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# Trace metals supplementation in anaerobic membrane bioreactors treating highly saline phenolic wastewater

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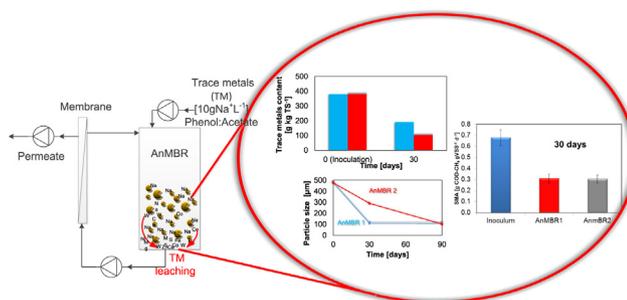
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## HIGHLIGHTS

- Metals partitioning in the biomass matrix in AnMBRs was determined.
- Tungsten supplementation improved the specific methanogenic activity by 17%.
- 50% inhibition of methanogenic activity was found at about 23 g Na<sup>+</sup> L<sup>-1</sup>.
- Sodium accumulation of about 36% and wash-out of the trace metals was experienced.
- AnMBRs reached 90% COD removal at 10 g Na<sup>+</sup> L<sup>-1</sup> by doubling the supply of trace metals.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Biomass requires trace metals (TM) for maintaining its growth and activity. This study aimed to determine the effect of TM supplementation and partitioning on the specific methanogenic activity (SMA), with a focus on cobalt and tungsten, during the start-up of two lab-scale Anaerobic Membrane Bioreactors (AnMBRs) treating saline phenolic wastewater. The TM partitioning revealed a strong accumulation of sodium in the biomass matrix and a wash-out of the majority of TM in the reactors, which led to an SMA decrease and a low COD removal of about 30%. The SMA exhibits a maximum at about 6 g Na<sup>+</sup> L<sup>-1</sup> and nearly complete inhibition at 34 g Na<sup>+</sup> L<sup>-1</sup>. The dose of 0.5 mg L<sup>-1</sup> of tungsten increases the SMA by 17%, but no improvement was observed with the addition of cobalt. The results suggested that TM were not bioavailable at high salinity. Accordingly, an increased COD removal was achieved by doubling the supply of TM.

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## 1. Introduction

An increasing number of industries generate wastewaters with high salinity and high concentrations of organic pollutants. Chemical and petrochemical industries have been identified as the major producers of these types of wastewaters. Recently, researchers have shown interest in the impact of salinity on anaerobic wastew-

ater treatment (Ismail et al., 2008; Vyrides and Stuckey, 2009; Yang et al., 2013). The presence of high concentrations of sodium chloride has a negative impact on the biological conversion with the major effect attributed to the sodium ions. Sodium is toxic when high intracellular concentrations are reached in microorganisms because of electrochemical and osmotic interactions with proteins and nucleic acids (Valentine, 2007). High salinity reduces the effectiveness of anaerobic processes (Lefebvre et al., 2007; Vallero et al., 2003) and induces the disintegration of flocs and granules, leading to a prominent biomass wash-out (Ismail et al.,

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2010; Pevere et al., 2007). The decrease in granule strength due to high sodium concentration results in a significant reduction in particle size in sludge bed reactor systems (Ismail et al., 2008; Jeison and van Lier, 2007).

In addition, saline chemical wastewaters often contain aromatic compounds such as phenolics that are not easily biodegradable (Ferrer-Polonio et al., 2016). Phenolic compounds interfere with the transmembrane electrochemical proton gradient and electron transport involved in energy production, which may lead to an inhibitory effect in the wastewater treatment process (Escher et al., 1996). Nevertheless, anaerobic biomass exhibits tolerance towards phenol (Fang and Chan, 1997). Phenol is a key intermediate in the anaerobic degradation of a wide variety of aromatics (Fuchs, 2008), and is therefore of particular interest for treating industrial chemical wastewater streams.

In order to optimize the function of microorganisms involved in anaerobic degradation pathways, attention to the trace metals (TM) content, fractionation and speciation is required (Fermoso et al., 2009; van Hullebusch et al., 2016). Especially under high salinity and presence of aromatic compounds, the supply of TM can have significant effects on the anaerobic biological treatment. Metals such as nickel, iron, cobalt, zinc, tungsten or molybdenum are contained in several enzymes, regulating the anaerobic pathways (Zandvoort et al., 2006). This work focused on the essential TM cobalt and tungsten. Cobalt was shown to play a key role in the methanogenic activity of high-rate anaerobic reactors treating phenolic wastewater and methanol (Flores et al., 1994; Melamane et al., 2007; Sharma and Singh, 2001). The polyphenol removal efficiency increased from 65 to 93% with the addition of 50 mg L<sup>-1</sup> of Co<sup>3+</sup> (Melamane et al., 2007). Similarly, tungsten plays an important role in the anaerobic degradation process (Schmidt et al., 2014). Tungsto-enzymes appear in catalytic reactions involving extremely low chemical potential that require anaerobic conditions, which is reported as stimulatory to the growth of many types of prokaryotes, including Archaea (Kletzin and Adams, 1996). Tungsten is antagonistic to molybdenum, and under certain conditions, tungsto-enzymes rather than molybdoenzymes catalyze the same degradation reactions (Boll et al., 2005). The concentration of TM that causes the inhibition or the stimulation of methanogenesis depends on their bioavailability (Osuna et al., 2004). Metals fractionation can be used for assessing the potential bioavailability of TM by taking into account the following four (4) operationally defined fractions: 1) exchangeable, 2) carbonates, 3) organic matter and sulfides, and 4) residual fraction. According to Tessier et al. (1979), these fractions are characterized by metal binding forms with declining solubility and reactivity in the order listed above.

However, only a few studies have focused on the effect on biomass activity of TM concentration, partitioning, and bioavailability (Worms et al., 2006). What is not yet well understood is the role and fate of TM and their partitioning and speciation in anaerobic bioreactors, particularly under high salinity conditions. Indeed, to the best of author's knowledge, no TM partitioning or bioavailability studies have been reported previously in AnMBR reactors.

AnMBRs have shown to be a promising technology for treating wastewaters under high salinity (Dereli et al., 2012; Jeison et al., 2008b; Vyrides and Stuckey, 2011). Therefore, the aim of this study was to determine the effect of TM supplementation and partitioning, on methanogenic biomass activity during the start-up of an AnMBR treating high salinity phenolic wastewater. The impact of sodium on the biomass and potential bioavailability of TM are evaluated by using laboratory-scale batch reactors and continuous flow AnMBRs. The findings of this study will contribute to the successful start-up and operation of AnMBRs, as treatment of hazardous and saline wastewaters is becoming more important.

## 2. Materials and methods

### 2.1. Batch reactor tests

#### 2.1.1. SMA response to sodium concentration

The effect of increasing sodium concentrations on the SMA was assessed using the AnMBR inoculum. A medium with only sodium chloride (NaCl) and one with NaCl and sodium bicarbonate (NaHCO<sub>3</sub>) (ratio 1:1 Na<sup>+</sup> w/w), both maintaining the same total sodium concentration. SMA tests were performed in duplicate using an automated methane potential test system (AMPTS, Bioprocess Control, Sweden). The initial pH of all SMA test bottles was adjusted to 7.0 (18 ± 0.4 °C).

#### 2.1.2. Phenol:acetate ratio effect on methanogenic activity

SMA tests using different ratios of phenol:acetate as substrate were carried out in triplicate. The ratio was varied from 0 (acetate as a sole substrate) to 1 (50% COD from acetate and 50% COD from phenol), while keeping a total COD concentration of 2 g COD L<sup>-1</sup>. The detailed concentrations are presented in Table 1.

#### 2.1.3. Effect of TM on methanogenic activity: tungsten and cobalt

The effect of cobalt, both in the form of CoCl<sub>2</sub> and vitamin B<sub>12</sub>, and tungsten on the methane production rate was investigated using SMA assays in triplicate. These assays were carried out with a mixture of phenol and sodium acetate in a COD ratio 1:4 and two different medium compositions A and B. In medium A, the sodium concentration was composed of 10 g NaCl L<sup>-1</sup> and 18.3 g NaHCO<sub>3</sub> L<sup>-1</sup> and the solution was buffered with 1.05 g K<sub>2</sub>HPO<sub>4</sub> L<sup>-1</sup> and 0.5 g NaH<sub>2</sub>PO<sub>4</sub> L<sup>-1</sup>. The macro and micro nutrients composition in the model wastewater was as follows (in mg L<sup>-1</sup> unless otherwise indicated): NH<sub>4</sub>Cl, 1020; CaCl<sub>2</sub>·2H<sub>2</sub>O, 48; MgSO<sub>4</sub>·7H<sub>2</sub>O, 54; FeCl<sub>3</sub>·6H<sub>2</sub>O, 1.2; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.3; CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.02; ZnCl<sub>2</sub>, 0.03; H<sub>3</sub>BO<sub>3</sub>, 0.03; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 0.05; Na<sub>2</sub>SeO<sub>3</sub>, 0.06; NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.03; EDTA, 0.6; resazurin, 0.3; yeast extract, 1.2; HCl 35%, 6·10<sup>-4</sup> ml L<sup>-1</sup>. In medium B, the composition of sodium compounds was changed while keeping the same total sodium concentration, i.e., 10 g Na<sup>+</sup> L<sup>-1</sup>, by using 16.7 g NaCl L<sup>-1</sup> and 9 g NaHCO<sub>3</sub> L<sup>-1</sup>. Typical sea water contains about 10.5 g Na<sup>+</sup> L<sup>-1</sup>. Micronutrients concentration in medium B were doubled from medium A, resulting in the following composition (in mg L<sup>-1</sup> unless otherwise indicated): NH<sub>4</sub>Cl, 1020; CaCl<sub>2</sub>·2H<sub>2</sub>O, 48; MgSO<sub>4</sub>·7H<sub>2</sub>O, 54; FeCl<sub>3</sub>·6H<sub>2</sub>O, 2.4; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.6; CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.04; ZnCl<sub>2</sub>, 0.06; H<sub>3</sub>BO<sub>3</sub>, 0.06; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 0.1; Na<sub>2</sub>SeO<sub>3</sub>, 0.12; NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.06; EDTA, 1.2; resazurin, 0.6; yeast extract, 2.4; HCl 35%, 6·10<sup>-4</sup> ml L<sup>-1</sup>. SMA assays were performed with different concentrations of cobalt and tungsten as indicated in Table 2. In the tungsten assays, the cobalt content was 0.29 mg Co L<sup>-1</sup> and 0.58 mg Co L<sup>-1</sup> for medium A and B, respectively. In the cobalt assays, no tungsten was added.

### 2.2. AnMBR

#### 2.2.1. Inoculum

Two reactors were seeded with concentrated anaerobic biomass obtained from a full-scale UASB reactor treating industrial wastewater (Shell, Moerdijk, The Netherlands) mainly containing acetate

**Table 1**  
Substrate phenol and sodium acetate concentrations in batch assays.

Phenol:acetate COD ratio	Phenol [mg L <sup>-1</sup> ]	CH <sub>3</sub> COONa·3H <sub>2</sub> O [mg L <sup>-1</sup> ]
0.00	0	4250
0.25	168.1	3400
0.43	252.1	2975
0.67	336.1	2553
1.00	420.2	2127

**Table 2**

Cobalt, as CoCl<sub>2</sub> or vitamin B<sub>12</sub>, and tungsten concentrations added to mediums A and B in SMA tests. All tests were carried out in triplicate.

Medium	Cobalt source	Cobalt Concentration [mg L <sup>-1</sup> ]	Tungsten Concentration [mg L <sup>-1</sup> ]
A	CoCl <sub>2</sub>	0	–
A	CoCl <sub>2</sub>	0.29	–
A	CoCl <sub>2</sub>	5	–
A	CoCl <sub>2</sub>	10	–
A	CoCl <sub>2</sub>	20	–
B	CoCl <sub>2</sub>	0.58	–
A	Vitamin B <sub>12</sub>	0.29	–
A	Vitamin B <sub>12</sub>	5	–
B	Vitamin B <sub>12</sub>	0.58	–
A	CoCl <sub>2</sub>	0.29	0
A	CoCl <sub>2</sub>	0.29	0.5
A	CoCl <sub>2</sub>	0.29	1
A	CoCl <sub>2</sub>	0.29	3
A	CoCl <sub>2</sub>	0.29	5
B	CoCl <sub>2</sub>	0.58	0.5

and benzoate. Three consecutive samplings were conducted before the inoculation to characterize the biomass. Total solids (TS), volatile solids (VS) and volatile suspended solids (VSS) concentrations of the inoculum used were 96 g L<sup>-1</sup>, 57 g L<sup>-1</sup>, and 55 g L<sup>-1</sup>, respectively. The alkalinity of the inoculum was 19 g CaCO<sub>3</sub> L<sup>-1</sup>, electrical conductivity was 22.5 mS cm<sup>-1</sup> (19.1 °C) and pH was 8.1 (19.2 °C).

### 2.2.2. Experimental set-up

The continuous flow experiments were performed using two laboratory scale AnMBR reactors with an effective volume of 6.5 L, in conjunction with ultra-filtration (UF) membrane module. The system was equipped with feed, recycle and effluent pumps (Watson-Marlow 120U/DV, 220Du), pH and temperature sensors (Endress & Hauser, Memosens), and a gas meter (Ritter, Milligas Counter MGC-1 PMMA). A biogas recirculation diaphragm pump (KNF) at a rate of 40 L h<sup>-1</sup> was used to mix the liquor. The temperature of the jacketed reactor was kept constant at 35.0 ± 0.8 °C by a thermostatic water bath (Tamson Instruments, The Netherlands). TMP was measured by three pressure sensors (AE Sensors ATM, The Netherlands). A tubular PVDF membrane (Pentair, The Netherlands) with 5.2 mm inner diameter, pore size of 30 nm and 0.64 m length was employed. The cross-flow velocity was kept constant at 0.6 m s<sup>-1</sup>. The two reactors were operated in parallel under similar conditions. The experimental system was controlled by a computer running LabView software (National Instruments, USA).

### 2.2.3. Start-up procedure

The reactors were inoculated at a concentration of 30 g VSS L<sup>-1</sup>. The model wastewater contained 10 g Na<sup>+</sup> L<sup>-1</sup> (medium A). The AnMBRs were continuously fed during a start-up period of three months. In the first month, acetate was used as the sole carbon source in the model wastewater. Hereafter, phenol was added, starting at 10 mg L<sup>-1</sup> and gradually increased once the start-up period was completed.

### 2.2.4. Particle size distribution

PSD analysis was carried out by using a DIPA-2000 Eyeteck particle analyzer (Donner Technologies) with a B100 laser lens (measuring range 10–2000 μm) and liquid flow cell DCM-104A (10x10 mm). Values D10, D50, and D90 from the volume particle size distribution were reported.

### 2.3. Metals partitioning in biomass matrix

A modified Tessier sequential extraction procedure proposed by Osuna et al. (Osuna et al., 2004) was used to assess the TM parti-

tioning in the exchangeable, the carbonate, the organic matter and sulfides, and the residual operationally defined fractions. For this purpose, 1 g TS of anaerobic biomass was treated as described in Table 3. Closed pressurized vessels and a microwave oven (Milestone MLS 1200 Mega) were used to carry out the extraction of the residual fraction. The heating period was 28 min at different microwave powers as follows: 2 min at 250 W, 2 min at 0 W, 5 min at 250 W, 5 min at 400 W and 14 min at 650 W.

### 2.4. Biomass and supernatant metal content determination

Inductively coupled plasma/optical emission spectrometry (ICP-OES) was used to characterize the main metals present in the biomass after the sequential extraction and in the supernatant. All samples were filtered through polyethersulfone syringe filters (0.45 μm, VWR), and two dilutions (1:50 and 1:1000) were made in a solution of nitric acid (HNO<sub>3</sub> 3% v/v) for analysis by the ICP-OES spectrometer (Spectro Arcos).

### 2.5. Statistical analysis

Means and standard deviations were calculated from the assays carried out in triplicate. The differences in SMA biomass activities between the test treatments were analyzed by one-way analysis of variance (ANOVA). Post hoc analysis was performed using the Tukey's honest significant difference (HSD) test of multiple means comparison at a significance level of α = 0.05. Two-way ANOVA was used for the evaluation of particle size distribution data. A four parameter logistic function, i.e.,  $f(x, (a, b, c, d)) = d + (a - d) / (1 + 10^{(x - \log c)^b})$ , was used to fit the experimental data from the sodium concentration-SMA response curves. The half maximal inhibition concentration (IC<sub>50</sub>) was estimated. The analysis was performed using OriginPro 8 (OriginLab Corporation, Northampton, USA).

## 3. Results and discussion

### 3.1. SMA response to sodium concentration and inoculum characterization

A deterioration of the biomass activity was observed during the start-up period in the AnMBRs 1 and 2. The SMA of the biomass decreased from 0.68 ± 0.07 g COD-CH<sub>4</sub> g VSS<sup>-1</sup> d<sup>-1</sup> to 0.31 ± 0.04 g COD-CH<sub>4</sub> g VSS<sup>-1</sup> d<sup>-1</sup> after one month in both reactors. Other studies also observed a decrease in removal performance due to the negative effect of the salinity increase on the bioreactor activity (Song et al., 2016). The SMA of the inoculum was about 0.80 g COD-CH<sub>4</sub> g VSS<sup>-1</sup> d<sup>-1</sup> at a sodium concentration of about 6 g Na<sup>+</sup> L<sup>-1</sup> as depicted in Fig. 1. As shown by Feijoo et al. (1995), a sodium concentration between 4.4 and 17.7 g Na<sup>+</sup> L<sup>-1</sup> may result in 50% inhibition of the methane production rate. According to the results, a sodium concentration of about 23 g

**Table 3**

Operating conditions required in sequential extraction method. Modified from Van Hullebusch et al. (2005).

Fraction	Extracting agent	Extraction conditions	
		Shaking time	Temperature
Exchangeable	10 ml NH <sub>4</sub> CH <sub>3</sub> COO 1 M	1 h	20 °C
Carbonates	10 ml CH <sub>3</sub> COOH 1 M	1 h	20 °C
Organic matter and sulfides	5 ml H <sub>2</sub> O <sub>2</sub> 30% (pH = 2)	3 h	35 °C
Residual	10 ml aqua regia (HCl:HNO <sub>3</sub> 3:1)		Microwave oven

$\text{Na}^+ \text{L}^{-1}$  decreased the methanogenic activity to half of the maximum value and was regarded as the 50% inhibition concentration ( $\text{IC}_{50}$ ). Almost complete inhibition was found at  $34 \text{ g Na}^+ \text{L}^{-1}$ . This exposure of the biomass to high salinity could result in cell plasmolysis and corresponding loss of activity (Yogalakshmi and Joseph, 2010).

The tendency observed in the SMA response to sodium concentration is similar to that found in previous studies (Jeison et al., 2008a). However, in our present study higher specific methanogenic activities were observed with a similar source of inoculum, with values of up to four times higher for sodium concentrations in the range of 0–12  $\text{g Na}^+ \text{L}^{-1}$ . For all sodium concentrations tested, the use of only sodium chloride resulted in a higher methanogenic activity. Nevertheless, from the inoculum supernatant metals characterization (Table 4) the mass ratio  $\text{Na}^+:\text{Cl}^-$  ( $4.65 \text{ mg mg}^{-1}$ ) was higher than expected ( $0.64 \text{ mg mg}^{-1}$ ) given that all sodium would have only originated from sodium chloride. Furthermore, it is shown in Table 4, that the presence of tungsten had a remarkable concentration variation ( $1.1\text{--}18.5 \text{ mg W}^{+6} \text{L}^{-1}$ ). The major variation (>40%) was found in the third sample for the ions  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ , and  $\text{W}^{+6}$ , whereas a less significant variation was observed for  $\text{Ca}^{2+}$ ,  $\text{K}^+$ , and  $\text{PO}_4^{3-}$ . These variations could be attributed to salinity swing fluctuations due to changes in operation from the production plant (Shell, Moerdijk). Additionally, the  $\text{K}^+/\text{Na}^+$  ratio, a key parameter for maintaining biodegradation activity under saline conditions, ranged from 0.14 to  $0.045 \text{ mg mg}^{-1}$  in the inoculum according to the ICP-OES analysis. The  $\text{K}^+/\text{Na}^+$  ratio was kept constant at 0.05 in the model wastewater.

### 3.2. Effect of phenol:acetate ratio on methanogenic activity

SMA tests were performed with inoculum biomass under varying phenol:acetate COD ratios in medium A to assess the potential toxicity of phenol on biomass activity in the reactor operation. The results (Fig. 2) show that the biomass methanogenic activity decreased while increasing the phenol:acetate COD ratio. An SMA of about  $0.25 \pm 0.01 \text{ g COD-CH}_4 \text{ g VSS}^{-1} \text{ d}^{-1}$  was obtained with a ratio of 1, i.e., adding  $420.2 \text{ mg Ph L}^{-1}$  to the medium, implying a reduction of about 43% of the SMA compared to the maximum found with no phenol addition ( $0.44 \pm 0.01 \text{ g COD-CH}_4 \text{ g VSS}^{-1} \text{ d}^{-1}$ ) within these assays. A similar SMA value was found by Razo-Flores et al. (2003) when starting-up a UASB reactor with biomass with acetate medium and subsequent addition of a mixture of phenolic compounds. The observed SMA reduction is in accordance with Fang and Chan (1997) who showed a decrease in SMA with the increase in phenol concentration. The concentration

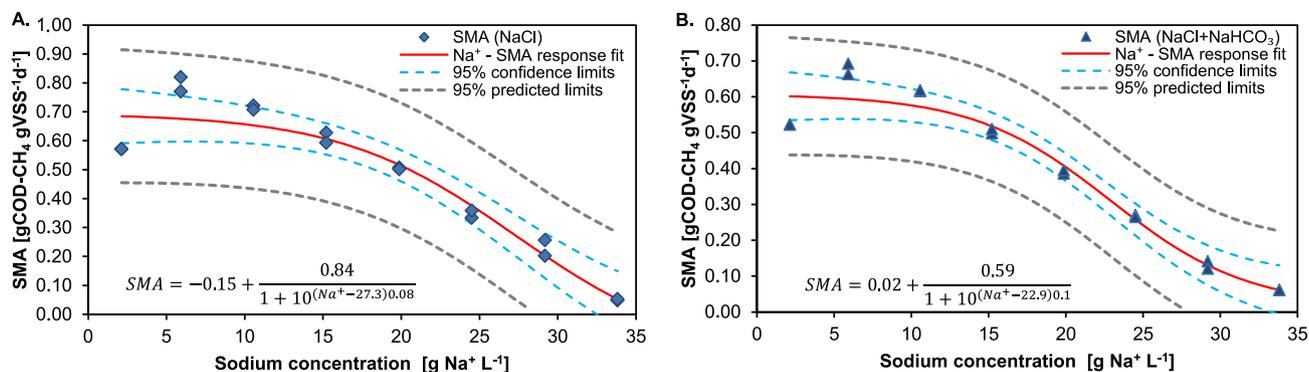
of phenol in these assays is in accordance with average medium-high phenol concentrations (from 120 to  $1200 \text{ mg L}^{-1}$ ) that are related to industrial wastewaters such as oil refineries ( $6$  to  $500 \text{ mg L}^{-1}$ ), coke ( $28$  to  $3900 \text{ mg L}^{-1}$ ) and petrochemical wastewaters ( $2.8$  to  $1220 \text{ mg L}^{-1}$ ) (Rosenkranz et al., 2013). However, the SMA obtained with a phenol:acetate COD ratio of 1 at the start of the test is still higher than that reported in previous studies using the same biomass source without any recalcitrant or toxic compound present but under similar salinity conditions (Ismail et al., 2008; Jeison et al., 2008a; Yang et al., 2013).

### 3.3. Effect of tungsten on methanogenic activity

Because tungsten stimulates the growth of many types of prokaryotes, including methanogenic Archaea (Kletzin and Adams, 1996; Plugge et al., 2009) the role of this trace metal and its dosage were assessed. Medium A was used in SMA tests with increasing concentrations of tungsten. A 17% increase in the methane production rate was achieved with the addition of 0.5 and  $1 \text{ mg L}^{-1}$  of tungsten in comparison with the reference test with no tungsten addition (Fig. 3. A). Feng et al. (2010) observed an increase in methane production efficiency from 7 to 15% for an anaerobic reactor treating food industry waste by adding a tungsten concentration of  $1.8 \text{ mg W L}^{-1}$ . However, in our current study, the presence of tungsten concentrations higher than  $1 \text{ mg W L}^{-1}$  caused a decrease in SMA, suggesting partial inhibition under the conditions applied in batch, resulting in about one-third of the maximum SMA with the addition of  $5 \text{ mg W L}^{-1}$ .

As the assessment of the potential bioavailability of trace metals by sequential extraction is based on the availability of metal binding forms for microbial uptake, metals associated with the exchangeable fraction are defined as the most available and those associated with the residual fraction as the least available (Tessier et al., 1979). Comparing the SMA results with the distribution of tungsten over the biomass fractions, it is postulated that particularly its presence in the exchangeable fraction, the most available one, caused a decline in SMA, as observed after the addition of  $5 \text{ mg W L}^{-1}$  (Fig. 3. A). The relative increase in tungsten concentration in the organic matter and sulfides fraction indicates its high affinity to this fraction.

The biomass in the presence of  $0.5 \text{ mg W L}^{-1}$  doubled the tungsten content in the organic matter and sulfides fraction in comparison with the inoculum, but with concentrations higher than  $1 \text{ mg W L}^{-1}$ , the content of tungsten in the organic matter and sulfides fraction was four times higher in comparison with no tungsten addition (Fig. 3. B). As expected, the increase in the tungsten concentration in the organic matter and sulfides fraction did not

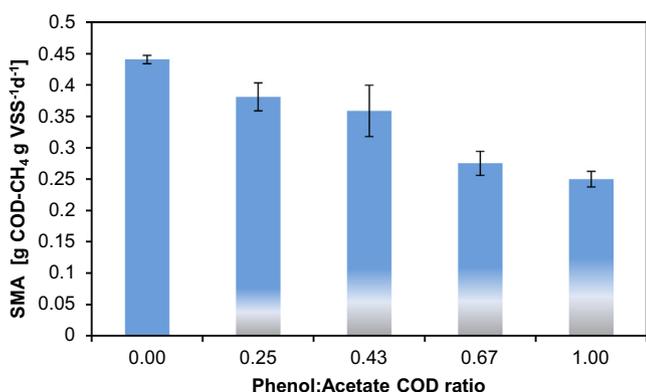


**Fig. 1.** Specific methanogenic activity response to sodium concentrations. A. Sodium concentration from NaCl with 95% confidence limits of SMA. The logistic model fit the data adequately,  $\chi^2 = 0.009$ , adj.  $R^2 = 0.965$ . The four parameters standard errors were  $SE_a = 0.05$ ,  $SE_b = 0.03$ ,  $SE_c = 3.01$ ,  $SE_d = 0.21$ .  $\text{IC}_{50\text{estimated}} = 27.3$ . B. Sodium concentration from NaCl and  $\text{NaHCO}_3$  (1:1  $\text{Na}^+$  w/w) with 95% confidence limits of SMA. The logistic model fit the data satisfactorily,  $\chi^2 = 0.005$ , adj.  $R^2 = 0.976$ . The four parameters standard errors were  $SE_a = 0.03$ ,  $SE_b = 0.02$ ,  $SE_c = 0.88$ ,  $SE_d = 0.03$ .  $\text{IC}_{50\text{estimated}} = 22.9$ . Both fitted curves significantly explained the SMA,  $R^2_{\text{NaCl}} = 0.97$ ,  $F_{\text{NaCl}}(4,15) = 119.08$ ,  $p < 0.001$ ;  $R^2_{\text{NaCl+NaHCO}_3} = 0.98$ ,  $F_{\text{NaCl+NaHCO}_3}(4,15) = 168.51$ ,  $p < 0.001$ .

**Table 4**

Main ions present in the inoculum supernatant characterized by ICP-OES. Samples 1–3 were taken in three different sampling campaigns. All concentrations in  $\text{mg L}^{-1}$ .

Sampling campaign	$\text{Ca}^{2+}$	$\text{K}^+$	$\text{Na}^+$	$\text{Cl}^-$	$\text{PO}_4^{3-}$	$\text{SO}_4^{2-}$	$\text{W}^{*6}$
1	871.7	1264.0	8956.5	1933.0	1859.7	201.1	18.5
2	800.0	1294.0	9632.0	1958.0	1815.0	174.1	6.6
3	833.3	844.0	18430.0	4189.5	1692.7	9.52	1.1



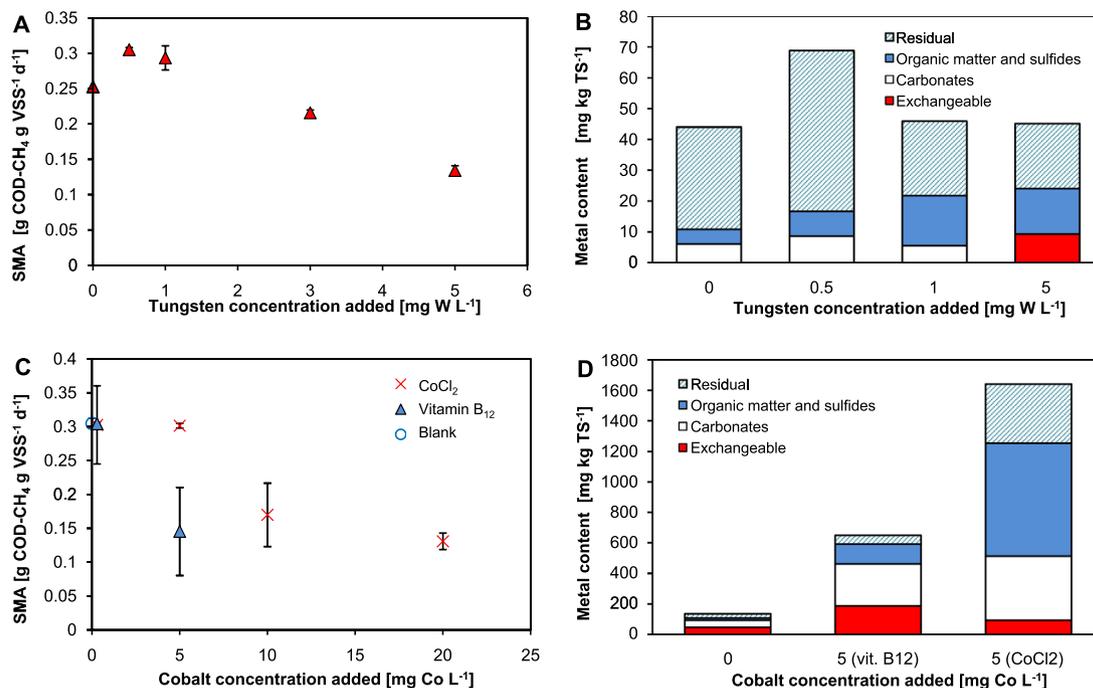
**Fig. 2.** Specific methanogenic activity under different phenol:acetate COD ratios. The means of all SMA were significantly different,  $F(4,10) = 33.46$ ,  $p < 0.001$ . Tukey's test for significance specified that at the 0.05 level, the means difference did not significantly differ between the phenol:acetate COD ratios of 0 ( $M = 0.44$ ,  $SD = 0.01$ ) and 0.25 ( $M = 0.38$ ,  $SD = 0.02$ ); 0.25 and 0.43 ( $M = 0.36$ ,  $SD = 0.04$ ); 0.67 ( $M = 0.28$ ,  $SD = 0.02$ ) and 1.0 ( $M = 0.25$ ,  $SD = 0.01$ ).

result in any improvement of methane production. Furthermore, at  $0.5 \text{ mg W L}^{-1}$ , an increase in the tungsten content of 36.4% and 53.4% in the residual fraction and 29.8% and 36% in the carbonate fraction was observed in comparison with the inoculum and biomass exposed to  $1 \text{ mg W L}^{-1}$ , respectively. Consequently, the

optimum dosage of tungsten was determined to be  $0.5 \text{ mg W L}^{-1}$  since it matched to the maximum SMA found.

### 3.4. Effect of cobalt on methanogenic activity

Similarly, the effect of cobalt addition on the methane production rate was evaluated through SMA assays using inoculum biomass under different concentrations of cobalt as  $\text{CoCl}_2$  and vitamin  $\text{B}_{12}$ . Cobalt-containing carbon monoxide dehydrogenase complex is involved in the acetate and phenol degradation via 4-hydrogenate and the benzoyl-CoA pathway (Levén et al., 2012). Furthermore, cobalt is present in methylcob(III)alamin:coenzyme M methyltransferase which is a key intermediate in methanogenesis (Zandvoort et al., 2006). The results are depicted in Fig. 3. C and D. Medium A was used as the macro/micronutrients solution. The maximum SMA was measured without cobalt (blank) in the presence of  $0.29 \text{ mg L}^{-1}$  of cobalt as vitamin  $\text{B}_{12}$  or  $\text{CoCl}_2$ , and  $5 \text{ mg L}^{-1}$  of cobalt as  $\text{CoCl}_2$ . The increase from 0 to  $5 \text{ mg Co L}^{-1}$  had no impact on the methane production rate. A decrease by approximately one-third in the SMA in comparison with the maximum SMA was observed with the addition of  $5 \text{ mg Co L}^{-1}$  as vitamin  $\text{B}_{12}$ , whereas  $20 \text{ mg Co L}^{-1}$  as  $\text{CoCl}_2$  was needed to achieve a similar decrease in the SMA. Results suggest that a likely higher bioavailability of cobalt as vitamin  $\text{B}_{12}$  caused the inhibition of the methane production with lower cobalt concentration



**Fig. 3.** A. Specific methanogenic activity of the inoculum using medium A at different concentrations of tungsten added. SMA results were statistically significant,  $F(4,10) = 220.59$ ,  $p < 0.001$ . Tukey's test revealed that at the 0.05 level, the difference of the means was not significant between  $0.5 \text{ mg W L}^{-1}$  ( $M = 0.30$ ,  $SD = 0.00$ ) and  $1 \text{ mg W L}^{-1}$  ( $M = 0.29$ ,  $SD = 0.01$ ). B. Partitioning of tungsten ( $\text{mg kg TS}^{-1}$ ) in the operationally defined fractions of the biomass from SMA assays. C. Specific methanogenic activity of the inoculum biomass using medium A at different concentrations of cobalt as  $\text{CoCl}_2$  and vitamin  $\text{B}_{12}$ . SMA results were statistically significant,  $F_{\text{CoCl}_2}(4,10) = 39.91$ ,  $p < 0.001$  and,  $F_{\text{vitamin B}_{12}}(2,6) = 17.53$ ,  $p = 0.003$ . Tukey's test inferred that the difference of the means between the blank and  $0.29 \text{ mg Co L}^{-1}$  was not significant at the 0.05 level for both  $\text{CoCl}_2$  and vitamin  $\text{B}_{12}$ . D. Partitioning of cobalt ( $\text{mg kg TS}^{-1}$ ) in the operationally defined fractions of the biomass from SMA assays.

compared to the  $\text{CoCl}_2$  test. Bhattacharya et al. (1995) observed an inhibition of methanogenesis of 7 and 17% when  $\text{CoCl}_2$  was added as cobalt source in a concentration of 600 and 800  $\text{mg Co L}^{-1}$ , respectively, demonstrating the importance of determining the optimum cobalt dosage.

Cobalt partitioning was assessed with biomass to which no cobalt was added and with biomass supplemented with 5  $\text{mg Co L}^{-1}$  as  $\text{CoCl}_2$  or vitamin  $\text{B}_{12}$ . Cobalt was present in all fractions in the reactors operated with no cobalt addition (Fig. 3. D). Apparently, the inoculum biomass contained the minimum cobalt levels to achieve maximum SMA values. An increase in total cobalt content was observed with the addition of  $\text{CoCl}_2$  or vitamin  $\text{B}_{12}$ , showing the availability of active sites in the biomass to adsorb and accumulate a higher quantity of cobalt. The affinity of cobalt in the form of  $\text{CoCl}_2$  to the carbonate, organic matter and sulfides, and residual fraction caused higher accumulation of cobalt in the presence of 5  $\text{mg Co L}^{-1}$  as  $\text{CoCl}_2$  in comparison to those with 5  $\text{mg Co L}^{-1}$  as vitamin  $\text{B}_{12}$ . In the presence of vitamin  $\text{B}_{12}$ , cobalt concentration in the exchangeable fraction was doubled, corroborating the higher affinity of vitamin  $\text{B}_{12}$  to the exchangeable fraction.

### 3.5. Particle size impacted by sodium

The particle size of the biomass significantly decreased during the membrane bioreactor's start-up period. The results of the particle size distribution analysis demonstrated a drastic reduction after three months of operation from an initial median size (D50) of  $424 \pm 73 \mu\text{m}$  to  $112 \pm 4.1 \mu\text{m}$  and  $100 \pm 2.5 \mu\text{m}$  for the AnMBRs 1 and 2, respectively (Fig. 4). Both the high shear force in the side stream AnMBR and the high sodium concentration may have contributed to the particle size reduction, the latter because of its negative impact on the particle strength (Ismail et al., 2008). This is in agreement with the observations made by Jeison et al. (2008a) who operated reactors at sodium concentrations of about 10, 12 and 7.5  $\text{g Na}^+ \text{L}^{-1}$  and found a significant reduction in particle size in comparison to the inoculum. The reduction in granule strength was higher in the reactors operated at a higher salinity level.

Unexpectedly, the reduction of particle size in reactor 2 was 48% less after one month than in reactor 1, indicating a different biomass matrix in the two reactors, but analogous distribution was observed in the third month with similar D10 and D90 values. It should be noted that free cells or smaller biomass particles are

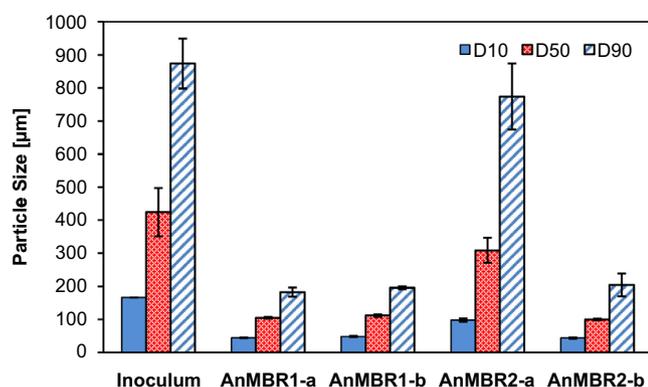


Fig. 4. Particle size distribution D10, D50, and D90 corresponding to the percentages 10%, 50%, and 90% of particles under the reported particle size. a. One month after inoculation. b. Three months after inoculation. Particle size results were statistically significant,  $p < 0.001$ . Tukey's test specified that the means difference is not significant at the level of 0.05 between the inoculum and AnMBR2 after one month, and between the AnMBR1 and AnMBR2 after three months.

subject to reduced mass transfer limitation and thus more sensitive to toxicity.

### 3.6. Effect of trace metals composition on AnMBR methanogenic activity

The response of biomass from the AnMBRs 1 and 2 in the presence of medium B was assessed in batch reactors after one and two months of operation. The inoculum biomass was used as the control. The experiments were carried out with the addition of  $\text{CoCl}_2$ , vitamin  $\text{B}_{12}$  or tungsten. Cobalt concentration was double (0.58  $\text{mg Co L}^{-1}$ ) in comparison to the concentration applied to the reactors, but for tungsten, the optimal supply determined in previous SMA assays (0.5  $\text{mg W L}^{-1}$ ) was added. A wash-out of essential TM from the biomass in the AnMBR reactors applying high salinity conditions could have been the cause for the observed lower SMA values after one month of reactor operation (Table 5). A similar effect of high salinity was observed by Ismail et al. (2010) with 38% decrease in  $\text{Ca}^{2+}$  biomass content after adding 20  $\text{g Na}^+ \text{L}^{-1}$  to the influent.

The assays with 0.5  $\text{mg W L}^{-1}$  showed a decrease in SMA compared to SMA with inoculum biomass. The latter indicated that the role of this metal changed during the reactor operation without any addition of tungsten, which could be attributed to changes in the distribution of metals concentration in biomass as observed by Espinosa et al. (1995) in a UASB reactor during different operational periods.

### 3.7. Metals partitioning and concentration from AnMBRs

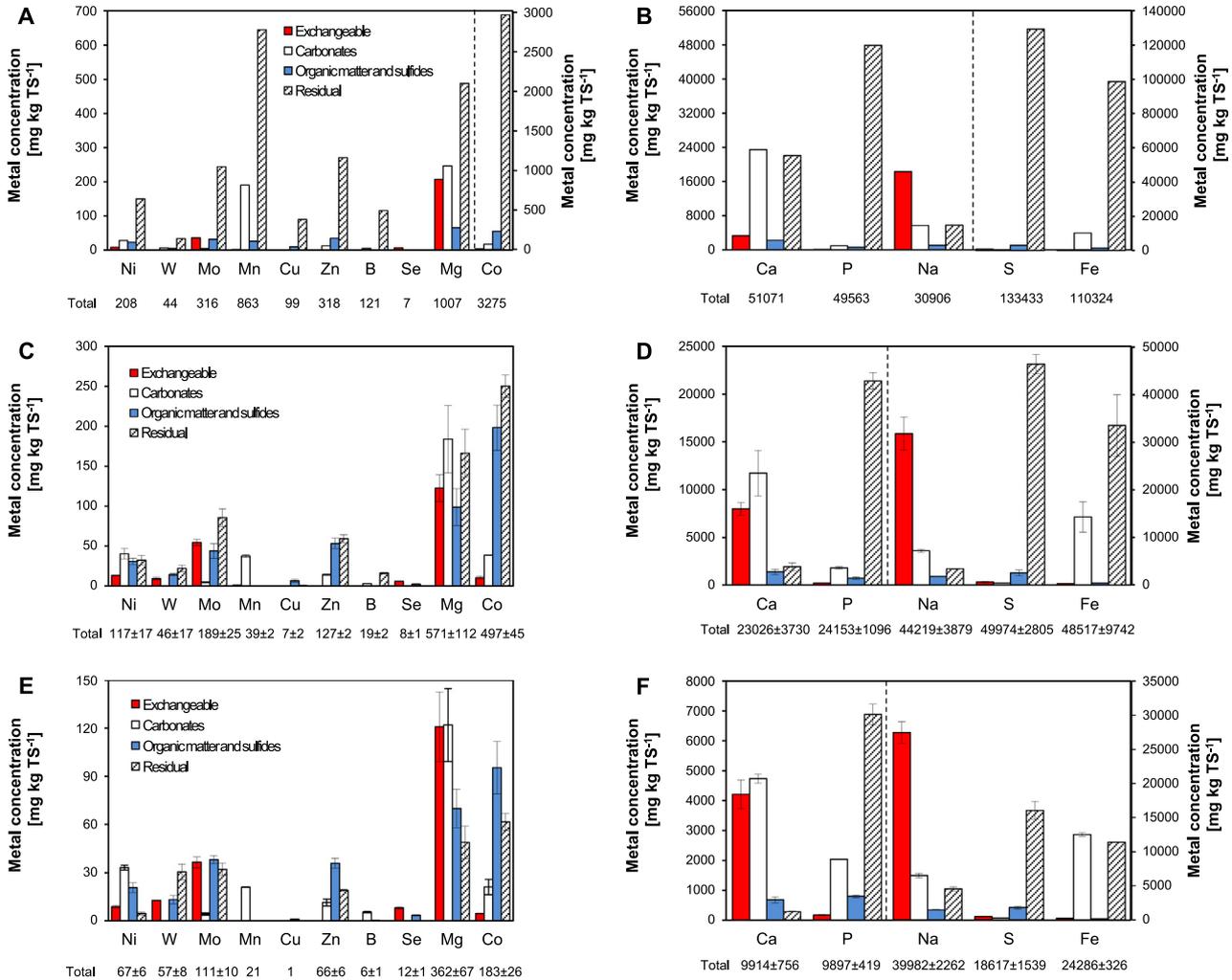
Fig. 5 shows the partitioning of trace and major elements in the operationally defined fractions and total concentrations in the inoculum and the biomass from the two AnMBRs. The inoculum biomass showed a high content of the TM cobalt and iron (higher than 3000  $\text{mg kg TS}^{-1}$ ) and a relatively high content of molybdenum, manganese, and zinc (about 300  $\text{mg kg TS}^{-1}$ ), which were mainly associated with the residual fraction. The content of tungsten was 44  $\text{mg kg TS}^{-1}$ , and it was bound to the carbonates (13.6%), organic matter and sulfides (10.9%), and residual (75.5%) fractions (Fig. 5. A). Phosphorus and magnesium were found to be predominantly associated with the residual fraction (96.7 and 48.4% of total amount, respectively), whereas sodium showed higher affinity to the exchangeable fraction (59.3%) and, calcium to the carbonates (45.9%) and residual (43.2%) fractions (Fig. 5. B). Even though a portion of about 11% of the total TM content in the inoculum biomass was found in the exchangeable and carbonates fractions (the more soluble ones), the TM amount found in these fractions was reasonable for ensuring bioavailability in the inoculum, except for copper.

A decrease in the total content of all metals in the biomass of AnMBRs was observed one month after inoculation, except for sodium and selenium. Moreover, a higher metal solubilization was observed in reactor 2, resulting in 46% less metal content in reactor 2 compared to reactor 1, whereas a different metal partitioning was found in each reactor. The major reduction in the total

Table 5

Specific methanogenic activity from inoculum and reactors biomass after one month of operation. Batch test using medium B with the addition of tungsten and cobalt as  $\text{CoCl}_2$  and Vitamin  $\text{B}_{12}$ .

Trace metal added	SMA [ $\text{g COD-CH}_4\text{:g VSS}^{-1} \text{d}^{-1}$ ]		
	Inoculum	AnMBR1	AnMBR2
0.58 $\text{mg Co L}^{-1}$ as $\text{CoCl}_2$	$0.43 \pm 0.01$	$0.10 \pm 0.01$	$0.11 \pm 0.01$
0.58 $\text{mg Co L}^{-1}$ as Vitamin $\text{B}_{12}$	$0.35 \pm 0.02$	$0.09 \pm 0.01$	$0.10 \pm 0.01$
0.5 $\text{mg W L}^{-1}$ as $\text{Na}_2\text{WO}_4$	$0.36 \pm 0.01$	$0.06 \pm 0.02$	$0.06 \pm 0.01$



**Fig. 5.** Trace and macro elements concentration [mg kg TS<sup>-1</sup>], and partitioning in the operationally defined fractions after one month of operation. A, B. Inoculum biomass. C, D. AnMBR1. E, F. AnMBR2. After dashed line the elements concentration must be read from the secondary y-axis.

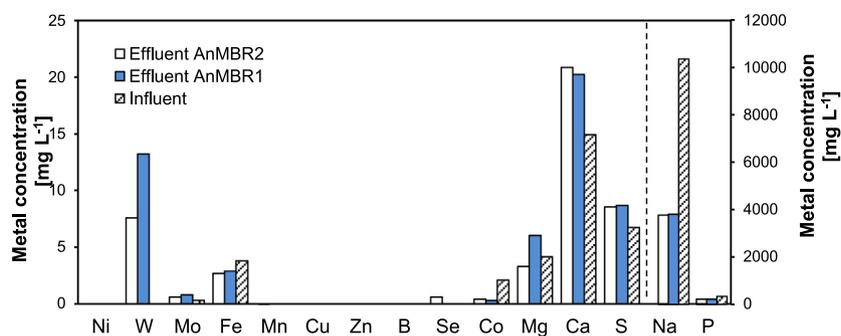
metal content was found in the residual fraction, 64% reduction in reactor 1 and 86% in reactor 2. This difference in the biomass metal matrix could also explain the observed discrepancy in particle size reduction in both reactors during this period of operation.

In contrast, an increase in the nickel and iron concentration in the exchangeable fraction was measured in both reactors, while cobalt and molybdenum concentrations only increased in this fraction in reactor 1 and selenium in reactor 2, indicating an increase in the bioavailability of these metals for the biomass during the start-up period (Fig. 5 C and E). Evidently, the metal composition of the reactor's influent impacted the chemical equilibrium of the initial biomass metal content, therefore affecting the biomass metal partitioning, as was also observed by Bhatti et al. (1995).

In spite of the high solubilization of metals into the reactors, relatively low concentrations of all TM were measured after ultrafiltration through the reactor's membrane after one month of operation (see Fig. 6), except for tungsten, molybdenum, and selenium. This suggests that the wash-out of the main portion of TM took place during the first month after inoculation. The concentration in the effluent of molybdenum and tungsten in both reactors was higher than in the influent (no addition of tungsten in the influent) showing that the solubilization and wash-out of these TM were slower than for the other metals and were still ongoing after one month of operation. According to Fig. 5. C and D, solubi-

lization of tungsten mainly occurred in the carbonates fraction, whereas the content in the exchangeable fraction increased from non-detectable to 9.2 and 12.8 mg kg TS<sup>-1</sup> in reactor 1 and 2, respectively. The content in the organic matter and sulfides fraction increased to nearly three times its original value in both reactors. The solubilization could have caused a change in the role played by tungsten in the anaerobic conversion of organic matter to methane, as shown by the SMA decrease (Table 5). With respect to the solubilization of molybdenum, similar molybdenum content was observed in the carbonates and the organic matter and sulfides fractions in the inoculum and reactors, showing that the major reduction in molybdenum was in the residual fraction. Selenium uptake in biomass was associated with the organic matter and sulfides fraction as was evidenced by the increase in selenium content in this fraction from 0 mg kg TS<sup>-1</sup> to 2.4 mg kg TS<sup>-1</sup> in reactor 1 and 3.5 mg kg TS<sup>-1</sup> in reactor 2.

Macroelements, except sodium, were also solubilized (Fig 5. D and F). The major solubilization of macroelements occurred in the residual fraction (64% reduction in total macroelements content in reactor 1 and 86% in reactor 2), while an increase in total macroelements content was measured in the exchangeable fraction (44% increase in reactor 1 and 30% in reactor 2). The main increase in the exchangeable fraction was associated with sodium that was mainly bound to the exchangeable and the organic matter



**Fig. 6.** Influent and effluent trace and major elements concentrations from AnMBRs after one month of operation. [W, Mo, Fe, Se, Co]  $\times 10$ . [Ni, Mn, Cu, Zn, B] below the level of detection [ $5 \mu\text{g L}^{-1}$ ]. After dashed line the elements concentration must be read from the secondary y-axis.

and sulfides fractions, with an increase in each fraction of about 40% in reactor 1 and 30% in reactor 2, showing the higher affinity of sodium to these fractions. This accumulation was caused by the elevated concentration of sodium in the model wastewater and the probable displacement of TM from the biomass metal matrix by this cation. Although the supply of TM provided should be sufficient, the metals may not be present in a bioavailable form that can be taken up by the microorganisms. Therefore, it was postulated that an apparently low amount of bioavailable TM due to the high sodium concentration caused the low COD removal observed during the start-up of the AnMBRs. The poorest performance was obtained (about 50% COD removal in reactor 1 and 30% removal in reactor 2) when wash-out of the majority of metals and the accumulation of sodium in the biomass occurred. Other studies researching anaerobic treatment under highly saline conditions completely relied on the microbial acclimatization process adapting the biomass to halotolerant conditions and gradually recovering the treatment performance (Luo et al., 2016; Qiu and Ting, 2013). However, the bioavailability of micronutrients impacted by the high concentration of sodium has never been considered. Affirmatively, after doubling the TM supplementation, an increasing COD removal was measured (90% of COD removal in both reactors on day 60, data not shown). Very likely, an increased TM concentration prevents depletion of bioavailable metals, preventing exhaustive removal of exchangeable compounds, ions substitution, or displacement of other TM by sodium. Similar changes in partitioning and bioavailability have been reported with other cations such as potassium and iron (Mulligan et al., 2010; Shakeri Yekta et al., 2014). It was beyond the scope of this study to examine the complexation reactions that play an important role in the AnMBRs making a particular metal either more or less bioavailable. The TM partitioning in the biomass matrix and determination of effluent metal concentration under high salinity conditions as performed in this study predicts TM leachability. Additional research is required to evaluate the long-term impact of high salinity conditions on reactor performance and TM speciation in the AnMBRs.

#### 4. Conclusions

The following conclusions could be drawn:

- The maximum SMA was obtained at  $6 \text{ g Na}^+ \text{ L}^{-1}$ . The high sodium content and reduced bioavailability of TM significantly decreased the SMA.
- The supply of tungsten increased the SMA by 17%, but no improvement was found with the addition of cobalt.
- High sodium concentration, mainly bound to the exchangeable fraction, resulted in different TM partitioning in the biomass matrix, with TM becoming more soluble and prone to wash-

out. In agreement with this, the particle size and COD removal decreased in the two AnMBRs. Accordingly, by doubling the TM supplementation a higher COD removal was achieved.

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