Effects of barium on the pathways of anaerobic digestion

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ABSTRACT

The sufficient presence of trace elements (TE) is essential for anaerobic digestion. Barium (Ba) is considered a non-essential trace element that can be collaterally added to digesters as part of low-cost trace element sources or because of its presence in some feedstocks, such as crude glycerol. In the present study, the impact of Ba supplementation (2–2,000 mg/L) on each stage of the anaerobic digestion (AD) process was evaluated using pure substrates (i.e., cellulose, glucose, a mixture of volatile fatty acids, sodium acetate and hydrogen) as well as a complex substrate (i.e., dried green

fodder). Hydrolytic activity was affected at dosages higher than 200 mg Ba/L, whereas cellulose degradation was completely inhibited at 2,000 mg Ba/L. The negative effects of the addition of Ba to methane production were observed only in the hydrolytic activity, and no effects were detected at any barium dosage in the subsequent anaerobic steps. Because Ba does not have a reported role as a cofactor of enzymes, this response could have been due to a direct inhibitory effect, a variation in the bioavailability of other trace elements, or even the availability of CO₂/SO₄ through precipitation as Ba-carbonates and sulphates. The results showed that the addition of Ba modified the chemical equilibrium of the studied system by varying the soluble concentration of some TEs and therefore their bioavailability. The highest variation was detected in the soluble concentration of zinc, which increased as the amount of Ba increased. Although little research has shown that Ba has some utility in anaerobic processes, its addition must be carefully monitored to avoid an undesirable modification of the chemical equilibrium in the system.

Keywords: Hydrolysis; trace elements; metal bioavailability; trace element dosing

1. INTRODUCTION

Anaerobic digestion (AD) constitutes an effective technology for organic waste treatment, management and valorisation through energy generation and fertiliser production. The AD process is carried out by microorganisms having an anoxic metabolism that degrades organic matter, ultimately releasing methane (CH₄), carbon dioxide (CO₂) and other minor components. AD consists of four main stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Appels et al., 2008; Merlin Christy et al., 2014).

Many studies have been carried out to understand the limiting factors of single groups of microorganisms that catalyse each stage in order to improve the cost and efficiency of AD (Merlin Christy et al., 2014). The adequate presence and availability of trace elements (TE) in enzymatic cofactors and metalloenzymes are prerequisites for the latter (Choong et al., 2016; Thanh et al., 2016). TE can be either stimulatory or inhibitory, and its imbalance may cause instability and even the complete failure of AD (Fermoso et al., 2008a).

In particular, several elements, such as iron (Fe), nickel (Ni), cobalt (Co), copper (Cu), zinc (Zn), molybdenum (Mo) and/or selenium (Se), have been reported to be essential in different stages of AD (Fermoso et al., 2008b; Gustavsson et al., 2013; Schmidt et al., 2014; Thanh et al., 2016; Worm et al., 2009). In consequence, supplementation with such elements has been shown to improve AD performance using different substrates (Choong et al., 2016). The addition of these elements was related to a decrease in volatile fatty acids (VFA) and a consequent increase in the biogas production rate and biogas yield (Nordell et al., 2016). In contrast, several studies found that inhibition and toxicity were attributed to the excess of TE (Bartacek et al., 2008; Chen et al., 2008; Pinto-Ibieta et al., 2016). Because of the complex pathways involved in AD, several studies examined the effects of TE on each step of AD (Fermoso et al., 2008a; Pinto-Ibieta et al., 2016). For example, Co was notably involved in the last (methanogenic) step. The importance of Co is related to its presence in CO dehydrogenase and methyl-H₄MPT:HS-CoM methyltransferase, which are enzymes involved in methanogenesis from acetate (Ferry, 1999; Pinto-Ibieta et al., 2016). Other TEs, such as Ni, Zn, Mg and Mn, have been reported to show important activity as metalloenzymes that catalyse hydrolytic reactions (Bertini and Luchinat, 1994). Despite their presence as cofactors, some elements may also exert further indirect effects. For

instance, the high presence of Fe increased methane yield when agricultural waste is used as a substrate, which was attributed to the capacity of its alkali treatment to break the cellulose structure (Cai et al., 2017).

For obvious reasons, non-essential TEs have not been studied as direct micronutrients in AD. However, the presence of some non-essential TEs may have effects on AD. Barium (Ba) is an element that belongs to the IIA group. It appears to be the least studied of the heavier alkali and alkaline earth metals in terms of its functionally in replacing elements in biological systems (Wackett et al., 2004). The presence of Ba in an anaerobic system could be related to its content in industrial wastewater, such as crude glycerol (Danilović et al., 2017). Furthermore, the purposeful addition of fly ash as low-cost source of TE to anaerobic systems may entail the collateral supplementation of Ba (Gertner et al., 2017; Montalvo et al., 2017). A recent study showed that hydrolytic enzymes were stimulated in the presence of Ba (Muñoz et al., 2016), whereas the mechanism remained unresolved. Many authors have reported that the concept of bioavailability is the key factor in the effects of trace metals. In fact, Ba has been used to enhance sulphate removal by precipitation as sulphates such as Barite (Navamani Kartic et al., 2018). Consequently, the addition of Ba may have a beneficial effect through the decreased formation of highly inhibitory sulphide, which is the metabolic end-product of sulphate reducers). However, the addition of Ba may negatively affect the substrate availability of the system because it may promote carbonate formation (e.g., Witherite); CO₂ is the substrate for hydrogenotrophs. To date, it has not been clearly shown how Ba affects AD or at which concentration the effects are beneficial or detrimental.

Therefore, the aim of this work was to study the influence of different concentrations of Ba dosage on different stages of AD using simple substrates (i.e.,

cellulose, glucose, a mixture of volatile fatty acids, sodium acetate and hydrogen) and a real complex substrate (i.e., dried green fodder).

2. MATERIALS AND METHODS

2.1 Anaerobic inoculum and substrates

Anaerobic inoculum was obtained from the wastewater treatment plant of a brewery company located in Seville, Spain. The inoculum was concentrated by sedimentation before characterisation (Table 1). To determine the effect of Ba supplementation on AD, different synthetic substrates were used to simulate each stage of the process. The stages of hydrolysis, acidogenesis, acetogenesis, acetoclastic methanogenesis and hydrogenotrophic methanogenesis were evaluated using cellulose (cellulose microcrystalline colloidal, Sigma Aldrich), glucose (D(+)-glucose anhydrous $\geq 99.5\%$, Panreac), a mix of short chain acids (propionic ($C_3H_6O_6$), butyric ($C_4H_8O_2$) and valeric ($C_5H_{10}O_2$), Sigma Aldrich), sodium acetate (sodium acetate anhydrous, Sigma Aldrich) and hydrogen (H_2 , SIAD Czech spol. s r.o.), respectively. The influence of the Ba conversion of a real complex substrate was evaluated by its addition to dried green fodder (DGF) (particle size $< 355 \mu m$). The main characteristics of the synthetic substrates and the DGF are shown in Table 1.

2.2 Biochemical methane potential assays

Biochemical methane potential (BMP) assays were performed in triplicate using different Ba supplementation doses on synthetic and DGF substrates. Ba was dosed as barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$ extra pure Sigma Aldrich) at concentrations of 2, 20, 200 and 2,000 mg Ba/L in the working volume of BMP assays (220 mL). In addition, the experimental design considered triplicates of BMP assays at 0 mg Ba/L (control) and without substrate (blank). The BMP assays were carried out until the

methane production was exhausted. At the end of the experiments, the liquid digestate of the BMP assays was characterised.

2.2.1 BMP assays: hydrolysis, acidogenesis, acetogenesis and acetoclastic methanogenesis stages

The BMP assays were performed in batch mode using a continuously stirred system in flasks of 250 mL. Temperature conditions were maintained at 35 ± 1 °C through a heated water bath. Methane production was quantified by soda volume displacement using soda 2 N in all experiments. A buffer solution of NaHCO_3 0.068 M (Holliger et al., 2016) and a micro-macronutrients solution (Table 2) (Angelidaki et al., 2009) were added. A glucose solution of 0.54 mM was added for sludge conditioning 24 hours before the substrates and Ba supplementation were added (Holliger et al., 2016). The air content in the system was displaced by nitrogen at the beginning of the assays to ensure anaerobic conditions.

The experiments were performed using substrate/inoculum ratios (measured in grams of total volatile solids) of 0.8, 0.3, 0.3, 0.3 and 0.5 for cellulose, glucose, mix of short chain acids (VFA), sodium acetate and DGF, respectively. The different substrate/inoculum ratios were selected to avoid the acidification of the process because of the easy biodegradability of the synthetic substrates.

2.2.2 BMP assays: hydrogenotrophic methanogenesis stage

The BMP assays were carried out using Pyrex® bottles of 500 mL as reactors. The temperature was maintained constant at 36 ± 1 °C in a temperature-controlled room. The methane production was quantified by chromatography. As in the other experiments, a buffer solution of NaHCO_3 and a micro-macronutrient solution were added. The concentration of the buffer solution was higher than in the other cases because it was used as a source of carbon dioxide. The sludge was conditioned for 24 hours using a

solution 0.54 mM glucose. The headspace of the reactors (420 mL) was flushed with nitrogen to remove the oxygen and then filled with hydrogen to overpressure up to 30 kPa.

2.3 Analytical techniques

The characterisation of the anaerobic inoculum and substrates as well as the characterisation of the samples at the end of the BMP assays were carried out according to the analytical techniques described below. Contents of total solids (TS g/kg), volatile solids (VS g/kg), alkalinity (mg CaCO₃/L) and pH were performed using standard methods (American Public Health Association [APHA], 2005). The soluble chemical oxygen demand (sCOD mgO₂/L) was measured by spectrophotometry at 600 nm after reaction with potassium dichromate (Raposo et al., 2008).

In the experiments carried out to evaluate the hydrogenotrophic methanogenesis stage, the decrease in pressure combined with the changes in gas composition were used to calculate the consumption of hydrogen. The biogas quality was measured using a GC-TCD (CE Instruments GC 8000 Top, Wigan, UK) equipped with a packed column (Porapak Q 80-100 mesh, Waters Corporation, Milford, Massachusetts, USA). Subsequently, the decrease in pressure combined with the changes in gas composition were used to calculate the share of hydrogen.

At the end of all BMP assays, the liquid phase was analysed to measure the concentration of dissolved metals. The liquid phase samples were prepared under anoxic conditions, filtered using 0.22 µm pore size and then fixed in a solution with a final concentration of 3% nitric acid (HNO₃ 65% semiconductor grade, Sigma Aldrich). Total metal concentrations in the inoculum and the substrates were also measured after the addition of nitric acid (HNO₃ 65% semiconductor grade, Sigma Aldrich). The analysis was performed using an 8800 qqq-ICP-MS system (Agilent, Basel,

Switzerland) with general-purpose operational settings. Quantification was performed using multi-element standards (Sigma Aldrich). Rh was used as internal standard to account for the matrix effects. To quantify $^{63}\text{Cu}^+$, $^{66}\text{Zn}^+$, $^{95}\text{Mo}^+$ and $^{135}\text{Ba}^+$, the ICP-MS was operated in single quad mode using helium as a collision gas, whereas Fe, Co, Ni and Se were measured in triple quad mass-shift mode using O_2 as the reaction gas (as $^{56}\text{Fe}^{16}\text{O}^+$, $^{59}\text{Co}^{16}\text{O}^+$, $^{60}\text{Ni}^{16}\text{O}^+$ and $^{80}\text{Se}^{16}\text{O}^+$).

2.5 Kinetic model

The kinetics parameters were determined by applying a non-linear regression approach using SigmaPlot™ version 11 (Systat Software). The experimental data fit with the transfer function (TF), equation 1 (Parra-Orobio et al., 2017). The application of the TF was previously reported in batch-mode biomethanisation processes of several organic substrates (Donoso-Bravo et al., 2010; Pinto-Ibieta et al., 2016; Wyman et al., 2017).

$$V_{\text{CH}_4}(t) = G_{\text{max}} \left(1 - \exp \left(\frac{-R_{\text{max}}(t - \lambda)}{G_{\text{max}}} \right) \right) \quad (\text{Equation 1})$$

where V_{CH_4} is the accumulated methane yield ($\text{mL}_{\text{STP}}/\text{gVS}$); G_{max} is the maximum methane yield ($\text{mL}_{\text{STP}}/\text{gVS}$); R_{max} is the maximum methane production rate ($\text{mL}_{\text{STP}}/(\text{gVSd})$); and λ is the lag phase period (d). All results are presented as average values and standard deviations at standard temperature and pressure (STP) conditions: 273.15 K and 101.33 kPa (Holliger et al., 2016).

3 RESULTS AND DISCUSSION

3.1 Effects of Ba on methane production

3.1.1 Effects of Ba on methanogenesis, acetogenesis and acidogenesis stages

The effects of Ba dosages on the accumulated methane production of acetoclastic methanogenesis (acetate), hydrogenotrophic methanogenesis (H_2),

acetogenesis (mix of short chain acids) and acidogenesis (glucose) stages are shown in Figure 1. In the acetoclastic methanogenesis stage (Figure 1A), it was observed that the maximum methane production was slightly decreased at Ba dosages of 200 and 2,000 mg Ba/L. The methane production using H₂ and CO₂ (hydrogenotrophic methanogenesis) did not show any inhibitory effect (Figure 2B). The acetogenesis and acidogenesis stages (Figure 1C and 1D) showed similar final methane yields in all the cases studied, varying in a short range from 313 ± 20 to 309 ± 10 (mL_{STP} CH₄/g COD), respectively.

The obtained coefficient values of the methane yield were close to the theoretical maximum methane yield coefficient of 350 mL_{STP} CH₄/g COD. The Ba doses did not result in an effect over the maximum methane yield coefficient. The trace metals that were mainly involved in these steps were Co and Ni (Gustavsson et al., 2013; Thanh et al., 2016), the concentrations of which did not increase as a consequence of the Ba addition (Table 3).

The effluents from the acetogenesis and acidogenesis assays showed pH values (Table 4) within the optimal range described for the anaerobic process: 7.1-7.7 (Wheatley, 1990). The assays in the methanogenesis step assays showed a pH slightly higher than those described as optimal, and the alkalinity values were higher than those obtained in the acetogenesis and acidogenesis assays (Table 4). These alkalinity values could be explained by the extra alkalinity provided by the synthetic substrate used, which was sodium acetate. However, the pH measured in the effluents of the assays carried out with hydrogen was around 9.1 (Table 4). This increase should not be attributed to the presence of Ba but to the excess of the NaHCO₃ added as a source of carbon dioxide.

The biodegradability values, which were measured as percentages of theoretical methane production from the total COD were in accordance with the high methane yield coefficient values described previously, reaching values higher than 83% in all cases. The high biodegradability was expected because of the use of readily digestible synthetic substrates. The COD values obtained oscillated between 105 ± 2 to 172 ± 5 mg O₂/L (Table 4), indicating that the soluble organic matter decreased during the experimental period.

3.1.2 Effects of Ba on the hydrolysis stage

The hydrolytic step was evaluated through the anaerobic digestion of cellulose as a simple synthetic substrate and DGF as complex real substrate. Figures 2A and 2B show the accumulated methane production in the cellulose and DGF assays at different doses of Ba. In both cases, the methane yield coefficient was sensitive to the presence of different Ba concentrations. Regarding the cellulose, the assay with the highest (2,000 ppm) concentration of Ba showed total inhibition, whereas the Ba dosages between 0 to 200 mg/L resulted in a mean methane yield coefficient of 264 ± 8 mL_{STP} CH₄/g COD (Figure 2A).

In the case of DGF, the methane yield coefficient slightly increased at dosages of 2 and 20 mg Ba/L, compared to the assay without the Ba addition (Figure 2B). This enhancement could not be explained because Ba is not an essential element. Because the methanogenic, acetogenic, acidogenic and cellulosic activities were not improved by the addition of Ba, it could be hypothesised that the enhancement of the methane yield coefficient could be the consequence of the improved activity of other hydrolytic enzymes. Ba dosages higher than 200 mg/L resulted in a decrease in the methane yield coefficient (Figure 2B). Regarding the complex substrate, DGF, the addition of 2,000 mg Ba/L did not result in the total inhibition of the methane production which was in

contrast to the results of the cellulose assays. This difference could be attributed to the complexity of the DGF, the biodegradation of which implied different hydrolytic pathways as well as the release of soluble compounds from the raw substrate that were easily methanised to avoid the hydrolysis stage.

As observed in the other stages of the AD process, the pH and alkalinity values obtained at the end of the experiments (Table 5) did not vary as the amount of Ba was increased. The values were close to the optimal range in the anaerobic metabolism and activity (Wheatley, 1990). However, in the hydrolysis stage, the biodegradability showed clear differences. In the case of the cellulose, the biodegradability was around $75.5 \pm 2.2\%$ at 0, 2, 20 and 200 mg Ba/L, whereas total inhibition occurred at 2,000 mg Ba/L. In the case of the DGF, the biodegradability values increased from $64.0 \pm 4.1\%$ at no Ba dosage to $75.1 \pm 1.2\%$ at 20 mg Ba/L. The values then decreased to $57.9 \pm 2.4\%$ at 200 mg Ba/L and $54.4 \pm 0.1\%$ at 2,000 mg Ba/L (Table 5). These sequential effects could be a consequence of the different sensitivities of the involved hydrolytic enzymes to the modifications of the chemical equilibrium and the bioavailability of TE by the addition of Ba.

The sCOD values obtained from the effluents of the digesters used to study the hydrolysis stage (Table 5) were around four times higher than those obtained in the other stages (Table 4). This behaviour could be explained by the complexity of the evaluated substrates. The highest sCOD values, around 920 mg O₂/L, were reached in the experiments using cellulose. In the DGF substrate, the values were around 416 mg O₂/L, indicating the higher degradation of soluble organic matter than in cellulose.

To compare the evolution of the methane production during the biomethanisation of DGF, the experimental data shown in Figure 3B were adjusted to the transfer function model (Table 6). As shown in Table 6, G_{max} presented values

similar to the experimental methane yield coefficients. Differences were shown between the theoretical and experimental values lower than 4.2% in all cases. These values showed that Ba concentrations from 0 to 20 mg/L did not entail variation in R_{max} , which showed a mean value of $7.2 \pm 0.4 \text{ mL}_{\text{STP}} \text{ CH}_4/(\text{g VS} \cdot \text{d})$ (Table 6). However, at dosages of 200 and 2,000 mg Ba/L, a marked drop in R_{max} values was observed, which were 23% and 35% lower, respectively, than in the assays performed without the addition of Ba (Table 6).

3.2 Effects of Ba on the final concentrations of soluble metals

Because Ba has not been reported to be a cofactor of enzymes involved in the AD process, the inhibition could be an indirect effect of the modification of the chemical equilibrium of other metals. The increasing Ba doses affected, to differing extents, the concentrations of the measured dissolved metals (Table 3). Most of the measured dissolved metals did not show any clear variation due to the addition of Ba except dissolved Ba and, to a lesser extent, dissolved Zn. Specifically, the Cu concentration varied widely from 2.49 ± 0.05 to $170 \pm 2.9 \text{ } \mu\text{g/L}$ (Table 3) with values lower than those reported as inhibitory, that is, from 70–400 mg/L (Karri et al., 2006). The determined variation in Cu concentrations did not follow a clear trend with the addition of Ba. It was not possible to establish any trend between Co, Ni and Fe concentrations against the Ba added to the assays (Table 3). In fact, the Co and Ni concentrations remained virtually constant in the differently measured samples. The measured range of concentrations of Co, Ni and Fe were within the required dissolved concentrations for each trace metal for anaerobic digestion: 5-22 $\mu\text{g/L}$ for Co, 8-146 $\mu\text{g/L}$ for Ni, and 0.3-8.3 mg/L for Fe (Choong et al., 2016; Matheri et al., 2016).

The concentration of dissolved Zn was increased by the addition of Ba (Table 3), showing a linear relation between the soluble Ba concentration and soluble Zn up to

values of around 1,000–1,200 µg/L (Figure 3). As shown in Table 3, the soluble Zn was lower than 0.2 mg/L in the reactors with low Ba supplementation (< 20 mg Ba/L). However, from 200 mg Ba/L, the variation in soluble Zn was increased more than 10 times compared to the other conditions (Table 3). It should be noted that because its input was measured as lower than 1%, the Zn concentration present in the Ba reagent as an impurity (Table 1) did not contribute to the final Zn concentration reported in the effluents.

Several studies reported that Zn is an essential element because of its participation as a cofactor in more than 300 enzymes (Zastrow and Pecoraro, 2014). For example, Zn participates significantly in the formation of metalloenzymes catalysing hydrolytic reactions (Bertini and Luchinat, 1994). Inhibition by Zn might occur in concentrations as low as 0.6 mg/L (Lo et al., 2007). Therefore, the addition of 2,000 mg Ba/L resulted in the increase in soluble Zn concentrations up to potentially toxic levels (Figure 3 and Table 3). Hence, it is possible to hypothesise that Zn could be the main inhibition agent because its concentration was above some previously reported inhibition concentrations (Lo et al., 2007).

4. CONCLUSIONS

The presence of Ba affected the anaerobic digestion pathways although these effects seemed to be indirect. The addition of Ba modified the chemical equilibrium of the studied system by varying the soluble concentrations of some TEs and therefore their bioavailability. The highest variation was detected in the soluble concentration of Zn, which increased as the amount of Ba increased. Hydrolytic activity was affected at Ba dosages higher than 200 mg/L, whereas cellulose degradation was completely inhibited at Ba dosages of 2,000 mg/L. The effects of the Ba addition were only on the hydrolytic

activity, and no effects were detected at any Ba dosage in the subsequent anaerobic steps. The determined inhibitory effects could be a consequence of a disturbance in the chemical equilibrium caused by the Ba supplementation instead of the direct effect of the Ba. Although responses were observed at high Ba concentrations, the knowledge of the role of Ba is important in contributing to the understanding of the AD process and the dynamics of chemical equilibrium in the anaerobic system. Similarly, although the addition of Ba has been reported to have some utility in anaerobic processes, its addition must be carefully monitored to avoid the undesirable modification of the chemical equilibrium of the system.

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FIGURE CAPTIONS

Figure 1. Accumulated methane yield obtained for synthetic substrates: (a) sodium acetate, (b) hydrogen, (c) mix of short chain acids, (d) glucose

Figure 2. Accumulated methane yield obtained for (a) cellulose and (b) dried green fodder (DGF).

Figure 3. Soluble Zn and Ba concentration detected in the effluent of the BMP assays using cellulose as a substrate.

Figure 1.

A)

B)

C)

D)

Figure 2.

A)

B)

Figure 3.

Table 1. Analytical characterization of the inoculum, reagent and the employed substrates

	Inoculum	BaCl₂· 2H₂O	Cellulose	Glucose	VFA mixture	Acetate	Dried green fodder
TS (g/kg)	29.1 ± 2.7	<i>n.a</i>	969.1 ± 1.7	999.2 ± 0.1	<i>n.a</i>	981.9 ± 1.9	895.9 ± 1.6
VS (g/kg)	18.1 ± 1.6	<i>n.a</i>	943.4 ± 1.4	999.2 ± 0.1	<i>n.a</i>	981.9 ± 1.9	804.7 ± 4.7
Alkalinity (mg CaCO₃/L)	3352 ± 920	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>
pH	7.9 ± 0.2	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>
Ba (mg/kg TS)	52.8 ± 5.1	>2000	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	49.7 ± 0.1
Co (mg/kg TS)	5.4 ± 0.5	<0.002	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	4.9 ± 0.1
Cu (mg/kg TS)	57.1 ± 4.5	0.205	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	55.6 ± 0.1
Fe (mg/kg TS)	5751.6 ± 6.4	<0.02	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	5478.2 ± 0.1
Ni (mg/kg TS)	42.0 ± 2.1	<0.005	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	39.7 ± 0.1
S (mg/kg TS)	8089 ± 733	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	7826.4 ± 0.1
Zn (mg/kg TS)	377.9 ± 15.3	0.789	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	<i>n.a</i>	378.9 ± 0.1

n.a., not available

1 **Table 2.** Composition of macronutrients and micronutrients solutions

Macronutrients	NH ₄ Cl (mg/L)	1400
	K ₂ HPO ₄ (mg/L)	1250
	MgSO ₄ ·7H ₂ O (mg/L)	500
	CaCl ₂ ·2H ₂ O (mg/L)	50
	Yeast (mg/L)	500
Micronutrients	FeCl ₂ ·4H ₂ O (µg/L)	2000
	CoCl ₂ ·6H ₂ O (µg/L)	2000
	MnCl ₂ ·4H ₂ O (µg/L)	500
	AlCl ₃ ·6H ₂ O (µg/L)	90
	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O (µg/L)	50
	H ₃ BO ₃ (µg/L)	50
	ZnCl ₂ (µg/L)	50
	CuCl ₂ ·2H ₂ O (µg/L)	38

2

3

Table 3. Concentration of soluble metals in the assays at the end of the BMP. Mo and Se were not detected in any case and, therefore, are not shown.

Substrate	Soluble metals						
	Ba added	Ba	Cu	Zn	Fe	Co	Ni
	mg/L	mg/L	µg/L	µg/L	µg/L	µg/L	µg/L
Hydrogen	0	0.6±0.2*	42.4±0.8	87.3±2.9	689.3±5.8	16.1±4.7	73±40
	2	0.4±0.1*	56.7±0.4	185.4±8.1	1740±20	22.3±9.8	145±12
	20	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>
	200	17.7±0.7	<i>n.d</i>	947±81*	5504±102	388±114*	700±140*
	2000	65.4±0.8	170.7±2.9	525±12	2285±41.6	20±12	146±9.7
Acetate	0	0.22±0.02*	5.8±0.6	31±18*	1490±543	6.5±1.3	15±7*
	2	1.4±0.1	32±15	113±11	3610±57	8.3±0.7	26±3*
	20	6.5±0.1	2.9±0.1	28.9±0.9	382±353	7.1±0.3*	15±4*
	200	239.5±3.2	130±4	171±9	1083±682	5.7±2.7*	29±2
	2000	353.6±3.1	<i>n.d</i>	161±18*	1861±2498	5.9±0.4	80±2*
VFA mixture	0	<i>n.d</i>	11.8 ±0.3	30±10	2065±2095	6.1±0.6	18±3*
	2	<i>n.d</i>	17±18	25.5±0.6	3443±4355	22±22*	42±4*
	20	9.8±1.4	19±18	518.0±2.1	1150±1136	6.4±0.4	16±2
	200	154.5±1.9	27.3±2.7	126±56	2135±301	5.9±0.1*	9±1
	2000	2720±367	4.51±0.03	998±27	875±726	5.5±0.4	12±5*
Glucose	0	<i>n.d</i>	15±12	79.9±2.5	1925±92	6.1±0.3	17±1*
	2	0.4±0.3*	20±23	44±19	6288±2963	6.2±0.4*	12±1*
	20	15.5±0.6*	<i>n.d</i>	25.2±0.2	965±376	4.9±0.1	39±44*
	200	238±99	16±13	157±29	1578±557	6.7±0.5	32±16*
	2000	2240±66	7.8±4.8	1259±112	891±892	5.6±0.1*	9±2*
Cellulose	0	1.8±0.3*	26.0±6.8	81.5 ± 3.3	8306±4587	8.0±0.1	26±1*
	2	1.9±0.2*	3.4±3.5	35.8 ± 8.0	513±144	5.9±0.4*	9.0±0.4
	20	14.9±6.2	20.7±0.3	110 ± 10	1096±364	5.8±0.2	23.8±1.7
	200	133±8*	39±52	195 ± 125	1279±1156	6.6±0.7*	61.6±4.2
	2000	2947±28	16±14	993 ± 32	1081±679	6.1±0.5*	31±2*
DGF	0	0.2±0.1*	<i>n.d</i>	37±22*	1070±576	8.1±0.3	25±10*
	2	0.6±0.1*	92.1±1.7	17±11*	1140±198	8.4±0.5	18±3*
	20	7.2±0.5	2.67±0.04	33.0±1.6	4550±3895	9.38±0.03	23±1*
	200	103±19	2.49±0.05	102.2±5.4	1322±1005	10.7±1.1	15.7±1.3
	2000	932±41	10.6±0.6	736±13	446±57.9	13.3±0.2	42±34*

n.d., not detected

n.a., not available

Values marked with * should be taken with care due to an increased standard deviation.

Table 4. pH, Alkalinity (mg CaCO₃/L), biodegradability (%), and soluble COD (CODs; mg O₂/L), in the BMP tests for acetate, hydrogen, VFA mixture and glucose at the end of the experiment.

		Acetate				
	Added Ba (mg/L)	0	2	20	200	2000
pH		8.01 ± 0.08	8.04 ± 0.03	8.00 ± 0.07	8.02 ± 0.03	7.81 ± 0.01
Alkalinity	(mg CaCO ₃ /L)	7805 ± 239	8049 ± 313	8049 ± 313	7882 ± 65	6951 ± 211
Biodegradability	(%, in CH ₄ -COD)	103.3±2.7	101.1±2.7	102.9±2.7	89.1±2.7	82.0±2.7
CODs	(mgO ₂ /L)	114±1	172±5	125±2	<i>n.d.</i>	<i>n.d.</i>
		Hydrogen				
	Added Ba (mg/L)	0	2	20	200	2000
pH		9.10±0.05	9.18±0.05	9.06±0.05	9.16±0.05	8.77±0.05
Biodegradability	(%, in CH ₄ -COD)	103.4±10.5	90.7±0.6	103.8±3.9	103.4±3.9	86.0±2.7
		VFA mixture				
	Added Ba (mg/L)	0	2	20	200	2000
pH		7.58 ± 0.12	7.57 ± 0.10	7.56 ± 0.16	7.56 ± 0.07	7.47 ± 0.12
Alkalinity	(mg CaCO ₃ /L)	5298 ± 57	5198 ± 192	5702 ± 455	5307 ± 13	5273 ± 120
Biodegradability	(%, in CH ₄ -COD)	92.1±0.0	83.5±14.10	88.3±0.0	85.9±2.35	97.8±0.0
CODs	(mgO ₂ /L)	114±11	140±16	138±12	<i>n.d.</i>	<i>n.d.</i>
		Glucose				
	Added Ba (mg/L)	0	2	20	200	2000
pH		7.36 ± 0.27	7.33 ± 0.23	7.34 ± 0.17	7.39 ± 0.19	7.23 ± 0.34
Alkalinity	(mg CaCO ₃ /L)	5304 ± 167	5343 ± 157	5046 ± 112	6115 ± 927	5124 ± 175
Biodegradability	(%, in CH ₄ -COD)	90.4±6.8	89.0±2.7	90.9±0.0	84.7±1.4	85.6±2.7
CODs	(mgO ₂ /L)	109±4	113±16	105±2	<i>n.d.</i>	<i>n.d.</i>

n.d., not detected

Table 5. pH, Alkalinity (mg CaCO₃/L), biodegradability (%), and COD soluble (mg O₂/L), in the BMP tests for cellulose and DGF at the end of the experimental time.

		Cellulose				
	Added Ba (mg/L)	0	2	20	200	2000
pH		7.70 ± 0.15	7.67 ± 0.12	8.09 ± 0.12	7.65 ± 0.08	7.82 ± 0.07
Alkalinity	(mg CaCO ₃ /L)	5199 ± 241	4827 ± 108	5105 ± 184	5137 ± 79	5153 ± 132
Biodegradability	(%, in CH ₄ -COD)	78.7± 3.4	73.8± 2.9	74.8± 2.6	74.6± 3.7	0.0± 0.1
CODs	(mgO ₂ /L)	883±0	920±7	871±55	<i>n.d</i>	<i>n.d</i>
		DGF				
	Added Ba (mg/L)	0	2	20	200	2000
pH		7.60 ± 0.06	7.58 ± 0.11	7.57 ± 0.08	7.53 ± 0.09	7.53 ± 0.07
Alkalinity	(mg CaCO ₃ /L)	5764 ± 88	7200 ± 0	5842 ± 17	5557 ± 219	5823 ± 143
Biodegradability	(%, in CH ₄ -VS)	64.0± 4.1	74.6± 7.1	75.4± 1.2	57.9± 2.4	54.40± 0.1
CODs	(mgO ₂ /L)	412±11	425±12	412±39	<i>n.d</i>	<i>n.d</i>

n.d., not detected

Table 6. Kinetics parameters obtained from the transfer function applied to the BMP tests of dried green fodder (DGF). σ_{est} , standard error of estimate.

Added Ba	0	2	20	200	2000
G_{max} (mL/g VS)	214.7	250.2	253.1	195.6	184.1
R_m (mL/ (gVS·d))	7.10	7.59	6.83	5.45	4.61
λ (d)	1.40 ± 0.47	0.50 ± 0.38	0.06 ± 0.48	4.87E-09 ± 0.70	2.80E-10 ± 1.09
r^2	0.9946	0.9971	0.9964	0.9913	0.9717
σ_{est}	6.07	5.02	5.59	6.61	11.00
Error (%)	4.2	4.1	4.1	3.5	3.3