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REVIEW PAPER

Methanogens, sulphate and heavy metals: a complex system

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Abstract Anaerobic digestion (AD) is a well-established technology used for the treatment of wastes and wastewaters 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 (e.g. 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 studies 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

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1 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) (Fig. 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

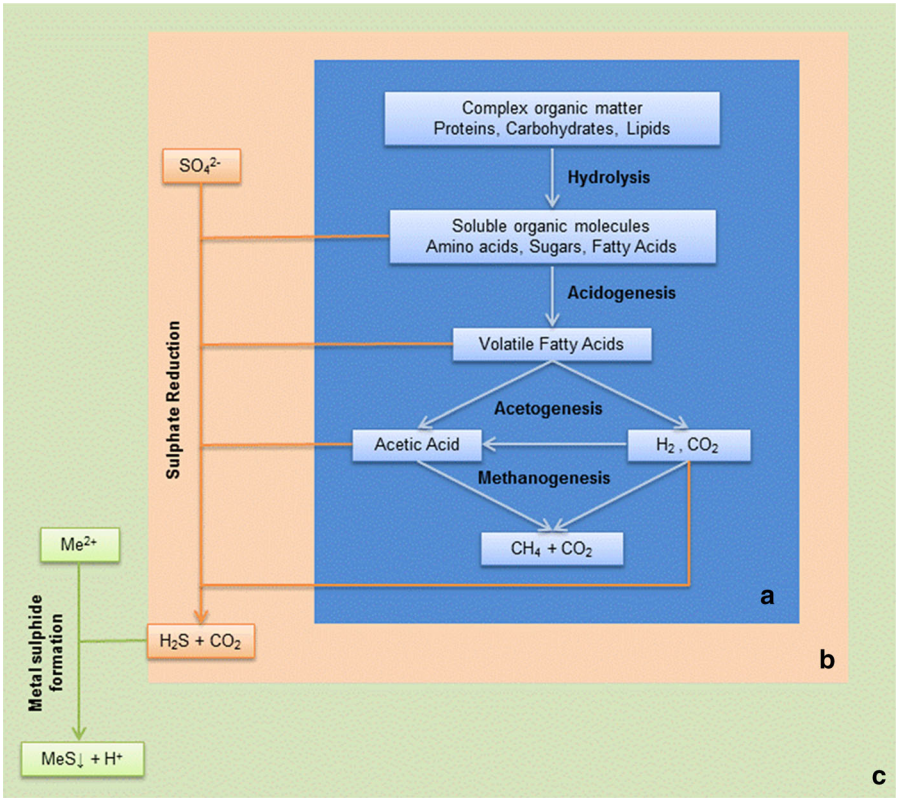

Fig. 1 Schematic representation of the anaerobic degradation of organic matter (*a*), in the presence of sulphate reduction (*b*) and coupled to metal sulphide formation (*c*). AD is generally divided in four steps: (1) hydrolysis, in which large molecules, such as carbohydrates, proteins and lipids, are converted in their monomers, i.e. simple sugars, amino acids, and glycerol plus long chain fatty acids; (2) acidogenesis, that consists in the conversion of fermentable compounds (e.g. sugars and glycerol) to volatile fatty acids; (3) 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 H_2/CO_2 by homoacetogenic bacteria; and, (4) methanogenesis, the final AD step in which simple compounds such as acetate and H_2/CO_2 are converted to biogas (*a*)

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 (Fig. 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 milimolar (mM).

2 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 (Fig. 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 to −400 mV (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).

2.1 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 [Eqs. 1–4; $\Delta G^{0\prime}$, 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:

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \quad \Delta G^{0\prime} = -151.9 \text{ kJ} \tag{1}$$

$$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2HCO_3^- \quad \Delta G^{0\prime} = -47.6 \text{ kJ} \tag{2}$$

Methanogenesis:

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \quad \Delta G^{0\prime} = -135.6 \text{ kJ} \tag{3}$$

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad \Delta G^{0\prime} = -31 \text{ kJ} \tag{4}$$

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. 1998a, b; 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 >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/SBR 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 10^4. 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. 1998a, b). 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:SO_4^{2-} ratios $\leq$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 aceticlastic 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 aceticlastic 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 m^3 (STP) for every kg of SO_4^{2-} reduced (Colleran et al. 1995).

2.2 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 H_2S 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 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. 1998a, b). 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. 1998a, b). 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.

3 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

Table 1 Sulphide inhibitory concentrations according to literature

Biological system	Substrate	Concentration H_2S	Notes	References
Anaerobic mixed culture	Cellulose	1.75 mM[a] 10 mM[b]	[a]Partial inhibition [b]Complete inhibition of cultures in sulphur-free medium and headspace with H_2/CO_2	Khan and Trottier (1978)
Inoculum from an upflow sludge blanket reactor	Acetate	1.75 mM[c] 6.25 mM[d]	[c]50 % inhibition [d]100 % inhibition	Kroiss and Plahl-Wabnegg (1983)
Anaerobic sludge from a reactor treating distillery wastewater	Acetate and acetate/ethanol	37.5 mM	50 % inhibition, 35 °C	Isa et al. (1986a)
Granular sludge from a UASB in a acetate-fed reactor	Acetate	7.8 mM[e] 2.8 mM[f]	[e]30 °C, pH 6.4–7.2 [f]30 °C, pH 7.8–8.0	Koster et al. (1986)
Unacclimated batch digester population		1.5 mM		Parkin et al. (1983)
Mixture of methanogens and SRB	Acetate, propionate (STR 25 days), lactate (STR 40 days)	1.76–2.2 mM[g] 5.9 mM[h]	CSTR and Anaerobic filters were used to test suspended and attached growth at 35 °C [g]concentrations causing stress in all suspended systems tested and higher concentrations caused failure in acetate and propionate systems [h]concentrations tested in propionate fed-filters systems causing none or little effect in the systems	Krishnanand et al. (1993)
Anaerobic sludge from a reactor treating distillery wastewater	Acetate and acetate/ethanol	43.75 mM	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 et al. (1986b)
Desulfotomaculum acetooxidans	Lactate	2.65 mM		Widdel (1988)
Desulfovibrio enrichment		17 mM	Total inhibition of the culture growth for lactate conversion, reversible after H2S stripping; 37 °C and automatic pH control	Reis et al. (1992)
Mixture of SRB and methanogens	Acetate, propionate	1.9 mM[i] 1.9 mM[j]	[i]Irreversible failure for systems using acetate [j]Irreversible failure for systems using propionate; sulphate reduction was shutdown before methanogenesis; in similar conditions, propionate-using systems failed before acetate-using systems	Parkin et al. (1990)
Granular sludge from a full-scale UASB reactor treating papermill wastewater	Synthetic influent with sucrose as carbohydrate	3.1 mM[k] 6.25 mM[l]	[k]30 % inhibition; pH 5; 55 °C; HRT 10 h; COD/SO_4^{2-} = 4 [l]65 % inhibition, pH 6, COD/SO_4^{2-} = 1	Lopes (2007)

Table 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, Devecey, 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 ± 0.23	–	5826–22,173
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	4632–17,627
Reference	Karvelas et al. (2003)	Chipasa (2003)	Sancey et al. (2011)

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 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, 2014) (Fig. 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. 1995a).

3.1 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 co-factors 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 7–15 % 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

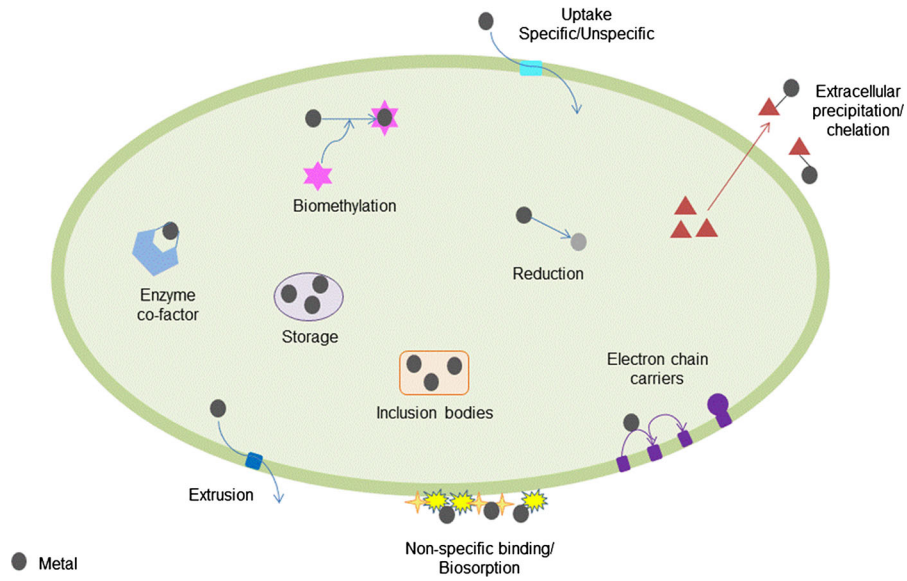

Fig. 2 Schematic representation the interactions between metals and cells

trace elements. A similar effect was observed after supplementing a UASB reactor treating swine wastewater with Zn: addition of extra 0.75–0.15 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 B_{12}-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 L^{-1} 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.

3.2 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).

3.3 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 (Fig. 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 *el 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 agent (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 *Penicillum 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).

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 *Methanosarcina acetivorans* that were exposed to 54 μM of Cd for 3.5 months and growing on methanol, were able to grow in the presence of high concentrations of Cd (0.63–2.5 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 thermautotrophicus* growing in H_2/CO_2, was able to reduce 0.2 and 0.4 mM Cr^{6+} completely (Lira-Silva et al. 2013). Singh et al. (2015) tested growing *M. thermautotrophicus* with higher concentrations of hexavalent chromium; amendment of 1, 3 and 5 mM of Cr^{6+} resulted in 43.6, 13 and 3.7 % reduction of the metal. The same methanogen was also able to reduce structural Fe^{3+} in smectite minerals at 65 °C although with low reduction extents (27 % for nontonite and 13–15 % for montmorillonite) (Zhang et al. 2013). *Methanosarcina barkeri* was also observed to be able to reduce Fe^{3+} in nontronite using methanol and H_2/CO_2 as substrates, but not with acetate (Liu et al. 2011). Microbial reduction of Fe^{3+} 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).

4 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 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 3). However, this is perhaps due to variations in the experimental conditions: differences in substrates, microorganisms, different oxidation states of the metal ion, pure versus co-culture, and environmental factors, such as pH (Chen et al. 2008).

4.1 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 formicicum* (Jarrell et al. 1987) showed that Zn and Cu were toxic at concentrations from 0.015 to 0.15 and 0.017–0.17 mM, respectively, while Ni was described as being the least toxic of the three metals; particularly, *M. formicicum* 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 where sensitive to concentrations between 4.25 and 20 mM. Using an anaerobic sludge from a UASB reactor treating wastewaters from a yeast factory,

Table 3 Toxic concentrations of metals according to literature

	pH	Temperature (°C)	Metal	Inhibition	Notes	Ref.
Anaerobic sludge	–	35	Cr^{3+}	(2.5 mM) 5 mM[a] <3.8 mM[b]	(Concentration at which the gas production started to decrease, step-fed conditions)	Hayes and Theis (1978)
			Cr^{4+}	(2.1 mM) 8.1 mM[a] <3.5 mM[b]	[a]70 % reduction in total gas production, step-fed conditions	
			Cu	(0.63 mM) 1.1 mM[a] <0.8 mM[b]	[b]70 % reduction in total gas production, pulse-fed conditions	
			Cd	>0.18 mM[a] >0.09 mM[b]		
			Ni	(0.17 mM) 0.5mM[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; 20,000 mgCOD/L; mix of acetic, propionic and n-butyric acid	Lin (1992)
			Cd	<0.03 mM[c]		
			Pb	0.35 mM[c]	[c]50 % inhibition of activity of VFA degradation over 30 h by metal dosage	
			Ni	1.7 mM[c]		
			Zn	0.26 mM[c]		
			Cu	0.11 mM[c]		
UASB granules	–	37	Cd	>4.9 mM[d]	[d]50 % inhibition of specific methanogenic activity of UASB granules	Fang and Hui (1994)
			Cr	12.1 mM[d]		
			Cu	2.5 mM[d]		
			Ni	2 mM[d]		
			Zn	1.5 mM[d]		
Sewage sludge	–	35	Cu	12.6 mM[e]	[e]50 % of inhibition of gas production	Wong and Cheung (1995)
			Ni	5.75 mM[e]	Sulphate metals were used	
			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 et al. (1995a)

Table 3 continued

	pH	Temperature (°C)	Metal	Inhibition	Notes	Ref.
Anaerobic sludge	7.0 – 7.2	35	Cd	0.53 mM[g]	Enrichments with acetic acid; SRT 50 days [g]100 % inhibition of gas production	Bhattacharya et al. (1995b)
Methanogenic granules	–	35	Cd	0.5 mM[h,i]		
			Cr	0.65 mM[h,i]		
			Cu	0.4 mM[h,i]		
			Ni	1.3 mM[h,i]		
			Pb	6.3 mM[h,i]		
			Zn	0.85 mM[h,i]		
Conventional granules	–	35	Cd	15.3 mM[h,j]	HRT 1 day; acclimation period 6 months	[i]Lin and Chen (1997)
			Cr	34 mM[h,j]	[h]50 % inhibition of gas production	[j]Lin and Chao (1996)
			Cu	5.4 mM[h,j]		[k]Lin (1993)
			Ni	52.3 mM[h,j]		
			Pb	49 mM[h,j]		
			Zn	104 mM[h,j]		
Acetogenic granules	–	35	Cd	8.8 mM[h,k]		
			Cr	8.4 mM[h,k]		
			Cu	4.4 mM[h,k]		
			Ni	24.7 mM[h,k]		
			Pb	26.8 mM[h,k]		
			Zn	12.6 mM[h,k]		
Anaerobic sludge	7.2	30	Cu	0.33 mM[l] 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	Karri et al. (2006)
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^{3+}	29.8 mM[n] 59.6 mM[o]		
Anaerobic sludge	–	30	Cu	0.28 mM[p]	[o]IC50 value for aceticlastic culture	Gonzalez-Estrella et al. (2013)
Anaerobic glucose-amended mixed culture	7	37	Zn	0.57 mM[p]	[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)
			Cu	0.16 mM[q]		
			Zn	0.6 mM[q]		
			Ni	1 mM[q]		

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).

4.2 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 0.3–0.8 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 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 Fe^{2+} 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.

5 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 (Eq. 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).

$$Me^{2+} + HS^- \rightarrow MeS\downarrow + H^+ \quad (5)$$

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 91–97 % 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 Cu^0, 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-del Risco 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 Cu^0 nanoparticles in aceticlastic 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.

6 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.

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