Anaerobic microbial processes for energy conservation and biotransformation of pollutants

Lara da Luz Ferreira Martins Paulo

Anaerobic processes for energy conservation and biotransformation of pollutants

Lara M. Paulo

Propositions

1. Metal supplementation can be used as a strategy to enhance environmental biotechnological processes. (this thesis)
2. Microbial community analysis provides crucial information to understand a particular biological system (this thesis)
3. Scientists are often unsure how to use statistical tools correctly.
4. With their statement "Life is a net exergonic chemical reaction, it releases energy to go forward.", Sousa *et al.* (Phil. Trans. R. Soc. B. 368: 20130088, 2013) ignored that a small input of energy is always required.
5. Besides scientific skills, good scientific collaborations are also necessary to finish a PhD.
6. The deprivation of sunlight has great impact on a person's happiness and mood.

Propositions belonging to the thesis, entitled

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Wageningen, 8 May 2017

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To my grandmother

Para a minha avó

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Chapter 1

General introduction and thesis outline

1.1. Anaerobic processes for methane production from waste and wastewater

Global energy demand continues to rise quickly. Currently, 86 to 88% of the energy supplied in the world is from fossil fuels, such as coal, oil or natural gas (Shah, 2014; Weiland, 2010). The reliance on fossil fuels to fulfil energy requirements, in a near future, is impracticable: reserves of this type of fuels are limited and depleting at a fast rate; and, CO₂ emissions derived from the utilization of fossil fuels are one of the major contributors for the greenhouse effect (Shah, 2014; Weiland, 2010; Scragg, 2009). It is time to shift towards more sustainable and renewable sources of energy. Biogas is a good alternative renewable energy source, as it can be used to produce power and heat, or used as transport fuel (Weiland, 2010). Biogas is produced anaerobically from organic waste and wastewater, or energy crops and it consists of methane (50-75%), CO₂ (25-50%), and small quantities of other components, such as water, H₂S, and O₂ (Gomez, 2013). Wastewaters store a high energy potential; it is estimated that municipal wastewater stores 3 to 10 times more energy than what is nowadays required for its treatment (Liu and Cheng, 2014; Gude, 2016). Recovering that energy is crucial to make the wastewater treatment process more sustainable and it will have a great impact on fulfilling the world's energy requirements. Anaerobic digestion (AD) has been applied for several decades for this, with installations spread all over the world. In recent years, Bioelectrochemical Systems (BESs) have gained interest as a promising technology to recover energy and resources from wastewaters as well. They can be applied alone or in combination with other processes, such as AD, algae treatment, or electrodialysis (Liu and Cheng, 2014). Both the conventional AD systems and the more *avant-garde* BES have some advantages and disadvantages that are summarised in Table 1.1.

Table 1.1 – Advantages and disadvantages of AD and BES for wastewater treatment and biogas production (Pham *et al.*, 2006; McCarty, 2001; Li *et al.*, 2014; Liu and Cheng, 2014; Pandey *et al.*, 2016).

	AD	BES
Advantages	<ul style="list-style-type: none"> • Well optimized reactors • High bioconversion efficiency (~90%) • Industrial scale feasibility • Well suited for wastewaters containing high chemical organic demand (COD) levels • Already widely applied in WWTPs • Low sludge production • Efficient removal of pathogens • Low operational costs 	<ul style="list-style-type: none"> • Can be applied at small scale • Enables direct electricity recovery • Low energy consumption • No need of temperature control • Low sludge production • Good effluent quality • Effective removal of some specific contaminants, such as nutrients, dyes or metals • Efficient for treating wastewaters containing low COD levels • Enables separation of processes between the anode and the cathode, decreasing the inhibitory effect of some compounds • Can be applied to produce different specific valuable compounds besides methane, e.g. hydrogen peroxide, acetate or ethanol
Disadvantages	<ul style="list-style-type: none"> • Not very efficient at low temperatures (below 30°C) • The quality of the biogas is often low due to H₂S contamination • Requires high organic load • Low effluent quality • High sensitivity of microbial community to environmental stress, like temperature changes or presence of inhibitory compounds, that leads to process failure 	<ul style="list-style-type: none"> • High cost of materials and maintenance • Problems with internal resistance and overpotentials • Poor long-term stability • Still difficult to scale-up • Low power production levels, especially in larger scales • Low methane production rate and purity

AD is a well-established process used for treating waste and wastewater; it can be applied almost everywhere and it does not require large space areas (Lettinga, 1995). AD is divided in four steps; in the first step, complex organic matter is hydrolysed to soluble organic molecules, such as sugars, amino acids and long chain fatty acids. Secondly, these macromolecules are converted to volatile fatty acids (VFAs) during the acidogenesis phase. VFAs are then converted to acetate, formate, H_2 and CO_2 , which are used by methanogens to produce methane and CO_2 (Speece, 1983; de Mes *et al.*, 2003; Abbasi *et al.*, 2012; Shah, 2014). Different groups of microorganisms are involved in each step, with diverse nutritional requirements and physiological properties. For a successful AD, a good balance between all the steps is required. Methanogens are a key group in AD. If methanogenesis is inhibited, the process stops at the acidogenesis step with incomplete degradation of organic matter and no biogas formation. Recent studies proved that methanogens can also be efficiently applied in BESs for methane production. Yet, this group of microorganisms is highly sensitive to alterations in the conditions, such as the presence of certain compounds (heavy metals, chlorinated compounds, antibiotics) and changes in pH or temperature. For this reason, they will be the primary target of study in this thesis.

1.2. Biochemistry of methanogenesis

Methanogenic microorganisms belong to the *Archaea* domain. They are strictly anaerobic microorganisms commonly found in anoxic and freshwater sediments, gastrointestinal tracts of ruminants and termites, hot springs, anaerobic sludge and flooded soils (Daniels, *et al.*, 1984; Liu and Whitman, 2008; Stams and Plugge, 2010; Angelidaki *et al.*, 2011). These microorganisms are a phylogenetically diverse group. They use a limited range of substrates, such as acetate, formate, H_2/CO_2 , CO and methylated compounds (Daniels *et al.*, 1984; Deppenmeir *et al.*, 1996; Liu and Whitman, 2008; Stams and Plugge, 2010). A general representation of the methanogenic pathways is shown in Figure 1.1.

The methanogenic communities present in AD and BES systems were characterized in several studies, although in the case of BESs not much information is available and more research in this topic is needed. A resume of the most common species found in AD and BES systems is presented in Table 1.2. Species from the *Methanoculleus* and *Methanospirillum* genera are the most common hydrogenotrophic methanogens found in AD systems, while in BES *Methanobacterium* and *Methanobrevibacter* species are among the most common. As for aceticlastic methanogens, *Methanosarcina* species are the most common in both systems. In AD systems, *Methanosaeta* species are also commonly abundant. So far, strict methylotrophic methanogens, such as *Methanomethylovorans* species, were only found in AD systems.

Figure 1.1 – Schematic representation of the different methanogenic pathways from formate, H₂/CO₂, acetate and methanol (adapted from Liu and Whitman, 2008; Stams and Plugge, 2010). The red/orange arrows indicate the pathway from formate/ H₂/CO₂; the blue arrows indicate the pathway from acetate and the green arrows indicated the pathway from methanol. The purple lines indicated the common steps for the three pathways. Fd_{red} – reduced form of ferredoxin; Fd_{ox} – oxidized form of ferredoxin; F₄₂₀H₂ – reduced form of the coenzyme F₄₂₀; MFR – methanofuran; H₄MPT – tetrahydromethanopterin; CoB-SH – coenzyme B; CoM-SH – coenzyme M; CoM-S-S-CoB – heterodisulphide of CoM and CoB; SH-CoA – coenzyme A.

Table 1.2 – Summary of the most commonly found methanogens in AD and BES using mixed cultures.

	Microorganism	AD	BES
Hydrogenotrophic	<i>Methanobacterium</i>	✓ ^{1,9}	✓ ^{2–8}
	<i>Methanobrevibacter</i>	✓ ^{1, 11, 13}	✓ ^{4, 6}
	<i>Methanoculleus</i>	✓ ^{1, 9, 10, 14, 15}	✓ ³
	<i>Methanospirillum</i>	✓ ^{1, 9, 10, 14, 15}	
	<i>Methanothermobacter</i>	✓ ^{1, 13}	
	<i>Methanocorpusculum</i>	✓ ^{9, 14, 15}	✓ ⁴
	<i>Methanocalculus</i>	✓ ⁹	
	<i>Methanogenium</i>	✓ ¹⁰	
	<i>Methanofollis</i>	✓ ¹⁵	
	<i>Methanolinea</i>	✓ ¹⁵	
	<i>Methanosarcina</i>	✓ ^{1,9–11, 14, 15}	✓ ^{2, 3, 4, 7}
Aceticlastic	<i>Methanosaeta</i>	✓ ^{1, 9, 11–15}	✓ ⁵
	<i>Methanosarcina</i>	✓ ^{1,9–11, 14, 15}	✓ ^{2, 3, 4, 7}
Methylootrophic	<i>Methanomethylovorans</i>	✓ ^{9, 11, 14}	
	<i>Methanosarcina</i>	✓ ^{1,9–11, 14, 15}	✓ ^{2, 3, 4, 7}

References: ¹Venkateshwaran *et al.*, 2016; ²van Eerten-Jansen *et al.*, 2013; ³Sasaki *et al.*, 2011; ⁴Shehab *et al.*, 2013; ⁵Siegert *et al.*, 2015; ⁶Siegert *et al.*, 2014; ⁷Bretschger *et al.*, 2015; ⁸Villano *et al.*, 2010; ⁹Leclerc *et al.*, 2004; ¹⁰Liu *et al.*, 2009; ¹¹McHugh *et al.*, 2003; ¹²Roest *et al.*, 2005; ¹³Supaphol *et al.*, 2011; ¹⁴Ziganshin *et al.*, 2013; ¹⁵Lee *et al.*, 2010.

1.2.1. Hydrogenotrophic methanogenesis

A wide diversity of hydrogenotrophic methanogens is described. They are able to use H₂ to reduce CO₂ to methane according with the Equation 1.1:

Formate can also be used by many, but not all hydrogenotrophic methanogens. Formate is converted to CH₄ according to Equation 1.2 (Stams and Plugge, 2010).

1.2.2. Aceticlastic methanogenesis

Methane resulting from acetate represents up to two thirds of the total methane produced during AD. There are only two known genera of methanogens that can use this substrate: *Methanosarcina* and *Methanosaeta* (Liu and Whitman, 2008; Stams and Plugge, 2010; Welte and Deppenmeier, 2014). *Methanosarcina* species are also able to use other substrates, such as methylated compounds and H₂/CO₂, while *Methanosaeta* can only use acetate (Liu and Whitman, 2008).

The carboxyl group of acetate is converted to CO₂ and the methyl group is converted to methane (Equation 1.3) (Liu and Whitman, 2008; Stams and Plugge, 2010).

1.2.3. Methylotrophic methanogenesis

Methylated compounds, such as methanol, dimethyl sulphide, methylated amines or methanethiol, can be used by methanogens from the order *Methanosarcinales* and *Methanosphaera* (Liu and Whitman, 2008; Stams and Plugge, 2010). For most methylotrophic methanogens the oxidation of methyl group to CO₂ in a reverse process of the hydrogenotrophic methanogenesis (Figure 1.1), provides the necessary electrons for the reduction of the methyl group to methane (Equation 1.4). On the other hand, *Methanomicrococcus blatticola* and *Methanosphaera* species reduce the methyl groups with external H₂ (Equation 1.5) (Liu and Whitman, 2008; Stams and Plugge, 2010).

1.3. Importance of metal ions in methanogenesis

Fe, Ni and Co are important trace metals for methanogens. The importance of Fe is related with its redox properties in a variety of redox enzymes. In methanogens, Fe is present in many enzymes, such as in Ech hydrogenase (Welte and Deppenmeier, 2014), $F_{420}H_2$ dehydrogenase (Deppenmeier, 2004), and many [Ni-Fe] hydrogenases. The methanogenesis metabolic pathways require several enzymes that are found exclusively in methanogens (Deppenmeier *et al.*, 1996). Despite their differences, all methanogenic pathways have a common step, the reaction between methyl-CoM (CH_3 -S-CoM) and coenzyme B (HS-CoB) to form methane and the corresponding heterodisulphide (CoM-S-S-CoB) (Hedderich and Whitman, 2006). This reaction is catalysed by methyl-coenzyme M reductase that has in its active site a prosthetic group, F_{430} , which is a Ni porphyrinoid (Hedderich and Whitman, 2006). The methyl- H_4 MPT:CoM-SH methyltransferase (Mtr) is a membrane complex composed of eight different subunits. In its subunit MtrA, this enzyme has a cob(I)amide prosthetic group which is believed to play an important role in the enzyme function (Hedderich and Whitman, 2006). Hydrogenases are essential for methanogens and five different types were identified so far (Hedderich and Whitman, 2006; Thauer *et al.*, 2010). Four of these hydrogenases are [Ni-Fe]-hydrogenases and the other is a [Fe]-hydrogenase (Thauer *et al.*, 2010). Moreover, many of the enzymes involved in methanogenesis possess [Fe-S] clusters (Deppenmeier *et al.*, 1996). Thus, enzymes containing metal ions in their active site have a fundamental role in the methanogenic pathways. For this reason, trace elements availability is a key factor for growth of methanogens. This topic is addressed in Chapter 2.

1.4. Factors affecting AD

The effectiveness of AD can be affected by several factors, such as pH, temperature, inhibitory compounds and presence of nutrients (Lettinga, 1995; Shah, 2014). Ideally, the pH for optimal biogas production should be around 6.5 and 7.5 (de Mes *et al.*, 2003). Accumulation of VFAs can lead to a decrease in pH, while accumulation of ammonia leads to an increase of the pH (Shah, 2014; Abbasi *et al.*, 2012). Depending on the temperature at which the process is occurring, AD can be considered psychrophilic (10-20°C), mesophilic (20-40°C) or thermophilic (50-60°C) (de Mes *et al.*, 2003). Thermophilic AD is generally more efficient since thermophilic methanogens have a higher growth rate than mesophilic methanogens, which makes the process faster. Moreover, high temperatures allow destruction of pathogens and reduce foaming (Ahring, 1995; Sung and Liu, 2003). On the other hand, thermophilic methanogens are more sensitive than mesophilic to temperature fluctuations and require longer times to adjust to a new temperature (Abbasi, 2012; Shah, 2014). Ammonia toxicity increases with temperature, which makes thermophilic operation more susceptible to ammonia inhibition (Weiland, 2010; Shah, 2014). The availability of nutrients is a key factor for the growth of the microorganisms involved in the process. The trace elements topic will be discussed further in Chapter 2. Several compounds can be present in wastewaters that can affect AD due to their toxic effect. Sulphate, detergents, heavy metals, antibiotics, organic solvents, chlorinated compounds and other micropollutants can be a cause of failure of AD. In particular, methanogens are sensitive to the presence of toxic/inhibitory compounds (Lettinga, 1995; Murphy and Thamsiriroj, 2013). This topic will be further discussed in section 1.5.

1.5. The effects of toxic/inhibitory compounds on AD

One of the main causes of failure during AD is the presence of toxic/inhibitory substances, which are commonly present in waste and wastewater. Ammonia, sulphide, heavy metals, organic compounds and chlorinated compounds, are among the substances that can inhibit AD (Chen *et al.*, 2008). In this section, an overview of the principal components affecting AD and their effects on the process, with special focus on the effects on methanogens, will be given.

1.5.1. Heavy metals

Heavy metals can be found in wastewaters, especially industrial wastewaters, such as the ones produced in metal plating, mining or paper industry. Heavy metals are non-biodegradable compounds that can accumulate up to toxic/carcinogenic concentrations in living organisms (Chen *et al.*, 2008; Fu and Wang, 2011; Chen *et al.*, 2014). Their toxic effect is caused by their ability to enter the cells and disrupt enzyme function and structure (Chen *et al.*, 2008). The toxicity of metals is influenced by different factors, such as pH, temperature, presence of chelating agents, redox potential, concentration and chemical form of the metal (Gadd and Griffiths, 1977; Collins and Stotzky, 1989; Chen *et al.*, 2008; Mudhoo and Kumar, 2013). Mainly the free form of the metal is toxic to the microorganisms (Oleszkiewicz and Sharma, 1990; Chen *et al.*, 2008).

One of the main consequences of metal toxicity for AD is the decrease in biogas production. Moreover, heavy metals can influence different physical-chemical processes that occur during an AD process, such as precipitation with carbonate, hydroxides and/or sulphide, sorption to the sludge, and formation of complexes with some of the compounds formed during AD (Mudhoo and Kumar, 2013). The available literature on this topic provides different information concerning the inhibitory concentrations of heavy metals, as for example 70 to 400 mg/L for Cu,

200 to 600 mg/L for Zn or 10 to 2000 mg/L for Ni (Chen *et al.*, 2014). The different experimental conditions used (different substrates, pH, temperatures, ionic forms of the metal, different sludges) are possibly one of the explanations for the differences observed between studies (Chen *et al.*, 2008). These differences make it difficult to compare the studies and to establish a toxic concentration for each heavy metal and a hierarchy of metal toxicity. In general, methanogens are considered to be more sensitive to toxic levels of metals than other anaerobes (Chen *et al.*, 2008). Microorganisms have developed protection strategies to deal with metals, such as biomethylation, active extrusion, excretion of precipitating or chelating agents (e.g. sulphide or melanin), formation of inclusion bodies, or reduction of the metal to a less toxic form (Oleszkiewicz and Sharma, 1990; Nies, 1999; Haferburg and Kothe, 2007; Gadd, 2009; Lemire *et al.*, 2013). In the case of high levels of heavy metals in the wastewaters, different technologies can be used prior to AD to decrease their concentrations and to avoid AD failure. Coagulation-flocculation, ion exchange, solvent extraction, adsorption, membrane filtration, complexation or precipitation are some of the technologies available to metal detoxification (Gadd and White, 1993). However, many of these treatments are costly and produce a chemical sludge that needs proper further treatment (Veeken and Rulkens, 2003). Another possible metal detoxification method is the use of biologically produced sulphide. Sulphide is produced by sulphate-reducing bacteria (SRB) via sulphate reduction. This method was proven to be useful in precipitating and recovering heavy metals from effluents resulting from mining activity (Hamack and Edenborn, 1992; Kaksonen and Puhakka, 2007; Huisman *et al.*, 2006). Yet, more studies are needed for the application of this method in AD systems and how the application of such method would affect methane production.

A more detailed review on the effects of heavy metals on AD and, more specifically, on methanogens is given in Chapter 2. The topic of biosulphide as detoxification method for metal toxicity is also addressed in that Chapter.

1.5.2. Ammonia

Ammonia is one of the products that result from the biological degradation of proteins and urea (Sung and Li, 2003; Chen *et al.*, 2008; Rajagopal *et al.*, 2013). It is an essential nutrient for microorganism, but at high concentrations it becomes an inhibitor (Chen *et al.*, 2014). Ammonium ion (NH_4^+) and free ammonia (NH_3) are the main forms in aqueous solution. The balance between the two forms is affected by pH and ammonia concentration increases with the pH (Chen *et al.*, 2014). Since bacterial cell membranes are highly permeable to NH_3 , this form is considered to be the main cause of inhibition, especially to methanogens (Rajagopal *et al.*, 2013). Once inside the cell, NH_3 is converted to NH_4^+ , absorbing protons for this. To keep the proton balance, the cells use an ATP-dependent potassium antiporter. Ammonia can also inhibit specific enzyme activities (Chen *et al.*, 2008; Rajagopal *et al.*, 2013; Chen *et al.*, 2014).

Methanogens are considered to be the most sensitive group in AD systems to ammonia (Chen *et al.*, 2008). For example, at ammonia concentrations of 4 to 5.7 g $\text{NH}_3\text{-N/L}$ acidogenic populations in granular sludge were almost unaffected, while methanogens lost more than 50% of their activity (Koster and Lettinga, 1988). Strong methanogenesis inhibition was observed in semi-continuous anaerobic digestion of food waste at concentrations above 2 g $\text{NH}_3\text{-N/L}$, while hydrolysis and acidification were only slightly affected (Chen *et al.*, 2016). Different inhibitory concentrations can be found in literature, possibly due to experimental differences; concentrations of 1.7 to 14 g $\text{NH}_3\text{-N/L}$ are described to cause 50% inhibition of methane production (Chen *et al.*, 2016). For example, 10 g $\text{NH}_3\text{-N/L}$ was lethal for

thermophilic aceticlastic methanogens (Liu and Sung, 2002). Similar concentrations were reported when using a continuous reactor treating pig manure co-digested with solid fractions separated from pig manure, organic load of 9.4 g VS/L_{digester}/day. A 50% decrease in methane yield in the presence of 11 g NH₄-N/L was observed (Nakakubo *et al.*, 2008). Also in agreement with these results, concentrations of 9 g NH₃-N/L totally inhibited methanogenesis in a reactor treating chicken manure and maize silage without water dilution (Sun *et al.*, 2016). Yet, in another study where the ammonia toxicity effects on granular sludge from upflow anaerobic sludge bed (UASB) and expanded granular sludge bed (EGSB) reactors were studied, only 2.35 and 2.75 g/L of ammonia nitrogen caused 50% inhibition of the specific methanogenic activity, respectively (Zhou and Qiu, 2006). They also reported that hydrogenotrophic methanogens are less sensitive to ammonia than aceticlastic methanogens. These examples show the wide range of inhibitory concentrations reported so far.

A peculiarity of methanogenesis in systems with high ammonia concentration is the predominance of syntrophic acetate oxidation. The first evidence for that was reported in an enrichment culture in the presence of 7 g NH₄⁺-N/L using acetate as only substrate (Blomgren *et al.*, 1990). Acetate was stoichiometrically converted to methane, yet, no aceticlastic methanogens could be detected. Later, using C¹⁴-labelled acetate in a triculture with a high ammonia concentration, pH 8, equal amounts of labelled methane and CO₂ were detected, which was attributed to oxidation of acetate followed by the reduction of CO₂ to methane (Schnürer *et al.*, 1994). The reduction of labelled carbonate (H¹⁴CO₃⁻) to labelled methane was also observed, indicating that hydrogenotrophic methanogenesis occurred. The pathway for methane formation from acetate was further investigated using the labelled substrates in sludges from 13 different biogas reactors with high levels of salts, mainly ammonium, and VFAs (Schnürer *et al.*, 1999). Syntrophic acetate oxidation

was reported to be the dominant mechanism of acetoclastic methanogenesis. The statistical analysis also showed a close relation between the degree of syntrophic acetate oxidation and the ammonium and potassium concentrations. Furthermore, the main methanogen identified in reactors with syntrophic acetate oxidation belonged to the genus *Methanoculleus*, a known hydrogenotrophic methanogen (Schnürer *et al.*, 1999). The changes in the microbial communities of syntrophic acetate-oxidizing bacteria and methanogens caused by the presence of high level of ammonia were investigated in laboratory-scale mesophilic biogas reactors using qPCR (Westerholm *et al.*, 2011). In the test reactor, the ammonia concentrations were increased from 0.65-0.90 g $\text{NH}_4^+\text{-N/L}$ to 0.8-6.9 $\text{NH}_4^+\text{-N/L}$. An increase in the populations of the syntrophic acetate oxidizers *Clostridium ultunense*, *Syntrophaceticus schinkii* and *Tepidanaerobacter acetatoxydans* was observed in the test reactor, compared with the control reactor. Moreover, a decrease in the abundance of the methanogenic families *Methanosaetaceae* and *Methanosarcinaceae* was observed in the test reactor. These results show a clear shift from acetoclastic methanogenesis to syntrophic acetate oxidation. Further research revealed that the shift was very distinct and occurred at $\text{NH}_4^+\text{-N}$ concentrations above 3 g/L (Schnürer and Nordberg, 2008).

Factors, such as pH, temperature, have a direct or indirect effect on the toxicity of ammonia (Yenigün and Demirel, 2013). At higher pH values, not only the NH_3 concentration is higher, but it is also reported that the biogas process becomes more sensitive towards ammonia. On the other hand, it was observed that thermophilic AD processes are more sensitive to ammonia than mesophilic ones (Chen *et al.*, 2014). Acclimation of the microorganisms to high concentrations is a successful strategy to deal with ammonia toxicity (Rajagopal *et al.*, 2013). The adapted cultures can survive at concentrations far exceeding the initial toxic concentrations (Chen *et al.*, 2008). However, a proper acclimation can take two months or longer (Rajagopal

et al., 2013). Since ammonia toxicity is highly influenced by pH, a proper pH control can help to reduce ammonia toxicity (Rajagopal *et al.*, 2013). Another possibility to mitigate the ammonia effect is to dilute the substrate/wastewater, but this leads to an increase of the volume to treat and is economically undesirable (Chen *et al.*, 2008; Rajagopal *et al.*, 2013). In terms of process operational parameters, increasing the biomass retention time is also a possibility to deal with ammonia toxicity. Immobilization of the microorganisms using inert materials, like clay or active carbon, is another possible strategy that was shown to reduce the effects of ammonia on biogas production (Sung and Liu, 2003; Rajagopal *et al.*, 2013). Finally, there is the possibility to use physico-chemical methods to remove ammonia from the influent (Chen *et al.*, 2008). The addition of material with ion exchange abilities, such as zeolites, phosphorite or glauconite, or inorganic absorbent materials, such as clay or manganese oxides, has shown good results in diminishing the toxic effect of ammonia on AD (Romero-Güiza *et al.*, 2016). A method that also has drawn attention is the coupling of AD with struvite precipitation in the same reactor by addition of Mg^{2+} and PO_4^{3-} (Romero-Güiza *et al.*, 2016).

1.5.3. Sulphate/Sulphide

The activity of many industries, as for example mining, paper, and petrochemical industries, is leading to an increase of sulphate levels in wastewater (Chen *et al.*, 2014). Although sulphate itself is not toxic, it can lead to inhibition of AD processes due to i) competition between sulphate-reducing bacteria (SRB) and other microorganisms involved in AD for common substrates; ii) inhibition of the microorganism by sulphide, the product of sulphate reduction (Chen *et al.*, 2008).

SRB can use a wide range of substrates, such as alcohols, VFAs, aromatic compounds, but in general not complex molecules, such as proteins or lipids. For this reason, they are able to compete with some of the other groups of

microorganisms involved in AD, especially acetogens and methanogens. The outcome of the competition will define the levels of sulphide in the media (Chen *et al.*, 2008). Many factors, such as COD/SO₄²⁻ levels, ratio between SRB and other microorganisms, temperature, affect the competition (Chen *et al.*, 2008).

Sulphate is reduced to sulphide which is highly undesired in an AD process because it is toxic to microorganisms, including SRB, is corrosive and malodorous (Colleran *et al.*, 1995). Sulphide can diffuse through the cell membrane and inside the cell can denature proteins, inhibit enzymes or interfere with the sulphur uptake mechanisms (Colleran *et al.*, 1995; Chen *et al.*, 2008). Like ammonia, sulphide toxicity is pH dependent due to the equilibrium between H₂S (present at lower pH values and the most toxic form) and HS⁻ (present at higher pH and less toxic) (Lens *et al.*, 1998). The toxic concentrations reported differ considerable, ranging from 100 – 800 mg/L of HS⁻ and 50 – 400 mg/L for H₂S (Chen *et al.*, 2008). Several techniques can be used for sulphide removal. These include stripping, coagulation, oxidation, precipitation or biological conversion to sulphur (Chen *et al.*, 2008). A more comprehensive review of this this topic is given in Chapter 2.

1.5.4. Chlorinated compounds

Anthropogenic chlorinated compounds are commonly present in wastewaters from industrial or domestic origin (Krzmarzick and Novak, 2014; Yang *et al.*, 2005). Chlorophenols, for example, are widely used as pesticides, antiseptics, fungicides and as preservatives for paint, leather, etc. (Leys *et al.*, 2013; Chen *et al.*, 2014). Highly chlorinated compounds are resistant to aerobic degradation, but they can be transformed under anoxic/anaerobic conditions (Holliger *et al.*, 2004). Although most of these compounds can be biodegraded, they are toxic to anaerobic microorganisms. Chlorophenols can disrupt the proton gradient across membranes and interfere with energy transduction in the cells (Chen *et al.*, 2008). Their toxicity

depends on the degree of chlorination and the position of the chlorine (Chen *et al.*, 2014). Pentachlorophenol is the most toxic of these compounds to methanogens and acidogenic bacteria with toxic concentrations of 0.5 to 10 mg/L, depending on the species (Patel *et al.*, 1991; Chen *et al.*, 2008). Available studies are not clear concerning the relationship between the number of chlorines and toxicity. Some studies report a positive correlation between the number of chlorines in the aromatic benzene rings and the higher toxicity for methanogens (Sierra-Alvarez and Lettinga, 1991; Jin and Bhattacharya, 1996), while other studies do not find this correlation (Blum and Speece, 1991). Regarding the position of substitution, toxicity increased from *ortho* << *para* < *meta* (Chen *et al.*, 2008; Chen *et al.*, 2014).

Polychlorinated aliphatics, such as dichloromethane (DCM), chloroform (CF), trichloroethene (TCE), tetrachloroethene (PCE) or perchloroethylene are common environment contaminants due to their large used as solvents (Yang *et al.*, 2005; Chen *et al.*, 2008). Yu and Smith (2000) studied the toxic effects of these compounds towards methanogenesis and found that CF is the most toxic; 0.09 mg/L of CF completely inhibited methane production. DCM and TCE inhibited methanogenesis at concentrations of 3.9 mg/L and 18 mg/L, respectively, while PCE did not inhibit methanogenesis at 14.5 mg/L.

1.3.4.1 Reductive dehalogenation

Under anaerobic conditions chlorinated compounds can be reductively dehalogenated to chlorinated compounds with less chlorines (Yang *et al.*, 2005). Reductive dehalogenation is the only mechanism for the anaerobic biological degradation of certain compounds, such as PCE or hexachlorobenzene, resulting in compounds that may be less toxic and easier to degrade further (Mohn and Tiedje, 1992). *Desulfomonile tiedjei* was the first anaerobic bacterium described to use a chlorinated compound as electron acceptor (Holliger and Schumacher, 1994;

McCarty, 2016). Nowadays, many different bacteria have been described that perform reductive dehalogenation. They can be classified as facultative or obligate organohalide-respiring bacteria (OHRB) (Krzmarzick and Novak, 2014, Atashgahi *et al.*, 2016). Halorespiring bacteria have been isolated from a wide range of environments and show great differences in their phylogeny (van Eekert and Schraa, 2001). *Dehalococcoides* species have been described to perform complete dehalogenation of PCE or vinyl chloride (VC) to ethane, while other bacteria can perform only incomplete dehalogenation, removing only one or two chlorine atoms (van Eekert and Schraa, 2001; Yang *et al.*, 2005; McCarty, 2016).

Facultative OHRB have a versatile metabolism and can use several electron acceptors, such as nitrate, nitrite, Fe(III), Mn(IV), DMSO, As(V), sulphate or thiosulphate, as well as different electron donors, such as acetate, butyrate, ethanol, lactate, formate, pyruvate, succinate or H₂. On the other hand, obligate OHRB have a more restrict metabolism and, for almost all studied cases, they are limited to H₂ as electron donor and use halogenated compounds as electron acceptors in their microbial energy metabolism (Mayer-Blackwell *et al.*, 2016). Several studies showed that halorespiring bacteria have higher affinity for hydrogen than methanogens, giving them an advantage in environments where hydrogen concentrations are low (Dolfing, 2016). However, when hydrogen concentrations are high, they are probably outcompeted by other microorganisms (Middeldorp *et al.*, 1999; Smidt and de Vos, 2004).

1.3.4.2 Co-metabolic reductive dehalogenation

Reductive dehalogenation can also be performed co-metabolically or facultatively (Holliger and Schumacher, 1994). For example, *Desulfitobacterium* can perform facultative dehalogenation, as well as some strains of *Desulfuromonas* or *Geobacter* (Krzmarzick and Novak, 2014; Mayer-Blackwell *et al.*, 2016). The enzyme systems

that were reported to be involved in the co-metabolic reductive dehalogenation include corrinoids, factor F₄₃₀, ferredoxins, iron (II) porphyrins or flavoprotein-flavin complexes (Middeldorp *et al.*, 1999; Holliger *et al.*, 2004).

Homoacetogens and methanogens can perform co-metabolic dehalogenation. Methanogens can dechlorinate compounds such as tetra- and trichloromethane, 1,2-dichloroethene (DCE), and PCE (Holliger *et al.*, 2004). DCE was described to be reductively dechlorinated by several methanogens, such as *Methanothermobacter thermoautotrophicum*, *Methanosarcina barkeri* or *Methanococcus mazei* (Holliger *et al.*, 1990). The work of Holliger *et al.* (1992) indicates that the reductive dechlorination of DCE to ethene and VC by methanogens is catalysed by the methyl-coenzyme M reductase, which contains F₄₃₀ as cofactor. Dechlorination of PCE by *Sporomusa ovata* and *Methanosarcina thermophila* involves enzymes that are part of the acetyl-CoA pathway (Middeldorp *et al.*, 1999). It was observed that vitamin B₁₂, cofactor F₄₃₀ and hematin catalysed the reductive dechlorination of polychlorinated ethenes and benzenes, while ferredoxins and azurin did not (Gantzer and Wackett, 1991). Vitamin B₁₂, and cofactor F₄₃₀ could sequentially dechlorinate PCE to ethene while hematin could only dechlorinate it to VC. In another work, it was reported that the corrinoid-containing carbon monoxide dehydrogenase of *Methanosarcina thermophila* was able to convert TCE to *cis*-, *trans*-, and 1,1-DCE, VC and ethene (Jablonski and Ferry, 1992). Although the presence of Fe²⁺ did not improve carbon tetrachloride degradation, the chloroform transformation rate by *M. thermophila* was enhanced when the cells were grown in the presence of Fe²⁺ (Andrews and Novak, 2001). The authors hypothesised that Fe²⁺ may influence the chloroform transformation rate by influencing the porphyrin and corrinoid activity. All these results point to the involvement and importance of corrinoids, porphyrins and cofactors in the co-metabolic dehalogenation. Many, if not all, of these compounds have metal ions in their structure. The importance of trace elements for microbial activities is

highlighted and points out that research on microbial trace elements requirements is needed. Amendment of trace elements can, most likely, not only improve biogas production, but also improve other microbial processes, such as dehalogenation.

1.5.5. Long chain fatty acids (LCFAs)

Lipids can cause clogging problems during AD processes and they can also interfere with substrate uptake since they can adsorb to the microbial biomass surface (Cirne *et al.*, 2007). During AD, lipids are hydrolysed to glycerol and LCFAs. Glycerol is further converted to acetate during acidogenesis and the LCFAs are converted to acetate, propionate (in the case of odd-number carbon LCFAs), and hydrogen by the β -oxidation pathway (Weng and Jeris, 1976). Most of the inhibitory problems associated with lipids are commonly attributed to LCFAs (Cirne *et al.*, 2007). Their toxic effect is related with their adsorption onto the cell wall and membrane, interfering with transport and protein function (Chen *et al.*, 2008; Chen *et al.*, 2014). The adhesion of biomass to LCFAs can lead to flotation and biomass washout (Hwu *et al.*, 1998; Cirne *et al.*, 2007; Chen *et al.*, 2008). The type of LCFAs, the microbial population, and temperature are factors that affect LCFAs toxicity towards AD processes (Palatsi *et al.*, 2012; Chen *et al.*, 2014). For example, oleic and lauric acid have similar toxicity with 4.3 mM causing 50% inhibition of aceticlastic methanogenesis (Koster and Cramer, 1987). On the other hand, granular sludge was reported to be more resistant to LCFAs than suspended or flocculent sludges (Hwu *et al.*, 1996). The work of Pereira *et al.* (2004) indicates that anaerobic sludge is able to mineralize LCFAs 1 g COD/ g volatile solids, contradicting the idea that LCFAs inhibition is permanent or that LCFAs have a bactericidal effect. Further work on the topic revealed that LCFAs form a physical barrier, inhibiting the transfer of substrates and products (Pereira *et al.*, 2005). Addition of calcium is a

possible strategy to decrease the LCFAs inhibitory effect. Other options are to increase the biomass/LCFA ratio, the addition of adsorbents, solubilisation of lipid waste via saponification, enzymatic pre-treatments, or addition of easily degradable co-substrates (Palatsi *et al.*, 2012; Chen *et al.*, 2014).

Regarding methanogens, they are considered to be highly sensitive to LCFAs. Kim *et al.* (2004) reported that the methane production from acetate decreased in the presence of LCFAs and that it was related to the concentration and number of double bonds of LCFAs. They reported 50% inhibitory concentrations of 3.1, 0.72, 5.71 and 5.37 mM for oleate, linoleate, palmitate and stearate, respectively. The effects of oleate, stearate and palmitate on methanogenic activity of enriched and pure cultures were evaluated by Sousa *et al.* (2013). The authors observed that *Methanobacterium formicum* could grow in oleate- and palmitate-degrading enrichments, while *Methanospirillum hungatei* could only grow in palmitate-degrading enrichments. *Methanosarcina mazei* and *Methanosaeta concilii* could survive in both enrichments. Further experiments using pure cultures of methanogens revealed that the methanogenic activity of *M. hungatei* on H_2/CO_2 was inhibited in 50% in the presence of 0.3, 0.4 and 1 mM of oleate, stearate and palmitate, respectively, while the hydrogenotrophic activity of *M. formicum* was inhibited in 50% with 1 mM of oleate and > 4 mM of palmitate or stearate. The effects of oleate and palmitate towards pure cultures of *M. concilii* and *M. mazei* was also performed (Silva *et al.*, 2016). A 50% inhibition of methane production from acetate was detected for 0.5 mM of oleate and total inhibition at 2 mM for both methanogens. Palmitate was better tolerated; 2 mM resulted only in 11% inhibition of methane production for cultures of *M. concilii* and 64% for cultures of *M. mazei*. A start-up strategy based on a sequence of step feeding and reaction cycles, was used for treating an oleate-rich wastewater (organic load of 21 kg COD m⁻³ day⁻¹, 50% oleate) (Cavaleiro *et al.*,

2009). These authors reported good treatment efficiency, with a 72% of methane recovery, using this strategy.

1.5.6. Emerging contaminants

The concept of emerging contaminants generally refers to surfactants, personal care products, pharmaceuticals, estrogens, perfluorinated compounds and phthalate esters (Stasinakis, 2012; Hamza *et al.*, 2016). The effects of these compounds on AD have been studied both in batch and in pilot-scale reactors. Most of the studies regard the effects of surfactants and pharmaceuticals, with a special focus on antibiotics (Stasinakis, 2012). Unfortunately, there is not much information available about the effects on AD of many of the emerging contaminants.

Linear alkyl benzene sulphonates, a type of surfactants, seem to affect more butyrate and propionate utilizing bacteria than methanogens (Angelidaki *et al.*, 2004). Garcia *et al.* (2006) reported an increase in the toxicity related with the increase of the alkyl chain length. On the other hand, the same authors proved in another study that for other surfactants group, the quaternary ammonium based compounds (QAC), the toxicity on methanogens decreases with the increase of the alkyl chain length (Garcia *et al.*, 1999). Concentrations higher than 25 mg/L of QAC were reported to inhibit methanogens (Tezel *et al.*, 2006). The same study reported that acidogenesis was less susceptible to inhibition by QAC than methanogenesis, which was explained by the fact that methanogens have no outer membrane, allowing QACs to easily reach the cytoplasmic membrane causing inhibition. The cytoplasmic membrane of methanogens is more hydrophobic than the one of bacteria, making methanogens more vulnerable to QACs; QACs also affect the proton motive force, which is a main driving force for ATP generation for methanogens, while energy conservation by fermenters is mostly based on substrate phosphorylation.

Pharmaceutical compounds can often be found in wastewaters and are one of the factors causing problems during AD, especially in wastewaters rich in these compounds, as the ones resulting from pharmaceuticals production industries (Ji *et al.*, 2013). Pharmaceutical compounds represent a large number of compounds. Sanz *et al.* (1996) studied the effect of several different antibiotics on AD. They observed that the inhibitory concentration and the inhibition mechanism depend on the type of compound. Doxycycline, streptomycin or neomycine were reported to have a strong inhibitory effect, affecting the activity of propionate and butyrate degrading bacteria. Chlortetracycline (40 mg/L) and chloramphenicol (15-20 mg/L) also showed a strong inhibitory effect, but in this case, towards acetoclastic methanogens. On the other hand, erythromycin only caused inhibition of biogas production at concentrations higher than 250 mg/L. β -lactamic antibiotics caused a 55% decrease of biogas production for concentration ranging from 10-500 mg/L. Other antibiotics, like kanamycin, gentamicin and spectinomycin, did not affect AD (Sanz *et al.*, 1996). A more specific study concerning the effects of several antibiotics on methanogenesis was performed by Fountoulakis *et al.* (2004). They determined that the IC_{50} was 30 mg/L for propranolol, 334 mg/L for ofloxacin, 120 mg/L for diclofenac, 220 mg/L for carbamazepine and more than 400 mg/L for clofibric acid and sulfamethoxazole. The effects of 16 antibiotics on methanogenesis were analysed; for most antibiotics studied, a moderate inhibitory effect for concentrations between 24 and 1000 mg/L was found, while metronidazol had a strong inhibitory effect causing 50% inhibition at 0.7 mg/L (Gartiser *et al.*, 2007). More recently, Ji *et al.* (2013) determined the IC_{50} after 15 min of antibiotic's treatment. They reported IC_{50} of 3.99, 5.11, 4.32 and 5.63 g/L for amoxicillin, kanamycin, lincomycin and ciprofloxacin, respectively. Another study showed that sulfamethazine and ampicillin have no impact on biogas production, while 201 mg/L of florfenicol inhibited in 75% (Mitchell *et al.*, 2013). Inhibitory concentrations of

tylosin for methanogenesis were recently reported; 49% inhibition with 0.01 mg/L and total inhibition with concentrations above 0.5 mg/L (García-Sánchez *et al.*, 2016). Moreover, it was observed that digesters acclimated to tylosin at concentrations from 0.01 to 0.065 mg/L did not suffer inhibition. In conclusion, pharmaceutical compounds represent a complex group of compounds, presenting a wide range of toxic concentrations, from a few mg/L like tylosin (0.01 mg/L), to several g/L, like ciprofloxacin (5.63 g/L). As new chemicals substances for pharmaceutical use continue to be developed every year, this topic will require continuous research and update.

Engineered nanomaterials are becoming more common in commercial products and some of the more used are TiO₂, Ag and ZnO (Demirel, 2016). Many studies have been performed to understand the impact of these emerging compounds on wastewater treatment and a review on the topic is given by Demirel (2016). The inhibitory concentrations for methane production reported go from 10 mg/L (CeO₂) up to 1680 mg/L (nano zero valent iron). One of the main causes for nanoparticles toxicity is the release of the metal ion that can reach inhibitory concentrations, as discussed previously in Section 1.5.1.

1.6. Bioelectrochemical systems (BESs)

In recent years, BESs for wastewater treatment gained more attention because, besides applications for energy production, they can also be used to produce chemicals (Hamelers *et al.*, 2010; Li *et al.*, 2014; Gude, 2016; Bajracharya *et al.*, 2016). This type of system is based on the ability of certain species of microorganisms to perform extracellular electron transfer (Rozendal *et al.* 2008; Rabaey and Rozendal, 2010; Malvankar and Lovley, 2014). Extracellular electron transfer can be performed indirectly through redox cycling of electron shuttling compounds between the microorganism and the electrode or directly through

contact between the microorganism and the electrode. Direct electron transfer was reported for *Geobacter* and *Shewanella* species and in mixed cultures using CO₂, O₂, Cr(VI), nitrate, fumarate or tetrachloroethene as electron acceptor (Huang *et al.*, 2011; Lovley, 2011; Patil *et al.*, 2012; Kato, 2015). *Geobacter* and *Shewanella* species are able to transfer electrons through a chain of c-type cytochromes across the cell envelope to extracellular electron acceptors (Rosenbaum *et al.*, 2011). Some microorganisms use nanowires (pili or cell appendages) to transfer the electrons to the electrode, such as *Geobacter sulfurreducens*, *Shewanella oneidensis* and the cyanobacterium *Synechocystis* (Patil *et al.*, 2012; Malvankar and Lovley, 2014; Kado, 2015). Microorganisms can also excrete redox-active compounds, such as flavins and phenazine compounds, to perform indirect electron transfer (Lovley, 2012; Kato, 2015; Tremblay and Zhang, 2015). For example, *S. oneidensis* and *Shewanella* sp. MR-4 were reported to excrete flavins as external electron transfer mediators (Marsili *et al.*, 2008). Moreover, microorganisms can also accept electrons from a solid electrode through the production of hydrogen or the reduction of added mediators, such as methylviologen, neutral red or anthraquinone-2,6-disulfonate (Huang *et al.*, 2011; Lovley, 2012). The addition of these external mediators, however, increases the operational costs and some of them are unstable and toxic. In the case of methanogens, in a recent study information about the electron transfer mechanism in *Methanococcus maripaludis* was obtained (Lohner *et al.*, 2014). The main mechanism was through indirect uptake via hydrogen as intermediate. Yet, in mutant cells lacking hydrogenase methane formation was still observed. The research on *M. maripaludis* showed evidence for the involvement of free, surface-associated redox enzymes, such as hydrogenases and possibly also formate dehydrogenases, facilitating electron transfer (Deutzmann *et al.*, 2015). The results indicate that these enzymes are released from the cells during culturing and that the enzymes catalyse the

formation of hydrogen and formate when sorbed to an appropriate redox-active surface (Deutzmann *et al.*, 2015).

In a BES, microorganisms can be attached to one or both bioelectrodes performing a reduction reaction at the biocathode and an oxidation reaction at the bioanode (Figure 1.2). If electricity is produced, the system is called a Microbial Fuel Cell (MFC). If energy needs to be supplied to the system, it is called Microbial Electrolysis Cell (MEC) (Rozendal *et al.* 2008; Hamelers *et al.*, 2010; Rabaey and Rozendal, 2010; Bajracharya *et al.*, 2016).

Figure 1.2 – Schematic representation of a Bioelectrochemical system with the different possible interactions in the anode and cathode.

BESs can be constructed using different materials and with different configurations. They can also be operated in a wide variety of conditions, such as different pHs, temperatures, sizes (Hamelers *et al.*, 2010). BESs can be applied to produce different valuable compounds, such as H_2 , H_2O_2 , methane or ethanol (Hamelers *et al.*, 2010).

BESs can also be used to treat wastewaters, allowing oxidation of the organic matter present in the wastewater in the anode compartment and producing valuable compounds by reduction in the cathode compartment. Specifically regarding methane production, and compared with AD, this process offers the possibility to separate organic matter degradation from methane production, avoiding the contact of methanogens with inhibitory compounds present in the wastewater (Villano *et al.*, 2010; 2011).

1.6.1. CO₂ reduction in a BES

Recent developments showed that methane can also be produced by electrochemical reduction of CO₂. In this case, a MEC with a biocathode is used with a hydrogenotrophic community. Methane can then be produced through two different mechanism: by the direct uptake of electrons according to Equation 1.6 or indirectly using hydrogen as an intermediary (Equations 1.7 and 1.8) (van Eerten-Jansen *et al.*, 2012). NHE stands for normal hydrogen electrode.

In the anode, the oxidation of water can provide the electrons and protons needed (Equation 1.9).

The overall process to produce methane from water and CO₂ is shown in Equation 1.10 (van Eerten-Jansen *et al.*, 2012).

The proof of concept was obtained using a biocathode and an abiotic anode in a MEC system without precious metals catalyst (Cheng *et al.*, 2009). Applying a potential of -0.7 to 1.2V (*vs.* Ag/AgCl), methane was produced at rates of 0.9 to 656 mmol CH₄ d⁻¹ m⁻². The biocathode microbial community was dominated by *Methanobacterium palustre*. A hydrogenotrophic culture was used in a biocathode and was able to reduce CO₂ to methane (up to 0.055 ± 0.002 mmol d⁻¹ mg VSS⁻¹) with potentials more negative than -650 mV (*vs.* SHE) (Villano *et al.*, 2010). They also observed that methane could be produced both from abiotically produced hydrogen and direct electron transfer. The relative contribution of the two mechanisms was dependent on the potential. *M. thermoautotrophicus* strain ΔH also produced methane in an electrochemical system using hydrogen produced according with Equation 1.7 with a current to methane conversion efficiency of 20% (Hara *et al.*, 2013). With an enriched culture from a wastewater treatment plant as inoculum for the biocathode, a simultaneous production of methane and acetate via direct and/or indirect electron transfer was obtained, and that the ratio of these end products was dependent on the cathode potentials (Jiang *et al.*, 2013). Although progress has been made and the results seem promising regarding methane production in BESs, still a lot of improvement needs to be done to make it an economically attractive technology.

An important fact that has to be taken into consideration regarding the efficiency of a MEC is the different energy losses that may lead to the need of providing additional energy to the system (van Eerten-Jansen *et al.*, 2012). In research using a MEC for methane production from CO₂ the resistances of the different elements of the system, such as cathode, anode, membrane and electrolyte, were investigated (van Eerten-Jansen *et al.*, 2012). The analysis of the internal resistance showed that cathode and anode losses were a major cause of efficiency loss, but also that pH and transport losses became important with time. Decreasing the energy losses in these systems is a crucial point and should be a topic of extensive research.

In many BES studies mixed cultures are used, but the role of the microbial community structure was not always well investigated. Van Eerten-Jansen *et al.* (2013) operated a biocathode producing methane at a maximum rate of 5.1 L methane m⁻² projected cathode day⁻¹ (1.6A m⁻²) at -0.7 V *vs.* NHE. The community was dominated by three phylotypes of archaea and six of bacterial phylotypes. The archaeal phylotypes were closely related to the hydrogenotrophic *M. palustre* and *Methanobacterium aarhusense*, while the bacterial phylotypes were related to *Desulfovibrio putealis*, *Hydrogenophaga caeni* and *Methylocystis*. It was hypothesized that the phylotype closely related to *D. putealis* could produce hydrogen at the working potentials used and that the hydrogen is consumed by the methanogens for methane production. The bioelectrochemical production of hydrogen catalysed by several *Desulfovibrio* sp. was previously reported at potentials of ≤ -0.44 V *vs* NHE (Aulenta *et al.*, 2012; Croese *et al.*, 2011). It was showed that bacteria can play a fundamental role in methane-producing biocathodes by promoting methane production through the production of hydrogen (Croese *et al.*, 2011). This conclusion is supported by the results from another study, which pointed to multiple mechanism of energy transduction between different members of the microbial community, including exchange of electron equivalents between the cathode and methanogens via *Desulfovibrionaceae* (Bretschger *et al.*, 2015).

BESs are a promising technology for the production of methane. Yet, the research in this topic has still a long way ahead; a better understanding of the microbial mechanisms of electron transfer is still needed to better exploit its potential and increase the conversion rates; decreasing the energy losses is a fundamental task, as well as to diminish the material costs for these systems, which are still very high. For these reasons, although BESs have shown some good results, they are still far from being able to compete with well-established technologies for methane production, such as AD.

1.7. Scope and outline of the thesis

In the past, several studies have been conducted to get insight on the effect of heavy metals on anaerobic processes, considering both their potential stimulatory or inhibitory effects. These studies were performed under a wide variety of experimental conditions, giving origin to an equally broad range of results that cannot be easily correlated. Sulphate is a common contaminant in wastewaters and its reduction, with the formation of sulphide, can lead to inhibition of methanogenesis. Yet, when metals are present in the wastewater, the produced sulphide can be used as a metal detoxification method. **Chapter 2** presents a review of the existing literature regarding heavy metal and sulphate effects on AD, with special focus on methanogenesis. Current knowledge on sulphide as metal detoxification method and possible applications was also reviewed. Few of the available studies tried to distinguish the differences in the response different groups of methanogens, *i.e.* acetoclastic, hydrogenotrophic and methylotrophic methanogens, to high metal concentrations. Furthermore, the changes in the microbial communities due to the metal presence are also poorly studied. The potential of biologically produced sulphide as metal detoxification method applied to anaerobic processes is still quite unexplored. The implications on methane production resulting from the application of such detoxification method are also not well studied. The aim of the research described in **Chapter 3** was to evaluate possible approaches to maximise methane production in wastewaters containing heavy metals. For that, the effects of low and high concentrations of Ni and Co added to anaerobic sludge were studied. The potential stimulatory and toxic effects of these metals on hydrogenotrophic and acetoclastic activities were evaluated. Further on, a detoxification strategy involving the precipitation of metals with sulphide was studied.

Chlorinated compounds are toxic compounds commonly present in wastewaters. Reductive dechlorination performed by OHRB is a well-studied process. Yet, and although several methanogenic metal-containing cofactors are known to be involved in this process, not many studies explored trace metal supplementation to enhance the dechlorination performance. **Chapter 4** reports the enrichment of methanogenic cultures able to perform PCE and DCE dechlorination using different inoculum sources and substrates. Moreover, the enriched cultures with the best dechlorination performance were selected and used for metal supplementation studies. Ni, Co, and Fe were supplemented to enriched cultures individually to access their effect in reductive dechlorination. **Chapter 5** describes a study to assess the effect of metal supplementation in DCE co-metabolism by *Methanosarcina barkeri*. The aim of this study was to access if the supplementation of the metal ions Fe, Ni, and Co, which are required for methanogenic activity, but also for co-metabolic reduction of DCE, could improve DCE dechlorination by pure cultures of *M. barkeri*. The presence of alternative electron acceptors in the cathode, such as sulphate, can affect the performance of methane-producing BES and this has not been previously studied. The research described in **Chapter 6** aims to understand the effect of sulphate on electromethanogenesis. The impact of sulphate in the system producing methane was evaluated in terms of variations in methane production, coulombic efficiency (CE) and current demand, and combined with information retrieved from the microbial community analysis.

Chapter 7 provides a general discussion, integrating the results observed in the previous chapters. Future lines of research regarding the impact of compounds, such as heavy metals, chlorinated compounds and sulphate, in microbial anaerobic systems are proposed.

Chapter 2

Methanogens, sulphate and heavy metals: a complex system

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2.1. Abstract

Anaerobic digestion (AD) is a well-established technology used for the treatment of wastewaters (and wastes) with high organic content. During AD organic matter is converted stepwise to methane-containing biogas – a renewable energy carrier. Methane production occurs in the last AD step and relies on methanogens, which are rather sensitive to some contaminants commonly found in wastewaters, such as heavy metals, or easily outcompeted by other groups of microorganisms (*e.g.* sulphate reducing bacteria, SRB). This review gives an overview of previous research and pilot-scale studies that shed some light on the effects of sulphate and heavy metals on methanogenesis. Despite the numerous on this subject, comparison is not always possible due to differences in the experimental conditions used and parameters explained. An overview of the possible benefits of methanogens and SRB co-habitation is also covered. Small amounts of sulphide produced by SRB can precipitate with metals, neutralising the negative effects of sulphide accumulation and free heavy metals on methanogenesis. Knowledge on how to untangle and balance sulphate reduction and methanogenesis is crucial to take advantage of the potential for the utilisation of biogenic sulphide as a metal detoxification agent with minimal loss in methane production in anaerobic digesters.

Keywords: heavy metals, syntrophy, methanogenesis, sulphate reducers, sulphide, inhibition

2.2. Introduction

Anaerobic digestion (AD) is a well-established and efficient process for waste and wastewater treatment. The process is based on the degradation of organic matter by a network of diverse microorganisms, with ultimate formation of methane-containing biogas (a renewable energy carrier) (Figure 2.1a). The different groups of microorganisms involved in AD (fermenters, volatile fatty acids (VFA) oxidizers, and methanogens) have diverse nutritional demands and growth properties. Methanogens are a key group in AD, because when methanogenic activity is inhibited digestion is blocked at the acidogenesis step leading to an incomplete degradation of the organic matter. Optimisation of methanogenesis is still a challenge, and that is mainly due to the low growth rates of methanogens and their high susceptibility to changes in environmental conditions and sensibility to toxic compounds (Chen *et al.*, 2008). Heavy metals are an important class of compounds that is known for its inhibitory effect towards methanogens. The effect of heavy metals such as Cr, Cd, Pb, Cu, Zn, and Ni on the activity of pure cultures of methanogens and methanogenic sludges is well reported in literature (*e.g.* Lin and Chen, 1999; Colussi *et al.*, 2009). One solution to overcome metal toxicity might be the precipitation of heavy metals, which can be done using biogenic hydrogen sulphide that is produced by sulphate-reducing bacteria (SRB) (Fu and Wang, 2011). Hydrogen sulphide is toxic to methanogens, but not after its complexation with metals (Figure 2.1c). Sub-stoichiometric amounts of sulphate entering the anaerobic digesters will not impair methanogenesis; the low amounts of hydrogen sulphide formed will precipitate in the form of metal sulphides decreasing both metal and sulphide toxicity. If sulphate is in excess though, SRB can outcompete methanogens for substrates such as acetate and hydrogen, resulting in decreased biogas production (Chen *et al.*, 2008; Colleran *et al.*, 1995; Dar *et al.*, 2008). This review will focus on the effects of sulphate and heavy metals in methanogenesis, as well as in the use of

biogenic hydrogen sulphide for metal detoxification and current state of research on this topic. Throughout the review, when needed and for the sake of comparison, we converted all the concentrations of metals from original literature to mM.

Figure 2.1 - Schematic representation of the anaerobic degradation of organic matter (a), in the presence of sulphate reduction (b) and coupled to metal sulphide formation (c). AD is generally divided in four steps: (i) hydrolysis, in which large molecules, such as carbohydrates, proteins and lipids, are converted in their monomers, i.e. simple sugars, amino acids, and glycerol plus long chain fatty acids; (ii) acidogenesis, that consists in the conversion of fermentable compounds (e.g. sugars and glycerol) to volatile fatty acids; (iii) acetogenesis, a process in which acetate is synthesized from the oxidation of, for example, fatty acids by syntrophic bacteria (in this case with the formation of hydrogen as well), or from the utilization of H₂/CO₂ by homoacetogenic bacteria; and, (iv) methanogenesis, the final AD step in which simple compounds such as acetate and H₂/CO₂ are converted to biogas (Figure 2.1a)

2.3. Sulphate reduction in anaerobic reactors

Mining and other industries that use sulphur compounds, like metallurgical, pulp and paper, and petrochemical industries, are responsible for an increase in sulphate concentrations in wastewaters. The presence of sulphate can have two major effects on methanogenesis: one resulting from the competition between methanogens and SRB, and the other due to sulphide toxicity (Figure 2.1b). Nevertheless, very low amounts of sulphate are beneficial for methanogenesis because sulphur is a required element for methanogenic archaea (O’Flaherty *et al.*, 1999). Moreover, the presence of sulphur compounds may lower the redox potential of the media, resulting in favourable conditions for methanogens, which need a redox potential of -200 mV to -400mV (Fetzer and Conrad, 1993; Hirano *et al.*, 2013). Optimal sulphur levels in AD processes range from 0.03 to 0.78 mM (Colleran *et al.*, 1995; Chen *et al.*, 2008).

2.3.1. Competition between methanogens and SRB

The physiology of SRB has been comprehensively reviewed (Castro *et al.*, 2000; Muyzer and Stams, 2008; Plugge *et al.*, 2011). SRB are able to use a broad range of substrates (such as alcohols, organic acids, fatty acids, hydrogen, etc.) and, in environments with low redox potential, SRB will compete with anaerobes, including methanogens, for common available substrates. Hydrogen and acetate conversion coupled to sulphate reduction has thermodynamical advantage over methanogenesis (equations 1 to 4; ΔG^0 , Gibbs free energies at 25°C calculated at standard conditions (*i.e.* solute concentrations of 1 M and gas partial pressure of 10^5 Pa)).

Sulphate reduction:

Methanogenesis:

Besides the favourable thermodynamics, SRB show higher affinity for H_2 than methanogens, which gives them an additional competitive advantage in the presence of excess of sulphate in the environment (Colleran *et al.*, 1995). The competition between acetate-utilising SRB and aceticlastic methanogens is not as clear because the differences in kinetic properties between the two groups are smaller. For example, acetate-utilising SRB show growth kinetic parameters only slightly better than *Methanosaeta*, a common methanogen in bioreactors (Oude Elferink *et al.*, 1998). Gupta *et al.* (1994) reported the prevalence of SRB over methanogens in acetate-fed chemostats, but there are also studies in which methanogens were not outcompeted by SRB (Omil *et al.*, 1996; O'Flaherty *et al.*, 1998; Rodriguez *et al.*, 2012). Aceticlastic methanogens can prevail in the presence of sulphate, even after long-term reactor operation, as shown in the study from Colleran *et al.* (1998). These authors studied SRB and methanogenic communities in a full-scale, fixed-bed digester treating a citric acid production wastewater (Chemical Oxygen Demand (COD)/sulphate ratio of 3-4:1) and observed that, although hydrogenotrophic methanogens and propionate syntrophs were outcompeted by SRB, aceticlastic methanogens were still playing an important role in acetate conversion after 5 years of reactor operation.

Different mechanisms have been suggested to explain the differences in competition between SRB and methanogens, namely the ratio between the concentration of

organic matter and sulphate (*i.e.*, COD/sulphate), the capacity for microbial aggregation, and process temperature. Theoretically, organic matter can be completely degraded via sulphate reduction for COD/sulphate ratios below 0.66 (Oude Elferink *et al.*, 1998). Nevertheless, a prediction on the competition outcome can only be done at much greater COD/sulphate levels: for COD/sulphate ratios greater than 10, sulphate reduction is minimal and methanogenesis is not affected (Rinzema and Lettinga, 1988); however, at COD/sulphate ratios below 1 methanogens are outcompeted by SRB (Visser *et al.*, 1993). The capacity of microbial communities to form biofilm or granulate, as well as the relative abundance of methanogens/SRB in the inoculum sludge, are also important factors that might influence the prevalence of one or other group of microorganisms. The predominance of methanogens over SRB in fixed-film reactors has been previously observed and explained by the lower attachment ability of SRB compared to methanogens (Isa *et al.*, 1986). Omil *et al.* (1996) also observed a partial washout of SRB in an upflow anaerobic sludge blanket (UASB) reactor operating at upflow velocities above 2 m.h⁻¹; additionally, an increase in methanogenic activity was detected at higher upflow velocities (4-6 m.h⁻¹) suggesting again a higher attachment of methanogens. A higher initial methanogens/SRB ratio in the inoculum sludge may also lead to a delay in SRB prevalence. Oude Elferink *et al.* (1994) simulated the competition between these two groups in bioreactors using a biomass retention time of 0.01 d⁻¹ and an initial methanogens/SRB ratio of 10⁴. They estimated a period of 1 year before the SRB could equal methanogens in number. Another factor that can influence the competition is temperature because methanogens and SRB have different optimal temperature ranges (O'Flaherty *et al.*, 1998). Madden *et al.* (2014) investigated the effect of sulphate in low-temperature (15°C) anaerobic expanded granular sludge bed (EGSB) bioreactors. At this lower temperature, methanogenesis seems to be affected only at COD:SO₄²⁻ ratios ≤ 1:2. The same authors also

investigated the community changes induced by the presence of sulphate; they suggested that at low temperatures, hydrogenotrophic methanogens were more sensitive than acetoclastic methanogens to the presence of sulphate.

As a general rule, one can argue that in the presence of excess of sulphate, the methanogens are likely to be outcompeted by SRB. Hydrogen utilisation by SRB at high COD/sulphate ratios is difficult to prevent, while acetoclastic methanogens are stronger players in the competition with SRB (Oude Elferink *et al.*, 1994), however, the outcome of the competition is highly dependent of many different conditions. SRB are associated with a decrease in methane yield of about 0.23 m³ (STP) for every kg of SO₄²⁻ reduced (Colleran *et al.*, 1995).

2.3.2. Inhibitory effect of sulphide

Although sulphate is considered to be non-toxic towards anaerobic microorganisms, the product of its reduction, hydrogen sulphide, is highly reactive and toxic towards methanogens and even SRB (Karhadkar *et al.*, 1987; Colleran *et al.*, 1995). Hydrogen sulphide can diffuse across the cell membrane and is responsible for protein denaturation, enzyme inhibition, and interference with the sulphur uptake metabolism (Speece, 1983; McCartney and Oleszkiewicz, 1993; Chen *et al.*, 2008). From an operational point of view, hydrogen sulphide causes malodour and corrosion problems (Colleran *et al.*, 1995).

Hydrogen sulphide toxicity is pH-dependent; at pH < 6 most of the hydrogen sulphide will be in the toxic H₂S form, whereas at higher pH (8-12) most of the hydrogen sulphide will be in the deprotonated less toxic HS⁻ form (Lens *et al.*, 1998). Information on medium pH is very often omitted in the literature, which makes the comparison of various studies of sulphide toxicity difficult. This could be a reason for the discrepancy on the reported sulphide-dependent inhibition of anaerobic microorganisms (Table 2.1). Parkin *et al.* (1990) observed that sulphate

reduction is inhibited before methanogenesis at high HS^- concentrations (4.5 and 6 mM of HS^- for acetate and propionate conversion, respectively). Some authors suggested a correlation between COD/sulphate ratio and sulphide toxicity towards SRB and methanogens. It has been shown that SRB are sensitive to an increase in the hydrogen sulphide concentration more than methanogens for a COD/sulphate ratio of 3.7 (McCartney and Oleszkiewicz, 1991). Yet, if the ratio was lowered to 1.6 or 0.8, SRB appeared to be less sensitive than methanogens (O'Flaherty *et al.*, 1998). For neutral pH values, a similar sensitivity to hydrogen sulphide of SRB and methanogens was observed (Visser *et al.*, 1993), but for higher pH ranges methanogens showed higher sensitivity (O'Flaherty *et al.*, 1998). Other factors that could affect these results are differences in the diffusion of unionized H_2S and dissolved sulphide (HS^-), microbial adaptation, and microbial assembly (biofilms, flocks, granules).

Hydrogen sulphide reacts with metal ions, forming an insoluble form of metal sulphide. The precipitation of trace metals, such as Co or Ni, which are essential as enzyme cofactors in methanogens, is an indirect form of methanogenesis inhibition by sulphide.

Table 2.1 – Sulphide inhibitory concentrations according to literature.

Biological system	Substrate	Concentration H ₂ S	Notes	References
Anaerobic mixed culture	Cellulose	1.75 mM ^a 10 mM	^a Partial inhibition Complete inhibition Cultures in sulphur-free medium. Headspace with H ₂ /CO ₂	Khan and Trottier, 1978
Inoculum from an upflow sludge blanket reactor	Acetate	1.75 mM ^b 6.25 mM ^c	^b 50% inhibition; ^c 100% inhibition;	Kroiss and Plahl-Wabnegg, 1983
Anaerobic sludge from a reactor treating distillery	Acetate and acetate/ ethanol	37.5 mM	50% inhibition, 35°C	Isa <i>et al.</i> , 1986
Granular sludge from a UASB in a acetate-fed	Acetate	7.8 Mm at pH 6.4-7.2 2.8 mM at pH 7.8-8.0	30°C	Koster <i>et al.</i> , 1986
Unacclimated batch digester population		1.5 mM		Parkin <i>et al.</i> , 1983
Mixture of methanogens and SRB	Acetate, propionate (STR 25 days), lactate (STR 40 days)	1.76-2.2 mM (150-200 mg DS/L) ^d 5.9 mM (1000 mg DS/L) ^e	CSTR and Anaerobic filters were used to test suspended and attached growth; 35°C; ^d concentrations causing stress in all suspended systems tested and higher concentrations caused failure in acetate and propionate systems; ^e concentrations tested in propionate	Krishnanand <i>et al.</i> , 1993
Anaerobic sludge from a reactor treating distillery wastewater	Acetate and acetate/ ethanol	43.75 mM ^f	^f higher concentration tested causing little or no effect on SRB. The little inhibition observed is justified by the authors as a result of lack of metal ions available; 35°C	Isa <i>et al.</i> , 1986

Biological system	Substrate	Concentration H ₂ S	Notes	References
<i>Desulfotomaculum acetoxidans</i>	Lactate	2.65 mM		Widdel, 1988
<i>Desulfovibrio</i> enrichment		17 mM ^g	^g total inhibition of the culture growth for lactate conversion, reversible after H ₂ S stripping; 37°C and automatic pH control.	Reis <i>et al.</i> , 1992
Mixture of SRB and methanogens	Acetate, propionate	1.9 mM (145 mg DS/L) ^h 1.9 mM (193 mg DS/L) ⁱ	^h irreversible failure for systems using acetate; ⁱ irreversible failure for systems using propionate; sulphate reduction was shutdown before methanogenesis; in similar conditions, propionate-using systems failed before acetate-using systems.	Parkin <i>et al.</i> , 1990
Granular sludge from a full-scale UASB reactor treating papermill wastewater	Synthetic influent with sucrose as carbohydrate	3.1 mM ^j 6.25 mM ^k	^j 30% inhibition; pH 5; 55°C; HRT 10h; COD/SO ₄ ²⁻ = 4 ^k 65% inhibition, pH 6, COD/SO ₄ ²⁻ = 1	Lopes, 2007

2.4. Heavy metals occurrence and toxicity

Heavy metals are usually defined as metals with a specific gravity above 5.0 (Collins and Stotzky, 1989; Fu and Wang, 2011; Mudhoo and Kumar, 2013). However, some elements included in this definition, *e.g.* the lanthanides (atomic number 57 – 71), are generally not considered as heavy metals. The development of certain industries, such as metal plating, mining, paper, pesticides and storage batteries, glass and ceramic, contributed for the increase of heavy metals concentration in wastewaters (Sarioglu *et al.*, 2010). In Table 2.2 the concentrations of some heavy metals found in wastewaters are mentioned. The removal of Pb, Hg, Cd, Cr, Zn, Cu and Ni from wastewaters has received major attention because these metals are considered to be toxic to the environment, including, plants, animals and microorganisms (Srivastava and Goyal, 2010; Fu and Wang, 2011). Heavy metals are not biodegradable and they tend to accumulate in living organisms until toxic or carcinogenic concentrations (Fu and Wang, 2011). The toxicity of heavy metals is related to their ability to disrupt enzyme functions and structures by binding with thiol and other groups on proteins or by replacing the natural existing metal cofactors in enzyme's prosthetic groups (Colussi *et al.*, 2009; Chen *et al.*, 2008; Chen *et al.*, 2014) (Figure 2.2). Metal toxicity is one of the main causes of bioreactors problems in bioreactors during the treatment of waste and wastewater (Fang and Hui, 1994; Bhattacharya *et al.*, 1995).

Table 2.2 – Concentrations of heavy metals detected in the municipal and industrial wastewaters.

Source of wastewater	Thessaloniki Wastewater Treatment Plant, Greece	Gdansk Wastewater Treatment Plant, Poland	Zindel, Devecsey, France
Type of wastewater	municipal and industrial wastewater	municipal and industrial wastewater	industrial effluent (surface finishing industry)
Metal concentration (μM)			
Cu	1.2 ± 0.55	~ 1.4	$0.7 - 9.6$
Cr	$0.77 \pm .23$	-	$5826 - 22173$
Ni	13 ± 3.4	-	$54.5 - 305$
Pb	0.19 ± 0.04	0.24	$3.4 - 31.9$
Mn	1.21 ± 0.21	-	$6.2 - 115$
Fe	8.6 ± 1.5	-	$95 - 919$
Cd	0.03 ± 0.009	0.18	$0.4 - 5.4$
Zn	7.2 ± 2.14	~ 7.2	$4632 - 17627$
Reference	(Karvelas <i>et al.</i> , 2003)	(Chipasa, 2003)	(Sancey <i>et al.</i> , 2011)

2.4.1. Biological importance of metals

It is important to mention that, despite their potential toxic effect, most metal ions are needed for structural and/or catalytic functions by microorganisms (Ehrlich, 1997; Mudhoo and Kumar, 2013; Lemire *et al.*, 2013). Fe, Mo and Mn are considered important trace metals with low toxicity, while Zn, Ni, Cu, V, Co, W and Cr have high to moderate importance in microbial metabolic functions and are often more toxic. Finally, As, Ag, Sb, Cd, Hg, Pb and U have been described as having limited biological function and are considered toxins (Ehrlich, 1997).

Many enzymes require metal-ions as cofactors for their functions. For example, Fe is the most abundant metal in cells and is essential for cytochromes and ferredoxin, whereas Cu is present in some superoxide dismutases, Zn and Se are common in hydrogenases, and Ni is needed for the synthesis of Coenzyme A (Oleszkiewicz and Sharma, 1990). Moreover, metals can also play a role as electron donors or acceptors in certain terminal electron accepting chains.

Some studies have shown that, up to a certain dosage, metals can work as stimulating agents in AD processes, with a consequent increase in methane production (Demirel and Scherer, 2011; Mudhoo and Kumar, 2013). Feng *et al.* (2010) used laboratory-scale reactors treating food industry waste to study the effects of different concentrations of trace elements mixtures (B/Mo/Ni; Se/W) and Co on biogas process and on the microbial community composition. The authors observed a 7 to 15% increase in methane production after the addition of a mixture of Se and W. Bacterial community composition was not significantly changed after metal supplementation, but dominant archaeal populations were influenced by the addition of trace elements, suggesting that archaea have a stronger response to variable concentrations of trace elements. A similar effect was observed after supplementing a UASB reactor treating swine wastewater with Zn: addition of extra 0.75 to 0.15 mM of Zn seemed to improve methanogenic performance and increased numbers of *Methanomicrobiales* in the system (Tuo *et al.*, 2014). A study that examined the conversion of a fatty acid cocktail (containing acetate, phenyl acetate, oleic acid or propionate, butyrate and valerate) to methane was also shown to be improved by the addition of trace metals, specifically Fe, Co and Ni (Karlsson *et al.*, 2012); because the conversion of fatty acids may rely upon syntrophic interactions between acetogenic bacteria and methanogens, the observed improvement in methane production was probably related with the stimulation of methanogens by the trace metals. Kida *et al.* (2001) observed a strong increase on aceticlastic methanogenic activity in the presence of Ni and Co. In a latter study, it was shown that aceticlastic *Methanosarcina* species have large Ni- and Co-dependent proteomes (including Ni/Co transporters, Ni-dependent proteins, and B₁₂-dependent proteins), which may explain this requirement (Zhang *et al.*, 2009). Lira-Silva *et al.* (2012) made an interesting observation regarding the positive effect of Cd on methane production by *Methanosarcina acetivorans*. Although Cd is not considered essential for methanogens,

the presence of Cd had a positive effect on methane production from acetate and methanol (9 and 6.5 fold, respectively). Growth of *M. acetivorans* on acetate was promoted in the presence of Cd although no effect was observed when this methanogen was grown on methanol. In addition to Cd, Co and Zn (100 μM) had also a positive effect on methane production by *M. acetivorans*; no effect was observed for the supplementation of Cu or Fe (Lira-Silva *et al.*, 2012). Hydrogenotrophic activity seems to be affected by the presence of metals as well, as shown by the improved methane production from formate by *Methanospirillum hungatei* after the addition of Mo and W (Plugge *et al.*, 2009). Several other studies showed that metals may stimulate methanogenesis even in the presence of high concentrations of S compounds. Gustavsson *et al.* (2011) studied the effect of metals supplementation during the digestion of bio-ethanol residues containing high sulphate levels. These authors concluded that daily supplementation with Co (8.5 μM), Ni (3.4 μM) and Fe (9 mM) were required for maintaining biogas process stability at the organic loading rate of 4.0 g volatile solids $\text{L}^{-1} \text{ day}^{-1}$. Similar results were later reported for bioreactors fed with stillage (Gustavsson *et al.*, 2013; Schmidt *et al.*, 2014).

Despite the favourable reports on metal supplementation, it is important to consider that each specific situation needs to be analysed *per se* and that adding metals is not always a rewarding solution. As an example, we can refer to the study by Park *et al.* (2010), in which nutrients supplementation (including metals) to full-scale mesophilic digesters did not show stimulatory effect on methane production, both in short and long term.

2.4.2. Factors affecting heavy metal toxicity

Metal ions can be present in different forms, depending on ionic strength of the medium, the presence of chelating agents (such as EDTA), the reduction potential, and temperature and pH. Some metals, such as Mn or Cr, can be present in more

than one valence state (Gadd and Griffiths, 1977; Collins and Stotzky, 1989). Metal bioavailability and reactivity are dependent upon metal speciation, and it can happen that just one or a small fraction of a metal form plays a role in microbial activity (Hughes and Poole, 1991; Lemire *et al.*, 2013; Olaniran *et al.*, 2013). pH variations can affect metal mobility and binding ability (Gadd and Griffiths, 1977; Collins and Stotzky, 1989) and may affect too the physiology of microorganisms and the way they are affected by metals. In the literature, distinction between the different forms of metals was rarely done, mainly due to lack of analytical techniques for metal-species separations and due to the complex interactions between metal and anaerobic sludge (Chen *et al.*, 2008). The oxidation-reduction potential (expressed as Eh) has an important effect on metal toxicity as well. Moreover, the Eh affects the valence state of a metal and some states are more toxic than others (Collins and Stotzky, 1989). Also, the presence of inorganic anions, such as OH⁻ or Cl⁻, which can form complexes with metals, can influence their toxicity (Gadd and Griffiths, 1977). In the case of inorganic cations, they affect the metal toxicity since they compete with cationic forms of the metals for anionic sites on cell surfaces (Collins and Stotzky, 1989). Some compounds, such as synthetic chelators (e.g. ethylenediaminetetraacetic acid (EDTA)), or natural chelators, like amino acids or humic acids, also influence the toxicity of metals (Collins and Stotzky, 1989).

2.4.3. Microbial resistance mechanisms to heavy metals

Normally, heavy metals need to enter the cell to play a physiological role or exert a toxic effect. Two systems are known for metal uptake by the cell: one is unspecific, usually driven by chemiosmotic gradients, and the other is highly specific and ATP-dependent (Nies, 1999). Because of large energy requirements, the specific metal uptake systems are used only if the microorganisms need a specific metal (during special metabolic needs or starvation); entrance of metals in the cell occurs mainly

through unspecific system. Especially in environments with high metal concentration, unspecific metal intake is promoted and metals inside the cell can reach toxic concentrations. This has created the need for microorganisms to develop resistance mechanisms to metals (Figure 2.2) (Nies, 1999; Gadd, 2009; Lemire *et al.*, 2013). One of the detoxification mechanisms consists in active extrusion of the metal ion from the cell (Nies, 1999). Some bacteria known to be heavy metal resistant, such as *Cupriavidus metallidurans*, have efflux transporters that allow the microbe to excrete metals when they are toxic or in excess (Haferburg and Kothe, 2007). Metal efflux proteins are well-known to be present in microorganisms. Some examples are the tetracycline-metal ion transporter TetL from *Bacillus subtilis*, the iron citrate exporter IceT in *Salmonella enterica*, the Cd transporter CadA from *Staphylococcus aureus* and *B. subtilis* or the Cu transporter CopA from *E. coli* (Bennett *et al.*, 2015). Recently, a Fe exporter, FeoE, was identified in *Shewanella oneidensis* MR-1 (Bennett *et al.*, 2015). It is also known that some ABC transporters are able to efflux metals out of the cells (Haferburg and Kothe, 2007). Another common heavy metal resistance mechanism is the excretion of precipitating or chelating agents by microorganisms. Sulphide is one of the main precipitating agents (Oleszkiewicz and Sharma, 1990; Nies, 1999). The excretion of chelating agents, such as melanin, carboxyl, deprotonated hydroxyl groups, has been reported (Haferburg and Kothe, 2007). Additionally, biomethylation of Hg, Pb, Tl, Pd, Pt, Au, Sn, Cr, As and Se has also been observed as a detoxification mechanism in microbial cells (Oleszkiewicz and Sharma, 1990). Certain microorganisms are also able to trap metals in internal inclusion bodies (Haferburg and Kothe, 2007), while others are able to reduce the ion to a less toxic oxidation state, as for example *Penicillium chrysogenum* that can reduce silver (Haferburg and Kothe, 2007). In some microorganisms a combination of more than one of these systems is present (Nies, 1999).

Figure 2.2 - Schematic representation the interactions between metals and cells.

In the case of methanogens, an *in silico* study showed that *Methanococcus maripaludis* C5 has in its genome 10 protein coding genes for cobalt transport and export. *Methanosarcina mazei* Go1 has in its genome the pathway to assimilate W, specifically, by two tungsten-specific transporter proteins, torB and torP (Chellapandi, 2011). In the presence of 100 μM of Cd, *Methanosarcina acetivorans* increases the intracellular levels of cysteine, sulphide and coenzyme M, indicating that this microorganism might have a metal resistance mechanism involving thiol molecules. On the other hand, cells of *M. acetivorans* that were exposed to 54 μM of Cd for 305 months and growing on methanol, were able to grow in the presence of high concentrations of Cd (0.63 to 2.5 mM CdCl_2). It was also observed that those pre-adapted cells, when exposed to 1.4 mM of Cd synthesised an extracellular matrix composed of DNA, proteins and carbohydrates to which the cells were attached and still producing methane (Lira-Silva *et al.*, 2013). *Methanothermobacter thermoautotrophicus* growing in

H₂/CO₂, was able to reduce 0.2 and 0.4 mM Cr⁶⁺ completely (Lira-Silva *et al.*, 2013). Singh *et al.* (2015) tested growing *M. thermoautotrophicus* with higher concentrations of hexavalent chromium; amendment of 1, 3 and 5 mM of Cr⁶⁺ resulted in 43.6, 13 and 3.7% reduction of the metal. The same methanogen was also able to reduce structural Fe³⁺ in smectite minerals at 65°C although with low reduction extents (27% for nontronite and 13-15% for montmorillonite) (Zhang *et al.*, 2013). *Methanosarcina barkeri* was also observed to be able to reduce Fe³⁺ in nontronite using methanol and H₂/CO₂ as substrates, but not with acetate (Liu *et al.*, 2011). Microbial reduction of Fe (III) was also observed in illite-smectite minerals by the methanogen *Methanosarcina mazei* using methanol as substrate (Zhang *et al.*, 2012).

Toxicity of metals towards microbial mixed cultures is often different than for individual microbial species. Microbial aggregation in granules can confer a way of protection for more sensitive microorganisms. Granular sludge shows higher resistance to toxicity than flocculent sludge (Lin and Chen, 1997). Such higher resistance of the granules is explained by their layered microstructure where the most sensitive microorganisms, such as methanogens, are found mainly in the interior while the exterior of the granule is mainly composed of fermentative bacteria which are more resistant to metal toxicity (Fang and Hui, 1994).

2.5. How do heavy metals affect AD?

An important consequence of AD disruption due to the presence of heavy metals is the decrease in biogas production and the accumulation of intermediate organic compounds (Hayes and Theis, 1978). In addition, heavy metals can be involved in different physico-chemical processes during AD. They can precipitate with sulphide, carbonate and hydroxides, they can form complexes with intermediate AD products, and they can also adsorb either to the solid fraction, biomass or inert matter (Chen *et*

et al., 2008). Concerning direct toxicity to microorganisms, it is thought that only the soluble free form of a metal is toxic (Oleszkiewicz and Sharma, 1990; Chen *et al.*, 2008). Similar to the ambiguity discussed earlier with respect of sulphide toxicity, the literature about toxic concentrations of metals also has discrepancies (Table 2.3). However, this is perhaps due to variations in the experimental conditions: differences in substrates, microorganisms, different oxidation states of the metal ion, pure *vs.* co-culture, and environmental factors, such as pH (Chen *et al.*, 2008).

2.5.1. Effect of heavy metals on methanogens

The effects of some metals, such as Zn, Ni or Cu, on methanogenesis have been extensively studied. However, the information about the effects of other metals, *e.g.* Co, Cd or Mn is much more limited, while studies on the effect of Hg, Al or Se are very scarce. *Methanospirillum hungatei* GP1 showed 95% inhibition with 15 μM of Cd and a total inhibition of methanogenesis using 50 μM of Hg, Cu and Zn, and a 49% inhibition was detected with 50 μM of Co (Pankhania and Robinson, 1984). However, in the same study it was observed that Mn and Mg, instead of having a toxic effect, in fact stimulated methanogenesis. The study of the effect of Ni, Zn and Cu on pure cultures of *Methanospirillum hungatei* JF1, *Methanosarcina barkeri* MS, *Methanothermobacter marburgensis* and *Methanobacterium formicum* (Jarrell *et al.*, 1987) showed that Zn and Cu were toxic at concentrations from 0.015 to 0.15 mM and 0.017 to 0.17 mM, respectively, while Ni was described as being the least toxic of the three metals; particularly, *M. formicum* was the most resistant of the methanogens towards Ni; for example, 0.26 M of Ni were needed to induce 50% inhibition to this microorganism while the other two microorganism were sensitive to concentrations between 4.25 and 20 mM. Using an anaerobic sludge from a UASB reactor treating wastewaters from a yeast factory, Sarioglu *et al.* (2010) evaluated the effect of Cu, Ni, Zn and Pb. They observed a decline in methane

production for heavy metal concentrations above 0.16 mM of Cu, 0.17 mM of Ni, 0.15 mM of Zn and 0.05 mM of Pb and a relative toxicity of Cu>Ni~Zn>Pb.

Due to significant variations in the experimental conditions evaluated and differences in results, it is hard to find a pattern and establish an average concentration at which the metals become toxic. In general, Cu is one of the most toxic metals while Pb is one of the most tolerated. Furthermore, it is interesting to note that metals that are considered important trace elements, such as Zn or Ni, and that are even used in small concentrations for stimulating methanogenesis, are often the most toxic ones when in excess. In mixed cultures, the interactions between the different microorganisms can offer a protective effect, which seems to attenuate the toxicity effect of some heavy metals (Gadd and Griffiths, 1977; Pankhania and Robinson, 1984; Jarrell *et al.*, 1987).

2.5.2. Effects of heavy metals on SRB

It is also evident from published work that the toxic concentrations of certain metals on SRB may vary depending on the experimental conditions used in the studies. It was observed that a pure culture of SRB can tolerate 0.3 to 0.8 mM of Cu (Booth and Mercer, 1963), similar to what was observed by Saleh *et al.* (1964), who also reported that SRB can tolerate around 1.5 mM of Zn. The use of 0.35 mM of Cd and 0.4 mM of Pb induced 50% inhibition in a SRB pure culture (Loka Bharathi *et al.*, 1990), while *Desulfovibrio desulfuricans* was reported to be sensitive to concentrations of Ni and Zn above 0.17 mM and 0.20 mM, respectively (Poulson *et al.*, 1997). The effects of Cu and Zn in a mixed culture of acetate-utilizing bacteria were analysed and observed a 50% inhibition of 0.17 mM for Cu and 0.25 mM for Zn (Utgikar *et al.*, 2001). However, certain SRB strains tolerated higher concentrations of metals. For example, a pure culture of *Desulfotomaculum* sp. was able to tolerate 9.5 mM of Ni, when Fe²⁺ was present (Fortin *et al.*, 1994). Different SRB strains were tested

and some showed resistance to concentrations of 50 mM of Al, 30 mM of Cr and/or 10 mM of Pb (Hard *et al.*, 1997). It is also described that some SRB, such as *Desulfovibrio desulfuricans* or *Desulfovibrio vulgaris*, are able to reduce metalloids oxyanions, as MoO_4^{2-} to MoO_2 , SeO_4^{2-} / SeO_3^{2-} to Se^0 or selenide (Se^{2-}), As^{5+} to As^{3+} , Pd^{2+} to elemental Pd, etc. (Hao, 2000). In comparison to methanogens, SRB appear to be resistant to higher concentrations of metals. The precipitation of metal ions with the sulphide excreted by these microorganisms is probably one of the main reasons for their higher tolerance of heavy metals.

2.6. Sulphide as a metal detoxification mechanism

Several physico-chemical technologies can be used for heavy metal removal from wastewaters, as for example, coagulation-flocculation, ion exchange, solvent extraction, adsorption, membrane processes, complexation and precipitation (Gadd and White, 1993). Many of these treatments have the disadvantage of producing concentrated chemical sludge that needs proper disposal (Veeken and Rulkens, 2003). These treatments are also not adequate for wastewaters with high organic content because of the interferences of organics with the physico-chemical processes (*e.g.* fouling problems, competitive adsorption, occurrence of side chemical reactions, etc.). Sulphide produced by SRB can be employed to assist in heavy metal detoxification (Hammack and Edenborn, 1992; Zayed and Winter, 2000) because it reacts with many heavy metals and forms metal sulphides (MeS), which are insoluble and sediment quickly (Equation 2.5), thus decreasing the amounts of sulphide and lowering the metal toxicity (Hao, 2000). This process can also facilitate the selective recovery of valuable metals from wastewaters in the form of metal sulphides since the precipitation of sulphides is pH-dependent (Kaksonen and Puhakka, 2007; Kieu *et al.*, 2011; Villa-Gomez *et al.*, 2012).

Equation 2.5

Table 2.3 – Toxic concentrations of metals according to literature.

	pH	Temperature	Metal	Inhibition	Notes	Ref.
Anaerobic sludge	-	35	Cr (III)	(2.5 mM) 5 mM ^a - < 3.8 mM (200 mg/L) ^b	(concentration at which the gas production started to decrease, step-fed conditions) ^a 70% reduction in total gas production, step-fed conditions ^b 70% reduction in total gas production, pulse-fed conditions	Hayes and Theis, 1978
			Cr (IV)	(2.1 mM) 8.1 mM ^a - <3.5 mM ^b		
			Cu	(0.63 mM) 1.1 mM ^a - <0.8 mM ^b		
			Cd	>0.18 mM ^a - >0.09 mM ^b		
			Ni	(0.17 mM) 0.5 mM ^a - >0.5 mM ^b		
			Pb	1.64 mM ^a - >1.2 mM ^b		
			Zn	(6.1 mM) 9.2 mM ^a - <26 mM ^b		
Seed sludge from a mesophilic sewage sludge digester	7.5	35	Cr	0.16 mM ^c	Sludge were acclimated for 2 months; HRT 10 days; 20000 mgCOD/L; mix of acetic, propionic and n-butyric acid ^c 50% inhibition of activity of VFA degradation over 30h by metal dosage	Lin, 1992
			Cd	<0.03 mM ^c		
			Pb	0.35 mM ^c		
			Ni	1.7 mM ^c		
			Zn	0.26 mM ^c		
			Cu	0.11 mM ^c		
			Cd	>4.9 mM ^d		
UASB granules	-	37	Cr	12.1 mM ^d	^d 50% inhibition of specific methanogenic activity of UASB granules	Fang and Hui, 1994
			Cu	2.5 mM ^d		
			Ni	2 mM ^d		
			Zn	1.5 mM ^d		

	pH	Temperature	Metal	Inhibition	Notes	Ref.
Sewage sludge	-	35	Cu	12.6 mM ^e	^e 50% of inhibition of gas production Sulphate metals were used.	Wong and Cheung, 1995
			Ni	5.75 mM ^e		
			Zn	17.6 mM ^e		
			Cr	6.7 mM ^e		
Anaerobic sludge	6.9-7.4	35	Co	16.1 mM ^f	SRT 50 days; 1120 mg/L COD; ^f 100% inhibition of gas production for enrichments with acetate or glucose	Bhattacharya <i>et al.</i> , 1995
Anaerobic sludge	7.0 - 7.2	35	Cd	0.53 mM ^g	Enrichments with acetic acid; SRT 50 days	Bhattacharya <i>et al.</i> , 1995
Anaerobic sludge	7.2	30	Cu	0.33 mM ^l	^g 100% inhibition of gas production	Karri <i>et al.</i> , 2006
				0.14 mM ^m	^l 50% of inhibition of gas production in methanogenic cultures with acetate	
					^m 50% of inhibition of gas production in methanogenic cultures with H ₂	
Anaerobic sewage Sludge	7.0 – 7.2	22	Hg	1.25 mM ⁿ – 2.5 mM ^o	HRT 30 days; ⁿ step-fed metal addition; ^o pulse-fed metal addition	Abdel-Shafy and Mansour, 2014
			Cd	3 mM ⁿ – 6 mM ^o		
			Cr (III)	29.8 mM ⁿ – 59.6 mM ^o		
Anaerobic sludge	-	30	Cu	0.28 mM ^p	^q IC50 value for acetitlastic culture	Gonzalez-Estrella <i>et al.</i> , 2013
			Zn	0.57 mM ^p		

pH	Temperature	Metal	Inhibition	Notes	Ref.
Methanogenic granules		Cd	0.5 mM ^{b,i}	HRT 1 day; acclimation period 6 months ^b 50% inhibition of gas production	ⁱ Lin & Chen, 1997 ^j Lin & Chao, 1996 ^k Lin, 1993
		Cr	0.65 mM ^{b,i}		
		Cu	0.4 mM ^{b,i}		
		Ni	1.3 mM ^{b,i}		
		Pb	6.3 mM ^{b,i}		
		Zn	0.85 mM ^{b,i}		
Conventional granules	35	Cd	15.3 mM ^{b,j}		
		Cr	34 mM ^{b,j}		
		Cu	5.4 mM ^{b,j}		
		Ni	52.3 mM ^{b,j}		
		Pb	49 mM ^{b,j}		
		Zn	104 mM ^{b,j}		
Acetogenic granules		Cd	8.8 mM ^{b,k}		
		Cr	8.4 mM ^{b,k}		
		Cu	4.4 mM ^{b,k}		
		Ni	24.7 mM ^{b,k}		
		Pb	26.8 mM ^{b,k}		
		Zn	12.6 mM ^{b,k}		

	pH	Temperature	Metal	Inhibition	Notes	Ref.
Anaerobic glucose- amended mixed culture	7	37	Cu	0.16 mM ^q	^q 50% inhibition of methanogenesis; mixed culture was enriched with anaerobic digester sludge from a beverage for 6 months, HRT 10 days	Zayed and Winter, 2000
			Zn	0.6 mM ^q		
			Ni	1 mM ^q		

The biological production of sulphide (biosulphide) by the existing SRB communities during anaerobic sludge treatment can reduce the costs of the addition of chemicals, like hydroxide or sulphide. Such an approach will also result in lower concentrations of sulphate in the effluents, and make it a more sustainable process (Kosolapov *et al.*, 2004; Huisman *et al.*, 2006, Kieu *et al.*, 2011). Main applications of this process are related with the treatment of acid mine drainage, but it can be applied to treat other types of wastewaters and other metal contaminated environments. Kieu *et al.* (2011) has already shown that it is possible to achieve heavy metal (Cu, Zn, Ni and Cr) removal efficiencies of 91 to 97% using semi-continuous stirred tank reactors by a consortium of SRB. Zinc sulphide precipitation inside of a full-scale reactor did not interfere with the achievement of a high rate of sulphate reduction (88%) and that methanogenesis was not suppressed (van Houten *et al.*, 2006).

Besides their toxicity effect, heavy metals can affect the competition between methanogens and SRB. It is reported that some metals may cause high and specific toxicity to SRB, which can favour methanogenesis (Capone *et al.*, 1983). Moreover, the protective effect of the sulphide production to methanogenesis in the presence of high concentrations of Cd or Cu (2 mM) was reported when analysing the effects of those metals, both in a pure culture and in a co-culture with a SRB (Mori *et al.*, 2000). In addition, it was observed that the presence of sulphide could induce the recovery of methanogenesis in cultures exposed to different concentrations of heavy metals (Zayed and Winter, 2000). The effects of simultaneous addition of sulphide and heavy metal in equimolar concentrations was investigated by Zayed and Winter (2000); their results suggest that both for Zn and Ni, the toxicity effect was totally prevented by sulphide amendment, and for Cu the toxicity could be eliminated for concentrations up to 0.47 mM, and minimized to concentrations up to 0.8 mM (Zayed and Winter, 2000). Metal sulphide precipitation can also be used as a strategy to control odour problems during AD due to the presence of volatile

organic sulphur compounds (Park and Novak, 2013). Engineered nanoparticles (NPs), for example ZnO and Cu⁰, are already widely used in industry, and it is expected that their utilisation will increase. Consequently, their concentration in wastewaters will also increase. A few studies, *e.g.* Mu *et al.* 2011; Gonzalez-Estrella *et al.*, 2013; Luna-delRisco *et al.*, 2011, have examined the toxic effects of metallic nanoparticles in AD and explored ways to reduce their effects. Biologically produced sulphide has been shown to be a good candidate to reduce the toxic effect of ZnO and Cu⁰ nanoparticles in acetoclastic methanogenesis by 14- and 7-fold, respectively (Gonzalez-Estrella *et al.*, 2015).

Inhibition by metal sulphides has also been reported. It was suggested that metal sulphides concentrate in the surface of the SRB creating a layer that blocks access to substrate and, consequently, inhibits microbial activity (Utgikar *et al.*, 2001). Metal sulphides generally present a specific gravity of around 4, which allows their separation from biomass by gravity settling; a solution to avoid their toxicity is their removal from the sludge before they reach inhibitory concentrations. Some systems have shown to operate well, even in the presence of metal sulphides (Utgikar *et al.*, 2002; Van Houten *et al.*, 2006). Van Houten *et al.* (2006) studied the start-up of a full-scale synthesis gas-lift reactor for treating metal and sulphate rich wastewater, and did not observe any interference from the zinc sulphide precipitates in the performance of the reactor. Microbial community analysis showed the presence of microorganisms closely related with *Methanobacterium* and *Methanospirillum*, suggesting that methanogenesis can coexist with sulphate reduction and metal precipitation.

2.7. Conclusions and future perspectives

The presence of sulphate and heavy metals in wastewaters can affect the performance of methanogens and therefore impact energy recovery (in the form of biogas) from

organic materials. Many different studies have been conducted to assess the toxicity and inhibition effects of these compounds. However, the variability of experimental conditions used in the studies and the omission of important data in some cases (*e.g.* pH values), makes their comparison difficult as the results are not always consistent. Studies have focused on only a few heavy metals (mainly Zn, Ni, and Cu). Biologically-produced sulphide can be employed for metal detoxification, while reducing the sulphide toxicity effect at the same time. The studies on this topic, however, are mainly focussed on the efficiencies of metal recovery (for example from mining-derived wastewaters) and not with the effects on methanogenic activity. It would be interesting to further study metal precipitation with biosulphide in wastewater treatment systems; biological reduction of sulphate occurs in wastewater treatment, starting in the sewers and lasting as long as sulphate is present. There is also limited information about the changes in the microbial communities induced by the presence of sulphate, heavy metals or metal sulphides. It is expected that certain microbial species are more sensitive to each of those compounds, which in turn can affect the dynamic of the microbial community. Also, the identification of microorganisms with high tolerance to elevated levels of those contaminants should be accomplished. A better insight on these aspects is important for the adaptation of AD for treating wastewaters with high metal concentration.

Chapter 3

Stimulation and inhibition of methanogenic enrichment cultures by nickel or cobalt

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3.1. Abstract

Heavy metals may have a dual effect in anaerobic digestion depending on their concentration. Anaerobic sludge was supplemented with nickel (2, 4 and 8 μM and 2, 4 and 8 mM) and cobalt (5.5, 10.5 and 25.5 μM and 2.5, 5, 10, 20 and 30 mM) to study the effect on methane production from H_2/CO_2 or acetate. Micro-molar levels of the metals did not stimulate methanogenesis significantly. Hydrogenotrophic activity was affected by high levels of metals, with 50% less methane produced from H_2/CO_2 (after 196 h incubation) in assays with 8 mM of Ni and 30 mM of Co, compared to controls. Methane production from acetate was also inhibited in $\sim 20\%$ with 8 mM of Ni and in $\sim 50\%$ with 30 mM of Co. Further on, metal precipitation with sulphide was tested as a possible means to alleviate their toxicity. Addition of sulphate resulted in sulphide production by sulphate-reducing bacteria, which could mitigate the inhibitory effect of Ni and Co on aceticlastic activity but not on hydrogenotrophic activity.

Keywords: heavy metals, anaerobic sludge, sulphate, sulphide, inhibition, stimulation

3.2. Introduction

Heavy metals are common pollutants in wastewaters, such as those from metal plating, mining or pulp and paper industries (Sarioglu *et al.*, 2010). Heavy metals may have a dual effect on microorganisms present in anaerobic wastewater treatment systems. Metals have a fundamental role in structural or catalytic functions in cells and can beneficiate certain biological processes (Ehrlich, 1997). On the other hand, high metal concentrations are often toxic to microorganisms (Chen *et al.*, 2008).

Positive effects of metals on the anaerobic digestion of wastewaters have been reported, especially for Ni and Co (Karlsson *et al.*, 2012; Gustavsson *et al.*, 2011; 2013). This is explained by the importance of these metals in enzymes involved in methanogenesis; Ni is present in [Ni-Fe] hydrogenases and in the cofactor F₄₃₀ (the prosthetic group of methyl coenzyme M reductase), while Co is present in cobalamides (DiMarco *et al.*, 1990). Cobalamides have an important role as methyl carriers in methanogenesis from methylated compounds as they are intermediates between methyl-H₄MPT and coenzyme M (DiMarco *et al.*, 1990). Moreover, methyl-H₄MPT:CoM-SH methyltransferase has in one of its subunits a cob(I)amide prosthetic group that is believed to be important for the enzymatic function (Hedderich and Whitman, 2006).

Recently, Chen *et al.* (2016) showed stimulation of anaerobic digestion by Co and Ni up to concentrations of 100 mg/L, above which toxic effects start to be noticeable. Distinct toxic concentrations of these metals have been reported in other studies, possibly due to the large variation in experimental setups used and of inocula tested (Chen *et al.*, 2008; Paulo *et al.*, 2015). For example, toxic concentrations for Ni range from 0.5 to 52.3 mM (Paulo *et al.*, 2015), while for Co 16.1 mM was reported (Bhattacharya *et al.*, 1995). The toxic effect of heavy metals can be due to their ability to disrupt enzyme functions and structures by binding with thiol and other groups in proteins. (Colussi *et al.*, 2009; Chen *et al.*, 2008). Co can inactivate

[Fe-S] proteins, interfere with sulphur metabolism and [Fe-S] cluster biogenesis, as well as cause formation of reactive oxygen species (Eitinger, 2013). As for Ni, it can replace Fe in many enzymes or bind to Cys, His or negatively charged residues in active sites of non-metal enzymes (Eitinger, 2013). Microorganisms have developed different strategies for metal resistance, including metal efflux proteins, excretion of chelating agents, biomethylation or ion reduction (Paulo *et al.*, 2015). These mechanisms are widely studied in bacteria. However, in methanogens, they are not well characterized yet (Maezato and Blum, 2012). One of the main mechanisms of metal resistance is active efflux. Three of the six identified Ni/Co transporters systems can be found in methanogens; the NiK/CbiMNQO, NikABCDE and NiCoT (Bini, 2010). The first two belong to the ABC transporters family and one of the NikABCDE components, the NikA, is suspected to be involved in Ni sequestration in case of metal excess conditions (Eitinger and Mandrand-Berthelot, 2000). RcnA is a nickel defence system and putative homologues were detected in archaea (Macomber and Hausinger, 2011). The expression of *rcnA* is regulated by RcnR, a nickel/cobalt responsive repressor (Macomber and Hausinger, 2011). CznABC efflux pumps are members of the RND family (Eitinger, 2013). They were identified in *Helicobacter* species, and export Cd, Zn and Ni (Sawers, 2013).

An alternative way to decrease toxic effect of metals in wastewater treatment systems is the microbial production of sulphide. In anaerobic digestion sulphide is formed from the degradation of S-containing organic compounds, but it can also be produced by sulphate-reducing bacteria (SRB), which are naturally present in the anaerobic sludge. Sulphide reacts quickly with several metal ions forming insoluble metal-sulphides (Zayed and Winter, 2000). Despite of its proven effectiveness to remove metals from wastewaters, the effects of using biologically produced sulphide on methane production or on the composition of microbial communities are not well studied.

In this study, low and high concentrations of Ni and Co were added to anaerobic sludge and the potential stimulatory and toxic effects of these metals on hydrogenotrophic and acetoclastic activities were evaluated. High metal levels were detrimental for methane production and, for this reason, a detoxification strategy involving the precipitation of metals with sulphide was further on studied. The aim of this work was to study possible approaches to maximise methane production in wastewaters containing heavy metals.

3.3. Materials and Methods

Inoculum sludge, medium composition and culture conditions

Granular anaerobic sludge was obtained from a Wastewater Treatment Plant treating food industry effluent (Delft, The Netherlands). The sludge was washed, and about 2 g of volatile suspended solids (VSS) was used to inoculate vials containing basal medium to a final volume of 50 mL. Medium was prepared according to the protocol previously described by Stams *et al.* (1993), with the following exceptions: no NaHCO_3 was added and buffering of the medium was done with 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH was corrected to 7.0); titanium citrate (0.2-0.3 mM) was amended as reducing agent instead of Na_2S (to avoid metal precipitation). HEPES was chosen because is not reacting with metal ions. The effect of metals on acetoclastic and hydrogenotrophic activities was tested in separate assays. Acetoclastic activity was measured in the presence of 20 mM of sodium acetate and using N_2 as the headspace gas (1.5 atm). Hydrogenotrophic activity was measured using as sole substrate 1.5 atm of H_2/CO_2 (80:20; % v/v). Metals, sulphate and sulphide were added at different final concentrations from anaerobic stock solutions, as detailed in the next section. All the materials (bottles, rubber stoppers) were previously washed with 3 M nitric acid to avoid any

contamination with other metals. All solutions were prepared with ultrapure water. The assays were performed in the dark at 30 °C and stirred at 100 rpm.

Metal, sulphate and sulphide supplementation tests

Effect of Ni and Co was assayed at low (supplementation test, Ni-L and Co-L) and high (toxicity test, Ni-H and Co-H) concentrations. Final concentrations of NiCl₂ were 2, 4 and 8 µM (Ni-L) and 2, 4 and 8 mM (Ni-H). For Co tests, CoCl₂ was added to final concentrations of 5.5, 10.5 and 25.5 µM (Co-L) and 2.5, 5, 10, 20 and 30 mM (Co-H). These final concentrations are already corrected for the Ni and Co present in the basal medium. The concentrations tested were chosen based on values described by others as reviewed by us (Paulo *et al.* 2015). Controls without metal addition were included. To study the potential synergistic effect of sulphide and sulphate, sulphate as Na₂SO₄ was added to the bottles with high metal concentrations; different metal to sulphate ratios were tested (i.e. 0.5:1; 1:1; 1.5:1, mol:mol). Moreover, bottles with Na₂S plus metals were also prepared (ratios 0.5:1; 1:1; 1.5:1, mol:mol). pH of Na₂S stock solution was corrected to 7.0. All conditions were tested in duplicate. A table with all the different conditions and codes for all the assays is included in supplementary material (Supplementary material, Table S1.1). Methane, fatty acids, sulphate and sulphide were monitored over a 6-days incubation period.

Analytical methods

Gas samples were analysed for H₂ and CH₄ with a Compact GC^{4.0} (Global Analyser Solutions, Breda, The Netherlands) equipped with Carbonex 1010 column (Supleco, 3 m x 0.32 mm) followed by a Mosieve 5A column (Restek, 30 m x 0.32 mm) and a Thermal Conductivity Detector (TCD). Argon was used as carrier gas at 0.8 mL

min⁻¹. Standard GC settings for measuring are: 300 kPa; valve (injection) oven: 60°C; column oven: 100 °C; TCD temperature: 100°C; filament: 175°C.

Liquid samples were analysed for volatile fatty acids (VFA) and alcohols with an Acella HPLC (Thermo Scientific, Waltham, MA) equipped with a Varian Metacarb 67H column (Agilent, 300 x 6.5 mm) and a refractive index detector. Column was kept at 45 °C and running with 0.01N of H₂SO₄ as eluent at a flowrate of 0.8 mL/min.

Sulphate concentrations were measured in an ICS-2100 Ion-Chromatograph system (Thermo Scientific) equipped with an AS19 column. Sulphide was measured using the Methylene Blue method, as described by Trüper and Schlegel (1964).

Microbial community analysis

At the end of the assays, 2 mL aliquots were withdrawn from the vials and frozen at -80°C for further DNA extraction. DNA was extracted using the FastDNA™ Spin Kit for Soil DNA Extraction (MPBio, Santa Ana, CA). After purification, DNA was amplified with bacterial and archaeal primers and the samples were screened using Denaturing Gradient Gel Electrophoresis (DGGE) and representative samples of each tested condition were chosen for sequencing in Hiseq platform. Barcoded amplicons targeting the V4 region of the 16S rRNA were generated using a 2-step PCR strategy in order to reduce the impact of barcoded primer on the outcome of the microbial profiling (Berry *et al.*, 2011). 10-20 ng of cDNA was used as template in the first PCR reaction (50 µL) which contained 10 µL HF buffer (Finnzymes, Vantaa, Finland), 1µL dNTP Mix (10 mM; Promega, Leiden, The Netherlands), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Finnzymes), 500 nM of each primer (UniTag1-515f (GAGCCGTAGCCAGTCTGC-GTGYCAGCMGCCG CGGTAA) and UniTag2-806r (GCCGTGACCGTGACATCG-GGACTACNVGGG TWTCTAAT)). The selection procedure of the UniTags is described elsewhere

(Tian *et al.*, 2016). PCRs were performed with a SensoQuest Labcycler (Göttingen, Germany) using an adaptation of the cycling conditions of Caporaso *et al.* (2012) due to the use of the 2-step protocol and the Phusion enzyme. The cycling conditions for the first step consisted of an initial denaturation at 98°C for 3 min, 25 cycles of: 98°C for 10 s, 50°C for 20 s and 72°C for 20 s, and a final extension at 72°C for 10 min. The size of the PCR products (~330 bp) was confirmed by agarose gel electrophoresis using 5 µL of the amplification-reaction mixture on a 1% (w/v) agarose gel containing 1× SYBR® Safe (Invitrogen, Carlsbad, CA).

5 µL of the first PCR reaction were used as template for the second PCR reaction (100 µL), which contained 20 µL HF buffer, 2 µL dNTP Mix, 2 U of Phusion® Hot Start II High-Fidelity DNA polymerase and 500 nM of UniTag1 (forward) and Unitag2 (reverse), each. UniTag1 and Unitag 2 were appended with an 8 nt sample specific barcode at the 5' end (Ramiro-Garcia *et al.*, 2016). The cycling conditions of the second step consisted of an initial denaturation at 98°C for 30s, followed by 5 cycles of: 98°C for 10s, 52°C for 20s and 72°C for 20s. A final extension at 72°C for 10 min followed the cycles. The incorporation of the specific barcodes (PCR product of ~350 bp) was confirmed by gel electrophoresis.

The final PCR products were purified with HighPrep™ (Magbio Genomics, Gaithersburg, MD) using 20 µL of Nuclease Free Water (Promega, Madison, WI) for elution and quantified using a Qubit in combination with the dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA). The purified products were mixed together in equimolar amounts to create a library pool and sent for sequencing on the HiSeq platform (GATC Biotech AG, Konstanz, Germany). Data was analysed using NG-Tax, a validated pipeline for 16S rRNA analysis (Ramiro-Garcia *et al.*, 2016).

Data analysis

A modified Gompertz model was used to fit the data from CH₄ measurements according to Equation 3.1.

$$M(t) = P \exp \left[- \exp \left[\frac{R_m e}{P} (\lambda - t) + 1 \right] \right] \quad \text{Equation 3.1}$$

Here M (mmol/L) is the cumulative methane production, P (mmol/L) is the methane production potential, R_m (mmol/L.h⁻¹) is the rate of production, λ (h) is the lag phase time, e is $\exp(1)$, and t (h) is time (Lay *et al.*, 1997). An example of data fit is given in the Supplementary material (Supplementary material, Figure S1.1).

For statistical comparison of different conditions, the probability distribution of the parameter vector $[P \ R_m \ \lambda]$ was obtained by a Markov chain Monte Carlo sampling method (ter Braak and Vrugt, 2008), and summarized by its 95% confidence region, following the methodology described by van Mourik *et al.* (2014). Sampling was carried out in log-space, i.e., after a log transformation of the parameter vector. For estimating the maximum likelihood of the parameter vector we used a hybrid algorithm consisting of a global search of the genetic search routine 'GA' in Matlab[®] software with a population of 200 with a maximum of 10³ generations, followed by a gradient based search with the 'lsqnonlin' routine in Matlab[®], starting from the optimum found by 'GA'. For Monte Carlo sampling, we used 3 chains, 2 · 10⁴ iterations per chain, 2 · 10³ burn-in iterations, and a thinning rate of 10. We used a log-uniform prior. The confidence regions were represented with 300 samples.

An example of the 95% confidence curves obtained for two of the studied conditions (Control and Ni-H-8, hydrogenotrophic) is given in Supplementary materials (Supplementary material, Figure S1.2). Three time points (t_{20} , t_{60} , t_{120}) were selected for comparison between different conditions; these time points are

representative of different growth phases (based on control curves, i.e. no metal addition).

Genomic searches

An analysis of the genomes of *Methanobacterium formicicum* JCM 10132 (Taxonomy ID: 1300163), *Methanosaeta concilii* GP6 (Taxonomy ID: 990316), *Methanosarcina barkeri* CM1 (Taxonomy ID: 796385) and *Methanolinea* sp. SDB (Taxonomy ID: 1735327) was performed using the online tool Rast (<http://rast.nmpdr.org/>) (Aziz et al., 2008; Overbeek et al., 2014; Brettin et al., 2015) and checked the presence of genes related with Ni/Co transport and metal resistance in these microorganisms. RcnA could not be found in Rast search for resistance subsystems. For this reason, RcnA protein sequence (NC-000913.3) (Rodrigue et al., 2005) was also searched using the online tool BLAST (<https://www.ncbi.nlm.nih.gov/blast/>) and compared with the genomes of the methanogens. These methanogens were chosen as they represent the abundant archaeal genera present in our samples. *M. barkeri*, is not abundant in our sludges, but it was included in the analysis. This microorganism is often present in anaerobic sludges (Venkiteshwaran et al., 2015; Ziganshin et al., 2013). Besides, this is one of the few methanogens with which metal toxicity tests were performed in pure cultures.

3.4. Results

Metal supplementation and toxicity tests

Cumulative methane production values measured after 20, 60 and 120 hours of incubation of the anaerobic sludge are shown in Table 3.1 (incubations with Ni) and Table 3.2 (incubations with Co). These values were obtained from the methane production curves. To illustrate this, some typical experiments for both stimulation and toxicity assays are shown in Figure 3.1.

Supplementation with 8 μM Ni improved hydrogenotrophic methanogenesis activity (11% higher cumulative methane production in Ni-L-8 at t_{120} , compared to the control), but that effect was not observed for lower amounts of Ni (*i.e.* 2 and 4 μM Ni). On the other hand, methanogenesis from H_2/CO_2 was negatively affected at high concentrations of Ni (*i.e.* 2, 4 and 8 mM). When compared with the controls, cumulative methane production (t_{120}) decreased in by approximately 20% when 2 mM of Ni were added and in 50% with 4 and 8 mM of Ni. Also Co, at concentrations ≥ 5 mM, had a negative effect on methanogenesis from H_2/CO_2 . Aceticlastic methanogenesis did not benefit from Ni supplementation but it was also negatively affected by higher concentrations of Ni with a decrease of 11% and 17.5% of cumulative methane production at concentrations of 4 and 8 mM respectively.

Regarding Co supplementation to hydrogen-consuming cultures, an improvement of 15% in cumulative methane production (t_{120}) was observed when 10.5 μM of Co was added. Conversely, at 10mM concentration, cumulative methane production decreased by $\sim 19\%$, and it got further reduced to 50% for higher concentrations (20 and 30 mM). Aceticlastic methanogenesis was not affected by supplementation but the toxic effect of Co is noticeable for concentrations ≥ 20 mM; cumulative methane production decreased by $\sim 11\%$ and $\sim 53.2\%$ for concentrations of 20 and 30 mM Co respectively.

Figure 3.1 - 95 % confidence curves between the Control and 4 μM of Ni (A and C) and 8 mM of Ni (B and D) supplementation in the presence of H_2/CO_2 (A and B) and acetate (C and D). The dark greys are the control curves.

Table 3.1 – Cumulative methane production measured at three different time points for each condition in the presence of Ni. A 95% confidence interval was considered and the deviations are indicated in the table. The production values for each time point were compared with the Control. Red and green shadow indicates decrease or increase of production values, respectively, when compared with the Control for the same time point.

Condition	Hydrogenotrophic (H ₂ /CO ₂ (80:20, %v:v), 1.7 atm)			Aceticlastic (20 mM Acetate)		
	t ₂₀	t ₆₀	t ₁₂₀	t ₂₀	t ₆₀	t ₁₂₀
Control	1.97±0.06	7.68±0.13	11.10±0.19	7.90±0.29	18.80±0.28	19.00±0.31
Ni-L-2	1.72±0.12	7.35±0.36	11.70±0.53	8.97±0.92	16.70±0.68	16.90±0.78
Ni-L-4	2.04±0.15	7.87±0.32	11.70±0.5	7.35±0.87	18.90±0.66	19.10±0.81
Ni-L-8	1.76±0.12	6.98±0.3	12.00±0.53	6.92±0.79	17.30±0.63	17.60±0.72
Ni-H-2	1.99±0.14	6.47±0.25	8.78±0.45	7.25±0.54	18.90±0.79	19.70±0.96
Ni-H-4	1.70±0.13	4.91±0.19	6.53±0.32	7.82±0.66	16.40±0.71	16.60±0.78
Ni-H-8	1.53±0.06	5.04±0.12	6.82±.017	5.61±.024	15.00±0.32	15.70±0.42

Table 3.2 – Cumulative methane production measured at three different time points for each condition in the presence of Co. A 95% confidence interval was considered and the deviations are indicated in the table. The production values for each time point were compared with the Control. The red shadow indicates a decreased and the green indicates an improvement of the production values, when compared with the Control for the same time point.

Condition	Hydrogenotrophic (H ₂ /CO ₂ (80:20, %v:v), 1.7 atm)			Aceticlastic (20 mM Acetate)		
	t ₂₀	t ₆₀	t ₁₂₀	t ₂₀	t ₆₀	t ₁₂₀
Control	1.97±0.06	7.68±0.13	11.10±0.19	7.90±0.29	18.80±0.28	19.00±0.31
Co-L-5.5	1.84±0.12	7.05±0.33	11.60±0.48	6.21±5.00	18.50±0.69	18.50±0.70
Co-L-10.5	2.12±0.14	8.55±0.40	12.30±0.60	5.78±3.50	16.60±0.69	16.70±0.73
Co-L-25.5	2.14±0.13	7.62±0.33	10.20±0.43	7.51±3.00	18.20±0.69	18.20±0.69
Co-H-2.5	1.76±0.13	7.46±0.37	11.10±0.47	4.82±3.80	18.40±0.69	18.50±0.73
Co-H-5	1.80±0.11	6.84±0.32	10.40±0.47	4.19±3.80	17.60±0.66	17.70±0.66
Co-H-10	2.06±0.14	6.95±0.29	9.00±0.43	4.42±4.00	18.20±0.70	18.20±0.71
Co-H-20	0.98±0.12	3.91±0.24	6.43±0.29	5.63±0.44	16.00±0.60	16.90±0.81
Co-H-30	1.21±0.08	4.29±0.16	6.60±0.22	2.11±0.15	7.34±0.23	8.89±0.33

Sulphide as metal detoxification method

To evaluate if sulphide produced by SRB could be a potential method for metal detoxification, anaerobic sludge was incubated with 8 mM of Ni or 30 mM of Co and sulphate. Incubations with sulphate (no metal) and with sulphide (Na₂S) plus metal were also performed as controls. The results are depicted in Table 3.3 and Figures 3.2 to 3.4.

Table 3.3 – Percentage of Na₂SO₄ consumption for the assays with Na₂SO₄, in the absence and presence of Ni or Co.

Condition/ Substrate	% of SO ₄ consumption	
	H ₂ /CO ₂	Acetate
SO ₄ -L-4	97.01	44.22
SO ₄ -M-8	76.86	35.49
SO ₄ -H-12	64.21	34.54
SO ₄ -L-15	46.62	25.57
SO ₄ -M-30	43.7	26.64
SO ₄ -H-45	10.7	5.44
Ni-SO ₄ -4	7.91	18.34
Ni-SO ₄ -8	2.04	23.19
Ni-SO ₄ -12	7.48	20.15
Co-SO ₄ -15	33.14	26.97
Co-SO ₄ -30	31.77	0
Co-SO ₄ -45	0.74	0

Figure 3.2 – Cumulative Methane Production values for the Ni detoxification assays at the three different time points (t_{20} ; t_{60} ; t_{120}) in A) in the presence of H_2/CO_2 and B) in the presence of acetate. The black bars indicate the 95% confidence intervals. H stands for High concentration, L for Low and M for Moderate. The numbers in front stand for the concentration tested.

Figure 3.3 – 95% confidence regions for the different conditions in the presence of H_2/CO_2 and 8 mM of Ni, with and without sulphate/sulphide, and control.

Figure 3.4 – Cumulative Methane Production values for the Co detoxification assays at the three different time points (t_{20} ; t_{60} ; t_{120}) in A) in the presence of H_2/CO_2 and B) in the presence of acetate. The black bars indicate the 95% confidence intervals. H stands for High concentration, L for Low and M for Moderate. The numbers in front stand for the concentration tested.

The addition of sulphate together with 8 mM of Ni or 30 mM of Co had no beneficial effect on methane production from H_2/CO_2 . Moreover, in the presence of metal, sulphate consumption is also lower than in the controls with sulphate alone. On the other hand, for both metals tested, the addition of moderate and high concentrations of Na_2S helped to restore methane production in comparison with those assays in which only metal is present. An example of comparison between the different conditions (Control, Metal, Metal + Sulphate, Metal + Na_2S) is depicted in Figure 3.3. For acetoclastic methanogenesis, the presence of only sulphate had no negative effect on methane production, except for the highest concentration tested (45 mM) where a lower value of cumulative methane produced ($\sim 6\%$, t_{120}) is observed (Figure 3.4B). When 4 mM of sulphate is added together with 8 mM of Ni, the cumulative methane produced is similar to the control (Figure 3.2B). On the other hand, 8 and 12 mM of sulphate together with Ni had a slight beneficial effect on methane production ($\text{max} \sim 13\%$; t_{120} , Ni- SO_4 -8), if compared with the situation with metal alone. The addition of 15 mM of sulphate together with Co had a beneficial effect in the first 60 h, but after that the cumulative methane production values are similar to the ones observed when the metal alone is present. If 30 mM of sulphate is added together with Co, the methane production improves. However, when sulphate concentration was of 45 mM, the values for methane produced are even lower than in the presence of only the metal. A synergistic effect of metal/sulphate was observed, for the majority of the conditions, both for hydrogenotrophic and acetoclastic conditions, with less methane being produced in those assays than when sulphate is present alone.

For hydrogen-consuming cultures in the presence of Ni, the addition of low concentration of Na_2S gives results similar to the ones observed for sulphide produced from sulphate. Yet, higher concentrations lead to a higher recovery of methane production. For acetoclastic conditions with Ni, the addition of Na_2S gave

similar results to the ones of sulphide formed from sulphate for all Na_2S concentrations tested. During the first 60h and for both substrates tested, the cumulative methane production for Co plus Na_2S is lower than Co alone. However, after 120 h, the values are higher in the presence of Na_2S and the shape of the curves indicates that the stationary phase was not reached yet (Figure 3.5).

Figure 3.5 – Comparison of the uncertainty curves for the different hydrogenotrophic conditions containing 30 mM of Co with and Na_2S and Control.

Microbial community analysis

The archaeal community (Figure 3.6) was dominated by species of *Methanobacterium* (hydrogenotrophic methanogens) and *Methanosaeta* (acetoclastic methanogens), which together represented 70 to 96% of the total archaeal community in the samples. *Methanolinea*, another genus of hydrogenotrophic methanogens, represented about 4-16% of the archaeal community. Unclassified species of *Halobacteriales* (1-23%) were also detected in most of the samples. In the presence of 8 mM of Ni, with H_2/CO_2 as

substrate, there was a change in the relative abundances of *Methanosaeta* population from 40-48% to ~21% while *Halobacteriales* relative abundances increased to 20%. The presence of sulphate leads to changes in *Methanosaeta* population to 27-32% and to 37-40% when sulphide was added. Relative abundances of *Methanobacterium* and *Methanolinea* populations showed little variations in the presence of Ni for all the conditions tested with H_2/CO_2 as substrate. In the presence of Co, *Methanosaeta* populations remained stable. When H_2/CO_2 was the substrate, *Methanobacterium* decreased from 42-46% to 28-35% when 30 mM of Co was in the medium, also in the presence of sulphate. *Methanolinea* increased from 5-11% to 13-18% when Co is present. When Na_2S was added, the numbers increased to 54-56%. If acetate was the substrate, the population of *Methanobacterium* decreased from ~45% to 24-31% in the presence of the metal, decreased to 34-38% when sulphate is added and to 31 to 46% if Na_2S was added. Moreover, with acetate, *Halobacteriales* are absent in the samples without or with low concentrations of metals, while they appear in samples with metal plus sulphate or sulphide in relative abundances up to 16%.

Figure 3.6 – Relative abundances for Archaeal diversity (genus level) in the presence of Ni (A) and Co (B).

Analysis of the genomes of M. formicicum, Methanolinea sp., M. barkeri and M. concilii

The analysis to the genomes of *M. formicicum* JCM, *M. concilii* GP6, *M. barkeri* CM1 and *Methanolinea* sp. SDB revealed that genes encoding for CbiMNQO and NikMQO transport systems could be found in all the four genomes. Furthermore, in the genome of *M. barkeri* it could also be found genes for the NiKABCDE system, including the NikA subunit. *M. concilii*, *M. formicicum* and *M. barkeri* possess genes encoding for NikN. In addition, all four genomes have multiple homologues encoding for CzcD, which is a known cobalt-zinc-cadmium resistance protein. The Ni and Co resistance gene from *E. coli* RcnA was also searched in the genomes of the methanogens without significant hits.

3.5. Discussion

Ni and Co are important metal ions for methanogenesis and their supplementation in anaerobic systems is considered to be beneficial for methane production (Gustavsson *et al.*, 2011; Evranos *et al.*, 2015). However, in our study, little or no increase of methane production could be observed as a result of their supplementation to anaerobic sludge. Ineffectiveness of trace metal supplementation to improve methane production was reported before. Park *et al.* (2010) tested the addition of different nutrients and different metals, including Ni and Co, and reported no stimulation of acetoclastic methanogenesis by metal supplementation. Furthermore, they observed a decrease in the total amount of methane produced, which became more severe at higher metal concentrations. Moreover, the substrate is also a factor to consider; Zandvoort *et al.* (2006) reported positive effects of trace metal addition, and especially Co, with sludge fed with methanol, but no effect with acetate and H₂/CO₂. The lack of positive effect observed in our study can be due to the fact that

the anaerobic sludge used might have had metals adsorbed in it. Metal adsorption in biofilms (including granules) is a known process (van Hullebusch *et al.*, 2003; Zandvoort *et al.*, 2006). The sorption kinetics and capacity of Ni and Co to granular sludge was studied by van Hullebusch *et al.* (2004; 2005) and it was observed that the sorption capacity of granular sludge was lower than that of other sorbents. However, from a microbial perspective, the amount of metal adsorbed is considerable. Although metal supplementation can be a good strategy to improve the system performance and increase methane production, it is important to emphasize that each system, set of conditions chosen for its operation (pH, temperature, operation times, etc.) and the microbial community are unique. The fact that metal supplementation is not always beneficial stresses the need for preliminary tests before its full scale implementation.

Heavy metals can accumulate in anaerobic bioreactors to toxic concentrations for microorganisms. In this study, the toxic effects of Co and Ni on hydrogenotrophic and acetoclastic methanogenesis were evaluated. Hydrogenotrophic activity showed higher sensitivity to the presence of Ni than the acetoclastic activity. For example, 8 mM of Ni reduced methane production by ~50% for hydrogenotrophic conditions and only by 18% for acetoclastic conditions. It can be hypothesised that the physiological observations could be linked with variations in the microbial community composition. However, in samples with or without Ni, no relevant changes could be observed in the relative abundances of the main methanogens. The fact that the analysis was based on DNA and that the assays time was only ~150 h could be a reason why changes could not be observed for some of the microorganisms. The doubling time of *Methanosaeta concilii*, one of the most common acetoclastic methanogens found in anaerobic digesters, is about 65 h (35°C, pH 7.8). The two species described of *Methanolinea*, *M. mesophila* have a doubling time of about 29 h (37°C, pH 7.0), while *M. tarda* has a doubling time of about 98 h (50°C,

pH 7.0). Most *Methanobacterium* species described in the methanogenic archaea database (<http://metanogen.biotech.uni.wroc.pl/>) (online August 2016) (Jablonski *et al.*, 2015) have doubling times around 20 h at optimal conditions. *Methanobacterium formicum*, for example, has a doubling time of 11 h with formate as substrate, pH 7.6, 37°C (Schauer and Ferry, 1980). Therefore, although some of the microorganisms present in our sludge could have long doubling times that would not allow to observe changes in their relative abundances with a DNA-based analysis, for other species with shorter doubling times, these changes should be visible. This suggests that some of the species present have some metal resistance characteristics. A study with pure cultures reported that *M. formicum* is more resistant to Ni than *M. barkeri* or *Methanothermobacter marburgensis* (Jarrell *et al.*, 1987). Similarly, it was reported that *M. formicum* was not affected by Ni concentrations up to 1200 mg/L (~20 mM), while *Methanosarcina thermophila* was inhibited with 500 mg/L of Ni (~8.5 mM) (Sanchez *et al.*, 1996). No information regarding toxicity of Ni towards *Methanosaeta* or *Methanolinea* species is presented in other studies. Co seems to be equally toxic for both hydrogen and acetate-consuming cultures, but compared to Ni, higher concentrations of metal (20 and 30 mM) are needed to cause negative effects. Less information is available about Co toxicity than about toxicity of other important metals. Bhattacharya *et al.* (1995) studied the effects of Co in enrichments with acetate and glucose and reported 100% inhibition of methane production in the presence of 16.1 mM of Co, which is a lower concentration than the one reported in the current study. *Methanobacterium* relative abundances were the only ones that decreased in the presence of Co and this result is corroborated by the decrease in methane production observed for hydrogenotrophic methanogenesis. Our results show a clear effect of high concentrations of Ni and Co on methanogenesis in granular sludge, but, in most cases, this could not be linked with variations in the microbial composition. The lack of changes in some of the populations indicates that

metal effect might not be lethal to these microorganisms and that some of them have developed resistance mechanisms to metal toxicity.

Different mechanisms might be involved in metal resistance in microorganisms. The resistance mechanism described for iron is the chemical reduction of the ion (Nies, 1992). Another resistance mechanism used by microorganisms is methylation, for example for Hg (Nies, 1992). However, in the case of Ni^{2+} and Co^{2+} these mechanisms cannot be applied. Active efflux is the most likely mechanism of resistance for these two metals. We performed an analysis to the genomes of *M. barkeri*, *M. concilli*, *Methanolinea* sp. and *M. formicum* in search of Ni and Co genes involved in metal resistance. The results revealed that all four examined microorganisms have homologues in their genomes for the main Ni and Co transport systems and for CzcD. These homologues seem to be equally distributed by the four species. This result indicates that the higher resistance of some species to Ni or Co cannot be explained by the presence of these systems. Metal resistance mechanisms and its regulation systems in archaea are still not well understood. Other metal transporters or resistance systems, that are not yet identified, can be the missing explanation.

Precipitation of metals with sulphide is a potential metal detoxification method, but the competition between SRB and methanogens for common substrates needs to be taken into account. In the case of hydrogenotrophic activity, the addition of sulphate to cultures with high metal concentration had little beneficial effect on methane production, while the addition of Na_2S helped to restore the methane production. This is possibly explained by the strong competition between methanogens and SRB for hydrogen. Not only the reduction of sulphate from hydrogen is thermodynamically more favourable than methane production, but it is also known that SRB have a higher affinity for hydrogen than methanogens and, for that, SRB are expected to outcompete methanogens. Moreover, the metal can also be toxic for the

SRB present in the sludge and inhibit their performance, which can be seen by the decrease of sulphate consumption in the presence of metal. Studies performed in pure cultures of *Desulfovibrio desulfuricans* showed that this microorganism is sensitive to Ni concentrations above 0.17 mM (Poulson *et al.*, 1997). *Desulfovibrio vulgaris* and *Desulfovibrio* sp. cultures were severely inhibited by similar Ni concentrations (0.14 mM) (Cabrera *et al.*, 2006). Other SRB species can tolerate higher concentrations, as for example *Desulfotomaculum* sp. that can tolerate 9.5 mM of Ni (Fortin *et al.*, 1994). In general, SRB are described to be more resistant to the presence of metals. The precipitation of metal ions by sulphide is probably one of the main metal resistance mechanisms in these bacteria (Paulo *et al.*, 2015). Yet, more studies regarding the effect of Ni and Co on SRB species are needed in order to verify these results. The presence of sulphide (bioproducted or Na₂S) in high Ni cultures with hydrogen resulted in higher numbers of *Methanosaeta*. This indicates that the presence of sulphide can help to diminish the toxic effects of the metal presence for some microorganisms and that metal toxicity can be reversed. In our assays with sulphate and acetate, no decrease in methane production was observed. Based on these findings, we hypothesized that homoacetogens convert H₂/CO₂ to acetate, which is then consumed by *Methanosaeta*, explaining the high abundance of this microorganism even such conditions. Moreover, sulphate consumption in the assays using acetate as substrate is relatively small, especially for high sulphate concentrations (less than 25%). The addition of sulphate together with high metal concentration had some beneficial effect as metal detoxification method, but the values of cumulative methane production are lower than when sulphate alone is present. On the other hand, Na₂S addition had a positive effect in restoring methane production. SRB can also be affected by the presence of the metal and they can present lower activity.

3.6. Conclusions

Addition of low concentrations of Ni and Co had no stimulatory effect on methane production from H_2/CO_2 and acetate by anaerobic sludges. It is possible that this effect is only observable in long term incubation, or enrichment procedures, when residual metals are extinguished from the medium. Because the effects of metal addition are variable, depending on inoculum and environmental conditions, the range on metals and conditions tested should be amplified and the differences between hydrogenotrophic, aceticlastic and methylotrophic methanogens should be considered. Our results show that sulphide can be an efficient method of metal detoxification. However, in the case of biological produced sulphide, the competition between SRB and methanogens for common substrates is a key factor that determines the efficiency of the method.

Chapter 4

Metal supplementation to enhance 1,2-dichloroethene and tetrachloroethene dechlorination in enriched cultures

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4.1. Abstract

Chlorinated compounds are common contaminants in wastewaters. They can be reductively dechlorinated under anaerobic conditions. This process is performed by several bacteria designated organohalide-respiring bacteria (OHRB). Methanogens are known to perform co-metabolic dechlorination and several methanogenic cofactors are described to be involved in the reductive dechlorination process. These cofactors are metal-dependent and, for this reason, it was hypothesized that metal supplementation could improve the reductive dechlorination. In this work, we aimed to enrich for methanogenic cultures degrading tetrachloroethene (PCE) and 1,2-dichloroethene (DCE). On a second phase, enrichment cultures with good dechlorination performance were supplemented with trace metals. We looked to the effects of inoculum source and substrate used (H_2/CO_2 , acetate and methanol), both on the performance of reductive dechlorination and on microbial community changes. Methanol was the most favourable substrate, while enrichments on acetate lost their viability after 3 transfers. The microbial community analysis showed that not only the inoculum origin, but also the substrate and the chlorinated compound used influenced the microbial composition. 20.5 μM of Co, 20 μM of Ni or 37 μM of Fe were supplemented to selected cultures. Our results showed that Fe and Ni supplementation can help to improve both methane production and reductive dechlorination, while Co had only a positive effect on reductive dechlorination. For example, TCE formation was 10x higher in the presence of Fe than in the control and VC formation started $\sim 400h$ earlier in the presence of Ni or Fe than with Co or not metal. Moreover, the results showed that the effect is not only dependent on the metal added but also on the metal concentration used.

Keywords: methanogenic, trace elements, reductive dechlorination, stimulation

4.2. Introduction

Chlorinated compounds are widely used as pesticides, pharmaceuticals, personal care products, flame retardants, cooling fluids, degreasing agents and solvents (van Eekert and Schraa, 2001; Smidt and de Vos, 2004; Krzmarzick and Novak, 2014). Due to their large use, they often end up in wastewater treatment plants from where they are transferred to the environment. Besides their toxicity, some chlorinated compounds are known to be endocrine disruptors, and this has raised a great concern about their fate in the environment (Krzmarzick and Novak, 2014). Under anaerobic conditions, chlorinated compounds can be reductively dechlorinated to less chlorinated compounds (Yang *et al.*, 2005). Bacteria performing reductive dehalogenation are classified as facultative or obligate organohalide-respiring bacteria (OHRB) (Krzmarzick and Novak, 2014, Atashgahi *et al.*, 2016; Mayer-Blackwell *et al.*, 2016). Obligate OHRB have a restricted metabolism; in most cases, they are constrained to the utilization of H₂ as electron donor and halogenated compounds as electron acceptors (Mayer-Blackwell *et al.*, 2016). Species from the genera *Dehalococcoides*, *Dehalogenimonas*, and *Dehalobacter* are examples of obligate OHRB (Mayer-Blackwell *et al.*, 2016). The more versatile metabolism of facultative OHRB is shown by their capability of using Fe(III), Mn(IV), DMSO or oxidized sulphur compounds as electron acceptors, in addition to chlorinated compounds. They can also use a wide range of electrons donors, such as acetate, butyrate, ethanol, formate, succinate or lactate (Mayer-Blackwell *et al.*, 2016). Well-studied facultative OHRB include members of the genera *Desulfitobacterium*, *Geobacter*, *Desulfovibrio*, and *Sulfurspirillum*. OHRB have been isolated from different environments and have different phylogenies (van Eekert and Schraa, 2001). Some bacteria can only perform incomplete dechlorination, removing just one or two chlorine atoms, while others, such as *Dehalococcoides* species, can perform dechlorination of PCE to ethene (van Eekert and Schraa, 2001; Yang *et al.*, 2005; McCarty, 2016).

Microbial reductive dechlorination is also found as a co-metabolic process in a variety of microorganisms, including methanogens (Holliger and Schumacher, 1994). Some methanogens can dechlorinate tetra- and trichloromethane, 1,2-dichloroethene (DCE) and tetrachloroethene (PCE) (Holliger *et al.*, 2004). For example, DCE is described to be reductively dechlorinated by *Methanosarcina barkeri*, *Methanococcus mazei* or *Methanothermobacter thermoautotrophicum* (former *Methanobacterium thermoautotrophicum*) (Holliger *et al.*, 1990), while PCE can be dechlorinated by *Methanosarcina thermophila* (Middeldorp *et al.*, 1990). Several studies pointed out the involvement of corrinoids, porphyrins, and co-factors in co-metabolic dechlorination in which metal ions are required. For instance, reductive dechlorination of DCE to ethene by methanogens is catalyzed by the methyl-coenzyme M reductase (Holliger *et al.* 1992). This enzyme requires the cofactor F₄₃₀, which has a Ni ion in its structure. Moreover, it was observed that highly chlorinated ethenes could be degraded by autoclaved sludge, although at low rates, which indicates the involvement of heat-stable cofactors, such as vitamin B₁₂ and cofactor F₄₃₀ (van Eekert *et al.*, 1999). The reductive dechlorination by metal-containing coenzymes was tested for different chlorinated compounds (Gantzer and Wackett, 1991). Vitamin B₁₂, cofactor F₄₃₀ and hemein were reported to be able to catalyze the reductive dehalogenation of polychlorinated ethenes and benzenes, while ferredoxins and azurin could not do (Gantzer and Wackett, 1991). Additionally, vitamin B₁₂ and cofactor F₄₃₀ could also sequentially dechlorinate PCE to ethene, but hemein could only perform the dehalogenation until vinyl chloride (VC).

PCE is a highly used compound. Due to its improper use and disposal, this compound is a common contaminant of soil and water (Aulenta *et al.*, 2002). During its dechlorination process, PCE is sequentially dechlorinated to trichloroethene (TCE), *cis*-1,2-dichloroethene (*cis*-DCE), VC and to ethene (Lohner and Tiehm, 2009). Although the complete dechlorination was already observed, both in field and

laboratory experiments, quite often VC accumulation is observed since the reduction of this compound seems to be a limiting step (Aulenta *et al.*, 2002). This represents a concern because VC is more toxic than the parent compound and is a known carcinogenic (Aulenta *et al.*, 2002).

Methanogenic cofactors seem to play an important role in reductive dechlorination. For this reason, in this work, we enriched for methanogenic cultures able of PCE and DCE reductive dechlorination. Three different substrates, methanol, acetate, and H_2/CO_2 , were used for the enrichments to evaluate the most favourable electron donor for this process. Cultures were monitored for methane production, intermediate chlorinated compounds, and microbial composition. The enriched cultures with the best dechlorination performance were selected and used for metal supplementation studies. Nickel (Ni^{2+}), cobalt (Co^{2+}), and iron (Fe^{3+}) were supplemented to enriched cultures individually to access their effect in reductive dechlorination.

4.3. Materials and Methods

Inoculum sludge, medium composition, and enrichment conditions

Anaerobic granular sludge was obtained from a wastewater treatment plant treating food industry effluent (Biothane, Delft, The Netherlands). Suspended sludge was obtained from municipal wastewater treatment (Apeldoorn, The Netherlands). 5 mL of well-mixed sludge were used to inoculate vials containing 50 mL of basal medium. Medium was prepared according to the protocol previously described by Stams *et al.* (1993). The assays were performed using three different substrates: acetate, methanol and H_2/CO_2 . Acetate was added to a final concentration of 20 mM and methanol was added to a final concentration of 40 mM. In both cases, bottles' headspace was flushed and pressurized with N_2/CO_2 (80:20 v/v, 1.5 atm). When H_2/CO_2 was used as substrate, bottles' headspace was flushed and pressurized

with 1.5 atm of H_2/CO_2 (80:20 v/v, 1.5 atm). *Trans*-1,2-DCE or PCE were dissolved in hexadecane and added to the serum bottles to a final concentration of 0.4 mM. The assays were performed in duplicate, and the bottles incubated at 30°C in the dark. The concentrations of substrates, PCE and DCE were monitored and supplied again when depleted. Methane was measured regularly to ensure that methanogenic microorganisms were present. The bottles were maintained in this way for four months without being transferred. After that, 5 mL of sample were taken to obtain the biomass for further microbial community analysis. The samples were centrifuged, and the pellet resuspended in phosphate buffered saline (PBS). Moreover, 5 mL of the liquid samples from each bottle were transferred to new medium with the same composition as described before and incubated at 30°C. The enrichments were monitored for substrate, PCE and DCE depletion and methane, acetate and the intermediate chlorinated compounds production. Four other similar transfers were performed regularly, making a total of five transfers. All enrichment cultures using acetate as substrate lost viability after the third transfer and for that reason those results are not shown. From the last transfer, after 30 days of incubation, 5 mL of sample were taken to obtain the biomass for further microbial community analysis.

For the sake of clarity, a system of acronyms was created to identify the different enrichment cultures, considering: the source of inoculum, G (granular sludge) or S (suspended sludge); the added chlorinated compound, P (PCE) or D (DCE); and, the methanogenic substrate used, H (H_2/CO_2), M (methanol). For example, G.D.H. for an enrichment started with granular sludge and incubated with DCE and H_2/CO_2 . To this acronym we added two numbers, G.D.H.#-† representing the replica (#, 1 or 2, assays done in duplicate) and number of transfers (†).

Metal supplementation tests

We evaluated the dechlorination performance of the enriched cultures after the last transfer and selected, for each chlorinated compound tested, the condition that presented the best dechlorination performance. In both cases, enriched cultures in methanol were selected for further incubations with metals. For incubations in DCE, culture G.D.M.1-5 was chosen, while for PCE, S.P.M.1-5 was the culture selected. 2.5 mL of sample were used to inoculate serum bottles containing mineral medium (prepared as described above). 125 mM of methanol were added as substrate, while the bottles headspace was flushed with N₂ (1.5 atm). *Trans*-1,2-DCE or PCE in hexadecane were added to the serum bottles in final concentration of 0.5 mM. Ni was added as NiCl₂, Fe as FeCl₃ and Co as CoCl₂ in final concentrations of 20, 37 and 20.5 µM, respectively (corrected with the considering the Ni, Fe and Co already available in the basal medium; in the case of Fe, it represents the total Fe (Fe²⁺ plus Fe³⁺). Controls without metal addition were included. All assays were performed at least in duplicates, incubated at 30°C in the dark. Assays with Ni supplementation and PCE lost viability and the results are not shown.

Analytical methods

Bottles' headspace was analysed for H₂ and CH₄ with a Compact GC^{4.0} (Interscience, Breda, The Netherlands) equipped with Carboxen 1010 column (Supleco, 3 m x 0.32 mm) followed by a Molsieve 5A column (Restek, 30 m x 0.32 mm), with argon as carrier gas at 0.8 mL min⁻¹, and a thermal conductivity detector (TCD). The temperature of the valve (injection) oven was kept at 60 °C, while column and detector were kept at 100 °C.

The chlorinated compounds concentration were measured by GC-2010 (Shimadzu, Kyoto, Japan) equipped with a CP-SIL5 CB column (Agilent, 30 mm x 0.25 mm)

and Innowax packing/coating of 0.5 μM . The GC setting were: column temperature: 60 $^{\circ}\text{C}$, injector temperature: 250 $^{\circ}\text{C}$, injector pressure: 124.6 kPa, injector total flow: 93.7 mL/min, detector temperature: 300 $^{\circ}\text{C}$, detector H_2 flow: 40 mL/min, and detector air flow: 400 mL/min. Before the headspace sample was analysed, the bottles were kept in a water bath at 30 $^{\circ}\text{C}$ for at least 30 minutes since the equilibrium of chlorinated compounds between liquid and gas phase is temperature dependent. Moreover, samples were also analysed by measuring samples of the headspace on Varian GC-FID (CP-3800) (Thermo Scientific) equipped with a split-splitless injector followed by an RT-Q Bond column (Restek, 30 m x 0.32 mm). Gas carrier was helium at a flow of 2 mL.min⁻¹. Temperature was set to 40 $^{\circ}\text{C}$ for 1 min, followed by a temperature ramp up to 200 $^{\circ}\text{C}$ in 4 min and additional 5 min at 200 $^{\circ}\text{C}$. Standards for chlorinated compounds were prepared by adding a known amount of chromatography-grade to a serum bottle with the same headspace to liquid ratio as the assay bottles.

Liquid samples were analysed with a Acella HPLC (Thermo Scientific, Waltham, MA) equipped with a Varian Metacarb 67H column (300 x 6.5 mm) column for Volatile Fatty Acids (VFA's) and alcohols. Column was kept at 45 $^{\circ}\text{C}$ and running with 0.01N of H_2SO_4 as eluent with a flow of 0.8 mL/min. The detector was a refractive index detector.

Microbial community analysis

DNA was extracted using the FastDNATM Spin Kit for Soil DNA Extraction (MPBio, Santa Ana, CA). After purification, DNA was amplified with bacterial and archaeal primers and the samples were screened using Denaturing Gradient Gel Electrophoresis (DGGE) and representative samples of each tested condition were chosen for sequencing in Hiseq platform. DNA amplification for sequencing was

prepared as described in Chapter 3. The amplification products were sent for sequencing on the Hiseq platform (GATC Biotech AG, Konstanz, Germany). Data was analysed using NG-Tax, a validated pipeline for 16S rRNA analysis (Ramiro-Garcia *et al.*, 2016).

4.4. Results

Physiological differences of cultures enriched in the presence of DCE and PCE using different substrates and with different inoculum origins

Two different sludges were used as inoculum source to enrich for cultures able to perform reductive dechlorination of PCE and DCE in methanogenic conditions using H_2/CO_2 , methanol and acetate as substrates. Acetate-grown cultures lost viability over time and no results are shown for those cultures. A total of five transfers were performed during one year. The last transfer (from here on named 5th transfer) was analysed for physiological differences under the different conditions tested. The 5th transfer was incubated for 30 days and during that time, H_2/CO_2 was supplemented for five times to G.D.H and G.P.H cultures, four times to S.D.H cultures and three times to S.P.H cultures. Methanol was supplemented in a total of three times to all methanol-grown cultures. After 30 days of incubation, the concentrations of the products formed (methane, acetate and chlorinated compounds) were measured (Figures 4.1 and 4.2). Due to differences between some of the duplicates for some measurements, the results for the duplicates are presented separately. The cultures incubated with DCE (Figure 4.1), in the presence of H_2/CO_2 , G.D.H.1-5, G.D.H.2-5 and S.D.H.2-5 produced ~26-28 mmol/L of methane, while S.D.H.1-5 produced 18 mmol/L. Moreover, G.D.H.1-5, S.D.H.1-5 and S.D.H.2-5 produced ~18 mM of acetate and G.D.H.2-5 produced only 10 mM. In the cultures incubated with methanol, the methane and acetate production were different depending on the

inoculum origin; G.D.M.1-5 and G.D.M.2-5 had a similar methane (14-17 mmol/L) and acetate production (14-17 mM), while S.D.M.1-5 and S.D.M.2-5 produced less methane (7.8-10 mmol/L) and more acetate (~22 mM). Furthermore, the cultures incubated with H₂/CO₂ showed some formation of VC; only 0.02-0.05 μM of VC could be detected after the 30 days of incubation. G.D.M.1-5 and G.D.M.2-5 showed a higher production of VC, producing 1.15-1.24 μM of VC in 30 days, while S.D.M.1-5 and S.D.M.2-5 produced 0.30-0.35 μM.

Figure 4.1 – Methane, acetate and VC concentrations formed after 30 days of incubation in the presence of 0.4 mM of DCE for the different conditions tested.

Figure 4.2 – Methane, acetate, *trans*-DCE, *cis*-DCE, TCE and VC concentrations formed after 30 days of incubation in the presence of 0.4 mM of PCE for the different conditions tested.

Regarding the cultures enriched with PCE, S.P.H.1-5 and S.P.H.2-5, although producing less methane (9.5-10 mmol/L), showed a good dechlorination performance; PCE was reduced to TCE (45-50 μM) and *cis*-DCE (190-204 μM). Acetate was also produced up to 17 mM. On the other hand, in G.P.H.1-5 and G.P.H.2-5, dechlorination was almost inexistent and only low concentrations (4.0-4.7 μM) of TCE could be measured. Methane was produced up to 34 mmol/L and no acetate was detected. The enrichments with methanol showed a better dechlorination performance, compared to the incubations with H₂/CO₂, for both types sludge used as inoculum. In S.P.M.1-5 and S.P.M.2-5, high concentrations of TCE (374-414 μM) and *cis*-DCE (441-471 μM) were measured. *Trans*-DCE (23-30 μM) and VC (0.14-0.16 μM) were also measured, indicating a complete degradation

of PCE to VC. Methane was produced up to 12 mmol/L and acetate up to 21.5 mM. G.P.M.1-5 and G.P.M.2-5 showed considerable differences regarding the TCE and *cis*-DCE concentrations: while in G.P.M.2-5 390 μ M of TCE and 115 μ M of *cis*-DCE could be measured, in G.P.M.1-5 only 195 μ M of TCE and 80 μ M of *cis*-DCE were found. 22.5-25 μ M of *trans*-DCE and 0.42-0.47 μ M of VC were measured in these cultures, which indicated that also in this case, PCE could be degraded to VC. Methane was produced up to 26 mmol/L, while no acetate was observed. No ethene or ethane could be detected in none of the incubations, neither in the presence of DCE or PCE.

Microbial community analysis

The microbial communities were analysed after the first and 5th transfer to observe the communities' evolution and differences originated by inoculum source and substrate used (Figures 4.3 to 4.6). In general, clear differences between the two inoculum sources were observed. Furthermore, even after one single transfer, the substrate, as well as the chlorinated compound had a clear impact on the microbial communities. Moreover, in some cases, like G.D.H, significant differences were observed between duplicates of the same condition.

In cultures from granular sludge incubated with DCE, after the 1st transfer, the archaeal community was composed mainly of *Methanocorpusculum* species for both substrates, while after the 5th transfer *Methanofollis* became one of the most abundant archaea. In the 1st transfer, *Methanosarcina* species were only detected in G.D.H.1-1 and G.D.H.2-1, while in the 5th transfer they were also present in G.D.M.1-5 and G.D.M.2-5. Moreover, in G.D.H.1-5 and G.D.H.2-5, *Methanospirillum* species became relatively abundant (29-36%), while in G.D.M.1-5 and G.D.M.2-5, *Methanomethylovorans* species became one of the predominant archaea. On the other

hand, cultures enriched from suspended sludge were initially composed of *Methanobacterium* and *Methanosaeta* species and no significant differences between the two substrates could be observed. However, in the 5th transfer the differences are clear; with H₂/CO₂, *Methanocorpusculum*, *Methanofollis* and *Methanospirillum* were the main archaea organisms found, while with methanol, archaeal community was mainly composed of *Methanobacterium* and *Methanosarcina*.

Figure 4.3 – Relative abundances for archaeal community in cultures enriched in the presence of 0.4 mM of DCE.

Figure 4.4 - Relative abundances for bacterial community in cultures enriched in the presence of 0.4 mM of DCE.

The bacterial composition of the communities also showed several differences between the different conditions tested. Considering the aim of this work, the focus will be kept on the OHRB present in the samples. From the microorganisms

classified as obligate OHRB, unclassified *Dehalococcoidetes* species could be detected in S.D.M.1 and S.D.M.2 in both transfers. Moreover, *Peptococcaceae* family members, most of them from unclassified genera, could be found in high abundance in cultures incubated with methanol, especially G.D.M.1-5 and G.D.M.2-5 where they represent most of the bacterial community. On the other hand, several facultative OHRB could be found in the 5th transfer. *Geobacter* and *Desulfovibrio* species were present in the majority of the samples, while *Desulfuromonas* species were only detected in G.D.H.1-5 and G.D.H.2-5.

In the presence of PCE, cultures enriched from granular sludge showed complete different archaeal communities at the 1st transfer; in G.P.H.1-1 and G.P.H.2-1, *Methanosaeta* and *Methanosarcina* were the main archaea, while in G.P.M.1-1 and G.P.M.2-1, *Methanocorpusculum* and unclassified Thermoplasmatales were the main organisms, although *Methanosarcina* was also present, but in lower relative abundances. Cultures from suspended sludge showed almost no differences between the two substrates at this point; *Methanobacterium* and *Methanosaeta* were the main archaea found. Unfortunately, only one of the replicates of the methanol incubations gave sufficient reads to be considered as valid. This was also encountered for one of the replicates from granular sludge with H₂/CO₂ for the 5th transfer data. On the 5th transfer, significant differences could be observed as a result of inoculum source and substrate used; culture G.P.H.1-5 was composed by *Methanofollis*, *Methanospirillum* and *Methanosaeta* species, while in G.P.M.1-5 and G.P.M.2-5, *Methanomethylovorans* constituted more than 93% of the archaeal community. In S.P.H.1-5 and S.P.H.2-5 *Methanobacterium* was still present in high percentages, while *Methanosarcina* relative abundances decreased and *Methanobrevibacter* and *Methanoculleus* relative abundances increased. S.P.M.1-5 and S.P.M.2-5 were mainly composed of *Methanobacterium* and *Methanosarcina*.

Figure 4.5 – Relative abundances for archaeal community in cultures enriched in the presence of 0.4 mM of PCE.

Unclassified *Dehalococcoidetes* species could be detected in low relative abundances in G.P.M.1-1, G.P.M.2-1 and G.P.M.1-5. Furthermore, *Peptococcaceae* family members could be found in almost all samples in the 5th transfer, except G.P.H.1-5. *Geobacter* could be found in all cultures originated from suspended sludge in the 1st transfer, but not in the 5th transfer. On the other hand, *Desulfovibrio* could be found in all samples from granular sludge in the 5th transfer.

Figure 4.6 – Relative abundances for bacterial community in cultures enriched in the presence of 0.4 mM of PCE.

Effect of metal supplementation on reductive dechlorination

G.D.M.1-5 was transferred to new medium and Fe, Co or Ni were added to the incubations. Ni and Fe supplementation had a clear beneficial effect on methane production, as is shown in Figure 4.7A. The effect is more noticeable in the initial methane production rates. For example, at t_{192h} , methane production in the control was of 0.8 ± 0.1 mmol/L, while it reached 1.6 ± 0.1 mmol/L in the presence of Fe and 1.9 ± 0.5 mmol/L when incubated with Ni. In contrast, Co supplementation inhibited methane production to almost 50%. Reductive dechlorination was enhanced by the presence of Ni and Fe, while Co had no significant effect (Figure 4.7B). Cultures incubated with Fe or Ni showed VC formation after 200h of incubation, while in the absence of metal or in incubations with Co, VC formation started only after 600h. Furthermore, Ni had a more stringent effect than Fe; at t_{360h} , VC concentration was 0.04 ± 0.00 μ M with Fe and 0.12 ± 0.02 μ M with Ni.

Hydrogen was also detected as product in these incubations (Figure 4.7C). In this case, Co supplementation enhanced hydrogen production, while Fe had no effect and Ni inhibited the production of hydrogen. At the end of the incubation, t_{792h} , hydrogen concentration in the control was 66 ± 11 μ mol/L, while in the cultures incubated with Co it reached 104 ± 2 μ mol/L and only 29 ± 5 μ mol/L in the presence of Ni.

A similar strategy of metal supplementation was applied to cultures incubated in the presence of PCE. S.P.M.1-5 was used as inoculum for further incubations with metal supplementation. Yet, the incubations with Ni failed to grow and, for that, only the results with Fe and Co are presented. Fe supplementation had a stringent effect on methane production (Figure 4.8A). For instance, at t_{360h} , methane production in the control is 3 ± 1 mmol/L, while with Fe supplementation it achieved 25 ± 3 mmol/L. Instead, Co supplementation had no effect on methane

production. The effect of metals supplementation on the reductive dechlorination was evaluated for the formation of each intermediate compound formed: TCE (Figure 4.8B), *cis*-DCE (Figure 4.8C), and VC (Figure 4.8D). Both supplemented metals enhanced TCE formation from PCE, but Fe had the strongest effect; for instance, at t_{360h} , TCE concentration in the control was $38 \pm 0 \mu\text{M}$ in the control, $96 \pm 20 \mu\text{M}$ with Co and $303 \pm 11 \mu\text{M}$ with Fe. Furthermore, in cultures incubated with Fe, *cis*-DCE formation could be observed after 192h, while in incubations with Co it was only detected after 600h and in the control it was almost not detectable. VC could only be detected in the incubations with Fe. Hydrogen production was not affected by metal supplementation (Figure 4.8E).

Figure 4.7 – Methane (A), VC (B) and hydrogen (C) production in cultures incubated in 0.5 mM of DCE, in methanol, without metal supplementation and supplemented with Fe, Co or Ni.

Figure 4.8 – Methane (A), TCE (B), *cis*-DCE (C), VC (D) and hydrogen (E) production in cultures incubated in 0.5 mM of PCE, in methanol, without metal supplementation and supplemented with Fe or Co.

4.5. Discussion

Methanogens are reported to be able to perform co-metabolic reductive dechlorination. Moreover, they are described to excrete compounds to the medium, such as corrinoids and cofactor F₄₃₀, that are said to be involved in reductive dechlorination. In this work we aimed to enrich for methanogenic cultures able to perform DCE and PCE dechlorination. Our results showed that reductive dechlorination, even if at a very slow rate, could be observed in all the enrichments with methanol or H₂/CO₂ and that the most efficient substrate was methanol. Several anaerobic non-methanogenic cultures growing on methanol have been described to perform dechlorination. For instance, PCE and TCE dechlorination was reported under methanogenic conditions and methanol was the most effective electron donor (Freedman and Gossett, 1989). Moreover, although complete dechlorination was observed, the limiting step was the conversion of VC to ethene. Furthermore, an anaerobic culture enriched with methanol was described to be capable of dechlorinating PCE to ethene at high rates, while methanogenesis did not occur (DiStefano *et al.*, 1991). A methanol-enriched anaerobic sediment consortium was reported to reductively dechlorinate PCE to TCE, 1,1-dichloroethene, VC, ethene and ethane (Skeen *et al.*, 1995). The absence of methanol or the addition of 2-bromoethanesulfonic acid, an inhibitor of methanogenesis, suppressed both methanogenic and dechlorination activity, suggesting that the dechlorination was strongly linked to methanogenesis. Another methanogenic sludge fed with methanol was reported to degrade PCE, TCE, DCE and VC (van Eekert *et al.*, 2001). It was also observed that the increase in the frequency of methanol additions improved the dechlorination, while H₂ supplementation had no effect. Furthermore, dechlorination by autoclaved sludge did not occur, implying that living microorganisms were responsible for dechlorination.

Anaerobic sludges are environments with high microbial diversity and they are a reservoir of microorganisms that are resistant to the presence of toxic compounds, such as chlorinated ones, and that are also able to degrade them. Each sludge, depending on its origin, can have a completely unique microbial composition. In our results we could observe that the microbial community composition is influenced by a combination of factors: inoculum source, substrate and other variants, such as the presence of a specific chlorinated compound. For instance, in cultures with DCE, after only one transfer we could observe that cultures from granular sludge had a complete different archaeal composition than the ones from suspended sludge. Furthermore, while at this point, for cultures from granular sludge, we could already observe that the substrate affected the archaeal composition, in cultures from suspended sludge, this was not visible yet. Moreover, after five transfers, the archaeal composition changed significantly. When looking to the 5th transfer of cultures incubated with the different chlorinated compounds, for the same substrate, cultures from granular sludge had similar archaeal composition. In contrast, cultures that originated from suspended sludge, with H₂/CO₂, had a different archaeal composition. The impact on microbial composition of different parameters, such as substrate concentration, C/N ratios, temperature or ammonia concentrations have been previously studied (Tay and Yan, 1996; Karakashev *et al.*, 2005; Lee *et al.*, 2009). For instance, in a study conducted in four lab-scale UASB reactors, fed with synthetic wastewater with different influent concentrations, it was observed that the substrate concentration had impact on the granules development, including granules' size and microbial composition (Tay and Yan, 1996). 1000 – 5000 mg COD/L influents gave origin to 2.5 – 3.4 mm granules dominated by *Methanosaeta*, while 10000 mg COD/L influents resulted in 0.54 mm granules dominated by *Methanosarcina* species (Tay and Yan, 1996). Karakashev *et al.* (2005) studied the impact of environmental conditions on methanogenic communities in 15 full-scale

biogas plants operating either with manure or sludge as feedstock. They observed clear differences between the reactors operating with manure and the ones operated with sludge; in the manure-operated reactors, the levels of ammonia and volatile fatty acids (VFAs) were high and the communities were dominated by *Methanosarcinaceae*, while in the reactors operated with sludge, the levels of ammonia and VFAs were lower and the main archaeal organisms found were *Methanosaetaceae*. Another study evaluated the impact of sulphate addition and temperature changes on methanogenic profiles of two hybrid reactors treating molasses (Pender *et al.*, 2004). At mesophilic conditions, in the presence or absence of sulphate, *Methanosaeta* sp. was predominant. At thermophilic conditions, in the absence of sulphate the dominant sequence isolated were closely related with *Methanocorpusculum parvum*, while in the presence of sulphate they were related with *Methanothermobacter thermoautotrophicum*. All these reported results are in agreement with our observations, showing that changes in environmental factors and cultivation parameters has great impact on the microbial communities.

OHRB, especially *Peptococcaceae* family members could be found in many of the enriched cultures and, in some of them, like G.D.M.1-5 and G.M.D.2-5, they constituted the majority of the bacterial population. Thermodynamic considerations predicted that hydrogenotrophic OHRB could outcompete methanogens in H₂-rich environments (Smidt and de Vos, 2004). Yet, OHRB need to be present in high abundance to lower the partial H₂ pressure and outcompete other hydrogenotrophic microorganisms present. When H₂ concentrations are high, they are probably outcompeted by other microorganisms (Middeldorp *et al.*, 1999; Smidt and de Vos, 2004). The cultures with the best PCE dechlorination, S.P.M.1-5 and S.P.M.2-5, presented *Methanobacterium* as the dominant archaea. *Methanobacterium* species were described to be involved in dechlorination, but they are not methylotrophic methanogens, which makes their presence in those enrichments unexpected.

Methanosarcina species could also be found in these enrichments. It is possible that either *Methanosarcina* or some bacterial species present in those cultures can convert methanol to H_2 that could be used by *Methanobacterium* to produce methane and *Peptococcaceae* family members to degrade PCE. Furthermore, it is known that OHRB depend on other community members, such as methanogens, to provide them with electron donors and growth factors due to their inability to perform *de novo* synthesis of some of these compounds, such as cobalamins (Atashgahi *et al.*, 2016). In fact, experimental data suggests that OHRB are often enriched in methanogenic and sulphate-reducing conditions (Atashgahi *et al.*, 2016). Some of our enriched cultures with methanol, such as G.P.M.1-5 and G.P.M.2-5, have their archaeal community dominated by *Methanomethylovorans* species (>90%), and they presented some of the best dechlorination performances for both chlorinated compounds. No information could be found regarding dechlorination assays with pure cultures of *Methanomethylovorans* species. *Methanomethylovorans* can be either involved directly in dechlorination, co-metabolically degrading the compounds, or indirectly by converting the methanol to H_2 which can be used by OHRB to perform reductive dechlorination. This last process was previously observed in a thermophilic reactor fed with methanol (Roest *et al.*, 2005). In that study, direct and indirect methanogenesis from methanol was observed and the results indicated that H_2 is an important intermediate in methanol conversion. Moreover, the most dominant archaea identified was closely related with *Methanomethylovorans hollandica*. Further assays with pure and co-cultures cultures of the dominant species present in our enrichments should be performed to understand the possible role of *Methanomethylovorans* in dechlorination.

The importance of cofactors, such as corrinoids or cofactor F_{430} , for reductive dechlorination has been shown in the past (Gantzer and Wackett, 1991; Holliger *et al.* 1992; van Eckert *et al.*, 1999). The cofactors contain in their structure metal ions,

such as Ni, Co or Fe, which can be in limiting concentrations in the medium. Metal supplementation could represent a good strategy to overcome this issue and increase dechlorination rates. Our results show a clear improvement of both, methanogenesis and dechlorination, when metals are supplemented to the medium. In DCE-enriched cultures, methane and VC production were enhanced by Fe and Ni supplementation, while Co had a negative effect on methane production and no effect in VC production. PCE degradation and methanogenesis were also enhanced by Fe supplementation, while Co had little or no effect. Fe supplementation has been reported to enhance the dechlorination by methanogens, most likely, by influencing the corrinoid and porphyrins activity (Andrews and Novak, 2001). Moreover, the effect of ferric iron on the dechlorination of TCE was evaluated (Wei and Finneran, 2011). The results in batch experiments showed that, in the presence of Fe(III)-NTA (nitrilotriacetic acid-Fe(III)), TCE degradation was faster. Furthermore, it was also observed that the presence of either Fe(III)-NTA or ferrihydrite helped to decrease the H_2 concentrations faster to a steady-state between 2 and 4 nM, which is considered to be within the optimal range of concentrations reported for mixed cultures degrading chlorinated compounds. Fe is present in many of the enzymes required for methanogenesis, which can explain its effect on this process (Glass and Orphan, 2012). Likewise, Ni is present in many methanogenic enzymes and cofactors, such as cofactor F_{430} . This cofactor was reported to be involved in the dechlorination of DCE (Holliger *et al.*, 1992), as well as polychlorinated ethenes and benzenes and PCE (Gantzer and Wackett, 1991). No other studies could be found where Ni was supplemented to enhance dechlorination, but our results show a beneficial effect resulting from such strategy. Yet, additional studies should be performed to clarify which enzymes or cofactors are affected by Ni supplementation. The little effect of Co amendment on methanogenesis and dechlorination was somewhat unexpected since this metal is required in the corrinoids structures. These

are fundamental cofactors for methanogenic enzymes (DiMarco, 1990), but they are also described to be involved in reductive dechlorination (Gantzer and Wackett, 1991). Yet, it might be that the nutritional requirements of the microorganism present in our sludge are fully fulfilled with the Co provided in the basal medium (0.5 μM). Another hypothesis is that Co supplementation alone is not enough. Methanol-coenzyme M methyltransferase, the enzyme involved in the first step of methylotrophic methanogenesis, requires Co and Zn as metal ions (Glass and Orphan, 2012). Further experiments with metal cocktails are required to confirm this hypothesis. Moreover, experiments with metal supplementation should be conducted in pure cultures of methanogens and OHRB, as well as in co-cultures of methanogens and OHRB. This would allow the application of techniques, as proteomics, to better understand which enzymes are affected by metal supplementation in both groups of microorganisms and if metal supplementation affects OHRB directly, by increasing the levels of some enzymes or cofactors, or indirectly by increasing the pool of methanogenic cofactors available for them to scavenge.

In summary, we enriched for DCE- and PCE degrading cultures at methanogenic conditions. Inoculum, substrate and chlorinated compound can be determinant factors affecting the microbial community composition. Methanol-enriched cultures presented the best dechlorination rates from all the conditions tested and the microbial composition seems to indicate that H_2 is an important intermediate in methanol conversion in those cultures. Moreover, we showed that metal supplementation can be a viable strategy to enhance dechlorination rates due to the involvement of several metalloproteins and cofactors. Yet, additional research, especially with pure cultures, is required to clarify the mechanisms involved.

Chapter 5

Nickel, cobalt and iron enhance co-metabolic 1-2-dichloroethene (DCE) dechlorination by *Methanosarcina barkeri*

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5.1. Abstract

Methanogenic cofactors are described to be involved in the co-metabolic reductive dechlorination of chlorinated compounds. These cofactors contain metal ions in their structure. For this reason, it was hypothesized that the supplementation of trace concentrations of metals could improve the co-metabolic dechlorination rates of DCE by pure cultures of *Methanosarcina barkeri*. Nickel, cobalt, and iron were supplemented to cultures growing on methanol. Our results showed that metal amendment can improve DCE dechlorination: 20 μM of Fe gave the best results increasing the vinyl chloride (VC) formation by more than 500%, while for 5.5 μM of Co and 5 μM of Ni improved VC formation up to 380%. Moreover, methane production was enhanced 5 μM Ni supplementation, but not by Co. Furthermore, we observed that cultures supplemented with Fe presented an enhancement of methane production during an initial stage of the assay, but after a certain time point, methane production stopped. The results also show that *M. barkeri* was able to reduce iron and that this inhibited methane production, while it triggered acetate production. These results show that metal supplementation is dependent on metal ion and concentration tested.

Keywords: co-metabolic dechlorination, methanogenesis, metals

5.2. Introduction

Chlorinated compounds from anthropogenic origin are commonly found in wastewaters (Yang *et al.*, 2005; Krzmarzick and Novak, 2014). These compounds are toxic and widely used as pesticides, fungicides, pharmaceutical agents, degreasing agents, cooling fluids, solvents for industry or flame retardants (van Eekert and Schraa, 2001; Krzmarzick and Novak, 2014). Highly chlorinated compounds, such as tetrachloroethene (PCE) or hexachlorobenzene, are resistant to aerobic degradation (Leys *et al.*, 2013). Under anaerobic conditions, chlorinated compounds can be reductively dehalogenated to chlorinated compounds with less chlorine atoms (Holliger *et al.*, 2004; Yang *et al.*, 2005; Atashgahi *et al.*, 2016). The first anaerobic microorganism described to perform reductive dechlorination in a process coupled to growth, was *Desulfomonile tiedjei* (Shelton and Tiedje, 1984). Nowadays, several bacteria, isolated from a wide range of environments, are described to be able to perform reductive dechlorination (Nijenhuis and Kuntze, 2016; Leys *et al.*, 2013). They can be classified as obligate or facultative organohalide-respiring bacteria (OHRB). Obligate OHRB have a restricted metabolism and can only use halogenated compounds as terminal electron acceptors, while facultative OHRB can use different electrons acceptors, such as nitrate, nitrite, Fe(III), Mn(IV), DMSO, As(V), sulphate or thiosulphate (Mayer-Blackwell *et al.*, 2016). Reductive dehalogenation can also take place as a co-metabolic process (Holliger and Schumacher, 1994). Methanogens are known to be able perform reductive dehalogenation of PCE or DCE as co-metabolic substrates. Several methanogens, such as *Methanosarcina barkeri*, *Methanococcus mazei* or *Methanothermobacter thermoautotrophicum* (former *Methanobacterium thermoautotrophicum*) are described to be able to dechlorinate DCE (Holliger, 1990), while *Methanosarcina thermophila* can also dechlorinate PCE (Middeldorp *et al.*, 1990).

Corrinoids, porphyrins and cofactors play an important role in co-metabolic dechlorination. It was observed that vitamin B₁₂, cofactor F₄₃₀ and hematin could catalyse the reductive dechlorination of polychlorinated ethenes and benzenes, but ferredoxins and azurin could not (Gantzer and Wackett, 1991). Vitamin B₁₂ and cofactor F₄₃₀ could also sequentially dechlorinate PCE to ethene, but hematin could only perform the dechlorination until vinyl chloride (VC). Jablonski and Ferry (1992) observed that the corrinoid-containing carbon monoxide dehydrogenase of *Methanosarcina thermophila* could degrade TCE to *cis*-, *trans*-, and 1,1-DCE, VC and ethene.

Most of these porphyrins, corrinoids and cofactors have metal, typically cobalt (Co), nickel (Ni) and iron (Fe), in their structure. These metals have a fundamental role in methanogens. For example, Ni and Fe are present in Ni-Fe hydrogenases. Ni is also present in cofactor F₄₃₀ and Fe is required for almost all enzymes involved in methanogenesis. Co is present in cobalamides that have an important role as methyl carriers in methanogenesis from methylated compounds. Moreover, cobalamides are also described to be intermediates between methyl-H₄MPT and coenzyme M (DiMarco *et al.*, 1990). Metal supplementation has proven to be a good strategy to improve several biological processes involving enzymes that require a metal ion, including methane production during anaerobic digestion of organic waste (Demirel and Scherer, 2011; Karlsson *et al.*, 2012; Gustavsson *et al.*, 2011; Gustavsson *et al.*, 2013; Schmidt *et al.*, 2014) and reductive dechlorination (Andrews and Novak, 2001).

The aim of this work was to access if the supplementation of metal ions Fe, Ni, and Co, which are required for methanogenic activity, but also for co-metabolic reduction of DCE, could improve DCE dechlorination by pure cultures of *M. barkeri*.

5.3. Materials and Methods

Source of culture and culture conditions

A pure active culture of *Methanosarcina barkeri* (DSM 800^T) was ordered from the German culture collection (DSMZ Braunschweig, Germany). Mineral basal medium was prepared according to the protocol previously described by Stams *et al.* (1993) and supplemented with 5 mg/L of yeast extract. 45 mL of mineral medium were dispensed into 120 mL serum bottles that were sealed with rubber stoppers and aluminium cramp caps. Bottles' headspace was flushed with N₂/CO₂ (80:20; % v/v; 1.5 atm). After sterilization of the medium, methanol was added to the bottles from a sterile stock solution to a final concentration of 125 mM. 1,2-dichloroethene (DCE) was added to a final concentration of 0.5 mM. Controls without DCE were also included. To test the effect of metals on the dechlorination of DCE, tests were performed with NiCl₂ (5, 10, 15 and 20 µM), CoCl₂ (5.5, 10.5, 15.5 and 20.5 µM), and FeCl₃ (22, 27, 32 and 37 µM). Metal concentration range was selected based on values described by others as reviewed by Paulo *et al.* (2015). Controls with DCE, but without metal addition were included. Bottles were inoculated with 2.5 mL of exponentially grown culture of *M. barkeri* and incubated at 30°C in the dark. The assays were performed in triplicate.

Analytical methods and calculations

Bottles' headspace was analysed for H₂ and CH₄ with a Compact GC^{4.0} (Interscience, Breda, The Netherlands) equipped with Carboxen 1010 column (Supleco, 3 m x 0.32 mm) followed by a Molsieve 5A column (Restek, 30 m x 0.32 mm), with argon as carrier gas at 0.8 mL min⁻¹, and a thermal conductivity detector (TCD). The temperature of the valve (injection) oven was kept at 60 °C, while column and detector were kept at 100 °C. The initial methane production rate was determined

for each test as the initial slope of the curve (as exemplified in Figure 5.1) obtained by plotting the measured methane in the triplicate assays.

Figure 5.1 - Example of methane production curve in a batch assay with 37 μM of Fe.

Concentrations of DCE and vinyl chloride (VC) were determined by analysing the bottles' headspace on a Varian GC-FID (CP-3800) (Thermo Scientific) equipped with a split-splitless injector followed by an RT-Q Bond column (Restek, 30 m x 0.32 mm). Gas carrier was helium at a flow of 2 mL/min. Temperature was set to 40°C for 1 min, followed by a temperature ramp up to 200°C in 4 min and additional 5 min at 200°C. Standards for chlorinated compounds were prepared by adding a known amount of chromatography-grade DCE and VC to a serum bottle with the same headspace to liquid ratio as the assay bottles.

Liquid samples were analysed for volatile fatty acids (VFA) and alcohols with an Acella HPLC (Thermo Scientific, Waltham, MA) equipped with a Varian Metacarb 67H column (Agilent, 300 x 6.5 mm) and a refractive index detector. Column was

kept at 45 °C and running with 0.01N of H₂SO₄ as eluent at a flowrate of 0.8 mL/min.

Fe (II) and Fe (III) were determined with the ferrozine method (Viollier *et al.*, 2000). In short, the ferrozine reagent reacts with the Fe²⁺ to form a stable magenta complex and the maximum absorbance is recorded at 562 nm. A reducing agent is then added to reduce all Fe³⁺ to Fe²⁺, given the total iron concentration in the sample. The absorbance is then recorded at the same wavelength. The concentration of Fe³⁺ is calculated between the difference of total iron minus the concentration of Fe²⁺. The calibration curve was built using FeSO₄ standards.

5.4. Results

Effect of metals and DCE on methane production from methanol

The effect of DCE towards methane production from methanol by *M. barkeri* was evaluated. Addition of 0.5 mM DCE to the cultures had a strong inhibitory effect on methane production; after 336 h of incubation methane production in assays with DCE was ~50% less than in the controls without DCE (Figure 5.2).

Several concentrations of Co, Fe or Ni were supplemented to pure cultures of *M. barkeri* incubated in the presence of DCE. Addition of Co resulted in lesser methane from methanol, compared to controls (Figure 5.2A). The lowest concentrations of Co tested (5.5 µM) caused a decrease of ~40% in the methane measured after 336 h of incubation, when compared with the same conditions without metal. Supplementation with higher Co concentrations had a more stringent inhibitory effect and, for 20.5 µM Co, ultimate methane production was only 70% of that in the control assay (Co-Ctr). The supplementation of 22 and 27 µM of Fe had a slight beneficial effect on the initial methane production rates, which increased from 0.0212 ± 0.005 mmol CH₄/h in the control with DCE to 0.0287 ± 0.009 mmol CH₄/h (Figure 5.2B). Furthermore, 32 µM and 37 µM of Fe increased the initial

methane production rate to 0.0336 ± 0.007 and 0.0395 ± 0.013 mmol CH₄/h, respectively. However, after 120h, in the presence of Fe, methane production seemed to stabilize in all conditions with this metal. Addition of Ni was the most effective strategy for increasing methane production rates and final methane concentrations. The supplementation of 5 and 10 μ M of Ni resulted in an increase in final methane production to values even higher than the ones from the control without DCE; methane production in these assays was 115-150% higher than in controls with DCE (Figure 5.2C). The beneficial effect of Ni on methane production rates was observed for added concentrations of up to 15 μ M. Addition of 20 μ M resulted in final higher methane production than in control with DCE, but no improvement was observed in the initial methane production rate.

Figure 5.2 - Methane production from the co-metabolism of methanol and DCE by *M. barkeri*. Assays were done with supplementation of different concentrations of Co (A), Fe (B) and Ni (C); curves corresponding to controls “without DCE” (red circles) and “with DCE and no metals” (blue circles) are also shown.

Figure 5.3 - Hydrogen (A, C, E) and acetate (B, D, F) production in the presence of Co (A, B), Fe (C, D) and Ni (E, F).

Figure 5.4 - Vinyl chloride production in the presence of Co (A), Fe (B) and Ni (C).

Effect of DCE and metals on hydrogen and acetate formation from methanol

In assays supplemented with Co, hydrogen production by *M. barkeri* occurred earlier in cultures containing DCE (192 h) than in the controls without DCE (355 h) (Figure 5.3A). Initial Co concentration affected the amount of hydrogen produced; higher amounts of hydrogen were formed with increasing Co concentrations (Figure 5.3A). Final acetate concentration was approx. 45% higher in assays with DCE than in controls without DCE, but no significant differences were obtained in the presence of different Co concentrations (Figure 5.3B). Supplementation of 22, 27 and 32 μM of Fe increased hydrogen production up to 60%, when compared to the control with DCE, whereas 37 μM of Fe led to an increase of 25% (Figure 5.3C). Fe amendment had a strong effect on acetate production, for all the initial Fe concentrations tested (Figure 5.3D). On the contrary to Co and Fe addition, supplementation of Ni did not stimulate hydrogen nor acetate production by *M. barkeri* from methanol (Figures 5.3E and 5.3F).

Effects of metals on the co-metabolic reduction of DCE

Co supplementation to cultures of *M. barkeri* in the presence of DCE improved DCE reduction to VC (Figure 5.4A). In the first 120 h of incubation, the effect of Co was similar for all the concentrations tested. However, after that, 5.5 and 10.5 μM had a stronger effect in the dehalogenation rate and final VC formation in these assays was approx. 250 and 380 % higher than in controls, respectively. In the case of Fe supplementation, 22, 27 and 32 μM of Fe had similar positive effects on the formation of VC (Figure 5.4B). 37 μM of Fe had even a stronger effect and could improve the VC formation in over 500% compared to control assays. The amendment of Ni improved VC formation since the beginning of the assay (Figure

5.4C). In the initial hours of assay, 5 μM of Ni increased VC formation up to 900%, but this effect was less stringent for the final VC formation. The Ni amendment effect was dependent on the metal concentration added and higher concentrations of metal had less or no effect than the lower concentrations. Moreover, even for 5 μM of added Ni, the VC formed is less than when 37 μM of Fe is supplemented.

Fe³⁺ and Fe²⁺ measurements

It is known that *M. barkeri* is able to reduce iron Fe³⁺ (van Bodegom *et al.*, 2004; Liu *et al.*, 2011). For this reason, the concentrations of Fe³⁺ and Fe²⁺ were measured at the end of the incubations with Fe (Table 5.1). Most of the Fe is in the form of Fe²⁺, indicating that there was reduction.

Table 5.1 – Concentrations of total Fe, Fe²⁺ and Fe³⁺ (μM) at the end of the incubations with Fe and in the control with DCE.

	Total Fe (μM)	Fe ²⁺ (μM)	Fe ³⁺ (μM)
Control DCE	2.24 $\pm$ 0.37	2.12 $\pm$ 0.29	0.12 $\pm$ 0.07
22 μM Fe	9.60 $\pm$ 1.20	8.01 $\pm$ 0.96	1.59 $\pm$ 0.24
27 μM Fe	12.76 $\pm$ 1.10	10.54 $\pm$ 0.88	2.22 $\pm$ 0.22
32 μM Fe	16.37 $\pm$ 0.81	13.43 $\pm$ 0.65	2.94 $\pm$ 0.16
37 μM Fe	24.15 $\pm$ 1.63	19.65 $\pm$ 1.31	4.50 $\pm$ 0.33

5.5. Discussion

Metal supplementation can be a good strategy to improve the performance of systems in which the reactions involve metal-depending enzymes and/or cofactors. Our results show that both methanogenesis from methanol, as well as the co-metabolic dechlorination of DCE by pure cultures of *M. barkeri* can be improved by metal amendment, but the effect depends on the metal ion and concentration. It was shown that the growth of *M. barkeri* is Co-dependent and that Ni stimulated

methanogenesis (Scherer and Sahm, 1981). Also, cells cultivated with methanol have three times more corrinoids than cells grown with acetate (Krzycki and Zeikus, 1980). In a previous study, supplementation of 45 μM of Co, Fe and Ni enhanced the formation of corrinoids, cofactor F₄₃₀ and cytochromes by *M. barkeri* cultures growing on methanol and about 70% of these compounds were excreted to the culture supernatant solution (Lin *et al.*, 1989). Metal supplementation to improve dehalogenation was previously studied (Andrews and Novak, 2001). Cells of *M. thermophila* were grown in the presence of Fe²⁺ and the rate of chloroform dehalogenation was improved. The authors hypothesised that Fe²⁺ may influence the dechlorination rate by influencing the porphyrin and corrinoid activity. Unfortunately, details of how metal supplementation affects co-metabolic dechlorination are not yet clear. In this work it was hypothesised that Co, Ni and Fe amendment could improve methane production from methanol and also the dechlorination of DCE. We observed that Co, Ni and Fe amendment can indeed help to improve DCE dechlorination: 20 μM of Fe gave the best results increasing the VC formation by more than 500%, while for 5.5 μM of Co and 5 μM of Ni improved VC formation up to 380%. These results are most likely due to increase in the levels of the cofactors that are involved in the co-metabolic dechlorination.

Methane production from methanol by *M. barkeri* was improved by all concentrations of Ni tested, when compared with the control with DCE. Yet, the improvement effect on methane production decreased as the metal concentration increased, which lead us to conclude that 5 μM of Ni is the optimal concentration required for both methanogenesis and reductive dechlorination. This shows the importance of Ni for the methanogenic pathway. It is also in agreement with other studies where similar concentrations of Ni were found to stimulate methanogenesis (Kida *et al.*, 2001; Gustavsson *et al.*, 2011; Evranos and Demirel, 2015). Moreover, the presence of the tested metal seems to play a role in directing the pathway in one direction. On the

other hand, Fe amendment affects the initial methane production rates, but had no effect for the rest of assay. It was observed an increase in acetate production from 2 mM, in the control, to 14-20 mM in the cultures supplemented with Fe. Hydrogen and acetate production started after 72 h of incubation, simultaneously with the halt in methane production. At the end of the incubation of these assays, most of the Fe in the bottles was in the form of Fe^{2+} . Inhibition of methane production due to iron reduction was studied in pure cultures of *Methanospirillum hungatei*, *M. barkeri* and *Methanosaeta concilii* (van Bodegom *et al.*, 2004). The presence of Fe^{3+} inhibited methane production in almost all conditions tested and *M. hungatei* was the most sensitive methanogen, being inhibited by 1 mM of Fe^{3+} . Moreover, *M. barkeri* growing on 25 mM of methanol presented a higher increase in Fe^{2+} concentrations than cultures growing with H_2/CO_2 . The relationship between methanogenesis and iron reduction in *M. barkeri* was further investigated (Liu *et al.*, 2011). In the presence of Fe^{3+} , inhibition of methanogenesis from methanol during the first 18 days was reported (Liu *et al.*, 2011). The reduction of Fe^{3+} is hypothesised to be one of the causes for the inhibition of methanogenesis due to a raise in the redox potential of the system. Moreover, electrons would go to Fe^{3+} reduction instead of to methane formation. However, in the same experiment, the authors observed that after 25 days, methanogenesis was stimulated by the presence of Fe^{3+} , which cannot be explained by changes in the redox potential. It is possible that at this point, the concentrations of Fe^{3+} were too low to accept electrons and to inhibit methanogenesis. Another hypothesis mentioned by the authors concerns the formation of intermediate substrates from methanol decomposition that might be more efficient in methanogenesis. Further studies revealed that *M. barkeri* can reduce iron and produce methane simultaneously, but that it can also switch from methanogenesis to iron reduction (Sivan *et al.*, 2016). Moreover, the authors also observed 80% less methane production in the presence of Fe^{3+} (Sivan *et al.*, 2016).

In the absence of H_2 , methanol is converted according to Equation 5.1. Three methyl groups are reduced to methane and the fourth methyl group is oxidized to CO_2 to produce the electrons needed for reduction of the other three methyl groups (Deppenmeier, 2002; Liu and Whitman, 2008).

Acetate production from methanol or H_2/CO_2 by *M. barkeri* was observed in the past; it was reported that *M. barkeri* could produce 30 to 75 μmol of acetate per mmol of CH_4 formed (Westermann *et al.*, 1989). Moreover, it was reported that *M. barkeri* growing on pyruvate produced acetate if bromoethanesulfonate (BES), an inhibitor of methyl-coenzyme M reductase, was added (Bock and Schönheit, 1995). The authors proposed that pyruvate would be oxidized to acetyl-CoA, which would then be converted to acetate via acetyl-phosphate, coupled to ATP production. From these results we hypothesized that the inhibition of methane production observed in our study can be due to iron reduction and that this inhibition triggered the production of acetate via acetyl-CoA oxidation. However, further tests need to be performed to confirm this hypothesis. For example, a proteomic analysis may indicate which enzymes are favoured by the presence of the Fe.

The addition of Co had a negative effect on methane production. Similar results were observed with the addition of 5 μM of Co to cultures of *M. barkeri* growing on methanol (Jiang, 2006). However, hydrogen production was enhanced by Co supplementation and acetate production also increased slightly. Furthermore, co-metabolic dechlorination was also enhanced by Co addition. In the methylotrophic pathway, Co is required for the first enzyme, methanol-coenzyme M methyltransferase and also for the $CH_3-H_4M(S)PT$ -coenzyme M methyltransferase, the first enzyme in the oxidative branch (Figure 5.5). Our results seem to indicate that Co amendment increases the levels of cofactors available, which leads to an

increase of the dechlorination rates. However, this is not reflected in an increase of methane production, which can be due to limitations of the levels of other metal ions, such as Ni, that are required for other enzymes of the pathway. Further tests with supplementation of a cocktail of metals would help to clarify this question.

Also important to notice is that only partial dechlorination is achieved; DCE is converted to VC, but no further dechlorination to ethane is observed. In a previous study inhibition of ethane formation by DCE was observed (Holliger *et al.*, 1990). VC is more toxic than the parent compound TCE. For this reason, achieving complete dechlorination is desirable. A co-culture with a microorganism able to perform complete dechlorination, such as *Dehalococcoides* species, could be a good solution for this issue. However, further studies are required to access the effect of metal supplementation in such co-culture.

In conclusion, our results show that metal ions can be a limiting factor in methanogenesis and dechlorination that require metal-depending enzymes or cofactors and that metal supplementation can lead to considerable improvement. However, the beneficial effect depends on the metal and concentration added, as well as which of the processes is intended to be enhanced. Yet, some questions remained open after this study that need to be further addressed to confirm some of the hypothesis that we made.

Chapter 6

Effect of sulphate on electromethanogenesis by an enriched microbial community

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6.1. Abstract

Microbial electrochemical cells (MECs) can be used to reduce CO₂ to methane (electromethanogenesis). The presence of alternative electron acceptors in the cathode, *e.g.* sulphate, can affect this process but this had not yet been studied. In this work, a stable MEC producing 0.33-0.42 mmol CH₄/day/L and with a coulombic efficiency (CE) of ~15% was disturbed by the injection of 15 mM of sodium sulphate. This addition caused a drop of 50% in methane production and the CE on the day that followed the addition. However, 3 days after the pulse, the system recovered to the initial methane and CE values. The sulphate concentration in the MEC rapidly (less than 20 min) decreased to ~2 mM, but no sulphide or elemental sulphur could be detected. An abiotic test suggested that sulphate removal could be biologically mediated. Microbial community analysis revealed the presence of sulphate-reducing species from the genera *Desulfomicrobium* and *Desulfovibrio* in the cathode. The archaeal community was dominated by species closely related to *Methanomethylovorans hollandica*, an obligatory methylotrophic methanogen, known for its ability to use methylsulphides, but unable to use H₂/CO₂. In further experiments it was observed that dimethylsulphide (up to 0.17 mM) transiently accumulated in the MEC upon sulphate addition. The archaeon related to *M. hollandica* seems to play a role in sulphur cycling in the MEC. This study indicates resilience of MEC and that sulphate only negatively affects electromethanogenesis for a short term.

Keywords: methanogenesis, microbial fuel cell, biocathode, sulphate removal

6.2. Introduction

Global emissions of CO₂ have steadily increased over the past decades and implications to global warming and climate change. In the COP21 summit held in Paris in 2015, 195 countries agreed to reduce CO₂ emissions by 50 % by the end of 2030. A major source of CO₂ emissions is the utilization of fossil fuels and sustainable alternatives to replace fossil fuels are required (Shah, 2014; Weiland, 2010). Currently much attention is paid to use CO₂ as a feedstock for production of renewable energy carriers and chemicals (Das and Wan Daud, 2014; Ampelli *et al.*, 2015; Passalacqua *et al.*, 2015; Claassens *et al.*, 2016). The capacity of archaea to produce methane from CO₂ in bioelectrochemical systems (BESs) was first investigated by Cheng *et al.* (2009). It was shown that a biofilm dominated by the methanogen *Methanobacterium palustre* could use electric current for direct biological reduction of CO₂ to methane. The process was termed electromethanogenesis (Cheng *et al.*, 2009). In that study, the biocathode was poised at a potential of -0.90 V vs. standard hydrogen electrode (SHE), a potential high enough to produce either biological or catalytic hydrogen (H₂). Later, Villano *et al.* (2010) proved that a cathode of a microbial electrolysis cell (MEC) inoculated with a hydrogenotrophic culture was able to reduce CO₂ to methane up to 0.055 ± 0.002 mmol d⁻¹.mg VSS⁻¹ at potentials lower than -0.65 V vs. SHE. *Methanothermobacter thermoautotrophicus* strain Δ H was also reported to produce methane in a BES using electrochemically-produced H₂ with a current to methane conversion efficiency of 20% (Hara *et al.*, 2013). Moreover, species closely related to *Methanobacterium palustre* and *Methanobacterium aarhusense* were identified in a biocathode producing methane (van Eerten *et al.*, 2013). *Methanosarcina* sp., *Methanobacterium* sp. and *Methanoculleus* sp. were also found in BESs without membranes (Sasaki *et al.*, 2011). These results show that different species of methanogens can be found in these systems.

Municipal wastewaters are estimated to store 3 to 10 times more energy than what is nowadays required for its treatment, which represents a great reservoir of energy (Liu and Cheng, 2014, Gude, 2016). Recovering that energy potential will make the treatment process more sustainable, and will contribute to some extent to the world's energy requirement. In the past years, MECs have gained attention for energy production, including biogas, from wastewater (Liu and Cheng, 2014). However, wastewaters contain contaminants such as sulphate, heavy metals or antibiotics, and some of these compounds can affect the performance of MECs. In a conventional anaerobic treatment, the presence of sulphate gives rise to two main issues: i) the competition between methanogens and sulphate-reducing bacteria (SRB) for common substrates, leading to a decrease in biogas production, and ii) the production of sulphide, which is toxic to some microorganisms and corrosive (Colleran *et al.*, 1995; Hulshoff Pol *et al.*, 1998). Biocatalysed sulphate reduction in MECs has been previously studied (Cordas *et al.*, 2008; Yu *et al.*, 2011; Su *et al.*, 2012; Coma *et al.*, 2013; Luo *et al.*, 2014). Different SRB species, such as *Desulfovibrio desulfuricans* and *Desulfobulbus propionicus*, were identified in the cathodic biofilms and were considered to be responsible for sulphate reduction (Cordas *et al.*, 2008; Su *et al.*, 2012). Sulphate reduction occurred at -400 mV (*vs.* Ag/AgCl) and sulphide accumulation was detected in the cathode compartment as a result of sulphate reduction and no methane production was detected (Su *et al.*, 2012). Metal sulphide precipitation was proposed as a mitigation method to avoid problems associated with the formation of sulphide (Coma *et al.*, 2013). However, this strategy would increase the costs of the process.

The main aim of this work was to study the effect of sulphate on electromethanogenesis. The general performance of the system was evaluated in terms of methane production, coulombic efficiency (CE) and current demand. Moreover, the microbial community was analysed to identify the key players.

6.3. Materials and Methods

Inoculum and enrichment conditions

Granular anaerobic sludge was obtained from a wastewater treatment plant treating food industry effluent (Delft, The Netherlands). A pre-inoculum was prepared by adding 0.5 mL of crushed granules to 45 mL of mineral medium containing 0.25 g/L of yeast extract. Medium was prepared as previously described by Stams *et al.* (1993) (Supplementary material, Table S2.1). Serum bottles (120 mL total volume, 45 mL liquid medium) were sealed with rubber stoppers and aluminium caps, and the headspace was pressurized with H₂/CO₂ (80:20; % (v/v); 1.5 bars). After inoculation, bottles were incubated at 30°C in the dark. When cultures were fully grown, 2.5 mL were transferred to fresh medium. Six transfers were performed to enrich for hydrogenotrophic microorganisms. The cathode compartment of the MEC was inoculated with 50 mL of the enriched culture.

Experimental setup and operation

A two chambers MEC was built, which consisted of a cathode and an anode placed at opposite sides of a single methacrylate rectangular chamber and separated by an anionic exchange membrane (Ami-7001, Membranes International Inc., Ringwood, NJ). The anode and cathode were filled with granular graphite (model 00514, diameter 1.5–5mm, EnViro-cell, Germany). The volume of each compartment was approximately 450 mL. Two thinner graphite electrodes (107 × 6 mm [anode] and 130 × 6mm [cathode], Sofacel, Sant Feliu de Llobregat, Spain) were connected to the potentiostat (BioLogic, Model VSP, Seyssinet-Pariset, France). Mineral medium (Supplementary material, Table S2.1) without resazurin and Na₂S was used to fill the chambers. An internal recirculation loop (150 L/d) was used in both compartments to ensure a proper mixing. The system was covered with aluminium foil throughout

the experiment to prevent phototrophic activity. Pure CO₂ (Praxair, Madrid, Spain) was flushed through the cathode compartment for 20 minutes every two weeks. The system was kept at 30 ± 2 °C. The gas produced in the cathode chamber was trapped in a methacrylate cylinder filled with water. The cathode potential was monitored with an Ag/AgCl reference electrode (+0.197 V vs. SHE, model RE-5B BASi, West Lafayette, IN). A voltage of -1V vs SHE was applied to the system to enable H₂ production. Once the cathode potential was poised, samples were taken after the system reached the steady state. Steady state conditions were assumed when current demand and voltage were maintained at constant values.

When methane production was stable, about 15 mM sulphate as Na₂SO₄ was added to the cathode chamber. Liquid and gas samples were taken from both compartments daily. The sulphate spike was repeated three times. A fourth spike of 25 mM of sulphate was performed at day 382. After 400 days of operation and five spikes of sulphate, the MEC was opened, and liquid and graphite granules sampled and used to inoculate a second MEC as a replicate, which was operated under similar experimental conditions.

Abiotic experimental setup

An abiotic MEC was built to confirm that sulphate reduction was a biologically mediated reaction at the cathode. The system was built and operated similarly as the biotic MEC, with the same proportion of graphite granules, but without inoculation. pH in the cathode was 9.03. About 15 mM of sulphate was injected in the cathode, and liquid samples were taken immediately before and after injection and after 1, 2 and 18 h. Identical abiotic MECs were built to test if dimethylsulphide (DMS), thiosulphate or polysulphides were formed by chemical reactions. In this case, 10 mM of Na₂S were added to the medium containing dissolved CO₂. Samples were

taken immediately before and after the spike at 0, 1, 2, 3, 6, 52, and 76 h. After that, samples were taken daily. Samples were stored in sealed vials with minimal headspace to avoid O₂ presence, and immediately frozen at -20°C to avoid DMS losses.

Analytical methods and calculations

Gas samples were analysed for H₂ and CH₄ with an Agilent technologies (Santa Clara, CA) 7890A GC system equipped with washed molecular sieve 5A and Porapak® Q columns and a thermal conductivity detector (TCD). Liquid samples were analysed with an Agilent technologies 7890A GC system equipped with an Agilent DB-FFAP nitroterephthalic-acid-modified polyethylene glycol (PEG) column for volatile fatty acids (VFA) and alcohols.

Sulphate was analysed with a Thermo Scientific (Waltham, MA) Dionex AS-AP Autosampler. Thiosulphate and sulphite were analysed with an ICS-2100 (Thermo Scientific) ion chromatography system equipped with an AS19 column. A colorimetric determination for sulphur and thiosulphate was performed according to the Sörbo method (Sörbo, 1957). Hydrogen sulphide was determined as described by Trüper and Schlegel (1964).

The DMS was analysed with an Acella HPLC (Thermo Scientific) equipped with general C18 column (250 x 4.6 mm, 5 µm particles), flow of 1 mL/min, eluent A: 0.1% formic acid; eluent B: 0.1% formic acid in acetonitrile. The detector used was a UV light detector set to 210 nm.

The efficiency of electron removal in MEC, generally referred as coulombic efficiency (CE), was calculated according to Equation 6.1 (Batlle-Vilanova *et al.*, 2015).

$$CE (\%) = \frac{8Fn_{CH_4}}{\int_{t_0}^{t_i} I(t)dt} \times 100 \quad \text{Equation 6.1}$$

Where 8 is the number of electrons consumed per mole of methane produced, F is the Faraday's constant (96485 C/mol), n_{CH_4} are the moles of methane produced between t_0 and t_i and $I(t)$ is the current (A) integrated over time (s).

Microbial community analysis

Liquid samples were collected from both the anode and cathode compartments at the end of the experiment. Samples of granular graphite, from both cathode and anode, and samples from the membrane were also taken. Phosphate-buffered saline solution (PBS) of pH 7.0 was added and the samples were placed in an ultra-sounds bath for 60s to release the biofilm attached to the graphite granules and membrane. The liquid was centrifuge at 4700 rpm for 10 min to collect the biomass. DNA was extracted using the FastDNA™ Spin Kit for soil DNA extraction (MPBio, Santa Clara, CA). Sequences were amplified by PCR using as forward primers the 25F (5'-CYG GTT GAT CCT GCC RG-3') for archaea and the 27F (5'-AGA GTT TGA TCC TGG CTC G-3') for bacteria, and universal reverse primer Uni1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Dojka *et al.*, 1998). PCR products were purified using GeneJetPCR Purification Kit from Thermo Fisher Scientific and used for ligation in the pGEM-T Easy vector system (Promega, Madison, WI). *Escherichia coli* XL-1 competent cells were used and transformed by thermal shock. Recombinant clones were grown on agar plates with Luria Broth medium containing IPTG 0.1M ampicillin 100 mg/mL and X-Gal 80 µg/mL and identify in a blue-white screening. The isolated clones were transferred to 0.5 mL of Luria Broth medium containing 100 mg/L of ampicillin and grown overnight at 37°C. 10 µL of culture was added to pre-ordered 96 wells plates and sent for sequencing with GATC Biotech AG

(Konstanz, Germany). Results were analysed using the online tool BLAST (<http://blast.ncbi.nlm.nih.gov/>) and compared with 16S ribosomal RNA sequences database for archaea and bacteria.

Scanning Electron Microscopy and Energy-dispersive X-ray spectroscopy

Scanning electron microscopy (SEM) analyses were performed at the end of the operational period. Graphite samples from the biotic MEC were extracted to compare the electrode surfaces. The samples were immersed in 2.5% (w/v) glutaraldehyde in a 0.1 M cacodylate buffer at pH 7.4 for a period of 4 h. The samples were washed and dehydrated in ethanol. Several washes were done with cacodylate buffer and with water. The fixed samples were dried with a critical-point drier (model K-850 CPD, Emitech, Lohmar, Germany) and sputter-coated with a 40 nm gold layer. The coated samples were examined with a SEM (model DSM-960; Zeiss, Oberkochen, Germany) at 20 kV and the images were captured digitally. Energy-dispersive X-ray spectroscopy (EDX) (QUANTAX Microanalysis System, Bruker, Billerica, MA) was also performed on the abiotic MEC graphite samples to identify the compounds deposited on the surface. Analysed samples were not pre-treated. Digital images of both SEM and EDX analysis were collected and processed by ESPRIT 1.9 BRUKER program (AXS Micro-analysis GmbH, Berlin, Germany).

6.4. Results

Sulphate removal in the microbial electromethanogenic system

The cathode compartment of a two-chambers MEC was inoculated and a potential of -1V *vs.* SHE was applied to ensure H₂ production either biologically or electrocatalytically (Batlle-Vilanova *et al.*, 2014). After an adaptation period of 7 days, methane production was detected, gradually increased and stabilized between 0.33 and 0.42 mmol CH₄/day.L after 24 days of operation, reaching a steady state

(data not shown). On day 38, 15 mM of sulphate were added to the cathode compartment. Sulphate spikes of 15 mM were repeated at days 70, 102 and 112. An additional spike of 25 mM of sulphate was done at day 382. Figure 6.1 depicts the methane production, current demand and CE during system operation, including the sulphate spikes at day 102 and 112 (A), and at day 382 (B).

A decrease in methane production and CE of ~50% was observed two days after the sulphate spikes (days 104 and 114). Nevertheless, the system was able to recover completely three days after the spikes (days 105 and 115). No significant variation in the current demand was observed, which was between 10.5 and 12.5 mA during the whole time of the MEC operation. Transient concentrations of hydrogen could be measured during the experimental period in the system with approximately 90 $\mu\text{mmol/L H}_2$ per day. The pH in the cathode was between 9.5 – 11 during the MEC operation, except after CO_2 flushing, when it dropped to around 7.5, increasing again in the following days (approximately 2-3 days to reach pH ~10). No changes in the pH were observed in the hours after the sulphate spikes.

When 25 mM of sulphate was injected, both methane production rate and CE decreased approximately 70 % one day after the spike (day 383). The system recovered and at day 384 both parameters were back to the values preceding the spike (0.25 mmol $\text{CH}_4/\text{day.L}$; 8.5%). The sulphate concentration decreased to 2 mM in approximately 20 min (Figure 6.2A). In the anode, the sulphate concentration (1.3 mM) remained constant throughout the experiment. During the MEC operation, sulphide was monitored after each sulphate spike. No sulphide could be detected in any of compartments during the experiment. Moreover, no other sulphur compounds (S_2O_3^- , SO_3^{2-}) could be detected in the analysed samples. EDX analysis of the most abundant elements was performed on the samples from the graphite granules of both cathode and anode of the biotic MEC. Sulphur was not detected in any of the samples, which indicated that no sulphur-containing

compound precipitated and accumulated on the granules and/or on the biofilms (Supplementary material, Table S2.2). An abiotic test was performed to determine if the sulphate removal could be electrochemically mediated. In the absence of biomass, sulphate concentration remained at 18 mM (Figure 6.2B), which suggested that microorganisms could be responsible for sulphate removal.

After approximately 400 days of operation, samples from the liquid phase, graphite granules, graphite electrodes and membrane samples of the MEC system were collected for further tests. The graphite granules samples were analysed by SEM (Figure 6.3). In the graphite granules collected from the cathode, rod shaped cells attached to the granules via extracellular polymeric substance EPS could be observed, in the biofilm. In contrast, no cells could be observed in the samples from the anode (Supplementary material, Figure S2.1).

Figure 6.1 - Methane production, current variation and coulombic efficiency (CE) in the cathode before and after the sulphate spikes of A) 15 mM and B) 25 mM. The dashed line indicates the time when sulphate was injected in the system.

Figure 6.2 –Sulphate concentrations in the cathode and anode of the MEC before and after a sulphate addition of 25 mM at time 0h (A) on day 382; and after a sulphate addition of 10 mM in biotic and abiotic conditions (B).

Figure 6.3 – Scanning electron microscopy images obtained from the analysis of the graphite granules from the MEC cathode compartment.

Microbial community characterization

The archaeal community analysis results for both cathode biofilms (graphite granules and membrane) were identical and almost all of the clones were identified as closely related to *Methanomethylovorans hollandica* with 99 to 100% identity (79/85 clones for the graphite granules biofilm sample and 87/87 for the membrane biofilm sample). Only a few clones (5/85 in the graphite granules biofilm) were identified as closely related with *Methasarcina* species. Furthermore, no DNA amplification product for archaea was detected in the suspended biomass of the cathode. The microbial community of the inoculum was analysed by high throughput sequencing and was composed *Methanocorpusculum* species (76.2 % of the whole

bacterial/archaeal community), which are hydrogenotrophic species (data not shown).

The predominant bacteria identified in the membrane biofilm, suspended biomass and graphite granules biofilm are summarized in Table 6.2. Most of the organisms in the biofilm on graphite granules were affiliated with *Desulfomicrobium* and *Hydrogenophaga* species. On the other hand, the microbial community of the suspended biomass was dominated by microorganisms closely related to *Azoarcus* and *Thiobacillus* species. The membrane biofilm was dominated by closely related species of *Azonexus*, *Pseudomonas* and *Thauera* species. Different species of *Hydrogenophaga* were also found in the sample from the membrane biofilm.

It was observed that i) sulphate removal could be biologically mediated, ii) no sulphide or sulphur compounds could be detected in any of the compartments, iii) sulphate reducers were detected in the microbial community analysis, and iv) the main archaea were *M. hollandica*, which is unable to use H_2/CO_2 , but is described as an obligatory methylotrophic methanogen. These results led to the formulation of a hypothesis where sulphate addition leads to the formation of methylsulphides, which can be used by *M. hollandica* to produce methane. To confirm the formulated hypothesis, additional experiments were implemented. An abiotic test was performed to determine if DMS formation was from a chemical reaction. In samples of the abiotic MEC no DMS could be detected. A second MEC system was built and inoculated with samples taken from the first one to repeat the sulphate reduction and to attempt to detect the presence of sulphate or other sulphur compounds. At day 28 of operation, after a period of 5 days where methane production stopped for a few days due to an unbalanced H_2 production and consumption (data not shown), sulphate was injected in the cathode. After 6h of the spike, 0.1 mM of DMS was measured and the concentration increased to 0.17 mM after 52h. Methane production also re-started and increased in the following days. Moreover, in the days

that followed the sulphate spike, transient concentrations of DMS (max. 0.1 mM) could be measured in some of the samples taken from the cathode. No sulphate or other sulphur compounds were detected in the samples.

Table 6.2 – Bacterial diversity in the graphite granules biofilm, liquid and membrane biofilm in the MEC cathode. The microorganisms indicated are the closest related ones to the clones. In parenthesis is indicated the number of clones found for each microorganism.

Cathode Biofilm (67)	Identity (%)	Cathode Liquid (63)	Identity (%)	Membrane (83)	Identity (%)
<i>Alistipes</i> (4)	84-85	<i>Alistipes</i> (1)	86	<i>Acetomicrobium</i> (1)	88
<i>Anaerobacterium</i> (1)	94	<i>Anaerocella</i> (1)	88	<i>Achromobacter</i> (2)	94-100
<i>Anaerocella</i> (1)	91	<i>Aquicola</i> (1)	93	<i>Alicyclophilus</i> (1)	99
<i>Azoarcus</i> (4)	93-98	<i>Azoarcus</i> (24)	93-97	<i>Alistipes</i> (1)	84
<i>Clostridium</i> (2)	85-92	<i>Azonexus</i> (3)	96-99	<i>Anaerocella</i> (4)	88-90
<i>Desulfomicrobium</i> (20)	98-100	<i>Catellibacterium</i> (2)	93	<i>Azoarcus</i> (1)	100
<i>Desulfovibrio</i> (2)	98-99	<i>Comamonas</i> (1)	96	<i>Azonexus</i> (9)	94-100
<i>Dethiosulfatibacter</i> (1)	90	<i>Desulfovibrio</i> (3)	99	<i>Bordetella</i> (1)	97
<i>Hydrogenophaga</i> (8)	88-99	<i>Desulfuribacillus</i> (3)	96-98	<i>Desulfomicrobium</i> (4)	98-99
<i>Malikia</i> (1)	94	<i>Flavobacterium</i> (1)	89	<i>Desulfovibrio</i> (2)	97-99
<i>Mariniphaga</i> (2)	88-93	<i>Fluviicola</i> (1)	84	<i>Desulfuribacillus</i> (1)	98
<i>Methylocystis</i> (2)	88-97	<i>Hydrogenophaga</i> (8)	96-99	<i>Diaphorobacter</i> (1)	100
<i>Prolixibacter</i> (1)	89	<i>Malikia</i> (1)	93	<i>Extensimonas</i> (3)	99
<i>Proteinivorax</i> (1)	93	<i>Prolixibacter</i> (1)	89	<i>Hydrogenophaga</i> (7)	98-99
<i>Pseudomonas</i> (3)	99-100	<i>Pseudomonas</i> (2)	99-100	<i>Limnohabitans</i> (1)	87
<i>Rhodobacter</i> (2)	98	<i>Sterolibacterium</i> (1)	94	<i>Mariniphaga</i> (1)	93
<i>Saccharofermentans</i> (1)	90	<i>Thauera</i> (3)	94-99	<i>Methylosinus</i> (1)	98
<i>Steroidobacter</i> (1)	89	<i>Thiobacillus</i> (6)	88-94	<i>Novosphingobium</i> (1)	97
<i>Thauera</i> (2)	98-99			<i>Paenibacillus</i> (1)	89
<i>Thiobacillus</i> (3)	98-99			<i>Pseudomonas</i> (9)	96-100
<i>Tissierella</i> (5)	92-96			<i>Rhizobium</i> (3)	95-99
				<i>Simplicispira</i> (2)	91-96
				<i>Stenotrophomonas</i> (2)	92-98
				<i>Sulfuricella</i> (1)	97
				<i>Sulfuritalea</i> (1)	94
				<i>Thauera</i> (15)	96-100
				<i>Thiobacillus</i> (7)	97-99
				<i>Thiomonas</i> (1)	99

6.5. Discussion

Sulphate is typically found in wastewater at concentrations of 1-6 g/L. At these concentrations, sulphate itself is not toxic for the microorganisms, but the product of its reduction, hydrogen sulphide, can be extremely toxic (Colleran *et al.* 1995). Furthermore, hydrogen conversion coupled to sulphate reduction is thermodynamically more favourable than methanogenesis, and SRB have a higher affinity for H_2 than methanogens (Muyzer and Stams, 2008). For these reasons, in conditions where sulphate is in excess, SRB are likely to outcompete methanogens. It could be hypothesized that, in an electrochemical system where both processes may occur simultaneously, methane production would be limited by the competition of SRB for H_2 and/or inhibited by the accumulated sulphide. Yet, in the system studied in this work, no sulphide accumulation or long-term inhibition was observed and the methane production was inhibited only for a short period of time (1-2 days) upon sulphate addition. Furthermore, sulphate was removed from the system in less than 20 minutes. Considering the potential applied to the system, sulphate conversion could be either chemically or biologically mediated. The results showed that sulphate conversion was biologically mediated, although the culture used as inoculum was enriched in the absence of sulphate. Sulphate reduction using mixed cultures in MEC cathodes was previously studied (Su *et al.* 2012; Coma *et al.*, 2013). In one of these studies, it was reported that sulphate concentrations decreased from 2 mmol/L to 0.4 mmol/L in 10 days (Su *et al.* 2012). This is a considerably slower rate than the sulphate conversion rate registered in our study. Our microbial community analysis revealed the presence of bacteria known to reduce sulphate, such as *Desulfomicrobium* (Copeland *et al.*, 2009; Krumholz *et al.*, 1999) and *Desulfovibrio* species. These microorganisms are present in the suspended biomass and in biofilms (membrane and graphite granules), but they are predominant in the biofilm attached to the graphite granules. In previous studies with BES, biologically

mediated sulphate reduction was attributed to microorganisms closely related to *Desulfobulbus propionicus* and was reported to occur at potentials of -0.4 V (Ag/AgCl) (Su *et al.* 2012). *Desulfomicrobium* sp. was reported to be able to reduced ~ 10 mM of sulphate in ~ 50 h using lactate as substrate (Azabou *et al.*, 2007). Furthermore, *Desulfovibrio desulfuricans* growing with lactate reduced ~ 41 mM of sulphate in approximately 78h in a microbial fuel cell (Cooney *et al.*, 1996). Both *Desulfovibrio vulgaris* and another *Desulfovibrio* sp. were able to reduced 10 mM of sulphate in approximately 7 days when tested in batch conditions (Cabrera *et al.*, 2006). These rates are much lower than the ones we observed, which might indicate that sulphate reduction could only account for a small part of the total sulphate removal in our system. At the given pH in the system, sulphate can precipitate in the form of CaSO_4 . However, the concentration of available Ca^{2+} is only ~ 1 mM, which cannot account for the removal of all sulphate that was added. Sulphate can also react with Mg^{2+} , but this would account for 0.5 mM of the total sulphate added. Another hypothesis is that sulphate could adsorb to the graphite. Yet, this generally requires an activation of the carbon, as for example with a positively charged polypyrrole (Hong *et al.*, 2014). Sulphate reduction in electrochemical systems was reported to lead to sulphide accumulation in the cathode compartment (Su *et al.* 2012; Coma *et al.*, 2013). Likewise, in studies regarding autotrophic sulphate reduction, sulphide accumulation was also observed in the cathode compartment (Luo *et al.*, 2014; Pozo *et al.*, 2015; 2016). At the given pH values found in the cathode of our system (10.5-11), most of the hydrogen sulphide should be in the form of HS^- and mainly in the liquid phase (Lens *et al.*, 1998; Hulshoff Pol *et al.*, 1998). Yet, in our system no sulphide could be detected in any of the compartments. Oxidation of sulphide to elemental sulphur occurs at potentials higher than -0.274V (SHE) (Rabaey *et al.*, 2006). Sulphur can then be further oxidized to less toxic compounds, such as polysulphides, thiosulphate and sulphate (Rabaey *et al.*, 2006). In most studies

regarding sulphide oxidation, the process occurred in the anode and elemental sulphur was detected (Ateya *et al.*, 2005; Rabaey *et al.*, 2006; Dutta, *et al.*, 2008). In our system no precipitated sulphur compounds could be detected. Studies of the fluid dynamics in BES showed that using graphite granules present a laminar flow and precipitation could occur in void volumes (Vilà-Rovira *et al.*, 2015). Our samples were collected in the middle of the compartment and, thus, the precipitation occurring in the system could have been overlooked, which could account for some of the sulphate removal observed.

Our microbial community analysis of the cathode suspended biomass revealed also the presence of bacteria closely related to *Thiobacillus*. *Thiobacillus* species are able to oxidized sulphide and thiosulphate (Townshend and White, 1960; Vlasceanu *et al.*, 1997) with oxygen (see below). Additionally, the archaeal community analysis revealed a few clones closely related to *Methanosarcina*, while the majority of the clones were closely related to *M. hollandica*. This microorganism was first isolated from fresh water sediments (Lomans *et al.* 1999). It uses methanol, dimethyl sulphide (DMS), methylamines and methanethiol as substrates, but not H_2/CO_2 . It is considered to be an obligatory methylotrophic methanogen. The microbial community analysis provided crucial information that combined with the results about sulphate/sulphide, led to the formulation of a hypothesis for biological and chemical reactions that could be taken place in the system in which thiosulphate and dimethylsulphide are important intermediate compounds (Figure 6.4).

Figure 6.4 – Schematic representation of the proposed reactions occurring inside the MEC system.

Before sulphate addition to the system, H_2O was split in the anode at the given potentials, providing the required electrons for the conversion of protons to H_2 in the cathode. The conversion of H_2 could be either biologically or chemically mediated. Subsequently, the H_2 , together with CO_2 dissolved in the media, could be further converted to CH_4 by *Methanocorpusculum* species, which were abundant during this stage of operation. The addition of sulphate introduced a new dynamic to the system and led to changes in the microbial community. In this second stage of operation, H_2 could be also scavenged by SRB species (*Desulfomicrobium* and *Desulfovibrio*), which produced S^{2-} as a result of sulphate reduction. The outcome of the competition between SRB and methanogens probably dictated the decrease in the abundance of *Methanocorpusculum* species and the disappearance of other hydrogenotrophic methanogens present in the initial enrichment, while SRB species became more abundant. The produced S^{2-} could be further oxidized to thiosulphate.

Formation of thiosulphate implies the presence of oxygen, but it has been previously observed that oxygen can diffuse from the anode to cathode causing parallel reactions that affect the performance of the MEC (van Eerten-Jansen *et al.*, 2012). Both thiosulphate and S^{2-} as well as hydrogen could be oxidized by *T. thioparus*. Moreover, S^{2-} could also be converted to DMS, which *M. hollandica* could convert to CH_4 . The presence of other species, such as *Azoarcus* and *Hydrogenophaga*, was detected in the system, but their roles in the system remained unclear. In particular, *Hydrogenophaga* species are known to use H_2 as electron donor. They were previously found in biocathodes producing H_2 , but their role in the process is not clear (van Eerten-Jansen *et al.*, 2013; Croese *et al.*, 2014). Some of the species detected, like *Azoarcus*, *Hydrogenophaga*, *Pseudomonas* or *Thaurea* are known to use oxygen as electron acceptor, which decreased the efficiency of the MECs. This also indicates that oxygen could diffuse from the anode (aerobic) to the cathode, also explaining why most of those species were found in the membrane biofilm. Moreover, the presence of oxygen might lead to parallel reactions, besides sulphate reduction and methane formation, leading to decreases in the CE.

Further abiotic tests showed that the formation of thiosulphate or DMS required the presence of microorganisms. Moreover, transient concentrations of DMS could be measured in the second MEC. This result confirmed the hypothesis that the reactions involved in DMS formation required the presence of microorganisms and explained the presence of *M. hollandica*. The complexity of the reactions and microbiology of the system could also explain the low rates of methane formation observed when compared with other similar MEC system (Villano *et al.*, 2010; Jiang *et al.*, 2013). Furthermore, the high pH in the cathode can also be a reason for the low methane production as methanogenesis should be inhibited at such pH levels; *M. hollandica* optimal pH range is between 6 and 8 (Lomans *et al.*, 1999).

In resume, we could observe a fast sulphate removal from the system that had limited impact on methane production. Our observations indicate that a part of the sulphate removal can be explained by microbial activity. Yet, the rate of removal is too fast to be explained by biological reduction of sulphate. Precipitation and adsorption can account for a small part of the removal observed, but not more than 1.5 mM. Several different methods were applied to measure any sulphur compounds that could be formed in the system, but without success. However, some of the sulphur compounds that can be formed are extremely reactive, volatile and for that, difficult to measure. Likely, we are missing crucial information that would allow us to make the complete sulphur balance and fully explain the fast sulphate removal observe. Further testing, for example improving sample collection and preservation to avoid evaporation of volatile compounds, should be pursued.

6.6. Conclusions

In this work we explored the resilience of an electromethanogenic system to high sulphate spikes. After the several spikes of sulphate that were performed, methane production was affected at short-time. The most intriguing question that raised was the fate of sulphate in the system; sulphate was rapidly removed, but no accumulation of sulphide in the cathode or of elemental sulphur in the anode was observed. The results obtained indicated that there was an adaptation of microbial population after the sulphate additions. The microbial community was formed by different microorganisms that are reported to be able to utilize sulphide, thiosulphate and DMS and to produce CH_4 . These results emphasized the importance of characterizing the reactor microbiome to understand the reactions that can be taking place.

Chapter 7

General Discussion

7.1 Introduction

The first anaerobic digestion (AD) plant was built in India in the 19th century, but it is from the 1970s onwards that anaerobic processes saw an increase in use for treating domestic and industrial wastes and wastewaters (Abbasi *et al.*, 2012). A considerable amount of research, both on applied and microbiological aspects of AD, have been done since then. Yet, several aspects, mainly considering the complex interactions that occur between microorganisms and other biotic and abiotic factors, are still not completely unravelled and there is still potential to improve the process further. In particular, methanogenesis is considered to be a crucial step in anaerobic processes; the failure of this last step in the anaerobic digestion chain leads to incomplete degradation of organic matter and no biogas formation. Methanogens, the key microorganisms in this step, are highly sensitive to changes in environmental conditions, such as temperature or pH, or to the presence of contaminants. The subjects studied in this thesis, are mainly concerned with the effects of heavy metals and sulphate on anaerobic processes. These are two common contaminants in wastewaters that may bring problems to AD processes. For example, when high concentrations of sulphate enter in AD systems it can lead to: i) competition between sulphate-reducing bacteria and other AD trophic groups, including methanogens, for common substrates; and ii) toxicity issues caused by the sulphide that generates from sulphate reduction (Chen *et al.*, 2008). On the positive side, sulphate helps to lower the potential which is essential in the AD reactors (Deublein and Steinhauser, 2010). Moreover, when high concentrations of heavy metals are present, production of sulphide can be a useful method for metal detoxification (metal precipitation in the form of metal sulphides). It is known that, at high concentrations, heavy metals are toxic for microorganisms, disrupting enzyme function and structure (Colussi *et al.*, 2009; Chen *et al.*, 2008). Nevertheless, low concentrations of metals are need for life, they are required for microbial growth

and microbial activity because they are present in enzymes and cofactors involved in electron transport chains. Some microorganisms can also use specific metals as electron donors or as external final electron acceptors.

In this thesis, we aimed to understand the toxic effect of sulphate and heavy metals towards methanogenesis, but also to explore the circumstances in which they may have potential benefits. A considerable part of this thesis deals with metal supplementation and how this could improve specific anaerobic processes.

7.2 Stimulation of anaerobic processes by heavy metals

Heavy metals are crucial for microorganisms; they are present as electron acceptors or donors in electron transport chains and in the structure of many enzymes and cofactors (Mudhoo and Kumar, 2013; Lemire *et al.*, 2013). As it was explained in **Chapter 2**, heavy metal supplementation in trace concentrations was reported to enhance biogas production in AD systems. Metal supplementation has been previously tested by others, and there is an array of data on e.g. the effect of individual metals or of varied cocktails of metals, the effect of metal concentration, and the effect of using different metal speciation forms (**Chapter 2**). Co, Ni and Fe are particularly important in methanogenesis because they are required for activity of several methanogenic enzymes and cofactors, such as Ni-Fe hydrogenases, cofactor F430 and cobalamides (DiMarco *et al.*, 1990). The effect of supplementation of Ni and Co on the methanogenic activity of anaerobic sludge was studied in **Chapter 3**. The supplementation resulted in little or no effect on methane production. The results observed contrasted with the results observed in other studies, in which metal supplementation enhanced AD (Demirel and Scherer, 2011; Karlsson *et al.*, 2012; Gustavsson *et al.*, 2011; Schmidt *et al.*, 2014; Abdelsalam *et al.*, 2016; Pinto-Ibieta *et al.*, 2016). However, when metals (Ni, Co, and Fe) were supplemented to enrichment cultures (**Chapter 4**) and pure cultures of *Methanosarcina barkeri*

(**Chapter 5**) converting 1,2-dichloroethene (DCE), an improvement in methanogenesis, or/and in reductive dechlorination of DCE, could be observed. Yet, the beneficial effect was dependent on the type of metal and the concentration added. Supplementation of Co had no beneficial effect on methanogenesis in any of the experiments performed; on the contrary, it inhibited methanogenesis in most conditions. However, it enhanced the dechlorination of 1,2-dichloroethene (DCE) (**Chapter 5**) and tetrachloroethene (PCE) (**Chapter 4**). The other metals tested, Ni and Fe, had a more stringent effect on reductive dechlorination, compared to experiments with Co supplementation; besides, supplementation of Ni and Fe also enhanced methanogenesis (**Chapters 4 and 5**). To illustrate this, in the assays with PCE-degrading enrichment cultures, trichloroethene (TCE) formation was 10x higher in the presence of Fe than in the control. Furthermore, vinyl chloride (VC) formation started ~400 h earlier in the presence of Ni or Fe than with Co or without metal (**Chapter 4**). Some methylotrophic methanogens are able to co-metabolise DCE, such as the case of *M. barkeri*, but some of the enzymes required for reductive dechlorination and for methanogenesis have different metal requirements: for example, reductive dechlorination requires vitamin B₁₂ (requiring Co), while methanogenesis involves [Ni-Fe] hydrogenases (requiring Ni and Fe) and methanol-coenzyme M methyltransferase that requires (requiring Co and Zn) (Glass and Orphan, 2012). For methanogens in general, metal requirements differ depending on the specific methanogenic pathway used because methylotrophic, hydrogenotrophic and acetoclastic pathways entail some different enzymes (Glass and Orphan, 2012). Supplementation of a single metal ion might be insufficient to suppress the requirements of the biological process in question. Moreover, methanogens can adjust their metal requirements under stress conditions, such as metal limitation conditions. For example, the hydrogenase Frh, which reduces coenzyme F₄₂₀, contains a [Ni-Fe] active site and four Fe₄S₄ clusters. Under Ni

limitation conditions, some methanogens without cytochromes replace Frh by Hmd, a Ni-free hydrogenase, reducing Ni requirements (Afting *et al.*, 1998). Nutrient-rich environments, such as anaerobic sludges, may have less requirements for metal supplementation, as many of these sludges have metals adsorbed in their matrices (van Hullebusch *et al.*, 2003; Zandvoort *et al.*, 2006). For this reason, metal supplementation to anaerobic digesters might bring no benefit (**Chapter 3**). However, in conditions where the metal stocks are depleted, such as long term incubations or enrichment procedures, metal supplementation can help to improve the methane production. This was observed in **Chapter 4**, where metal supplementation to enriched cultures had a positive effect on methane production. On the other hand, interspecies transfer of metabolites, including enzymes and cofactors, might lead to indirect benefits of metal supplementation. For instance, it is known that organohalide-respiring bacteria (OHRB) scavenge cofactors from sulphate-reducing bacteria (SRB) and methanogens (Atashgahi *et al.*, 2016). It can be hypothesized that metal supplementation would lead to an increase of the methanogenic cofactors levels, which could be transferred to OHRB, indirectly affecting the dechlorination rates. Yet, this hypothesis requires further testing using, for instance, assays with pure and co-cultures of OHRB and methanogens.

In summary, metal supplementation represents a promising strategy to enhance biological reactions dependent on metalloproteins and/or metal-containing cofactors. However, the results shown in this thesis indicate that there is no general rule. Each case should be individually evaluated and tests are required to determine metal requirements of the system/microorganisms in question.

7.3 Inhibition of methanogenic activity by heavy metals

Above certain concentrations, heavy metals become toxic to microorganisms, disrupting enzyme activity and structure (Colussi *et al.* 2009; Chen *et al.* 2008). Several studies were dedicated to understanding the effects of heavy metals towards AD. However, the comparison between these studies is difficult due to the differences in the experimental conditions used (**Chapter 2**). In **Chapter 3** the inhibitory concentration of Ni and Co towards an anaerobic granular sludge was determined. The results obtained for Ni are in the range of inhibitory concentrations (0.5 – 52.3 mM) that are reported in literature, and reviewed in **Chapter 2**. However, the concentrations found for Co are much higher than the 16.1 mM reported in literature (Bhattacharya *et al.*, 1995). Moreover, the sensibility of hydrogenotrophic and acetoclastic methanogens to each metal was different. Metal resistance mechanisms are well studied in bacteria and can comprise different strategies, such as biomethylation, active efflux, excretion of precipitating or chelating agents, such as sulphide, formation of inclusion bodies or reduction of the metal ion to a less toxic form. However, these mechanisms are not fully understood in archaea yet (Maezato and Blum, 2012). In the case of Ni and Co, the most likely resistance mechanism is active efflux. An analysis of the genome of four model methanogens, *Methanosarcina barkeri*, *Methanosaeta concilli*, *Methanolinea* sp. and *Methanobacterium formicicum* was performed in search for differences that could account for the differences observed between hydrogenotrophic and acetoclastic methanogens to the presence of high metal concentrations (**Chapter 3**). The genomes were searched for presence of genes related with Ni/Co transport as well as with the nickel resistance system RcnA. However, the results of such analysis were inconclusive as no relevant differences could be observed in the distribution of these systems in the studied methanogens. The knowledge available on metal resistance mechanisms in archaea is limited and further research should be conducted

in this topic. For example, differential proteomic studies using pure cultures of model methanogens incubated with different metal ions could reveal new transport and/or metal-resistance systems. That information could then be used to update the genome annotations and to perform genome comparison to analyse the distribution of such systems in other microorganisms.

Operational parameters, such as pH or temperature, affect metal toxicity. In anaerobic systems applied to wastewater treatment a great variety of parameters is applied, depending on the type of wastewater that is being treated, influent quality and temperature, effluent quality requirements, and other process characteristics, such as the type of reactor. Furthermore, microorganisms have different tolerance limits towards each metal and the fact that they are isolated or in mixed cultures and that they can adopt different organizational structures, such as anaerobic granules, can also affect their metal tolerance. All these considerations lead to conclude that each anaerobic system is unique and, as a consequence, its response to metal toxicity will be also unique. Moreover, a unique inhibitory concentration for each metal cannot be found; it will be always a range of concentrations that will depend on the conditions applied to the system and microbial community present. Further research with pure cultures, especially regarding the metal resistance systems in methanogens will, possibly, bring further answers in this topic and it will help to optimise metal detoxification strategies.

7.4 Effect of sulphate and/or sulphide in methanogenic systems

Sulphate is not toxic by itself, but the product of its reduction, sulphide, can be highly toxic to microorganisms. Similar to what was observed for heavy metals, the great variety of experimental conditions resulted in a wide range of inhibitory concentrations (**Chapter 2**). Yet, the presence of sulphide in an anaerobic system may not always represent a problem. In metal-rich environments, sulphide can be an

advantage since it reacts with metals ions, forming insoluble metal-sulphides, alleviating the toxic effect of metals (Hammack and Edenborn, 1992; Zayed and Winter, 2000). As it was shown in **Chapter 3**, this strategy can work for metal detoxification in methanogenic sludges, restoring methane production up to levels similar to the ones observed in the absence of metal. Yet, if the sulphide is from biological origin, i.e. the product resulting from microbial sulphate reduction, the competition between sulphate reducing bacteria (SBR) and methanogens should be taken into account. Once more, each system needs to be evaluated individually. The operational parameters, like pH or temperature, will influence the microbial community, metal speciation form, the solubility of sulphide and all these factors will affect the outcome of the competition between SBR and methanogens and the success of sulphide as detoxification method.

In **Chapter 6**, the effects of sulphate in an electromethanogenic system were studied. Bioelectrochemical systems (BES) are promising technologies for wastewater treatment (Li *et al.*, 2014; Pandey *et al.*, 2016). Their application for methane production has been the focus of several recent studies (Villano *et al.*, 2010; 2011; van Eerten-Jansen *et al.*, 2012; Jiang *et al.*, 2013). Moreover, sulphate reduction was also studied in these type of systems (Cordas *et al.*, 2008; Coma *et al.*, 2013), but the effects of sulphate as contaminant in a methane-producing system were never analysed before. As it was previously mentioned, sulphate leads to competition for substrate and toxicity issues related with sulphide production. For those reasons, it could be expect that the addition of sulphate to a BES producing methane would affect methane production permanently. However, in the system studied in this thesis methane production was inhibited, but only for a short period (2-3 days). Moreover, a fast sulphate removal was observed (less than 20 min), which could only partially be explained by microbial activity, as SRB could be identified as well as closely related species to *Thiobacillus thioparus* (known

thiosulphate and sulphide oxidisers). Also, transient concentrations of dimethylsulphide could be measured during the operation of the system after sulphate addition. Before sulphate addition, the archaeal community was dominated by species closely related to *Methanocorpusculum*. After sulphate spikes, *Methanomethylovorans hollandica* became the dominant archaeon. This microorganism is a methylotrophic methanogen, able to use methanethiol, methanol and dimethylsulphide to produce methane, and this was the first time this microorganism was detected in a BES. Although sulphate removal was too fast to be only explained by microbial activity, these results demonstrate how microbial communities are able to evolve to new operational conditions and perform even with unfavourable conditions, such as the high pH (~10) that should have inhibited methanogenesis.

7.5 Importance of microbial community analysis

Microbial community analysis can be a key tool to better understand conversions taking place in a certain biological system. In particular, in wastewater treatment, knowing the microbial composition and understanding the functions of the different species allows a better control of the biological processes (Narihiro and Sekiguchi, 2007; Hu *et al.*, 2012; Shchegolkova *et al.*, 2016). In **Chapter 3** the microbial community analysis not only gave insights in who were the main key players in that specific sludge, but also helped to understand which populations were more affected by the presence of heavy metals. Furthermore, in **Chapter 4** the microbial community analysis allowed to observe the evolution of the communities when enriched in the presence of specific conditions and to observe the differences between distinct conditions tested, such as different substrates or chlorinated compounds. The results obtained can also be used to determine if further experimental work towards isolation of new microorganisms should be performed. In the case of organohalide-respiring bacteria, most of the genera detected in the

enriched cultures were reported as unclassified, which might indicate that new species are present that have the ability to perform reductive dechlorination. In this case, further work is recommended to try to isolate and characterize them. The microbial community analysis in the work performed in **Chapter 6** helped to understand the process and to understand the reactions taking place inside the microbial fuel cell. Without knowing the microbial community, many of the questions that were raised during the experiment would have remained unanswered. These results showed that the analysis of the microbial communities can help to better understand how the systems are affected by different parameters, such as substrates, or how they react to disturbances, such as the presence of contaminants. Moreover, it helps to create a more complete picture of the reactions that can be possibly occur in a system and to design new experiments to confirm the hypotheses formed.

7.6 Concluding remarks and future perspectives

From the review work in **Chapter 2** it became clear that further research regarding the inhibitory effect of heavy metals towards anaerobic processes needed to be performed. The range of metals tested is mainly limited to Cu, Ni and Zn, while other metals, such as Co, are little studied. Furthermore, few studies analysed the changes that high concentrations of metals induce in the microbial communities. When it comes to microbial communities' analysis, DNA-based technologies allow to overcome some of the limitations of culture-dependent technologies. For example, artificial culturing conditions normally fail to fully recreate the natural environmental conditions that would allow the growth of all the microorganisms present, acting as a selective process. Moreover, other methods, such as the ones involving probes design for specific organisms imply previous knowledge of closely related species. The complementary use of both types of technologies, culture-

dependent and culture-independent, allows to have a more complete picture of the main players in a certain system. The application of such technologies to the study of the impact of contaminants in anaerobic systems treating wastewater can increase the knowledge about the response of the systems to different disturbances. For example, it was demonstrated in this thesis that hydrogenotrophic and acetoclastic methanogenesis can have a different response to the same metal concentration. Similar studies should be considered, not only to other metals, but also for other contaminants. Microbial resistance mechanisms are well understood for bacteria whereas as for archaea they are still not well studied. Studies with pure cultures, combining laboratory experiments with genome analysis, could help discover new metal resistance systems and verify their distribution in other archaea. For example, proteomics studies in cultures in the presence of high metal concentration could allow to observe an increase in the levels of certain enzymes involved in metal resistance mechanisms. In resume, future research should consider to extend the range of metals and concentrations tested, understand how the microbial communities are affected by these different combinations and search for still unknown metal resistance systems. The combination of all that knowledge would allow a better understanding of AD systems response to high metals concentrations, which can be applied to predict issues and improve the systems operation in real case scenarios.

Heavy metal supplementation represents a promising strategy to enhance the performance of several biological reactions and should be further investigated. In particular, heavy metal supplementation can be a useful strategy to enhance reductive dechlorination, a relatively slow process. Also, high-throughput technologies applied to fields like proteomics could help to better understand the effect of metal supplementation and which enzymes/pathways are affected by metal supplementation.

To finalize, emerging technologies, such as BES, have been made promising advances in the past years and their application to wastewater systems might happen in a near future. Yet, more research is needed to overcome the limitations of these technologies, such as the high costs of materials, decrease energy losses and still low methane production rates. Furthermore, wastewaters have a complex composition of compounds, many of them can interfere with the processes and performance of these bioelectrochemical systems. Research on the impact of contaminants in the performance of BES performing defined processes, such as methane formation, is still very limited and needs to be considered and investigated.

Supplementary material

Supplementary material Chapter 3

Table S1.1 – Metal, sulphate and sulphide conditions.

Short name	Metal Conc.	Sulphate Conc.	Sulphide Conc.	Sample sent to sequentiation
No	-	-	-	X
Ni-L-2	2 μ M Ni	-	-	-
Ni-L-4	4 μ M Ni	-	-	X
Ni-L-8	8 μ M Ni	-	-	-
Co-L-5	5 μ M Co	-	-	-
Co-L-10	10 μ M Co	-	-	X
Co-L-25	25 μ M Co	-	-	-
Ni-H-2	2 mM Ni	-	-	-
Ni-H-4	4 mM Ni	-	-	-
Ni-H-8	8 mM Ni	-	-	X
Co-H-2.5	2.5 mM Co	-	-	-
Co-H-5	5 mM Co	-	-	-
Co-H-10	10 mM Co	-	-	-
Co-H-20	20 mM Co	-	-	-
Co-H-30	30 mM Co	-	-	X
SO ₄ -L-4	-	4 mM	-	-
SO ₄ -M-8	-	8 mM	-	-
SO ₄ -H-12	-	12 mM	-	-
SO ₄ -L-15	-	15 mM	-	-
SO ₄ -M-30	-	30 mM	-	-
SO ₄ -H-45	-	45 mM	-	-
Ni-SO ₄ -4	8 mM Ni	4 mM	-	-
Ni-SO ₄ -8	8 mM Ni	8 mM	-	X
Ni-SO ₄ -12	8 mM Ni	12 mM	-	-
Ni-Na ₂ S-4	8 mM Ni	-	4 mM	-
Ni-Na ₂ S-8	8 mM Ni	-	8 mM	X
Ni-Na ₂ S-12	8 mM Ni	-	12 mM	-
Co-SO ₄ -15	30 mM Co	15 mM	-	-
Co-SO ₄ -30	30 mM Co	30 mM	-	-
Co-SO ₄ -45	30 mM Co	45 mM	-	X
Co-Na ₂ S-15	30 mM Co	-	15 mM	-
Co-Na ₂ S-30	30 mM Co	-	30 mM	X
Co-Na ₂ S-45	30 mM Co	-	45 mM	-

Figure S1.1 – Example of the output from Matlab[®] for the parameter uncertainty regions for the Control and hydrogenotrophic with 30 mM of Co.

Figure S1.2 – Comparison between a control condition (blue) and a test condition (pink). Points depict experimental data; line represents the fitting model and in grey shadows the respective 95% confidence intervals for each condition. The dashed lines indicate the time points selected for comparison of the different conditions, i.e. 20, 60 and 120 hours.

Figure S1.3 – Relative abundances for Bacterial diversity (family level) in the presence of Ni (A) and Co (B).

Supplementary material

Bacterial communities were dominated by *Anaerolineaceae* (3 to 13%), *Syntrophomonadaceae* (3 to 12.5%), *Nitrospiraceae* (2 to 18%), *Geobacteraceae* (5 to 19%), and *Syntrophaceae* (1.7% to 17.5%), while other families are present in smaller abundances. *Carnobacteriaceae*, which in hydrogenotrophic samples can reach up to 13% of the population, was not present in samples containing high concentrations of Co, Ni, sulphate, or sulphide. Relative abundances of *Eubacteriaceae*, also present in higher percentage in the hydrogenotrophic samples (up to 7%), also changes in the presence of metal, sulphate, or sulphide. *Rhodocyclaceaea*, specifically, the genus *Azospira*, shows up in the hydrogenotrophic samples containing Ni and sulphate, reaching 8 to 12% of the population in these samples. On the other hand, *Desulfovibrio* is only present in the samples containing 45 mM of sulphate and Ni.

Supplementary material Chapter 6

Table S2.1 – Medium Composition

Compound	Final concentration (g/L)
Na ₂ HPO ₄ . 2 H ₂ O	0.53
KH ₂ PO ₄	0.41
NH ₄ Cl	0.3
CaCl ₂ . 2 H ₂ O	0.11
MgCl ₂ . 6H ₂ O	0.1
NaCl	0.3
NaHCO ₃	80
Reazurin	0.005
Vitamins	1 mL. *)
Trace elements	1+1 mL. *)

(*) Vitamins and trace elements are added from stock according to protocol of Stams et al., 1993

Table S2.2 – Chemical composition of the cathode and anode measured by SEM-EDX.

Element	Cathode - Wt. (%)	Anode - Wt. (%)
Carbon	88.72	92.01
Oxygen	11.28	7.54
Sodium	0.00	0.27
Aluminium	0.00	0.00
Phosphorus	0.00	0.19
Sulphur	0.00	0.00

Figure S2.1 - Scanning electron microscopy images obtained from the analysis of the graphite granules from the MEC anode compartment

Appendices

References

Summary

Acknowledgments

About the author

List of publications

Overview of completed
training activities

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Summary

Anaerobic microbial processes are commonly applied in the treatment of domestic and industrial wastewaters. Anaerobic digestion (AD) of wastewater has received a great deal of attention, but many aspects related to the complex interactions between microorganism, and how that is affected by the presence of certain toxic, are not yet fully understood. A particular case of this is the effect of heavy metals or chlorinated compounds. These compounds are known to have a strong impact in methanogens, a phylogenetic diverse group responsible for the last step of the AD process. The negative effect of sulphate towards methanogenesis is mainly related to outcompetition of methanogens by sulphate-reducing bacteria (SRB), or to toxicity caused by the sulphide generated from sulphate reduction. Heavy metals are part of many enzymes and cofactors and, in low concentrations, may beneficiate microbial activity. However, high concentrations of metals may disrupt enzyme function and structure. In cases where metal concentration is high, the presence of sulphate or sulphide might be favourable because sulphide precipitate with metals and detoxify the environment. In **Chapter 2** we provide a review on the current knowledge on the effects of heavy metals and sulphate on AD, with special focus on methanogenesis. From this literature study, it came out that the influence of some metals, such as Co, is not extensively studied and that the potential of biologically produced sulphide as metal detoxification method in AD is still quite unexplored. In **Chapter 3** we explored different strategies to improve methane production. Low concentrations of Ni and Co were supplemented to anaerobic sludge and the impact on methane production was evaluated. Although in contrast with other studies, no beneficial effect of metal supplementation was observed. Further on, the impact of high concentrations of Ni and Co added to anaerobic sludge was evaluated, as well as the use of sulphide as a detoxification strategy. This was evaluated in terms of impact on methane production and in changes in the microbial communities. The results

showed that sulphide can be used as a method for metal detoxification, but in the case of biological produced sulphide, the competition between SRB and methanogens needs to be considered.

Chlorinated compounds are widely used and commonly found in wastewaters. Several methanogenic metal-containing cofactors are reported to be involved in reductive dechlorination. Therefore, in **Chapters 4** and **5** the potential of metal supplementation to enhance the dechlorination process was studied. In **Chapter 4**, the enrichment of methanogenic cultures able to perform reductive dechlorination of 1,2-dichloroethene (DCE) and tetrachlorethene (TCE) using different inoculum sources and substrates is described. Differences in physiological performance and in the microbial communities were evaluated. The results showed that the microbial community can be influenced by inoculum and substrate as well as by the chlorinated compound used. The enriched cultures presenting the best dechlorination performance were selected and used for metal supplementation studies with Ni, Co, and Fe. The results showed a clear positive impact of metal addition, both on methane production and reductive dechlorination. Further research on metal supplementation to enhance dechlorination was performed in pure cultures of *Methanosarcina barkeri*, a methanogen known to be able to reduce DCE (**Chapter 5**). In this case, it was observed that metal supplementation could improve methane production and reductive dechlorination, but the effect is dependent on the metal and concentration used. It was found that methanogenesis and reductive dechlorination can be affected in a different way by the same metal.

Finally, in **Chapter 6** the impact of sulphate on a methane-producing bioelectrochemical system (BES), an emerging technology that can be applied to wastewater treatment, was studied. The results showed an unexpected fast sulphate removal in the system and a limited impact caused by sulphate addition on methane production. The sulphate removal could only partially be explained by microbial

activity, but the results demonstrated the ability of microbial communities to evolve and adapt to new operational conditions.

In conclusion, the work presented in this thesis gave insights on the impact of heavy metals and sulphate in methanogenic systems. Furthermore, different approaches to maximise methane production were evaluated. In particular, it was shown that metal supplementation can be a promising strategy to improve anaerobic microbial processes, such as methanogenesis and reductive dechlorination.

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*“They do not know, nor dream of,
That dreaming commands life.
That whenever a man dreams
the world leaps forth
like a colourful ball
into a child’s little hands.”*

António Gedeão, Philosopher's stone.

Life gives many turns, but you can't stop dreaming. Always follow your dreams. When, in the beginning of 2012, I was told that I should consider a change in my life, I would have never guessed where that would lead me. A few months later I landed in The Netherlands, moved to a place I knew little about (Wageningen), and embraced the PhD challenge. Being abroad is not always easy. You miss birthdays, parties, dinners, activities with friends and family, celebrations; you miss births and the growing up of children; you miss the last years of life of dear ones. Yet, you gain so many other things in return! During these 4.5 years I had the opportunity to meet so many nice people from all over the world, I went to so many different, amazing places. I have developed myself, not only professionally, but also at personal level. The PhD gave me much more than the professional knowledge and I am thankful for the whole experience! This journey was not made alone and I want to acknowledge and say 'thank you' to several people who helped me in this journey, some even before it started. So brace yourselves, because the list is long.

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António Feio

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Lara

About the author

Lara da Luz Ferreira Martins Paulo, was born on April 6th 1984 in Barreiro, Portugal. In 2008, she finished her Integrated Master degree in Biological Engineering at Instituto Superior Técnico de Lisboa, Portugal. In January 2009, she started to work within the Metalloproteins and Bioenergetics Unit at Instituto de Tecnologia Química e Biológica, Oeiras, as Research Fellow under the supervision of Dr. Manuela Pereira. Her work was focused on membrane-proteins from the respiratory chain, their over-expression, purification and enzymatic activities. In April 2012, she started to work in the Protein-Nucleic Acid Interactions Group, Instituto Gulbenkian de Ciência, Oeiras, as Research Technician under the supervision of Dr. Alekos Athanasiadis. There, she focused on the study of Z α domains from KOI Herpes Virus and the formation of homo and heterodimers with other Z domains by electrophoretic mobility shift assay and size exclusion chromatography. In August 2012, she moved to The Netherlands and started her PhD at the Laboratory of Microbiology, Microbial Physiology Group at Wageningen University. During her PhD, she studied anaerobic microbial processes for energy conservation as part of the SANITAS project under the supervision of Prof. Dr. Alfons J.M. Stams and Dr. Diana Z. Sousa.

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D I P L O M A

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The Netherlands Research School for the
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The SENSE Research School declares that Ms Lara Paulo has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 36.8 EC, including the following activities:

SENSE PhD Courses

- o Environmental research in context (2012)
- o Research in context activity: 'Organising SANITAS workshop on: Emerging challenges for a sustainable and integrated urban water system management, Verona, Italy' (2014)

Other PhD and Advanced MSc Courses

- o Modelling and control of urban wastewater system - 2nd SANITAS training week, University of Lund and the Technical University of Denmark (2012)
- o Practical field work - 3rd SANITAS training week, Ghent University and Aquafin (2013)
- o New frontiers in microbial ecology, Wageningen University and University of Groningen (2013)
- o ARB/Silva basic training: Basic training in ARB - a software environment for sequence data, Wageningen University (2014)
- o Decision-making and multicriteria for urban water systems - 4th SANITAS training week, Universitat de Girona (2014)
- o Techniques for writing and presenting a scientific paper, Wageningen University (2015)

Management and Didactic Skills Training

- o Assisting practicals of the course 'Research methods in microbiology' (2013-2016)
- o Supervising MSc student with thesis entitled 'Effects of metal supplementation in co-metabolic dehalogenation by methanogens' (2015-2016)

Oral Presentations

- o *The dual effect of nickel on methanogenesis*. 2nd International Conference on Biogas, 10-12 June 2014, Uppsala, Sweden
- o *Optimisation of sustainable biological wastewater treatment*. 2nd International Water Association Specialist Conference on EcoTechnologies for sewage treatment plants, 23-27 June 2014, Verona, Italy
- o *Driving methane or acetate production in anaerobic sludge enrichments by cofactors engineering*. 14th World Congress on Anaerobic Digestion, 15-18 November 2015, Viña del Mar, Chile

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