. The importance of Fe, Co, and Ni for biogas formation

It has long been recognised that the addition of metals, including Fe, Co, and Ni, to biogas reactors improves the efficiency and stability of biogas production, while their absence leads to inefficient biogas formation and process instability (e.g. Wilkie et al. 1986, Takashima and Speece 1989). The stimulatory effects of Fe, Co, and Ni additions on biogas formation from a variety of organic substrates have been reported. Jarvis et al. (1997) showed that the addition of Co during the anaerobic digestion of grass-clover silage stabilised the digester pH and improved the conversion of acetate. As a result, the addition of Co enabled an increase in organic loading rate (OLR) and, consequently, higher methane production as compared to a process which did not incorporate Co. Gustavsson et al. (2011) indicated that problems associated with the anaerobic digestion of sulfate-rich grain stillage, such as process instability, low methane production, and the accumulation of volatile carboxylic acids (VCA), could be resolved by the addition of Fe, Co, and Ni. In addition to the stimulated conversion of intermediate fermentation products and methane production, Karlsson et al. (2012) showed that inhibiting effect of phenyl acetate on methane formation was reduced by the presence of Fe, Co, and Ni. Furthermore, they suggested that the addition of Fe, Co, and Ni may assist the acetoclastic methanogens in overcoming ammonia inhibition. Further evidence of the effectiveness of adding Co as a strategy for improving biogas formation at high ammonia concentrations is also shown elsewhere, in e.g. Banks et al. (2012) and Moestedt et al. (2013).

The need for Co and Ni is related to their function as parts of the enzymes and cofactors of anaerobic metabolisms. For instance, Co occurs as a centre ion in the corrinoid structure of methylcobalamin-dependent methyltransferase enzymes in acetogenic and methanogenic microorganisms, while Ni is essential for synthesis and the activity of cofactor F₄₃₀ catalysing methane formation via methyl-CoM reduction during methanogenesis (Diekert et al. 1981, Banerjee and Ragsdale 2003). Iron is also an essential nutrient for anaerobic microorganisms (Williams and Fraústo Da Silva, 2000). In addition to its importance as a micronutrient, Fe is commonly added to biogas reactors as a means of reducing sulfide levels in both the reactors and the biogas, and is present in much higher concentrations than other trace metals (Lens et al.

1998, Lindorfer et al. 2012). Stimulation of the anaerobic digestion process through the addition of Fe is generally attributed to sulfide removal, mitigating either the toxicity of sulfide to microorganisms or its negative effect on micronutrient bioavailability (e.g. Ziganshin et al. 2011).

2.2. Regulation of metals bio-uptake

In aquatic environments, the fraction of metals available for microbial uptake is controlled by interactive physicochemical and biological processes, which enable the transport and association of metal species to the cell membrane and further internalisation of the metals by the cells (Worms et al. 2006). It is generally accepted that the microbial uptake of metals involves the association of free metal ions and certain metal complexes to the metal-binding ligands of the membrane (i.e. active and facilitated transport mechanisms), or passive transport of neutral and non-polar metal species across the cell membrane (Hudson 1998). The processes and reactions involved in the uptake of metals by microbial cells are summarised in Figure 1.

Regulation of the bio-uptake processes in terms of the kinetic and thermodynamic factors involved depends on the relative rates of the diffusion of metal species from the bulk solution to the cell surface, and the internalisation of metals by the cell (Batley et al. 2004). When the diffusion rate of metals is faster than the microbial internalisation rate, metals in the vicinity of the metal-binding sites of the membrane are able to reach a pseudo-equilibrium with the bulk solution. Under these conditions, metal species at the solution-membrane interface are the same as those in the bulk solution (cf. uptake routes i and ii in Figure 1). On the other hand, when the microbial internalisation of metals is faster than their diffusive transport, a concentration gradient is created near to the cell. Consequently, labile metal complexes dissociate as a response to perturbed equilibrium (cf. uptake route iii in Figure 1). Under these circumstances, the association/dissociation kinetics of the labile metal complexes and/or the dissolution rate of less stable metal-bearing minerals control the metal bio-uptake processes. Metal uptake may also involve diffusion and direct transport of metal complexes, mainly as neutral inorganic or lipophilic organic complexes, across the cell membrane (cf. uptake route iv in Figure 1). The bio-uptake of metals via passive transport is generally derived by the cross-membrane concentration gradient of metal species, and depends on their permeability in the lipid membrane. Permeability is usually low for charged metal species, while neutral inorganic and

Figure 1. Metal uptake processes by microorganisms: i) diffusion of free metal ions (M) through the diffusive boundary layer around the biological membrane surface followed by complexation with biotic ligands (BL); ii) diffusion of metal-ligand complexes (ML) through the diffusive boundary layer followed by ternary complex formation with BL; iii) diffusion of labile ML through the diffusive boundary layer and ML dissociation to M, followed by complexation with BL; iv) passive diffusion of neutral and non-polar ML through the diffusive boundary layer and the biological membrane (summarised from Hudson 1998, Batley et al. 2004, and Mason 2013). Molecular charges are removed for simplicity.

organic metal species are more hydrophobic and, thus, prone to permeating through membranes (Mason 2013).

Typical microbial mechanisms responsible for the bio-uptake of Co and Ni involve either non-specific ion transporters, or those that specifically transport Co and Ni ions (Eitinger and Mandrand-Berthelot 2000, Okamoto and Eltis 2011). The bio-uptake processes are therefore linked to the uptake capacity and affinity of the transporter systems. This may vary according to the concentration of metals in the aquatic environment (De Pina et al. 1995). It has been demonstrated that the uptake efficiencies of Co by *Methanosarcina barkeri* are disproportional to the total Co concentration of the growth medium, suggesting applications of different microbial transport mechanisms with dissimilar capacities and affinities at different concentrations of Co (Jiang 2006). Similarly, the roles of the bulk solution concentration and speciation of Ni in regulating the Ni-uptake mechanisms by microorganisms e.g. by the

regulation of the Ni transporters gene transcription, are well-known (Eitinger and Mandrand-Berthelot 2000, Mulrooney and Hausinger 2003).

In CSTBR systems, reactor materials are suspended by extensive mixing. This ideally facilitates the diffusive transport of the metal species in the bulk solution towards the cell membrane surface. Thus, it is reasonable to assume that the concentrations of metal species at the solution-membrane interfaces are the same as in the bulk solution, and that the thermodynamic factors which determine the chemical speciation of the metals in the bulk solution play a decisive role in the regulation of the metal bio-uptake. Accordingly, determining the magnitudes and dynamics of the metal complexes and free metal ions in the bulk solution is a prerequisite for the assessment of the bio-uptake processes in CSTBRs.

2.3. Major chemical reactions involved in Fe, Co, and Ni speciation in biogas reactors

The nature of metal species in biogas reactors largely depends on the available metal-binding ligands, which involve in chemical reactions such as (co)precipitation, dissolution, aqueous complex formation, adsorption, and desorption. Under anaerobic conditions, the inorganic ligands including carbonate, phosphate, and sulfide are assumed to control the chemical speciation of metals (Callander and Barford 1983). These ligands are formed through microbial mineralisation of organic matter. Furthermore, inorganic P and S in a substrate contribute to the presence of phosphate and sulfide ligands. For example, the anaerobic digestion of primary and activated sewage sludge, containing a large quantity of P in the form of Fe-phosphate precipitates, results in a high concentration of phosphate in the reactors (Carliell-Marquet et al. 2010). An example of a sulfate-rich substrate is stillage, a by-product of bioethanol production, which is associated with extensive biogenic hydrogen sulfide production due to the microbial reduction of sulfate during anaerobic digestion (Gustavsson et al. 2011). In addition, the degradation of organic matter gives rise to dissolved organic molecules with strong metal-binding affinities, such as carboxylic, amino, alcohol, and thiol groups (Smith et al. 2002, Shakeri Yekta et al. 2012).

Observations of natural environments such as anoxic soils and sediments, in which organic matter is subjected to microbial decomposition in a manner similar to that of biogas reactors,

have shown that reduced S compounds have a regulatory role in the interactions between metals and microorganisms (Luther and Rickard 2005). Previous studies have also pointed to the importance of reduced S, particularly sulfide, for chemical speciation and the bioavailability of metals during anaerobic digestion (e.g. Callander and Barford 1983, Gonzalez-Gil et al. 1999, Barber and Stuckey 2000, Aquino and Stuckey 2007, Jansen et al. 2007, Gustavsson et al. 2011). Sulfide is generally considered to limit the bioavailability of metals by the formation of poorly soluble metal sulfides. The role of aqueous metal sulfide complexes for bio-uptake is generally less recognized. In spite of the fact that the interrelationships between metals and sulfide have been studied in great depth in the natural system (e.g. Rickard and Luther 2006), information regarding the effects of reduced S compounds on the dynamics of Fe, Co, and Ni, and their relationship with the bio-uptake processes in biogas reactors, is limited.

Cobalt and Ni additives to biogas reactors are commonly salts, dissolving as free ions with oxidation state of two. These metals form a number of solid precipitates with hydrogen sulfide, as well as mono- and multinuclear aqueous complexes of $\text{Co}_n(\text{HS})_m$ and $\text{Ni}_n(\text{HS})_m$ (Rickard and Luther 2006). The formation of nano-crystalline Co- and Ni-sulfide species under sulfate-reducing conditions has also been observed (Sitte et al. 2013). Iron additives are in the form of Fe(II), Fe(III), or a mixture of the two. Under sulfidic conditions, Fe(III) is reduced to Fe(II), which reacts with excess sulfide to form FeS(s) precipitates. Iron(II) is able to form structurally different precipitates with sulfide, including amorphous Fe-monosulfide (FeS), greigite (Fe_3S_4), and pyrite (FeS_2) (Rickard and Luther 2007). Iron is also able to form Fe-sulfide complexes and clusters in the aqueous phase. Davison et al. (1999) demonstrated that, in a solution saturated with respect to FeS(s), the formation of aqueous $\text{Fe}(\text{HS})_2$ and $\text{Fe}_n(\text{HS})_{2n}$ ($n \geq 2$) complexes may explain the solubility. Using an alternative approach, Rickard (2006) suggested that the dissolution of FeS(s) involves the formation of aqueous FeS nano-clusters (i.e. $\text{FeS}(\text{aq})$) of an estimated 1:1 stoichiometry.

In addition to hydrogen sulfide, organic matter which includes thiol functionalities (RSH) has a high affinity for Fe(II), Co(II), and Ni(II), which form both dissolved and adsorbed complexes with these metals (Smith et al. 2002). Elemental S, which also occurs in anaerobic environments, contributes to the formation of aqueous polysulfides (i.e. S_n^{2-} , $n \geq 2$) in chemical equilibrium with rhombic S (Wang and Tessier 2009). The polysulfide ions are able to form complexes with Fe(II), Co(II), and Ni(II), thereby affecting their chemical speciation (Chadwell et al. 2001). Furthermore, the complexity of sludge chemistry in biogas reactors in terms of the

properties of organic and inorganic particulates may also cause processes other than precipitation/dissolution and the aqueous complexation of metals. For instance, the FeS(s) formed subsequent to biogenic sulfide production has high metal-adsorbent properties and, therefore, association of Co(II) and Ni(II) to FeS(s) phases may occur (Watson et al. 1995). The occurrence of dissolved and particulate organic functional groups may contribute to the complexation and adsorption of these metals with organic matter. Metal speciation may also include compounds formed as a result of cell lysis, the production of metal-containing and metal-chelating microbial byproducts, and biomass-bound metals in the solid fraction (e.g. adsorbed on the cell walls or incorporated into protein structures; Aquino and Stuckey 2007).

3. Specific research objective and questions

In relation to the background information provided, it is hypothesised that the concentration and different types of S forms influence the chemical speciation and bio-uptake of Fe, Co, and Ni in biogas reactors. Accordingly, the main objective of the thesis is to assess the role of reduced S species, in particular sulfide, in controlling the chemical speciation and bio-uptake of Fe, Co, and Ni. To pursue the main objective of the thesis, the following research questions are formulated:

1. What is the composition and contribution of reduced S forms to S speciation in biogas reactors?
2. Which are the major processes that control the chemical speciation and potential bioavailability of Fe, Co, and Ni under sulfidic conditions?
3. To what extent do sulfide concentrations in biogas reactors control the chemical speciation of metals and their bio-uptake processes?

Samples were taken from three laboratory and eight full-scale continuous stirred tank biogas reactors (CSTBR), the most common reactor type in Sweden for industrial biogas production. Sulfur chemical speciation was studied by use of chemical extraction and S K-edge absorption spectroscopy. These methods were used to address research question one. The chemical speciation and potential bioavailability of Fe, Co, and Ni under sulfidic conditions, as put forward in research question two, were approached through chemical extraction of metal fractions pertaining to solid phases, analyses of aqueous phase concentrations of metals and their removal over time, and thermodynamic modelling. The answer to research question three was provided by an analysis of the chemical speciation of metals and their partitioning between solid and aqueous phases at different sulfide concentrations. Based on the chemical nature of the major metal fractions and species identified in the reactors, their potential contribution to overall metal bio-uptake processes was assessed in relation to current theories of uptake mechanisms.

4. Material and methods

4.1. Case studies - biogas reactors

Three laboratory and eight full-scale CSTBRs were included as case-studies (Table 1). Stillage was chosen as the substrate for the laboratory CSTBRs (designated R1, R2) as it contains a high concentration of sulfate and its usage in anaerobic digestion process results in the production of a large amount of biogenic sulfide. Furthermore, efficient and stable biogas production using stillage requires the addition of Fe, Co, and Ni (Gustavsson et al. 2011). Accordingly, a high sulfide concentration in stillage-fed biogas reactors, together with the essential addition of metals, provided a system highly suitable for the assessment of the interrelationships between S, Fe, Co, and Ni chemical speciation, as reported in Papers I-III. The effects of different sulfide concentrations on the chemical speciation of Fe, Co, and Ni were also evaluated using a stillage-fed laboratory CSTBR (R3), which was supplied with increasing amounts of Fe resulting in a decline in sulfide concentrations (Paper V).

Table 1. Operational conditions and substrate profiles of the laboratory and full-scale CSTBRs. Abbreviations: HRT (hydraulic retention time), OLR (organic loading rate), FIW (food industry waste), OFMSW (organic fraction of municipal solid waste), PBSS (primary and biological sewage sludge), ShW (slaughterhouse waste), and VS (volatile solid).

Reactor	Temp. (°C)	OLR (kg VS m ⁻³ d ⁻¹)	HRT (d)	Substrate (% of total substrate)
R1	37	2.0 - 2.5	20	Grain stillage (100%)
R2	37	4.0	20	Grain stillage (100%)
R3	37	2.0	20	Grain stillage (100%)
CD2T	53	3.6	20	OFMSW (62%), dry fodder (13%), ShW (9%), not specified (16%)
CD4	37	3.4	25	Fat (3%), Manure (32%), OFMSW (20%), ShW (45%)
CD5	38	3.6	26	Manure (67%), OFMSW/ShW (32%), Sludge (1%)
CD7	37	2.6	30	Fat/manure (10%), FIW (21%), OFMSW (57%), ShW (12%)
CD8T	53	3.5	20	Fat (5%), OFMSW (95%)
SS1	37	3.0	39	FIW (58%), PBSS (42%)
SS2T	52	2.5	12	PBSS (100%)
SS4	37	2.4	18	PBSS (100%)

The studied full-scale CSTBRs covered a range of sulfide and Fe concentrations common for the operational conditions of Swedish biogas reactors. These varying substrates and conditions provided excellent cases for comparing the S and metal speciation at different reduced S concentrations (Paper IV). The reactors included three sewage sludge digesters (SS), designated SS1, SS2T, and SS4, as well as five co-digesters (CD) fed by different combinations of organic wastes, designated CD2T, CD4, CD5, CD7, and CD8T (T signifies reactors operated under thermophilic conditions; Table 1). The analytical methods used for the assessment of the S, Fe, Co, and Ni speciation and dynamics are briefly presented in the following section.

4.2. Analytical methods

Solid phase S speciation was determined using S K-edge X-ray absorption near edge structure spectroscopy (XANES) at the semi-conductive wiggler beamline I811 at the MAX Laboratory, Lund University, Sweden. The method offers an analytical tool with low disturbance of the samples, and provides chemical information on S oxidation states (Jalilehvand 2006). In combination with statistical methods, XANES enables quantitation of the major S forms in complex samples (D'Amore et al. 2005). A modified least square fitting procedure was applied using Gaussian peaks as pseudo-model compounds to identify and quantify dominant S species (cf. Xia et al. 1998). The modification included an arctan step function to model the background for each of the pseudo-model compounds FeS, zero-valent S (elemental S, polysulfide, and possible traces of FeS₂), organic reduced S (RSH and RSR), organic disulfide (RSSR), sulfoxide, sulfonate, and sulfate. Further, one Gaussian peak was used to model the post-edge structure of organic reduced S, and one was used to model the sum of FeS, zero-valent S, and organic disulfide. Long-term exposure of the samples to X-rays may lead to changes in the original S speciation of the samples (cf. Farges et al. 2009). To avoid possible damage to the sample by the beam, a quick scan mode (with an exposure time of 40 s) was implemented, and 3-10 replicate scans were recorded for each sample. The analysis of the spectroscopy data on the replicate spectra indicated no damage to the samples, indicating a good reproducibility for the analyses.

By use of sequential extraction (SE), and determination of acid volatile sulfide (AVS) and simultaneously extracted metals (AVS-M), operationally-defined chemical fractions of metals were addressed. The SE method was originally developed by Stover et al. (1976) and Tessier

et al. (1979), and several different extraction methods have been developed since. The method developed by Tessier et al. (1979), later modified by Begoña Osuna et al. (2004), has been shown to be more accurate with respect to reproducibility and repeatability as compared to the other methods (cf. van Hullebusch et al. 2005). As a result, this method was applied to address the chemical fractionation of metals in the samples. Metal fractions, chemical reagents, and experimental conditions are summarised in Table 2.

Table 2. A summary of operationally-defined metal and S fractions, as well as the extraction reagents and conditions implemented. The metal analyses were performed using either atomic absorption spectroscopy (1100 Atomic Absorption Spectrophotometer, Perkin Elmer, USA) or inductively coupled plasma mass spectrometry (NexION 300, Perkin Elmer, USA). Abbreviations: AVS/AVS-M (acid volatile sulfide/simultaneously extracted metals), SE (sequential extraction).

Extracted metal fraction	Reagent (volume)	Extraction conditions
*Soluble fraction (from sludge samples)	-	Centrifugation followed by filtration, pore size 0.2/0.45 μm , inside the anaerobic box
SE1: Exchangeable (from sludge solid phase)	1 mol L ⁻¹ ammonium acetate (10 ml)	pH 7, shaken for 1 h, 150 rpm, 20°C, inside the anaerobic box
SE2: Acid soluble (from solid phase after SE1)	1 mol L ⁻¹ sodium acetate (10 ml)	pH 5.5, shaken for 1 h, 150 rpm, 20°C, inside the anaerobic box
SE3: Oxidisable (from solid phase after SE2)	30% hydrogen peroxide (5 ml)	pH 2 (adjusted by nitric acid), shaken for 3 h, 150 rpm, 37°C
SE4: Residual (from solid phase after SE3)	7 mol L ⁻¹ nitric acid (20 ml)	Autoclaved for 30 min., 120°C
AVS/AVS-M (from solid phase after SE1-SE3)	2 mol L ⁻¹ NaOH + 0.1 mol L ⁻¹ ascorbic acid + 0.1 mol L ⁻¹ EDTA (10 ml) / 1 mol L ⁻¹ hydrochloric acid (25 ml)	Stirred for 1 h, 150 rpm, inside the anaerobic box
Total (from sludge samples)	7 mol L ⁻¹ nitric acid (20 ml)	Autoclaved for 30 min., 120°C

*The contribution of small colloidal metal species (particle size <0.2 or 0.45 μm), as well as the effects of centrifugation and filtration, is not apparent. The soluble metal fraction is therefore an operationally-defined fraction, and should not be misinterpreted as an entirely dissolved metal species.

The sulfide fraction and the metals associated with this fraction were quantified using AVS and AVS-M (Hsieh and Yang 1989, Brouwer and Murphy 1994, Leonard et al. 1999). The applications of AVS/AVS-M extraction as a method with which to assess the bioavailability of Fe, Co, and Ni under anaerobic conditions have been previously discussed (e.g. Jong and Parry 2004, van der Veen et al. 2007). These analyses were therefore considered as a complement to

SE. The results of the chemical fractionation studies were further implemented to evaluate the potential bioavailability and chemical forms of Fe, Co, and Ni. The removal kinetics of the added metals were studied using time-intensive measurements of their soluble fraction immediately following their addition to the reactors. A thermodynamic model was constructed to simulate the chemical speciation of Fe, Co, and Ni in the biogas reactors. The Visual MINTEQ 3.0 software was used for calculations (Gustafsson JP 2012, <http://www2.lwr.kth.se/English/OurSoftware/Vminteq/index.html>). Details regarding the thermodynamic parameters, model inputs, and modelling approaches are presented in Paper IV. The overall performance of the laboratory reactors was monitored by measuring methane production, total solid (TS) and volatile solid (VS) contents, pH, and VCAs. The methods used for the measurements of the monitoring parameters are presented in Gustavsson et al. (2011) and Paper V. For comprehensive descriptions of the methods, the reader is referred to Papers I-V.

5. Outcomes and discussion

5.1. Sulfur speciation in biogas reactors

Analyses of S in biogas reactors have generally been limited to quantification of total concentration, although a few studies have focused on the chemical speciation of S, which mainly includes the anaerobic digestion of wastewater (e.g. Sommers et al. 1977, Du and Parker 2013). Determining S speciation is of importance as it has a direct influence on the chemical speciation and dynamics of trace metals. As discussed in the background section, various S species, including inorganic sulfides, organic thiols, and polysulfides, are able to form a number of aqueous, adsorbed and solid-bound compounds with metals. In this section, information regarding the major S species, along with the processes which contribute to their formation, and their conversion in CSTBRs across a broad range of substrate profile and operational conditions is provided.

5.1.1. Major S species in laboratory and full-scale CSTBRs

The analysis of the total S content of the samples demonstrated that the studied laboratory reactors, R1 and R2, which were digesting S-rich grain stillage, had the highest concentrations among the reactors; 16 and 13 mg S gTS⁻¹ respectively (Paper I). The CD8T had the lowest total S concentration, with 7.5 mg S gTS⁻¹, while total S concentrations in the other reactors were in the range of 10 – 12 mg S gTS⁻¹ (Paper IV). The relative concentrations of major S species in the solid phase of the samples from ten CSTBRs are presented in Figure 2 (Papers I and IV). The S speciation was dominated by FeS(s) in most of the reactors, ranging between 27 and 62% of the total S. The second most abundant S group was reduced organic S (RSR and RSH), with concentrations of 22 – 46% of the total S. This group includes inherited S-containing organic compounds such as amino acids, polypeptides, and proteins. Furthermore, biogenic sulfide production under anaerobic conditions could promote reactions between sulfide and organic molecules, which form RSH groups, through what has been termed the "sulfurisation process" (Vairavamurthy and Mopper 1987, Eglinton et al. 1994). Using methylmercury as a probe, Qian et al. (2002) demonstrated that RSH groups comprised approximately 30% of the reduced organic S moieties (as determined by S XANES) in natural organic matter of soils and streams.

Figure 2. Results from the least square fitting of pseudo-model compounds to XANES spectra for samples from two laboratory and eight full-scale CSTBRs. The major S species included S bound to Fe (as FeS(s)), reduced organic S (as organic thiol and organic monosulfide moieties), zero-valent S (as polysulfides, elemental S, and possible traces of pyrite), organic disulfide moieties having S-S bridges, as well as oxidised S compounds with sulfonate and sulfate functionalities.

Zero-valent S was identified in the samples, and accounted for 3 – 25% of the total S. This S group is attributed to the presence of elemental and polysulfidic S. In sulfidic environments, zero-valent S species can be produced in situ via partial oxidation of aqueous sulfide as a result of chemical and/or microbial processes (Steudel 1996, Yang et al. 2005, Sher et al. 2008). For example, elemental S is formed through a series of reactions which consist of the sequential formation of polysulfides from aqueous sulfide (Steudel 1996). The chemical reduction of Fe(III) to Fe(II) in the presence of sulfide also involves a series of intermediate polysulfidic and orthorhombic S formation stages (Tekin et al. 1999). Organic disulfide (RSSR), sulfonate, and sulfate functional groups (RSO_3 and ROSO_3) were also observed, each with a concentration in the range of <1 – 12% of the total S in the reactors. Inorganic sulfate (SO_4) cannot be separated from organic sulfate esters, and may have been present at low concentrations under anaerobic conditions. The presence of disulfide species may be related to the tendency of thiols to form mono- and di-sulfidic S bridges (Sinninghe Damste and De Leeuw 1990).

5.1.2. Regulation of S speciation by Fe

Comparison of the S speciation in the samples revealed that the relative quantity of zero-valent S in the SS reactors was higher than in the CD and laboratory reactors, while the opposite was true for FeS(s), with the exception of CD8T (Figure 2). In the SS reactors, the quantity of Fe exceeded that of S and induced Fe(II)-dominated conditions under anaerobic conditions (termed as ferruginous), while sulfide dominated in the CD and laboratory reactors due to an excess of S over Fe (Paper IV). A pattern was discerned, wherein the relative quantity of FeS(s) decreased when zero-valent S concentrations increased in line with a decrease in the S:Fe molar ratio (Paper IV). This suggested that the formation of polysulfide and elemental S is potentially at the expense of FeS(s) under conditions at which the concentration of Fe(II) dominates over sulfide, for example through the oxidation of S bonded to Fe via the "polysulfide mechanism" (Sand et al. 2001). The substrate of the SS reactors is rich in Fe(III), which is reduced to Fe(II) by the biogenic sulfide and, thus, causes the formation of zero-valent S (Tekin et al. 1999). The CD8T reactor had an exceptionally low Fe content in relation to S (S:Fe molar ratio = 2.8, Paper IV). As a result, it is possible that biogenic sulfide is involved in the formation of FeS(s), in that the excess sulfide would be available to react with organic compounds via the sulfurisation process. This may be the reason why the CD8T reactor had the largest pool of reduced organic S among the studied CSTBRs, comprising 46% of the total S (Figure 2).

To summarise, reduced S compounds, including inorganic sulfides, organic sulfides, elemental S, and polysulfides, dominate the S speciation in biogas reactors. The occurrence of these compounds, in particular inorganic sulfide, polysulfides and thiols, which have high metal binding affinities, suggests a regulation of the chemical speciation of metals by reduced S species. Furthermore, the observations suggested that Fe content in relation to S highly influences the S speciation in CSTBRs and, therefore, indirectly affects the chemical speciation of trace metals. These results motivated further investigation of the Fe, Co, and Ni speciation in relation to S and Fe concentrations in the biogas reactors.

5.2. Chemical speciation and potential bioavailability of Fe, Co, and Ni under sulfidic conditions

The use of S-rich grain stillage as the substrate for biogas production allowed an establishment of sulfidic conditions (i.e. concentrations of sulfide highly exceeding concentrations of Fe(II)) in the laboratory reactors. The hydrogen sulfide (as HS⁻) concentration in R1 and R2 was 0.7 and 1.5 mmol L⁻¹, respectively (Paper III). Cobalt and Ni additions to these reactors stimulated and stabilised the biogas production performance, and a limited supply of either resulted in process disturbances (Gustavsson et al. 2013). Thus, the observed positive effects of metal supplementation on the process performance of the reactors indicated that the sulfidic conditions did not inhibit the bioavailability of metals (Paper II). These observations therefore called for further assessments of the processes controlling the chemical speciation of Fe, Co, and Ni under sulfidic conditions, and their potential bioavailability.

5.2.1. Major chemical fractions of Fe, Co, and Ni in solid phase under sulfidic conditions

The chemical fractions of Fe, Co, and Ni in the solid phases of R1 and R2 were operationally defined as exchangeable (e.g. metals adsorbed on particulates), acid-soluble (e.g. metals associated to carbonate), oxidisable (e.g. metals associated to organic matter and sulfide), and residual (i.e. unspecified metal fractions with strong chemical bindings; Table 2; Papers I and II). The results demonstrated that approximately 6% and 10% of the total Fe and Co were extracted as the sum of the exchangeable and acid-soluble fractions, respectively. Nickel did not occur in these forms. The oxidisable fraction accounted for approximately 65%, 70%, and 100% of total Fe, Co, and Ni, respectively. This fraction was largely dominated by sulfide-bound metals, as demonstrated by analyses of S K-edge XANES and AVS-M (Papers I and II), but also included organically-bound metal fractions of e.g. biomass origin. Approximately 30% and 4% of total Fe and Co occurred in the residual fraction, respectively, while Ni was not present in this fraction.

It should be emphasised that the results from the SE, in a manner similar to the majority of the wet extraction methods, are subjected to artefacts related to an uncertain selectivity of the reagents or an occurrence of secondary and unknown reactions in samples with complex matrices (D'Amore et al. 2005). To identify the potential effects of the SE procedure on metal

fractionation, changes in the chemical speciation of S along with the sequential addition of extracting reagents, and their effects on metal fractionation, were investigated (Paper I). The results demonstrated that the first step of the SE, which aimed to extract exchangeable metal fractions, oxidised approximately 30% of the reduced inorganic S content of the samples to zero-valent S. The reduced inorganic S is mainly bound to Fe (Figure 2). The partial oxidation of the Fe-bound S did not result in a mobilisation of Fe from the solid phase, showing that the released Fe reacted with the sludge matrix (Paper I). Thus, the first SE step altered the binding forms of the sulfide-bound Fe in the samples and potentially the binding forms of Co and Ni, which also occur as sulfide minerals in the solid phase. In relation to these observations, metals extracted subsequent to the exchangeable fraction (i.e. acid soluble metals) may partially be the product of the unintended oxidation of the metal sulfides and secondary phases formed.

Furthermore, the combined results of AVS/AVS-M and SE suggest that the extraction of the oxidisable metal fraction may result in the formation of a secondary Fe phase (Paper I). Oxidation of the samples may convert sulfide to sulfate at low pH during the extraction (i.e. SE3 in Table 2). In the presence of Fe, this could potentially promote the formation of jarosite, $(\text{H,K,Na})\text{Fe}_3(\text{OH})_6(\text{SO}_4)_2(\text{s})$, and schwertmannite, $\text{Fe}_8\text{O}_8(\text{OH})_6\text{SO}_4(\text{s})$ (Bigham et al. 1996). Both minerals are able to incorporate Co and Ni into their structures via ion substitution with Fe and/or adsorption reactions (Dutrizac and Chen 2004). Thus, their potential formation may affect the chemical binding forms of Co and Ni during the course of the sequential extraction procedure. Accordingly, the concentration of Fe and Co measured as a residual fraction may be related to the formation of secondary phases, originating from the oxidation of the metal sulfides.

To summarise, the evaluation of the effects of the SE procedure on the chemical fractionation of the S and the metals in the samples suggested that the metals which were measured as acid-soluble and residual fractions may originally be bonded to sulfide (Paper I). Together with a large fraction of metals occurring as oxidisable, this indicates that the chemical speciation of Fe, Co, and Ni in their solid phase were dominated by metal sulfides. It was therefore concluded that biogenic sulfide controlled the chemical speciation of the metals under sulfidic conditions in the stillage-fed CSTBRs to a considerable extent (Paper II). This is in agreement with the results reported by van der Veen et al. (2007) and Jansen et al. (2007) for similar conditions in up-flow anaerobic sludge bed reactors and enriched methanogenic cultures, respectively.

Figure 3. The major chemical reactions which regulate the partitioning of metals between the solid and aqueous phases under sulfidic conditions, as suggested by the analysis of the chemical fractions of metals, removal kinetics of metals after supplementation, and thermodynamic calculations. Dashed lines represent processes which were hypothesised to occur based on observations.

5.2.2. Regulation of the aqueous Fe, Co, and Ni speciation under sulfidic conditions

Similar to the results obtained from the analysis of the chemical fractions of metals in the solid phase, analyses of the kinetics and thermodynamics of the added Fe, Co, and Ni in the aqueous phase further suggested the critical role of sulfide (Paper III). Figure 3 summarises the main chemical reactions which regulate the speciation of supplemented metals under sulfidic conditions. More than 95% of the Fe added to R1 and R2 was near-instantaneously (<1min after addition) removed from the aqueous phase through precipitation with sulfide and the formation of FeS(s) (Paper III). Thermodynamic calculations suggested that the formation of aqueous Fe-sulfide species, as well as Fe-organic complexes, as represented by thiol functional groups in the model, dominated the aqueous phase of Fe (Paper III). Because Fe(II), Co(II), and Ni(II) form complexes with O, N, and S functionalities with an approximately similar stability, as demonstrated by Smith et al. (2002), the thiol groups included in the thermodynamic model

may be seen as a representative for a mixture of strong metal-binding organic functionalities including carboxyl, phenol, amino, and thiol groups. The results also indicated that changes in the pH of sludge (caused by e.g. variation in the influent substrate pH) may contribute to a temporary alteration of the solubility of the metal-bearing minerals. In R1 and R2, the addition of acidic stillage as substrate led to a temporary change in the solubility of FeS(s) due to a short-term drop in pH at the time of substrate addition (Paper III).

Cobalt was initially and almost instantaneously removed by sulfide at the time of addition to R1 and R2, via precipitation with sulfide. Despite a dominance of CoS(s) minerals in the solid phase, the calculated concentration of aqueous Co species in equilibrium with CoS(s) could explain less than 7% of the measured concentrations (Paper III). Therefore, it is suggested that unknown process(es) enhanced the solubility of CoS(s). The soluble Co fraction comprised up to 20% of the total Co content of R1 and R2 (Paper III). This fraction was apparently very stable, given the observed high solubility of Co in the presence of high aqueous HS⁻ concentrations (up to 1.5 mmol L⁻¹) and predominance of CoS(s) in the solid phases of R1 and R2 (Paper III). The formation and release of Co-containing vitamin B₁₂ into methanogenic slurries, reported by Zhang et al. (2004), may be the reason behind the observed high solubility of Co. Furthermore, specific organic ligands with high affinities for binding Co may be produced by microorganisms as a response to either Co deficiency or excess (Saito et al. 2002, Waldron and Robinson 2009). Microbial responses to Co stress, such as up-regulation of Fe siderophore transporters for compensating high cellular Co concentration by Fe, or Co efflux proteins, which expedite the export of Co from the cell, have also been observed (Stadler and Schweyen 2002, Ranquet et al. 2007). Furthermore, the response of microorganisms to nutrient limitation and stress may in many cases result in an enhanced dissolution of nutrient-bearing minerals (Banfield et al. 1999). It is noteworthy that nano-crystalline Co- and Ni-sulfide particles may be formed under sulfidic conditions and thus contribute to the measured soluble fraction of these metals (Sitte et al. 2013)

Based on the thermodynamic calculations, soluble Ni forms were dominated by Ni-sulfide complexes in equilibrium with NiS(s) in the solid phase. A positive correlation between changes in the concentrations of aqueous Ni and Fe after the addition was observed (Paper III). This suggested that the removal of Ni from the aqueous phase was related to chemical processes of Fe, such as reactions involving co-precipitation, ion substitution, and/or adsorption with FeS(s) (cf. Morse and Arakaki 1993). Furthermore, studies of the adsorbing capacity of FeS(s) particles

formed subsequent to microbial sulfate reduction and biogenic sulfide production have shown that these minerals can effectively scavenge Co(II) and Ni(II) from the aqueous phase (Watson et al. 1995). In addition, the presence of metal-binding organic functionalities in the solid phase may promote the association of Fe, Co, and Ni to particulate organic matter at the time of addition. The importance of particulate organic compounds, such as extracellular polymeric substances and cell surfaces, for binding metals has been previously demonstrated (Borrok et al. 2005, van Hullebusch et al. 2006). The results from the S K-edge XANES analysis of the samples from R1 and R2 demonstrated that 30% of the total S in the solid phases occurred as reduced organic S, representing the sum of the RSR and RSH species (Figure 2). The occurrence of thiol groups is an indication of the presence of metal-binding organic groups pertaining to the particulate matter. In addition to the thiol groups, other metal-binding organic compounds with O and N functionalities may be present (Shakeri Yekta et al. 2012). Unfortunately, very little is known about the characteristics and effects of these ligands on the metal speciation in CSTBRs, and further in-depth research on this subject is highly needed.

5.2.3. Cobalt and Ni potential bioavailability under sulfidic conditions

It is generally assumed that less stable metal forms associated with the solid phase (e.g. adsorbed metals) have a higher potential availability for microbial uptake as compared to more stable minerals (e.g. metal sulfides; Jong and Parry 2004, van der Veen et al. 2007). Thus, metal fractions in the solid phase as specified by the SE method are (potentially) available for microbial uptake in the order of exchangeable > acid soluble > oxidisable > residual. Assertive bindings of Co and Ni to the oxidisable fraction, containing mainly metal sulfides (Paper II), suggest that the sulfidic conditions of R1 and R2 restrained the potential bioavailability of these metals. The essential addition of Co and Ni, the absence of which would have led to process instability and failures (cf. Gustavsson et al. 2013), suggested that metals could be utilised by microorganisms and, therefore, microbial uptake of these metals occurred in spite of the limitations set by the sulfidic conditions (Paper II).

Thermodynamic modelling of the chemical speciation of Co and Ni in R1 and R2 demonstrated that free Co^{2+} and Ni^{2+} ion concentrations were at the level of pmol L^{-1} . Assuming cellular concentrations of $0.1 - 100 \text{ nmol L}^{-1}$ for Co and Ni as rough estimates (Williams and Fraústo Da Silva, 2000), the cell requirements for these metals are substantially higher than the concentrations of free Co^{2+} and Ni^{2+} ions that were participating in active bio-uptake in R1 and

R2. However, by combining experimental data and a modelling approach for the analysis of Co and Ni bio-uptake by cultures of *Methanosarcina bakeri*, Jansen (2004) argued that concentrations of free Co^{2+} and Ni^{2+} ions of as low as $0.1 - 0.5 \text{ pmol L}^{-1}$ may be enough to maintain reasonable bio-uptake fluxes. Accordingly, the free Co and Ni ion concentrations of R1 and R2 could potentially support the cellular requirements of these metals, even at the prevailing sulfide concentrations.

Little is known about the bio-uptake mechanisms other than free Co and Ni ion transports across the cell-membrane. Aqueous metal sulfide complexes in the form of $\text{Co}(\text{HS})_2$ and $\text{Ni}(\text{HS})_2$ reached concentrations at the level of $\mu\text{mol L}^{-1}$ in R1 and R2 (Paper III). The high concentration of these complexes in the bulk solution as compared to the intracellular concentration therefore creates a sharp cross-membrane gradient, which may in turn promote passive uptake of these metal species by the microorganisms. The potential bio-uptake of neutral metal sulfides depends on the permeability of these complexes through the lipid membrane. Benoit et al. (2001) demonstrated that the diffusive membrane permeability of neutral Hg-sulfide complexes was sufficient to support the passive transport of these species by the sulfate-reducing bacterium *Desulfobulbus propionicus*. It has also been suggested that inorganic complexes such as HgCl_2 , AgCl , and CdCl_2 are assimilated via passive uptake mechanisms (Mason et al. 1996, Mason 2013). Even if information regarding the permeability of $\text{Co}(\text{HS})_2$ and $\text{Ni}(\text{HS})_2$ in lipid media (e.g. octanol – water partitioning) or through cell membranes is scarce, the concentrations of these complexes in the studied biogas reactors should be high enough to support any potential passive uptake of Co and Ni.

The results also demonstrated that a high sulfide concentration and the formation of CoS(s) minerals did not control the high solubility of Co in the studied biogas reactors. The aqueous concentration of Co is regulated by as-yet unidentified mechanisms, which are presumably related to the production of cobalamin-like biomolecules (Paper III). The incorporation of the complex structures, which are similar to cobalamin, into the membrane-bound transporters and their further uptake is well-known (Chimento et al. 2003). Thus, the pool of soluble Co fraction in R1 and R2 may also be accessible forms of Co for microorganisms due to their high abundance.

In relation to the arguments presented above, it is concluded that the microorganisms access to metals is restricted at high sulfide concentrations. However, the free Co and Ni ion

concentrations, the potential passive uptake of neutral metal sulfide complexes in the aqueous phase, and the high overall solubility of Co allowed metal acquisition by the microorganisms using different transport mechanisms. This may explain why Co and Ni additions stimulated and stabilised the process performance, despite the high sulfide concentrations in R1 and R2.

5.3. Effects of sulfide concentrations on the chemical speciation of metals and their bio-uptake processes

Under the anaerobic conditions in CSTBRs, different concentrations of S relative to Fe result in different free sulfide concentrations in the aqueous phase. In the full-scale CD reactors, higher S relative to Fe content gave rise to HS^- concentrations ranging between 40 and 150 $\mu\text{mol L}^{-1}$, while the higher concentration of Fe than S in the SS reactors resulted in relatively low HS^- concentrations of 1 – 5 $\mu\text{mol L}^{-1}$ (Paper IV). Furthermore, HS^- concentration continuously declined (from ~2000 to 200 $\mu\text{mol L}^{-1}$) in the R3 reactor as a result of the addition of Fe (Paper V). These experiments provided a gradient of inorganic sulfide levels, enabling further evaluation of the effect of sulfide on the chemical speciation of Fe, Co, and Ni.

5.3.1. Chemical speciation of Fe, Co, and Ni in full-scale CSTBRs

The analysis of Fe, Co, and Ni concentrations in the solid and aqueous phases of the samples from eight full-scale CSTBRs demonstrated that the aqueous phase of Co comprised between 4 and 18% of the total Co, while aqueous Fe and Ni contributed less than 2% and 5% of the total Fe and total Ni, respectively. To investigate the extent of the effects of different sulfide concentrations on the chemical speciation of metals, a thermodynamic equilibrium model was constructed which took the roles of important environmental factors such as temperature, pH, and concentrations of major metal-binding ligands into account (Paper IV). The model included chemical equilibrium reactions between metals and dominant reduced S compounds including sulfides, polysulfides, and thiols, as well as carbonate, phosphate, hydroxide, and chloride (cf. Paper IV). As discussed above, the thiol groups may be regarded as proxies for a group of organic ligands, possibly involving O and N functionalities which have a particular high affinity for Fe(II), Co(II) and Ni(II) (Smith et al., 2002). The thermodynamic modelling was conducted in a three-step fashion. In the first two steps, the solubility and chemical speciation of Fe, which controlled the HS^- concentration, were modelled and, in the last, the solubility and chemical speciation of Co and Ni were simulated (Paper IV).

The results suggest that metal sulfide precipitation is the major process behind the removal of metals from the aqueous phase, with the exception of Fe in the low sulfide SS reactors, where only 8 – 18% of the total Fe was precipitated as FeS(s). Siderite (FeCO_3) and ferrous phosphate ($\text{Fe}_3(\text{PO}_4)_2$) are Fe(II)-minerals which potentially occur during anaerobic digestion of Fe-rich sewage sludge. Based on the modelling results, siderite and FeS(s) were the two major solid Fe phases in the SS reactors (Paper IV). In agreement with these results, Mamais et al. (1994) and Zhang et al. (2010) demonstrated that Fe, when added to anaerobic reactors treating sludge, was primarily involved in the precipitation of $\text{FeCO}_3(\text{s})$ and $\text{FeS}(\text{s})$. However, a more diverse composition of Fe species might be expected, depending on the overall physicochemical conditions in the reactors. Cobalt and Ni were undersaturated in relation to their solid phases with carbonate, phosphate, hydroxide, and chloride in all reactors, and were present mainly in form of sulfide minerals (Paper IV).

In the aqueous phase, sulfide and thiol complexes dominated the Fe speciation in the sulfidic (CD) reactors. In the SS reactors, a relatively high solubility of Fe was observed as compared to the CD reactors, largely represented by complexes with phosphate. Neutral sulfide, thiols, and carbonate complexes were the major aqueous Co and Ni forms in the CD reactors. In the SS reactors, complexes with carbonate were the major aqueous forms, but thiols, phosphates, and free Co^{2+} and Ni^{2+} ions also made considerable contributions. The model ability to predict the solubility of Co and Ni was evaluated (Paper IV). The modelled concentration of aqueous Ni showed a reasonable merit-of-fit in all CD and SS reactors, but the model was less successful in predicting the aqueous concentration of Co. Accordingly, it is suggested that thermodynamic reactions included in the model properly captured the major chemical speciation of Ni, but several important aqueous components were missing for Co. This finding was in line with the results from the SE (Paper I and II) and the kinetic studies (Paper III). The presence of Co in soluble fractions may be related to processes which enhance $\text{CoS}(\text{s})$ solubility (cf. Figure 3) and/or the formation of Co-containing biomolecules. The extent of these processes likely differs among full scale CSTBRs due to differing substrate and operational conditions, causing variation in Co partitioning between the solid and aqueous phases.

5.3.2. The effects of sulfide concentration on the chemical speciation of Fe, Co, and Ni

The effects of the concentration of sulfide on chemical speciation of Co and Ni were examined by increasing the influent Fe concentration in R3, from 5 to 14 mmol L⁻¹ over the course of 150 days of operation (Paper V). During the same period, the R1 reactor operated in parallel as a control, with an influent Fe concentration of 5 mmol L⁻¹. The primary outcome of the increase in influent Fe concentration was an accumulation of sulfide as FeS(s). This was confirmed by an elevated AVS concentration and a decrease in the hydrogen sulfide content of the biogas overtime (Paper V). An initial increase in total Fe concentration to 7 mmol L⁻¹ did not considerably change the aqueous concentration of Fe (between 4 and 5 μmol L⁻¹). This was followed by a dramatic increase in the aqueous Fe concentration to 36 μmol L⁻¹, when a doubling of the total Fe concentration was reached. In parallel, aqueous Co and Ni concentrations in R3 demonstrated a decreasing pattern; from 20% to 10% of total Co, and 2% to <1% of total Ni by the end of the experiment, despite their unchanged total concentrations in the reactor (Paper V).

To explore the chemical processes which caused the observed shifts in the solid-aqueous partitioning of Fe, Co, and Ni, the chemical interactions between metals and inorganic ligands of carbonate, phosphate, hydroxide, chloride, and sulfide were modelled. The model included temperature, pH, total P, and Fe, Co, and Ni concentrations, as well as H₂S(g) and CO₂(g) concentrations in the biogas as inputs (Paper V). The modelling results demonstrated that the addition of Fe led to a decrease in HS⁻ concentration from approximately 2.0 mmol L⁻¹ at the beginning of the experiment down to 0.2 mmol L⁻¹ at the end. The declining HS⁻ concentration caused a substantial decrease in the concentration of aqueous Co- and Ni-sulfide complexes (i.e. Co(HS)₂ and Ni(HS)₂), while aqueous complexes of Co and Ni formed with other inorganic ligands, as well as their free ion forms, increased by up to two orders of magnitude over the duration (Paper V). Nevertheless, the aqueous Co- and Ni-sulfide complexes dominated over the course of the reactor operation and contributed to >99% of the aqueous concentrations. Accordingly, the decrease in the aqueous concentrations of Co and Ni could mainly be attributed to the decrease in the concentration of sulfide and its related aqueous metal sulfide complexes. Furthermore, it may be argued that an increase in the Fe content and precipitation of FeS(s) in R3 may have triggered co-precipitation and adsorption of Co and Ni with the FeS(s) structure, which could ultimately decrease the overall solubility of Co and Ni (Watson et al. 1995, Paper V).

The observed increase in the aqueous Fe concentration was likely due to an emerging competition for binding Fe between other available ligands with sulfide when the sulfide concentration changed from high to low. During periods of high sulfide concentration, the solubility of Fe in equilibrium with FeS(s) is primarily dominated by aqueous Fe-sulfide species. As a result of the Fe addition and the decrease in the HS⁻ concentration, a formation of free Fe²⁺ ions and aqueous Fe(II) complexes with phosphate and carbonate will predominate the aqueous phase speciation of Fe (Paper V).

It is concluded that the dynamic decrease in the sulfide concentration under the specified conditions of the R3 reactor lowered the overall solubility of Co and Ni due to a decrease in the concentrations of the dominant aqueous Co- and Ni-sulfide complexes. This was likely exacerbated by the interactions of Co and Ni with FeS(s), which was the result of an elevated concentration of precipitated FeS(s). Simultaneously, the depletion of the aqueous HS⁻ enabled more extensive reactions between Fe(II) and other major ligands in the reactor, such as phosphate and carbonate, which ultimately enhanced the Fe solubility.

5.3.3. The effects of sulfide concentration on Co and Ni bio-uptake processes

The above assessment of Co and Ni chemical speciation provided information regarding the magnitudes and dynamics of major metal species interacting with the biological interface at different sulfide concentrations. The main bio-uptake processes as presented in Figure 1 involve three major categories of inorganic metal species as potential contributors, i.e. free metal ions, labile and easily dissociable compounds (e.g. metal complexes with phosphate, carbonate, hydroxides, and chloride), and non-labile and neutral metal species (e.g. neutral metal sulfide complexes of Co(HS)₂ and Ni(HS)₂). The relationship between the concentrations of these metal species and the sulfide concentration are presented in Figure 4. This includes the results of the chemical speciation modelling for full-scale CSTBRs as well as the R3 reactor (Papers IV and V).

The chemical speciation of Co and Ni in the aqueous phase of the studied CSTBRs is dominated by their complexes with sulfide at the point at which HS⁻ concentration exceeds ~30 μmol L⁻¹ (Figure 4). The concentration of labile complexes and free ions exceeded the sulfide complexes only in the SS reactors, which were characterised by low HS⁻ concentrations due to

Figure 4. Results of the thermodynamic modeling of the Co and Ni chemical speciation in full-scale CSTBRs (A and C) and the R3 reactor (B and D) for free metal ions, labile metal complexes with phosphate, carbonate, hydroxide, and chloride ligands, as well as non-labile and neutral metal sulfide complexes of $\text{Co}(\text{HS})_2$ and $\text{Ni}(\text{HS})_2$ (Papers IV and V).

the high Fe content of the influent sewage sludge (Paper IV). From a thermodynamic point of view, the bio-uptake of metals via ion transporters can be expressed as surface-complexation reactions between metal ions (M^{2+}) and membrane-bound organic acids (RAH), the metal-binding properties of which depend on the equilibrium constants of the protonation/deprotonation reactions of the organic acids ($\text{R-AH} = \text{R-A}^- + \text{H}^+$) and complexation of metal ions with the negatively-charged functional groups ($\text{R-A}^- + \text{M}^{2+} = \text{R-AM}^+$; Flynn et al. 2014). It is therefore postulated that the high free metal ion concentration at low sulfide concentrations stimulates the binding of metals to the membrane-bound organic acids and increases the fraction of metals adsorbed on the cell surface.

Moreover, according to the free ion activity model that describes the kinetics of microbial metal uptake (Hudson 1998), the bio-uptake rates of metals are also regulated by the concentration of free metal ions at the membrane-solution interface. The bio-uptake rate (J) depends on the uptake capacity of the microorganisms (J_{max}) and the characteristic affinity of the microbial transport systems (K_m) for free metal ions (C_m ; Worms et al. 2006). The bio-uptake rate is commonly related to the concentration of free metal ions by applying Michaelis-

Menten kinetics as $J/J_{max} = C_m/(K_m + C_m)$, where J/J_{max} is taken to be the relative bio-uptake rate (van Leeuwen and Pinheiro 2001). According to this kinetic approach, the relative bio-uptake flux of metals would increase (i.e. $J \rightarrow J_{max}$) for lower sulfide and consequent higher free ion concentrations (Paper V). In addition, the bio-uptake of free metal ions is assisted by the diffusion of labile metal complexes towards the cell membranes and their further dissociation in the vicinity of membrane-bound ligands (Hudson 1998, Figure 1). In this regard, the bio-uptake of Co and Ni is facilitated due to the increase in the concentration of labile Co- and Ni-complexes at lower sulfide concentrations (Figure 4).

It is therefore concluded that the extent of the adsorption of Co and Ni on the cell surface, as well as their bio-uptake rates, are potentially higher in the Fe-rich SS reactors and/or following the addition of Fe due to the increased concentration of labile complexes and free ions. It should also be noted that the transport of metal ions across the cell membrane is highly dependent on the quantity of other competing divalent cations in the solution (Mason 2013). It has been shown that Co(II) and Ni(II) compete with each other to bind to the metal transporter sites (Degen et al. 1999). Therefore, the potential antagonistic effects of Co and Ni on each other's bio-uptake are likely exacerbated at low concentrations of sulfide and high ionic concentrations of these metals.

As discussed above, neutral metal complexes may be assimilated through passive uptake processes. The passive uptake is derived from the concentration gradient of metals across the membrane, and depends on the permeability of metal species into the membrane layer (Mason 2013). As is evident in Figure 4, neutral metal sulfide complexes have concentrations which are up to 4 orders of magnitude higher than those of free ion and labile forms under sulfidic conditions (e.g. in the R3 reactor, with an HS^- concentration of up to 2.0 mmol L^{-1}). At low sulfide and consequently low aqueous Co- and Ni-sulfide concentrations, the concentration gradient and thus the diffusive forces across the cell membrane diminish, which will decrease the bio-uptake of metals via diffusion-driven mechanisms.

6. Concluding remarks

In this thesis, focus was given to the role of reduced S species, in particular sulfide, in controlling the chemical speciation of Fe, Co, and Ni in CSTBRs. An important outcome was the detailed chemical speciation of S using S K-edge XANES. The major organic and inorganic S species and processes contributing to their formation/conversion have been highlighted for the various operational conditions of CSTBRs. The results reveal that the molar ratio of S to Fe controls the S and Fe speciation, e.g. by regulating the formation of FeS(s) and zero-valent S. The former species control concentrations of aqueous sulfide, aqueous metal sulfide complexes, and the potential co-precipitation or adsorption of metals on the FeS(s) particles, while the latter is a prerequisite for metal-polysulfide formation.

The major processes which control the chemical speciation and potential bioavailability of Fe, Co, and Ni under sulfidic conditions were further investigated. The results demonstrated that the dynamics of Co and Ni in biogas reactors have different features, although both metals are strongly affected by the extent of sulfide present through the precipitation of metal sulfide phases and have relatively similar inorganic chemistry in the aqueous phase. High Co solubility was observed in some cases, which appeared to be independent of complexation with inorganic and organic sulfides and the precipitation/dissolution of CoS(s). It is suggested that microorganisms are able to enhance the solubility of the pool of CoS(s) in the reactors by, for example, releasing specific Co-binding organic ligands. It is also possible that vitamin B₁₂ is a part of this phenomenon. It is concluded that the prevailing sulfidic conditions in biogas reactors restrict the accessibility of metals for microorganisms. Nonetheless, it is evident that, even under sulfidic conditions, free metal ion concentrations, the potential passive uptake of aqueous neutral metal sulfide complexes, and high Co solubility were able to support the bio-uptake of metals.

Moreover, the regulatory role of sulfide concentrations in biogas reactors in the chemical speciation of metals and their bio-uptake processes were studied. It has been shown that decreasing levels of sulfide (as induced through the addition of Fe) may initially lower the concentration of soluble Co and Ni in reactors. This is partially due to a reduction in the formation of aqueous Co- and Ni-sulfide species, which were dominant primarily in the CD reactors or those receiving S-rich substrate (e.g. grain stillage). However, non-sulfide metal

species such as free metal ions and labile metal complexes with phosphate and carbonate in the aqueous phase increase at lower sulfide concentrations. The corresponding effects of changes in the sulfide level on the bio-uptake of Co and Ni by microorganisms could be either positive or negative, depending on the microbial uptake mechanisms involved. The uptake of free metal ions by active/facilitated ion transport mechanisms increases as sulfide is removed from the aqueous phase. This in turn enables a more efficient free metal ion acquisition by the metal transporter systems. However, a lower aqueous concentration of neutral metal sulfide complexes may reduce the cross-membrane concentration gradient, which may diminish diffusion-driven metal uptake mechanisms.

The observed presence of reduced organic S suggested an occurrence of thiol functional groups. The chemical speciation of metals in the aqueous phases is largely affected by dissolved organic matter such as thiols, which in turn affect the chemical nature of the pool of soluble metal species in contact with the cell surface. Furthermore, the contribution of complex processes which involve interactions between metals and FeS(s) in biogas reactors to the solubility of metals is suggested. The specific effects of such processes on the regulation of Co and Ni speciation and bioavailability need to be further studied.

Additionally, the findings of this thesis offer analytical tools and fundamental knowledge for an improved assessment of the chemical speciation and potential bioavailability of metals in biogas reactors. The results revealed critical limitations to the application of the SE and AVS/AVS-M methods that were used for the assessment of the trace metal chemical fractions in the biogas reactors, enabling more accurate interpretation of the extraction results. In particular, the importance of taking the chemistry and quantity of Fe into consideration when applying chemical extraction methods has been emphasised.

The chemical speciation modelling approach should be considered as a point of departure to further link the speciation of trace metals to their microbial uptake, and to clarify the role of different metal species in the nutrition of the microorganisms in relation to the performance of biogas reactors. Finally, future research may make use of the comprehensive picture of Co and Ni chemical dynamics provided in this thesis for elucidating the microbial response to variation in the chemical speciation of metals under the different operational conditions of the biogas reactors.

6.1. Practical recommendations

This thesis provides a rational context for evaluating the bio-uptake processes of metals in biogas reactors. The application of the SE methods for the assessment of the potential bioavailability of metals added to biogas reactors produces valuable information regarding the chemical fraction of micronutrient metals. However, the preparation and performance of the extraction procedures are time-consuming, making frequent implementations at biogas plants impractical. In addition, there are uncertainties regarding the undesirable effects of extracting reagents on the original metal speciation which are related to the complexity and heterogeneity of the sludge samples. Addressing the major uptake processes involved in metal acquisition by microorganisms in CSTBRs, the bulk solution chemistry and speciation of metals are directly linked to the bio-uptake processes. Therefore, it is suggested that the soluble fraction of metals is the most relevant fraction for consideration, in addition to the total metal concentrations. The soluble fraction of metals can be separated by simple centrifugation and filtration procedures, which represent the dissolved and most mobile metal species in the reactors. Thus, in combination with chemical speciation modelling, the bioavailability of metals in relation to the major bio-uptake processes can be evaluated.

For the common configuration of the CSTBRs, the pool of metals, which largely pertain to the solid phase as metal sulfides, is continuously lost in the effluent, necessitating a continuous addition of metals in order to maintain a steady level in the reactors. It has been demonstrated that a high sulfide concentration in biogas reactors and extensive precipitation of metal sulfides do not essentially hinder the bio-uptake of metals. It has been argued that low concentrations of free metal ions and labile complexes, and/or relatively high concentrations of neutral metal sulfide species under sulfidic conditions are sufficient to support the bio-uptake of metals. In the case of Co, it has also been suggested that the solubility of CoS(s) minerals can be enhanced under anaerobic digestion conditions. Accordingly, the pool of metal sulfides in the biogas reactors may be regarded as a source of metals for microorganisms. It is therefore suggested that the recovery and utilisation of this fraction of metals may offer the possibility of minimising the metal dosing concentrations. Thus, a means of avoiding the loss of metals in CSTBRs may be the recirculation of the sludge solid phase, which is rich in metals, to the reactor. Further research is recommended to link optimisation of the metal dosing to the CSTBRs to the recirculation of the sludge and its effect on the chemical speciation of metals and reactor performance.

The concentration of Fe resulting from its addition to biogas reactors in relation to S is a critical factor in regulating the chemical speciation of Co and Ni. Thus, Fe addition should be given attention as a means of controlling the bioavailability of trace metals in practice. The concentration of free metal ions and labile complexes, regarded as the most bioavailable metal species in the aqueous phase, is considerably higher at low HS^- concentration. The HS^- concentration in turn is directly controlled by the influent Fe concentration to the reactors. For circumstances in which sulfide removal is needed for practical purposes and metals are present in the influent substrate, adjustment of the Fe concentration may be the first option to test to achieve process optimisation, prior to metal dosing.

The concentration of Fe additives in the form of Fe(II) and Fe(III) should also be considered in relation to the concentration of sulfide in the reactor. Theoretically, the reduction of Fe(III) to Fe(II) involves the oxidation of HS^- to elemental S, followed by a reaction between Fe(II) and the excess HS^- , and the formation of FeS(s) precipitates. The addition of Fe(II) only involves the precipitation of FeS(s) . Thus, the addition of Fe(III) for sulfide removal may be more economical. However, this causes an increase in elemental S concentration in the reactors, and the formation of polysulfide species. The contribution of Co and Ni polysulfide complexes to the solubility and chemical speciation of these metals was insignificant (cf. Paper IV). However, the chemical speciation of other micronutrient metals such as copper (Cu) may be influenced to a great extent by the formation of stable Cu-polysulfide complexes (Paper IV). Finally, the dosing of the metals in complexed forms to maintain a sufficient free metal ion concentration in the aqueous phase of the sludge may also be considered. The application of strong metal-complexes hinders precipitation and association of metals to the solid phase in the reactor, which in turn will enhance their overall solubility. Future research should therefore explore the possibility of using commercially-viable metal-complexing agents for dosing purposes.

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