Intermittent operation of UASB reactors treating wastewater polluted with organic solvents: process performance and microbial community evaluation

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Abstract

The effect of intermittent feeding on the treatment of wastewater polluted with ethanol, ethyl acetate and 1-ethoxy-2-propanol in anaerobic upflow sludge blanket reactors was investigated. Three laboratory-scale reactors, one periodically supplemented with chitosan, were operated in an intermittent pattern (16 hours/day; 5 days/week) during 5 months. Removal efficiencies higher than 94% were obtained at organic loading rates up to 50 kgCOD m^{-3} d^{-1}. The addition of chitosan positively affected the specific methanogenic activity of the granular sludge. Although partial deterioration of the granules was observed, it was not correlated with variations in the production of extracellular polymeric substances, the percentage of granules remained between 57 and 84%. Microbial community analysis showed the prevalence of bacteria of the genus *Geobacte*r and archaea of the *Methanocorpusculum* genus were the most abundant methanogens, suggesting that hydrogenotrophic methanogenesis, with the syntrophic oxidation of the substrate, was an important pathway for the solvent degradation.

Keywords: Anaerobic reactors; intermittent feeding; solvents; DGGE; High-throughput sequencing.

1 Introduction

High rate anaerobic reactors are an effective technology for the treatment of industrial wastewater. The advantages of the high rate anaerobic reactors compared to the conventional aerobic process (such as lower energy requirements, lower sludge generation, the recovery of bioenergy as methane, and the application of high loading rates) has consolidated this technology for the treatment of medium and high strength wastewater. Of these, sludge bed reactors (such as the upflow anaerobic sludge bed (UASB) reactor), have been widely applied to the treatment of food, beverages and agro-based industrial wastewater[1]. Nowadays, their application includes the treatment of wastewater from other sectors, such as those polluted with organic solvents from chemical[2], petrochemical[3] or pharmaceutical industries[4].

Despite the advantages of the high rate reactors, the sensitivity of the anaerobic process to system imbalances and the instability of transitory phases are drawbacks that limit the widespread use of anaerobic technology compared to aerobic processes[5]. In anaerobic treatment, a delicate balance exists between the hydrolysis-acidogenesis phases and the acetogenesis-methanogenesis phases. This balance remains mostly stable for effluents with a steady composition, concentration and flow rate. However, in practice, industrial effluents are subjected to organic and flow rate fluctuations that may adversely affect the stability of the reactor and the efficiency of the treatment[6]. Process imbalances often cause deterioration in COD removal, reduce biogas production, change biogas composition and reduce effluent quality and sometimes, in a temporary higher sludge washout[5, 6]. The properties of the granular sludge affects, or even governs, the overall performance of the process[7], thus maintaining a robust granular structure with varying conditions is highly desirable to ensure an effective treatment. Although sludge bed anaerobic reactors have been shown to be feasible systems for the treatment of wastewater

64 polluted with organic solvents, one drawback has been pointed out in several studies, i.e.
65 the partial or total disintegration of the aggregates as a consequence of perturbations in
66 operational conditions, such as the shift in wastewater composition and strength[8], the
67 exposure to specific solvents[9], the application of high organic loads[2] or fluctuations in
68 wastewater supply[10]. Physical disruption of granules could result in the loss of
69 methanogenic activity because of the decrease in the syntrophic interactions which are
70 favored by the granular structure[11] and, in most cases, it may lead to the washout of active
71 biomass[12].

72 The shift in the microbial population, as a result of disturbances caused by hydraulic
73 and organic shock loads, has been observed through molecular techniques such as
74 denaturing gradient gel electrophoresis (DGGE) and next generation sequencing (NGS).
75 The adaptability of the microorganisms to varying conditions, as well as the maintenance
76 of individual populations during periodic fluctuation determines the effectiveness of long-
77 term treatment and the robustness of the anaerobic reactors[13]. Several studies have
78 addressed the effects of variations in flow or concentration on the operation of microbial
79 communities in anaerobic reactors treating molasses, carbohydrates or dairy effluents[14,
80 12, 15], but there is still a lack of information about the effect of such disturbances on the
81 anaerobic treatment of wastewater polluted with organic solvents.

82 The main objective of this study was to evaluate the robustness of sludge bed
83 reactors treating solvent-polluted wastewater under intermittent feeding, caused by
84 typical shutdown periods at industrial facilities. For this purpose, we evaluated: 1) the
85 performance of three UASB reactors fed with synthetic wastewater polluted with ethanol,
86 ethyl acetate and the glycol ether 1-ethoxy-2-propanol, at an intermittent pattern of 16 h
87 day^{-1}; 5 days per week; 2) the effect of the intermittent operation on the stability of
88 granular sludge, by assessing the dynamics of the physicochemical characteristics of the

sludge; and 3) the effect of the intermittent operation on the microbial community structure. We also assessed the effects of adding the cationic polymer chitosan, which is proven to be effective in assisting granulation in sludge bed reactors treating solvent-polluted wastewater[16].

2 Materials and methods

2.1 Experimental set-up

2.1.1 Reactors configuration and feed characteristics

Three identical UASB reactors (R1, R2 and R3), with an effective volume of 7.8 L, were used to perform the experiments. The schematics of the reactor configuration are shown in Fig. S1. The reactors consisted of two PVC parts: a bottom zone of 6.5 cm in diameter and 120 cm in height and a settling zone containing the gas-liquid-solid separator, with a diameter of 20 cm and a height of 24 cm. Water (containing nutrients and alkalinity) was pumped from a tank with a peristaltic pump (Watson-Marlow, USA). The macronutrients N and P were added in a COD ratio of 300:2:1. Micronutrients were supplemented according to the compositions shown in Table S1. Ca^{+2} and Mg^{+2} were added as $CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$ to ensure 100 and 40 mg L^{-1} in the influent, respectively, and $NaHCO_3$ was then added in order to maintain a pH between 7.0 and 7.5. The inlet stream of each reactor was contaminated with a mixture of ethanol, ethyl acetate and 1-ethoxy-2 propanol (E2P), as the major constituents of the emission from the flexographic industry, in a mass ratio of 7:2:1, by using a syringe pump (New Era, 1000 model, USA). The upflow velocity was regulated by adjusting the liquid recirculation flow rate using a peristaltic pump (Watson-Marlow, USA). The biogas produced was passed through a NaOH solution (3M) to absorb the CO_2 content before being conducted to the gas flow meter (AMPTS II, Bioprocess Control, Sweden).

2.1.2 Source of inoculum

Each reactor was seeded with 2.5 L of granular sludge, obtained from a previous experiment which studied UASB reactors treating a synthetic wastewater polluted with the same organic solvents and in which the addition of chitosan was evaluated by studying the formation of anaerobic granules[16]. The sludge from reactor R1 was obtained without chitosan, whereas the sludge from the reactors R2 and R3 was granulated with the addition of polymer doses of 2.4 mg g VSS^{-1} two times. The reactors, from which the sludge was obtained, were working at a continuous OLR of 20 kg COD m^{-3} d^{-1} for more than 30 days. Before the start of this study, the sludge was sieved through a 50-mesh sieve to remove fine particles and standardize particle size in the three reactors. The percentage of granules, which is defined as the percentage of aggregates with a particle size greater than 300 μm, was 73.2% for R1, 76.0% for R2 and 74.7% for R3, with a mean particle size of 500, 570 and 625 μm for R1, R2 and R3, respectively.

2.1.3 Experimental procedure and operational conditions

The UASB reactors were started up simultaneously and operated under intermittent feeding at room temperature (26.1 ± 1.1 °C). In order to evaluate the effect of chitosan on the reactors' performance and on the biomass characteristics under intermittent feeding, reactor R2 was supplied with 2.4 mg g VSS^{-1} of chitosan at the seeding point and with a frequency of 21 days, thereafter. R1 and R3 were operated without the addition of chitosan. Chitosan was applied using a stock solution of 1% commercial grade chitosan powder (medium molecular weight: 75% deacetylation grade, Sigma-Aldrich, Spain) with 1% acetic acid.

Synthetic solvent-based wastewater was fed to the reactors in an intermittent pattern of 16 hours per day, 5 days per week. Wastewater supply was stopped during nights and weekends, simulating typical shutdown periods of industrial facilities related to

manufacturing shift work. Recirculation was maintained during the shutdown periods. To determine the transient response of the reactors to feeding resumption, the characteristics of the effluents (COD concentration, volatile fatty acid (VFA) concentration and solvent composition) and methane production were measured every 2 hours, from the recommencement of feeding resumption until 8 hour later. The transient response was evaluated twice per week: on Mondays, after 56 h without substrate supply (weekend shutdown) and on Thursdays, after an 8 h feedless period (night shutdown). The experiment was performed in four phases, each phase corresponding to an increasing OLR and the HRT was set at 10 h. Table 1 summarizes operational conditions in each phase. Since the inoculum of each reactor was adapted to the organic solvents, the reactors were started up and operated during phase I (days 0 to 48) at the high OLR of 20 kg COD m^{-3} d^{-1}. The OLR was increased in each phase up to 50 kg COD m^{-3} d^{-1} for all reactors. In phase I, the liquid upflow velocity (U_L) was 0.5 m h^{-1} during the feeding periods. From the first day of phase II onwards, it was adjusted to 1 m h^{-1}.

2.2 Analytical methods

The soluble COD of effluent samples filtered by 0.22 μm, TSS and VSS were measured according to the Standard Methods for the Examination of Water and Wastewater[17]. The VFA and alkalinity of centrifuged samples were determined using a titrator (848 Titrino Plus, Metrohm, Switzerland). The VFA represents the concentration of short chain volatile fatty acids, expressed as acetic acid (mg HAc L^{-1}). The solvent effluent content of samples filtered by 0.22 μm was analyzed in a gas chromatograph (Agilent GC 7890A, Spain) equipped with a Restek Rtx-VMS column (30 m × 0.25 mm × 1.4 mm) and a flame ionization detector. Biogas composition was measured in a gas chromatograph (Agilent GC 7820A, Spain) with thermal conductivity detector and

equipped with two columns connected in series: HP-Plot/U (30 m × 0.32 mm × 10 mm) and HPMolisieve (30m × 0.32mm × 12 mm). Methane production was monitored by using the volumetric gas meter of an automatic methane potential test system (AMPTS II, Bioprocess Control, Sweden).

2.3 Granular sludge properties

2.3.1 Specific Methanogenic Activity (SMA)

SMA tests of the biomass sampled from the reactors on day 126 were conducted in an AMPTS II (Bioprocess Control, Sweden). The tests were carried out at 25 ºC in flasks of 500 mL intermittently stirred (1 min on/1 min off) at 112 rpm. Flasks were filled with biomass and medium at a ratio of 2.1 g VSS g COD^{-1}. The medium consisted of synthetic wastewater contaminated with the ternary mixture of solvents at a concentration of 2.5 g COD L^{-1}. The medium was supplemented with macro and micronutrients and buffered with $NaHCO_3$ to maintain the pH between 7 and 7.5.

2.3.2 Particle size distribution

Particle size distribution (by volume) was measured every 2 to 3 weeks by laser diffraction using a Mastersizer 2000 (Malvern Instruments Ltd, UK) with a detection range of 0.02–2000 μm. The sludge samples were taken from each reactor and filtered through a 2 mm sieve and the fraction < 2 mm was then measured in triplicate.

2.3.3 Extraction and characterization of EPS

Two EPS fractions, slime EPS (S-EPS) and tightly bound EPS (T-EPS), were extracted from the sludge samples taken from the reactors on days 0, 29, 58, 100, 126 and 147. Sludge samples of 50 mL were centrifuged at 8000 g for 15 min at 4 °C; the supernatant was filtered by 0.45 μm and then collected as the S-EPS fraction. The sludge pellets were re-suspended and diluted to the original volume by adding a buffer solution

(pH 7.0) to extract the T-EPS. The extraction was carried out using the sonication and cationic exchange resin (CER) method of D'Abzac et al.[18]. The solution was sonicated at 42 kHz for 1 minute using a Branson ultrasonic (MT-1510, USA). Cationic exchange resin (Dowex 20–50 mesh, Sigma-Aldrich, Spain) was then added at a ratio of 70 g resin g VSS^{-1} and the mixture was stirred at 600 rpm for 3 h at 4 °C, followed by centrifugation at 15,000 g for 30 min at 4 °C; the supernatant was filtered by 0.45 μm and collected as the T-EPS fraction from the sludge samples. Polysaccharides (PS) and proteins (PN) of both EPS fractions were determined by the Dubois et al.[19] and Lowry et al.[20] colorimetric method, respectively.

2.4 Microbial community analysis

Microbial community analysis was performed on the samples taken from the reactors on days 0, 58, 100, 126 and 147. DNA was extracted using the Power Soil DNA Isolation Kit (MOBIO Laboratories, USA) and stored at -20 ºC. PCR and DGGE were carried out according to the method proposed by Bravo et al.[21], adapting the conditions of the linear denaturant gradient: from 40 to 55% for archaeal DGGE and from 35 to 50% for bacterial DGGE. Electrophoresis was performed at a constant voltage of 100 V and a temperature of 60ºC for 14 h. The sequencing results were compared with the 16S rRNA sequences in the GenBank™ Database using the Basic Local Alignment Search Tool (BLAST). For the high-throughput sequencing of the samples taken on day 0 and 147, the V4 hyper-variable region of the extracted DNA was amplified with the universal primers 515F (5'-GTG CCA GCMGCC GCG GTA A-3') and 806R (5'-GGACTA CHV GGGTWT CTA AT-3'). Sequencing was performed using a MiSeq System (Illumina, USA). The raw 16S rRNA gene sequences obtained were screened and trimmed by using the Quantitative Insights Into Microbial Ecology (QIIME) software with a sequence length (200 nt) and mean quality score cut-off of 25 nt.

3 Results and discussion

3.1 Performance of the reactors

The OLR_{16h} applied to the reactors during the feeding periods and their performance in terms of the COD removal efficiency, effluent VFA concentration and methane production are depicted in Fig. 1a, 1b and 1c, respectively. The values correspond to measurements taken 8 hours after the resumption of feeding. As the seed sludges were adapted to the solvents, COD removal efficiencies greater than 94% were obtained from the beginning of the experiment. Removal efficiencies remained in these ranges during phases II and III also (with OLR_{16h} of 25 and 35 kg COD m^{-3} d^{-1}, respectively), with the exception of slight transitory decreases in response to the OLR_{16h} increase. The evolution of the VFA concentration also showed the effect of the OLR_{16h} increase, with values up to 200 mg HAc L^{-1} on applying OLR_{16h} steps, and progressively decreasing to values lower than 10 mg HAc L^{-1} in R1 and R2, and lower than 30-60 mg HAc L^{-1} in R3.

The OLR_{16h} was increased to 50 kg COD m^{-3} d^{-1} at the beginning of phase IV. The COD removal efficiency of R1 showed a sharp decrease up to 70% on day 119, with the concomitant increase in the effluent VFA concentration up to 1750 mg HAc L^{-1} and the pH dropping to 5.0, showing the incapability of this reactor to treat an OLR so high. In order to avoid complete inhibition in this reactor, the OLR_{16h} was decreased to the previous value of 35 kg COD m^{-3} d^{-1} on day 120 and, soon after, the performance of R1 was restored. R2 and R3 showed stable performances at the OLR_{16h} of 50 kg COD m^{-3} d^{-1}, with COD removal efficiencies higher than 90%, although VFA concentrations (average values of 225 and 452 mg HAc L^{-1} for R2 and R3, respectively) were higher than in previous phases (average value lower than 50 mg HAc L^{-1}). R2 exhibited better performance than R3, in terms of VFA concentrations. The better performance of reactor

R2 can be attributed to the addition of chitosan, which may have contributed to greater biomass retention, just like it was shown by the VSS in the effluent (Fig. S2). The higher retention capacity of R2 would explain the lower VFA concentration in its effluent, so that the specific methanogenic activity was kept and the microbial community was able to treat the applied OLR with a high performance. At the end of phase IV, the OLR_{16h} was further increased from 50 to 75 kg COD m^{-3} d^{-1} in R2 and R3. The OLR increase led to the accumulation of VFA and the failure of the degradation process. After two operational cycles of 16 hours, the VFA were accumulating, reaching values of 6615 and 6220 mg HAc L^{-1} in R2 and R3 (data not shown), respectively, with a pH drop to 5.0 and decrease in the COD removal efficiency of up to 55% in both reactors. These results indicated that the treatment capacity of these systems was exceeded and the experiment was finalized. Taking into account the performance results of the three reactors operating at 35 to 50 kg COD m^{-3} d^{-1}, it can be concluded that the UASB is a robust reactor configuration to treat a synthetic solvent-polluted wastewater in an intermittent pattern (16 h per day; 5 days per week). The application is suitable for the treatment of wastewater polluted with organic solvents, such those from the flexographic sector, up to an OLR_{16h} of at least 50 kg COD m^{-3} d^{-1}.

The methane production of the three reactors increased as the OLR_{16h} was applied, reaching relatively stable values at the end of each operational phase. During phase I, the methane production of the reactors whose inoculum was obtained with the addition of chitosan (R2 (45.1±5.1 L d^{-1}) and R3 (44.4±5.1 L $d^{-)}$)) was higher than that of R1 (39.3±4.4 L d^{-1}). In phase II, the three systems showed a similar methane production. In phases III and IV, reactor R2, to which chitosan was added periodically every 3 weeks, showed a methane production 4 to 7% higher than that of the other reactors (without considering the de-stabilization period in R1 during phase IV). Thus, experimental results

suggest that the methanogenic activity of a UASB reactor operated at a high OLR can be improved by the periodic addition of chitosan. These results are consistent with the SMA of the sludge of the three reactors, which was evaluated on day 126. For the reactor R2, a higher SMA of 530 NmL CH_4 g VSS^{-1} d^{-1} was obtained compared to the values 465 and 450 NmL CH_4 g VSS^{-1} d^{-1} obtained for R1 and R3. These results represent an improvement of the SMA of the chitosan-assisted reactor of 12 to 15%.

The average methane yields obtained throughout the experiment were 0.256±0.051, 0.282±0.032 and 0.268±0.035 Nm^3 CH_4 kg $COD_{removed}^{-1}$ for R1, R2 and R3, respectively. In the three reactors, a decrease in methane yield was observed as the organic load increased and the values were lower than those obtained during the continuous treatment of solvent-polluted wastewater, which were closer to the theoretical value of 0.350 Nm^3 CH_4 kg $COD_{removed}^{-1}$ [16] showing a shift in the response of the anaerobic biomass under intermittent conditions. Nevertheless, methane yield values were similar to those reported in other studies of sludge bed anaerobic reactors, operated under periodic organic and/or hydraulic loading shocks[10, 12].

Throughout the experiment, the effluent of the three reactors was characterized by the presence of 1-ethoxy-2-propanol; the other solvents, ethanol and ethyl acetate, were almost completely degraded with COD removal efficiencies higher than 99% (except during process failure of R1 on day 119 and of R1 and R3 at the end of the experiment) and the removal efficiency of E2P was lower. The applied OLR_{16h} of E2P and the removal efficiency in the three reactors is illustrated in Fig. 2. In phase I, when operating at an OLR_{16h} of E2P of 2.1 kg COD m^{-3} d^{-1}, the E2P removal efficiency of the reactor R3 was higher than in the other reactors, with an average of 83±4% compared to values of 77±4% and 75±8% for R1 and R2, respectively. In phases II and III, the three reactors achieved similar removal efficiencies between 80 and 85%, operating at an OLR_{16h} of E2P 3.7 kg

285 COD m^{-3} d^{-1}. These removal efficiencies were maintained for R2 and R3 operating at 5.7
286 kg COD m^{-3} d^{-1} during phase IV. For R1, the process disturbance on day 119 led to the
287 decrease of the E2P elimination capacity; although the same operational conditions before
288 the overloading were re-established, the removal efficiencies were lower than those
289 obtained during phase III, with an average value of 70±8%. This may have been caused
290 by the decrease in pH that could adversely affect the populations of microorganisms
291 capable of carrying out the degradation of this organic solvent. The byproducts acetone
292 and isopropanol were detected in the effluent of the three reactors at low concentrations
293 (< 30 mg L^{-1} for acetone and < 10 mg L^{-1} for isopropanol). Acetone has been proposed as
294 an intermediate product in the anaerobic degradation of glycol ethers such as E2P and
295 isopropanol has been reported to appear by reversible reduction of acetone in the presence
296 of H_2[22, 23].

297 3.2 Transient response to substrate resumption

298 The continuous monitoring of methane production was performed over 4 operation
299 cycles (106 hours), from day 98 to day 102 (Fig. S3). The reactors showed a nearly
300 constant methane production during the feeding periods, with a slightly higher production
301 for R2 (with the periodic addition of chitosan). The resumption of methane production
302 after the feedless periods, as well as the conversion of the remaining organic matter when
303 the feeding was stopped, occurred in less than 1.5 hours. This result indicates that
304 substrate was not accumulated during the feeding periods and supports the idea that the
305 reactors were well adapted to the operational cycles. In contrast, Nadais et al.[15] reported
306 that 25% of the total methane was produced during the feedless periods in the intermittent
307 treatment of dairy wastewater. The quick shutdown and recovery of methane production
308 in the present study can be attributed to the characteristics of the wastewater mostly being
309 composed of a readily biodegradable solvent, such as ethanol.

The transient response of the reactors to the wastewater supply resumption was evaluated during all of the experimental phases. VFA concentration and methane yield were evaluated every 2 h from the feeding resumption until 8 h later, see Fig. 3. For methane yield, average values for each phase have been depicted. For VFA, the figures include data averages, excluding phase IV where an imbalance in the anaerobic process resulted in high VFA concentrations. After periods of 8 h without substrate supply (Fig. 3a), VFA concentration showed similar variations in the three reactors, increasing from values of 0 mg HAc L^{-1} to maximum values of <75 mg HAc L^{-1} after 2 to 4 hours of operation and then decreasing at the end of the monitoring period. Methane yield increased after 2 hours from resumption of the feeding. There were no notable differences between the three reactors, all reaching values of approximately 0.280 Nm^3 CH_4 kg $COD^{-1}_{removed}$ after 8 hours of the substrate supply resumption.

The feedless periods of 56 hours affected the stability of the reactors to a greater extent. The VFA concentration reached values around 150 mg HAc L^{-1} during the transitory period, showing a higher variability compared to the 8 hours shutdown periods. The methane yield after shutdown periods of 56 hours indicated a slower recovery of the reactors, with values after 2 hours being significantly lower compared to those of the 8 hours feedless periods. At the end of the monitoring period, the methane yields were 0.250, 0.280 and 0.270 Nm^3 CH_4 kg $COD^{-1}_{removed}$ for R1, R2 and R3, respectively; slightly lower than those in the shorter shutdown periods, as previously reported by Lafita et al.[10].

3.3 Effect of intermittent feeding on granule characteristics

3.3.1 Particle size distribution

Table 2 summarizes the percentage of granules (> 300 μm) and the mean diameter of the sludge samples taken during the study. For more detail, size distribution of the

sludge samples is shown in Fig. S4. The percentage of granules was not affected during the first four weeks of intermittent operation, with values in the range of 71.7 to 78.3% and stable values of mean diameter. Afterwards, the flotation and washout of big granules in the upper zone of all of the reactors was observed, which led to a decrease in the percentage of granules with a diameter greater than 650 μm**.** Consequently, the mean diameter in all the reactors decreased on day 43 (Table 2) as well as the percentage of granules (56.4, 64.3 and 52.7% for R1, R2 and R3, respectively). From this day onwards, the U_L was increased from 0.5 to 1.0 m h^{-1} in order to reconcile the operational conditions at laboratory scale to those recommended at an industrial scale. The shift in U_L seemed to favor the maintenance of the sludge bed in the systems, since the particle size of the granules increased at the end of phase II. Operating at an OLR of 35 kg COD m^{-3} d^{-1} in phase III (day 100), the granular size of the sludge from R2 and R3 decreased, but not that from R1. This could be related to higher shear forces derived from the higher biogas production in R2 and R3 during phase III, promoting higher abrasive action with partial disintegration of granules and biomass washout, as previously reported by Syutsubo et al.[24]. However, R2 was less susceptible to biomass washout than R3, most probably because of the sludge retention induced by the addition of chitosan. On day 126 of phase IV, the percentage of granules and the mean diameter had increased in reactor R1, operating at 35 kg COD m^{-3} d^{-1}, and in R2, operating at 50 kg COD m^{-3} d^{-1}. Meanwhile, in R3 at the same OLR, the size parameters remained almost at the same values as in the previous phase. Finally, on day 147, the extreme OLR of 75 kg COD m^{-3} d^{-1} imposed on R2 and R3, led to a decrease in the granules' mean diameter. Except at the highest OLR applied, the results obtained in this study indicated that a dynamic balance existed between the deterioration or/and loss of bigger particles and the growth of the smaller

ones, promoting the maintenance of a high percentage of granules in the reactors during the intermittent operation.

3.3.2 EPS production

Slime EPS (S-EPS) and tightly-bound EPS (T-EPS) were extracted from different sludge samples taken from the reactors during the experiment and the polysaccharide (PS) and protein (PN) content was quantified. Fig. 4 shows the results. The EPS of all the samples were mainly accumulated in the T-EPS and have been identified as the skeleton of granules mediating the cohesion and adhesion of cells, while the S-EPS are distributed in the bulk solution[7]. The higher T-EPS content indicates granules with high strength and mechanical stability that resist external disturbances. The content of PN was higher than the PS content in both fractions.

The T-EPS values of almost all the sludge samples from R1 and R2 were higher than the values of each seeding sluge, while the values corresponding to the sludge from R3 were somewhat more variable. T-EPS of the sludge from R3 on day 147 showed a value a half than the previous one (on day 100), which was related to a lower protein content, and coinciding with the decrease in particle size and the loss of structural stability in this reactor at the end of the study. The S-EPS values showed a similar dynamic in the three reactors, increasing in phase I and then decreasing as the OLR increased.

The results obtained herein suggest that other factors, not the EPS excretion, could be associated in the disintegration and/or flotation of the granules during the intermittent operation. Ding et al.[25] suggested that the loss of aggregate stability is not necessarily related to EPS excretion, but could also be a mechanism for microorganisms to survive in stressful environmental conditions. Under starvation conditions, for example, the disintegration of granules could occur to facilitate access to the substrate by the

microorganisms inside the granule. As a majority of the substrate is utilized near the granule surfaces, starvation may result in substrate limitation at the core of the lager granules, leading to hollowed cores and, thus, granule flotation[26]. In this respect, reactor R3, whose mean particle diameter was higher at the beginning of the study, showed more biomass flotation and washout, especially during phases II and III (Fig. S2).

3.4 Microbial community analysis

3.4.1 DGGE

The microbial populations of the sludge samples taken from the three UASB reactors on days 0, 58, 100, 126 and 147 were evaluated through DGGE. Fig. 5 shows the DGGE banding patterns for the archaeal and the bacterial populations in each reactor. The bands marked in Fig. 5 were excised and sequenced. Table 3 summarizes the designation of the bands and the phylogenetic affiliation of the 16S rRNA gene sequences along with the degree of similarity to related GenBank sequences. Five predominant bands were observed for the archaeal community of the UASB reactors (Fig. 5a). Three of them (A1, A3, A4) were affiliated with hydrogenotrophic methanogens and two (A2, A5) with acetotrophic methanogens. The archaeal community in R1 remained stable despite the intermittent feeding pattern and the increase in OLR; it showed a slight shift in reactors R2 and R3, with a greater number of bands, indicating greater diversity. This result could be related to the better performance of these reactors in terms of methane yield production compared to R1, since high diversity can play a major role in the performance of anaerobic reactors that are subject to organic loading variations[12].

The predominant bands in the three reactors were A1 and A2, which were closely related to *Methanocorpusculum labreanum* and *Methanosaeta concilii*, respectively. These microorganisms kept their dominance throughout the experiment in all of the

406 biomass samples from the reactors, so they were not affected by the high OLR or the
407 intermittent feeding pattern applied. *Methanocorpusculum*-like microorganisms have
408 been reported to be predominant in high rate granular sludge bed anaerobic reactors
409 operated at sub-optimal mesophilic or psychrophilic temperatures[27, 16].
410 *Methanocorpusculum labreanum* is a hydrogenotrophic methanogen which uses H_2-CO_2
411 and formate as substrates to produce methane[28]. *Methanosaeta* is a well-known
412 acetoclastic methanogen and it is considered to play a key role in the formation and
413 maintenance of the granules[29]. The prevalence of both populations of hydrogenotrophic
414 and acetoclastic microorganisms throughout the experiment can explain the good
415 performance of the reactors regarding the substrate conversion and the low concentration
416 of VFA in the effluents, even when an intermittent OLR up to 35 for R1 and 50 kg COD
417 m^{-3} d^{-1} for R2 and R3 was applied. The band A5, which was observed only in the sludge
418 samples from R3 (days 0 and 58), was identified as *Methanosaeta harundinacea*. The
419 increase in the OLR, along with the biomass washout, may have caused the disappearance
420 of this microorganism and could be related to the worse evolution of the granular
421 characteristics in this reactor. The disappearance of *Methanosaeta*-like cells has
422 previously been reported to contribute to anaerobic granule dispersion/rupture in UASB
423 reactors[7].

424 The bands A3 and A4 were associated with archaea of the *Methanobacteriales* order.
425 A3 was related to *Methanobacterium formicicum* and was detected in reactor R2,
426 operating at an OLR of 50 kg COD m^{-3} d^{-1}. *Methanobacterium* species are
427 hydrogenotrophic methanogens that have been found in methanogenic granules under low
428 and mesophilic temperatures[27, 21]. Wang et al.[30] observed the predominance of
429 *Methanobacterium* species in the treatment of pre-hydrolyzed pig manure in an EGSB
430 reactor when the OLR was drastically increased. The band A4, found in R2 and R3 at

high OLR, was identified as *Methanobrevibacter arboriphilus*, a hydrogenotrophic methanogen whose only growth substrate is H_2-CO_2[31]. These results are in agreement with other studies in which it has been shown that hydrogen-utilizing methanogens play an important role in granular anaerobic systems operating under shock conditions, making hydrogenotrophic methanogenesis the main pathway for methane production[14]. Nevertheless, it can be pointed out that the archaeal community was little affected by the intermittent operation or the increases in the OLR, at least in qualitative terms.

Regarding the bacterial community, a total of eleven bands were retrieved and sequenced (Fig. 5b and Table 3). The dominant bands seemed to remain in all three reactors during the experiment. The phylum *Bacteroidetes* was represented by bands B1, B2, B8, B9 and B11. *Bacteroidetes* are commonly found in anaerobic reactors, where they are involved in the hydrolytic-acidogenic step of the anaerobic digestion process[32]. These microorganisms were present in the seed sludge and remained for most of the following days in the reactors R2 and R3. B1, B2 and B11 almost disappeared by the end of the study in R1 (B11 also disappearing in R3 as well), which suggests a different impact of the intermittent feeding and increases in OLR on granular sludge from reactor R2, where the chitosan addition could promote the retention of these microorganisms. The bands B3 and B7 were related to species of the order *Clostridiales* (phylum *Firmicutes*). B3 was identified as *Clostridium* sp. and the band B7 was closely linked to the homoacetogenic bacteria *Acetobacterium wodii*. *Acetobacterium* sp. have been reported as degrading some methyl esters to methanol and the corresponding carboxylic acids[33] as well as performing the enzymatic cleavage of the ether bond of glycol ethers, such as polyethylene glycol or 2-phenoxyethanol[34, 35]. Thus, the presence of these microorganisms in the anaerobic sludge from the three reactors could be associated with the degradation of ethyl acetate and 1-ethoxy-2-propanol. The band B4 was affiliated with

Pelobacter Propionicus, a strictly anaerobic microorganism that is able to produce propionate and acetate from ethanol fermentation[36]. The bands B5, B6 and B10 were identified as species of the genus *Geobacter*. *Geobacter* sp. can oxidize substrates as ethanol or acetate to carbon dioxide, coupled to the reduction of iron or manganese oxides, and can grow under mesophilic temperatures. *Geobacter* species are predominant in anaerobic reactors treating wastewater with a high content of ethanol, either synthetic[37] or brewery wastewater[38], where they have been found to participate in syntrophic methanogenesis with organisms such as *Methanosaeta,* through the mechanism of direct interspecies electron transfer (DIET) for the reduction of carbon dioxide to methane. Since ethanol was the main solvent in the inlet of our reactors, as well as a possible intermediary in the degradation of the other solvents in the ternary mixture, and *Methanosaeta* was in the samples from the three reactors, it could be expected that this type of interaction would take place in the granular sludge.

3.4.2 High- throughput sequencing

High-throughput sequencing was performed to elucidate the microbial community structure of the sludge at the beginning of the study and after 147 days operating under intermittent feeding. Fig.6a shows the microbial community structure of the three reactors at phylum level, with the phyla detected in relative abundance (higher than 1%) in at least one sample analyzed. *Proteobacteria* and *Euryarchaeota* were the most abundant phyla in all the samples. The *Proteobacteria* phylum significantly raised its relative abundance with respect to the seed sludge, accounting for between 16.6% and 23.3% and then rising to values of 54.5%, 33.4% and 31.7% of the population in R1, R2 and R3, respectively. The increase of this phylum in the three reactors can be related to the increase in the OLR during the experiment. Some species belonging to this phylum, more specifically to the class *Deltaproeobacteria*, are known to carry out the syntrophic degradation of ethanol

481 and VFA in methanogenic reactors[37]. Otherwise, the relative abundance of the
482 *Euryarchaeota* phylum remained at values about 30% throughout the experiment in the
483 reactors R2 and R3, while in R1 it dropped to 17.4%. The *Euryarchaeota* phylum includes
484 methanogenic archaea, which explains the lower removal rate capacity and the VFA
485 accumulation observed in R1 at an OLR of 50 kg DQO m^{-3} d^{-1}; there is a lower population
486 of methanogens in comparison with R2 and R3, which showed a stable performance
487 operating at this OLR. Other predominant phyla in the three reactors were *Bacteroidetes*
488 and *Firmicutes,* with relative abundances in the range of 8.2% to 11.0%, and 2.8% to
489 4.7%, respectively. The dominance of *Bacteroidetes* and *Firmicutes* has been reported in
490 methanogenic reactors operating at high OLR for the treatment of organic substrates[39,
491 30]. After 147 days of operation, the abundance of both phyla decreased with respect to
492 the seed sludge. The high VFA concentrations in the final phase of the experiment could
493 lead to the decrease of species belonging to this phylum, as Luo et al.[39] suggested.

494 At the genus level of bacteria (Fig. 6b), electrogenic microorganisms belonging to
495 *Geobacter* were the most abundant in the three reactors, with high relative abundances
496 ranging between 18.9% and 41.2%. Sulfate-reducing bacteria (*Desulfovibrio*) were also
497 abundant, with relative abundances between 5.3% and 7.3%. In addition to oxidizing
498 substrates as ethanol or hydrogen with sulfate reduction, *Desulfovibrio* species can grow
499 in syntrophic association with hydrogenotrophic methanogens for the degradation of
500 ethanol or lactate[40]. Furthermore, they are capable of switching between a sulfidogenic
501 and syntrophic metabolism[41]. Both of these syntrophic genera are predominant in
502 anaerobic rectors treating wastewater polluted with ethanol[37, 38]. Their relative
503 abundances increased considerably after 147 days of operation, which indicated that they
504 were performing an important role in the treatment of the substrate fed to the reactors.
505 Other microorganisms, such *Paludibacter* and *Syntrophomonas* (belonging to the

506 *Bacteroidete*s and *Firmicutes* phyla, respectively), decreased in abundance, to values less
507 than 0.5% in all three reactors. Such a decrease also suggests that the intermittent
508 operation and/or the high OLR that was applied, induced a selection pressure in the
509 microbial communities, since a similar trend was observed in all the three reactors. The
510 dominance of the ethanol-degrader syntrophic communities suggests they were less
511 sensitive to the stress conditions applied, as the prevalence of syntrophic communities in
512 non-steady state conditions has been previously reported[12]. The organic substrate could
513 also have exerted a microbial selection. In this way, other syntrophic communities such
514 as *Syntrophomonas,* which are able to syntrophically degrade long-chain fatty acids along
515 with hydrogenotrophic methanogens[42], almost disappeared after 147 days of exposure to
516 a mixture of solvents, mainly composed of ethanol.

517 The archaeal microbial community structure at genus level revealed that
518 *Methanocorpusculum* was the most abundant methanogen in the reactors, which is
519 consistent with DGGE results, accounting for 15.4%, 25.5% and 27.8% of the total
520 sequences in R1, R2 and R3, respectively, by the end of the experiment. *Methanosaeta*
521 had low relative abundances ranging between 0.4% and 1.1%. In spite of the intermittent
522 operation and the high OLR applied, the reactors maintained a high percentage of
523 granules, which can be associated with the presence of *Methanosaeta*. A greater
524 abundance of hydrogenotrophic methanogens was also observed by Song et al.[43] in the
525 granular sludge from a pilot-scale UASB reactor treating swine wastewater and, despite
526 *Methanosaetaceae* showing no significant growth, its abundance contributed to granule
527 sustainability. *Methanobrevibacter* and *Methanobacterium* accounted for relative
528 abundances ranging between 0.2% and 0.8% and 0.8 and 2.8%, respectively, with the
529 highest values for the chitosan assisted reactor (R2), which indicated that the polymer had
530 some influence in the prevalence of the methanogenic community.

The shift in the microbial community structure, especially for bacteria population, was weaker in reactor R2, where chitosan was periodically applied, and more severe for reactor R1, whose granules were developed without the addition of the polymer. The dominance of hydrogenotrophic methanogens indicated that the methane produced from the hydrogen utilization pathway played a significant role in the syntrophic oxidation of the substrates to methane. Considering the low VFA concentration in the effluent of the reactors at OLR below 50 kg COD m^{-3} d^{-1} (R2 and R3) and the predominance of hydrogenotrophic over acetoclastic methanogens, it could be hypothesized that syntrophic acetate-oxidation is the most likely degradation pathway[39].

4 Conclusion

UASB has been proven to be robust for the intermittent treatment of a mixture of ethanol, ethyl acetate and 1-ethoxy-2-propanol. Stable performance was achieved at an OLR of 50 kg COD m^{-3} d^{-1} with removal efficiencies higher than 94%. The addition of chitosan improved performance when operating at the highest OLR. Feedless periods of 56 hours affected microorganism activity to a greater extent than feedless periods of 8 hours. Intermittent feeding led to partial granule disintegration without performance deterioration. Microbial community analysis showed the prevalence of *Geobacter* bacteria and the dominance of *Methanocorpusculum* archaea, indicating that hydrogenotrophic methanogenesis, with the syntrophic oxidation of the substrate, was the main pathway for methane production.

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Figure captions

Fig. 1. Performance of the reactors in each operational phase **a)** Organic Loading Rate applied in an intermittent pattern of 16 h per day and 5 days per week (Line R1, Dash Line R2&R3) and COD removal efficiency (Symbols), **b)** Effluent VFA concentration (Symbols) and **c)** Methane production (Symbols). Symbols: ● R1; ◊ R2 and ▲ R3.

Fig. 2. Applied E2P Organic Loading Rate (Line R1, Dash Line R2&R3) and E2P removal efficiency (● R1; ◊ R2 and ▲ R3).

Fig. 3. Transient response of the reactors to wastewater supply resumption. a) VFA concentration and methane yield after 56 h without wastewater supply (weekend shutdown periods), b) VFA concentration and methane yield after 8 h without wastewater supply (night shutdown periods). Symbols: ● R1; ◊ R2 and ▲ R3.

Fig. 4. Variation with time of the EPS production of the different sludge samples from the reactors in terms of protein (PN) and polysaccharide (PS) content. a) Tightly-bound EPS (T-EPS) and b) Slime EPS (S-EPS).

Fig. 5. Variation with time of the DGGE profiles of biomass samples from the three reactors. **a)** Archaeal DGGE profiles, **b)** Bacterial DGGE profiles.

Fig. 6. Microbial community structure in each reactor on days 0 and 147: a) At phylum level, b) At genus level.

Table 1. Operational parameters of the UASB reactors.

Day	Phase I (0–48)	Phase II (49–90)	Phase III (91–108)	Phase IV (109–147)	
	R1-R2-R3	R1-R2-R3	R1-R2-R3	R1	R2-R3
OLR_{16h} (kg COD m^{-3} d^{-1})[a]	20	25	35	35 - 50	50 - 75
OLR_{24h} (kg COD m^{-3} d^{-1})[b]	13.3	16.7	23.3	23.3 - 33.3	33.3 - 50
Influent COD (g L^{-1})	8.3	10.4	14.6	14.6 - 20.8	20.8 - 31.3
OLR_{E2P} (kg COD m^{-3} d^{-1})	2.1	2.6	3.7	3.7 - 5.3	5.3 - 7.9
Influent E2P (g COD L^{-1})	0.9	1.1	1.5	1.5 - 2.2	2.2 - 3.3
U_L (m h^{-1})	0.5	1	1	1.0	1.0

[a]OLR_{16h}: organic loading rate applied during 16 hours per day; [b]OLR_{24h}: daily organic loading rate.

Table 2. Evolution of particle size of the sludge samples from all reactors.

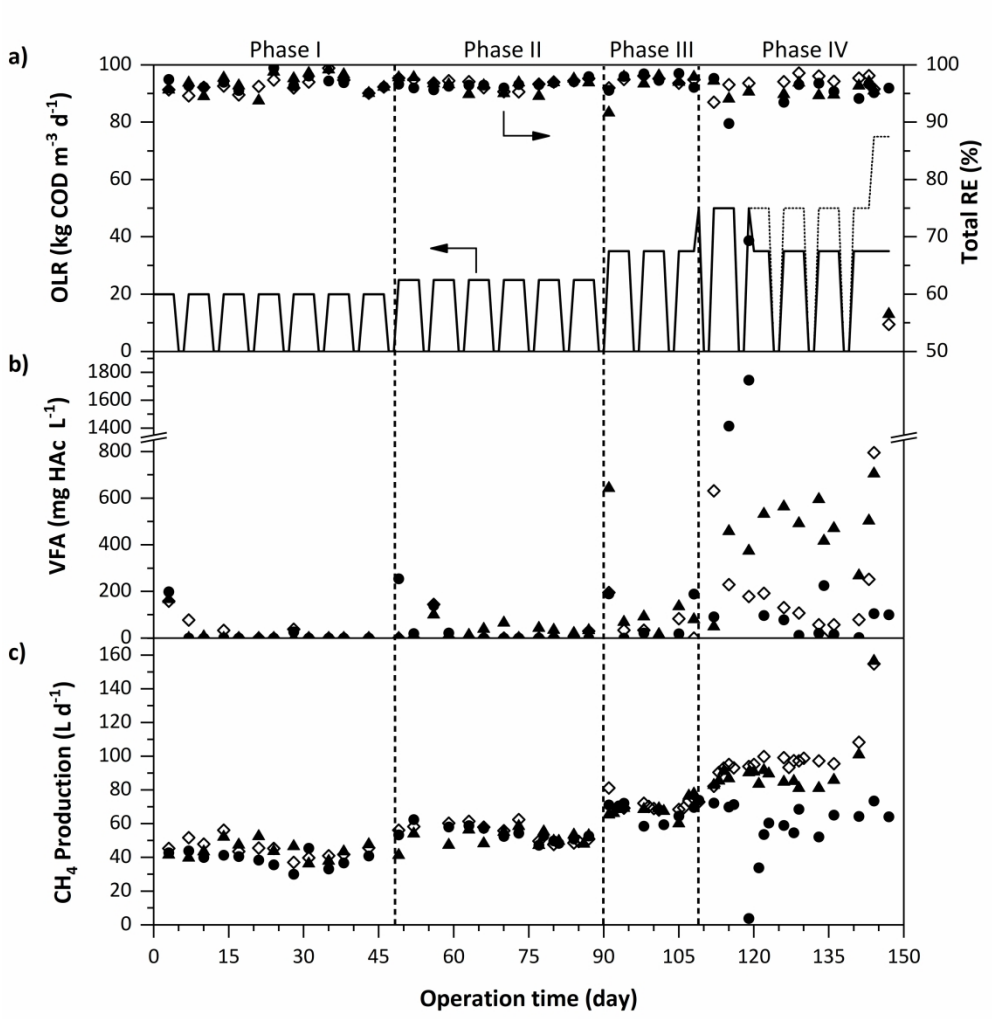
		Granules (%)			Mean diameter (μm)		
	Day	R1	R2[a]	R3	R1	R2[a]	R3
Phase I	0	73.2	76.0	74.7	498	570	625
	15	73.0	71.7	71.9	496	592	682
	29	72.9	78.3	75.3	469	596	627
	43	56.4	64.3	52.7	341	506	469
Phase II	58	51.4	69.4	54.1	347	589	429
	79	67.6	81.8	71.0	439	614	530
Phase III	100	67.1	63.6	56.4	471	481	419
Phase IV	126	74.8	68.1	55.5	516	526	409
	147	83.8	62.7	56.9	616	401	371

[a]Reactor assisted with chitosan each three weeks.

Table 3. Phylogenic affiliation of bacterial and archaeal sequenced bands from DGGE profiles (Fig. 5).

Band	Closest organism (accession number)	Similarity %	Phylogenetic group
A1	*Methanocorpusculum labreanum (NR_074173.1)*	100	*Methanomicrobiales*[a]
A2	*Methanosaeta concilii (NR_102903.1)*	100	*Methanosarcinales*[a]
A3	*Methanobacterium formicicum (NR_115168.1)*	100	*Methanobacteriales*[a]
A4	*Methanobrevibacter arboriphilus (NR_042783.1)*	99	*Methanobacteriales*[a]
A5	*Methanosaeta harundinacea (NR_043203.1)*	99	*Methanosarcinales*[a]
B1	*Paludibacter propionicigenes (NR_074577.1)*	89	*Bacteroidetes*[b]
B2	*Capnocytophaga haemolytica (NR_113562.1)*	84	*Bacteroidetes*[b]
B3	*Clostridium limosum (NR_104825.1)*	91	*Firmicutes*[b]
B4	*Pelobacter propionicus (NR_074975.1)*	98	*Proteobacteria*[b]
B5	*Geobacter chapellei (NR_025982.1)*	96	*Proteobacteria*[b]
B6	*Geobacter psychrophilus (NR_043075.1)*	88	*Proteobacteria*[b]
B7	*Acetobacterium woodii (NR_026324.1)*	100	*Firmicutes*[b]
B8	*Bifidobacterium hapali (NR_147762.1)*	93	*Bacteroidetes*[b]
B9	*Ornithobacterium rhinotracheale (NR_102940.1)*	88	*Bacteroidetes*[b]
B10	*Geobacter uraniireducens (NR_074940.1)*	91	*Proteobacteria*[b]
B11	*Bifidobacterium longum (NR_145535.1)*	98	*Bacteroidetes*[b]

[a]Order; [b]Phylum



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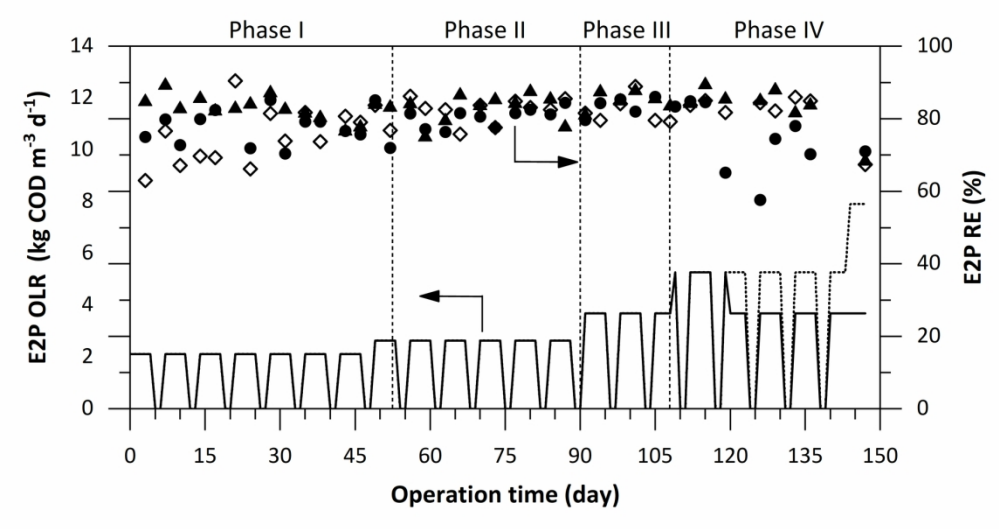

Fig. 2. Applied E2P Organic Loading Rate (Line R1, Dash Line R2&R3) and E2P removal efficiency (● R1; ◇ R2 and ▲ R3).

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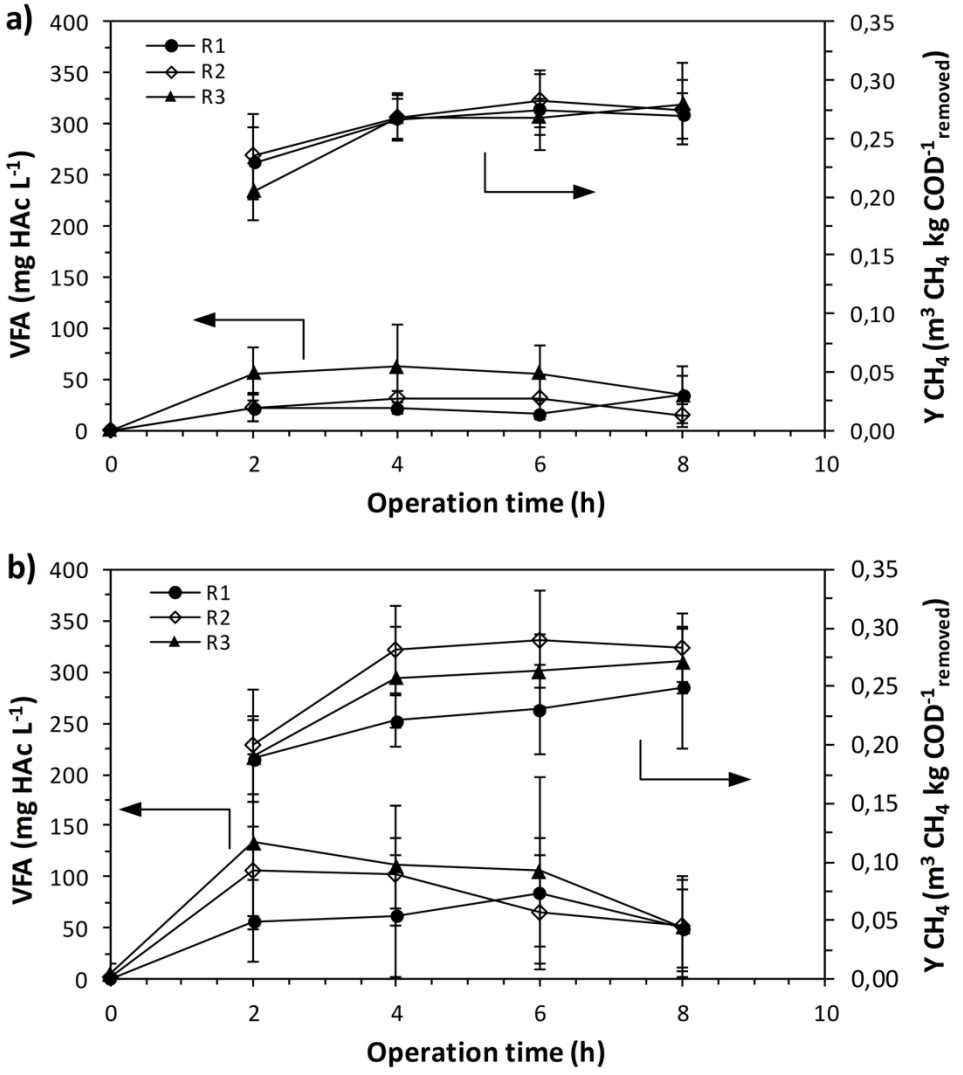

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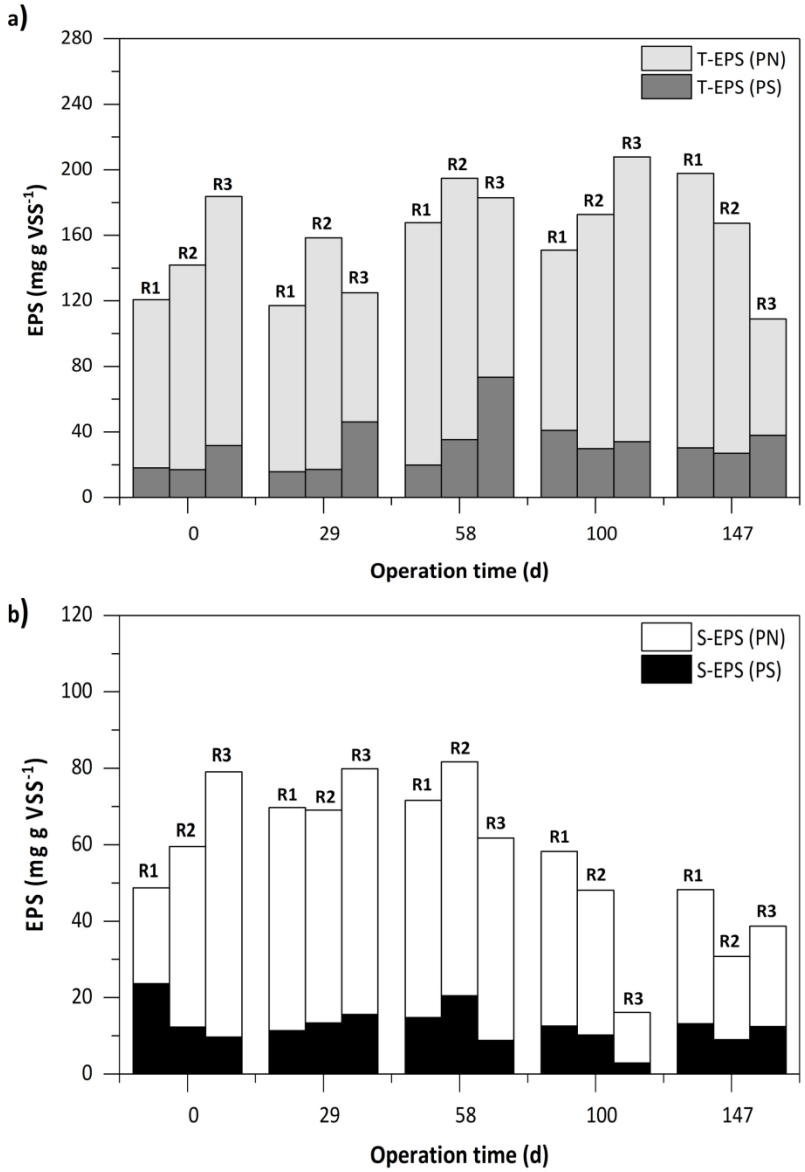

Fig. 4. Variation with time of the EPS production of the different sludge samples from the reactors in terms of protein (PN) and polysaccharide (PS) content. a) Tightly-bound EPS (T-EPS) and b) Slime EPS (S-EPS).

89x131mm (600 x 600 DPI)

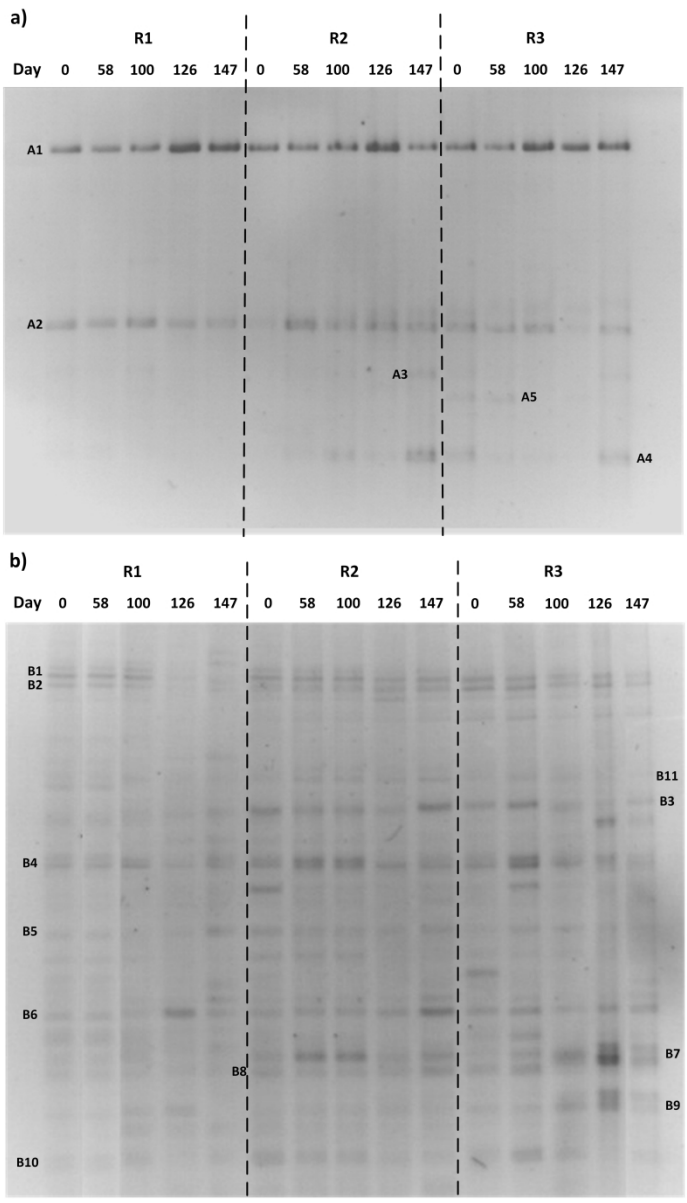

Fig. 5. Variation with time of the DGGE profiles of biomass samples from the three reactors. a) Archaeal DGGE profiles, b) Bacterial DGGE profiles.

136x237mm (600 x 600 DPI)

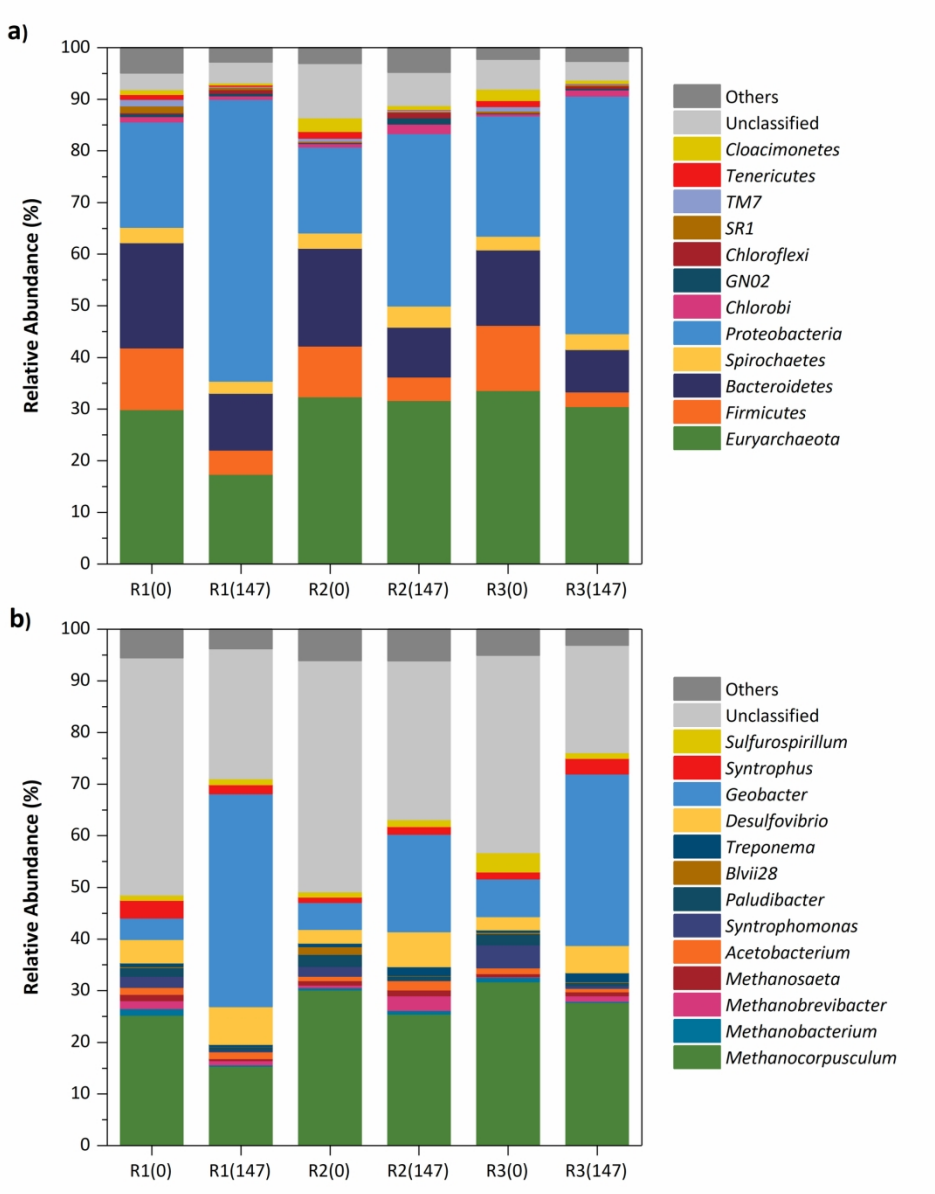

Fig. 6. Microbial community structure in each reactor on days 0 and 147: a) At phylum level, b) At genus level.

170x218mm (300 x 300 DPI)

Supplementary material

Table Sup1. Macro- and micro-nutrients supplementation.

Macro-nutrients	mg g COD^{-1}	Micro-nutrients	mg g COD^{-1}
NH_4Cl	15.3	$FeCl_3 \cdot 6H_2O$	0.42
$(NH_4)_2HPO_4$	9.5	H_3BO_3	0.11
KCl	3.8	$ZnSO_4 \cdot 7H_2O$	0.01
Yeast extract	7.5	$CuCl_2 \cdot 2H_2O$	0.01
Alkaline-earth Metals		$MnCl_2 \cdot 4H_2O$	0.14
Mg^{+2} as $MgCl_2 \cdot 6H_2O$	40 mg Mg L^{-1}	$(NH_4)6Mo_7O_{24} \cdot 4H_2O$	0.06
Ca^{+2} as $CaCl_2 \cdot 2H_2O$	100 mg Ca L^{-1}	Al_2O_3	0.06
		$CoCl_2 \cdot 6H_2O$	0.16
		$NiSO_4 \cdot 6H_2O$	0.04
		$EDTANa_2$	0.1

Fig. S1. Schematic diagram of the reactor configuration.

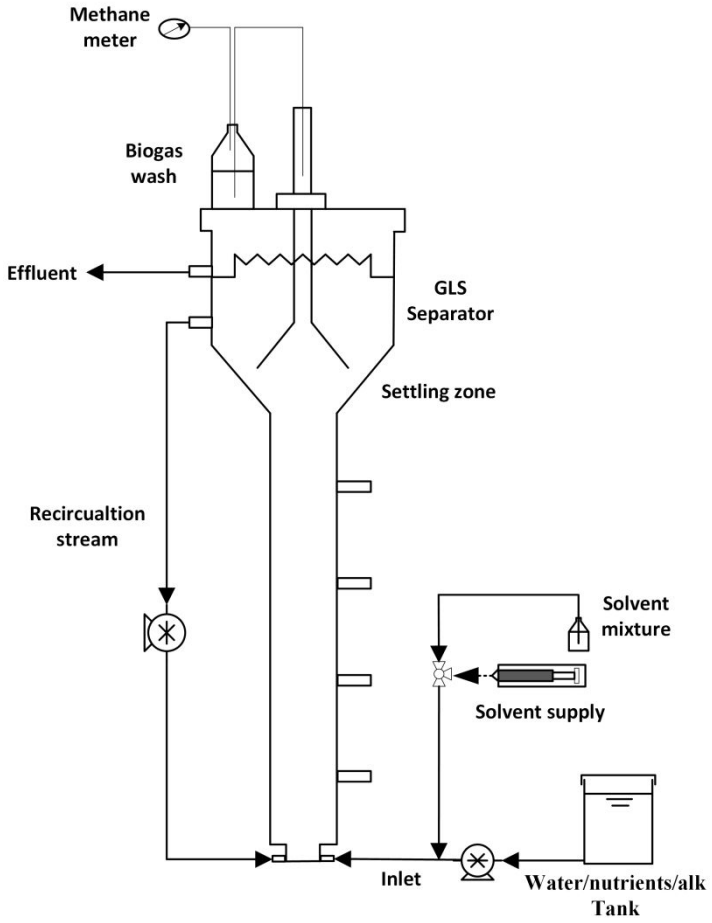

Fig. S2. Variation with time of the Volatile Suspended Solids (VSS) concentration of the effluent of the reactors.

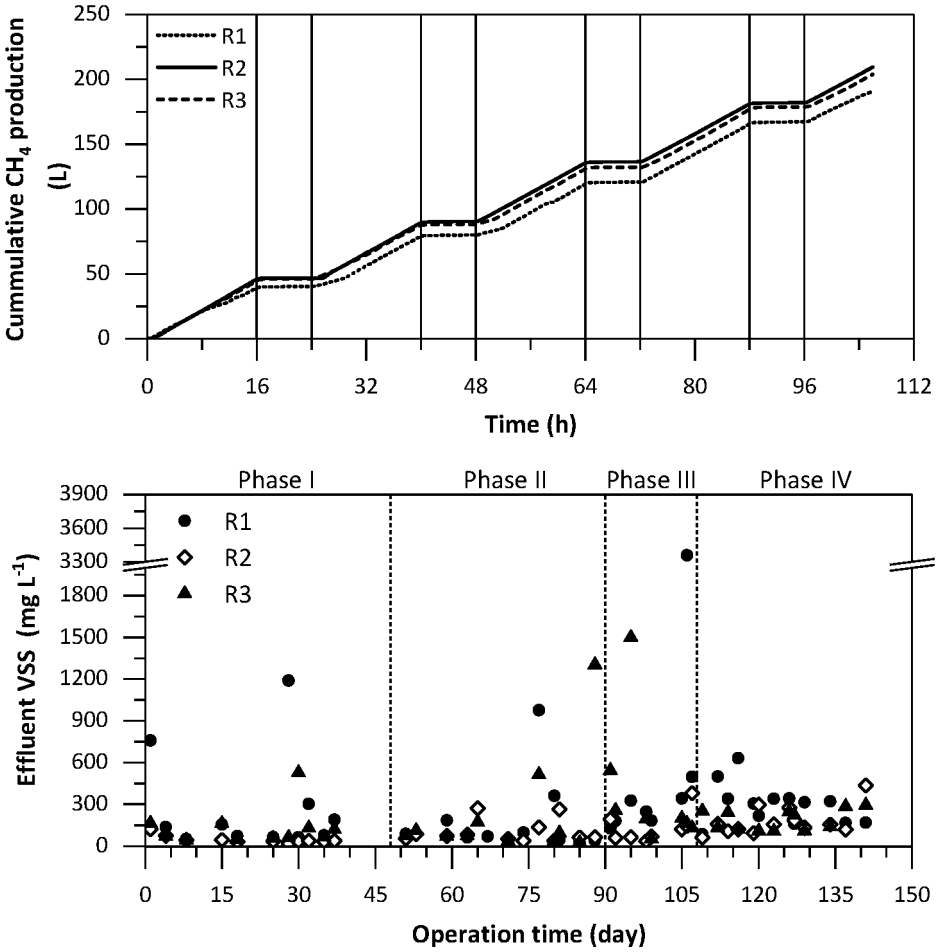

Fig. S3. Cumulative methane production of the UASB reactors during 106 h of intermittent operation.

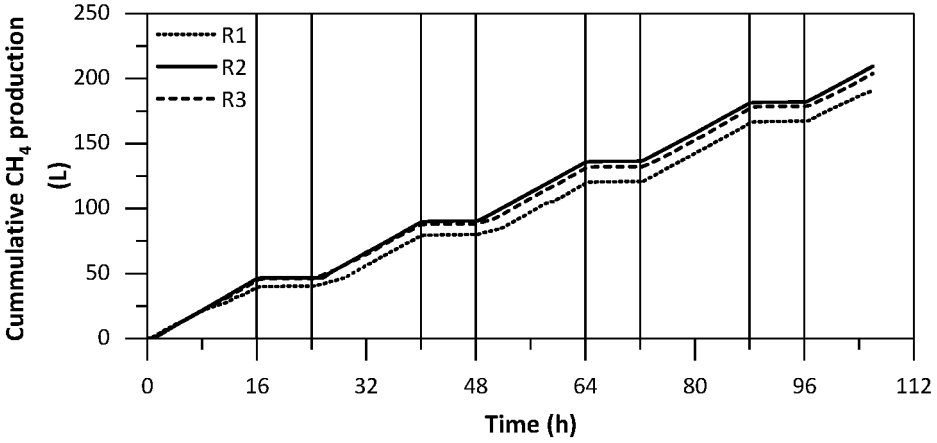

Fig. S4. Variation of the particle size distribution of the sludge from the reactors throughout the experiment.