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Anaerobic Granulation at high salinities

Autor:

Rodríguez Antolín, Raquel

Fernández-Polanco, María

Wageningen University & Research

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TÍTULO: Anaerobic Granulation at high salinities

ALUMNO: Raquel Rodríguez Antolín

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TUTOR: MSc. Dainis Sudmalis y dr. ir. Hardy Temmink

RESUMEN

El tratamiento de aguas residuales salinas se lleva a cabo a través de procesos físicos y químicos que son costosos. La digestión anaeróbica se presenta como una alternativa potencial de estos métodos, pero se ve inhibida a altas salinidades.

Una investigación en ETE ha logrado una granulación exitosa en altas salinidades a escala de laboratorio con distintos substratos, triptona, gelatina y sacarosa. En este informe, se estudia el rendimiento de estos sustratos en la granulación anaeróbica con una salinidad de 20 g de Na^+ / L.

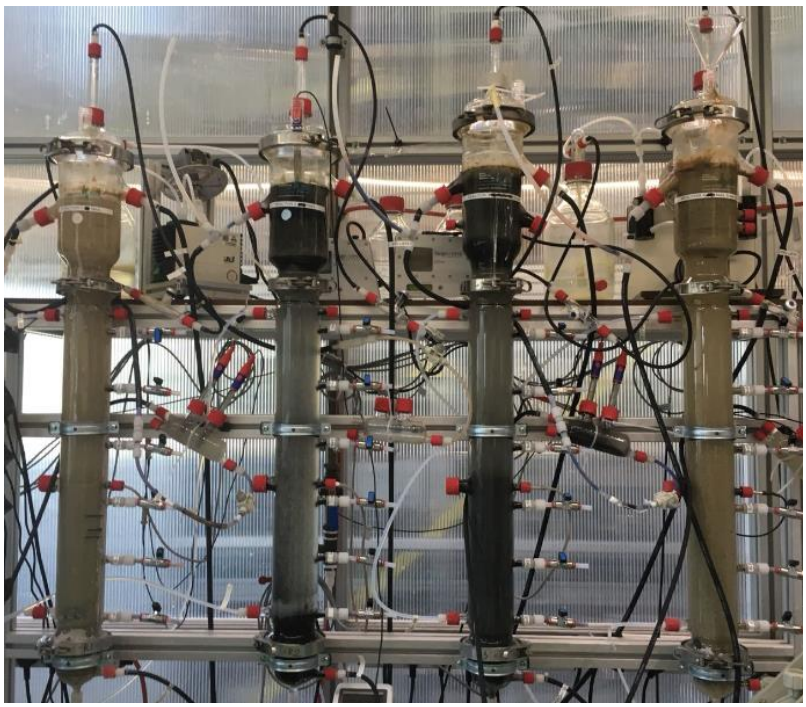
Tanto en el reactor alimentado con triptona como en el otro alimentado con gelatina, se logró la granulación. En el caso del reactor alimentado con almidón no se logró la granulación y el rendimiento fue menor.

PALABRAS CLAVES

Granulación, anaeróbico, salinidades, aguas residuales, substrato

Anaerobic Granulation at high salinities

Bachelor Thesis	Rodríguez Antolín, Raquel
Registration No	950628-699-020
Supervisors	MSc. Dainis Sudmalis
	dr. ir. Hardy Temmink



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Abstract

The saline wastewater treatment is carried out though physical and chemical processes which are expensive and energetically intense. Anaerobic digestion, with upflow anaerobic sludge blanket reactors, is presented as a potential alternative of these methods but it is known to be inhibited by high salinity.

A research in ETE have achieved a successful granulation at high salinities when lab-scale reactors were fed with tryptone or gelatin in addition to glucose and sodium acetate. In this report, the performance of these substrates was studied in the anaerobic granulation at high salinities, 20 g Na^+/L.

Both in the reactor fed with tryptone and in the other fed with gelatin, the granulation was achieved. However, the one containing tryptone showed better activity in sludge characteristics and reactor performance. Moreover, a granules disintegration and less biogas production were observed when the amount of tryptone was decreased. The role of calcium ion was studied, when it was added together with tryptone in the reactor and it was concluded that a lack of Ca^{2+} can be harmful for the granulation.

Other reactor was started-up with starch as substrate. In this case, granulation was not achieved and the reactor performance was lower than in the other reactors.

Finally, it was hypothesized that tryptone could increase the glucose degradation rate. After batch experiments, it was concluded that tryptone could improve the glucose degradation when biomass was acclimated to 5 g Na^+/L and up-shock to 20 g Na^+/L, but not when the biomass was only acclimated to 20 g Na^+/L.

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List of abbreviations

COD: Chemical Oxygen Demand

EPS: Extracellular Polymeric Substance

HPLC: High Performance Liquid Chromatography

OLR: Organic Loading Rate

PAC: Powdered Activated Carbon

SMA: Specific Methanogenic Activity

TS: Total Solids

TSS: Total Suspended Solids

UASB: Upflow Anaerobic Sludge Blanket

VFA: Volatile Fatty Acid

VS: Volatile Solids

VSS: Volatile Suspended Solids

Contents

1. Introduction

1.1 The process of anaerobic digestion

Among the different forms that exit to treat saline wastewater, anaerobic digestion could be important due to the production of biogas, the net energy recovery and less excess of sludge production if it is compared with aerobic process (Vieira, Sérvulo et al. 2005, Vyrides, Santos et al. 2010, Xiao and Roberts 2010).

The process of anaerobic digestion has four steps: hydrolysis, acidogenesis or fermentation, acetogenesis and methanogenesis (Batstone, Keller et al. 2002).

During the first step, hydrolysis, organic particles and macromolecules (peptides, carbohydrates, lipids) are degraded to monomers and dimers (amino acids, sugars and glycerol). Hydrolysis is needed to allow the absorption of the substrate for the microorganism. It is one of the rate-limiting steps in the process of anaerobic digestion. There are some factors that can affect hydrolysis, such as temperature because every enzyme has their own optimum temperature. Other important factors are pH, inhibiting compounds, formed during this step, substrate composition, solids retention time (SRT) and particle size (Sanders 2001).

Acidogenesis consists on the conversion of amino acids and sugars to simple compounds. Ammonium is formed as a degradation product (Batstone, Keller et al. 2002).

Next step is acetogenesis which is the conversion of fermentation products into acetic acid, CO_2 and H_2. The main fermentation products are ethanol, butyric acid and propionic acid (Batstone, Keller et al. 2002).

And finally, methanogenesis step produces methane and CO_2. The most important substrates for the methanogens are hydrogen and acetate (Lu, Zhen et al. 2015).

1.2 Development of UASB reactor and its advantages over flocculent sludge AD

Since the end of the 19th century, anaerobic digestion has been applied for the treatment of wastewaters. It can be considered one of the oldest wastewater treatment technology. The development from 1970 till 2000 of high-rate reactor designs, in which biomass retention and liquid retention is uncoupled, has produced an increase in the use of anaerobic digestion (Lettinga 1995). In the mid 70's anaerobic treatment for industrial wastewater was first applied on a commercial scale in a sugar industry.

The anaerobic filter (AF), developed by James C. Young in 1968, was the first anaerobic treatment system and it is used to rely on solids-liquid separation. In this form, biomass is immobilized to an inert porous support material, allowing the slow-growing anaerobic microbes to remain within the bioreactor (McHugh, O'reilly et al. 2003). During initial experiment with the anaerobic filter, it was observed the aggregation of a large proportion of the sludge present to form granules (Lettinga 1995, Lettinga 2001). Recognition of the sludge granulation concept was a significant milestone in anaerobic wastewater treatment and has greatly enhanced both the efficiency and applicability of the technology (McHugh, O'reilly et al. 2003).

In USA, scientists combined an upflow sludge bed tank with AF, and they concluded the importance of the bacterial immobilisation due to the granulation phenomenon. Later, Lettinga (2001) noticed that for retaining the anaerobic sludge no packing material was required. For these reasons, UASB was converted in the focus of the investigation. Finally, in 1977, the first full-scale UASB reactor was put into operation (Lettinga 2001). It was one of the earliest systems to rely on the establishment of a granular biomass and it is currently the most

widely applied reactor technology for high rate anaerobic treatment of industrial effluents (Frankin 2001). This kind of reactor has been employed in the treatment of wastewater during decades. However, the long start-up period required for the development of granules constitutes a serious impediment (Morgenroth, Sherden et al. 1997).

The phenomenon of granulation achieves high biomass concentration and rich microbial diversity. For these reasons, contaminant transformation is rapid and large volumes of organic waste can be treated (Liu, Xu et al. 2003).

The reason of the granulation is that the upflow velocity contributes to create in the reactor a selective pressure due to which, the organism can be washed out or bind together and form granules (Morgenroth, Sherden et al. 1997). These granules are dense particles, consisting of a mixture of the symbiotic anaerobic microorganisms. A typical granule may include millions of organisms per gram of biomass (McHugh, O'reilly et al. 2003). However, none of the individual species in these micro-ecosystems are capable of completely degrading influent wastes (Liu & Tay 2002).

Anaerobic granulation is a complex process, in which microbiological, other biotic and abiotic factor are combined (O'flaherty, Lens et al. 1997). Granulation may be initiated by bacterial adsorption and adhesion to inert matter, to inorganic precipitates and other physical-chemical interactions (Yu, Fang et al. 2001). All these substances serve as an initial precursor to bacterial growth. Many mechanisms have been proposed to explain the phenomenon of granulation (McHugh, O'reilly et al. 2003):

1. Physical-chemical theories such as inert nuclei model (Lettinga, Van Velsen et al. 1980).
2. Ecological theories where spaghetti model can be found (Wiegant 1988).
3. Thermodynamic theories like surface tension model (Thaveesri, Daffonchio et al. 1995).
4. Others such as general four-step model (Liu, Xu et al. 2003).

1.3 Salty wastewater streams and the current strategy to treat them

Currently, the wastewaters are more concentrated and increasingly characterised by harsh environmental conditions such as high salinity and high temperatures due to the increase of water efficiency in industrial processes (Ismail, Gonzalez et al. 2008). Saline wastewaters are discharged daily from many industries such as seafood processing or textile dyeing. Environmental management of the saline and hypersaline wastewaters is becoming more stringent and the treatment is becoming more necessary (Xiao and Roberts 2010). This treatment could represent as much as 5% of worldwide effluent treatment requirements (Lefebvre, Quentin et al. 2007).

At present, the processing of saline wastewater is carried out through physical and chemical processes. However, they are energy intensive and expensive (Fakhru'l-Razi, Pendashteh et al. 2009). Anaerobic digestion is a good alternative to physical and chemical processes to treat saline wastewater due to the specific advantages that it offers (Lettinga 1995).

Anaerobic digestion achieves that the phenomenon of granulation is produced. It has advantages such as more efficient microbial proliferation which can be preserved unfed for long periods of time without any serious deterioration of their activity (Lettinga 1995), generation of a reactor effluent with low suspended

solids and continuous operation of reactors beyond normal washout rates (McHugh, O'reilly et al. 2003). Besides, less production of secondary contaminants is achieved if it is compared with other physical and chemical processes (Xiao and Roberts 2010).

Successful implementation of the technology, which can be applied at practically any place and at any scale (Lettinga 1995), requires the retention of high levels of active biomass in the system by self-immobilisation of the microbes in the form of sludge aggregates or granules (Lettinga and Pol 1991). The retention of a high biomass concentration allows the application of very high organic loading rates resulting in a compact wastewater treatment plant (McHugh, O'reilly et al. 2003).

Other advantages must be found in the cost. System designs in anaerobic treatment focus on increase process control to secure optimal operating conditions and system compactness in order to reduce costs of investment (Frankin 2001). Low operating costs and low surplus sludge production result in overall favourable economics (McHugh, O'reilly et al. 2003). Technically plain and inexpensive reactor are used and anaerobic treatment systems generally can be operated with little grade of energy (Lettinga 1995).

One of the disadvantages is the relatively high susceptibility of methanogens and acetogens to a variety of xenobiotic compounds. However, currently, more is known about the extent of the toxicity, and a better insight is gained in the counter measures that can be taken. Besides, if the system is not properly managed, anaerobic digestion could be unstable (Lettinga 1995).

It is known, among other things, that the presence of high Na^+ concentrations is negatively impacting the anaerobic treatment process (Ismail, Gonzalez et al. 2008). However, the presence of calcium ions has influence on the granulation process. In low concentrations, they have a positive effect enhancing the mechanical strength and the settle ability of the granules (Yu, Fang et al. 2001).

Finally, the anaerobic treatment can be accompanied with mal-odorous nuisance problems but they can be prevented with microaerophilic methods (Lettinga 1995).

Due to the mentioned advantages and considering that the disadvantages are being solved, it can be concluded that the anaerobic treatment is an appropriate option.

1.4 Effects of high levels of salinity in biological treatment and anaerobic granulation

As it has been said, saline wastewater is discharged by many industries (Xiao and Roberts 2010). However, many of them only increases the salinity during certain short periods of the year, while in others, salinity can suddenly increase due to different parts of the process (Vyrides, Santos et al. 2010). This could generate stress conditions that can result in dehydrated cells which lose viable biofunctions (Oren 2008). Microorganisms have to adapt to moderate and high salt environments, using a variety of solutes, organic and inorganic, to counter external osmotic pressure (Roberts 2005).

For cells survival under osmotic stress, two strategies can be held:

1. An increase of the intracellular ion concentration to balance the external osmotic pressure. The intracellular enzymes have to adapt to the new conditions (Vyrides, Santos et al. 2010).

2. Many microorganisms accumulate organic solutes intracellularly. With organic solutes, the high external osmotic pressure is balanced without the need for any special adaptation of the intracellular enzymes. These solutes can be synthesized by the cell or provided by the medium (Vyrides 2015).

Lefebvre et al. found that increase in the salinity can have a little effect on the microbial diversity of anaerobic biomass and the salinity influences the degradation rates (Lefebvre, Quentin et al. 2007).

Anaerobic digestion is known to be inhibited by high salinity mainly due to the presence of cations (Lefebvre, Quentin et al. 2007). Inorganic cations, such as K^+ in small quantities, are often important in osmotic balance and in the response to osmotic stress (Roberts 2005). However, elevated potassium concentrations can produce an osmotic stress which causes the inhibition of methane production (De Vrieze, Coma et al. 2016).

Low concentrations of Na^+ are necessary for the methanogenesis (Feijoo, Soto et al. 1995). However, high concentrations of it result in a deterioration of granule strength (Ismail, Gonzalez et al. 2008), and also inhibit the granule formation in anaerobic digesters due to the dramatic increase in osmotic pressure (Liu, Xu et al. 2003, Vyrides 2015). Besides, this high concentration of Na^+ can affect the physiology of the microorganisms as well as the morphology of the granules. It has been proven that Na^+ concentration exceeding 5 g/L, at neutral pH, inhibits methanogenesis (Rinzema, van Lier et al. 1988, Ismail, Gonzalez et al. 2008).

De Vrieze, Coma et al. (2016) observed that an increase in salinity resulted in a complete inhibition of methanogenesis, affecting granular sludge stability which resulted in a washout of the sludge and a clear shift in microbial community composition. Basically, there was a clear enrichment of Methanomicrobiales in the reactor sludge compared to the effluent and a decrease in the abundance of Methanosaetaceae and Methanobacteriales which are crucial for a stable methanogenesis.

Many of the salt-tolerance mechanisms available to aerobic organisms are energetically expensive and are not feasible in anaerobic environments (Xiao and Roberts 2010). For this reason, there are a lot of studies which have investigated the formation of granules at high salinity, however, they have not achieved their goal (Feijoo, Soto et al. 1995, Vallero, Lettinga et al. 2003, Lefebvre, Quentin et al. 2007, Ismail, Gonzalez et al. 2008, De Vrieze, Coma et al. 2016).

Only a study within ETE has shown a successful granulation at high salinities when it is working at lab-scale. During the realization of this research UASB reactors were used. These reactors were fed with glucose, sodium acetate and tryptone/ gelatin in COD proportions of 3:2:1. Two different concentrations of sodium were used in this study, namely 5 and 20 g Na^+/L. During all the research, the performance of the process was stable and formation of granules was achieved in all of the reactors, irrespective to Na^+ concentrations (Sudmalis, Gagliano et al. 2018).

Other research shows that if the reactor is fed with amino acids instead of tryptone, less amount of biomass was obtained and granules were not formed, however, the activity was suitable. This behaviour suggests the tryptone or proteins in general play an important role in the formation of granules. Besides, tryptone may provide building blocks for extracellular polymeric substances (EPS) (Jen 2018).

1.5 Biological strategies to deal with osmotic stress

It is known there are two different strategies for cells to survive under osmotic stress. They are the "salt-in" strategy and the compatible solute strategy (Vyrides 2015).

In the "salt-in" strategy, the physiology of the anaerobic bacteria has been adapted to high saline environments (Müller, Spanheimer et al. 2005). Cells increase the intracellular ion concentration, mainly K^+ and Cl^- (Oren 2002), in order to balance the external osmotic pressure (Vyrides 2015). There is a research which proves the usefulness of low concentrations of potassium to reduce the inhibition of sodium to methanogens (Vyrides 2015). However, this strategy requires far-reaching adaptations of intracellular machineries to the high salt concentrations and limits growth to certain salinities (Müller, Spanheimer et al. 2005).

With the compatible solute strategy, many microorganisms accumulate organic solutes called "compatible solutes" which can balance the high external osmotic pressure and also serve as protein stabilisers in the presence of high ionic strength inside the cell (Müller, Spanheimer et al. 2005). These solutes can be provided with the medium which is energetically more favourable than synthesis (Vyrides 2015).

Apart from compatible solutes, other important part in the granulation are the extracellular polymeric substances (EPS) which help to adhere and connect the sludge particles together, increasing the granulation (Li, Wang et al. 2012).

EPS are secreted by microbial cells and produced by anaerobic biomass (Vyrides 2015). They mainly consist of various organic substances such as polysaccharides, proteins, nucleic acids and lipids (Lu, Zhen et al. 2015). EPS are not affected by the high salinity but a significant drop in granule strength can be observed (Ismail, Gonzalez et al. 2008).

They can help cells to survive under sodium toxicity (Vyrides, Santos et al. 2010). Moreover, they can be generated as a protective barrier around the bacteria specially under harsh conditions (Vyrides 2015).

The production of EPSs under salinity can be employed by the anaerobic biomass simultaneously with the generation of compatible solutes, in other words, higher amounts of salinity result in higher amounts of EPSs (Vyrides and Stuckey 2009). Part of the energy is consumed for the production of EPSs, so less substrate is available for methane production (Vyrides 2015). They can also serve as carbon and energy source to ensure the survival and normal growth of microorganisms during starvation periods (Lu, Zhen et al. 2015).

EPS play a key role in anaerobic granulation formation and long-term stability of UASB system (Li, Wang et al. 2012). The granules with high amount of EPS have a more stable three dimensional structure to maintain the structural integrity (Lu, Zhen et al. 2015). They can potentially contribute to the COD content in the effluent too (Sudmalis, Gagliano et al. 2018)

1.6 Effects of organic substrate on granular sludge formation

Morgenroth, Sherden et al. (1997) determined that the composition of the substrate is an important factor for granule formation. The granules had an uneven surface probably resulting from the degradable substrate and the biomass in the center began to decay when substrate was limited. This biological phenomenon is influenced by the choice of the substrate (Dolfing, Luijten et al. 1987).

Lefebvre, Quentin et al. (2007) showed that the type of organic substrate determines the type of microorganisms evolving in the reactors and their sensitivity to sodium toxicity. In this research, two different substrats were used, distillery vinasse and ethanol. The conclusion was that the reactor operating with distillery vinasse appeared to be inhibited at a lower salt concentration than the reactor operating with ethanol. The impact of NaCl was different according to the nature of the substrate: NaCl inhibition was observed at lower concentrations when using a complex substrate. A higher diversity of bugs for the reactor operating with distillery vinasse was shown than for the reactor operating with ethanol. This could be a consequence of the nature of the substances, the distillery vinasse might require the intervention of a higher number of microorganisms for its degradation.

In conclusion, according to the nature of the substrate, the sludge can show different ways to increasing salinity, the performance of anaerobic digestion under saline conditions depends on the type of methanogenic substrate used and the impact of the NaCl can change if the substrate changes (Lefebvre, Quentin et al. 2007).

The more easily biodegradable substrates make the inoculum more tolerant to salts. Salt-tolerance is greater when the substrate to be degraded is more easily degraded or when there is more energy in the substrates (Xiao and Roberts 2010).

1.7 Hypothesis and research questions

To this day, different start-ups have been tried at ETE with different substrates in the same amount to observe differences in granules formation. Reactors have been fed with different carbon sources in COD proportions of:

Table 1 Start-ups tried in ETE with different substrates and their COD proportion 3:2:1

COD	3	2	1
Case 1	Glucose	Acetate	Tryptone
Case 2	Glucose	Acetate	Gelatin
Case 3	Glucose	Acetate	Asp:Glut Ac
Case 4	Glucose	Acetate	Leuc:Pro

In all the cases, amino acids participate. But, in cases 1 and 2, hydrolysis occurs and granules are formed. However, in cases 3 and 4, hydrolysis does not occur and granules are not formed. Moreover, the diversity of amino acids found in tryptone and gelatin is much higher than the one used in case 3 and 4 (Table 1).

To test if the need for hydrolysis within the substrate can drive the granulation without added amino acids, a new reactor will be used. It will be fed with COD proportions of:

Table 2 Start-ups of R2 in ETE with starch as substrate and their COD proportion 3:2:1

3	2	1
Glucose	Acetate	Starch

With this, hydrolysis and no peptides are got in the reactor because of the using of starch. Of this form, the following research questions will be answered:

RQ1: When is hydrolysis, in general, needed for the granulation?

RQ2: What is the performance of the process when amino acids/proteins are replaced with polysaccharides?

RQ3: How solids washout compares for gelatin or tryptone reactor?

RQ4: How affect little amounts of calcium ion to the rector activity?

2. Materials and methods

2.1 Experimental set-up

2.1.1 Growth medium

The nutrient medium used for reactor 1, 3 and 4 (R1, R3, R4) consisted of the following salts at final concentrations in g/L: NH_4Cl (1.02) , $MgSO_4 \cdot 7H_2O$ (0.05), $CaCl_2 \cdot 2H_2O$ (0.05), KH_2PO_4 (0.22), $NaHCO_3$ (1.5) and in mg/L: $FeCl_2 \cdot 4H_2O$ (1.2), HBO_3 (0.03), $ZnCl_2$ (0.03), $CuCl_2 \cdot 2H_2O$ (0.02), $MnCl_2 \cdot 4H_2O$ (0.3), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.05), $CoCl_2 \cdot 6H_2O$ (1.2), $NiCl_2 \cdot 6H_2O$ (0.03), EDTA (0.6), Resazurin (0.3), $Na_2SeO_3 \cdot 5H_2O$ (0.3) and 0.216 ml/L of 36% HCl (Sudmalis, Gagliano et al. 2018). In case of R1 additional dosing of $CaCl_2 \cdot 2H_2O$ (100 mg Ca/L) was added after 449 days of continuous operation.

The COD was soluble and consisted of D-Glucose monohydrated, Na-Acetate and Tryptone (Sigma-Aldrich, Microbiologically tested, N content 11-16%) in the case of R1 and R4 and D-Glucose monohydrated, Na-Acetate and Gelatine (Emprove® exp, Ph Eur, BP, NF) in R3. The COD proportions were 3:2:1 in all cases. The COD concentration in the influent was increased in steps from 3 g/L to 12 g/L to obtain a final organic rate of approximately 16 g COD/$m^3 \cdot$d.

Analytical grade NaCl (Merck Suprapur®) was used to adjust sodium concentration in each reactor's effluent at first and afterwards it was replaced by technical grade NaCl (VWR, minimum purity 98%). Final sodium concentrations of 20 g Na^+/L include the sodium originating from sodium acetate (Sudmalis, Gagliano et al. 2018).

For the R2, the nutrient medium was similar and consisted of the following salts:

- KH_2PO_4 (0.22), $NaHCO_3$ (1.5) and $(NH_4)_2CO_3$ (0.17).

Macro and micro nutrients at final concentrations in mg/L:

- $MnCl_2 \cdot 4H_2O$ (0.3), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.05), $CoCl_2 \cdot 6H_2O$ (1.2), $NiCl_2 \cdot 6H_2O$ (0.03), EDTA (0.6), Resazurin (0.3), $Na_2SeO_3 \cdot 5H_2O$ (0.3) and 0.216 ml/L of 36% HCl.

Tryptone consists of salts and peptides which were measure by ICP-OES. To compensate the ions present in tryptone, 3 metal stocks were used. These 3 metal stocks were added in the medium. They were made up of, in g/L:

- Metal stock 1: NH_4Cl (90.96), KH_2PO_4 (7.77) and Na_2SO_4 (5.58).
- Metal stock 2: $FeCl_2 \cdot 4H_2O$ (0.018).
- Metal stock 3: $MgSO_4 \cdot 7H_2O$ (0.37), $CaCl_2 \cdot 2H_2O$ (0.69), H_3BO_3 (0.01), $ZnCl_2$ (0.05), $CuCl_2 \cdot 2H_2O$ (0.01) and $AlCl_3$(0.05).

The COD was soluble and consisted of D-Glucose, Na-Acetate and Starch (Merck KGaA, pro analysi, ISO). The COD proportions were 3:2:1 and the concentration in the influent was 3 g_{COD}/L at first. As for the rest of the reactors, final sodium concentrations of 20 g Na^+/L included the sodium originating from sodium acetate.

2.1.2 Inoculum, set-up and operation

Four double-walled glass UASB bioreactors (R1, R2, R3 and R4) of 1.73 L active volume, were inoculated with 6 g VSS/L. The biomass was a full scale UASB treating wastewater from styrene and propene-oxide production plant (Shell, Moerdijk, The Netherlands).This sludge was acclimated at 8 g Na^+/L and it had a SMA of 0.2-0.3 g_{COD}/g_{VSS} with acetate as carbon source (Ismail 2013).

The inoculum of all the reactors was dispersed by forcing it through a 125 μm sieve under nitrogen gas flow before adding to the UASB reactor and then, it was mixed with PAC of 0.1% w/w.

All the reactors were operated with circulation after a few weeks of operation and the upflow velocity was increased in steps from 0.2 m/h to 1 m/h. This velocity was imposed by peristaltic circulation pumps. The temperature in all the reactors was 35 $\mp$1℃.

2.2 Process performance analysis

2.2.1 Analytical methods

Biogas, temperature, redox and pH was monitored and annotated every day by using the online measurements.

Total and soluble COD measurements were carried out with LCK 314, LCK 514 and LCK 1414 kits (HACH GMBH, Germany) after sample dilution with milli-Q water to prevent chloride interference. The dilution factor was 25 in R2, R3 and R4 and 30 in R1. The differences between total COD (tCOD) and soluble COD (sCOD) was achieved by filtration. sCOD fraction was defined by a prewashed 0.45 μm membrane filter. tCOD is measured in homogeneous conditions of effluent samples. The homogeneous conditions were achieved by mixing with a blender (Waring, Commercial Blendor).

Volatile and total suspended solids (VSS, TSS) were measured every week to quantify the biomass washout of the reactor. The effluent bottles were filtered through microfiber filters (Whatman™ Glass Microfiber Prefilter 2 µm, 47 mm, GE Healthcare Life Sciences). At the end of the experiment, the percentage of TSS and ASH was achieved.

$$\mathrm{TSS} = \frac{\mathrm{m}(105℃) - \mathrm{m}(\text{treated filter})}{\mathrm{m_0} - \mathrm{m}} = \frac{\mathrm{g_{TSS}}}{\mathrm{g_{effluent}}} \cdot 1000 = \frac{\mathrm{g_{TSS}}}{\mathrm{L}}$$

$$\mathrm{ASH} = \frac{\mathrm{m}(550℃) - \mathrm{m}(\text{treated filter})}{\mathrm{m_0} - \mathrm{m}} = \frac{\mathrm{g_{ASH}}}{\mathrm{g_{effluent}}} \cdot 1000 = \frac{\mathrm{g_{ASH}}}{\mathrm{L}}$$

$$\mathrm{VSS} = \mathrm{TSS} - \mathrm{ASH}$$

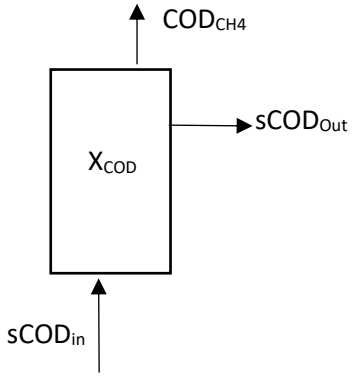
Where:

- $m_0 - m$: amount of effluent sample filtered, g.
- $m(treated\ filter)$: grams of treated tray and filter. The treated trays were in the oven during 2 hours at 550℃ and the filters at 105℃ during the same time.
- $m(105℃)$: grams of sample, treated tray and filter after staying in the oven during the night at 105℃.
- $m(550℃)$: grams of sample, treated tray and filter after staying in the oven during 2 hours at 550℃.

Samples of DNA, raw effluent and FISH were kept regularly and the turbidity was measured every week.

For FISH experiments, samples taken from each reactor were fixed with 37% w/w formaldehyde. After fixation, samples were washed 3 times with PBS and stored at -20℃ in ethanol: PBS (1:1).

2.2.2 Soluble COD balance at steady state conditions



Of this form, the balance is:

$$\mathrm{x_{COD}} = \mathrm{Q} \cdot (\mathrm{sCOD_{in}} - \mathrm{sCOD_{out}}) - \mathrm{COD_{CH4}}$$

Assuming steady state conditions, and where:

- Q: flow, $\frac{\mathrm{L}}{\mathrm{d}}$. The same in sCOD$_{In}$ and sCOD$_{out}$.
- $\mathrm{sCOD_{in}}$: soluble COD in the influent, $\frac{\mathrm{gCOD}}{\mathrm{L}}$.
- $\mathrm{sCOD_{out}}$: soluble COD in the effluent, $\frac{\mathrm{gCOD}}{\mathrm{L}}$.
- $\mathrm{x_{COD}}$: biomass production, $\frac{\mathrm{gCOD}}{\mathrm{L}}$.
- $\mathrm{COD_{CH4}}$: biogas COD-CH_4 $\frac{\mathrm{gCOD}}{\mathrm{d}}$.

For the biogas COD, it was necessary to calculate the biogas production rate:

$$\mathrm{P.R_{biogas}} = \frac{\mathrm{BP_{t_1}} - \mathrm{BP_{t_0}}}{\mathrm{t_1} - \mathrm{t_0}}$$

Where:

- $\mathrm{BP_{t_1}}$: biogas production after same time of composite sampling, m^3.
- $\mathrm{BP_{t_0}}$: biogas production when the gas accumulation started, m^3.
- $\mathrm{t_1} - \mathrm{t_0}$: gas accumulation time, d.

As the biogas production rate was calculated in normal units, the ideal gas law could be used to calculate the number of moles of gas and finally the biogas COD$_{CH4}$ with the methane fraction.

$$\mathrm{p} \cdot \mathrm{V} = \mathrm{n} \cdot \mathrm{R} \cdot \mathrm{T}; \quad \mathrm{n} = \frac{\mathrm{p} \cdot \mathrm{P.R_{biogas}}}{\mathrm{R} \cdot \mathrm{T}}$$

$$\mathrm{COD_{CH4}} = \mathrm{n} \cdot \mathrm{f_{CH_4}} \cdot 64 = \frac{\mathrm{p} \cdot \mathrm{P.R_{biogas}}}{\mathrm{R} \cdot \mathrm{T}} \cdot \mathrm{f_{CH_4}} \cdot 64$$

Where:

- p: pressure, 1 atm.
- P. R_{biogas}: biogas production rate, $\frac{L}{d}$.
- n: moles of gas, $\frac{molgas}{d}$.
- R: Ideal gas constant, 0.082 $\frac{L \cdot atm}{mol \cdot K}$
- T: temperature, 298.15 K.
- f_{CH_4}: methane fraction.
- 64: grams of COD per mole of CH_4

The yield of biomass is calculated with this equation:

$$\eta_{biomass} = \frac{g_{biomass\ COD}}{g_{COD\ converted}} = \frac{x_{COD}}{Q \cdot (sCOD_{in} - sCOD_{out})}$$

The percentage of COD converted in biogas is:

$$\%\ COD\ converted = \frac{COD_{CH4}}{Q \cdot (sCOD_{in} - sCOD_{out})}$$

2.2.3 Soluble COD removal efficiency and biogas production rate

The sCOD removal efficiency was calculated with the following formula:

$$\eta = (1 - \frac{sCOD_{out}}{sCOD_{in}})$$

$sCOD_{in}$ was determined by the chemical recipe of medium. $sCOD_{out}$ was measured in the effluent.

The biogas production rates were monitored with μFlow gas flow meter for R2 and R3 (Bioprocess Control Sweden AB) and biogas composition was measured periodically. For R1 and R4, the biogas production rates were taken for readings of biogas meters (Ritter, Trommel-Gaszähler).

2.2.4 Total COD removal efficiency

The total COD removal efficiency is deriving from the tCOD concentrations between influent and effluent.

$$\eta_{tCOD} = (1 - \frac{tCOD_{out}}{tCOD_{in}})$$

Where:

- $tCOD_{in}$: total COD in the influent, $\frac{gCOD}{L}$
- $tCOD_{out}$: total COD in the effluent, $\frac{gCOD}{L}$

2.2.5 Mass balance of biomass

The mass balance of biomass in the reactor is:

$$BM_f + BM_{washout} = BM_i + BM_{produced}$$

BM_i (biomass in the influent) and BM_f (biomass in the effluent) were measured at the beginning and the end of the operation, respectively. $BM_{washout}$ was the cumulative VSS from the effluent and the waste from recirculation. With these values given in VSS, $BM_{produced}$ (g_{VSS}) was calculated from the mass balance. All the measures must be done in duplicate.

2.3 Granular sludge characteristics

2.3.1 Particle size distribution and settling properties

Particle size distribution of the microbial granules was performed for R4 with a Nikon SMZ 800 macroscope where the images were taken with Euromex Cmex-5 pro camera using Image Focus alpha software. At first, a preparation had to be done. It was achieved with 0.5 ml of sludge and 6 ml of tap water in a petri dish. The minimum number of photos that had to be taken were 10, which means around 100 particles.

To measure the settling properties in R3 and R4, 8 ml of sample was taken and 1.5 g of TS was measure for duplicate. These samples were filtered and entered in the oven during 3 hours at 105℃. Consequently, the concentrations of TS (g/g) were calculated following this equation:

$$TS_{g/g} = \frac{m_{filt\ 3h} - m_{dry\ cruicable+filter}}{m_{sample\ filtered}}$$

Where:

- $m_{filt\ 3h}$: grams of sample, treated tray and filter after staying in the oven during 3 hours at 105℃.
- $m_{dry\ cruicable+filter}$: grams of treated tray and filter. The treated trays were in the oven during 2 hours at 550℃ and the filters at 105℃ during the same time.
- $m_{sample\ filtered}$: grams of sample filtered, approx. 1.5 g.

The amount of sample that needed to be added in settling column was calculated considering 0.2 g of TSS:

$$\text{Sample volume} = \frac{0.2}{\text{average(TS)}}$$

Where:

- $Average(TS)$: the average of both samples taken, g.

The next step was to fill up the column with tap water and add the amount of sample calculated (sample volume). Then, the mass of empty collection tanks was measured and the needed upflow velocity was applied (changing the rpm of the pump). This procedure was done for 4 different velocities. For each rpm, the pump was pumping for 15 minutes approximately, except for the highest rpm, which was pumping for 7 minutes only.

The mass of the full collection tanks was measured and filtered. These samples were entered in the oven (105℃) and after one night, they were weight. Of this form, the amount of suspended solids were achieved.

$$SS_x = m_{dry\ cruicable+filter+supernatant,x} - m_{dry\ cruicable+filter,x}$$

Where:

- SS_x: suspended solids for 4 different rpms, g.

- $m_{\text{dry cruicable+filter+supernatant,x}}$: grams of sample, treated tray and filter after staying in the oven all the night at 105℃ for each rpm.
- $m_{\text{dry cruicable+filter+supernatant,x}}$: grams of treated tray and filter for each rpm. The treated tray was in the oven during 2 hours at 550℃ and the filters at 105℃ during the same time.

It was necessary to measure the pellet (the amount of sample that was in the column after the 4 different rpms) and calculate the suspended solids with the same method.

Knowing these values, the flow and the upflow velocity can be calculated following these equations:

$$Q = \frac{g_{\text{full collection tank}} - g_{\text{empty collection tank}}}{\text{time}}$$

$$V_{up} = \frac{Q}{S} \cdot \frac{60}{10^6}$$

Where:

- Q: flow for each rpm, $\frac{\text{ml}}{\text{min}}$.
- $g_{\text{empty collection tank}}$: mass of the collection tank before pumping with each rpm, g.
- $g_{\text{full collection tank}}$: mass of the collection tank after pumping with each rpm, g.
- time: time that the pump was pumping for each rpm, min.
- V_{up} : upflow velocity, $\frac{\text{m}}{\text{h}}$.
- S: surface of the column, $9.85 \cdot 10^{-4}$, m^2.
- $\frac{60}{10^6}$: conversion factor to get, $\frac{\text{m}}{\text{h}}$.

The fraction of total suspended solids for each rpm can be calculated and the fraction that survived the flushing was known too.

$$\text{Fraction of total SS} = \frac{SS_x}{\text{total SS}}$$

$$\text{Fraction not flushed out} = 1 - \text{Fraction total SS}$$

Where:

- SS_x: suspended solids for 4 different rpms, g.
- $total\ SS$: sum of all the SS, g.

2.3.2 EPS extraction yields and composition

This protocol is based on Felz, Al-Zuhairy et al. (2016) protocol where before the extraction, the sludge is concentrated by a centrifugation (3620 RCF, 5 ℃, 20 min). Part of the residue is used to measure the total and the volatile solids (TS, VS). The rest of it is precipitated and added with Na_2CO_3 to get 0.5% w/w concentration.

The blend is mixed at 80℃ and 400 rpm for 35 minutes. Finally, the mix is purified with a 3500D dialysis membrane with demi water for 24 hours to obtain the extracted EPS.

All the samples have to be measured in duplicate. Then, the TS and VS extraction yields are calculated with the following formulas:

$$\text{TS yiel} = \frac{\text{TS}_{\text{extraction}} \cdot \text{sludge}_{\text{TS/VS}}}{\text{TS}_{\text{sludge}} \cdot \text{sludge}_{\text{extraction}}}$$

$$\text{VS yiel} = \frac{\text{VS}_{\text{extraction}} \cdot \text{sludge}_{\text{TS/VS}}}{\text{VS}_{\text{sludge}} \cdot \text{sludge}_{\text{extraction}}}$$

The organic fraction of samples is:

$$\text{Organic fraction} = \frac{\text{VS}}{\text{TS}}$$

Proteins and carbohydrate are measured and given as fractions of the EPS. Carbohydrate quantification is done with the phenol-sulphuric method, using glucose. Protein quantity is measured from nitrogen quantity multiplied by a conversion factor of 6.25 and the nitrogen is measured using 3-5 times diluted extracts by Laton Total Nitrogen cuvette (20-100 mg/L) of Hach. The rest of EPS could be other polymers.

2.4 Batch experiments

The objective of these experiments was to study the effect of a chosen osmoprotectant, tryptone, on activity glucose fermenters. Of this form, the glucose removal rate was achieved.

This experiment was performed using serum bottles. In total, two sets of bottles were prepared. Each of the sets was prepared with a different biomass originating of 2 UASB reactors of 1.73 L of active volume. These biomasses were kept at 4 degrees before using in batch experiments.

One of the biomass was taken on 05/09/2016 from R2 of a previous study (Sudmalis, Gagliano et al. 2018). It was adapted to 5 g Na^+/L (R5). The objective of R5 was to study what happen when the salinity was increased until 20 g Na^+/L. The other biomass was taken on 08/12/2017 from R1 in the same research with a salinity of 20 g Na^+/L (R20), to study if the activity can be improved using tryptone. Both types of biomass were exposed to 20 g Na^+/ L in the experiment.

In each batch set, three sample types were tested: blank, GL and GLT. The blank, which was done in duplicate, did not contained COD and osmoprotectants and it was used to measure the pressure. GL samples contained COD but no osmoprotectants and GLT contained COD and tryptone because addition of tryptone to the granular sludge could improve the methanogenic activity (unpublished data ETE). Ammonium carbonate [$(NH_4)_2CO_3$] was added in GL samples to compensate the nitrogen pressure caused by tryptone in GLT samples. The COD was provided from sodium acetate.

All the samples were done in duplicate, in one of them, the pressure was controlled in triplicate and in the other, samples to analyse the VFA, glucose and COD were taken in triplicate. Some of these bottles were used only to measure the pressure because the volume changed in those that were used to take liquid samples and the pressure changed too. Of this form, 14 serum bottles had to be used for each batch set (2 blanks, 6 GL, 6 GLT).

For these experiments, 11 stocks solutions were prepared using two different medium matrixes. 5 of these stocks solutions were prepared in 20 g Na^+ /L matrix, 3 with a salinity lower than 20 g Na^+ /L and 3 containing

more than 20 g Na^+ /L. The salinity was obtained by adjusting the amount of NaCl into matrix. The lab matrix composed of macro and micronutrients and buffer. The composition of the stocks solutions is in Table 3 and Table 4, Appendix.

The amount of tryptone as osmoprotectant that had to be applied was estimated with the osmotic pressure generated from Na^+ and Cl^-. The osmotic pressure follows this equation:

$$\pi = \sum \gamma \cdot M_{solution} \cdot R \cdot T$$

Where:

- π: osmotic pressure, atm.
- γ: van´t hoff factor.
- $\mathrm{M_{solution}}$: solution molarity, M.
- R: gas constant, $0.082\ \frac{\mathrm{L \cdot atm}}{\mathrm{mol \cdot K}}$.
- T: temperature, K.

Of this form:

$$\mathrm{M_{trypton}} = \frac{\pi}{\gamma \cdot \mathrm{R} \cdot \mathrm{T}}$$

Where:

- $\mathrm{M_{trypton}}$: tryptone molarity, M.

The final Na^+ was 20 g/L, so the molarity of Na^+ was 0.87 M and the molarity of Cl^- 0.86 M. These two ions were the main responsible in the salinity. Van´t hoff factor was considered as 1 and the temperature was 35°C, so 308.15 K. Of this form, the osmotic pressure was estimated to be 43.71 atm. The tryptone molarity was calculated assuming equal temperature. It was estimated in 1.73 mol/ kg of biomass that was needed.

All the bottles were inoculated with a different amount of sludge to achieve 1 g VSS/L.

A pre-condition step was needed to avoid lag phase during the experiment. In this phase, the ionic composition did not change. The COD/VSS ratio was of 0.4 (w/w). During this part, the bottles were filled using the stock solutions and one of the 2 diverse types of biomass (Figure 1). The salinity was set at 5 and 20 g Na^+/L for R5 and R20, respectively. The amount of each serum bottle in the pre-condition step is in Table 5, Appendix.

After the addition, the bottles were flushed with N_2 gas to create an N_2 atmosphere. Then, the bottles were sealed and cultivated in the incubator (Innova 44, Incubator Shaker Series) at 35°C and 120 rpm. Since that moment, pressure was taken each 2 hours to check that the biomass was active.

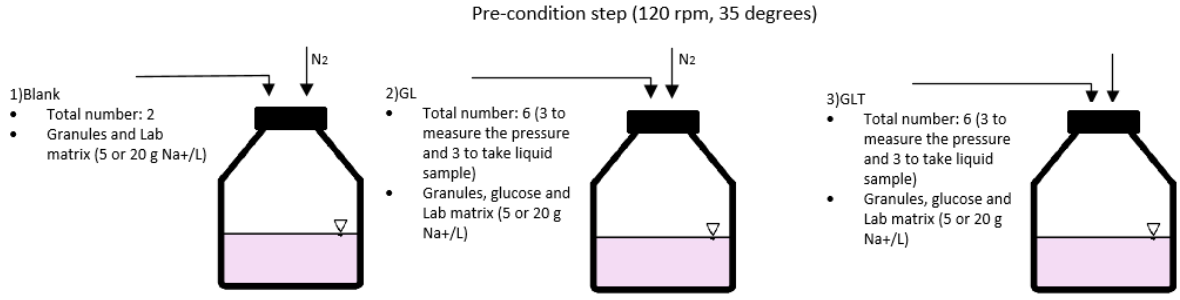


Figure 1. Pre-condition step. All the process was done in serum bottles with a salinity of 5 g Na^+/L (R5) or 20 g Na^+/L (R20). The pressure was measure in triplicate during 24 hours.

After 24 hours, the up-shock experiment was initiated. For this phase, the level of Na^+ and COD were increased into 20 g Na^+/L and 4 g COD/L, respectively. The COD/VSS ratio, in this case, was set to 4 (w/w). The Na^+ level was adjusted by adding a volume of saline lab matrix for R5 granules. The other components, sodium acetate, ammonium carbonate and tryptone, were added to the bottles too (Figure 2). The concentration of these components in all the serum bottles are in the Table 6, Appendix.

The pH was measured in all the bottles and samples of these bottles were taken to measure the initial COD. Finally, all of them were flushed with N_2 gas, sealed and incubated in the same conditions as the pre-condition step. This part finished when the pressure in the bottles were stable. For this reason, the pressure in headspace was measured over time, around 5 times a day with a digital pressure meter (GMH3151, Greisinger Electronic, Germany). When the pressure was more than 230 kPa, it had to be reduced not to overcome the meter limit. In batch bottles designated for sampling of liquid aliquots, 8 millimetres of liquid sample were taken and centrifugated at 4500 rpm, 5℃ and 10 min (FirlabO SW12R-FACENSW12001). The liquid part was kept in 12 ml tubes and introduced in the freezer, to choose and analyse the more representative ones, considering the pressure build-up plot, when the experiment was finished.

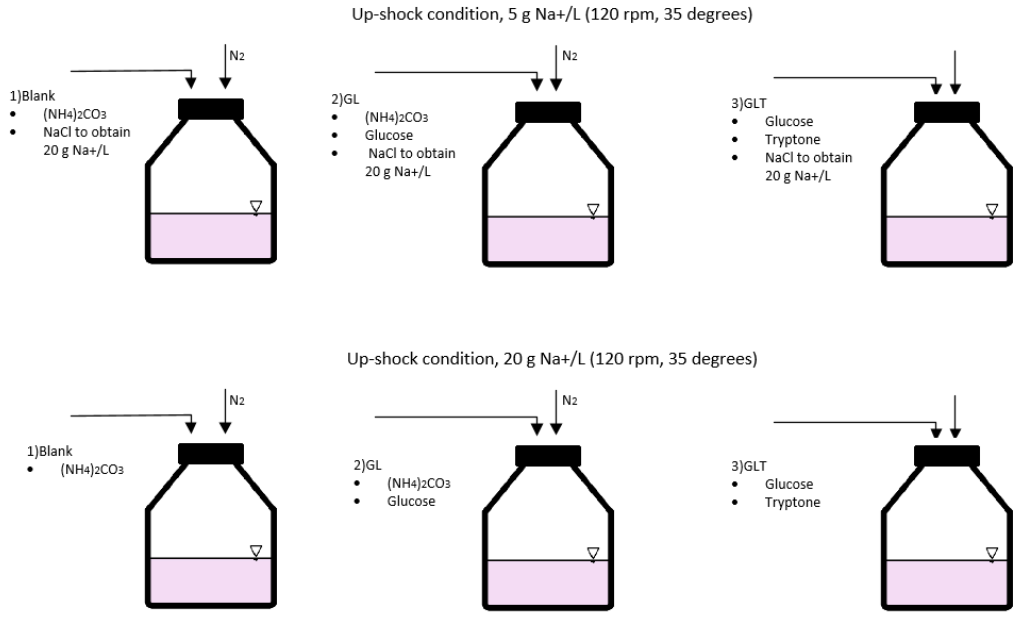


Figure 2. Up-shock condition. All the serum bottles had a salinity of 20 g Na+/L. The pressure was measure in triplicate and liquid samples were taken in triplicate during all the process until steady state.

At the end of the experiment, the pressure and pH was measured to get the final value and liquid samples were taken for all the bottles to measure the final sCOD. The biogas composition was analysed using gas chromatography in triplicate (Shimadzu GC-2010). This chromatograph uses two columns which are connected in parallel (Porabond Q and Molsieve 5A). The carrier gas is Helium 5.0 (He) with a pressure of 0.95 bar. The detection is produced by a thermal conductivity detector. The operational temperatures were set to 120 ℃ for the injection port, 80 ℃ for the column and 150 ℃ for the detector. The injection volume is 50μL and the software that was used is Chromeleon 6.80 SR13.

Volatile fatty acids were studied too. For this part, different liquid samples must be chosen in duplicate at the beginning and when it attained the steady state conditions but also when the slope of pressure build-up curve was maximum because that was the moment of maximum VFA production rate. VFAs were measure with a gas chromatography (Agilent 7890B). Samples must be diluted before putting them in the chromatography. To prepare the chromatography liquid samples were filtered with 0.45 μm filter. After that, 1.35 ml of sample and 0.15 ml of formic acid were mixed. The carrier gas is Helium 5.0. The injection volume is 1μL and the constant temperature is 250 ℃. This chromatograph has one column(HP-FFAP). The software that was used is Chromeleon 6.80 SR13.

Glucose measurement were done by means of high performance liquid chromatography (HPLC). Liquid samples were measured in duplicate at the beginning and in steady state but also when the slope of the pressure build-up curve was maximum because that was the moment of maximum glucose use. The samples were the same that were used for volatile fatty acids. Glucose were detected by means of Refractive Index detector (RI). The quantification is done by a three point of calibration. For this calibration it was necessary to prepare 6 standard solutions and a blank. Pure glucose (D-(+)-Glucose, 99.5%) and sulphuric acid (1.25 mM) were used for that. To prepare the chromatography, liquid samples were filtered with 0.45 μm filter. After that, samples had to be diluted with sulphuric acid (1.25mN) before putting them in the chromatography. The column used was Hi-PLEX H, Agilent partnr, 1F70-6830. Results are acquired and processed by Chromeleon v6.8.

2.4.1 Specific methanogenic activity

The specific methanogenic activity is calculated as follows:

$$\mathrm{SMA} = \frac{\Delta \mathrm{P} \cdot \mathrm{V}}{\mathrm{R} \cdot \mathrm{T}} \cdot \mathrm{f}_{\mathrm{CH_4}} \cdot \frac{64}{\mathrm{VSS}} = \frac{\mathrm{g}_{\mathrm{CH_4-COD_{produced}}}}{\mathrm{g}_{\mathrm{VSS}} \cdot \mathrm{d}}$$

Where:

- ΔP: maximum slope of pressure build-up curve, $\frac{kPa}{h}$. To simplify, the maximum value of pressure build-up in the plot was taken assuming that maximum rate was been calculated.
- R: Ideal gas constant, $8.314 \cdot 10^3 \frac{kPa \cdot m^3}{mol \cdot K}$.
- T: Temperature, $308.15\ K$.
- V: headspace volume, $44.775 \cdot 10^{-6}\ m^3$.
- f_{CH_4}: Methane fraction in biogas.
- VSS: volatile suspended solids, $0.2\ g_{VSS}$.
- 64: grams of COD per mole of CH_4.

3. Results and discussion

3.1 Comparison between R3 and R4

R3 was started on 20/12/2017. This reactor was working with gelatin as osmoprotectant. On the other hand, R4 was started on 07/11/2016 with tryptone as osmoprotectant. The amount of tryptone was reduced between days 367 and 409 since the start-up of R4. To compare the operation of both reactors, the date used will be 27/12/2017, 7 days after the start-up of R3 and on day 415 since the start-up of R4. Total amount of tryptone (1.68 g/L) was used in R4 from that day. Besides, the R4 performance was studied to analyse what happened with changes of tryptone in the biomass and biogas production, granules strength and solids washout.

3.1.1 Soluble COD balance

The balance was done at steady state with an organic loading rate of 9 g COD/L·d. For R4, it is interesting to compare the differences in the percentages of COD converted in biogas when the amount of tryptone was different. Figure 3 shows these differences.

Between days 274 and 332 since the start-up of R4, the amount of tryptone was 1.68 g/L, the average of the percentages of soluble COD converted was 84.26% ±1.53%. When the amount of tryptone decreased, the average of these percentages was 82.23%±4.05%, the values were fluctuating enough and granules were decomposing. Finally, the amount of tryptone were 1.68 g/L other time, on day 416, the average was 79.28%±3.8% and granules recovered the strength.

The overall yields of mixed anaerobic consortia have been reported to be between 4 and 20%. In our study, we found between 15 and 21%, 15.74%±1.53% in the first range (1.68 g tryptone/L), 17.77%±4.05% when the amount of tryptone decreased and 20.72%±3.8 when the amount of tryptone recovered its normal value. These values are in line with Sanders (2001).

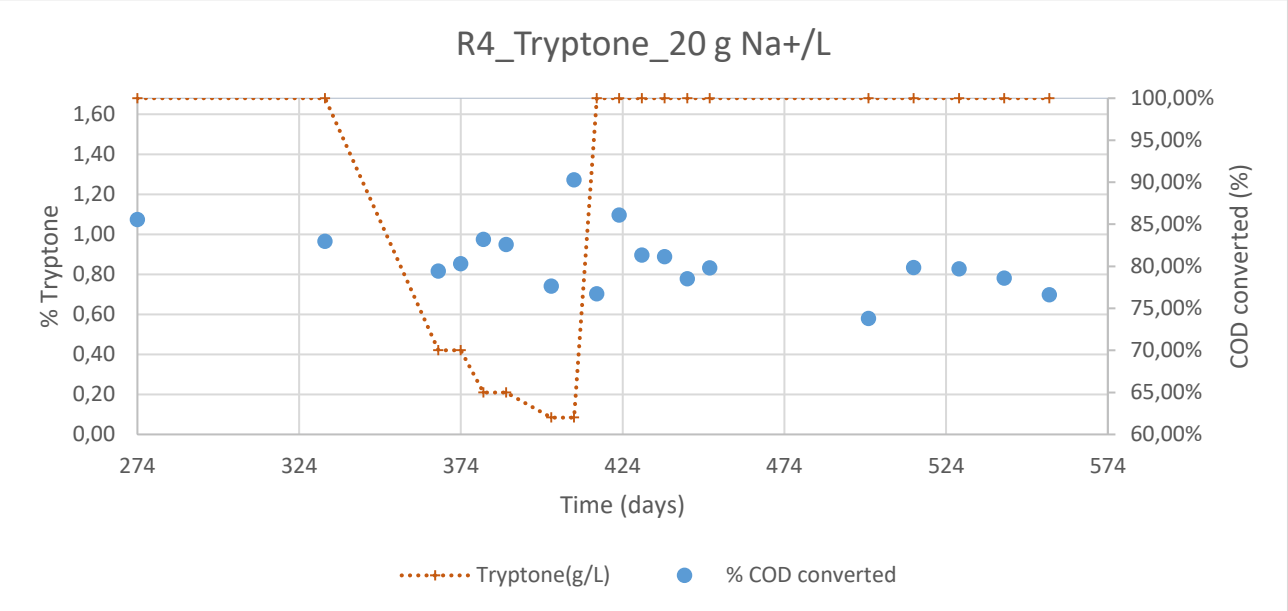


Figure 3 Percentage of soluble COD converted in biogas for R4 with different amounts of tryptone (1.68 g/L, 0.42 g/L, 0.21 g/L and 0.08 g/L). The balance was done at steady state with an OLR of 9 g COD/L·d. Average: 84.26% ±1.53% until day 332, 82.23%±4.05% between days 332 and 416 and 79.28%±3.8% since day 416.

With the biogas meter of R3 the progression of biogas can show perfectly, however, this meter is not accurate to calculate de COD converted and the yield of biomass.

3.1.2 Soluble COD removal efficiency and biogas production rate

In this section, a comparison of the soluble COD in R3 and R4 has been done to look the difference between two substrates, gelatin and tryptone.

The average removal efficiencies based on sCOD, when the COD in the influent was fixed on 12 g/L and the OLR around 9 gCOD/L·d in both reactors, were 94.20%±1.7% and 98.29%±0.8% for R3 and R4 respectively, showing that R4, operating with tryptone, performed slightly better than R3, operating with gelatin.

Figure 4A shows the removal efficiency of the sCOD in R3. When R3 was started, the COD in the influent was fixed on 3g/L and the OLR was 1.31 gCOD/L·d, so the removal efficiency was 83.8%. To increase the COD_{in} value, the OLR was incremented manually. When the OLR was boosted from 1.31 to 5.17 gCOD/L·d and the COD_{in} was fixed on 7 g COD/L, the removal efficiency dropped to 77.86% the first week. This decrease on the removal efficiency may mean that conversion of soluble COD to biomass or biogas has been weakened due to a suddenly change so more time is needed to restore this efficiency. Finally, the COD_{in} was fixed on 12 g/L and the removal efficiency achieved values between 90 and 95%.

Figure 4B shows a stable removal efficiency despite the changes in the OLR for R4, which has been feeding with tryptone. As this reactor had been working during one year before changing tryptone the COD in the influent is always 12 g COD_{in}/L. The amount of tryptone was changed between days 367 and 409 since the start-up of the reactor, however, the efficiency did not change a lot, maintaining between 96 and 98%. These values were higher than R3 in the same conditions. It can be explained because gelatin is enzymatically digestive meaning more amino acids. The chain of peptides in tryptone are shorter than in gelatin producing an easier biodegradation of tryptone than gelatin.

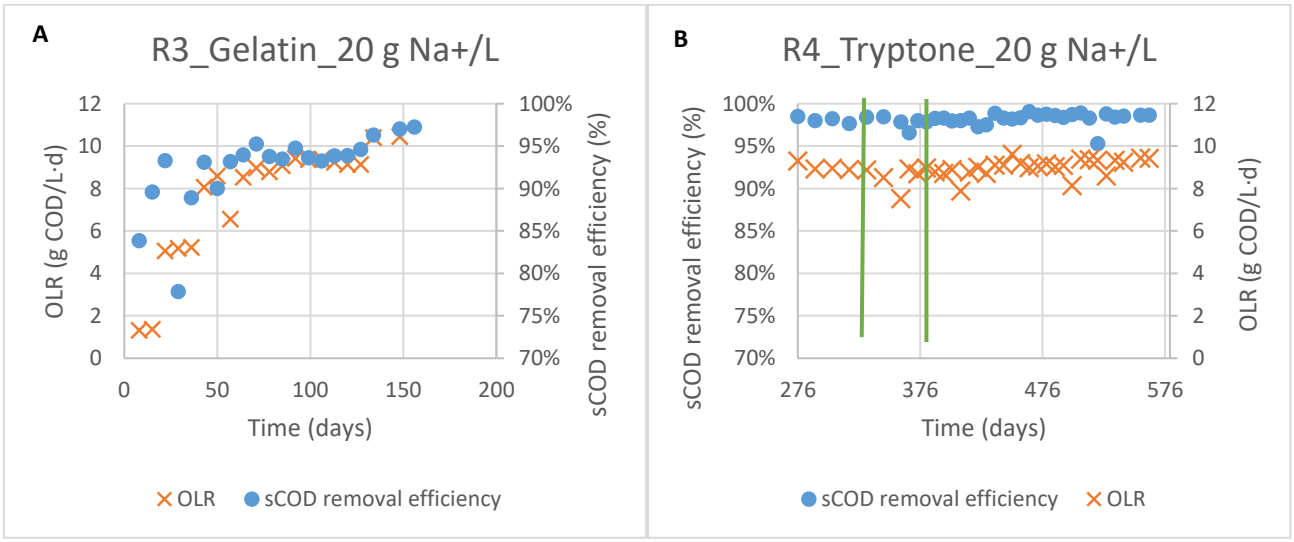


Figure 4 A: Performance of sCOD removal efficiency (%) of R3 (gelatin) with different values of OLR (g COD/L·d). When the OLR uploaded, the removal efficiency grew too until stable values. Average: 94.20%±1.7%, when the OLR was around 9 g COD/L·d and the COD in the influent was 12 g/L. B: Performance of sCOD removal efficiency (%) of R4 (tryptone) with values of OLR around 9 g COD/L·d. Average: 98.29%±0.8% when the OLR was around 9 g COD/L·d and the COD in the influent was 12 g/L. Between the two green lines is shown the moment with less tryptone. The efficiency maintained stable despite changes in tryptone.

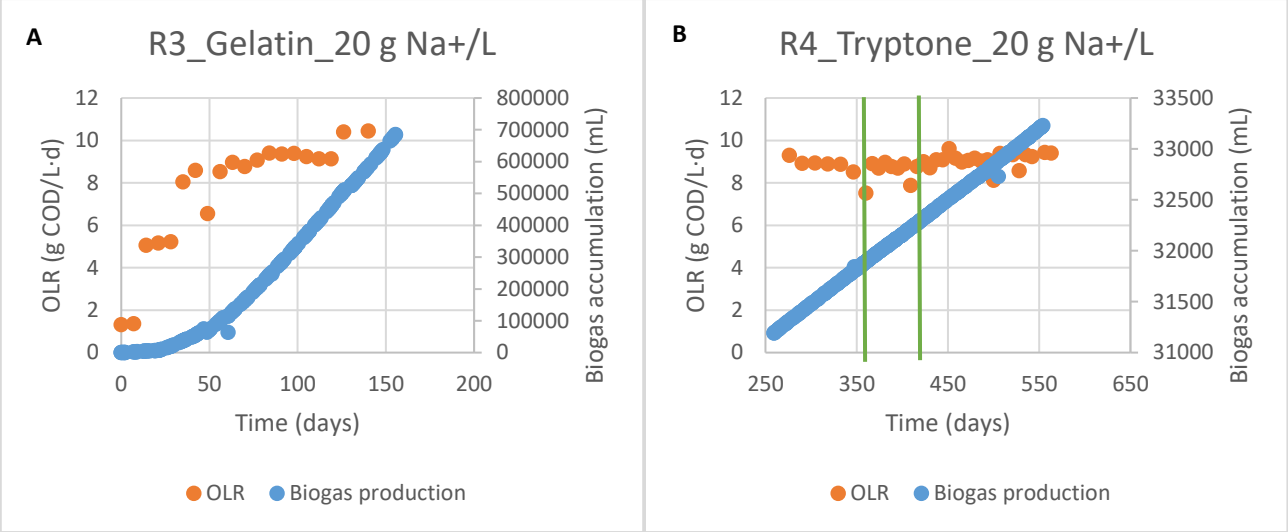


Figure 5 A: Biogas production (mL) for R3 (gelatin) with different values of OLR (g COD/L·d). The biogas production was 80% higher when OLR was increasing. B: Biogas production (mL) for R4 (tryptone) with different values of OLR (g COD/L·d). The moment with less tryptone is shown between the two green lines. R4 produced 3% less amount of biogas during the period with changes in tryptone.

Figure 5 shows the biogas production rate with gelatin for R3 and tryptone for R4. Despite of the small changes in the organic loading rate, the biogas production continued growing linearly for both reactors. In case of R3, 80% more biogas was producing when the COD in the effluent was increasing. Besides, R4 produced 3% less amount of biogas during the period of changes in tryptone.

Methane content over the whole period was high in the reactor operated with tryptone. The average for each reactor was 66.6%±5.1% and 67,15%±1% for R3 and R4, respectively. This fact may be due to the different substrate used for each reactor which needed different conversions, therefore, different amount of CH_4 were achieved (Wagner, Hohlbrugger et al. 2012).

3.1.3 Turbidity and total COD removal efficiency

Figure 6 shows a comparison in the total COD removal efficiency between R3 and R4. Volatile suspended solids and total COD are related with a correlation of 1.42 g COD/g VSS. tCOD contain colloidal COD which have a high effect in the turbidity. For this reason, the turbidity has been added in the plots.

The average removal efficiencies based on tCOD, when the COD in the influent was 12 g/L and the OLR around 9 g COD/L·d, were 90.05%±2.17% and 95.43%±0.98% for R3 and R4 respectively, concluding that R4 contained less number of solids in the effluent, which showed that solids retention within R4 was better that in R3.

When R3 contained only 3 g/L of COD in the influent, the removal efficiency was 58.33%, the activity was very low, and there were a lot of suspended solids in the effluent. When the COD_{in} was increased, the total COD removal efficiency dropped initially and increased to 90% after 35 days of operation.

Figure 6B showed the removal efficiency for R4. It was stable during all the process, between 94 and 97%. These values were higher than R3. The plots show 25% less turbidity in R4 than in R3 during the experiment. That might mean the granules were stronger in R4 probably because of the influence of tryptone.

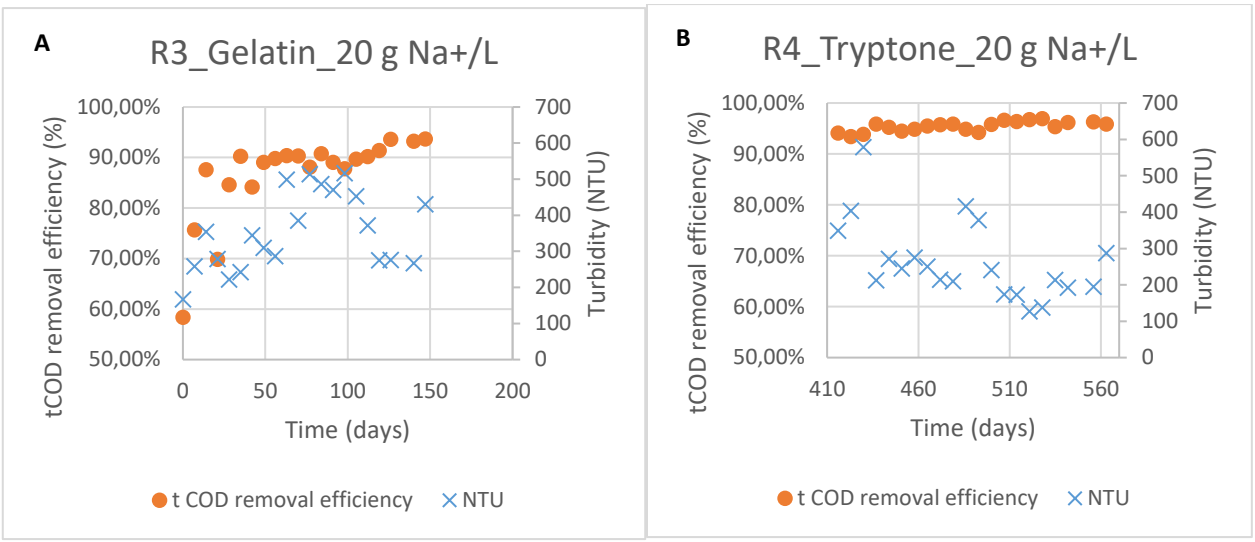


Figure 6 A: Turbidity (NTU) and total COD removal efficiency (%) of R3 (gelatin as substrate). The removal efficiency increased when OLR grew. Average: 90.05%±2.17%, when the COD in the influent was 12 g/L and the OLR around 9 g COD/L·d. B: Turbidity and total COD removal efficiency of R4 (tryptone as substrate). Average: 95.43%±0.98% when the COD in the influent was 12 g/L and the OLR around 9 g COD/L·d. There was 25% less turbidity in R4 than in R3.

It is interesting to compare the differences in the turbidity when the amount of tryptone changed (Figure 7). Before reducing the amount of tryptone, the efficiency in R4 was working in an effective way. When the amount of tryptone was decreased, the turbidity increased considerably which shows that there are more solids in the effluent. Adding 1.68 g tryptone/L, was enough to recover the normal activity of the reactor and decrease the turbidity.

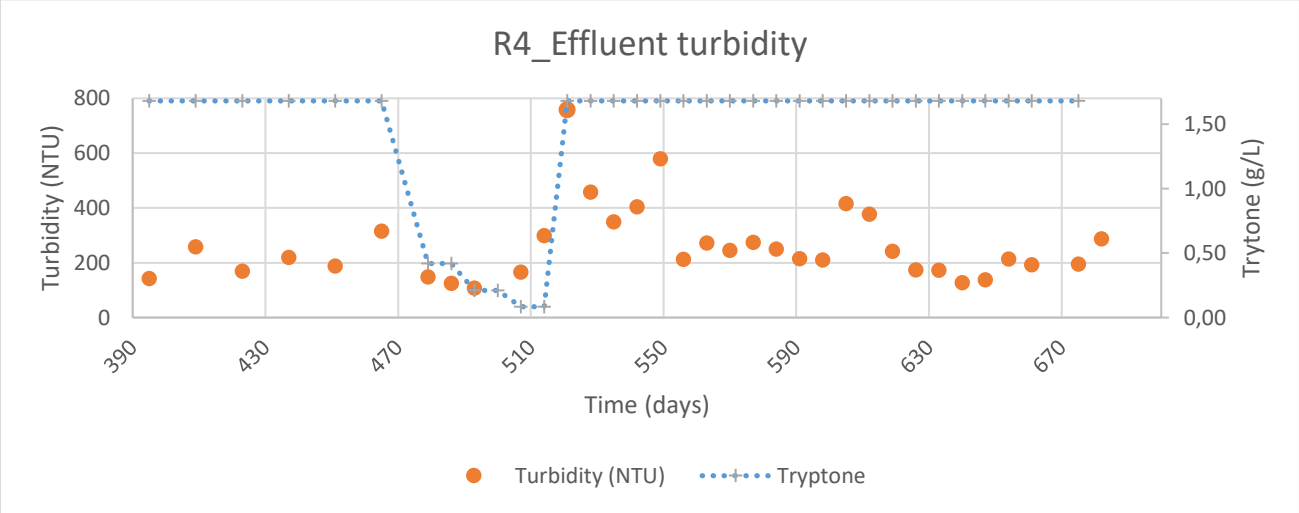


Figure 7 Changes in the turbidity (NTU) when the amount of tryptone changed (1.68 g/L, 0.42 g/L, 0.21 g/L and 0.08 g/L). The turbidity grew when the amount of tryptone decreased. Adding 1.68 g tryptone/L, was enough to recover the normal activity of the reactor and decrease the turbidity.

The fractions contributing to total effluent COD are represented in Figure 8. Particulate and colloidal COD were calculated with the difference between total and soluble COD. These values were calculated on day 119 since R3 was started for R3 and on day 535 for R4. tCOD was 1.04 g/L for R3 and 0.56 g/L for R4. Soluble COD was 0.65 g/L and 0.19 g/L for R3 and R4, respectively. The particulate and colloidal fractions contributing to total effluent COD were similar for both reactors, 0.39 g/L for R3 and 0.37 g/L for R4.

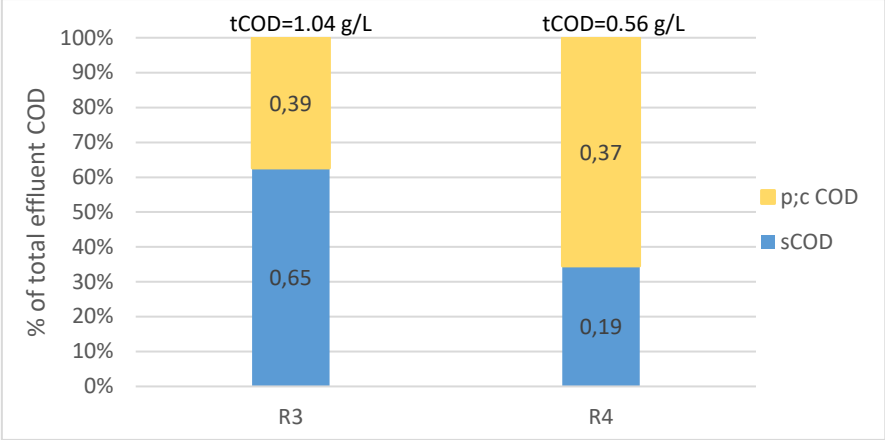


Figure 8 Sample COD fractions (g/L) in the effluent collected on day 119 for R3 and day 535 for R4. Fractions contributing to total effluent: tCOD: R3: 1.04 g/L, R4:0.56 g/L. sCOD: R3: 0.65 g/L, R4:0.19 g/L. p,c COD: R3: 0.39 g/L, R4:0.37 g/L.

3.1.4 Solids washout

Table 7 shows the average washout rate in the effluent during the experiment for R3, with gelatin, and R4, with and without total addition of tryptone. Values of washout rate were very similar for all the situations in this case. However, the washouts for R4 were a bit higher than R3. It means an increased washout. The source of this extra washout may be a disintegration of the granules due to the strength loss. The loss of strength could be the case when the amount of tryptone was less than 1.68 g/L. Also, new biomass could be produced in form of fluffy materials instead of granules which was probably the case at the end of the experiment as it is shown in Figure 9.

Table 7 Total solids washout (g VSS/d). R3: the average washout rate was since the start-up until day 162. R4: the average washout was done from day 346 until day 570. Amount of tryptone less than 1.68 g /L between days 367 and 409 and 1.68 g tryptone/L since day 409.

	Average washout rate (g VSS/d)
R3_Gelatin	0,1324
R4_1.68gTryptone/L	0,1596
R4_<1.68gTryptone/L	0,1776

Figure 9 R4 on day 563 since the start-up of the reactor. 1.68 g/L tryptone connected. A disintegration of granules was observed despite the total amount of tryptone probably because new biomass was being produced in form of fluffy materials.

3.1.5 Particle size distribution and settling properties

Before the total addition of tryptone to R4, granules started disintegrating. Figure 10 shows how tryptone affected to the granule properties. With 1.68 g tryptone/L, the fraction that cannot be flushed was higher than R4 with only 0.42 g tryptone/L. It means that granules are retained better in the reactor.

Besides, Figure 11 shows the settling properties of R3. If fraction that cannot be flushed in R3 were compared with those of R4, the result was similar than in case of R4_0.42gTryptone/L. Granules were retained in the reactor, but the performance was better when reactor had tryptone in a total amount of 1.68 g/L.

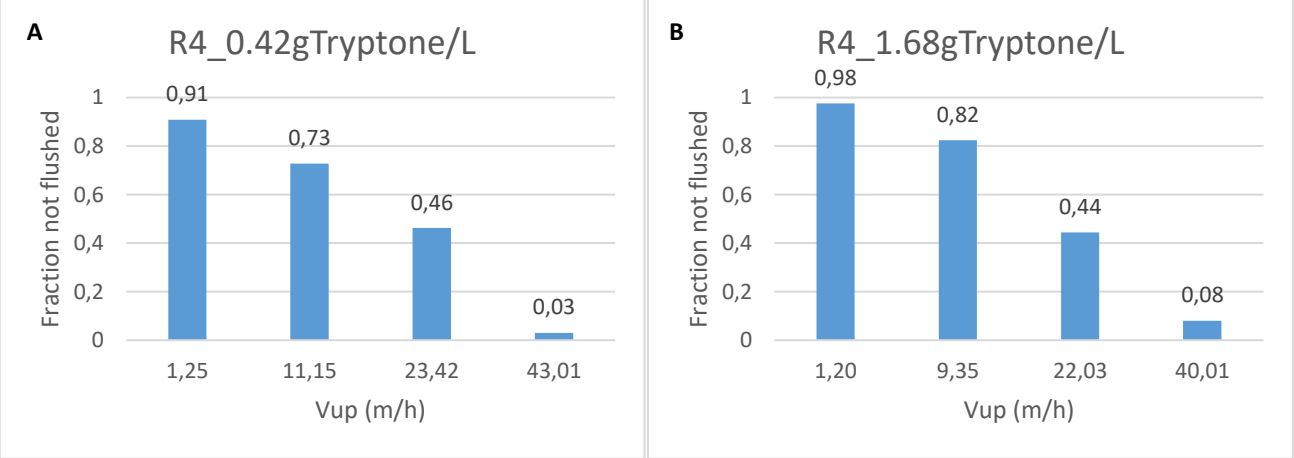


Figure 10 A: Settling properties of R4 on day 379 since the start-up (0.42 g tryptone/L). Different RPM was fixed in the pump to get different upflow velocities (m/h). B: Settling properties of R4 on day 527 since the start-up (1.68 g tryptone/L). Different RPM was fixed in the pump to get different upflow velocities (m/h).

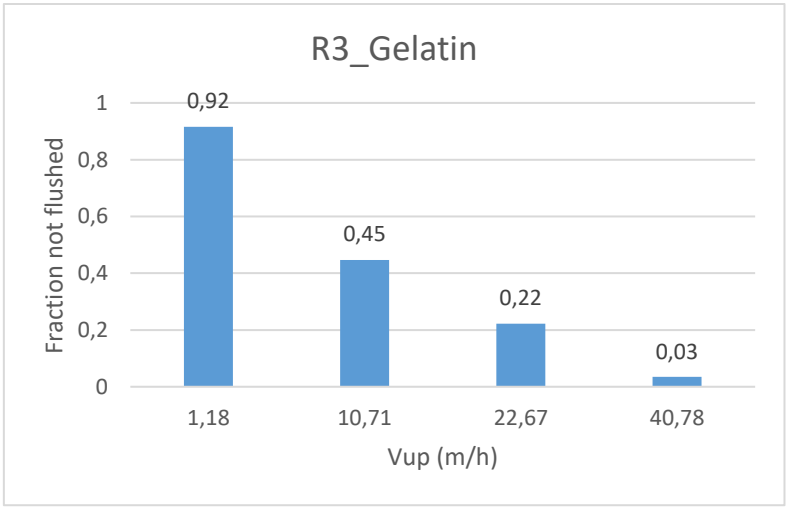


Figure 11 Settling properties of R3 feeding with gelatin on day 117 since the start-up of the reactor. Different RPM was fixed in the pump to get different upflow velocities (m/h)

For R4, the particle size distribution was done during the two different periods, with total amount of tryptone (1.68 g/L) and with an amount of tryptone lower. Figure 12 shows the performance of the granules during the experiment. It can be observed the average in the equivalent projection diameter had a similar performance in all the cases despite changes in the amount of tryptone.

In Figure 13, it is possible to observe only two days of analysis, one of them with 0.08 g tryptone/L on day 396 since the start-up of the reactor (Figure 13A), and the other with 1.68 g tryptone/L, on day 527 of analysis

(Figure 13B). Particles in R4 when the amount of tryptone was 1.68 g/L had a lower projection equivalent diameter. It could mean new biomass produced in form of fluffy materials instead of granules.

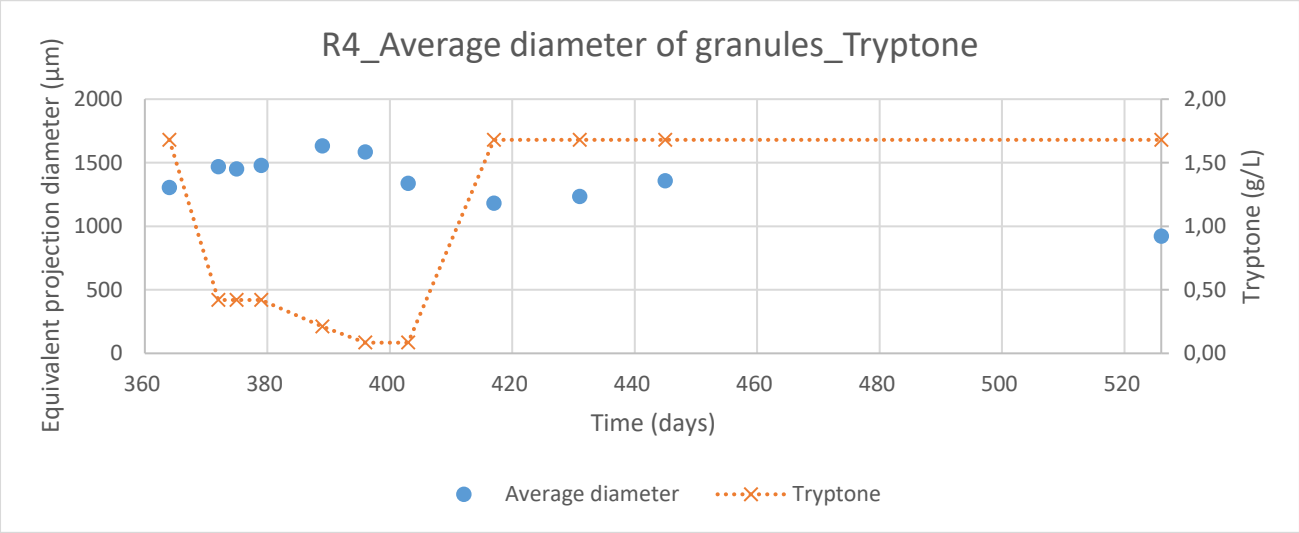


Figure 12 Particle size distribution of R4 with different grams of tryptone per litre. The plot shows a similar average in the equivalent projection diameter (µm) despite changes in tryptone.

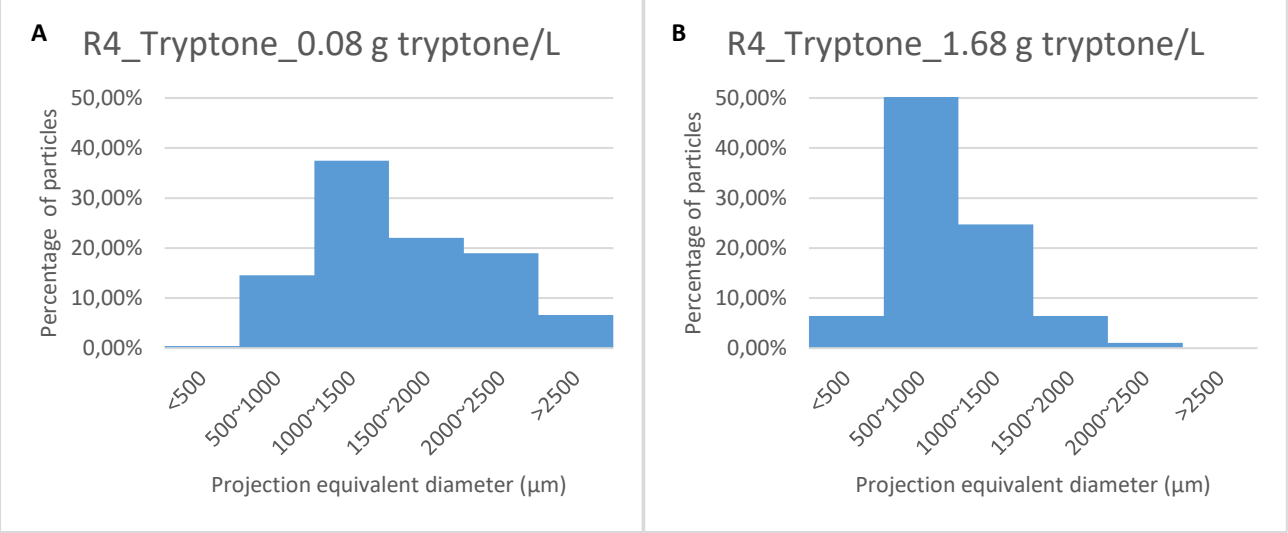


Figure 13 Particle size distribution of R4 in two different situations. A: R4 with 0.08 g tryptone/L (day 396). 227 particles were analysed. B: R4 with 1.68 g tryptone/L (day 527). 93 particles were analysed. Particles had a lower projection equivalent diameter in this case.

3.2 Start-up of R2 in comparison with start-up of R3

To compare the operation of R2 and R3, the 3 g COD/L period was taken in both cases. R3 was started on 20/12/2017. This reactor was working with gelatin. During the first 15 days, R3 was working with 3 g COD/L. On the other hand, R2 was started on 9/04/2018 with starch. During the first 53 days, R2 was working with 3 g COD/L.

3.2.1 Turbidity and total COD removal efficiency

The upflow velocity was the same in both reactors during this period (0.4 m/h) since recirculations in R2 and R3 were connected, but we did not achieve organic loading rates as high as in R3 for R2 at the same time.

It can be explained because gelatin can provide osmolites and EPS so bacterias do not need to produce it again providing a higher OLR.

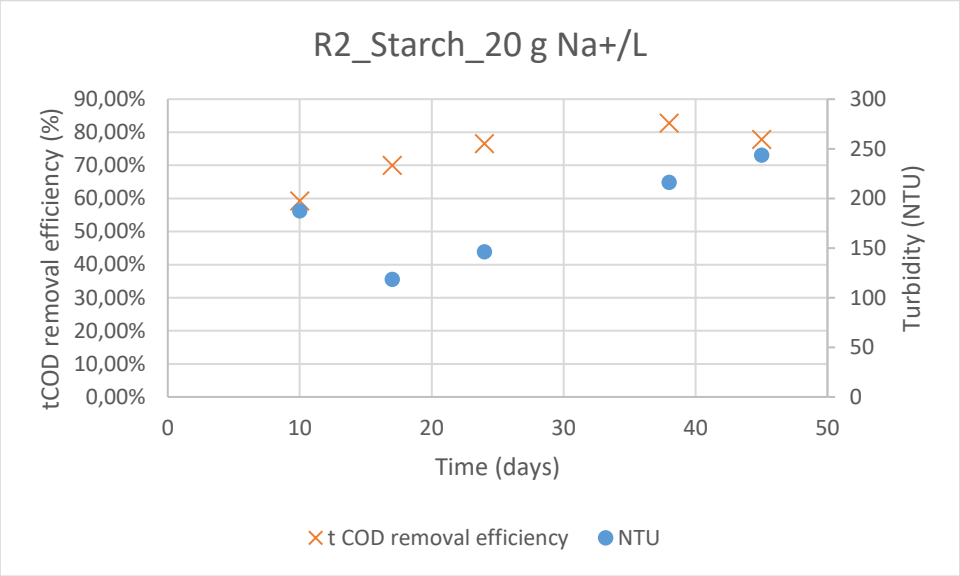


Figure 14 Turbidity (NTU) and total COD removal efficiency (%) of R2. This plot was compared with Figure 6, A, until day 15 since the start-up of R3. Average: R3-67% (gelatin), R2-73.23% (starch). The total COD removal efficiency was higher in R2 in the same period. However, OLR was higher in R3.

Figure 14 shows the total COD removal efficiency for R2 together with the turbidity. At first, in R2, the total removal efficiency was very low, 59.25%, but the efficiency was increasing during the weeks. The average removal efficiencies based on tCOD were 67% and 73.23% for R3 and R2 respectively, during the same period, 3 g COD/L. R2 contained less solids in the effluent, probably because of the lower organic loading rates.

Different substrate together with different OLR may be the reason why in R3 there were granules and in R2 not in the 3 g COD/L period. We cannot explain more about that with the data that we have now.

The turbidity was 30% higher in R3. It was a logical performance because of the higher organic loading rates.

3.2.2 Soluble COD balance

One balance was done on R2 to observe the yield of biomass and the percentage of soluble COD converted. It is necessary to consider R2 was not working in steady state because of technical issues with the feeding system, so this value should be only an indicator for future results.

The balance was done on day 52 since the start-up of R2. That day, the methane fraction was 69.3%. The COD in the influent was 3 g/L and in the effluent, it was 0.293 g/L. The COD converted in biogas was 30.44% and the yield of biomass was 69.56%. It is an abnormal value if it is compared with other in the bibliography, where the overall yields of mixed anaerobic consortia have been reported to be between 4 and 20% (Sanders 2001).

3.2.3 Soluble COD removal efficiency and biogas production rate

Figure 15 shows the soluble COD removal efficiencies for R2. It can be compared with values in R3, which are in Figure 4, A, period of 3 g COD/L (first 15 days of operation).

The average sCOD removal efficiency during the operation period studied, first 53 operation days of R2 and first 15 operation days of R3, was 75.41%±8.05% for R2. If this value is compared with R3, 91.43%±1.85%, then R3 performed at a higher removal efficiency, showing that R3, which is working with gelatin, was working better than reactor operated with starch.

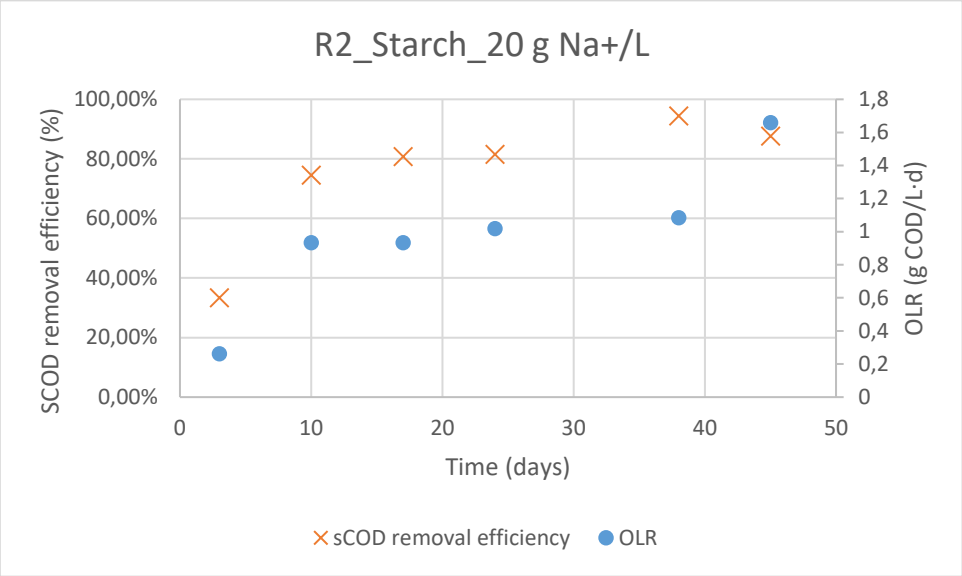


Figure 15. Performance of sCOD removal efficiencies (%) of R2 with different values of organic loading rate (g COD/L·d). This plot has been compared with Figure 4, A until day 15 since the start-up of R3. Average: R2-75.41%±8.05%. R3-94.20%±1.7%.

When R2 was started, the COD in the influent was fixed on 3 g/L and OLR was 0.26 g COD/L·d, so the removal efficiency was 33.42%. The efficiency continued increasing until values around 85%. On day 38 since the starting of R2, the removal efficiency was 94.45%, an abnormal value due to technical issues with the feeding system.

During the same period of R3, the removal efficiencies were higher than R2, around 85 and 90%. This fact can be explained because gelatin can alleviate salt stress better than starch. Besides, R2 was only working for 53 days and the feeding tube was clogged many times, which meant less organic loading rate. If the organic loading rate is lower, the soluble COD removal efficiency should be higher, but in this case, we found the opposite case. It could mean that methanogens were working less with starch as substrate.

Figure 16 shows the biogas production with starch. Despite changes in the organic loading rate, the biogas production was growing during all the experiment. However, the biogas production was 30% less efficiency in R2 if it was compared with R3, probably due to lower applicable organic loading rates.

The average for the methane content in R2 was 69.6±2.42%. In the same period of R3, 3 g COD/L in the effluent, the methane content was 73%±2.4%. The different may be due to the different substrate used for each reactor, which produce different amount of CH_4 (Wagner, Hohlbrugger et al. 2012).

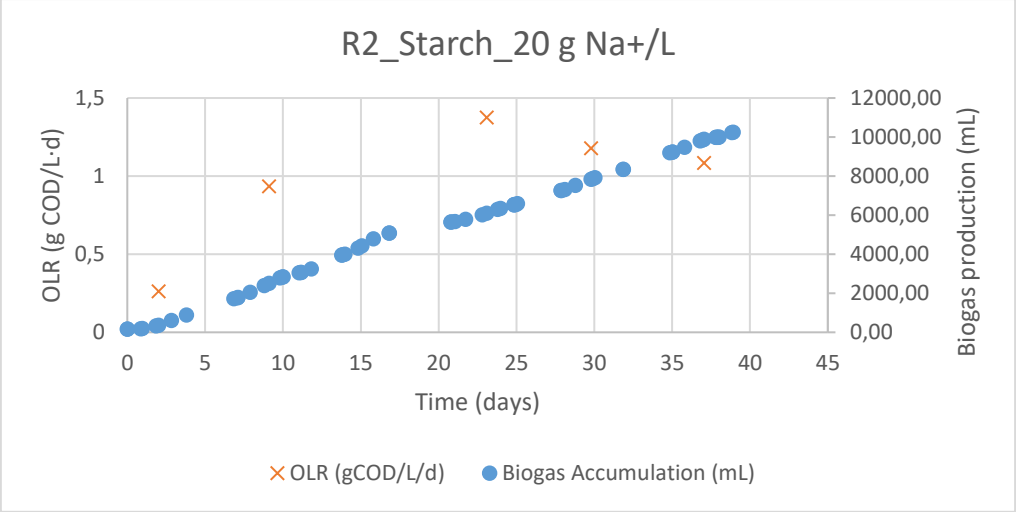


Figure 16 Biogas production (mL) for R2 with different values of organic loading rate (g COD/L·d). This plot was compared with Figure 5, A until day 15 since the start-up of R3. The biogas production continued growing despite changes in the organic loading rate. R3 produced 80% more biogas when OLR was increasing. R3 produced 30% more amount of biogas during the same period of R2.

3.2.4 Solids washout

To compare the solids washout rate in R2 and R3, it is necessary to use the same period: with 3 g COD/L in the influent, both recirculations connected and the same upflow velocity (0.4 m/h). The recirculation in R2 was started on day 25 since the start-up of the reactor and R3 recirculation was started on day 13 since the start-up of the reactor. The average of washout rate in R2 was lower than R3 as it is shown in Table 8. This is a normal performance, because in R2 the organic loading rate was lower, as it was explained in section 3.2.1. If the organic loading rate is lower, the effluent contains less solids because less medium is used. Other explanation could be the different substrate, but it is not possible to explain more about that, because we do not have enough data.

Table 8 Total solids washout (g VSS/d). The average washout rate was done in the same period: 3 g COD/L in the influent, recirculations connected and same upflow velocities (0.4 m/h).

	Average washout rate (g VSS/d)
R2_Starch	0,0092
R3_Gelatin	0,0525

3.3 Effects of Ca^{2+} dosing in R1

In this section a comparison between R1 without and with dosing of 100 mg Ca^{+2}/L will be done. Calcium ion was added to R1 on day 391 of stable operation. R1 was decreasing its activity until day 495, when the reactor was stopped. Tryptone was added in the same amounts during all the process (1.68 g/L). Granules disintegrated after adding Ca^{2+}. White precipitates were observed on the top of the reactor once Ca^{2+} was dosed. An XRD was done, and the results suggested that these precipitates contained calcium ion and phosphate ion. It is known that small amounts of these ions can contribute to the granulation so the precipitates may suggest the lack of these ions could be harmful for the activity.

3.3.1 Turbidity and total COD removal efficiency

A comparison of total COD removal efficiency between both situations in R1 was done in Figure 17.

Before adding Ca^{2+}, total COD was maintained stable. The average was 94.74%±1.36%. However, when Ca^{2+} was added to R1 medium, the total COD efficiency started to fluctuate and decrease. The average was 88.20%±3.6% in that moment, showing that there were less solids before the addition of Ca^{2+}.

Turbidity in the effluent was displayed in the plot too. Changes in the turbidity was observed during all the process. However, before adding Ca^{2+}, turbidity was lower than after that which means that granules were stronger before the addition. Turbidity in day 161 and day 196 was 1036 NTU, the highest value. During final days, the recirculation of this reactor was locked continually and it had to be cleaned manually, producing changes in the turbidity in the effluent.

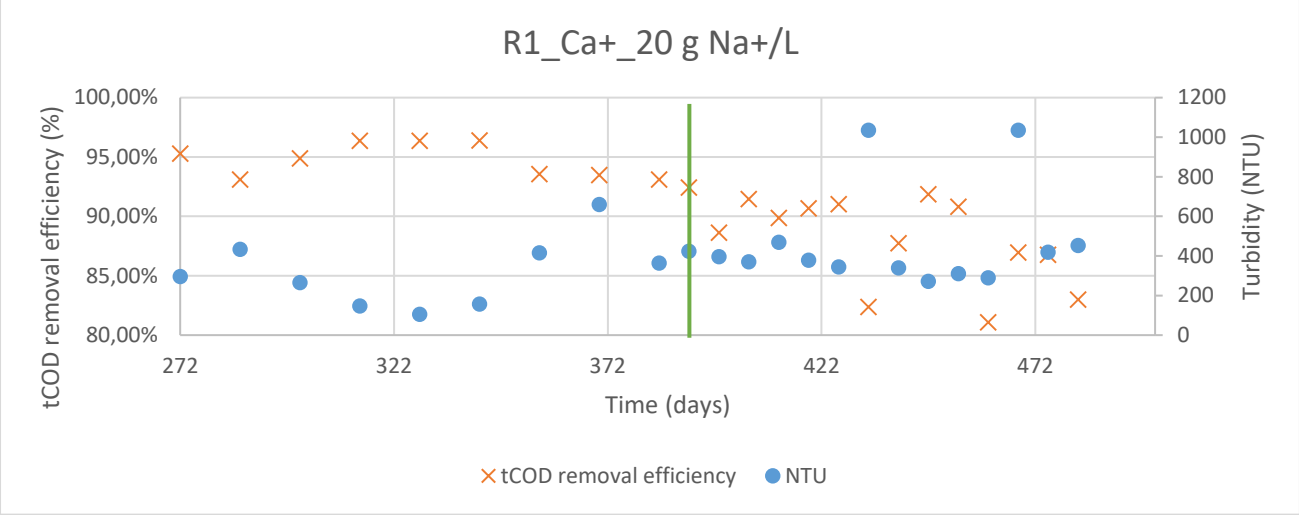


Figure 17 Turbidity (NTU) and tCOD removal efficiency (%) for R1. Green line shows the day when calcium ion was added (day 391 of stable operation). Average of tCOD: R1 without Ca^{+2} 94.74%±1.36%, R1 with Ca^{+2} 88.20%±3.6%.

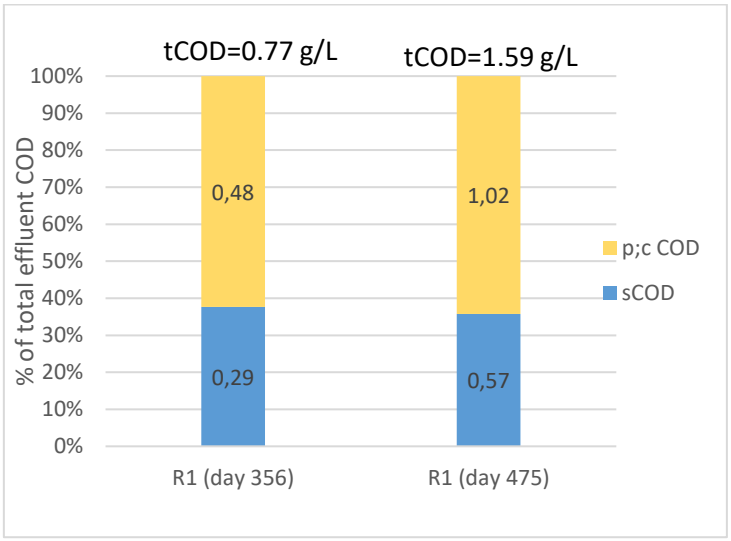


Figure 18 Sample COD fractions (g/L) in the effluent collected on days 356, without Ca^{2+}, and 454, with Ca^{2+}, of stable operation of R1. Fractions contributing to total effluent: tCOD: Day 356: 0.77 g/L, Day 475:1.59 g/L. sCOD: Day 356: 0.29 g/L, Day 475:0.57 g/L. p,c COD: Day 356: 0.48 g/L, Day 475:1.02 g/L.

As in section 3.1.3, particulate and colloidal COD were calculated with the difference between total and soluble COD (Figure 18). The values are taken on day 356 and day 475 of stable operation of R1. On day 356, R1 was feeding without Ca^{2+} and on day 475 R1 was feeding with Ca^{2+}. tCOD was 0.77 g/L on day 356 and 1.59

g/L on day 475. Soluble COD was 0.29 g/L and 0.57 g/L on days 356 and 475 of stable operation, respectively. The particulate and colloidal fractions were quite different in each case, 0.48 g/L when R1 did not have Ca^{2+} in the medium and 1.02 g/L when Ca^{2+} was added. It means that might be more polymers or salts floating around the effluent.

3.3.2 Solids washout

It is interesting knowing the average washout rate in g VSS/d in R1 for the two different situations. In this case, the average of washout rate when R1 did not contained Ca^{2+} was lower than R1 with Ca^{2+}, as it is shown in Table 9 It could mean a disintegration of the granules due to the strength loss after adding Ca^{2+}.

Table 9 Total solids washout (g VSS/d). The average washout rate is since day 292 until day 391 for R1 without Ca^{2+}. The average washout rate is since day 392 until the stop of R1 with Ca^{2+}.

	Average washout rate (g VSS/d)
R1_Without Ca^{2+}	0,0492
R1_With Ca^{2+}	0,3635

This fact can be observed in Figure 19, where a comparison between both situations was done. In Figure 19A, granules were observed perfectly. However, after adding Ca^{2+}, a total decomposition of these granules was shown (Figure 19B). It might be because the calcium ion in the granules get exchanged by sodium ion, weakened their structures. Besides, as it was suggested in the XRD, the precipitates which were found contained calcium ion. It means less amount of dissolved Ca^{+2} in the medium and a probably deterioration of the granulation.

Figure 19. A: Granules before adding Ca^{2+}. B: Disintegration of granules after adding Ca2+.A disintegration of the granules is shown.

3.3.3 Soluble COD balance

In R1, different COD balances were done at steady state with an organic loading rate around 9 g COD/L. These balances were calculated before and after the addition of calcium ion to the medium to compare the amount of soluble COD converted to biogas between both cases. Figure 20 shows the differences.

Before adding Ca^{2+}, the average of the percentages of soluble COD converted was $68.40\% \pm 8.12\%$. After the addition of Ca^{2+}, two different situations were observed. At first, the soluble COD converted increased, but when granules were decomposed, this conversion dropped until only 17.24% of soluble COD converted. It was a normal result because R1 was not working in stable state in that moment.

The yield of biomass was higher before adding Ca^{2+}. It may mean that less calcium ion dissolved could contribute to a granules disintegration or decline their activity.

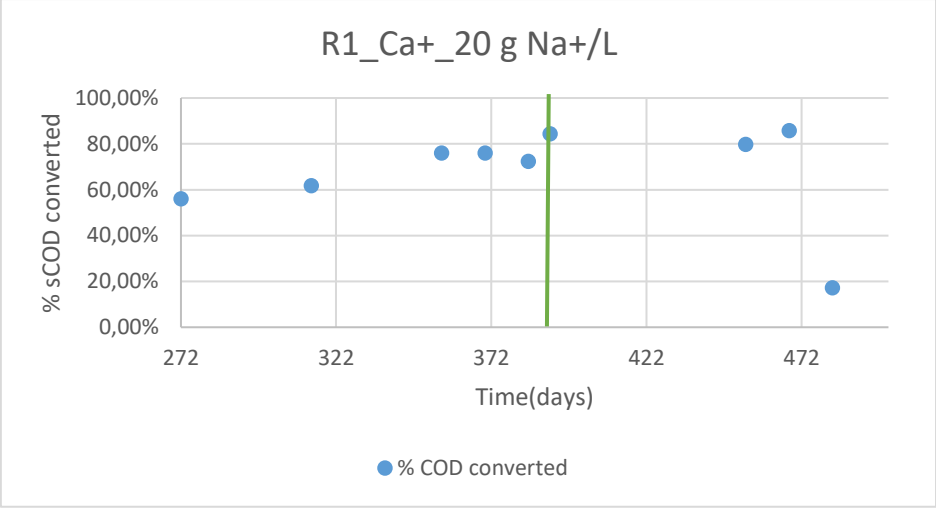


Figure 20 Percentage of COD converted in biogas. The green line divides the plot in two regions, on the left, R1 before the addition of Ca^{2+}, on the right, R1 after the addition of Ca^{2+} on day 391 of stable operation. The soluble COD experimented a lot of changes after the addition of Ca^{2+}, probably because the reactor was not working in stable conditions in that moment.

3.3.4 Soluble COD removal efficiency and biogas production rate

Changes have been produced in the soluble COD removal efficiency after adding Ca^{2+} to R1. Tryptone was the osmoprotectant during all the research in this reactor.

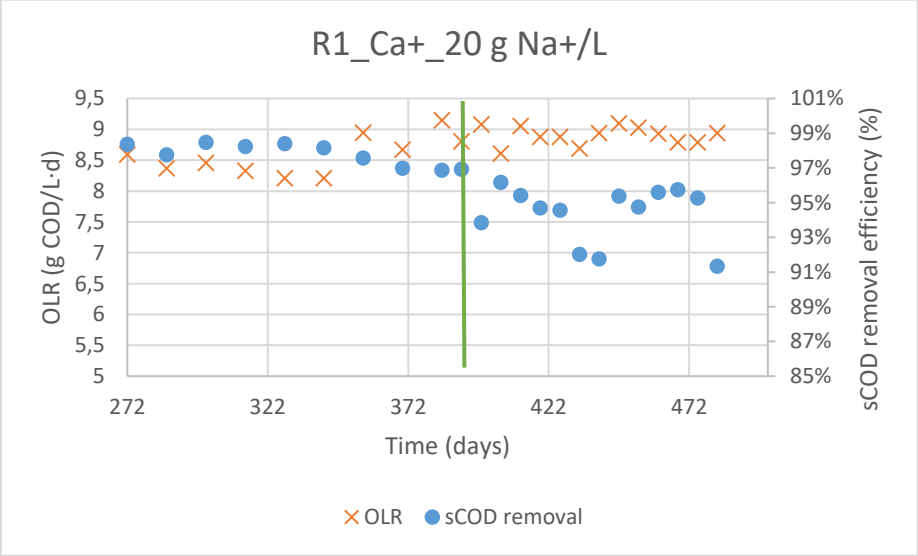


Figure 21 sCOD removal efficiency (%) in R1 for different organic loading rates (g COD/L·d). The green line divides the plot in two regions, on the left, R1 before the addition of Ca^{2+}, on the right, R1 after the addition of Ca^{2+} on day 391 of stable operation. The efficiency was approximately 3% lower than before adding Ca^{2+}.

Figure 21 shows the sCOD removal efficiency during all the process. The COD in the influent was always 12 g COD_{in}/L and Ca^{2+} was added in R1 medium on day 391 of stable operation. The removal efficiency started fluctuating greatly when Ca^{2+} was added despite the stable values in the organic loading rate. At the end of the process, the efficiency was approximately 3% lower than before adding Ca^{2+}. The decrease on the removal efficiency showed more solids in the effluent. It means a decomposed of the granules due to the lack of dissolved Ca^{+2} and PO_4^{-2} as suggested the XRD done to the precipitates where these ions were found. This fact

may be confirmed with the pH drop in the reactor which shows that methanogens were not working correctly since the addition of Ca^{2+}.

In Figure 22, the biogas production can be observed. Despite of the addition of Ca^{2+}, the biogas production continued growing during all the process in this reactor despite granules are decomposed and the reactor was not in stationary state. It could be because there was enough biomass in the reactor during this time to continue producing biogas. In Figure 23 the biogas production during the last part of experiment, when granules were disintegrated, is shown. The biogas production was 17% more slowly at that moment.

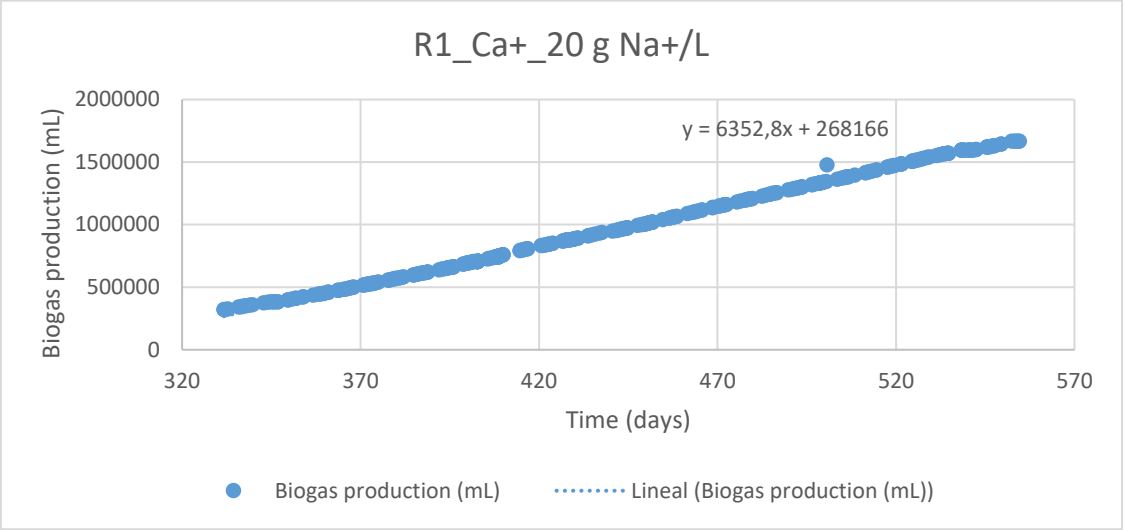


Figure 22 Biogas production (mL) of R1 since day 331 until the end of the operation period. A biogas production 17% more slowly with addition of Ca2+ can be observed. It is shown in Figure 23.

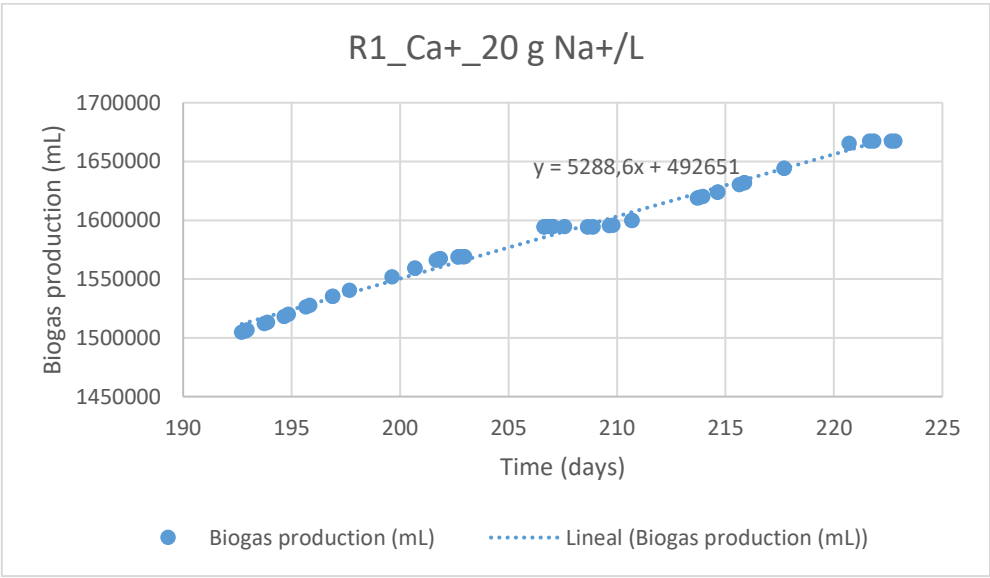


Figure 23 Biogas production (mL) of R1 during the last part of the experiment), after the disintegration of granules, since day 192. 17% less biogas was produced.

3.4 Batch experiments

3.4.1 Pressure build-up

Produced pressure during all the process was measuring to know when the experiment reached the stable state. In that moment, the process was ended. The produced pressure was calculated with:

$$P_{biomass} = P_{bottle} - P_{atm} - average(P_{blank1}; P_{blank2})$$

Where:

- $P_{biomass}$: biomass produced pressure, kPa.
- P_{bottle}: total pressure in the bottle, kPa.
- P_{atm}: atmospheric pressure, kPa.
- P_{blank1}: pressure in the blank 1, kPa.
- P_{blank2}: pressure in the blank 2, kPa.

Most of the triplicates showed similar trends. In Figure 24 produced pressure can be observed until stable state in R5. In the figure, a lag phase was observed for 7 hours. R5 process lasted around 311 hours, but 180 hours after starting, the process was stable.

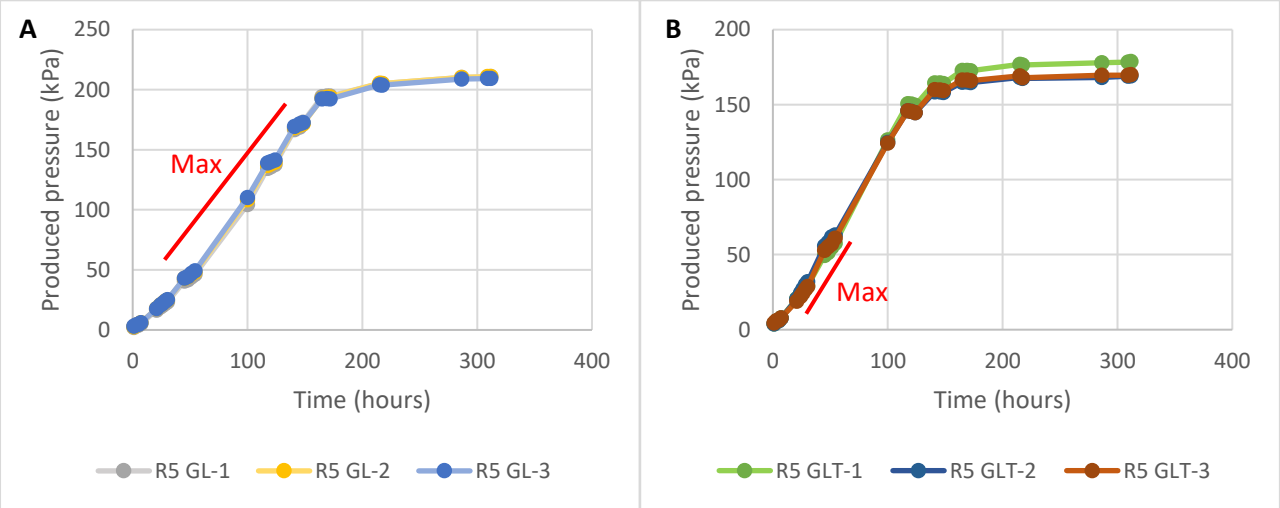


Figure 24. A: pressure build-up R5 for bottles with only glucose (GL). B: pressure build-up R5 for bottles with glucose and tryptone (GLT). The maximum slope (kPa/h) is indicated in both cases with the word "max" and a red line. The experiment ended after 311 hours, but 180 hours after starting the process was stable.

Figure 25 shows the produced pressure during the experiment in R20. In this case, lag phase was not observed, which means biomass is more active from the start of the experiment than R5. In R20, there were higher slopes than in R5, which indicates more methane production. R20 process lasted around 311 hours, but 150 hours after starting, the process was stable.

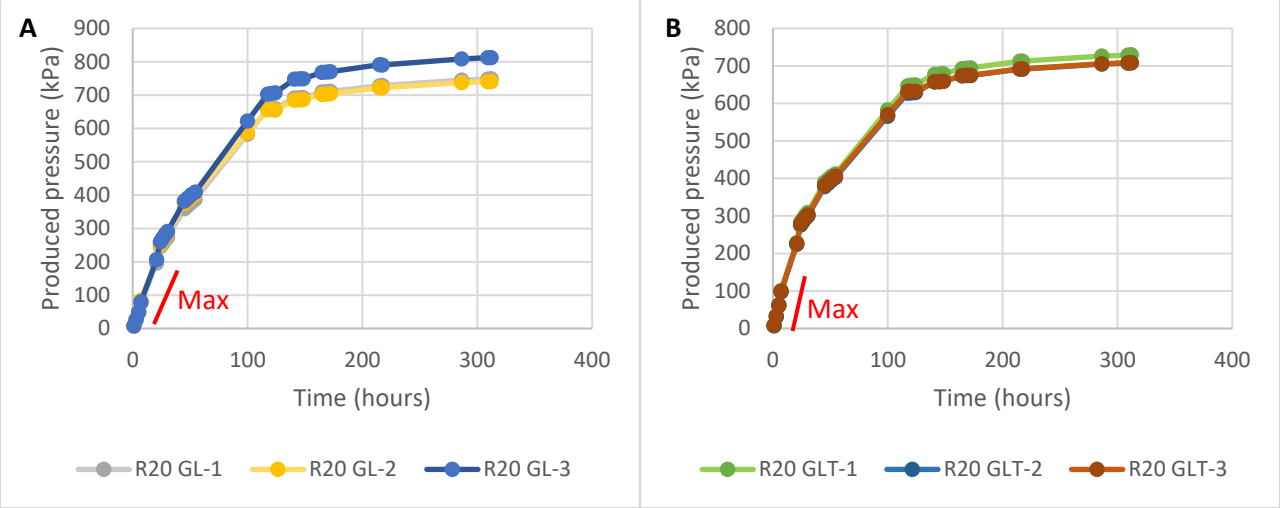


Figure 25 A: pressure build-up R20 for bottles with only glucose (GL). B: pressure build-up R20 for bottles with glucose and tryptone (GLT). The maximum slope (kPa/h) is indicated in both cases with the word "max" and a red line. The process ended after 311 hours, but 150 hours after starting the process was stable.

3.4.2 Effect of tryptone

Acidifiers are responsible to produced propionate and acetate from glucose and then, acetate is converted into methane by methanogens. To obtain the glucose and VFA results, liquid samples were measured in duplicate in different moments: at the beginning of the experiment, at the end and when the pressure slope was maximum because that was the moment of maximum VFA and glucose production rates. The averages and standard errors was calculated to study the performance. Glucose results are specified in Annex, Table 12 and VFA results are shown in Annex, Table 13 and Table 14.

Methane fractions (Table 10) were obtained with a gas chromatography and SMAs was calculated using the maximum slope for pressure build-up curve, section 3.4.1, Figure 24 and 25. The pH was measured at the beginning and at the end of the experiment (Table 11).

Table 10 Methane fractions at the end of the experiment. Methane composition is higher in the bottles without tryptone in R5. However, methane composition is higher in the bottles with tryptone in R20. It indicated more methanogenic activity in R20.

Methane fraction			
Biomass 5 g Na+/L		Biomass 20 g Na+/L	
Blank1	0,092	***Blank1***	0,106
Blank2	0,08	***Blank 2***	0,121
GL-1	0,116	***GL-1***	0,484
GL-2	0,12	***GL-2***	0,492
GL-3	0,123	***GL-3***	0,491
GLT-1	0,087	***GLT-1***	0,514
GLT-2	0,076	***GLT-2***	0,519
GLT-3	0,067	***GLT-3***	0,518

Table 11 A-pH before the incubation. B-pH after the incubation. There was a higher difference in R5 where the pH at the end of the experiment was lower, meaning that methanogens could not have been working in a good way. The difference in R20 was very low meaning that methanogens worked in a good way.

A	R5		R20	
	Gas	Liquid	Gas	Liquid
Blank1	7,83	7,83	7,66	7,66
Blank2	7,84	7,84	7,69	7,69
GL-1	7,36	7,39	7,12	7,2
GL-2	7,36	7,39	7,12	7,1
GL-3	7,38	7,4	7,13	7,12
GLT-1	7,23	7,25	7,09	7,1
GLT-2	7,22	7,25	7,11	7,1
GLT-3	7,22	7,24	7,11	7,1

B	R5		R20	
	Gas	Liquid	Gas	Liquid
Blank1	7,59	7,59	7,38	7,38
Blank2	7,6	7,6	7,36	7,36
GL-1	4,91	5,13	6,04	6,2
GL-2	4,91	5,23	6,03	6,21
GL-3	4,93	5,32	6,02	6,22
GLT-1	4,89	5,15	6,09	6,24
GLT-2	4,85	5,25	6,09	6,24
GLT-3	4,81	5,29	6,09	6,24

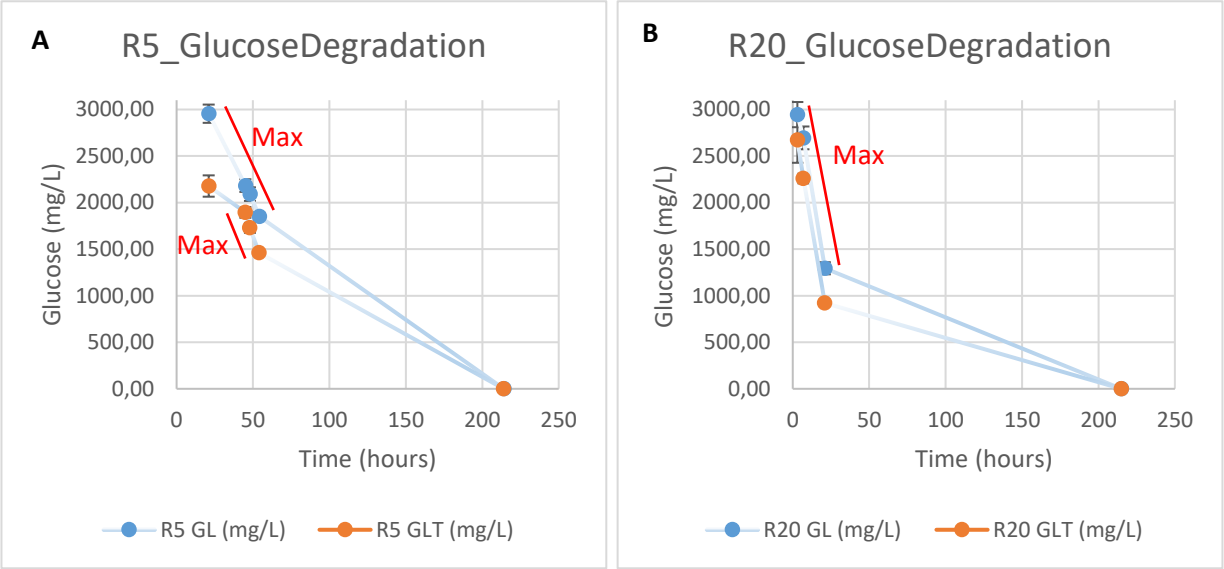


Figure 26 Glucose degradation. A: R5- GL and GLT after the lag phase (hour 21), in the maximum pressure slope (hours 45, 48 and 54) and in steady state conditions (hour 214). The glucose degradation rate was obtained in the maximum slope (indicated in the plot with a red line and the word "max"): -32.056 mg/L·d (GL) and -47.859 mg/L·d (GLT). B: R20-GL and GLT at the beginning of the experiment (hour 3), in the maximum pressure slope (hours 7 and 21) and in steady state conditions (hour 215). The glucose degradation rate was obtained in the maximum slope (indicated in the plot with a red line and the word "max"): -93.875 mg/L·d (GL) and -96.893 mg/L·d (GLT). The black bars are the bar errors and they indicate maximum and minimum value measured during the experiment. The values are shown in Annex, Table 12.

Figure 26A shows the glucose degradation during the experiment in R5. At the beginning, there were a big amount of glucose and the amounts of acetate and propionate (Figure 27A and Figure 28A) were very low. During the process, glucose decreased its value and acetate and propionate started to increase, meaning that acidifiers were working in a good way. At the end of the experiment, there was no glucose, and an accumulation of volatile fatty acids (acetate and propionate) was shown.

VFAs accumulation could explain the low values of specific methanogenic activity (Figure 29A). It suggested that acidifiers had a higher activity than methanogens, more accumulation of H_2, acidifying the medium before methanogens could use the substrate. In other words, the activity of methanogens could have been inhibited by the acidifiers. The higher activity of acidifiers was confirmed when pH was measure and low values were registered. It dropped from 7.3 to 5.2, and with this pH, methanogens cannot work.

The glucose removal-rate were higher in the bottles with glucose and tryptone, -47.859 mg/L·d, compared with the ones with only glucose, -32.056 mg/L·d, which meant that the glucose fermentation was 40% faster when tryptone was present. Volatile fatty acids were higher in the bottles with tryptone too, and of this form, the specific methanogenic activity was lower, 0.014 g COD_{CH4}/g VSS·d, compared with the ones with only glucose, 0.021 g COD_{CH4}/g VSS·d. That fact involved a faster inhibition of methanogens by acidifiers.

In R5, the granules were acclimated to 5 g Na^+/L and up-shocked to 20 g Na^+/L and it was concluded that tryptone could have improved the glucose fermentation rate.

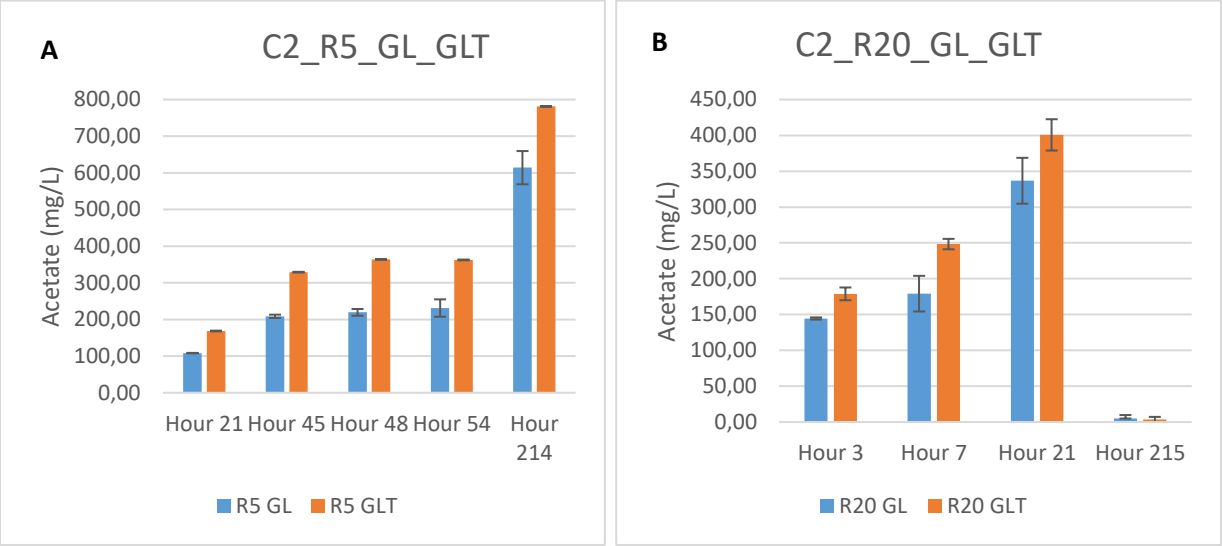


Figure 27 VFA. Acetate. A: R5- GL and GLT after the lag phase (hour 21), in the maximum pressure slope (hours 45, 48 and 54) and in steady state conditions (hour 214). A bigger amount of acetate in the bottles with tryptone is shown. B: R20-GL and GLT at the beginning of the experiment (hour 3), in the maximum pressure slope (hours 7 and 21) and in steady state conditions (hour 215). A bigger amount of acetate in the bottles with tryptone is shown, except at the end of the experiment. The black bars are the bar errors and they indicate maximum and minimum value measured during the experiment. The values are shown in Annex, Table 13.

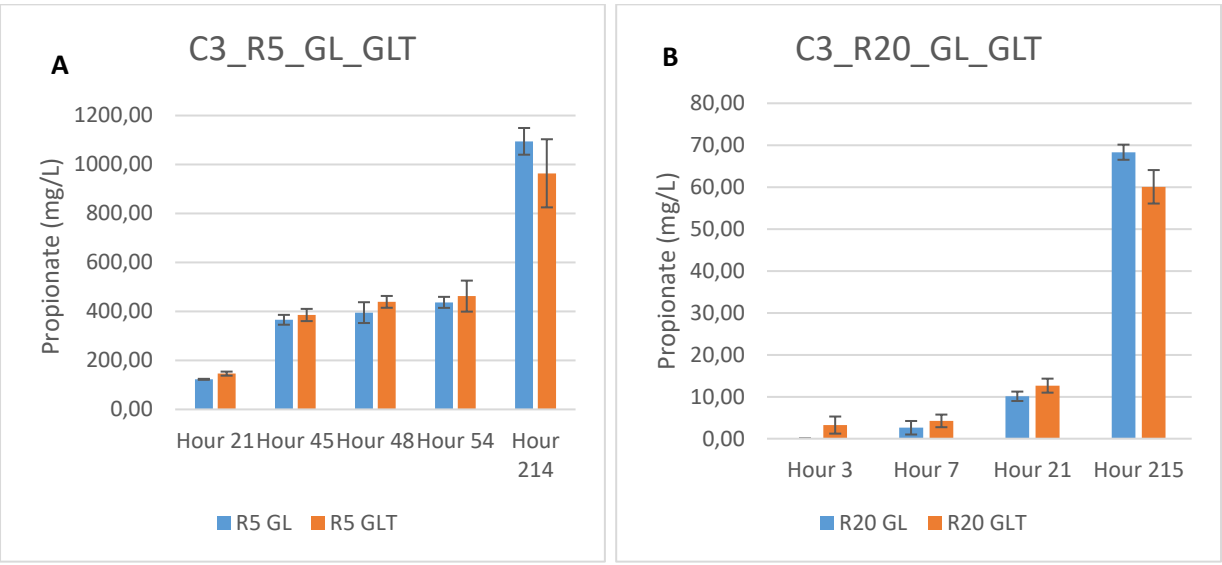


Figure 28 VFA. Propionate. A: R5- GL and GLT after the lag phase (hour 21), in the maximum pressure slope (hours 45, 48 and 54) and in steady state conditions (hour 214). A bigger amount of propionate in the bottles with tryptone is shown. B: R20-GL and GLT at the beginning of the experiment (hour 3), in the maximum pressure slope (hours 7 and 21) and in steady state conditions (hour 215). A bigger amount of propionate in the bottles with tryptone is shown, except at the end of the experiment. The black bars are the bar errors and they indicate the standard error. The values are shown in Annex, Table 14.

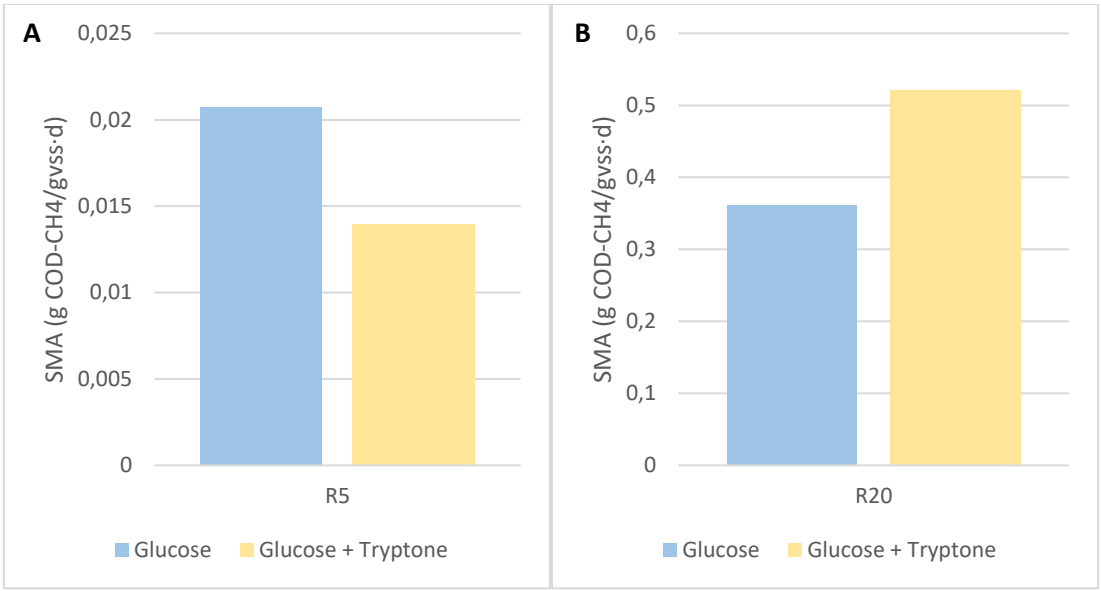


Figure 29 Specific methanogenic activity. A: R5, glucose and glucose+ tryptone. Methanogens were working better in the bottles without glucose, however the activity was very low in both cases. B: R20, glucose and glucose+ tryptone. Methanogens were working better in the bottles with glucose.

Respect R20, Figure 26B shows the glucose degradation during the experiment. At the beginning, there were a high amount of glucose, however, the amounts of acetate and propionate (Figure 27B and Figure 28B) were very low. During the process, glucose decreased its value and acetate and propionate started to increase as it happened in R5. At the end of the experiment, we did not find glucose and acetate and only a small of propionate were found.

When methane production was analyzed, R20 granules had a very significant specific methanogenic activity (Figure 29B), meaning that methanogens were working during all the process until the acetate and propionate was converted. These results can also be explained studying the pH, which decreased only from 7.1 to 6.2.

In R20, granules were acclimated of 20 g Na^+/L of matrix and with no salinity stock solution applied. The glucose removal rate was similar in both cases, -93.875 mg/L·d in the bottles with glucose and -96.893 mg/L·d in the bottles where tryptone was added, indicating a similar glucose degradation so it was concluded that tryptone could not have improved the glucose fermentation rate.

The amount of acetate was higher in the bottles with tryptone, though, at the end of the experiment, acetate was not present in the samples. The plots showed a faster accumulation of acetate when tryptone was present but SMA values are higher too, 0.521 g COD_{CH4}/g VSS·d compared with the bottles with only glucose, 0.362 g COD_{CH4}/g VSS·d. It means that tryptone could have improved the methanogenic activity under an elevated salinity of 20 g Na^+/L, reducing sodium toxicity and increasing the methane production.

4. Conclusions and recommendations

During this experiment, four UASB reactors were working with a salinity of 20 g Na^+/L. UASB reactor started-up with tryptone (R4) showed a better performance (COD removal efficiency and biogas production) and stronger granules that the one which was working with gelatin (R3). However, reactor feeding with starch showed lower removal efficiency and less biogas production compared with the one with gelatin. It was easier to get higher organic loading rates in the reactor with gelatin as substrate instead of starch, producing more solids in the effluent.

After some weeks of operation, the reactor feeding with tryptone showed a disintegration of the granules and new biomass was produced in form of fluffy materials. When the amount of tryptone was decreased in the reactor, the turbidity increased, showing less strength in the granules or even a disintegration of them. The biogas production decreased too.

Different amounts of CH_4 were achieved with different substrate.

With an organic loading rate of 3 g COD/L in the influent, granules were not observed in the reactor started-up with starch.

One of the reactors was feeding with calcium chloride dehydrate together with tryptone. After some weeks of study, it was concluded that the lack of Ca^{2+} and PO_4^{+-} was harmful for the reactor performance (COD removal and biogas production) producing a disintegration of the granules. Methane production decreased too.

Maintaining a salinity of 20 g Na^+/L, batch experiments showed a total glucose degradation. A higher specific methanogenic activity was got when tryptone was added as osmoprotectant which were related with more methane production and a higher pH. The glucose removal rate was similar with and without tryptone. When the salinity was increased from 5 g Na^+/L to 20 g Na^+/L, a total glucose degradation was observed. A drop in the pH was measured and less specific methanogenic activity was calculated. It was related with more accumulation of volatile fatty acids and less methane production.

The activity in reactor 2 should be monitored during more time to understand the role of a polysaccharide in anaerobic granulation under saline conditions.

To compare the differences between different substrates in the production of EPS, a study of the amount and composition of EPS is recommended.

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Appendix

For all the stock solutions the procedure was the same. At first, an original medium matrix was prepared without COD and osmoprotectants and with the properly salinity. Then, the stocks solutions were prepared with different volumes of this matrix and different substances (glucose monohydrate, ammonium carbonate, tryptone or sodium chloride).

Table 3. Original medium matrix and stocks solution with 20 g Na+/L.

Original Medium Matrix

Substance	To add per L
Macro, ml	6.00
Micro, ml	0.60
D-Gluc·H_2O, g	0.00
Na-Ac, g	0.00
Trypt, g	0.00
KH_2PO_4, g	0.22
$NaHCO_3$, g	1.50
NaCl, g	49.83

Stocks solution	Substance	Concentration (g/L)	Final Volume (ml)
20_a	-	-	1600
20_Glu	D-Gluc·H_2O	103.13	250
20_b	$(NH_4)_2CO_3$	0.25	600
20_c	Tryptone	0.61	600
20_blank	$(NH_4)_2CO_3$	0.23	500

Table 4. Original medium matrix and stocks solutions with salinity lower and higher than 20 g Na+/L.

Original Medium Matrix

Substance	To add per L
Macro, ml	6.00
Micro, ml	0.60
KH_2PO_4, g	0.22
$NaHCO_3$, g	1.50

Stock solution (<20 g/L)	Substance	Concentration (g/L)	Final Volume (ml)
5_a_blank	NaCl	11.67	500
5_a	NaCl	11.79	1200
5g_Glu	D-Gluc·H_2O	103.13	50

Stock solution (>20 g/L)	Substance	Concentration (g/L)	Final Volume (ml)
20_up_bl	NaCl	87.98	500
	$(NH_4)_2CO_3$	0.23	
20_up	NaCl	91.28	700
	$(NH_4)_2CO_3$	0.25	
20_up_T	NaCl	90.99	700
	Tryptone	0.60	

Table 5. Pre-condition step. 5 g Na^+/L and 20 g Na^+/L. This step lasted around 24 hours

5 g Na^+/L

To add, g	_Blank	GL	GLT
Biomass	14.80	14.80	14.80
5g_Glu	0.00	0.80	0.80
5_a_blank	85.20	0.00	0.00
5_a	0.00	84.40	84.40
Total volume	100	100	100

20 g Na^+/L

To add, g	_Blank	GL	GLT
Biomass	6.70	6.70	6.70
20_Glu	0.00	0.80	0.80
20_a	93.30	92.50	92.50
Total volume	100	100	100

Table 6. Up-shock conditions. This step lasted until steady states.

From 5 g Na+/L to 20 g Na+/L			
To add, g	**_Blank**	**GL**	**GLT**
20_Glu	0.00	7.98	7.31
20_up_bl	100	0.00	0.00
20_up	0.00	92.02	0.00
20_up_T	0.00	0.00	92.69
Total volume	200	200	200

20 g Na+/L			
To add, g	**_Blank**	**GL**	**GLT**
20_Glu	0.00	8.04	7.38
20_b	0.00	91.96	0.00
20_c	0.00	0.00	92.62
20_blank	100	0.00	0.00
Total volume, g	200	200	200

Table 12 Glucose average and standard error for R5, GL and GLT, and R20, GL and GLT. In all the cases a glucose degradation can be observed. In R5, the glucose removal-rate are higher in the bottles with glucose and tryptone, however, in R20, the glucose removal-rate are similar in the bottles. Figure 26 shows these values in a plot.

		R5 GL	**R5 GLT**
Hour 21	Average (mg/L)	2954,99	2178,20
	Standard error	±97,93	±114,62
Hour 45	Average (mg/L)	2183,04	1895,01
	Standard error	±68,27	±57,52
Hour 48	Average (mg/L)	2091,29	1728,97
	Standard error	±73,10	±53,48
Hour 54	Average (mg/L)	1852,33	1459,79
	Standard error	±42,52	±8,93
Hour 214	Average (mg/L)	1,00	0,00
	Standard error	±1,00	0,00

		R20 GL	**R20 GLT**
Hour 3	Average (mg/L)	2945,37	2674,46
	Standard error	±135,19	±246,72
Hour 7	Average (mg/L)	2695,14	2259,01
	Standard error	±122,50	±52,08
Hour 21	Average (mg/L)	1294,76	921,68
	Standard error	±64,96	±12,28
Hour 215	Average (mg/L)	0,00	0,00
	Standard error	0,00	0,00

Table 13 Acetate average and standard error for R5, GL and GLT, and R20, GL and GLT. In R5, more acetate can be observed in GLT during all the process. In R20, more acetate can be observed in GLT at the beginning and in the maximum slope, however, at the end of the experiment the amount of acetate is lower in the bottles with tryptone. Figure 27 shows these values in plots.

		R5 GL	***R5 GLT***
Hour 21	Average (mg/L)	108.07	168.42
	Standard error	±0.65	±0.02
Hour 45	Average (mg/L)	208.47	328.93
	Standard error	±4.55	±26.23
Hour 48	Average (mg/L)	219.31	363.73
	Standard error	±9.20	±26.29
Hour 54	Average (mg/L)	231.09	362.41
	Standard error	±23.72	±39.02
Hour 214	Average (mg/L)	614.15	781.14
	Standard error	±45.30	±9.91

		R20 GL	***R20 GLT***
Hour 3	Average (mg/L)	144,50	178,86
	Standard error	±1.51	±8.91
Hour 7	Average (mg/L)	179.15	248.32
	Standard error	±24.86	±7.28
Hour 21	Average (mg/L)	336,67	400.69
	Standard error	±32.00	±21.84
Hour 215	Average (mg/L)	4.88	3.59
	Standard error	±4.88	±3.59

Table 14 Propionate average and standard error for R5, GL and GLT, and R20, GL and GLT. In R5, more propionate can be observed in GLT during all the process. In R20, more propionate can be observed in GLT at the beginning and in the maximum slope, however, at the end of the experiment the amount of propionate is lower in the bottles with tryptone. Figure 28 shows these values in plots.

		R5 GL	***R5 GLT***
Hour 21	Average (mg/L)	122.80	146.05
	Standard error	±2.03	±8.23
Hour 45	Average (mg/L)	365.94	385.45
	Standard error	±20.06	±24.92
Hour 48	Average (mg/L)	395.14	439.14
	Standard error	±42.48	±24.14
Hour 54	Average (mg/L)	437.08	462.53
	Standard error	±22.48	±63.36
Hour 214	Average (mg/L)	1094.55	964.03
	Standard error	±54.53	±139.12

		R20 GL	***R20 GLT***
Hour 3	Average (mg/L)	-	3.25
	Standard error	-	±2.04
Hour 7	Average (mg/L)	2.61	4.25
	Standard error	±1.61	±1.51
Hour 21	Average (mg/L)	10.11	12.65
	Standard error	±1.12	±1.67
Hour 215	Average (mg/L)	68.33	60.08
	Standard error	±1.81	±3.99