Interrelating EPS, soluble microbial products and metal solubility in a methanogenic consortium stressed by nickel and cobalt

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ABSTRACT

The relationships between extracellular polymeric substances (EPS), soluble microbial product production, metal solubility, and methanogenic activity were investigated. The individual, and joint, toxic effects of nickel and cobalt on methanogenic consortia fed with glucose as model substrate were studied using biomethane potential assays. Cobalt was found to be less toxic to methanogens than nickel at each concentration tested, and the combined effects of Ni and Co on methane production in the bimetal experiment was higher than the sum of the effects of each metal alone. The protein content of EPS, and extracellular soluble protein fractions, decreased with increasing concentrations of total metals. Meanwhile, no significant change in response to metal stress was apparent for carbohydrate content of EPS or extracellular soluble carbohydrate. Decreasing protein content of EPS was accompanied by reduced methanogenic activity and an increase in the soluble metal fraction. The strong associations observed between these variables could be due to the critical role of EPS in protecting microbial cells against nickel and cobalt stress, possibly by capturing metal cations through their functional groups, thus reducing metal availability to the microbial cells in the methanogenic consortia underpinning the anaerobic digestion process.

1. Introduction

Nickel (Ni) and cobalt (Co) are two important metals required as trace elements by microorganisms in anaerobic digestion (AD) (Paulo et al., 2015; Šafarič et al., 2020; Tsapekos et al., 2018). Therefore, an adequate concentration of Ni and Co in the digestion system is needed for stable microbial metabolism. Despite the essential role in AD of Ni and Co, at comparatively high concentrations they can alter the optimum biochemistry and performance of the processes by generating intermediate compounds and creating cytotoxic effects (Paulo et al., 2015). Ni and Co are widely used, particularly in the metallurgical industry and lithium-ion batteries. Therefore, a significant proportion of these metals are likely to end up in the wastewater treatment plants and affect the function of the microbial consortia underpinning AD (Gikas, 2008; Y. Liu et al., 2021). So far, little research has been reported on Ni and Co toxicity in AD (Ashley et al., 1982; Bartacek et al., 2008; Bhattacharya and Safferman, 1989; Fang and Hui, 1994). Moreover, the comparative biological activity of Ni and Co, assessed by several previously published studies, showed contradictory results, as reviewed by Gikas (2008). Therefore, the biological activities of these metals can be compared only between identical biological systems. When considering metal toxicity in biological systems, three metal fractions are important: the total metal, soluble metal and free metal concentration (Pinto-Ibieta et al., 2016). The free metal concentration is reported as the fraction of metal that could be taken up by cells and so is thought to be responsible for metal toxicity (Zhu et al., 2014). However, because it is difficult to measure the free metal concentration in complex systems, such as in AD, it has been suggested to measure the dissolved metal concentration to predict microbial responses (Pinto-Ibieta et al., 2016).

When the diverse microbial community in AD is exposed to toxic concentrations of metals, several tolerance strategies are adopted by microbial cells to tackle the metal stress (Igiri et al., 2018). Some of these responses adopted by microorganisms to survive and grow in metal-stressed conditions have been reported as active efflux,

intracellular sequestration, enzymatic transformation, excretion of chelating agents, biomethylation and oxidoreduction of toxic metal ions (Igiri et al., 2018). All these mechanisms help the microbial cells under stress to change the balance between total, dissolved and free metal concentration to reduce the availability for the cells of the metal ions (Fermoso et al., 2015). So far, few studies have targeted the effect, under metal excess, of microbial stress responses on metal solubilization in AD systems (Ashley et al., 1982; Bartacek et al., 2008; Pinto-Ibieta et al., 2016; Sarioglu et al., 2010).

Soluble microbial products (SMPs) play an important role in metal solubility in biological systems, such as AD, due to their chelating properties that could affect metal availability (Kuo et al., 1996). It has been demonstrated that SMPs may be changed in response to stress conditions. This may be due to a strategic response of microbial cells to stress or due to release of cell components caused by cell lysis (Aquino and Stuckey, 2004). To the best of our knowledge, limited information is available about the production of SMPs in response to metal stress. Extracellular polymeric substances (EPS) comprise another important group of compounds affecting metal solubility. EPS production has been reported as an important defence mechanism for bacteria to thrive under metal-stressed conditions (Mohite et al., 2018). EPS are mainly comprised of polysaccharides, proteins, humic substances and nucleic acids, and plays an important role in protecting microbial cells against metal toxicity by chelating metal cations, and hindering the immediate contact between the cells and free metal ions (Ding et al., 2018). The capacity of EPS to bind and sequester metals from contaminated environments has been extensively demonstrated in the literature (Gadd, 2010; Guibaud et al., 2003; J. Li et al., 2019; Mathivanan et al., 2021; Mikutta et al., 2012; Nouha et al., 2016; Pagliaccia et al., 2022; Wei et al., 2019), while the relationship in AD between EPS content, dissolved metal concentration and, in turn, metal toxicity is still limited, specifically in response to different metals. Clarification of this relationship could help reveal the protective mechanism on the AD microbial community of EPS against metal toxicity, possibly influenced by changing metal bioavailability.

To date, no studies have assessed the effect of Ni or Co – or both – on EPS and SMP production in AD. Therefore, the main hypothesis of this work was that metal toxicity in AD is related to metal solubility which, in turn, is associated with EPS content and SMPs. The aim of this study was, thus, to investigate the effects of Ni and Co toxicity on AD using glucose as model substrate, and focusing on the relationship between EPS content, extracellular soluble protein and soluble carbohydrate production, methanogenic activity and metal solubility.

2. Materials and methods

2.1. Source of biomass

Sludge samples were collected directly from the anaerobic sewage sludge stabilisation tank at a wastewater treatment plant in Seville, Spain, transported to the laboratory as soon as possible and maintained at 4 °C. The sewage sludge was characterised by measuring total solids (TS), volatile solids (VS), pH, alkalinity and the total and dissolved contents of Ni and Co.

2.2. Biomethane potential (BMP) assays

BMP assays were used to determine the effect of Ni and Co on the methanogenic activity of the biomass. Three sets of experiments were designed to evaluate the individual, and simultaneous, effect of Ni and Co on AD. For each metal, three concentrations (460, 920, 1840 mg/L) were used. Control incubations were also set up, without any metal. Subsequently, and to assess the simultaneous effect of Ni and Co, an additional experiment comprising of a single concentration of Ni (460 mg/L), a single concentration of Co (920 mg/L) and a bimetal system (460 mg/L Ni + 920 mg/L Co) was also designed. The concentrations of

metals at this second stage of the experiment were selected according to the results obtained from assessing the individual effects of Co and Ni i.e. the lowest toxic concentration of each metal was selected for the combined Co and Ni tests.

Experiments were done at 35 °C in 250-ml, glass, serum bottles with 200 ml working volume. Each of the serum bottles contained sewage sludge (as 14 g VS/L), glucose (7 g/L) and Vanderbilt mineral medium added according to Altaş (2009). Thus, the ratio of inoculum (g VS/L) to substrate (g/L) was 2:1 in each bottle. The serum bottles were sealed with a rubber screw cap, and continuously stirred at 120 rpm. The methane production was measured using a sodium hydroxide solution (3%, w/v) displacement system. All the biomethane volumes were transformed into standard conditions before analysing. Experiments were finished after 46 h incubation, once the methane production was exhausted. Samples from suspended anaerobic sludge were taken at the end of the BMP assays (after 46 h) for the following analysis.

2.3. EPS extraction

The EPS could be extracted by different physical and chemical methods. Some studies found that heating was a gentle and efficient method to extract EPS from sludge because did not introduce exogenous substances during the EPS extraction process and that the destruction of cells from this method was relatively slight (Sun et al., 2018). It is also less time consuming than other methods. The extraction was done using heating method according to Li and Yang, (2007). Previously to extraction, 5 g (~0.07 g of VS) of BMP effluent were centrifuged (5800 g for 25 min). The biomass was resuspended in 0.05% NaCl solution and kept in a water bath at 70 °C for 30 min. Finally, the samples were centrifuged at 20,000 g and 4 °C for 20 min and supernatant was collected. Protein and carbohydrate were measured as two main components of EPS using bicinchoninic acid (BCA) assay and Anthrone method, respectively. Total organic carbon (TOC) of each EPS solution was measured. Considering the presence of compounds i.e. humic like substances and nucleic acid, other than protein and carbohydrate, in EPS, the TOC of EPS provide information about the variation in the total content of EPS because it comprises carbon from organics substances include proteins, polysaccharides, humic acids, etc.

2.4. SMPs measurement

In this work, the changes in the concentration of soluble proteins and carbohydrates, as two of the main fractions of SMPs, in response to Ni and Co concentrations were investigated using the methods in the following section. The theoretical chemical oxygen demand (COD) of the soluble protein and carbohydrate was calculated to determine the different fractions of soluble COD. As the precise chemical formula of the carbohydrates detected was not determined, the percentage of soluble COD represented by carbohydrates was estimated using the coefficient obtained from glucose oxidation reaction (Dionisi et al., 2018). The theoretical COD of soluble protein content was calculated using the coefficient obtained from bovine serum albumin oxidation reaction (Dionisi et al., 2018).

2.5. Analytical methods

Samples were taken from anaerobic sludge and centrifuged at 4000 rpm for 20 min. After filtering through 0.45 μm nylon filters, the filtrate was used to analyse soluble COD, soluble metal concentration and soluble protein and carbohydrate content. For measuring the soluble metal concentration, after acidifying with HNO_3 to 1%, the Ni and Co in the supernatant were measured using Inductive Coupled Plasma Mass Spectrometry (ICPMS-Agilent-7800). Total trace elements concentrations in sludge samples were determined through the same procedure after digestion with nitric acid in a microwave oven. Carbohydrate content of the filtrates and EPS solutions were measured by the

Anthrone colorimetric method, using a spectrophotometer at 620 nm (Biorad iMark Microplate Reader, Hercules, CA, USA), and expressed as mg of glucose equivalents/L. Total protein content of the filtrates and EPS solutions was measured by BCA assay using bovine serum albumin as standard and the same spectrophotometer at 562 nm. The determination of pH, alkalinity, the concentration of TS and VS, and soluble COD were developed following the recommendations of the Standard Methods of APHA et al. (2017). The TOC concentration of EPS was measured by a TOC analyser TOC-L (SHIMADZU, Japan).

2.6. Statistical analysis and calculations

Interaction Factors (IF) was defined as the ratio of the mixture (Ni with Co or Co with Ni) activity (methane production) over the summation of their effects which occur when the metals are applied individually (Yu et al., 2018). All the data were analysed by SPSS Statistics software (Version 27.0, IBM Corp.), with a significance level of P < 0.05. The significance of variation between different tested conditions were determined by one-way ANOVA (alpha = 0.05) with Tukey's multiple correction method. All data in the figures and text were given as the mean value of triplicate experiment ± standard deviation. Pearson analysis (P < 0.05) was used to investigate the association between EPS content, soluble metal concentration and methanogenic activity.

3. Results

3.1. Ni and Co solubility

Less than 1% of total Ni and Co at the concentrations tested was in soluble form (Fig. 1). At the highest total concentration (1840 mg/L) of each metal, the dissolved fraction was 19.0 ± 1.2 and 8.5 ± 2.1 mg/L for Ni and Co, respectively (Fig. 1). The soluble fraction of Ni and Co increased disproportionately with increasing total metal concentrations (Fig. 1). At each of the tested concentrations, the proportion of soluble Ni was higher than for Co, and this difference increased with increasing total metal concentration (Fig. 1). The concentrations of soluble Ni and Co in the bimetal test (460 mg/L Ni + 920 mg/L Co) were 1.16 ± 0.01 and 0.64 ± 0.02 mg/L, respectively, whilst in the single Ni and single Co experiments the concentration of soluble Ni and Co was 0.66 ± 0.00 and 0.23 ± 0.00 mg/L, respectively (data not shown). Therefore, in the bimetal experiment, the soluble proportion of Ni was 3.7 times higher than the soluble proportion of Co. Moreover, the solubilization of Ni and Co was increased two and three times, respectively, in the bimetal experiment compared to the single-metal systems.
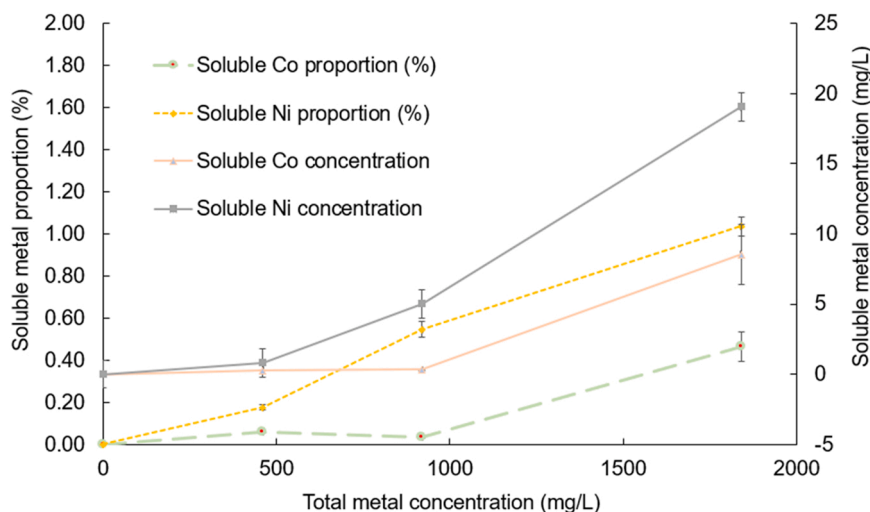
3.2. Effects of Ni and Co on methane production and COD removal

Theoretical biochemical methane potential of glucose (1.4 g) added to each bioreactor was estimated to be 0.52 L under standard condition based on Buswell equation (Zulkifli et al., 2019). The average experimental methane production volume in the no-metal controls was 0.34 L, around 66% of the theoretical value. Both Ni and Co at the tested concentrations reduced the methane production but the decrease in methane production was higher for Ni compared to Co at the same concentrations (Fig. 2a). The inhibition of methane production was 24%, 43% and 88.5% with increasing total Ni concentrations of 460, 920 and 1840 mg/L, respectively, whilst with 460, 920 and 1840 mg/L total Co, methane production was inhibited by 7%, 21% and 45.2%, respectively (Fig. 2a). The IC50 values, the metal concentrations impairing methane production by 50% during the test (over 46 h), were estimated to be 1274 mg/L total Ni and 1933 mg/L total Co.

This decrease in methane production was followed by an increase in soluble COD. Soluble COD removal was impaired with increasing total Ni and Co concentrations (Fig. 2a). Inhibition of soluble COD removal was significantly greater with Ni than Co at all tested concentrations. Methane production in BMP assays with single-metal and bimetal addition was impaired by 16%, 24% and 48% for single Ni, single Co and the bimetal experiment, respectively (Fig. 2b). The calculated IF for Co and Ni at 2:1 ratio in this experiment was 1.25. Therefore, the sum of the effects of Ni and Co on methane production in the bimetal experiment was higher than the sum of the effects of each metal alone. Soluble COD removal was impaired, by single Ni, single Co and the bimetal concentrations (Fig. 2b).

3.3. Effects of Ni and Co on EPS content

At 460 mg/L total Ni and Co, the EPS protein content decreased 28% and 24%, respectively, compared to the no-metals controls. The EPS protein content was declined 64.7% for Ni and 72% for Co by increasing the total metals concentration to 1840 mg/L (Fig. 3). The EPS carbohydrate production did not significantly change in response to Ni and Co except for a 20% increase at 920 mg/L total Ni (Fig. 3). The protein to carbohydrate ratio in EPS was around 3:1 in control and this ratio was changed to around 1:1 at the highest concentration of Ni and Co. The TOC of EPS extracted from experiment with 0, 460, 920 and 1840 mg/L of total Co were 0.49 ± 0.01, 0.47 ± 0.01, 0.46 ± 0.01 and 0.36 ± 0.01 mg/L, respectively (data not shown). According to these results, at 1840 mg/L total Co concentration there was a 26.55% reduction in TOC of EPS compared to the control without metal supplementation.
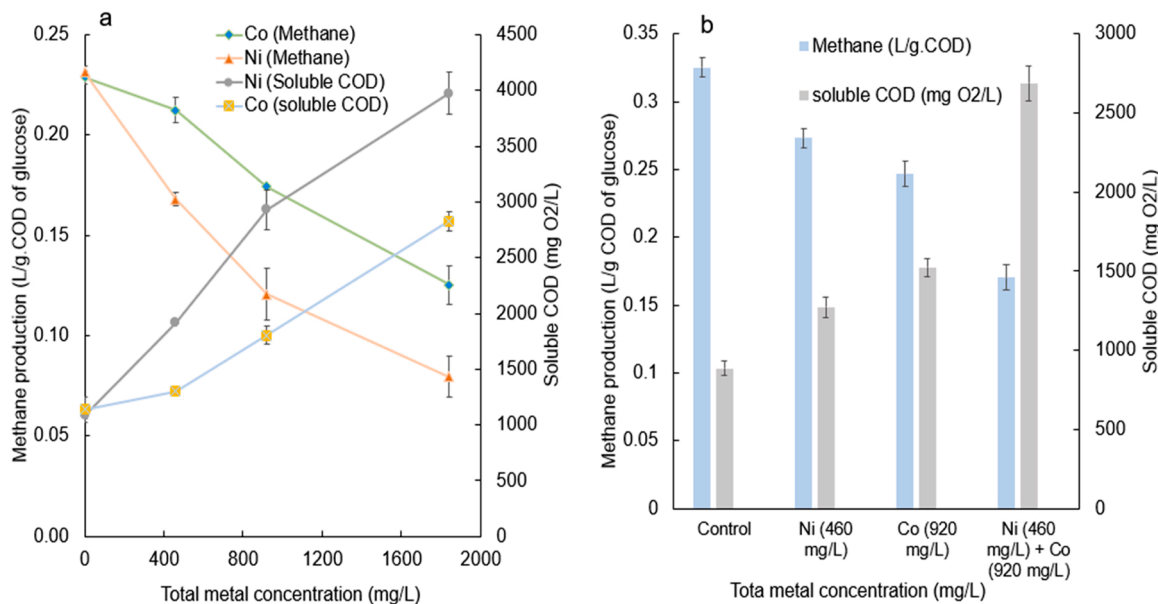

Fig. 1. Soluble Ni and Co concentrations and proportions (%) in BMP assays containing different metal concentrations (0–1840 mg/L). Error bars represent standard deviations of triplicate assays.

Fig. 2. Methane production and soluble COD in BMP assays with Ni and Co at total concentrations of 0–1840 mg/L (a) and Ni (460 mg/L), Co (920 mg/L) and both metals (460 mg/L Ni and 920 mg/L Co) after 46 h incubation (b).
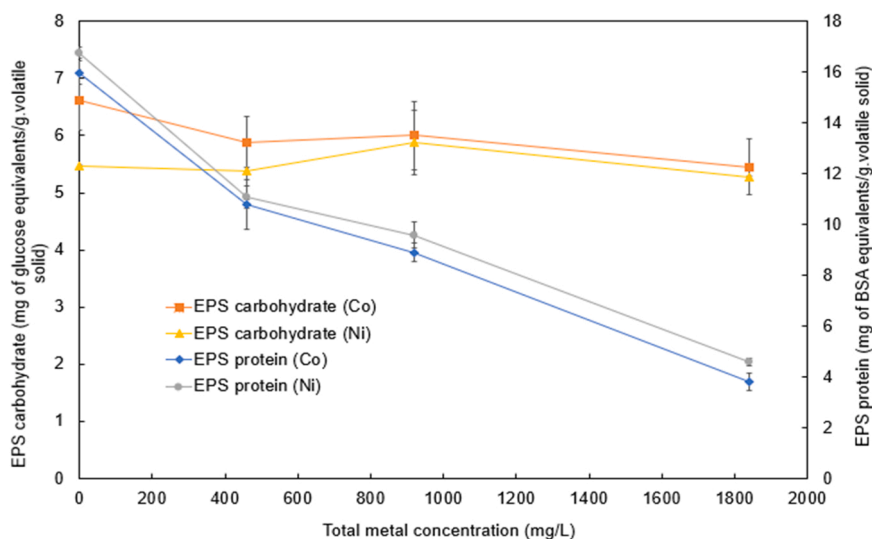

Fig. 3. The effects of Ni and Co concentrations on EPS protein and carbohydrate content.

The EPS production in the bimetal experiment was measured as TOC of the EPS solutions. The TOC of EPS extracted from control, single Ni, single Co and the bimetal experiment were 0.59 ± 0.01, 0.48 ± 0.01, 0.44 ± 0.02 and 0.39 ± 0.01 mg/g VS, respectively. According to these results, the TOC of EPS decreased significantly compared to the control in both single-metal conditions and bimetal test (19%, 25% and 36% for Ni, Co and the bimetal test, respectively).

3.4. Effects of Ni and Co on SMPs

Extracellular soluble protein production was impaired by both Ni and Co concentrations, while no significant changes was observed in the soluble carbohydrate content in response to Ni and Co stress.

3.4.1. Soluble carbohydrate
The measured soluble carbohydrate concentration was not affected in response to different concentrations of Ni but decreased 25% at 460 mg/L of total Co and then remained constant with increasing total

Co concentrations (Fig. 4a). The results of experiments with single and bimetal treatments showed that the soluble carbohydrate concentration was not changed in the presence of 460 mg/L Ni while a small decrease (16%) was observed in bimetal and single Co experiment (Fig. 4b).

3.4.2. Extracellular soluble protein
The extracellular soluble protein production was significantly affected by adding 460 mg/L of Ni or Co. This decrease was 33.0% and 47.5% for Ni and Co, respectively, compared to the control without metal supplementation (Fig. 4a). From this concentration onwards, the protein content decreased slightly with increasing total metal concentration. The results of BMP assays with both metals showed that extracellular soluble protein production was also lower in the bimetal experiment (Fig. 4b). This reduction was 38%, 22% and 35% for bimetal, single Ni and single Co conditions, respectively. The reduction in extracellular protein content caused by metal stress was higher for Co than Ni at the same concentrations (Fig. 4a and b).
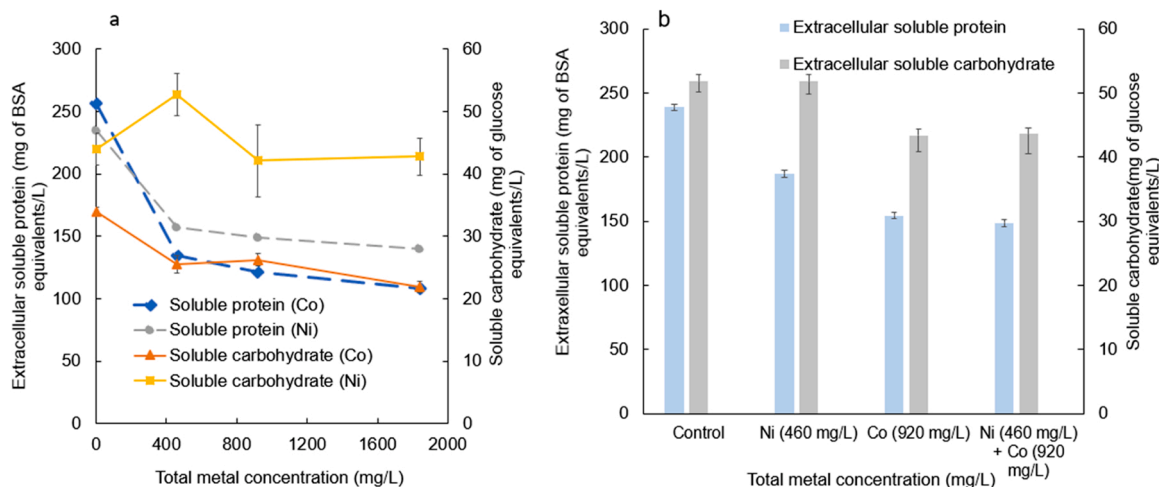
Fig. 4. Soluble carbohydrate and extracellular soluble protein content in BMP assays with 0–1840 mg/L total concentrations of Ni and Co (a) and Ni (460 mg/L), Co (920 mg/L) and both metals (460 mg/L Ni and 920 mg/L Co) (b).

3.4.3. Other SMPs and pH

The total soluble COD increased with increasing Ni or Co concentrations (Fig. 2a). The soluble carbohydrate concentration expressed in terms of COD was negligible compared to the total soluble COD measured in each tested condition (Fig. 5a and b), indicating likely that most of the glucose was consumed. The soluble protein COD was accounted for a large fraction of the total soluble COD in no-metal controls (average 37%) while the fraction of soluble protein COD was less important with increasing total metal concentration (Fig. 5a and b). The pH value of the media decreased from 7.7 ± 0.1 in control to 6.7 ± 0.1 in experiment with 1840 mg/L total concentration of Ni (Fig. 5b) and from 7.3 ± 0.0 in control to 6.9 ± 0.1 in experiment with 1840 mg/L total concentration of Co after incubation period (Fig. 5a).

3.5. Relationships between SMPs, metal solubility, methanogenic activity and EPS

3.5.1. EPS, dissolved metal concentration and methanogenic activity

The increase in the soluble fraction of Ni and Co highly correlated with lower EPS protein production (R = −0.89 and −0.86, respectively, p < 0.01). No correlation was found between EPS carbohydrate and

soluble Ni or Co (Table 1).

3.5.2. SMPs and dissolved metal concentration

There was no correlation between soluble carbohydrate and dissolved Ni concentration. However, a moderately negative association (R = −0.67, p < 0.05) was found between soluble carbohydrate and soluble Co concentration. No correlation was observed between

Table 1
Pearson's correlation results between EPS content, methane production, soluble COD and metal solubility.

EPS fraction	Metal	Methane production	Soluble COD	Soluble metal
TOC	Co	0.92[a]	-0.95[a]	-0.96[a]
	Ni	NM	NM	NM
Protein	Co	0.97[a]	-0.94[a]	-0.86[a]
	Ni	0.98[a]	-0.97[a]	-0.89[a]
Carbohydrate	Co	NC	NC	NC
	Ni	-0.57[b]	NC	NC

NM: not measured; NC: no correlation was found between the two variables.
[a] Correlation is significant at the 0.01 level (2-tailed).
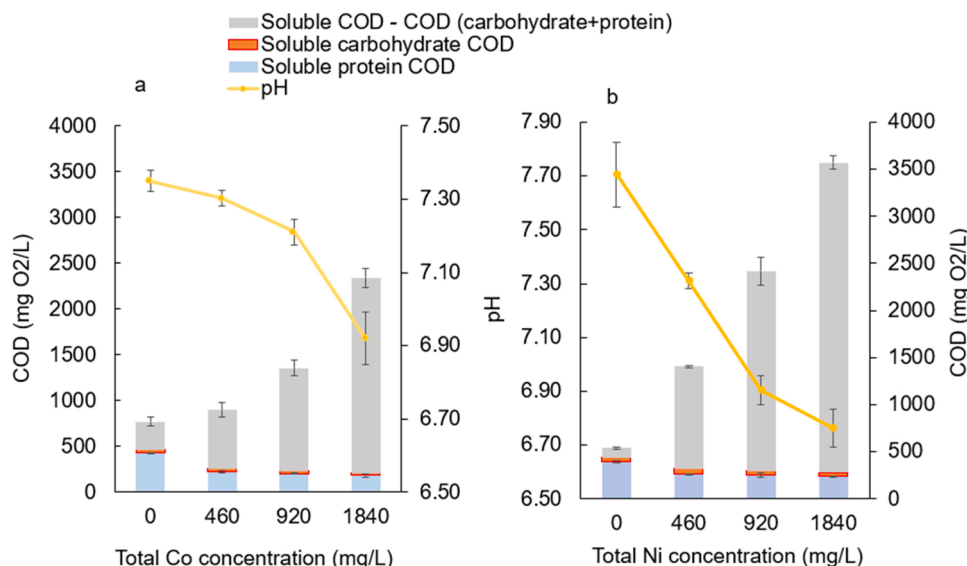[b] Correlation is significant at the 0.05 level (2-tailed).

Fig. 5. pH and soluble COD fractions in BMP assays with Co (a) and Ni (b).

extracellular soluble protein and soluble Co, but a moderately negative correlation (R = −0.58, p < 0.05) was found between soluble Ni and extracellular soluble protein (Table 2). The zero-order correlations showed that the negative association between EPS protein (or TOC) and soluble Ni or Co was still strong after controlling for the SMPs (Table 2).

4. Discussion

4.1. Effects of Ni and Co on methanogenic activity

The few available studies on the effect on AD of Ni and Co reported diverse toxic concentrations for both metals (Fang and Hui, 1994; Lin, 1993; Zayed et al., 2000), which were significantly different from the toxic concentrations determined by the current study. Most previous studies reported only on the concentration of the added metal without considering total, soluble and free metal concentrations. Only very few studies have considered dissolved metal concentrations when evaluating Ni and Co toxicity in AD (Ashley et al., 1982; Bartacek et al., 2008). Ashley et al. (1982) reported that dissolved Ni at concentrations above 1 mg/L were toxic to the methanogenic process. Indeed, the lowest concentration of dissolved Ni to effect inhibition of the methanogenesis in the present study was 0.8 mg/L (Fig. 1). We submit it is, therefore, more relevant to measure soluble metal concentrations in such complex systems.

Some of the most relevant Ni and Co toxicity mechanisms, which could explain the reduced methane production and COD removal under Ni and Co stress in this study, include the replacement of essential metals in metalloproteins; binding to catalytic sites of non-metalloproteins; and indirectly causing oxidative stress (Chen et al., 2008; Mudhoo et al., 2013). The higher observed Ni than Co toxicity could be related to the higher dissolved concentrations of Ni than of Co even when the total concentration of each metal was the same.

The combined toxic effects of Ni and Co was higher than the sum of the toxic effects in the single-metal experiments (Fig. 2b). The higher combined effects of Ni and Co could be related to enhanced solubilization of the metals in the bimetal experiments compared to single-metal treatments, which might have increased the combined toxic effects compared to the sum of the toxic effects in the single-metal experiments.

4.2. Effects of Ni and Co on EPS content

EPS content and composition have frequently been reported to change in response to metal stress (Aquino and Stuckey, 2004; Mu et al., 2012; Ozturk et al., 2008). Reports on the effect of metals on EPS content provide contradictory results, including reports of increased, decreased or unaffected EPS production (Mu et al., 2012; Tian et al., 2019). It appears the concentration of metals plays a critical role in determining the effect on the EPS (Mu et al., 2012; Tian et al., 2019; Zeng et al.,

Table 2
Pearson's correlation results between soluble protein and carbohydrate content and metal solubility.

		Metal	Soluble metal
Bivariate correlation (Pearson)	Soluble protein	Co	N
		Ni	-0.59[a]
	Soluble carbohydrate	Co	-0.67[a]
		Ni	N
Zero-order (partial) correlation	EPS protein (controlled for soluble protein)	Co	-0.88[b]
		Ni	-0.98[b]
	EPS protein (controlled for soluble carbohydrate)	Co	-0.93[c]
		Ni	-0.91[b]

N: no correlation was found between the two variables.
[a] Correlation is significant at the 0.05 level (2-tailed).
[b] Correlation is significant at the 0.001 level (2-tailed).
[c] Correlation is significant at the 0.01 level (2-tailed).

2020b). The lower EPS protein and EPS TOC production we observed could be due to the shock-loading of metals, which possibly transcended the capacity of EPS to bind the cations so that the concentration of soluble and, in turn, free metal ions was increased and affected microbial activity including the EPS protein production (Ding et al., 2018; Sheng et al., 2005). Moreover, it has been reported that during oxidative stress certain proteases are activated, which degrade cellular proteins including EPS proteins (Ding et al., 2018).

Our observations of lower EPS protein support those of Ozturk et al. (2008) and Mu et al. (2012), who found EPS protein content reduced under metal-stressed conditions caused by chromium (15–35 ppm) and zinc. Equally, our observations of EPS carbohydrates support those of Pereira et al. (2011) that neither soluble nor EPS carbohydrate were either inhibited nor stimulated under metal-stressed conditions. This could be related to the higher resistance of carbohydrate structures to oxidative stress compared to the proteinaceous compounds (Ding et al., 2018; Mohite et al., 2018).

4.3. Relationships between metal solubility, EPS content and methanogenic activity

EPS have been reported to capture metals mainly by complexation and ion exchange mechanisms (Hawari and Mulligan, 2006; Zeng et al., 2020a). Soluble Ni and Co concentrations were found to be strongly associated with EPS protein content while no correlation was found between EPS carbohydrates and metal solubility (Table 1). Therefore, according to these results, protein components in EPS seem to be responsible for metal sequestration by EPS. It has been reported that the adsorption capacity of EPS towards metal cations is highly associated with protein content of EPS (Liu et al., 2015). The carbonyl and hydroxyl groups of proteins have been indicated to act as ligands in adsorption of Pb^{2+}, Cd^{2+}, and Zn^{2+} by EPS from anaerobic granular sludge (Liu et al., 2015). Moreover, according to Li et al. (2020), in the EPS extracted from anammox granules, carboxyl groups of proteins showed a faster response in binding Cu^{2+} compared to polysaccharides and hydrocarbons. Therefore, the reduction in EPS protein content may explain the elevated soluble metal fraction when the total metal concentrations were high. A high Pearson's coefficient (R = −0.96, p < 0.01) was also found between TOC of EPS and soluble Co concentration. This was higher than the correlation between EPS protein and soluble Co, which may be due to the impacts of other components of EPS not measured in this study (e.g., humic substances).

According to the obtained results, there was a significant association between methane production, EPS protein, EPS TOC, and soluble metal concentration (Table 1). Considering the protective role of the EPS against free metal ions, it follows that reduced production of EPS protein, and, in turn, increased soluble and free metal concentrations, could expose sensitive methanogens to free metal species, thus impairing methane production. Moreover, several catabolic enzymes have been identified in EPS structure, such that it appears to serve as an important matrix for enzymatic activities. Methyl-coenzyme M reductase (MCR) was one of the main proteins found in EPS from anaerobic sludge, and is a critical enzyme in AD combining the methyl group of coenzyme M with hydrogen from the coenzyme B to generate methane (Dubé and Guiot, 2019). One of the possible reasons for reducing methane production and COD removal under metal stress could be reduction in EPS protein which contain such necessary enzymes.

4.4. Effects of Ni and Co on SMPs

The soluble carbohydrate content of each tested concentration was less than 1% of the amount of glucose added to each reactor. Therefore, almost all amount of glucose that was added to the bioreactors was consumed at the tested concentrations of Ni and Co. This result showed that Ni, Co and both metals did not affect the acidogens function at the tested concentrations, so they converted all amount of added glucose to

intermediate products i.e. VFAs (Fig. 5a and b). This result is in line with previous reports that methanogens are the most vulnerable group in AD to toxic compounds (Mudhoo et al., 2013). That low amount of soluble carbohydrate present in the bulk media could be produced as a microbial product (i.e. EPS release) or could be the remaining amount of the glucose that was used as substrate.

Considering the low toxicity of Co at 460 mg/L, the significant decrease in protein production showed that change in extracellular soluble protein concentration seems to be one of the first responses of microbial cells in AD to metal stress that starts even at low metal toxicity level. Hassen et al. (1998) reported that the 0.1 mM of Co decreased the synthesis of proteins by approximately 24%. It has been reported that the global protein synthesis is reduced under stress condition and this helps reduce the cellular burden during stress conditions. The stress response is displayed as changes in the metabolic function of the cell, arise from the suppression of synthesis of most of the proteins produced in the cell under normal physiological conditions, and induction of the synthesis of a specific group of proteins empowering the cell to cope with the new conditions (Świeciło and Zych-Wezyk, 2013). Besides, this reduction in extracellular soluble protein could be a consequence of oxidative stress caused by metals which lead to protein degradation and affect negatively protein production pathways (Mohite et al., 2018; Palma et al., 2002).

The increase in total soluble COD with increasing total metal concentration could be due to the increase in intermediate products of glucose metabolism i.e. VFA; macromolecules resulting from cell lysis; or molecules secreted from the cells for specific purposes (Aquino and Stuckey, 2004). Indeed, although almost all of the glucose supplied to the experiments was consumed at each metal concentration tested, it was not all converted to methane implying some VFA accumulation. In fact, this was evidenced by lower pH coinciding with increasing metal concentrations (Fig. 5), and points to the overall effect on the AD process.

4.5. Relationships between metal solubility and soluble microbial products

The extracellular soluble protein and carbohydrate concentration seems not to be related to soluble metal concentration in this study (Table 2). However, the proteins produced under metal-stressed condition have been reported to be an effective factor in metal solubilization, especially for Ni (Liu et al., 2021). It has been reported that dissolution of Ni was enhanced by VFAs accumulation (Molaey et al., 2021). VFAs can act as monodentate ligand bonding to metal atoms and thus might have decreased the adsorption affinity of Ni due to weak covalent bonds with functional groups of the microbial cells.

4.6. Research limitations and future perspectives

The observed higher solubility of Ni could be related to the presence of specific ligands for Ni among SMPs which avoid this cation to be precipitated or it could be due to lower affinity of Ni to bind with precipitating agents, i.e. EPS functional groups. It has been reported that binding capacity of EPS is different for different metals (J. Wang et al., 2014; T. Wang et al., 2022). According to Yang et al. (2020), Ni had lower binding affinities to EPS compared to Co in oxalate solution that could lead to a decrease in recovery of Ni compared to Co. The higher solubility of Ni could also be related to the proteins which have been reported to be produced and released in extracellular environment under Ni-stressed conditions (Liu et al., 2021). The above-mentioned mechanisms for higher Ni solubility, could be promising for selectively recover Ni and Co through AD. Considering the specificity of protein-metal interactions compared to other cellular compounds, extracellular and EPS proteins could be potential candidates for such selectively solubilization of Ni. Therefore, an interesting direction for future work would be metaproteomic analysis of exoproteome and EPS proteins with the aim of searching for the specific proteins that are produced under Ni stress and are responsible for its higher solubility.

5. Conclusion

The results of this study revealed that Ni and Co stress in short term affect negatively the EPS and extracellular soluble protein production. The decrease in EPS content caused by Ni and Co affect their protective role on the inner microorganisms of anaerobic flocs, which was correlated with the detected lower general physiological activity of the microbial community in AD. More than 99% of the total metal was precipitated in each condition and the soluble metal faction increased along with increasing total metal concentration. Due to the EPS role in metal chelating, the increase in dissolved metal concentration possibly could be related to the decline in EPS protein content; however, the possible role of SMPs, such as VFAs, in metal solubilization should also be considered. Ni showed higher solubility and toxicity than Co at all tested conditions, which may have been due to the formation of soluble protein-Ni complexes. Therefore, future research should focus on characterization of extracellular proteins with the aim of identifying proteins with selective metal binding affinities.

CRediT authorship contribution statement

Parvin Hasani Zadeh: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Antonio Serrano:** Conceptualization, Writing – review & editing. **Gavin Collins:** Funding acquisition, Conceptualization, Writing – review & editing. **Fernando G. Fermoso:** Funding acquisition, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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