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# Biofilm formation and granule properties in anaerobic digestion at high salinity

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ABSTRACT

For the anaerobic biological treatment of saline wastewater, Anaerobic Digestion (AD) is currently a possibility, even though elevated salt concentrations can be a major obstacle. Anaerobic consortia and especially methanogenic archaea are very sensitive to fluctuations in salinity. When working with Upflow Sludge Blanket Reactor (UASB) technology, in which the microorganisms are aggregated and retained in the system as a granular biofilm, high sodium concentration negatively affects aggregation and consequently process performances. In this research, we analysed the structure of the biofilm and granules formed during the anaerobic treatment of high salinity (at 10 and 20 g/L of sodium) synthetic wastewater at lab scale. The acclimated inoculum was able to accomplish high rates of organics removal at all the salinity levels tested. 16S rRNA gene clonal analysis and Fluorescence In Situ Hybridization (FISH) analyses identified the acetoclastic *Methanosaeta harundinacea* as the key player involved acetate degradation and microbial attachment/granulation. When additional calcium (1 g/L) was added to overcome the negative effect of sodium on microbial aggregation, during the biofilm formation process microbial attachment and acetate degradation decreased. The same result was observed on granules formation: while calcium had a positive effect on granules strength when added to UASB reactors, *Methanosaeta* filaments were not present and the degradation of the partially acidified substrate was negatively influenced. This research demonstrated the possibility to get granulation at high salinity, bringing to the forefront the importance of a selection towards *Methanosaeta* cells growing in filamentous form to obtain strong and healthy granules.



## 1. Introduction

High concentrations of salt occur in several natural and man-made hypersaline environments, and also several industries generate saline wastewater (Gunde-Cimerman and Oren, 2005; Xiao and Roberts, 2010). It is estimated that 5% of industrial effluents are saline or hypersaline (Le Borgne et al., 2008; Lefebvre et al., 2007). For these reasons, the biological treatment of saline wastewater has been an emerging topic in the last decade and currently has drawn a great interest. The anaerobic digestion (AD) of wastewater is an efficient and sustainable technology applied for organics degradation together with energy recovery in the form of biogas. AD involves several microbial groups forming interdependent microbial consortia (O'Flaherty et al., 2006). Methanogenesis is the final step and methanogenic archaea are very sensitive to fluctuations in salinity (Feijoo et al., 1995). Sodium concentrations exceeding 10 g/L are known to inhibit methanogenesis (Kugelman and McCarty, 1965; Chen et al., 2008), and in particular acetoclastic methanogens (Rinzema et al., 1988; Oren, 2010). Generally, the high concentrations of salt can cause cell plasmolysis and cell death due to a dramatic increase in osmotic pressure and as a result the organic compounds in saline wastewaters often are poorly biodegraded (Rinzema et al., 1988; Vyrides and Stuckey, 2009; Lay et al., 2010; Kimata-Kino et al., 2011). The use of a salt- adapted

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microbial consortia in the anaerobic degradation process could be the best approach to overcome this issue (Aspé et al., 1997; Hierholtzer and Akunna, 2014). On the other hand, some studies have demonstrated the effectiveness of potassium addition to alleviate sodium toxicity on methanogenesis (Kugelman and McCarty, 1965; Feijoo et al., 1995; Vyrides and Stuckey, 2009; Vyrides et al., 2010). In fact, potassium addition can promote the "salt in" strategy, in which the cells increase their intracellular ion concentration to balance the external osmotic pressure. Different halophilic microorganisms maintain extremely high salt concentrations inside their cells to balance the osmotic pressure outside (Welsh, 2000; Roeßler and Müller, 2001), and in most cases potassium is the main intracellular cation, with the negative-inside membrane potential as the driving force for the massive accumulation (Oren, 1999).

Granular sludge based anaerobic high-rate reactors, such as the UASB reactor, are commonly applied to treat high-strength industrial wastewaters (Van Lier et al., 2015). The microorganisms usually aggregate into granules of 0.5–3 mm. Strong and healthy granules with a high sedimentation velocity are beneficial for a successful operation of UASB reactors because there is no wash-out of viable biomass and allows a compact reactor design (Lu et al., 2013). In granules, the microorganisms are packed as a spherical biofilm, forming a microbial ecosystem with a characteristic internal architecture (Sekiguchi et al., 1999). Although granule formation has been studied intensively, the literature is often contradictory about the responsible mechanisms (Hulshoff Pol et al., 2004). Probably, several factors contribute to granulation and multiple mechanisms are involved (Ahn et al., 2009). One of the most interesting hypotheses is the "Spaghetti theory" (Wiegant, 1987) where granule formation is explained by a selection towards acetoclastic archaeal *Methanosaeta* cells, and by promoting the filamentous growth of this microorganism (Tay et al., 2010). Besides the microbial composition of granules, divalent cations such as calcium play an important role in the aggregation of microorganisms (Yu et al., 2001), forming positively charged bridges between negatively charged bacterial surfaces and extracellular polymeric substances (EPS) (Veiga et al., 1997; Pevere et al., 2007). Ismail et al. (2010) showed that high sodium concentrations cause calcium leaching from anaerobic granules, resulting in a loss of granule strength. The calcium-augmented granules were more resistant to shear than granules in a similar reactor without calcium augmentation. Thus, high salinity can reduce granule strength, causing viable biomass to be washed out from the reactor. Application of UASB reactors for treatment of high salinity wastewater only was investigated in a few studies (e.g. Rinzema et al., 1988; Vallero et al., 2003; Ismail et al., 2008, 2010; Li et al., 2014; Aslan and Şekerdağ, 2015). In this work we investigated biofilm formation and functionality of granular anaerobic sludge in UASB reactors at 10 and 20 g/L sodium. The experiments focused on attachment of cells to non-woven fabric sheets as well as on the stability of mature granules under elevated saline conditions in a lab-scale UASB reactor. Potassium and calcium addition was investigated to assess their effect on biomass activity and granule strength.

## 2. Materials and methods

### 2.1. Reactor operation and performance

#### 2.1.1. Inoculum

Anaerobic sludge granules from a full-scale UASB reactor treating wastewater from the Shell plant in Moerdijk, the Netherlands, was used in this study. The sludge was already adapted to a sodium concentration of approximately 8 g $Na^+$/L, with acetic and benzoic acids as the main sources of chemical oxygen demand (COD), for more than 10 years.

#### 2.1.2. Biofilm formation

The experiments were performed in a temperature controlled room at 30 ± 2 °C, using four up-flow fixed bed glass biofilm column reactors (Fig. 1). The reactors had an inner diameter of 60 mm, a height of 330 mm and a volume of 0.9 L. Four non-woven fabric sheets (38 × 3.0 × 0.05 cm; Hanotex B.V., Joure, the Netherlands) were used as removable support material in each reactor, placed vertically in PVC plastic frames (Fig. 1). The up-flow liquid velocity was controlled by liquid recirculation at 0.8 m/h with a peristaltic pump (Watson Marlow 202, Falmouth, UK). The reactors were seeded with 2 g volatile suspended solids (VSS)/L of crushed sludge granules. The reactors were operated as a fed batch, and influent concentrations are given in Table 1. The media were prepared according to Vallero et al. (2003). To evaluate the effect of sodium on the process, reactor B1 was operated at 10 g $Na^+$/L while reactors B2, B3 and B4 were operated at 20 g $Na^+$/L. Additional calcium ($CaCl_2 \cdot 2H_2O$) was added to reactor B3 at a concentration of 1 g $Ca^{2+}$/L. In reactor B4, 0.7 g/L of potassium (KCl) was added, to obtain a $K^+/Na^+$ ratio of 0.037, similar to seawater (Chester and Jickells, 1990). The reactors were fed with sodium acetate each time it was depleted, based on the results of COD analyses. Acetate was chosen as a substrate to simplify the process and to be able to directly follow the (acetoclastic) methanogenic performance.

#### 2.1.3. Granular sludge reactor operation

The experiments were performed in a temperature controlled room at 30 ± 2 °C, using two glass UASB reactors with a working volume of 3 L. The up-flow liquid velocity was controlled by liquid recirculation at 0.8 m/h with a peristaltic pump (Watson Marlow 202, UK). The reactors were inoculated with 41 g VSS/L of anaerobic sludge granules. The hydraulic retention time (HRT) was 1.34 days for the entire experimental period (120 days). The organic loading rate (OLR) was gradually increased from 6 to 24 g COD/L·d by feeding the reactors with synthetic wastewater consisting of NaCl at a concentration of 20 g $Na^+$/L, a solution of macro and micronutrients (Vallero et al., 2003), and a partially acidified substrate (PAS) containing acetate, gelatine and starch in a 7:2:1 COD ratio. The PAS substrate was chosen to study granule formation while treating complex wastewater. In both reactors potassium was added with a final concentration of 0.7 g/L. Additional calcium ($CaCl_2 \cdot 2H_2O$) was added to reactor G2 at a concentration of 1 g/L. Operational parameters are given in Table 1.

#### 2.1.4. Chemical analyses

Volatile fatty acids (VFAs) were analysed in a Hewlett Packard 5890A gas chromatograph equipped with a 2 m × 6 mm × 2 mm glass column packed with Supelco port, 100–120 mesh, coated with 10% Fluorad FC 431, as described in Ismail et al. (2008). COD and VSS analysis were carried out according to Standard Methods (APHA, 1998).

### 2.2. Granule properties

#### 2.2.1. Sampling of UASB solids and fines

Solids from the sludge blanket of the UASB reactors were sampled via a valve at a height of 22 cm (liquid height was 67 cm). Fines in these samples were defined as particles that did not settle within 1 min.

#### 2.2.2. Particle size distribution (PSD)

PSD of sludge blanket solids and fines (see above) was determined for each reactor by light scattering using a Mastersizer 2000 (Malvern, UK). Each sample was measured in triplicate and PSD

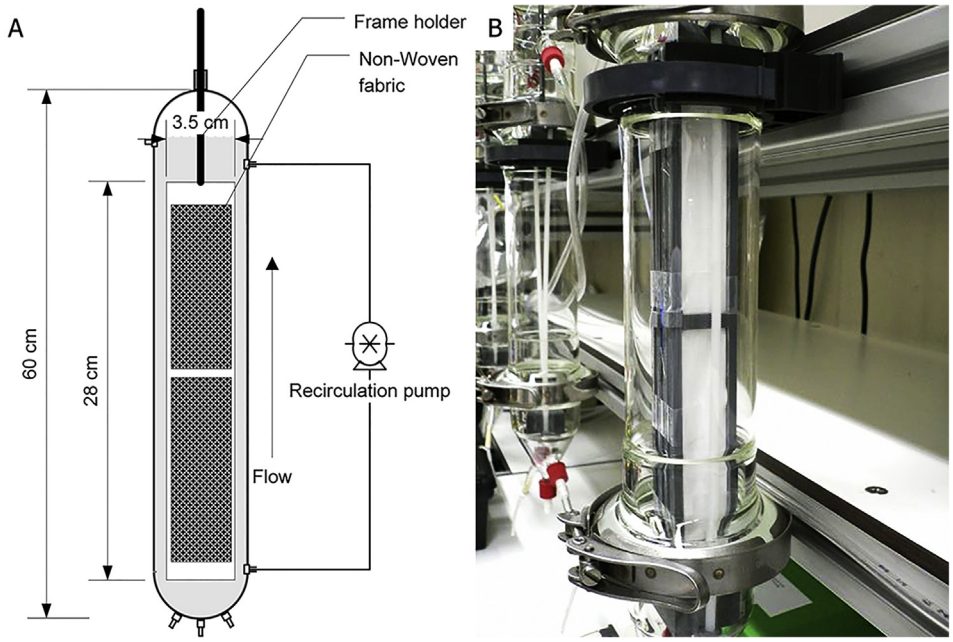


**Fig. 1.** Schematic representation (A) and picture (B) of an up-flow fixed-bed column reactor used to evaluate microbialadhesion on fixed materials.

**Table 1**
Characteristics of the biofilm and granular sludge reactors. Background calcium and potassium concentrations in the feed were 5 mg/L and 55 mg/L, respectively.

| ***Biofilm reactors*** | B1 | B2 | B3 | B4 |
|---|---|---|---|---|
| Sodium (g/L) | 10 | 20 | 20 | 20 |
| Calcium (g/L) | – | – | 1 | – |
| Potassium (g/L) | – | – | – | 0.7 |
| ***Granular sludge reactors*** | | G1 | | G2 |
| Sodium (g/L) | | 20 | | 20 |
| Calcium (g/L) | | 0 | | 1 |
| Potassium (g/L) | | 0.7 | | 0.7 |

based on particle volume was calculated using the instrument software. Granules were macroscopically investigated using a SMZ800 (Nikon, Melville, USA) stereomicroscope, fitted with a Nikon camera (Nikon Coolpix5000).

### 2.3. Scanning electron microscopy (SEM)

Before the analysis, biofilm and granules samples were fixed, dried and pre-treated as described in Ismail et al. (2010). Samples were glued on a sample holder by carbon adhesive tabs (EMS, USA) or with carbon-glue (Leit-C, Germany). Biofilm samples were sputter coated with 2–4 nm Tungsten in a MED 020 High Vacuum Metal Evaporation Coater (Leica, Austria). Granules samples were sputter coated with 10 nm platinum in a dedicated preparation chamber (CT 1500 HF Oxford Instruments). All the samples were analysed with a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, The Netherlands) at room temperature at a working distance of 4 mm with SE detection at 2 kV, coupled to a back-scattered electron (BSE) detector to determine surface morphology.

### 2.4. Microbial community analysis

#### 2.4.1. Fluorescent in situ hybridization (FISH)

FISH analysis was performed on paraformaldehyde-fixed biomass samples taken from biofilm and UASB reactors, according to the procedure described in Amann et al. (1995). Oligonucleotide probes applied are listed in Table S1. Biofilm samples were examined by epifluorescence microscopy using CLSM (Carl Zeiss, LSM 510, Germany) coupled with an Ariron laser (488 nm) and HeNe laser (543 nm). Z-sectioning was performed on whole granules and 3-D rendered with the LSM 510 Viewer software (Zeiss). Granules samples were examined under a Zeiss Axioplan 2 Imaging epifluorescence Photomicroscope. The images were captured by a Photometrics Sensys 1305 × 1024 pixel CCD camera and analysed with the Genus Image Analysis software (Applied Imaging Corporation). Image optimization was conducted using Adobe Photoshop CS (Adobe Systems Inc., USA). To identify glucose/mannose rich glycoconjugates associated with the microbial cells in fines of UASB reactors, lectin staining with FITC–labelled Concanavalin A (Con A, Sigma Aldrich, St. Louis, Missouri) was applied after the FISH procedure, according to Neu et al. (2001).

#### 2.4.2. DNA extraction

Biofilm sampling was done at three different time points (0, 40 and 90 days). A sheet of non–woven fabrics was gently removed from the reactor, cut into small pieces (5 × 5 mm), and directly placed in Eppendorf tube containing 2 mL anoxic reactor medium. Tubes with the samples were directly placed in a sonicator for 1 min at low power for at least five times, to detach the biomass from the fabric. The samples were centrifuged for 1 min at 14,000 rpm. For the UASB reactors, liquid samples were collected at the end of the process and centrifuged for 1 min at 14.000 rpm. In both cases, cell pellets, containing the biomass, were stored in RNAlater® (Applied Biosystems/Ambion, Austin, TX) at −20 °C. Total genomic DNA was extracted from all samples using a FastDNA® SPIN kit for soil (MPBio, USA) according to the manufacturer's instructions. DNA concentration and purity were measured with the NanoDrop® spectrophotometer.

#### 2.4.3. Cloning and sequencing

For biofilm and granular sludge samples PCR-amplicons of bacterial and archaeal 16S rRNA genes were obtained using primers

listed in Table S1. PCR amplification was carried out as described in Gagliano et al. (2015). Cloning of PCR products was performed using pGEM-T Easy Vector System (Promega, USA) into *E.coli* JM109 competent cells (Promega, USA) according to the manufacturer's instructions. After blue-white screening, white colonies were randomly selected and transferred to 1 mL liquid LB medium with 100 mg $L^{-1}$ ampicillin. After overnight incubation at 37 °C, clones were transferred to a GATC 96 well agar plate with 100 mg $L^{-1}$ ampicillin and sent to GATC (GATC Biotech AG, Konstanz, Germany) for sequencing. Sequences were manually trimmed and checked for chimeras using VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) and Decipher (http://decipher.cee.wisc.edu/FindChimeras.html). Sequence similarity was checked using NCBI MegaBlast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Data analysis of amplicons was carried out with SilvaNGS (https://www.arb-silva.de/ngs). 16S rRNA sequences were submitted to GenBank database under the accession numbers reported in Tables 2 and 3.

## 3. Results and discussion

### 3.1. Anaerobic biofilm formation at high salinity

#### 3.1.1. COD removal

Fig. 2 shows acetate-COD concentrations in the four biofilm reactors during the 80 days of operation. Reactor B1 (Fig. 2a), operated at 10 g $Na^+$/L, achieved the highest acetate conversion rates (670–1300 mg COD/L.d) among the four reactors. From the trend of the acetate COD removal rate in reactor B2 operated at 20 g $Na^+$/L (210–320 mg COD/L.d) (Fig. 2b), it can be concluded that this higher sodium concentration had a strong negative effect on the acetate conversion rate. However, when 0.7 g $K^+$/L was added to reactor B4 this negative effect of a higher sodium concentration was less severe with acetate conversion rates of 500–900 mg COD/L·d (Fig. 2d). Surprisingly, reactor B3, supplemented with 1 g $Ca^{2+}$/L, exhibited the lowest acetate removal rates of 15–40 mg COD/L·d (Fig. 2c). The enhanced acetate conversion rates of reactor B4 in comparison to B2 clearly shows that a low amount of potassium is required when anaerobic biomass is exposed to a high sodium concentration. The applied $K^+/Na^+$ ratio in our experiments was similar to the ratio in seawater. This is supported by Feijoo et al. (1995) who found that the use of seawater instead of synthetic wastewater without potassium resulted in a better performance of anaerobic digestion, probably because the microorganisms need potassium to effectively operate their sodium-potassium pumps to maintain sufficiently low cytoplasmic sodium levels (Sleator and Hill, 2002). Also Vyrides et al. (2010) showed a slight decrease of sodium inhibition by adding potassium during anaerobic batch experiments at sodium concentrations up to 16 g/L. Despite the prevalent role that is claimed for calcium ions to bridge EPS and thus to stimulate biofilm formation (Patrauchan et al., 2005), the addition of 1 g/L of calcium to reactor B3 had a strong detrimental effect on the microbial activity. This negative effect will be further discussed in sections 3.1.2 and 3.3.

#### 3.1.2. Microbial community composition of the biofilms

SEM analysis carried out on samples taken after 40 days of operation revealed that mainly rods and small cocci were present, embedded in an EPS matrix. Surface coverage in reactor B3 (Fig. 3c) was significantly lower than in the other three reactors, indicating a reduced microbial attachment in the presence of calcium. Surface coverage in the other two reactors operated at 20 g $Na^+$/L (reactors B2 and B4) was not visibly lower or higher than in reactor B1 operated at 10 g $Na^+$/L, suggesting that the higher salinity did not have a negative impact on microbial attachment. CLSM analysis confirmed the attachment of microbial cells to the non-woven fibre material (Fig. S1). FISH-CLSM analysis (Fig. 4) highlighted the dominance of archaeal filamentous cells, positive to the ARC915 probe (in yellow), in reactors B1 and B4 after 40 days of operation (Fig. 4a and c). In the sample of reactor B3 (Fig. 4b), which received additional calcium, mainly bacterial cells were observed (in red). There is an evident difference in the morphology of the archaeal cells identified by FISH. In reactor B1 robust and long filaments were highlighted (Fig. 4a). In reactor B3 (Fig. 4b) the fluorescence signal from archaeal cells, which mainly had a single or multiple rod morphology, was very weak (yellow) and bacterial cells (in red) were present in a higher proportion. Finally, archaeal filaments observed in reactor B4 (Fig. 4c) were similar to the ones detected in R1, but shorter and less thick. The microscopic observations discussed above and the low acetate removal rate in B3 indicate that the presence of 1 g/L of calcium prevents, or at least slows down, attachment of methanogens. Biofilm samples from reactor B1 after 40 days and from reactor B3 at the end of the experimental period (90 days) were used for DNA extraction, archaeal 16S rRNA gene amplification and cloning (Table 2). The 16S rRNA gene sequences identified in both reactors were classified as close relatives of the archaeal genera *Methanosaeta*, *Methanobacterium, Methanolinea* and the family *Methanomicrobiaceae*. 83% of the clones from reactor B1 and 78% from reactor B3 were closely related to *Methanosaeta*

**Table 2**
16S rRNA gene clonal analysis of biofilm reactors B1 (day 40) and B3 (day 90).

| no. of clones | Accession number | Affiliation | Closest relative (accession no.) | Similarity (%) |
|---|---|---|---|---|
| **B1** | | | | |
| 54 | KX018770, KX018772 | *Uncultured Methanosaeta* | *Methanosaeta harundinacea 6Ac* (NR_102896.1) | 99 |
| 3 | KX018776 | *Uncultured Methanobacterium* | *Uncultured Methanobacterium clone B9* (LN717044.2) | 99.2 |
| 1 | KX018773 | *Uncultured Methanobacterium* | *Methanobacterium aarhusense strain H2-LR* (NR_042895.1) | 99 |
| 6 | KX018771 | *Uncultured Methanolinea* | *Uncultured archeaon clone HsA47fl* (AB266915.1) | 100 |
| 1 | KX018777 | *Uncultured Methanomicrobiales* | *Uncultured Methanomicrobiales archaeon clone KO-Buty-B* (AB236052.1) | 97 |
| 1 | KX018774 | *Uncultured Methanomicrobiales* | *Uncultured archaeon clone SYNH02_C3-07A-033* (JQ245666.1) | 94 |
| Total 66 | | | | |
| no. of clones | Accession number | Affiliation | Closest relative (accession no.) | Similarity (%) |
| **B3** | | | | |
| 60 | KX018781, KX018780 | *Uncultured Methanosaeta* | *Methanosaeta harundinacea 6Ac* (NR_102896.1) | 97–99 |
| 7 | KX018779 | *Uncultured Methanobacterium* | *Methanobacterium subterraneum strain A8p* (NR_028247.1) | 99 |
| 1 | KX018786 | *Uncultured Methanobacterium* | *Methanobacterium aarhusense strain H2-LR* (NR_042895.1) | 99 |
| 1 | KX018785 | *Uncultured Methanobacterium* | *Methanobacterium beijingense strain 8-2* (NR_028202.1) | 99 |
| 6 | KX018784 | *Uncultured Methanolinea* | *Uncultured Methanomicrobiales archaeon clone KO-Buty-B* (AB236052.1) | 99 |
| 1 | KX018787 | *Uncultured Methanoculleus* | *Methanoculleus* sp.*Clone A3* (AJ133793.1) | 98 |
| 1 | KX018788 | *Uncultured Methanomethylovorans* | *Uncultured archaeon clone R27A15* (KC676298.1) | 99 |

**Table 3**
16S rRNA gene clonal analysis of archaea and bacteria in reactor G2 at the end of the experimental process.

| no. of clones | Accession number | Affiliation | Closest relative (accession no.) | Similarity (%) |
|---|---|---|---|---|
| **Archaea** | | | | |
| 85 | KX018757, KX018758 | *Uncultured Methanosaeta* | *Methanosaeta harundinacea 6Ac* (NR_102896.1) | 99 |
| **Bacteria** | | | | |
| 50 | KX018759 | *Uncultured Marinilabiaceae* | *Uncultured bacterium clone SWM-5-5* (KR107290.1) | 99.7 |
| 1 | KX018768 | *Uncultured Proteiniphilum* sp. | *Proteiniphilum* sp. *S2* (KP178480) | 99.5 |
| 2 | KX018763 | *Uncultured bacterium* | *Uncultured bacterium clone SYNH02_ew01B-094* (JQ245635) | 95 |
| 1 | KX018766 | *Uncultured Bacteroidetes* | *Uncultured bacterium clone EMIRGE_OTU_s6b4a_8166* (JX224181) | 90.2 |
| 1 | KX018765 | *Uncultured bacterium* | *Uncultured bacterium clone YC12* (KJ734898) | 92 |
| 6 | KX018762 | *Uncultured Clostridiales* | *Uncultured bacterium clone N0076* (JX391512) | 99.4 |
| 4 | KX018764 | *Uncultured Clostridiales* | *Uncultured bacterium clone B19CH1_61_65* (HF558553) | 95.8 |
| 1 | KX018760 | *Uncultured Clostridiales* | *Uncultured bacterium clone ARDBACWH19* (EU869412) | 96.6 |
| 1 | KX018761 | *Uncultured Alkaliphilus* | *Uncultured bacterium clone BAC_SB_233* (NR_116395.1) | 99.3 |
| 1 | KX018767 | *Uncultured Dethiosulfatibacter* | *Uncultured bacterium clone: YWB38* (AB294307) | 96.7 |
| 1 | KX018769 | *Uncultured Spirochaetaceae* | *Uncultured bacterium clone b57* (HM468049) | 99.6 |
| Total 69 | | | | |

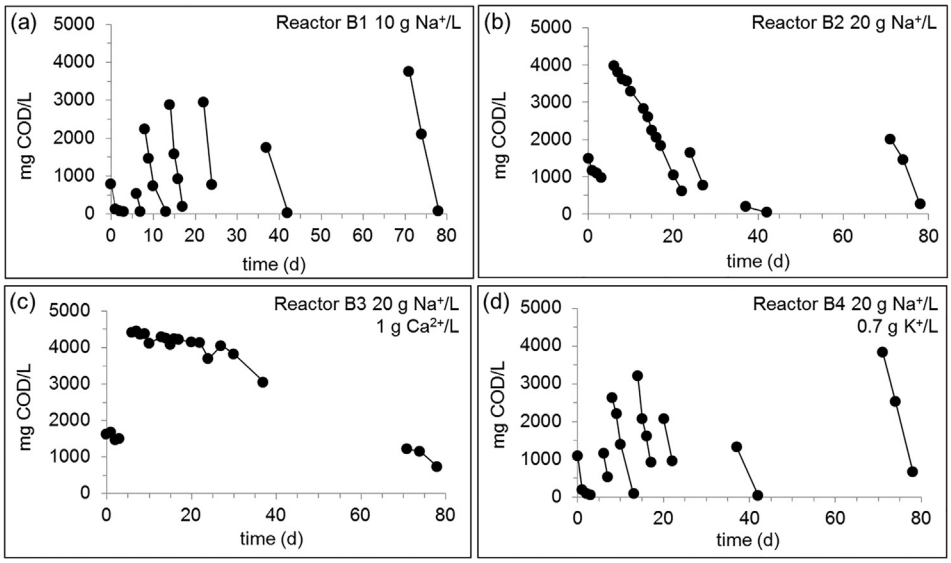


**Fig. 2.** - Acetate-COD concentrations in reactors B1 (a), B2 (b) B3 (c) and B4 (d).

*harundinacea* species. This methanogenic archaeon was previously isolated from the granules of an up-flow anaerobic sludge bed granules and it is usually occurring as single rods or in pairs/filaments (Ma et al., 2006). *M.harundinacea* was also visualized as the filamentous archaeon by FISH (Fig. 4a). Although acetate was the sole energy substrate, sequences affiliated to hydrogenotrophic methanogens were found in both reactors, and they were more abundant and higher in diversity in the calcium augmented reactor B3 (23% in comparison to 18% in reactor B1) (Table 2). In reactor B3, members of the genus *Methanobacterium* represented 13% of total clones. *Methanobacterium subterraneum*, present in several anaerobic systems (Hwang et al., 2008; Kobayashi et al., 2009; Yamane et al., 2011), was detected in reactors B1 and B3. *M. subterraneum* is an alkaliphilic and halotolerant methanogen, and the first example of a methanogen isolated from a deep granitic aquifer (Kotelnikova et al., 1998), phenotypically adapted to an extreme environment. Members of the order *Methanomicrobiales* (as *Methanolinea* and *Methanomicrobiaceae*) are known as $H_2/CO_2$ and formate using methanogens (Imachi et al., 2008; Cheng et al., 2008). Several 16S rRNA gene surveys have retrieved *Methanolinea* related clones from a variety of anaerobic environments (Sakai et al., 2012), indicating the widespread distribution of these methanogens. While both reactors showed high similarity in the archaeal population composition, by merging the FISH and clonal analysis results we can state that in reactor B1 the high acetate conversion rate was related to the dominance of *M. harundinacea,* which was the only archaeon that hybridized with the probe ARC915 and could be further identified based on its unique filamentous morphology. The low acetate degradation rate in calcium augmented reactor B3 (Fig. 2c) can be explained by a very low surface coverage by the acetotrophic *Methanosaeta*: the archaeal biomass showed a weak fluorescence, and the typical filamentous form of *Methanosaeta* was not detected (Fig. 5b). A basal activity of the hydrogenotrophic methanogens in reactor B3 cannot be excluded, which possibly was driven by the hydrogen that was released from the fermentation of decaying biomass. The slow acetate degradation (Fig. 2c), the poor microbial attachment of cells (Fig. 3c) and the weak *Methanosaeta* fluorescence (Fig. 5b) in reactor B3 highlights the importance of this archaeon in biofilm formation process, as will be further discussed in section 3.3.

### 3.2. Anaerobic granule formation

To study the effect of high salinity on granule formation two

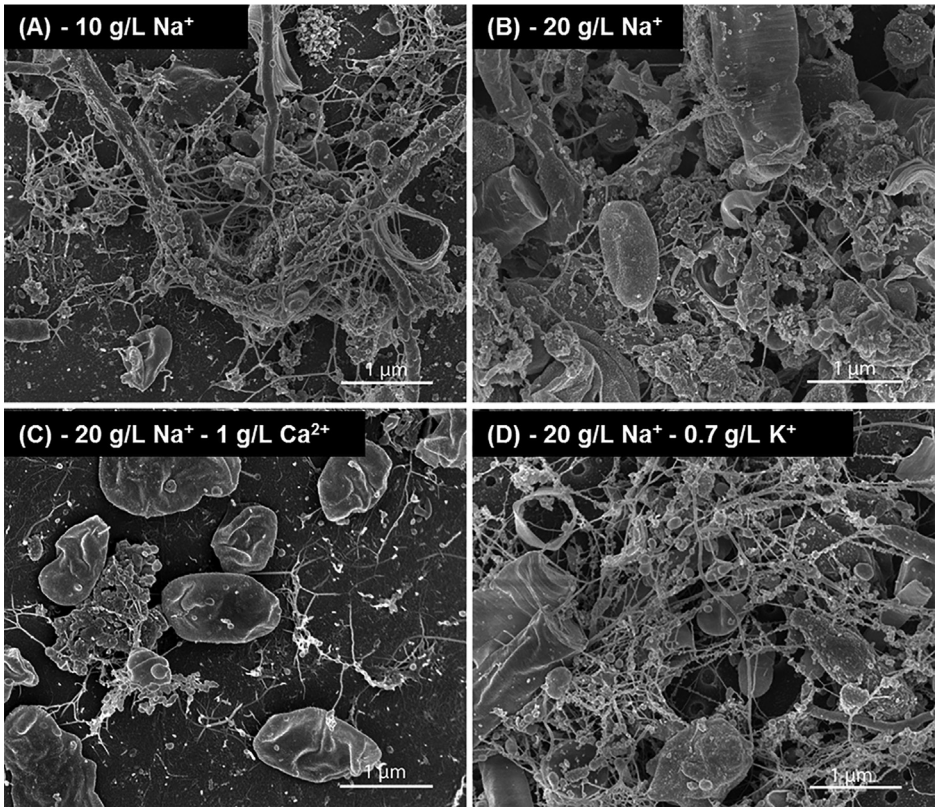


**Fig. 3.** SEM images of biofilms samples from the reactors B1 (a), B2 (b) B3 (c) and B4 (d) after 40 days of operation.

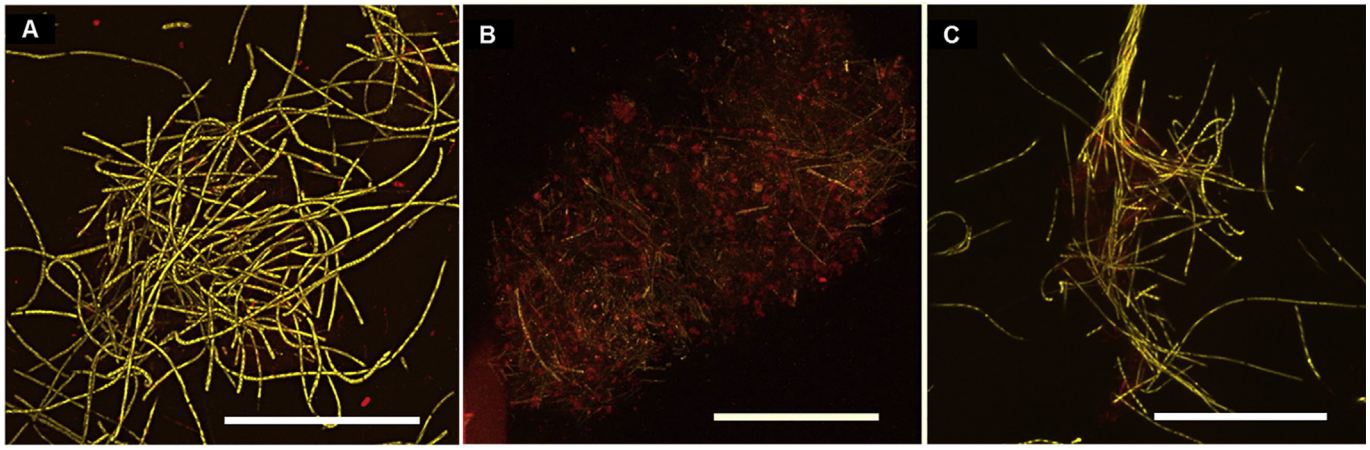


**Fig. 4.** FISH-CLSM images of biofilm sampled from reactors B1 (a), B3 (b) and B4 (c) after 40 days of operation. The samples were simultaneously hybridized with the probes for bacterial (EUB338-Cy3, red) and archaeal domain(ARC915-Cy5, yellow-orange). White bar = 50 µm. A FISH-CLSM image of reactor B2 with a sufficiently high quality unfortunately was not available. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

UASB reactors, G1 and G2, were inoculated with the same granular sludge that was used to inoculate the biofilm reactors described in the previous section. The reactors were operated for 120 days and operational parameters are given in Table 1.

### 3.2.1. UASB reactor performances

Fig. 5 shows influent soluble COD (sCOD), effluent sCOD concentrations, the imposed organic loading rate (OLR) to the reactors and the effluent VFA concentrations. Until day 60, at an OLR of 14 g COD/L·d, effluent COD concentrations in reactor G1 were slightly lower and more stable than in reactor G2 (Fig. 5a and b). Average sCOD removal efficiencies during the first 60 days for reactor G1 and reactor G2 (with extra calcium) were 95 and 92%, respectively. The OLR that was applied during the first 60 days fits well in the feasibility range for a practical application of UASB reactors (Van Lier, 2008) and such high removal efficiencies at similar salinities have not been documented before (Lefebvre and Moletta, 2006). This demonstrates that anaerobic granular sludge technology is feasible at a salinity of 20 g $Na^+$/L, provided a sufficient amount of potassium is added to the influent to alleviate sodium toxicity. This is in agreement with the previous observations for the biofilm reactors (section 3.1.1). From day 60 onwards, the increase in OLR by

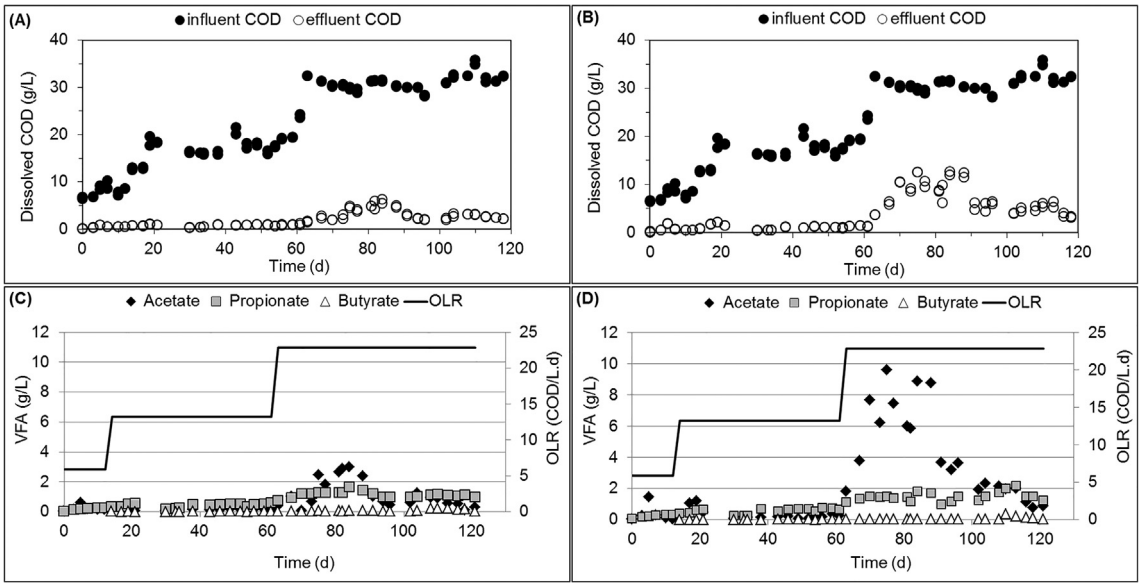


**Fig. 5.** Influent and effluent dissolved COD concentrations, and VFA concentrations for reactor G1 without additional calcium (A and C) and for reactor G2 with additional calcium (B and D).

more than 70%, from 14 to 24 g COD/L·d, resulted in lower and less stable sCOD removal efficiency for both reactors. Effluent sCOD concentrations initially were considerably higher than in the first phase of the process, especially for reactor G2 (Fig. 5b), but afterwards gradually decreased towards the end of the operational period. In particular, reactor G2 had a COD removal efficiency 3–10% lower than at the lower OLR (indicatively comparing day 65 to day 95, Fig. S2), while again achieving a good removal efficiency towards the end of the experimental period. The worst COD removal in both reactors (Fig. S2) was a temporary effect, indicating that the process could work also at higher OLR. This temporary higher effluent COD concentrations after the OLR increase can be explained by higher effluent VFA concentrations (Fig. 5c and d), i.e. propionate and butyrate concentrations of 1.2–2.4 g/L compared to 200–800 mg/L at an OLR of 14 g COD/L·d, and in particular in reactor G2 by an acetate concentration that increased to a maximum concentration of about 9.6 g/L. In reactor G1 this peak in acetate concentration was significantly lower (4 g/L). Apparently the addition of 1 g/L of calcium had a negative impact on the response time of the system to adapt to the higher OLR.

### 3.2.2. Granule and fines properties

Volume median diameters (50%) of reactor granular sludge and fines in samples taken on day 120 show that the fines in reactors G1 and G2 had similar diameters (75 and 82 μm, respectively) and that the granule size in both reactors had increased considerably compared to the inoculum (412 μm) with 763 μm for granules in reactor G1 and 581 μm for granules in reactor G2. The PSD (Fig. 6) showed that the distribution of fines in reactor G1 was binomial. This could indicate that the granules in reactor G1 not only suffered from abrasion by shear, but also were breaking up into smaller granules. Results suggested that calcium augmentation in reactor G2 reduced the granule size but improved the granule strength at high sodium concentration, which is in agreement with previous findings by Ismail et al. (2010). In addition, calcium carbonate precipitates, that can negatively affect granule strength when in excess (Dang et al., 2014), were not detected when the inorganic fraction of G2 granules surface was characterized by SEM-BSE and SEM-EDX (Fig. S3). As seen in Fig. S3 B, precipitates on the surface of these granules were rich in iron and sulfur, while calcium is very low. This suggests iron sulphide precipitates are more abundant than calcium carbonate.

### 3.2.3. Microbial community composition of granules

Microscopic investigations carried out using SEM for granules and FISH for fines, highlighted the main role of archaeal filamentous cells, as already described for the biofilm reactors in section 3.2. Filamentous cells were distributed on the surface of granules in reactors G1 (Fig. 7a), whereas the granule surface of calcium augmented granules of reactor G2 (Fig. 7b) was characterized by the presence of several single cells that were embedded in a “spider web” shaped matrix of EPS (magnification of insets in Fig. 7). The surface texture of the G1granules was smooth and densely packed, while G2 granules exhibited smooth as well as less densely packed regions. In fines from reactor G1 (Fig. 7c), a higher fraction of bacteria (in red) was detected than in reactor G2 (Fig. 7d). On the other hand, archaeal cells in reactor G1 (Fig. 7c, in green) mostly grouped in small aggregates, were significantly less than in fines from reactor G2, in which only single rod cells were detected by the ARC915 probe (Fig. 7d). These microscopic observations indicate that in reactor G2 the selective washout of archaeal cells was higher. Glycoconjugate groups, visualized by Con A staining and important in EPS structures (in purple) were more abundant in the fines from reactor G1. Sequence analysis of 16S rRNA genes of granules samples taken from reactor G1 at the end of the experimental period for Archaea (85 clones) (Table 3), revealed the acetotrophic *Methanosaeta harundinacea* as the only methanogenic archaeon, confirming the identity of the filamentous cells as already highlighted for the biofilm formation in section 3.2. All 85 archaeal clones were highly similar to *M. harundinacea*, with an identity between 98 and 99%. Although the granular reactors were fed with a partially acidified substrate, no hydrogenotrophic methanogens were detected, and the results of the clonal analysis are in line with the microscopic observations, showing exclusively the *Methanosaeta* rod/filament like morphology. The presence of this methanogen and of acetate as the only readily available substrate in the PAS mixture could have selected towards acetoclastic methanogenesis, while the hydrogen/formate generated from the hydrolysis and fermentation of organic matter could have been metabolized by bacteria. The identification of *Methanosaeta* in both

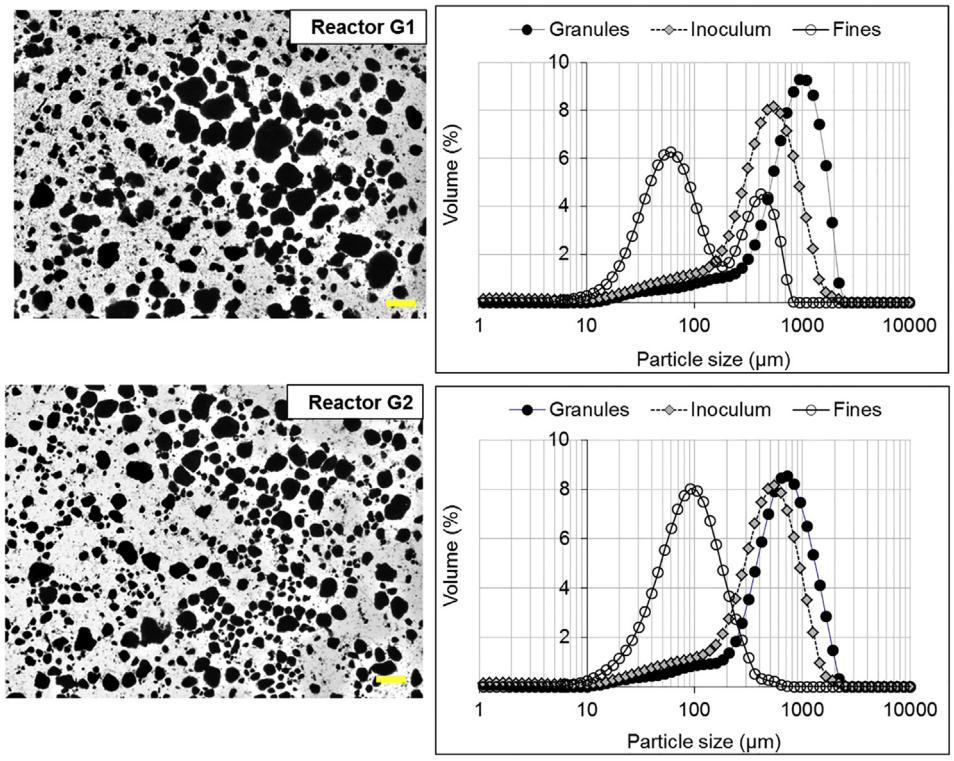


**Fig. 6.** Microscopic images and particle size distribution of sludge from the two UASB reactors as well as for the fines produced in these reactors. Samples were taken after 120 days. Yellow bars in microscopic images are 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biofilm and granular reactors underlines for the first time the importance of this particular methanogen in the aggregation of microbial communities under high salinity conditions. Sequencing the 16S rRNA amplicons for Bacteria (69 clones) (Table 3) revealed a higher diversity within the bacterial community. The majority of the bacterial sequences affiliated with the phylum *Bacteroidetes*, and the dominant phylotype (50 out of 69 clones) was affiliated to the family *Marinilabiaceae*. Most of the members of this family are hydrolytic polycarbohydrate degraders (Zhilina et al., 2004; Zhao and Chen, 2012; Zhao et al., 2012), with formate, acetate and propionate as main end products, while basic protease activity was detected (Miyazaki et al., 2012). The other 14 clones were distantly related with 16S rRNA genes of uncultured bacteria from marine or hypersaline environments, classified into the order *Clostridiales* (*A.halophilus*, *S. alkalica* and *D. aminovorans*). These are mainly mono- and disaccharides fermenters, producing formate, acetate and hydrogen as end products.

### 3.3. Effect of calcium and filamentous growth of M.harundinacea

The beneficial effect of calcium on the mechanical stability of biofilms is known (Körstgens et al., 2001; Patrauchan et al., 2005), and several studies reported a positive effect of calcium on anaerobic granulation, granule strength and biomass retention (Mahoney et al., 1987; Batstone and Keller, 2001; de Graaff et al., 2011). Apparently, calcium addition also has a beneficial effect at high sodium levels when sodium can (partially) replace the calcium ions in the extracellular polymeric matrix (Ismail et al., 2010). The effect of calcium on anaerobic digestion is still not clearly understood (Ahn et al., 2006) and opinions differ about the "cut-off" limit during the process. (Sanjeevi et al., 2013). Kugelman and McCarty (1965) already found that calcium has a negative effect on methanogenic activity in the presence of sodium. However, Ahn et al. (2006) showed that low calcium concentrations (up to 3 g/L) could stimulate the methanogenic activity of granular sludge, while Dang et al. (2014) showed that when the calcium concentration increased from 500 to 1000 mg/L this activity in static anaerobic tests was significantly reduced. Our results indicate that calcium can have a detrimental effect on microbial activity, on biofilm attachment (reactor B3) and on granule size (reactor G2). However, in UASB reactor G2 calcium had a positive effect on granule strength. The negative effect of calcium in biofilm reactor B3 and granular sludge reactor G2 was particularly evident regarding the aggregation of archaeal filamentous cells belonging to *M.harundinacea* species, which we speculate have an essential role in the main structure of biofilms and granules in this study. *M.harundinacea* is usually found as a single rod cell or in pairs, but when grown at the optimum acetate threshold (>100 mM) and at high cell densities (Zhang et al., 2012) it forms long filaments (Ma et al., 2006). The filamentous form consists of cells encapsulated in a protein sheath (Beveridge et al., 1986), which can better withstand unfavourable environmental conditions outcompeting other acetate utilizing microorganisms. In the filamentous form there is an altered carbon metabolic flux that favors the conversion of acetate to methane and gives a reduced biomass yield (Zhang et al., 2012). Filaments formation of *M.harundinacea* is initiated by a quorum sensing signal pathway involving the production of carboxylated acyl homoserine lactones (AHLs) molecules, with a chemical structure similar to medium chain fatty acids (Fig. S4). Calcium can bind to medium and long fatty acids (Galbraith et al., 1971; Roy et al., 1985). Thus, the high calcium content in reactor B3 during biofilm formation and in reactor G2 during granulation may have caused the precipitation of the signal molecules, preventing the formation of filaments. The latter may explain the poor performance of reactor B3 and slower adaptation of G2 to an increased OLR in comparison with the other reactors which did not receive additional calcium. Additionally, weaker and less thick *Methanosaeta* filaments were detected in biofilm reactor B4 (Fig. 3c) fed

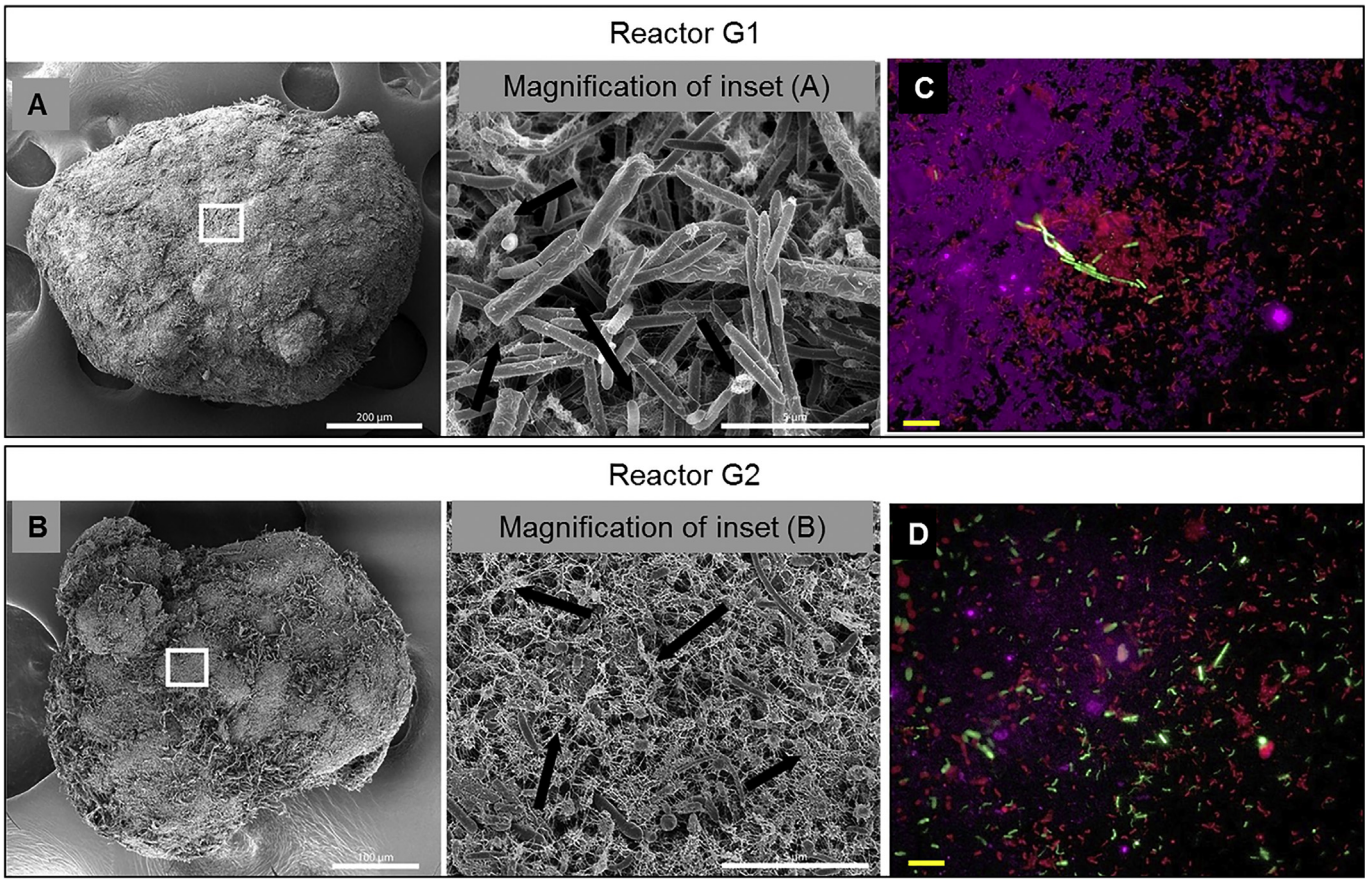


**Fig. 7.** SEM images of granules (A and B) and FISH images of fines (C and D) sampled from the two UASB reactors after 120 days. Aggregates of microorganisms on the surface of the granules at higher magnification (insets) are shown for each SEM image. Black arrows indicate arrangements of microorganisms on the surface of granules embedded in an EPS matrix. In C and D, FISH samples were hybridized with specific probes for Archaea (ARC915-FITC, green), Bacteria (EUB338-Cy3, red) and glycoconjugates (Con A, purple). Bars: 200 µm in A, 100 µm in B, 5 µm in the other images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with 20 g $Na^+$/L in comparison to reactor B1 (Fig. 3a) supplemented with 10 g $Na^+$/L, and long filaments were not detected in the granular sludge reactor G1 (20 g $Na^+$/L). This means that *M.harundinacea* can adapt and grow at high salinity while a concentration higher than 20 g $Na^+$/L can likely negatively influence its filamentous growth. The importance of filaments formation in granular sludge reactors by *Methanosaeta* sp. was highlighted in several studies. Li et al. (2015) showed that external addition of AHLs into the UASB system facilitated filaments of *M. harundinacea* to contribute to the granulation and performance, likely through immobilizing other microorganisms involved in syntrophic interactions, enhancing COD removal and performance. Calli et al. (2003) found that an increase in the ammonia concentration and pH during different anaerobic processes resulted in a shift from long to shorter filaments of *Methanosaeta* sp., and aggregating properties were positively related to the stability of the system and to the formation of flocs or granules. Overall, the conditions imposed to our initial inoculum promoted the establishment of a stable community of *Methanosaeta* in all reactors studied, but the aggregation of the microbial community was positively affected only when filamentous growth occurred. Due to the general importance of *Methanosaeta* in forming complex mixed communities biological structures (Wiegant, 1987; MacLeod et al., 1990; Tay et al., 2010) further studies should be focussed on determination of the range of the sodium tolerance when grown in mixed cultures, in order to apply it in high salinity wastewater treatment systems.

## 4. Conclusions

- Anaerobic biofilm formation at high salinity (10 and 20 g $Na^+$/L) is possible, even with a simple organic substrate such as acetate.
- UASB reactors achieved high removal efficiencies at an OLR of 14–25 g COD/L.d, at a salinity as high as 20 g $Na^+$/L.
- Potassium (0.7 g/L) alleviates the negative effect of 20 g $Na^+$/L on biofilm formation and UASB performance.
- Calcium (1 g/L) has a negative effect on biofilm formation and reactor performance, although it has a positive effect on granular strength.
- The acetoclastic methanogen *M. harundinacea* dominates biofilm and granule communities in all reactors, showing the ability to grow at a sodium concentration up to 20 g $Na^+$/L, and its filamentous form is important for biofilms and granule formation.
- Calcium has a negative effect on the aggregation abilities of this archaea.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2017.05.016.

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