Microbial community dynamics reflect reactor stability during the anaerobic digestion of a very high strength and sulfate-rich vinasse

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DOI
10.1002/jctb.5449

Publication date
2018

Document Version
Final published version

Published in
Journal of Chemical Technology and Biotechnology

Citation (APA)
Jiménez, J., Barrera, E. L., De Vrieze, J., Boon, N., DeMeester, S., Spanjers, H., Romero, O. R., & Dewulf, J. (2018). Microbial community dynamics reflect reactor stability during the anaerobic digestion of a very high strength and sulfate-rich vinasse. *Journal of Chemical Technology and Biotechnology*, *93*(4), 975-984. https://doi.org/10.1002/jctb.5449

and reactor efficiencies.[10,11,15–17] Only few studies have focused on microbial community characteristics.[18,19] Knowledge on the characteristics of the microbial community is essential to control and/or enhance process efficiency.[20–22] For instance, the study of the microbial community dynamics could help in early detection of operational difficulties (e.g. acidification, toxicity by intermediate metabolic accumulation, microbial washout, bacteria or methanogens inhibition, etc.), making the application of preventive actions possible.[20,23–26]

The production and characteristics of vinasse are variable and dependent on the feedstocks and the ethanol production process. Wash water used to clean the fermenters, cooling water blow down, and boiler water blow down may all be combined with the vinasse and contribute to its variability in concentration.[27] Variations of the COD and SO_4^{2-} concentrations of vinasse cause dynamical responses in the sulfate reduction process during anaerobic treatment, influencing reactor performance.[11] However, little research has been done on how the variability of vinasse concentration may affect the microbial community dynamics during the anaerobic digestion process. Therefore, the aim of this work was to study the microbial community dynamics in the anaerobic digestion of a very high strength and sulfate-rich vinasse; linking to experimental observations about product yields and organic matter degradation.

For the purpose of this research, an upflow anaerobic sludge blanket (UASB) reactor was fed with sugar cane vinasse by means of giving COD and SO_4^{2-} pulses at different SO_4^{2-}/COD ratios. The observed product yields and organic matter degradation are discussed; and later explained from the microbial community dynamics based on molecular techniques approaches.

MATERIAL AND METHODS

Experimental setup and process operation

Experimental observations from a characterization study of the sulfate reduction process in the anaerobic digestion of a very high strength and sulfate-rich vinasse[11] were used for the calculation of products yields and degradation of the organic matter. Samples taken during the experiment of Barrera *et al.*[11] were used for the microbial community analysis. During these experiments, a lab-scale upflow anaerobic sludge blanket (UASB) reactor with an internal diameter of 8 cm, height of 70 cm and working volume of 3.5 L, was operated for 75 days under dynamic conditions. The experimental set-up, the analytical methods, the vinasse and sludge characteristics, as well as the operating conditions are described in detail in Barrera *et al.*[11]

The operational conditions during the different phases of the experiment (Table 1) can be summarized as follows (E-codes represent successive experimental phases, carried out under different operating conditions): from E-1 to E-3, the concentration of influent COD and SO_4^{2-} was gradually increased, while the SO_4^{2-}/COD ratio was kept at 0.05; from E-3 to E-4, the concentration of influent SO_4^{2-} was increased, while the concentration of influent COD was decreased so as to increase the SO_4^{2-}/COD ratio up to 0.10; from E-4 to E-6, the concentration of influent COD and SO_4^{2-} was increased, while the SO_4^{2-}/COD ratio was kept at 0.10; in E-7, the concentration of influent COD and SO_4^{2-} was reduced to control toxicity, while the SO_4^{2-}/COD ratio was kept at 0.10; and at E-8 and E-9, the concentration of influent SO_4^{2-} was increased, while keeping a constant influent COD concentration to increase the SO_4^{2-}/COD ratio to 0.15 and 0.20, respectively. After day 75, the feed of vinasse ceased until the reactor was disassembled on day 83 (E-10).

Table 1. Operational conditions for each experimental phase

Experiment	Duration (d)	SO_4^{2-}/COD (g SO_4^{2-} g^{-1} COD)	Influent COD (g COD L^{-1})	Influent SO_4^{2-} (g SO_4^{2-} L^{-1})
E-1	1-7	0.05	38	1.75
E-2	8-15	0.05	48	2.20
E-3	16-26	0.05	58	2.65
E-4	27-36	0.10	38	3.65
E-5	37-45	0.10	48	4.60
E-6	46-49	0.10	56	5.50
E-7	50-58	0.10	48	4.60
E-8	59-68	0.15	38	5.65
E-9	69-75	0.20	38	7.50
E-10	76-83	-	-	-

Product yield

Methane and sulfides were considered as the major end products from the degradation of the organic matter in anaerobic treatment. The methane and sulfide yields were calculated during steady state conditions of the experimental periods, the steady state conditions being characterized by a constant gas production rate (±5%).[28] The methane yield was calculated based on the methane produced in the biogas per unit of COD removed (mL CH_4 (g $COD_{removed}$)$^{-1}$). Theoretically, 1 g of COD removed always corresponds with 350 mL CH_4 when the COD is used only by methanogens.[29,30] However, if part of this COD is used also by SRB to produce H_2S, then 1 g of COD removed inside the reactor will produce less than 350 mL CH_4 and this methane production decreases when SRB activity increases. To account for the SRB activity, the sulfide yield was calculated from the aqueous and gas phase hydrogen sulfide production (H_2S_{aq} and H_2S_{gas}) per unit of SO_4^{2-} fed to the reactor and expressed as mg H_2S-S (g SO_4^{2-})$^{-1}$.

Degradation of the organic matter

The degradation of organic matter was calculated based on the COD removed by SRB and M, by means of Equations (1), (2) and (3).[10,31] Equation (1) was used to calculate the total amount of COD removed (COD_{conv}, as g COD d^{-1}), with φ the influent flow (L d^{-1}), COD_{in} the COD concentration of the influent (g COD L^{-1}), and COD_{eff} the COD concentration of the effluent (g COD L^{-1}).

$$COD_{conv} = \phi\ (COD_{in} - COD_{eff}) \qquad (g\ COD\ d^{-1}) \qquad (1)$$

The amount of COD degraded by SRB (COD_{conv_SRB}, as g COD d^{-1}) was calculated by Equation (2), where influent and effluent SO_4^{2-} are the sulfate concentrations (g SO_4^{2-}-S L^{-1}) of the influent and effluent, respectively, and the factor 2 is the number of grams of COD needed to produce 1 g H_2S-S.

$$COD_{conv\_SRB} = 2\phi\ (\text{influent } SO_4^{2-} - \text{effluent } SO_4^{2-}) \qquad (g\ COD\ d^{-1}) \qquad (2)$$

The amount of COD degraded by M (COD_{conv_M}, as g COD d^{-1}) was calculated by Equation (3), where, Q_g is the methane gas production flow rate (L d^{-1}), f is the COD of the methane gas (g COD L^{-1}), and g is the solubility of methane (g COD L^{-1}) in water.

$$COD_{conv\_M} = Q_g \cdot f + g \cdot \phi \qquad (g\ COD\ d^{-1}) \qquad (3)$$

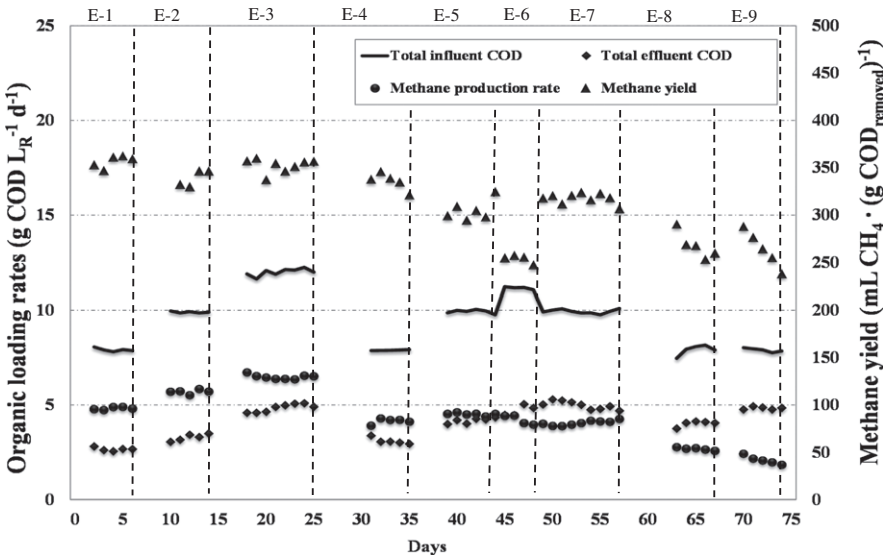

Figure 1. Methane yield and organic loading rates in the UASB reactor during the steady state conditions for E-1 to E-9.

Finally, from these calculations, the COD removed (expressed as percentage) by SRB and M were calculated as the ratios $COD_{conv\_SRB}/COD_{conv}$ and COD_{conv_M}/COD_{conv}, respectively.

Microbial community characterization

To characterize the microbial community during the experiments, sludge samples were taken during the steady state conditions of E-3, E-4, E-5, E-6, E-7, E-8, E-9 (at the end of these periods), and after the experimental period (E-10). Because physicochemical parameters were stable during E-1, E-2 and E-3,[11] E-3 was considered as representative of them, and no samples were taken at the end of E-1 and E-2. Total DNA was extracted from the sludge samples following the protocol of Vilchez-Vargas *et al.*[32]

The analysis of the quality and quantity of the DNA was carried out using agarose gel electrophoresis and spectrophotometry, using a Nanodrop ND-1000 Spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands), and 100-fold dilutions of the DNA extracts were prepared to reach a final DNA concentration between 1 and 10 ng μL^{-1}.

To perform the real-time PCR (qPCR) on triplicate DNA extracts, a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) was used. A reaction mixture of 15 μL was prepared by means of the GoTaq qPCR Master Mix (Promega, Madison, WI, USA), and contained 10 μL of GoTaq® PCR Master Mix, 3.5 μL of nuclease-free water, and 0.75 μL of each primer (final concentration of 375 nmol L^{-1}). To this mixture, 5 μL of template DNA was added.

The qPCR program followed a two-step thermal cycling procedure, which comprised a predenaturation step of 10 min at 94 °C, followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C for total bacteria, using the general bacterial primers P338F and P518r that were described by Ovreas *et al.*[33] For the methanogenic order Methanobacteriales and the families Methanosaetaceae and Methanosarcinaceae, the qPCR program consisted of a predenaturation step of 10 min at 94 °C, followed by 40 cycles of 10 s at 94 °C and 1 min at 60 °C. In order to quantify the Methanomicrobiales order, the annealing temperature was set to 63 °C. The primers for the methanogenic orders Methanomicrobiales and Methanobacteriales, and the families Methanosaetaceae and Methanosarcinaceae were described by Yu *et al.*[34] The qPCR data were represented as copies per gram of wet sludge. The PCR product length was verified on a 1% agarose gel.

Denaturing gradient gel electrophoresis (DGGE) was carried out following the protocol of Boon *et al.*,[35] using the total bacterial primers P338f-GC and P518r.[36] A 2720 thermal cycler (Applied Biosystems) was used for the PCR, using the Taq DNA Polymerase (Thermo Fisher). The PCR product (5 μL) quality was verified on a 1% agarose gel. Consistent with the protocol of Boon *et al.*,[35] an INGENY phorU2X2 DGGE-system (Goes, The Netherlands) was used to prepare the 8% (w/v) polyacrylamide DGGE gel with a denaturing gradient ranging from 45% to 60%. The DGGE gel obtained was processed with the Bionumerics software 5.1 (Applied Maths, Kortrijk, Belgium); only bands with an intensity over 1% were considered. A matrix of similarities between the densiometric curves of the band patterns was determined based on the Pearson product–moment correlation coefficient. The bacterial population was characterized considering the Richness (*Rr*) and Community organization (*Co*) parameters based on the microbial resource management (MRM) concept described by Marzorati *et al.*[37] The *Rr* was determined as the number of bands in each DGGE pattern, with the aim to reflect the number of dominant species. The *Co* was calculated as the percentage of the Gini coefficient, with the aim to describe a specific degree of evenness of a microbial community by measuring the normalized area between a given Pareto–Lorenz curve and the perfect evenness line.[37,38]

RESULTS AND DISCUSSION

Products yields and substrate utilization

Observations on the methane yield

At a SO_4^{2-}/COD ratio of 0.05 (E-1 to E-3), the increase of the total influent COD caused variations on the methane yield between 330 and 362 mL CH_4 (g $COD_{removed}$)$^{-1}$ showing an average value of 350 ± 6.9 mL CH_4 (g $COD_{removed}$)$^{-1}$ (Fig. 1). Similar results were reported in the literature. A methane yield of 381 mL CH_4 (g $COD_{removed}$)$^{-1}$ was obtained during the treatment of undiluted vinasse in a modified UASB reactor at a SO_4^{2-}/COD ratio of 0.04,[39] whereas a methane yield of 373 mL CH_4 (g $COD_{removed}$)$^{-1}$ was also reported by Harada *et al.*[40] during the treatment of diluted vinasse at a SO_4^{2-}/COD ratio of 0.04.

Once the SO_4^{2-}/COD ratio increased to 0.10 (E-4), an average methane yield of 335 ± 8.1 mL CH_4 (g COD $_{removed}$)$^{-1}$ was obtained (Fig. 1). By comparing E-1 with E-4 (experiments with similar organic loading rate (OLR) of 7.66 and 7.90 g COD L_R^{-1} d^{-1}), a reduction of the average methane yield from 356 ± 5.8 to 335 ± 8.1 mL CH_4 (g COD $_{removed}$)$^{-1}$ was observed. This suggested the existence of microorganisms capable of degrading a larger fraction of the organic matter to form products other than methane at the higher SO_4^{2-}/COD ratio of 0.10 (i.e. at a higher sulfate loading rate (SLR)). A similar reduction [(20 mL CH_4 g $COD_{removed}$)$^{-1}$] of the methane yield was obtained by Erdirencelebi *et al.*,[17] when the SO_4^{2-}/COD ratio increased from 0.05 to 0.10 during the treatment of a synthetic wastewater.

The fluctuation in vinasse concentration at a SO_4^{2-}/COD ratio of 0.10 (E-4 to E-6) caused a gradual decrease in the methane yield, which was on average 335 ± 8.1, 305 ± 9.9 and 267 ± 3.8 mL CH_4 (g COD $_{removed}$)$^{-1}$ during E-4, E-5 and E-6, respectively (Fig. 1). A decrease in the reactor efficiency (in terms of COD removal) was observed at the SO_4^{2-}/COD ratio of 0.10, showing higher effluent COD concentrations for the experimental conditions E-4, E-5 and E-6 with respect to E-1, E-2 and E-3, respectively. Consequently, a remarkable decrease (up to 30%) in the methane production rate was observed in E-4, E-5 and E-6 with respect to E-1, E-2 and E-3, respectively. In contrast, Erdirencelebi *et al.*[17] showed similar COD removal efficiencies at SO_4^{2-}/COD ratios of 0.05 and 0.10, when OLR and SLR increased in the same order as this experiment. It was likely because they used a medium strength synthetic wastewater (6000 mg COD L^{-1}), which decreased the concentrations of $[H_2S]_{free}$ and H_2S_{aq} (both are known inhibitors of anaerobic digestion[9–11]) in the experiment of Erdirencelebi *et al.*,[17] leading to a higher COD removal efficiency in comparison with this experiment. However, Annachhatre and Suktrakoolvait[41] showed a 15% reduction of the COD removal efficiency when the SO_4^{2-}/COD ratios changed from 0.05 to 0.10. They worked also with a medium strength synthetic wastewater (6000 mg COD L^{-1}), but with an OLR and SLR three times higher than this experiment.

A reduction of the OLR to control toxicity (E-7) at the same SO_4^{2-}/COD ratio of 0.10 increased the methane yield to 317 ± 5.0 mL CH_4 (g $COD_{removed}$)$^{-1}$ (Fig. 1). In spite of that, a decrease in the reactor efficiency was observed, as the total effluent COD increased and the methane production rate decreased in E-7, when comparing E-7 with E-5, suggesting that methanogens were affected during the toxicity exposure under the experimental condition E-6.

During E-8, the SO_4^{2-}/COD ratio was changed to 0.15, causing a decrease in the average methane yield to 262 ± 12.5 mL CH_4 (g $COD_{removed}$)$^{-1}$, as well as a decrease in the reactor efficiency and the methane production rate, when comparing those results with experimental conditions at a similar OLR (e.g. E-1 and E-4).

To reach a SO_4^{2-}/COD ratio of 0.20 (E-9), the SLR was increased while maintaining the OLR. Under these conditions, a reduction in the methane yield to 237 mL CH_4 (g $COD_{removed}$)$^{-1}$ (without reaching steady state conditions) showed a tendency to methanogenesis failure, because an increase of the effluent COD together with a decrease in the methane production rate was observed. A tendency to methanogenesis failure was also reported during the treatment of sugar cane vinasse in a UASB reactor at a SO_4^{2-}/COD ratio of 0.23 when the OLR was increased to 6.1 g COD L_R^{-1} d^{-1}, showing methane production rates in the same order of magnitude as found in this experiment (1 L L_R^{-1} d^{-1}) after the first hydraulic retention time.[42]

A reduction in the methane yield was observed at SO_4^{2-}/COD ratios ≥0.10 suggesting: (i) inhibition of the methanogenic consortium, likely because of the formation of inhibitor compounds when the SLR was increased; and (ii) degradation of the organic matter by other bacterial groups to form products other than methane when the SLR was increased. A summary of the main observations with respect to the methane yield (observations 1 to 5) during the reactor operation can be found in the Appendix.

Observations on the sulfide yield

Sulfide yield and sulfur loading rates in the UASB reactor during the steady state conditions for E-1 to E-9 are shown in Fig. 2. At a SO_4^{2-}/COD ratio of 0.05, similar values for the average sulfide yield were observed, being 257 ± 5.3, 269 ± 5.6 and 262 ± 6.9 mg H_2S-S (g SO_4^{2-})$^{-1}$ for E-1, E-2 and E-3, respectively. An increase in total sulfide proportional to the influent sulfate concentration was observed during these periods, which accumulated as gas phase sulfide rather than as effluent sulfide, because of the increased stripping effect of a higher biogas production rate.[11,27] As effluent sulfide remained constant (50 mg H_2S-S L_R^{-1} d^{-1}) and below the inhibitory levels (150 and 590 mg H_2S-S L^{-1} for $[H_2S]_{free}$ and H_2S_{aq} respectively[9–11]), the methane and sulfide yields remained stable during E-1, E-2 and E-3 (Figs 1 and 2).

An increase in the SO_4^{2-}/COD ratio from 0.05 to 0.10 (E-4), resulted in an average sulfide yield of 260 ± 5.4 mg H_2S-S (g SO_4^{2-})$^{-1}$ which is similar to that obtained in E-1 to E-3. As more sulfide was produced in E-4 compared with E-1 (experimental conditions with similar OLR), this indicates that a higher fraction of the organic matter was degraded by SRB to form sulfides at the SO_4^{2-}/COD ratio of 0.10 (Fig. 2). Sulfate removal efficiencies (not shown) in the same order of magnitude (80–85%) were obtained at the SO_4^{2-}/COD ratios of 0.05 and 0.10, as reported earlier.[17,41]

The fluctuations of the COD and SO_4^{2-} concentrations in vinasse within the SO_4^{2-}/COD ratio of 0.10 (E-4 to E-7) showed: (i) an average sulfide yield in the same order of magnitude in E-4 and E-5 (262 ± 5.4 and 273 ± 5.9 mg H_2S-S (g SO_4^{2-})$^{-1}$, respectively), with an increase of total sulfide produced, suggesting an increase of the organic matter fraction degraded by SRB; (ii) a decrease in the average sulfide yield in E-6 [(255 ± 7.1 mg H_2S-S (g SO_4^{2-})$^{-1}$], suggesting inhibition of SRB; and (iii) a lower average sulfide yield in E-7 (261 ± 4.0 mg H_2S-S (g SO_4^{2-})$^{-1}$ compared with E-5 (experimental conditions with similar SRL and OLR), suggesting also that the SRB were affected during E-6 (Fig. 2).

Although total sulfide increased in E-8 (SO_4^{2-}/COD ratio of 0.15), a reduction of the average sulfide yield to 244 ± 7.6 mg H_2S-S (g SO_4^{2-})$^{-1}$ was observed, suggesting partial inhibition of SRB likely because of the increase of effluent sulfide (Fig. 2). Sulfate removal efficiencies (not shown) remained between 80 and 85%, in contrast to values around 70% reported[17,41] when more than ten times higher SLR values were used.

During E-9, a drastic reduction in the average sulfide yield was observed (178 ± 10.9), whereas the increase in the influent sulfate concentration did not increase the total sulfide produced (Fig. 2). The influent sulfate increase was not converted to sulfides and left the reactor as effluent sulfates and sulfurous precipitates.[11,41]

The average sulfide yield was stable at SO_4^{2-}/COD ratios of 0.05 and 0.10 with no significant differences. Only when the phase E-6 was conducted was a decrease in the average sulfide yield observed. As the total sulfide production increased during experiments E-1 to E-6, a higher fraction of the organic matter was removed by SRB, which explains the reduction in the methane

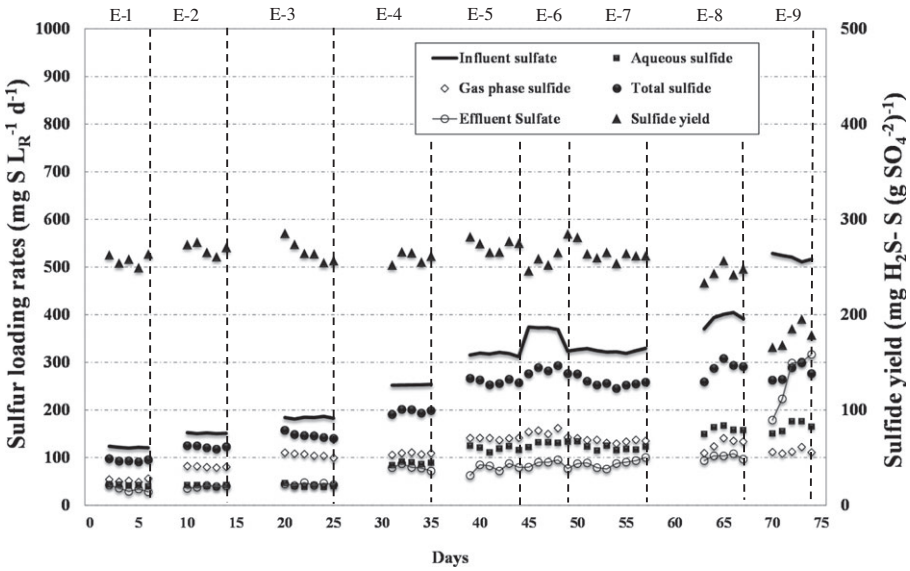

Figure 2. Sulfide yield and sulfur loading rates in the UASB reactor during the steady state conditions for E-1 to E-9.

yield discussed earlier. The increase of effluent sulfides caused inhibition of SRB, with 32% reduction compared with E-1, at a SO_4^{2-}/COD ratio of 0.20. A summary of the main observations with respect to the sulfide yield (observations 6 to 12) during the reactor performance can be found in the Appendix.

Observations on degradation of the organic matter

The fractions of organic matter (in terms of COD percentages) degraded by methanogens and SRBs are depicted in Fig. 3. Although the SLR increased during experiments E-1, E-2 and E-3 (SO_4^{2-}/COD ratio of 0.05), the COD fractions degraded by methanogens and SRBs remained constant, with average values of 95.5 ± 0.3% and 4.5 ± 0.3%, respectively. These observations were attributed to the concentrations of the inhibitor compounds (H_2S_{aq} and $[H_2S]_{free}$) below the inhibitory concentration.[11] At this SO_4^{2-}/COD ratio, similar values were reported[16,17,43] when similar OLR and SLR values were applied. Operation at a higher SLR caused 85% and 15% conversion of the COD by M and SRB, respectively.[41]

From experiments E-4 to E-6 (SO_4^{2-}/COD ratio of 0.10), the rise of the OLR and SLR increased the average COD fraction converted by SRBs from 9.3 ± 0.3% to 13.5 ± 0.5% (Fig. 3). The comparison among experiments with similar OLR and higher SLR (E-1 with E-4, E-2 with E-5, and E-3 with E-6) suggested that SRBs can outcompete methanogens when the SLR is increased.[9,10] When comparing E-5 with E-7 (experiment with similar OLR and SLR), two aspects were observed: (i) the organic fractions converted by methanogens decreased slightly, confirming that they were partially affected during the toxicity exposure under the experimental condition E-6; and (ii) the organic fraction converted by SRBs increased slightly, because of the small increase in SLR.

Towards the end of the experiments, the increase in SLR during E-8 and E-9 caused an increase in the average fraction of organic matter converted by SRB to 20.3 ± 0.8% and 27.1 ± 0.6%, respectively. These results were in between the lower (11.7%) and the higher values (58%) reported by Erdirencelebi *et al.*[17] and Annachatre and Suktrakoolvait,[41] when similar and higher SLR values were applied at a SO_4^{2-}/COD ratio of 0.20.

In general, during the different phases of the experiment, the COD fraction removed by SRB increased from 4.5 ± 0.3% to 27.1 ± 0.6% with increasing SLR. A summary of the main observations with respect to the degradation of the organic matter (observations 13 and 14) during the reactor performance can be found in the Appendix.

Microbial community characterization

The microbial community diversity and dynamics during the anaerobic digestion of a very high strength and sulfate-rich vinasse are discussed in this section, and linked with the experimental observations concerning product yields and organic matter degradation.

Effect on the dynamic of bacterial and archaeal community

Throughout the experiment, bacterial 16S rRNA gene copy numbers were higher than archaeal gene copy numbers (Fig. 4). This was expected considering that three of the four anaerobic digestion steps are carried out by the bacterial community and, moreover, the carbon source of the methanogenic archaea (responsible only for the fourth step), from acetate or CO_2, limits their growth rate due to the metabolic energy consumption required for their macromolecules and cell structure synthesis pathways.[44] The substrate characteristics (sulfate-rich waste stream) and the observations described above on the sulfate reduction process confirm the presence of hSRB, aSRB and pSRB and their contribution to the higher total bacteria abundance in the reactor, due to the substrate competition between SRB and M in the anaerobic digestion of vinasse.[6,12]

The methane and sulfide yields were maximum and stable during E-1, E-2 and E-3 (observation 1 and 6, Appendix), where the ratio total methanogens gene copies /total bacterial gene copies was 0.2 (Fig. 4), suggesting a suitable balance of the microbial community for reactor stability. Total bacterial gene copies remained constant in E-3 and E-4 (276.2 and 249.1 × 10^9 copies g^{-1}, respectively), showing stability of the bacterial groups, which agreed with the similar sulfide yields obtained during both periods. From E-4 to E-5, total bacteria increased five times, probably because of the favorable conditions for SRB demonstrated by the 30% increase of

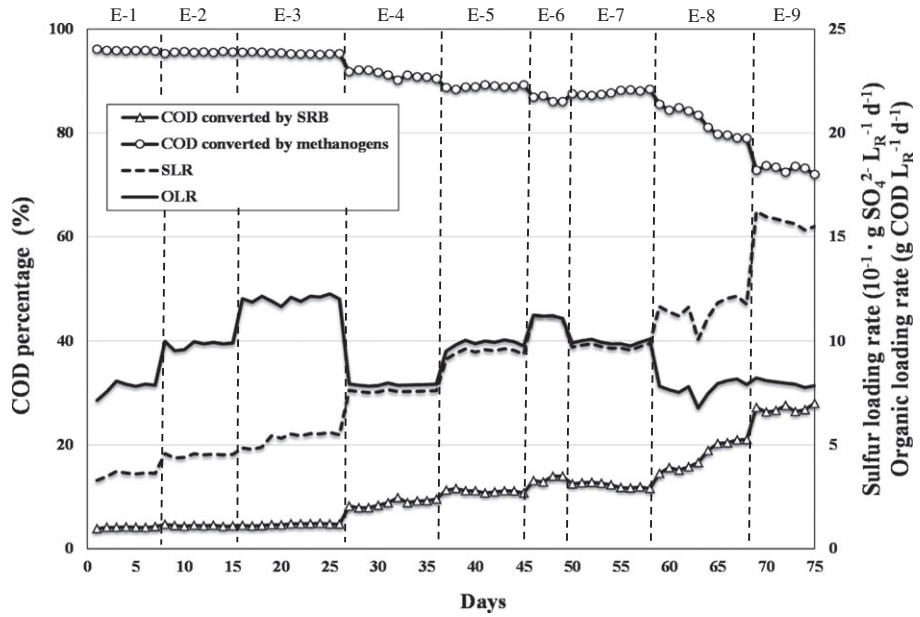

Figure 3. Fractions of organic matter degraded by M and SRB during the dynamics experiments with variations of the sulfur loading rate (SLR) and the organic loading rate (OLR).

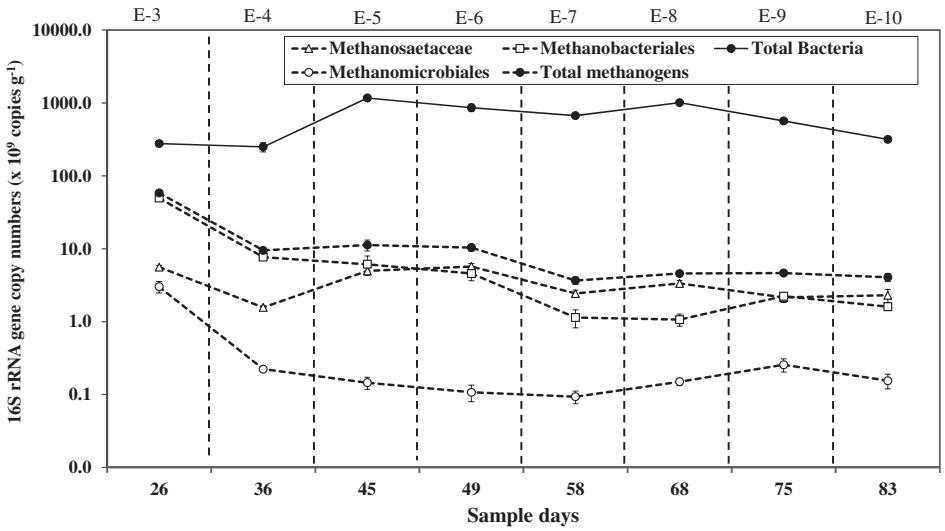

Figure 4. 16S rRNA gene (*rrs*) copy numbers within UASB reactor at different sampling times at the end of each experimental phase, i.e. on day 26, day 36, day 45, day 49, day 58, day 68, day 75, and day 83, determined by group-specific quantitative real-time PCR using total microbial DNA as template. The vertical error bars indicate the standard deviations of three replicates.

the total sulfide produced with sulfide yields in the same order of magnitude. The slight decrease of total bacteria in E-7 with respect to E-5 agreed with the response of SRB during the toxicity exposure under the experimental condition E-6 (observation 10, Appendix). Thus, the total bacterial dynamics during these experimental conditions suggested a strong contribution of SRB to the variation of the 16S rRNA gene copy numbers.

Although the sulfide yield decreased during E-8 suggesting partial inhibition for SRB (observation 11, Appendix), total bacterial number and total sulfide increased (Figs 2 and 4). Considering that the increase in total bacterial number was associated with an increase of SRB, this increase was not sufficient to degrade the excess of sulfates in the influent vinasse, and for that reason, a lower sulfide yield was obtained. The results of the microbial community dynamics rendered additional information to explain the observations at the SO_4^{2-}/COD ratio of 0.15. Towards the end of the experiments (E-9), a decrease of total bacteria was observed, possible due to a severe inhibition of SRB, agreeing with the values of the sulfide yield (observation 12, Appendix). Even when vinasse feeding ceased (E-10), the decrease in the total bacteria 16S rRNA gene copy numbers indicated unfavorable conditions for SRB (Fig. 4).

In general, the archaeal community showed a decrease of the total methanogens towards the end of the experiments, being similar for E-4, E-5, and E-6 ($9.5–11.3 \times 10^9$ copies g^{-1}) as well as for E-7, E-8, E-9 and E-10 ($3.67–4.64 \times 10^9$ copies g^{-1}) (Fig. 4). During E-3, higher total methanogens numbers were achieved, with the dominant species belonging to the order Methanobacteriales (hydrogenotrophic methanogenic archaea). Total methanogens decreased by a factor of six from E-3 to E-4, which corresponds

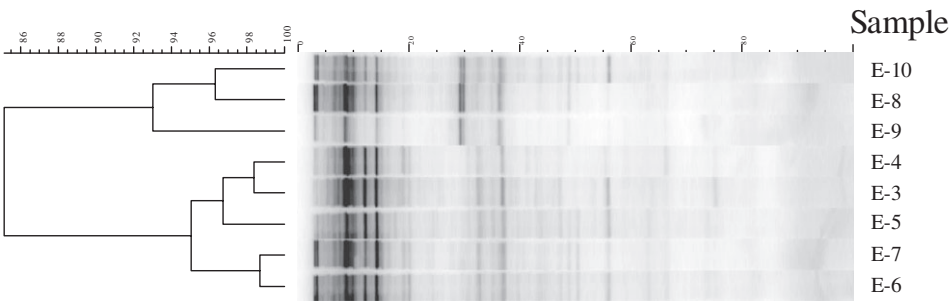

Figure 5. Cluster analysis of the DGGE fingerprint of the bacterial community in the UASB reactor at the end of each phase, i.e. E-3 (day 26), E-4 (day 36), E-5 (day 45), E-6 (day 49), E-7 (day 58), E-8 (day 68), E-9 (day 75), and E-10 (day 83). Cluster analysis (WARD algorithm) of the DGGE patterns performed based on the Pearson correlation coefficient, and expressed as percentage.

to the reduction of the methane yield described in observation 2 (Appendix). Although no significant differences were observed for total methanogens during E-4, E-5 and E-6 (based on the standard deviations), the 16S rRNA gene copy numbers for the species belonging to the order Methanomicrobiales and Methanobacteriales (hydrogenotrophic methanogenic archaea) decreased slightly (Fig. 4). In contrast, species belonging to the Methanosaetaceae (acetoclastic methanogenic archaea) were favored among the total methanogens (Fig. 4). Therefore, the reduction in methane yield from E-4 to E-6 (observation 2, Appendix) could be associated with the slight decrease in the hydrogenotrophic methanogenic archaea. These results suggested that a higher fraction of the organic matter in previous steps of the anaerobic digestion processes (e.g. propionate degradation) was removed by SRB (e.g. pSRB) to produce hydrogen sulfide, carbon dioxide and acetic acid, rather than by pDB to produce carbon dioxide, acetic acid and hydrogen, causing a reduction in the available hydrogen for Methanomicrobiales and Methanobacteriales. This consequently reduced their 16S rRNA gene copy numbers (Fig. 4). The acetic acid produced from this process supported the increase in the 16S rRNA gene copy numbers of Methanosaetaceae.

Although SLR and OLR were reduced to control toxicity in phase E-7, a decrease of the 16S rRNA gene copy numbers for total methanogens was observed in comparison with E-4, E-5 and E-6, which confirmed the toxic effect on methanogens during phase E-6. The total methanogen copy numbers remained similar from E-7 to E-10, showing an increase of the hydrogenotrophic methanogenic archaea (Methanomicrobiales and Methanobacteriales) and a decrease of acetoclastic methanogenic archaea (Methanosaetaceae) in E-9. These favorable conditions for hydrogenotrophic methanogenic archaea would be associated with a severe inhibition of SRB as described above (observation 12, Appendix). However, from E-8 to E-10 the remarkable decrease in methane yield suggested a tendency of methanogenesis failure, which apparently could not be explained based on the results of the archaeal 16S rRNA gene copy number (Fig. 4). Nevertheless, operational conditions, such as the acetic acid concentration, perhaps stimulated metabolic changes in the methanogenic population without cell lysis, influencing the methane yield. The relationships between microbial groups can impact on their metabolic patterns and the amount of methane generated.[45] In addition, the high level of stability of microbial DNA does not confirm a fast response in the 16S rRNA gene copy number in reflection of a decrease in methanogenic activity.

Despite the fact that Methanosarcinaceae are considered the most robust methanogen from metabolic and physiologic points of view,[46] in this study no species belonging to Methanosarcinaceae were detected. Acetoclastic methanogenic archaea species belonging to Methanosaetaceae were not affected by the increase in the SO_4^{2-}/COD ratio. Several authors have recognized *Methanosaeta spp*. as indicators for well-balanced anaerobic digestion processes, considering their dominance under stable operating conditions.[47,48] In contrast, species belonging to Methanomicrobiales and Methanobacteriales order were severely affected. Three aspects can explain these findings: (i) the low acetic acid concentrations detected during most of the experiments (below 2000 mg L^{-1} until day 64);[11] considering the high affinity of Methanosaetaceae for acetate; (ii) the high substrate competition between aSRB and acetoclastic methanogenic archaea (Methanosaetaceae), leading to low acetic acid concentrations, due to their similar Gibbs Free Energy;[49–52] and (iii) the fact that hSRB outcompete hydrogenotrophic methanogenic archaea (Methanomicrobiales and Methanobacteriales) for the available hydrogen.

Cluster analysis of the bacterial community DGGE patterns

To obtain an overview of the bacterial community dynamics during the different phases of the experiment, cluster analysis of the DGGE patterns was carried out (Fig. 5). Similar DGGE patterns were observed for E-3 and E-4 (Pearson correlation > 98%), suggesting no significant changes of the bacterial community when the SLR was increased from 0.53 to 0.76 g SO_4^{2-} L_R^{-1} d^{-1}, and the OLR decreased from 12.00 to 7.90 g COD L_R^{-1} d^{-1}. The variation of the DGGE pattern from E-4, to E-5 and to E-6, could be explained by the likely development of SRB when the SLR was increased.

Similar DGGE patterns were observed for E-6 and E-7 (Pearson correlation ≈ 99%), indicating no changes of the bacterial community, because the changes in the community during the toxicity exposure during E-6 persisted during E-7. The increase in the SLR in E-8 and E-9 affected the bacteria community (Pearson correlation < 85%). Therefore, the increase of the SO_4^{2-}/COD ratio above 0.10 shifted the bacterial community, suggesting that substrate composition determines bacterial community organization and dynamics in this process, as described in the literature.[46,53]

Bacterial richness and community organization

The bacterial richness (*Rr*) and community organization (*Co*) (Table 2) were revealed based on the DGGE patterns. The *Rr* of the bacterial community decreased from E-3 to E-4, which corresponded with a reduction in influent COD concentration (from 58 to 38 g COD L^{-1}), while the *Rr* increased from E-4 to E-6 (SO_4^{2-}/COD ratio of 0.10), after the influent COD was increased, suggesting that bacterial richness is associated with the substrate

Table 2. Bacterial richness and community organization revealed by DGGE analysis of the bacterial community in the USAB reactor under different experimental conditions, i.e. E-3 (day 26), E-4 (day 36), E-5 (day 45), E-6 (day 49), E-7 (day 58), E-8 (day 68), E-9 (day 75), and E-10 (day 83)

	Experimental conditions							
	E-3	E-4	E-5	E-6	E-7	E-8	E-9	E-10
Richness (*Rr*)	34.7	24.0	47.3	45.5	29.1	17.5	6.8	17.8
Community organization (*Co*)	40.2	49.1	27.2	35.1	46.9	41.1	59.3	46.9

COD concentration at SO_4^{2-}/COD ratios ≤0.10. A decrease of *Rr* was observed during phase E-7 due to the toxicity exposure at E-6. Consequently, at SO_4^{2-}/COD ratios of 0.15 (E-8) and 0.20 (E-9), *Rr* decreased from 17.5 to 6.8, indicating an inverse relationship between *Rr* and SLR at SO_4^{2-}/COD ratios >0.10, together with a toxicity to the bacterial community. When the feeding of vinasse was stopped (E-10), the *Rr* increased from 6.8 (E-9) to 17.8 (E-10), suggesting the possibility of bacterial population diversity retrieval after a short period of sulfide toxicity, likely because the COD remaining in the reactor liquid bulk from the nourished medium, which could stimulate alternative metabolic pathways and the growth of multiple different species.

The *Rr* values in this study were similar to those reported by Dar *et al.*,[54] who studied two sulfidogenic reactors. The lower *Rr* values obtained in this work could be explained by the use of vinasse as mono-substrate, despite the fact that vinasse contain various organic molecules. The high strength and sulfate-rich vinasse assayed in this study strongly reduced or limited the bacterial richness; as postulated by several authors the substrate characteristics determine the bacterial richness in anaerobic reactors (Table 2).[55–57]

Table 2**.** Bacterial richness and community organization revealed by DGGE analysis of the bacterial community in the USAB reactor under different experimental conditions, i.e. E-3 (day 26), E-4 (day 36), E-5 (day 45), E-6 (day 49), E-7 (day 58), E-8 (day 68), E-9 (day 75), and E-10 (day 83).

The community organization (*Co*) values are an indication of evenness, and inform on the functional organization of the microbial community.[37,38] Low (20–25) and high (>80) *Co* values indicated a highly even and specialized community, respectively. Average *Co* values (45–60) represent balanced communities that can potentially deal with changing environmental conditions.

In this study, most of the *Co* values were between 40 and 59.3 (Table 2), which were representative of balanced microbial communities and functional stability.[37] The *Co* showed similar values throughout the different phases of the experiment, except for E-5, E-6 and E-9. During E-5, the *Co* value was the lowest, when OLR and SLR were increased at the same time, and no toxicity was observed yet (E-6), suggesting a tendency to a more even community. After the apparent toxic effect (from E-6 to E-9), *Co* values increased, which showed a tendency to a more uneven community, suggesting that uneven communities might not be indicative of a well-functioning reactor. The increase in SRB activity toward the end of the experiment (from E-6 to E-9) supported by the increase in its organic fraction conversion (observation 14, Appendix) and the reduction in the total methanogens (Fig. 4), could represent a community specialization. Therefore, the electron-flow to SRB increases with increasing SO_4^{2-}/COD ratio as was indicated by Wang *et al.*[58]

CONCLUSIONS

The microbial community dynamics during the anaerobic digestion of a very high strength and sulfate rich vinasse was investigated. The methane and sulfide yields decreased with increasing SO_4^{2-}/COD ratio, while the fraction of organic matter degraded by sulfate reducing bacteria increased to 27.1 ± 0.6%. The dynamics of the archaeal community showed that Methanosaetaceae were little affected by the increase of SO_4^{2-}/COD ratio, in contrast to Methanomicrobiales and Methanobacteriales. The variation of the sulfide yield was explained from the total bacteria dynamics through the 16S rRNA gene copy numbers. Therefore, the bacterial diversity was influenced mainly by substrate composition, showing that the increase of SO_4^{2-}/COD ratio above 0.10 shifted the bacterial community to a lower richness. These results provide knowledge on the dynamics of the microbial communities, which can be useful to control anaerobic digestion of sulfate-rich vinasses, showing that reactor stability equates to the higher ratios between total methanogens and total bacteria gene copy numbers, whereas operational difficulties (e.g. reduction of methane yield) can be associated to lower bacterial richness and higher community organization. Further metagenomics studies are needed concerning the taxonomic identification and metabolic potential of these bacterial communities to ensure a better understanding of which species are involved in the sulfate reduction process, but this was outside the scope of this research.

ACKNOWLEDGEMENTS

Janet Jiménez and Ernesto L. Barrera were supported financially by the VLIR Program, Project ZEIN2009PR362. Jo De Vrieze is supported as postdoctoral fellow from the Research Foundation Flanders (FWO-Vlaanderen).

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Appendix. Summary of the main observations during the reactor performance.

No. Observations	SO_4^{2-}/ COD ratio	Experimental condition
Observations on the methane yield		
1. Maximum and stable values for methane yields.	0.05	E-1 to E-3
2. Reduction of the methane yield with respect to E-3, suggesting inhibition for M and degradation of the organic matter by other bacterial groups to form other products than methane.	0.10	E-4 to E-6
3. Affectation of methanogens during the toxicity exposure at the experimental condition E-6.	0.10	E-7
4. Remarkable decrease of the methane yield suggesting severe inhibition of methanogens.	0.15	E-8
5. Remarkable decrease of the methane yield suggesting a tendency to methanogenesis failure.	0.20	E-9
Observations on the sulfide yield		
6. Maximum and stable values for the sulfide yield.	0.05	E-1 to E-3
7. Sulfide yield of the same order of magnitude as the one obtained in E-3.		E-4
8. Increasing of the sulfide yield suggesting an increase of the organic matter fraction degraded by SRB.	0.10	E-5
9. Decreasing of the sulfide yield suggesting inhibition of SRB.	0.10	E-6
10. Affectation of SRB during the toxicity exposure at the experimental condition E-6.	0.10	E-7
11. Reduction of the sulfide yield advising partial inhibition of SRB.	0.15	E-8
12. Drastic reduction of the sulfide yield.	0.20	E-9
Observations on degradation of the organic matter		
13. Gradual decrease of the COD fraction converted by M.	0.05 to 0.20	E-1 to E-9
14. Gradual increase of the COD fraction converted by SRBs.	0.05 to 0.20	E-1 to E-9