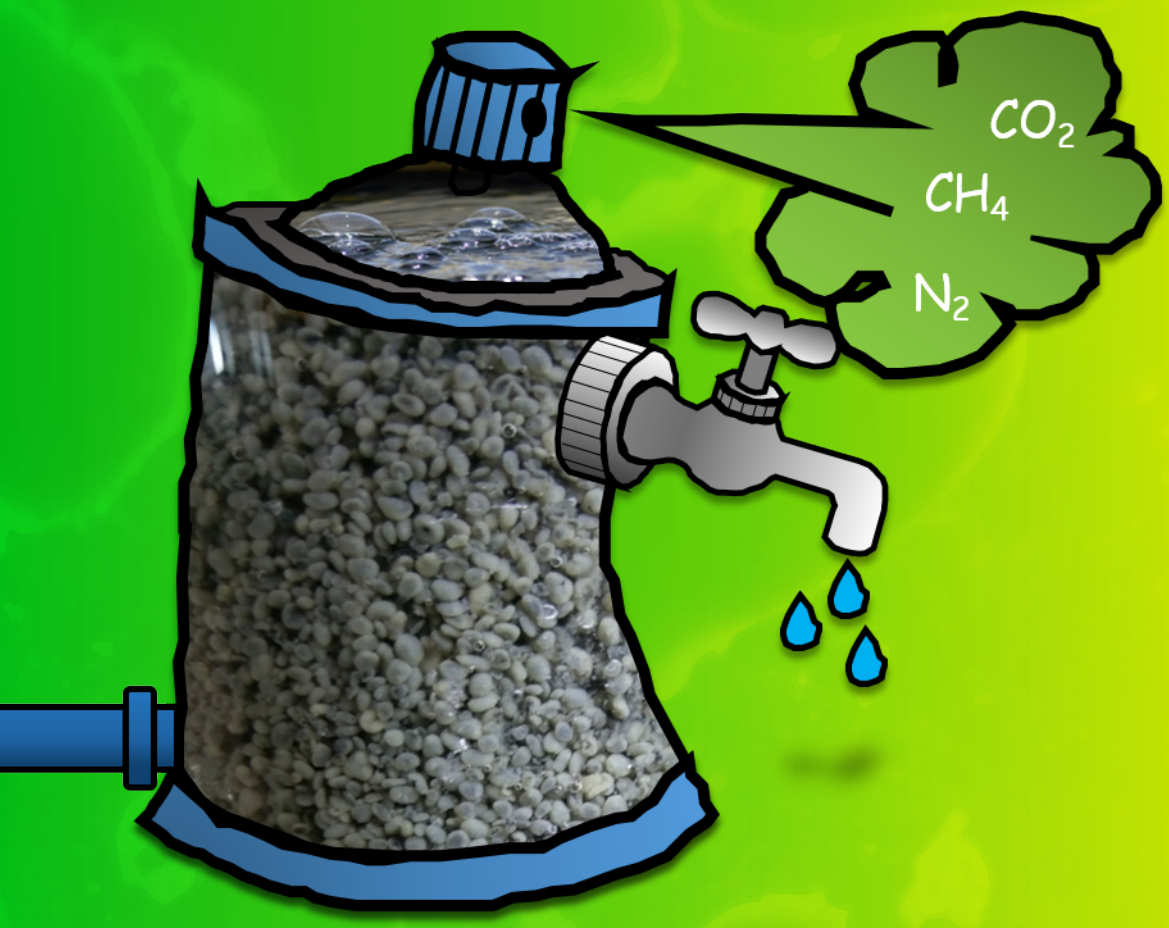
Anaerobic Sludge Granulation at High Salinity



Dainis Sudmalis

Anaerobic Sludge Granulation at High Salinity

Dainis Sudmalis

Thesis committee

Promotors

Prof. Dr H.H.M. Rijnaarts
Professor of Environmental Technology
Wageningen University & Research

Prof. Dr G. Zeeman
Personal chair, Environmental Technology
Wageningen University & Research

Co-promotor

Dr B.G. Temmink
Associate Professor, Environmental Technology
Wageningen University & Research

Other members

Prof. Dr A.J.M. Stams, Wageningen University & Research
Prof. Dr I. Smets, KU Leuven, Belgium
Prof. Dr M.C.M. van Loosdrecht, Delft University of Technology
Dr. J.C.T. Vogelaar, Paques Technology B.V., Balk

This research was conducted under the auspices of the Graduate School for Socio-Economic and Natural Sciences of the Environment (SENSE)

Anaerobic Sludge Granulation at High Salinity

Dainis Sudmalis

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 6 March 2020
at 1:30 p.m. in the Aula.

Dainis Sudmalis
Anaerobic Sludge Granulation at High Salinity
246 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2020)
With references, with summary in English

ISBN: 978-94-6395-223-1
DOI: https://doi.org/10.18174/507538

Table of Contents

Chapter 1. General Introduction 7

Chapter 2. Fast Anaerobic Sludge Granulation at Elevated Salinity 29

Supplementary Information of Chapter 2 48

Chapter 3. The potential of osmolytes and their precursors to alleviate osmotic stress of anaerobic granular sludge. 55

Supplementary Information of Chapter 3 75

Chapter 4. Proteins and Peptides in Wastewater Enhance Anaerobic Sludge Granulation at High Salinity 79

Supplementary Information of Chapter 4 100

Chapter 5. Protein Requirement for Anaerobic Sludge Granulation at High Salinity 105

Supplementary Information of Chapter 5 120

Chapter 6. Microbial community drivers in anaerobic granulation at high salinity 123

Supplementary Information of Chapter 6 143

Chapter 7. Cation exchange membrane behaviour of extracellular polymeric substances (EPS) in salt adapted granular sludge 157

Supplementary Information of Chapter 7 176

Chapter 8. General Discussion and Outlook 181

Summary 205

References 213

Acknowledgements 237

About the author 243

Chapter 1.
General Introduction

1. The Water Nexus programme and this thesis

The increasing worldwide fresh water scarcity and intrusion of saline water into fresh water aquifers, especially in delta areas, has served as a driver to create a unique research consortium – Water Nexus. This consortium aims to create a paradigm shift with respect to use of fresh water, i.e., to only use freshwater when necessary and to use saline water when possible instead. Achieving real breakthroughs with respect to the way fresh and saline water is used requires a holistic approach and involvement of different research disciplines. The Water Nexus consortium has taken such interdisciplinary group (companies, research institutes, universities and local governments) of participants under one umbrella, and the consortium as a whole deals with fresh and saline water management at different levels: i) monitoring, modelling and management of saline and fresh water at large and local scale; ii) development of technologies for sustainable treatment of saline water that enables further use of it; iii) development of integral solutions, natural systems (such as wetlands) and dynamic real time models for matching supply and demand of water with various quality requirements.

This thesis is an integral part of the technology development block within the Water Nexus consortium that deals with biological "bulk" nutrient removal from saline water streams. The work package is called "Biological nutrient removal" and was focused on the state-of-the-art biological wastewater treatment technologies for removal of macro pollutants, i.e., organics expressed as chemical oxygen demand (COD), nitrogen (N), and phosphorous (P). The removal of bulk pollutants can enable efficient further physical-chemical treatment of the water for reuse in industry or to ensure safe discharge. While this PhD research was focused on anaerobic granular treatment for sustainable removal of organics from wastewater, a second PhD project was carried out by the Technical University of Delft and focused on aerobic granular treatment of saline wastewater allowing for simultaneous COD, N and P removal. In the course of the research programme also collaborative investigations were performed to, e.g., study the potential of creating biological treatment trains of saline wastewater, where effluent of the anaerobic reactor was further treated with the aerobic technology. The microbiological aspects of the research in this thesis were studied in close collaboration with the Laboratory of Microbiology of Wageningen University, also a member of the Water Nexus consortium.

2. Wastewater treatment and upflow anaerobic sludge blanket (UASB) reactors for high strength industrial wastewater treatment

Various human activities at a household and industrial level generate polluted water streams – wastewater. These streams require treatment (purification) prior to release to the environment or reuse (Tchobanoglus et al., 2003). Usually wastewater is treated by a combination of physical, chemical and biological processes and often the biological treatment units are the core processes of the treatment facilities to remove dissolved and colloidal organic matter as well as nutrients, such as nitrogen and phosphorous (Tchobanoglus et al., 2003). This is because biological removal of bulk pollutants is often economically more viable compared to physical chemical processes (Tchobanoglus et al., 2003; Fakhru'l-Razi et al., 2009). Biological wastewater treatment relies on the activity of microorganisms that use the organic pollutants and nutrients (phosphorous and nitrogen) for energy generation and growth (metabolism) (Tchobanoglus et al., 2003; Grady Jr et al., 2011). In terms of redox conditions prevailing in these reactors, two major biological treatment technologies are currently applied: aerobic biological treatment and anaerobic biological treatment and sometimes combinations hereof (Tchobanoglus et al., 2003; Grady Jr et al., 2011). Aerobic treatment allows for removal of organic contaminants and nutrients (N and P), whereas anaerobic treatment is efficient for removal of organics and retains nutrients for potential recovery and reuse (Lettinga et al., 1980; Lettinga, 2010; Grady Jr et al., 2011). The main difference is that aerobic biological wastewater treatment requires oxygen as an electron-acceptor for oxidation of organic compounds into carbon dioxide (CO_2,) whereas anaerobic treatment does not require oxygen as an electron-acceptor and converts the organic compounds into biogas, i.e. a mixture of methane (CH_4) and CO_2 (Tchobanoglus et al., 2003). The methane that is produced can be used for energy production: approximately 1.5 kWh of electric energy can be generated per kg of COD removed and additional energy can be recovered as heat if combined heat and power turbines are used for combustion of the produced methane (Van Lier et al., 2015).

Depending on the amount of organic matter (COD), in a wastewater stream, it can be classified as low strength (COD below 1000 mg/L) or high strength (COD above 4000 mg/L)(Grady Jr et al., 2011). Typically, aerobic wastewater treatment is applied for treatment of low strength wastewaters with moderate temperatures, especially in the municipal wastewater treatment domain (Wan et al., 2016) whereas the anaerobic treatment can be applied for high strength and warm low strength wastewaters (Chan et al., 2009; Grady Jr et al., 2011). The advantages of anaerobic over aerobic process are increasing at higher COD concentrations due to smaller energy consumption and less surplus sludge produced in anaerobic compared to aerobic processes (Chan et al., 2009). This thesis will focus on anaerobic biological treatment of high strength wastewaters.

Anaerobic wastewater treatment is a well-established treatment option for industrial high strength wastewaters (Chan et al., 2009; Grady Jr et al., 2011). It can be performed in various types of reactors. A common reactor type, especially for slurries and waste streams is a continuously stirred tank reactor (CSTR) in which the hydraulic residence time (HRT) is equal to sludge residence time (SRT) and the conversion of the substrate is directly linked to the growth rate of the microorganisms. Since anaerobic microorganisms have a slow growth rate (μ_{max} 0.3 to 0.38 d-1) (Tchobanoglus et al., 2003), such systems must have a relatively long HRT. Until 1960s CSTR was the most applied system for anaerobic treatment of wastewater (Van Lier et al., 2015). Nowadays such systems (HRT=SRT) are termed as "low rate" and are mainly applied to treat streams with a high particulate chemical oxygen demand (COD) concentration, such as domestic sewage sludge, animal manure or food waste (De Mes et al., 2003).

Minimizing the size of a bioreactor and increasing its capacity in principle requires increased amounts of biocatalysts – in this case anaerobic microorganisms - in it. This can be achieved by making the SRT much longer compared to HRT (McHugh et al., 2003). Anaerobic bioreactors in which the SRT is much larger than the HRT are termed "high rate" (De Mes et al., 2003). Examples of high rate anaerobic reactors are anaerobic contact process (Schroepfer et al., 1955), anaerobic filters (Young and McCarty, 1969), anaerobic membrane bioreactors (Vrieze et al., 2014) and upflow anaerobic sludge blanket (UASB) reactors (Lettinga and Hulshoff Pol, 1991). A UASB reactor, similar to other reactors with sludge retention, relies on the formation of sludge with excellent settling properties containing highly active methanogenic microorganisms and depending on the type of wastewater the developed sludge will be granular or flocculent (Lettinga et al., 1980). When the wastewater contains a high concentration of suspended solids and these solids are entrapped by the sludge flocs, a well-settling flocculent instead of granular sludge in UASB reactors can be developed. In this case the fraction of active microorganisms in the sludge will be smaller than in granular reactors, which implies that lower volumetric loading rates should be applied (Lettinga and Hulshoff Pol, 1991; Hulshoff Pol et al., 2004). The UASB reactor design can be simple and compact (**Figure 1**). Wastewater in such a reactor flows through a sludge bed from the bottom of the reactor at an upflow velocity of 1-2 m/h for granular sludge and 0.5-1 m/h for flocculent sludge and the treated effluent is separated from the sludge and biogas at the top of the bioreactor with a three phase separator (**Figure 1**) (Lettinga et al., 1980; Lettinga and Hulshoff Pol, 1991). Since its development, granular UASB reactors and its various derivatives are by far the most applied high rate anaerobic wastewater treatment systems worldwide for treatment of various industrial wastewaters containing high soluble COD concentration (McHugh et al., 2003; Van Lier et al., 2015). The focus of this thesis is on the granular type of UASB reactors.

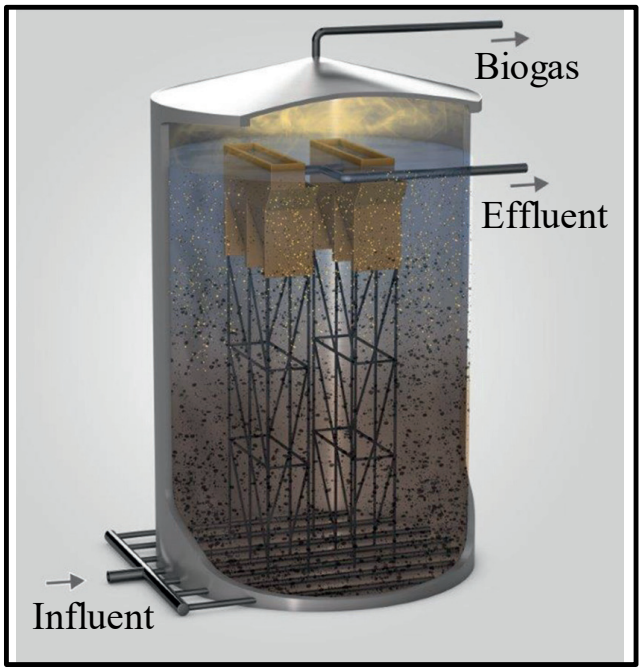


Figure 1. A schematic representation of a UASB reactor. This is a modified image of Biopaq® plus reactor from Van Lier et al. (2015).

3. Granular sludge and UASB

3.1. Mechanisms of granulation

A significant amount of research has been addressed to the underlying mechanisms of sludge granulation. These mechanisms have been discussed in excellent review articles (Liu et al., 2003; Hulshoff Pol et al., 2004). Nevertheless, even after more than 40 years of full-scale application of granular sludge technology there is no common consensus on a unified theory for sludge granulation. A common agreement though is that granular sludge is essentially a globular shaped biofilm in which a complex microbial consortium is entangled in a three dimensional matrix of extracellular polymeric substances (EPS) that are produced by the microbial consortium itself (**Figure 2**) (Lu et al., 2013). There are three commonly known theories, two of which are directly related to this structure of granular sludge.

One of the first theories, commonly known as the “selection pressure” theory was proposed by Hulshoff Pol et al. (1983) and is related to the operational mode and physical conditions in UASB reactors. The “selection pressure” hypothesis explains the granulation process as a continuous selection of heavier sludge particles that settle better and eventually serve as a nucleus for granules formation. Such selection occurs due to hydraulic and gas loading rates encountered in the UASB

reactors forcing the more voluminous and light sludge to wash-out from the reactor, while retaining the heavier particles that grow in time and eventually form granules (Hulshoff Pol et al., 1983).

The second theory relates granulation to the properties of EPS. These EPS and microorganisms are known to carry a net negative charge due to functional groups, such as carboxyl groups (Liu and Fang, 2003). It has been hypothesised that multivalent cations can reduce the electrostatic repulsion between microorganisms and EPS and bind the negatively charged groups of EPS, thereby strengthening the structure and allowing for granulation. This theory is often supported, because multivalent cations, such as Ca^{2+}, have been shown to increase the mechanical strength of biofilms (Yu et al., 2001; Flemming and Wingender, 2010).

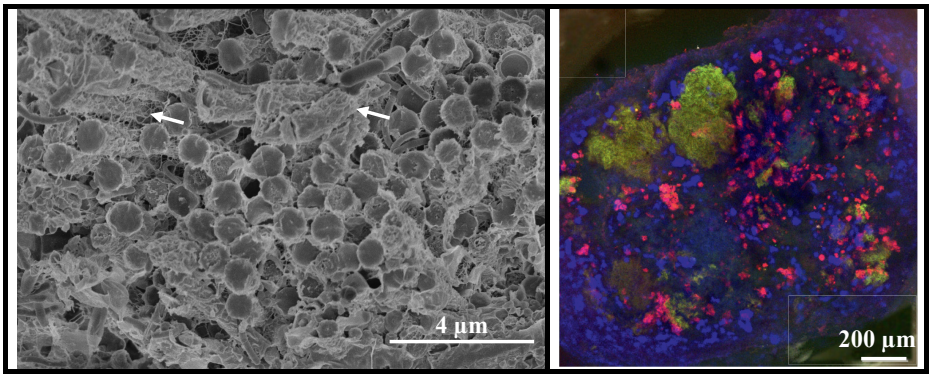


Figure 2. Scanning electron microscopy (SEM) micrograph of a sliced microbial anaerobic granule (left) and a confocal laser scanning microscopy (CLSM) micrograph of a sliced anaerobic microbial granule (right). In the SEM micrograph various morphologies of microorganisms can be distinguished and the white arrows are indicating polymeric sheets and webs within the structure. In the CLSM micrograph: blue - the distribution of methanogens, green – the distribution of fucose residues of the extracellular polymers and the red - mannose residues of extracellular polymers. SEM imaging from this thesis by D. Sudmalis. CFLSM image from Gagliano et al. (2018).

The third theory relates granulation to particular microorganisms often found in the granular sludge. Wiegant (1988) introduced the "spaghetti theory", which states that the formation of granular sludge depends on the growth of acetoclastic methanogens belonging to *Methanosaeta* (formerly known as *Methanothrix*) species in its filamentous form. These would attach to inert nuclei or small bacterial flocs and form three dimensional structures, in which other microorganisms could grow. It must be noted that also Wiegant (1988) admitted the possible important role of EPS covering the cells of *Methanosaeta* and affecting their aggregation properties. This theory is also often supported, because in most full-scale UASB reactors the filamentous acetoclastic *Methanosaeta* is found as the dominant methanogen (Rinzema et al., 1988; Narihiro et al., 2008; van Haandel et al., 2014). This is probably due to the fact that full-scale reactors are often operated at low effluent concentrations of acetate and *Methanosaeta* has a much lower affinity constant (K_s) compared to *Methanosarcina* - another wide-spread acetoclastic methanogen (Welte and Deppenmeier, 2014). This provides *Methanosaeta* with a competitive advantage to proliferate in full-scale UASB reactors.

3.2. Role of EPS in granular sludge

The roles and composition of EPS in biofilm systems, such as granular sludge, have been extensively reviewed (Harrison et al., 2007; Flemming and Wingender, 2010; Seviour et al., 2019). In short, the EPS provide a complex matrix consisting of various polymeric molecules, such as polysaccharides, proteins, nucleic acids and glycoproteins providing mechanical stability to the anaerobic granular sludge (Dolfing, 1986; Seviour et al., 2019) and acting as a protective barrier against fluctuating environmental conditions, such as pH and temperature (Strelkova et al., 2013) or against toxic compounds, such as heavy metals (Harrison et al., 2007). It has also been shown, that exposure of microorganisms to high salinity leads to increased production of EPS (Vyrides and Stuckey, 2009a; Corsino et al., 2017; Campo et al., 2018). It has been suggested this is an adaptive microbial response to osmotic stress (Vyrides and Stuckey, 2009a). At low heavy metal concentrations it is clear that toxicity is reduced due to binding of the metals to the EPS, which hinders their diffusion towards the microorganisms (Hu et al., 2007). However, the mechanism by which EPS reduce osmotic stress of microbial communities exposed to high salinities is thus far poorly studied and understood.

3.3. Enhancing the rate of granulation

One drawback of UASB reactor technology is that sludge granulation proceeds slowly and the development of granular sludge from flocculent biomass can take as long as several months (Lettinga et al., 1980; Hulshoff Pol, 1989; El-Mamouni et al., 1998; Tiwari et al., 2005). Thus, if suitable granular sludge is not available for inoculation, long start-up times are needed. This explains why many researchers have investigated factors/strategies for enhancing the rate of granulation. These strategies are often directly related to one of the abovementioned theories on granulation and granular structure and include: i) dosing of multivalent cations, such as calcium (Mahoney et al., 1987; Hulshoff Pol, 1989; Yu et al., 2001; Ahmad et al., 2011) for binding the negatively charged groups of EPS and preventing electrostatic repulsion; ii) dosing of polymers (El-Mamouni et al., 1998; Show et al., 2004) for bridging the microorganisms; iii) adding an inert carrier (Yoda et al., 1989) or small amounts of (seed) granular sludge (Hulshoff Pol, 1989) as a nucleus for initial granulation. With respect to reactor operational conditions it has been reported that the initial sludge loading rate should be kept between 0.2-0.3 gCOD/gVSS·d to enhance granulation (Ghangrekar et al., 1996; Ghangrekar et al., 2005). Finally, a superficial liquid upflow velocity between 0.5 and 2.2 m/h in UASB reactors has been reported to promote the granulation and growth of granules in size (Alphenaar et al., 1993; Arcand et al., 1994; O'Flaherty et al., 1997).

3.4. Effect of organic substrate on granulation

Obviously, the type of organic substrate together with the environmental and operational conditions will determine the microbial community in a bioreactor (Batstone et al., 2004; Lefebvre et al., 2007), and this can in turn affect the success of granulation as well as the properties of granular sludge (Batstone and Keller, 2001). The influence of organic substrate has been extensively investigated, both in terms of the rate of granulation as well as in terms of properties of granular sludge developed (e.g., size, strength) (Fukuzaki et al., 1991a; Fukuzaki et al., 1991c; Chang et al., 1993; Fang et al., 1994; Thaveesri et al., 1994; Fukuzaki et al., 1995; Tay and Yan, 1996; O'Flaherty et al., 1997; Zhou et al., 2006). However, it is difficult to interpret and compare the results of these studies because of different reactor operational parameters, various inocula that were used, supplementation of vitamin solutions or not, and differences in the composition of the nutrient medium. The common consensus seems to be that dissolved energy rich substrates, such as carbohydrates favour faster granulation compared to low energy content substrates, such as volatile fatty acids (Vanderhaegen et al., 1992; O'Flaherty et al., 1997). Also, it has been shown that granules treating saccharide-rich wastewater are stronger and have a better settleability than those treating wastewater rich in organic acids (Batstone and Keller, 2001).

4. Saline wastewaters and granular UASB reactor application

4.1. Where does saline wastewater come from and what are the challenges for granular anaerobic technology application?

Many industrial activities, such as leather tanning, vegetable pickling, dairy, fisheries, petroleum and olive oil milling produce wastewaters containing high concentration of organic pollutants and salt (NaCl) (Lefebvre et al., 2005; Lefebvre and Moletta, 2006; Sundarapandiyan et al., 2010; Xiao and Roberts, 2010; Giustinianovich et al., 2018). Fakhru'l-Razi et al. (2009) estimated that globally approximately 39.5 million m^3 of saline wastewater is generated daily by the petroleum industry. In the European Union (EU) the production of olive oil reached 10.2 million tonnes in 2017, out of which 91% was produced in Italy, Spain and Greece (Eurostat, 2018a). Production of 1 kg of olive oil, generates around 7.5 kg of wastewater (Vlyssides et al., 2004). This means that approximately 76.5 million tonnes of saline wastewater is generated by this industry in the EU alone. Approximately 56 million tonnes of milk were used for cheese production in EU in 2016 (Eurostat, 2018b). Carvalho et al. (2013) reported that the amount of cheese whey wastewater generated is roughly four times the volume of the milk processed, meaning that annually EU generates 224 million m^3 of cheese whey wastewater. The amount of wastewater generated by fish processing plants can vary in a broad range depending on the fish and the type of end-product (Chowdhury et al., 2010). Nonetheless, also this

industry is expected to generate a large amount of saline wastewater considering that the annual fish catch of the EU was 5.14 million tonnes in 2015 (Eurostat, 2018c). Leather tanneries are also expected to produce a significant amount of saline wastewater. Thirteen EU member states produced 290 million m^2 of tanned leather in 2013 (accounting for 27.4 % of the global production), whereas the average amount of wastewater produced per ton of raw hide processed is 30 – 35 m^3 (Lofrano et al., 2013). The salinity and ionic composition of different wastewaters can vary in a broad range. For example, the salt concentration expressed as total dissolved solids (TDS) in the petroleum industry wastewater can be as low as 2.6 g/L, but can also reach up to 360 g/L of TDS (Fakhru'l-Razi et al., 2009). Similarly, in the fish processing industry the wastewater salinity can reach up to 150 g/L (59 g Na^+/L) of NaCl due to use of brines in the canning process (Corsino et al., 2017), but can also be approximately 5 – 10 g Na^+/L in processes without use of brines (Feijoo et al., 1995). The highest salinity level of 20 g Na^+/L (~51 g NaCl/L) that was studied in this thesis was selected based on the experiences of the industrial project partners in Water Nexus consortium and their inventory on known saline wastewater streams.

Many of the abovementioned wastewater streams contain high COD concentrations, therefore anaerobic biological treatment is considered an attractive option for removal of organics and conversion to biogas, especially if compact reactors such as granular UASB could be applied. Granular UASB reactors for treatment of wastewaters containing high (above 10 g Na^+/L) monovalent salt concentrations are frequently considered as unsuitable (Jeison et al., 2008; Van Lier et al., 2015; De Vrieze et al., 2016a). This is typically attributed to (suspected) negative effects of high salinity on the two key requirements for application of anaerobic granular technology, i.e., microbial activity and the sludge granulation itself. However, some evidence in scientific literature suggests that these negative effects can be overcome. In *Section 4.2.* the microbial activity inhibition by salt, various ways of overcoming this inhibition and wastewater characteristics affecting the salt inhibition are presented. The reason why sludge granulation at high salinity is often reported unachievable and evidence that granulation could potentially be possible even at such extreme conditions is explained in *Section 4.3*.

4.2. Inhibition of methanogenic activity: why can salt be a challenge?

The anaerobic digestion process involves a complex microbial community in which methanogens perform the last conversion step of acetate and/or H_2 plus CO_2 into methane (O'Flaherty et al., 2006). Acetoclastic methanogens have been frequently reported to be the most sensitive microorganisms of the anaerobic consortia and methanogenic activity is often inhibited when exposed to high salinity (Na > 10 g/L) (McCarty and McKinney, 1961; Rinzema et al., 1988; Liu and Boone, 1991; Boardman

et al., 1995; Jeison et al., 2008; Fang et al., 2011; De Vrieze et al., 2016a). Thus, one of the focuses of this research is on methanogenic activity upon exposure to high salinity. The decrease of methanogenic activity is typically related to the osmotic stress that methanogenic cells experience. When a microbial cell is surrounded by a low concentration of solutes as shown in **Figure 3-A**, water can diffuse through the semipermeable cytoplasmic membrane (permeable to water but not to many solutes) of the cell thereby exerting pressure (turgor) on the cell wall. Turgor pressure is known to be important for cell growth and division (Oren, 2013). Microbial cell walls are rigid and can withstand pressures up to 100 atm (Csonka, 1989). Exposure of the microbial cell to a high concentration of solutes in the surrounding environment (**Figure 3-B**) can cause shrinkage of the cytoplasm due to diffusion of water outside of the cell, a process known as plasmolysis. This process decreases water activity and thereby also turgor pressure within the microbial cell and can impair physiological processes, including nutrient uptake and cell division (Csonka, 1989).

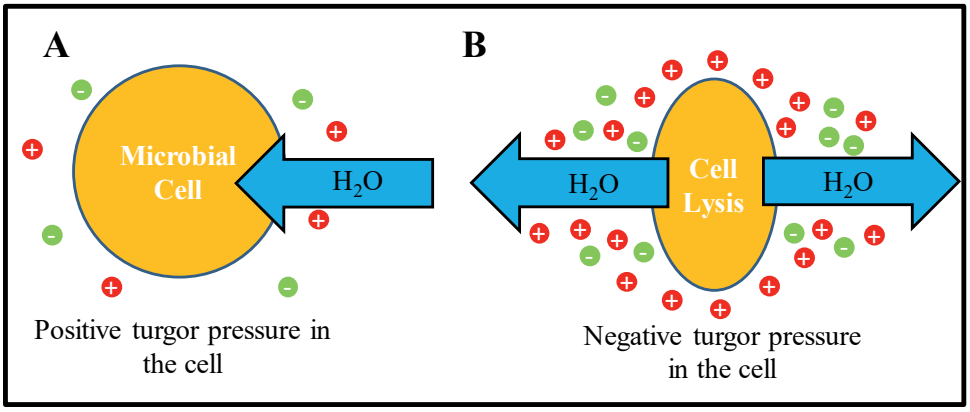


Figure 3. A microbial cell with a positive (A) and a negative (B) turgor pressure. (N.B. This is a simplified graphical representation, based on Csonka (1989)).

4.2.1. Microbial strategies to overcome osmotic stress

The salinity level at which cell plasmolysis would occur can vary in a wide range, depending on the type of microorganism. Actually, growth of many prokaryotic cells is even dependent on a certain salt concentration in the surroundings and microorganisms exist that thrive at levels as high as salt saturation (Oren, 2013). Thus, microorganisms can be classified based on their growth dependence on salt, i.e., at which salt concentration they grow best (**Table 1**). A special group of microorganisms in this classification scheme are the halotolerant microbes. In principle, they are non-halophiles (**Table 1**), i.e., do not require high salt concentrations for growth, but are able to tolerate salt and grow at very high salinity levels (higher than 2.5 M) (Oren, 2013).

Two very different strategies for prokaryotic cells to adapt to increasing salinity have been reported in literature. The first is called "salt in" strategy. Microorganisms using this adaptation mechanism accumulate high concentrations of KCl salts (in rare exceptions NaCl) in order to balance the osmotic pressure between the surrounding environment and cell cytoplasm. The accumulation of KCl requires for adaptation of the cell's enzyme machinery and is restricted to extremely halophilic *Halobacteriales* (aerobic Archaea) and halophilic anaerobic *Haloanaerobiales* (bacteria) (Oren, 1999; Roeßler and Müller, 2001; Oren, 2013). These microorganisms cannot efficiently adapt to changing environmental conditions, because most of their salt-adapted enzymes and proteins require constant presence of salt for their structural stability (Oren, 2013). Upon exposure to salt concentrations below 1-2 M, the proteins of such microorganism denature (Oren, 2013). To date no extreme halophilic acetoclastic methanogen relying on "salt in" strategy has been isolated (**Table 2**). However this mechanism is less relevant for the wastewater types considered in this thesis (*Section 4.1*).

Table 1. Classification of microorganisms based on optimum salinity for growth. Table from Oren (2013)

Category	Grows best at, M of salt
Non-halophilic	Less than 0.2
Slight halophile	0.2-0.5
Moderate halophile	0.5-2.5
Borderline extreme halophile	1.5-4.0
Extreme halophile	2.5-5.2
Halotolerant	Non-halophile which can tolerate salt. Those growing above 2.5 M are extremely halotolerant

The second strategy of dealing with salinity by microbial cells involves accumulation of small extremely soluble organic molecules, named compatible solutes or osmolytes, and this is a mechanism used by most prokaryotic cells. This strategy can be divided into two phases (**Figure 4**). During the first phase, a fast uptake of potassium into the microbial cell is triggered. During the second phase the intracellular potassium concentration is decreased by replacing it with compatible solutes (osmolytes) (Pflüger et al., 2005). Compatible solutes are preferred over potassium, because they do not interfere with the central metabolism of the cells and allow them to survive in a broader range of salinities (Pflüger et al., 2005). For example, the non-marine methanogen *Methanosarcina thermophila* can grow in a salinity range between 0.05 and 1.5 M of NaCl (1.5 – 34.5 g Na^+/L), while accumulating compatible solutes for osmoadaptation (Sowers et al., 1990). Also, *Methanosarcina barkeri*, *Methanosarcina mazei* and *Methanosarcina vacuolata* (strains of *Methanosarcina spp.*) were isolated from sewage sludge digesters (salinity below ~ 0.15 M of external solutes) and shown to be able to grow at salinity levels of up to 1.0 M of NaCl (23 g Na^+/L) by accumulating compatible solutes

(Sowers and Gunsalus, 1995). Similar behaviour has been reported by a thermophilic methanogen *Methanococcus thermolithotrophicus* (Martin et al., 2000).

Table 2. Methanogenesis at extreme salinities.

Methanogenic Substrate	NaCl concentration, M	NaCl, g/L	Microorganism	Reference
$H_2 + CO_2$	2.05	120	*Methanobacterium alcaliphilus*	(Ollivier et al., 1998)
Methylotrophic	3.4	200	Related to genus *Methanohalophilus*	(Ollivier et al., 1998)
Methylotrophic	3.5	*	*Methanosalsum*	(Sorokin et al., 2015)
Acetoclastic	1.54	90	Closely related to *Methanosaeta harundinacea* 6Ac	(Kita et al., 2016)

*The sodium originates from a carbonate buffer

An interesting feature of compatible solutes is that microorganisms do not have to necessarily produce them *de novo*, but can also take them up from the bulk liquid. In fact, this is preferred over synthesis by most microbial cells due to bioenergetic considerations (Müller et al., 2005). For example, transport of the compatible solute betaine from the bulk liquid into *Methanohalophilus portucalensis* FDF1 has an energetic cost as little as 2 ATP molecules, whereas *de novo* synthesis would require as much as 36 ATP (Roberts, 2005).

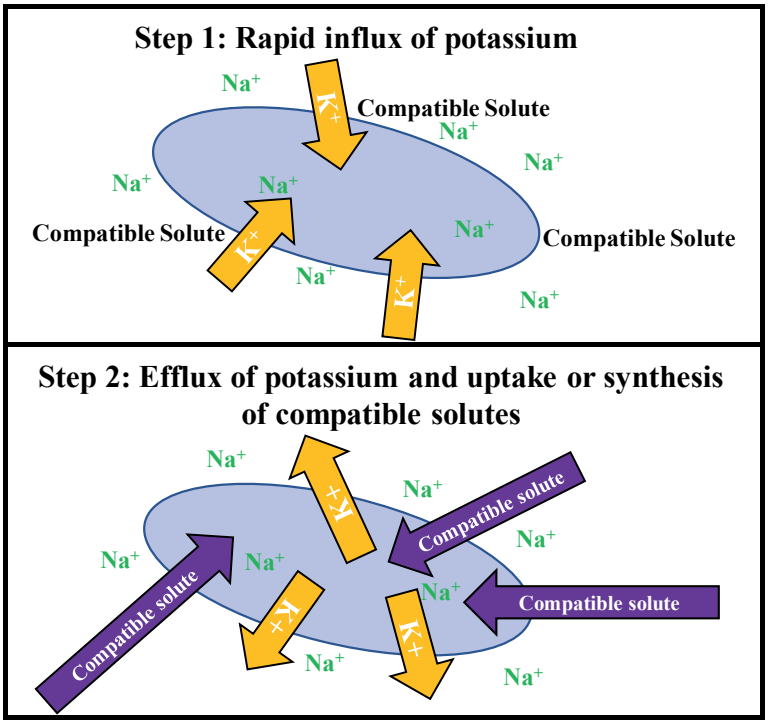


Figure 4. Mechanism of adaptation to high salinity with accumulation of compatible solutes based on Martin et al. (2000).

Many types of molecules can act as osmolytes, such as sugars and their derivatives, amino acids and their derivatives as well as dipeptides (Roberts, 2005). Methanogenic non-halophilic archaea have mostly been reported to accumulate/synthesize nitrogen-containing negatively charged or zwitterionic solutes (**Figure 5**) and often accumulate several of these molecules simultaneously (Sowers et al., 1990; Sowers and Gunsalus, 1995; Martin et al., 2000).

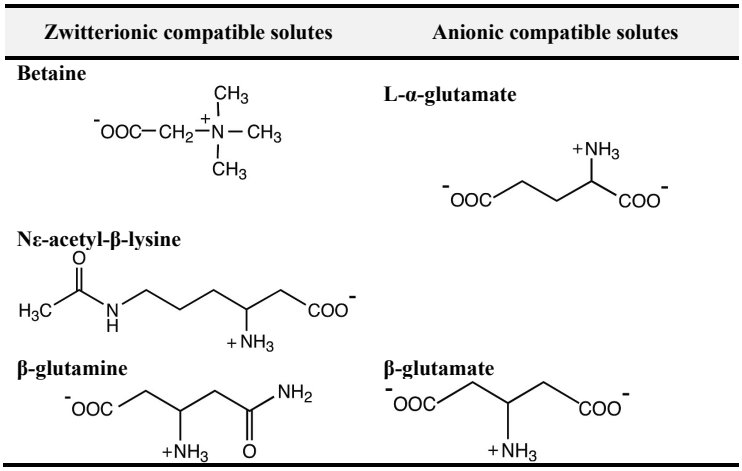


Figure 5. Frequently reported methanogenic compatible solutes. A modified figure as reported in Roberts (2005).

4.2.2. Inoculum adaptation to salinity for overcoming osmotic stress

Microbiological studies on accumulation of compatible solutes in pure cultures of microorganisms and the salt tolerance of these compatible solute accumulating microbial cells (*Section 4.2.1*) have mainly been performed by gradual transfer of the cultures from lower to higher salinity. This allows for acclimatisation of the cells to the new environment, without causing cell plasmolysis, via (gradual) accumulation of compatible solutes.

Several researchers have reported gradual acclimatisation of anaerobic sludge as a possible way to overcome salt toxicity. De Baere et al. (1984) showed that gradual adaptation of biomass from a sewage sludge digester can lead to salt tolerance as high as 24 g Na^+/L. Feijoo et al. (1995) demonstrated that 50% inhibition of methanogenic activity only occurred at 16.2 g Na^+/L in flocculent sludge from an anaerobic filter (AF) reactor adapted to mussel (5 – 10 g Na^+/L) wastewater for more than 2 years. The reference methanogenic activity against which inhibition was calculated in this study was the activity of the same sludge exposed to 2 g Na^+/L. Also, Roberts et al. (2016) recently showed that digested sewage sludge can be adapted to sea-water salinity while operating laboratory scale completely stirred tank reactors (CSTR) for more than a year with gradual increases of salinity. Thus, it becomes clear that adaptation of anaerobic biomass to high salinity is possible. These results contradict methanogenesis inhibition described in *Section 4.2* and indicate, that factors leading to

such different outcomes should be investigated and clarified. It should also be noted that in the field of wastewater treatment the terms "sludge acclimatisation" or "adaptation to salinity" are often interchangeably used without having a true mechanistic meaning, i.e., it is not clear whether halophilic microbial communities are gradually selected for or the existing inoculum microbes are halotolerant and start accumulating compatible solutes. For example, in the work of Feijoo et al. (1995) described above, the term "adapted sludge" was used for the biomass treating mussel wastewater, whereas batch experiments showed that this biomass already contained moderate halophile methanogens with an optimum methane production between 5 and 9 g Na^+/L (see **Table 2**). Use of halophilic (not extreme halophiles) microorganisms as an inoculum has been extensively studied for aerobic wastewater treatment with good results in terms of process performance (Lefebvre and Moletta, 2006). For anaerobic wastewater treatment this approach has been less explored and resulted in varying process performance from one study to another (Lefebvre and Moletta, 2006; Xiao and Roberts, 2010).

4.2.3. Overcoming the osmotic stress in biological wastewater treatment by dosing compatible solutes

Although gradual sludge adaptation to high salinity wastewater is possible, in some cases the acclimation of biomass to salinity can require up to a year (Roberts et al., 2016), which limits practical application of such a strategy (Vyrides and Stuckey, 2009b). Therefore, a number of researchers have tried to alleviate the osmotic stress in anaerobic wastewater treatment systems by dosing compatible solutes. This is a relatively unexplored approach and has been reviewed in a recent work by Vyrides and Stuckey (2016). The most studied molecule for such purposes is betaine (see **Figure 5**), which has shown to be effective in alleviating osmotic stress mostly in batch experiments at concentrations from 1 mM (0.12 g/L) up to 12.8 mM (1.5 g/L). Only in few investigations dosing of osmolytes to continuously operated anaerobic reactors has been reported. The results in the early work of Yerkes et al. (1997) showed that continuous addition of 10 mM (1.2 g/L) of betaine in a fluidized bed reactor operated at 17.2 g Na^+/L resulted in a decreased propionate concentration in the effluent compared to a reactor at the same salinity without added betaine. However, the effluent still contained a considerable amount (1.3 g/L) of propionate. In the same study betaine (10 mM) was also continuously dosed to a UASB reactor operated at 17.2 g Na^+/L. This resulted in methane production rates higher compared to a reactor without dosing of betaine, but lower compared to a reference reactor operated at low salinity (Yerkes et al., 1997). In a more recent study Vyrides et al. (2010) showed that continuous dosing of 1 mM (0.12 g/L) of betaine did not have noticeable positive effects on continuously operated submerged membrane bioreactors at 14 g Na^+/L. Thus, the results of osmolyte dosing to continuous anaerobic reactors are somewhat contradicting. A major drawback of

adding betaine to the bioreactors would be the high cost of such a strategy, especially if continuously dosed at concentrations as high as reported by Yerkes et al. (1997).

As mentioned in *Section 4.2.1*, microbial cells can often accumulate a "cocktail" of osmolytes as a response to high salinity and the osmolytes can vary from one microbial cell to another. For some osmolytes mostly found in methanogenic archaea, such as N-acetyl-β-lysine, the biosynthesis pathways are nowadays known. Pflüger et al. (2003) reported that this zwitterionic molecule can be synthesized from aspartate as a starting substrate with lysine as an intermediate. Galinski and Trüper (1994) suggested that most nitrogen containing osmolytes are likely to be synthesized from aspartate or glutamate. In theory, even addition of biosynthesis precursor compounds, such as aspartate and glutamate, should alleviate the osmotic stress of microorganisms. This is because biosynthesis of amino acids from substrates such as glucose and ammonium is energetically expensive (Akashi and Gojobori, 2002). Addition of amino acids could allow to save this energy. A similar effect could be provided by soluble, easily hydrolysable proteinaceous substrates, because after hydrolysis of proteins amino acids are released (De Mes et al., 2003) and these could potentially be taken up by microbial cells to synthesize osmolytes. As mentioned in *Section 3.2*, also EPS are produced as a microbial response to osmotic stress. The presence of proteinaceous substrate could also provide energetically favourable "building blocks" for the EPS protein fraction. Thus far, there are no studies investigating the efficiency of such potential precursor compounds to alleviate osmotic stress during anaerobic digestion of highly saline wastewater. Finally, it seems that the choice of the concentration of osmolytes to add to the treatment system is trivial and tools are required to assess an optimal dosage (Vyrides and Stuckey, 2016). Typically, the osmolyte dose is reported in mM (or g/L) of osmolyte dosed at the beginning of a batch experiment, or continuously added to a reactor influent (Vyrides and Stuckey, 2016). However, the osmotic balance needs to be achieved in active microbial cells exposed to high salinity and therefore tools to assess the necessary osmolyte dose should be directly related to the biomass concentration in the reactors.

4.2.4. Wastewater characteristics potentially affecting salt toxicity

Not only a proper choice or "adaptation" of anaerobic inoculum, but also the wastewater ionic composition may affect salt toxicity. For example, Kugelman and McCarty (1965) reported that other cations, such as potassium can have antagonistic effects on sodium toxicity. In the investigation of Feijoo et al. (1995) sodium chloride had a more severe detrimental effect on methanogenic activity when added as NaCl salt to the test medium compared to addition of sea water. Also, the organic substrate in the wastewater has been shown to affect the salt tolerance of microorganisms. Lefebvre et al. (2007) reported higher salt tolerance of methanogens when fed with ethanol compared to

distillery vinasses. In batch reactors fed with ethanol and vinasses 90% methanogenic inhibition occurred at 60 g NaCl/L (~ 24 g Na/L) and 10 g NaCl/L (~ 4 g Na/L), respectively. Similar observations were made by Feijoo et al. (1995), who reported that methanogenic activity is less negatively influenced with glucose as carbon and energy source compared to volatile fatty acids. Despite these early observations, systematic studies on the effects of organic substrate on the anaerobic wastewater treatment process at high salinity are still lacking. As explained in *Section 4.2.1*, the most commonly applied strategy of prokaryotic cells to adapt to high salinity is synthesis or uptake of compatible solutes and synthesis of these solutes is energetically expensive (Oren, 2013). Thus, potentially saline wastewaters containing more energy-rich substrates, such as glucose, may allow for a better microbial adaptation to salinity compared to substrates yielding a little amount of energy upon their conversion into methane, such as volatile fatty acids. Furthermore, given that methanogens typically synthesize/accumulate nitrogen containing osmolytes (see *Sections 4.2.1* and *4.2.3*), proteinaceous substrates in the wastewater could allow for energetically more favourable microbial adaptation to high salinity even compared to energy-rich substrates, such as glucose.

4.3. Granular sludge disintegration at high monovalent salt concentrations

As mentioned in *Section 2*, both flocculent and granular UASB reactors can be applied for high rate anaerobic wastewater treatment. However, the latter allows a higher biomass concentration in the reactor and thereby also higher applicable organic loading rates (Lettinga and Hulshoff Pol, 1991). Due to the more compact design, reactors relying on granular sludge formation have become especially appealing for treatment of industrial wastewaters with low suspended solids content (Hulshoff Pol et al., 2004; Van Lier, 2008; Van Lier et al., 2015).

Most investigations on sludge granulation at high monovalent salt concentrations suggest that sludge granulation at such (saline) conditions is not possible. This is because inoculation of UASB reactors with intact granular sludge and exposure to high monovalent salt concentrations has typically resulted in deterioration of granular strength, slow disintegration of the granules and subsequent biomass washout (Gomec et al., 2005; Lefebvre et al., 2006; Ismail et al., 2008; Jeison et al., 2008; De Vrieze et al., 2016a; Muñoz Sierra et al., 2019). Thus, granular sludge technology for treatment of highly saline wastewater is often considered unsuitable. The decreasing strength and disintegration of granules is usually explained by the displacement of multivalent cations, such as calcium, from the granular structure by monovalent cations (Na^+) (Jeison et al., 2008; Muñoz Sierra et al., 2019). Such displacement can result in a weaker granular sludge structure and disintegration (see **Figure 6**).

Although the explanation for the decreasing granular sludge strength upon exposure to monovalent salts as shown in **Figure 6** is widely accepted and proven to be valid, this scheme is oversimplified.

In reality the EPS matrix stability can also be affected by various other physical-chemical interactions such as, electrostatic interactions, hydrogen bonding, van der Waals interactions, polymer entanglement and ionic attractive forces (Flemming and Wingender, 2010). Granular sludge in UASB reactors is exposed to hydrodynamic shear forces caused by upward flowing liquid and the biogas that is produced in the reactor (Liu and Tay, 2002). If the attractive interaction forces between EPS and microbial cells are of sufficient strength to withstand these shear forces, the formation of granular sludge should also be possible. In fact, despite a decreasing granular strength, Ismail et al. (2010) observed an increasing size of granular sludge accompanied with good process performance in a continuous UASB reactor at 20 g Na^+/L. Also Li et al. (2014) reported increasing granule's size at salinity of 11 g Na^+/L. The experiments of Ismail et al. (2010) were performed at liquid upflow velocities of 0.8 m/h, which is slightly lower than typically applied in granular sludge UASB reactors. The positive result of increasing granule's size in the investigation of Li et al. (2014) was offset by severe inhibition of methanogenic activity. Still, such an increase in granular size is an indication for the possibility to achieve sludge granulation at high salinity and therefore justifies a more detailed investigation.

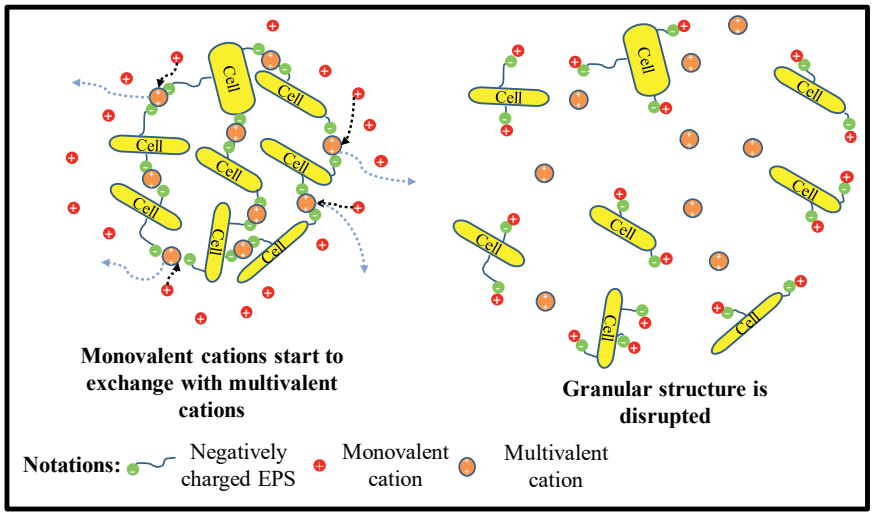


Figure 6. A schematic representation of the mechanism by which monovalent cations can decrease the strength of granular sludge via replacement of multivalent cations in the granular structure. Based on Jeison et al. (2008).

4.3.1. Is granular sludge formation at high salinity possible? An open field for research.

As explained in *Section 4.3*, typical research on anaerobic sludge granulation at high salinity has been performed while seeding reactors with a granular inoculum. There is no work performed to study the effects of high concentrations of monovalent salts on the process of sludge granulation itself, i.e. when inoculating bioreactors with dispersed sludge. This has prevented clear conclusions on the possibility to form granular sludge at high salinity and to gain insights in the mechanisms of

granulation (including microbial community development) at these conditions. Thus, systematic research on factors affecting granulation at high salinity (assuming it is possible), is needed. As shown in **Figure 7** there are many interrelated factors, which can all affect sludge granulation and process performance at high salinity. Amongst these factors organic substrate may play a crucial role, because it can potentially affect the osmoadaptation of the microbial community (*Section 4.2.4*), and the development of active microbial communities in time (*Section 3.4*). Since the active microorganisms are producing EPS – the binding material of granules – the organic substrate may also indirectly affect the granulation process itself by providing energetically favourable EPS "building blocks" (*Section 4.2.3*). Also other measurements can be taken to promote granular sludge formation at high salinity. For example, addition of a support (seed) material can potentially enhance sludge granulation (*Section 3.3*), and using a moderately halophilic inoculum may improve the initial microbial activity and reduce the time needed for reactor start-up (*Section 4.2.2*). Besides studying the process of granulation, also mechanistic insights into osmoadaptation of mixed microbial consortia treating saline wastewater are needed. For example, studies on the osmolytes (*Section 4.2.1*) accumulated by microbial consortia at high salinity, may help to select the best compounds to add (*Section 4.2.3*) for osmotic stress alleviation. Finally, the role of EPS in osmoprotection of granular microbial consortia at high salinity should be further elucidated (*Section 3.2*).

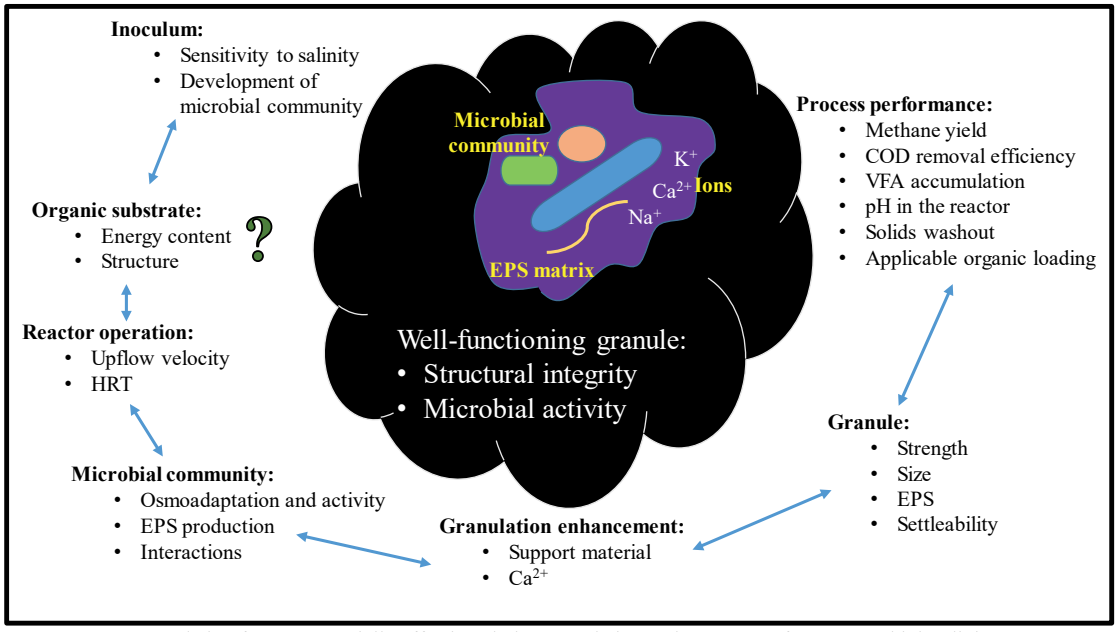
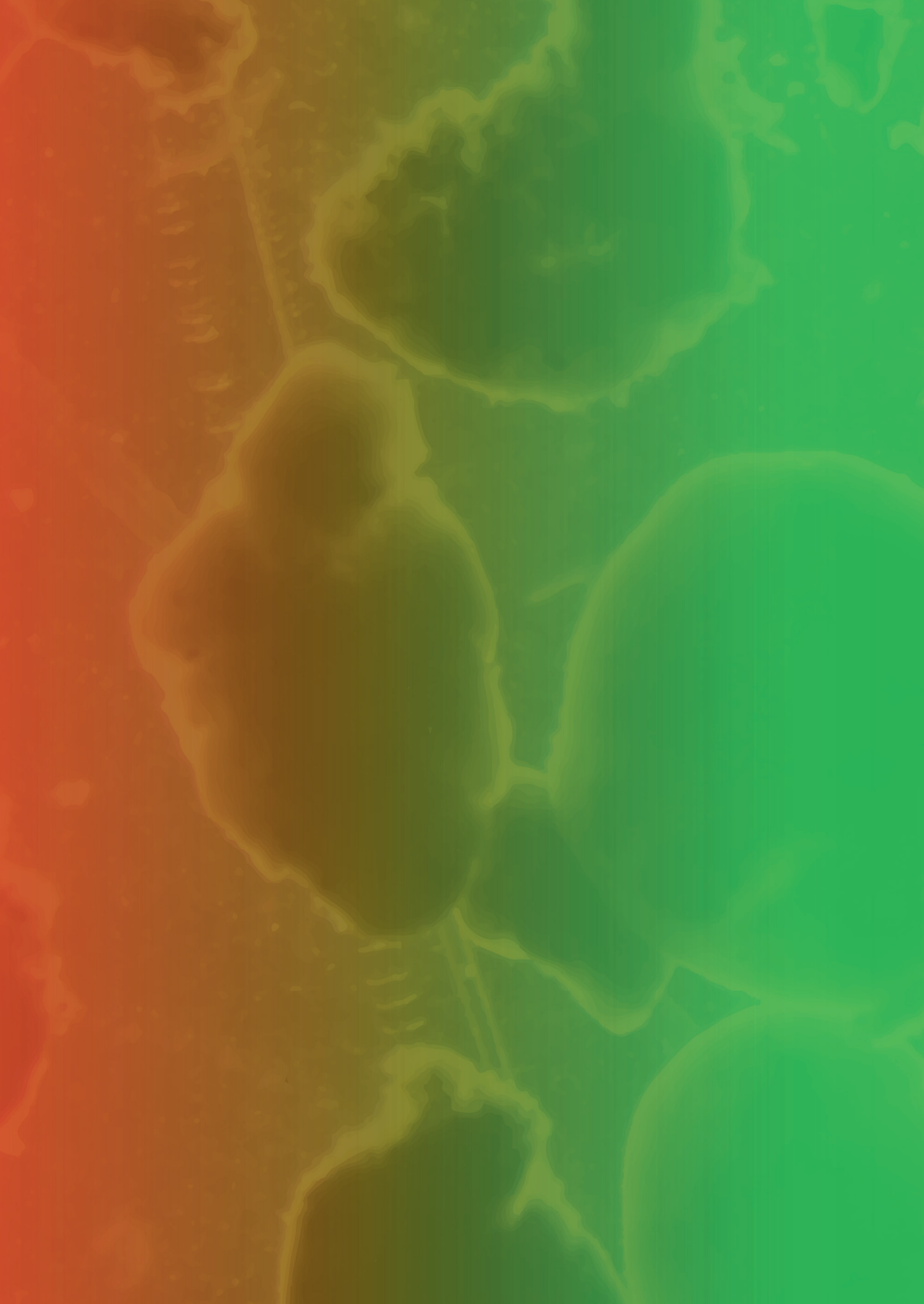


Figure 7. Interrelating factors potentially affecting sludge granulation and process performance at high salinity

5. This thesis

This thesis aimed to investigate mechanisms of anaerobic sludge granulation at high salinity, microbial osmoadaptation and the applicability of anaerobic granular sludge technology for treatment of saline wastewater streams. In **Chapter 2**, the possibility to form anaerobic granular sludge from dispersed inoculum at 5 and 20 g Na^+/L in UASB reactors was investigated. The hypothesis was that with proper reactor operating conditions granulation should be possible even at salinity as high as 20 g Na^+/L. The reactors were fed with wastewater containing a mixture of energy rich (glucose), acidified (acetate) and proteinaceous complex organic substrate to increase chances of granulation and osmo-protection. Also, the reactor start-up procedure (e.g., initial organic loading, addition of support material and upflow velocity) was adapted from positive experiences with fresh water granulation. Surprisingly, in this manner even at 20 g Na^+/L rapid formation of robust granules was accomplished from dispersed sludge while achieving good process performance. In **Chapter 3**, the hypothesis that proteinaceous organic substrates after hydrolysis can help to alleviate osmotic stress was tested. First, nitrogen-containing osmolytes accumulated by microbial consortium in anaerobic granular sludge adapted to 20 g Na^+/L were identified. Further, application of these osmolytes and their potential biosynthesis precursor compounds, such as amino acids and proteins, for salt stress alleviation in granular sludge was evaluated. In **Chapter 4**, the hypothesis that the fast granulation observed in **Chapter 2** was achieved in part by addition of proteinaceous organic substrate in the wastewater was tested because proteins might provide energetically favourable EPS biosynthesis building blocks. The effect of organic substrate on the process of granulation and treatment performance at 20 g Na^+/L was systematically investigated. The results showed that proteinaceous substrate indeed enhances the rate of granulation at high salinity compared to, for example carbohydrates. In **Chapter 5**, the required concentration of proteinaceous substrate, necessary for rapid granulation at high salinity, was estimated. Approximately a 9 fold decrease of protein in the wastewater compared to the amount used in previous experiments was shown to be possible while still obtaining good sludge granulation and process performance. The organic substrate in wastewater at non-saline conditions affects the composition of the microbial community, which in turn can affect the success of granulation. Therefore, in **Chapter 6** the development of microbial communities in granular sludge of reactors operated at different salinities and with different organic substrates was studied. The results showed that the dominant active methanogenic (archaeal) community is less affected as a response to changing salinity or organic substrate than the bacterial community. Several researchers have suggested that increased EPS production as a response to increasing salinity could be a microbial strategy to adapt to osmotic stress. However, the mechanism of this osmotic stress alleviation by EPS is poorly understood. **Chapter 7** provides insights into ion-exchange properties

of salt adapted granular sludge EPS and a hypothesis was proposed on how EPS and osmoadaptation could be related. Finally, in **Chapter 8** the results obtained in this thesis are discussed in a broader context and future research directions are proposed.



Chapter 2. Fast Anaerobic Sludge Granulation at Elevated Salinity

A modified version of this chapter has been published as:

Sudmalis, D., Gagliano, M.C., Pei, R., Grolle, K., Plugge, C.M., Rijnaarts, H.H.M., Zeeman, G. and Temmink, H., 2018. Fast anaerobic sludge granulation at elevated salinity.
Water Research 128(Supplement C), 293-303
https://doi.org/10.1016/j.watres.2017.10.038.

Abstract. It is commonly accepted that high salt concentrations negatively affect microbial activity in biological wastewater treatment reactors such as upflow anaerobic sludge blanket (UASB) reactors. Microbial aggregation in such reactors is equally important. It is well documented that anaerobic granules, when exposed to high salinity become weak and disintegrate, causing wash-out, operational problems and decreasing process performance. In this research, the possibility of microbial granule formation from dispersed biomass was investigated at salinity levels of 5 and 20 g Na^+/L. High removal efficiencies of soluble influent organics were achieved at both salinity levels and this was accompanied by fast and robust formation of microbial granules. The process was found to be stable for the entire operational period of 217 days. As far as we know this is the first time it has been demonstrated that stable granule formation is possible at a salinity level as high as 20 g Na^+/L. *Methanosaeta* was identified as the dominant methanogen at both salinity levels. *Streptococcus spp*. and bacteria belonging to the family *Lachnospiraceae* were identified as the dominant microbial population at 5 and 20 and g Na^+/L, respectively.

1. Introduction

Saline wastewater streams are generated by several industries such as the fish processing (Soto et al., 1991), chemical, agro-food, petroleum and leather industry (Lefebvre and Moletta, 2006). Biodegradation of organics in such saline waste streams is reported to be poor due to toxic effects of sodium on the bacterial cells and Archaea causing cell lysis and death (Sleator and Hill, 2002; Vyrides and Stuckey, 2009a; Sochacki et al., 2011). Physical and chemical treatment processes are energy intensive and more expensive compared to biological processes (Fakhru'l-Razi et al., 2009), hence the need to explore the limits of biological wastewater treatment regarding salinity and to stretch these limits where possible.

Because of a lower sensitivity of aerobic biomass to salt toxicity compared to anaerobic biomass, most research is done on aerobic treatment (Freire et al., 2001; Tellez et al., 2002; Tellez et al., 2005; Zhao et al., 2006; Ozgun et al., 2013). Anaerobic biological wastewater treatment has several advantages over aerobic, such as net energy recovery, reduced sludge production and the possibility to handle higher volumetric loading rates if formation of granular or immobilised biomass is possible (Vieira et al., 2005b; Vyrides et al., 2010; Xiao and Roberts, 2010).

In the field of anaerobic wastewater treatment it is generally accepted that methanogenic activity is inhibited above sodium levels of 5 g Na^+/L (McCarty and McKinney, 1961; Patel and Roth, 1977; Rinzema et al., 1988; Liu and Boone, 1991; De Vrieze et al., 2016b). However, sodium toxicity may depend on the composition of wastewater due to possible antagonistic and synergistic effects caused by other ions (McCarty and McKinney, 1961). Moreover the type of organic substrate generally

determines the type of microorganisms evolving in the reactors and thus their sensitivity to sodium toxicity (Patel and Roth, 1977; Lefebvre et al., 2007). Hence, the potential of anaerobic treatment of saline wastewaters thus far may have been largely underestimated.

If high rate anaerobic bioreactors such as upflow anaerobic sludge blanket (UASB) for treatment of wastewater are applied, both microbial activity and formation of granular microbial aggregates plays a crucial role on the process performance (O'Flaherty et al., 1997; Yu et al., 1999; Liu et al., 2009; Lu et al., 2013). After the initial formation of granules it is of equal importance to maintain a robust granular structure (McHugh et al., 2003). Microbial granules of sufficient strength and weight then allow the decoupling of hydraulic and sludge retention times, achieve high biomass concentrations and permit the use of compact reactors (O'Flaherty et al., 1997; Liu et al., 2009).

The most commonly used methods to enhance the rate of granulation are addition of multivalent cations, such as calcium (150-300 mg/L) (Zeeuw, 1984; Mahoney et al., 1987; Hulshoff Pol, 1989; Yu et al., 2001) and/or addition of a support material, such as powdered activated carbon (PAC) (Wirtz and Dague, 1996; Yu et al., 1999).

Only a few studies have reported on the effect of monovalent salt stress on anaerobic granules. All of these studies were performed by inoculating (intact) granular sludge and - without exception - showed subsequent granule disintegration, biomass washout and loss of microbial activity (Rinzema et al., 1988; Jeison et al., 2008; Ismail et al., 2010; Li et al., 2014; De Vrieze et al., 2016a). As far as we know, formation of anaerobic granules from dispersed biomass under saline conditions has not been reported before.

In this chapter granulation from dispersed biomass, accompanied by stable UASB process performance, at sodium concentrations as high as 20 g Na^+/L is demonstrated for the first time. The rate of granulation was compared to granulation at a reference level of 5 g Na^+/L. The effect on granulation of small amounts of powdered activated carbon (PAC) was studied. Calcium was only fed at extremely low concentrations of 13 mg/L. Microscopic analyses coupled with fluorescent in situ hybridization (FISH) showed dominance of filamentous *Methanosaeta,* irrespective of the salinity (5 or 20 g Na^+/L) at which the UASB reactors were operated.

2. Materials and methods

2.1. Experimental set –up

2.1.1. Growth medium

The nutrient medium used was adapted from Vallero et al. (2002) and 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.

The COD of the influent was soluble and consisted of D-Glucose, Na-Acetate and Tryptone (Sigma-Aldrich, Microbiologically tested, N content 11-16%) in COD proportions of 3:2:1. The COD concentration in the influent was increased in steps from 3 g/L to 12 g/L to reach a final organic loading 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 influent for the first 3 months and afterwards was replaced by technical grade NaCl (VWR, minimum purity 98%). Final sodium concentrations of 5 or 20 g Na^+/L include 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) were inoculated with 6 g VSS/L. Origin of the biomass was a full scale mesophilic UASB treating wastewater from a styrene and propene-oxide production plant (Shell, Moerdijk, the Netherlands). The sludge was adapted to approximately 8 g Na^+/L for almost 10 years and has a specific methanogenic activity of approximately 0.2 – 0.3 g COD/ gVSS·d with acetate as carbon source (Jeison et al., 2008; Ismail, 2013). The COD in this wastewater mainly consisted of benzoic and acetic acid. The inoculum for R1, R2 and R4 was dispersed by forcing it through a 125 µm sieve before adding to the UASB, while in R3 it was left intact. The inoculum of R2, R3 and R4 was mixed with powdered activated carbon (PAC) (0.1% of inoculum mass; 100-400 mesh, Sigma-Aldrich).

Figure 1 shows a schematic drawing of R4. The set – ups of R1 - R3 (**Figure S1**) had external circulation tanks while R4 was operated with an internal circulation. The upflow velocity of 0.2 – 1 m/h, was controlled by peristaltic circulation pumps (Watson – Marlow 530U, UK). **Table 1** contains a summary of start-up and operational conditions for each bioreactor.

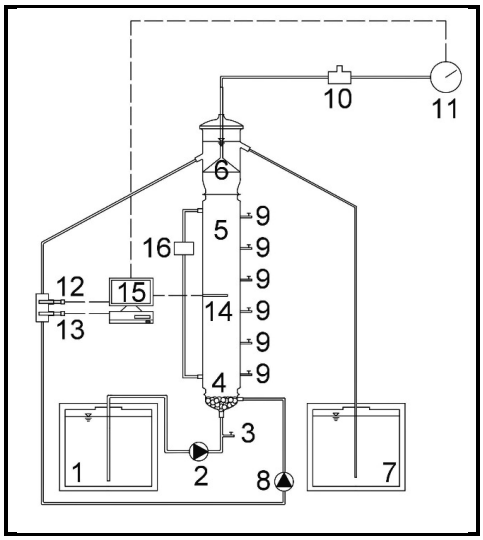


Figure 1. Diagram of experimental UASB reactor setups. 1 – influent tank at 1 ºC; 2 – peristaltic influent pump; 3 – influent sampling port; 4 – supporting material; 5 – UASB reactor; 6 – settler; 7 – effluent tank at 4 ºC; 8 – peristaltic recirculation pump; 9 – sampling ports; 10 – biogas sampling port; 11 – gas flow meter; 12 – oxidation-reduction (redox) sensor; 13 – pH sensor; 14 – temperature sensor; 15 – computer for data logging; 16 – heating bath

Table 1. Summary of reactors operation parameters and dimensions. COD_{inf} – influent COD concentration. SB – sieved biomass; IB – intact biomass; PAC – powdered activated carbon

Parameters	R1	R2	R3	R4
Inoculum	SB	SB + 0.1% PAC	IB + 0.1% PAC	SB + 0.1% PAC
Operating Temperature, ºC	35 ± 1	35 ± 1	35 ± 1	35 ± 1
Salinity, g Na^{+}/L	5	5	20	20
Total Reactor volume prior height increase, mL	680	730	730	2940
Additional Gas – Solids Liquid separator volume added on day 102, mL	190	190	190	Not needed
Reactor volume prior sedimentation zone prior height increase, mL	500	550	550	1960
Reactor height prior sedimentation zone, m	0.24	0.26	0.26	0.65
Reactor Diameter, m	0.052	0.052	0.052	0.062
Loading rate during start – up, g COD/L·d	1	1	1	1.4
Final loading rate after 100 days, g COD/L·d	11	11	11.5	9.11
Day of increase of upflow velocity from 0.2 m/h to 1 m/h	30	30	30	18
Day of increase of COD_{inf} from 3g/L to 7g/L*	31	31	31	26
Day of increase of COD_{inf} from 7g/L to 12g/L*	52	52	52	60

*Increase of COD_{inf} corresponds to increasing loading rates

2.2. Process performance analysis

2.2.1. Analytical methods

Daily biogas production was monitored with µFlow gas flow meter (Bioprocess Control Sweden AB). Biogas composition was measured periodically as described in Steinbusch et al. (2008).

Volatile fatty acids (VFAs) were quantified in soluble fraction of effluent samples on Agilent 7890B gas chromatograph equipped with FID detector and HP-FFAP column (25m x 0.32 mm, 0.5 µm film thickness). Helium (purity 5.0) was used as the carrier gas (1.25 ml/min for first 3.5 min and 2 ml/min further). The injection volume (split injection 1:25) was 1µL and the injection temperature 250 ºC. The oven temperature had the following temperature ramp: 60 ºC for 3 min; 21 ºC/min up to 140 ºC; 8 ºC/min up to 150 ºC; 1.5 min constant 150 ºC; 120 ºC/min up to 200 ºC; 1.25 min constant 200 ºC; 120 ºC/min up to 240 ºC; 3 min 240 ºC constant. The detector temperature was constantly kept at 240 ºC. The data acquisition was done with Chromeleon 6.80 SR13 software.

COD measurements were performed with LCK314, LCK514 and LCK1414 kits (HACH GMBH, Germany) after sample dilution with milli-Q to reach chloride levels of 0.2 - 1.1 g/L. Differentiation between total (tCOD), colloidal and supracolloidal (cCOD) and soluble fractions (sCOD) of COD was achieved by filtration. Raw effluent samples were filtered through 4 -7 µm paper filters (Whatman™, grade 595 ½) and part of the filtrate was further passed through a pre-washed 0.45 µm cellulose acetate membrane filter (VWR® Syringe Filters). Soluble fraction is defined as filtrate through 0.45 µm filter (Park et al., 2006). Colloidal and supracolloidal fraction is calculated from the difference between 4-7 µm and 0.45 µm filtrates (Dulekgurgen et al., 2006). Total COD is measured in homogenized effluent samples. Particulate COD (pCOD) is defined as difference between 4-7 µm filtrate COD and total COD.

During the entire operation period grab samples of effluent COD were collected and sCOD removal efficiency was measured in singleton per sample. 40 hour composite effluent samples were collected on day 179 in R1, R2 and R3 (day 100 for R4). COD analysis on different fractions of the samples was performed in triplicate.

2.2.2. Sludge retention time

Suspended solids were measured in duplicate according to APHA standard method Nr.2540D. 24 hour composite effluent samples to determine the sludge retention time (SRT) were collected on days 178 and 179. Sampling of the sludge from bottom and top of the reactor columns was performed while flushing them with N_2 gas to achieve good mixing.

The SRT was calculated as follows:

$$SRT = \frac{TSS_{Reactor}}{TSS_{wasted}} = \frac{TSS_{Reactor}}{TSS_{eff} + TSS_{discharged}}$$

Where: *SRT* – sludge retention time (days); $TSS_{Reactor}$ – the amount of solids within a bioreactor (gTSS); TSS_{wasted} – the amount of solids wasted per day (gTSS/day); TSS_{eff} - the amount of solids leaving with the effluent (gTSS/day); $TSS_{discharged}$ - the amount of solids discharged from the bioreactor (gTSS/day). Due to low frequency and low amounts of solids sampled and no intentional sludge wasting from reactors, the term $TSS_{discharged}$ was neglected.

2.3. Granular sludge characteristics

2.3.1. Particle size distribution (PSD) and strength

Particle size distribution of the microbial granules was measured as described in Ismail et al. (2010). The measurement was performed in triplicate on a Mastersizer 2000 (Malvern, UK). Sampling was done as described above. The volume and surface based mean particle sizes were calculated based on total volume or total surface of the particles, respectively.

Strength of the microbial aggregates was measured as described in Xiao et al. (2008). Approx. 0.12 g of suspended solids were used in each test. The microbial aggregates were put in 50 mL centrifuge tubes, filled with reactor medium up to 30 mL and vortexed at 2500 rpm for 2, 4 and 8 minutes. After each period of vortexing, the aggregates were allowed to settle for 1 min and the supernatant was decanted to quantify the amount of fines produced as total suspended solids. To minimize biomass removal from running bioreactors, singletons were measured at each time point for strength characterization.

The integrity coefficient IC is calculated as follows:

$$IC_x = \left(1 - \frac{SS_x}{SS_0}\right) * 100$$

Where IC_x – the integrity coefficient after x minutes of vortexing, SS_x – the amount of solids quantified after x minutes of vortexing, SS_0 - the total amount of solids used for the test. The higher IC value corresponds to stronger aggregates.

2.4. Microscopy and image analysis

2.4.1. Scanning electron microscopy (SEM)

Samples of granules for SEM analysis were prepared as described in Ismail et al. (2010) and imaged at an acceleration voltage of 2 kV and beam current of 6 pA, at room temperature in a field emission scanning electron microscope (Magellan 400, FEI Company, Oregon, USA).

2.4.2. Fluorescence in situ hybridization (FISH)

Samples taken at the end of continuous experiment from each UASB reactor sludge bed were directly fixed with 37% w/w formaldehyde according to the procedure described in Amann et al. (1995). After fixation, samples were washed several times with PBS 1X and then stored at -20 ºC in ethanol:PBS 1x (1:1). Before further FISH analysis fixed granules were gently crushed through a 0.7 mm diameter needle and diluted (Amann et al.,1995). Oligonucleotide probes applied are listed in **Table S2.** Probes were labelled with Cy3-red or Alexa488-green fluorophores. Samples were examined by epifluorescence microscopy (Olympus BX41, Olympus, Japan) equipped with Infinity Camera (Lumenera corporation, Canada). FISH images were modified using the ImageJ software package (version1.37v, Wayne Rasband, National Institute of Health, Bethesda, MD, USA).

3. Results

3.1. Granulation

3.1.1. Size, strength and retention

During start-up fast granulation (R1, R2, R4) and growth of particle size (R3) was observed in all bioreactors, irrespective of the salinity level or whether PAC was added to the inoculum. As an example, **Figure 2** illustrates the development of granules over time in R4. Microbial aggregation was already observed on day 23 (**Figure 2 – B**), while on day 33 the majority of the sludge bed consisted of granules (**Figure 2 – C**). By day 43 the sludge bed was completely filled with granular biomass (**Figure 2 – D**).

Particle size distribution (PSD) was followed over time and was similar for R1 and R2. **Figure 3-A-B** shows an increase of particle sizes in R1 and R2 operated at 5 g Na^+/L compared to the maximum particle size of 125 µm of the inoculum. **Figure 3-C-D** shows that also in R3 and R4, operated at 20 g Na^+/L the particle size increased. **Table 2** summarizes the development of volume and surface based average particle sizes in all four bioreactors. Addition of PAC to the inoculum did not result in a faster development of granules compared to the inoculum where no PAC was added (R1 compared to R2). Also, no observable difference in the rate of granulation was found when comparing the start –up of reactors with sieved biomass at 5 g Na^+/L (R2) and 20 g Na^+/L (R4).

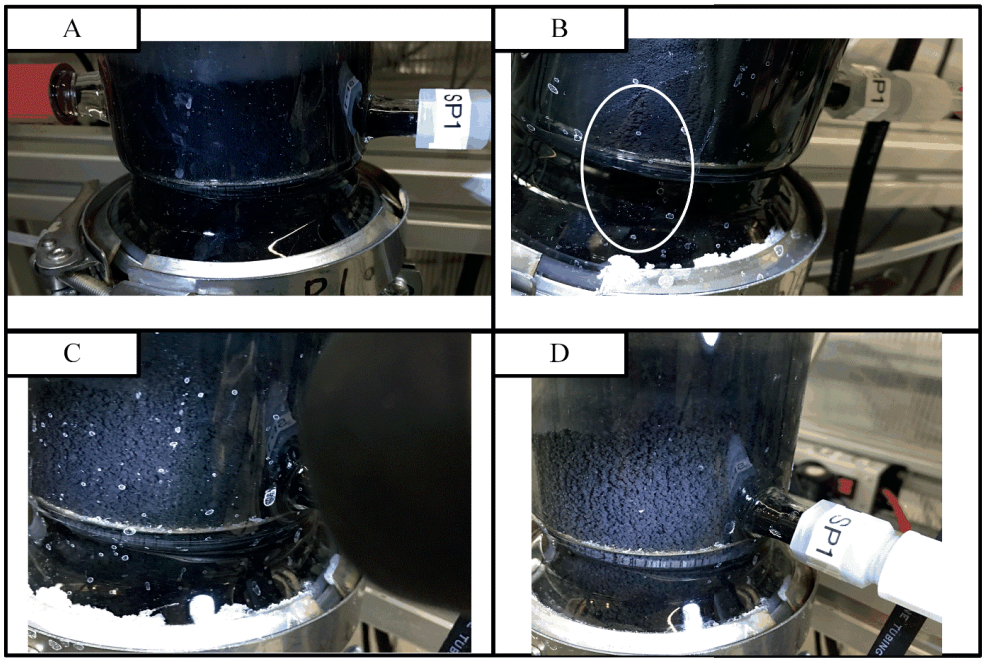


Figure 2. Images of granular sludge formation in R4. A – day 4; B – day 23; C – day 33; D – day 43. White circle in B for guiding the reader's attention to the aggregates appearing in the bioreactor. Reactor inoculated with 125 um sieved biomass and operated at 20 g Na^+/L

On Day 79 the volume based average size of granules in R3 was higher than in R1 and R2. This difference is due to larger particles inoculated in R3. Compared to the inoculum, the average volume based size increased by 141.1 µm, 124.7 µm and 121.4 µm in R1, R2 and R3, respectively. Thus, the rate at which particle size increased in R1, R2 and R3 was similar. The SRTs were 74.9, 98.6 and 70 days for reactors R1, R2 and R3, respectively. For R4 no solids balance was made, as it was merely started up to study if the process of granulation from dispersed biomass is possible at 20 g Na^+/L.

Table 2. Development of volume and surface based average particle sizes within bioreactors. S – Sieved; I – Intact; PAC – powdered activated carbon. Sizes given in µm.

Reactor	Na^+, g/L	Inoculum Type[a,b]	Day 79	Day 87	Day 148	Day 205	Day 79	Day 87	Day 148	Day 205
			Surface Based				Volume based			
R1	5	S	96.3	-	74.4	75.8	220.7	-	303.8	329.6
R2	5	S + PAC	64.3	-	61.5	73.5	204.0	-	295.20	289.1
R3	20	I+ PAC	84.7	-	98.2	103	426.9	-	368.4	384.7
R4	20	S + PAC	-	74.5	-	-	-	250.5	-	-
-	8	I								
-	8	S								

[a]Surface and volume based average diameter of intact inoculum is 82.3 and 305.5 µm, respectively; [b]Surface and volume based average diameter of sieved inoculum is 37.9 and 79.3 µm, respectively.

In **Table 3** strength measurements of the granules indicate that in all bioreactors aggregates of higher strength compared to the inoculum developed. The strength of microbial granules in R1, R2 and R3 was similar, implying that strength was not affected by the sodium concentration or by addition of PAC. Also, the strength of granules formed from sieved biomass (R1 and R2) was not different from the strength of the granules that developed from intact granules (R3). The integrity coefficients during strength tests in R1 – R3 stabilised with vortexing time while in R4 a decreasing trend remained. These outcomes suggest that granules in R4 were less resistant to shear forces compared to R1-R3. This difference may be caused by the scaled – up set – up of R4, which may have resulted in sludge exposure to different shear forces and mixing patterns of influent.

Table 3. Integrity coefficient measurements of sludge from reactors and of intact inoculum sludge. n.d. not determined

Reactor	Intact inoculum	R1	R2	R3	R4	R1	R2	R3	R4
Day	0	148	148	148	126	205	205	205	n.d
IC_2, %	45.3	60.2	66.5	61.6	74.7	61.3	59.9	58.4	n.d
IC_4, %	34.9	49.6	51.9	53.1	48.9	49.7	45.3	47.0	n.d
IC_8, %	30	44.5	43.9	48.2	27.4	44.7	38.6	39.7	n.d

3.1.2. Microscopic analyses

Microscopic analysis carried out using FESEM and Fluorescence microscopy coupled with FISH visualized the differences between granules operated at 5 and 20 g Na^+/L. SEM images of granules showed that the dominant morphology of the microorganisms was filamentous (**Figure 4**). In R1 and R2 operated at 5 g Na^+/L the most abundant morphologies were coccus-shaped microorganisms organized in chains (**Figure 4 – A, B, C, D**). In R3, operated at 20 g Na^+/L, filaments were also observed but these mainly consisted of aggregated rod shaped cells (**Figure 4 – E, F**). The difference in morphology at 5 g Na^+/L and 20 g Na^+/L suggests that different microbial communities were "selected" in response to salinity. For further analysis FISH probes were used to target the microbial groups commonly found in anaerobic treatment systems (**Table S2**) and in anaerobic environments.

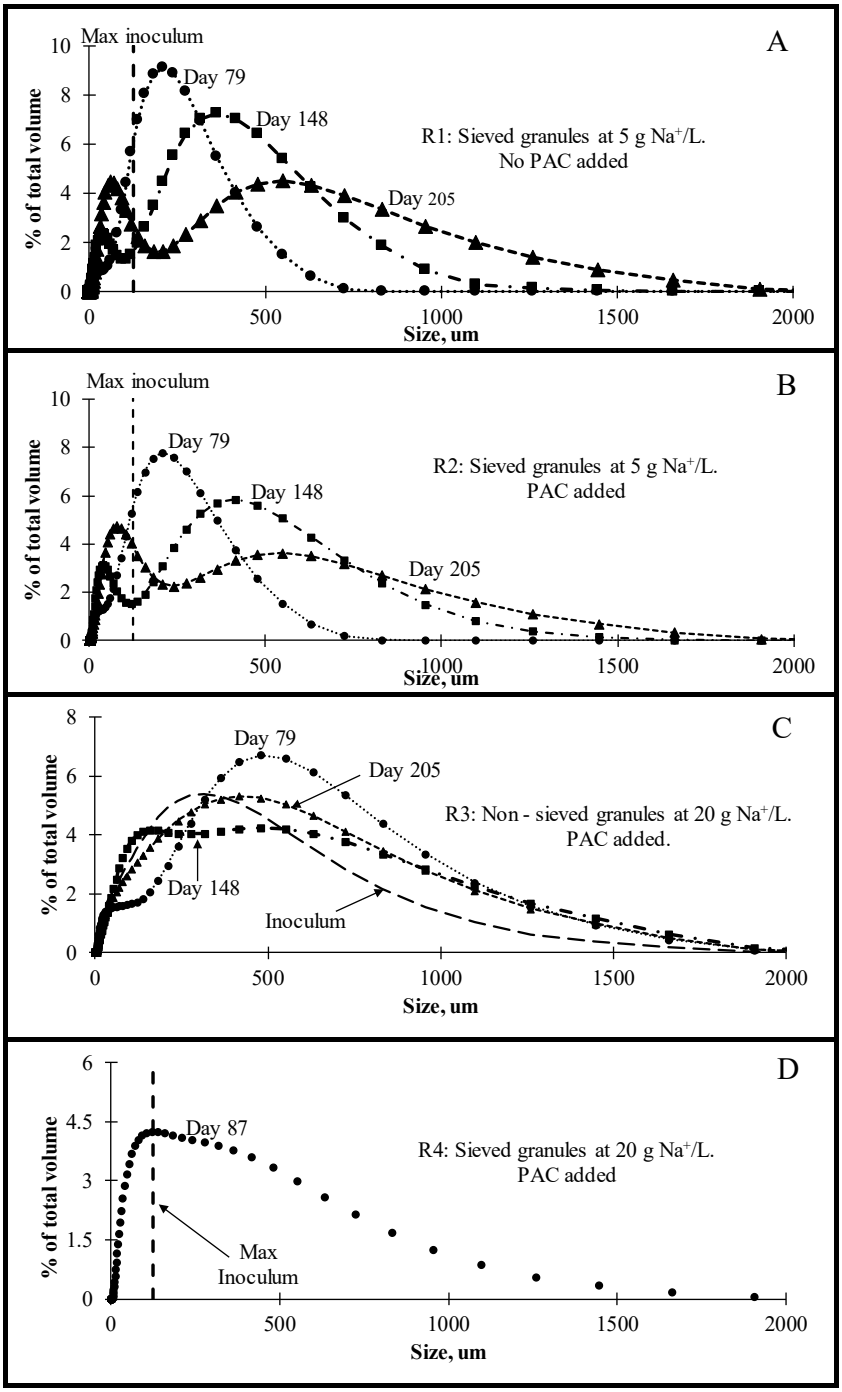


Figure 3. Particle size distribution in time. Particle size distribution presented as % of total volume of the measured particles. The vertical lines in A, B, D represent the maximum particle size of inoculum for R1, R2 and R4. Samples were taken from top sampling ports of the bioreactors, while mixing the reactor with nitrogen gas.

The use of ARC915 archaea domain specific probe, combined with the application of specific probes for *Methanosarcinales, Methanosaetaceae* and *Methanomicrobiales* (**Table S2**) revealed acetoclastic *Methanosaetaceae* as the dominant methanogen at both salinities (**Figure 5 - A, C, E and F** in green), while members of the hydrogenotrophic order of *Methanomicrobiales* were present in much lower amounts in both R1 (**Figure 5 - E**) and R3 (**Figure 5 - F**) populations. In R1 rods and filaments appeared, whereas only few rods hybridized with the probes in R3. Within the bacterial population in all samples taken at the end of the operational runs (day 217), *Streptococcus spp*. dominated in low salinity reactors (5 g Na^+/L) (**Figure 5-B**), which corresponds to the twisted chain shaped microorganism shown by SEM at the exterior of the granule (**Figure 4 A-D**), while a long filamentous bacterium belonging to the family *Lachnospiraceae* was the most represented in the granules grown at 20 g Na^+/L (**Figure 5-D**). Both belong to the phylum *Firmicutes* and are reported as carbohydrate fermenting bacteria (Cotta and Forster, 2006; Milinovich et al., 2008). Apparently, salinity resulted in a shift in the bacterial and hydrogenotrophic methanogens populations, while the other methanogenic biomass was not affected by increasing sodium concentration. No other specific group of microorganisms could be visualized when applying all probes listed in **Table S2**.

3.2. COD removal efficiency, fractionation in the effluent and methane production

Figure 6 shows that all reactors accomplished high removal efficiencies regardless of the salinity level and COD loading rate. The average removal efficiencies based on sCOD during the entire operation period were 97.2 ± 2.1%, 96.6 ± 3.3%, 94.0 ± 2.2% and 92.8 ± 5.4% for R1, R2, R3 and R4, respectively, showing that the reactors operated at 5 g Na^+/L (R1 and R2) performed slightly better than the ones operated at 20 g Na^+/L (R3 and R4). No clear differences were observed between reactors seeded and not seeded with PAC (R1 and R2) at any stage of the continuous operation, thus showing no effect of additional PAC on start – up of R1 and R2.

Effluent tCOD was higher in reactors operated at 20 g Na^+/L. In composite effluent samples at day 179 (day 100 for R4), tCOD was 257 mg/L, 330 mg/L, 769 mg/L and 1170 mg/L for R1, R2, R3 and R4, respectively. The pCOD concentrations in the effluent were 31, 89.5, 120.8 and 222.5 mg/L for R1, R2, R3 and R4, respectively. The fractions contributing to total effluent COD were similar for all reactors and are depicted in **Figure 7-A**.

The detected VFAs in the effluent were acetate and propionate. The sum of these VFAs contributed between 29.5 and 60.5% to the effluent soluble COD (**Figure 7-B**). The highest VFA fraction was found in R3 (operated at 20 g Na^+/L) and the lowest fraction in R2 (operated at 5 g Na^+/L). These results show that between 39.5 and 70.5% of the soluble effluent COD consisted of organic material other than VFAs.

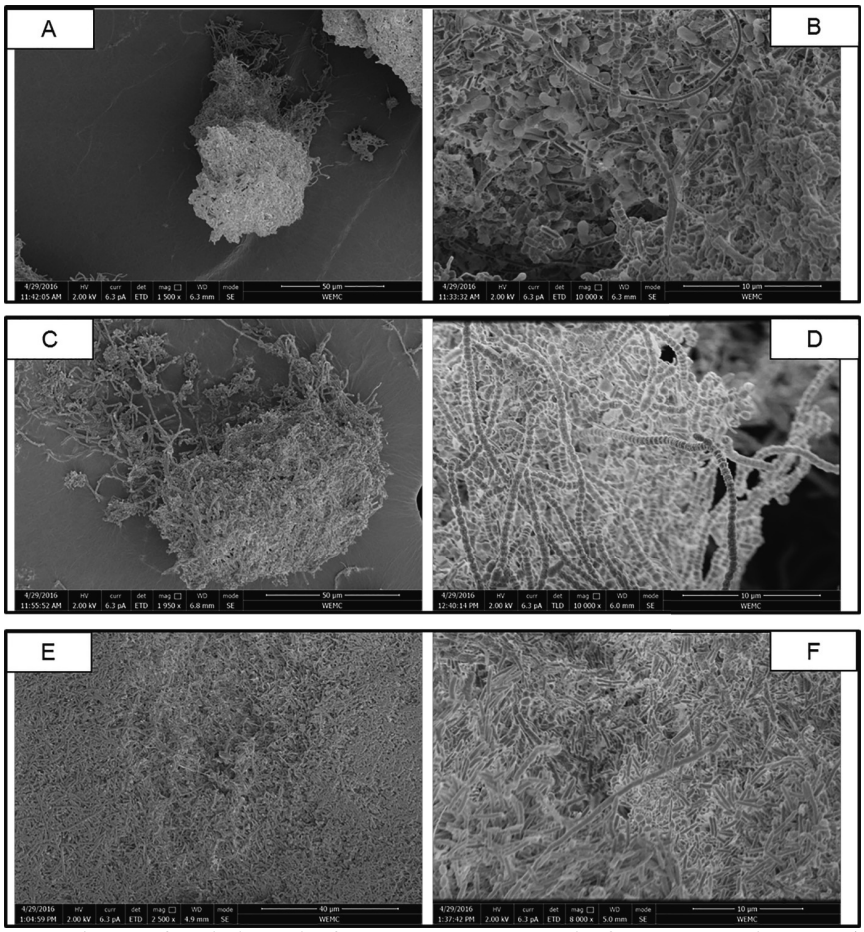


Figure 4. FESEM images of crushed granules from reactors. A, B – samples from R1 operated at 5 g Na^+/L; C, D – samples from R2 operated at 5 g Na^+/L and seeded with PAC upon start-up; E, F – samples from R3 operated at 20 g Na^+/L and seeded with PAC upon start-up.

The profiles of effluent VFAs are shown in **Figure S3**. Generally, lower effluent acetate and propionate concentrations were measured in R1 and R2 compared to R3 and R4. However, both in R3 and R4 the effluent VFA concentrations showed a downward trend, indicating an improvement of reactor performance over time. The effluent acetate concentration at the end of operational runs of R3 and R4 were 85 and 34.7 mg/L, respectively, whereas propionate concentration was 34.8 mg/L in R3 and could not be detected in R4.

Cumulative biogas production as well as biogas composition were monitored over time. Biogas composition over the whole period was similar in the reactors operated at both 5 g (R1 and R2) and 20 g Na^+/L (R3 and R4): 63.6 ± 4.6%, 65.9 ± 6.5%, 68.4 ± 4.3% and 70 ± 2.9% for R1, R2, R3 and R4, respectively. Biogas production curves are presented in **Figure S4** and show continuous biogas production with increasing slopes as the loading rates increase.

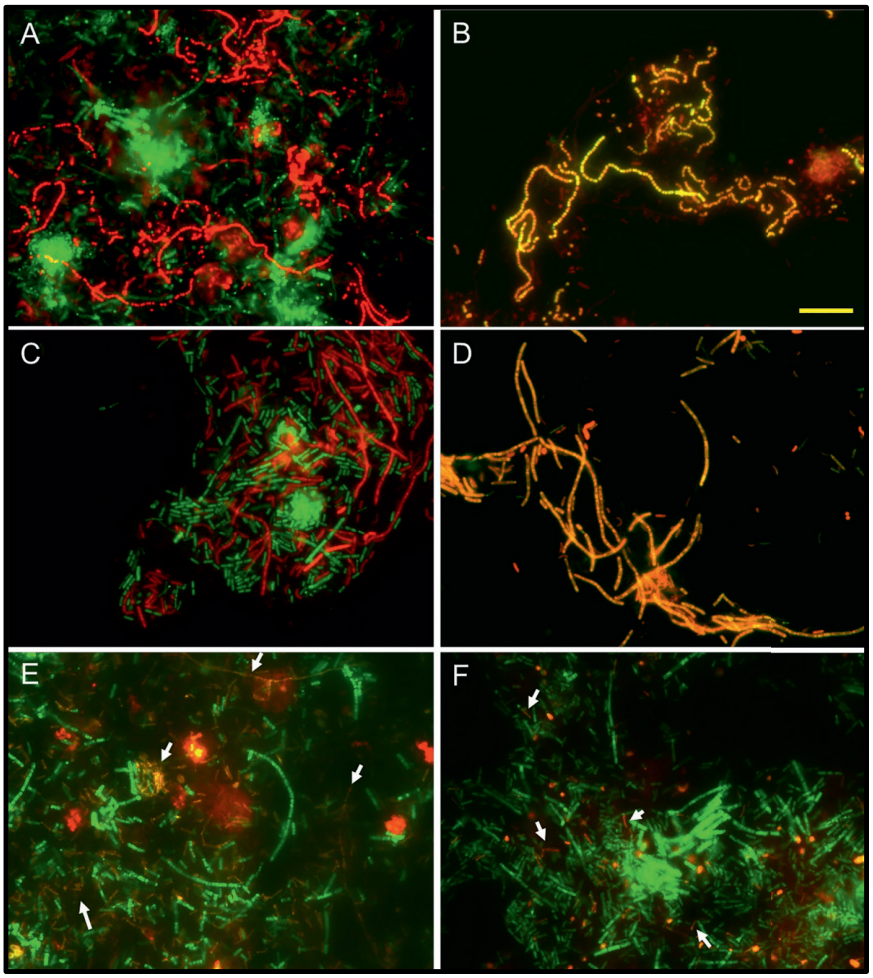


Figure 5. FISH images of samples taken from R1 (5 g Na^+/L, in A, B and E) and R3 (20 g Na^+/L, in C, D and F) at the end of the reactor runs. In A and C samples were hydribized with the EUB338mix probe (domain Bacteria, red) and the ARC915 probe (domain Archaea - green). In B and D the EUB338mix probe (red) was applied together with specific probes. In B, *Streptococcus sp.* was identified in reactor R1 samples by using Strept probe (in green). In D, filamentous bacteria of reactor R3 were further identified with the probe LAC435 (green). In E and F, ARC915 probe (green) was applied together with *Methanobacteriales* specific probe MG1200b, and arrows indicate archaeal cells positive to both probes. Details on the probes are reported in **Table S2**. Yellow bar is 10 µm.

4. Discussion

4.1. Granulation and start – up period

4.1.1. Rapid granulation and start –up at high salinity: role of cations and PAC

In our UASB reactors a rapid granular sludge formation from dispersed biomass at 5 g Na^+/L and even at 20 g Na^+/L occurred (**Figure 2** and **Figure 3)**. The first granules were already observed after 23 days (**Figure 2**). This is in contrast with Ismail et al. (2008) who showed that considerable washout of (granular) biomass from UASB reactors took place, even at salinity levels as low as 5 g Na^+/L.

Robust granules were formed, even though the wastewater only contained a (background) concentration of 13 mg/L of calcium during the first 3 months of operation. Multivalent cations are known to play a crucial role in the formation of microbial granules/biofilms by bridging negatively charged groups of EPS (Mahoney et al., 1987; Yu et al., 2001; Vlyssides et al., 2009; Flemming and Wingender, 2010), although their role with respect to effects on rates of granulation is somewhat controversial. Mahoney et al. (1987) and Yu et al. (2001) reported that dosing of 100 – 300 mg Ca^{2+}/L increases the rates of granulation and biomass retention during UASB start – up, whereas Hulshoff Pol (1989) and Guiot et al. (1988) found no positive effect upon addition of 150 to 640 mg Ca^{2+}/L at non – saline conditions. Since at high Na^{+} concentrations Ca^{2+} is displaced in the EPS matrix of granules, thereby weakening the granular structure (Jeison et al., 2008; Ismail et al., 2010), it would be expected that at highly saline conditions additional Ca^{2+} dosing would be needed to promote granulation. Here we demonstrated that at highly saline conditions granulation rate can be fast without dosing of additional calcium, probably due to gelling of EPS with sodium ions, as discussed in paragraph *4.1.3*.

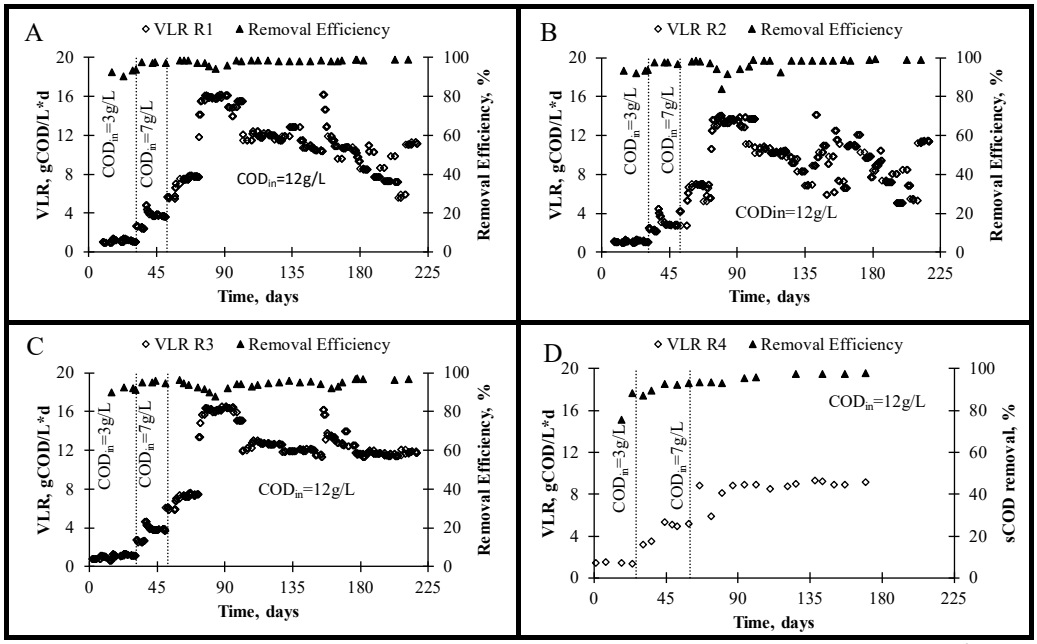


Figure 6. COD loading rates and sCOD removal efficiencies. VLR – volumetric loading rate. A – R1 (at 5 g Na^{+}/L), B – R2 (at 5 g Na^{+}/L), C – R3 (at 20 g Na^{+}/L), D – R4 (at 20 g Na^{+}/L). Dotted lines indicate the periods with different influent COD concentrations.

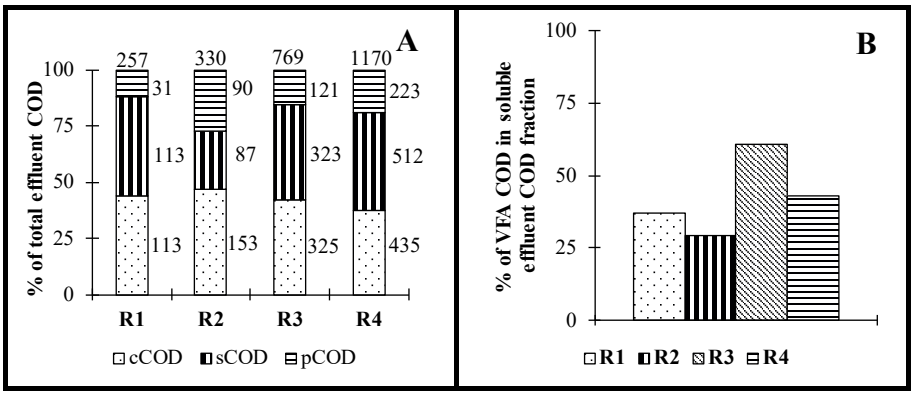


Figure 7. 40 hour composite effluent sample COD fractions collected on day 179 in R1, R2, R3 and on day 100 in R4. A – COD fractions in the effluent; B – fraction of effluent sCOD as acetate and propionate. Numbers above bars in A show total effluent COD in mg/L, numbers next to the fractions show their concentrations in mg/L.

For all reactors a start – up period (as defined by Ghangrekar et al. (1996)) of only 3 weeks was necessary at both salinity levels and regardless of the inoculum being sieved or intact (**Figure 6 A-D**). This period coincided with the appearance of the first granules (**Figure 2 – B**) and was as fast as reported at non – saline conditions, with operational parameters chosen for enhanced UASB start - up (Ghangrekar et al., 1996).

Interestingly, during the start-up no sizable difference was observed in process performance between R1 (no PAC added) and R2 (0.1% of inoculum mass PAC) and in the development of the particle size (**Figure 3-A, B**) as opposed to Yu et al. (1999), who reported faster granulation upon addition of PAC to inoculum. However, the amount of PAC that was mixed with the inoculum in our study was only 0.02 compared to 0.24 g PAC/gVSS in experiments of Yu et al. (1999). Zhou et al. (2015) demonstrated that the diameter of the activated carbon can be a determining factor for enhancing the granulation process of sludge due to velocity field differences between the flocs and activated carbon. It was concluded that 0.33 g GAC/gVSS with a diameter of 0.2 mm enhances microbial granulation and reactor start-up. Also Yu et al. (1999) used PAC with a diameter of 0.2 mm, whereas in our study the diameter of PAC was 0.037 to 0.149 mm. Due to differences in both type and quantity of PAC used in our study, a definitive conclusion on, why additional PAC had no effect on process performance and granulation cannot be made.

4.1.2. Microbial key players

SEM (**Figure 4**) and FISH (**Figure 5**) analysis show that different bacterial communities developed at different salinity levels, even though the same organic substrate and inoculum were used. Within the archaeal population, a member of the *Methanosaetaceae* was the dominant methanogen in all

reactors studied. Meanwhile, the hydrogenotrophic *Methanomicrobiales* decreased in abundance with increasing salinity.

It has been hypothesized by Wiegant (1988), that a precursor of granulation is formation of filamentous *Methanosaeta* nuclei, to which other microbial cells can attach. The aggregation properties of *Methanosaeta* were also found to be crucial during anaerobic digestion in several other studies (Calli et al., 2003; Angenent et al., 2004; Li et al., 2015b; Gagliano et al., 2017a). In R1 and R3 granules, *Methanosaeta*-like cells and filaments were always detected in large clusters, forming a nucleus for aggregation, to which a bacterial population could attach (**Figure 5**). This demonstrated the possible fundamental role of this acetoclastic methanogen in granulation, as already discussed by Gagliano et al. (2017b). However, the presence of the two bacteria as filaments may also have contributed to the aggregation.

This is the first time that *Streptococcus sp*. and *Lachnospiraceae* family members were identified at salinity levels of 5 to 20 g Na^+/L, and as key population within anaerobic granules. FISH analysis confirmed their association with *Methanosaeta* clusters (**Figure 5-A, C**). In the 20 g Na^+/L granules the filamentous members of the family *Lachnospiraceae* and *Methanosaeta* clusters were uniformly distributed within the granule (**Figure 4 E-F**), with a clear dominance of the methanogens along the granular structure (**Figure 5-C**). The close connection between the methanogenic and bacterial community in the granules suggests the presence of a syntrophic metabolism which, given the substrate composition of the synthetic wastewater stream and the microbial metabolic characteristics, could be based on glucose fermentation coupled with methane production. The presence of *Methanomicrobiales* was already shown in highly saline natural environments and wastewater treatment systems (Webster et al., 2014; De Vrieze et al., 2016b) and suggests that hydrogenotrophic methanogenesis was taking place, most likely coupled with either protein or glucose fermentation (Stams, 1994). However, FISH analysis could not clarify the utilization of the proteinaceous substrate, which could be partially used for the EPS build-up by one of microbial protagonists.

4.1.3. Strength of granules

The strength of the granules at low and high salinity was similar and the granules that were formed were stronger than the granules in the inoculum (**Table 3**). This is in conflict with the results of others using the same inoculum (Ismail et al., 2008; Jeison et al., 2008; Ismail et al., 2010). Also, the high values of 70 to 98.6 days found for SRTs confirmed that robust granules were formed. The different outcomes between ours and the other studies may be explained by different carbon sources used, a factor shown to affect chemical composition of EPS (Wolfaardt et al., 1998), which can in turn affect the gelling properties of these polymers.

Both monovalent and divalent cations can form gels with microbial polysaccharides (e.g., gellan) (Chandrasekaran and Radha, 1995) and alginate-like EPS are likely to bind both calcium and sodium ions (Seviour et al., 2012; Stewart et al., 2014), meaning that both can play a role in granulation at high salinity. Calcium ions will form stronger gels than monovalent cations because carboxylate---Ca^{2+}---carboxylate interactions are stronger than those of, for example carboxylate---Na^{+}---water---Na^{+}---carboxylate (Chandrasekaran and Radha, 1995). Due to a much higher concentration of sodium over calcium in the influent wastewater of all reactors in our study, sodium is very likely to play an important role in the process of granulation, thus explaining the similar strength of granules measured both at 5 g Na^{+}/L and 20 g Na^{+}/L. **Figure S5** shows that the concentration of sodium is 5.75 and 22.79 g/L in granular sludge of R1 and R3, respectively, whereas the concentration of calcium is 0.159 and 0.037 in the granular sludge of R1 and R3. The preliminary outcomes of metal analysis within the granular sludge indeed indicate, that further investigation of the abovementioned hypothesis is needed to further understand the gelling properties of EPS formed at high monovalent salt concentration.

4.2. Process performance

4.2.1. Performance of bioreactors at 5 and 20 g Na^{+}/L: role of substrate and inoculum biomass

The reactors R1, R2 and R3 have been continuously operated for more than 200 days and reactor R4 for more than 170 days with stable process performance (**Figure 6**). This shows that with an appropriate inoculum selection, start-up procedure and operation, highly saline waste streams can be treated in UASB reactors at loading rates as high as 16 g COD/m^3·d.

Even though good COD removal efficiencies were obtained at 5 and 20 g Na^{+}/L (**Figure 6 A-D**), it becomes clear that total effluent COD concentrations around 1000 mg/L require a further polishing step. The higher effluent COD concentration at 20 g Na^{+}/L can be explained by a lower methanogenic activity (Li et al., 2014; De Vrieze et al., 2016a), sloughing of loosely bound EPS, a higher production of soluble EPS (Corsino et al., 2017), washout of individual cells and by a lower affinity of the microorganisms to the substrate, since different microbial communities developed at 5 and 20 g Na^{+}/L (**Figure 5**). **Figure 7** shows, that colloidal and soluble fractions were contributing to the largest effluent COD difference between 5 and 20 g Na^{+}/L reactors. In these fractions both EPS and microorganisms can potentially contribute to the COD content.

Although deteriorating process performance and operational problems were found by other authors at levels close to or below 5 g Na^{+}/L (Patel and Roth, 1977; Rinzema et al., 1988; Lefebvre et al., 2007; Jeison et al., 2008; Li et al., 2014; De Vrieze et al., 2016a), our findings are in agreement with De Baere et al. (1984), who have reported salt tolerance of anaerobic biomass similar to the one demonstrated here. Potentially both the substrate and inoculum (adapted to 8 g Na^{+}/L) used, may have

influenced the positive outcome of this research (McCarty and McKinney, 1961; Patel and Roth, 1977; Lefebvre et al., 2007).

From bioenergetics point of view in most prokaryotes, uptake of osmoprotectants from surrounding liquid is preferred over de novo synthesis (Oren, 1999; Roeßler and Müller, 2001; Gunde-Cimerman et al., 2006). Since also amino acids and their derivatives can act as osmolytes (Roberts, 2005), tryptone fed to the reactors during our study may have acted as source of "cheap" osmoprotectants after undergoing hydrolysis. Also in experiments of De Baere et al. (1984), who reported salt tolerance of anaerobic biomass similar to one presented here, peptone (tryptic soy) was added to synthetic wastewater, in contrast to other studies reporting sodium inhibition in the absence of such substances.

Use of salt adapted microorganisms as inoculum was suggested as one of the most suitable strategies to overcome sodium toxicity in anaerobic wastewater treatment (Vyrides, 2015). The inoculum used in our study had been adapted to 8 g Na^+/L, which is considerably lower than the 20 g Na^+/L applied in R3 and R4. However, in the few studies where high methanogenic activity was found at comparable salinities, the inoculum was adapted to similar salinity as reported here (Ismail et al., 2010; Gagliano et al., 2017b), demonstrating the importance of inoculum at high salinities.

5. Conclusions

Granulation of anaerobic biomass from dispersed inoculum is possible, even at sodium concentrations as high as 20 g Na^+/L, which was not reported in literature before. Moreover, calcium concentration as low as 13 mg/L is sufficient to form microbial granules at 20 g Na^+/L and is around ten times lower than the traditionally reported 100 – 150 mg Ca^{2+}/L in non-saline conditions. The granulation process at 20 g Na^+/L can be accompanied by efficient soluble COD removal, however the removal efficiency is still lower (92.8 ± 5.4% to 94.0 ± 2.2%) compared to reactors operated with the same substrate at 5 g Na^+/L (96.6 ± 3.3% to 97.2 ± 2.1%). Interestingly, addition of powdered activated carbon to inoculum does not guarantee a faster reactor start-up. High salinity acted as a selective pressure on the dominant active microbial (*Streptococcus spp* at 5 g Na^+/L and bacterium belonging to *Lachnospiraceae* at 20 g Na^+/L) and hydrogenotrophic archaeal community (decreasing abundance of *Methanomicrobiales* at elevated salinity). Interestingly, the active acetoclastic archaeal community remained similar within the range of 5 to 20 g Na^+/L (*Methanosaeta* as dominant methanogen at both salinities).

.

Supplementary Information of Chapter 2

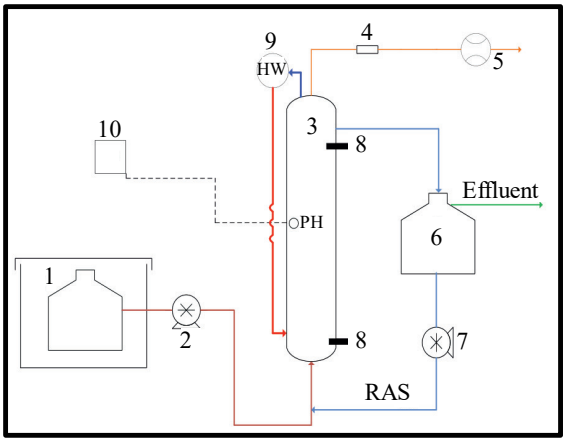


Figure S1. Schematic drawing of set-ups for reactors R1-R3. 1 – refrigerated influent tank at 4 ºC; 2 – peristaltic influent pump; 3 – double walled glass bioreactor; 4 – biogas sampling port; 5 – gas flow meter; 6 – effluent/recirculation tank; 7 – peristaltic effluent recirculation pump; 8 – sampling ports; 9 – heating bath for temperature control at 35 ºC; 10 – pH meter.

Table S2. Oligonucleotide probes used in FISH analysis of granules in R1 and R3

Name	Target group	Probe sequence (5'-3')	Formamide (%)	Reference
ARCH915	*Archaea*	GTGCTCCCCCGCCAATTCCT	20 or 35	(Stahl, 1991)
EUB338, EUB338-II, EUB338-III	*Bacteria*	GCTGCCTCCCGTAGGAGT, GCAGCCACCCGTAGGTGT, GCTGCCACCCGTAGGTGT	20 or 35	(Amann et al., 1990)
BET42a	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	35	(Manz et al., 1992)
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	35	(Manz et al., 1992)
ALF968	*Alphaproteobacteria*	GGTAAGGTTCTGCGCGTT	20	(Neef et al., 1998)
CF319a	*Bacteroidetes*	TGGTCCGTGTCTCAGTAC	35	(Manz et al., 1992)
DELTA485A, DELTA495B, DELTA495C	*Deltaproteobacteria*	AGTTAGCCGGTGCTTCCT, AGTTAGCCGGCGCTTCCT, AATTAGCCGGTGCTTCCT	35	(Lücker et al., 2007)
CFX1223	*phylum Chloroflexi*	CCATTGTAGCGTGTGTGTMG	35	(Björnsson et al., 2002)
SYN961	*Synergystetes*	GTTCTTCGGTTTGCATCG	20 or 35	(Gagliano et al., 2015)
NLIMI91	*Nostocoida Liimicola type I (Thricococcus spp)*	CGCCACTATCTTCTCAGT	35	(Liu and Seviour, 2001)
STREPT	*Streptococcaceae*	CACTCTCCCCTTCTGCAC	30	(Trebesius et al., 2000)
LAC435	*family Lachnospiraceae*	TCTTCCCTGCTGATAGA	35	(Kong et al., 2010)
MSMX860	*Methanosarcinales*	GGCTCGCTTCACGGCTTCCCT	45	(Raskin et al., 1994)
MX825	*Methanosaetaceae**	TCGCACCGTGGCCGACACCTAGC	50	(Raskin et al., 1994)
MX825b	*Methanosaetaceae subgroup**	TCGCACCGTTGCCGACACCTAGC	50	(Crocetti et al., 2006)
MX825c	*Methanosaetaceae subgroup**	TCGCACCGTGGCTGACACCTAGC	50	(Crocetti et al., 2006)
MG1200b	*most Methanomicrobiales*	CRGATAATTCGGGGCATGCTG	20	(Crocetti et al., 2006)
MB311	*Methanobacteriales*	ACCTTGTCTCAGGTTCCATCTCC	30	(Crocetti et al., 2006)

*(except *M.harundinacea*)

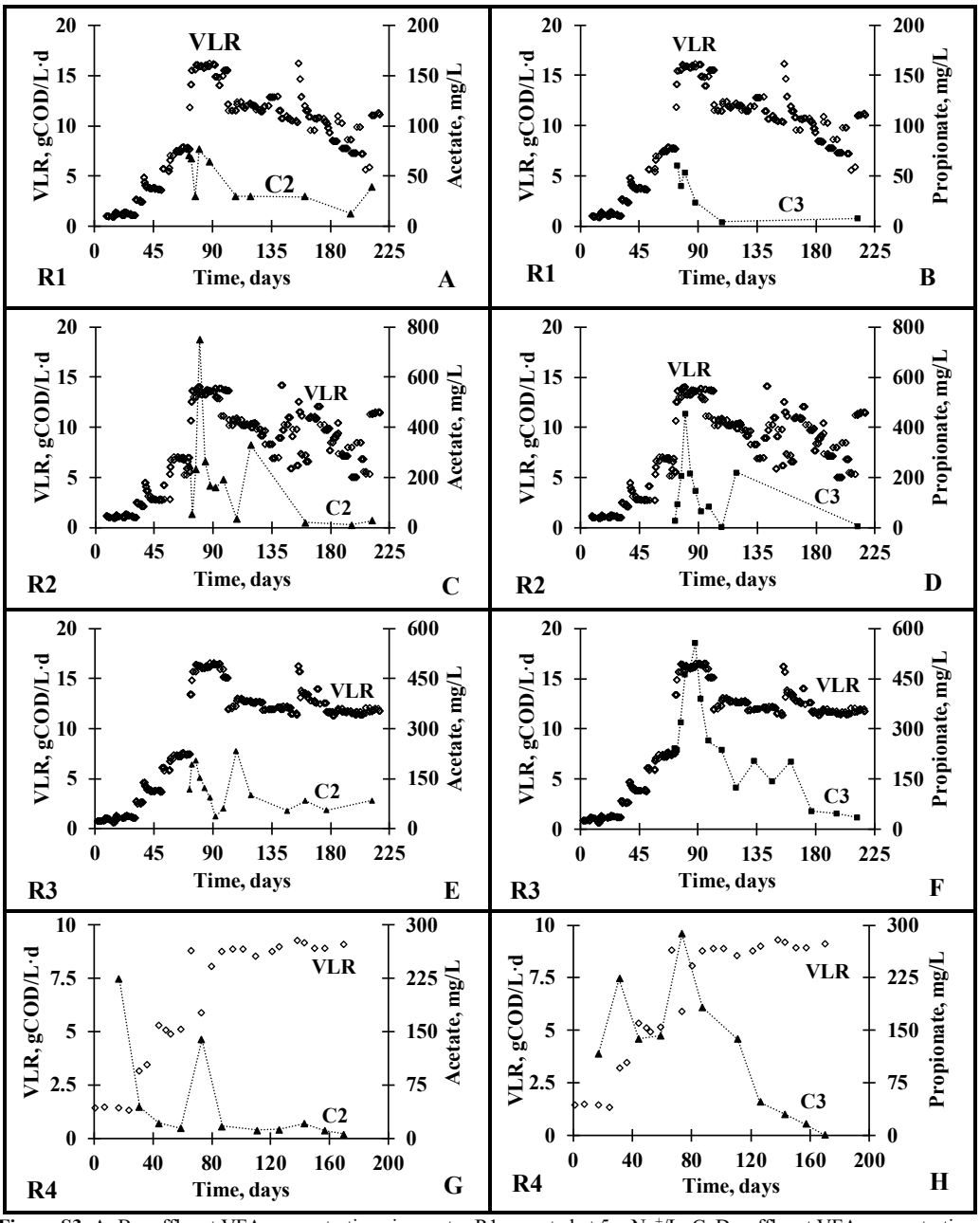


Figure S3. A, B – effluent VFA concentrations in reactor R1 operated at 5 g Na^+/L; C, D - effluent VFA concentrations in reactor R2 operated at 5 g Na^+/L, E, F – effluent VFA concentration in reactor R3 operated at 20 g Na^+/L and inoculated with granular biomass, G, H – effluent VFA concentration in reactor R4 operated at 20 g Na^+/L and inoculated with dispersed biomass. VLR – volumetric loading in gCOD/L·d; C2- acetate; C3- propionate.

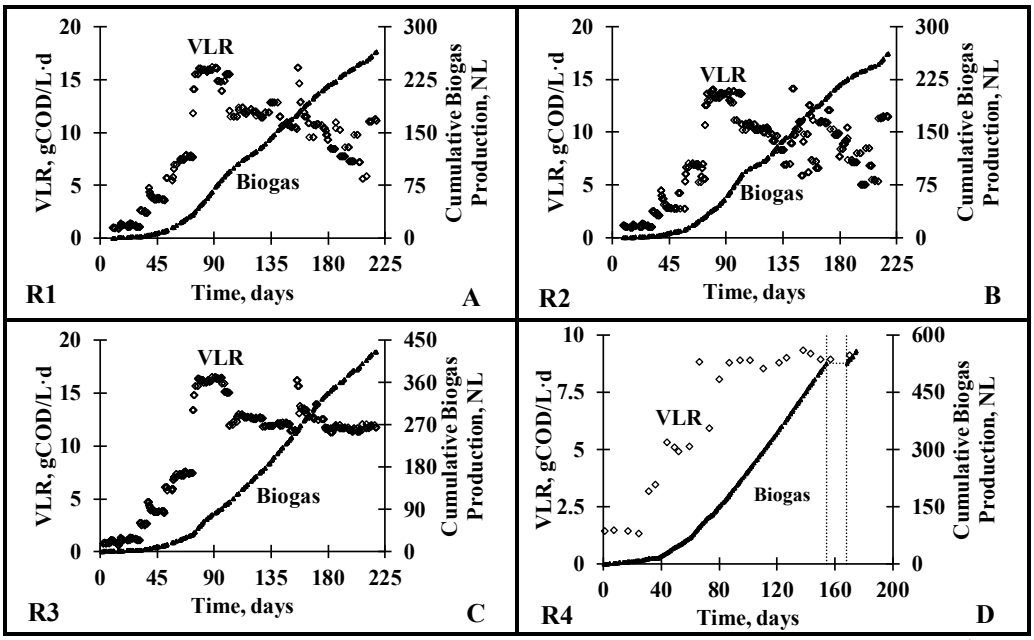


Figure S4. Biogas production profiles in continuously operated bioreactors. A – reactor R1 operated at 5 g Na^+/L; B – reactor R2 operated at 5 g Na^+/L; C – reactor R3 operated at 20 g Na^+/L and inoculated with granular biomass; D - reactor R4 operated at 20 g Na^+/L and inoculated with dispersed biomass. Vertical lines in D represent a period of testing a tailor made gas meter during which no quantification with Bioprocess control was performed. NL – normalized litres (for standard conditions of 0 ºC and 1 atm).

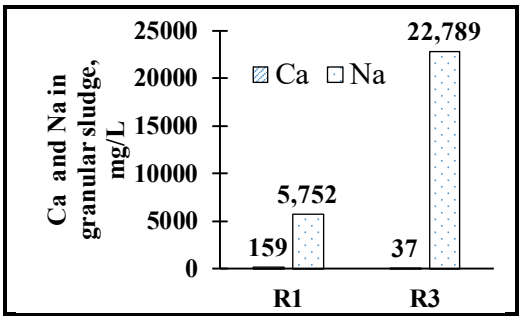
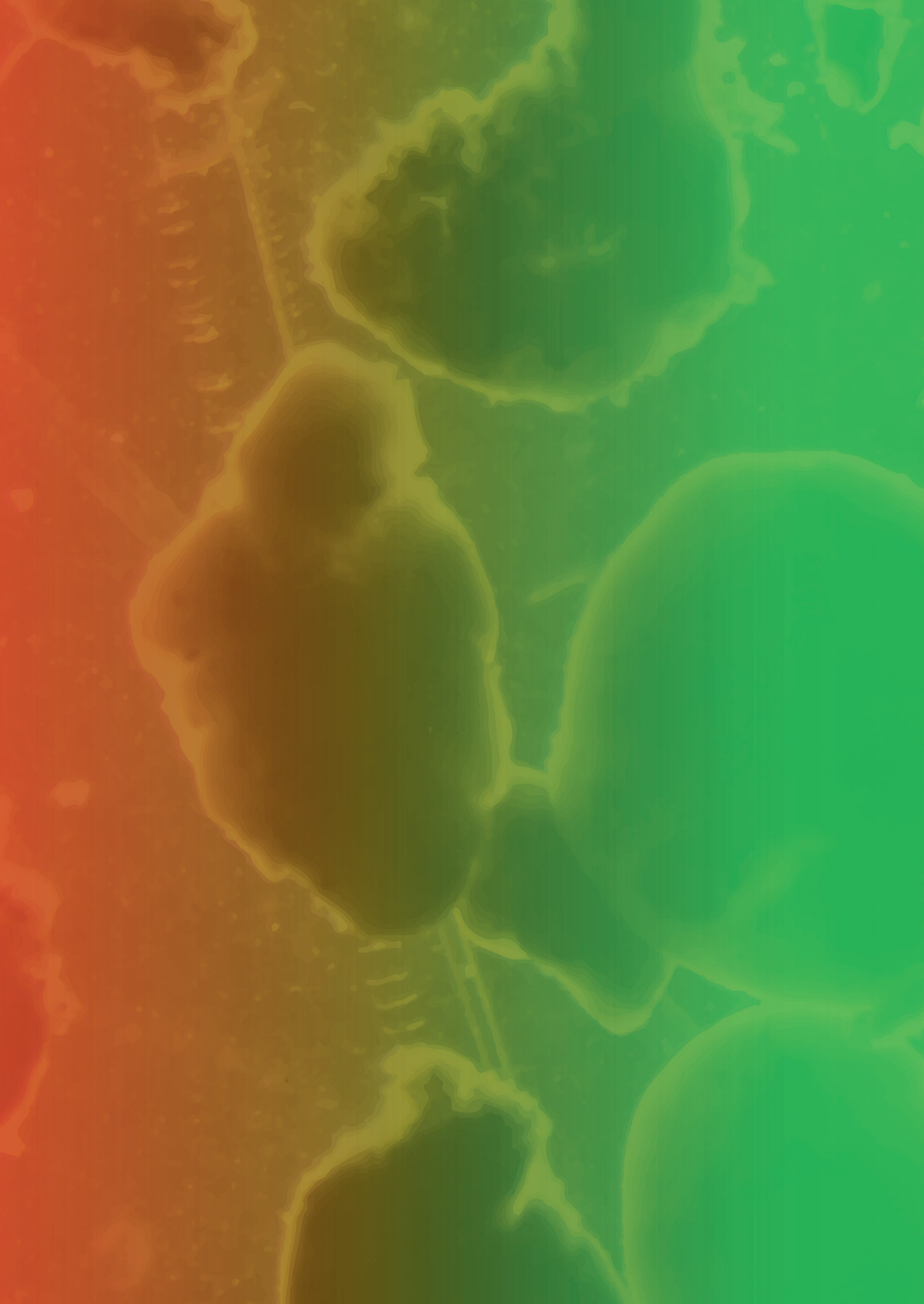


Figure S5. Concentration of calcium and sodium within granular sludge of R1 (operated at 5 g Na^+/L) and R3 (operated at 20 g Na^+/L).

Figure S5. The samples were collected, while vigorously mixing reactor content with nitrogen gas. Granular sludge was separated from the liquid phase by centrifugation at 10000xg and 4 ºC for 15 min. The supernatant after centrifugation was removed. The pellets were carefully homogenised with a spatula. Approximately 0.7g of solids per sample were taken for microwave digestion (ETHOS 1 - Advanced Microwave Digestion Labstation, Milestone S.r.l., Italy) with 7.5 mL of 37% HCl (For Analysis Emsure® Acs,Iso,Reag. Ph Eur) and 2.5 mL 65% HNO_3 (For Analysis Emsure® ISO). As a blank for ionic composition measurements mili-Q was treated in the same way as samples. Digested samples were brought up to 50 mL with mili-Q water and further diluted for analysis. Ion analysis was carried out by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian, Australia) as described in (Ismail, 2013). All measurements were performed in duplicate.



Chapter 3. The Potential of Osmolytes and their Precursors to Alleviate Osmotic Stress of Anaerobic Granular Sludge

A modified version of this chapter has been published as:

Sudmalis, D., Millah, S.K., Gagliano, M.C., Butré, C.I., Plugge, C.M., Rijnaarts, H.H.M., Zeeman, G. and Temmink, H., 2018. The potential of osmolytes and their precursors to alleviate osmotic stress of anaerobic granular sludge.
Water Research 147, 142-151
https://doi.org/10.1016/j.watres.2018.09.059.

Abstract. Increasing amounts of saline wastewater with high concentrations of organic pollutants are generated globally. In the anaerobic wastewater treatment domain, high salt concentrations are repeatedly reported to inhibit methanogenic activity and strategies to overcome this toxicity are needed. Current research focuses on the use of potential osmolyte precursor compounds for osmotic stress alleviation in granular anaerobic sludges upon exposure to hypersalinity shocks. Glutamic acid, aspartic acid, lysine, potassium, gelatine, and tryptone were tested for their potential to alleviate osmotic stress in laboratory grown and full – scale granular sludge. The laboratory grown granular sludge was adapted to 5 (R5) and 20 (R20) g Na^+/L. Full-scale granular sludge was obtained from internal circulation reactors treating tannery (waste)water with influent conductivity of 29.2 (Do) and 14.1 (Li) mS/cm. In batch experiments which focused on specific methanogenic activity (SMA), R5 granular sludge was exposed to a hypersalinity shock of 20 g Na^+/L. The granular sludge of Do and Li was exposed to a hypersalinity shock of 10 g Na^+/L with sodium acetate as the sole carbon source. The effects on R20 granular sludge were studied at the salinity level to which the sludge was already adapted, namely 20 g Na^+/L. Dosing of glutamic acid, aspartic acid, gelatine, and tryptone resulted in increased SMA compared to only acetate fed batches. In batches with added glutamic acid, the SMA increased by 115% (Li), 35% (Do) and 9% (R20). With added aspartic acid, SMA increased by 72% (Li), 26% (Do), 12% (R5) and 7% (R20). The addition of tryptone resulted in SMA increases of 36% (R5), 17% (R20), 179% (Li), and 48% (Do), whereas added gelatine increased the SMA by 30% (R5), 14% (R20), 23% (Li), and 13% (Do). The addition of lysine, meanwhile, gave negative effects on SMA of all tested granular sludges. Potassium at sea water Na/K ratio (27.8 w/w) had a slight positive effect on SMA of Do (7.3%) and Li (10.1%), whereas at double the sea water ratio (13.9% w/w) had no pronounced positive effect. R20 granular sludge was also exposed to hyposalinity shock from 20 down to 5 g Na^+/L. Glutamate and N-acetyl-β-lysine were excreted by microbial consortium in anaerobic granular sludge adapted to 20 g Na^+/L upon this exposure to hyposalinity. A potential consequence when applying these results is that saline streams containing specific and hydrolysable proteins can be anaerobically treated without additional dosing of osmolytes.

1. Introduction

Economic sectors such as leather tanning, chemical and agro-food industries generate large amounts of saline (waste)water that is both rich in salt (NaCl) and organic matter (Lefebvre and Moletta, 2006). Le Borgne et al. (2008) reported that around 5% of the globally produced industrial (waste)water is either saline or hypersaline. This amount of saline industrial (waste)water is increasing due to growing industrial activities and recycling of (waste)water (Rozzi et al., 1999; Giustinianovich et al., 2018). A viable option for removing bulk organic pollutants from this water is anaerobic biological

treatment. This approach also generates energy and reduces the amount of waste sludge. If sludge aggregation into microbial granules can be achieved, high rate anaerobic reactors such as upflow anaerobic sludge blanket (UASB) should be considered. The excellent settling properties of granular sludge allows for a reduction in the bioreactor footprint (Vieira et al., 2005a; Xiao and Roberts, 2010) and the high rate anaerobic reactors are already widely applied for industrial (waste)water treatment (Van Lier et al., 2015).

There are two factors of particular importance when treating (waste)water with high concentrations of soluble chemical oxygen demand (COD) with high rate anaerobic treatment: i) microbial activity; and ii) microbial sludge granulation and stability (O'Flaherty et al., 1997; McHugh et al., 2003; Liu et al., 2009; Lu et al., 2013). However, high concentrations of monovalent salts have been repeatedly reported to inhibit the methanogenic activity (Rinzema et al., 1988; Liu and Boone, 1991; De Vrieze et al., 2016b) and cause granular sludge disintegration with subsequent biomass washout from the bioreactors (Rinzema et al., 1988; Ismail et al., 2008; Jeison et al., 2008; De Vrieze et al., 2016a).

Microbial cells have two different strategies for adapting to high osmotic pressure. The first is by increasing their intracellular ion concentration (mainly K^+ and Cl^-). The second is by up taking and/or synthesizing small organic molecules called compatible solutes (Sowers et al., 1990; Oren, 1999; Roberts, 2005). The first strategy is used by *Halobacteria* (Archaea) and *Haloanaerobiales* (Bacteria) and requires energy for adaptation of the cells enzyme machinery (Oren, 1999; Roeßler and Müller, 2001; Müller et al., 2005).

The second strategy, which is used by the majority of the living cells (Pfluger, 2005), does not require such adaptation. Additionally, microorganisms applying this mechanism can survive in a broader range of salinity levels (Roeßler and Müller, 2001; Müller et al., 2005; Pflüger et al., 2005). In compatible solute accumulating bacteria, the response to an osmotic shock can be divided into two phases. During the first phase, a rapid influx of potassium ion into the microbial cell is triggered. During the second phase, cellular levels of potassium are decreased and compatible solutes are accumulated instead (Pflüger et al., 2005). Similar responses to osmotic stress have been demonstrated in pure cultures of the methanogenic archaea *Methanococcus thermolitotrophicus* (Martin et al., 2000). Since salinity of (waste)water often fluctuates in time (Vyrides, 2015), this mechanism is likely to be utilized by the microbial consortium of bioreactors treating several types of saline (waste)water.

Several types of molecules can act as compatible solutes: sugars (trehalose, betaine), amino acids (glutamic acid, proline), derivatives of amino acids (N-ε-acetyl-β-lysine, ectoine) (Roberts, 2005), and polyols (Roeßler and Müller, 2001). Uptake from the bulk liquid of such molecules is bio-

energetically more favourable than synthesis *de novo* (Oren, 1999). This explains why several studies focused on the potential of dosing osmolytes in anaerobic (waste)water treatment systems. Molecules like glycine betaine, α-glutamate, β-glutamate, trehalose and, N-acetyl-β-lysine (Yerkes et al., 1997; Vyrides and Stuckey, 2009a; Vyrides et al., 2010; He et al., 2012) have all been demonstrated to have the potential of increasing methanogenic activity when flocculent biomass was exposed to osmotic stress. In a recent review Vyrides and Stuckey (2016) reported that glycine betaine is by far the most studied osmolyte to date. The effects of osmolytes on methanogenic activity of granular sludge are not researched so far. Moreover, the bottle neck for application of osmolytes in practice is the relatively high cost of such molecules (Vyrides and Stuckey, 2016).

In **Chapter 2**, robust anaerobic sludge granulation at high salinity levels (up to 20 g Na^+/L) was reported. The microbial activity was sufficient to achieve a high soluble COD removal efficiency (94%) at a loading rate of 16 g COD/L·d. The source of carbon in the synthetic (waste)water was a mixture of glucose, acetate, and tryptone (**Chapter 2**). Proteins contain a broad range of amino acids as their natural building blocks (Smith and Friedman, 1984; Di Gioia and Guilbert, 1999). These amino acids are known to act as osmo-protectant precursors in anaerobic microorganisms (Roberts, 2005; Zaprasis et al., 2014). It was hypothesised that, after hydrolysis, tryptone (a mixture of peptides formed by digesting casein with trypsin) supplied the microorganisms with the "building-blocks" for the osmo-protectants uptake/synthesis. Thus hydrolysable proteins are potentially a cheaper alternative to osmolytes if indeed they can alleviate osmotic stress.

In this work, the osmolytes present in granular sludge adapted to 20 g Na^+/L were first identified. Next it was investigated if these osmolytes, their precursors, and proteins were able to alleviate osmotic shock stress in four types of granular sludge. Furthermore, the potential of potassium to alleviate osmotic shock stress was studied, since its rapid uptake is reported as the first response to increase in salinity. The granular sludge was obtained from two full-scale anaerobic reactors treating saline tannery (waste)water and two from laboratory-scale reactors treating synthetic (waste)water at salinity levels of 5 and 20 g Na^+/L.

The results show that not only osmolytes, but also their precursors and proteins can alleviate osmotic stress in all four types of the anaerobic granular sludges.

2. Materials and methods

2.1. Inoculum

Full scale granular sludge was obtained from two internal circulation (IC) anaerobic reactors (Waterstromen, The Netherlands) treating tannery (waste)water in Dongen (Do) and Lichtenvoorde (Li) (The Netherlands). The average influent (waste)water conductivity was 14.1 and 29.2 mS/cm for the IC reactors in Lichtenvoorde and Dongen, respectively. The average influent total COD concentration was 6.8 g/L and 5 g/L in Lichtenvoorde and Dongen, respectively. The average influent chloride concentration was 4 g/L in Lichtenvoorde and 6.7 g/L in Dongen. The granular sludges from the full-scale reactors were pre-washed with 5 g Na^+/L nutrient medium (composition as described in **Table 1**) and were stored in the same medium at 4ºC before use in batch experiments. This was done in order to have a defined liquid phase ionic composition during the batch experiments.

Table 1. Nutrient medium with final salt concentrations used in all batch experiments. *NaCl was used to adjust sodium concentration depending on the type of experiment. **$(NH_4)_2CO_3$ was used to compensate for additional nitrogen originating from osmo-protectants used in experiments with increasing salinity.

Macronutrients, alkalinity, Na, NH_4-N		Micronutrients	
Salt	**Final Concentration, g/L**	**Salt**	**Final Concentration, mg/L**
NH_4Cl	1.02	$FeCl_2 \cdot 4H_2O$	1.20
$MgSO_4 \cdot 7H_2O$	0.05	HBO_3	0.03
$CaCl_2 \cdot 2H_2O$	0.05	$ZnCl_2$	0.03
KH_2PO_4	0.22	$CuCl_2 \cdot 2H_2O$	0.02
$NaHCO_3$	1.5	$MnCl_2 \cdot 4H_2O$	0.30
NaCl	Variable*	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.05
$(NH_4)_2CO_3$	Variable**	$CoCl_2 \cdot 6H_2O$	1.20
		$NiCl_2 \cdot 6H_2O$	0.03
		EDTA	0.60
		HCl, mM	0.007
		Resazurin	0.30
		$Na_2SeO_3 \cdot 5H_2O$	0.06

The laboratory-scale granular sludge adapted to 5 and 20 g Na^+/L was formed from dispersed biomass, as described in detail in **Chapter 2** and was kept at 4 ºC before use in batch experiments. From this point forward, the granular sludge formed at 5 and 20 g Na^+/L is referred to as R5 and R20, respectively.

2.2. Batch experiments

Three types of batch experiments were performed in this study. The first involved use of tryptone at high COD proportion to study its potential for alleviating salt stress in R5 granular sludge. The second involved an abrupt decrease in salinity and identification of osmolytes in R20 granular sludge. The third type of experiments was performed to study the potential of potassium and potential precursors

of osmolytes to alleviate osmotic stress in R5, R20, Do and Li granular sludge. A detailed description of each type of experiment is given in *Sections 2.2.1., 2.2.2. and 2.2.3.*

The batch experiments with laboratory grown granules (R5 and R20) were performed in 118 mL serum bottles with a working volume of 50 mL. The experiments were performed in an incubator at 35 ºC and with a 120 RPM mixing speed. The VSS concentration was set to 1 g/L and the COD:VSS ratio was 4:1 (w/w).

The experiments with full scale granules (Do and Li) were performed in Automatic Methane Potential Test System. (AMPTS) II (Bioprocess Control, Sweden) at a stirring speed of 30 rpm (30 seconds on and off) and at 35 ºC. Due to the overall lower activity of the full-scale as compared to the laboratory grown biomass, the working volume of batch experiments was set to 200 mL. Additionally, the biomass concentration for batch experiments was chosen accordingly and was set to 2.5 and 5 g VSS/L for Do and Li, respectively. The COD concentration during experiments was the same as for R5 and R20, namely 4 g/L.

The level of sodium in each experiment was adjusted by varying the NaCl concentration (VWR, minimum purity 98%) of nutrient medium as presented in **Table 1**. Sodium originating from sodium acetate was taken into account for the final concentration calculations. Parallel to all batch experiments, blank experiments were performed at corresponding salinity without the addition of COD to correct for biogas production originating from the inoculum. The headspace before each batch experiment was flushed with nitrogen gas. An overview, containing all of the batch experiments in this study and the main parameters, is presented in **Table 2**.

Table 2. Summary of performed batch experiments. Preconditioning salinity – salinity at which biomass was incubated with 0.4 g/L acetate COD prior the experiment. Experimental salinity - salinity to which granular sludge was exposed for SMA determination with and without added potential osmo-protectant. Total COD – initial COD concentration during the experiment.

	Effectiveness of potential osmo-protectants and their precursors				Experiment with tryptone added in excess in R5	Identification of osmo-protectants in R20
	R5	**R20**	**Do**	**Li**	**R5**	**R20**
Preconditioning salinity, g Na^+/L	5	20	5	5	5	20
Experimental salinity, g Na^+/L	20	20	10	10	20	5
Total COD, g/L	4	4	4	4	4	4
COD/VSS	4	4	1.6	0.8	4	4
% of COD added as osmo-protectant during the experiments and conc. of K^+ in g/L						
Lysine,%	2.1	2.1	2.5	4.5	-	-
Glutamate, %	2.8	2.7	3.4	6.7	-	-
Aspartate, %	1.7	1.6	2.1	4.2	-	-
Tryptone, %	9.7	9.7	12.1	23.8	25	-
Gelatine, %	1.7	1.7	4.4	8.5	-	-
Potassium, g/L	0.72	0.72	0.36	0.36	-	-
Potassium, g/L	1.44	1.44	0.72	0.72	-	-

Previously it was demonstrated that the dominant active methanogen was the acetoclastic *Methanosaeta* in both R5 and R20 granular sludge (Gagliano et al., 2018; Sudmalis et al., 2018a). Therefore, acetate was chosen as the main electron donor. Furthermore, data from one of the full-scale IC reactors operator revealed, that the reactor was operated at 7.6 ± 3.8 meq/L of effluent volatile fatty acids (VFAs), potentially requiring additional acetoclastic activity for conversion into methane.

2.2.1. Tryptone for alleviation of hypersalinity shock at high COD proportion

A preliminary experiment was performed to study if peptides can alleviate an osmotic shock in acetoclastic methanogens. This was done by exposing R5 granular sludge to an abrupt increase in salinity from 5 g Na^+/L to 20 g Na^+/L with and without a dosing of tryptone. In the batches with added tryptone, the COD consisted of 75% sodium acetate and 25% tryptone. In the batches without added tryptone, the COD consisted solely of sodium acetate. Similar experiments with R5 granular sludge were performed at a constant salinity level of 5 g Na^+/L. These experiments were performed to investigate if specific methanogenic activity (SMA) of R5 granular sludge would increase at a stable 5 g Na^+/L salinity with added tryptone compared to only sodium acetate fed batches.

2.2.2. Identification of osmolytes present in 20 g Na^+/L adapted biomass (R20)

To identify osmolytes present in 20 g Na^+/L (R20) adapted granular biomass, this sludge was exposed to a sudden decrease of salinity down to 5 g Na^+/L with sodium acetate as the only carbon source. After exposure to the lower salinity, liquid aliquots were sampled with an interval of two hours. This experiment was designed to study if amino acids or their derivatives would be released to the bulk liquid. This would indicate their use as osmo-protectants in the biomass (Pflüger et al., 2005). As a control for osmolyte excretion, batch experiments at a stable salinity of 20 g Na^+/L with sodium acetate as the carbon source were performed.

To study the specific methanogenic activity (SMA) upon a sudden exposure to hyposalinity as well as at stable salinity of 20 g Na^+/L, batch experiments for biogas production were run in parallel to those for osmolyte analysis.

2.2.3. Potential osmolytes and their ability to alleviate hypersalinity shocks

The third set of experiments was used to study the SMA of granular sludge fed with acetate along with addition of a potential osmo-protectant precursor upon exposure to hypersalinity of 10 or 20 g Na^+/L. The selection of osmo-protectant precursors is detailed in the following steps. Firstly, osmo-protectants were detected in laboratory scale granules adapted to 20 g Na^+/L and their biosynthesis precursors were chosen to study as the model compounds. Secondly, the addition of tryptone and gelatine was chosen to assess if peptides and proteins can also alleviate hypersalinity shock in granular sludge. Finally, potassium was chosen as a known transition element upon exposure of anaerobic microorganisms to hypersalinity shocks (Martin et al., 2000).

The selected compounds were: tryptone (Sigma Aldrich-New Zealand), gelatine (Type B from bovine bone, Merck-United Kingdom), glutamic acid (Merck-Germany), aspartic acid (TCI chemicals-Japan), lysine (Merck-France), and potassium (Merck-Germany).

To shorten the lag phase of these experiments, all batches were preconditioned for 1 to 3 days at an acetate COD level of 0.4 g/L in 75% of the final working volume. The salinity level during preconditioning was set to 5 g Na^+/L for R5, Do and Li granular sludge and to 20 g Na^+/L for R20 granular sludge. After the preconditioning period, additional nutrient medium, sodium acetate and potential osmo-protectants were spiked, resulting in a final COD concentration of 4 g/L and salinity levels of 10 g Na^+/L (Do, Li) and 20 g Na^+/L (R5, R20).

A lower salinity for ‘full scale granules’ Do and Li was chosen after preliminary experiments had shown almost no activity when these were exposed to 20 g Na^+/L. The nitrogen concentration

originating from addition of tryptone, gelatine, glutamic acid, aspartic acid, or lysine was compensated in batches fed with only acetate. This was done through the dosing of $(NH_4)_2CO_3$ in the nutrient medium, shown in **Table 1**.

2.3. Estimation of applicable osmo-protectant concentration for hypersalinity shock experiments

Since Na^+ and Cl^- ions were by far the predominant ions in the experiments, the amount of osmo-protectant to dose in the third set of batch tests was estimated based on the concentration of these two ions. For example, the concentration of Na^+ and Cl^- at 20 g Na^+/L equals 0.87 mol/L and 0.86 mol/L, respectively. It was assumed, that the density of the biomass is close to 1g/cm^3 and the fraction of bacteria accounts for about 40% of VSS in the granular sludge (m/m) (Chung and Neethling, 1990). The amount of osmo-protectant required to balance the pressure of Na^+ and Cl^- would be 0.69 mmol per gram VSS if it is assumed that the efficiency of uptake for these molecules is 100%. This calculation was not corrected for the fact that osmo-protectants can carry more than one charge at physiological pH (McNeil et al., 1999).

In the case of tryptone, it was assumed that after hydrolysis into amino acids, mostly glutamic acid acts as the osmo-protectant precursor (Roberts, 2005). In fact, tryptone is a pancreatic digest of casein, where glutamic acid accounts for around 20.2% of amino residues (Smith and Friedman, 1984). This was assumed to be similar in tryptone.

In the case of gelatine, the amount to dose was estimated based on the nitrogen content of gelatine, assuming that one mole of nitrogen corresponds to one mole of osmo-protectant. Potassium was dosed both at seawater Na/K ratio (w/w) of 27.8 (Zhu et al., 2004) and double this ratio resulting in a Na/K of 13.9 (w/w).

2.4. Identification of osmolytes as amino acids

The liquid samples were filtered through 0.45 µm acetate cellulose membrane filters (VWR® Syringe Filters). 0.5 mL of the filtrate was mixed with 0.4 mL of methanol and 0.1 mL of internal standard (0.4 mM Norleucine) and centrifuged to remove proteins. High Performance Liquid Chromatography (HPLC) was performed with Thermo Fisher (Dionex) Ultimate 3000 HPLC equipped with Thermo Fischer TCC3000 column compartment, Variable wavelength detector 3400RS, Acquity UPLC BEH C18 1.7 um, 2.1x150 mm column (Waters 186002350) and VanGuard Acquity UPLC 1.7 um, 2.1x5 mm guard column (Waters 186003975). The derivatisation procedure was based on (Hanczko et al., 2007). The data was analysed using Chromeleon 7.1 software.

2.5. Identification of amino acid derivatives as osmolytes

During HPLC analysis fractions containing unknown peaks, were collected and concentrated five times for further hydrophilic interaction liquid chromatography (HILIC) analysis. Acetonitrile (ACN) and trifluoroacetic acid (TFA) were added to the samples to reach final concentrations of 10 % ACN and 0.1 % TFA. Samples were centrifuged for 5 min (19000 xg, 20ºC) prior to injection. Samples were analysed on an Acquity UPLC System (Waters, Milford, MA, USA) using an Acquity UPLC BEH amide column (2.1 x 150 mm, 1.7 µm particle size) with an Acquity BEH amide Vanguard precolumn (2.1 x 50 mm, 1.7 µm particle size). Eluent A was 1 % (v/v) ACN containing 0.1 % (v/v) (TFA) in Millipore water and eluent B was 100 % ACN containing 0.1 % (v/v) TFA. Supernatants (8 µL) were injected into the column at 35 °C. The elution profile used was: 0-2 min isocratic on 90 % B; 2-3 min linear gradient from 90 % to 80 % B; 3-6 min linear gradient from 80 to 65 % B; 6-8 min linear gradient from 65 % to 60 % B; 8-10 min isocratic on 40 % B, 10-12 min linear gradient from 40 % to 90 % B and 12-30 min isocratic on 90 % B. The flow rate was 350 µL/min. Detection was performed using a PDA, which scanned the absorbance from 200-400 nm at a 1.2 nm resolution with 20 spectra per second.

The mass spectra of the samples separated by HILIC were obtained with an online Synapt high definition mass spectrometer G2-Si (Waters), coupled to the UPLC system, equipped with a z-spray electrospray ionization (ESI) source, a hybrid quadrupole and an orthogonal time-of-flight (Q-TOF). The system was calibrated using sodium iodide. The capillary voltage was set to 3 kV with the source operation in positive ion mode and the source temperature at 150 °C. The sample cone was operated at 35 V. Nitrogen was used as desolvation gas (500 °C, 800 L/h) and cone gas (200 L/h). The trap gas was set at 2 mL/min. In the first scanning of the samples, MS and MS/MS (MSe continuum method) were performed between m/z 100-2000 with a scan time of 0.3 seconds. The trap collision energy was set at 4 V in single MS mode and ramped from 20 to 30 V in MSe mode. The transfer collision energy was set at 2 V in MS and switched between 2 and 10 V in the MSe mode. The ion of interest had a m/z of 189.13. To tentatively identify the compound, MS/MS method was performed on the fixed mass m/z 189.13. The trap collision energy ramped from 15 to 35 V and the MS and MS/MS were recorded for m/z 50 to 500. Online lock mass data (angiotensin II, $[M+2H]^{2+}$ 523.7751) were collected in parallel of the measurements and used to correct the measured m/z. UV and MS data were acquired using MassLynx software v 4.1 (Waters).

2.6. Biogas composition and amount of methane

Biogas composition at the end of the batch experiments was measured as described in Steinbusch et al. (2008). To quantify the amount of methane produced in the serum bottles, a pressure meter with an absolute pressure probe (GMH3151, Greisinger Electronic, Germany) was used to follow pressure build-up in time. The amount of methane produced was calculated using the ideal gas law. The amount of methane produced in AMPTS II system was automatically recorded through the AMPTS web-based software application (Bioprocess Control, Sweden) and corrected for the actual biogas composition measured.

3. Results and discussion

3.1. Tryptone at high COD proportion as an effective compound to alleviate osmotic stress

Tryptone in a COD ratio tryptone/acetate of 1:4 was added to the batches, upon exposure to an abrupt increase in salinity from 5 to 20 g Na^+/L. This resulted in a clear alleviation of osmotic stress for laboratory scale R5 granular sludge (**Figure 1-A**). Methane production curves of only acetate fed batches compared with acetate and tryptone fed batches clearly show a strong reduction of lag-phase from approximately 350 hours to approximately 150 hours before a significant amount of methane was produced (**Figure 1-A**). The maximum slope of the methane production curve increased by 86% from 0.051 NmL-CH_4/h in the acetate fed batch to 0.095 NmL-CH_4/h in the batch with added tryptone (**Figure 1-A**).These results demonstrate a positive effect of tryptone addition to alleviate salt stress in anaerobic granules.

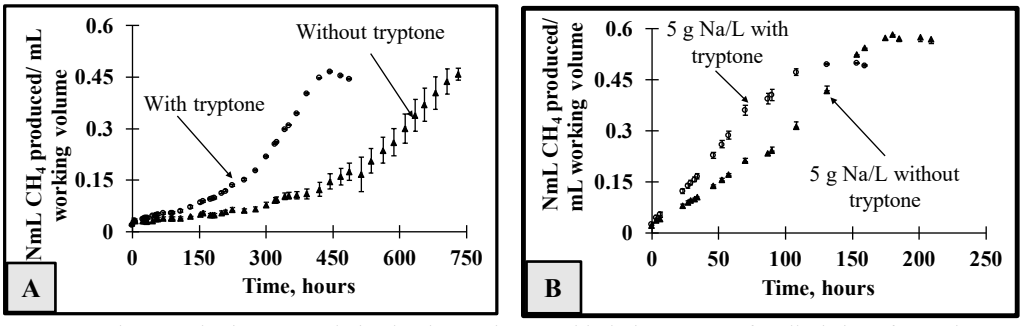


Figure 1. Methane production curves during batch experiments with dosing tryptone for alleviation of osmotic stress. NmL – methane volume normalized to 0 °C and 1 atm. A – R5 granular sludge exposed to an abrupt increase in salinity B - R5 granular sludge exposed to a stable 5 g Na^+/L salinity. Error bars in the graphs indicate the maximum and minimum measured values from duplicate samples

It is assumed that the increase of methanogenic activity is mostly related to the conversion of acetate rather than the conversion of the hydrogen produced from tryptone fermentation. Indeed, based on stoichiometry, the theoretical amount of methane produced from hydrogen during casein fermentation

only accounts for approximately 10% of the total produced methane (Ramsay and Pullammanappallil, 2001).

The results of the experiment with stable salinity i.e., granular sludge adapted to 5 g Na^+/L (R5) and exposed to 5 g Na^+/L are shown in **Figure 1-B**. The slope of the methane production curve increased by 37% from 0.16 to 0.22 NmL-CH_4/h due to the addition of tryptone. The improvement of methanogenic activity is much smaller compared to exposing the same granular sludge to 20 g Na^+/L (86%). However this still shows that tryptone can improve the methanogenic activity of granular sludge that is already adapted to a certain salinity level.

3.2. Amino acids and their derivatives as osmo-protectants in 20 g Na^+/L adapted granular sludge

When exposed to a sudden decrease in osmotic pressure, bacteria activate the so called mechanosensitive channels located in cytoplasmic membranes. These channels allow to rapidly restore cell turgor by either selectively releasing small osmolytes or - if membrane tension increases - by non-selectively releasing osmolytes together with small proteins (Berrier et al., 1992; Pflüger et al., 2005). Archaea have also shown to possess such mechanosensitive channels (Kloda and Martinac, 2002). Since the granular sludge used in these experiments with decreasing salinity was previously adapted to 20 g Na^+/L, a rapid release of accumulated osmolytes upon exposure to a hyposalinity shock was expected.

HPLC chromatogram of amino acids in **Figure S1-A** shows that during an abrupt decrease in salinity from 20 g Na^+/L to 5 g Na^+/L glutamic acid and another compound containing amine bonds were excreted by the granular biomass. These compounds were not detected at the stable 20 g Na^+/L salinity as shown in **Figure S1-B**. The unknown compound was further analysed with HILIC coupled to ESI-Q-TOF-MS and tentatively identified as N-acetyl-β-lysine. Both molecules have been previously reported as compatible solutes in anaerobic microorganisms. This is true for moderate salinity environments, particularly in methanogenic archaea, which accumulate α-glutamate, β-glutamate, β-glutamine, and N^{ε}-acetyl-β-lysine (Müller et al., 2005; Schlegel and Müller, 2011).

Uptake of osmolytes is more bio-energetically favourable than synthesis *de novo* (Oren, 1999). Therefore, glutamic acid - an abundant amino residue in tryptone - can potentially be taken up by microbial cells after hydrolysis of tryptone, where under physiological pH it would take its anionic form of glutamate (Sowers and Gunsalus, 1995). Glutamic acid in its anionic form (glutamate) can counter balance the charge of accumulated potassium in microorganisms as well as balance the osmotic pressure (Roberts, 2005). One must keep in mind that glutamate can be also synthesized *de*

novo without exogenous addition of casamino acids (Csonka, 1989; Sowers and Gunsalus, 1995). However, given the positive effect of tryptone on methanogenic activity (**Figure 1-A**), it is likely that in R20 granular sludge glutamate is taken up after hydrolysis of tryptone.

N^{ε}-acetyl-β-lysine is a compatible solute unique to methanogenic archaea (Pflüger et al., 2003) and some halophilic bacteria (Joghee and Jayaraman, 2014). This osmolyte has shown to accumulate in many methanogenic archaea such as different *Methanosarcina* subspecies, *Methanogenium cariaci, and Methanohalophilus* strain *FDF1*. (Sowers et al., 1990; Roberts et al., 1992; Sowers and Gunsalus, 1995).

In pure cultures of methanogenic archaea *Methanosarcina mazei Gö1* and *Methanococcus maripaludis* it was demonstrated that the synthesis of N^{ε}-acetyl-β-lysine starts with α-lysine production from pyruvate and aspartic acid semi-aldehyde (Pflüger et al., 2003; Schlegel and Müller, 2011). Tryptone contains both lysine (6.96 mole %) and aspartic acid (6.87 mole %) (Smith and Friedman, 1984). It is expected that both molecules may have acted as potential substrate for N^{ε}-acetyl-β-lysine synthesis. In fact, it appears that most of the nitrogen containing osmolytes are synthesized either via aspartic acid pathway or via the glutamic acid pathway (Galinski and Trüper, 1994). This suggests that proteins or peptides containing these amino acids have the potential to provide microorganisms with the "building blocks" of osmolytes.

In our recent work (Gagliano et al., 2018) we showed that the dominant microorganism in granules sampled from the R20 laboratory - scale reactor was the acetoclastic *Methanosaeta harundinacea*. Parallel with our findings, the in silico analysis of the complete genome of *M.harundinacea strain 6Ac* showed that this microorganism possesses a series of genes which products are most likely involved in the pathway for the synthesis of N^{ε}-acetyl-β-lysine, both from aspartate or lysine (**Figure S2**) (Gagliano et al., 2018). In particular, two locus encode for the L-lysine 2,3-aminomutase enzyme (**Figure S2**), which activity is essential for the biosynthesis of this osmolyte (Saum et al., 2009).

Figure 2 illustrates that the sudden decrease in salinity down to 5 g Na^+/L gave a steeper slope of the methane production curve as compared to R20 granular sludge exposed to the adapted salinity of 20 g Na^+/L (**Figure 2**). This result is logical because response to hyposalinity shock both in bacteria and archaea has been shown to be a mechanistic reaction (Berrier et al., 1992; Kloda and Martinac, 2002) and does not interfere with energy metabolism. The increase in activity can be explained with lower energy requirements to maintain cell turgor at decreased salinity. For practical applications, this means that problems with granular sludge activity are expected with a rapid increase (not decrease) in salinity.

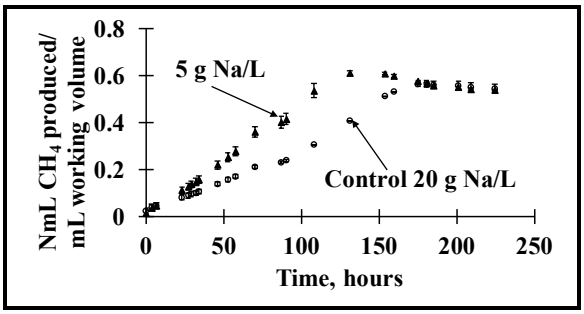


Figure 2. Methane production curves during the decreasing salinity experiments with 20 g Na^+/L adapted (R20) granular sludge. NmL – volume normalized to 0 ºC and 1 atm. Error bars indicate maximum and minimum measured values in duplicate samples.

3.3. Effect of amino acids on SMA of laboratory-scale and full-scale derived biomass

Three amino acids were tested for their potential to alleviate osmotic stress of full-scale and laboratory-scale granular sludge. The first two are potential precursors for production of N^{ε}-acetyl-β-lysine, namely aspartic acid and lysine (Pflüger et al., 2003). The third was glutamic acid – a precursor for glutamate found in R20 biomass. N-acetyl-β-lysine was not considered in this study due to its high price compared to the other tested molecules.

Figure 3 shows that glutamic acid and aspartic acid were both efficient in alleviating osmotic stress with all tested granular sludges. This is an important observation because it shows that both osmo-protectants themselves and also their precursors can indirectly alleviate osmotic stress. Similarly, evidence of a positive effect of osmolyte precursors was recently shown in *Bacillus subtilis*, which imports proteogenic amino acids and converts them into proline to enhance its growth under otherwise osmotically unfavourable conditions (Zaprasis et al., 2014).

The improvement of SMA by dosing glutamic acid and aspartic acid (**Figure 3-A, B**) also demonstrates the efficiency of osmolytes other than commonly used glycine betaine (Vyrides and Stuckey, 2016).

The addition of glutamic acid was more efficient in alleviating salt stress than aspartic acid (**Figure 3-A, B**). This is in line with Pflüger et al. (2003), who found that in *Methanococcus maripaludis JJ* glutamate is the main osmo-protectant up to 376 mM NaCl (approx. 10 g Na^+/L) and within microbial cells its concentration is unaffected up to salinity levels of 800 mM NaCl (approx. 20 g Na^+/L). The dominant osmolyte at 800 mM was N^{ε}-acetyl-β-lysine (Pflüger et al., 2003). Upon increasing salinity, a similar pattern of osmolytes was found in eight strains representing five species of *Methanosarcina spp.* (Sowers and Gunsalus, 1995). Aspartate is known as a precursor for biosynthesis of N^{ε}-acetyl-β-lysine. However, glutamate can act as an osmolyte directly within the entire range of investigated salinities in this study and, therefore, the higher observed effect of dosing of glutamic acid. Moreover,

full-scale granular sludge was exposed to 10 g Na^+/L which is a salinity level at which glutamate has been shown as predominant osmolyte (Pflüger et al., 2003).

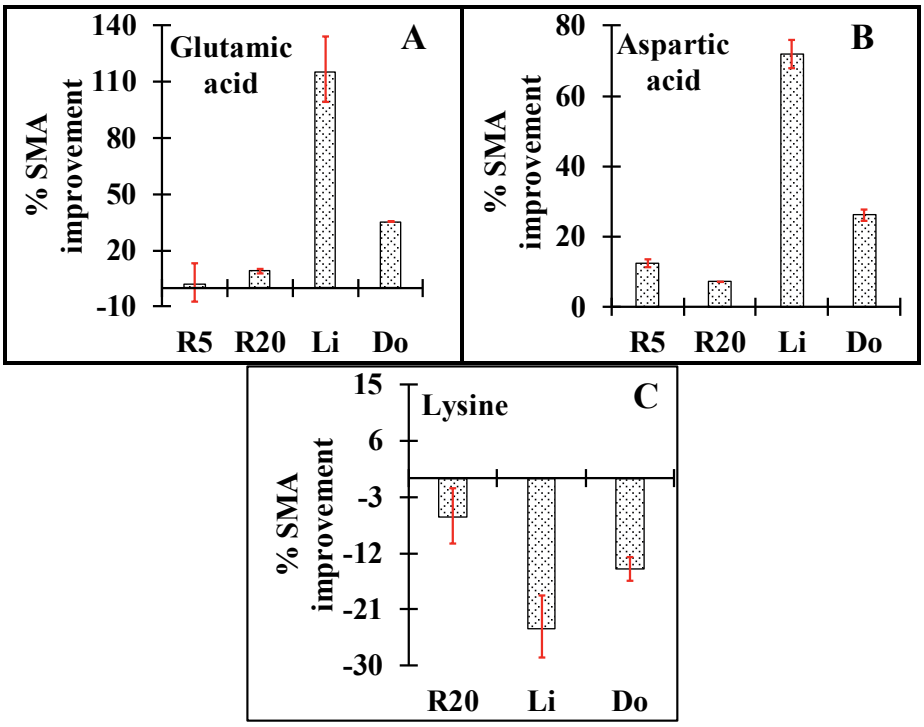


Figure 3. Effect of added amino acids on SMA of full-scale and laboratory-scale granular sludge, when exposing the biomass to hypersalinity shocks. The error bars indicate the minimum and maximum measured effect with respect to control samples without additional dosing of amino acids.

Addition of glutamic acid and aspartic acid had a more pronounced effect on Li and Do granules from full scale ICs than on laboratory scale R5 and R20 granular sludge. Compared to acetate fed granular sludge, glutamic acid increased the SMA by 115%, 35% and 9% in Li, Do and R20, respectively (**Figure 3-A**). Similarly, aspartic acid increased the SMA by 72%, 26%, 12% and 7% in Li, Do, R5 and R20, respectively (**Figure 3-B**). Because the amount of added amino acids was calculated based on the VSS amount used in the experiment, more glutamic and aspartic acid was added to full-scale granular sludge Do and Li compared to R5 and R20 (**Table 2**). This difference may partially explain the general trend of a stronger positive effect of glutamic acid and aspartic acid on Do and Li sludge as compared to R5 and R20. The larger difference of osmo-protectant concentration between granules and bulk liquid can favour faster transport in the granules by diffusion, thereby also allowing for a faster and more pronounced positive effect. Additionally, R5 granular sludge was exposed to a salinity increase from 5 g Na^+/L to 20 g Na^+/L compared to the case of Do and Li where the increase was from 5 to 10 g Na^+/L. The combination of R5 granules exposure to higher salinity (as compared to Do and Li) with potentially slower diffusion of amino acids due to the concentration gradient may

have contributed to the less pronounced effect of glutamic acid and aspartic acid on the SMA. The lowest effect on R20 granular sludge can be explained by the fact that this sludge had already been adapted to 20 g Na^+/L for almost two years.

Figure 3-C shows that the addition of lysine gave a negative effect on the SMA of all tested granules, as already observed in anaerobic batch experiments by Wagner et al. (2012). The decrease in SMA was 6 %, 24% and 14.6% for R20, Li and Do, respectively. No explanation for this result could be found.

3.4. Effect of tryptone and gelatine on SMA of laboratory-scale and full-scale biomass

Tryptone and gelatine both had a positive effect on the SMA for all granular sludges tested, with tryptone giving a higher improvement of SMA compared to gelatine (**Figure 4**). The average increase of SMA by addition of tryptone was 36%, 17%, 179% and 48% for R5, R20, Li and Do, respectively. The addition of gelatine resulted in an SMA increase of 30%, 14%, 23% and 13% for R5, R20, Li and Do, respectively.

The more pronounced improvement of SMA with tryptone compared to gelatine (**Figure 4**) can be explained in several ways. Firstly, gelatine needs to undergo more hydrolysis steps compared to tryptone before its amino residues can be taken up by microbial cells. This may be a rate limiting step. This also implies gelatine would be less efficient than tryptone to alleviate osmotic stress upon abrupt increase in osmotic pressure.

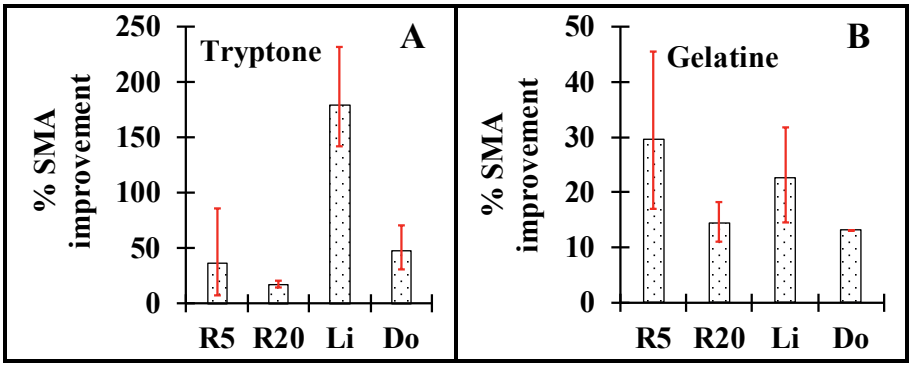


Figure 4. Effect of added tryptone and gelatine on SMA of full-scale and laboratory-scale biomass, when exposing the biomass to hypersalinity shocks. The error bars indicate the minimum and maximum measured effect with respect to control samples without additional dosing of these compounds.

Secondly, tryptone was added in a higher concentration compared to gelatine (See **Table 2**). This was done because the dose of tryptone was based only on its glutamic acid content whereas the dose of gelatine was based on its nitrogen content. As a result, potentially more osmolyte precursors in a form of other amino acids were supplied by tryptone. Thirdly, tryptone also differs from gelatine in its

amino acid residue composition. The most abundant amino acid sequences in gelatine are triplets of glycine, proline and hydroxyproline (Karim and Bhat, 2009), whereas the most abundant amino residue in tryptone is glutamic acid (18.5 %) (Smith and Friedman, 1984). Tryptone also contains more (6.9 %) aspartic acid compared to gelatine (5 %) (Smith and Friedman, 1984; Karim and Bhat, 2009). As showed previously (**Figure 3-A, B**), both glutamic acid as well as aspartic acid are efficient in alleviating osmotic stress. Therefore, tryptone can potentially provide more of these amino acids as osmo-protectants or as osmo-protectant precursors compared to gelatine.

The positive effect of a peptide such as tryptone and a protein such as gelatine on the activity of anaerobic biomass, when exposed to high salinity is a very important outcome in this study, because addition of a cheap (waste) protein can be economically more viable than addition of osmolytes.

Additionally, a number of saline (waste)waters, (e.g., cheese, olive oil milling, and fish industry) contain proteins (Paredes et al., 1999; Ramsay and Pullammanappallil, 2001). In fact, these saline (waste)water streams have been shown to be anaerobically treatable without dosing of osmolytes (Gutiérrez et al., 1991; Khoufi et al., 2006; Chowdhury et al., 2010). In literature, no adequate explanations were yet reported for sufficient microbial activity to achieve high efficiencies of organics conversion when treating these saline streams. Our results show that the presence of certain proteins, which after hydrolyses can be taken up as osmo-active organic chemicals in the granules, probably play an important role in salt stress alleviation when treating these types of (waste)waters.

3.5. Effect of potassium on SMA of laboratory-scale and full-scale biomass

Figure 5 indicates that the addition of potassium at sea water Na/K ratio (27.8 w/w) resulted in a slight positive effect on SMA in Do and Li granules. With the “laboratory-scale granules” no clear positive effect of potassium was detected. Potassium at double the sea water Na/K ratio (13.9 w/w) gave no positive effect on any of the sludges. At the salinity levels investigated in our study (10 – 20 g Na^+/L) organic osmolytes are predominant within non-halophilic methanogenic archaea. Since potassium only alleviates a part of the osmotic pressure (Sowers and Gunsalus, 1995), the low effect of potassium can be explained.

Gagliano et al. (2017b) reported that potassium can alleviate the osmotic stress in a continuously operated anaerobic system at 20 g Na^+/L at a seawater Na/K ratio. In batch experiments with a Na/K ratio higher than sea water (359 w/w), a positive effect was only observed after approximately 3 weeks of incubation at 14 g Na^+/L (Vyrides et al., 2010).

In the current study the maximum applied incubation time was 10 days. Potassium had no positive effect during the first 10 days of batch incubation.

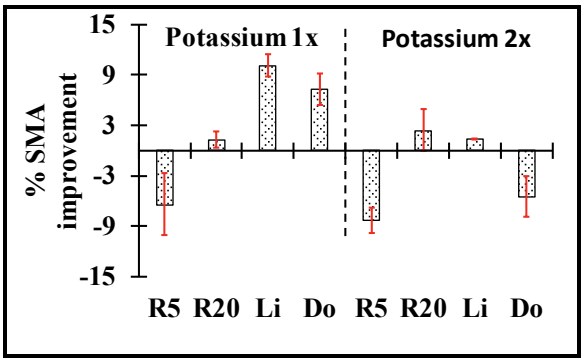


Figure 5. Effect of added potassium on SMA of full-scale and laboratory-scale biomass, when exposing the granular sludge to hypersalinity shocks. The error bars indicate the minimum and maximum measured effect with respect to control samples without additional dosing of potassium. Potassium 1x – Na/K ratio of 27.8 (w/w); Potassium 2x – Na/K ratio of 13.9 (w/w).

3.6. The potential of precursor compounds as osmo-protectants for anaerobic treatment in practice.

Vyrides and Stuckey (2016) recently reported the bottleneck for application of osmolytes for (waste)water treatment is the costs of these molecules. In this contribution we demonstrated the potential of precursor compounds, such as proteins and amino acids, to alleviate osmotic stress in methanogenic archaea. However, dosing of proteins or amino acids for anaerobic treatment of saline (waste)waters still may not be an economically viable option. However, the existing saline (waste)waters containing proteins, for example, can be identified and potentially treated anaerobically. Moreover, when possible, mixing (waste)water streams can be considered to avoid external dosing of osmolytes. Vlyssides et al. (2004) suggested recovery of all marketable compounds from olive oil milling (waste)water to make the olive oil production more economically viable. Our findings suggest that not all proteins from existing (waste)water streams should be recovered if anaerobic treatment is to be applied.

There is a large potential for anaerobic biological treatment application in the saline (waste)water domain. In the EU, the annual fish catch in 2015 was reported to be 5.14 million tonnes (Eurostat, 2018c). The annual production of olive oil in 2017 for the EU was 10.2 million tonnes. 91% of this was produced in Greece, Spain, and Italy (Eurostat, 2018a). Similarly, within the EU approximately 56 million tonnes of milk were used to produce cheese in 2016 (Eurostat, 2018b). The large amounts of goods produced by these industries are expected to generate even larger quantities of saline (waste)water. As an example, production of 1 kg of olive oil generates around 7.5 kg of saline (waste)water (Vlyssides et al., 2004). This means that, during harvest time, around 70 million tonnes of heavily polluted saline (waste)water needs to be treated in Greece, Spain, and Italy. Anaerobic treatment technology could become an attractive alternative for treatment of these (waste)waters.

Fish processing and olive oil industry wastewaters are known to contain high amounts of suspended solids (Paredes et al., 1999; Palenzuela-Rollon et al., 2002). Such (waste)waters are not suitable for direct treatment with granular sludge reactors. However, flocculent UASB reactors can be applied instead. If granular sludge UASB is the desired treatment technology, suspended solids, such as lipids, need to be removed prior the anaerobic treatment (Palenzuela-Rollon et al., 2002). Most of the suspended solids in fishery wastewater are proteins and lipids (Palenzuela-Rollon et al., 2002). This means that only dissolved proteins would remain in the (waste)water after removing these solids. Further anaerobic treatment with granular sludge reactors would then only be possible if a sufficient amount of dissolved proteins remain in the (waste)water. Similar considerations as for fish processing (waste)water should also be made for other (waste)waters with high suspended solids content.

Within the microbial consortia of anaerobic reactors, acetoclastic methanogens are often mentioned as the most sensitive microorganisms to high salinity (Feijoo et al., 1995; Chen et al., 2008a; Gagliano et al., 2017b). As such, this study focuses only on methanogenic activity. However, effective anaerobic treatment of complex wastewater is achieved by a diverse microbial community (O'Flaherty et al., 2006) and all microorganisms involved in the biodegradation process should be able to cope with the osmotic stress for successful treatment. This means that both archaea and bacteria need to synthesize/accumulate osmo-protectants. A potential advantage of precursor compounds like tryptone and gelatine for osmo-protection lies in their chemical composition. After hydrolysis, potentially diverse groups of microorganisms can be supplied with substrates for osmolyte synthesis. In **Chapter 2**, successful sludge granulation in UASB reactors, treating wastewater with a mixture of glucose, acetate, and tryptone at salinity levels as high as 20 g Na^+/L was shown. The high bacterial and methanogenic activity within these UASB reactors is explained by the presence of tryptone in the influent, which may have indirectly supplied the microbial community with precursors of osmolytes. Further research should be targeted to study the effects of compounds like gelatine and tryptone on specific microbial groups driving granules formation, upon exposure to osmotic stress. Furthermore, it is important to understand shifts in microbial communities when different osmoprotectant precursors/substrates are used during long-term continuous flow studies, because microbial communities can explain effectiveness of different precursor compounds of osmolytes as well as potentially reveal the key microorganism driving the granulation process.

4. Conclusions

The results obtained in these batch experiments allow us to conclude the following. Glutamic acid and N-acetyl-β-lysine are the sole osmo-protectants in anaerobic granular biomass, grown in a UASB on a mixture of glucose, acetate, and tryptone (3:2:1 on COD basis) and adapted to 20 g Na^+/L. Glutamic acid and aspartic acid, as well as the non-hydrolysed tryptone and gelatine, can alleviate salt toxicity of methanogens in anaerobic granular sludge. Lysine cannot alleviate salt toxicity of methanogens in anaerobic granular sludge. Potassium added at sea water ratio can slightly alleviate osmotic stress of methanogens in anaerobic granular sludge during incubation period of 10 days. Potassium added at double the sea water ratio cannot alleviate salt stress of methanogens in anaerobic granular sludge during incubation period of 10 days. When adequate amounts and types of proteins are present in the (waste)water, streams with high salt content could be anaerobically treated without external dosing of osmolytes. Hyposalinity shock from 20 g Na^+/L down to 5 g Na^+/L does not negatively affect methanogenic activity in 20 g Na^+/L adapted granular sludge.

Supplementary Information of Chapter 3

Figure S1. To identify the osmolytes present in 20 g Na^+/L (R20) adapted granular sludge, this sludge was exposed to a sudden decrease of salinity down to 5 g Na^+/L in batch experiments fed with acetate as the sole carbon source (COD/VSS of 4:1). As a control, batch experiments at a stable 20 g Na^+/L salinity were performed. Liquid aliquots were sampled with an interval of 2 hours from both types of batch experiments.

HPLC analysis in **Figure S1-A** showed two peaks of compounds containing amino bonds appearing at a retention time of approximately 3 min in batch experiments with biomass exposed to hyposalinity shock. The first smaller peak could be identified as glutamic acid and the second large peak was further tentatively identified as N-acetyl-β-lysine with HILIC coupled to ESI-Q-TOF-MS. The control sample at stable salinity (**Figure S1-B**) did not contain these peaks, therefore their appearance in **Figure S1-A** indicates a rapid efflux of amino acids and their derivatives as osmolytes upon 20 g Na^+/L adapted biomass exposure to hyposalinity shock.

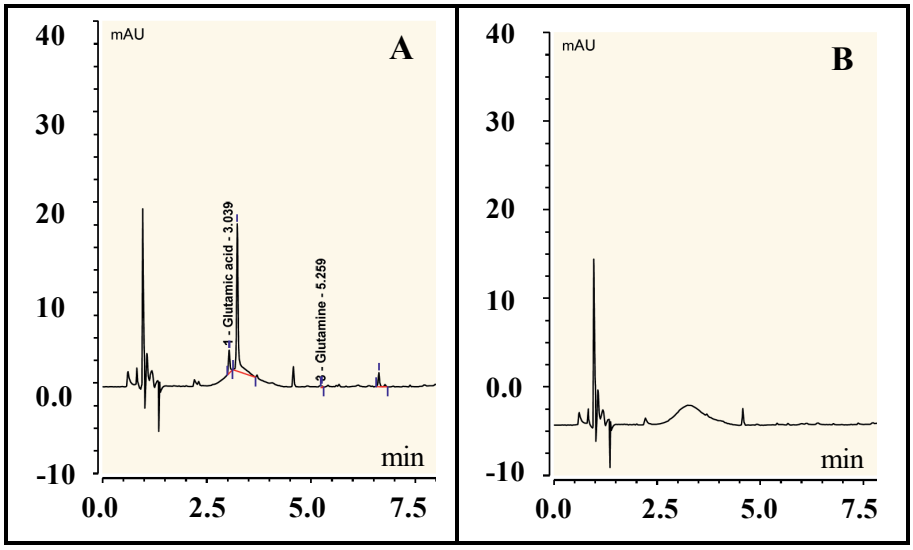


Figure S1. HPLC amino acid chromatograms of liquid samples collected during decreasing salinity experiments. A – 20 g Na^+/L adapted biomass exposed to a sudden decrease in salinity to 5 g Na^+/L after 2 hours of incubation. B - 20 g Na^+/L adapted biomass exposed to 20 g Na^+/L after 2 hours of incubation.

Figure S2. In a recent study (Gagliano et al., 2018) we have analysed *in silico* the complete genome of *M. harundinacea 6Ac (Zhu et al., 2012)*. A series of genes was identified, end products of which are likely to be involved in the pathway of N^{ε}-acetyl-β-lysine synthesis from aspartate. In **Figure S2** a schematic representation of possible N^{ε}-acetyl-β-lysine production pathway from aspartate is shown. The presence of these genes in *M. harundinacea* may explain the positive effect of aspartic acid on acetoclastic methanogenic activity found in our study. Furthermore, N-acetyl-β-lysine was indeed identified as an osmolyte in granular sludge adapted to 20 g Na^{+}/L.

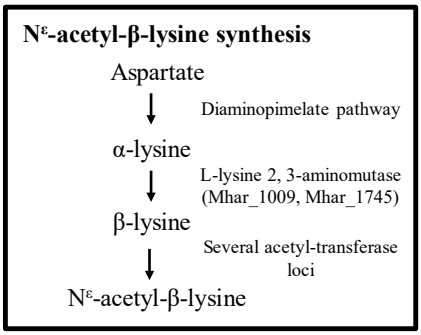
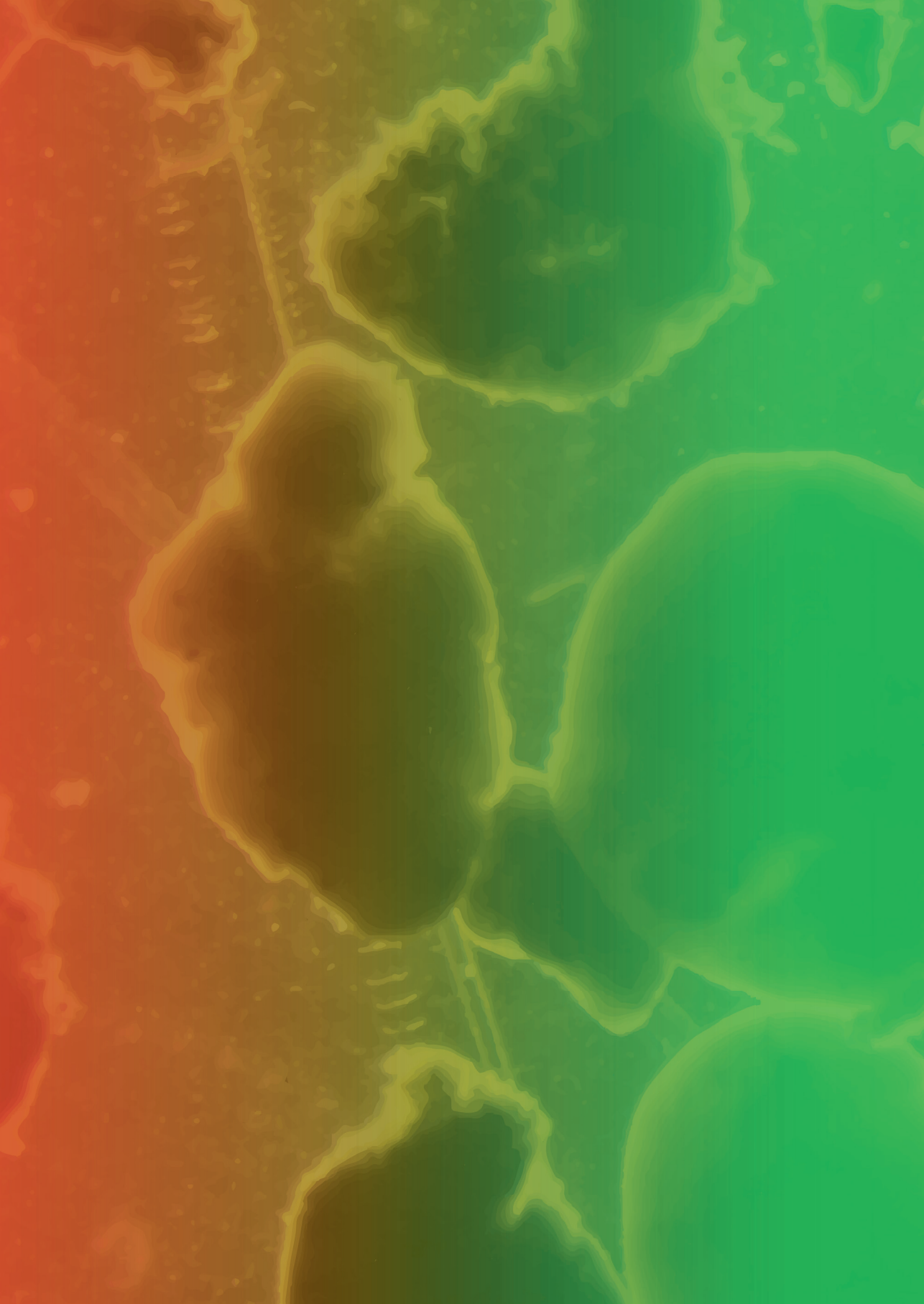


Figure S2. Schematic representation showing the presence in the *M.harundinacea 6Ac* genome of the genes encoding for the acetyl-β-lysine synthesis, with two loci encoding for the crucial activity of the L-lysine 2,3-aminomutase.



Chapter 4. Proteins and Peptides in Wastewater Enhance Anaerobic Sludge Granulation at High Salinity

Authors:

D. Sudmalis[a], M.C. Gagliano[b,c], M.A. Kabel[d], P. de Gijsel[d], G. Zeeman[a], H.H.M. Rijnaarts[a], H. Temmink[a]

[a] Department of Environmental Technology, Wageningen University and Research, Bornse Weilanden 9, 6708 WG, Wageningen, The Netherlands

[b] Laboratory of Microbiology, Wageningen University and Research, Stippeneng 4, 6708 WE, Wageningen, The Netherlands

[c] Wetsus, Centre of Excellence for Sustainable Water Technology, Agora 1, P.O. Box 1113, 8900 CC Leeuwarden, The Netherlands

[d] Wageningen University & Research, Laboratory of Food Chemistry, Bornse Weilanden 9, 6708 WG, Wageningen, The Netherlands.

Abstract. The objective of this research was to study the influence of organic substrate on anaerobic sludge granulation at high salinity. Several anaerobic sludge blanket (UASB) reactors operating at 20 g Na^+/L were fed with glucose and acetate. In addition, they received either starch, gelatine, a peptide mixture (tryptone) or a mixture of amino acids as co-substrates with a contribution of 17% to the total COD of the feed. At similar loading rates of 7 - 8 g COD/L·d all the reactors exhibited good removal of soluble COD (90-98%) and a high methane recovery (80-89%), indicating the different co-substrates did not affect the (overall) microbial activity. However, large differences in microbial aggregation were observed. With the amino acid mixture granulation was absent, but instead suspended biomass and a thick biofilm formed. With all the other co-substrates strong granules were formed. The rate of granule formation with tryptone and gelatine was much faster (first granules already after 50 days) than with starch (first granules not before 150 days). The morphology of the granules also was very different. Removing tryptone from the feed resulted in rapid granule disintegration and biomass wash-out. As extracellular polymeric substances (EPS) were expected to play an important role in granule formation, the amino acid and sugar composition of the EPS in the starch and tryptone fed reactors were determined. The sugar composition of the EPS was slightly different but surprisingly the amino acid composition of the EPS was the same, implying that the proteinaceous EPS is produced *de novo*. *De novo* production of EPS may explain faster granulation with proteins in the (waste)water due to potentially more energetically favourable building blocks of EPS contained in them.

1. Introduction

Due to increasing industrial activities and recycling of (waste)water, the volume of produced saline (waste)water is rising (Rozzi et al., 1999; Giustinianovich et al., 2018). Anaerobic biological treatment of these saline water streams should be considered, to recover energy in the form of biogas from the organic pollutants, and reduce the amount of required chemicals and produced waste sludge (Vieira et al., 2005b; Vyrides et al., 2010; Xiao and Roberts, 2010). For application of simple and compact bioreactors capable to handle high volumetric and organic loading rates, anaerobic sludge granulation should be achieved (Nicolella et al., 2000; Van Lier et al., 2015), and accompanied with a sufficiently high microbial activity (Liu et al., 2009).

With a recent exception in **Chapter 2**, where successful anaerobic sludge granulation from dispersed biomass accompanied with good process performance was achieved, the salinity effects on anaerobic microbial granules have mainly been studied by exposing granules, “pre-formed” under non-saline conditions, to high salinity levels. Generally it is concluded that, upon exposure to high monovalent salt concentrations, the microbial granules become weak, methanogenic activity decreases and

eventually washout of biomass from bioreactors occurs, leading to failure of the treatment process (Rinzema et al., 1988; Jeison et al., 2008; Ismail et al., 2010; Li et al., 2014; De Vrieze et al., 2016a).

At non – saline conditions, it has been shown that the organic substrate concentration and composition can influence the rate of granulation and properties of pre-formed granular sludge (Fukuzaki et al., 1991a; Fukuzaki et al., 1991c; Chang et al., 1993; Fang et al., 1994; Thaveesri et al., 1994; Fukuzaki et al., 1995; Tay and Yan, 1996; O'Flaherty et al., 1997; Zhou et al., 2006). The results obtained under non-saline conditions are not always consistent amongst different researchers. However, the common consensus is that compared to lower energy substrates, such as volatile fatty acids, energy rich substrates, such as glucose, stimulate the granulation process (Vanderhaegen et al., 1992; O'Flaherty et al., 1997). This is attributed to improved production of microbial extracellular polymeric substances (EPS) – the "gluing material" for structural integrity of granules (Vanderhaegen et al., 1992; Fukuzaki et al., 1995; Zhou et al., 2006). Thus far there is no systematic study reporting the effect of organic substrate on anaerobic sludge formation under saline conditions, while under such conditions the organic substrate may play an even bigger role for granulation. This is because besides production of EPS for aggregation, the microbial cells also need to produce small organic molecules, called compatible solutes to maintain the cells turgor pressure under increased osmotic stress (Csonka, 1989; Pflüger et al., 2005; Campo et al., 2018).

Interestingly, a major fraction (41 to 90%) of EPS in anaerobic granular sludge is reported to be protein, when grown with various organic substrates, such as starch, sucrose, ethanol, propionate, lactate or phenol containing (waste)water (Fukuzaki et al., 1991a; Fukuzaki et al., 1991b; 1995; Liu and Fang, 2002). While not elucidated thus far, this abundance of proteins in the EPS of anaerobic granular sludge suggests their importance for granulation. Furthermore, the compatible solutes of archaea and anaerobic bacteria are often reported to be nitrogen containing compounds, such as amino acids or their derivatives (Roberts, 2005; Zaprasis et al., 2014). Production of proteinaceous EPS and osmolytes, is expected to be highly energy demanding (Akashi and Gojobori, 2002). Since proteins may potentially supply the proper "building blocks" for EPS and osmoprotectant synthesis (**Chapter 3**), a hypothesis was proposed that presence of peptides or proteins in the waste stream may promote the rate of granular sludge formation under saline conditions.

To investigate this hypothesis, laboratory-scale UASB reactors were operated with saline (waste)water (20 g Na^+/L) containing glucose, acetate and different amino acids, peptides (tryptone), a protein (gelatine) or complex sugars (starch) as co-substrates. A combination of l-leucine and l-proline was selected as they are abundant amino residues in tryptone (Smith and Friedman, 1984) and proline is known as an osmoprotectant in anaerobic microorganisms (Roberts, 2005; Zaprasis et al.,

2014). The protein gelatine was tested to study if soluble peptides/proteins in general enhance anaerobic sludge granulation at saline conditions. Both, peptides and proteins, require hydrolytic activity for conversion into amino acids and finally methane. Starch was selected to study if addition of a complex substrate, requiring hydrolytic activity but not containing amino acids could result in fast granulation at high salinity. Finally, to study the differences of EPS composition in a protein fed reactor and a starch fed reactor sludge, amino acid and sugar contents and composition of the EPS extracts were studied.

Results of the present study show that peptides and soluble proteins in (waste)water, compared to single amino acids and starch, significantly reduce the time needed to form granules at 20 g Na^+/L and are required to maintain the granular sludge structure. Furthermore, the results suggest that this faster granulation is due to energetically more favourable EPS building blocks contained in proteins and peptides compared to single amino acids or polysaccharides.

2. Materials and methods

2.1. Inoculum, reactors

All bioreactors were seeded with 6 g VSS/L of granular biomass which was forced through a 125 µm metal sieve as described in **Chapter 2**. The source of the inoculum was a full scale UASB reactor treating (waste)water from a styrene and propene-oxide production plant (Shell, Moerdijk, the Netherlands) (**Chapter 2**). A simplified scheme and a detailed description of reactor set-ups is given in **Chapter 2**. In short, the reactors were double walled glass UASB reactors with an internal diameter of 6 cm and a volume of 2 L. The settler volumes were approximately 1 L and the exact volumes are specified for each experiment in **Table 2**. The reactors were operated at 35 ^oC. The superficial upflow velocity was controlled by peristaltic circulation pumps (Watson Marlow 530u, UK) and was increased in steps from 0.2 to 1 m/h. All bioreactors were fed with synthetic (waste)water with a total sodium concentration of 20 g Na^+/L. The composition of the nutrient medium is reported in **Chapter 2**.

2.2. The role of peptides in maintaining granular sludge structure at 20 g Na^+/L

The first experiment was designed to verify if peptides in the (waste)water are important for maintaining good granular structure at high salinity. This was done by stepwise removal of tryptone from the feed of a UASB reactor (**R_{tryp}**) operated at 20 g Na^+/L and containing granular sludge. The feed COD consisted of D-glucose, sodium acetate and tryptone (Sigma Aldrich) in a COD proportion of 3:2:1 and a total COD concentration of 12 g/L. The reactor was started-up with dispersed biomass

at 20 g Na^+/L as described in **Chapter 2** and operated for more than 300 days at a stable loading rate of 7.9 g COD/L·d.

The proportion of tryptone COD was decreased in steps from 1 down to 0.05. To keep the influent COD concentration constant, the tryptone was compensated by addition of D-glucose and sodium acetate in a COD ratio of 3:2. **Table 1** shows the changes in substrate composition and organic loading rate. Ammonium chloride was used to compensate for the nitrogen that was removed with tryptone. Because cations are reported to play an important role in the process of granulation (Yu et al., 2001; Vlyssides et al., 2009; Ahmad et al., 2011), the ionic composition of tryptone salt was measured (**Table S1**), and the major cations removed with tryptone were compensated to the influent upon decreasing the tryptone concentration. Finally, after gradually decreasing the tryptone fraction, the feed tryptone was increased back to the original concentration. The reactor was subsequently operated for an additional 135 days.

Table 1. Changes of organic substrate composition in the UASB reactor with gradual tryptone removal (R_{tryp})

Day	Glucose COD, g/L	Acetate COD, g/L	Tryptone COD, g/L	Total COD, g/L	VLR, g COD/L·d
300-366	6	4	2	12	7.9 ± 0.4
366-380	6.9	4.6	0.5	12	7.8 ± 0.04
380-394	7.05	4.7	0.25	12	7.9 ± 0.01
394-415	7.14	4.76	0.1	12	7.6 ± 0.5
415-550	6	4	2	12	7.9 ± 0.2

2.3. Importance of organic substrate for granulation and sludge retention at high salinity

The second set of experiments was designed to study how different organic substrates would affect granular sludge formation and biomass retention under saline conditions. This was done by performing four continuous flow experiments in laboratory scale UASB reactors. All reactors were fed with D-glucose (Merck, Germany) and acetate (Merck, Germany) in a COD proportion of 3:2. Each reactor was supplied with a third co-substrate (along with glucose and acetate) to reach the final COD proportion of 3:2:1. The following co-substrates were used: i) a combination of l-leucine (Sigma Aldrich) and l-proline (Sigma Aldrich) (**R_{leuc1}**), ii) gelatine (Merck, Germany. From bovine bone) (**R_{gel}**), and iii) starch (Merck, Germany, soluble grade for analysis) (**R_{star}**). To confirm the outcome of the first experiment with leucine and proline, this experiment was performed twice (**R_{leuc2}**). A reactor with a combination of l-lysine and glutamic acid was also studied. However, the process performance of this reactor was poor and therefore the results will not be further discussed here. The main operational parameters including the organic substrates of all reactors are summarized in **Table 2**. The initial influent COD of all reactors was set to 3 g COD/L and was increased in steps to 7 g COD/L and finally to 12 g COD/L. The increase in influent COD concentration corresponded to increasing loading rates. The loading rate was increased in a reactor once the soluble COD (sCOD)

removal efficiency was approximately 90%. Because recent results in **Chapter 2** have shown rapid granulation at 20 g Na^+/L with accompanying high sCOD removal efficiency, the reactors were only operated for longer than 50 days in two cases: i) if visible granulation within the reactors occurred; ii) if a rapid increase in loading rate was possible. The initial upflow velocity was 0.2 m/h. To study the performance of the reactors at upflow velocity typical for UASB reactors, the final operating upflow velocity was increased to 1 m/h.

Table 2. The main operational parameters and start-up strategy of the UASB reactors with various organic substrates.

Parameter	R_{leuc1}	R_{leuc2}	R_{gel}	R_{star}
COD proportion of D-glucose and acetate	3:2	3:2	3:2	3:2
Co-substrate and its COD proportion of total influent COD	leucine 0.5 proline 0.5	leucine 0.5 proline 0.5	Gelatine 1	Starch 1
Operating temperature, °C	35	35	35	35
Salinity, g Na^+/L	20	20	20	20
Empty bed active reactor volume, L	1.98	1.98	1.95	1.98
Internal Reactor Diameter, m	0.062	0.062	0.062	0.062
Settler Volume, L	1.12	1.12	1.28	1.12
Loading rate during start-up	1.07 ± 0.15	1.1 ± 0.36	1.18 ± 0.02	1.02 ± 0.21
Day of increase of COD_{inf} from 3 g/L to 7 g/L	27	27	21	60
Day of increase of COD_{inf} from 7 g/L to 12 g/L	60	n.d.	42	122
Day of increase of upflow velocity from 0.2 m/h to 1 m/h	18	40	79	136

2.4. Analytical methods

2.4.1. Chemical oxygen demand (COD), biogas flow and composition, turbidity, volatile suspended solids

Effluent chemical oxygen demand (COD), effluent turbidity (NTU) and effluent volatile suspended solids were measured on a weekly basis in 24 hour composite effluent samples. COD measurements were done in singleton with LCK314 and LCK1414 kits (HACH GMBH, Germany). Samples for COD analysis were diluted with mili-Q water to reach chloride concentrations below 1.5 g/L. Soluble COD (sCOD) is defined here as a sample filtered through Polyethylene Sulfonate (PES) filter with a pore size of 0.45 µm (CHROMAFIL® Xtra). To measure the total COD (tCOD), samples were homogenized with a thorax (Ultra-Turrax T25, Janke & Kunkel, The Netherlands) prior dilution for analysis. Effluent turbidity (NTU) was measured with a turbidity meter WTW Turb 550 (USA) in duplicate. Effluent volatile suspended solids (VSS) were measured in duplicate according to APHA standard method Nr.2540D.

Biogas flow was monitored with μFlow gas flow meters (Bioprocess Control Sweden AB). To improve the cumulative gas measurement accuracy in continuous reactors R_{gel}, R_{star} and R_{tryp}, μFlow gas flow meters were replaced with a tailor-made gas measuring system. The gas system was only connected to reactors in which granular sludge developed in order to perform COD mass balances. A schematic drawing of this system is shown in **Figure S1**. Biogas composition was measured as described earlier (Steinbusch et al., 2008).

2.4.2. EPS extraction and quantification of sugar and protein content in the EPS

Extracellular polymeric substances were extracted from biomass of all bioreactors running with different substrates. The polymers were extracted using an alkaline extraction method as described in Felz et al. (2016). After dialysis (SnakeSkin™, 3.5K MWCO) against mili-Q for 24 hours the total nitrogen (TN) content of EPS was measured with LCK314, LCK514 and LCK1414 kits (HACH GMBH, Germany). To estimate protein content of the EPS, the TN values were multiplied by a factor of 6.25 (Williams et al., 1987). The sugar content was measured with the phenol sulphuric acid method (Dubois et al., 1956) as D-glucose equivalents.

2.4.3. Determination of amino acid and sugar content and composition of extracted EPS

For analysis of amino acid composition of EPS, 0.1 to 1 mg of lyophilised EPS samples were first hydrolysed with 6 N HCl containing 1% (w/v) phenol for 24 hours. Hydrolysis was performed under vacuum and at 110 ºC. After hydrolysis, excess HCl was removed while drying samples under vacuum for approximately 30 min. Further samples were re-suspended in 1 mL of solution containing 0.2 mL of internal standard (0.4 mM Norleucine) and 0.8 mL of ethanol, centrifuged at 15 000 RPM, filtered through 0.2 μm PES membrane filters and diluted with mili-Q. The diluted samples were further analysed with HPLC for their amino acid contents. Detailed HPLC method and hydrolysis description is available elsewhere (Meussen et al., 2014).

For analysis of neutral sugars, lyophilised EPS samples were pre-treated for 1 hour at 30 ºC with 72% (w/w) H_2SO_4 and further hydrolysed with 1 M H_2SO_4 for 3 hours at 100 ºC, according to Englyst and Cummings (1984). Neutral monosaccharides released were derivatised and analysed as their alditol acetates with gas chromatography (ThermoScientific, Waltham, MA, USA) (Englyst and Cummings, 1984). The internal standard was inositol and samples were treated and analysed in duplicate.

2.4.4. Light microscopy and settling velocity of granules

Light microscopy images were taken with Nikon SMZ800 microscope equipped with CMEX-5 PRO USB 3.0 camera. The images were processed with Image Focus Alpha software. Before imaging, approximately 2.5 mL of samples taken from the bottom sampling port (H = 7.5 cm) of the reactor were diluted with 10 mL of tap water.

During the first experiment in R_{tryp}, settling properties of granular sludge were measured bi-weekly in one replicate. Settling properties of the sludge were measured in a settling column with a diameter of 1.8 cm and a height of 31 cm. 0.2 g of TSS sampled from the bottom sampling port (H = 7.5 cm) of the reactor were added to the column filled with tap water and exposed to upflow velocities, of 1.3 and 10.8 m/h. The upflow velocity was obtained by pumping tap water through the column with a peristaltic pump (Watson Marlow 323, UK). Effluent of the column was gathered and analysed for its TSS content. Finally, after applying an upflow velocity of 10.8 m/h, the remainder solids in the column were measured and fractions of solids settling faster than a particular upflow velocity were calculated as follows:

$$f = \left(1 - \frac{\sum_{i=1.3}^{i=10.8} SS_{V,i}}{\sum SS}\right)$$

Where $SS_{V,i}$ – the amount of suspended solids washed out of the settling column after applying particular upflow velocities, ∑SS – the total amount of suspended solids used in the experiment.

3. Results

3.1. Peptide-rich substrates are important to maintain granular sludge structure at high salinity

In R_{tryp} adapted to 20 g Na^+/L with stable granular performance, a gradual decrease of the influent tryptone COD proportion down to 0.05 resulted in clear granules disintegration, and biomass washout (**Figure 1-C, D**). **Table 3** summarizes the process performance during the experiment. As a result of a reduced influent tryptone concentration down to a proportion of 0.05, the effluent turbidity increased from 194 ± 68 to 521 ± 211 NTU, whereas tCOD removal efficiency decreased from 95.4 ± 1.4 to 93.7 ± 1.7 %. The effluent NTU remained high for another 50 days after increase of tryptone COD fraction back to 1. Finally, the turbidity stabilized at 223 ± 88 NTU and tCOD removal efficiency increased back to 95.9 ± 0.8 %. The decrease of effluent turbidity and increase of tCOD removal efficiency coincided with gradual formation of granules, as shown in **Figure 1-E**.

Figure 1-B shows the sludge bed appearance before decrease of influent tryptone concentration. The sludge had clearly defined and smoothed edges. In **Figure 1-C, D** the sludge bed during increased

biomass washout is shown. The sludge bed looked less dense, the granular structure disintegrated and clearly defined granules were no longer visible. Increase of influent tryptone fraction back to 1 resulted in reoccurrence of the granular sludge bed. First, after approximately 50 days, clearly shaped small granules formed (**Figure 1-E)** and finally the granular sludge bed could be fully restored (**Figure 1-F**).

Above 90% of the sludge in the reactor could settle faster than 1.2 m/h throughout the experiment (**Figure 1-A**). However, analysis of the settling properties at an upflow velocity of 10.8 m/h revealed deteriorating settleability after the tryptone COD fraction was decreased to 0.05. The fraction of sludge settling faster than this velocity decreased from 88 % down to 67 % and slowly recovered to 82 % after increasing the influent tryptone COD fraction back to 1 (**Figure 1-A**). Improvement of the settleability coincided with formation of granular sludge in the reactor (**Figure 1-E, F**).

A decreasing tryptone concentration did not result in notable process performance deterioration in terms of influent sCOD conversion and the methane content of biogas remained around 67% throughout the experiment (**Table 3**). Also the conversion of sCOD into methane COD remained stable, i.e. around 80% of converted influent sCOD could be recovered as methane throughout the experiment (**Table 3**).

Table 3. Process performance of a UASB reactor operated at 20 g Na^+/L during gradual decrease of tryptone fraction in the feed from 1 down to 0.05. Values given with standard deviations between weekly composite effluent samples.

Sludge bed	Tryptone COD Fraction	Period, days	tCOD removal, %	sCOD removal, %	Biogas CH_4 content	Conversion $sCOD_{removed}$ to CH_4, %	Effluent Turbidity, NTU
Granular	1	300 - 366	95.4 ± 1.4	97.9 ± 0.7	67.8 ± 1.3	81.1 ± 4.2	194 ± 68
Granular	0.25-0.125	366 - 394	96.8 ± 0.4	97.7 ± 0.8	66.9 ± 0.5	81.5 ± 2.2	174 ± 87
Disintegration	0.05	394 - 415	**93.7 ± 1.7**	98.1 ± 0.2	66.4 ± 0.5	81.7 ± 6.1	**521 ± 211**
Re-granulation	1	415 - 465	**94.7 ± 0.9**	98.2 ± 0.7	67.3 ± 0.8	80.5 ± 1.2	**319 ± 130**
Granular	1	465 - 550	95.9 ± 0.8	98.3 ± 1.0	67.2 ± 0.5	78.6 ± 3.6	223 ± 88

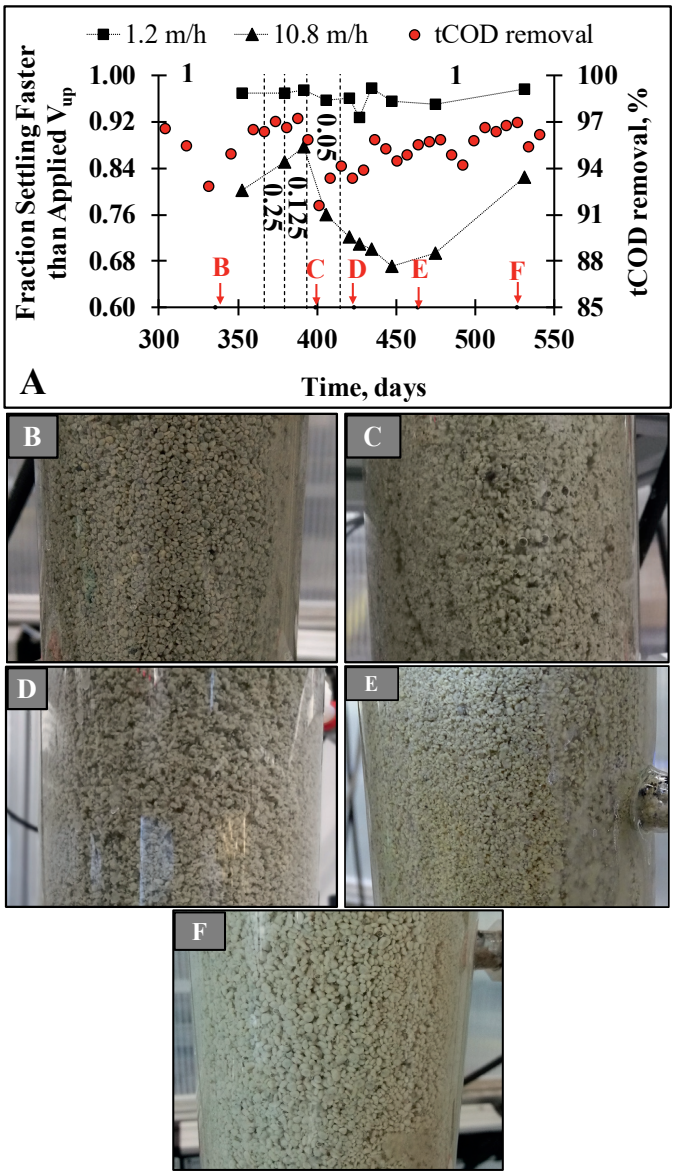


Figure 1. A – tCOD removal efficiency and granular settling properties of 20 g Na^+/L adapted UASB reactor during the experiment with decreasing influent tryptone concentration. Dashed vertical lines represent changes in influent tryptone concentration. Numbers at the top of the graph show the influent tryptone COD fraction. Red arrows indicate the time at which corresponding images of the sludge bed in B-F were taken. B-F – images of the sludge bed in time.

3.2. Process performance with various organic co-substrates: soluble COD conversion, applicable loading rates, and biogas composition

Similar to earlier experience with tryptone, a rapid increase of organic loading rate, accompanied with a high sCOD removal efficiency, could only be achieved with gelatine (R_{gel}) and a combination of leucine and proline (R_{leuc}) as co-substrates (**Figure 2-A-C**). In R_{star} the increases of organic loading rate could be achieved more gradually compared to R_{gel} and R_{leuc} (**Figure 2-D**).

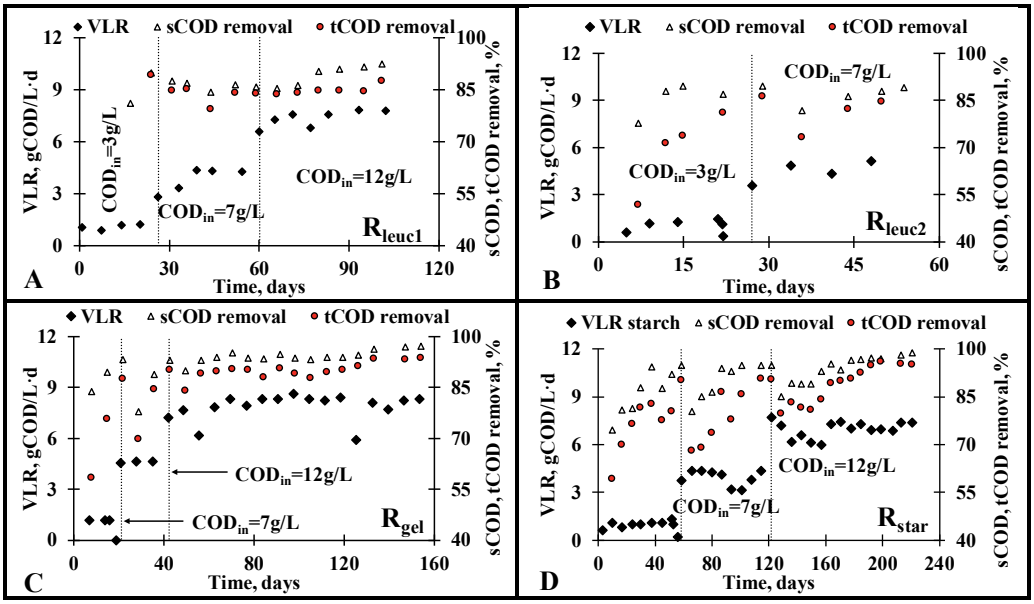


Figure 2. Volumetric loading rate and COD removal efficiency of UASB reactors fed with different co-substrates. A – R_{leuc1}, B – R_{leuc2}, C - R_{gel}, D – R_{star}. sCOD – soluble COD; tCOD – total COD. The vertical dashed lines represent increases of influent COD concentration.

A summary of the process performance at different loading rates is shown in **Table 4**. The final sCOD removal efficiencies were 92.5, 88.9, 97.3 and 98.8% in R_{leuc1}, R_{leuc2}, R_{gel} and R_{star}, respectively (**Figure 2**). The final total COD (tCOD) removal efficiency was 87.4%, 93.6% and 96.1% in R_{leuc1}, R_{gel} and R_{star}, respectively. A higher tCOD removal efficiency was accomplished in reactors with formed granules or granule-alike aggregates, i.e. in R_{gel} and R_{star} (**Table 4**).

With amino acids or proteins as co-substrates, the biogas contained more methane compared to starch. The average methane content of biogas throughout the operational period was 67.4 ± 4.4%, 70.3 ± 4.1%, 66.8 ± 3.3%, 63.5 ± 4.3% for R_{leuc1}, R_{leuc2}, R_{gel} and R_{star}, respectively. These results are logic in terms of oxidation status of substrates, because more reduced carbon forms in amino acids and protein can yield a higher methane content in the biogas than less reduced carbon forms such as starch (De Mes et al., 2003). In R_{gel} more than 85% of removed sCOD could be recovered as methane, whereas in R_{star} the methane recovery reached only around 66% (**Table 4**).

Table 4. COD removal efficiency in R_{leuc1}, R_{leuc2}, R_{gel} and R_{star} at different loading rates and % conversion of sCOD into methane COD in reactors with formed granules. n.a – non applicable, n.d. – data not available, *only one tCOD measurement was performed at the end of VLR1.

	Days	Loading, gCOD/L·d	sCOD, %	tCOD, %	Conversion $sCOD_{removed}$ to CH_4 at final loading, %
			VLR1		
R_{leuc1}	0-26	1.1 ± 0.15	85.4 ± 6.2	89.1*	n.a
R_{leuc2}	0-27	1.1 ± 0.36	85.4 ± 5.3	69.4 ± 12.5	n.a
R_{gel}	0-21	1.2 ± 0.02	86.7 ± 4.1	67.0 ± 12.3	n.a
R_{star}	0-58	1.0 ± 0.21	85.6 ± 7.0	75.5 ± 8.4	n.a
			VLR2		
R_{leuc1}	26-60	3.8 ± 0.71	86.1 ± 1.2	83.2 ± 2.7	n.a
R_{leuc2}	27-54	4.5 ± 0.68	86.2 ± 3.4	81.5 ± 5.8	n.a
R_{gel}	21-42	4.6 ± 0.05	86.7 ± 7.9	80.6 ± 9.5	n.a
R_{star}	58-121	3.9 ± 0.49	90.5 ± 5.6	80.3 ± 9.2	n.a
			VLR final		
R_{leuc1}	60-103	7.3 ± 0.48	**89.5 ± 3.0**	**84.8 ± 1.4**	n.d.
R_{leuc2}	n.d.	n.d.	n.d.	n.d.	n.d.
R_{gel}	42-160	7.9 ± 0.75	94.3 ± 1.8	90.2 ± 2.4	84.8 ± 11.8
R_{star}	121-224	7.0 ± 0.52	93.9 ± 4.1	88.9 ± 5.8	65.6 ± 3.3

3.3. Protein increases the rate of granulation at high salinity

Figure 3-A shows that the sludge bed of R_{gel}, similarly to experience with tryptone, after 42 days of reactor operation already contained granules. Sludge granulation could not be achieved in R_{leuc1} and R_{leuc2}. As an example, **Figure 3-B** shows the sludge bed of reactor R_{leuc2} after 50 days of operation. Compared to R_{gel} (**Figure 3-A**), the height of the sludge bed was lower and no granules were observed. The lower height of the sludge bed in R_{leuc2} compared to R_{gel} is unlikely to be related to a better settleability, but should be related to retention of biomass. This is because in R_{gel} granular sludge bed formed (**Figure 5**), whereas in R_{leuc2} the sludge was flocculent (**Figure S2**). The same was true for R_{leuc1}, even after an extended reactor operation for 118 days. Similarly to R_{leuc1} and R_{leuc2}, also in R_{star} granulation could not be observed for more than 100 days. However, after 150 days, small granule-alike aggregates started to appear, together with dispersed EPS (**Figure 3-D, Figure 5-B, D**).

The biomass retention was better in R_{gel} compared R_{star}. The cumulative volatile suspended solids (VSS) washout data in **Figure 4** show that after 160 days of operation 33.1 g and 41.1 g of VSS were lost from R_{gel} and R_{star}, respectively. Unlike in R_{gel}, the biomass washout rate in R_{star} increased rapidly upon increases of the organic loading rate. Such increases were not measured in R_{gel}. After formation of the granule-like aggregates in R_{star} (around day 160), the slope of the cumulative VSS washout rate decreased (**Figure 4-B**). The settling properties of the formed aggregates were similar to those of

sludge in R_{tryp}. Approximately 75 % of sludge in R_{star} was settling faster than 10.5 m/h (**Figure S3**), whereas in R_{tryp} this fraction was 88% before removal of tryptone (**Figure 1-A)**.

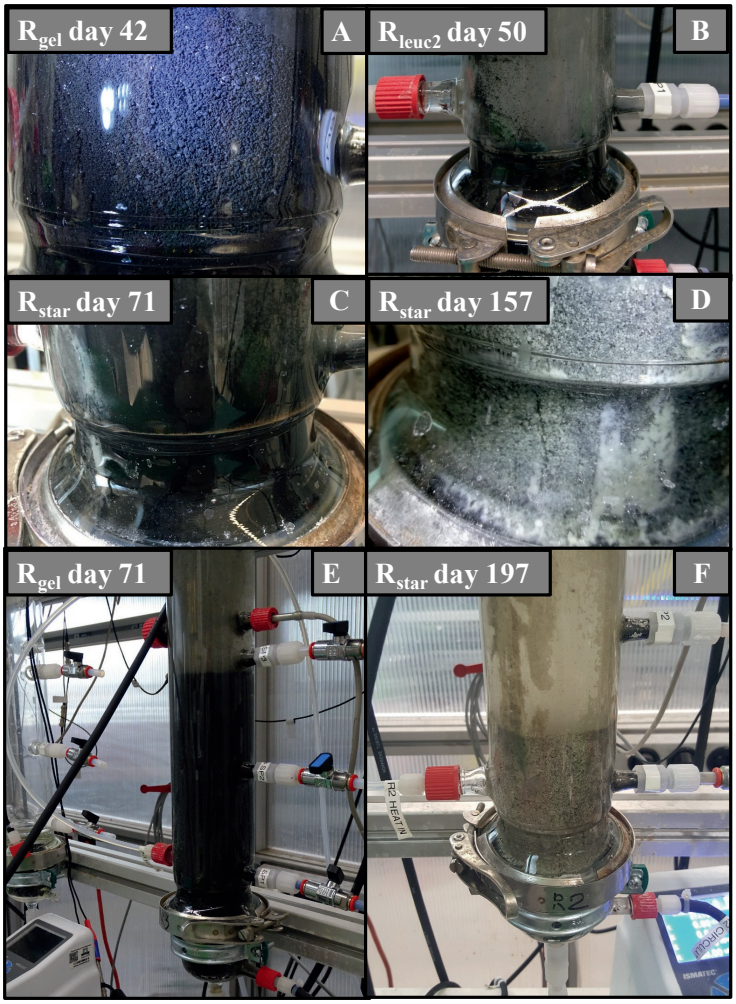


Figure 3. Images of sludge bed appearance in the bioreactors (A-D) and height of the sludge bed in the reactor with gelatine as a co-substrate after 71 day (E) and starch as a co-substrate after 197 days (F).

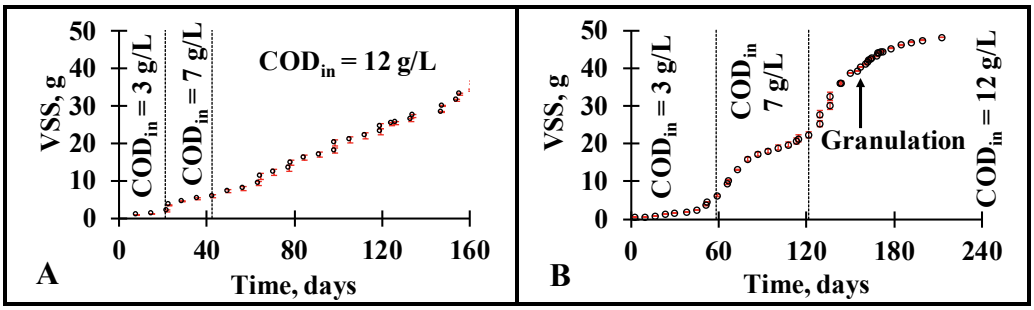


Figure 4. Cumulative VSS washout data. A – R_{gel}, B - R_{star}. Error bars (in red) represent the absolute deviation from duplicate measurements.

The morphology of the sludge in R_{gel} and in R_{star} is compared in **Figure 5**. Initially, the granular sludge in R_{gel} had a more regular shape and smoothed edges (**Figure 5-A, C**) compared to R_{star} (**Figure 5-B, D**). R_{star} contained more flocs compared to R_{gel} (**Figure 5**). The granule-alike aggregates in R_{star} matured further and developed smoothed edges (**Figure 5-E, F**) with a smaller amount of flocs in-between, similarly to R_{gel}. Such morphological changes match with the VSS data in **Figure 4-B**. In reactors R_{leuc1} and R_{leuc2} large flocs of irregular shapes formed. As an example, the morphology of sludge in R_{leuc2} at the end of experiment is shown in **Figure S2**. Interestingly, in R_{leuc1} a thick biofilm was formed on the walls of the reactor, probably contributing significantly to the overall COD conversion in the bioreactor (**Figure S4**).

3.4. Sugar and protein content of extracellular polymeric substances (EPS)

Figure 6-A shows the sugar content of EPS as D-glucose equivalents and protein content of EPS as total nitrogen equivalents. Irrespective of the co-substrate, the proteinaceous EPS fraction in the sludge was high, with 0.69 ± 0.02 in R_{leuc2} up to 0.85 ± 0.01 g protein per gram EPS in R_{gel}. The weight fraction of sugar ranged from 0.075 ± 0.0002 in R_{tryp} up to 0.124 ± 0.001 g per gram EPS in R_{star} (**Figure 6-A**). The yield of extracted protein was similar in R_{tryp}, R_{leuc1}, R_{leuc2}, R_{gel} and R_{star} and ranged from 352 ± 4 in R_{star} to 460 ± 17 mg/$g_{sludge\text{-}VS}$ in R_{gel} (**Figure 6-B**).

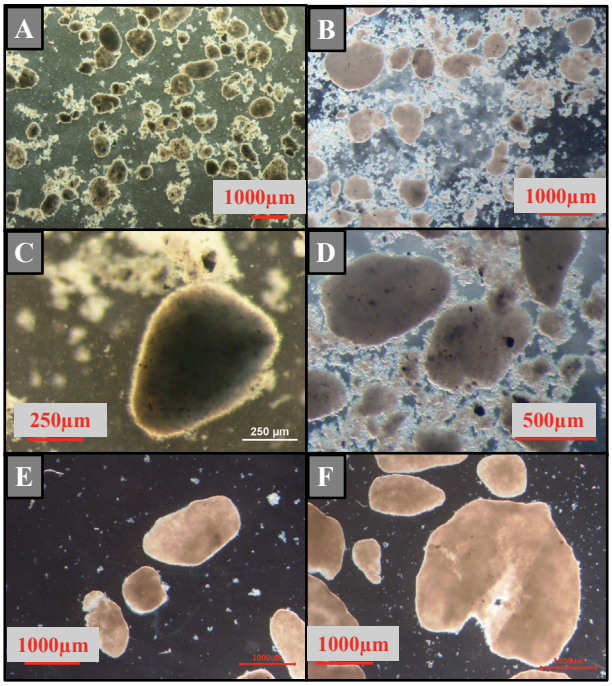


Figure 5. Macroscopy images of granular sludge formed with gelatine as a co-substrate (A, C) and granule-alike aggregates formed with starch as a co-substrate (B, D). Aggregates after maturation with starch as a co-substrate are depicted in E, F.

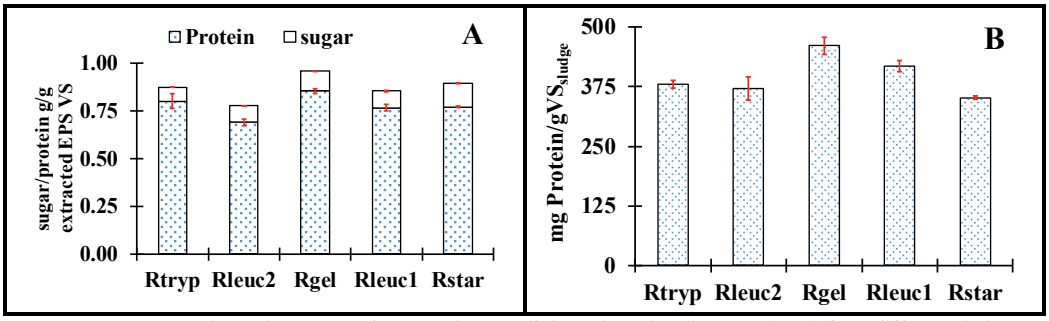


Figure 6. A - Sugar and protein content of extracted extracellular polymeric substances (EPS) from different sludges. B – the yield of extracted EPS protein normalised per gram of biomass volatile solids. R_{tryp} – tryptone as a co-substrate, $R_{leuc1,2}$ – first and second experiment with leucine as a co-substrate, R_{star} – experiment with starch as a co-substrate. Error bars on the columns represent the absolute deviation of duplicates from the average.

3.5. Amino acid and sugar composition of EPS in rapidly (R_{tryp}) and slowly (R_{star}) formed granules

Figure 7 shows that the amino acid composition of EPS extracted from R_{tryp} and R_{star} was similar and showed a good linear fit ($R^2 = 0.93$). The most abundant EPS amino acids were glutamic and aspartic acid with concentrations of 8.1 ± 0.5, 8.6 ± 0.3 and 7.8 ± 0.4, 9.0 ± 0.4 wt% of EPS in R_{tryp} and R_{star}, respectively. Also, both types of EPS had a similar fraction of hydrophobic amino acids with 44.9 ± 0.5% and 44.6 ± 0.8% of EPS amino acids being hydrophobic in R_{star} and R_{tryp}, respectively.

The measurements of sugar content (**Figure 8**) showed that EPS of R_{star} and R_{tryp} had similar weight fractions of rhamnosyl (0.22 ± 0.01 w/w % and 0.20 ± 0.01 w/w %) and arabinosyl (0.12 ± 0.01 w/w % and 0.14 ± 0.002 w/w %) residues. All other sugar residues were measured in higher abundance in R_{star} compared to R_{tryp}. For example, 0.53 ± 0.02 w/w % of R_{star} EPS were composed of fucosyl residue, whereas in R_{tryp} EPS this sugar could not be detected. Glucosyl residues were the most abundant in both R_{star} (2.75 ± 0.25 w/w %) and R_{tryp} (1.49 ± 0.07 w/w %) EPS (**Figure 8**).

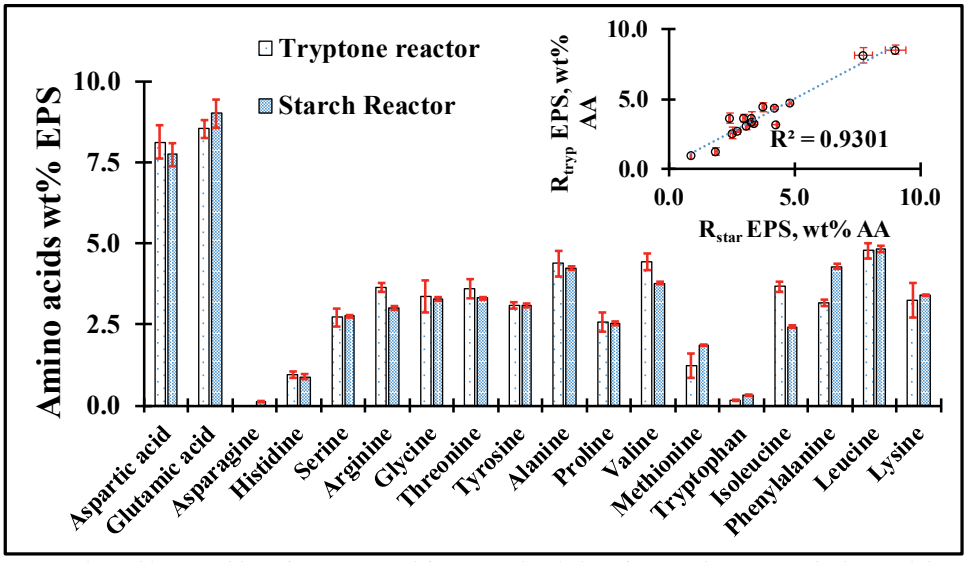


Figure 7. Amino acid composition of EPS extracted from granular sludge of R_{tryp} and R_{star}. Insert in the top right corner of the graph shows the linear correlation between amino acid compositions of the two types of EPS. Error bars show standard deviation between triplicate measurements.

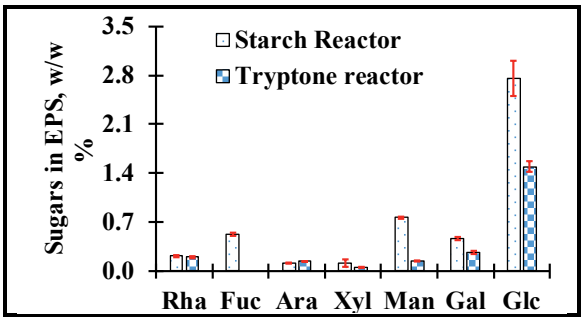


Figure 8. Sugar content (% w/w per dry matter EPS) of EPS extracted from granular sludge of R_{tryp} and R_{star}. Error bars show absolute deviation between duplicate measurements. Rha – rhamnose, Fuc – fucose, Ara – arabinose, Xyl – xylose, Man – mannose, Gal – galactose, Glc – glucose. Carbohydrates represent anhydrous sugars.

4. Discussion

4.1. Are proteins and peptides the "glue" or are they converted to act as such?

The results in *Paragraph 3.1* (**Figure 1**) clearly indicate the importance of peptides to provide and maintain structure of anaerobic granules at high salinity. Fast sludge granulation could be achieved with gelatine and tryptone in the influent. In R_{leuc1}, biofilm formed on the walls of the reactor (**Figure S4**) and granulation could not be achieved within 110 days. Albeit much slower, granulation could also be achieved with addition of starch to the influent after prolonged reactor operation periods. This means that the results of the experiments with amino acids as co-substrates should be viewed from the perspective of incubation time needed for granulation to start. It cannot be concluded that granulation with amino acids as co-substrates at high salinity is impossible. However, the granulation was much faster with gelatine (**Figure 3**) and tryptone compared to all other substrates**.** The final settling properties of aggregates in R_{star} (**Figure S3)** were similar to those in R_{tryp} (**Figure 1-A**), showing that the faster formation of granules does not compromise their settleability.

Under non-saline conditions, energy rich organic substrates, such as sugars, have been shown to promote granular sludge formation from flocculent sludge, whereas waste streams exclusively consisting of volatile fatty acids are mostly reported to yield poor granulation or no granulation at all (Jhung and Choi, 1995; Zhou et al., 2006). Some researchers have also suggested that complex waste streams may promote granulation due to activity of EPS producing bacteria (Vanderhaegen et al., 1992; Fukuzaki et al., 1995; Zhou et al., 2006). The results of this study show that under saline conditions (20 g Na^+/L) the presence of energy rich compounds, such as D-glucose is insufficient for improving the granulation and addition of protein and peptide to the influent promotes formation of granular sludge (**Figure 3**). Thus, both the organic substrate and adequate inoculum are important for fast granulation at high salinity.

Most likely, proteins, peptides and amino acids contained in them have several roles in the process of granulation under saline conditions. Firstly, microorganisms at high salinity have to cope with the osmotic pressure of the surrounding liquid (Campo et al., 2018). The osmotic pressure within microbial cytoplasm needs to be greater than that of the surrounding liquid to maintain cells turgor pressure (Csonka, 1989). In most non-halophilic microorganisms the osmotic balance is achieved by accumulating small organic molecules, called compatible solutes (Pflüger et al., 2005). Recently, it has been demonstrated, that addition of both tryptone and gelatine can alleviate osmotic stress in anaerobic granular sludges, most likely by providing aspartic acid and glutamine after hydrolysis of these proteins (**Chapter 3**). Secondly, addition of protein supplies the microbial community with organic nitrogen for production of cell material. Thus, addition of both proteins and peptides can allow to save energy for EPS production. Thirdly, in order to maintain the granular structure, extracellular polymeric substances (EPS) need to be produced (Schmidt and Ahring, 1996; Liu et al., 2003; Hulshoff Pol et al., 2004; Liu et al., 2009). In a UASB reactor the sludge is continuously subjected to liquid and gas shear forces shearing the EPS (Tiwari et al., 2006), meaning that this production should be continuous. In this study EPS were extracted from sludges of all reactors. **Figure 6-A** shows that, irrespective of formation of granules and the organic co-substrate, the EPS of all sludges contain a large fraction of protein (69 to 85%). The yield of extracted EPS protein was also comparable in all reactors (**Figure 6-B**). From a bioenergetic point of view, production of proteins from amino acids contained in proteins and peptides is much more favourable compared to production from ammonium and an organic substrate, such as glucose (Akashi and Gojobori, 2002). This means, that proteins and peptides can potentially supply the microbial community with energetically favourable "building blocks" that can be included into the EPS and therefore lead to a faster granulation compared to other substrates. Finally, peptides can spontaneously form gels with other peptides or proteins when present at sufficient concentrations (Doucet et al., 2003; Saiani et al., 2009). Since the EPS in the anaerobic sludges consist mainly of proteins (**Figure 6**), upon local increases of peptides or proteins at the aggregate/water interface, gelling could potentially occur due to physical-chemical interactions. It is difficult to predict the nature of such interactions due to the complex composition of e.g., tryptone and largely unknown chemistry of EPS (Seviour et al., 2019). However, the data provided in **Figure 7** clearly suggest the production of EPS protein *de novo* in this study. This is because the amino acid composition of EPS was similar in a reactor with added peptide (R_{tryp}) and without added peptide or protein (R_{star}). Furthermore, even though tryptone contains all amino acids found in the R_{tryp} EPS, the similarity of the amino acid composition in tryptone and EPS could not be found (**Figure S5**). Interestingly, glucosyl residues were the main blocks of the EPS in R_{star} and R_{tryp}, while other sugar residues, such as fucosyl differed in their relative amounts (**Figure**

8). It is unclear how the sugar composition of EPS could have affected the granulation in this study. However, their importance for granulation cannot be excluded.

With the experimental design presented in this study, it is not possible to definitively conclude what is the critical concentration of peptide or protein needed for maintenance of stable granular sludge. In fact, this concentration may depend on the sludge concentration in the bioreactor, assuming there is a certain amount of peptide per gram VSS necessary to form stable hydrogels. Further research should focus on minimal protein/peptide requirements for granular sludge formation at high salinity and maintenance of stable granules. The authors also acknowledge the importance to study microbial community in order to correlate granulation with certain microorganisms. However, currently tools to accurately identify functional EPS (e.g., structural EPS) are lacking and therefore production of these EPS cannot be assigned to certain microorganisms in mixed culture systems (Seviour et al., 2019). Therefore separate inter-disciplinary studies need to be performed in the future to clearly address the relations between microbial communities, granulation and EPS responsible for granulation. Finally, dedicated experiments should be performed to clarify, if physical-chemical interactions between EPS and peptides or proteins cause hydrogel formation in granules.

4.2. Is microbial activity a real issue or can microbes adapt?

Several authors have reported loss of methanogenic activity and subsequent process failure already at salinity levels well below those reported here (Rinzema et al., 1988; Jeison et al., 2008; Li et al., 2014). In UASB reactors with all tested co-substrates in this study high COD removal efficiencies could be achieved (**Figure 1-A, Figure 2**). Such good process performance could be a result of proper choice of inoculum already adapted to 8 g Na^+/L. Our previous work showed that from this inoculum, with an organic substrate as in R_{tryp}, the salt tolerant acetoclastic *Methanosaeta harundinacea* develops as the dominant methanogen responsible for methane production (Gagliano et al., 2018). In addition to this, the presence of osmolytes or precursor compounds in the influent can explain sufficient microbial activity to achieve good COD removal efficiencies in R_{leuc1}, R_{leuc2}, R_{gel} and R_{tryp}. In fact, influent of reactors R_{leuc1} and R_{leuc2}, potentially contained osmolytes in the form of proline (Roberts, 2005; Zaprasis et al., 2014). In R_{gel} and R_{tryp}, gelatine and tryptone could potentially act as precursors for osmolytes (**Chapter 3**). Decrease of tryptone fraction in R_{tryp} did not result in notable process deterioration in terms of sCOD conversion (**Table 3**). This is probably due to the prior adaptation of microbial community to 20 g Na^+/L. Also, most of the sludge could settle faster than 1.2 m/h throughout the experiment, therefore allowing to retain sufficient amount of biomass, capable of handling the applied organic loading rate.

The successful start-up of R_{star} with subsequent high COD removal efficiency (**Figure 2-D**) provides evidence that anaerobic biomass, previously adapted to 8 g Na^+/L, is able to further acclimate to salinity levels of 20 g Na^+/L without external dosing of osmolytes. This acclimation is possible due to the ability of microbial cells to synthesize osmolytes *de novo* (Sowers and Gunsalus, 1995; Roberts, 2005).

The finding that dosing of osmolytes for successful UASB operation at 20 g Na^+/L is not a prerequisite can even further broaden the application of high rate anaerobic digestion in saline industrial (waste)water treatment domain. The formation of granules is much slower with starch compared to tryptone and gelatine and less sCOD gets converted into methane. However, high sCOD removal efficiency at relevant loading rates can be achieved (**Table 4**) and in practice no additional costs of dosing osmolytes or their precursor compounds should be considered.

4.3. Practical implications

The start-up time of UASB reactors is very important. A number of authors have studied strategies to speed up granular sludge formation, for faster UASB reactor start-up under non-saline conditions: appropriate (initial) organic loading rates (Ghangrekar et al., 2005), manipulating the liquid upflow velocity (Arne Alphenaar et al., 1993), dosing multivalent cations (Vlyssides et al., 2009), adding support materials (Yoda et al., 1989) and dosing natural and synthetic polymers (El-Mamouni et al., 1998; Tiwari et al., 2005). Here we demonstrate the importance of proteins and peptides in the (waste)water stream at high salinity if fast sludge granulation is desired. The addition of protein to the (waste)water for its treatment is expected to be too expensive. However, saline types of (waste)water containing proteins, such as olive oil milling, cheese and fish industry (waste)water (Paredes et al., 1999; Ramsay and Pullammanappallil, 2001) can be identified as suitable for anaerobic treatment. Furthermore, where applicable, mixing of several (waste)waters can be considered. Alternatively, we demonstrated that with prolonged start-up periods granulation can be achieved without proteins being present in the (waste)water given that an appropriate inoculum is selected. Future research should focus on finding minimum requirements of protein to achieve rapid granulation and on possibility to stop dosing proteins as soon as granulation has been achieved.

Figure 2 shows good sCOD removal efficiency at relatively high organic loading rates with most of the tested co-substrates, even in reactors where sludge granulation was not achieved. This means that reactors with flocculent sludge can also be applied at high salinity, where granulation is not possible. However, granular sludge is desired in reactors operated at higher up-flow velocities, such as expanded granular sludge bed reactors.

5. Conclusions

Soluble proteins in (waste)water enhance the rate of anaerobic sludge granulation at salinity levels of 20 g Na^+/L. However, formation of well settling granule-alike biomass at 20 g Na^+/L is possible without application of proteins to enhance the granulation, but requires prolonged reactor start-up time. Possibly, addition of soluble proteins in saline (waste)water streams results in faster granulation due to more energetically favourable EPS building blocks provided. High soluble COD removal efficiencies (above 90%) can be achieved at salinity of 20 g Na^+/L while anaerobically treating (waste)waters of various compositions. These findings provide important guidelines for applicability of high rate anaerobic digestion at high salinity depending on the organic substrate.

Supplementary Information of Chapter 4

Table S1. Cations (especially multivalent) have been repeatedly reported to play an important role in the process of granulation. To exclude the possibility of fast granulation with tryptone due to the ions contained in its salt, the major ions measured in tryptone (Sigma Aldrich) were compensated in the experiment with tryptone removal.

A dissolved tryptone sample was acidified to reach 1% HNO3 (w/v) concentration. The ion content was measured on Thermo iCAP-6500 DV (Thermo Fisher Scientific) ICP-AES in radial direction. The coefficient of variance for the measurements of laboratory standards was within 2% range. Iron content was measured with LCK320 iron kits (HACH, UK).

Ion	Conc., mg/g tryptone	% compared to nutrient medium
K (766.490)	13.28	26.27
Mg (285.213)	0.22	5.13
Na (589.592)	25.23	0.16
P (177.495)	8.10	20.23
Al (394.401)	0.06	N/A
Ca (317.933)	1.13	10.87
Cu (324.754)	0.02	469.19
Pb (220.353)	0.01	N/A
S (182.034)	7.49	134.36
Zn (213.856)	0.14	1227.66
Fe*	1.19	11.12
B (249.773)	0.01	305.47

Figure S1. Our experience showed that recovery of converted soluble COD as methane COD with µFlow gas meters was often difficult and inaccurate due to specifics of biogas release in laboratory scale UASB reactors. The gas is often released from the sludge bed as large bubbles after periods of accumulation. This results in more gas released through the counter, than the meter actually counts (8 NmL per release). Below a simplified custom-made scheme for biogas quantification used to close mass balances in reactors with formed granules (R_{tryp}, R_{gel} and R_{star}) is presented. 1 – gas sampling port; 2 – water lock with a volume of 3L; 3 – mechanical pressure sensor with a relay (FEMA DCM4016, Germany) set to 1 mbar; 4 – power source; 5 – gas pump (KNF N86KT.18, France); 6 – drum-type gas meter (Ritter TG0.5/6, Germany)

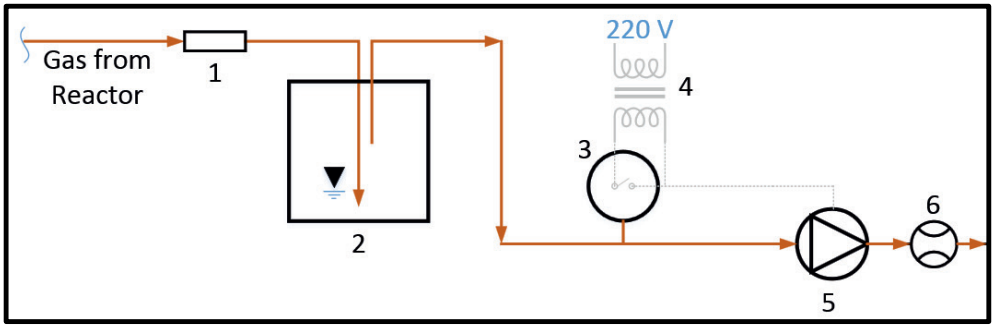


Figure S1. Custom-made gas measurement system

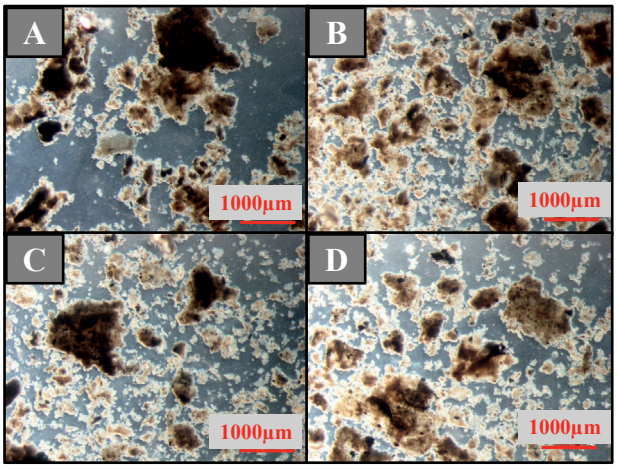


Figure S2. At the end of experiment in R_{leuc2} heavy flocks of irregular forms had formed at the bottom of the reactor. Images taken after diluting approximately 2.5 mL of biomass with 10 mL of tap water. The reactor was operated for 54 days. Scale bars correspond to 1000 µm in the micrographs.

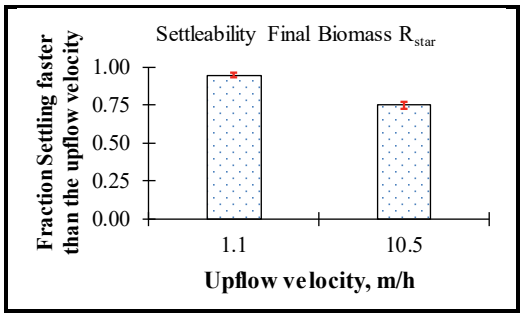


Figure S3. At the end of experiment, the granule alike aggregates from R_{star} were collected and their settling properties measured as described in the materials and methods section of the manuscript. Red error bars represent the absolute deviation from the average of duplicate measurements.

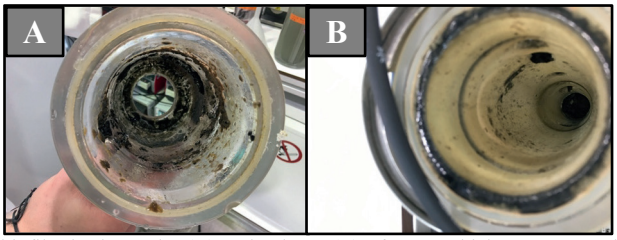


Figure S4. The formed biofilm in the settler (A) and column (B) of R_{leuc1} which was operated at 20 g Na^+/L using D-glucose, sodium acetate, proline, and leucine on day 112. This biofilm was potentially largely contributing to the COD conversion in the bioreactor.

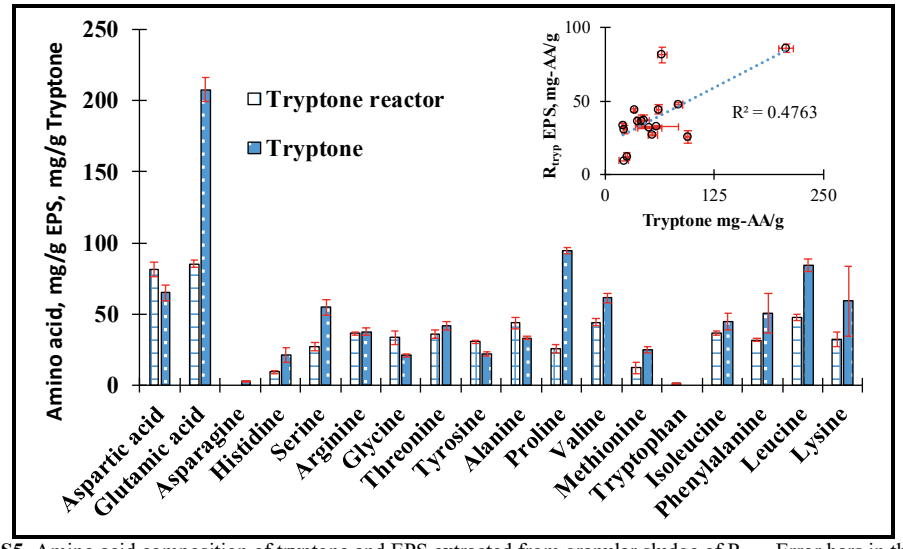
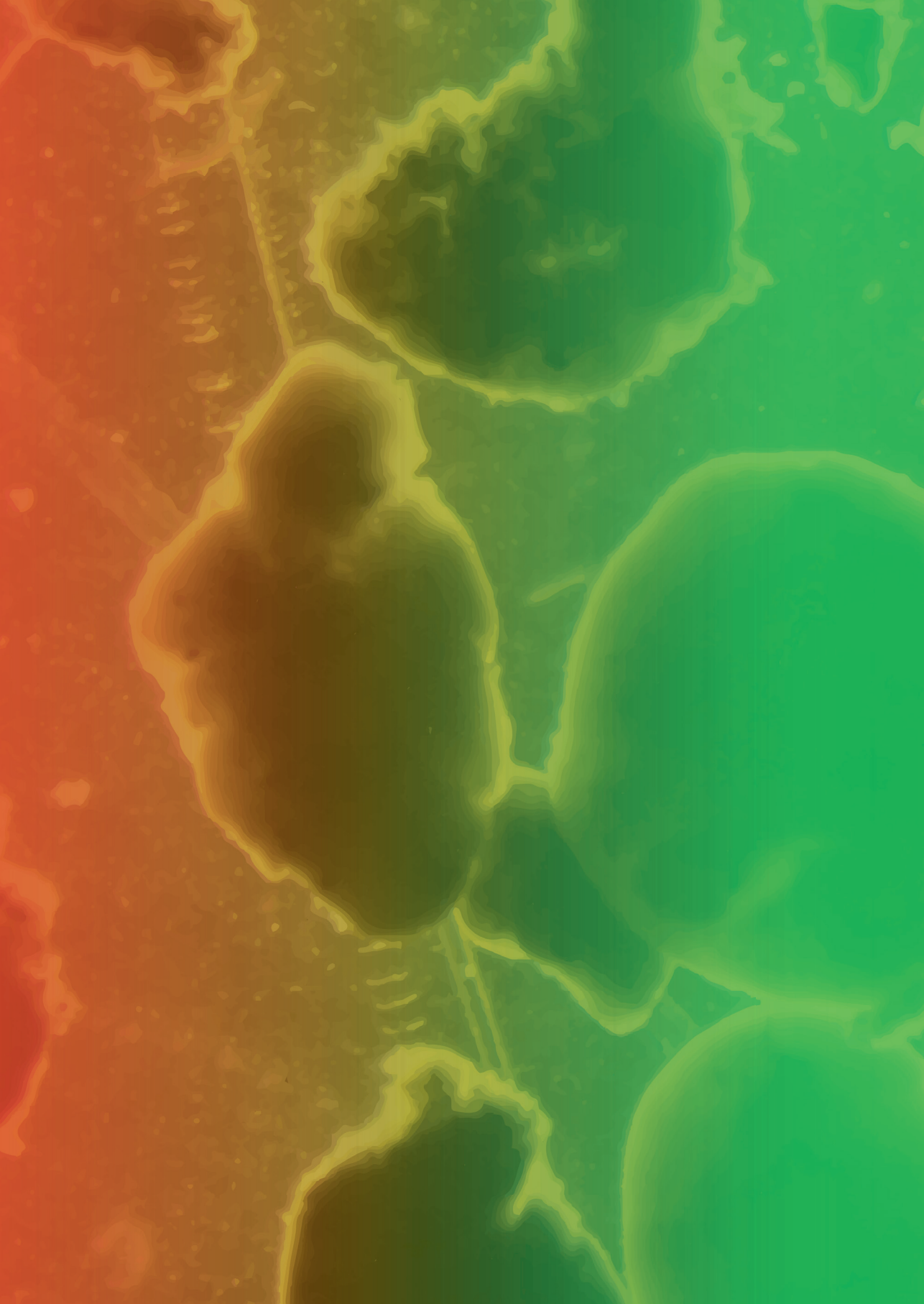


Figure S5. Amino acid composition of tryptone and EPS extracted from granular sludge of R_{tryp}. Error bars in the figure show standard deviation in triplicate measurements. The insert in the right upper corner of the figure shows poor linear correlation between EPS and tryptone amino acid composition.



Chapter 5. Protein Requirement for Anaerobic Sludge Granulation at High Salinity

Authors:

D. Sudmalis[a], L. Zeng[a], M. C. Gagliano[b], H.H.M. Rijnaarts[a], G. Zeeman[a], H. Temmink[a]
[a] Department of Environmental Technology, Wageningen University and Research, Bornse Weilanden 9, 6708 WG, Wageningen, The Netherlands
[b] Wetsus, Centre of Excellence for Sustainable Water Technology, Agora 1, P.O. Box 1113, 8900 CC Leeuwarden, The Netherlands

Abstract: Recently it was shown that when proteinaceous substrate is fed to an UASB reactor at a 16.7% COD contribution to saline wastewater, this enhanced sludge granulation. It was also shown that two amino acids contained in proteins – aspartate and glutamate – can alleviate osmotic stress of methanogens. However, continuous dosing of such high concentrations of proteins may limit the practical feasibility of this strategy. The objective of this contribution was to estimate the minimum amount of proteinaceous substrate that is needed to achieve good granulation in UASB reactors at high salinities. Three UASB reactors were started with a dispersed inoculum and saline wastewater (20 g Na^+/L). In the first reactor $R_{16.7\%}$ the influent proteinaceous substrate COD fraction was 16.7% - an amount reported before to yield good granulation and methanogenic activity. In the second reactor $R_{1.8\%}$ the proteinaceous influent COD fraction was 1.8%. This was an amount calculated to obtain sufficient osmoprotection, based on the protein's aspartate and glutamate content. The third reactor $R_{0.46\%}$ received 25% of the calculated amount of $R_{1.8\%}$. The results showed that excellent granulation and long sludge retention times (SRT) could only be achieved in $R_{16.7\%}$ (SRT = 239 ± 47 days) and $R_{1.8\%}$ (SRT = 226 ± 47 days). The sludge in $R_{0.46\%}$ was flocculent and was poorly retained in the reactor (SRT = 3.2 ± 0.2 days). The results of reactors performance at an organic loading rate of 8 g COD/L·d showed good soluble COD removal efficiency in both, $R_{16.7\%}$ (97.1 ± 0.5%) and $R_{1.8\%}$ (96.6 ± 0.4%) with COD conversion efficiencies into methane of 87.0% ± 4.3% and 81.2% ± 4.5%, respectively. Reactor performance of $R_{0.46\%}$ was poor (COD removal efficiency 82.9 ± 0.2%) and deteriorating in time with increasing concentration of effluent volatile fatty acids. These results show that the amount of proteinaceous substrate for good granulation and process performance at high salinity can be decreased at least nine fold compared to what we found before. This information can help to identify saline (waste)water streams that are amenable for high rate granular anaerobic treatment.

1. Introduction

Many industries generate (waste)water with high concentrations of sodium chloride and organic pollutants (Woolard and Irvine, 1995; Lefebvre and Moletta, 2006; Lefebvre et al., 2006; Lefebvre et al., 2007; Corsino et al., 2017; Campo et al., 2018). Anaerobic biological treatment in compact granular sludge reactors of such streams should be considered, because this process allows for recovery of energy in the form of methane gas, produces small amounts of surplus sludge and reduces the footprint (space requirements) of the bioreactors (Van Lier, 2008; Chan et al., 2009; Xiao and Roberts, 2010).

However, application of anaerobic granular biological reactors for treatment of highly saline wastewater streams is often reported to be unfeasible. Firstly, this is because high concentrations of

sodium inhibit anaerobic microbial activity, especially that of acetoclastic methanogens (Kugelman and McCarty, 1965; Rinzema et al., 1988; Boardman et al., 1995; Pollice et al., 2000; De Vrieze et al., 2016a). Secondly, high monovalent salt concentrations are reported to cause weakening and disintegration of the anaerobic granules (Ismail et al., 2008; Jeison et al., 2008; De Vrieze et al., 2016a; Muñoz Sierra et al., 2019). This is because monovalent cations displace multivalent cations in the EPS matrix. Whereas the latter are known to strengthen the granular structure by bridging negatively charged groups of extracellular polymeric substances (EPS) contained in the granular sludge, monovalent cations are unable to form such bridges (Higgins Matthew and Novak John, 1997; Jeison et al., 2008; Ismail et al., 2010). Both, granular sludge integrity and microbial activity are prerequisites for application of compact anaerobic bioreactors (Hulshoff Pol et al., 2004; Van Lier et al., 2015).

Sodium toxicity towards methanogens may be overcome by gradual adaptation of inoculum sludge to increasing salinity (Feijoo et al., 1995; Roberts et al., 2016), by inoculating bioreactors with moderately halophilic biomass (Le Borgne et al., 2008; Ismail et al., 2010; Gagliano et al., 2017b; Muñoz Sierra et al., 2019) or by dosing small organic molecules called osmolytes (or compatible solutes) for osmotic stress alleviation (Vyrides et al., 2010; He et al., 2012; Vyrides and Stuckey, 2016). The latter strategy relies on the ability of microorganisms to take up specific molecules, such as betaine, trehalose or amino acids from the bulk liquid in order to balance the osmotic pressure between the cell's cytoplasm and the bulk liquid. However, in practice dosing of such molecules would be expensive because of the high prices of these osmolytes. Besides, this approach is still in early stages of research (Vyrides and Stuckey, 2016).

Our recent work (**Chapter** 2) was the first to demonstrate successful anaerobic sludge granulation with good process performance at high monovalent salt concentrations from dispersed biomass although several researchers have reported increasing size of (halotolerant) anaerobic granular sludge inocula in UASB reactors upon exposure to sodium levels as high as 20 g Na^+/L (Ismail et al., 2010; Gagliano et al., 2017b). Increasing granular sludge size has also been reported in a UASB reactor operated at Na^+ concentration of 11 g/L (Li et al., 2014). However, the positive result of increasing granule's size in the latter research was offset by severe inhibition of methanogenic activity at this sodium concentration.

The reactors in **Chapter 2** were operated with synthetic wastewater consisting of D-glucose, sodium acetate and tryptone (a peptide derived from casein) in a COD ratio 3:2:1. Further investigations in **Chapter 3** showed that methanogenic activity of granular sludge upon exposure to high salinity can be positively influenced by proteinaceous organic substrates, such as tryptone or gelatine. This is

probably due to release of amino acids (aspartate and glutamate), that can be taken up from the bulk liquid to act as osmolytes after hydrolysis of tryptone and gelatine. Thus, proteinaceous substrates in the wastewater may act as precursors for osmolytes. **Chapter 4** showed that soluble proteinaceous substrates, such as tryptone or gelatine, besides improving osmoadaptation of methanogens, can also enhance the rate of anaerobic sludge granulation at high salinity (20 g Na^+/L) compared to, for example, the granulation rate with soluble starch. This is probably because proteins provide energetically more favourable building blocks for proteinaceous EPS production. It was also shown that a gradual decrease of the influent fraction (from 16.7% to 0.8% as COD fraction) of a proteinaceous substrate (tryptone) of a UASB reactor with stable granular sludge resulted in rapid disintegration of the sludge granules. However it was not possible to estimate the minimum amount of protein that is required for good granulation. For a practical application, it would be of importance to know this concentration, because it determines the economic feasibility of dosing proteins. Also, some saline wastewaters may contain enough protein for anaerobic granular treatment without additional dosing. These can be identified if the minimum protein requirements are known.

The aim of this study was to determine the minimum amount of proteinaceous substrate that is needed to achieve good granulation at high salinity. Firstly, a minimum amount was calculated based on the osmotic pressure originating from Na^+ and Cl^- ions and amount of tryptone needed to provide enough aspartate and glutamate for balancing this osmotic pressure between the bulk liquid and microbial cells. Secondly, the process performance of UASB reactors operated at 20 g Na^+/L was compared at three influent proteinaceous substrate concentrations: i) higher (same as in **Chapter 2**) than the calculated amount; ii) the calculated amount; iii) 75% less than the calculated amount.

2. Materials and methods

2.1. Inoculum, influent composition, reactor set-up and operation

The biomass inoculum was sampled from a full-scale UASB reactor adapted to approximately 8 g Na^+/L. Details of the inoculum can be found in (Jeison et al., 2008; Ismail et al., 2010). Before the experiments, the biomass was sieved through a 125 μm metal sieve. All reactors were inoculated with 6 gVSS/L of the biomass that passed the sieve.

Continuous laboratory scale experiments were performed in three double-walled glass UASB reactors. The main operational parameters are summarised in **Table 1** and a schematic representation of the reactors is shown in **Figure 1**. The volumetric loading rates in the UASB reactors were increased by a gradual increase of the influent COD concentration from 3 to 12 g COD/L to reach the final loading rate of 7.9 g COD/L·d (**Table 1**). The upflow velocity was increased stepwise from 0.01

m/h up to 1 m/h (**Table 1**) by circulation of the effluent with a peristaltic circulation pump (**Figure 1**, unit Nr. 8).

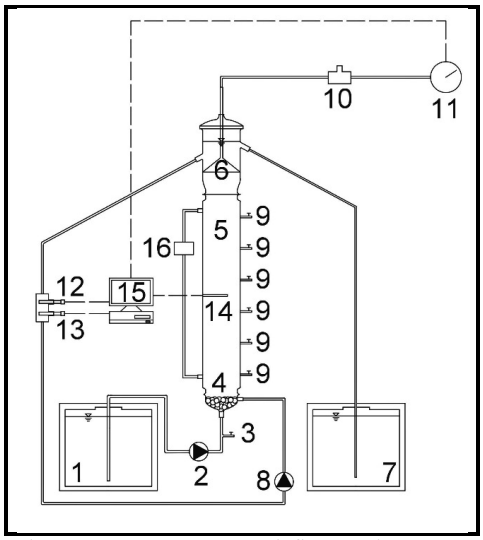


Figure 1. Diagram of experimental UASB reactor setups. 1 – influent tank at 1 ºC; 2 – peristaltic influent pump; 3 – influent sampling port; 4 – supporting material; 5 – UASB reactor; 6 – settler; 7 – effluent tank at 4 ºC; 8 – peristaltic recirculation pump; 9 – sampling ports; 10 – biogas sampling port; 11 – gas flow meter; 12 – oxidation-reduction (redox) sensor; 13 – pH sensor; 14 – temperature sensor; 15 – computer for data logging; 16 – heating bath

The influent COD of all reactors was soluble and was composed of D-glucose, Na-acetate and tryptone. A different COD proportion of tryptone was used in each one of the three UASB reactors. The first UASB reactor was operated as a positive control for granulation and COD removal performance. The COD ratio of D-glucose : Na-acetate : tryptone was 3: 2: 1 for this reactor, as previously reported in **Chapter 2**. This ratio corresponds to 16.7 % of influent COD as tryptone and the reactor will be further referred to as $R_{16.7\%}$. The influent COD proportion of tryptone in the second reactor was 1.8% ($R_{1.8\%}$) and was based on a calculation such that enough glutamate and aspartate would be provided for osmoprotection of microorganisms. A detailed explanation of this calculation is provided in **Appendix 1**. Finally, the third reactor ($R_{0.46\%}$) was operated at 25% of the calculated tryptone concentration of $R_{1.8\%}$.

The influent COD concentration for the three different reactors was kept the same by compensating decreasing amounts of tryptone with D-glucose and sodium acetate in a COD proportion of 3:2. The decreasing nitrogen concentration due to removal of tryptone was compensated with NH_4Cl. Other cations contained in tryptone were compensated as described in **Chapter 4**. The nutrient medium was the same as described in **Chapter 2**. The sodium concentration in all reactors was 20 g Na^+/L and included sodium originating from addition of Na-acetate. The COD composition of all reactors for the different applied influent COD concentrations is shown in **Table 2**.

Table 1. Main operational parameters of the UASB reactors. V_{up} – upflow velocity; COD_{in} – influent COD concentration

Parameter	$R_{16.7\%}$	$R_{1.8\%}$	$R_{0.46\%}$
Temperature, °C	34.9 ± 0.9	35.7 ± 0.5	33.9 ± 1.1
Volume (reactor + settler), L	2.94	3.23	3.25
Volume of reactor, L	1.96	1.95	2.00
Diameter, m	0.06	0.06	0.06
Initial VLR, g COD/L·day	1.2	1.2	1.2
Initial COD_{in}, g/L	3	3	3
Day of COD_{in} increase to 7 g/L	31	31	31
Day of COD_{in} increase to 12 g/L	67	53	67
Initial V_{up}	0.01	0.01	0.01
Period (days) at the final VLR of 7.9 g COD/L·day	67-109	53-109	67-109
Day of V_{up} increase to 0.2 m/h	11	11	11
Day of V_{up} increase to 0.7 m/h	32	32	32
Day of V_{up} increase to 1.0 m/h	78	78	78

Table 2. The ratio of D-glucose, Na-acetate, and tryptone of each reactor at different influent COD concentrations

CODinfluent, g/L	COD ratio D-glucose : Na-acetate: tryptone		
	R16.7%	R1.8%[a]	R0.46%[a]
3	3:2:1	31.9: 21.3:1	129.3:86.2:1
7	3:2:1	32.3:21.5:1	130.9:87.3:1
12	3:2:1	32.8:21.9:1	133.0:88.7:1

[a] The ratios were rounded to 1 digit behind the comma.

2.2. Analytical techniques for process performance monitoring

Effluent total and soluble chemical oxygen demand (COD), effluent total suspended solids (TSS) and volatile suspended solids (VSS) and effluent volatile fatty acid (VFA) concentrations were measured in 24 hour composite effluent samples in duplicate on a weekly basis. The COD was measured with LCK 314, LCK514 and LCK1414 kits (Hach GMBH, Germany). Prior analysis samples were diluted to reach non-interfering chloride concentrations (below 1500 mg/L). For tCOD measurements, effluent samples were homogenised with an IKA T25 Ultra-Turrax homogeniser (JANKE & KUNNEL, Germany) at 24000 rpm. Soluble COD (sCOD) is defined here as a sample centrifuged at 15000 rpm for 5 minutes followed by filtration through a 0.45 µm polyethylene sulfonate (PES) membrane filter (CHROMAFIL® Xtra, Germany). Effluent total suspended solids (TSS) and VSS were determined in duplicate according to standard methods 2540 D and 2540 E, respectively (APHA, 2005). Analysis of effluent VFA concentration was carried out using Agilent 7890B gas chromatograph as described in **Chapter 2**. Sludge retention times (SRT) in the UASB reactors were determined from slopes of the volatile suspended solids washout between day 96 and 103 and the final VSS concentration in the reactors on day 109.

Biogas production of $R_{16.7\%}$ and $R_{1.8\%}$ was measured with a drum-type gas meter (Ritter, Germany) as described in **Chapter 4**. Biogas production of $R_{0.46\%}$ was measured with a µFlow gas flow meter (Bioprocess Control Sweden AB). Biogas composition was determined on a weekly bases in singleton by using a GC-2010 gas chromatograph (Shimadzu Corporation, Japan) as described in Steinbusch et al. (2008).

2.3. Sludge characterization

2.3.1. Light microscopy and sludge volume index (SVI)

Sludge samples for light microscopy were taken from the bottom sampling ports of the UASB reactors at a height of ~ 7.5 cm from the bottom of the reactor. Prior imaging, approximately 2.5 mL of a sample was diluted with 7.5 mL of tap water. Sludge samples were examined with a Nikon SMZ800 Stereo Zoom Microscope (Nikon, Japan) equipped with a CMEX-5 PRO USB 3.0 mounted camera. Images were processed with ImageFocusAlpha software (Euromex, the Netherlands). Photos of the sludge bed of each reactor were also taken daily with a digital camera.

At the end of the experiments, 15 minute sludge volume index (SVI_{15}) measurements of sludge samples taken from reactors $R_{16.7\%}$ and $R_{1.8\%}$ were conducted. This was done in triplicate using Imhoff cones according to the standard method 2450F (APHA, 2005). The measurements were not performed for $R_{0.46\%}$ due to loss of sludge from the bioreactor after final increase of the volumetric loading rate.

2.3.2. Extraction and characterisation of extracellular polymeric substances (EPS)

At the end of experiments, the EPS of $R_{16.7\%}$ and $R_{1.8\%}$ were extracted and characterised for their sugar and protein content. This could not be done for reactor $R_{0.46\%}$ due to sludge washout applying a high volumetric loading rate. Approximately 20 g of the sludge was sampled from the bottom sampling ports of the reactors $R_{16.7\%}$ and $R_{1.8\%}$ and separated from the bulk liquid by centrifugation at 4000 rpm and 4℃ for 20 min. The sludge samples were homogenised with a spatula and EPS were extracted twice from ~3 g of the homogenised wet sludge samples with an alkaline extraction method as described in Felz et al. (2016) and dialysed (SnakeSkin™, 3.5K MWCO) with milli-Q water for 24 hours

Total nitrogen content of the EPS samples was measured with LCK338 total nitrogen kits (HACH GMBH, Germany) and was used to estimate the protein content of EPS with 6.25 $g_{protein}/g_{nitrogen}$ as a conversion factor (Williams et al., 1987). Carbohydrates content was measured in duplicate with phenol-sulphuric acid method as D-glucose equivalents (Dubois et al., 1956).

3. Results and discussion

3.1. Reactor performance

Figure 2 shows total and soluble COD removal efficiencies of $R_{16.7\%}$, $R_{1.8\%}$ and $R_{0.46\%}$. Reactors $R_{16.7\%}$ and $R_{0.46\%}$ were accidently overloaded (day 30-50) during the first increase of the volumetric organic loading rate (VLR) from 1.2 to 4.5 gCOD/L·d and this resulted in a rapid decrease of COD removal efficiency (**Figure 2-A, C**) and accumulation of volatile fatty acids (VFA) in the reactors (**Figure 3-A, C**). However, extending the period of a lower VLR allowed for further stepwise increase of VLR to achieve a similar organic loading rate in all reactors (~ 7.9 g COD/L·d) (**Figure 2**). In the following, reactor performances will only be compared for the final two measurements of the effluent samples (day 100 and 108) at the final loading rate of 7.9 g COD/L·d. The COD removal efficiencies during this period reached stable high values in $R_{16.7\%}$ and $R_{1.8\%}$, but showed a downward trend in $R_{0.46\%}$.

The sCOD removal efficiencies were clearly better in $R_{16.7\%}$ and $R_{1.8\%}$ compared to $R_{0.46\%}$ with average values and absolute deviations of 97.1 ± 0.5 %, 96.6 ± 0.4 % and 82.9 ± 0.2 % for $R_{16.7\%}$, $R_{1.8\%}$ and $R_{0.46\%}$, respectively. The difference in tCOD removal efficiencies between $R_{0.46\%}$ and the other two reactors was even more pronounced (**Figure 2**). The tCOD removal efficiencies were 94.5 ± 1.7 %, 94.8 ± 0.1 % and 74.3 ± 0.5 % for $R_{16.7\%}$, $R_{1.8\%}$ and $R_{0.46\%}$, respectively. The large difference (8.5%) between soluble and total COD removal efficiencies in reactor $R_{0.46\%}$ indicates a strong biomass washout from this reactor.

High concentrations of VFAs, such as acetate and propionate, in the effluents of anaerobic reactors indicate organic overloading (Powell and Archer, 1989; Parawira et al., 2006). The effluent VFA concentrations throughout reactor operation are shown in **Figure 3**. At the VLR of 7.9 g COD/L·d, the effluent VFA concentrations of $R_{16.7\%}$ and $R_{1.8\%}$ (**Figure 3-A, B**) were much lower compared to $R_{0.46\%}$ (**Figure 3-C**). The effluent propionate concentrations showed downward trends for $R_{16.7\%}$ (**Figure 3-A**) and $R_{1.8\%}$ (**Figure 3-B**) but an upward trend in $R_{0.46\%}$ (**Figure 3-C**). At the end of the reactor operation, the acetate and propionate concentrations had decreased to 15.5 ± 0.3 mg/L and 104.6 ± 1.1 mg/L in $R_{16.7\%}$, and to 43.2 ± 0.4 mg/L and 94.7 ± 0.6 mg/L in $R_{1.8\%}$. The effluent acetate and propionate concentrations in $R_{0.46\%}$ were 777.9 ± 5.8 mg/L and 783.1 ± 4.5 mg/L, respectively. The biogas methane fractions at the end of reactor operation were 67.4 ± 0.3 %, 66.0 ± 0.5 % and 60.2 ± 0.1 % in $R_{16.7\%}$, $R_{1.8\%}$ and $R_{0.46\%}$ respectively. Conversion of removed sCOD into methane at the final loading rate was 87.0% ± 4.3% in $R_{16.7\%}$ and 81.2% ± 4.5% in $R_{1.8\%}$. Due to technical issues conversion efficiencies in $R_{0.46\%}$ were not measured.

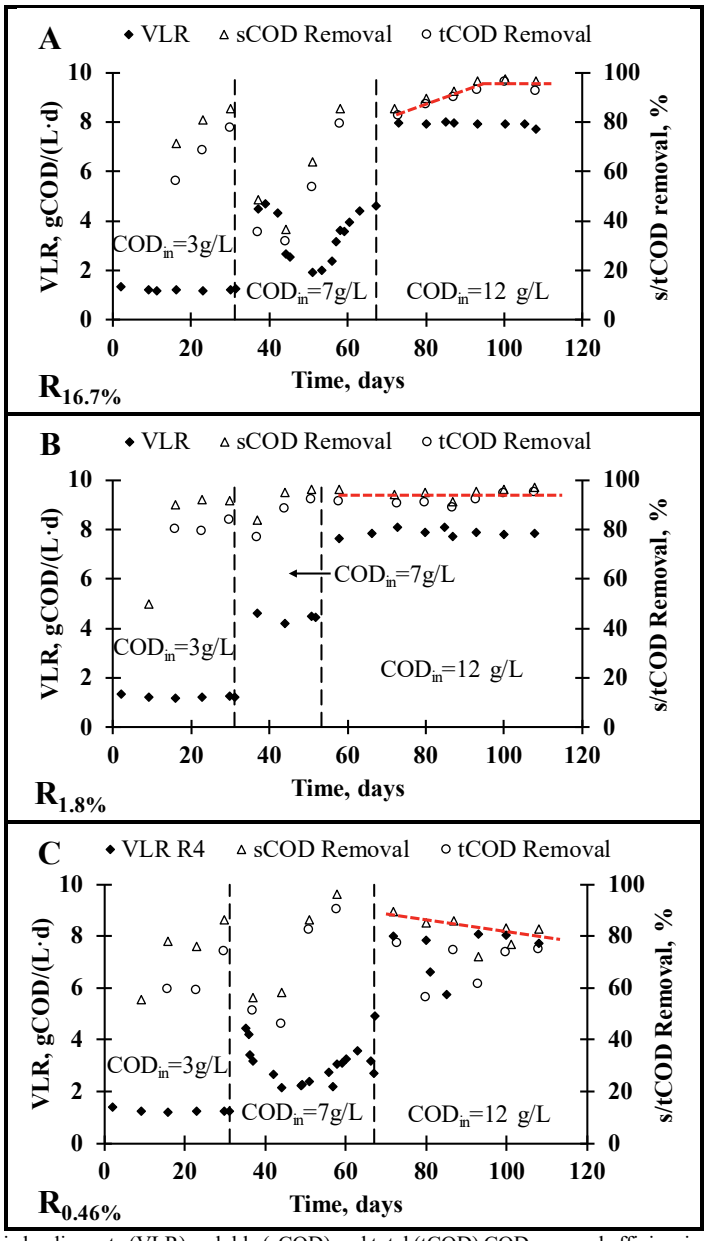


Figure 2. Volumetric loading rate (VLR), soluble (sCOD) and total (tCOD) COD removal efficiencies of $R_{16.7\%}$ (A), $R_{1.8\%}$ (B) and $R_{0.45\%}$ (C). Red dotted lines added to guide the reader's eye for the sCOD removal trends.

The results above clearly show that the addition of proteinaceous substrate, such as tryptone, to mitigate a negative effect of high salt concentration, can be much lower than the 16.7% that was used previously (**Chapter 2**). For successful UASB operation at 20 g Na^+/L, apparently a tryptone fraction between 1.8% and 0.46% is needed. In **Chapter 4** it was shown that also gelatine (16.7% influent COD) enhances the granulation and reactor start-up at 20 g Na^+/L. However, the requirements of gelatine and other proteins may be different and depend on their amino acid composition.

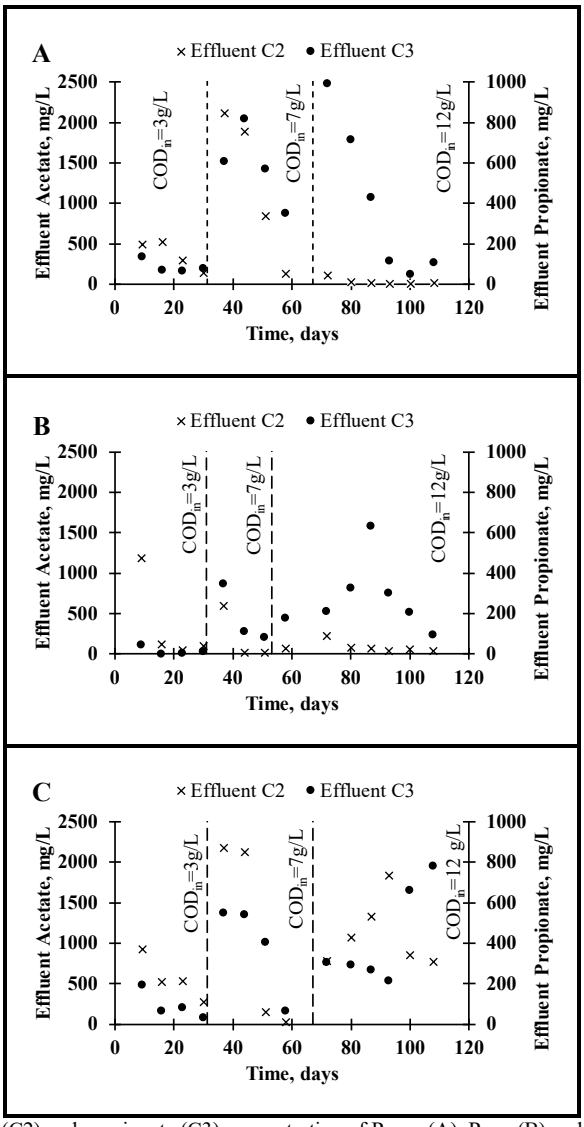


Figure 3. Effluent acetate (C2) and propionate (C3) concentration of $R_{16.7\%}$ (A), $R_{1.8\%}$ (B) and $R_{0.45\%}$ (C).

3.2. Granulation and biomass retention

The tryptone fraction had a clear effect on granulation (**Figure 4**) and biomass retention (**Figure 5, Figure 6**). After 45 days sludge beds of all UASB reactors contained aggregates of irregular shapes larger than the inoculum (max 125 μm) (**Figure 4-A, C, E**). In **Chapter 2** it was reported that fully granular sludge can be formed already after 45 days with an influent substrate composition as reported here for $R_{16.7\%}$. The sludge bed of $R_{16.7\%}$ during this study was not fully granular after 45 days, probably due to overloading of the reactor at the initial stage of the start-up (**Figure 2**). Such overloading is known to cause ingrowth of fluffy and less dense granules (Ghangrekar et al., 1996;

Ghangrekar et al., 2005). At the end of the experiment a full granular sludge bed had developed in $R_{16.7\%}$ and $R_{1.8\%}$ (**Figure 4-B, D**) showing that with the calculated amount of tryptone in $R_{1.8\%}$ formation of granular sludge was possible. Granules of $R_{1.8\%}$ had smoother edges and looked more dense compared to those formed in $R_{16.7\%}$, probably still as a consequence of the initial reactor overloading in $R_{16.7\%}$. Granular sludge did not develop in $R_{0.46\%}$. Instead, the sludge in this reactor mainly consisted of flocs, with some aggregates of irregular shapes in-between (**Figure 4-F**).

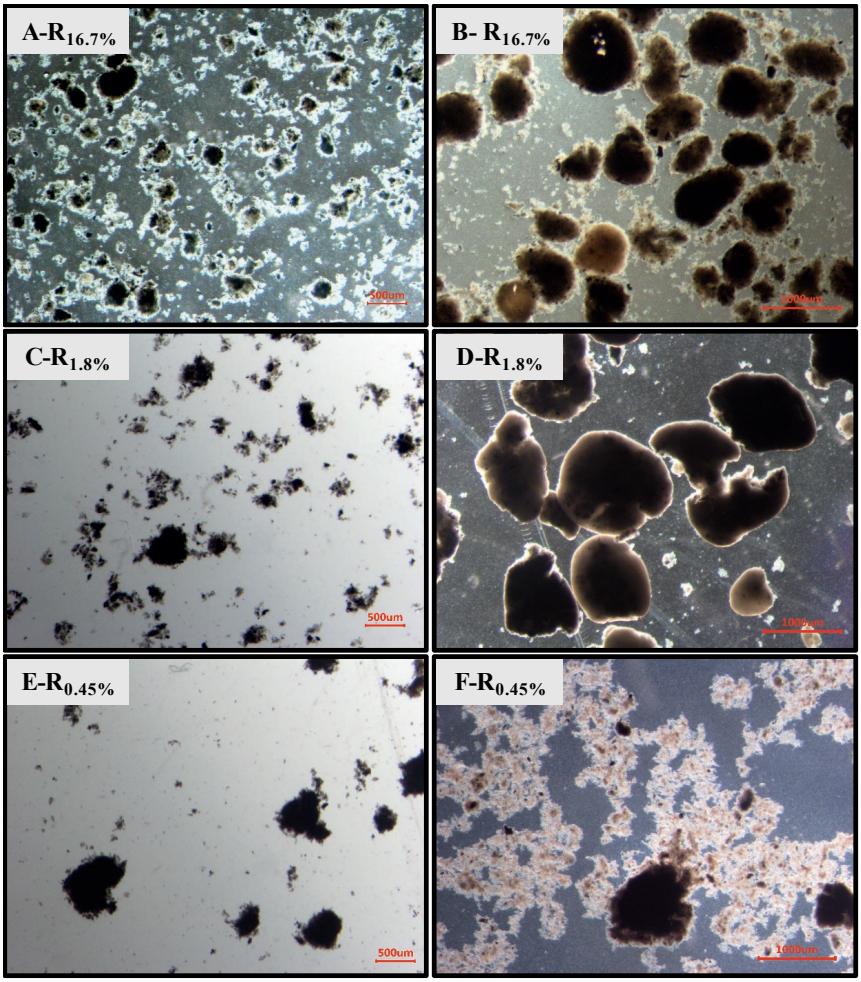


Figure 4. Macroscopy images of sludge developing in the UASB reactors. A, C, E – sludge morphology after 45 days of continuous reactor operation. B, D, F – sludge morphology at the end of experiments. The size bars are 500 µm in A, C, E and 1000 µm in B, D, F.

Cumulative effluent VSS results (**Figure 5**) show a clear difference in solids retention between reactors $R_{16.7\%}$, $R_{1.8\%}$ and $R_{0.46\%}$ after the final increase of the organic loading rate. In $R_{16.7\%}$ the solids washout rate from day 40 until the end of experiment remained almost unaffected at 0.076 ± 0.002 gVSS/d (**Figure 5-A**), while in $R_{1.8\%}$ the washout rate initially increased from 0.069 ± 0.003 (days

33-53) to 0.177 ± 0.0001 (days 53-86) gVSS/d, but subsequently decreased to 0.082 ± 0.005 (day 86-end of experiment) gVSS/d (**Figure 5-B**). In $R_{0.46\%}$ the VSS washout rate sharply increased from 0.052 ± 0.001 (days 42 - 67) to 0.47 ± 0.01 (day 67 onwards) gVSS/d and this negative trend did not reverse until the end of experiment (**Figure 5-A**). The corresponding solids retention times (SRT) at the end of experiment were 238.7 ± 46.7 days, 225.8 ± 46.6 days and only 3.2 ± 0.2 days in $R_{16.7\%}$, $R_{1.8\%}$ and $R_{0.46\%}$ respectively. Thus, whereas with 16.7 and 1.8% tryptone granulation and excellent solid retention was obtained, 0.46% tryptone was too low for this. Visual observations of sludge beds in **Figure 6** support these results. In reactors $R_{16.7\%}$ (**Figure 6-A, B**) and $R_{1.8\%}$ (**Figure 6-C, D**) a clear development and accumulation of well settling granular sludge was observed after the final increase in VLR. Contrary, biomass washout and decreasing height of sludge bed was observed in $R_{0.46\%}$ (**Figure 6-E, F**).

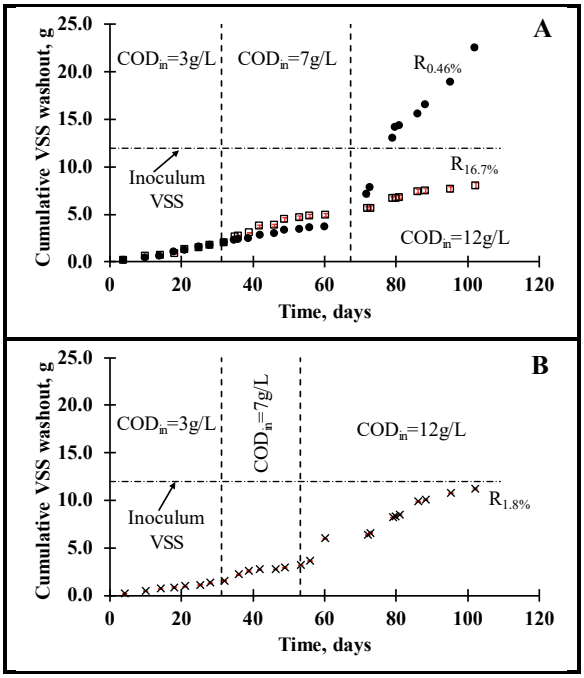


Figure 5. Cumulative VSS washout from the UASB reactors. A – reactors $R_{0.46\%}$ and $R_{16.7\%}$. B – reactor $R_{1.8\%}$. The error bars (in red) show absolute deviation of duplicate effluent VSS measurements from the average.

Good (granular) sludge settleability is essential for compact bioreactors, such as UASB or expanded granular sludge blanket (EGSB) reactors that are operated at upflow velocities between 1.2 to 1.5 m/h and 7-10 m/h (Souza, 1986; Jeison and Chamy, 1999), respectively. The 15 minute sludge volume indexes (SVI_{15}) at the end of experiments were 38.8 ± 1.1 and 31.8 ± 1.5 ml/g for granules of reactors $R_{16.7\%}$ and $R_{1.8\%}$, respectively. This is well within the range reported for granular sludges formed under non-saline conditions (9.6 to 39.3 mL/g) after a longer settling time of 30 min (SVI_{30})

(Ghangrekar et al., 2005; Molina et al., 2008; Sanjeevi et al., 2013). The sludge washout in $R_{0.46\%}$ (**Figure 6**) did not allow for SVI measurements of this sludge.

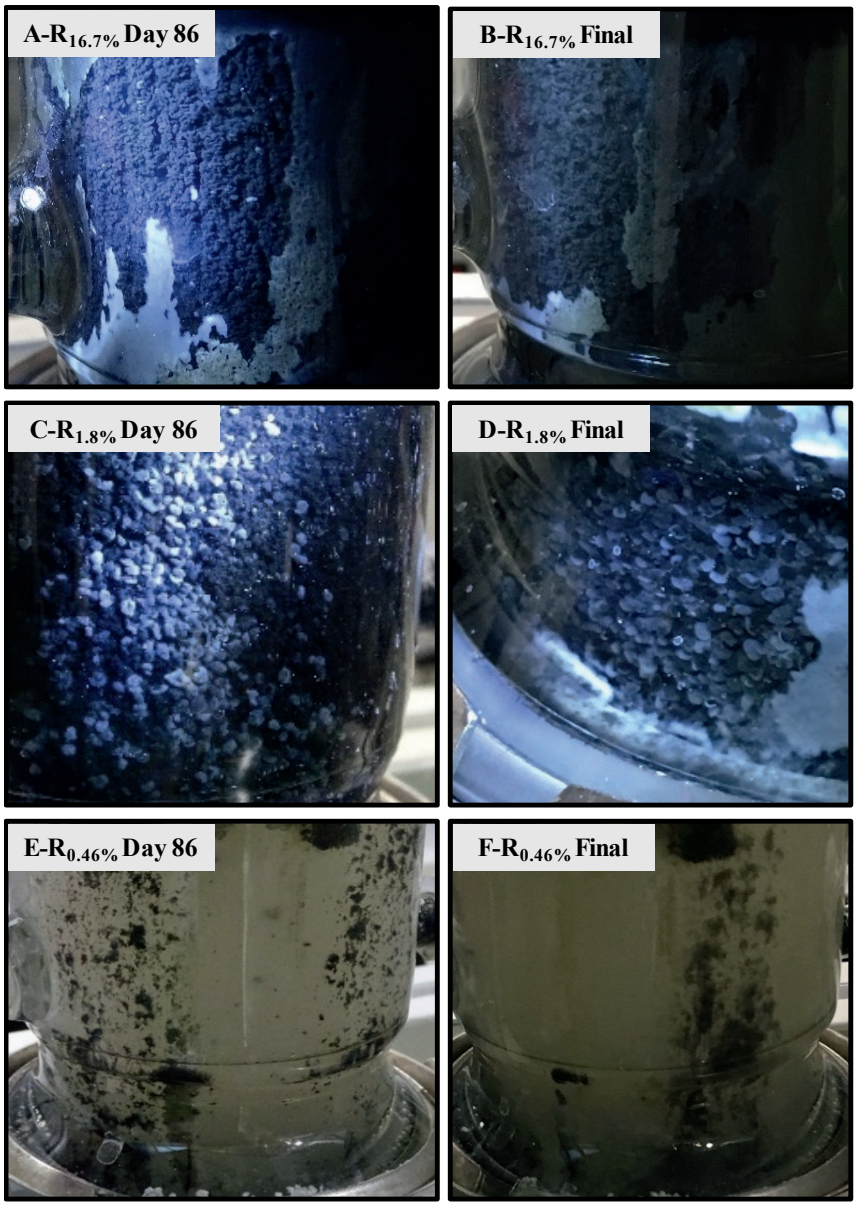


Figure 6. Appearance of the sludge bed in time in the UASB reactors. Images show biomass accumulation In $R_{16.7\%}$ (A, B) and $R_{1.8\%}$ (C, D) and washout in $R_{0.46\%}$ (E, F). All images taken at ~7.5 cm from the bottom of the reactors (1st sampling port in Figure 1).

3.3. The protein requirements to obtain good granulation can be estimated

The results of this study are in agreement with the results of **Chapter 4**. There it was shown that decreasing the influent tryptone COD fraction from 16.7 % to 0.8 % in a UASB reactor operated at 8 g COD/L·d with a stable granular performance, resulted in granular sludge disintegration. This investigation shows that excellent granulation can be achieved at an influent tryptone COD fraction of 1.8 % (**Figure 4-D**), but not at an influent tryptone COD fraction of 0.45 % (**Figure 4-F**) Interestingly, the results in **Chapter 4** showed that after prolonged reactor operation (approximately 180 days) granule-alike sludge formed in a reactor without protein in the feed, but with soluble starch instead (16.7% of influent COD apart from D-glucose and sodium acetate). The protein fraction of the EPS and the amino residue composition of the EPS of these granules were similar to the granules formed with protein in the feed. In fact it has been recently shown that in granular sludge, the proteins in the EPS matrix may play an important role for granule's integrity (Xiong and Liu, 2013). Continuous dosing of proteins even at influent COD fraction as low as 1.8% may be unfeasible in practice due to high costs. In future it should be investigated, if dosing of proteins can be applied as a strategy to initiate fast granulation, after which the protein dosing would be stopped. Such an approach could be a much cheaper alternative, as long as good granular structure can be sustained.

The question to be answered is why the dose of tryptone calculated for osmoprotection also yields robust granules, whereas a 75% lower concentration does not. It is very likely that tryptone not only provides osmolytes but also energetically favourable "building blocks" for protein-rich EPS biosynthesis, as was already hypothesized in **Chapter 4**. The yields of EPS proteins and polysaccharides extracted from granular sludges of reactors $R_{16.7\%}$ and $R_{1.8\%}$ are summarised in **Table 3**. Approximately 0.3 grams of protein were extracted per gram of volatile solids contained in the granular sludge of $R_{1.8\%}$ and $R_{16.7\%}$. In **Appendix 1** the calculated dose of tryptone per gram of biomass VS was 0.49 grams out of which only 25% (glutamate and aspartate) were assumed to act as osmolytes. This means that after hydrolysis approximately 0.37 grams of amino acids (contained in tryptone) per gram of biomass VS would remain available for uptake into microbial cells and synthesis of either cell material or EPS. In fact, both aspartic and glutamic acid were shown to be abundant in EPS of granular sludge grown with tryptone and starch at 20 g Na^+/L (**Chapter 4**). The calculations presented here show, that theoretically EPS biosynthesis in granular sludges of $R_{1.8\%}$ and $R_{16.7\%}$ can occur from amino acids after hydrolysis of tryptone. Further research should be performed to clarify if such incorporation of amino acids into EPS is indeed possible by performing for example ^{13}C carbon labelled experiments.

Table 3. The yield of EPS protein and polysaccharide extracted from granular sludge of $R_{16.7\%}$ and $R_{1.8\%}$ at the end of experiment.

Reactor	Protein yield, $g_{protein}/g_{sludge\text{-}VS}$	Polysaccharide yield, $g_{sugar}/g_{sludge\text{-}VS}$
$R_{16.7\%}$	0.33 ± 0.03	0.043 ± 0.003
$R_{1.8\%}$	0.34 ± 0.01	0.040 ± 0.001

4. Conclusions

Previous investigations had shown that a relatively high (16.7%) proteinaceous fraction of influent COD can enhance anaerobic sludge granulation at high salinity. The findings of this contribution could narrow this down to a protein fraction of at least 1.8%. This is of significance for practical purposes, when identifying saline (waste)water streams suitable for anaerobic granular treatment and/or to estimate required protein additions to the wastewater.

Supplementary Information of Chapter 5

Appendix 1. Calculation of osmolyte dose for continuous UASB reactors

In **Chapter 3** it was demonstrated, that two abundant amino acids of tryptone, i.e., glutamate and aspartate, can alleviate osmotic stress of methanogens in granular sludges. The influent COD proportion of tryptone in the second reactor was 1.8% ($R_{1.8\%}$) and was based on an a simple calculation such that enough glutamate and aspartate would be provided for osmoprotection of microorganisms. In the calculation sodium and chloride are the dominant ions in the influent of all reactors. The osmotic pressure π originating from Na^+ and Cl^- is:

$$\pi = i \times [Na^+] \times R \times T + i \times [Cl^-] \times R \times T \cong 44\ atm$$

Where π is the osmotic pressure in atm, i is the Van't Hoff factor (1 for monovalent ions), R is the universal gas constant (0.0821 atm·L/(mol·K)), T is the absolute temperature (308 K), [Na^+] and [Cl^-] are the concentrations of sodium and chloride in the medium (mol/L).

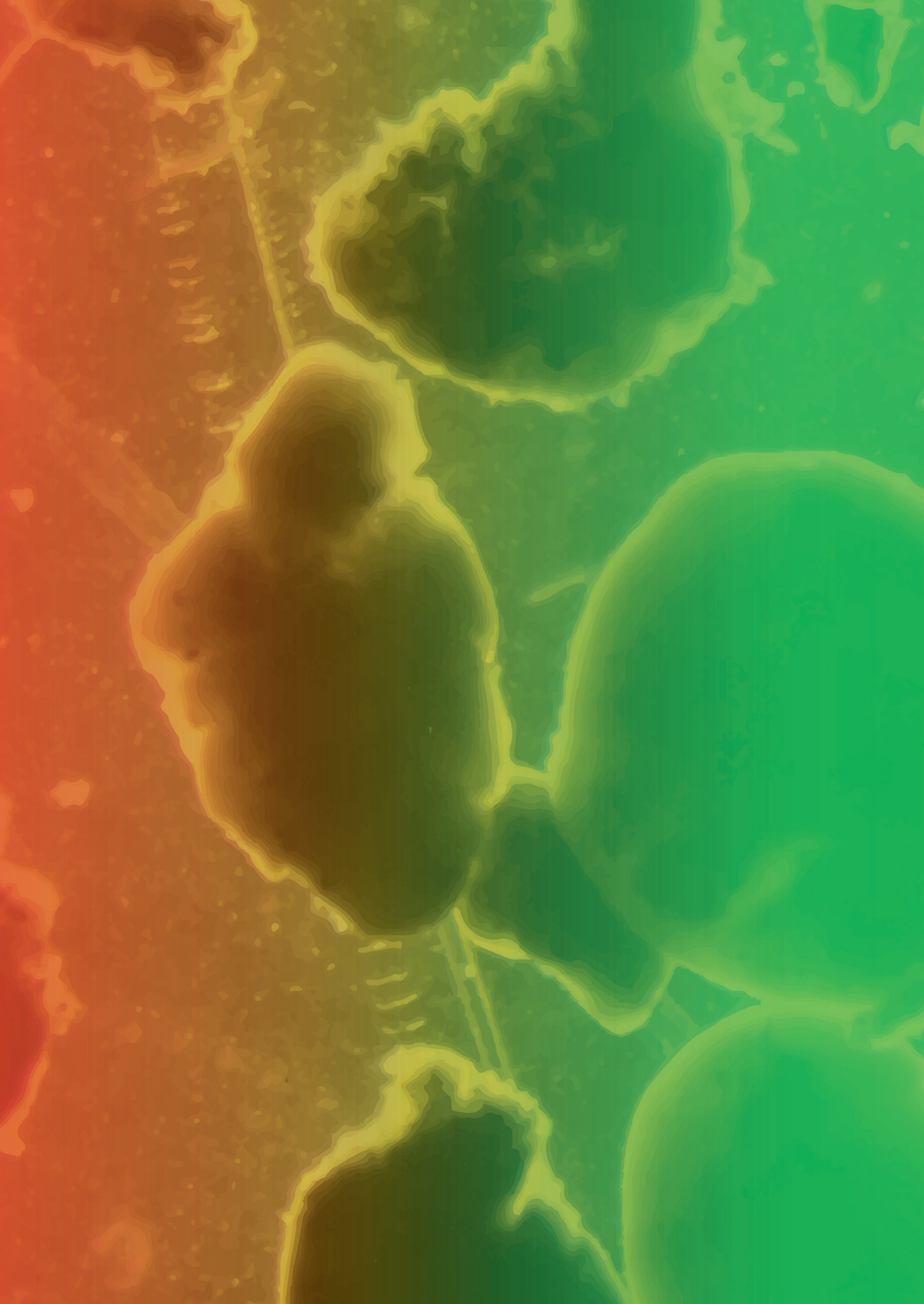
The Van't Hoff factor of glutamate and aspartate is 2, because these molecules carry 2 charges at physiological pH. To balance the osmotic pressure between the bulk liquid and the cytoplasm of microorganisms by these 2 molecules, their concentration in the microbial cells should be:

$$M_{osmo} = \frac{\pi}{i \times R \times T} \cong 0.87 \left(\frac{mol_{glu+asp}}{L_{cell-volume}}\right)$$

In tryptone, glutamate and aspartate content is 18.7% (w/w) and 6.52% (w/w), respectively. This corresponds to 1.27 mmol glutamate and 0.49 mmol aspartate per gram of tryptone (1.76 $mmol_{glu+asp}/g_{trypt}$). This means that 0.49 g of tryptone per mL of cell volume in the reactor would be necessary.

To estimate concentration of tryptone for a continuous UASB reactor, one mL of cell volume was approximated to be equivalent with one gram of biomass volatile suspended solids ($VSS_{biomass}$) and the tryptone dose was calculated based on theoretical biomass yield at a given loading rate. Following parameters were chosen to estimate this: i) Biomass yield, 0.05 g VSS-COD/g $COD_{converted}$ (Tchobanoglus et al., 2003); ii) COD biomass, 1.42 g COD/gVSS (Tchobanoglus et al., 2003); iii) Removal efficiency in the reactor, 90%.

The resulting influent tryptone COD fraction was 1.8%.



Chapter 6. Microbial Community Drivers in Anaerobic Granulation at High Salinity

A modified version of this chapter has been accepted for publication in *Frontiers in Microbiology*
https://doi.org/ 10.3389/fmicb.2020.00235
Authors:

M.C. Gagliano[a,b,1], D. Sudmalis[c,1], R. Pei[b], H. Temmink[b,c] and C.M. Plugge[a,b]
[a] Laboratory of Microbiology, Wageningen University and Research, Stippeneng 4, 6708 WE, Wageningen, the Netherlands
[b] Wetsus – European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911MA Leeuwarden, The Netherlands
[c] Department of Environmental Technology, Wageningen University and Research, Bornse Weilanden 9, 6708 WG, Wageningen, the Netherlands
[1] both authors contributed equally

Abstract. In the recent years anaerobic sludge granulation at elevated salinities in upflow anaerobic sludge blanket (UASB) reactors has been investigated in few engineering based studies, never addressing the microbial community structural role in driving aggregation and keeping granules stability. In this study, a combination of different techniques was applied in order to follow the microbial community members and their structural dynamics in granules formed at low (5 g/L Na^+) and high (20 g/L Na^+) salinity conditions. Experiments were carried out in four UASB reactors fed with synthetic wastewater, using two experimental set-ups. By applying 16S rRNA gene analysis, the comparison of granules grown at low and high salinity showed that acetotrophic *Methanosaeta harundinacea* was the dominant methanogen at both salinities, while the dominant bacteria changed. At 5 g/L Na^+, cocci chains of *Streptoccoccus* were developing, while at 20 g/L Na^+ members of the family *Defluvitaleaceae* formed long filaments. By means of Fluorescence in Situ Hybridization (FISH) and Scanning Electron Microscopy (SEM), it was shown that aggregation of *Methanosaeta* in compact clusters and the formation of filaments of *Streptoccoccus* and *Defluvitaleaceae* during the digestion time were the main drivers for the granulation at low and high salinity. Interestingly, when a complex proteinaceous substrate (tryptone) in the synthetic wastewater was substituted with single amino acids (proline, leucine and glutamic acid), granules at high salinity (20 g/L Na^+) were not formed. This corresponded to a decrease of *Methanosaeta* relative abundance and a lack of compact clustering, together with disappearance of *Defluvitaleaceae* and consequent absence of bacterial filaments within the analysed dispersed biomass. In these conditions, a biofilm was growing on the glass wall of the reactor instead, highlighting that a complex proteinaceous substrate such as tryptone can contribute to granules formation at elevated salinity.

1. Introduction

Granular sludge bed based technologies such as the upflow anaerobic sludge blanket (UASB) reactor (Lettinga et al., 1980) are considered the most cost-effective at industrial scale for high-rate anaerobic treatment of concentrated soluble organic wastewater (Van Lier et al., 2015). The characteristic phenomenon in this process is sludge granulation, i.e., granular-shaped sludge is spontaneously formed within the reactor (Narihiro et al., 2008). Granular sludge is a spherical biofilm, rich in extracellular polymeric substances (EPS), possessing all the microbial trophic groups to complete the anaerobic degradation of organic matter (Sekiguchi et al., 1998; Seviour et al., 2009).

Many industrial processes produce substantial amounts of saline wastewater (Giustinianovich et al., 2018), for which high-rate treatment via anaerobic biofilm-based technologies (such as UASB reactors) are the most interesting, because of a successful biomass immobilization and retention (Xiao and Roberts, 2010; Kobayashi et al., 2018). However, high salinity is regarded as a stress factor that

severely hampers the performance of biological systems, negatively affecting biomass retention (Hemmelmann et al., 2013; Fang et al., 2018). In sludge granulation, granule strength and stability is considerably reduced in presence of high concentrations of Na^+, leading to displacement of the bridging Ca^{2+} ions within the EPS matrix (Lambert et al., 1975; Bruus et al., 1992; Ismail et al., 2010). As a consequence, non-adapted granules disintegrate, causing biomass wash-out and deteriorating process performance (Rinzema et al., 1988; Vallero et al., 2003; Ismail et al., 2008). Still, utilization of salinity adapted biomass was successfully applied to overcome this issue and obtain aggregates/granules (Hierholtzer and Akunna, 2014; Gagliano et al., 2017b).

The aggregation of microorganisms is a key event for granule development (Ding et al., 2015), positively influenced by the cell surface hydrophobicity, which enhances cell-to-cell adhesion and thereby the formation of aggregates (Wilén et al., 2018). Some studies suggested that proteins as substrate can increase hydrophobicity, favouring granule formation and improving their stability (McSwain et al., 2005; Zhu et al., 2015a; Li et al., 2016). Also, often anaerobic granules stability is strongly related to the presence of structured microbial clusters, mostly dominated by methanogens (El-Mamouni et al., 1997; Sekiguchi et al., 1999; Gonzalez-Gil et al., 2001; Satoh et al., 2007). This is particularly true for the filamentous methanogenic archaeon *Methanosaeta,* which during the initial development of granules firstly attaches on precursors, and subsequently forms a 3D network in which other microorganisms are entrapped (Wiegant, 1988; Liu et al., 2003; Angenent et al., 2004). Granulation increases rapidly when *Methanosaeta* is present in UASB reactors, ensuring the preservation of the granular structure and an optimal methane production (Uemura and Harada, 1993; El-Mamouni et al., 1995; El-Mamouni et al., 1997; van Haandel et al., 2014). *Methanosaeta harundinacea* cells were shown to significantly enhance granulation and performance of UASB reactors (Li et al., 2015b). Our group demonstrated that *M. harundinacea* is a key-microorganism to obtain compact granules in UASB reactors at low and high salinity conditions, when using a salt-adapted, *Methanosaeta*-rich inoculum (Gagliano et al., 2017b; Gagliano et al., 2018).

Previous research investigating UASB reactors operated at high salinity (Rinzema et al., 1988; Vallero et al., 2003; Ismail et al., 2008; Ismail et al., 2010; Aslan and Şekerdağ, 2016; Gagliano et al., 2017b; Sudmalis et al., 2018a) did not carefully address the microbial ecology and its importance in driving aggregation and granules formation.

In the present study, two experimental set-ups were used to monitor granulation in UASB reactors working at low (5 g/L Na^+) and high (20 g/L Na^+) salinity. Furthermore, a comparison with respect to aggregation performance with or without proteinaceous substrate was made. By combining comparative analyses of bacterial and archaeal 16S rRNA genes (by means of next generation

sequencing (NGS) and clone libraries), together with microscopy techniques (scanning electron microscopy (SEM) and fluorescence microscopy), the objective of this work was to obtain a comprehensive overview and understanding of *Methanosaeta* and possible other microbial key-players favouring granulation under saline conditions.

2. Materials and methods

2.1. Reactor operation and performance

2.1.1. Inoculum

The start-up biomass originated from a full-scale UASB reactor from the Shell plant in Moerdijk (the Netherlands). The sludge was adapted to a sodium concentration of ≈8 g Na^+/L for more than 10 years, with acetic and benzoic acids as the main substrate (Jeison et al., 2008). Before the reactors inoculation, the biomass was dispersed by forcing it through a 125 µm sieve.

2.1.2. Granular sludge reactor operation

In total, four double-walled glass UASB bioreactors were inoculated with 6 g VSS/L of anaerobic inoculum. Reactors R1 (R1 in **Chapter 2**) and R2 (R3 in **Chapter 2**), with 0.7 L active volume and 1 L total volume, were operated for 217 days. Reactors R3 (R4 **Chapter 2**) and R4 (R_{leuc1} **Chapter 4**), with 1.98 L active volume and 3.13 L total volume, were operated for 120 days. The reactors were running at 35 ± 1 ˚C and fed with a synthetic wastewater consisting of macro and micro-nutrients (**Chapters 2 and 4**). In all reactors, the upflow velocity at final loading rate was 1 m/h, while the pH remained close to neutral throughout reactor operation. A summary of operational conditions and performances is shown in **Table 1**.

Salinity effect experiment

To evaluate the salinity effect on granules formation and process performances, reactor R1 was operated at 5 g Na^+/L, while reactor R2 was operated at 20 g Na^+/L. In both reactors, the substrate was composed of soluble COD (sCOD) D-glucose, acetate, and tryptone in a 3:2:1 COD ratio. The influent COD concentration was increased in steps to reach a final organic loading rate (OLR) of approximately 16 g COD/L·d.

Substrate effect experiment

To evaluate the influence of proteinaceous substrate (tryptone) on granulation, reactor R3 was fed with the same substrate as described in the previous experiment, while in reactor R4 tryptone was substituted by the amino acids proline and leucine from day 0 to day 70, and by proline and glutamic

acid from day 70 onwards. The influent COD concentration was increased in steps to reach a final OLR of approximately 8.8 g COD/L·d.

2.1.3. Analytical procedures

Daily biogas production was monitored with µFlow gas flow meter (Bioprocess Control, Sweden). Biogas composition was measured periodically as described in Steinbusch et al. (2008). Volatile fatty acids (VFAs) were quantified in the soluble fraction of effluent samples as described in **Chapter 2**. COD measurements were performed with LCK314, LCK514 and LCK1414 kits (HACH GMBH, Germany) on total (tCOD), and soluble fractions (sCOD) as described in **Chapter 2** and **Chapter 4**.

Table 1. The main operational parameters and performance of the four UASB reactors

Experiment	Salinity effects		Substrate effects	
Reactor	R1	R2	R3	R4
Operating Temperature, ºC	35 ± 1	35 ± 1	35 ± 1	35 ± 1
Start-up Inoculum concentration, g VSS/L	6	6	6	6
Salinity, g Na^+/L	5	20	20	20
COD ratio D-glucose: acetate	3:2	3:2	3:2	3:2
Third organic substrate to yield the final COD ratio of 3:2:1	Tryptone	Tryptone	Tryptone	Leucine 0.5* Proline 0.5
Initial organic loading rate, g COD/L·d	1	1	1	1
Day of Increase of influent COD from 3 to 7 g/L**	31	31	26	26
Day of Increase of influent COD from 7 to 12 g/L**	52	52	60	60
sCOD removal at the final loading, %	98.4 ± 0.4	95.3 ± 1.5	94.2 ± 1.2	89.5 ± 3.0
Average Biogas Methane content	63.6 ± 4.6%	68.4 ± 4.3%	70 ± 2.9%	67.4 ± 4.4%
Total Experiment Time, days	217	217	120	120

*On day 71 leucine was replaced with glutamic acid
**Increase of influent COD corresponds to increasing loading rates

2.2. Microbial community analysis

2.2.1. Sample collection and DNA extraction

Fresh granules were sampled at different time points from all reactors and stored at – 20 °C until DNA extraction. Genomic DNA was extracted from ≈ 500 mg of granules sampled from all reactors using a FastDNA® SPIN kit for soil (MPBio, USA) according to the manufacturer's instructions. Prior the DNA extraction, granules were washed with PBS 1X several times and pre-treated by sonication (40 kHz, 50 W, 30 s). DNA concentration and purity were measured with the NanoDrop® spectrophotometer (Thermo Fisher Scientific, Germany).

2.2.2. Amplification, cloning and sequencing of 16S rRNA genes

16S rRNA genes were amplified using primers 27F (AGAGTTTGATCMTGGCTCAG) and 1369R (GCCCGGGAACGTATTCACCG) for Bacteria and primers 25F (CYGGTYGATYCTGCCRG) and 1386R (GCGGTGTGTGCAAGGAGC) for Archaea, using the GoTaq DNA polymerase kit (Promega, USA). PCR amplification of 16S rRNA genes of Archaea was carried out as described in Borrel et al. (2012). PCR reaction conditions for bacterial 16S rRNA genes were pre-denaturation at 94 °C for 10 min, 30 cycles of denaturation at 94 °C for 30 sec, annealing at 52 °C for 40 sec, elongation at 72 °C for 3 min and post-elongation at 72 °C for 10 min. Cloning of purified PCR products was performed using pGEM-T Easy Vector System into *E.coli* JM109 competent cells (Promega, USA) according to the manufacturer's instructions. A total of 96 clones from each reactor were selected for 16S rRNA amplicon sequencing and data analysis, as described in Gagliano et al. (2017b). The sequences were reconstructed and deposited in GenBank, and accession numbers are listed in **Table S1**, **S2, S3 and S4**. The affiliation was determined by the SILVA version 132 16S reference database (Quast et al., 2012) and the closest relatives were checked through the NCBI Blast alignment tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.3. Next generation sequencing (Illumina sequencing and computational analysis)

Universal primers based on the V3-V4 hypervariable region of the prokaryotic 16S rRNA genes were applied for simultaneous amplification of Bacteria and Archaea in the reactor samples. Details on primers and the protocol applied are reported in (Takahashi et al., 2014). Sequencing of PCR products was conducted on an Illumina MiSeq sequencing system, and the reads generated were processed (i.e., filtered, clustered, taxonomically assigned and aligned) using the QIIME pipeline version 1.9.1 (Caporaso et al., 2010). Further details are reported in Supplementary Information. For the data presented in this study, we considered the most significant OTUs per each sample out of the total number of sequences, setting a cut-off value of 1%. The sequences reported have been deposited in the European Nucleotide Archive (ENA) database (accession nos. ERR3237939, ERR3237938, ERR3237937, ERR3237936, ERR3237935, ERR3237934, ERR3237933, ERR3237932 and ERR3237931).

2.2.4. Fluorescent in situ hybridization (FISH) and microscopy analysis

At the end of the reactor runs, granules from each of the UASB reactors were fixed with 37% formaldehyde (w/w) according to (Amann et al., 1995). After fixation, granules were washed with PBS to remove the excess salinity, and then stored at -20 ˚C in ethanol/ PBS (1:1). Granules were gently crushed by flushing in 1 ml syringe with a 0.7 mm diameter needle, before FISH analysis

(Hugenholtz et al., 2002). To visualize the organization and clustering of microorganisms in high salinity granules (R2 reactor granules, 20 g/L Na^+), FISH analysis was carried out on 10 µm slices obtained after cryosectioning, as described in (Gagliano et al., 2018). All oligonucleotide probes applied, labelled with Cy3-red or Alexa488-green fluorophores, are listed in **Table S5**. Oligonucleotide probes were selected on the basis of the collected 16S rRNA sequences and the probeBASE database (Greuter et al., 2015).

Samples were examined using an epifluorescence microscope BX41 (Olympus, Japan) equipped with Infinity Camera (Lumenera corporation, Canada). For the image analysis, the FIJI software package (version 1.51g, Wayne Rasband, NIH, Bethesda, MD, USA) was used (Schindelin et al., 2012).

2.3. Scanning electron microscopy (SEM)

Freshly sampled granules were fixed, dehydrated and sputter coated with tungsten before analysis, as described in Ismail et al. (2010). For samples visualization, a Magellan 400 SEM (FEI Company, OR, USA) was used, at an acceleration voltage of 2 kV and a beam current of 6 pA at room temperature.

3. Results

3.1. Salinity effect experiment

3.1.1. Reactors operation and granules formation

Biogas production and a high COD removal of >95% were achieved in both reactors R1 and R2 throughout the 217 days operation, regardless of the salinity level (R1 (5 g Na^+/L), R3 (20 g Na^+/L) in **Chapter 2**). The biogas was continuously produced in both R1 and R2 (**Chapter 2**), with a net increase during the third phase of the process (with an OLR of 12 gCOD/L·d). A detailed description of the performance of these two reactors is described in (Sudmalis et al., 2018a). In both reactors R1 and R2, the black particulate inoculum gradually aggregated into granules with a whitish gel-like external EPS layer (**Figure 1-A, B, C**), highlighted with crystal violet and FITC staining in **Figure 1-D, E**.

In reactor R2 (20 g/L Na^+), the appearance of this whitish gel-layer was observed immediately after increasing the influent COD concentration from 7 to 12 g COD/L (≈ day 80), while in reactor R1 (5 g/L Na^+) it started to appear only after 135 days of operation. At the end of the process, visual and microscopic analysis evidenced that R2 granules were bigger than those developed in R1, and had a thicker EPS gel layer (**Figure 1-B, C**). Thus, higher Na^+ concentration may have been beneficial for biomass aggregation, and the growth of this EPS layer can be seen as an adaptive response of the microbial community to the increased salinity.

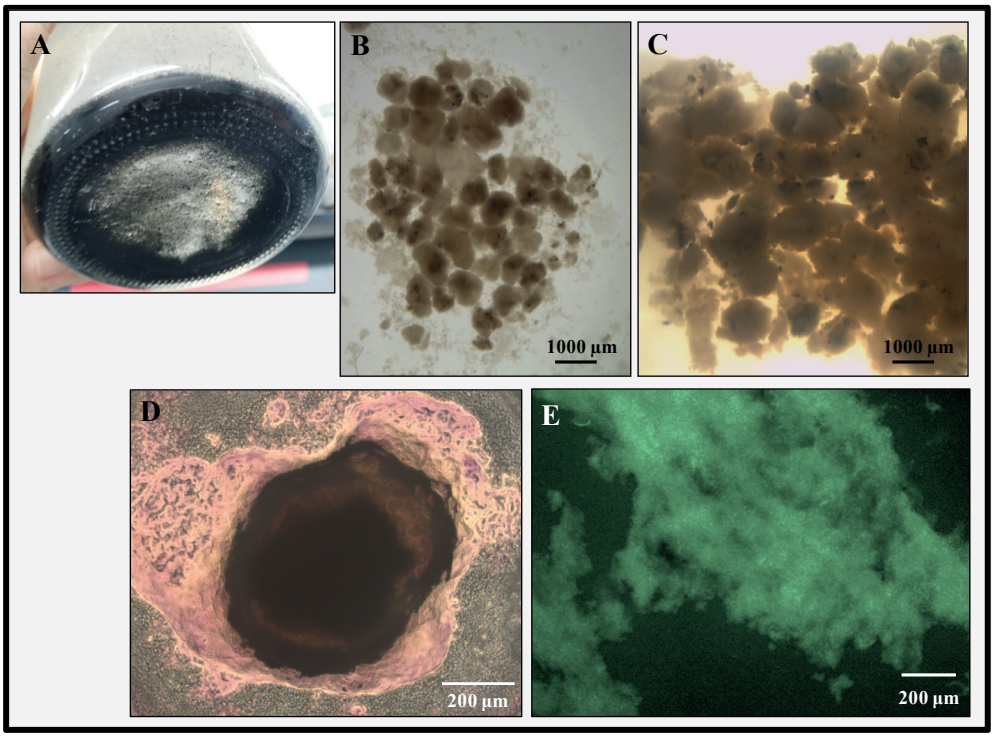


Figure 1. The initial inoculum taken from the Shell plant of Moerdijk (The Netherlands) (A), and granules grown during the anaerobic process in reactor R1 (5 g/L of Na^+, in B) and reactor R2 (20 g/L of Na^+, in C). In D and E, crystal violet (total EPS) and FITC (EPS proteins) staining of a high salinity granule from R2, respectively. Crystal violet and FITC staining procedures are described in Supplementary information section.

3.1.2. Microbial community composition using NGS and clonal analysis

Microbial community analysis by NGS was carried out on the initial inoculum sample and on granules taken from both reactors R1 and R2 on day 74 i.e., after the last increase of the OLR, and at the end of the operation period (day 217), and the results are reported in **Figure 2**. The microbial community composition of reactor R1 and R2 at the end of the process changed significantly compared to the inoculum (**Figure 2-A**).

All of the most important groups in the inoculum were falling into the phylum *Firmicutes* (*Caldicoprobacter, uncultured Clostridiales* and *Anoxynatronum*) with the exception of the family *Anaerolinaceae* (7%) (**Figure 2-A**). *Atribacteria* was the second most abundant bacterial phylum in the inoculum (10%), but its presence was notably lower in the reactors (1.8% in R1 and 1.4% in R2 at day 217). In the inoculum, the relative abundance of the acetotrophic *Methanosaeta* (25.6%) and the hydrogenotrophic *Methanobacterium* (22.2%) was similar, while the methanogenesis turned predominantly acetoclastic within R1 and R2, with a clear dominance of *Methanosaeta* (18.7% and 23.6% at the end of the digestion period, respectively) over *Methanobacterium* (0.4% and 2.4% at the end of the digestion period, respectively).

Regarding the bacterial population, in reactor R1 (5 g/L Na^+), *Enterococcus* was the dominant genus at day 74 (27%), followed by members of the family *Rikenellaceae* (genera vadinBC27 at 4.08% and Blvii28 at 3.79%), *Synergistaceae*, *Atribacteria*, *Syntrophomonas, Desulfuromonas* and *Streptococcus* (**Figure 2-A**). By the end of the experimental period, the family *Rikenellaceae* (vadinBC27 at 13.8% and Blvii28 at 4.4%), the genus *Streptococcus* (10.8%) and *Synergistaceae* (6.7%) increased and were the most abundant, with a stable presence of *Syntrophomonas* (3.5%) and the appearance of *Syntrophobacter* (4.5%) and *Oceanotoga* (2.4%) (**Figure 2-A**). All the other main groups were decreasing or disappearing after 217 days of process. Most of the bacterial population in reactor R2 was affiliated to the family *Defluviitaleaceae* (also classified as *Lachnospiraceae* by Greengenes taxonomy and reported as such in **Chapter 2**), which relative abundance was increasing, ranging from 32% at day 74 to 34% at the end of the digestion. An increasing trend was also observed for the other main OTUs, such as *Enterococcus*, *Caldicoprobacter* and vadinBC27 *(Rikenellaceae)* (**Figure 2-A**). Members of *Atribacteria,* Blvii28 (*Rikenellaceae*) and *Draconibacteriaceae* were decreasing in relative abundance (**Figure 2-A**).

To increase the accuracy and the resolution of the taxonomical classification, clonal analysis of 16S rRNA gene was carried out on samples taken at the end of the reactor operation, and the results are shown in **Tables S1** and **S2**. The dominant Archaea in R1 and R2 granules had a high similarity (99% based on 16S rRNA) to *Methanosaeta harundinacea*, with 90% and 97% of the total clones, respectively (**Table S1**). Just a few other identified and unidentified Archaea were detected (**Table S1**). The bacterial 16S rRNA gene sequences obtained from the clonal analyses were closely related to *Rikenellaceae*, *Streptococcus* and *Synergistaceae* in reactor R1 and of *Defluvitaleaceae*, *Enterococus* and *Clostridiales* in reactor R2, confirming the NGS results (**Table S2**).

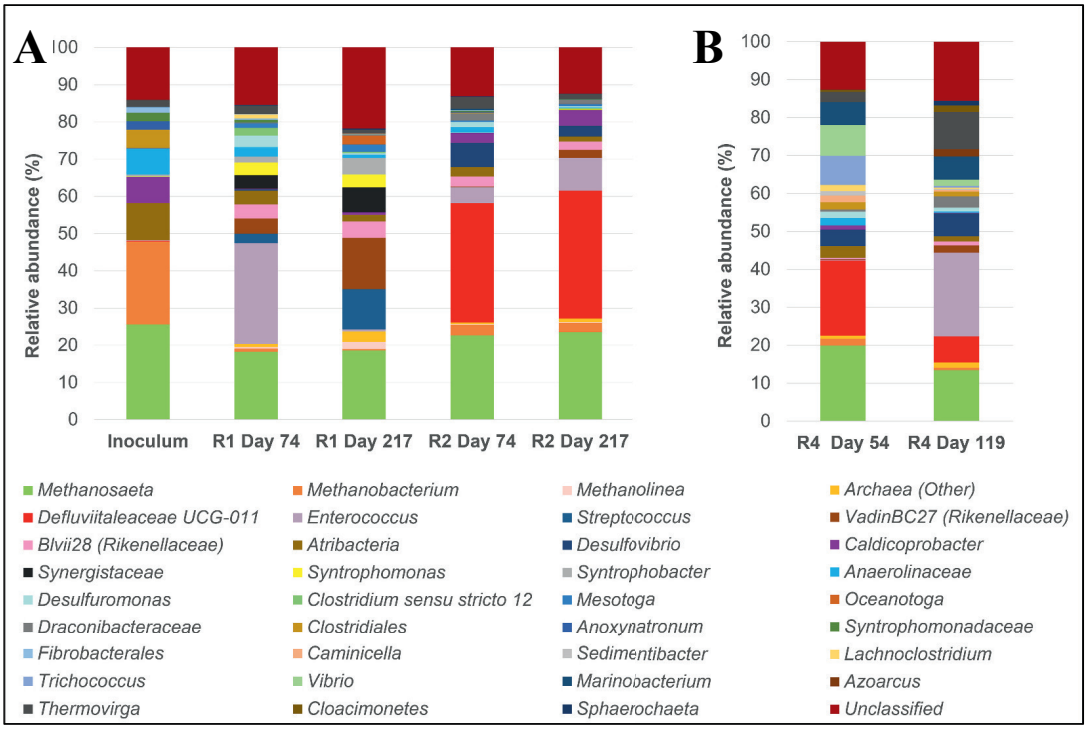


Figure 2. Relative abundance of core OTUs (>1 % relative abundance, 100% occupancy) and their taxonomy classification at the identified level (mostly genus level). All OTUs <1% are summed together and presented as unclassified. (A): the start-up inoculum and samples taken from both reactor R1 and R2 at day 74 and day 217 (end of the digestion period) are represented. (B): samples taken from R4 reactor at day 54 and day 119 (end of the operational period).

3.1.3. Microbial population observation by microscopy

To determine the importance of the microbial community in driving the granulation process at the two different salinities, SEM and FISH analysis were applied. Granules were sampled from both reactors on day 88 (appearance of EPS layers in R2 granules) and on day 217 (end of the reactor run), and observed through SEM. On day 88 (**Figure 3-A, C**), in both reactors microorganisms mainly appeared as single cells, with few pairs and filaments. On day 217, granules were composed by tightly aggregated filaments, covered by a thick EPS layer (**Figure 3-B, D**).

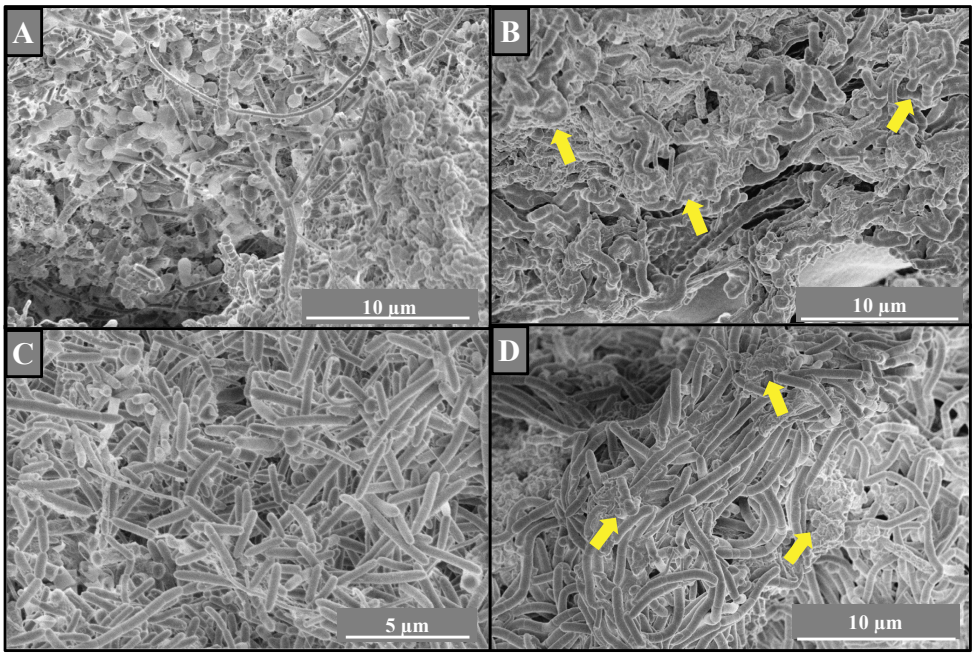


Figure 3. Scanning electron microscope (SEM) images of samples taken from reactor R1 operated at 5 g/L of Na^+ (A and B) and reactor R2 operated at 20 g/L of Na^+ (C and D). The sampling was done on day 88 (A and C) and day 217 (B and D). Yellow arrows indicate some spots more rich in EPS.

The morphology of the dominant filamentous microorganisms in the granules from both reactors was different: in R1, mostly twisted chains of coccus-shaped microorganisms were observed (**Figure 3-B**), while in R2, filaments mainly consisted of aggregated rod shaped cells (**Figure 3-D**). On the basis of the collected 16S rRNA sequences, the coccus- and rod-shaped morphologies were further identified by FISH analysis as *Streptococcus spp.* (**Figure S1 - A**) and members of the family *Defluvitaleaceae* (**Figure S1 - B**), respectively. FISH analysis and SEM are in agreement with NGS results (**Figure 2**), confirming both the increase in time of *Streptococcus* in reactor R1 and the dominance of *Defluvitaleaceae* in the microbial population of reactor R2. The granule formation trend described in *Section 3.1.1*, as well as the cells assembly visualized by SEM, were further investigated by following aggregation in time of the above mentioned bacterial morphologies by FISH. For R1, chains of *Streptococcus* cells were detected from June-July (day 135), while in R2 the rod-shaped *Defluvitaleaceae* cells were starting to cluster in April-May (from day 80 onwards) (**Figure 4**).

On the other hand, the acetoclastic *Methanosaeta spp.* was the most abundant aggregated microorganism in both granules from the initial stages of the process (**Figure 5-A, C**), detected in both reactors in its round-shaped and "fibrous" clustered forms (an example in **Figure 5-A, B**). When growing at 20 g/L Na^+, the aggregation of these clusters was tighter than at 5 g/L Na^+ (**Figure 5**). The dominance of *Methanosaeta* cells in the total archaeal population was confirmed via FISH with ARC915, MSMX860 and MX825mix probes (See supplementary information section). FISH on

cryosections (**Figure 5-E, F**) showed the uniform distribution of *Methanosaeta* within the R2 granules and its structural role in establishing connections with bacterial clusters. The latter was clear also looking at the F_{420} autofluorescence emission of granules (**Figure S2**), where the signal was uniformly distributed throughout the granule's structure, with some dense spots of clustered cells.

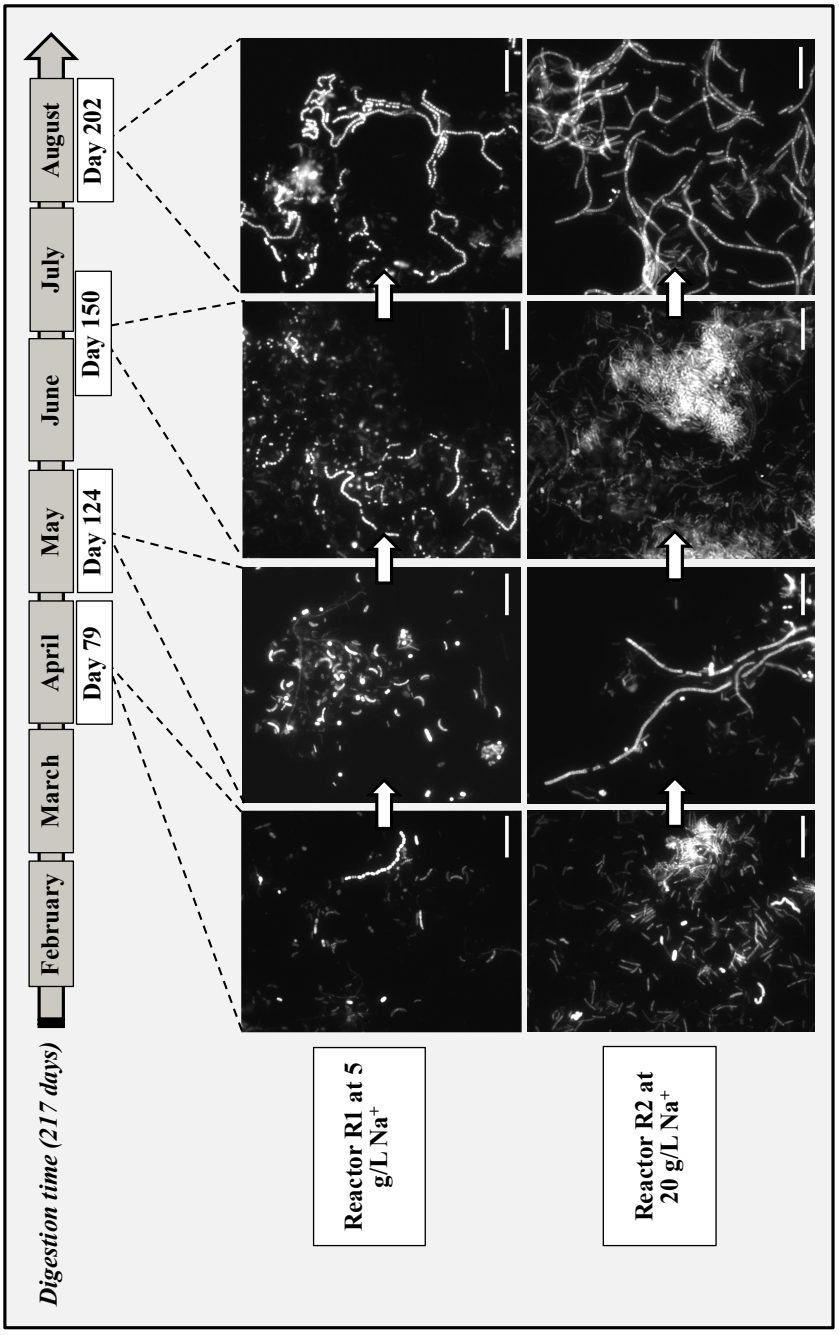


Figure 4. Evolution of bacterial aggregation in time during the digestion process surveyed by FISH, with the development of dominant populations of *Streptococcus* (5 g/L Na^+) and *Defluviitaleaceae* (20 g/L of Na^+). At both salinities, the filamentous bacteria emitted high fluorescence signal compared to the other community members. Size bar is 10 µm

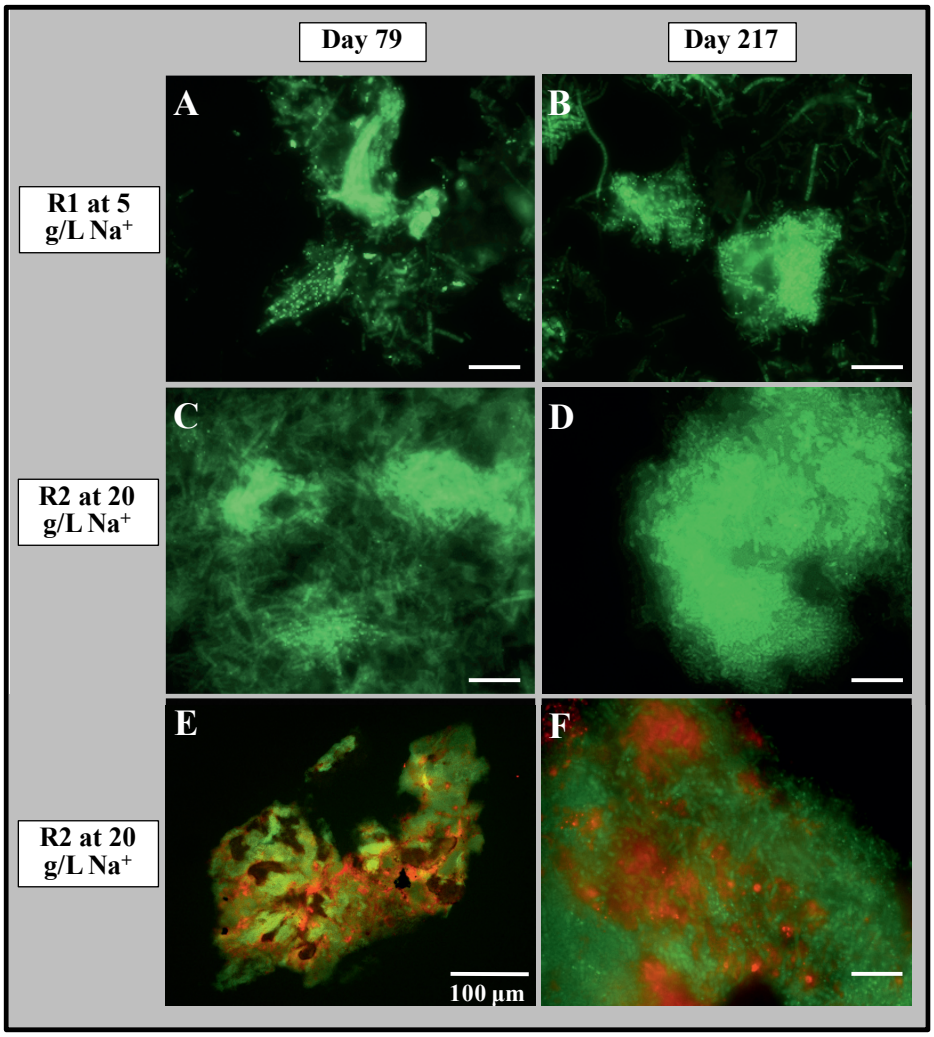


Figure 5. From A to D, FISH images of samples taken on day 79 and day 217 from reactor R1 operated at 5 g/L of Na^+ (A and B) and reactor R2 operated at 20 g/L of Na^+ (C and D). The FISH images show *Methanosaeta*-like clusters positive to the ARC915 probe (Archaea). In E and F, FISH on cryosections from R2 reactor granules sampled at day 217, with Archaea in green and Bacteria (EUB338) in red. Size bar is 10 µm or indicated otherwise.

3.2. Substrate effect experiment

3.2.1. Reactor operation and biomass granulation

In the second experiment reactors R3 (R4 in **Chapter 2**) and R4 (R_{leuc1} in **Chapter 4**), both working at 20 g/L Na^+, were fed either with or without tryptone, respectively. Reactors performances were followed in terms of sCOD removal and biogas production (See **Chapter 2** and **Chapter 4**) throughout the reactors run. In the start-up period, when the OLR was around 1.4 g COD/L·day, the sCOD removal efficiencies in R3 and R4 were 81.9 ± 6.4% and 85.4 ± 6.2%, respectively. After day 24, with the gradual OLR increase to 7.5 g COD/L·d, in R3 the sCOD removal efficiency reached

92.5 ± 2.8 %. In R4, where proline and leucine instead of tryptone were used as the carbon source (together with acetate and D-glucose), the sCOD removal was lower than in R3 (85.7 ±1.0%). On day 71, leucine was replaced by glutamic acid, and the sCOD removal efficiency increased to 90.2 ± 2.5%. The increasing OLRs resulted in a higher VFA accumulation in R4 compared to R3 (**Table S6**), most likely due to higher capacity of R3 to convert influent sCOD into methane compared to R4.

Visual observation showed that granules occurred only in reactor R3, fed with tryptone as protein source (**Figure 6**). In reactor R3, the sludge bed started to form from day 23 onwards, and the reactor was fully filled by granules after ≈40 days. Meanwhile, within 130 days of operation of R4, fed with proline and leucine, and from day 71 onwards with proline and glutamic acid (as replacement of tryptone), no granular sludge formed, and a biofilm was growing on the glass wall of the reactor instead (**Figure 6**). This indicates that a complex proteinaceous substrate such as tryptone contributes to granules formation at high salinity conditions (20 g/L Na^+).

3.2.2. Microbial community composition using NGS and clonal analysis

In order to understand the lack of granulation in reactor R4 at the microbial level, microbial community analysis using 16S rRNA amplicon sequencing was carried out on biomass sampled on day 54 and at the end of the operation period (day 119) (**Figure 2-B**). A comparison between R3 and R4 microbial population was made through clonal analysis, and the results are shown in Supplementary Information (**Table S1, S3** and **S4**). Similar to R1 and R2, *Methanosaeta* was the dominant methanogen detected at the end of the process in both, R3 and R4 (**Table S1**). However, considering the NGS data presented in **Figure 2-B**, in reactor R4 the relative abundance of *Methanosaeta* decreased from 20% (Day 54) to 13.5% (end of the process). Hydrogenotrophic methanogens were represented by *Methanobacterium* (1.8%) at day 54, but these had disappeared at day 119 (**Figure 2-B**). Similarly as observed for reactor R2 (20 g/L Na^+), dominant bacteria in the R4 reactor at day 54 were members of *Defluvitaleaceae*, but their relative abundance decreased from 19.8% (Day 54) to 6.9% at the end of the process, with a concomitant appearance of *Enterococcus* (22% relative abundance) (**Figure 2-B**). Other bacterial groups, such as *Trichococcus, Vibrio* and *Atribacteria,* followed the same decreasing trend from day 54 to the end (**Figure 2-B**). On the other hand, the genus *Thermovirga* increased from 2.6% at day 54 to 10% at the end of the process. Changing the substrate from leucine to glutamic acid (at day 74) could have influenced the bacterial population composition in reactor R4.

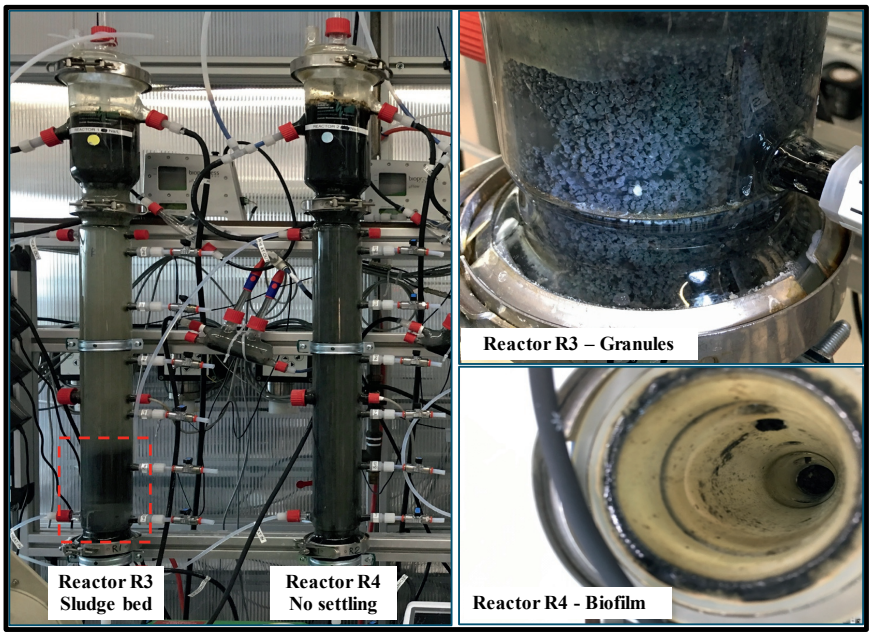


Figure 6. Comparison between reactor R3, with an evident sludge bed (red box), and reactor R4, were the biomass remained suspended and washed out. In reactor R3 granules formed, while in reactor R4 the biomass adhered onto the glass wall as biofilm.

The NGS results were confirmed by clonal analysis, as shown in **Table S4**. In reactor R3, at day 54 *Defluviitaleaceae* (35%) and *Exiguobacterium* (20%) were the dominant bacteria, followed by *Enterococcus* (9.8%), *Synergistaceae*, *Atribacteria* and *Marinobacterium* (**Table S4**). At the end of the process, *Exiguobacterium* drastically reduced to 1% relative abundance, while *Defluviitaleaceae*, *Enterococcus* and *Synergistaceae* were stable (**Table S4**), differently than what was observed for reactor R4 (**Figure 2** and **Table S4**).

3.2.3. Microbial population observation by FISH

In reactor R3 (fed with tryptone), after 40 days of process and the appearance of granules, the aggregated form of *Methanosaeta* was detected (**Figure 7**). This aggregation became more compact from day 66 onwards, with tight clusters of short filaments of different shapes (**Figure 7**), which were observed until the end of the experiment (**Figure 7**, day 119). In reactor R4 (without tryptone), at the beginning of the process non-aggregated, long *Methanosaeta* filaments were detected in otherwise dispersed sludge (**Figure 7**, day 40). This loose aggregation was observed throughout the experimental period, with small aggregates only. Just at the end of the process, a higher amount of aggregated clusters of *Methanosaeta* was present in the dispersed biomass (**Figure 7**, day 119), with "fibrous" shaped clusters as dominant ones. Regarding the bacterial population, as was also observed for reactor R2 (20 g/L Na^+), in reactor R3 members of *Defluvitaleaceae* gradually changed from rod

shaped cells into filaments (**Figure S3**). In reactor R4, without tryptone addition, no members of this family were detected by FISH. On day 119, in reactor R4 no bacterial filaments were present in the dispersed sludge (**Figure 7**), but instead big aggregates and short chains of the dominant *Enterococcus sp.* had appeared (**Figure 7**). In the biofilm growing on the R4 reactor walls, and sampled at the end of the experiment, *Methanosaeta* cells were aggregated more tightly than in the dispersed biomass, but filamentous bacteria were not detected (**Figure 7**, biofilm at day 119).

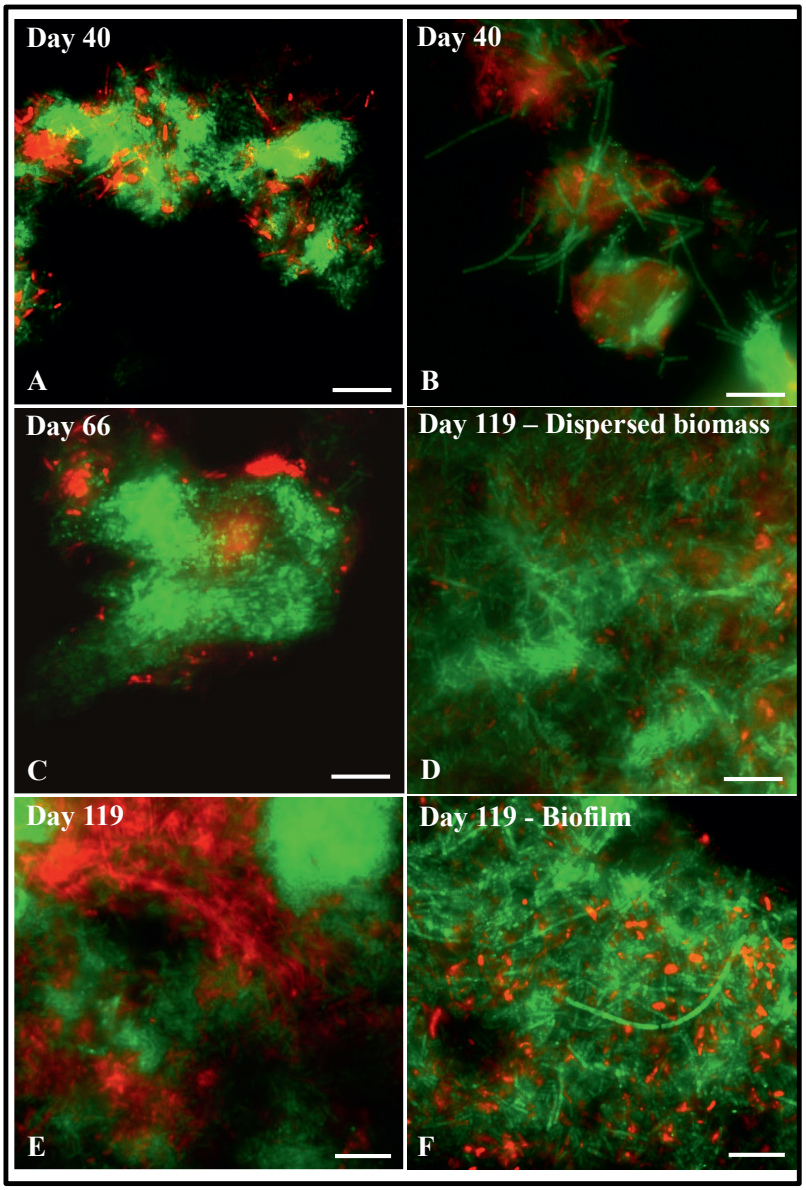


Figure 7. FISH images of samples taken from reactor R3 with tryptone (A, C, E) and R4 with amino acids (B, D, F) at different time points. The sampling was done on day 40, day 66 and at the end of the digestion (day 119). FISH was carried out also on the biofilm sampled from the R4 reactor wall at day 119. The images show the ARC915 probe (Archaea) in green and the EUB338 mix probes (Bacteria) in red. Size bar is 10 µm.

4. Discussion

4.1. Salinity and substrate influence on microbial community and EPS producers

In R1 and R2 UASB reactors rapid granular sludge formation from dispersed biomass at 5 g Na^+/L and even at 20 g Na^+/L occurred. Considering the microbial composition of the start-up inoculum (**Figure 2**), it is clear that while *Methanosaeta* abundance was not influenced by the salinity, the salt concentration was working as a selective pressure for the bacterial community. The key-players in this community changed in response to low (5 g/L) and high (20 g/L) sodium levels. In both cases, the microbial community produced a surrounding EPS layer, which was thicker at higher salinity (**Figure 1**). EPS gel layer has been suggested to function as a protective barrier towards salinity stress (Decho and Gutierrez, 2017). Thus, the thicker EPS layer may be a microbial response to osmotic stress.

With tryptone as a co-substrate, metabolically diverse bacteria in the granules were involved in EPS production and granulation. In reactor R1 granules (5 g/L Na^+) *Streptococcus* filaments were dominant (**Figure 3, Figure 4**), and members of this species are reported as both peptone and carbohydrate fermenters, producing EPS (Tagg et al., 2011; Sanalibaba and Çakmak, 2016). The other dominant group of *Rikenellaceae*, together with *Syntrophobacter* (**Figure 2**), were positively related to anaerobic sludge granulation driven by quorum sensing mechanisms (Ma et al., 2017). At higher salinity (reactor R2 and R3, 20 g/L Na^+), members of *Defluviitaleaceae* were prevailing **(Figures 2** and **4**), with relative abundances of 34% in reactor R2 and 30% in reactor R3 at the end of the process. The lactic acid bacterium *Enterococcus,* known to synthesize EPS from sugars (Mostafa et al., 2009; Bhat and Bajaj, 2018), was the most abundant after *Defluvitaleaceae*.

When tryptone was replaced by single amino acids, the abundance of *Defluvitaleaceae* in reactor R4 was negatively affected (**Table S4**). Also taking the microscopic observations in *Section 3.1.1* into account, it is evident that tryptone availability is a key factor to promote growth and maintenance of granule forming microorganisms such as *Defluvitaleaceae*. *Enterococcus* presence and abundance along time was observed in reactor R4 instead, and this can be related to the EPS production and formation of a biofilm (**Figure 7**) rather than granules. This is in line to the relative hydrophilicity possessed by *E. faecium* in comparison to the microbial community members present in UASB reactors hydrophobic granules (Daffonchio et al., 1995). Other abundant bacterial groups detected in R4 (without tryptone) and previously associated with biofilms, rather than granules formation, were *Thermovirga* (Lenhart et al., 2014), *Desulfovibrio* (Heggendorn et al., 2018) and *Marinobacterium* (Erable et al., 2009).

4.2. *Methanosaeta* clustering and bacterial filaments formation as key events in granulation

The microscopy investigations clearly showed the importance of both *Methanosaeta* clusters and bacterial filaments. The role of *M. harundinacea* is in line with previous studies demonstrating its potential to improve sludge granulation in UASB reactors, both under fresh water (Satoh et al., 2007; Li et al., 2015b) and high salinity levels (Gagliano et al., 2017b; Sudmalis et al., 2018a). In this study, *M. harundinacea* was shown to provide structure to the granules, as indicated by its uniform distribution (**Figure 5-E** and **S2**). *Methanosaeta* cells in both granule types were aggregated in two sorts of clusters: round shaped, rich in rods, and fibrous, with short filaments approaching each other (**Figure 5**). The development of *Methanosaeta* compact clusters (**Figure 5-A to D**) is in contrast to what is usually observed under mesophilic, non-saline conditions, where *Methanosaeta* filaments are suspected to drive granulation (Zheng et al., 2006; Li et al., 2015b), as described by the *"spaghetti theory"* (Wiegant, 1988; Liu et al., 2003). In this study, granules in reactor R1 and R2 increased in size over time when filamentous bacteria *Streptococcus* and *Defluviitaleaceae* were gradually aggregating around the *Methanosaeta* clusters (**Figure 3, Figure 4**). This microbial arrangement is similar to the one observed inside granules exposed to thermophilic (stress) conditions, where long filaments of *Methanosaeta* were replaced by dispersed cells forming microcolonies (Uemura and Harada, 1993; Syutsubo et al., 1997; Sekiguchi et al., 1999). In these thermophilic granules, filamentous bacteria were developing, thereby improving granule settleability (Sekiguchi et al., 2001). Similarly, a salinity stress response in our UASB reactors could direct the aggregation towards the clustering of *Methanosaeta* and the filamentation of bacteria.

The aggregation properties of *Methanosaeta* likely rely on the peculiar glycoproteic sheet surrounding filaments and its hydrophobic characteristics (Beveridge et al., 1986; Pellerin et al., 1990; Nomura, 2012). Interestingly, fluorescence protein specific staining showed the presence of a proteinaceous outer layer not only on *M. harundinacea* cells (**Figure S5 - A**), but also on *Defluviitaleaceae sp.* (**Figure S5 – B, C**). On the other hand, our previous studies applying lectin staining showed that *Streptococcus* chains in low salinity granules (5 g/L Na^+) were covered by a capsular EPS rich in mannose (Gagliano et al., 2018). *Streptococcus* species showing a similar capsular EPS glycoconjugates pattern are known to promote bioaggregation (Tawakoli et al., 2017). Overall, *Methanosaeta* clusters were working as "granulation initiator" – a hydrophobic nuclei – on which *Streptococcus* and *Defluviitaleaceae* where aggregating as filaments, driving further the process by increasing the granules size in time (**Figure 4**).

Interestingly, with the lack of a complex mixture of proteins (tryptone) in the synthetic wastewater fed into reactor R4, the percentage of the granulation key players *Methanosaeta* and above all

Defluvitaleaceae was drastically reduced in comparison to the other high salinity reactors (**Figure 7** and **Table S4**). Moreover, no active *Defluvitaleaceae* cells were detected by FISH at any time in reactor R4, either in dispersed biomass or in the biofilm. Cultured representatives of the family *Defluviitaleaceae* are thermophilic, anaerobic and sugar-fermentative bacteria (Jabari et al., 2012; Ma et al., 2017). Their presence in biogas plants and anaerobic processes has been demonstrated in several studies by 16S rRNA gene sequence diversity analysis (Fardeau et al., 2017). The species type of the family, *Defluviitalea saccharophila*, was isolated from a UASB reactor (Jabari et al., 2012). *Defluviitalea phaphyphila,* isolated from coastal sediment*,* is the only strain able to tolerate up to 5% NaCl (w/v), but not able to grow on proteinaceous substrates such as casamino acids (Ji et al., 2016). Thus, a new mesophilic species was represented in this study, with high salinity tolerance and most likely involved in protein fermentation and/or EPS production.

4.3. Granules vs biofilm growth: what about proteins?

In this framework, above all tryptone, a mixture of peptides and amino acids resulting by the digestion of casein via trypsin protease, could be an essential substrate for granulation, as observed in R2 and R3 reactors (20 g/L of Na^+) in connection to the growth of the filamentous *Defluvitaleaceae.* In general, casein hydrolysate positively influences EPS production (Jolly et al., 2002). Tryptone is an essential nitrogen source to culture lactic acid bacteria (LAB) for EPS production (Leroy and De Vuyst, 2016), and a potent growth enhancer for EPS producing *Bifidobacterium* strains (Poch, 1991; Prasanna et al., 2012). Possibly the lack of tryptone caused lower EPS production, thereby decreasing the granulation rate in reactor R4. A number of studies have demonstrated that casein hydrolysis can produce biologically active peptides promoting microbial growth (Phelan et al., 2009). For instance, the low-molecular-weight peptide fraction (< 3 kDa) of casein hydrolysates was found to specifically promote the activity of different LAB enzymes leading to higher EPS production (Zhang et al., 2014). Interestingly, the trypsin hydrolysate of casein applied in the study of Zhang and colleagues was rich in glutamic acid, leucine and proline (0.843, 0.483 and 0.314 g kg^{-1} of casein hydrolysates, respectively). However, when these three amino acids where applied in this study as substrates in reactor R4, no granulation was observed, suggesting the importance of the entire peptide fraction to promote aggregation.

Besides the promotion of EPS production, protein availability as growth substrate can result in diverse EPS secreted, with different chemical characteristics, influencing the overall hydrophobicity of granules (Liu et al., 2004; Zhang et al., 2007). The latter is likely true, considering that in reactor R4 (without tryptone) part of the biomass formed a thick biofilm on the borosilicate glass inner reactor walls (**Figure 6**), a very hydrophilic surface. Thus, tryptone can also be considered a source of the

building-blocks needed for the microbial synthesis of specific EPS that drives the cells aggregation towards the formation of spherical, rather than flat, biofilms. Some studies addressed the importance of proteins for aerobic granulation by modifying sludge surface properties, and found them to be essential for granules stability (McSwain et al., 2005; Xiong and Liu, 2013). Two recent investigations on anaerobic granules properties (Zhu et al., 2015b; Li et al., 2016) suggested that proteins are responsible for hydrophobicity, especially due to key components such as aromatic amino acids and their nonpolar groups. Surface proteins with nonpolar sites are known to be fundamental for cell attachment to hydrophobic substrata (Donlan, 2002) and herewith for self-aggregation. This could likely be the characteristic of the proteinaceous outer layer surrounding *Methanosaeta* and *Defluvitaleaceae* cells growing with tryptone (**Figure S5**). A very recent study (Dubé and Guiot, 2019) found that *Methanosaeta* proteins were by far the most represented in the EPS extracted from three different granular anaerobic sludges, further proving its key role within the granules matrix.

5. Conclusive remarks

Granulation of anaerobic biomass from dispersed inoculum was observed at sodium concentrations of 5 and 20 g Na^+/L when feeding UASB reactors with glucose, acetate and tryptone as substrate. The salinity was acting as a selective pressure on the overall microbial community composition and on the dominant active bacteria (*Streptococcus* at 5 g Na^+/L and *Defluvitaleaceae* at 20 g Na^+/L), while *M. harundinacea* was the dominant methanogen at both salinities. The two bacteria were growing as filaments and aggregating together with *Methanosaeta* clusters, shaping granules along time. The substitution of a complex protein substrate as tryptone with single amino acids (proline, leucine and glutamic acid) caused the lack of granules development, with a concomitant decrease in *Methanosaeta* and *Defluvitaleaceae* populations and the absence of compact clusters/filaments. Overall, both microbial community composition and substrate were shown to be important for anaerobic granulation at high salinity, a phenomenon yet to be fully explored in its complexity. Further insights into the metagenomics profiles and the protein expression patterns could further clarify the role of each member of the microbial community in terms of substrates utilization and EPS production, confirming a series of fundamental knowledge about microbial consortia applicability for high salinity wastewater treatment through UASB systems.

Supplementary Information of Chapter 6

Table S1 – Archaeal 16S rRNA gene clonal analysis of R1, R2, R3 and R4 UASB reactors at the end of each reactors run.

No. of clones	Accession number	Affiliation	Closest relative (accession no.)	Similarity (%)
R1 Reactor				
85	MG062723	Uncultured *Methanosaeta*	*Methanosaeta harundinacea* 6Ac (CP003117.1)	99
1	MG062729	*Methanosaeta concilii*	*Methanosaeta concilii* strain GP6 (NR_102903.1)	99
5	MG062728	Uncultured *Methanobacteriales*	Uncultured archaeon clone ARCSB_122 (JQ738707.1)	99
	MG062726		Uncultured archaeon clone AP0-62 (KF564599.1)	99
3	MG062727	Uncultured *Methanolinea*	Uncultured *Methanolinea* sp. clone R1T2_A6 1(KX018771.1)	99
1	MG062725	Uncultured *Thermoplasmata*	Uncultured *Methanomassiliicoccales* archaeon clone: Alpha-MMA (LC200514.1)	99
95				
R2 Reactor				
92	MG062730	Uncultured *Methanosaeta*	*Methanosaeta* sp. HA (LC006969.1)	99
3	MG062724	Uncultured *Euryarchaeota*	Uncultured archaeon clone ARCSB_122 (JQ738707.1)	98
95				
R3 Reactor				
79	MN434996	Uncultured *Methanosaeta*	Uncultured *Methanosaeta* sp. clone D003011I03 (EU721747.1)	99
	MN434997	Uncultured *Methanosaeta*	Uncultured archaeon clone: AR80A32 (AB539923.1)	99
9	MG062724	Uncultured *Euryarchaeota*	Uncultured archaeon clone ARCSB_122 (JQ738707.1)	98
88				
R4 Reactor				
85	MN435003	Uncultured *Methanosaeta*	Uncultured *Methanosaeta* sp. clone D003011I03 (EU721747.1)	99
	MN435004		Uncultured *Methanosaeta harudinacea* clone S3_F10 (MG062730.1)	99
2	MN435001	Uncultured *Methanocalculus*	Uncultured archaeon clone PL-9C11 1(AY570669.1)	99
	MN435002		Uncultured *Methanocalculus* sp. clone Z3ALLARC44 (KX062751.1)	99
87				

Table S2 – Bacterial 16S rRNA gene clonal analysis of R1 and R2 UASB reactors after 217 days of digestion process.

R1 Reactor				
No. of clones	**Accession number**	**Affiliation (SILVA)**	**Closest relative (accession no.) (NCBI)**	**Similarity (%)**
24	MK637498	Uncultured *Rikenellaceae*	Uncultured bacterium clone: Niigata-03 (AB243814.1)	99%
	MK637501		Uncultured bacterium clone ALM-360-38 (MH734889.1)	100%
20	MK637499	*Streptococcus* sp.	*Streptococcus henryi* strain OZK31 (KT716262.1)	99%
15	MK637493	Uncultured *Synergistaceae*	Uncultured *Synergistetes* bacterium clone: 3CP(-)_78 (AB908690.1)	99%
12	MK637506	Uncultured *Clostridia*	Uncultured organism clone MAT-CR-H4-A11 (EU245202.1)	89%
4	MK637494	Uncultured *Mesotoga*	Uncultured *Thermotogales* bacterium clone FC.C HM003079.1	99%
4	MK637496	*Sphaerochaeta* sp.	*Spirochaeta* sp. enrichment culture clone (KP178482.1)	99%
3	MK637495	Uncultured *Pelolinea*	Uncultured bacterium clone Nit5A0650_587 (FJ628288.1)	97%
2	MK637508	Uncultured *Syntrophobacter*	Uncultured *Syntrophobacter* sp. clone B16 (EU888828.1)	99%
1	MK637497	Uncultured *Latescibacteria*	Uncultured bacterium clone TDNP_Wbc97_110_1_21 (FJ516997.1)	96%
2	MK637509	Uncultured *Firmicutes*	Uncultured bacterium clone Er-LAYS-38 GU180170.1	99%
	MK637504		Uncultured bacterium clone 01f12 GQ132255.1	99%
1	MK637510	Uncultured *Propionibacteriales*	Uncultured bacterium clone D1 KJ808114.1	99%
88				

Table continued in the next page

Table S2 continued – Bacterial 16S rRNA gene clonal analysis of R1 and R2 UASB reactors after 217 days of digestion process.

R2 Reactor				
No. of clones	**Accession number**	**Affiliation (SILVA)**	**Closest relative (accession no.) (NCBI)**	**Similarity (%)**
48	MK637476	Uncultured *Defluviitaleaceae*	Uncultured bacterium clone H3098 (JX391238.1)	99%
13	MK637479	*Enterococcus* sp.	Enterococcus avium strain E6844 (NR_028748.1)	99%
10	MK637482	Uncultured *Clostridiales*	Uncultured bacterium clone DangY118-A25 (KC333944.1)	99%
5	MK637480	Uncultured *Rikenellaceae*	Uncultured Bacteroidetes/Chlorobi group bacterium clone De1156 (HQ183936.1)	99%
	MK637481		Uncultured Bacteroidetes bacterium clone RII-AN097 (JQ580476.1)	98%
4	MK637483	Uncultured *Synergistes*	Uncultured Synergistes sp. clone D004025G05 (EU721828.1)	98%
2	MK637489	Uncultured *Anaerovorax*	Uncultured bacterium clone NK-L29 (JN685456.1)	99%
6	MK637488	Uncultured bacterium	Uncultured OP9 bacterium clone QEDN10BG12 (CU927230.1)	99%
	MK637492		Uncultured bacterium clone P5-b21 (FR853013.1)	94%
1	MK637490	Uncultured *Geotoga*	Geotoga aestuarianus strain BPX-5 1 (MG696672.1)	99%
1	MK637478	*Sporolactobacillus* sp.	Sporolactobacillus nakayamae subsp. Racemicus (AB362636.1)	99%
1	MK637485	Uncultured *Atribacteria*	Uncultured bacterium clone De1870 (HQ184012.1)	99%
1	MK637486	*Sphaerochaeta* sp.	Spirochaeta sp. enrichment culture clone (KP178482.1)	99%
1	MK637487	Uncultured *Desulfuromonas*	Uncultured bacterium clone ALM-360-18 (MH734878.1)	99%
93				

Table S3– Bacterial 16S rRNA gene clonal analysis of R3 UASB reactor after 54 days and at the end of the reactors runs (day 119).

Day 54				
No. of clones	**Accession number**	**Affiliation (SILVA)**	**Closest relative (accession no.) NCBI**	**Similarity (%)**
33	MN270919	Uncultured *Defluviitaleaceae*	Uncultured bacterium clone H3098 (JX391238.1)	99
21	MN270922	*Exiguobacterium* sp.	*Exiguobacterium* sp. Pb-WC11088 (JX913842.1)	100
9	MN270917	*Enterococcus* sp.	*Enterococcus avium* strain FDAARGOS_184 (CP024590.1)	100
5	MN270923	Uncultured *Atribacteria*	Uncultured bacterium clone De1870 (HQ184012.1)	99
4	MN270916	Uncultured *Marinobacterium*	Uncultured bacterium clone M2_56_F2 (JN683981.1)	100
4	MN270906	Uncultured *Clostridiales*	*Clostridia* bacterium P221(2) (GU370091.2)	96
4	MN270914	Uncultured *Synergistaceae*	Uncultured *Synergistetes* bacterium clone D121231H04 (GU180064.1)	98
3	MN270901	Uncultured *Rikenellaceae*	Uncultured *Bacteroidetes/Chlorobi* group bacterium clone De1156 (HQ183936.1)	98
3	MN270900	Uncultured *Trichococcus*	Uncultured bacterium clone RS-E76 (KC541372.1)	99
2	MN270920	Uncultured *Thermovirga*	Uncultured bacterium clone VHW_D_R9 (JQ085712.1)	100
1	MN270915	Uncultured *Lachnoclostridium*	*Clostridium amygdalinum* strain Marseille-P2095 (LT223651.1)	95
1	MN270913	Uncultured *Zixibacteria*	Uncultured organism clone SBZC_6039 (JN509545.1)	98
1	MN270908	Uncultured *Anaerolineaceae*	Uncultured bacterium clone Er-MLAYS-59 (EU542479.1)	97
1	MN270910	*Rhodococcus* sp.	*Rhodococcus* sp. NJ-530 (CP034152.1)	100
92				

Table continued in the next page

Table S3 continued – Bacterial 16S rRNA gene clonal analysis of R3 UASB reactor after 54 days and at the end of the reactors runs (day 119).

Day 119				
No. of clones	**Accession number**	**Affiliation (SILVA)**	**Closest relative (accession no.) NCBI**	**Similarity (%)**
28	MN413122	Uncultured *Defluviitaleaceae*	Uncultured *Clostridiales* bacterium clone Gran.bac.B09 1 (KX018762.1)	99
11	MN413128	*Enterococcus* sp.	*Enterococcus avium* strain FDAARGOS_184 (CP024590.1)	100
10	MN413123	Uncultured *Alkalibacter*	Uncultured *Alkalibacter* sp. clone De3229 (HQ183778.1)	99
8	MN413143	Uncultured *Synergistaceae*	Uncultured *Synergistes* sp. clone D004025G05 (EU721828.1)	98
7	MN413119	Uncultured *Clostridiales*	Uncultured *Clostridiales* bacterium clone Gran.bac.A05 (KX018760.1)	99
5	MN413124	Uncultured *Anaerolineaceae*	Uncultured organism clone MAT-CR-M7-G09 (GEU245894.1)	94
4	MN413144	Uncultured *Rikenellaceae*	Uncultured bacterium clone: Niigata-03 (AB243814.1)	99
3	MN413137	Uncultured *Mesotoga*	Uncultured *Thermotogales* bacterium clone FC.C (HM003079.1)	100
2	MN413129	Uncultured *Draconibacteriaceae*	Uncultured bacterium clone: 6T1cBa12 (LC183852.1)	99
2	MN413131	Uncultured *Lentimicrobiaceae*	Uncultured bacterium clone SSW62Au (EU592366.1)	99
2	MN413135	Uncultured *Christensenellaceae*	Uncultured bacterium clone DangY118-A25 (KC333944.1)	99
2	MN413118	Uncultured *Peptococcaceae*	Uncultured bacterium clone: 21ARB9 (AB668495.1)	99
2	MN413121	Uncultured *Desulfuromonadaceae*	Uncultured bacterium clone ALM-360-18 (MH734878.1)	99
1	MN270902	Uncultured *Exiguobacterium*	Uncultured bacterium clone: Niigata-24 (AB243992.1)	94
1	MN413125	Uncultured bacterium	Uncultured bacterium clone SA_58 (JQ738994.1)	94
1	MN413132	*Staphylococcus* sp.	*Staphylococcus pasteuri* strain JS7 (CP017463.1)	100
1	MN413145	Uncultured *Caldicoprobacteraceae*	Uncultured bacterium clone B19CH1_61_65 (HF558553.1)	95
1	MN413140	Uncultured *Acetoanaerobium*	Uncultured bacterium clone AB140 (KU667219.1)	98
1	MN413120	Uncultured *Spirochaetaceae*	*Spirochaeta* sp. enrichment culture clone (KP178482.1)	99

92

Table S4– Bacterial 16S rRNA gene clonal analysis of R4 UASB reactor after 54 days and at the end of the digestion process (day 119).

Day 54				
No. of clones	**Accession number**	**Affiliation (SILVA)**	**Closest relative (accession no.) NCBI**	**Similarity (%)**
40	MN414338	Uncultured *Defluviitaleaceae*	Uncultured bacterium clone JCC_RecOTU_41 (KX550184.1)	100
10	MN414330	*Trichococcus* sp.	Uncultured bacterium clone: 1, note: B2, *Carnobacterium* sp (AB495354.1)	100
8	MN414348	Uncultured *Atribacteria*	Uncultured bacterium clone De1870 (HQ184012.1)	99
5	MN414352	*Marinobacterium* sp.	*Marinobacterium* sp. IC961 strain: IC961 (AB196257.1)	100
5	MN414332	Uncultured *Clostridiaceae*	Uncultured bacterium clone Ll142-7F3 (FJ672758.1)	100
3	MN434991	Uncultured *Cloacimonetes*	Uncultured *Planctomycetales* bacterium clone B50-16 (KC555220.1)	97
3	MN414350	*Vibrio* sp.	*Vibrio cincinnatiensis* strain ATCC 35912 1 (NR_026122.1)	99
2	MN414337	Uncultured *Anaerolineaceae*	Uncultured bacterium clone Er-MLAYS-59 (EU542479.1)	97
2	MN414329	Uncultured *Sphaerochaeta*	Uncultured *Spirochaetes* bacterium clone D010011A22 (GU179806.1)	95
2	MN414346	Uncultured *Synergistaceae*	Uncultured *Synergistetes* bacterium clone D120231C06 (GU180061.1)	99
1	MN414331	Uncultured *Draconibacteriaceae*	Uncultured bacterium gene clone: 6T1cBa12 (LC183852.1)	99
1	MN414334	Uncultured *Caldicoprobacteraceae*	Uncultured bacterium clone B19CH1_61_65 (HF558553.1)	95
1	MN414336	Uncultured *Sedimentibacter*	Uncultured *Sedimentibacter* sp. clone Ced_Swamp_G3 (MG367117.1)	96
1	MN414339	Uncultured *Alkalibacter*	Uncultured bacterium gene clone: AS_B09 (LC214868.1)	100
1	MN414340	*Eggerthella* sp.	*Eggerthella lenta* strain AUH-Julong365 (JN874873.1)	100
1	MN414341	Uncultured *Peptococcaceae*	Uncultured bacterium clone Er-LAYS-38 1 (GU180170.1)	100
1	MN414343	Uncultured *Desulfuromonadaceae*	Uncultured bacterium clone ALM-360-18 (MH734878.1)	100
1	MN414345	Uncultured *Marinimicrobia*	Uncultured eubacterium AB16 (AF275926.2)	96
1	MN414335	Uncultured *Firmicutes*	Uncultured bacterium clone Er-MLAYS-91 (EU542485.1)	99
1	MN414342	Uncultured *Sulfurovum*	Uncultured bacterium clone GXU-03 (GU583971.1)	97
1	MN434990	Uncultured *Rhodococcus*	*Rhodococcus ruber* strain R1 (CP038030.1)	96
91				

Table continued in the next page

Table S4 continued – Bacterial 16S rRNA gene clonal analysis of R4 UASB reactor after 54 days and at the end of the digestion process (day 119).

Day 119				
No. of clones	**Accession number**	**Affiliation (SILVA)**	**Closest relative (accession no.) NCBI**	**Similarity (%)**
39	MN414182	*Enterococcus* sp.	*Enterococcus avium* strain FDAARGOS_184 (CP024590.1)	100
9	MN414190	Uncultured *Defluviitaleaceae*	Uncultured bacterium clone H3098 (JX391238.1)	99
8	MN414188	Uncultured *Synergistales*	Uncultured *Synergistes* sp. clone D004025G05 (EU721828.1)	99
4	MN434989	Uncultured *Cloacimonetes*	Uncultured *Planctomycetales* bacterium clone B50-16 (KC555220.1)	97
3	MN414180	Uncultured *Rikenellaceae*	Uncultured *Bacteroidetes* bacterium clone RII-AN097 (JQ580476.1)	98
3	MN414181	Uncultured *Sphaerochaeta*	Uncultured bacterium clone LC65 (FJ024715.1)	99
3	MN414185	*Rhodococcus* sp.	*Rhodococcus ruber* strain R1 (CP038030.1)	100
3	MN414187	Uncultured *Atribacteria*	Uncultured bacterium clone De1870 (HQ184012.1)	100
3	MN414198	Uncultured *Draconibacteriaceae*	Uncultured bacterium clone Asc-w-36 (EF632714.1)	97
2	MN414197	Uncultured *Marinobacterium*	Uncultured bacterium clone M2_56_F2 (JN683981.1)	96
2	MN414184	Uncultured *Piscirickettsiaceae*	Uncultured *Methylophaga* sp. clone DOM14 (HQ012276.1)	96
1	MN414196	Uncultured *Azoarcus*	Uncultured bacterium clone M1_146_B6 (JN683949.1)	99
1	MN414179	Uncultured *Clostridiales*	Uncultured bacterium isolate FBP407, clone 127A (LN680034.1)	99
1	MN434992	Uncultured *Desulfuromonas*	Uncultured bacterium clone ALM-360-18 1(MH734878.1)	99
1	MN414186	Uncultured *Lentimicrobiaceae*	Uncultured bacterium clone SSW62Au (EU592366.1)	93
1	MN414199	Uncultured bacterium	Uncultured Unclassified bacterium clone QEDS3AH04 (CU921882.1)	87
1	MN414193	Uncultured *Thermovirga*	Uncultured bacterium clone BA141 (AF323769.1)	99

85

Table S5 - Oligonucleotide probe sequences, target microbial groups and stringency conditions used in this study.

Name	Target Group	Probe sequence (5'-3')	Formamide (%)	Reference
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	20 or 35	Stahl and Amann, 1991
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	20 or 35	Amann et al., 1990
CF319a	*Bacteroidetes*	TGGTCCGTGTCTCAGTAC	35	Manz et al., 1996
DELTA485A		AGTTAGCCGGTGCTTCCT		
DELTA495B	*Deltaproteobacteria*	AGTTAGCCGGCGCTTCCT	35	Lücker et al., 2007
DELTA495C		AATTAGCCGGTGCTTCCT		
CFX1223	*Phylum Chloroflexi*	CCATTGTAGCGTGTGTGTMG	35	Björnsson et al., 2002
SYN961	*Synergystetes*	GTTCTTCGGTTTGCATCG	20 or 35	Gagliano et al., 2015
STREPT	*Streptococcaceae*	CACTCTCCCCTTCTGCAC	30	Trebesius et al., 2000
LAC435	*Family Lachnospiraceae* [1]	TCTTCCCTGCTGATAGA	35	Kong et al., 2010
MSMX860	*Methanosarcinales**	GGCTCGCTTCACGGCTTCCCT	45	Raskin et al., 1994
MG1200b	*Methanomicrobiales*	CRGATAATTCGGGGCATGCTG	20	Crocetti et al., 2006
LAB158	*Lactobacilli-Enterococci*	GGTATTAGCAYCTGTTTCCA	25	Harmsen et al. 1999
LGC354A		TGGAAGATTCCCTACTGC		
LGC354B	*Firmicutes*	CGGAAGATTCCCTACTGC	35	Meier et al. 1999
LGC354C		CCGAAGATTCCCTACTGC		
MX825	*Methanosaetaceae***	TCGCACCGTGGCCGACACCTAGC	50	Raskin et al., 1994
MX825b	*Methanosaetaceae* subgroup**	TCGCACCGTTGCCGACACCTAGC	50	Crocetti et al., 2006
MX825c		TCGCACCGTGGCTGACACCTAGC	50	Crocetti et al., 2006

[1] (The probe was used to target *Defluviitaleaceae*. Sequences classified by the SILVA taxonomy as *Defluviitaleaceae* were also classified as *Lachnospiraceae* by the Greengenes taxonomy).

* (including *M.harundinacea*)

** (except *M.harundinacea*)

Additional Information on Materials and Methods

Next generation sequencing -QIIME 16s rDNA analysis

The paired-end MiSeq reads were merged based on the overlap between the two reads. Only merged sequences were used in subsequent analyses, and the non-overlapping pairs were discarded. The merged sequences were filtered, clustered, taxonomically assigned and aligned using the QIIME pipeline version 1.9.1 (Caporaso et al., 2010). The process consisted of quality checking, de-noising, and a microbial diversity analysis. The primer sequences were removed from the raw reads, and then placed in a single Fasta file using the add_qiime_labels.py script. OTU picking was performed with the script pick_open_reference_otus.py using the SILVA database version 128 (Quast et al., 2012) and the clustering method UCLUST (Edgar, 2010). The RDP classifier (version 2.2) (Wang et al., 2007) was used to classify the OTUs on the basis of the same SILVA database. The QIIME script core_diversity_analyses.py was used to calculate alpha- and beta-diversity statistics of the samples.

Staining of EPS and proteins

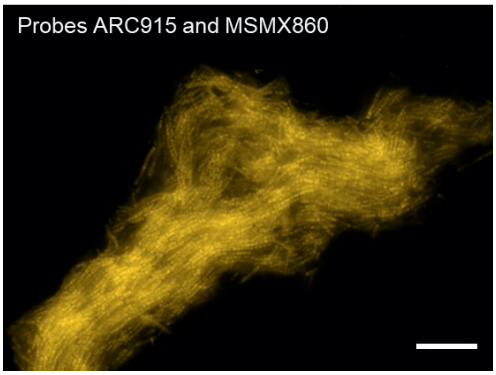
Freshly sampled granules were periodically analyzed to check the EPS production and aggregation. Crystal violet 0.1% (v/v) staining was used to visualize the EPS layer on granules (O'Toole et al., 1999). Samples were observed by bright field microscopy using a Leica EZ 4D Stereomicroscope equipped with a Coach DSC webcam (Leica microsystems, Germany).

FITC staining (ThermoFisher Scientific, Germany) was used to visualize the protein portion of granules' EPS. The staining was carried out on freshly sampled granules in their reactor liquid-phase for 30 min at room temperature (RT) in the dark. Granules were washed with PBS/30% sacharose solution before microscopic observation. Samples were examined using an epifluorescence microscope BX41 (Olympus, Japan) equipped with Infinity Camera (Lumenera corporation, Canada).

FISH identification of *Methanosaeta harundinacea*

In this work, the inoculum used was characterized several times for the microbial composition, showing the presence and dominance of *M.harundinacea* (Gagliano et al., 2017b; Gagliano et al., 2018; Sudmalis et al., 2018a). From our previous work (Gagliano et al., 2018) we know that the 16S rRNA of the subspecies *M. harundinacea* is not positive (does not hybridize) to the three MX825 (a, b and c) probes available from literature. However, MSMX860 probe (*Methanosarcinales*) works well. In the samples analysed in this study, all the *Methanosaeta* cells were negative to MX825, but positive to MSMX860. Given this, and the results of our previous works, we were safe in driving out the discussion about *M. harundinacea*. Thus, the dominance of *Methanosaeta* cells out of the total archaeal population was confirmed via FISH with ARC915, MSMX860 and MX825mix probes.

The hybridization carried out with ARC915 (total Archaea, red) and MSMX860 (*Methanosarcinales*, green) (stringency 45% of Formamide) gave a yellow signal (see image below), indicating that most of the archaeal population probably belongs to this *Methanosaeta spp.* The peculiar *Methanosaeta* morphology was widespread, the only one detected when applying the two probes ARC915 and probe MSMX860. Moreover, a lot of microscopic characterization and work has been done on this sludge, and even the application of probes MG1200b (*Methanomicrobiales*) and MB311 (*Methanobacteriales*) didn't show any significant result.



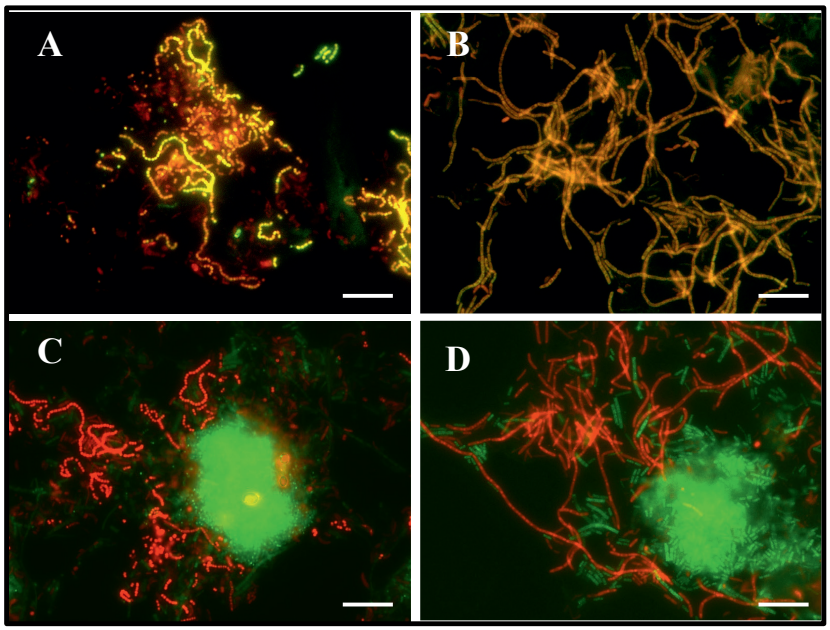


Figure S1 – Epifluorescence images after FISH analysis of anaerobic granules. In A, *Streptococcus sp.* was detected in reactor R1 samples by using Strept probe. In B, filaments *Defluvitalea sp.* were further identified in reactor R2 with the probe LAC435 (green). In A and B, the EUB338mix probe (red) was applied together with specific probes (in green), resulting in a yellow signal. In C and D, the EUB338mix probe (red) was applied together with ARC915 probe (in green) to show the association between the dominant filamentous bacteria and *Methanosaeta* clusters in reactor R1 (C) and reactor R2 (D). Scale bar is 10 µm.

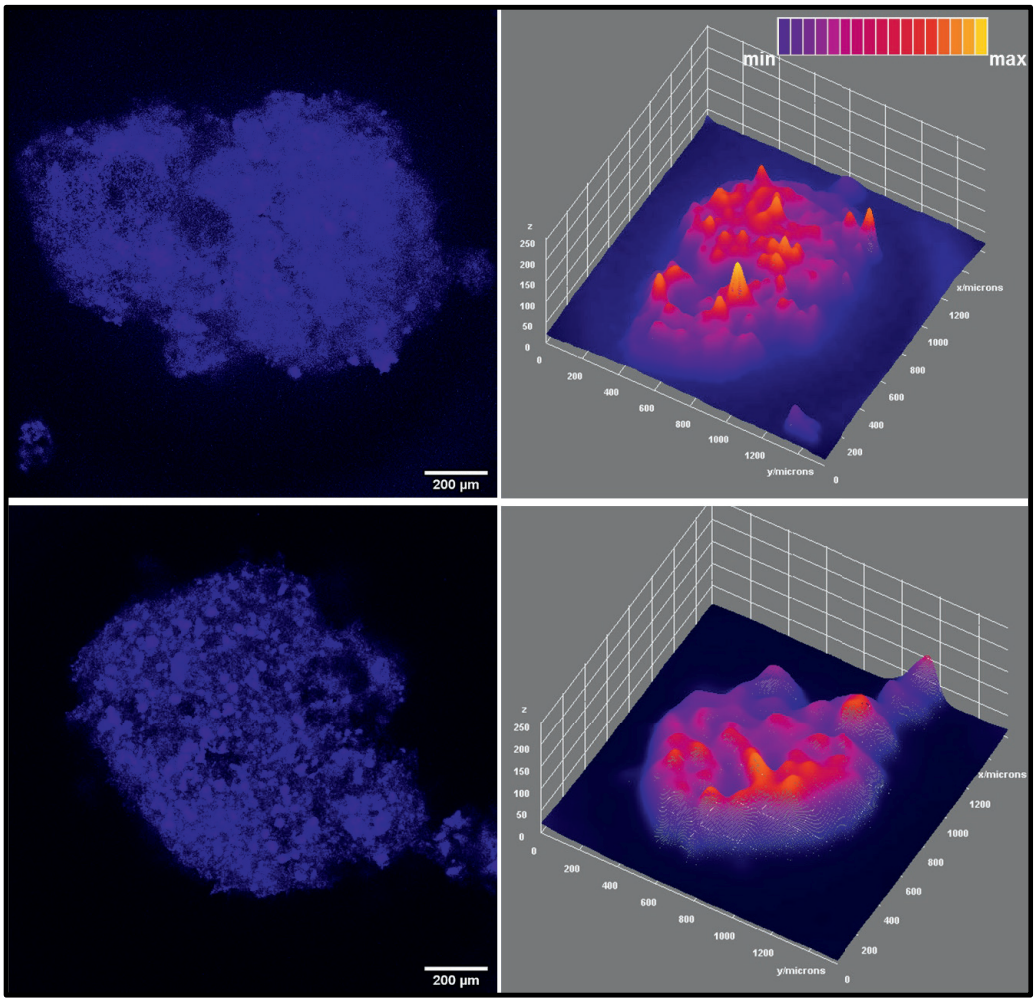


Figure S2 – CLSM images (left) and the relative signal distribution (right) of F_{420} autofluorescence emission in high salinity granules, highlighting the presence of active *Methanosaeta* clusters throughout their structure.

CLSM analysis was carried out using a TCS SP5 (Leica, Wetzlar, Germany). CLSM datasets were recorded from granules in the multichannel mode taking advantage of F_{420} autofluorescence (Doddema and Vogels, 1978)

The FIJI software package (version1.51g, Wayne Rasband, NIH, Bethesda, MD, United States) was used to analyze/modify the CLSM stacks and to construct the signal distribution maps.

Table S6 – Volatile fatty acids levels in reactors R3 and R4 along the digestion time. After day 75, in R4 the substrate leucine was substituted with glutamic acid.

		VFA concentration (mg/L)						
	day	***Acetate***	***Propionate***	***Isobutyrate***	***Butyrate***	***Isovalerate***	***Valerate***	***Isocaproate***
	18	224.1	116.3	14.8	0	24.1	0	16.4
	33	44.1	223.5	27.9	11.6	48.4	11.0	5.1
	46	21.5	137.5	23.9	0.0	28.6	0	1.1
R3	*61*	14.2	142.1	11.2	0.8	31.5	0	2.9
	75	139.3	287.2	5.5	1.1	51.4	0	3.7
	89	17.1	182.4	3.8	1.0	4.7	0	3.1
	106	78.2	234.8	4.6	0	28.1	0	3.4
	18	46.5	63.0	0	0	33.3	11.0	10.7
	33	231.0	170.4	7.0	1.2	90.8	26.0	2.8
	46	191.7	420.4	14.5	6.4	182.0	49.6	1.2
R4	*61*	24.2	314.2	12.9	0.8	142.4	5.2	3.1
	75	82.9	910.9	23.7	1.7	98.4	13.1	3.4
	89	49.2	664.2	15.0	0	6.4	7.4	1.3
	106	43.7	561.6	13.8	0	5.7	5.2	0.8

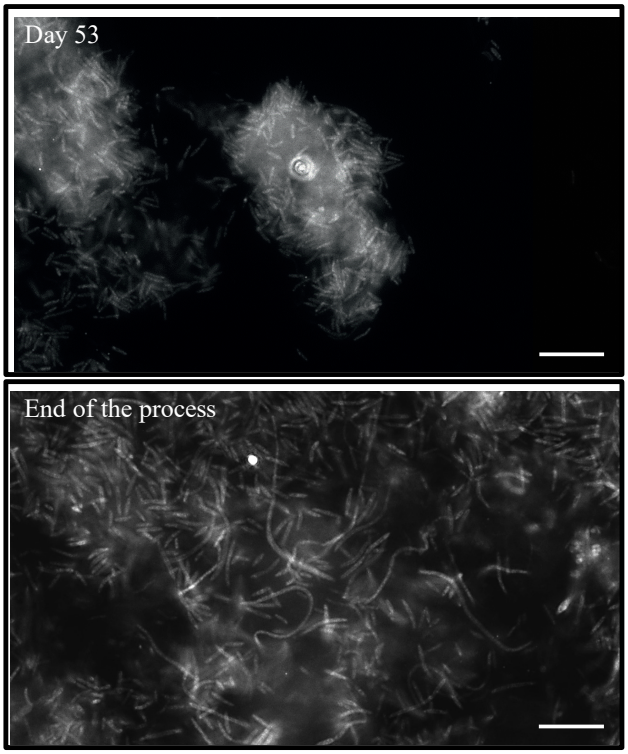


Figure S3 – Epifluorescence images after FISH analysis of R3 reactor anaerobic granules at two different stages of the process, showing members of the family *Defluvitalea sp.* identified with the probe LAC435. Size bar is 10 µm.

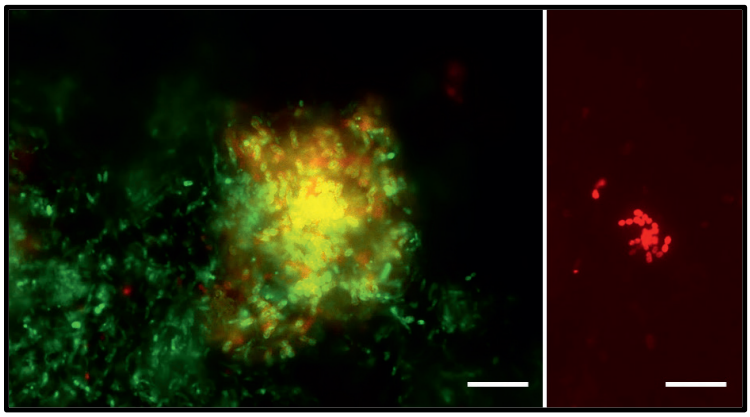


Figure S4 – Epifluorescence images after FISH analysis of R4 reactor anaerobic biomass at the end of the process, showing *Enterococcus sp.* identified with the probe LAB158 (in red). In green, total bacteria detected by EUB338 probe. Yellow signal corresponds to the double positivity to both probes. Size bar is 10 μm.

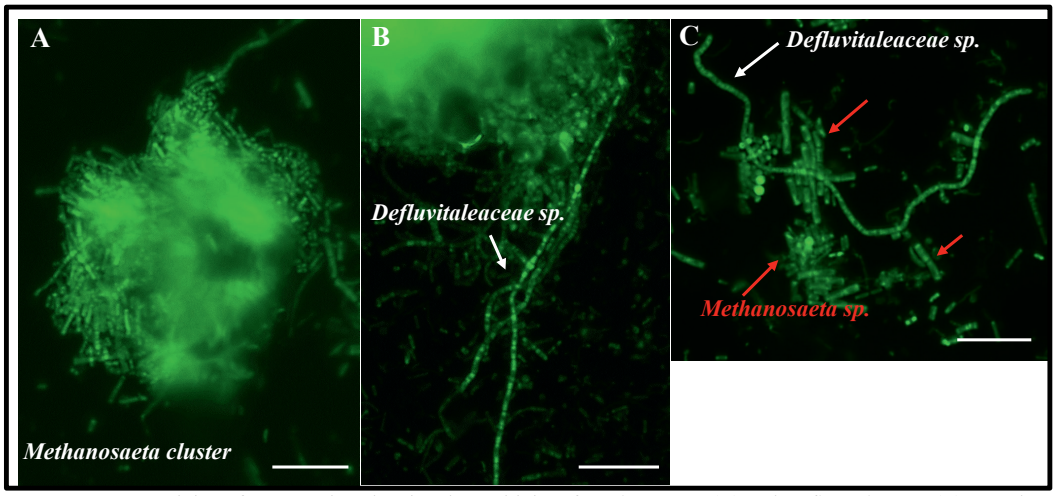
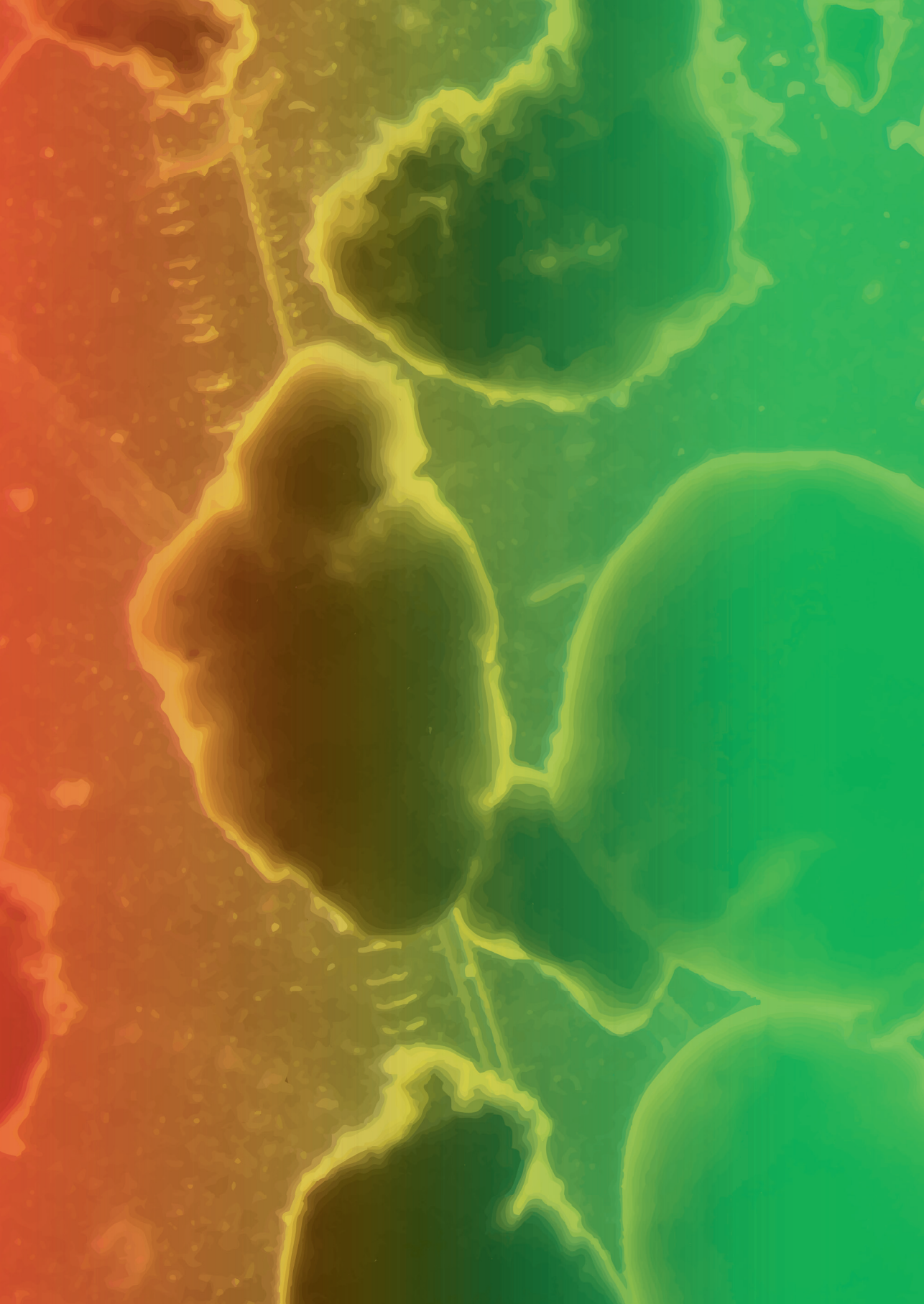


Figure S5 – FITC staining of R2 granules, showing the positivity of *Methanosaeta* (A) and *Defluvitaleaceae* (B) outer layer. In C, the typical morphology of *Methanosaeta* (red arrow) and *Defluvitaleaceae* (white arrow) cells, previously highlighted by FISH (Fig. S2), are shown together. Size bar is 10 μm.



Chapter 7. Cation Exchange Membrane Behaviour of Extracellular Polymeric Substances (EPS) in Salt Adapted Granular Sludge

Authors:

D. Sudmalis[a,1], T. Mubita[a,b,1], M.C. Gagliano[b], E. Dinis[a,b],
G. Zeeman[a], H.H.M. Rijnaarts[a], H. Temmink[a]

[a] Department of Environmental Technology, Wageningen University and Research, Bornse Weilanden 9, 6708 WG, Wageningen, The Netherlands

[b] Wetsus, Centre of Excellence for Sustainable Water Technology, Agora 1, P.O. Box 1113, 8900 CC Leeuwarden, The Netherlands

[1] both authors contributed equally

Abstract: This paper aims to elucidate the role of EPS in regulating ion concentrations in anaerobic granular sludges adapted to high salinity. Equilibrium concentrations of Cl^- in granular sludge adapted to 0.22 (R5) and 0.87 (R20) M sodium chloride solutions, were found to be 31.2 and 35.8 % of the concentration in the bulk liquid, respectively. The intra-granular concentration of sodium was found to be 9% higher in the R5 and 17% lower in the R20 granular sludge, as compared to bulk liquid concentrations. The interactions of EPS and alginate, a model compound of EPS, with Na^+, K^+ and Cl^- were studied using freshly prepared EPS and alginate-based ion exchange membranes placed in electrodialysis cells. The properties of these membranes were compared to a commercial cation exchange membrane. The current efficiency of both EPS and alginate-based membranes was ~80%, showing their ability to partially repel anions. The commercial cation exchange membrane exhibited a current efficiency of 92%. Both, EPS and alginate-based membranes, had higher transport selectivity for potassium over sodium with separation factors (S_{K^+/Na^+}) of 1.3 and 1.9 for EPS and alginate based membranes, respectively. In contrast, the commercial membrane showed no selectivity for these cations. Effects of monovalent ions (Br^-, K^+, Na^+ and Cl^-) on specific methanogenic activity (SMA) of salt adapted granular sludge were also examined. The SMA of R5 granular sludge decreased by 25% when exposed to equimolar (0.22 M) concentration of potassium chloride and by 49% when exposed to increased concentration (0.43 M) of potassium. Exposure of R5 granular sludge to 0.43 M of sodium resulted in 20% decrease of SMA. Bromide salts appeared to have slightly smaller negative effects on SMA compared to chloride salts. These results suggest that EPS of granular sludge adapted and exposed to high salinity function as a protective barrier against high concentrations of ions, such as Cl^- and Na^+, but are less protective against K^+.

1. Introduction

In anaerobic wastewater treatment systems, high monovalent salt concentrations are considered to negatively affect microbial activity, especially the methanogenic activity (Rinzema et al., 1988; Vyrides et al., 2010; Fang et al., 2011; De Vrieze et al., 2016a). It was only recently shown that anaerobic granules adapted to approximately 0.35 M NaCl can increase in size (Gagliano et al., 2017b) and can even be formed from dispersed biomass (Sudmalis et al., 2018a) at NaCl concentrations as high as 0.87 M. This can be achieved while maintaining high methanogenic activity for successful high rate anaerobic reactor operation. The high methanogenic activity could in part be explained by production of osmolytes that balance the osmotic pressure between the microbial cells and the bulk liquid (Sudmalis et al., 2018b). The osmo-toxic effect of highly saline environments on anaerobic microbial consortia has mainly been attributed to cations rather than anions (McCarty and McKinney, 1961; Kugelman and McCarty, 1965; Lin and Chen, 1999; Karri et al., 2006; Lefebvre et al., 2007; Altaş, 2009; Fang et al., 2011) and this toxicity of cations has been explained by various

mechanisms, i.e., their ability to replace metallic enzyme cofactors thereby disrupting the biological function of these cofactors, induction of redox reactions with cellular thiols, provoking Fenton-type reactions that produce reactive oxygen species and by interference with membrane transport processes (Harrison et al., 2007; Chen et al., 2014).

In anaerobic granular sludge, microorganisms are entangled in a matrix of extracellular polymeric substances (EPS) forming a dense spherical biofilm structure (Zhou et al., 2006; Seviour et al., 2012). Extracellular polymeric substances generally have a net negative charge (Liu and Fang, 2003; Ajao et al., 2018), which enables them to adsorb (multivalent) heavy metals (Toner et al., 2005; Comte et al., 2006c; Comte et al., 2008; d'Abzac et al., 2013; Li and Yu, 2014; Dobrowolski et al., 2017). Also sorption of monovalent cations by EPS of pure cultures of marine bacteria has been studied. While the EPS of *Halomonas sp.* were shown to poorly adsorb monovalent cations (Gutierrez et al., 2012), the EPS of *Pseudoalteromonas* have a high affinity towards K^+ sorption (Gutierrez et al., 2008). Extracellular polymeric substances can alleviate toxicity of low concentrations (mg/L range) of heavy metals towards microorganisms (Teitzel and Parsek, 2003; Harrison et al., 2007; Flemming and Wingender, 2010; Wang et al., 2013) by binding and coordination reactions between the metals and the negatively charged functional groups of EPS, which prevents their diffusion into the deeper parts of the biofilm (Teitzel and Parsek, 2003; Horn and Morgenroth, 2006; Hu et al., 2007). A recent microscopy study of our group has shown that mannose-rich EPS surrounding methanogenic cells in high salinity adapted granular sludge adsorb high concentrations of sodium, while the concentration within active cells remains low (Gagliano et al., 2018). These results suggested that even at high salinity EPS may have a protective role against monovalent cations such as Na^+ by hindering their diffusion into microbial cells (Gagliano et al., 2018).

At high concentrations (0.2 M) Cl^- has been reported to cause toxicity to aerobic acidophilic microorganisms because the flux of Cl^- into microbial cells alters the intracellular pH (Suzuki et al., 1999). In general anions are typically reported to be less toxic for anaerobic microorganisms than cations. However there seem to be no attempts on investigating, why this is the case. In principle anions and cations both contribute to osmotic pressure in water (Smith et al., 2016), and therefore microorganisms should have a strategy to cope with high concentrations of either of them. Theoretically the negative charge of EPS should prevent or hinder the transport of anions to microbial cells, thereby lowering their toxicity towards the microorganisms present in porous biofilm structures, such as sludge granules. However, this ability of EPS to partially repel anions has not been experimentally demonstrated. Also, thus far, only few studies focused on the transport rather than sorption of monovalent ions (e.g., K^+, Na^+, Cl^-) in EPS layers. In one study Siegrist and Gujer (1985) experimentally determined the diffusion coefficients of Br^- and Na^+ through a heterogeneous biofilm

and found that the diffusion coefficient of both ions in the biofilm was smaller compared to the diffusion coefficients in water and that the diffusion coefficient of Br^- in biofilm was slightly higher compared to Na^+. In another study, Horn and Morgenroth (2006) studied the diffusion of NaCl and $NaNO_3$ in biofilms. However, in the latter study the transport of individual ionic species could not be distinguished because conductivity rather than individual ion concentrations was measured.

In the current study, the distribution of monovalent ions (K^+, Na^+ and Cl^-) was investigated in microbial granules adapted to 0.87 M and 0.22 M of NaCl. Initial results indicated that Cl^- is repelled from the granular sludge by EPS surrounding the microorganisms. To verify this, the ion-exchange nature of EPS was investigated. This was done with a novel approach, where EPS extracted from granular sludge were used to fabricate EPS layers (EPS-membranes) and these were tested in an electrodialysis cell for transport of K^+, Na^+ and Cl^-. Finally, to study, if the ions with the highest transport rate would also more negatively affect methanogenic activity of salt adapted granular sludge, the effect of different concentrations of NaCl and KCl towards specific methanogenic activity (SMA) of 0.87 M and 0.22 M NaCl adapted granular sludge was determined.

2. Materials and methods

2.1. Source of anaerobic granular sludge

Anaerobic granular sludge was obtained from laboratory scale Upflow Anaerobic Sludge Bed (UASB) reactors. The reactors were operated at sodium concentrations of 0.22 (R5) and 0.87 (R20) M as described in **Chapter 2**.

2.2. Ion distribution and concentration within salt adapted granular sludge

2.2.1. Scanning electron microscopy with energy dispersive X- ray spectroscopy (SEM-EDX)

Samples of granules for SEM-EDX analysis were prepared with a modified procedure described in Ismail et al. (2010). The modifications included dehydration with graded series (10, 30, 50, 70 and two times 100%) of ethanol instead of acetone and sputter coating with tungsten instead of platinum. The granules were imaged at an acceleration voltage of 10 kV and a beam current of 0.4 nA, at room temperature in a field emission scanning electron microscope (Magellan 400, FEI Company, Oregon, USA) equipped with energy dispersive X-ray detector. The images were processed using AZtec software (OXFORD Instruments).

2.2.2. Ion concentration in hydrated granular sludge

Approximately 20 g of sludge samples for determination of ion concentrations in hydrated granular sludge were taken directly from R5 and R20 through a sampling port located 7.5 cm from the bottom of the reactor columns with a total column height of 65 cm. Additionally, the distribution of Cl^-, Na^+, and K^+ in granules of R20 upon granular sludge exposure to equimolar KCl (0.87 M) concentration was investigated. To do this, 1 g of R20 granular sludge was immersed in 1L of a modified ($NaHCO_3$ and NaCl replaced with $KHCO_3$ and KCl, respectively) nutrient medium of R20 (Sudmalis et al., 2018a) for 24 hours before analysis of ionic composition. As a control R20 granular sludge was also exposed to its original medium containing 0.87 M of NaCl.

For ionic composition measurements of hydrated granular sludge, the sludge was first separated from the liquid phase by centrifugation at 10000 xg and 4 ºC for 15 min. Further, the sludge samples were carefully homogenised with a spatula and two samples (approximately 0.5 g each) of homogenised solids were taken for microwave digestion (ETHOS 1 - Advanced Microwave Digestion Labstation, Milestone S.r.l., Italy) with 10 mL of 65% HNO_3 (For Analysis Emsure® ISO). Digested samples were brought up to 50 mL with mili-Q water and further diluted for analysis. As a blank for ionic composition of solids mili-Q was treated in the same way as the samples. The supernatant after centrifugation was filtered through 0.2 µm cellulose acetate membrane filter (VWR® Syringe Filters) and diluted with mili-Q for further analysis. Measurements of bulk liquid were made in one replicate to confirm the expected bulk liquid concentrations of ions. Differences between measurements and expected values were below 2% in all cases.

Sodium and potassium analysis was carried out by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian, Australia) as described in Gagliano et al. (2018). Chloride content was measured on Dionex ICS-2100 Ion Chromatography System (Breda, The Netherlands) equipped with a Dionex IonPac AS19 column (4 x 250 mm) and data were processed with Chromeleon 6.80 SR13 software. The results are presented on weight bases ($mmol_{ion}/g_{medium}$ and $mmol_{ion}/g_{wet\ sludge}$) to prevent introduction of errors due to differences in the density of the medium and the granular sludge.

2.3. Ion exchange properties of EPS

2.3.1. Extraction and purification of EPS

Extracellular polymeric substances (EPS) were extracted from granular sludge adapted to 0.87 M of Na^+ (R20) by using an alkaline extraction method as reported by Felz et al. (2016). In short, 3 g of granular sludge (wet weight) were put into a 0.5% (w/v) Na_2CO_3 solution, heated to 80 ºC and stirred at 400 rpm for 35 min. After extraction, the cell debris and insoluble fraction of the extract were

separated by centrifugation at 4000 xg and 4 ºC for 20 min. Finally the EPS solution was dialysed (SnakeSkin™, 3.5K MWCO) against milli-Q for 24 hours. To obtain purified EPS in a dry state, the EPS solution was further lyophilised at -84 ºC and 0.001 mbar.

2.3.2. Fabrication of alginate gel membranes (ALG – membranes)

Alginate is a well-known EPS model compound (Körstgens et al., 2001; Flemming and Wingender, 2010). Therefore, alginate membranes were prepared as a control for comparison with ion exchange membranes with EPS extracted from granular sludge. The alginate gel layers (ALG-membranes) were casted with an external gelation method adapted from Li et al. (2015a) in following steps: i) dissolving the polymer in 10 mL milli-Q; ii) degassing the solution for 24 hours at 4ºC; iii) casting the solution on a petri dish (ID = 5.7 cm) and drying at 40ºC in a levelled oven; iv) crosslinking the casted layers in a 2% (w/v) calcium chloride solution with 30% (v/v) of ethanol. For casting a mixture of 0.4 g of sodium alginate (SA) and 0.004 g of guar gum (GG) as a plasticiser were used, resulting in 0.016 g SA/cm^2. The resulting membrane was washed with demi-water and equilibrated in a solution with 10 mM NaCl and 10 mM KCl for 8 h before ion selectivity tests.

2.3.3. Fabrication of membranes with EPS (EPS-membranes)

First, a polymeric solution was prepared by dissolving poly(vivylidene fluoride-co-hexafluoropropene) (PVDF-HFP) in dimethyl acetamide (DMAc) in a ratio 1:9.5 w/v for 3h at 80 °C. Then, a weighed amount of EPS was dispersed in the polymeric solution and the materials were blended in a ball mill for 4 h. The resulting mixture contained 70:30 weight ratio of PVDF-HFP to EPS. The mixture was cast onto a glass plate at 50 °C until complete evaporation of the solvent. The resulting membrane/layer was washed with demi-water and equilibrated in a solution with 10 mM NaCl and 10 mM KCl for 8 h before ion selectivity tests. The content of EPS in the membranes was approximately 0.0027 g EPS/cm^2 of dry membrane. This was calculated from the total area of the membrane by assuming a uniform EPS distribution.

2.3.4. Confocal laser scanning microscopy (CLSM) for visualisation of EPS distribution in the PVDF

To visualize the EPS distribution in the PVDF-EPS membranes they were first equilibrated in a 10 mM KCl and 10 mM NaCl solution (buffer solution) overnight. For the staining of the protein fraction of EPS, the EPS-PVDF membranes were placed in PBS 1X and SYPRO Ruby for 1 hour in the dark. Finally, the membranes were washed with the buffer solution (KCl and NaCl) and immediately observed under CLSM. To guarantee that SYPRO Ruby does not stain PVDF, a pure PVDF membrane was processed in the same way as PVDF-EPS membranes. Microscopy analysis was

performed with an inverted AxioObserver Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss, Germany) with a 40×/1.3 Oil DIC M27 Plan-Apochromat objective lens. PVDF in the membranes was visualized with 488 nm excitation wavelength, at a maximum emission of 510 nm. EPS protein portion stained with SYPRO Ruby was visualised with 458 nm excitation wavelength at a maximum emission of 656 nm. The argon laser was set to 1% power for both excitation wavelengths. A series of Z-axis images (212.5 µm x 212.5 µm x 70 µm) were generated by optical sectioning with a slice thickness of 1 µm. Maximum projection intensity and orthogonal 3D reconstruction were generated with Zen Blue software (Zen imaging Software, ZEISS, Germany). The choice of emission wavelengths and the dye for EPS protein staining is explained in detail in **Appendix 1**.

2.3.5. Electrochemical characterization and ion selectivity

Performance of the membranes was evaluated in a six-compartment electrodialysis cell as shown in **Figure 1**. Different membranes separated the compartments inside the cell: i) the membrane under investigation, placed in between compartments A and B. This membrane was either EPS-membrane (EPS-PVDF), ALG-membrane (alginate layer) or a commercial membrane (Neosepta CMX, ASTOM Corporation, Tokyo, Japan) used as a comparison. ii) Commercial ion exchange membranes, i.e., Neosepta AMX and CMX, which separated the other compartments. Characterization of the membranes was done while using a mixture containing two different monovalent cations, i.e., potassium (K^+) and sodium (Na^+) as chloride salts, allowing for direct comparison of monovalent ion transport through the membranes in multi-ion solutions.

Compartments A, B, and C were filled with a solution containing 10 mM NaCl and 10 mM KCl, whereas compartments D were filled with 0.1 M Na_2SO_4 solution. Compartments A and B were filled with 130 mL solutions that were continuously stirred. For compartments C and D one-litre solutions were circulated at a flow rate of 170 mL/min to keep the ion concentrations constant throughout the experiment. A current density of 10 A/m^2 was applied to the cell for 1h. Samples from compartments A and B were taken over time and ion concentration was measured by ion chromatography.

An increase in chloride concentration was calculated from compartment A, whereas increases in sodium and potassium concentration were calculated from compartment B. Ion selectivity (**S**) in the membranes is based on the concentration changes between potassium and sodium and is given by

$$S_{K^+/Na^+} = \frac{\Delta c_{K^+}}{\Delta c_{Na^+}},$$

and

$$\Delta c = c_t - c_{initial},$$

where t, $c_{initial}$ and c_t are the sampling time, the initial concentration and the concentration at time t measured in compartment B (mol/L), respectively.

The ability of the membrane to allow the transport of only one type of ion (cations or anions), the permselectivity, was characterised as current efficiency (η):

$$\eta = \sum_{i}^{i=2} \frac{(c_{t,i} - c_{initial,i}) \cdot V_{cell} \cdot F}{I \cdot t}$$

where subscript *i* refers to cation species in solution, i.e., potassium and sodium, V_{cell} is the volume of liquid in compartment B (L), F is Faraday's constant (96485 C/mol), and I is the applied current (C/s) and t is time (s).

The current efficiency gives an indication on how selectively ions are transported across the membrane (Sadrzadeh and Mohammadi, 2009), i.e., it indicates if the ionic current is mainly transported by potassium and sodium (cation-exchange membrane), or the membranes also allow the transport of chloride.

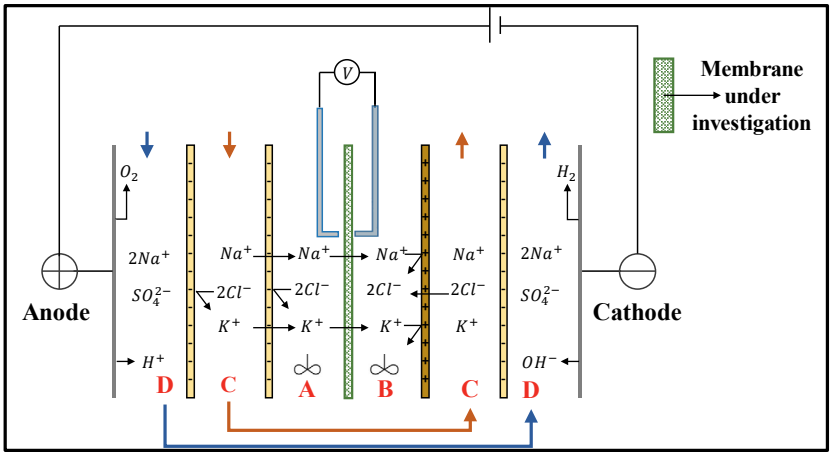


Figure 1. Schematic representation of the electrochemical cell used to evaluate the performance of the membranes. The membrane under investigation separates compartments A and B. After applying a potential difference, cations move from compartment A to B. For an ideal cation-exchange membrane, the transport of chloride from compartment B to A is zero. Water splitting was not relevant in these experiments.

2.4. Effect of anions and cations on specific methanogenic activity

To study the effect of monovalent anions and cations on specific methanogenic activity (SMA) of salt adapted granular sludge, batch experiments in 118 mL serum bottles at a working volume of 50 mL, 120 RPM mixing speed and 35 ºC were performed. The VSS concentration was set to 1 g/L and the COD:VSS ratio was 4:1 (w/w). Sodium acetate was used as the electron donor and carbon source. The SMA was calculated through measurements of pressure build-up curves with a pressure meter equipped with an absolute pressure probe (GMH3151, Greisinger Electronic, Germany). The biogas composition at the end of experiments was measured as described in Steinbusch et al. (2008). Unless stated otherwise, the experiments were performed in triplicate. All results were corrected for the atmospheric pressure and pressure build-up in blank experiments without added acetate.

The first set of experiments was performed to study the SMA of granular sludge adapted to 0.22 (R5) and 0.87 (R20) M of Na as NaCl, when exposed to equimolar concentration of cations by addition of NaBr, KCl and KBr. The second set of experiments was performed to study the SMA of R5 granular sludge upon exposure to hyper-salinity shocks (i.e., an abrupt increase of salinity by spiking a nutrient medium with increased ion concentration) of NaCl, NaBr, KCl and KBr. The final cation concentration in these experiments was 0.43 M. Bromide salts were tested (NaBr and KBr), to investigate if different anions at very high salinity would also have a substantial effect on SMA, as is frequently reported for cations (Kugelman and McCarty, 1965; Lefebvre et al., 2007; Fang et al., 2011). All experiments contained 0.08 M of Na^+ originating from inocula and sodium acetate as a

carbon source. This was taken into account when calculating the desired concentration of cations in the experiments.

The nutrient medium consisted of the following salts 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, $Na_2SeO_3 \cdot 5H_2O$ and 0.216 mL/L of 36% HCl. NaCl, NaBr, KCl and KBr salt amounts were calculated to reach the desired cation concentration for each experiment.

3. Results

3.1. Equilibrium of sodium, chloride and potassium in granules at different salinities

The measured ion concentrations (**Figure 2**) and distributions (**Figure 4)** in the anaerobic, salt adapted, granular sludges suggest a cation exchange nature of EPS in the granules. **Figure 2-A, B** show the sodium and chloride concentrations in bulk liquid and in granular sludges of R5 and R20, respectively. Sodium concentration in the granular sludge was 0.24 ± 0.004 (~0.24 M) and 0.71 ± 0.01 (~0.71 M) mmol/$g_{wet\ sludge}$ in R5 and R20, respectively. The sodium concentration in R5 granular sludge was 9% higher compared to the bulk liquid, whereas in R20 it was 17% lower compared to the bulk liquid, probably due to metal cations other than sodium also being present in the granular structure and interacting with the negatively charged EPS groups. The chloride concentration was 0.11 ± 0.002 (~0.11 M) and 0.52 ± 0.001 (~0.52 M) mmol/$g_{wet\ sludge}$ in R5 and R20, respectively (**Figure 2-A, B**). These concentrations correspond to a 31.2 and 35.8% lower chloride concentration compared to the bulk liquid in R5 and R20, respectively. Thus, apparently EPS of granular sludge acclimated to 0.22 and 0.87 M of Na^+ preferentially repel anions, such as Cl^-, probably due to anionic nature of EPS, as will be discussed in more detail in *Section 4.1*.

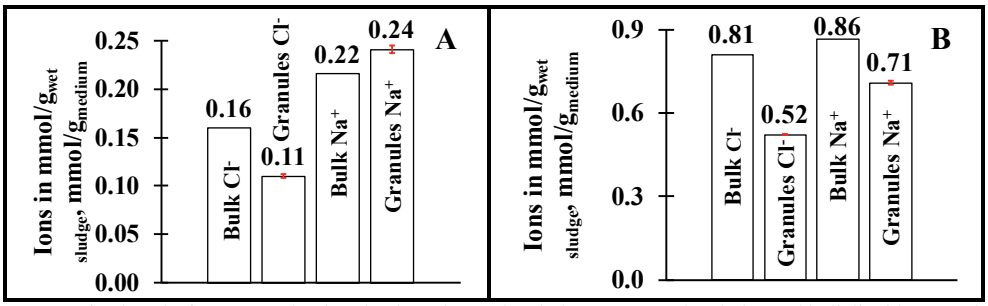


Figure 2. Na^+, Cl^- and K^+ concentration in salt adapted granular sludge. A – granular sludge and bulk liquid composition of R5 adapted to 0.22 M Na^+; B - granular sludge and bulk liquid composition of R20 adapted to 0.87 M Na^+. The error bars show absolute deviation from measurements of two separately prepared and digested 0.5 g sludge samples.

Potassium concentration in the R20 granular sludge before changing the medium was 0.05 mmol/$g_{wet\ sludge}$ (~ 0.02 g/$g_{sludge\ VS}$) (**Figure 3**). This corresponds to a 28 fold increase compared to the growth medium. Such a high concentration of potassium can be found within methanogenic cells upon exposure to high osmotic stress, even after accumulation of osmolytes within the microbial cells (Sowers and Gunsalus, 1995). When placing the R20 granules, previously exposed to 0.87 M NaCl, in a nutrient medium with KCl (0.87 M) the Cl^- concentration in the granular sludge did not change (**Figure 3**). However, Na^+ had almost completely been displaced with K^+ within 24 hours (**Figure 3**). After the medium change, the potassium concentration within the granular sludge reached a concentration of 0.81 ± 0.04 mmol/$g_{wet\ sludge}$ (~0.32 g/$g_{sludge\ VS}$), which is higher compared to the Na^+ concentration of 0.73 ± 0.002 mmol/$g_{wet\ sludge}$ in the control sample. This indicates a higher affinity of K^+ towards EPS in granular sludge compared to Na^+.

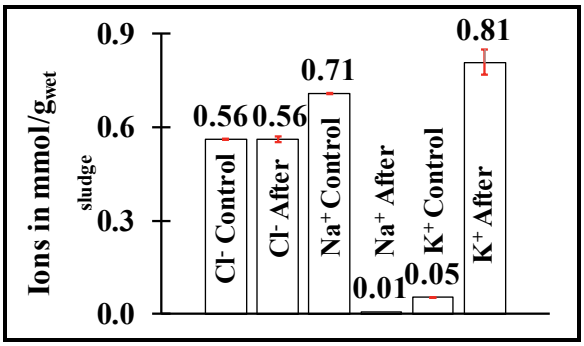


Figure 3. Ionic composition of R20 granular sludge after 24 hours in 0.87 M Na^+ medium (control) and 0.87 M K^+ medium (after). The error bars show absolute deviation from duplicate measurements.

Scanning electron microscopy with energy dispersive X- ray spectroscopy (SEM-EDX) of sliced microbial granules adapted to 0.87 M of NaCl showed a uniform distribution of sodium ions throughout the granules (**Figure 4-A**), confirming the ionic composition results in **Figure 2**. In contrast to the ion chromatography results (**Figure 2**), chloride could not be detected in the granules with SEM-EDX imaging (**Figure 4-B**). This could be a result of chloride ions removal together with water from the pores of the granular sludge during the dehydration steps of the SEM samples preparation (see Materials and Methods). This removal of chloride, coupled with sodium retention in the granules, suggests that sodium was bound to the EPS, whereas chloride was mainly present in the water phase of the granule pores as will be further discussed in *Section 4.2*.

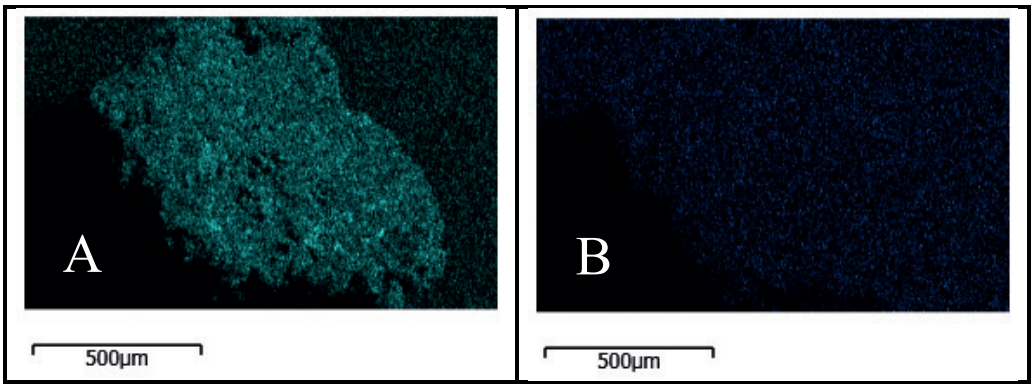


Figure 4. SEM – EDX micrographs of a sliced microbial granule adapted to 0.87 M NaCl. A – distribution of sodium throughout the granular structure. B – distribution of chloride throughout the granular structure. The black corner in the bottom left of the micrographs is a region from which the detector could not receive any signal due to the structure of the granule. No clear shape of a granule in B indicates that the background signal was as strong as the signal from the granule.

3.2. The ion exchange properties of EPS-membrane and ALG-membrane

The CLSM showed that binding of EPS with the inert binder PVDF-HFP was successful. Micrographs in **Figure 5-A, C** show a uniform distribution of EPS (in red, stained with Sypro RUBY) as a thin layer on the surface of the PVDF-HFP (auto-fluorescence in green). Such a distribution is likely due to the hydrophobic nature of the inert binder (PVDF-HFP), which could interact with the hydrophobic moieties of EPS, but did not allow for mixing of EPS throughout the depth of the membrane. The negative control micrographs of PVDF-HFP membranes (**Figure 5-B, D**) confirmed that SYPRO Ruby did not stain the binder and that the signal in **Figure 5-A, C** indeed originated from Sypro Ruby bound to EPS.

After successful preparation of the membranes as shown in **Figure 5-A, C**, their selectivity towards transport of potassium, sodium and chloride was tested in an electrodialysis cell. **Figure 6-I, II, and III** show changes in cation concentration (potassium and sodium) in compartment B and of chloride concentration in compartment A, for the ALG-membrane, EPS-membrane, and the commercial membrane, respectively. Cations (sodium and potassium) were preferentially transported across the membranes, whereas chloride ions were partially repelled. In addition, a preferential transport of potassium over sodium across the ALG and EPS-membrane was measured (**Figure 6-I**, **II**). The rate of change in ion concentration (determined by the slopes of the curves, m value) shows that in the ALG-membrane the potassium transport was 11 % higher and sodium transport was 23 % lower than in the EPS-membrane. The resulting potassium selectivity ($S_{K^+/Na^+} > 1$) is depicted in **Figure 6-IV** and was higher for the ALG-membrane compared to EPS-membrane. With the commercial ion exchange membrane, there was no markedly difference in the transport between potassium and sodium (**Figure 6-III**, **IV)**. In **Figure 6-IV,** the current efficiency shows how much of the current

was transported by cations. Despite the non-ideal cation selectivity of the “bio-membranes”, the current efficiency was relatively high: 79% for the ALG-membrane and 83% for the EPS-membrane. The commercial membrane gave the highest current efficiency of 92%.

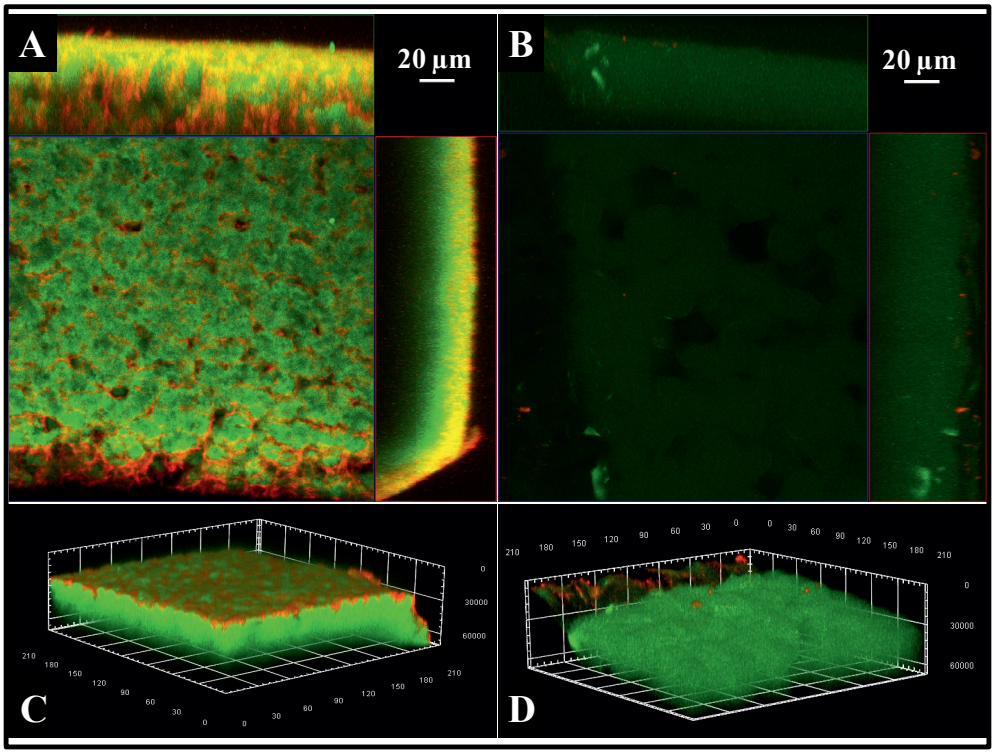


Figure 5. A, B - Orthogonal view of CLSM micrographs of membranes using maximum intensity projection and the respective 3D reconstructions in C, D. The membrane with the EPS in A, C. The green signal shows auto-fluorescence of the binder. The red signal shows EPS proteins stained with SYPRO Ruby. The bright yellow signal originates from the combination of green and red. The reference membrane composed solely of inert binder (PVDF-HFP) in B, D.

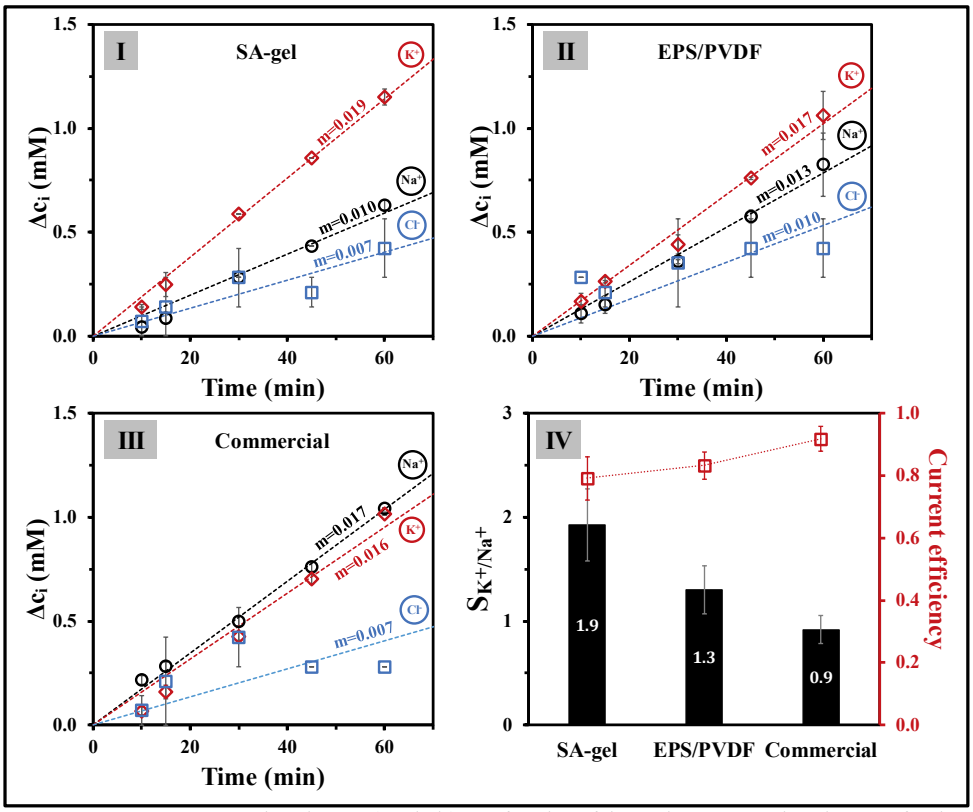


Figure 6. I, II, III - Ion concentration changes (symbols) as a function of time. The concentration changes of cations are measured in compartment B, whereas the concentration changes of anions was measured in compartment A. The m value indicates the slope of the dashed lines. IV - Potassium selectivity (S_{K^+/Na^+}) and current efficiency of the membranes. The error bars show absolute deviation in duplicate measurements.

3.3. Effect of anions and cations on specific methanogenic activity

Figure 7 – A shows that exposure of 0.22 M NaCl adapted granular sludge to equimolar concentration of KCl and KBr salts resulted in a decrease of methanogenic activity by 25.3 and 11.7 %, respectively, when compared to the blanc at 0.22 M of NaCl. Doubling the ion concentration to 0.43 M of KCl and KBr resulted in SMA decrease by 49.2 and 44.1%, respectively. The negative effect of sodium salts on the SMA of granular sludge at increased molarity of 0.43 M was considerably less pronounced compared to potassium salts (**Figure 7 – A**). The SMA decreased by 19.9 and 11.1 % with NaCl and NaBr salts, respectively. It seems that in general bromide salts had a slightly smaller negative effect on SMA compared to chloride salts (**Figure 7-A**). However, the results obtained with NaBr at 0.22 M (**Figure 7-A**) did not follow the overall trend, therefore additional experiments are needed in the future to confirm this.

Finally, the 0.87 M Na^+ adapted granular sludge could retain its methanogenic activity when exposed to equimolar concentration of NaBr (**Figure 7-B**). NaBr had no clear negative effect on the SMA,

whereas 0.87 M of potassium salts resulted in complete loss of methanogenic activity. Overall, the results of the SMA tests clearly show that at equimolar concentrations of monovalent salts, the methanogenic activity of salt (NaCl) adapted granular sludge was more influenced by changing the cations (Na^+ to K^+) than by changing the anions (Cl^- to Br^-).

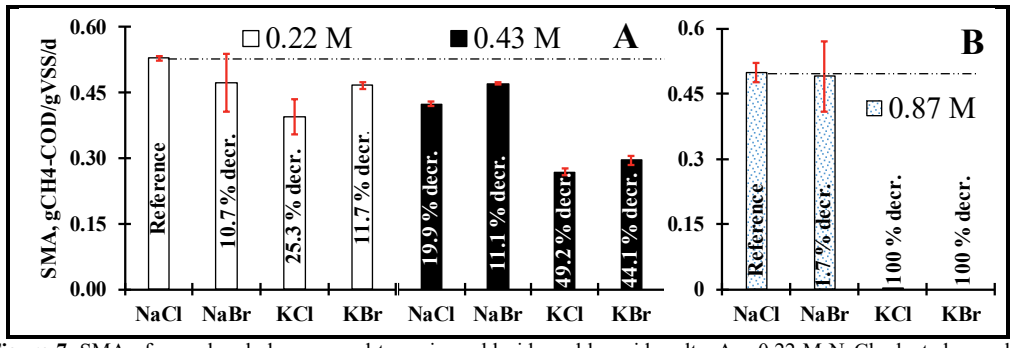


Figure 7. SMA of granular sludge exposed to various chloride and bromide salts. A – 0.22 M NaCl adapted granular sludge exposed to equimolar concentration and double concentration of NaCl, NaBr, KCl and KBr; B – 0.87 M NaCl adapted granular sludge exposed to equimolar concentration of NaBr, KCl and KBr. Error bars show standard deviations from triplicate experiments in B and Cl salts in A. Error bars with Br salts in A show absolute deviations from average in duplicate experiments. % decr. - % SMA decrease with respect to the reference. Horizontal dashed lines show the SMA of the reference.

4. Discussion

4.1. Cation exchange membrane properties of EPS and potassium selectivity

In one of our previous studies mannose-rich EPS of anaerobic granular sludge adapted to high salinity was shown to bind sodium (Gagliano et al., 2018). The current results clearly demonstrate that EPS extracted from UASB reactor granules treating saline wastewater and entangled in an inert binder (PVDF-HFP) behave as cation exchange membranes (CEM), i.e., partially repel anions, such as chloride and selectively transport cations, such as potassium and sodium (**Figure 2, Figure 4, Figure 6-I, II**). This is probably the result of the abundant presence of different negatively charged functional groups such as carboxylic, phenolic and phosphoric groups in EPS (d'Abzac et al., 2013). The same CEM ability was shown also for the ALG-membranes, most likely because of abundance of negatively charged carbonyl groups at neutral pH in alginate (Nestle and Kimmich, 1996; Lee and Mooney, 2012).

The EPS and ALG-membranes were not fully cation selective (**Figure 6**) since the transport of cations (sodium and potassium) represented ~ 80% of the total ionic current (**Figure 6-IV**). Thus, 20% of the ionic current can be attributed to transport of chloride. Such non-ideal cation exchange nature of EPS would be desirable from a microbial perspective, because ideal behaviour would prevent diffusion of important substrates, such as phosphate or acetate, to the microbial cells. From a physical-chemical perspective some transport of anions, such as chloride, through EPS-membranes and alginate-

membranes is expected due to their hydrogel nature. Hydrogels are known for their hydrophilic structure, and hence high sensitivity to water: high water uptake, leading to swelling or even dissolution (Lee and Mooney, 2012; Ahmed, 2015). The swelling in the membranes can create non-selective paths/channels for ion (K^+, Na^+, and Cl^-) passage. This is due to decrease of charge density caused by swelling in a membrane with a fixed amount of charges. Such decreased charge density may lead to decreased ion-functional group interactions. Optical coherence tomography (OCT) analysis (**Figure S2**) showed that a hydrated PVDF-HFP membrane was 50 µm thick, while addition of 10% w/w EPS increased the membrane thickness by 89 µm, clearly showing that swelling of EPS indeed took place.

Surprisingly, the EPS and particularly the ALG-membrane were more selective to potassium than to sodium (**Figure 6-IV**). Such selectivity was not measured in the commercial membrane (**Figure 6-IV**) and is known to be difficult to achieve with commercial membranes in general (Luo et al., 2018). It is not clear, which EPS and alginate properties resulted in such a selectivity and this certainly should be further looked into.

The current efficiency of ALG-membrane was very similar to that of the EPS-membrane (**Figure 6-IV**). Measurements of charge density show a markedly difference between alginate and EPS (**Figure S3**). In principle, membranes with high charge density favour the conductivity of counter-ions (Geise et al., 2013). Alginate had a charge density of 7.0 meq/g and EPS a charge density of 2.5 meq/g as measured for free dissolved polymers in solution. These values may be significantly different in the membranes themselves due to charge shielding. In ALG-membranes, the crosslinking with calcium ions may have led to a reduction of charged functional groups available for ion-exchange. In the EPS-membranes, some functional groups may have been completely embedded in the matrix of the supporting polymers (PDVF). Therefore, in future studies the real charge densities in the membranes themselves should be assessed, for example by acid-base titration (Hosseini et al., 2012).

4.2. Methanogenic activity inhibition by various ions

The methanogenic activity of 0.87 M NaCl adapted granular sludge was completely inhibited when this sludge was exposed to equimolar concentrations of KCl (**Figure 7- B**). Also, exposure of 0.22 M NaCl adapted granular sludge to 0.22 M of KCl led to a 25 % decrease in methanogenic activity. The decrease of SMA was smaller (19.9 %) when exposing the same granular sludge to 0.43 M NaCl (**Figure 7-A**). Kugelman and McCarty (1965), showed that potassium had a higher negative effect on methanogenic activity than sodium, using low salinity adapted sludge and equimolar concentrations of sodium and potassium bicarbonates. Our results extend this finding with chloride salts and high salinity adapted sludge and demonstrate that anions at equimolar concentrations have

little influence on the SMA (**Figure 7**). The mechanism of a potential Br^- or Cl^- toxicity on methanogens is not known. Possibly anion toxicity is "masked" by the ability of EPS to repel these anions, as was shown in this study (**Figure 2, Figure 6**). This "masking" would occur if granular structure could be viewed upon as a membrane (**Figure 8**), electroneutrality in which is achieved by the net negative charge of the EPS (**Figure S3**), balancing the positive charges of cations in the granules. The concentration of ions in membranes is determined by the concentration of fixed charged functional groups in it and meets the requirement of electroneutrality according to the following equation (Galama et al., 2013):

$$c_{counter-ion} - c_{co-ion} - X = 0$$

Where $c_{counter\text{-}ion}$ is the concentration of the ion with the charge opposite to the functional groups of the membrane (cations in the case of EPS), $c_{co\text{-}ion}$ is the concentration of ions with the same charge as the functional groups of a membrane (anions in the case of EPS), and X is the concentration of charged functional groups (negative for EPS). Due to the fixed negative charges of EPS with a concentration X, the concentration of negatively charged co-ions in the porous granular structure can potentially be reduced, while positively charged counterions are allowed to accumulate (**Figure 8**). The extent to which the amount of co-ions is reduced in the granular structure compared to the bulk liquid depends on X. Due to the fixation of the charged functional groups in EPS, the osmotic pressure experienced by the microbial cells can be reduced.

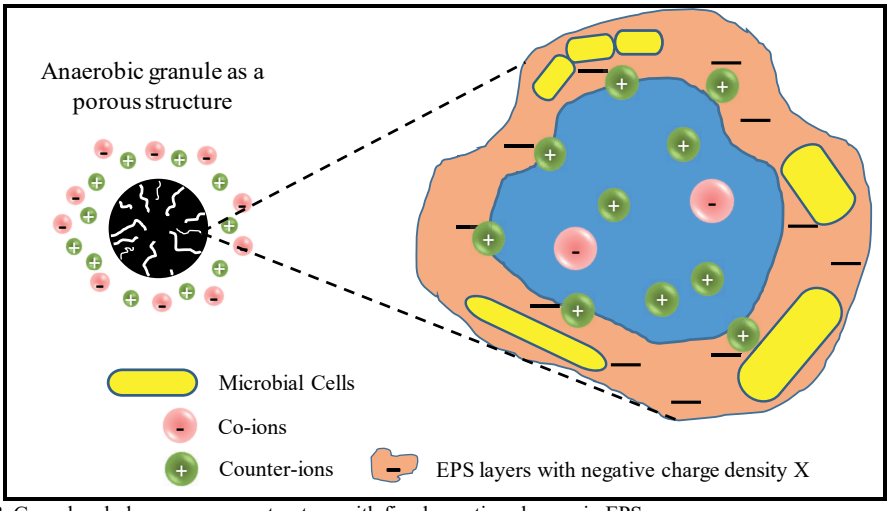


Figure 8. Granular sludge as a porous structure with fixed negative charges in EPS

This hypothesis can be tested with EPS deficient mutants of methanogens in toxicity experiments, and by comparing their sensitivity to different anions with EPS covered methanogens. Such an approach has been used by Wang et al. (2013) to study the role of EPS in osmo-protection upon exposure of *Klebsiella pneumoniae* to 100 mM of $CaCl_2$. This showed that in EPS deficient microbial cells the turgor pressure decreased upon exposure to salt, whereas in cells covered with EPS the turgor pressure remained unaffected. This suggests that EPS helps to counteract the osmotic pressure experienced by microbial cells in high salinity environments.

The mechanism by which high potassium concentrations inhibit methanogenic activity is not exactly known. Toxicity studies with high concentrations of metallic cations and other types of microorganisms demonstrated interference with membrane transport processes, either by competitively inhibiting membrane transporter proteins or by affecting the membrane potential (Harrison et al., 2007). Acetoclastic methanogens couple sodium transport across the cytoplasmic membrane with the transfer of the methyl group from tetrahydrosarcinapterin (H_4SPT) to coenzyme M (HS-CoM). This precedes the final reduction step for methane production from acetate (Welte and Deppenmeier, 2014). Also, acetoclastic methanogens make use of both proton and sodium gradients across the cell membrane for ATP synthesis (Welte and Deppenmeier, 2014). Abrupt exposure of the microorganisms to high concentrations of potassium could potentially interfere with these processes. Prolonged periods (more than a month) of exposure can result in adaptation of methanogens to K^+ concentrations of up to 0.19 M (Chen and Cheng, 2007). However, further increases of K^+ concentration in completely stirred continuously operated tank reactor resulted in decreasing methanogenic activity, similar to results of batch experiments in this study (Chen and Cheng, 2007). This suggests that adaptation of methanogens to high K^+ concentration is more difficult than adaptation to Na^+.

4.3. Can the EPS-membranes be applied for separation of sodium and potassium in practice?

Separation of two monovalent cations is a serious challenge, even for the most advanced technologies, such as electro-membrane processes. This is because these ions possess similar physicochemical properties, e.g., hydrated size. Most state-of-the-art ion exchange membranes lack selectivity for one specific ion (Luo et al., 2018). With the mimicked "bio-membranes" (ALG and EPS-membranes) as prepared in this study, a relatively high selectivity towards potassium over sodium ($S_{K^+/Na^+} > 1$), was observed, particularly for the ALG-membrane. Such selectivity was absent in a commercial cation-exchange membrane (**Figure 6-IV**). However, the membranes prepared in this study cannot be considered for commercial application at this stage due to their limited stability. Approximately 48 hours exposure of membranes to water leads to EPS detachment from the PVDF EPS-membrane

and to water leakage in the ALG-membrane (data not shown). In the EPS-membrane, this is likely due to weak adhesion between EPS (hydrophilic with hydrophobic moieties) and PVDF (hydrophobic), whereas in the ALG-membrane this is likely due to Ca^{2+} exchange with sodium, leading to disintegration of 3D structure of the gel (Lee and Mooney, 2012). Future studies need to focus on the EPS and sodium alginate properties that dictate the selective transport of potassium over sodium, which may help to develop analogous synthetic membranes.

5. Conclusions

An electrochemical method with EPS entrapped in a membrane like configuration was shown to be effective in characterising EPS affinity and selectivity towards monovalent ions. This allowed to demonstrate that EPS of anaerobic granular sludge adapted to high salinity behaved as a cation exchange membrane. Also, a well-studied EPS model compound – alginate – had similar properties, therefore the results may potentially be extrapolated to biofilms other than anaerobic granular sludge. The cation exchange nature of EPS together with the ionic composition measurements of granular sludge, in which considerably smaller amounts of Cl^- were measured compared to the bulk liquid, suggest that EPS of the granules function as a protective barrier against anions, such as Cl^-. For cations, such as Na^+ the extent to which EPS reduce their toxicity seems to be dependent on how well the cations can diffuse through the EPS. This protection was demonstrated to be much less for specific cations such as potassium.

Supplementary Information of Chapter 7

Appendix 1

For the visualization of PVDF-EPS membranes under CLSM, the auto-fluorescence of PVDF-HFP was tested on pure a PVDF-HFP membrane, previously hydrated in a solution containing 10 mM KCl and 10 mM NaCl. The PVDF membrane was analysed for autofluorescence with an inverted AxioObserver Zeiss LSM 880 confocal laser scanning microscope (CLSM; Carl Zeiss, Germany) using fluorescence excitation/emission filters with a 20×/0.8M27 Plan-Apochromat objective lens. The emission scans were performed with 9 nm bandwidth increments in the range of 410-695 nm excitation wavelength. The argon laser power was set to 1% for 488 and 458 nm excitation wavelengths. Acquired CLSM images were processed with the Zen Blue software (ZEISS, Germany). The obtained images are shown in **Figure S1**.

The PVDF membrane has high emission intensity in the range from 486 nm to 575 nm at excitation wavelength of 458 nm (**Figure S1-A**). The emission spectrum at excitation wavelength of 488 nm showed a maximum emission intensity in the range from 477 to 521 nm (**Figure S1-B**). This excludes the possibility to apply on the PVDF-EPS membranes fluorescent dyes falling in the same emission range.

SYPRO Ruby was chosen for EPS protein staining, because it has its maximum excitation at 458 nm and its maximum emission at 656 nm. This allowed to simultaneously scan the auto-fluorescence of PVDF (excitation 488 nm, max. emission 510 nm) and the protein portion of EPS by the fluorescence of SYPRO Ruby (excitation 458 nm, max. emission 656 nm).



Figure S1. Single pictures show the isolated emission spectra of the PVDF membrane after excitation with 458 nm (A) and 488 nm (B) lasers in the emission range from 415 nm to 691 nm. In red the emission spectra with highest intensities are identified.

Figure S2

To study the thickness of the hydrated (in 10 mM NaCl + 10 mM KCl) membranes, a spectral domain Optical Coherence Tomograph (OCT) (Thorlabs Ganymede OCT System, USA) was used. The OCT was equipped with a 5x telecentric scan lens (Thorlabs LSM03BB, USA) with a maximum scan area of 100 mm2. The OCT engine was configured to provide high-resolution images with a sensitivity of 106 dB at 1.25 kHz A-scan rate.

The results in **Figure S2** show a large increase of membrane swelling upon mixing PVDF with EPS. Upon addition of 10% (w/w) of EPS to PVDF the thickness of the PVDF membrane increased from 50 μm (**Figure S2-A**) to 139 μm (**Figure S2-B**).

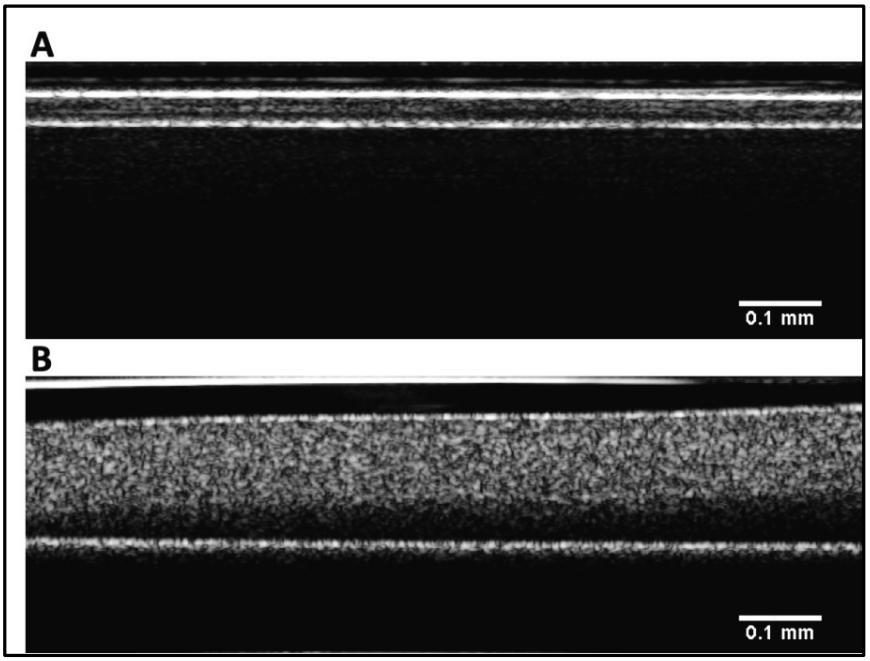


Figure S2 – OCT micrographs of PVDF membrane (A) and PVDF mixed with 10% (w/w) EPS (B). The thickness of the membrane is 50 μm in A and 139 μm in B.

Figure S3

To compare the charge density of EPS and alginate used for preparation of the EPS-PVDF and SA-gel membranes, a colloid titration method was used, as described in Ajao et al. (2018). In short, 10 mL of samples (1 mg/L) were titrated against 0.01 mM poly-diallyldimethylammonium chloride (pDADMAC) using a Mütek Particle Charge Detector (PCD03) with an automatic titrator (Metrohm titrando 888). The titrant was added in steps of 0.02 mL, while simultaneously recording the streaming potentials (mV). The charge density was then calculated on the basis of titrant volume added to reach the point of 0 charge (streaming potential equals to 0 mV). The charge densities of samples were measured at their original pH (below 6) in mili-Q and at pH adjusted to 7. 0.1 M of NaOH/HCl was used for the pH adjustment.

In **Figure S3** the charge density of EPS extracted from several sludges grown in laboratory scale reactors at 20 g Na^+/L and 5 g Na^+/L and of alginate used to prepare SA-gel membranes. The charge density of EPS used to prepare EPS-PVDF membranes corresponds to the sample *Tryptone 20 g Na*. A variety of EPS was tested to confirm if EPS from anaerobic sludge of various reactors would have similar charge density.

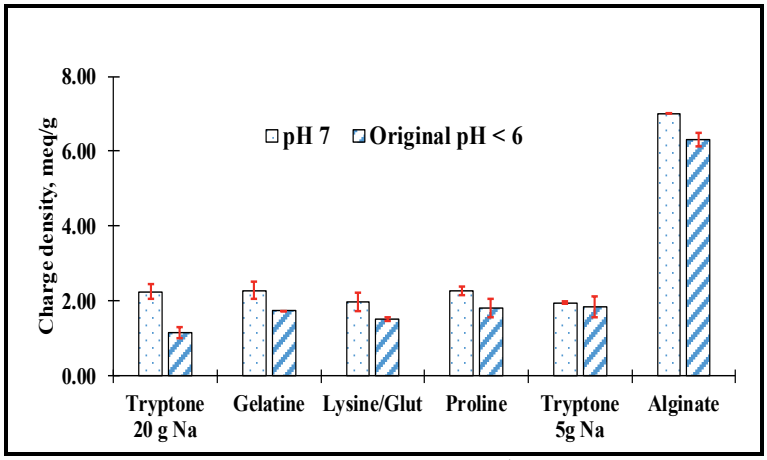
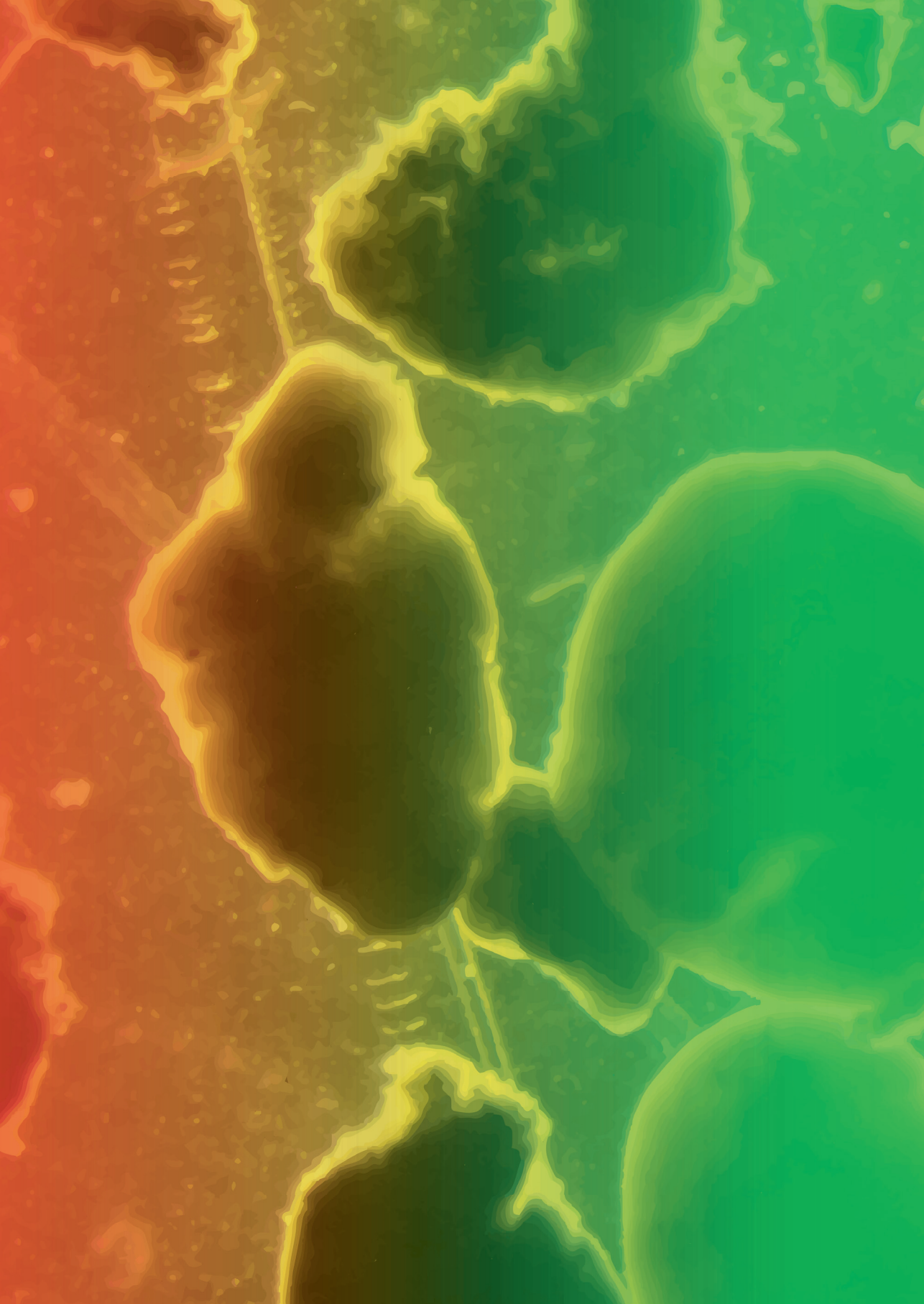


Figure S3. Charge density of various anaerobic sludges grown at 20 g Na^+/L (Tryptone 20 g Na, Gelatine, Lysine/Glut, Proline), at 5 g Na^+/L (Tryptone 5 g Na) and of alginate used for SA-gel membrane preparation.



Chapter 8. General Discussion and Outlook

1. Rationale behind this research

Several industrial sectors, such as petroleum, natural gas, agro-food, fisheries, leather tanning and petrochemical industries produce highly saline wastewater and it is estimated that globally approximately 5% of the total amount of generated wastewater is hypersaline (salinity above 3.5%) (Lefebvre and Moletta, 2006; Xiao and Roberts, 2010; Ali et al., 2019). Since many of these streams have a high soluble organics content, anaerobic biological treatment is a good alternative because it converts the organic pollutants into energy rich biogas and produces relatively small amount of surplus sludge. For removal of soluble organics at non-saline conditions the most compact, simple and cost-effective anaerobic industrial wastewater treatment plants nowadays rely on granular sludge formation, i.e., sludge with excellent settling properties and a high methanogenic activity (Hulshoff Pol et al., 2004; Van Lier, 2008; Van Lier et al., 2015).

For treatment of wastewaters containing high monovalent salt (NaCl or KCl) concentrations granular sludge technology has often been regarded unsuitable due to (suspected) inhibition of methanogenic activity (Jeison et al., 2008; Fang et al., 2011; De Vrieze et al., 2016a) and disintegration of the granules (Gomec et al., 2005; Jeison et al., 2008; De Vrieze et al., 2016a). However there is increasing evidence, that sufficient methanogenic activity can be sustained also at saline conditions (NaCl concentrations above 25 g/L) and some evidence that granular sludge exposed to high salinity can increase in size in upflow anaerobic sludge blanket (UASB) reactors (De Baere et al., 1984; Ismail et al., 2010; Roberts et al., 2016). Despite these findings, before this research, nobody had focused on anaerobic sludge granulation at high salinity from dispersed biomass, which prevents clear conclusions about the applicability of granular sludge technology at high salinity. Thus, the core focus of this PhD research was on the mechanisms of sludge granulation from dispersed biomass at high salinity, microbial strategies to deal with osmotic stress at such conditions and from this identify methods to mitigate problems preventing granulation in practice.

A systematic approach was followed to study anaerobic sludge granulation at saline conditions. This resulted in scientific findings regarding the mechanisms of this granulation process and in findings that will help to implement anaerobic granular treatment of saline wastewaters in practice. In **Chapter 1** of this thesis we introduced the complex structure of anaerobic sludge granules and complex interactions that exist within this structure. **Figure 1** gives an overview of various aspects with respect to sludge granulation and microbial osmoadaptation that were investigated in the different experimental chapters of this thesis. In *Section 2* of this discussion chapter, the main outcomes of each experimental chapter of the thesis will be briefly described in the context of the existing knowledge, whereas in *Section 3* the necessity to study toxicity of other compounds in combination

with high salinity and to explore stability of granular sludge process under salt varying conditions is discussed. Also, the strategy of continuous dosing of proteins for granulation and the influence of inoculum biomass on granulation at high salinity is considered. Finally, the findings of this thesis with respect to granulation and osmoadaptation mechanisms are reviewed and possible approaches to further elucidate on these findings are proposed. *Sections 4,5 and 6* will explain the relevance of the results for fresh water granulation, for combining anaerobic-aerobic biological wastewater treatment that could allow to remove organics, nitrogen and phosphorous and relevance for applications in the domestic wastewater domain, respectively. Finally, in *Section 7* it is discussed how the results of the executed research can reduce the environmental impact of industries producing highly saline wastewater.

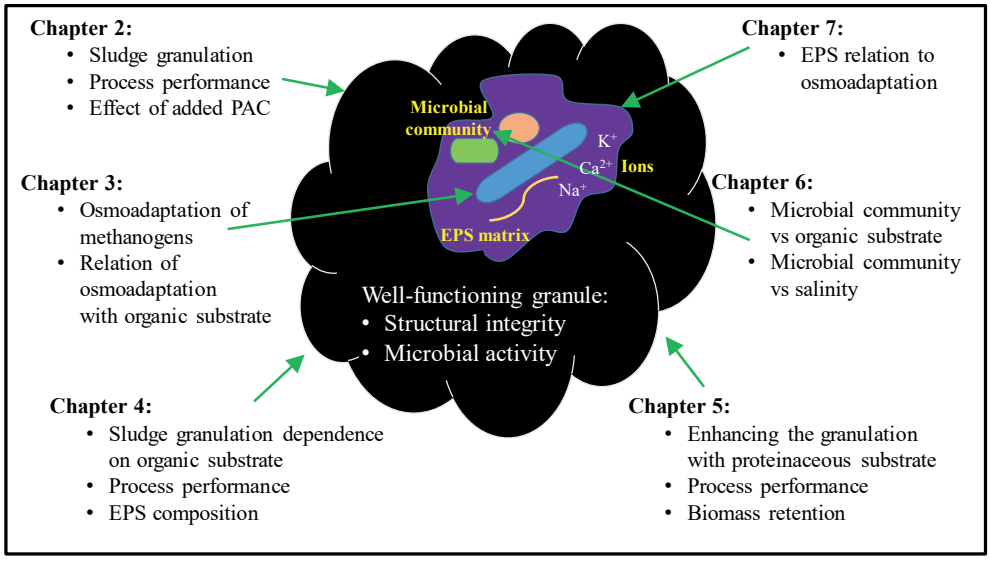


Figure 1. Various levels of detail, investigated with respect to sludge granulation at high salinity. EPS – extracellular polymeric substances; PAC – powdered activated carbon.

2. State of the art knowledge and the outcomes of this research

In **Chapter 2** it was demonstrated that with a reactor start-up procedure shown to favour fresh-water granulation and, when treating a complex energy rich organic substrate consisting of glucose, tryptone and acetate, anaerobic granular sludge formation from dispersed biomass can be achieved at high (20 g/L of Na^+) salinity levels and this is accompanied with good process performance. Granulation was achieved surprisingly fast (after 45 days of reactor operation) and the results confirmed the hypothesis that with wastewaters containing energy rich organic substrates and with a moderately halophilic (adapted to 8 g Na^+/l) inoculum granulation from dispersed biomass is possible. As illustrated in **Figure 2** and summarized in **Table 1**, the first investigations in the field of wastewater treatment suggesting that methanogens can tolerate high salinity, and anaerobic wastewater treatment at high

salinities (Na^+ above 10 g/L) should be feasible, were published as early as in the 90s of the 20th century (De Baere et al., 1984). The first publication suggesting that also granular sludge technology might be applicable at such high salinity was published only 25 years later by Ismail et al. (2010), who reported an increase of the size of sludge granules, but deteriorating strength properties (**Table 1** and **Figure 2**). The results presented in this thesis have shown that sludge granulation at saline conditions can be achieved from dispersed biomass. Thus, granular sludge technology is clearly shown to be feasible at such saline conditions.

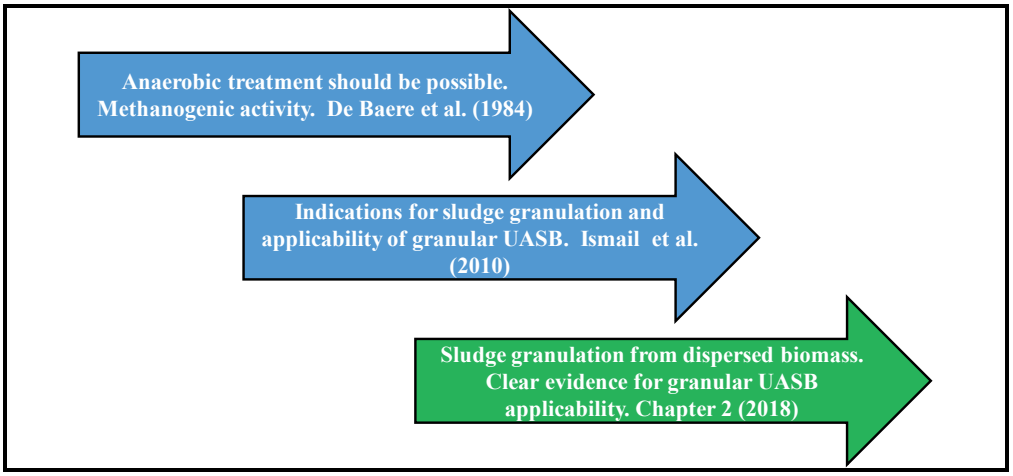


Figure 2. Build-up of knowledge towards application of granular UASB reactors for treatment of saline wastewater

The positive, but perhaps unexpected outcome of the methanogenic sludge activity and sludge granulation at high (20 g Na^+/L) salt concentrations as presented in **Chapter 2** required for a mechanistic explanation. This is of particular importance, because also very recent research results have shown the opposite i.e., inhibition of methanogenic activity and/or disintegration of sludge granules at high salinity. For example, Muñoz Sierra et al. (2019) reported granular sludge disintegration and UASB reactor failure, while treating phenolic wastewater at 26 g Na^+/L salinity. In the same study it was shown that in anaerobic membrane bioreactors with efficient sludge retention, sufficient methanogenic activity can be achieved to treat such wastewater (see **Table 1**). Ali et al. (2019) reported poor methanogenic activity and process performance in anaerobic baffled reactors treating mono-ethylene glycol based wastewater at 14 g Na^+/L.

Table 1. The build-up of positive experience with anaerobic wastewater treatment at high salinity before. TOC – total organic carbon; VLR – volumetric organic loading rate; COD – chemical oxygen demand.

Findings	Reference
Good methanogenic activity	
In semi continuous anaerobic filters TOC removal above 90% can be achieved at Na^+ conc. as high as 22 g/L and VLR of 20 g COD/L/d after 45 days of gradual increment of salinity. Inoculum from a conventional sewage sludge digester.	(De Baere et al., 1984)
Sludge inoculum adapted to fish canning wastewater can tolerate up to 15 g Na^+/L without methanogenic inhibition in laboratory scale batch reactors. VLR and COD removal efficiency for the enrichement culture not reported.	(Balslev-Olesen et al., 1990)
Pure cultures of *Methanosarcina* can grow at salinity levels as high as 23 g Na^+/L (Sowers and Gunsalus, 1995)	(Sowers and Gunsalus, 1995)
Acetoclastic *Methanosaeta* can grow at 35 g Na^+/L with minor inhibition compared to growth at 10 g Na^+/L	(Kita et al., 2016)
In an anaerobic membrane reactor inoculated with moderately halophilic inoculum (adapted to 8 g Na^+/L) gradually (in a year) further adapted to 24 g Na^+/L above 90% COD removal effciency treating phenolic wastewater at VLR of 6.6 g COD/L/d	(Muñoz Sierra et al., 2019)
Indications for applicability of granular sludge technology	
Size of granules in UASB reactors operated at 0.8 m/h, 20 g Na^+/L and VLR of 17.5 g COD/L/d increases, but the strength decreases. COD removal efficiency above 90 %, but with a decreasing trend. After ~ 130 days of reactor operation, abrupt sludge washout and sharp decrease in COD removal. Inoculum moderataly halophilic (adpated to 8 g Na^+/L)	(Ismail et al., 2010)
Increasing granule size in a UASB reactor at 11 g Na^+/L and VLR of ~ 6 g COD/L/d[a]. Poor COD removal efficiency of ~ 50%. Upflow velocity not reported. Granular inoculum treating brewery wastewater. Reactor operated for 240 days with gradual increments of salinity.	(Li et al., 2014)
Size of granules in UASB reactors operated at 0.8 m/h, 20 g Na^+/L and VLR of 12.5 g COD/L/d increases. COD removal above 90%. Addition of calcium (1g/L) can increase strength of granules but decreases activity. Inoculum adapted to 8 g Na^+/L and reactors operated for 120 days	(Gagliano et al., 2017b)
Clear evidence for applicability of granular sludge technology at high salinity	
Sludge granulation is achieved at 20 g Na^+/L from dispersed biomass in UASB reactors operated at an upflow velocity of 1 m/h. Calcium only supplied with the nutrient medium (13 mg/L). COD removal efficiency above 90% at VLR of 16 gCOD/L/d. Inoculum adapted to 8 g Na^+/L. Reactors operated for 220 days	This thesis. Chapter 2 (2018)
Granulation at 20 g Na^+/L from dispersed biomass in a UASB reactor operated at 1 m/h. Inoculum adapted to 8 g Na^+/L. COD removal 95.4 % at VLR of 7.9 g COD/L/d. Reactor operated for 1 year.	This thesis. Chapter 4 (2019)

[a]Calculated from reported hydraulic residence time, influent COD and reactor volume

The experiments in **Chapter 3** and **Chapter 4** were designed to test the hypothesis (see **Chapter 1**) that proteinaceous organic substrates, after their hydrolysis, play a crucial role for osmoadaptation of methanogenic archaea and can enhance the granulation at saline conditions by providing energetically favourable "building blocks" for extracellular polymeric substances (EPS). This hypothesis was based on the knowledge that:

- in response to osmotic stress pure cultures of methanogens accumulate nitrogen containing osmolytes, such as alpha and beta amino acid derivatives or the zwitterionic N-acetyl-β-lysine (Sowers et al., 1990; Sowers and Gunsalus, 1995; Roberts, 2005);
- uptake of osmolytes is favoured over synthesis *de novo* due to bioenergetic considerations (Oren, 1999; Roberts, 2005; Oren, 2013);
- nitrogen containing osmolytes can be synthesized from amino acids (Galinski and Trüper, 1994; Pflüger et al., 2003);
- addition of nitrogen-containing osmolytes (mainly betaine) to batch experiments alleviates osmotic stress of methanogens (Vyrides and Stuckey, 2016);
- EPS of anaerobic granules consist mainly of proteins (Fukuzaki et al., 1995; Zhou et al., 2006; Ismail et al., 2010).

Figure 3 summarizes our key findings reported in **Chapters 3, 4 and 5**, about the role proteinaceous substrate has on the performance of anaerobic granular reactors operated at high salinity. Nitrogen containing osmolytes were identified to be the key compounds to achieve stable granules, good sludge methanogenic activity and reactor performance at a salinity of 20 g Na^+/L. Osmolyte biosynthesis precursor compounds (amino acids) contained in proteins and proteinaceous substrates can alleviate the negative effect of a hyperosmotic shock on methanogenic activity (**Chapter 3**). The results of **Chapter 4** showed that proteinaceous organic substrates can dramatically enhance the rate of granular sludge formation at high salinity. Finally, it was experimentally validated that the amount of proteinaceous substrate needed for enhancing granular sludge formation and process performance can be estimated based on very simple osmotic pressure calculations (**Chapter 5**). These calculations were directly related to wastewater salinity and theoretical biomass yield allowing for a significant decrease of proteinaceous substrate in the wastewater compared to the arbitrary chosen concentrations in **Chapter 2** and **Chapter 4**. Besides demonstrating the positive effects of proteins on methanogenic activity, the results obtained in **Chapter 3** revealed glutamate and N-acetyl-β-lysine as the osmolytes accumulated by microbial community of salt adapted granules. Typically, osmolytes have been studied in pure cultures of microorganisms and before this research osmolytes accumulated by mixed anaerobic consortia were examined only by Vyrides et al. (2010), who also found N-acetyl-β-lysine

along with trehalose and betaine as the main osmolytes in anaerobic membrane reactors exposed to high salinity.

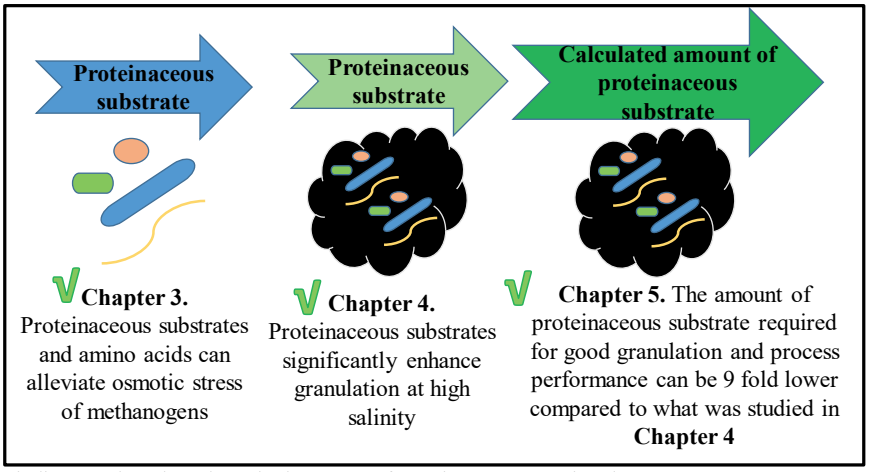


Figure 3. Findings on the role and required amounts of proteinaceous organic substrate.

Under non-saline conditions, *Methanosaeta* cells have often been reported as the most abundant methanogens in granular sludge (Rinzema et al., 1988; Narihiro et al., 2008; van Haandel et al., 2014) and are known to enhance granulation in their filamentous form (Wiegant, 1988; Li et al., 2015b). Molecular microbial community and microscopy analyses presented in **Chapter 6** revealed the morphology and identity of microorganisms in relation to different salinity levels (5 and 20 g Na^+/L) and organic substrates (**Figure 4**). It was shown that similarly to non-saline conditions, *Methanosaeta* in its filamentous form was the most abundant methanogen, irrespective of the type of organic substrate or salinity (5 or 20 g Na^+/L). The dominant active bacteria at 5 g Na^+/L belonged to the genus *Streptococcus* and at 20 g Na^+/L the dominant active bacteria were members of the family *Defluvitaleaceae*. Both of these bacteria appeared as filaments while the granules developed in time. Replacement of complex proteinaceous substrate with amino acids (leucine and proline) at 20 g Na^+/L resulted in the disappearance of *Defluvitaleaceae* and consequent absence of filamentous bacteria which negatively affected granule formation from the dispersed inoculum. Thus, even though the mechanism still remains unclear, this suggests that not only filamentous *Methanosaeta,* but also filamentous bacteria are important for granulation (**Figure 4**).

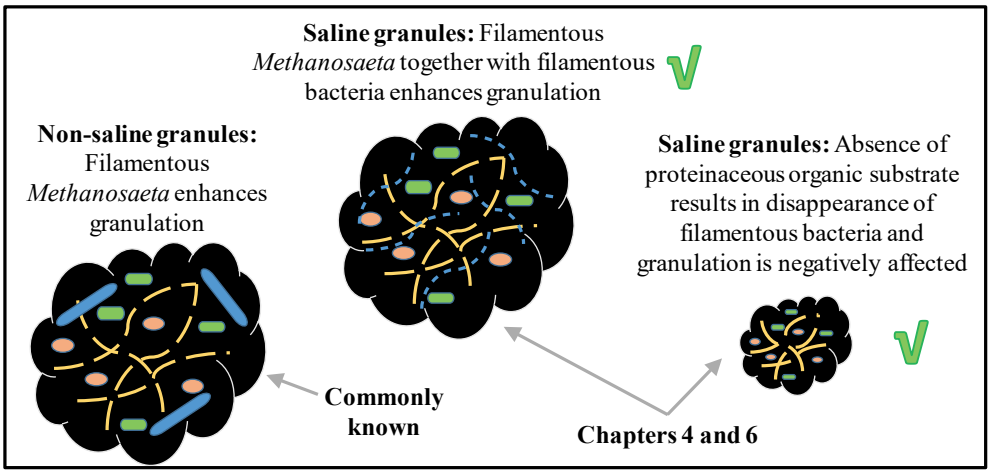


Figure 4. Microbial community in response to salinity and organic substrate. References for non-saline conditions in the paragraph above this figure.

Microbial EPS synthesis is reported to increase upon exposure of microbial communities to high salinity and this increase is attributed to microbial adaptation to osmotic stress (Vyrides and Stuckey, 2009a; Corsino et al., 2017; Campo et al., 2018). However, why increased EPS production could alleviate salt stress was not explored. Work done previously by Gagliano et al. (2018) suggested that EPS rich in mannose residues could bind and immobilize sodium ions, thereby lowering the osmotic stress (Gagliano et al., 2018). At high salt concentrations microorganisms are exposed to both, cations and anions and also the role of microbial EPS in acclimation to high anion concentrations required further elucidation. For this purpose, in **Chapter 7** ion-exchange properties of EPS extracted from high salinity adapted granular sludge were investigated with EPS-based ion exchange membranes. This showed that EPS behave as cation exchange membranes and partially repel anions such as chloride. Thus, EPS can act as a protective barrier against anions. Interestingly, the EPS more selectively transported potassium than sodium. The former was also shown to be more toxic towards methanogens, suggesting that EPS properties may also determine the extent to which microorganisms are exposed to certain cations.

3. Anaerobic granular treatment of hypersaline wastewater streams: a research field to further explore

Although **Chapters 2-7** resulted in a number of scientific and practical insights into the process of sludge granulation and applicability of high rate anaerobic digestion at high salinity (*Section 2*), many research questions remain unexplored. Also, several more applied laboratory or pilot-scale experiments are needed before anaerobic granular sludge treatment of saline wastewaters can be put to practice with real wastewater. Below some of these questions/topics will be discussed in more detail.

3.1. Combining high sodium concentration with other potentially toxic compounds as they occur in saline wastewater streams

Saline wastewaters may contain toxic compounds other than sodium, which can potentially affect microbial activity and thereby also sludge granulation. For example, cheese whey wastewater can contain very high concentration of proteins (up to 34 g/L) (Carvalho et al., 2013). Anaerobic conversion of protein leads to release of ammonium (Stams, 1994; De Vrieze et al., 2015), a compound in its unionised form occurring at elevated pH (above 7.5) known to inhibit methanogenesis. The concentration at which inhibition occurs varies broadly and has been reported at total ammonium nitrogen concentrations between 1.4 and 14 g/L (Chen et al., 2008a). It must also be noted that similarly to adaptation to salinity, methanogenic adaptation to high total ammonium concentrations is also possible (Koster, 1986; Pechan et al., 1987). Leather tanning industry wastewater may contain high concentrations of sulphates (up to 1.2 g/L), sulphides (up to 0.9 g/L) and trivalent chromium (up to 0.3 g/L) (Lofrano et al., 2013). In anaerobic bioreactors sulphate is reduced to hydrogen sulphide by sulphate reducing bacteria (SRB), therefore sulphide can potentially reach high concentrations in the bioreactors (Roberts et al., 2016). Unionised sulphide (H_2S) is known to negatively affect methanogenic activity at concentrations between 50 to 400 mg/L (Chen et al., 2008a). Trivalent chromium has been reported to cause strong inhibition of acidogenesis at concentrations of approximately 50 mg/L (Yu and Fang, 2001). Olive oil milling wastewater can contain high concentrations of potassium (up to 6.9 wt%) (Paredes et al., 1999), toxicity of which was demonstrated in **Chapter 7** and was also reported elsewhere (Onodera et al., 2013).

Also high concentrations of multivalent cations, such as magnesium and calcium were not studied in this thesis, while they are abundant in, for example, seawater (Oren, 2013) and therefore often in fishery wastewater. Petroleum industry wastewater can contain as much as 51 g/L and 4 g/L of calcium and magnesium, respectively (Fakhru'l-Razi et al., 2009). On a molar basis, multivalent cations have been shown to inhibit methanogenic activity even more compared to monovalent cations

(Kugelman and McCarty, 1965). For sludge granulation a small amount (e.g., up to 300 mg/L of Ca^{2+}) of multivalent cations can be beneficial, whereas much higher concentrations can lead to cementation (calcium carbonate precipitation) of the sludge bed and further decrease of methanogenic activity (Yu et al., 2001). The examples listed above highlight the need to further investigate sludge granulation and microbial activity at high salinity, while including potentially toxic compounds as they would occur in industrial wastewaters. Probably the best approach to tackle this would be the use of a well-characterized industrial wastewater of interest in pilot or laboratory scale studies. This would allow for inclusion of all possible synergistic and antagonistic effects of different compounds present in the stream as they would occur in practice. However, to gain more fundamental understanding, the use of synthetic streams, while including such potentially toxic compounds is recommended. It could be that addition of small amount of proteins can alleviate the osmotic stress of cations other than sodium because it allows for production of osmolytes thereby preventing excessive influx of these ions in the microbial cells. This should be further investigated.

3.2. Time varying salinity in granular bioreactors

All reactor experiments were performed at a constant salinity level. However, the composition and concentration of industrial wastewaters can vary in time and increases or decreases in salinity can occur throughout the year (Lefebvre and Moletta, 2006). Thus far only one study on abrupt salinity fluctuations in continuously operated anaerobic reactors has been published. Vyrides et al. (2010) reported deteriorating process performance when exposing submerged membrane bioreactors to salt increases from 0.2 to 35 g/L of NaCl and improving process performance upon subsequent salinity decreases back to 0.2 g NaCl/L. The effects on granular sludge stability and process performance under salinity varying conditions in continuous reactor experiments have not been reported. Also during this research preliminary experiments on the effect of salt fluctuation on granular sludge adapted to 5 and 20 g Na^+/L were performed. The experimental setup and methods is briefly described in **Box 1**.

Box 1. Two UASB reactors were started up at 5 and 20 g Na^+/L following the same procedure, in the same setup and with the same medium composition as described in **Chapter 2**. After an operation period of 90 days, wastewater salinity was fluctuated by switching the influent feed of the two reactors. During the first experiment the influent salinity was changed from 5 to 20 g Na^+/L and vice versa and kept stable (at 5 or 20 g Na^+/L) for two weeks before changing it back to the level at which the reactor was adapted. During the second experiment the influent salinity of the UASB reactors was changed every 48 hours for three weeks. The effluent COD analysis was performed as described in **Chapter 4**.

The results showed that process performance in terms of COD removal efficiency of 20 g Na^+/L adapted granular sludge was less negatively affected compared to the reactor adapted to 5 g Na^+/L, especially after frequent salinity fluctuations for a period of three weeks (**Figure 5).** Also, clear differences between the two reactors were found with respect to granule stability after salinity fluctuations every 48 hours for three weeks. The sludge in 20 g Na^+/L adapted UASB reactor remained granular, whereas in 5 g Na^+/L adapted UASB reactor the granules disintegrated, causing significant biomass washout (**Figure 6**).

Obviously, such experiments need to be repeated to ensure reproducibility. However, the initial outcomes have important practical implications that need to be further investigated, i.e., they indicate that granular sludge adapted to high salinity is robust when exposed to decreasing salinity followed by exposure to high salinity to which the granules were adapted. It would be interesting to examine, whether exposure of 20 g Na^+/L adapted granules to fluctuations of increased salinity would yield similar results as observed with 5 g Na^+/L adapted granules when exposed to 20 g Na^+/L.

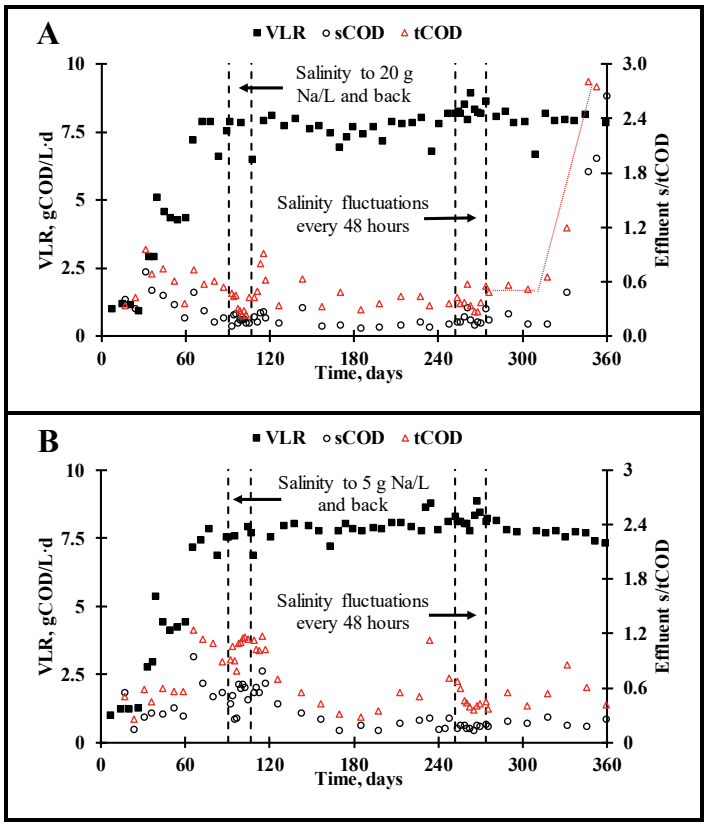


Figure 5. Effect of salinity fluctuations on effluent total (tCOD) and dissolved (sCOD) chemical oxygen demand. UASB reactor adapted to 5 (A) and 20 (B) g Na^+/L. The dotted red line in A is provided to guide the reader's eye. VLR – volumetric organic loading rate, g COD/L/d.

In practice short term fluctuations of wastewater composition can be levelled off by installing a buffer tank in front of the biological reactor (Lettinga and Hulshoff Pol, 1991; Chen et al., 2008b), although this requires additional space and higher investment costs. The results in **Figure 5** and **Figure 6** suggest that if the wastewater of a particular industry is expected to be highly saline throughout most of the year with decreasing salinity during some periods, the buffer tank may not be needed. This is, of course, assuming salinity is the most important environmental variable. For wastewaters with other fluctuating parameters, such as pH or organics concentration, some buffering capacity will already be included in the design of a treatment plant (Lettinga and Hulshoff Pol, 1991; Chen et al., 2008b). It would be of interest to investigate how large a buffering capacity is needed to reduce the negative effects of salinity, particularly when sludge is adapted to low salinity and exposed to increases of salt concentrations.

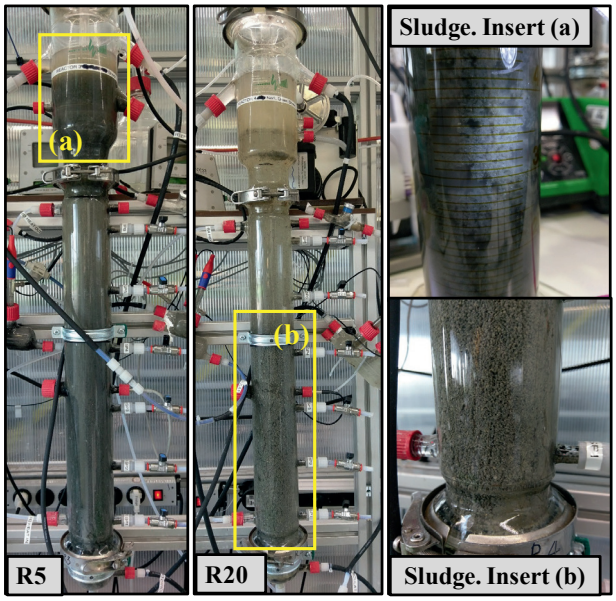


Figure 6. Sludge in UASB reactors adapted to 5 (R5) and 20 (R20) g Na^+/L after 3 weeks of receiving varying influent salinity.

3.3. Proteins for anaerobic granular reactor start-up instead of continuous dosing

Besides alleviating osmotic stress, at high salinity, proteins also enhance the rate of granulation. To accomplish this approximately 1.8% of the wastewater COD should be proteinaceous (**Chapter 5**). Thus, for a bioreactor designed to treat 100 m^3 of wastewater per day with an influent COD of 5000 mg/L and a salinity of 20 g Na^+/L, approximately 2.8 tons of protein per year is required. This is equivalent to 90 mg/L of proteinaceous COD. Dosing such amounts of protein may still not be feasible. However, many wastewaters, especially from industries producing goods of animal origin are likely to contain sufficient amount of protein without the need for additional dosing.

In **Chapter 4** it was found that after prolonged UASB reactor operation (~180 days) dense granule-alike sludge can also develop at high salinity without dosing protein. Even though not truly granular, the sludge had excellent settling properties and provided a high COD removal efficiency. The question remains whether it is possible to temporarily dose proteins/peptides to stimulate and speed-up the granulation process and omit the addition of proteins thereafter. Such a strategy is much cheaper and less laborious than continuous dosing of proteins. Thus, a systematic study is required to elucidate how such an approach affects the synthesis of EPS (type/amount), the active microbial population and granulation process.

3.4. Can granulation at high salinity also be achieved with freshwater inoculum?

In all executed reactor experiments presented in **Chapters 2, 4 and 5** the same inoculum biomass (adapted to ~ 8 Na^+/L) was used. Experiences with fresh water inocula have thus far shown that the acclimation time to higher salinities can vary between 40 days (De Baere et al., 1984) up to a year (Roberts et al., 2016). Unfortunately, the microbial community composition was not monitored in these studies. Our experience has shown that as a response to salinity bacterial communities shift, whereas methanogenic communities remain similar at different salinity levels (**Table 2**). Thus, it should be investigated if certain bacterial communities present in the inoculum allow for more prompt adaptation to salinity and thereby also faster granulation and reactor start-up. Such knowledge would allow for better selection of inocula for seeding reactors operated at high salinity. Albeit the results suggest that different outcomes with respect to granulation in our experiments were obtained solely due to the conditions applied in the reactors, it would be interesting to perform similar experiments, with a different inoculum, for example digested sewage sludge or septic tank sludge. This is of practical and scientific importance as the availability of salt adapted inocula, such as used in our study, probably is limited.

3.5. Osmoadaptation in granular microbial communities

In **Chapter 3** osmolytes were identified that were used by the microbial community and the efficiency of proteins and amino acids to alleviate hypersalinity shocks on acetoclastic methanogens was investigated. **Figure 7** illustrates the knowledge that was gained and points out research questions that still need to be addressed. The nitrogen-containing compounds glutamate and N-acetyl-β-lysine were shown to act as osmolytes. Also, addition of proteins or amino acids, such as aspartic acid and glutamic acid can alleviate osmotic stress on acetoclastic methanogens in anaerobic granular sludge. *In silico* genome analyses of biomass sampled from our reactors suggests that N-acetyl-β-lysine may be synthesized from aspartic acid (**Figure 8**) by the dominant methanogen in the granules (closely

related to *Methanosaeta harundinacea*) (Gagliano et al., 2018), thereby explaining the efficiency of aspartic acid to reduce osmotic stress (**Chapter 3**).

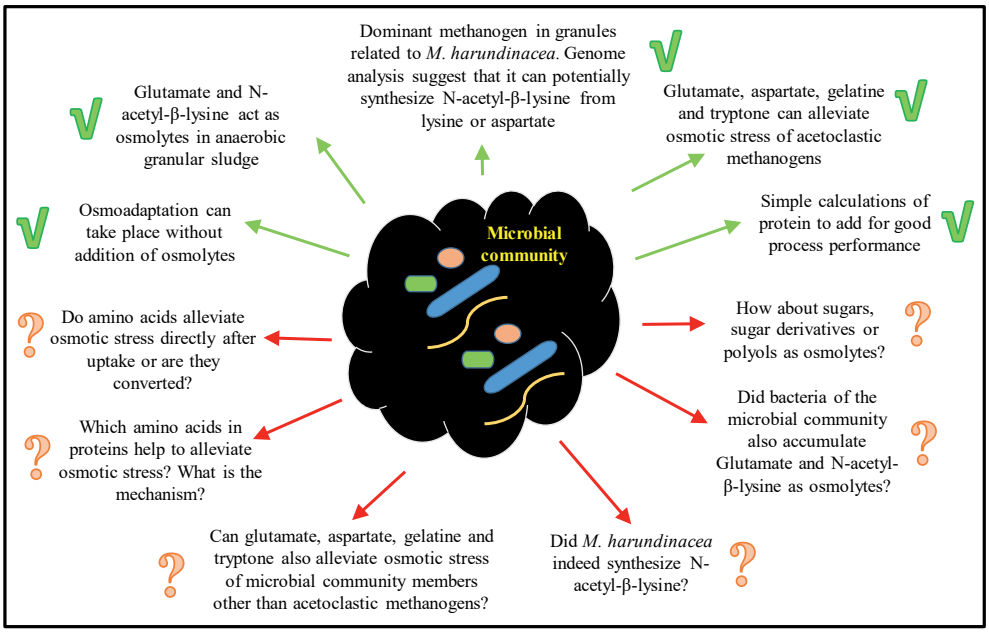


Figure 7. Osmoadaptation results and remaining research questions.

It must be noted osmolytes from pure cultures of *Methanosaeta* strains have never been isolated, implying that we may have shown another methanogenic archaeal genus synthesizing and accumulating this zwitterionic osmolyte. However, the experiments were performed with mixed cultures (granular sludge) and further investigations are necessary to prove that N-acetyl-β-lysine was indeed accumulated by *Methanosaeta harundinacea* cells and not by other microbial community members, and that during hypersalinity shocks aspartic acid was further converted into N-acetyl-β-lysine. Direct proof of the ability to synthesize N-acetyl-β-lysine by *Methanosaeta* cells can be obtained by isolating the microorganism and analysing extracts of cells grown at high salinity, as has been done with pure cultures of methanogens by e.g., Martin et al. (2000). Unfortunately isolation of pure cultures of microorganisms can be very difficult or even impossible (Keegan et al., 2016). A more effective approach is offered by metagenome and metatranscriptomics analysis (Keegan et al., 2016). This requires extraction and sequencing of RNA and good knowledge of bioinformatics to analyse the obtained results (Peimbert and Alcaraz, 2016). Complementary to molecular techniques experiments with 13^{C}-labelled compounds can be performed in order to follow the fate of e.g., amino acids during hypersalinity experiments. This technique relies on the possibility to detect mass differences between isotopes with the use of modern instruments, such as mass spectrometers and, similarly to metatranscriptomics and metagenomics, would require sophisticated analysis of the

obtained data (Tang et al., 2009). The tools required to answer the questions in **Figure 7** emphasize the need for collaborative research between process engineers, microbiologists, experts in modern analytical techniques (e.g., mass spectrometry) and bioinformaticians.

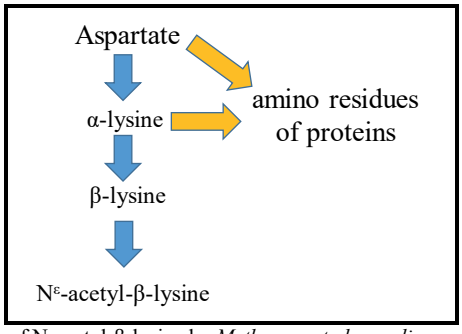


Figure 8. Putative synthesis steps of N-acetyl-β-lysine by *Methanosaeta harundinacea*.

In this study the focus was on osmotic stress alleviation of methanogens. This was motivated by the fact that methanogens are often reported to be inhibited at high salinity (Rinzema et al., 1988; Liu and Boone, 1991; De Vrieze et al., 2016a). However, some researchers have suggested that propionate utilising bacteria are even more sensitive to high salinity than methanogens (Feijoo et al., 1995; Vyrides and Stuckey, 2009a). Such conclusions are debatable due to the dependency of propionate oxidising bacteria on syntrophic hydrogen consumers (Stams and Plugge, 2009) and therefore the difficulty to identify which group is inhibited most. Results in this thesis in **Chapters 2 and 5** did not show increasing effluent propionate concentration in time. However, batch activity experiments to study salt inhibition of propionate degraders in comparison to methanogens were not performed. It is therefore of interest to further study osmotic stress alleviation not only of methanogens, but also of the bacterial community. The first step could be to perform hyposalinity experiments with high salinity adapted granular sludge and analysing, if osmolytes other than glutamate and N-acetyl-β-lysine, such as sugars or their derivatives, are released. This may give an idea of the pool of osmolytes used by high salinity adapted anaerobic granular sludge. The individual molecules subsequently can be tested for their efficacy to alleviate osmotic shock of, for example, sugar fermenters. It must be noted that glutamate – an osmolyte released during the batch experiments described in **Chapter 3** – can potentially be accumulated by both, methanogenic archaea and also a wide diversity of bacteria (Roeßler and Müller, 2001). For example it was shown to be accumulated by mixed cultures of aerobic granular sludge that are expected to consist mainly of bacteria (Wan et al., 2014). Also, a molecule very similar to N-acetyl-β-lysine (N-acetyl-α-lysine) was recently found to be synthesized by marine bacteria (*Planococcus maritimus*) using aspartate as its precursor (Joghee and Jayaraman, 2014). Therefore both, glutamate and aspartate, can serve as the first compounds for testing their efficacy for alleviation of osmotic stress in microorganisms other than methanogens.

3.6. Unravelling the mechanisms of sludge granulation

In **Chapters 4-5** the effect of the organic substrate on sludge granulation and EPS composition was investigated and in **Chapter 6** development of microbial communities was studied. Also, the granulation process and microbial community development was compared at different salinities, but for the same influent composition (**Chapters 2 and 6**). The most important findings of these chapters are explained in *Section 2* and summarised in **Table 2**.

Table 2. Granulation at high salinity with respect to organic substrate, the dominant active microorganisms in granular sludge at the end of experiments and EPS sugar and protein content of granules in wt%. n.d. – data not available. *results from flocculent sludge

Organic substrate and COD ratio	Length of Experiment, days	g Na^+/L	Granulation/ day sludge bed formed	wt % EPS protein/wt % EPS sugars	Dominant active methanogen at the end of experiment	Dominant active bacteria at the end of experiment
Glucose: Acetate: **Tryptone** (3:2:1)	216	20	**Yes/43**	80/7.5	*Methanosaeta (23.6%)*	*Defluvitaleaceae (34%)*
Glucose: Acetate: **Tryptone** (3:2:1)	216	5	**Yes/~45**	76/9.0	*Methanosaeta (18.7%)*	*Streptococcus (10.8%)*
Glucose: Acetate: **Gelatine** (3:2:1)	160	20	**Yes/42**	85/10.4	n.d.	n.d.
Glucose: Acetate: **Starch** (3:2:1)	240	20	**Yes/180**	77/12.4	n.d.	n.d.
Glucose: acetate: **leucine: proline** (3:2:0.5:0.5)	120	20	**No/*Biofilm*** formed on reactor walls	77/8.7*	*Methanosaeta (13.5%)**	*Enterococcus (22%)**

From an engineering perspective, this overview (**Table 2**) gives a guideline for decision-making, when considering granular sludge reactors for treatment of saline wastewater streams. It becomes clear that soluble-protein-containing wastewaters allow for faster sludge granulation compared to wastewaters that do not contain proteins. This in turn allows for a prompt reactor start-up, i.e., for reaching the design organic loading rate, if 'adapted' granular sludge is not available.

Proteins have been demonstrated to be the major components of EPS, irrespective of the salinity at which the reactors were operated, the organic substrate in the wastewater or the type of sludge developed (granular or flocculent). Also the sugar content of all EPS was similar (**Table 2**), showing that traditional "bulk" component characterisation cannot provide sufficient information about the relation between EPS and granulation. A step further was made in **Chapter 4** by analysing the amino acid and sugar composition of EPS, which showed similar amino acid composition in the EPS of granules in the reactor fed with starch and tryptone. The sugar composition in both cases was different. This suggests that EPS proteinaceous components have a more generic composition, whereas sugars vary depending on the type of organic substrate. However, also the monomer

composition does not allow gaining mechanistic insights into interactions between different EPS macromolecules and microbial communities.

To better understand the mechanisms of granulation, key is to gain more knowledge about the *relation* between *organic substrate, EPS, granulation and composition of the microbial community* (**Figure 9**). Obviously, the active microbial community converts the organic substrate into biomass, including EPS and biogas. But are the *dominant microorganisms* also those which produce the *key EPS components for granulation*? The most challenging task is to characterize the chemical structure of the EPS macromolecules, because such characterisation requires EPS solubilisation, which can in turn cause structural changes of these molecules compared to the insoluble form found in the granules (Seviour et al., 2019). Development of methods to achieve this chemical characterisation should be the key focus in future, because in combination with modern molecular tools it could potentially allow to assign EPS production to certain microorganisms (*Section 3.5*). Furthermore, knowledge of the chemical structure of these EPS macromolecules can allow a better study of the interactions amongst them and assign their functions, such as providing mechanical stability to the granules.

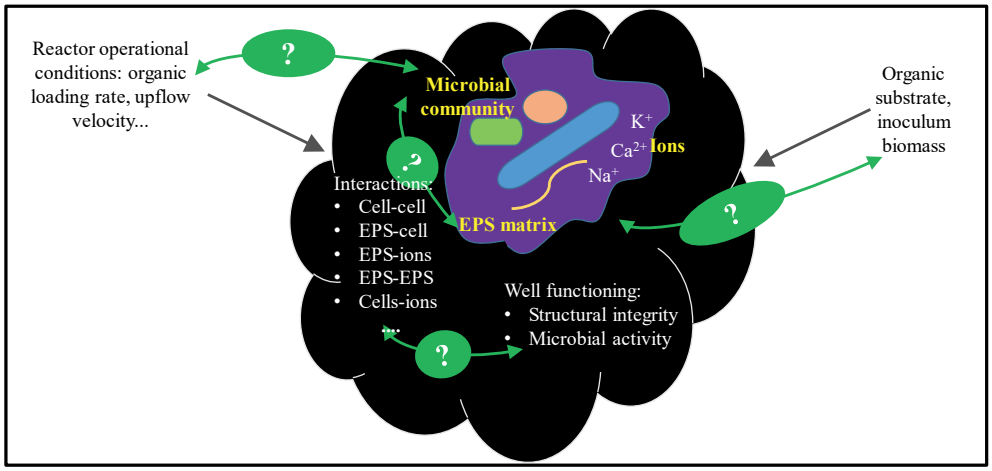


Figure 9. Sludge granulation: the "coctail" of interactions.

The results of collaborative research between the Department of Environmental Technology and the Laboratory of Microbiology, as reported in **Chapter 6,** show that also techniques such as fluorescence in situ hybridisation (FISH) coupled to epifluorescence microscopy can provide substantial understanding of which active microorganisms are aggregating and how their morphology changes in time. Additionally, confocal laser scanning microscopy (CLSM) coupled to lectin staining and FISH can provide information on 3D organisation of EPS components and microorganisms in hydrated granular sludge (Reichhardt and Parsek, 2019). Such research was performed by Gagliano

et al. (2018) to understand this organisation in granular sludges formed at different salinities during the experiments of **Chapter 2**. These kinds of analyses do not provide definitive information on which EPS component is produced by which microorganism and does not allow reconstructing the exact chemical structure of EPS. However, it can deepen our understanding on organisation of microbial communities and EPS in aggregates that are eventually formed with different substrates (**Chapter 6**) and at different salinities (Gagliano et al., 2018). Future studies should focus on the initial stages of granulation, while applying the abovementioned techniques. The challenge associated to such an approach will be the need of iterative screening of different FISH probes and lectin stains as EPS composition and microbial communities change in time.

Studies on how EPS interact (e.g., form gels) and what the bulk properties of EPS are can also be done with extracted exopolymers "outside of the granules" as shown in **Chapter 7.** However, the outcomes of this should still be interpreted with care and if possible confirmed in hydrated granules because the extraction procedure may change the quantity (Comte et al., 2006a) and properties (Comte et al., 2006b) of the EPS. Such an approach together with knowledge about the chemical composition of EPS, their (mechanical) properties and interactions may lead to potential commercial applications. As an example of such properties selectivity of extracted EPS towards potassium was already shown in **Chapter 7**.

4. From anaerobic sludge granulation at saline conditions to fresh water conditions

Anaerobic sludge granulation at non-saline conditions has been studied for decades and a large number of full-scale granular sludge reactors treating various industrial wastewaters have been installed (Van Lier, 2008; Van Lier et al., 2015). UASB reactor start up can be very rapid (less than a week) when the reactors are seeded with granular sludge (Van Lier, 2008). However, even with an increasing number of full-scale reactors, large projects still may require start-up with flocculent biomass if granular sludge is unavailable (Paques personal communications, 2018). In this case tools/strategies to achieve rapid sludge granulation would be very helpful. Also, knowledge on how the organic substrate may influence the granulation process is important in this context (**Chapters 4 and 5**). But can the findings of this research also be applied for sludge granulation at non-saline conditions? The first step for answering such a question should be a comprehensive literature review summarising the past findings on granulation with various organic substrates and operational parameters. Some excellent earlier work has shown that granular sludge can be formed within 40 to 100 days in reactors fed with a mixture of volatile fatty acids (VFAs) (Hulshoff Pol et al., 1983; Fukuzaki et al., 1991c; Fukuzaki et al., 1995), a mixture of VFAs and ethanol (O'Flaherty et al., 1997), sugars (Fukuzaki et al., 1995; Zhou et al., 2006), ethanol (Fukuzaki et al., 1995), starch

(Fukuzaki et al., 1995), and lactate (Fukuzaki et al., 1991a). From these studies several observations can me made:

- Sludge granulation in these studies was achieved, while supplying the reactors with a vitamin mixture or yeast extract (100 mg/L);
- Without exception, EPS of all granular sludges analysed consisted mainly of proteins;
- The lack of consistency with respect to operational parameters (e.g., influent COD, loading rates, type of inoculum biomass (granular/flocculent), concentration of multivalent cations) across different studies (and sometimes also within one study) does not allow for a clear comparison with respect to time needed for granulation depending on the organic substrate.

Albeit this still needs to be researched in depth, these observations suggest that a small amount of proteins in the wastewater may also be crucial for granulation under freshwater conditions. This is worthwhile investigating in experiments similar to those performed in this study.

5. Integrated solutions: granular sludge technologies for COD, N and P removal from industrial wastewater

Anaerobic granular treatment technology cannot remove nitrogen (N) and phosphorous (P) (Tchobanoglus et al., 2003), and its effluent may still contain some biodegradable COD (see **Chapters 2, 4 and 5**). Thus, a polishing step is required, especially if the effluent is eventually released to a receiving water body or considered for re-use. As mentioned in **Chapter 1**, this particular project was a part of the bigger Water Nexus project, the aim of which is to develop technology trains for saline wastewater treatment. To gain insights in the potential of further biologically polishing the effluent of UASB reactors operated at high salinity, experiments were performed with sea water adapted aerobic granular sludge – a technology developed by a Water Nexus consortium member at TU Delft (**Figure 10**). Aerobic granular sludge technology is an attractive alternative for conventional activated sludge (CAS) processes because, similarly to anaerobic granular sludge, it relies on formation of dense microbial aggregates with excellent settling properties (Bengtsson et al., 2018). This allows for significant reduction of the wastewater treatment plants (WWTP) volume (33%), approximately 75% reduction of the area occupied by the WWTP , and to save approximately 58-63% of electric energy compared to CAS systems (de Bruin et al., 2004; Pronk et al., 2015; Bengtsson et al., 2018).

The results of these experiments (not published) showed that the phosphate concentration of the UASB reactor effluent could be decreased from 36.3 down to 25.4 mg PO_4-P/L and the concentration of COD was decreased by approximately a half (from 400 to 200 mg/L). Also, the results suggested

that the nitrifying bacteria were inhibited by the high sodium concentration in the UASB reactor effluent. Nevertheless, these outcomes showed promising possibilities to couple anaerobic-aerobic granular treatment of saline industrial wastewater for removal of COD and phosphorous. In principle, also nitrification should not present a problem as others have already shown that both partial nitrification (Wan et al., 2014) and simultaneous nitrification and denitrification (Corsino et al., 2016) can be achieved at salinity levels as high as 20 g Na^+/L, while the more relevant anammox process seems to be inhibited at salinity levels above 12 g Na^+/L (Kartal et al., 2006; He et al., 2019). Further studies should elucidate if long term exposure of aerobic granular sludge to saline effluents of UASB reactors would result in development of microbial communities capable of efficient nitrogen removal.



Figure 10. Granular UASB reactor (left), the aerobic granular sludge cultivated in TU Delft (right) and the PhD candidates (in the middle) with the author of this thesis in front and Danny de Graaf at the back.

6. Thinking beyond industrial wastewater: relevance for domestic sewage treatment

Currently most modern domestic sewage treatment plants rely on conventional activated sludge (CAS) treatment processes mineralizing organics into CO_2, removing nitrogen and phosphorous and generating large amounts of bio-solids (Wan et al., 2016). Also domestic sewage treatment plants in some cases face the need to treat saline wastewater due to salt water intrusion into the sewer in the coastal areas or runoff of salty water in the municipal sewer during de-icing of roads (van den Akker et al., 2015; Bengtsson et al., 2018). In these cases conventional treatment plants are not designed to receive saline wastewater and the salinity may inhibit the biological reactors. However, the growing global fresh water scarcity has already forced some densely populated areas, such as Hong Kong, to consider the use of seawater for toilet flushing (Liu et al., 2019) and pilot plants relying on aerobic granular sludge technology have already been tested for this purpose (personal communication Mario Pronk, Royal HaskoningDHV, The Netherlands, 2017). Obviously, the future infrastructure development should be focused on decentralised sanitation with low freshwater use and recovery of

nutrients, such as phosphorous and nitrogen, recovery of biogas and reuse of stabilised sludge in agriculture (de Graaff et al., 2011; Zeeman, 2012). However, with current infrastructure in place for most developed countries, the transition to new sanitation will require time. This means that in the largely populated and developed coastal areas domestic sewage in the near future may become more saline. In this scenario the results of this thesis may become relevant in terms of the anaerobic microbial activity attainable at high salt concentrations (**Chapters 2, 4**), when applying for primary and secondary sludge digestion, that is produced in large excess (Appels et al., 2008; Duan et al., 2012; Wan et al., 2016). The salinity of these sludge streams is expected to be similar to that of the wastewater. Thus, the finding of sufficiently high microbial activity at high salinity to convert complex organic stream into CH_4 (**Chapter 2, 4 and 5**), together with possibilities to further increase these activities by addition of osmolytes (**Chapter 3**) become relevant (Appels et al., 2008; De Vrieze et al., 2015). It must be recognised, that WAS typically contains high concentrations of particulate COD (5-6 wt% dry solids after thickening) and the hydrolysis of particulate COD at high salinity was not investigated during this research. Since hydrolysis of particulate COD is the rate limiting step (and not methanogenesis) in anaerobic conversion of complex particulate wastes into biogas (Appels et al., 2008), it is important to study if this conversion step at high salinity can proceed successfully. It is known, that for particulate substrate the available substrate surface area limits the hydrolysis rate, whereas for soluble substrate the enzyme activity (Sanders et al., 2000; Sanders et al., 2002; Vavilin et al., 2008). During this research sufficient hydrolytic activity was achieved for hydrolysis of proteins and carbohydrates but it is not known if a similar outcome will be found for the digestion of particulate substrates.

7. Reducing the environmental footprint of industrial wastewater treatment

The aim of this thesis was to investigate the possibility of expanding the application of high rate anaerobic industrial wastewater treatment to saline conditions. The results showed that anaerobic wastewater treatment of hypersaline (salinity above 35 g/L of NaCl) streams is possible (**Chapters 2, 4 and 5**). In the European Union (EU) industrial water uptake for production of goods is approximately 30 billion m^3 per year, excluding water used for irrigation in agriculture. Additional 170 billion m^3 of water are used to support some industrial processes, for example as a heat carrier in cooling towers for electricity production. After use by the industry the quality of this water is deteriorated and requires treatment (EEA, 2018). A recent report by the European Environment Agency showed that only approximately 40% of European surface water bodies are in good ecological state (EEA, 2018). Thus, it is of utmost importance to protect the water bodies from influx of pollutants and for this purpose environmentally friendly technological solutions for the treatment of industrial effluents are needed. It is not clear what fraction of the total amount of industrial

wastewater is saline. However, our estimations of the amounts of only few wastewater streams in **Chapter 1** and also estimations of others (Lefebvre and Moletta, 2006; Fakhru'l-Razi et al., 2009) show that this volume is considerable. Assuming that the outcomes of this thesis with respect to granulation and process performance (**Chapters 2, 4 and 6**), can be extrapolated to full-scale applications, the impact would be enormous. To illustrate this, below two treatment options for removal of organic contaminants from saline cheese whey wastewater as an example will be compared: 1) conventional activated sludge (CAS); 2) anaerobic biological treatment. Cheese whey wastewater, depending on the productions process, can contain between 0.5 and 10% (wt/vol) of salt. This range of possible salinity explains why anaerobic digestion of cheese whey wastewater has been reported before (Prazeres et al., 2012). Also, recovery of proteins from this wastewater is becoming more attractive than conversion into biogas (Prazeres et al., 2012). However, even after recovery of proteins this wastewater can still contain a considerable concentration of COD in the form of lactose (0.18 – 60 g/L) and therefore requires treatment before discharge (Prazeres et al., 2012).

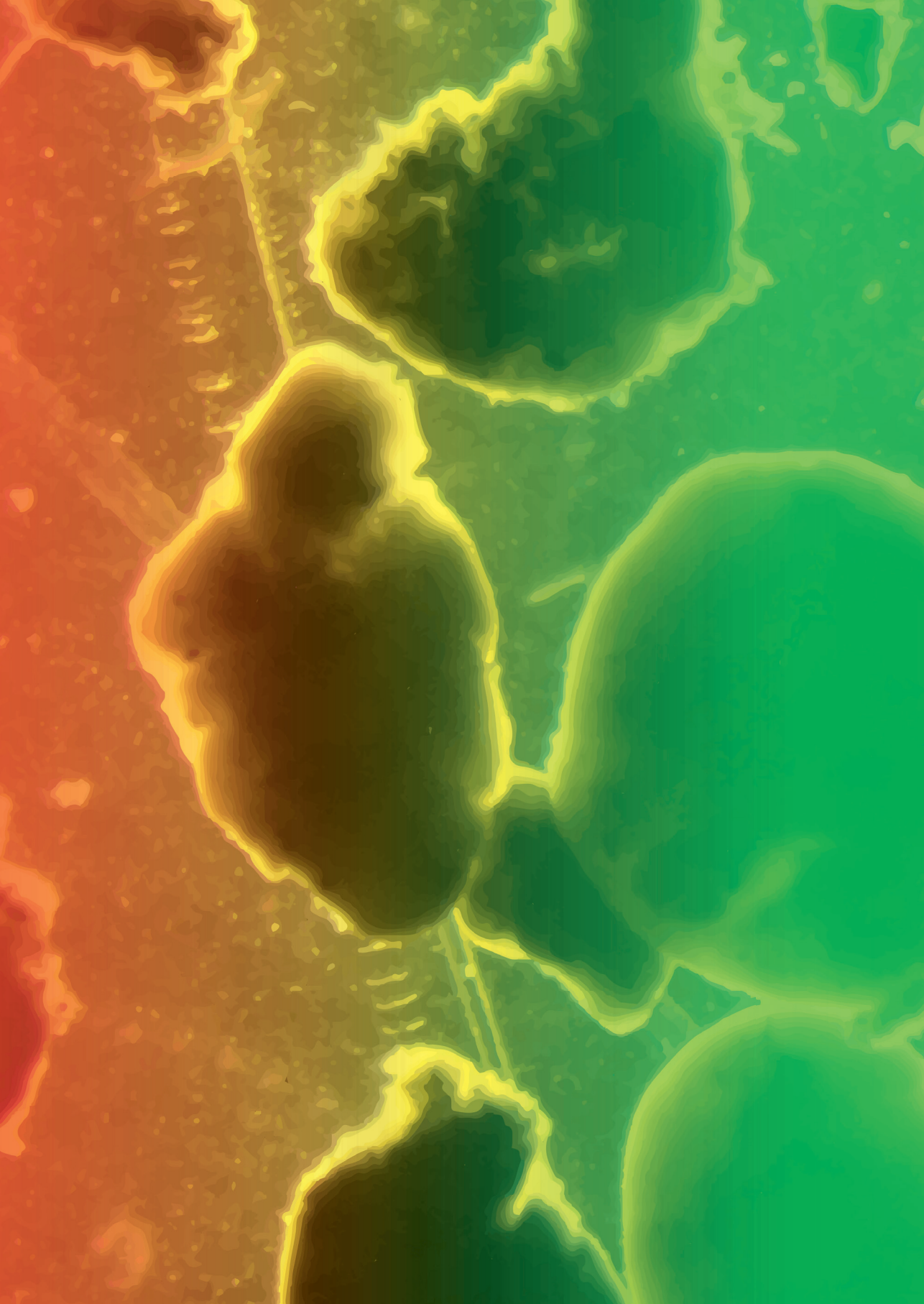
In **Chapter 1** it was estimated that within the EU alone approximately 224 million m^3 of cheese whey wastewater is generated annually. It is assumed that all of this wastewater contains 10 g/L (can reach up to 100 g COD/L) of biodegradable chemical oxygen demand (bCOD) (Carvalho et al., 2013) and that both types of treatment techniques can achieve 98% bCOD removal efficiency. It is estimated that on average 1 kWh of fossil energy is saved per kg of COD removed, if CAS treatment is replaced by anaerobic treatment (Van Lier et al., 2015). This means that anaerobic treatment of such a stream can save approximately 2.2 TWh of fossil energy, i.e. enough energy to supply all households of a country like Latvia with approximately two million residents with electricity (Eurostat, 2019a; b). Global warming due to emission of greenhouse gases such as CO_2 is a well-recognised problem and these emissions should be minimised (Peter, 2018). If this electricity would be produced in a modern plant using coal as a fuel with CO_2 emissions of 751.8 g CO_2/kWh (Chiesa et al., 2005), approximately 1.65 million tons of CO_2 would end up in the atmosphere. Furthermore, the CAS treatment would result in considerably higher production of surplus sludge compared to anaerobic treatment and would require more extensive treatment prior to disposal (Appels et al., 2008). The aerobic surplus sludge production can be estimated with a conservative yield of 0.4 kg of sludge produced per kg of COD converted (Tchobanoglus et al., 2003). Anaerobic biomass yield value can be calculated from mass balances presented in **Chapter 5** and the yield is approximately 0.1 kg biomass produced per kg COD converted. This would result in 0.88 and 0.22 million tons (dry weight) of surplus sludge produced by aerobic and anaerobic treatment, respectively. Thus, anaerobic treatment would save considerable costs with respect to sludge treatment and disposal.

It must be noted, that the calculations above, are not intended to yield exact numbers of CO_2 emissions or energy savings, but only to indicate the impact anaerobic digestion would have when implemented for saline industrial wastewater treatment.

Anaerobic biological treatment in UASB reactors can be performed with well settling flocculent sludge as well as with granular sludge. If granulation can be accomplished, as was demonstrated in this thesis, this would offer huge advantages in terms of smaller sized bioreactors due to increased biomass concentrations, which in turn reduces the investment costs to build the treatment plants (Hulshoff Pol et al., 2004). The maximum loading rate studied in the granular sludge reactors of this research, was approximately 16 g COD/L/d (**Chapter 2**), while achieving 98% COD removal efficiency. At this loading rate the treatment of all cheese whey wastewater (224 million m^3 per year) would require a total bioreactor volume equivalent to approximately 150 Olympic swimming pools (2500 m^3 each). Typical applicable organic loading rates in CAS with high rate aeration system ranges between 1.2 and 2.4 g COD/L/d (Tchobanoglus et al., 2003). This means that the required reactor volume of CAS would range between 1002 and 2005 Olympic swimming pool equivalents. Construction of the bioreactors requires materials, such as concrete – a mixture of sand, gravel, cement and water. The cement industry is one of the largest anthropogenic sources of CO_2 emissions (Deja et al., 2010). Hence, the application of more compact anaerobic bioreactors can even further reduce the CO_2 footprint of wastewater treatment plants. It must be noted that the emerging aerobic granular sludge technology is expected to reduce the volume and energy consumption of aerobic treatment reactors (Pronk et al., 2015; Bengtsson et al., 2018).

8. Concluding remarks

The outcomes of this research showed that high rate anaerobic wastewater treatment is expandable to hypersaline conditions. For further advancement of the technology to practice, future research should focus on pilot studies with saline wastewaters of interest and salt fluctuations in time. Albeit not studied in this thesis, the results on microbial activity suggest that not only high rate granular treatment of soluble wastewaters, but also low rate treatment of saline wastewaters with high solids content should be possible. This could be of interest for the fresh water stressed delta areas, where the aerobic municipal wastewater treatment plants may need to treat saline sewage in the near future and such knowledge contributes to the overall goal of the Water Nexus consortium to reduce the fresh water uptake. The core findings of this thesis may even further contribute to this mission, if saline industrial wastewaters need to be reused. It seems that scientifically a breakthrough to investigate the mechanisms of granulation in relation to particular microorganisms and EPS components requires development of EPS chemical characterisation techniques and should be one of the future focuses.



Summary

Summary

Many industries, such as leather tanning, agro-food, fisheries, petroleum, petrochemical and textile dyeing industries generate highly polluted saline wastewater. It is estimated that approximately 5% of the globally generated wastewater is hypersaline (salinity above 3.5%). For example, it has been assessed that the petroleum industry alone already generates approximately 39.5 million m^3 of wastewater per day. Because salts have a negative effect on microbial activity, biological treatment processes usually are not considered for such wastewaters. Such wastewater streams therefore are generally treated with physical-chemical processes. However, physical-chemical methods are energy intensive and require a lot of chemicals. Hence, there is a need to explore possibilities to replace them by more sustainable biological treatment methods. In particular anaerobic biological treatment should be considered because it: i) converts the organic pollutants in the wastewater into energy-rich biogas, consisting mainly of CH_4 and CO_2; ii) only requires a small amount of energy for operation; iii) produces a small amount of bio-solids requiring further disposal.

Amongst the anaerobic biological treatment technologies those based on formation of granular sludge are of special interest for treatment of industrial wastewater. Sludge granules are spherically shaped biofilms in which microorganisms are entangled in a matrix of extracellular polymeric substances (EPS) providing the structural integrity of these biofilms. Because they have superb settling velocities and a high methanogenic activity, these granules allow for a compact reactor design with high concentrations of active biomass, i.e. can handle high volumetric organic loading rates. Before this research, anaerobic granular sludge was reported to be unsuitable for treatment of highly saline wastewater because methanogens are inhibited by the high osmotic pressure caused by the salinity and sludge granulation was impossible, probably because of the negative effects of monovalent ions on the structural integrity of the EPS matrix. However there is increasing evidence that salt inhibition of methanogens can be overcome by inoculating bioreactors with moderately halophilic inocula and/or by gradual adaptation to higher salinity levels. Also, some evidence in literature showed that anaerobic granules could increase in size in upflow anaerobic sludge bed (UASB) reactors at saline conditions, although these reactors were operated at upflow velocities lower than those typically applied in UASB reactors, the strength of the granules decreased and methanogenic activity became lower. Still, these observations indicated sludge granulation at saline conditions might be possible and justified further research. In fact, research on sludge granulation at high salinity had not been yet been performed from scratch, i.e. starting with dispersed biomass. The main objective of this thesis was to accomplish anaerobic granulation at high salinity from dispersed biomass and to find ways to stimulate osmoprotection of anaerobic biomass.

Earlier microbiology studies had shown that most prokaryotic microbial cells, including those expected to occur in anaerobic wastewater treatment systems, cope with osmotic stress by accumulating small, highly soluble organic molecules called osmolytes which balance the osmotic pressure between cell cytoplasm and the environment. Methanogens can synthesize or take up from the environment (mainly) nitrogen containing osmolytes, such as amino acid derivatives. Uptake from the environment of such compounds energetically is much more favourable than synthesis *de novo*. It was also shown that these molecules can be synthesized from amino acids as the starting substrate. Additionally, under fresh water conditions EPS – the gluing material of granules – are often reported to contain up to 90% weight fraction of proteins and granulation proceeds faster with wastewaters containing complex energy rich substrates. Based on these observations we hypothesized that at saline conditions proteinaceous substrates in wastewater could enhance sludge granulation by providing energetically favourable EPS "building blocks" and after hydrolysis improve microbial adaptation to high salinity by providing precursors for osmolyte synthesis (amino acids).

In **Chapter 2** we investigated the possibility to form granules at low (5 g Na^+/L) and high salinity (20 g Na^+/L) in UASB reactors, starting with moderately halophilic dispersed biomass, while operating the reactors at conditions (e.g., organic loading rate and upflow velocity) known to improve fresh water granulation. The wastewater contained a complex, energy rich and proteinaceous substrate – a mixture of glucose, acetate and tryptone. This allowed a surprisingly fast development of anaerobic granules, even at 20 g Na^+/L (~ 50 g/L NaCl). The sludge bed already turned granular after 45 days of continuous reactor operation. Although the COD (chemical oxygen demand, a measurement for organic pollution) removal efficiency was slightly better at 5 g Na^+/L compared to 20 g Na^+/L, at both salinities the removal efficiency exceeded 98% at organic loading rates as high as 16 g COD/L/d.

Inhibition of anaerobic activity and granule disintegration at high monovalent salt concentrations has been reported, even in very recent publications. Thus, further exploration in this thesis was dedicated to factors and mechanisms allowing for the successful but somewhat surprising outcome of **Chapter 2.** The focus was on testing the abovementioned hypothesis that amino acids and proteinaceous substrates may play an important role in both, osmoadaptation and granulation at high salinity. In **Chapter 3** proteins and amino acids were inspected for their potential to alleviate osmotic shock stress of acetoclastic methanogens in granular sludge sampled from two full scale high rate anaerobic treatment reactors (so called IC or internal circulation reactors) and from laboratory scale reactors operated at 5 and 20 g Na^+/L. Aspartate, glutamate, gelatine and tryptone all could alleviate the negative effects of high salinity on methanogens. For example, addition of glutamate to batch experiments with an abrupt increase in salinity enhanced the methanogenic activity of full-scale granular sludge by 115%. Similarly, addition of the peptide tryptone raised the methanogenic activity

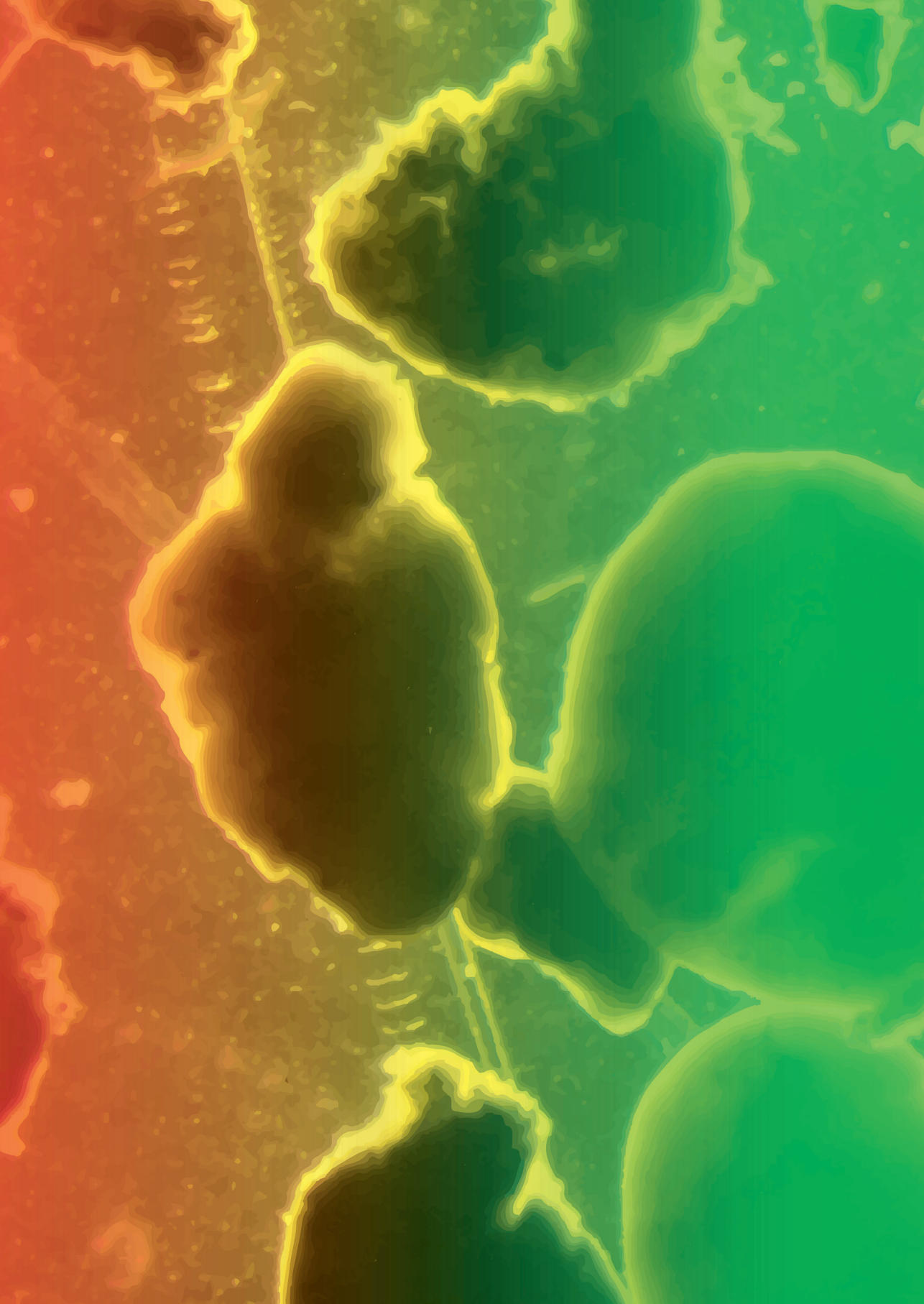
in granules from the full scale reactors by 48% and 179%. These results indicated osmoadaptation of methanogens may be faster if saline wastewater contains soluble proteinaceous substrates. Analysis of nitrogen containing osmolytes accumulated by the 20 g Na^+/L adapted granular sludge revealed glutamate and N-acetyl-β-lysine as the major osmolytes. This could in part explain the positive effect of amino acids on methanogenic activity: glutamate could be taken up directly from the bulk liquid, while N-acetyl-β-lysine could be synthesized from aspartate after uptake in the cell. Hydrolysis of a protein (gelatine) and a peptide (tryptone) potentially could also provide both of these amino acids, thereby explaining their positive effect on methanogenic activity.

In **Chapter 4** the (positive) effect of proteinaceous substrates on anaerobic sludge granulation was further explored. In all reactor experiments glucose and acetate were present in the wastewater, together with a third substrate (at 16.7% contribution to the wastewater COD) that was different for each reactor. If proteinaceous compounds (tryptone or gelatine) were added as the third substrate granulation at 20 g Na^+/L already was observed after 40-50 days. With starch as the third substrate also formation of dense, well settling granule-alike aggregates took place and this was accompanied with more than 98% soluble COD removal at a loading rate of 7.5 gCOD/L/d. However, this was only possible after a much longer period (~180 days) than with the proteinaceous substrate. Still, apparently methanogenic adaptation and sludge granulation can be achieved at high salinity even without addition of proteins. This implies a broad spectrum of saline wastewaters is amenable for treatment by anaerobic granular sludge reactors without the need to dose proteins. However, inoculation with saline adapted anaerobic sludge is not always possible, and in these cases the start-up period can be significantly reduced by (temporary) addition of proteinaceous substrate. In **Chapter 5**, the minimum amount of proteinaceous substrate (tryptone in this case) needed to support sludge granulation and process performance at 20 g Na^+/L was found to be 1.8% of the wastewater COD. It was also shown this amount can be easily estimated from relatively simple osmotic pressure calculations. It should be remarked that in many cases wastewaters, in particular those related to the food industry, already contain such small amounts of proteins.

In fresh water granules acetoclastic methanogenic archaea belonging to the genus *Methanosaeta* are often reported to be the most abundant methanogens performing the conversion of acetate into methane. In its filamentous form *Methanosaeta* also positively influences the sludge granulation by forming 3D webs and acting as a granulation nucleus on which other bacteria can grow. An elaborate study on the development of the microbial community in anaerobic granular sludge exposed to high salinity was still lacking. The microbial molecular and microscopy analyses in **Chapter 6** revealed that in reactors supplied with proteinaceous substrate (16.7% as COD tryptone) the dominant methanogenic archaea at both 5 and 20 g Na^+/L belonged to *Methanosaeta*, which was growing in its

filamentous form. The dominant bacteria at 5 g Na^+/L were *Streptoccoccus* whereas at 20 g Na^+/L the dominant bacteria belonged to the family *Defluvitaleaceae*. Interestingly, also these dominant bacteria were present as filaments. An experiment was also performed in which the granulation at 20 g Na^+/L from dispersed biomass was studied without a proteinaceous substrate, but with amino acids leucine (8.3% COD fraction) and proline (8.3% COD fraction) instead. In this reactor, the bacteria belonging to *Defluvitaleaceae* disappeared. Consequently, also filaments of bacteria did not form in this reactor and the granulation was not achieved after 120 days. These outcomes suggested the importance of filamentous bacteria for granulation at saline conditions.

As a response to high salinity microbial cells can produce increased amounts of EPS, suggesting EPS help to alleviate osmotic stress. A confocal laser scanning microscopy (CLSM) study of granules grown during the experiments of **Chapter 2** suggested that EPS rich in mannose residues can bind sodium ions and in this manner reduce the osmotic stress on the microorganisms. However, microbial cells need to cope not only with cations but also with anions. To examine if EPS could also act as a protective barrier against high concentrations of anions, ion exchange membranes were prepared with EPS extracted from high salinity adapted granules and their selectivity to transport cations and anions was tested (**Chapter 7**). The EPS acted as cation exchange membranes which selectively transported sodium and potassium cations and partially repelled anions (chloride). With Scanning Electron Microscopy coupled to Energy Dispersive X-ray Spectroscopy (SEM-EDX) and ionic composition measurements of granules it was shown that the concentration of anions was significantly lower in granules compared to the bulk liquid. Together these observations imply that EPS can indeed act as a protective barrier against high concentration of anions. Interestingly, EPS exhibited a higher selectivity for potassium than for sodium ions, even though potassium and sodium have the same valence and similar physical-chemical properties. As potassium selectivity has commercial relevance, future studies focusing on the reason for this selectivity perhaps can result in the development of commercial potassium selective membranes. For microbial cells such improved transport of ions through EPS seems to have a negative effect. In methanogenic activity assays potassium was much more toxic compared to sodium suggesting that cation toxicity may be influenced by properties of EPS, i.e. the better the ion can diffuse through the EPS, the more toxic it is (**Chapter 7**). Finally, in **Chapter 8** the results of this research are discussed in a broader context and future research directions are proposed.



References

References

GRiSP Research Solutions. [updated cited 2019.24-9-2019]. Available from: http://www.grisp.pt/docs/gcm23-tryptone.pdf

Ahmad, A., Ghufran, R. and Wahid, Z.A., 2011. Role of calcium oxide in sludge granulation and methanogenesis for the treatment of palm oil mill effluent using UASB reactor. Journal of Hazardous Materials 198, 40-48 http://dx.doi.org/10.1016/j.jhazmat.2011.10.008.

Ahmed, E.M., 2015. Hydrogel: Preparation, characterization, and applications: A review. Journal of Advanced Research 6(2), 105-121 https://doi.org/10.1016/j.jare.2013.07.006.

Ajao, V., Bruning, H., Rijnaarts, H. and Temmink, H., 2018. Natural flocculants from fresh and saline wastewater: Comparative properties and flocculation performances. Chemical Engineering Journal 349, 622-632 https://doi.org/10.1016/j.cej.2018.05.123.

Akashi, H. and Gojobori, T., 2002. Metabolic efficiency and amino acid composition in the proteomes of Escherichia coli and Bacillus subtilis. Proceedings of the National Academy of Sciences 99(6), 3695 https://doi.org/10.1073/pnas.062526999.

Ali, M., Elreedy, A., Ibrahim, M.G., Fujii, M., Nakatani, K. and Tawfik, A., 2019. Regulating acidogenesis and methanogenesis for the separated bio-generation of hydrogen and methane from saline-to-hypersaline industrial wastewater. Journal of Environmental Management 250, 109546 https://doi.org/10.1016/j.jenvman.2019.109546.

Alphenaar, P., Visser, A. and Lettinga, G., 1993. The effect of liquid upflow velocity and hydraulic retention time on granulation in UASB reactors treating wastewater with a high sulphate content. Bioresource Technology 43(3), 249-258 https://doi.org/10.1016/0960-8524(93)90038-D.

Altaş, L., 2009. Inhibitory effect of heavy metals on methane-producing anaerobic granular sludge. Journal of Hazardous Materials 162(2), 1551-1556 https://doi.org/10.1016/j.jhazmat.2008.06.048.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology 56(6), 1919

Amann, R.I., Ludwig, W. and Schleifer, K.-H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological reviews 59(1), 143-169 https://doi.org/10.1016/j.jip.2007.09.009.

Angenent, L.T., Sung, S. and Raskin, L., 2004. Formation of granules and Methanosaeta fibres in an anaerobic migrating blanket reactor (AMBR). Environmental microbiology 6(4), 315-322 https://doi.org/10.1111/j.1462-2920.2004.00597.x.

APHA, 2005. Standard methods for the examination of water and wastewater. , American Public Health Association, Washington, DC

Appels, L., Baeyens, J., Degrève, J. and Dewil, R., 2008. Principles and potential of the anaerobic digestion of waste-activated sludge. Progress in Energy and Combustion Science 34(6), 755-781 https://doi.org/10.1016/j.pecs.2008.06.002.

Arcand, Y., Guiot, S., Desrochers, M. and Chavarie, C., 1994. Impact of the reactor hydrodynamics and organic loading on the size and activity of anaerobic granules. The Chemical Engineering Journal and the Biochemical Engineering Journal 56(1), B23-B35

Aslan, S. and Şekerdağ, N., 2016. Salt inhibition on anaerobic treatment of high salinity wastewater by upflow anaerobic sludge blanket (UASB) reactor. Desalination and Water Treatment 57(28), 12998-13004 https://doi.org/10.1080/19443994.2015.1059369.

Balslev-Olesen, P., Lynggaard-Jensen, A. and Nickelsen, C., 1990. Pilot-Scale Experiments on Anaerobic Treatment of Wastewater from a Fish Processing Plant. Water Science and Technology 22(1-2), 463-474 https://doi.org/10.2166/wst.1990.0170.

Batstone, D.J. and Keller, J., 2001. Variation of bulk properties of anaerobic granules with wastewater type. Water Research 35(7), 1723-1729 https://doi.org/10.1016/S0043-1354(00)00446-2.

Batstone, D.J., Keller, J. and Blackall, L., 2004. The influence of substrate kinetics on the microbial community structure in granular anaerobic biomass. Water Research 38(6), 1390-1404 https://doi.org/10.1016/j.watres.2003.12.003.

Bengtsson, S., de Blois, M., Wilén, B.-M. and Gustavsson, D., 2018. Treatment of municipal wastewater with aerobic granular sludge. Critical Reviews in Environmental Science and Technology 48(2), 119-166 https://doi.org/10.1080/10643389.2018.1439653.

Berrier, C., Coulombe, A., Szabo, I., Zoratti, M. and Ghazi, A., 1992. Gadolinium ion inhibits loss of metabolites induced by osmotic shock and large stretch-activated channels in bacteria. The FEBS Journal 206(2), 559-565 https://doi.org/10.1111/j.1432-1033.1992.tb16960.x.

Beveridge, T.J., Patel, G.B., Harris, B.J. and Sprott, G.D., 1986. The ultrastructure of Methanothrix concilii, a mesophilic aceticlastic methanogen. Canadian Journal of Microbiology 32(9), 703-710 https://doi.org/10.1139/m86-128.

Bhat, B. and Bajaj, B.K., 2018. Hypocholesterolemic and bioactive potential of exopolysaccharide from a probiotic Enterococcus faecium K1 isolated from kalarei. Bioresource Technology 254, 264-267 https://doi.org/10.1016/j.biortech.2018.01.078.

Björnsson, L., Hugenholtz, P., Tyson, G.W. and Blackall, L.L., 2002. Filamentous Chloroflexi (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. Microbiology 148(8), 2309-2318 https://doi.org/10.1099/00221287-148-8-2309.

Boardman, G.D., Tisinger, J.L. and Gallagher, D.L., 1995. Treatment of clam processing wastewaters by means of upflow anaerobic sludge blanket technology. Water Research 29(6), 1483-1490 https://doi.org/10.1016/0043-1354(94)00303-O.

Borrel, G., Lehours, A.-C., Crouzet, O., Jézéquel, D., Rockne, K., Kulczak, A., Duffaud, E., Joblin, K. and Fonty, G., 2012. Stratification of Archaea in the Deep Sediments of a Freshwater Meromictic Lake: Vertical Shift from Methanogenic to Uncultured Archaeal Lineages. PLOS ONE 7(8), e43346 https://doi.org/10.1371/journal.pone.0043346.

Bruus, J.H., Nielsen, P.H. and Keiding, K., 1992. On the stability of activated sludge flocs with implications to dewatering. Water Research 26(12), 1597-1604 https://doi.org/10.1016/0043-1354(92)90159-2.

Calli, B., Mertoglu, B., Tas, N., Inanc, B., Yenigun, O. and Ozturk, I., 2003. Investigation of variations in microbial diversity in anaerobic reactors treating landfill leachate. Water Science and Technology 48(4), 105-112

Campo, R., Corsino, S.F., Torregrossa, M. and Di Bella, G., 2018. The role of extracellular polymeric substances on aerobic granulation with stepwise increase of salinity. Separation and Purification Technology 195, 12-20 https://doi.org/10.1016/j.seppur.2017.11.074.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J. and Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7(5), 335-336 https://doi.org/10.1038/nmeth.f.303.

Carvalho, F., Prazeres, A.R. and Rivas, J., 2013. Cheese whey wastewater: Characterization and treatment. Science of The Total Environment 445-446, 385-396 https://doi.org/10.1016/j.scitotenv.2012.12.038.

Chan, Y.J., Chong, M.F., Law, C.L. and Hassell, D.G., 2009. A review on anaerobic–aerobic treatment of industrial and municipal wastewater. Chemical Engineering Journal 155(1), 1-18 https://doi.org/10.1016/j.cej.2009.06.041.

Chandrasekaran, R. and Radha, A., 1995. Molecular architectures and functional properties of gellan gum and related polysaccharides. Trends in Food Science & Technology 6(5), 143-148 http://dx.doi.org/10.1016/S0924-2244(00)89022-6.

Chang, Y.-J., Nishio, N., Maruta, H. and Nagai, S., 1993. Characteristics of granular methanogenic sludge grown on glucose in a UASB reactor. Journal of Fermentation and Bioengineering 75(6), 430-434 https://doi.org/10.1016/0922-338X(93)90090-U.

Chen, J.L., Ortiz, R., Steele, T.W.J. and Stuckey, D.C., 2014. Toxicants inhibiting anaerobic digestion: A review. Biotechnology Advances 32(8), 1523-1534 https://doi.org/10.1016/j.biotechadv.2014.10.005.

Chen, Y. and Cheng, J.J., 2007. Effect of Potassium Inhibition on the Thermophilic Anaerobic Digestion of Swine Waste. Water Environment Research 79(6), 667-674 http://doi.org/10.2175/106143007x156853.

Chen, Y., Cheng, J.J. and Creamer, K.S., 2008a. Inhibition of anaerobic digestion process: a review. Bioresource Technology 99(10), 4044-4064 https://doi.org/10.1016/j.biortech.2007.01.057.

Chen, Z., Ren, N., Wang, A., Zhang, Z.-P. and Shi, Y., 2008b. A novel application of TPAD–MBR system to the pilot treatment of chemical synthesis-based pharmaceutical wastewater. Water Research 42(13), 3385-3392 https://doi.org/10.1016/j.watres.2008.04.020.

Chiesa, P., Consonni, S., Kreutz, T. and Robert, W., 2005. Co-production of hydrogen, electricity and CO2 from coal with commercially ready technology. Part A: Performance and emissions. International Journal of Hydrogen Energy 30(7), 747-767 https://doi.org/10.1016/j.ijhydene.2004.08.002.

Chowdhury, P., Viraraghavan, T. and Srinivasan, A., 2010. Biological treatment processes for fish processing wastewater – A review. Bioresource Technology 101(2), 439-449 https://doi.org/10.1016/j.biortech.2009.08.065.

Chung, Y.-C. and Neethling, J., 1990. Viability of anaerobic digester sludge. Journal of Environmental Engineering 116(2), 330-343 https://doi.org/10.1061/(ASCE)0733-9372(1990)116:2(330).

Comte, S., Guibaud, G. and Baudu, M., 2006a. Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties: Part I. Comparison of the efficiency of eight EPS extraction methods. Enzyme and Microbial Technology 38(1–2), 237-245 http://dx.doi.org/10.1016/j.enzmictec.2005.06.016.

Comte, S., Guibaud, G. and Baudu, M., 2006b. Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS: Part II. Consequences of EPS extraction methods on Pb2+ and Cd2+ complexation. Enzyme and Microbial Technology 38(1–2), 246-252 http://dx.doi.org/10.1016/j.enzmictec.2005.06.023.

Comte, S., Guibaud, G. and Baudu, M., 2006c. Biosorption properties of extracellular polymeric substances (EPS) resulting from activated sludge according to their type: Soluble or bound. Process Biochemistry 41(4), 815-823 https://doi.org/10.1016/j.procbio.2005.10.014.

Comte, S., Guibaud, G. and Baudu, M., 2008. Biosorption properties of extracellular polymeric substances (EPS) towards Cd, Cu and Pb for different pH values. Journal of Hazardous Materials 151(1), 185-193 https://doi.org/10.1016/j.jhazmat.2007.05.070.

Corsino, S.F., Capodici, M., Morici, C., Torregrossa, M. and Viviani, G., 2016. Simultaneous nitritation–denitritation for the treatment of high-strength nitrogen in hypersaline wastewater by aerobic granular sludge. Water Research 88, 329-336 https://doi.org/10.1016/j.watres.2015.10.041.

Corsino, S.F., Capodici, M., Torregrossa, M. and Viviani, G., 2017. Physical properties and Extracellular Polymeric Substances pattern of aerobic granular sludge treating hypersaline wastewater. Bioresource Technology 229, 152-159 http://dx.doi.org/10.1016/j.biortech.2017.01.024.

Cotta, M. and Forster, R., 2006. "The family Lachnospiraceae, including the genera Butyrivibrio, Lachnospira and Roseburia" in The Prokaryotes, pp. 1002-1021, Springer, Dordrecht.

Crocetti, G., Murto, M. and Björnsson, L., 2006. An update and optimisation of oligonucleotide probes targeting methanogenic Archaea for use in fluorescence in situ hybridisation (FISH). Journal of Microbiological Methods 65(1), 194-201 https://doi.org/10.1016/j.mimet.2005.07.007.

Csonka, L.N., 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiological Reviews 53(1), 121-147

d'Abzac, P., Bordas, F., Joussein, E., van Hullebusch, E.D., Lens, P.N.L. and Guibaud, G., 2013. Metal binding properties of extracellular polymeric substances extracted from anaerobic granular sludges. Environmental Science and Pollution Research 20(7), 4509-4519 http://doi.org/10.1007/s11356-012-1401-3.

Daffonchio, D., Thaveesri, J. and Verstraete, W., 1995. Contact angle measurement and cell hydrophobicity of granular sludge from upflow anaerobic sludge bed reactors. Applied and Environmental Microbiology 61(10), 3676

De Baere, L.A., Devocht, M., Van Assche, P. and Verstraete, W., 1984. Influence of high NaCl and NH4Cl salt levels on methanogenic associations. Water Research 18(5), 543-548 http://dx.doi.org/10.1016/0043-1354(84)90201-X.

De Bruin, L.M.M., de Kreuk, M.K., van der Roest, H.F.R., Uijterlinde, C. and van Loosdrecht, M.C.M., 2004. Aerobic granular sludge technology: an alternative to activated sludge? Water Science and Technology 49(11-12), 1-7 https://doi.org/10.2166/wst.2004.0790.

De Graaff, M.S., Temmink, H., Zeeman, G. and Buisman, C.J.N., 2011. Energy and phosphorus recovery from black water. Water Science and Technology 63(11), 2759-2765 https://doi.org/10.2166/wst.2011.558.

De Mes, T., Stams, A., Reith, J. and Zeeman, G., 2003. "Methane production by anaerobic digestion of wastewater and solid wastes" in Bio-methane & Bio-hydrogen. 58-102

De Vrieze, J., Gildemyn, S., Vilchez-Vargas, R., Jáuregui, R., Pieper, D. and Verstraete, W., 2015. Inoculum selection is crucial to ensure operational stability in anaerobic digestion. Appl Microbiol Biotechnol 99 https://doi.org/10.1007/s00253-014-6046-3.

De Vrieze, J., Coma, M., Debeuckelaere, M., Van der Meeren, P. and Rabaey, K., 2016a. High salinity in molasses wastewaters shifts anaerobic digestion to carboxylate production. Water Research 98, 293-301 https://doi.org/10.1016/j.watres.2016.04.035.

De Vrieze, J., Regueiro, L., Props, R., Vilchez-Vargas, R., Jáuregui, R., Pieper, D.H., Lema, J.M. and Carballa, M., 2016b. Presence does not imply activity: DNA and RNA patterns differ in response to salt perturbation in anaerobic digestion. Biotechnology for Biofuels 9(1), 244 https://doi.org/10.1186/s13068-016-0652-5.

Decho, A.W. and Gutierrez, T., 2017. Microbial Extracellular Polymeric Substances (EPSs) in Ocean Systems. Frontiers in Microbiology 8(922) https://doi.org/10.3389/fmicb.2017.00922.

Deja, J., Uliasz-Bochenczyk, A. and Mokrzycki, E., 2010. CO2 emissions from Polish cement industry. International Journal of Greenhouse Gas Control 4(4), 583-588 https://doi.org/10.1016/j.ijggc.2010.02.002.

Di Gioia, L. and Guilbert, S., 1999. Corn protein-based thermoplastic resins: effect of some polar and amphiphilic plasticizers. Journal of Agricultural and Food Chemistry 47(3), 1254-1261 https://doi.org/10.1021/jf980976j.

Ding, Z., Bourven, I., Guibaud, G., van Hullebusch, E.D., Panico, A., Pirozzi, F. and Esposito, G., 2015. Role of extracellular polymeric substances (EPS) production in bioaggregation: application to wastewater treatment. Applied microbiology and biotechnology, 1-23 https://doi.org/10.1007/s00253-015-6964-8.

Dobrowolski, R., Szcześ, A., Czemierska, M. and Jarosz-Wikołazka, A., 2017. Studies of cadmium(II), lead(II), nickel(II), cobalt(II) and chromium(VI) sorption on extracellular polymeric substances produced by Rhodococcus opacus and Rhodococcus rhodochrous. Bioresource Technology 225, 113-120 https://doi.org/10.1016/j.biortech.2016.11.040.

Doddema, H.J. and Vogels, G.D., 1978. Improved identification of methanogenic bacteria by fluorescence microscopy. Applied and Environmental Microbiology 36(5), 752

Dolfing, J., 1986. Granulation in UASB reactors. Water Science and Technology 18(12), 15-25

Donlan, R.M., 2002. Biofilms: microbial life on surfaces. Emerging infectious diseases 8(9), 881-890 https://doi.org/10.3201/eid0809.020063.

Doucet, D., Otter, D.E., Gauthier, S.F. and Foegeding, E.A., 2003. Enzyme-Induced Gelation of Extensively Hydrolyzed Whey Proteins by Alcalase: Peptide Identification and Determination of Enzyme Specificity. Journal of Agricultural and Food Chemistry 51(21), 6300-6308 https://doi.org/10.1021/jf026242v.

Duan, N., Dong, B., Wu, B. and Dai, X., 2012. High-solid anaerobic digestion of sewage sludge under mesophilic conditions: Feasibility study. Bioresource Technology 104, 150-156 https://doi.org/10.1016/j.biortech.2011.10.090.

Dubé, C.-D. and Guiot, S.R., 2019. Characterization of the protein fraction of the extracellular polymeric substances of three anaerobic granular sludges. AMB Express 9(1), 23 https://doi.org/10.1186/s13568-019-0746-0.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P. and Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Analytical chemistry 28(3), 350-356

Dulekgurgen, E., Doğruel, S., Karahan, Ö. and Orhon, D., 2006. Size distribution of wastewater COD fractions as an index for biodegradability. Water Research 40(2), 273-282 https://doi.org/10.1016/j.watres.2005.10.032.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19), 2460-2461 https://doi.org/10.1093/bioinformatics/btq461.

EEA, 2018. Industrial wastewater treatmentr - pressures on Europe's environmnet, European Environmnet Agency, Copenhagen, Denmark https://doi.org/10.2800/496223.

El-Mamouni, R., Leduc, R., Costerton, J.W. and Guiot, S.R., 1995. Influence of the microbial content of different precursory nuclei on the anaerobic granulation dynamics. Water Science and Technology 32(8), 173-177 https://doi.org/10.1016/0273-1223(96)00022-4.

El-Mamouni, R., Leduc, R. and Guiot, S.R., 1997. Influence of the starting microbial nucleus type on the anaerobic granulation dynamics. Applied Microbiology and Biotechnology 47(2), 189-194 http://doi.org/10.1007/s002530050911.

El-Mamouni, R., Leduc, R. and Guiot, S.R., 1998. Influence of synthetic and natural polymers on the anaerobic granulation process. Water Science and Technology 38(8), 341-347 https://doi.org/10.1016/S0273-1223(98)00710-0.

Englyst, H.N. and Cummings, J.H., 1984. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. Analyst 109(7), 937-942 https://doi.org/10.1039/an9840900937.

Erable, B., Roncato, M.-A., Achouak, W. and Bergel, A., 2009. Sampling Natural Biofilms: A New Route to Build Efficient Microbial Anodes. Environmental Science & Technology 43(9), 3194-3199 https://doi.org/10.1021/es803549v.

Eurostat, Olives by production. [updated 2018.05.30.; cited 2018.05.31]. Available from: http://ec.europa.eu/eurostat/tgm/table.do?tab=table&init=1&plugin=1&language=en&pcode=tag00122

Eurostat, What is the milk in the EU used for? [updated 2018.01.29; cited 2018.05.31]. Available from: http://ec.europa.eu/eurostat/en/web/products-eurostat-news/-/DDN-20180129-1

Eurostat, Catches in all fishing regions. [updated 2018.05.03; cited 2018.05.31]. Available from: http://ec.europa.eu/eurostat/tgm/table.do?tab=table&init=1&plugin=1&language=en&pcode=tag00076

Eurostat, Electricity consumption. [updated 2019.07.03.; cited 2019.10.12]. Available from: https://ec.europa.eu/eurostat/web/products-datasets/-/nrg_cb_e

Eurostat, Population on 1 January. [updated 09.09.2019.; cited 2019.10.12]. Available from: https://ec.europa.eu/eurostat/web/products-datasets/-/tps00001

Fakhru'l-Razi, A., Pendashteh, A., Abdullah, L.C., Biak, D.R.A., Madaeni, S.S. and Abidin, Z.Z., 2009. Review of technologies for oil and gas produced water treatment. Journal of Hazardous Materials 170(2), 530-551

Fang, C., Boe, K. and Angelidaki, I., 2011. Anaerobic co-digestion of desugared molasses with cow manure; focusing on sodium and potassium inhibition. Bioresource Technology 102(2), 1005-1011 https://doi.org/10.1016/j.biortech.2010.09.077.

Fang, F., Yang, M.-M., Wang, H., Yan, P., Chen, Y.-P. and Guo, J.-S., 2018. Effect of high salinity in wastewater on surface properties of anammox granular sludge. Chemosphere 210, 366-375 https://doi.org/10.1016/j.chemosphere.2018.07.038.

Fang, H.H.P., Chui, H.K. and Li, Y.Y., 1994. Microbial structure and activity of UASB granules treating different wastewaters. Water Science and Technology 30(12), 87-96 https://doi.org/10.2166/wst.1994.0587.

Fardeau, M., Postec, A. and Ollivier, B., 2017. Bergey's Manual of Systematics of Archaea and Bacteria. Whitman, W.B., Rainey, F., Kämpfer, P., Trujillo, M., Chun, J., DeVos, P., Hedlund, B. and Dedysh, S. (eds), pp. 1-5.

Feijoo, G., Soto, M., Méndez, R. and Lema, J.M., 1995. Sodium inhibition in the anaerobic digestion process: Antagonism and adaptation phenomena. Enzyme and Microbial Technology 17(2), 180-188 https://doi.org/10.1016/0141-0229(94)00011-F.

Felz, S., Al-Zuhairy, S., Aarstad, O.A., van Loosdrecht, M.C.M. and Lin, Y.M., 2016. Extraction of Structural Extracellular Polymeric Substances from Aerobic Granular Sludge. Journal of Visualized Experiments : JoVE (115), 54534 https://doi.org/10.3791/54534.

Flemming, H.-C. and Wingender, J., 2010. The biofilm matrix. Nature Reviews Microbiology 8(9), 623-633

Freire, D., Cammarota, M. and Sant'Anna, G., 2001. Biological treatment of oil field wastewater in a sequencing batch reactor. Environmental Technology 22(10), 1125-1135

Fukuzaki, S., Chang, Y.-J., Nishio, N. and Nagai, S., 1991a. Characteristics of granular methanogenic sludge grown on lactate in a UASB reactor. Journal of Fermentation and Bioengineering 72(6), 465-472 https://doi.org/10.1016/0922-338X(91)90056-M.

Fukuzaki, S., Nishio, N. and Nagai, S., 1991b. Chemical composition and kinetic properties of granular methanogenic sludge grown on propionate. Journal of Fermentation and Bioengineering 72(5), 405-407 https://doi.org/10.1016/0922-338X(91)90097-Z.

Fukuzaki, S., Nishio, N., Sakurai, H. and Nagai, S., 1991c. Characteristics of methanogenic granules grown on propionate in an upflow anaerobic sludge blanket (UASB) reactor. Journal of Fermentation and Bioengineering 71(1), 50-57 https://doi.org/10.1016/0922-338X(91)90303-X.

Fukuzaki, S., Nishio, N. and Nagai, S., 1995. High rate performance and characterization of granular methanogenic sludges in upflow anaerobic sludge blanket reactors fed with various defined substrates. Journal of Fermentation and Bioengineering 79(4), 354-359 https://doi.org/10.1016/0922-338X(95)93994-U.

Gagliano, M., Braguglia, C., Gianico, A., Mininni, G., Nakamura, K. and Rossetti, S., 2015. Thermophilic anaerobic digestion of thermal pretreated sludge: Role of microbial community structure and correlation with process performances. Water Research 68, 498-509

Gagliano, M., Gallipoli, A., Rossetti, S. and Braguglia, C., 2017a. Efficacy of methanogenic biomass acclimation in mesophilic anaerobic digestion of ultrasound pretreated sludge. Environmental Technology (just-accepted), 1-25

Gagliano, M., Ismail, S., Stams, A., Plugge, C., Temmink, H. and Van Lier, J., 2017b. Biofilm formation and granule properties in anaerobic digestion at high salinity. Water Research 121, 61-71 https://doi.org/10.1016/j.watres.2017.05.016.

Gagliano, M.C., Neu, T.R., Kuhlicke, U., Sudmalis, D., Temmink, H. and Plugge, C.M., 2018. EPS glycoconjugate profiles shift as adaptive response in anaerobic microbial granulation at high salinity. Frontiers in Microbiology 9, 1423 https://doi.org/10.3389/fmicb.2018.01423.

Galama, A.H., Post, J.W., Cohen Stuart, M.A. and Biesheuvel, P.M., 2013. Validity of the Boltzmann equation to describe Donnan equilibrium at the membrane–solution interface. Journal of Membrane Science 442, 131-139 http://dx.doi.org/10.1016/j.memsci.2013.04.022.

Galinski, E.A. and Trüper, H.G., 1994. Microbial behaviour in salt-stressed ecosystems. FEMS Microbiology Reviews 15(2-3), 95-108 https://doi.org/10.1111/j.1574-6976.1994.tb00128.x.

Geise, G.M., Hickner, M.A. and Logan, B.E., 2013. Ionic Resistance and Permselectivity Tradeoffs in Anion Exchange Membranes. ACS Applied Materials & Interfaces 5(20), 10294-10301 https://doi.org/10.1021/am403207w.

Ghangrekar, M., Asolekar, S., Ranganathan, K. and Joshi, S., 1996. Experience with UASB reactor start-up under different operating conditions. Water Science and Technology 34(5), 421-428

Ghangrekar, M., Asolekar, S. and Joshi, S., 2005. Characteristics of sludge developed under different loading conditions during UASB reactor start-up and granulation. Water Research 39(6), 1123-1133 https://doi.org/10.1016/j.watres.2004.12.018.

Giustinianovich, E.A., Campos, J.-L., Roeckel, M.D., Estrada, A.J., Mosquera-Corral, A. and Val del Río, Á., 2018. Influence of biomass acclimation on the performance of a partial nitritation-anammox reactor treating industrial saline effluents. Chemosphere 194, 131-138 https://doi.org/10.1016/j.chemosphere.2017.11.146.

Gomec, C.Y., Gonuldinc, S., Eldem, N. and Ozturk, I., 2005. Behavior of an Up-flow Anaerobic Sludge Bed (UASB) reactor at extreme salinity. Water Science and Technology 51(11), 115-120 10.2166/wst.2005.0397.

Gonzalez-Gil, G., Lens, P.N.L., Van Aelst, A., Van As, H., Versprille, A.I. and Lettinga, G., 2001. Cluster Structure of Anaerobic Aggregates of an Expanded Granular Sludge Bed Reactor. Applied and Environmental Microbiology 67(8), 3683 https://doi.org/10.1128/aem.67.8.3683-3692.2001.

Grady Jr, C.L., Daigger, G.T., Love, N.G. and Filipe, C.D., 2011. Biological wastewater treatment, CRC press, New York

Greuter, D., Loy, A., Horn, M. and Rattei, T., 2015. probeBase—an online resource for rRNA-targeted oligonucleotide probes and primers: new features 2016. Nucleic Acids Research 44(D1), D586-D589 https://doi.org/10.1093/nar/gkv1232.

Guiot, S., Gorur, S., Bourque, D. and Samson, R., 1988. Metal effect on microbial aggregation during upflow anaerobic sludge bed-filter (UBF) reactor start-up. Granular Anaerobic Sludge: Microbiology and Technology, 187-194

Gunde-Cimerman, N., Oren, A. and Plemenitaš, A., 2006. Adaptation to life at high salt concentrations in Archaea, Bacteria, and Eukarya, Springer Science & Business Media

Gutiérrez, J.L.R., Encina, P.A.G. and Fdz-Polanco, F., 1991. Anaerobic treatment of cheese-production wastewater using a UASB reactor. Bioresource Technology 37(3), 271-276 https://doi.org/10.1016/0960-8524(91)90194-O.

Gutierrez, T., Shimmield, T., Haidon, C., Black, K. and Green, D.H., 2008. Emulsifying and Metal Ion Binding Activity of a Glycoprotein Exopolymer Produced by Pseudoalteromonas sp. Strain TG12. Applied and Environmental Microbiology 74(15), 4867 https://doi.org/10.1128/aem.00316-08.

Gutierrez, T., Biller, D.V., Shimmield, T. and Green, D.H., 2012. Metal binding properties of the EPS produced by Halomonas sp. TG39 and its potential in enhancing trace element bioavailability to eukaryotic phytoplankton. BioMetals 25(6), 1185-1194 https://doi.org/10.1007/s10534-012-9581-3.

Hanczko, R., Jámbor, A., Perl, A. and Molnar-Perl, I., 2007. Advances in the o-phthalaldehyde derivatizations: comeback to the o-phthalaldehyde–ethanethiol reagent. Journal of Chromatography A 1163(1-2), 25-42 https://doi.org/10.1016/j.chroma.2007.06.013.

Harrison, J.J., Ceri, H. and Turner, R.J., 2007. Multimetal resistance and tolerance in microbial biofilms. Nature Reviews Microbiology 5, 928

He, Q., Kong, X.-J., Chai, H.-X., Fan, M.-Y. and Du, J., 2012. Efficiency influence of exogenous betaine on anaerobic sequencing batch biofilm reactor treating high salinity mustard tuber wastewater. Environmental Technology 33(15), 1695-1699 https://doi.org/10.1080/09593330.2011.643317.

He, S., Yang, W., Li, W., Zhang, Y., Qin, M. and Mao, Z., 2019. Impacts of salt shocking and the selection of a suitable reversal agent on anammox. Science of The Total Environment 692, 602-612 https://doi.org/10.1016/j.scitotenv.2019.07.215.

Heggendorn, F.L., Fraga, A.G.M., Ferreira, D.d.C., Goncalves, L.S., Lione, V.d.O.F. and Lutterbach, M.T.S., 2018. Sulfate-Reducing Bacteria: Biofilm Formation and Corrosive Activity in Endodontic Files. International Journal of Dentistry 2018, 12 https://doi.org10.1155/2018/8303450.

Hemmelmann, A., Torres, A., Vergara, C., Azocar, L. and Jeison, D., 2013. Application of anaerobic membrane bioreactors for the treatment of protein-containing wastewaters under saline conditions. Journal of Chemical Technology & Biotechnology 88(4), 658-663 https://doi.org/10.1002/jctb.3882.

Hierholtzer, A. and Akunna, J.C., 2014. Modelling start-up performance of anaerobic digestion of saline-rich macro-algae. Water Science and Technology 69(10), 2059-2065 https://doi.org/10.2166/wst.2014.100.

Higgins Matthew, J. and Novak John, T., 1997. Characterization of Exocellular Protein and Its Role in Bioflocculation. Journal of Environmental Engineering 123(5), 479-485 https://doi.org/10.1061/(asce)0733-9372(1997)123:5(479).

Horn, H. and Morgenroth, E., 2006. Transport of oxygen, sodium chloride, and sodium nitrate in biofilms. Chemical Engineering Science 61(5), 1347-1356 https://doi.org/10.1016/j.ces.2005.08.027.

Hosseini, S.M., Gholami, A., Madaeni, S.S., Moghadassi, A.R. and Hamidi, A.R., 2012. Fabrication of (polyvinyl chloride/cellulose acetate) electrodialysis heterogeneous cation exchange membrane: Characterization and performance in desalination process. Desalination 306, 51-59 https://doi.org/10.1016/j.desal.2012.07.028.

Hu, Z., Jin, J., Abruña, H.D., Houston, P.L., Hay, A.G., Ghiorse, W.C., Shuler, M.L., Hidalgo, G. and Lion, L.W., 2007. Spatial Distributions of Copper in Microbial Biofilms by Scanning Electrochemical Microscopy. Environmental Science & Technology 41(3), 936-941 https://doi.org/10.1021/es061293k.

Hugenholtz, P., Tyson, G.W. and Blackall, L.L., 2002. Gene Probes: Principles and Protocols, pp. 29-42, Humana Press, Totowa, NJ.

Hulshoff Pol, L., 1989. The phenomenon of granulation of anaerobic sludge, Landbouwuniversiteit te Wageningen.

Hulshoff Pol, L., de Castro Lopes, S., Lettinga, G. and Lens, P., 2004. Anaerobic sludge granulation. Water Research 38(6), 1376-1389 https://doi.org/10.1016/j.watres.2003.12.002.

Hulshoff Pol, L.W., de Zeeuw, W.J., Velzeboer, C.T.M. and Lettinga, G., 1983. Granulation in UASB-Reactors. Water Science and Technology 15(8-9), 291-304 https://doi.org/10.2166/wst.1983.0172.

Ismail, S., Gonzalez, P., Jeison, D. and Van Lier, J., 2008. Effects of high salinity wastewater on methanogenic sludge bed systems. Water Science and Technology 58(10), 1963-1970 https://doi.org/10.2166/wst.2008.528.

Ismail, S., de La Parra, C., Temmink, H. and Van Lier, J., 2010. Extracellular polymeric substances (EPS) in upflow anaerobic sludge blanket (UASB) reactors operated under high salinity conditions. Water Research 44(6), 1909-1917 https://doi.org/10.1016/j.watres.2009.11.039.

Ismail, S.B., 2013. Anaerobic wastewater treatment of high salinity wastewaters: impact on bioactivity and biomass retention, Wageningen University, Wageningen

Jabari, L., Gannoun, H., Cayol, J.-L., Hamdi, M., Fauque, G., Ollivier, B. and Fardeau, M.-L., 2012. Characterization of Defluviitalea saccharophila gen. nov., sp. nov., a thermophilic bacterium isolated from an upflow anaerobic filter treating abattoir wastewaters, and proposal of Defluviitaleaceae fam. nov. International Journal of Systematic and Evolutionary Microbiology 62(3), 550-555 https://doi.org/10.1099/ijs.0.030700-0.

Jeison, D. and Chamy, R., 1999. Comparison of the behaviour of expanded granular sludge bed (EGSB) and upflow anaerobic sludge blanket (UASB) reactors in dilute and concentrated wastewater treatment. Water Science and Technology 40(8), 91-97 https://doi.org/10.1016/S0273-1223(99)00613-7.

Jeison, D., Rio, A.D. and Lier, J.V., 2008. Impact of high saline wastewaters on anaerobic granular sludge functionalities. Water Science and Technology 57(6), 815-820 https://doi.org/10.2166/wst.2008.098.

Jhung, J.K. and Choi, E., 1995. A comparative study of UASB and anaerobic fixed film reactors with development of sludge granulation. Water Research 29(1), 271-277 https://doi.org/10.1016/0043-1354(94)E0123-N.

Ji, S.-Q., Wang, B., Lu, M. and Li, F.-L., 2016. Defluviitalea phaphyphila sp. nov., a novel thermophilic bacterium that degrades brown algae. Applied and Environmental Microbiology 82(3), 868 https://doi.org/10.1128/aem.03297-15.

Joghee, N.N. and Jayaraman, G., 2014. Metabolomic characterization of halophilic bacterial isolates reveals strains synthesizing rare diaminoacids under salt stress. Biochimie 102, 102-111 https://doi.org/10.1016/j.biochi.2014.02.015.

Jolly, L., Vincent, S.J.F., Duboc, P. and Neeser, J.-R., 2002. Lactic Acid Bacteria: Genetics, Metabolism and Applications: Proceedings of the seventh Symposium on lactic acid bacteria: genetics, metabolism and applications, 1–5 September 2002, Egmond aan Zee, the Netherlands, pp. 367-374, Springer Netherlands, Dordrecht.

Karim, A.A. and Bhat, R., 2009. Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. Food Hydrocolloids 23(3), 563-576 https://doi.org/10.1016/j.foodhyd.2008.07.002.

Karri, S., Sierra-Alvarez, R. and Field, J.A., 2006. Toxicity of copper to acetoclastic and hydrogenotrophic activities of methanogens and sulfate reducers in anaerobic sludge. Chemosphere 62(1), 121-127 https://doi.org/10.1016/j.chemosphere.2005.04.016.

Kartal, B., Koleva, M., Arsov, R., van der Star, W., Jetten, M.S.M. and Strous, M., 2006. Adaptation of a freshwater anammox population to high salinity wastewater. Journal of Biotechnology 126(4), 546-553 https://doi.org/10.1016/j.jbiotec.2006.05.012.

Keegan, K.P., Glass, E.M. and Meyer, F., 2016. Microbial Environmental Genomics (MEG), pp. 207-233, Springer New York, New York, NY.

Khoufi, S., Aloui, F. and Sayadi, S., 2006. Treatment of olive oil mill wastewater by combined process electro-Fenton reaction and anaerobic digestion. Water Research 40(10), 2007-2016 https://doi.org/10.1016/j.watres.2006.03.023.

Kita, A., Suehira, K., Miura, T., Okamura, Y., Aki, T., Matsumura, Y., Tajima, T., Nishio, N. and Nakashimada, Y., 2016. Characterization of a halotolerant acetoclastic methanogen highly enriched from marine sediment and its application in removal of acetate. Journal of Bioscience and Bioengineering 121(2), 196-202 https://doi.org/10.1016/j.jbiosc.2015.05.018.

Kloda, A. and Martinac, B., 2002. Common evolutionary origins of mechanosensitive ion channels in Archaea, Bacteria and cell-walled Eukarya. Archaea 1(1), 35-44 http://dx.doi.org/10.1155/2002/419261.

Kobayashi, T., Hu, Y. and Xu, K.-Q., 2018. Impact of cationic substances on biofilm formation from sieved fine particles of anaerobic granular sludge at high salinity. Bioresource Technology 257, 69-75 https://doi.org/10.1016/j.biortech.2018.02.078.

Kong, Y., He, M., McAlister, T., Seviour, R. and Forster, R., 2010. Quantitative fluorescence in situ hybridization of microbial communities in the rumens of cattle fed different diets. Applied and Environmental Microbiology 76(20), 6933 https://doi.org/10.1128/aem.00217-10.

Körstgens, V., Flemming, H., Wingender, J. and Borchard, W., 2001. Influence of calcium ions on the mechanical properties of a model biofilmof mucoid Pseudomonas aeruginosa. Water Science & Technology 43(6), 49-57

Koster, I.W., 1986. Characteristics of the pH-influenced adaptation of methanogenic sludge to ammonium toxicity. Journal of Chemical Technology & Biotechnology 36(10), 445-455

Kugelman, I.J. and McCarty, P.L.C.F.p.d.J., 1965. Cation Toxicity and Stimulation in Anaerobic Waste Treatment. Journal (Water Pollution Control Federation) 37(1), 97-116

Lambert, P.A., Hancock, I.C. and Baddiley, J., 1975. The interaction of magnesium ions with teichoic acid. Biochemical Journal 149(3), 519 https://doi.org/10.1042/bj1490519.

Le Borgne, S., Paniagua, D. and Vazquez-Duhalt, R., 2008. Biodegradation of organic pollutants by halophilic bacteria and archaea. Journal of Molecular Microbiology and Biotechnology 15(2-3), 74-92 https://doi.org/10.1159/000121323.

Lee, K.Y. and Mooney, D.J., 2012. Alginate: Properties and biomedical applications. Progress in Polymer Science 37(1), 106-126 https://doi.org/10.1016/j.progpolymsci.2011.06.003.

Lefebvre, O., Vasudevan, N., Torrijos, M., Thanasekaran, K. and Moletta, R., 2005. Halophilic biological treatment of tannery soak liquor in a sequencing batch reactor. Water Research 39(8), 1471-1480 https://doi.org/10.1016/j.watres.2004.12.038.

Lefebvre, O. and Moletta, R., 2006. Treatment of organic pollution in industrial saline wastewater: a literature review. Water Research 40(20), 3671-3682 https://doi.org/10.1016/j.watres.2006.08.027.

Lefebvre, O., Vasudevan, N., Torrijos, M., Thanasekaran, K. and Moletta, R., 2006. Anaerobic digestion of tannery soak liquor with an aerobic post-treatment. Water Research 40(7), 1492-1500 https://doi.org/10.1016/j.watres.2006.02.004.

Lefebvre, O., Quentin, S., Torrijos, M., Godon, J.J., Delgenes, J.P. and Moletta, R., 2007. Impact of increasing NaCl concentrations on the performance and community composition of two anaerobic reactors. Appl Microbiol Biotechnol 75 https://doi.org/10.1007/s00253-006-0799-2.

Lenhart, T.R., Duncan, K.E., Beech, I.B., Sunner, J.A., Smith, W., Bonifay, V., Biri, B. and Suflita, J.M., 2014. Identification and characterization of microbial biofilm communities associated with corroded oil pipeline surfaces. Biofouling 30(7), 823-835 https://doi.org/10.1080/08927014.2014.931379.

Leroy, F. and De Vuyst, L., 2016. Advances in production and simplified methods for recovery and quantification of exopolysaccharides for applications in food and health. Journal of Dairy Science 99(4), 3229-3238 https://doi.org/10.3168/jds.2015-9936.

Lettinga, G., van Velsen, A.F.M., Hobma, S.W., de Zeeuw, W. and Klapwijk, A., 1980. Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. Biotechnology and Bioengineering 22(4), 699-734 https://doi.org/10.1002/bit.260220402.

Lettinga, G. and Hulshoff Pol, L., 1991. UASB-process design for various types of wastewaters. Water science and technology 24(8), 87-107

Lettinga, G., 2010. Challenges of a feasible route towards sustainability in environmental protection. Frontiers of Environmental Science & Engineering in China 4(2), 123-134 https://doi.org/10.1007/s11783-010-0028-1.

Li, J., Yu, L., Yu, D., Wang, D., Zhang, P. and Ji, Z., 2014. Performance and granulation in an upflow anaerobic sludge blanket (UASB) reactor treating saline sulfate wastewater. Biodegradation 25(1), 127-136 https://doi.org/10.1007/s10532-013-9645-2.

Li, J., He, J., Huang, Y., Li, D. and Chen, X., 2015a. Improving surface and mechanical properties of alginate films by using ethanol as a co-solvent during external gelation. Carbohydrate Polymers 123, 208-216 https://doi.org/10.1016/j.carbpol.2015.01.040.

Li, L., Zheng, M., Ma, H., Gong, S., Ai, G., Liu, X., Li, J., Wang, K. and Dong, X., 2015b. Significant performance enhancement of a UASB reactor by using acyl homoserine lactones to facilitate the long filaments of Methanosaeta harundinacea 6Ac. Applied Microbiology and Biotechnology 99(15), 6471-6480 https://doi.org/10.1007/s00253-015-6478-4.

Li, W.-W. and Yu, H.-Q., 2014. Insight into the roles of microbial extracellular polymer substances in metal biosorption. Bioresource Technology 160, 15-23 https://doi.org/10.1016/j.biortech.2013.11.074.

Li, Y., Zheng, P., Zhang, M., Zeng, Z., Wang, Z., Ding, A. and Ding, K., 2016. Hydrophilicity/hydrophobicity of anaerobic granular sludge surface and their causes: An in situ research. Bioresource Technology 220, 117-123 https://doi.org/10.1016/j.biortech.2016.08.012.

Lin, C.-Y. and Chen, C.-C., 1999. Effect of heavy metals on the methanogenic UASB granule. Water Research 33(2), 409-416 https://doi.org/10.1016/S0043-1354(98)00211-5.

Liu, H. and Fang, H.H., 2002. Extraction of extracellular polymeric substances (EPS) of sludges. Journal of Biotechnology 95(3), 249-256 https://doi.org/10.1016/S0168-1656(02)00025-1.

Liu, J.R. and Seviour, R.J., 2001. Design and application of oligonucleotide probes for fluorescent in situ identification of the filamentous bacterial morphotype Nostocoida limicola in activated sludge. Environmental Microbiology 3(9), 551-560 https://doi.org/10.1046/j.1462-2920.2001.00229.x.

Liu, X.-W., Sheng, G.-P. and Yu, H.-Q., 2009. Physicochemical characteristics of microbial granules. Biotechnology Advances 27(6), 1061-1070 https://doi.org/10.1016/j.biotechadv.2009.05.020.

Liu, X., Dai, J., Ng, T.-L. and Chen, G., 2019. Evaluation of potential environmental benefits from seawater toilet flushing. Water Research 162, 505-515 https://doi.org/10.1016/j.watres.2019.07.016.

Liu, Y. and Boone, D.R., 1991. Effects of salinity on methanogenic decomposition. Bioresource Technology 35(3), 271-273 https://doi.org/10.1016/0960-8524(91)90124-3.

Liu, Y. and Tay, J.-H., 2002. The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. Water Research 36(7), 1653-1665

Liu, Y. and Fang, H.H.P., 2003. Influences of Extracellular Polymeric Substances (EPS) on Flocculation, Settling, and Dewatering of Activated Sludge. Critical Reviews in Environmental Science and Technology 33(3), 237-273 https://doi-org.ezproxy.library.wur.nl/10.1080/10643380390814479.

Liu, Y., Xu, H.-L., Yang, S.-F. and Tay, J.-H., 2003. Mechanisms and models for anaerobic granulation in upflow anaerobic sludge blanket reactor. Water Research 37(3), 661-673 http://dx.doi.org/10.1016/S0043-1354(02)00351-2.

Liu, Y., Yang, S.-F., Tay, J.-H., Liu, Q.-S., Qin, L. and Li, Y., 2004. Cell hydrophobicity is a triggering force of biogranulation. Enzyme and Microbial Technology 34(5), 371-379 https://doi.org/10.1016/j.enzmictec.2003.12.009.

Lofrano, G., Meriç, S., Zengin, G.E. and Orhon, D., 2013. Chemical and biological treatment technologies for leather tannery chemicals and wastewaters: A review. Science of The Total Environment 461-462, 265-281 https://doi.org/10.1016/j.scitotenv.2013.05.004.

Lu, Y., Slater, F., Bello-Mendoza, R. and Batstone, D.J., 2013. Shearing of biofilms enables selective layer based microbial sampling and analysis. Biotechnology and Bioengineering 110(10), 2600-2605 https://doi.org/10.1002/bit.24947.

Lücker, S., Steger, D., Kjeldsen, K.U., MacGregor, B.J., Wagner, M. and Loy, A., 2007. Improved 16S rRNA-targeted probe set for analysis of sulfate-reducing bacteria by fluorescence in situ hybridization. Journal of Microbiological Methods 69(3), 523-528 https://doi.org/10.1016/j.mimet.2007.02.009.

Luo, T., Abdu, S. and Wessling, M., 2018. Selectivity of ion exchange membranes: A review. Journal of Membrane Science 555, 429-454 https://doi.org/10.1016/j.memsci.2018.03.051.

Ma, S., Huang, Y., Wang, C., Fan, H., Dai, L., Zhou, Z., Liu, X. and Deng, Y., 2017. Defluviitalea raffinosedens sp. nov., a thermophilic, anaerobic, saccharolytic bacterium isolated from an anaerobic batch digester treating animal manure and rice straw. International journal of systematic and evolutionary microbiology 67(5), 1607-1612 https://doi.org/10.1099/ijsem.0.001664.

Mahoney, E., Varangu, L., Cairns, W., Kosaric, N. and Murray, R., 1987. The effect of calcium on microbial aggregation during UASB reactor start-up. Water Science & Technology 19(1-2), 249-260

Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.-H., 1992. Phylogenetic Oligodeoxynucleotide Probes for the Major Subclasses of Proteobacteria: Problems and Solutions. Systematic and Applied Microbiology 15(4), 593-600 https://doi.org/10.1016/S0723-2020(11)80121-9.

Martin, D.D., Ciulla, R.A., Robinson, P.M. and Roberts, M.F., 2000. Switching osmolyte strategies: response of Methanococcus thermolithotrophicus to changes in external NaCl. Biochimica et Biophysica Acta (BBA) - General Subjects 1524(1), 1-10 https://doi.org/10.1016/S0304-4165(00)00131-8.

McCarty, P.L. and McKinney, R.E.C.F.p.d.A., 1961. Salt Toxicity in Anaerobic Digestion. Journal (Water Pollution Control Federation) 33(4), 399-415

McHugh, S., O'reilly, C., Mahony, T., Colleran, E. and O'flaherty, V., 2003. Anaerobic granular sludge bioreactor technology. Reviews in Environmental Science and Biotechnology 2(2-4), 225-245 https://doi.org/10.1023/B:RESB.0000040465.45300.97

McNeil, S.D., Nuccio, M.L. and Hanson, A.D., 1999. Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. Plant Physiology 120(4), 945-949 https://doi.org/10.1104/pp.120.4.945.

McSwain, B.S., Irvine, R.L., Hausner, M. and Wilderer, P.A., 2005. Composition and Distribution of Extracellular Polymeric Substances in Aerobic Flocs and Granular Sludge. Applied and Environmental Microbiology 71(2), 1051 https://doi.org/10.1128/aem.71.2.1051-1057.2005.

Meussen, B.J., van Zeeland, A.N.T., Bruins, M.E. and Sanders, J.P.M., 2014. A Fast and Accurate UPLC Method for Analysis of Proteinogenic Amino Acids. Food Analytical Methods 7(5), 1047-1055 https://doi.org/10.1007/s12161-013-9712-7.

Milinovich, G.J., Burrell, P.C., Pollitt, C.C., Bouvet, A. and Trott, D.J., 2008. Streptococcus henryi sp. nov. and Streptococcus caballi sp. nov., isolated from the hindgut of horses with oligofructose-induced laminitis. International journal of systematic and evolutionary microbiology 58(1), 262-266

Molina, F., García, C., Roca, E. and Lema, J.M., 2008. Characterization of anaerobic granular sludge developed in UASB reactors that treat ethanol, carbohydrates and hydrolyzed protein based wastewaters. Water Science and Technology 57(6), 837-842 http://doi.org/10.2166/wst.2008.067.

Mostafa, H., El-Mezawy, A., Geis, A. and Heller, K., 2009. Exopolysaccharide production by Enterococcus faecium. Milchwissenschaft 64(4), 361-365

Müller, V., Spanheimer, R. and Santos, H., 2005. Stress response by solute accumulation in archaea. Current Opinion in Microbiology 8(6), 729-736 https://doi.org/10.1016/j.mib.2005.10.011.

Muñoz Sierra, J.D., Oosterkamp, M.J., Wang, W., Spanjers, H. and van Lier, J.B., 2019. Comparative performance of upflow anaerobic sludge blanket reactor and anaerobic membrane bioreactor treating

phenolic wastewater: Overcoming high salinity. Chemical Engineering Journal 366, 480-490 https://doi.org/10.1016/j.cej.2019.02.097.

Narihiro, T., Terada, T., Kikuchi, K., Iguchi, A., Ikeda, M., Yamauchi, T., Shiraishi, K., Kamagata, Y., Nakamura, K. and Sekiguchi, Y., 2008. Comparative Analysis of Bacterial and Archaeal Communities in Methanogenic Sludge Granules from Upflow Anaerobic Sludge Blanket Reactors Treating Various Food-Processing, High-Strength Organic Wastewaters. Microbes and Environments 24(2), 88-96 https://doi.org/10.1264/jsme2.ME08561.

Neef, A., Amann, R., Schlesner, H. and Schleifer, K.-H., 1998. Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. Microbiology 144(12), 3257-3266 https://doi.org/10.1099/00221287-144-12-3257.

Nestle, N. and Kimmich, R., 1996. Heavy metal uptake of alginate gels studied by NMR microscopy. Colloids and Surfaces A: Physicochemical and Engineering Aspects 115, 141-147 https://doi.org/10.1016/0927-7757(96)03608-4.

Nicolella, C., van Loosdrecht, M.C.M. and Heijnen, J.J., 2000. Wastewater treatment with particulate biofilm reactors. Journal of Biotechnology 80(1), 1-33 https://doi.org/10.1016/S0168-1656(00)00229-7.

Nomura, T., 2012. Control of microbial adhesion using fine particle technology. Advanced Powder Technology 23(5), 532-537 https://doi.org/10.1016/j.apt.2012.05.003.

O'Flaherty, V., Lens, P., De Beer, D. and Colleran, E., 1997. Effect of feed composition and upflow velocity on aggregate characteristics in anaerobic upflow reactors. Applied Microbiology and Biotechnology 47(2), 102-107 https://doi.org/10.1007/s002530050896.

O'Toole, G.A., Pratt, L.A., Watnick, P.I., Newman, D.K., Weaver, V.B. and Kolter, R., 1999. Methods in Enzymology, pp. 91-109, Academic Press.

O'Flaherty, V., Collins, G. and Mahony, T., 2006. The microbiology and biochemistry of anaerobic bioreactors with relevance to domestic sewage treatment. Reviews in Environmental Science and Bio/Technology 5(1), 39-55 https://doi.org/10.1007/s11157-005-5478-8.

Ollivier, B., Fardeau, M.-L., Cayol, J.-L., Magot, M., Patel, B.K.C., Prensier, G. and Garcia, J.-L., 1998. Methanocalculus halotolerans gen. nov., sp. nov., isolated from an oil-producing well. International Journal of Systematic and Evolutionary Microbiology 48(3), 821-828 https://doi.org/10.1099/00207713-48-3-821.

Onodera, T., Sase, S., Choeisai, P., Yoochatchaval, W., Sumino, H., Yamaguchi, T., Ebie, Y., Xu, K., Tomioka, N., Mizuochi, M. and Syutsubo, K., 2013. Development of a treatment system for molasses wastewater: The effects of cation inhibition on the anaerobic degradation process. Bioresource Technology 131, 295-302 https://doi.org/10.1016/j.biortech.2012.12.126.

Oren, A., 1999. Bioenergetic aspects of halophilism. Microbiology and Molecular Biology Reviews 63(2), 334-348 http://www.ncbi.nlm.nih.gov/pubmed/10357854.

Oren, A., 2013. Life at high salt concentrations. The prokaryotes: Prokaryotic communities and ecophysiology, 421-440

Ozgun, H., Ersahin, M.E., Erdem, S., Atay, B., Kose, B., Kaya, R., Altinbas, M., Sayili, S., Hoshan, P. and Atay, D., 2013. Effects of the pre-treatment alternatives on the treatment of oil-gas field produced water by nanofiltration and reverse osmosis membranes. Journal of Chemical Technology and Biotechnology 88(8), 1576-1583

Palenzuela-Rollon, A., Zeeman, G., Lubberding, H., Lettinga, G. and Alaerts, G., 2002. Treatment of fish processing wastewater in a one-or two-step upflow anaerobic sludge blanket (UASB) reactor. Water Science and Technology 45(10), 207-212 https://doi.org/10.2166/wst.2002.0332.

Parawira, W., Murto, M., Zvauya, R. and Mattiasson, B., 2006. Comparative performance of a UASB reactor and an anaerobic packed-bed reactor when treating potato waste leachate. Renewable Energy 31(6), 893-903 https://doi.org/10.1016/j.renene.2005.05.013.

Paredes, C., Cegarra, J., Roig, A., Sánchez-Monedero, M.A. and Bernal, M.P., 1999. Characterization of olive mill wastewater (alpechin) and its sludge for agricultural purposes. Bioresource Technology 67(2), 111-115 https://doi.org/10.1016/S0960-8524(98)00106-0.

Park, C., Muller, C.D., Abu-Orf, M.M. and Novak, J.T., 2006. The effect of wastewater cations on activated sludge characteristics: effects of aluminum and iron in floc. Water environment research 78(1), 31-40

Patel, G. and Roth, L., 1977. Effect of sodium chloride on growth and methane production of methanogens. Canadian journal of microbiology 23(7), 893-897

Pechan, Z., Knappová, O., Petrovičová, B. and Adamec, O., 1987. Anaerobic digestion of poultry manure at high ammonium nitrogen concentrations. Biological Wastes 20(2), 117-131 https://doi.org/10.1016/0269-7483(87)90162-5.

Peimbert, M. and Alcaraz, L.D., 2016. Field Guidelines for Genetic Experimental Designs in High-Throughput Sequencing, pp. 313-342, Springer International Publishing, Cham.

Pellerin, P., Fournet, B. and Debeire, P., 1990. Evidence for the glycoprotein nature of the cell sheath of Methanosaeta-like cells in the culture of Methanothrix soehngenii strain FE. Canadian Journal of Microbiology 36(9), 631-636 https://doi.org/10.1139/m90-109.

Peter, S.C., 2018. Reduction of CO2 to Chemicals and Fuels: A Solution to Global Warming and Energy Crisis. ACS Energy Letters 3(7), 1557-1561

Pflüger, K., Baumann, S., Gottschalk, G., Lin, W., Santos, H. and Müller, V., 2003. Lysine-2, 3-aminomutase and β-lysine acetyltransferase genes of methanogenic archaea are salt induced and are essential for the biosynthesis of Nε-acetyl-β-lysine and growth at high salinity. Applied and Environmental Microbiology 69(10), 6047-6055 https://doi.org/10.1128/AEM.69.10.6047-6055.2003.

Pflüger, K., Wieland, H. and Müller, V., 2005. "Osmoadaptation in methanogenic archaea: recent insights from a genomic perspective" in "Adaptation to Life at High Salt Concentrations in Archaea, Bacteria, and Eukarya", pp. 239-251, Springer, Dordrecht.

Phelan, M., Aherne, A., FitzGerald, R.J. and O'Brien, N.M., 2009. Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. International Dairy Journal 19(11), 643-654 https://doi.org/10.1016/j.idairyj.2009.06.001.

Poch, M.A.P.M.A.B.A., 1991. Bovine milk kappa-casein trypsin digest is a growth enhancer for the genus Bifidobacterium. Journal of agricultural and food chemistry v. 39(no. 1), pp. 73-77-1991 v.1939 no.1991 https://doi.org/10.1021/jf00001a013.

Pollice, A., Rozzi, A., Tomei, M.C., Di Pinto, A.C. and Limoni, N., 2000. Monitoring the Inhibitory Effect of NaCl on Anaerobic Wastewater Treatment Processes by the Rantox Biosensor. Environmental Technology 21(5), 535-544 https://doi.org/10.1080/09593332408618095.

Powell, G.E. and Archer, D.B., 1989. On-line titration method for monitoring buffer capacity and total volatile fatty acid levels in anaerobic digesters. Biotechnology and Bioengineering 33(5), 570-577 https://doi.org/10.1002/bit.260330509.

Prasanna, P.H.P., Grandison, A.S. and Charalampopoulos, D., 2012. Effect of dairy-based protein sources and temperature on growth, acidification and exopolysaccharide production of Bifidobacterium strains in skim milk. Food Research International 47(1), 6-12 https://doi.org/10.1016/j.foodres.2012.01.004.

Prazeres, A.R., Carvalho, F. and Rivas, J., 2012. Cheese whey management: A review. Journal of Environmental Management 110, 48-68 https://doi.org/10.1016/j.jenvman.2012.05.018.

Pronk, M., de Kreuk, M.K., de Bruin, B., Kamminga, P., Kleerebezem, R. and van Loosdrecht, M.C.M., 2015. Full scale performance of the aerobic granular sludge process for sewage treatment. Water Research 84, 207-217 https://doi.org/10.1016/j.watres.2015.07.011.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41(D1), D590-D596 https://doi.org/10.1093/nar/gks1219.

Ramsay, I.R. and Pullammanappallil, P.C., 2001. Protein degradation during anaerobic wastewater treatment: derivation of stoichiometry. Biodegradation 12(4), 247-256 https://doi.org/10.1023/A:1013116728817.

Raskin, L., Stromley, J.M., Rittmann, B.E. and Stahl, D.A., 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Applied and Environmental Microbiology 60(4), 1232

Reichhardt, C. and Parsek, M.R., 2019. Confocal Laser Scanning Microscopy for Analysis of Pseudomonas aeruginosa Biofilm Architecture and Matrix Localization. Frontiers in Microbiology 10(677) https://doi.org/10.3389/fmicb.2019.00677.

Rinzema, A., van Lier, J. and Lettinga, G., 1988. Sodium inhibition of acetoclastic methanogens in granular sludge from a UASB reactor. Enzyme and Microbial Technology 10(1), 24-32 https://doi.org/10.1016/0141-0229(88)90094-4.

Roberts, K.P., Heaven, S. and Banks, C.J., 2016. Quantification of methane losses from the acclimatisation of anaerobic digestion to marine salt concentrations. Renewable Energy 86, 497-506 https://doi.org/10.1016/j.renene.2015.08.045.

Roberts, M., Lai, M. and Gunsalus, R., 1992. Biosynthetic pathways of the osmolytes N epsilon-acetyl-beta-lysine, beta-glutamine, and betaine in Methanohalophilus strain FDF1 suggested by nuclear magnetic resonance analyses. Journal of Bacteriology 174(20), 6688-6693 https://doi.org/10.1128/jb.174.20.6688-6693.1992.

Roberts, M.F., 2005. Organic compatible solutes of halotolerant and halophilic microorganisms. Saline Systems 1(1), 5 https://doi.org/10.1186/1746-1448-1-5.

Roeßler, M. and Müller, V., 2001. Osmoadaptation in bacteria and archaea: common principles and differences. Environmental Microbiology 3(12), 743-754 https://doi.org/10.1046/j.1462-2920.2001.00252.x.

Rozzi, A., Malpei, F., Bonomo, L. and Bianchi, R., 1999. Textile wastewater reuse in northern Italy (Como). Water Science and Technology 39(5), 121-128 https://doi.org/10.1016/S0273-1223(99)00093-1.

Sadrzadeh, M. and Mohammadi, T., 2009. Treatment of sea water using electrodialysis: Current efficiency evaluation. Desalination 249(1), 279-285 https://doi.org/10.1016/j.desal.2008.10.029.

Saiani, A., Mohammed, A., Frielinghaus, H., Collins, R., Hodson, N., Kielty, C.M., Sherratt, M.J. and Miller, A.F., 2009. Self-assembly and gelation properties of α-helix versus β-sheet forming peptides. Soft Matter 5(1), 193-202 https://doi.org/10.1039/b811288f.

Sanalibaba, P. and Çakmak, G.A., 2016. Exopolysaccharides production by lactic acid bacteria. Appl. Microbiol. Open Access 2, 115 http://dx.doi.org/10.4172/2471-9315.1000115.

Sanders, W., Geerink, M., Zeeman, G. and Lettinga, G., 2000. Anaerobic hydrolysis kinetics of particulate substrates. Water Science and Technology 41(3), 17-24

Sanders, W.T.M., Zeeman, G. and Lettinga, G., 2002. Hydrolysis kinetics of dissolved polymer substrates. Water Science & Technology 45(10), 99-104 https://doi-org.ezproxy.library.wur.nl/10.2166/wst.2002.0301.

Sanjeevi, R., Abbasi, T. and Abbasi, S., 2013. Role of calcium (II) in anaerobic sludge granulation and UASB reactor operation: a method to develop calcium-fortified sludge outside the UASB reactors. Indian Journal of Biotechnology 12(2), 246-253

Satoh, H., Miura, Y., Tsushima, I. and Okabe, S., 2007. Layered Structure of Bacterial and Archaeal Communities and Their In Situ Activities in Anaerobic Granules. Applied and Environmental Microbiology 73(22), 7300 https://doi.org/10.1128/aem.01426-07.

Saum, R., Mingote, A., Santos, H. and Müller, V., 2009. A novel limb in the osmoregulatory network of Methanosarcina mazei Gö1: Nε-acetyl-β-lysine can be substituted by glutamate and alanine. Environmental Microbiology 11(5), 1056-1065 https://doi.org/10.1111/j.1462-2920.2008.01826.x.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nature Methods 9(7), 676-682 https://doi.org/10.1038/nmeth.2019.

Schlegel, K. and Müller, V., 2011. "Extremophiles Handbook", pp. 327-342, Springer, Dordrecht.

Schmidt, J.E. and Ahring, B.K., 1996. Granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors. Biotechnology and Bioengineering 49(3), 229-246 https://doi.org/10.1002/(SICI)1097-0290(19960205)49:3<229::AID-BIT1>3.0.CO;2-M.

Schroepfer, G.J., Fullen, W.J., Johnson, A.S., Ziemke, N.R. and Anderson, J.J.C.F.p.d.A., 1955. The Anaerobic Contact Process as Applied to Packinghouse Wastes. Sewage and Industrial Wastes 27(4), 460-486

Sekiguchi, Y., Kamagata, Y., Syutsubo, K., Ohashi, A., Harada, H. and Nakamura, K., 1998. Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis. Microbiology 144(9), 2655-2665 https://doi.org/10.1099/00221287-144-9-2655.

Sekiguchi, Y., Kamagata, Y., Nakamura, K., Ohashi, A. and Harada, H., 1999. Fluorescence In Situ Hybridization Using 16S rRNA-Targeted Oligonucleotides Reveals Localization of Methanogens and Selected Uncultured Bacteria in Mesophilic and Thermophilic Sludge Granules. Applied and Environmental Microbiology 65(3), 1280

Sekiguchi, Y., Takahashi, H., Kamagata, Y., Ohashi, A. and Harada, H., 2001. In Situ Detection, Isolation, and Physiological Properties of a Thin Filamentous Microorganism Abundant in Methanogenic Granular Sludges: a Novel Isolate Affiliated with a Clone Cluster, the Green Non-Sulfur Bacteria, Subdivision I. Applied and Environmental Microbiology 67(12), 5740 https://doi.org/10.1128/aem.67.12.5740-5749.2001.

Seviour, T., Pijuan, M., Nicholson, T., Keller, J. and Yuan, Z., 2009. Gel-forming exopolysaccharides explain basic differences between structures of aerobic sludge granules and floccular sludges. Water Research 43(18), 4469-4478 https://doi.org/10.1016/j.watres.2009.07.018.

Seviour, T., Yuan, Z., van Loosdrecht, M.C. and Lin, Y., 2012. Aerobic sludge granulation: a tale of two polysaccharides? Water Research 46(15), 4803-4813

Seviour, T., Derlon, N., Dueholm, M.S., Flemming, H.-C., Girbal-Neuhauser, E., Horn, H., Kjelleberg, S., van Loosdrecht, M.C.M., Lotti, T., Malpei, M.F., Nerenberg, R., Neu, T.R., Paul, E., Yu, H. and Lin, Y., 2019. Extracellular polymeric substances of biofilms: Suffering from an identity crisis. Water Research 151, 1-7 https://doi.org/10.1016/j.watres.2018.11.020.

Show, K.-Y., Wang, Y., Foong, S.-F. and Tay, J.-H., 2004. Accelerated start-up and enhanced granulation in upflow anaerobic sludge blanket reactors. Water Research 38(9), 2293-2304 https://doi.org/10.1016/j.watres.2004.01.039.

Siegrist, H. and Gujer, W., 1985. Mass transfer mechanisms in a heterotrophic biofilm. Water Research 19(11), 1369-1378 https://doi.org/10.1016/0043-1354(85)90303-3.

Sleator, R.D. and Hill, C., 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS microbiology reviews 26(1), 49-71

Smith, G. and Friedman, M., 1984. Effect of carbohydrates and heat on the amino acid composition and chemically available lysine content of casein. Journal of Food Science 49(3), 817-820 https://doi.org/10.1111/j.1365-2621.1984.tb13219.x.

Smith, W.R., Moučka, F. and Nezbeda, I., 2016. Osmotic pressure of aqueous electrolyte solutions via molecular simulations of chemical potentials: Application to NaCl. Fluid Phase Equilibria 407, 76-83 https://doi.org/10.1016/j.fluid.2015.05.012.

Sochacki, K.A., Shkel, I.A., Record, M.T. and Weisshaar, J.C., 2011. Protein diffusion in the periplasm of E. coli under osmotic stress. Biophysical journal 100(1), 22-31

Sorokin, D.Y., Abbas, B., Geleijnse, M., Pimenov, N.V., Sukhacheva, M.V. and van Loosdrecht, M.C.M., 2015. Methanogenesis at extremely haloalkaline conditions in the soda lakes of Kulunda Steppe (Altai, Russia). FEMS Microbiology Ecology 91(4) https://doi.org/10.1093/femsec/fiv016.

Soto, M., Méndez, R. and Lema, J., 1991. Biodegradability and toxicity in the anaerobic treatment of fish canning wastewaters. Environmental Technology 12(8), 669-677

Souza, M.E., 1986. Criteria for the Utilization, Design and Operation of UASB Reactors. Water Science and Technology 18(12), 55-69 https://doi.org/10.2166/wst.1986.0163.

Sowers, K.R., Robertson, D.E., Noll, D., Gunsalus, R.P. and Roberts, M.F., 1990. N epsilon-acetyl-beta-lysine: an osmolyte synthesized by methanogenic archaebacteria. Proceedings of the National Academy of Sciences 87(23), 9083-9087 https://doi.org/10.1073/pnas.87.23.9083.

Sowers, K.R. and Gunsalus, R.P., 1995. Halotolerance in Methanosarcina spp.: Role of N (sup (epsilon))-Acetyl-(beta)-Lysine,(alpha)-Glutamate, Glycine Betaine, and K (sup+) as Compatible Solutes for

Osmotic Adaptation. Applied and Environmental Microbiology 61(12), 4382-4388 https://www.ncbi.nlm.nih.gov/pubmed/16535193.

Stahl, D., 1991. Development and application of nucleic acid probes. Nucleic acid techniques in bacterial systematics, 205-248

Stams, A.J., 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie van Leeuwenhoek 66(1-3), 271-294

Stams, A.J. and Plugge, C.M., 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nature Reviews Microbiology 7(8), 568-577

Steinbusch, K.J.J., Hamelers, H.V.M. and Buisman, C.J.N., 2008. Alcohol production through volatile fatty acids reduction with hydrogen as electron donor by mixed cultures. Water Research 42(15), 4059-4066 http://dx.doi.org/10.1016/j.watres.2008.05.032.

Stewart, M.B., Gray, S.R., Vasiljevic, T. and Orbell, J.D., 2014. The role of poly-M and poly-GM sequences in the metal-mediated assembly of alginate gels. Carbohydrate Polymers 112, 486-493 http://dx.doi.org/10.1016/j.carbpol.2014.06.001.

Strelkova, E.A., Pozdnyakova, N.V., Zhurina, M.V., Plakunov, V.K. and Belyaev, S.S., 2013. Role of the extracellular polymer matrix in resistance of bacterial biofilms to extreme environmental factors. Microbiology 82(2), 119-125 https://doi.org/10.1134/s002626171302015x.

Sudmalis, D., Gagliano, M.C., Pei, R., Grolle, K., Plugge, C.M., Rijnaarts, H.H.M., Zeeman, G. and Temmink, H., 2018a. Fast anaerobic sludge granulation at elevated salinity. Water Research 128(Supplement C), 293-303 https://doi.org/10.1016/j.watres.2017.10.038.

Sudmalis, D., Millah, S.K., Gagliano, M.C., Butré, C.I., Plugge, C.M., Rijnaarts, H.H.M., Zeeman, G. and Temmink, H., 2018b. The potential of osmolytes and their precursors to alleviate osmotic stress of anaerobic granular sludge. Water Research 147, 142-151 https://doi.org/10.1016/j.watres.2018.09.059.

Sundarapandiyan, S., Chandrasekar, R., Ramanaiah, B., Krishnan, S. and Saravanan, P., 2010. Electrochemical oxidation and reuse of tannery saline wastewater. Journal of Hazardous Materials 180(1), 197-203 https://doi.org/10.1016/j.jhazmat.2010.04.013.

Suzuki, I., Lee, D., Mackay, B., Harahuc, L. and Oh, J.K., 1999. Effect of Various Ions, pH, and Osmotic Pressure on Oxidation of Elemental Sulfur by Thiobacillus thiooxidans. Applied and Environmental Microbiology 65(11), 5163

Syutsubo, K., Harada, H., Ohashi, A. and Suzuki, H., 1997. An effective start-up of thermophilic UASB reactor by seeding mesophilically-grown granular sludge. Water Science and Technology 36(6), 391-398 https://doi.org/10.1016/S0273-1223(97)00547-7.

Tagg, J.R., Wescombe, P.A. and Burton, J.P., 2011. Lactic Acid Bacteria: Microbiological and Functional Aspects, Fourth Edition. Lahtinen, S., Ouwehand, A.C., Salminen, S. and Wright, A.v. (eds), pp. 123-145, CRC Press.

Takahashi, S., Tomita, J., Nishioka, K., Hisada, T. and Nishijima, M., 2014. Development of a Prokaryotic Universal Primer for Simultaneous Analysis of Bacteria and Archaea Using Next-Generation Sequencing. PLOS ONE 9(8), e105592 https://doi.org/10.1371/journal.pone.0105592

Tang, Y.J., Martin, H.G., Myers, S., Rodriguez, S., Baidoo, E.E.K. and Keasling, J.D., 2009. Advances in analysis of microbial metabolic fluxes via 13C isotopic labeling. Mass Spectrometry Reviews 28(2), 362-375 https://doi.org/10.1002/mas.20191.

Tawakoli, P.N., Neu, T.R., Busck, M.M., Kuhlicke, U., Schramm, A., Attin, T., Wiedemeier, D.B. and Schlafer, S., 2017. Visualizing the dental biofilm matrix by means of fluorescence lectin-binding analysis. Journal of Oral Microbiology 9(1), 1345581 https://doi.org/10.1080/20002297.2017.1345581.

Tay, J.-H. and Yan, Y.-G., 1996. Influence of substrate concentration on microbial selection and granulation during start-up of upflow anaerobic sludge blanket reactors. Water Environment Research 68(7), 1140-1150 https://doi.org/10.2175/106143096X128540.

Tchobanoglus, G., Burton, F. and Stensel, H.D., 2003. Wastewater engineering: Treatment and reuse, McGraw-Hill, New York

Teitzel, G.M. and Parsek, M.R., 2003. Heavy Metal Resistance of Biofilm and Planktonic Pseudomonas aeruginosa. Applied and Environmental Microbiology 69(4), 2313 https://doi.org/10.1128/aem.69.4.2313-2320.2003.

Tellez, G.T., Nirmalakhandan, N. and Gardea-Torresdey, J.L., 2002. Performance evaluation of an activated sludge system for removing petroleum hydrocarbons from oilfield produced water. Advances in Environmental Research 6(4), 455-470 http://doi.org/10.1016/S1093-0191(01)00073-9.

Tellez, G.T., Nirmalakhandan, N. and Gardea-Torresdey, J.L., 2005. Kinetic evaluation of a field-scale activated sludge system for removing petroleum hydrocarbons from oilfield-produced water. Environmental progress 24(1), 96-104

Thaveesri, J., Gernaey, K., Kaonga, B., Boucneau, G. and Verstraete, W., 1994. Organic and ammonium nitrogen and oxygen in relation to granular sludge growth in lab-scale UASB reactors. Water Science and Technology 30(12), 43-53 https://doi.org/10.2166/wst.1994.0579.

Tiwari, M.K., Guha, S., Harendranath, C.S. and Tripathi, S., 2005. Enhanced granulation by natural ionic polymer additives in UASB reactor treating low-strength wastewater. Water Research 39(16), 3801-3810 https://doi.org/10.1016/j.watres.2005.07.004.

Tiwari, M.K., Guha, S., Harendranath, C. and Tripathi, S., 2006. Influence of extrinsic factors on granulation in UASB reactor. Applied Microbiology and Biotechnology 71(2), 145-154 https://doi.org/10.1007/s00253-006-0397-3.

Toner, B., Manceau, A., Marcus, M.A., Millet, D.B. and Sposito, G., 2005. Zinc Sorption by a Bacterial Biofilm. Environmental Science & Technology 39(21), 8288-8294 https://doi.org/10.1021/es050528+.

Trebesius, K., Leitritz, L., Adler, K., Schubert, S., Autenrieth, I.B. and Heesemann, J., 2000. Culture independent and rapid identification of bacterial pathogens in necrotising fasciitis and streptococcal toxic shock syndrome by fluorescence in situ hybridisation. Medical Microbiology and Immunology 188(4), 169-175 https://doi.org/10.1007/s004300000035.

Uemura, S. and Harada, H., 1993. Microbial characteristics of methanogenic sludge consortia developed in thermophilic UASB reactors. Applied Microbiology and Biotechnology 39(4), 654-660 https://doi.org/10.1007/bf00205070.

Vallero, M., Pol, L.H., Lens, P. and Lettinga, G., 2002. Effect of high salinity on the fate of methanol during the start-up of thermophilic (55 C) sulfate reducing reactors. Water science and technology 45(10), 121-126

Vallero, M.V.G., Treviño, R.H.M., Paulo, P.L., Lettinga, G. and Lens, P.N.L., 2003. Effect of sulfate on methanol degradation in thermophilic (55°C) methanogenic UASB reactors. Enzyme and Microbial Technology 32(6), 676-687 https://doi.org/10.1016/S0141-0229(03)00031-0.

van den Akker, B., Reid, K., Middlemiss, K. and Krampe, J., 2015. Evaluation of granular sludge for secondary treatment of saline municipal sewage. Journal of Environmental Management 157, 139-145 https://doi.org/10.1016/j.jenvman.2015.04.027.

van Haandel, A., De Vrieze, J., Verstraete, W. and dos Santos, V.S., 2014. Methanosaeta dominate acetoclastic methanogenesis during high-rate methane production in anaerobic reactors treating distillery wastewaters. Journal of Chemical Technology & Biotechnology 89(11), 1751-1759 https://doi.org/10.1002/jctb.4255.

Van Lier, J., Van der Zee, F., Frijters, C. and Ersahin, M., 2015. Celebrating 40 years anaerobic sludge bed reactors for industrial wastewater treatment. Reviews in Environmental Science and Bio/Technology 14(4), 681-702 https://doi.org/10.1007/s11157-015-9375-5.

Van Lier, J.B., 2008. High-rate anaerobic wastewater treatment: diversifying from end-of-the-pipe treatment to resource-oriented conversion techniques. Water Science and Technology 57(8), 1137-1148

Vanderhaegen, B., Ysebaert, E., Favere, K., Van Wambeke, M., Peeters, T., Pánic, V., Vandenlangenbergh, V. and Verstraete, W., 1992. Acidogenesis in Relation to In-Reactor Granule Yield. Water Science and Technology 25(7), 21 http://hdl.handle.net/1854/LU-206962.

Vavilin, V.A., Fernandez, B., Palatsi, J. and Flotats, X., 2008. Hydrolysis kinetics in anaerobic degradation of particulate organic material: An overview. Waste Management 28(6), 939-951 http://dx.doi.org/10.1016/j.wasman.2007.03.028.

Vieira, D., Sérvulo, E. and Cammarota, M., 2005a. Degradation potential and growth of anaerobic bacteria in produced water. Environmental technology 26(8), 915-922

Vieira, D.S., Sérvulo, E.F.C. and Cammarota, M., 2005b. Degradation potential and growth of anaerobic bacteria in produced water. Environmental Technology 26(8), 915-922 https://doi.org/10.1080/09593332608618499.

Vlyssides, A., Barampouti, E.M. and Mai, S., 2009. Influence of ferrous iron on the granularity of a UASB reactor. Chemical Engineering Journal 146(1), 49-56 https://doi.org/10.1016/j.cej.2008.05.011.

Vlyssides, A.G., Loizides, M. and Karlis, P.K., 2004. Integrated strategic approach for reusing olive oil extraction by-products. Journal of Cleaner Production 12(6), 603-611 https://doi.org/10.1016/S0959-6526(03)00078-7.

Vrieze, J., Hennebel, T., Brande, J., RiM, B.i.l.a.d., Bruton, T.A. and Vankelecom, I.F.J., 2014. Anaerobic digestion of molasses by means of a vibrating and non-vibrating submerged anaerobic membrane bioreactor. Biomass Bioenergy 68 https://doi.org/10.1016/j.biombioe.2014.06.009.

Vyrides, I. and Stuckey, D., 2009a. Adaptation of anaerobic biomass to saline conditions: Role of compatible solutes and extracellular polysaccharides. Enzyme and Microbial Technology 44(1), 46-51 https://doi.org/10.1016/j.enzmictec.2008.09.008.

Vyrides, I. and Stuckey, D.c., 2009b. Effect of fluctuations in salinity on anaerobic biomass and production of soluble microbial products (SMP s). Biodegradation 20(2), 165-175

Vyrides, I., Santos, H., Mingote, A., Ray, M. and Stuckey, D., 2010. Are compatible solutes compatible with biological treatment of saline wastewater? Batch and continuous studies using submerged anaerobic membrane bioreactors (SAMBRs). Environmental Science & Technology 44(19), 7437-7442 https://doi.org/10.1021/es903981k.

Vyrides, I., 2015. "Anaerobic Treatment of Organic Saline Waste/Wastewater: Overcome Salinity Inhibition by Addition of Compatible Solutes" in Environmental Microbial Biotechnology, pp. 105-117, Springer, Dordrecht.

Vyrides, I. and Stuckey, D.C., 2016. Compatible solute addition to biological systems treating waste/wastewater to counteract osmotic and other environmental stresses: a review. Critical Reviews in Biotechnology, 1-15 https://doi.org/10.1080/07388551.2016.1266460.

Wagner, A.O., Hohlbrugger, P., Lins, P. and Illmer, P., 2012. Effects of different nitrogen sources on the biogas production–a lab-scale investigation. Microbiological Research 167(10), 630-636 https://doi.org/10.1016/j.micres.2011.11.007.

Wan, C., Yang, X., Lee, D.-J., Liu, X., Sun, S. and Chen, C., 2014. Partial nitrification of wastewaters with high NaCl concentrations by aerobic granules in continuous-flow reactor. Bioresource Technology 152, 1-6 https://doi.org/10.1016/j.biortech.2013.10.112.

Wan, J., Gu, J., Zhao, Q. and Liu, Y., 2016. COD capture: a feasible option towards energy self-sufficient domestic wastewater treatment. Scientific Reports 6, 25054

Wang, H., Wilksch, J.J., Lithgow, T., Strugnell, R.A. and Gee, M.L., 2013. Nanomechanics measurements of live bacteria reveal a mechanism for bacterial cell protection: the polysaccharide capsule in Klebsiella is a responsive polymer hydrogel that adapts to osmotic stress. Soft Matter 9(31), 7560-7567 https://doi.org/10.1039/c3sm51325d.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R., 2007. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73 https://doi.org/10.1128/aem.00062-07.

Webster, G., O'sullivan, L.A., Meng, Y., Williams, A.S., Sass, A.M., Watkins, A.J., Parkes, R.J. and Weightman, A.J., 2014. Archaeal community diversity and abundance changes along a natural salinity gradient in estuarine sediments. FEMS microbiology ecology 91(2), 1-18

Welte, C. and Deppenmeier, U., 2014. Bioenergetics and anaerobic respiratory chains of aceticlastic methanogens. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1837(7), 1130-1147 https://doi.org/10.1016/j.bbabio.2013.12.002.

Wiegant, W.M., 1988. The spaghetti theory on anaerobic granular sludge formation, or the inevitability of granulation. Granular anaerobic sludge, 146-152

Wilén, B.-M., Liébana, R., Persson, F., Modin, O. and Hermansson, M., 2018. The mechanisms of granulation of activated sludge in wastewater treatment, its optimization, and impact on effluent quality. Applied Microbiology and Biotechnology 102(12), 5005-5020 https://doi.org/10.1007/s00253-018-8990-9.

Williams, K., Percival, F., Merino, J. and Mooney, H., 1987. Estimation of tissue construction cost from heat of combustion and organic nitrogen content. Plant, Cell & Environment 10(9), 725-734 https://doi.org/10.1111/1365-3040.ep11604754.

Wirtz, R.A. and Dague, R.R., 1996. Enhancement of granulation and start-up in the anaerobic sequencing batch reactor. Water environment research 68(5), 883-892

Wolfaardt, G., Lawrence, J., Robarts, R. and Caldwell, D., 1998. In situ characterization of biofilm exopolymers involved in the accumulation of chlorinated organics. Microbial ecology 35(3-4), 213-223

Woolard, C.R. and Irvine, R.L., 1995. Treatment of hypersaline wastewater in the sequencing batch reactor. Water Research 29(4), 1159-1168 https://doi.org/10.1016/0043-1354(94)00239-4.

Xiao, F., Yang, S. and Li, X., 2008. Physical and hydrodynamic properties of aerobic granules produced in sequencing batch reactors. Separation and Purification Technology 63(3), 634-641

Xiao, Y. and Roberts, D.J., 2010. A review of anaerobic treatment of saline wastewater. Environmental Technology 31(8-9), 1025-1043 https://doi.org/10.1080/09593331003734202.

Xiong, Y. and Liu, Y., 2013. Importance of extracellular proteins in maintaining structural integrity of aerobic granules. Colloids and Surfaces B: Biointerfaces 112, 435-440 https://doi.org/10.1016/j.colsurfb.2013.07.060.

Yerkes, D., Boonyakitsombut, S. and Speece, R., 1997. Antagonism of sodium toxicity by the compatible solute betaine in anaerobic methanogenic systems. Water Science and Technology 36(6-7), 15-24 https://doi.org/10.1016/S0273-1223(97)00502-7.

Yoda, M., Kitagawa, M. and Miyaji, Y., 1989. Granular sludge formation in the anaerobic expanded micro-carrier bed process. Water Science & Technology 21(4-5), 109-120 https://doi.org/10.1016/B978-1-4832-8439-2.50015-8.

Young, J.C. and McCarty, P.L., 1969. The Anaerobic Filter for Waste Treatment. Water Pollution Control Federation 41(5), 160-173

Yu, H., Tay, J. and Fang, H.H., 1999. Effects of added powdered and granular activated carbons on start-up performance of UASB reactors. Environmental technology 20(10), 1095-1101

Yu, H., Tay, J. and Fang, H.H., 2001. The roles of calcium in sludge granulation during UASB reactor start-up. Water Research 35(4), 1052-1060 https://doi.org/10.1016/S0043-1354(00)00345-6.

Yu, H.Q. and Fang, H.H.P., 2001. Inhibition by chromium and cadmium of anaerobic acidogenesis. Water Science and Technology 43(11), 267-274 https://doi.org/10.2166/wst.2001.0691.

Zaprasis, A., Bleisteiner, M., Kerres, A., Hoffmann, T. and Bremer, E., 2014. Uptake of amino acids and their metabolic conversion into the compatible solute proline confers osmoprotection to Bacillus subtilis. Applied and Environmental Microbiology 81(1), 250-259 https://doi.org/10.1128/AEM.02797-14.

Zeeman, G., 2012. New sanitation: bridging cities and agriculture, Wageningen University, Wageningen

Zeeuw, W., 1984. Acclimatization of anaerobic sludge for UASB-reactor start-up. Editora: Agricultural University of Wageningen

Zhang, L., Feng, X., Zhu, N. and Chen, J., 2007. Role of extracellular protein in the formation and stability of aerobic granules. Enzyme and Microbial Technology 41(5), 551-557 https://doi.org/10.1016/j.enzmictec.2007.05.001.

Zhang, Q., Yang, B., Brashears, M.M., Yu, Z., Zhao, M., Liu, N. and Li, Y., 2014. Influence of casein hydrolysates on exopolysaccharide synthesis by Streptococcus thermophilus and Lactobacillus

delbrueckii ssp. bulgaricus. Journal of the Science of Food and Agriculture 94(7), 1366-1372 https://doi.org/10.1002/jsfa.6420.

Zhao, X., Wang, Y., Ye, Z., Borthwick, A.G.L. and Ni, J., 2006. Oil field wastewater treatment in Biological Aerated Filter by immobilized microorganisms. Process Biochemistry 41(7), 1475-1483 http://doi.org/10.1016/j.procbio.2006.02.006.

Zheng, D., Angenent, L.T. and Raskin, L., 2006. Monitoring granule formation in anaerobic upflow bioreactors using oligonucleotide hybridization probes. Biotechnology and Bioengineering 94(3), 458-472 https://doi.org/10.1002/bit.20870.

Zhou, J.-h., Zhao, H., Hu, M., Yu, H.-t., Xu, X.-y., Vidonish, J., Alvarez, P.J.J. and Zhu, L., 2015. Granular activated carbon as nucleating agent for aerobic sludge granulation: Effect of GAC size on velocity field differences (GAC versus flocs) and aggregation behavior. Bioresource Technology 198, 358-363 http://dx.doi.org/10.1016/j.biortech.2015.08.155.

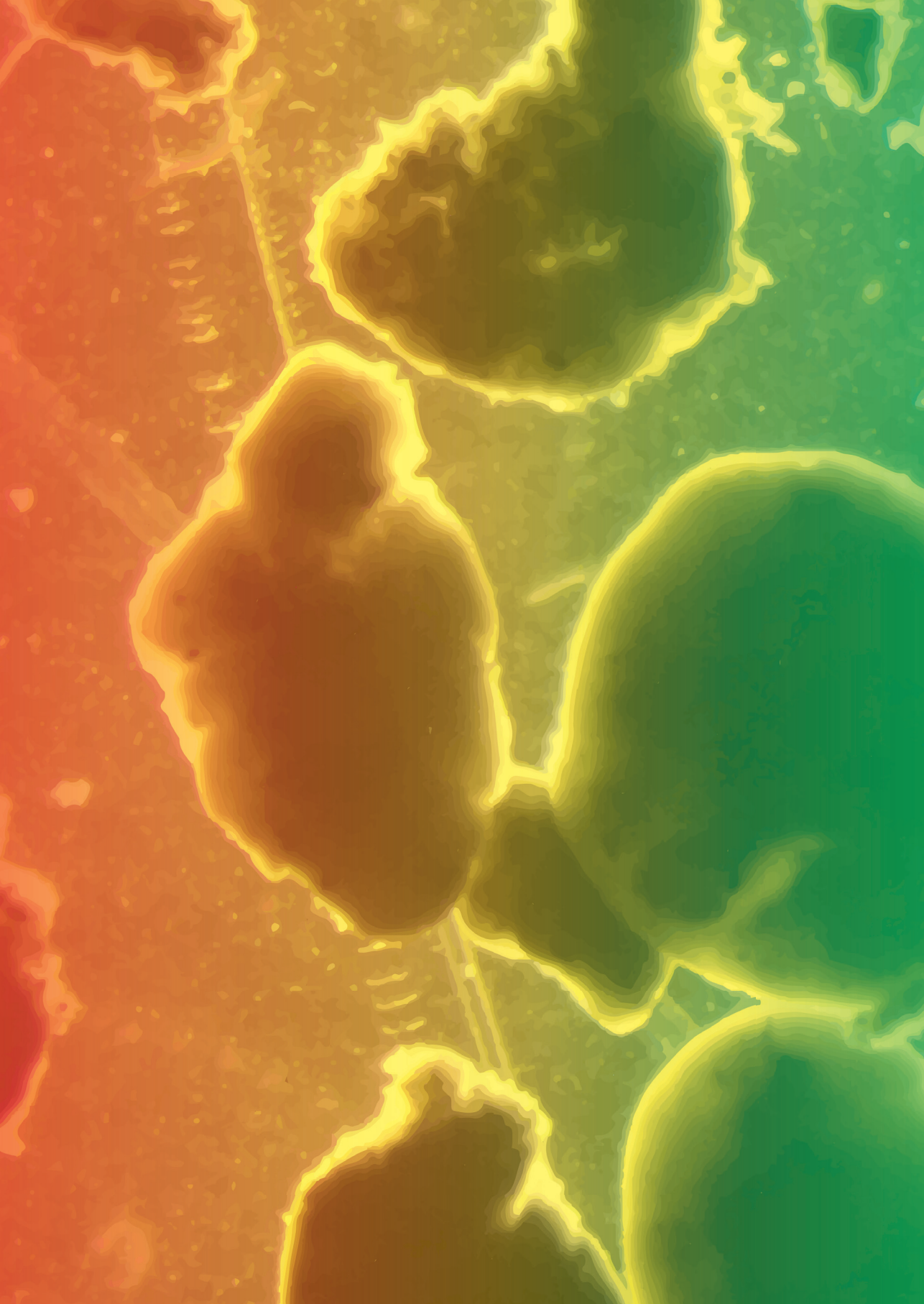
Zhou, W., Imai, T., Ukita, M., Sekine, M. and Higuchi, T., 2006. Triggering forces for anaerobic granulation in UASB reactors. Process Biochemistry 41(1), 36-43 http://dx.doi.org/10.1016/j.procbio.2005.02.029.

Zhu, C., Dong, S., Wang, F. and Huang, G., 2004. Effects of Na/K ratio in seawater on growth and energy budget of juvenile Litopenaeus vannamei. Aquaculture 234(1-4), 485-496 https://doi.org/10.1016/j.aquaculture.2003.11.027.

Zhu, J., Zheng, H., Ai, G., Zhang, G., Liu, D., Liu, X. and Dong, X., 2012. The Genome Characteristics and Predicted Function of Methyl-Group Oxidation Pathway in the Obligate Aceticlastic Methanogens, Methanosaeta spp. PLOS ONE 7(5), e36756 https://doi.org/10.1371/journal.pone.0036756

Zhu, L., Yu, H., Liu, Y., Qi, H. and Xu, X., 2015a. Optimization for extracellular polymeric substances extraction of microbial aggregates. Water Science & Technology 71(7), 1106-1112

Zhu, L., Zhou, J., Lv, M., Yu, H., Zhao, H. and Xu, X., 2015b. Specific component comparison of extracellular polymeric substances (EPS) in flocs and granular sludge using EEM and SDS-PAGE. Chemosphere 121, 26-32 https://doi.org/10.1016/j.chemosphere.2014.10.053.



Acknowledgements

Acknowledgements

The time has passed in a blink of an eye and, like many others have done before me, I also get to write this piece of slightly sentimental and yet important text. It is very important, in fact, because many deserve to be acknowledged besides the author of this book and these lines. So many have to be mentioned that I am actually worried somebody will feel left out. If this happens you should contact me to request your well-deserved homebrew from me :).

Firstly, I would like to say a very big thank you (Really big. Imagine huge and then multiply it by a factor of 10^6 or bigger) to Hardy, Cristina, Huub and Grietje. Thank you for all of the supervision, reviewing of my manuscripts, suggestions, collaboration, endless number of questions to which even Wikipedia has no answers, for the help and guidance with my future steps, for the nice talks and interest in my life. A special thank you to Caroline for your enthusiasm and involvement in the project, especially during the first half of it. And finally thank you all for pushing me to get this book written. Another amusement worthy fact is that Cristina managed to keep her sanity while working with the "engineers". Thank you for that Cri and also for your enthusiasm throughout this project. Looking forward to what we will do next.

Secondly, I have to say a thank you as big as above or even larger to whom is by now known as my wife and the mother of our awesome Amanda Louise. Tania, you were carrying Amanda in your belly and I was a huge baby in the outside world that you were supporting, advising, reading a big deal of nonsense I was writing down and taking care of me during the busy writing period. My pizza consumption would have probably increased tremendously if it weren't for you, which would result in a gravitational field around my belly. I cannot say enough thank you for everything and simply for you being around... I also hope we get our paper published. Not only because of what we investigated is cool, but also the fact itself is rather nice. Also, I will try to do my best to prevent any fields forming around your belly later this year, when you will be aiming for the PhD finish line yourself.

Ludo and Koen, my senior "examples" and buddies throughout these years. It is actually awesome to know that I have made real friends in The Netherlands and that you guys are there if and when needed. Looking forward to our next trip where ever it takes us and to our friendship in the years to come. For now we can all remember THE surfing morning in Fuerteventura and the HAPPY BABY in Morocco. For full fairness, also pole dances should not be forgotten. And do not worry, your useful advise for both my PhD and the future is also truly appreciated. @Carlos: you have been an excellent "surf dude group" addition. Cheers for that.

Katja fortunately you will not qualify for a homebrew due to being left out in these lines, but you are always welcome to visit me, Amanda and Tania for sharing one. I am really grateful for the nice talks we had during my PhD period, for all of the tips and tricks in the lab and quite a few discussions on my research. You are a true mood booster and I hope you will never change. I am also very happy to have become a part of the "poop on the ceiling of the experimental cabinet" club. It is a great honour to be a member and I hope we will (actually I am sure we will) have some interesting endeavours in the future.

To my parents and my brothers I would like to say thank you for supporting my path of "endless studying". If we would spin the time back and reflect to how I ended up where I am, it would never have been possible without you all. Thank you for creating opportunities for the youngest (and arguably the coolest) family member. Vilis and Mikus, it is also always a pleasure to receive the finger combination pictures from you two. Lets' keep the proud tradition alive and continue confusing people of what's so funny about it.

A huge thank you to Ruizhe, Chih-Yuan, Diana, Raquel, Millah, Bohong, Lin and Aaron for all your hard work invested in this project. I really enjoyed supervising your thesis/internships and seeing how all of you became more experienced and confident in doing research. It was also nice to get to know each one of you a little better during the process. Bohong additional thanks for the contraband of a fermenter all the way from China. I can now report that it does the job very well.

The research would be quite difficult without having a proper experimental set-up. Thank you Vinnie and Bert for helping out with all of the set-up related issues, especially with biogas quantification. I see it has already been approved and even adopted by our colleagues. Analytical tools are also rather important in the process. The support and help of Ilse, Hans, Livio, Pieter, Jean, and Katja to make this research possible is very much appreciated.

We often seek for collaborations and someone, who could help us with making a step forward with our research. Often such collaborators are not located in the same city or even country. I was quite fortunate to discover that many enthusiastic researchers would be located rather close in my case. In fact quite a few were neighbours from within the Axis building. Peter (Wierenga) it was a real pleasure meeting you and seeing how scientists from seemingly unrelated research fields can become enthusiastic about the research of others. I was very pleased how you tried to integrate me within Laboratory of Food Chemistry even at the very end of my PhD stretch. It was also nice to get a fresh "outside view" on our research from you. Claire and Yuxi, thank you a lot for the help with the analysis and also for the nice discussions. Claire, I was very impressed how you could pull off something that would be a mission impossible for myself in a relatively short time and communicated

the results with me in a very clear and open way. As it turned out, there were even more enthusiasts at the Laboratory of Food Chemistry, who were ready to get their "equipment dirty" and minds busy with the polymers of sludge granules. Mirjam, thank you for your kind welcome into the world of sugar and uronic acid analysis and for your time devoted to our discussions. Peter (de Gijsel), your practical assistance with the analysis is really appreciated. It was very nice of you to guide me through the procedures and to make my life in your labs comfortable. Other departments were also involved. Thank you: Suzan for the help with amino acid analysis and your time to train me in using the hydrolysis equipment, as well as your tips on where we should watch out with data interpretation; Harry for the particle size distribution measurements; Marcel for training me into scanning electron microscopy. Also my Wetsus colleagues Victor and Emmanuel are appreciated for our discussions and analysis we could perform together. Besides collaborations also communication of our results with a good group of people is important. Cees, thank you for having me in the biorecovery meetings and also cheers to the group for the presentations and discussions. It was also very nice to communicate our progress with all of the members of Water Nexus and especially with the members of work package 2.1: Nadine, Arjen, Paul, Georg, Danny, Jaap, Mario, Mark, Frank and Dennis. Danny, I enjoyed our experiments in Delft and all of the discussions we have had.

The life of any PhD candidate at ETE would be a disaster without the members of the secretary's office being always there to help us with everyday organisational issues. Liesbeth, I do not remember an organisational/practical question you wouldn't know how to solve. Even my defence date was made possible due to your creative solutions for those who do not read the emails carefully enough. This is truly appreciated and we are lucky to have you at ETE. Gea, you are not there anymore, but it was very nice of you to help me with all of the bookings for traveling abroad. Wies, thanks for being our financial wizard to help organising the PhD trip to Chile and for your patience with my "timely" hour registration. The secretary office is rather dynamic. Therefore, many thanks to Marjolein and Janine - the current sidekicks of Wies and Liesbeth - and also to those that were contributing before.

Our department is known for loads of togetherness and I would like to thank all of my fellow PhDs and other colleagues for the nice atmosphere in the department. Especially thank you to my officemates Thomas, Joeri, Arnoud, Adrian, Annemerel and Koen for all of the chitty chats we have had. The togetherness crew does not stop within the office. Carlos, Andrea, Merijn, Laura, Leire, Emilius, Sanne (de Smit), Sanne (Raes), Kasper, Bingnan, Dandan, Momo, Rieks, Rosanne, Ilse, Silvi, Pradip, Margo, Jess, Farzaneh, Livio, Jean, Jouke, Pieter, Vinnie, Nora, Pim it was very nice to meet you at a togetherness birthday event, soccer training, WE day or any other social gathering. Miriam, thanks for promoting me to a professor since my second year of the PhD and also for your genuine ability to improve the mood of people, when it's not their best day.

A special group of people I would like to acknowledge are those involved in organising the PhD trip to Chile. I need to mention Paulina here, because you are not mentioned above. It was a hectic and yet an exciting exercise. Hans without your contacts to get the funding and organise our site visits in Chile, this would have been much more difficult.

Finally, thanks to Joeri for the help with one of the final tasks to make this book finished. It was very nice of you to help with the cover and other design elements of this book. Besides this, you have been constantly helping me with practical “Dutch life” tips and suggestions throughout my PhD. This was also very nice of you (and also of you Koen, Thomas and Arnoud). Thomas, did you sell your wetlands to buy a house? I really appreciate your happy/funny/sarcastic spirit and looking forward to sharing our next office. Laura, also thanks to you for teasing me once in a while amongst others. :)

About the author



Dainis Sudmalis was born in Riga, Latvia on the 11th of October, 1988. After birth of Dainis, the family lived for a few years in the city of Bauska, and later on moved for settling in Riga. This is where Dainis graduated from Riga Secondary School 47 in the summer of 2008 with an honorary diploma signed by the prime minister of Latvia and immediately pursued his engineering education on Water, Heat and Gas Technology in the Riga Technical University (RTU). During his studies at RTU Dainis applied for an Erasmus grant for spending a study exchange period abroad. The application was successful and this brought him for an exchange period in the Cyprus University of Technology from the late summer of 2010 until the end of January 2011, where he took courses from both, environmental and mechanical engineering, departments. Despite language barrier challenges, the final exams were passed with very good results and gave the opportunity to apply for another exchange grant in the future. The year 2011 was spent in studies at Riga Technical University and settling additional courses that were not available in Cyprus during the exchange period. In February 2012 Dainis was granted his second Erasmus exchange grant and joined the PILLS project team of Glasgow Caledonian University for his internship to study the correlation between wastewater toxicity and its micropollutants content. After seven months of research work he returned to Riga for finalizing his studies at RTU with a thesis entitled *Chemical Composition and Toxicity of Urban Wastewater*. Dainis was included in the "Golden Fund of Graduates of RTU" – graduates with exceptional academic performance during their studies.

Following the graduation Dainis was hired by a construction firm as an assistant project manager for construction of drinking water/sewage networks and a wastewater treatment plant for the village of Vainode. By the end of the project Dainis had decided to continue with his studies and strengthen his knowledge in the field of water technology. This idea brought him to Wetsus Academy in Leeuwarden, The Netherlands. Dainis graduated *cum laude* from the Wetsus Academy in the summer of 2015 with a thesis entitled *Biological Treatment of Water Produced by Oil and Gas Industry*. In October 2015 he started with the PhD research on anaerobic sludge granulation at high salinity at Wageningen University.

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

D I P L O M A

for specialised PhD training

The Netherlands research school for the
Socio-Economic and Natural Sciences of the Environment
(SENSE) declares that

Dainis Sudmalis

born on 11th of October 1988 in Riga, Latvia

has successfully fulfilled all requirements of the
educational PhD programme of SENSE.

Wageningen, 6 March 2020

The Chairman of the SENSE board

Prof. dr. Martin Wassen

the SENSE Director of Education

Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that **Dainis Sudmalis** has successfully fulfilled all requirements of the educational PhD programme of SENSE with a work load of 48.6 EC, including the following activities:

SENSE PhD Courses

- Environmental research in context (2016)
- Research in context activity: 'Co-organizing the International Conference on Biogas Microbiology, 1-3 May 2017, Wageningen'

Other PhD and Advanced MSc Courses

- Advanced Biofilm Course, Karlsruhe Institute of Technology (2016)
- Basic Scanning Electron Microscopy (SEM), Wageningen University (2017)
- Open & Reproducible Microbiome Data Analysis, Wageningen University (2018)
- Project and time management, Wageningen Graduate Schools (2018)

External training at a foreign research institute

- "Predict it" workshop on modelling anaerobic wastewater treatment systems, TU Delft, The Netherlands (2019)

Selection of Management and Didactic Skills Training

- Organising meetings with several stakeholders (2015)
- Teaching in the BSc course 'Introduction to environmental technology' (2016)
- Organising the practicals of the MSc course 'Water Treatment' (2016-2018)
- Supervising 5 MSc students and one BSc student with thesis (2016-2019)
- Supervising two MSc students with internship (2018)
- Co-organising a PhD knowledge exchange trip to Chile (2018-2019)

Selection of Oral Presentations

- *High rate anaerobic wastewater treatment at saline conditions: is it possible?*. 3rd International Conference on Biogas Microbiology, 1-3 May 2017, Wageningen, The Netherlands
- *Anaerobic sludge granulation in a UASB reactor at high salinity and subsequent process performance*. 15th IWA World Conference on Anaerobic Digestion, 17-20 October, Beijing, China
- **Best presentation award:** *Effect of powdered activated carbon and substrate on anaerobic sludge granulation and activity at high salinity*. IWA Granular Sludge Conference, 18-21 March 2018, Delft, The Netherlands
- *Anaerobic Granular Sludge Technology at High Salinity – Why Protein May be Crucial*. 16th IWA World Conference on Anaerobic Digestion, 23-27 June 2019, Delft, The Netherlands

SENSE coordinator PhD education



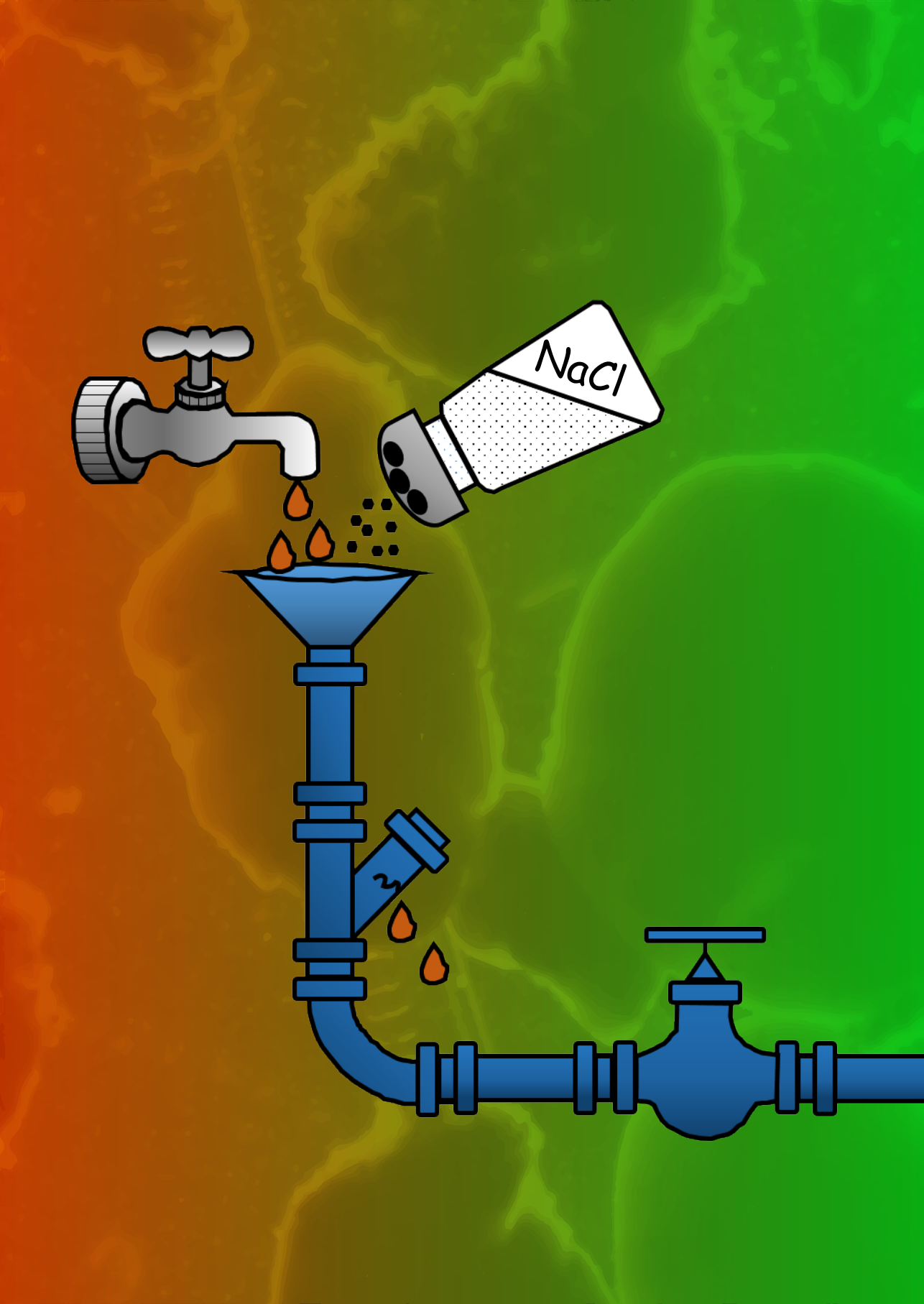
Dr. ir. Peter Vermeulen

This research was financed by the Netherlands Organisation for Scientific Research (NWO), which is partly funded by the Ministry of Economic Affairs and Climate Policy, by the Ministry of Infrastructure and Water Management, and partners of the Dutch Water Nexus consortium (project nr. STW 14300 Water Nexus 2.1).

Cover design concept by Dainis Sudmalis and Joeri Willet

Final cover design by Proefschriftmaken

Printed by Proefschriftmaken on FSC-certified paper


NaCl