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Abstract

In this study, changes in bacterial and archaeal communities involved in anaerobic digestion processes operated with high solid contents were investigated. Batch tests were performed within a range of total solids (TS) of 10% - 35%. Between 10% and 25% TS, high methanogenic activity was observed and no overall specific structure of active bacterial communities was found. At 30% and 35%, methanogenesis was inhibited as a consequence of volatile fatty acids accumulation. Here, a specific bacterial signature was observed with three main dominant bacteria related to *Clostridium* sp., known for their ability to grow at low pH. Additionally, archaeal community was gradually impacted by TS content. Three archaeal community structures were observed with a gradual shift from *Methanobacterium* sp. to *Methanosarcina* sp. according to the TS content. Overall, several species were identified as biomarkers of methanogenesis inhibition, since bacterial and archaeal communities were highly specific at high TS contents.

Keywords

Anaerobic digestion; Biohydrogen inhibition; CE-SSCP; Dry solids; Methanogenesis

1. Introduction

Anaerobic digestion (AD) is a biotechnology that converts organic matter into a methane-rich biogas which represents a wide source of renewable energy. According to their water content, the AD processes have been classified in three types: “wet”, “semi-dry” and “dry” AD. Currently, the main dominant technology is the “wet” digestion system where the proportion of total solids (TS) is lower than 10%. This technology is mainly applied to treat anaerobically sewage sludge produced in wastewater treatment plants. “Semi-dry” anaerobic digestion (SDAD) (10% < TS < 20%) and “Dry” anaerobic digestion (DAD) (TS > 20%) are well-suited to the treatment of solid waste and more particularly municipal solid waste (MSW) (Bolzonella et al., 2006; Forster-Carneiro et al., 2008; Guendouz et al., 2008; Mata-Alvarez, 2000; Montero et al., 2008). These last two technologies are attractive because less water and energy are required for the AD process, and the size of the digester is smaller to treat a same amount of organic matter. However, these two types of AD suffer from biological and technological drawbacks mainly due to the excessive content in solids and the small amount of water.

Overall, the water content strongly affects AD performances. When the TS content increases from 20% to 30%, substrate degradation decreases significantly (Forster-Carneiro et al., 2008). In comparison to 20% TS, TS content higher than 30% reduces methane production by about 17% (Fernandez et al., 2008). Corroborating this result, Le Hyaric et al. (2011) showed that

methanogenic activity increased linearly by a factor of 3.5 when the moisture content increased from 65% to 82%. Water content can also affect the rheological behaviour of digested media for high TS values (Garcia-Bernet et al., 2011).

So far, only little research has been dedicated to clarifying the impact of TS content on microbial communities. Nevertheless, it has been shown that not only the abundance but also the composition of microbial communities is an important factor in DAD. Both the source of inoculum and TS contents are responsible for obtaining a rapid onset of a balanced microbial population in DAD , and, consequently, better COD removal, higher cumulative methane production and faster start-up of the bioprocess (Forster-Carneiro et al., 2008). When focusing on methanogenic *Archaea*, a high content of 23% of hydrogenotrophic methanogens in inoculum was shown favourable to fasten the start-up of DAD bioprocesses, likely because H_2 was immediately consumed by these micro-organisms during the hydrolysis and acidogenesis steps (Montero et al., 2008). Interacting directly with syntrophic acetate-oxidizing bacteria that transform acetate into CO_2 and H_2 (Goberna et al., 2009), the activity of hydrogenotrophic methanogens is decreasing after the start-up period, likely because of a drop in acidogenic activity and, therefore, H_2 production rates. This leads to an increase in acetotrophic methanogens and consequently to a change in the hydrogenotrophic/acetotrophic methanogens ratio. Montero et al. (2008) showed a decrease of the hydrogenotrophs/acetotrophs ratio from 11:1 to 7:32 at start and at steady state of a thermophilic DAD bioreactor, respectively. Moreover, in SDAD processes ranging from 11% to 16% TS, VFA concentrations increase concomitantly with TS contents (Li et al., 2010). Such high VFA concentration with high acetate levels could then

favour mixotrophic *Methanosarcinaceae* methanogens which consume acetate faster than pure acetotrophic methanogens such as *Methanosaetaceae* (Karakashev et al., 2006).

The aim of this work was to determine the effect of TS concentration on AD bacterial and archaeal communities. Different levels of TS content from semi-dry anaerobic digestion (SDAD) to DAD, i.e. 10% to 35 % TS, respectively, were tested in batch tests operated at mesophilic temperature (35°C).

2. Material and Methods

2.1 Anaerobic batch tests

The experiments were carried out in 600 mL batch flasks with a working volume of 100 mL, as described previously by Abbassi-Guendouz et al. (2012). The substrate corresponded to a compact cardboard with a density of 1.42 $kg.m^{-3}$, shredded and sieved at 2 mm. Six different TS contents were tested to represent "wet" to "dry" anaerobic digestion conditions: TS = 10, 15, 20, 25, 30, 35 %. An initial substrate to biomass ratio S/X of 20 (w/w) was used; S and X representing the initial TS concentration of the substrate and the microbial inoculum, respectively. The flasks were inoculated with a leachate of pressed MSW digestate sampled in an industrial plant treating MSW. One mL of an oligo-element solution was added to the mixture. The composition of the oligo-element solution is detailed in Abbassi-Guendouz et al. (2012). The experiments were run 298 days at 35°C and four replicates were carried out for each condition.

2.2 Chemical analysis

The volume of biogas was measured by a water displacement method and then normalized according to the ambient temperature, as explained by Abbassi-Guendouz et al. (2012). Biogas composition was determined using a gas chromatograph (Varian µGC-CP4900) equipped with two columns: a Molsieve 5Å PLOT column for O_2, N_2, CH_4 and CO and a HayeSep A column for CO_2 quantification, with Helium as carrier gas. The sample volume injected was approximately 1 mL. Volatile Fatty Acids (VFA) were analysed at the end of each batch test. After three-fold dilution with distilled water and centrifugation (13000 rpm, 15 min), VFA concentration was measured with a gas chromatograph (Varian µGC-CP3900), as described elsewhere by Ganesh et al. (2010). pH was measured directly on the digestate using a pH meter (Eutech Instruments pH510) and Mettler Toledo InLab® Expert Pt1000 pH electrodes.

2.3 Microbial community fingerprinting analysis.

2.3.1 DNA extraction and 16S rRNA gene amplification

Microbial diversity was analysed at the end of each batch test, i.e. after 298 days of experiment. 0.5 g of digestate were sampled and stored at -20°C. Total DNA was extracted using the QIAamp DNA stool (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. Amplification of partial 16S rRNA gene was performed using primer pairs W49 – W104 for Bacteria and W274 - W275 for *Archaea* (Table 1). Each reaction tube contained 0.5 µM of each primer, 1 µL of purified DNA, 0.5 U of Pfu Turbo DNA polymerase (Stratagene), 1 x Pfu Turbo DNA polymerase buffer, 200 µM for each dNTP, adjusted with ultra-pure water (CDM Lavoisier, France) to a total volume of 50 µL for Bacteria amplification and 25 µL for *Archaea* amplification. The reaction mixture was placed in a Mastercycler ep gradient S thermal cycler

(Eppendorf). After an initial denaturation step at 94°C for 2 min, 25 temperature cycles for Bacteria and 30 cycles for *Archaea* were carried out at 94 °C for 30 seconds, 51 °C for 30 seconds for Bacteria and 61°C for *Archaea*, 72 °C for 30 second, and 72 °C for 10 min. PCR products providing bands of the proper size were confirmed on a 2% agarose gel.

[Table 1]

2.3.2 *CE-SSCP DNA fingerprinting.*

Capillary electrophoresis and fluorescent dye-labelled PCR products were used for CE-SSCP fingerprinting. One microliter of PCR products was diluted 5- to 500-fold and mixed with 18.8 mL of formamide and 0.2 mL of internal standard GeneScan ROX (Applied Biosystems). Samples were denatured at 95 °C for 5 min and then immediately cooled in ice. CE-SSCP electrophoresis was carried out in an ABI Prism 3130 genetic analyser (Applied Biosystems) with 50-cm-long capillary tubes filled with a non-denaturing 5.6% conformation analysis polymer (Applied Biosystems). CE-SSCP data were analysed using GeneScan software (Applied Biosystems) and the StatFingerprints R library (Michelland et al., 2009) as described by Quéméneur et al. (2011).

2.3.3 Cloning and sequencing of 16S rRNA gene

For identifying CE-SSCP peaks, amplification of partial 16S rRNAgene was done with primer pairs W18–W31 for *Bacteria* and W274-W280 for *Archaea*. Each reaction tube contained 0.5 µM of each primer, 1 µL of purified DNA (0,5 µL for *Archaea*), 1 µL of Red Taq polymerase (Sigma), 5 µL of Red Taq buffer, 200 µM for each dNTP, adjusted with pure H_2O to a total volume of 50 µL for *Bacteria* amplification and 25 µL for *Archaea*. The reaction mixtures were placed in a Mastercycler ep gradient S thermal cycler (Eppendorf). After an initial denaturation at

94°C for 2 min, 25 temperature cycles for *Bacteria* and 30 cycles for *Archaea* were carried out at 94 °C for 1 min, 50 °C for 30 seconds for *Bacteria* and 65°C for *Archaea*, 72 °C for 1 min, and 72 °C for 10 min. The size of PCR products was checked on a 2% (w/v) agarose gel. Amplified DNA was then purified using the Qiaquick kit (Qiagen) in accordance with the manufacturer's instructions. The purified PCR products were cloned and transformed using the PCR4-TOPO plasmid and TOP10 *E. coli* competent cells, as indicated by the manufacturer (TOPO TA Cloning Kit, Invitrogen). Recombinant cells were selected by kanamycin resistance and ccdB gene killer inactivation before cultivation at 37°C for 24 h in LB medium. Clones were analysed by CE-SSCP of the V3 region with the same PCR conditions as those described above. Their electrophoretic mobility was compared to the complex fingerprint of the original sample. For each peak of interest on the CE-SSCP fingerprint, a number of clones (3 on average) were chosen for sequencing (Wery et al. 2010). Sequences were deposited in the GenBank database under the accession numbers ranging from HE613791 to HE613797.

2.4 Quantitative PCR

The quantitative amplification reactions were carried out in a total volume of 25 µL. All reaction mixtures contained 5 µL of template DNA and 12.5 µL of Express qPCR Supermix with Premixed ROX (Invitrogen) and primers and probe. For the quantification of the bacterial 16S rRNA gene, the primers used were W208 (250 nM) and W209 (250 nM) and probe was W210 (50 nM). For *Archaea*, the 16S rRNA gene primers were W211 (250 nM) and W212 (250 nM) and the probe was W213 (50 nM), according to Yu et al. (2005). The reactions were run on a Mastercycler realplex 2S (Eppendorf). The reaction sequence was 20 s at 95°C, followed by 40 cycles of 15 s at 95°C and 1min at 60°C. All tests were performed in duplicate. Results were then

compared with a standard curve to obtain the number of target copies in the sample. The standard DNA curves were generated by amplification of serial 10-fold dilutions of a reference clone (Snell-Castro et al., 2005). The cycle threshold (CT) corresponded to the cycle number at which the reaction became exponential. The CT of each sample was compared with a linear standard curve corresponding to CT *vs*. number of gene copies. The total number of *Bacteria* or *Archaea* were finally expressed as the number of target copies per gram of VS. The total abundance of the population generating one CE-SSCP peak was obtained by multiplying the percentage of relative abundance, calculated from the CE-SSCP chromatogram, and the total number of gene copies obtained from qPCR data.

2.5 Phylogenetic Analysis

A neighbour-joining phylogenetic tree of the most important *Archaea* previously identified in batch tests operated with different TS content was built. The tree was generated using TREECON software with a neighbour joining distance method using a Jukes-Cantor algorithm. *Sulfolobus acidocaldarius* was used as root outgroup. One thousand bootstrap calculations was performed to evaluate the consistency of the final tree.

1. Results and Discussion

1.1 Methanogenic performance of the microbial ecosystem in high-solids batch tests

Table 2 presents the cumulated methane production monitored throughout the experiment, and the final VFA concentration at day 298. Methanogenic activity was closely related to TS concentration, and higher water content enhanced methane production. Indeed, the average

cumulated methane production decreased from 141 to 129 mL gTS^{-1} when TS increased from 10% to 25%. Two distinct behaviour patterns were observed at 30% TS. Two replicates (30%-a) showed performances similar to the 10%-25% TS tests, with an average methane yield of 109 mL gTS^{-1}. The two other replicates (30%-b) were similar to the 35%-TS tests with a low methane yield of 28 mL gTS^{-1}, whereas at 35% TS the methane yield was about 19 mL gTS^{-1}. For all 30%-b and 35% replicates, high VFA accumulation (between 29 g L^{-1} and 36 g L^{-1} of water in the digestate) was observed. Acetate was the main dominant VFA, followed by propionate (Table 2). Batch tests between 10% to 30-a% TS presented effective methanogenic activity and their pH remained neutral (pH 7.4 ± 0.3). Where AD was inhibited, i.e. at 30-b% and 35% TS, pH values were more acid due to VFA accumulation (pH 5.9 ±0.1). On the basis of total methane yield, two groups were distinguished: group A corresponded to the batch tests with a active AD methanogenic process (10, 15, 20, 25, 30-a% TS) while group B corresponded to the batch tests where AD was inhibited through VFA accumulation and subsequent low pH value (30-b, 35% TS).

[TABLE 2]

As observed here, anaerobic digestion performances were closely related to the TS concentration, and methane production decreased at low water content. This result is consistent with a previous study of Fernandez et al. (2008) which also observed better performances, *i.e.* higher methane production and shorter reaction time at 20% TS than 30% TS. In our study, two distinct behaviours were observed at 30% TS. One half (30%_a) behaved as in the 10%-25% TS batch tests, and the second half (30%_b) like the 35% TS batch tests. For this second group,

methanogenesis was clearly inhibited. The difference between these two groups of replicates suggested that 30% TS corresponded to a critical value where mass transfer limitation occurs, especially through local accumulation of CO_2 and H_2 and, consequently, an increase in inhibitory VFAs compounds, as discussed elsewhere in Abbassi-Guendouz et al. (2012). Abbassi-Guendouz et al. (2012) concluded that a value of 30% TS was closely related to a critical TS value for methanogenesis. Above this value, methane production is affected by the overall mass transfer limitation and leads to inhibition of methanogenesis. Around this value, the different behaviors observed for each replicates might be related to a slight difference in the initial TS concentration (Abbassi-Guendouz et al., 2012). Staley et al. (2011) reported similar trends in refuse with critical values around 30% TS for methane production.

When anaerobic digestion was blocked, high VFA accumulation was observed. An insufficient amount of methanogenic archaea may be the cause of such high concentration of VFAs: methanogens are indeed very sensitive to VFAs accumulation. As is widely known, total VFAs specifically affect methanogens rather than bacteria (Amani et al., 2010). Bacteria are less sensitive to VFAs except when amounts exceed about 10 g L^{-1} (Amani et al., 2010). Indeed, high VFAs concentrations induce acidification of the medium, and this itself leads to the presence of VFAs in their undissociated form which is more toxic for microorganisms (Amani et al., 2010).

1.2 Characterisation of the bacterial community based on CE-SSCP fingerprints analysis: Shift of the bacterial community structure when methanogenesis was inhibited

At the end of the experiments, DNA fingerprinting of all batch samples as well as the inoculum was carried out. For each test, all CE-SSCP profiles of the replicates presented a high degree of

similarity. Therefore, an average profile of the four replicates performed at the same TS concentration was built-up, and corresponded to the average relative abundance of each peak within the original profiles. At 30% TS, where two different methanogenic behaviours were observed, the average profiles were assessed by gathering the CE-SSCP profiles according to AD performance (two replicates of 30-a% and two others of 30%-b). The average profiles are given as e-supplementary material (Figure S1). With regard to the profiles obtained in batch tests between 10% and 30% TS (group A), the bacterial diversity was high with many peaks, illustrating the high activity of the bacterial populations. No common peaks, ie. shared and abundant peaks, were distinguished due to a large number of peaks and their close migration position leading to high overlapping of the peaks. Overall, within group A which represented the most active methanogenic consortia, no direct link between individual population abundance and AD performance was found. The CE-SSCP profiles obtained from the batch tests of group B, where anaerobic digestion was inhibited i.e. 30% and 35-b% TS, were highly similar with a strict concordance of several dominant peaks (see Supplementary Material Fig. S1). The relative abundance of these peaks was calculated by considering the area of the peak divided by the total SSCP profile area and is presented in Figure 1a. Three predominant peaks were distinguished within the batch tests of group B, and represented more than 24% in relative abundance of the sum of the three peaks, namely *Bac1, Bac2, Bac3*. Also present in group A batch tests, these three bacteria represented less than 5%. The abundance of *Bac1, Bac2* and *Bac3* was statistically higher in group B. Moreover, the closest BLAST sequences were *Clostridium thermobutyricum* (NR_044849) at 85 % for *Bac1*, *Acetanaerobacterium elongatum* strain Z7 (NR_042930) at 98 % for *Bac2 and Bac3*. All these bacterial species were affiliated to the order *Clostridiales*. In the inoculum, *Bac1* and *Bac2* were present at a low relative abundance, less than 2.5%. *Bac3*

represented around 5.3 % of the total bacterial population in the inoculum. Although *Bac3* was not one of the most abundant bacteria, *Bac3* was in a higher amount than *Bac1* and *Bac2* in the inoculum. Fig. 1a summarizes the relative abundance of the three peaks *Bac1, Bac2* and *Bac3* between group A and group B. Each of these three peaks was present at less than 2.5% in group A and more than 9% in group B, except for *Bac2* present at only 2.2 % in 30%-b. Analyses of variance (ANOVA) between groups A and B were performed on the basis of relative abundances of *Bac1, Bac2* and *Bac3* peaks. Groups A and B were statistically very different, with p-value always lower than 0.001 for peaks *Bac1, Bac2* and *Bac3*. Similarly to AD performances, the relative abundances of *Bac1, Bac2* and *Bac3* were therefore significantly different between group A, where efficient AD occurred (high methane yield), and group B, where AD was inhibited (low methane accumulation). In conclusion, *Bac1, Bac2* and *Bac3* were always in higher relative abundance when AD was blocked (group B). By considering the total bacterial growth and assessing the associated amounts of *Bac1, Bac2* and *Bac3* that were produced, it was concluded that the emergence of *Bac1, Bac2* and *Bac3* in group B resulted from a selective and significant biomass growth, in comparison with group A (Fig. 1b).

[FIGURE 1]

In group B, where VFAs were highly concentrated and pH was lower, only three species were associated with low methane production. They were in all likelihood the bacteria most resistant to acid environment since the other species disappeared during the course of the experiment. The relative abundances of *Bac1, Bac2* and *Bac3* showed that these bacteria were the most representative microorganisms in group B, but these bacteria were not only resistant to acidic environment but could likely grow in such acid medium. This observation is in accordance with

their affiliated genus, i.e. *Clostridium sp.* Mostly, the persistence of these microorganisms in the environment is attributed to their readiness to sporulate. However, the relative abundance of these *Bac 1, Bac2* and *Bac3* increased as well as the number of total bacteria despite low pH and high VFA contents, meaning that these bacteria were most likely in their vegetative and active form. Actually, many studies have shown the resistance of the vegetative forms of *Clostridium* sp. to acidic pH and members of the *Clostridium* genus can maintain hydrolytic fermentative activity even at low pH (Ye et al., 2007). Our results are consistent with Staley (2009) who identified very similar bacteria (*Clostridium* sp. GQ453533.1) in methanogenic dry AD of refuse with high solids content, presenting 99% and 97% similarity with *Bac1* and *Bac2*, respectively.

1.3 Characterisation of the archaeal methanogenic community based on CE-SSCP fingerprints analysis: TS content responsible of gradual shifts of the archaeal populations

The archaeal diversity, represented by the number of peaks in the CE-SSCP profiles, was lower than for bacteria (with less than 10 peaks). Thus, higher percentages of relative abundance of the most abundant peaks were observed. By comparing the average CE-SSCP profiles, three groups were distinguished (see Supplementary Material S1): the group A previously determined for the bacterial population was split into two groups: Group A1, corresponding to 10% and 15% TS (semi-dry AD), and Group A2 corresponding to dry AD, ie. 20%, 25%, and 30-a % TS. The third group, Group B (30-b, 35% TS), was similar to the group found for bacteria and corresponded to inhibited AD. The relative abundance of each peak was calculated using the area of the peaks divided by the total SSCP profiles area (Fig. 2a). Each most dominant archaeal

species was clearly identified and represented more than 10% in relative abundance. These peaks were named *Arc1, Arc2, Arc3, Arc4*. Relative abundance of *Arc1* was 34% and 22%, respectively, in batch tests operating with 10% and 15% TS. For other TS contents, *Arc1* abundance was lower than 4%. In addition, the average relative abundances of *Arc2* and *Arc3* ranged between 14% and 25% in batch tests operating with a TS concentration higher than 20%. *Arc2* and *Arc3* abundances were lower than 3% for the other TS contents. Finally, *Arc4* presented a relative abundance of 18% and 33% at 30-b% and 35% TS, respectively. *Arc4* abundance was lower than 3% for whatever the other TS contents. Interestingly, the change in abundance of each species was gradual all along the TS contents (Fig. 2a). CE-SSCP profiles of the archaeal community in the inoculum showed that the archaeal community found in the digestate at day 298 differed substantially from the inoculum (see supplementary material S1). All of the most abundant *Archaea* identified at the end of the experiments in Groups A1, A2 and B were present in very low abundance in the inoculum, indicating the actual growth of these archaeal species whatever the operating conditions. In the inoculum, peaks *Arc1, Arc2, Arc3* and *Arc4* represented, respectively, only 0.6%, 2%, 0.3% and 2.8% of the total abundance (Fig 2.a). On the basis of relative abundances of *Arc1, Arc2, Arc3* and *Arc4*, analysis of variance ANOVA was done to differentiate groups A1, A2 and B. The results showed that these groups were statistically different, with a significant confidence index ($p<0.001$). These results confirm the coherence of the groups selected by observations based on CE-SSCP profiles.

Quantification of total archaea was carried out for all samples and amounts of *Arc1, Arc2, Arc3* and *Arc4* were assessed by calculation (Fig. 2b). By comparing the values of *Arc1, Arc2, Arc3* and *Arc4* according to the TS content, it was shown that *Arc1* was likely the most abundant

archaeon at the lowest TS values of 10% and 15% but decreased gradually as the TS concentration increased. Similarly, the highest biomass growth of *Arc2* and *Arc3* was at 20%, 25%, and 30-a% TS, which corresponded to dry anaerobic digestion. At higher TS concentrations (10%, 15%, 30%-b and 35%), smaller quantities of *Archaea*, and consequently, *Arc2* and *Arc3* were produced. Interestingly, the behaviour of *Arc4* was out of the ordinary, since *Arc4* showed the highest relative abundance when methanogenesis was inhibited, but no significant difference in the total calculated amount, due to the lower amount of total *Archaea* at 35 % (Fig. 2b).

[FIGURE 2]

As to peak identification, *Arc1*, *Arc2* and *Arc4* were affiliated to a genus of hydrogenotrophic methanogens, *Methanobacterium sp*., with respectively 100%, 100% and 99% similarity. *Arc3* corresponded to an acetoclastic methanogen, *Methanosarcina sp*. (100% similarity) (Fig. 3).

[FIGURE 3]

Low methane production at high levels of TS content showed that methanogenic *Archaea* were deactivated or inhibited. Total abundance of methanogenic *Archaea* in group B was one log lower than that at other TS content. The most abundant *archaea* at these TS concentrations (30-b% and 35% TS) was *Arc4* which corresponded to an hydrogenotrophic methanogen *Methanobacterium* sp.. In addition, *Arc4* had 100% similarity with *Methanobacterium* sp. (GQ453588) previously identified by Staley (2009) and found in methanogenic environment at 31% TS and acidic pH. Our results are consistent with Staley's findings. According to Blume et al. (2010), *Methanobacteriales* have higher abundance compared to the families *Methanomicrobiales*, *Methanosarcinaceae* and *Methanosaetaceae* in environments with high

total acid concentrations and low pH. *Methanobacteriales* are even able to grow at a pH about 5 (Garrity and Holt, 2001). As our results showed, McMahon et al. (2004) reported that, at high acetate concentrations, ie. up to 8 g L^{-1}, *Methanosarcina* sp. and *Methanosaeta* sp. were strongly inhibited, with a significant increase in members of the *Methanobacteriales* family. In our experiments carried out at solid concentrations higher than 30% TS, *Methanobacterium* sp. was the most abundant methanogen, and despite resistance of this archaea to environmental acidity, methanogenesis was inhibited. This inhibition resulted from VFA and more precisely acetate accumulation at concentrations above 29 g L^{-1}. Methanogens were therefore highly influenced by the TS concentration through VFA accumulation and pH lowering. In semi-dry AD (10% - 15% TS), the most abundant archaea was *Arc1* and corresponded to *Methanobacterium*. Karakashev et al. (2006) showed a strong correlation between the absence of *Methanosaetaceae* (acetoclastic methanogens) and the involvement of bacteria in the acetate oxidation pathway. In solid waste refuse, Staley et al. (2012) showed that *Methanosarcinales* had relative abundances from 1.4 to 14 times lower in the leachate than in the solid fraction. The authors concluded that many acetate-oxidative bacteria are motile and are less preferentially attached to particulate matter. In our study, this fact may explain why there is a significant dominance of hydrogenotrophic archaea at 10% and 15% TS: more water led to a selective advantage for motile acetate-oxidative bacteria. Although no hydrogen was detected, this does not exclude the presence of dissolved hydrogen in a very localised bacterial environment and dissolved hydrogen concentration plays a direct role in the predominance of the hydrogenotrophic methanogens (Hori et al., 2006). More specifically, *Methanobacterium* sp. is favoured compared to other hydrogenotrophic methanogens at high dissolved H_2 concentrations (Hori et al., 2006). For dry AD, a shift was observed with a co-dominance of *Methanosarcina* and *Methanobacterium* genera. The coexistence of acetoclastic

and hydrogenotrophic methanogens has also been reported to occur in natural environmental ecosystems (Kemnitz et al., 2004) and more especially in animal rumen (Kim et al., 2011). Leclerc et al. (2004) studied 44 digesters located in eight different countries and treating different kinds of waste (effluent from agriculture, food processing, petro-chemical industries, pulp and paper plants, breweries, slaughterhouses and municipal waste leachates). The authors showed that the most frequent archaeal sequences were affiliated to *Methanosaeta* sp. and *Methanobacterium* sp. in 84% and 73% of the digesters, respectively. In this study, the acetoclastic methanogens corresponded to *Methanosarcina* sp.. Moreover, it has been generally reported that acetate cleavage is performed by *Methanosarcinaceae* at high acetate concentrations and by *Methanosaetaceae* at low acetate concentrations (Hori et al., 2006). In the present study, the acetate concentrations were very low but *Methanosarcina* sp. was the most dominant archaeal species, probably because: (1) *Methanosarcina* is the most metabolically-diverse methanogen (Garcia et al., 2000) and could have another pathway for producing methane such as the hydrogenotrophic pathway (Kotsyurbenko et al., 2004); or (2) although no acetate was measured at a macroscopic level, the localised bacterial environment was rather different, especially at the interface with the solid substrate. At this very localised level, acetate concentration can be much higher and pH values much lower than those measured, as already discussed elsewhere (Abbassi-Guendouz et al., 2012). In a compact and unmixed medium, the existence of microenvironments with high VFA accumulation is the most probable explanation for the proliferation of *Methanosarcina* sp. which, at acetate concentrations above 1 mmol L^{-1}, presents higher growth rate than the widespread *Methanosaeta* sp. which grows preferentially at lower acetate concentrations (Hori et al., 2006). The presence of *Methanobacterium* (*Arc2*) confirmed this assumption since similar *Methanobacterium sp*. (DQ677518) was detected by Kotsyubenko et al.

(2007) at a pH below 4.5. Also, the predominance of such *Methanosarcina* sp. in a dry anaerobic digestion environment was probably due to the resistance of this methanogen to high acid concentrations, since the total amount of VFA accumulated at 30% TS was three times higher than at 10% TS.

2. Conclusions

This work showed a significant impact of TS concentrations on microbial communities. The most abundant bacteria growing in presence of high solid concentrations were from the genus *Clostridium sp.* The structure of the archaeal communities was also specifically influenced with a gradual shift of the most important *Archaea* between semi-dry and dry AD conditions. At the highest TS concentrations, environmental conditions did not allow the growth of acetoclastic methanogens or acetate-oxidizing bacteria on account of high VFA concentration and low pH values. Monitoring microbial communities to prevent methanogenesis failure constitutes therefore a good alternative for operational DAD.

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Table captions

Table 1. Primers and quantification probes used for DNA amplification. Underlined primers and probes corresponded to archaeal 16S rDNA amplification, and not underlined primers and probes for bacterial 16S rDNA amplification.

Table 2. Main performance parameters characterizing the anaerobic digestion process. The tests were carried out in batch tests at 37°C, with cardboard as substrate. VFAs and pH were analysed at the end of the reaction (day 298). Biogas volume and methane content were monitored throughout the reaction. "nd" : not detected. Total VFAs correspond to the sum of individual VFA amount, when detected.

Figure captions

Figure 1. Relative (a) and total (b) abundance of main bacterial dominant peaks (*Bac1, Bac2 and Bac3)* found in inhibited batch tests after 298 days of experiment. *(1.a) Calculated relative abundance of the peaks Bac1, Bac2 and Bac3 (most abundant bacteria in group B) according to TS content. Error bars represent the standard deviation between profiles of four replicates. (1.b) Quantification of the Bac1, Bac2 and Bac3 according to TS content. Total amounts of bacteria were determined by multiplying the relative abundance of each species by the total Bacteria quantification obtained by qPCR.*

Figure 2. Relative (a) and total (b) abundance of main archaeal dominant peaks (*Arc1, Arc2, Arc3 and Arc4)* found in inhibited batch tests after 298 days of experiment. *(2.a) Calculated relative abundance of peaks Arc1, Arc2, Arc3, Arc4 in batch tests according to TS concentration. The error bars represent the standard deviation between profiles of the four replicates. (2.b) Quantification of Arc1, Arc2, Arc3 and Arc4*

according to the TS content. Total amounts of Archaea were determined by multiplying the relative abundance of each species by the total Bacteria quantification obtained by qPCR.

Figure 3. Neighbour-joining phylogenetic tree of most important *Archaea* (*Arc1*, *Arc2*, *Arc3*, *Arc4*) identified in batch tests with different TS content. The tree was generated using TREECON and a neighbour joining distance method using a Jukes-Cantor algorithm. *Sulfolobus acidocaldarius* was used as root outgroup. The size bar indicates the Jukes-Cantor evolutionary distance. Numbers at the nodes indicate the bootstrap values for 1000 bootstrap calculations.

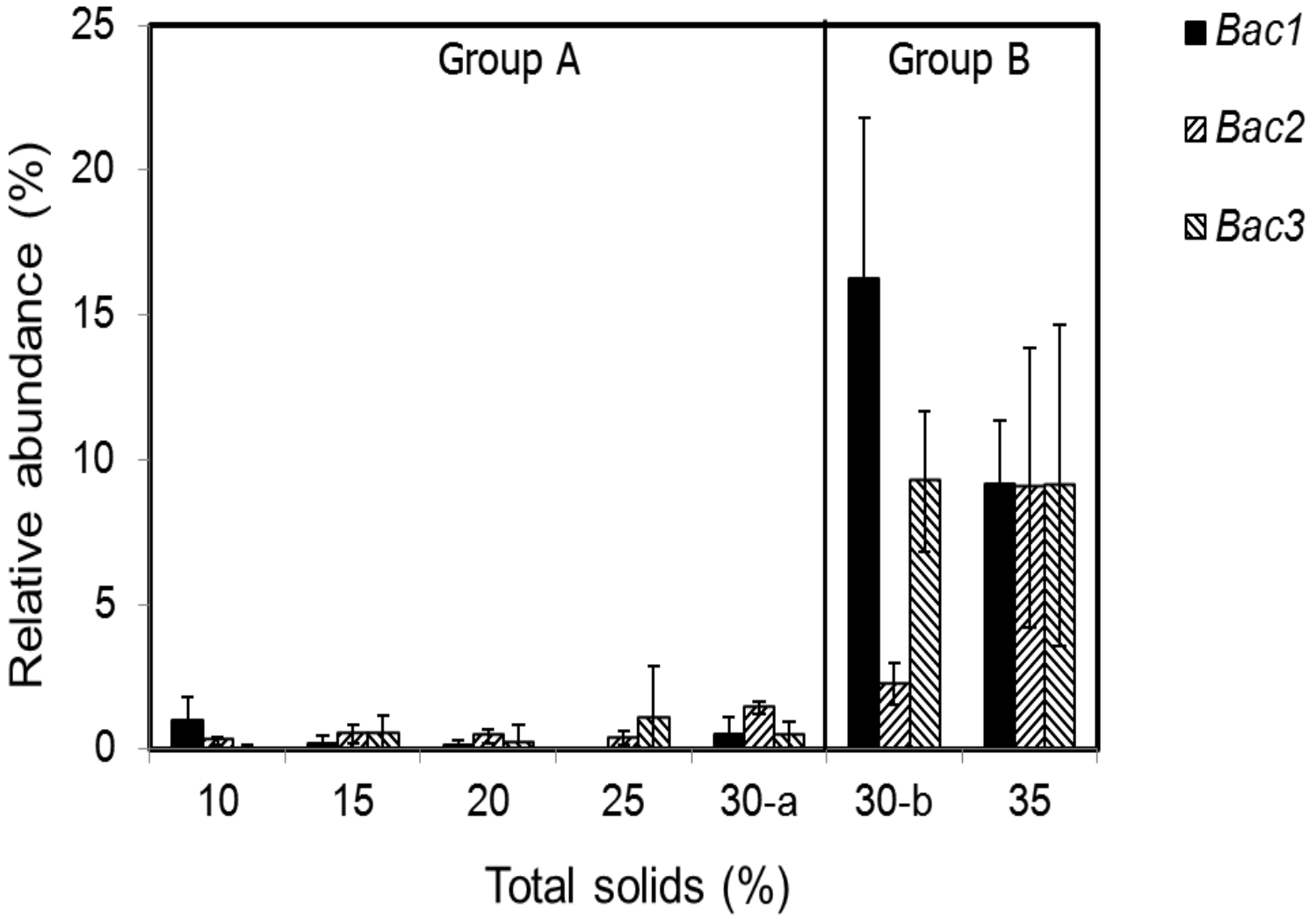
Abbassi_et_al_Table_1_R2.pdf

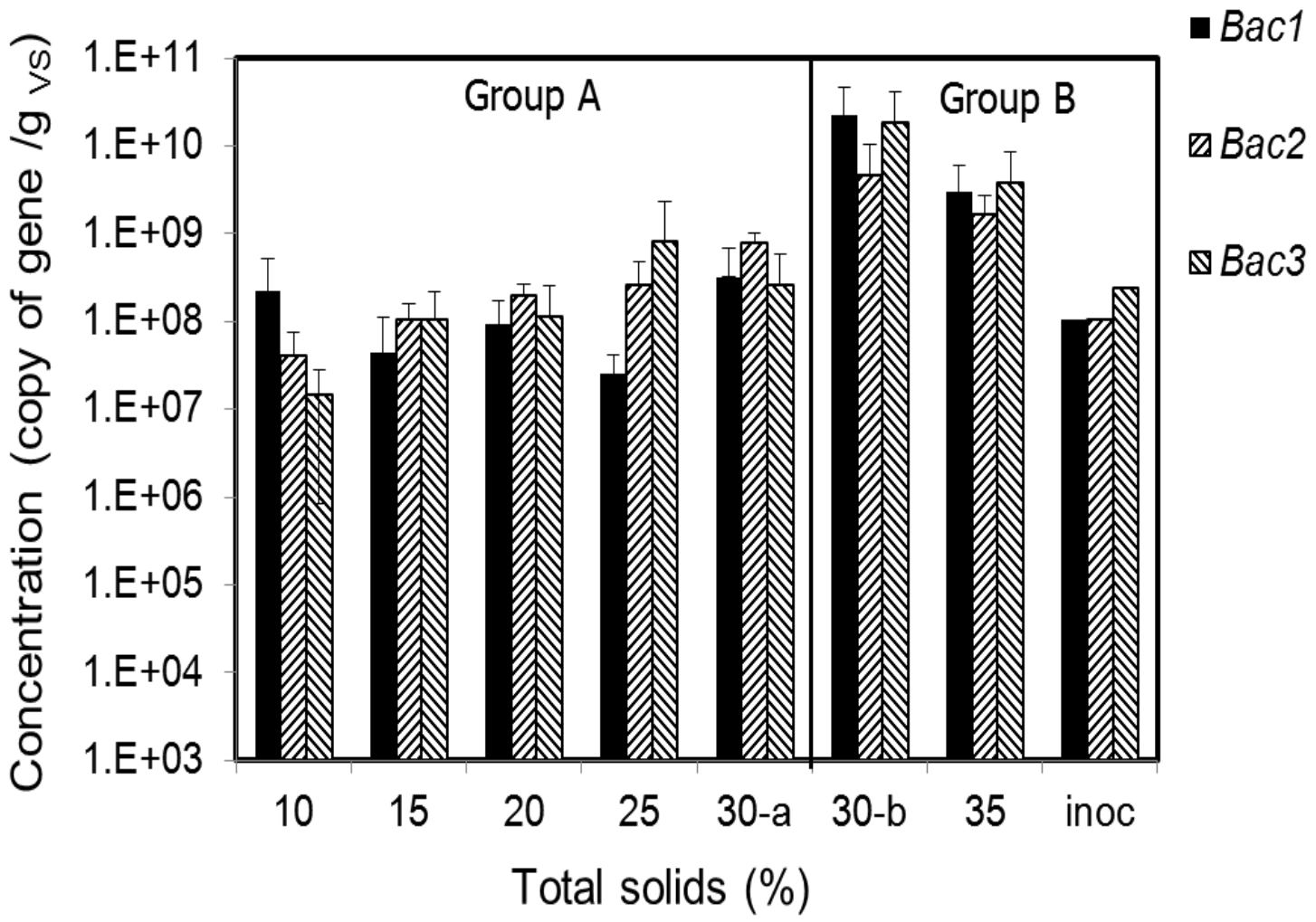
	Sequence (5'–3')	Position[a] (*Escherichia coli*)	Reference
W49	ACGGTCCAGACTCCTACGGG	F330	Delbes *et al.* (2001)
W104	FAM-TTACCGCGGCTGCTGGCAC-Tamra	R533	Delbes *et al.* (2001)
W274	CCCTACGGGGCGCAGCAG	F340	Ovreas *et al.* (1997)
W275	FAM-TTACCGCGGCGGCTG-Tamra	R519	Ovreas *et al.* (1997)
W18	GAGTTTGATCMTGGCTCAG	F9	Brosius *et al.* (1981)
W31	TTACCGCGGCTGCTGGCAC	R500	Delbes *et al.* (2001)
W280	TTACCGCGGCGGCT	R500	Delbes *et al.* (2001)
W208	ACTCCTACGGGAGGCAG	F338	Yu *et al.* (2005)
W209	GACTACCAGGGTATCTAATCC	R805	Yu *et al.* (2005)
W210	Yakima Yellow- TGCCAGCAGCCGCGGTAATAC-Tamra	F516	Yu *et al.* (2005)
W211	ATTAGATACCCSBGTAGTCC	F787	Yu *et al.* (2005)
W212	GCCATGCACCWCCTCT	R1059	Yu *et al.* (2005)
W213	FAM-AGGAATTGGCGGGGGAGCAC-Tamra	F915	Yu *et al.* (2005)

Abbassi_et_al_Table_2_R2.pdf

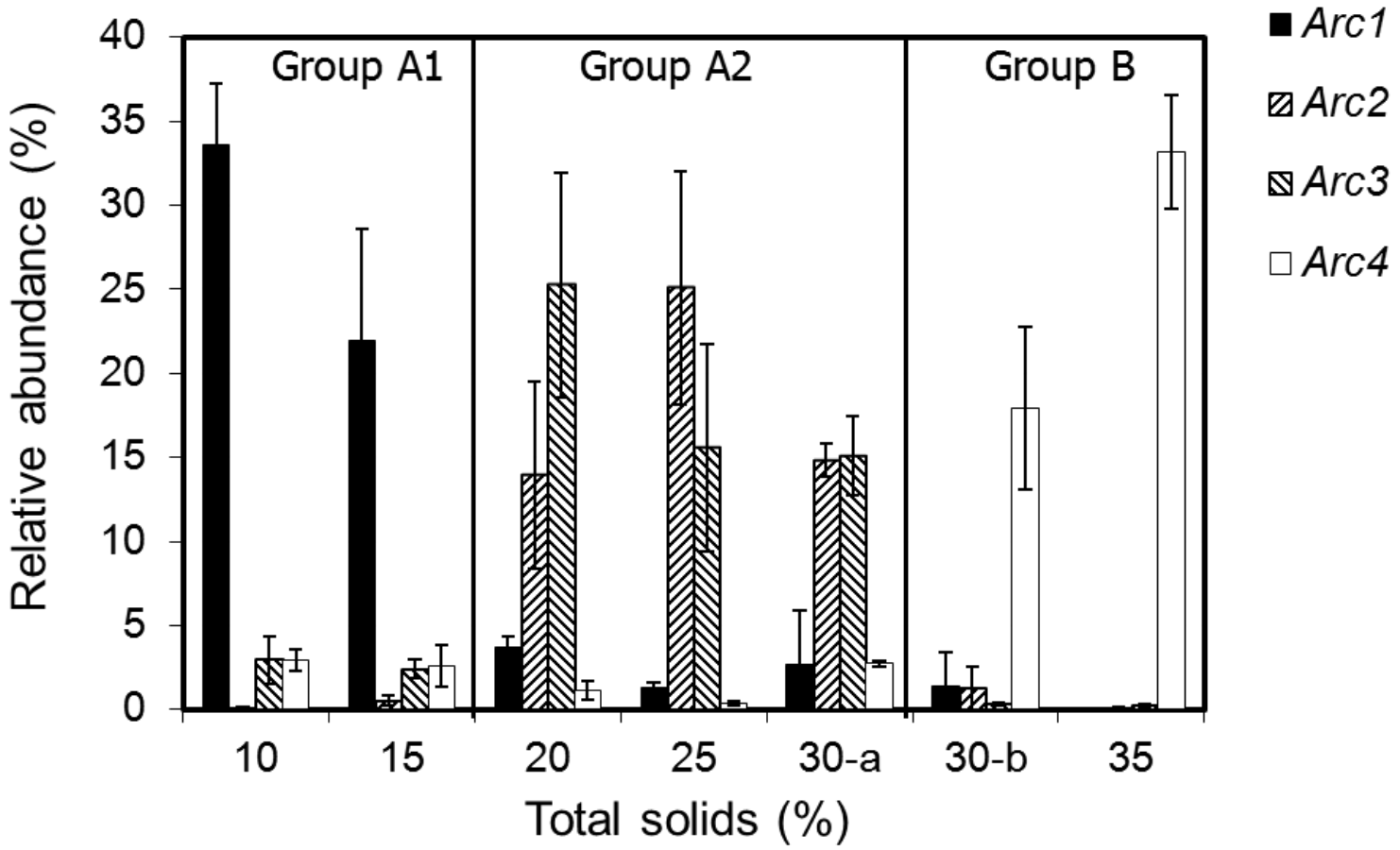
TS (%)	V_{CH4} (mL gTS^{-1})	% CH4 (%)	Acetate (g L^{-1})	Propionate (g. L^{-1})	Butyrate (g. L^{-1})	isoButyrate (g. L^{-1})	Valerate (g. L^{-1})	isoValerate (g. L^{-1})	Total VFA (g L^{-1})	Final pH
10	141±9	51±2	nd	nd	nd	nd	nd	nd	-	7.4
15	139±8	58±5	nd	nd	nd	nd	nd	nd	-	7.4
20	132±8	58±2	nd	nd	nd	nd	nd	nd	-	6.9
25	129±9	60±1	nd	nd	nd	nd	nd	nd	-	7.8
30-a	109±1	63±1	nd	nd	nd	nd	nd	nd	-	7.5
30-b	28±7	42±1	25±7	8±2	2±0.5	4±0	1±0	0.2±0	36±10	5.9
35	19±2	4±2	18±4	9±3	1±0.5	3±0	1±0	0.1±0	29±8	6.0

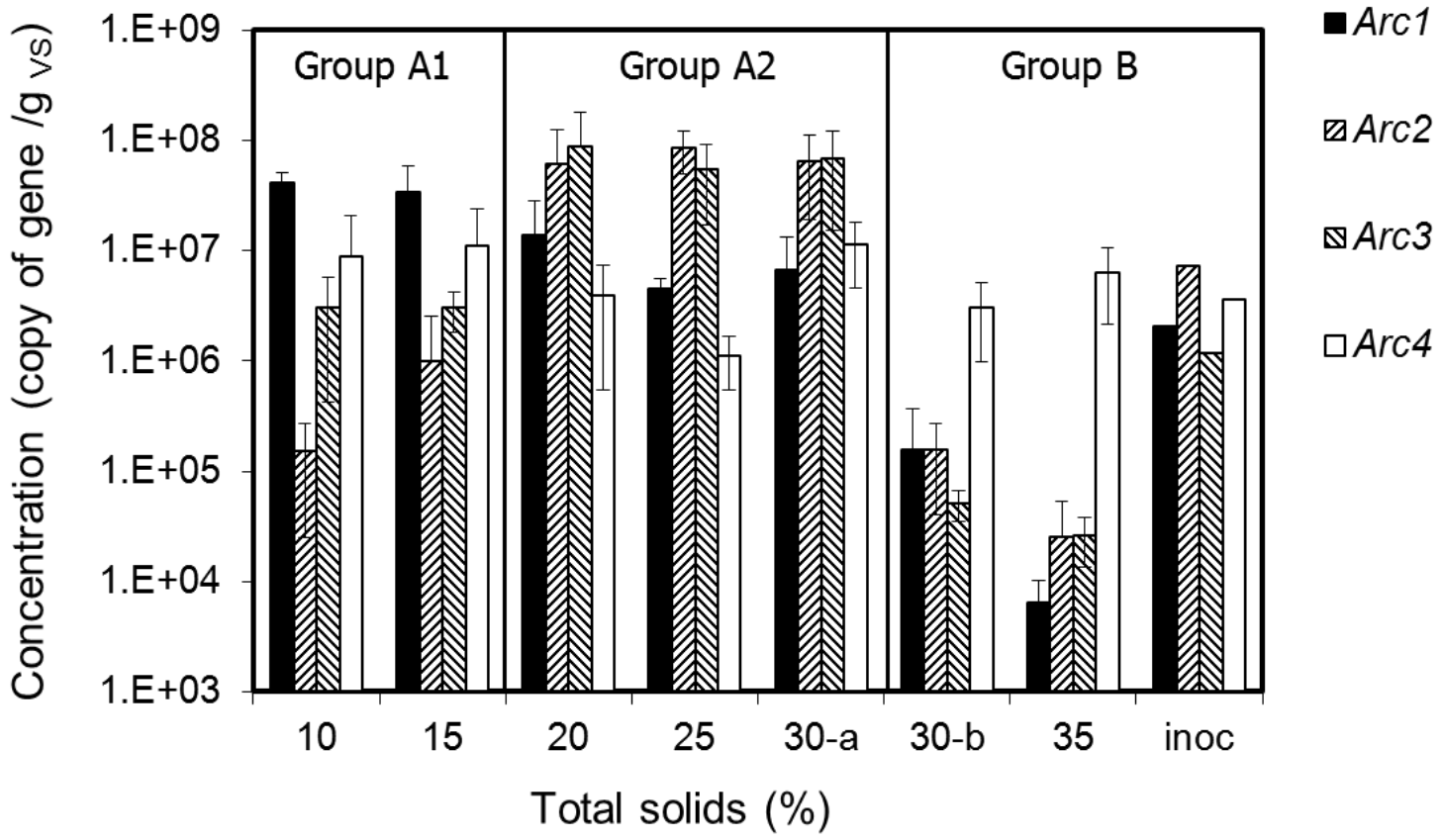
Abbassi_et_al_Figure1_R4.pdf




Concentration (copy of gene /g VS)
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
Group A
Group B
Bac1
Bac2
Bac3
10
15
20
25
30-a
30-b
35
inoc
Total solids (%)

Abbassi_et_al_Figure2_R4.pdf




Concentration (copy of gene /g VS)
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
Group A1
Group A2
Group B
10
15
20
25
30-a
30-b
35
inoc
Total solids (%)
Arc1
Arc2
Arc3
Arc4

Abbassi_et_al_Figure3_R2.pdf