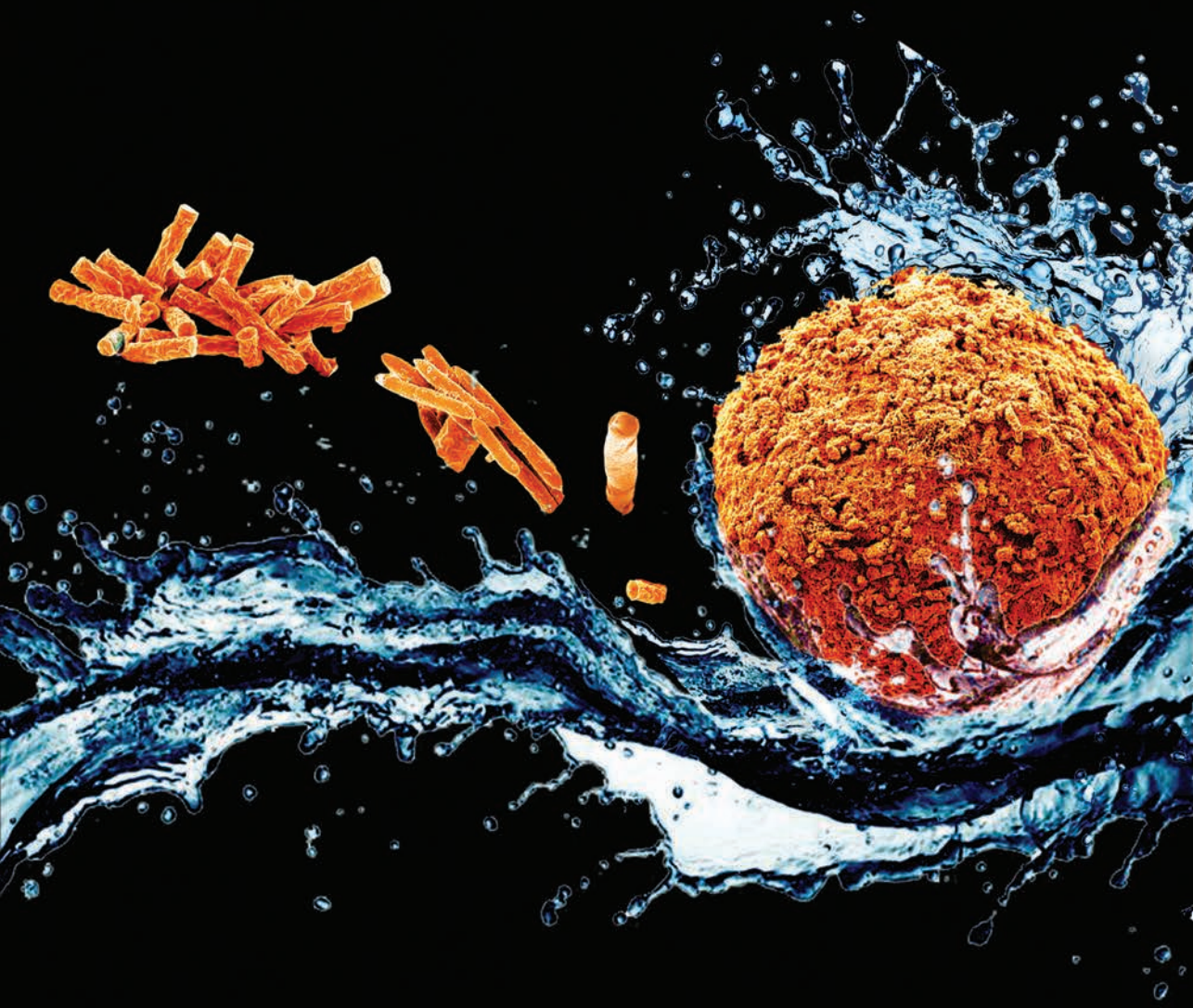
Anaerobic Wastewater Treatment of High Salinity Wastewaters:

Impact on Bioactivity and Biomass Retention



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ANAEROBIC WASTEWATER TREATMENT OF HIGH SALINITY WASTEWATERS: IMPACT ON BIOACTIVITY AND BIOMASS RETENTION

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Anaerobic wastewater treatment of high salinity wastewaters: Impact on bioactivity and biomass retention

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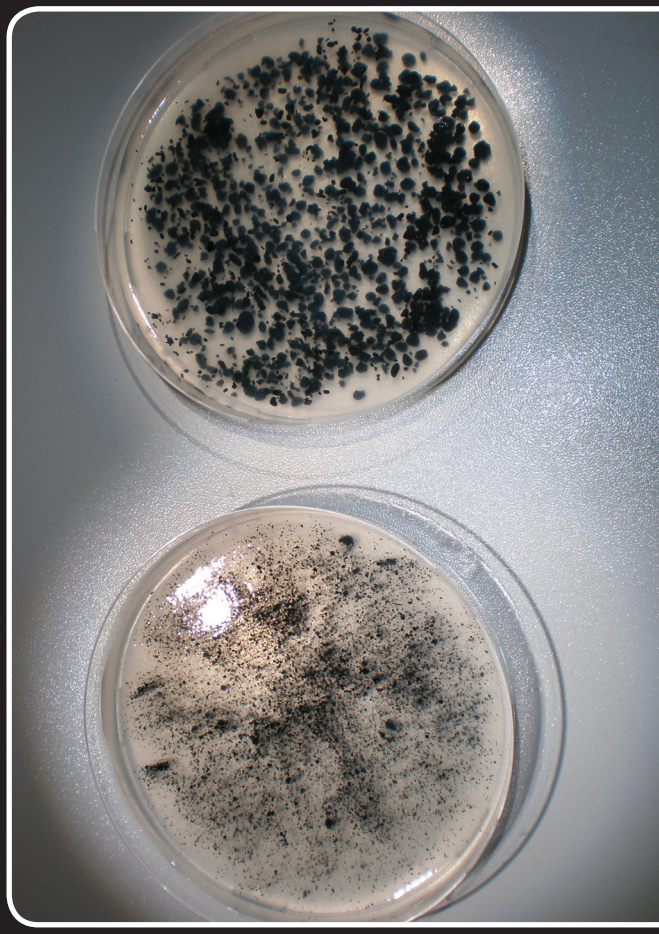
Chapter 1



General Introduction: High Rate Anaerobic Treatment of High Salinity Wastewaters

"De molen gaat niet om met wind die voorbij is," The windmill does not care for the wind that has gone past.
Dutch proverb

According to United Nations predictions, between two and seven billion people will face water shortages by the year 2050 (UN, 2007). Recently reported data for 2012, mentioned that 1 out of 6 people worldwide, i.e. about 783 million, do not have access to improved drinking water sources (UNICEF, 2013). This has become a global issue, and not only a problem relevant to arid zones. Because this scarcity is a threat to human activity, water reuse strategies deserve major attention (Fritzmann et al., 2007).

Closed water loop approaches in industry minimize the amount of fresh water used for production processes, thereby protecting water resources from depletion, and reducing the volume of wastewater that is discharged into the environment (Figure 1). However, this closure of industrial water loops drastically changes the characteristics of the resulting wastewater streams. A high degree of 'closure' even may results in extreme conditions with respect to pH, temperature, and salinity, with values beyond the known operational conditions for biological wastewater treatment systems (Pevere et al., 2007).

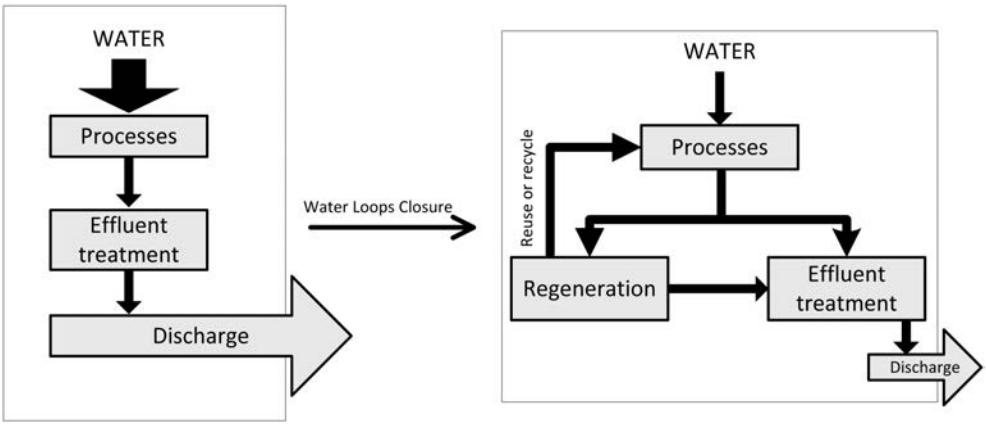


Figure 1 | Optimization of water network by reuse and/ recycling of wastewater (adapted from de Faria et al., 2009).

Verstraete et al. (2009) suggested that the recovery and maximum use of both the organic matter enclosed chemical energy and nutrients present in 'used water' is a more sustainable approach than a direct discharge of treated wastewater. With respect to this, biological anaerobic wastewater treatment (AnWT) has a much better sustainability score than alternative treatment methods. Van Lier (2008) reviewed the anaerobic treatment technology, including its advantages with respect to sustainability as well as cost effectiveness on a variety of applications.

One of the examples where a 'closed water loop' approach in combination with AnWT has been successfully applied is the pulp and paper industry (Figure 2). Paper mill effluents contain starch, volatile fatty acids (VFA) and cellulose, which are all readily bio-degradable and thus, very good substrates for AnWT. The organic matter is mineralized to considerable amounts of methane, which is subsequently used to produce steam. After an aerobic post-treatment stage and filtration system, the treated water is reused in the process (Vogelaar et al., 2002). However, the disadvantage of such a closed system is that fine particles, colour related compounds, and/or salt can accumulate, which in the case of high quality paper production also would requires a tertiary treatment (Habets and Knelissen, 1985, Pramod, 2012).

The accumulation of salt, in particular Na^+, in industrial effluents can be considered a new challenge for anaerobic wastewater treatment. The toxic effects of Na^+ on anaerobic treatment have been studied by various authors, e.g. Lefebvre et al. (2012). High Na^+ concentrations also have an impact on the anaerobic granulation process (Lefebvre and Moletta, 2006), which is a requirement for operating high rate anaerobic reactor systems.

This chapter focuses on the treatment of high salinity wastewaters using AnWT. The type of industry producing high salinity wastewaters and the bottlenecks of the AnWT application for such wastewaters are reviewed. Microbiological aspects as well as granulation theories related to high salinity conditions are discussed.

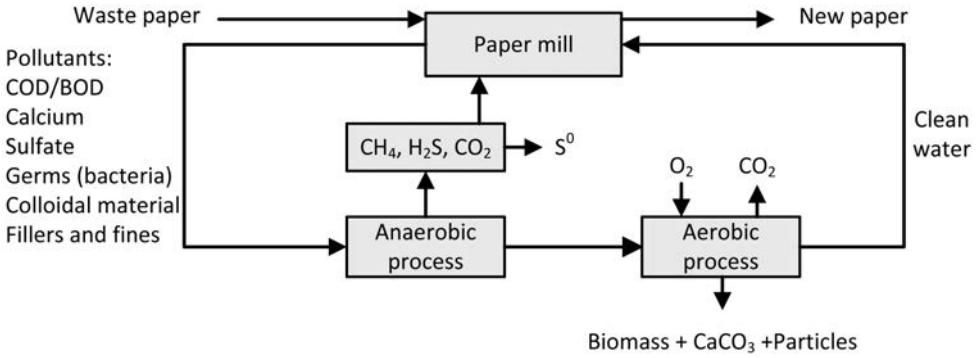


Figure 2 | 'Zero discharge' paper mill with in line anaerobic–aerobic treatment (Habets and Knelissen, 1985).

1.1 Anaerobic wastewater treatment

1.1.1 Anaerobic digestion

Anaerobic digestion refers to the treatment of organic materials, which are degraded into biogas composed of mainly methane and carbon dioxide. Nowadays, it can be considered an established technology, successfully applied for the treatment of many kinds of industrial wastewaters as well as municipal sewage. Anaerobic treatment offers the combination of efficient wastewater treatment at low operational costs with energy recovery (Lettinga et al., 1997). The success of modern high-rate anaerobic wastewater treatment systems can be attributed to an efficient uncoupling of the solids retention time (SRT) from the hydraulic retention time (HRT) through biomass immobilization, which is usually accomplished by biofilm and/or granule formation. With this strategy, high concentrations of biomass are obtained, leading to high treatment capacities. From the available technologies, sludge bed reactors, such as the up-flow anaerobic sludge blanket reactor (UASB) and expanded granular sludge bed reactor (EGSB) are mostly applied. Van Lier (2008) reported that almost 80 % of the full-scale anaerobic wastewater treatment installations are sludge bed reactors in which biomass retention is achieved by the formation of sludge granules.

The anaerobic digestion pathway of organic matter involves several biochemical reactions, performed by different microbial trophic groups, which sequentially degrade a variety of substrates and intermediate compounds (Figure 3). The microbial consortia that are involved ultimately mineralize the organic matter into methane (CH_4), carbon dioxide (CO_2), ammonium (NH_3), hydrogen sulphide (H_2S), phosphates (PO_4^{3-}) and water (H_2O).

Anaerobic digestion can be divided in four phases, related to the characteristic microorganisms and main conversion processes taking place: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 3). Firstly, hydrolytic enzymes are excreted by fermentative bacteria, the so called 'exo-enzymes' and fermentative bacteria actively colonise the organic matter. The non-dissolved material is converted into less complex, dissolved compounds, which can be taken up by the fermentative bacteria. Secondly, during acidogenesis, the dissolved compounds present are converted by fermentative bacteria into a number of simple compounds, which are then excreted. The compounds produced during this phase include volatile fatty

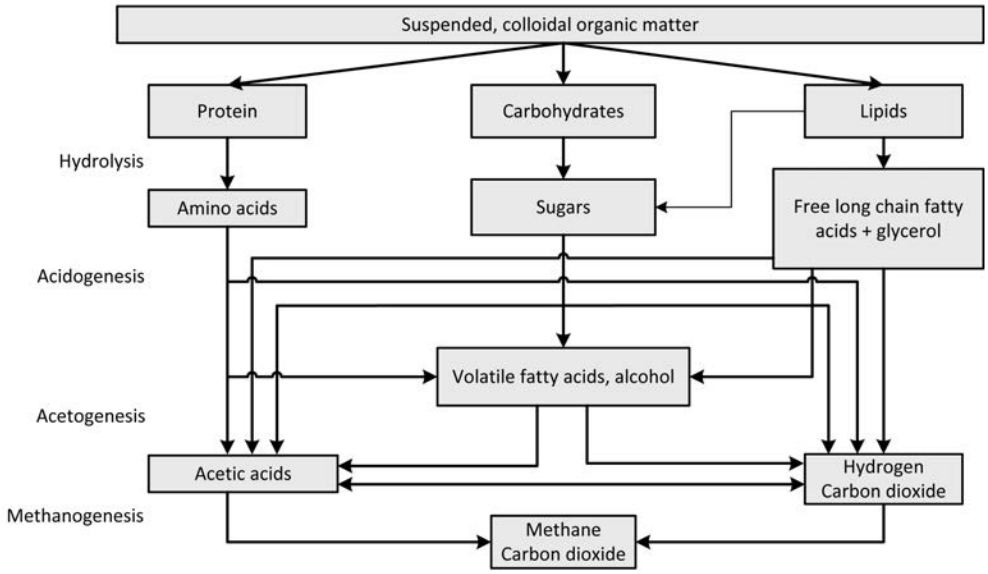


Figure 3 | Simplified schematic representation of anaerobic digestion process (adapted from van Lier, 2008).

acids (VFA), alcohols, lactic acid, CO_2, H_2, NH_3, and H_2S. Thirdly, the acetogenic phase, or intermediary acid-production, converts the digestion products into acetate, hydrogen (H_2) and CO_2. Lastly, methanogenesis converts acetate, hydrogen plus CO_2, formate or methanol into methane.

1.1.2 Anaerobic granulation

Granulation is a key factor in the operation of high rate anaerobic reactors. It is a complex process that involves physicochemical as well as biological mechanisms. There is no clear and defined distinction between different types of conglomerates such as granules, pellets, flocs, and flocculent sludge. Dolfing (1987) defined flocs or flocculant sludge as conglomerates with a loose structure, which often settle as a homogenous layer at a settling velocity at 0.1 – 1 m/h. Pellets are conglomerates with a more dense structure than flocs, and are visible as separate entities after settling. According to Lettinga (1995), the UASB reactor inventor, anaerobic granules are particulates enveloped by biofilms, which are formed spontaneously in a UASB reactor by the auto-immobilization of anaerobic microorganisms. Structurally, granules are particles, consisting of an intertwined mixture of symbiotic anaerobic microorganisms. Granules are also defined as a functional unit in itself, comprising of all the different microorganisms necessary for the methanogenic degradation of organic matter (Bhunia and Ghangrekar, 2008, Zheng et al., 2006).

The diameter of sludge granules varies from 0.15 to 5 mm and the granules possess higher shear strength than flocculated sludge (Jeong et al., 2005, Show et al., 2004), depending on the type of wastewater that is treated and the operational conditions applied. Granules grown on acidified substrates, such as acetate, are generally smaller than granules grown on partly or non-acidified substrates, e.g. glucose (Fukuzaki et al., 1991, Grotenhuis et al., 1991a). Alt-

hough the granules can vary widely in shape (Schmidt and Ahring, 1996), they usually have a spherical form.

The upward liquid flow rate at which a granule with a given volume and buoyant density will be washed out from a reactor can be estimated by Stoke's law. However, granules with different volumes and densities are present in a reactor; i.e. both small granules with high densities and larger granules with low and high densities. It was reported that a settling velocity of 60 m/h is considered to be very good for granular sludge (Hulshoff Pol et al., 2004). Granular sludge can therefore be divided into three fractions: a poor settling fraction, a satisfactory settling fraction, and a good settling fraction, with settling velocities of up to 20 m/h, from 20 to 50 m/h, and over 50 m/h, respectively (Schmidt and Ahring, 1996).

1.1.3 Application of anaerobic granular sludge bioreactors

The proper selection of reactor technology determines the success of anaerobic wastewater treatment. Industrial wastewaters with easily degradable organic matter, like those coming from food and beverages industry, were the first to be treated by anaerobic technology as a feasible treatment technology (Austermann-Haun et al., 1999, van der Lubber et al., 2010). The upflow anaerobic sludge blanket (UASB) reactor is the most widely applied anaerobic wastewater treatment system among the anaerobic granular sludge bioreactor (van Lier, 2008). The UASB reactor has positively contributed to the acceptance and implementation worldwide of the anaerobic technology for the treatment of a wide range of agricultural and industrial wastewaters (Latif et al., 2011).

The key factor for the success of the UASB reactor is the uncoupling of the hydraulic retention time from the solids retention time. This uncoupling results from the growth of anaerobic microorganisms in densely packed flocs or granules following the self-immobilization process without any carrier material. The high settleability of the granules prevents the granules from being washed out from the reactor applying a specific upflow velocity. Another specific feature of the UASB reactor is the instalment of a gas, liquid, solids, separator (GLSS) at the top part of the reactor, in which the gas bubbles are captured. In the quiescent zone above the GLSS the solids particles are settled and slide back in the reactor to the sludge blanket.

1.2 High salinity wastewater

High salinity wastewaters are characterised by high concentrations of inorganic salts, mostly NaCl, and are generated in various kind of industries. Some high salinity wastewaters are also discharged from drinking water treatment plants, i.e. concentrates from reverse osmosis (RO) processes or from ion-exchange units (Alameddine and El-Fadel, 2007). Wastewaters are classified in the high salinity range based on the Na^+ concentration (Madigan et al., 2000):

- highly saline, >7.9 g Na^+/L
- hyper saline, 15.8 – 47.2 g Na^+/L
- extreme saline, >47.2 g Na^+/L

The typical Na^+ concentrations seawater ranges between: 12 – 16 g Na^+/L , with 11.8 g Na^+/L on average (Madigan et al., 2000). The presence of high salt concentrations in these wastewa-

Table 1 | Anaerobic treatment of high salinity wastewaters (adapted from Xiao and Roberts, 2010).

Target	Influent (g/L) Salt % w/v NaCl	Reactor	Performance	Inoculum	Remark	Ref.
Seafood processing wastewater	COD: 1–6 Salt: 1.3–3.1	Pilot-plant industrial V = 15 m^3	COD removal: 70–90 %	Anaerobic sludge from the treatment of non-saline wastewaters		1, 2
Fishery wastewater	COD: 5–32 Salt: 1.5–1.8	Lab-scale completely mixed flow reactor V = 1.5 L, HRT = 3–30 d	Methane in biogas: 45 % COD removal: 40 %	Marine sediment	Methane production was inhibited at COD/SO_4^{2-} ratios lower than 0.5.	3
Fishery wastewater	COD: 6 Salt: 3.9	Lab-scale continuously mixed system packed with filter medium V = 1.95 L, HRT = 0.86–3.9 d	Methane in biogas:>80 % COD removal: 60–96 %	Marine sediment	Methane production was inhibited at NH_3-N >100 mg/L	4
Sludge from fish farm effluents	COD: 60–74 Salt: 1–1.5	Lab-scale completely mixed batch reactor V = 15 L, HRT = 25–65 d	Methane yields: 0.114 – 0.184 L/g COD COD removal: 36–55 %	Sludge from an anaerobic digester originally inoculated with municipal sewage sludge	The process was strongly inhibited, presumably by sodium, and unstable	5
Sludge from a salmon smolt hatchery	COD: 110–193 Salt: 1.4	Lab-scale completely mixed batch reactor V = 15 L, HRT ≈ 60 d	Methane in biogas:~60 % Methane yields: 0.14–0.154 L/g COD COD removal: 44–54 %	Sludge from anaerobic digester	The process was inhibited by volatile fatty acid with concentrations of up to 28 g/L	6
Dye Reactive Brilliant Red K-2BP	Dye: 550 mg/L Salt: 15	Lab-scale batch HRT = 100 h	Dye removal >90 %	A salt-tolerant culture acclimated in the lab		7
Synthetic wastewater consisting of sucrose, acetic acid, urea and other minerals	COD: 0.5 Salt: 3	Two lab-scale anaerobic/ anoxic/aerobic reactors HRT = 2/2/12 h	Non-acclimated: COD removal 60 %, Acclimated: COD removal 71 %	Salt acclimated: a local salt-rich tannery wastewater	One reactor inoculated with salt-acclimated seeds, the other with non-acclimated seed	8

Table 1 | Anaerobic treatment of high salinity wastewaters (adapted from Xiao and Roberts, 2010) …cont.

Synthetic wastewater containing diluted piggery manure	COD: 1.1–2.9 Salt: 1.5	A lab-scale down-flow anaerobic fixed bed reactor (DFAFBR) V = 1.4 L, HRT = 96, 48, 24 and 12 h	At HRT = 24 h COD removal: 72 %	Anaerobic digested sludge from a pilotscale cattle-manure digester		9
Synthetic wastewater consisting of glucose, acetic acid, ammonium chloride and other minerals	COD: 1.9–6.3 Salt: 3	A lab-scale upflow anaerobic packed bed reactor V = 3 L, HRT = 11–30 h	COD removal: 60–84 %	*Halanaerobium lacusrose*		10
Synthetic wastewater consisting of molasses, urea and other minerals	COD: 1.12 Salt: 3.8 (TDS)	An upflow multistage biofilm reactor V = 54 L, HRT = 8–24 h	COD removal: >85 %	Sludge from an anaerobic baffled reactor treating non-alcoholic beer wastewater		11
Synthetic sewage wastewater consisting of peptone, meat extract and urea	COD: ~0.465 Salt: 3.5	A submerged anaerobic membrane reactor (SAMBR) V = 3 L, HRT = 8–20 h	DOC removal: 99 %	Anaerobic biomass from a conventional sewage sludge digester	Membrane: polyethylene, pore size: 0.4 μm Flux through membrane: 5–8 L/(m^2·h)	12, 13
Synthetic high salinity wastewater	COD: 3 Salt: 2	Modified UASB reactor with hollow-cylinder electrode	COD removal: 93 %	Sludge from laboratory scale UASB reactor		14
Aquaculture wastewater	-	Waste stabilization ponds and lab-scale UASB reactor	BOD removal: 91 - 97 %	Sludge from brackish/marine recirculation aquaculture system		15

[1](Omil et al., 1995); [2](Omil et al., 1995); [3](Aspé et al., 1997); [4](Aspé et al., 2001); [5](Gebauer, 2004); [6](Gebauer and Eikebrokk, 2006); [7](Guo et al., 2007); [8](Panswad and Anan, 1999); [9](Rovirosa et al., 2004); [10](Kapdan and Erten, 2007); [11](Ghaniyari-Benis et al., 2009); [12](Vyrides and Stuckey, 2009); [13](Vyrides et al., 2010);[14](Zhang et al., 2012); [15](Mirzoyan et al.)

ters has been observed as inhibitory for conventional biological treatment processes, especially anaerobic treatment. Therefore, the environmental management of these wastewaters is becoming more stringent and their treatment is becoming a major concern for researchers, regulators and engineers.

1.2.1 Industries generating saline wastewaters

Many agro-food industries such as seafood processing, vegetable canning, pickling, and cheese processing, as well as fish processing industries generate large volumes of high salinity wastewaters with high concentrations of organic pollutants and nutrients. For example, in the fish processing industry, the sources of pollution are related to the fish unloading activities which contribute to a considerable amount of seawater. Fishery industries generate wastewaters rich in organic suspended solids, proteins, and salinity close to seawater (Vidal et al., 1997, Aloui et al., 2009).

Tanning, which turns raw hides and skins into finished leather products, is a lengthy process that involves several steps, many of which involve the addition of reactive chemicals and salt (Lefebvre et al., 2005). The tanning process is a wet process that generates large amounts of wastewater. Certain streams are hyper saline, such as the chromium tanning effluents or the soak liquor generated by the soaking of hides and skins that can contain as much as 80 g/l of NaCl (Lefebvre and Moletta, 2006). Therefore, discharge of tannery effluents has a huge environmental impact.

Oil and gas industries produce large volumes of wastewaters containing high concentrations of salt and oil (Tellez et al., 2002). The salt concentration of the produced water may range from a only few mg's/L to 300 g/L (Neff, 2002). The applied extraction technology and reservoir characteristics both affect the amount of produced water. At some sites, it may be even 10-fold the quantity of oil production (Campos et al., 2002). The discharge of produced water without proper treatment might cause pollution to soil, water ways and underground water (Pendashteh et al., 2010).

1.2.2 Previous research on anaerobic treatment of saline wastewaters

Examples of earlier documented anaerobic treatment of saline industrial effluents are shown in Table 1. Anaerobic digestion of seafood-processing wastewater already has been studied for the past 20 years. Besides that, Rovirosa et al. (2004) conducted a study about anaerobic digestion of a piggery effluent diluted in a saline synthetic water (15 g/L of salt), using a lab-scale down-flow anaerobic fixed-bed reactor to obtain a final total COD concentration in the range of 1100 – 2900 mg/L. The COD removal efficiency exceeded 90 % for a hydraulic retention time (HRT) of 96 h and 68 % for an HRT of 12 h. Vyrides and Stuckey (2009) used a submerged anaerobic membrane reactor to treat a synthetic saline wastewater, consisting of peptone, meat extracts, urea, using anaerobic biomass from a conventional sewage sludge digester as the inoculum. At a salinity of 35 g NaCl/L and an HRT of 8 h or 20 h, with membrane fluxes ranging from 5 to 8 L/m^{2}h, 99 % removal of dissolved organic carbon (DOC) was achieved, although only 40 – 60 % removal of DOC was attributed to biodegradation with the remaining removal determined by membrane rejection. Lefebvre et al. (2006) studied the anaerobic digestion of tannery soak liquor in a UASB reactor and concluded that the COD

removal efficiency was effected by very low organic loads. However, the granules formation at high salinity wastewater has not been discussed and remained unclear.

1.3 Microbiology in high salinity wastewater

In nature, many saline environments can be found, such as saltern pond brines and natural salt lakes. The presence of large amounts of salt is known to inhibit the growth of microorganisms. However, in these extreme environments, the microorganisms present are completely adapted to cope with the high salinity. It would be relevant to use these extremophiles in biotechnological processes, including the biological treatment of high salinity wastewaters (Claros et al., 2010). However, research has yet not focussed on using this natural potential.

1.3.1 Halophilic/halotolerant microorganisms and their application in anaerobic wastewater treatment

Lefebvre et al. (2006) reported that only little knowledge is available regarding the microbial diversity of biological wastewater treatment systems operating at a high salinity. The general phylogenetic diversity in natural environment was reviewed by Oren (2010). Halophilic and halotolerant microorganisms are able to grow in the presence of high salt concentrations and are found in all three domains of life: Archaea, Bacteria, and Eukarya (Ma et al., 2010). Microorganisms living at high salt concentrations can be divided into two groups: halophilic and halotolerant microorganisms.

Halophiles are extremophilic microorganisms that require high salt concentrations to sustain their growth. Halophilic microorganisms can be divided into four main categories as shown in Table 2. The halophiles were found within the Domain Bacteria such as the phyla Cyanobacteria, Proteobacteria, Firmicutes, Actinobacteria, Spirochetes, and Bacteroidetes. The most salt-requiring Archaea are found in the class Halobacteria, that require over 100 g/L to 150 g/L of salt (NaCl) for growth and structural stability (Oren, 2008). Halophilic microorganisms have been successfully used in lab-scale anaerobic reactors for the treatment of high salinity wastewaters (Kapdan and Erten, 2007). However, the microbial diversity involved in anaerobic digestion particularly at high salinity levels is largely unknown (Oren, 2010). For example, halophilic methanogens obtained from mangrove soil were used to treat high salinity wastewaters in laboratory–scale anaerobic filters, resulting in an increased COD removal efficiency, exceeding 80 % (Riffat and Krongthamchat, 2007).

Table 2 | Categories of microorganisms according to the optimal growth range in Na^+ concentration (adapted from Lay et al., 2010).

Category	Na^+ range (g/L) for optimal growth
Non-halophilic	<4
Marine or slightly halophilic	4 – 12
Moderate halophilic	12 – 60
Extremely halophilic	>60

Halotolerants are non–halophilic microorganisms, which are able to grow in the absence as well as, to a limited extent, in the presence of salt. Halotolerants that are able to grow above approximately 15 % (w/v) NaCl (2.5 M) are sometimes referred to as extremely halotolerant

(Pikuta et al., 2007). Table 3 lists some methanogenic and acetogenic conversions performed by halotolerant species, together with the upper limit for the salt concentration. Some studies report about use of salt tolerant species, which are added to anaerobic biomass for the treatment of high salinity wastewaters. For example, Kapdan and Erten (2007) applied Halanaerobium lacusrosei in an upflow multistage biofilm reactor for the treatment of high salinity wastewaters at concentrations up to 40 g Na^+/L. The results of that study indicated that addition of those salt tolerant microorganisms enhances COD removal efficiencies, exceeding 85 % (Table 1). In this thesis we focus on methanogenic and acetogenic microorganisms, since they represent the core metabolism of the bio-methane producing biomass treating soluble wastewater.

1.3.2 Strategies of microorganisms to adapt to saline environments

High concentration of salt can cause cell plasmolysis and cell death due to the dramatic increase in osmotic pressure across the bacterial membrane (Mahajan and Tuteja, 2005). Two different strategies enable microorganisms to survive under this condition: the "salt in" and the "compatible solute" strategy. The salt in strategy refers to the process where the cell increases the intracellular ion concentration (mainly K^+) to balance the external osmotic pressure. This means that all intracellular enzymes have to be able to cope with the elevated intracellular cation concentrations. The compatible solute strategy is employed by many microorganisms. They accumulate organic compatible solutes within their cytoplasm to balance the high external osmotic pressure. β–glutamine, α–glutamate, N-acetyl–β–lysine and glycine betaine are among the solutes found in isolated methanogenic archaea from moderately saline environments (Sowers et al., 1990, Müller et al., 2005). In some studies, the use of compatible solutes as osmoprotectants for anaerobic biomass treating high salinity wastewaters was tested, resulted in significantly higher methane production rates (Vyrides and Stuckey, 2009).

Table 3 | The upper salt concentration limit of several methanogenic and acetogenic conversions performed by microorganisms in pure culture (adapted from Oren, 2010).

Process	Most halotolerant reprentative	Approximate upper limit in culture
Methane formation from $H_2 + CO_2$	*Methanocalculus halotolerans*	47 gNa^+/L
Methane formation from acetate	*Methanosarcina acetovorans*	4 – 12 gNa^+/L
Methane formation from methylated amines or methanol	*Methanohalobium evestigatum*	12 – 60 gNa^+/L
Formation of acetate from $H_2 + CO_2$	*Acetohalobium arabaticum*	>60 gNa^+/L

1.4 Anaerobic granulations and effects of salinity

Microorganisms tend to adhere to any interface and, if the right conditions prevail, they grow out into biofilms or granules. This adhesion phenomenon also is the basis of anaerobic granulation and many theories have been postulated in literature to better understand this process. Monds and O'Toele (2009) provide a critical review on the recent study on the biofilm formation (as illustrated by Figure 4).

In contrast to sludge granulation, biofilm formation has been intensively investigated. Formation of a layered structure of an anaerobic biofilm has been proposed (Picioreanu et al., 2005, Picioreanu et al., 2007), based on the differences between the relative kinetic rates of the

different steps in anaerobic digestion using the anaerobic digestion model proposed by the International Water Association (IWA) task group (Batstone et al., 2002).

1.4.1 Theories on granules formation

Liquid and gas upflow, suspended solids in the influent or seed sludge, attrition, and removal of excess sludge from the reactor are all considered as the main physical factors involved in granulation. The selection pressure theory combined with microbial factors is selected to be explained in more details. According to Hulshoff Pol et al. (2004), the granulation process in a UASB reactor is believed to be the continuous selection between lighter and heavier sludge particles that occurs in the reactor. This selection pressure is caused by the sum of the hydraulic loading-rate and the gas loading-rate, which depends on the sludge loading rate. When the hydraulic and gas-loading rates are high, the lighter and dispersed sludge will be washed-out from the reactor, while heavier components will be retained. Thus, growth of finely dispersed sludge (known as consortia) is minimised and microbial growth is delegated to a limited number of growth nuclei that consist of inert organic and inorganic carrier materials, or small bacterial aggregates present in the seed sludge (Hulshoff Pol et al., 1987). Filamentous microorganisms are known to act as bridge between different aggregates to produce compact granules (Li et al., 2007). MacLeod at el. (1990) hypothesized that Methanosaeta, a filamentous acetotrophic methanogen functions as a central nucleus that initiates the development of densely packed sucrose degrading granules. Therefore, combining the selection pressure and proper type of microorganisms will form a strong granule with a higher ability to settle and retain in bioreactor.

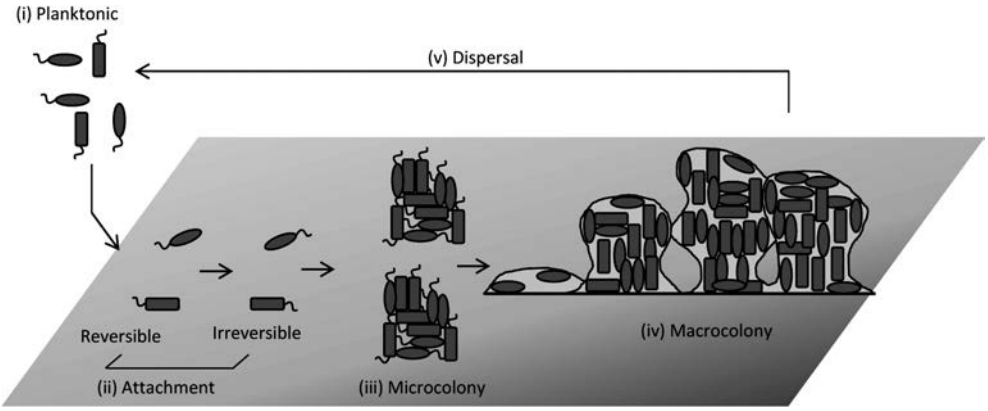


Figure 4 | Developmental model of biofilm formation. The biofilm formation begins with the attachment of cell to the substratum. Later on, the formation of discrete cell clusters starts that are referred to as microcolony. Growth of the microcolony proceeds either by clonal growth of attached cells or by active translocation of cells across the surface. This microcolony will grow in size and integrate to form macrocolonies. Macrocolonies can dissolve, releasing cells from the biofilm that are subsequently returned to the planktonic phase (adapted from Monds and O'Toele, 2009).

1.4.2 The role of type of substrates, extracellular polymeric substances (EPS) and Ca^{2+} binding on granule formation

Three aspects on the anaerobic granulation processes, particularly at high salinity, are illustrated in Figure 5. The first hypothesis is that different types of substrates will determine

the microbial populations as well as EPS production. Secondly, EPS production is crucial and plays an important role for bacterial adhesion in granulation processes. Lastly, Ca^{2+} augmentation is necessary for polymer binding and to prevent Ca^{2+} leaching in high salinity wastewaters. The details about those aspects are discussed below.

The success of sludge granulation is directly related to the composition of wastewater (Tay et al., 2002). Also Ahn et al. (2000) states that the type of wastewater is the most important factor determining the physicochemical and microbial characteristics of anaerobic granules. Several other authors noticed the positive effect of high-energy substrates, mainly partially acidified substrate (PAS) such as carbohydrates, on granulation (Thaveesri et al., 1994, Schmidt and Ahring, 1996, Imai et al., 1997). Thaveesri et al. (1995) studied the addition of high-energy substrate on the formation of anaerobic granular sludge and found that when there were insufficient amounts of sugars, i.e. less than 7 % of the influent COD, influent no granulation was observed, whereas when sufficient sugars were made available by the addition of sucrose, biomass started to granulate. With 27 % (Thaveesri et al., 1995) and 20 % (Grootaerd et al., 1997) of the COD of the influent as high energy substrates (carbohydrates), granular growth and sludge bed stability were enhanced significantly. Apparently, when more steps in the anaerobic food chain are required to degrade the COD and thus, when a more diverse microbial population is present, the properties of the granular biomass will be improved.

Granulation was reported in reactors fed with VFAs, but generally, the formation of granules takes much more time and the granules are smaller, more fragile and less stable during storage (Tiwari et al., 2006, Grotenhuis et al., 1992, Schmidt and Ahring, 1996, Morvai et al., 1992). Imai et al. (1997) observed that the development of granular sludge on VFA mixture was slower than that of in reactors fed with glucose.

Dafonchio et al. (1995) reported that the presence of influent sugars resulted in hydrophilic acidogens on the surfaces of the granules, whereas VFA as substrate tended to enrich for more hydrophobic methanogens. Moosbrugger et al. (1990) observed small and fragile granules when fed with protein and Batstone and Keller (2001) found that protein-grown granules had a poor strength and low settling velocity. Van der Haegen et al. (1992) also showed that high levels of protein produced a very fluffy sludge bed and reduced the in-reactor granular sludge build-up strongly, which was tentatively attributed to the growth of species not producing EPS or repressing EPS production. Thaveesri et al. (1994) observed that the substitution of carbohydrates by proteins resulted in a deterioration of the sludge characteristics, which was attributed to a reduction in the liquid surface tension caused by the proteins, rather than to the presence of NH_4^+, the final product of protein degradation.

In general, it can be concluded that reactor systems utilizing partially acidified substrates are characterized by a successful granulation process. In this thesis, the effects of different type of substrates (i.e. partially and fully acidified) on anaerobic granulation focussing on high salinity wastewaters will be discussed in more details.

Extracellular polymeric substances (EPS), are sticky materials and complex mixture of biomolecules (proteins, humic-liked substances, polysaccharides, uronic acid, nucleic acid, lipids, glycoprotein) of various molecular weight, excreted by microorganisms, produced from cell lysis and hydrolysis and adsorbed organic matter from wastewater (Adav and Lee,

2011, Sheng et al., 2010). EPS have been detected in significant amounts in anaerobic granules, forming a three-dimensional matrix in which bacteria and other particles are embedded (Quarmby and Forster, 1995, Fang et al., 2000). There is strong evidence that EPS are strongly involved in adhesion phenomena and formation of matrix structures (Li and Yu, 2011, Liu et al., 2011, Batstone and Keller, 2001). Hence, EPS play a key role in the anaerobic granulation process (Figure 5).

The accumulation of EPS as capsular material and peripheral slime has been correlated with biological adhesion and aggregation processes (Liu and Fang, 2002, Tay et al., 2001). The metabolic blocking of extracellular polysaccharide synthesis was found to prevent microbial aggregation (Yang et al., 2004, Cammarota and Sant'Anna Jr, 1998). These organic polymers are assumed to play a role in the formation of larger and dense anaerobic granules and are associated with divalent cations, particularly Ca^{2+} known as the bridging agent, as shown schematically in Figure 5.

Abundantly available Na^{+} replaces Ca^{2+} in the granule matrix, resulting in a weak granule structures. Previously, Grotenhuis and coworkers (1991a) showed a deterioration of granule strength upon specific Ca^{2+} displacements. A similar finding was made by Bruss et al. (1992) who observed Ca^{2+} displacement in aerobic activated sludge flocs when exposing the flocs to high Na^{+} concentrations.

Therefore, Ca^{2+} augmentation could be a solution to prevent leaching of multivalent cations from the granules, for example if they are exposed to high salinity wastewaters. The role of Ca^{2+} is already known in biogranulation processes, especially for strength improvement. This can be explained by the divalent cation bridging (DCB) theory which has been proposed by McKinney (1952) and Tezuka (1969). The bivalent cation, Ca^{2+} binds to negatively charged groups present on bacterial surfaces and extracellular polymers molecules, and acts as a bridge to interconnect these components (Sobeck and Higgins, 2002, Flemming and Wingender, 2010), thus promoting bacterial aggregation and matrix stability (Wloka et al., 2004 and Subramanyam, 2013).

Batstone et al. (2001) encountered a partial linear correlation between granule strength and Ca^{2+} concentration. 100 – 200 mg/l of Ca^{2+} was found to exert a positive impact on the size, settleability and on the rate of granulation (Mahoney et al., 1987 and Liu at al., 2002). Liu and Sun (2011) demonstrated that even at low concentration at 50 mg Ca^{2+}/L helped to strengthen the structure of denitrifying granules.

However, the presence of too much calcium could damage the environment required for maintenance of the granular structure and the bacterial activity, as previously studied on anaerobic granulation by van Langerak et al. (2000). Yu et al. (2001) postulated that an immobilized calcium concentration higher than 5350 mg/L would impose a severe restraint on the specific activity of the granules and is detrimental to granule formation due to the cementation and encapsulation of biomass inside the granules, mainly by the formation of $CaCO_3$.

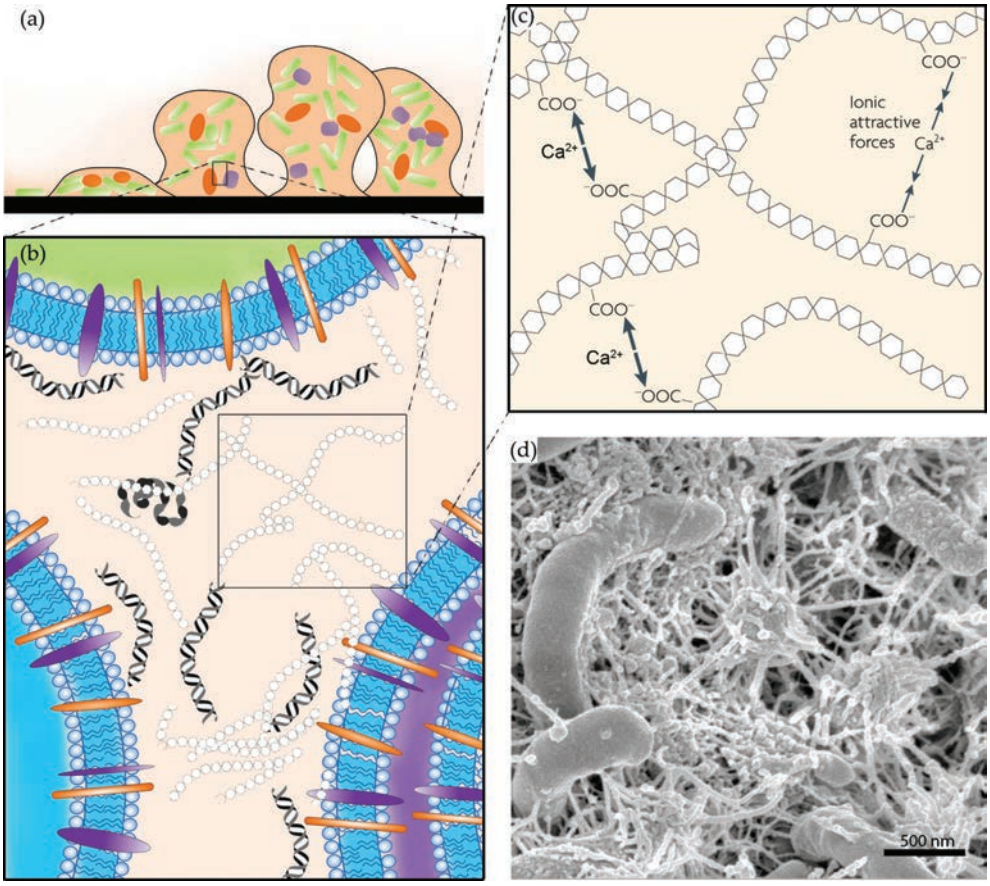


Figure 5 | The role of extracellular polymeric substances (EPS) matrix and Ca^{2+} in biofilms formation. (a) A graphical representation of a bacterial biofilm attached to a solid surface. Biofilm formation starts with the attachment of a cell to a surface. A micro colony forms through division of the bacterium, and production of the biofilm matrix is initiated. Other bacteria then can be grown as the biofilm expands owing to cell division and the further production of EPS components. (b) The major EPS components—polysaccharides, proteins and DNA—are distributed between the cells in a non-homogeneous pattern, setting up differences between regions of the matrix. (c) The interactions of Ca^{2+} bridging with EPS that dominate the stability of the EPS. (d) SEM image shows the arrangement of bacterial cells on the surface of granule surrounded by EPS matrixes (adapted from Fleming and Wengender, 2010).

Other constraints concerning high Ca^{2+} concentrations is the risk of cementation of the entire sludge bed in the UASB reactors due to the formation of mineral precipitates such as $CaCO_3$ and $Ca_5OH(PO_4)_3$ (van Langerak et al., 2000). Ca^{2+} concentrations above 320 mg/l do not cause a further strengthening of anaerobic granules (Yu et al., 2001). Similarly, Yu et al. (2001) reported that calcium concentrations up to 300 mg/l had a positive influence on sludge granulation process and that higher calcium concentrations (above 600 mg/l) had a negative influence.

1.4.3 Effects of high salinity

The presences of high Na^+ and Cl^- concentrations traditionally have been considered as inhibitory for anaerobic wastewater treatment (Lefebvre and Moletta, 2006, Rinzema et al., 1988).

According to De Baere et al. (1984) and Speece (2008), Na^+ was shown to be the strongest inhibitor on activity of bacteria among the cations, on the molar basis. Na^+ concentrations exceeding 10 g/L showed strong inhibitory impacts in anaerobic treatment with non-adapted sludge, resulting in lower methane production (Kugelman and McCarty, 1965, Rinzema et al., 1988). Meanwhile, Waldron et al. (2007) suggested that Cl^- concentrations higher than 35.5 g/L could inhibit aceticlastic methanogenesis.

Varies studies focussed on the negative impact of high Na^+ concentrations on the microbiological processes in anaerobic digestion (Kimata-Kino et al., 2011, Uygur and Kargi, 2004, Riffat and Krongthamchat, 2007, Lefebvre et al., 2007). Feijo et al. (1995) reported that when 11 g Na^+/L was abruptly added to a mesophilic UASB reactor supplied with the neutralized volatile fatty acids (VFAs) acetic, propionic, and n-butyric acid, the VFA-removal efficiencies decreased, as well as the methane production, by 90 %. In addition, Gebauer and Eikebrokk (2006) found that Na^+ concentrations of 10 g/L could have inhibited the anaerobic process, being responsible for the decrease in COD removal efficiencies from 55.2 to 36.7 %. Meanwhile, Kimata-Kino et al. (2011) demonstrated that the adaptation period can be shorten to only 30 days by increasing the salinity level abruptly to 7.9 g Na^+/L, followed by gradual adaptation to 12.6 g Na^+/L for anaerobic granules in mesophilic UASB reactors. The reduction in methane production at 12.6 g Na^+/L was only 13 % compared with the control.

In addition to the impact on microbiological processes, high Na^+ concentrations also directly impact the physicochemical environment of the microbes. Particle size and density will be reduced in high salinity wastewaters due to cell plasmolysis and death of microorganisms (Kargi and Dincer, 1997) and high Na^+ concentrations in medium, preventing the cross linking between surface bound negatively charged functional groups, like carboxylic groups. Lefebvre et al. (2006) noted that degranulation occurred in a UASB reactor treating saline tannery wastewater, while van Leerdam et al. (2007) observed small aggregates in a UASB reactor treating methanethiol at high salinity levels (0.8 M NaCl). However, the exact mechanisms and cause for the instability of anaerobic granules at high salinity levels have not been identified.

1.5 Problem statement and thesis outlook

This thesis presents studies on the development of anaerobic granular sludge at high salinity. Emphasis was given on the development of tools, methods, and/or strategies to stabilize the granular consortia. The salinity level applied in this study was up to 20 g Na^+/L, i.e. exceeding sea water concentrations. In the past decades, the effects of high salinity in wastewater treatment systems particularly in biological processes were thoroughly studied. However, the effect of high Na^+ concentration on the property and stability of anaerobic granular sludge has been only limitedly addressed. In the underlying research several topics were defined:

- Effect of high salinity on activity and functionality of anaerobic granular sludge.
- Impact of salinity on EPS production in anaerobic granular sludge. The structural role of EPS in anaerobic granular sludge has been thoroughly discussed in literature. However, to our knowledge, the production of EPS under high salinity conditions has hardly been studied.

- Important of bivalent cations, i.e. Ca^{2+} augmentation to prevent granule deterioration. High Na^{+} concentrations may impact Ca^{2+} binding in the granules. Therefore by augmenting Ca^{2+} into the system, the ratio of divalent to monovalent cations will increase, possibly stabilizing the consortia.
- Research on initial adhesion and microbial characterization of anaerobic consortia at high salt concentrations. The initial attachment of specific microbes, reveal important information to start and operate UASB reactor treating high salinity wastewater.
- Enhancing anaerobic granule properties at optimized conditions. Continuous flow UASB reactors will be operated using optimized conditions from the previous experiments.

In Chapter 2 the effect of high sodium concentrations on the biological and physical properties of anaerobic granular sludge in continuously fed UASB reactors is studied. The biological properties of the obtained sludge were determined by assessing 1) the specific methanogenic activities (SMA) on defined substrates and 2) the quantity and composition of the extracellular polymeric substances (EPS). Characterization of the physical properties was done by determining the granule strength and size distribution. Furthermore, the effect of high Na^{+} concentration on Ca^{2+} leaching from methanogenic granules is assessed in batch tests. The production and function of EPS in high sodium concentration wastewaters pertaining to anaerobic granule properties is described in Chapter 3. Chapter 4 describes whether Ca^{2+} augmentation could prevent the Ca^{2+} leaching from anaerobic granular sludge in high salinity wastewater. Chapter 5 investigates the early stages of anaerobic biofilm development, under different salinity levels, using different feed compositions in sequenced batch reactor. The results from UASB reactor operation, operated under the envisaged optimum conditions is discussed in Chapter 6. Finally, the results of this study are summarized and the future research prospect is given in Chapter 7.

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Chapter 2

This chapter has been adapted from published version: S.B. Ismail, P. Gonzalez, D. Jeison and J.B. van Lier – "Effects on High Salinity on Methanogenic Sludge Bed Systems", *Water Science and Technology*, vol. 58, no. 10, pp.1963-1970, July 2008.

Effects of High Salinity Wastewater on Methanogenic Sludge Bed Systems

"Untung sabut timbul, untung batu tenggelam," It is the fate of the coconut husk to float, of the stone to sink.
Malay proverb

Abstract - The attainable loading potentials of anaerobic sludge bed systems are strongly dependent on the growth of granular biomass with a particular wastewater. Experiments were conducted to determine the effects of high salinity wastewater on the biological and physical properties of methanogenic sludge. Sodium concentrations of 5 and 15 g/L were added to the influent of upflow anaerobic sludge blanket (UASB) systems. After 100 days of operation, the methanogenic activity and granular strength were analysed. The results showed a high removal of organic matter but with accumulating propionate concentrations in the effluents. Meanwhile, wash-out of active methanogenic biomass in the effluent of the reactors was observed, likely as a result of the high Na^+ concentrations. The washed-out biomass was characterized by a considerable specific methanogenic activity (SMA) on acetate, propionate and hydrogen as the substrates. On the other hand, results showed that the SMA evolution, particularly with respect to acetate, was not affected by high salt concentrations. However, results clearly show a sharp drop in the granule strength as a result of high Na^+ concentration.
Keywords: Bacterial immobilization, granule strength, high salinity, sodium, UASB reactor.

2.1 Introduction

The biological treatment of high salinity wastewater is becoming a topic of increasing importance in the industrialized world. Lefebvre et al. (2007) reported that the treatment of saline and hypersaline wastewater could represent as much as 5 % of worldwide effluent treatment requirements. This concerns both seawater-based agro food industries, such as fish processing, as well as concentrated wastewaters, such as those coming from chemical industries and distilleries. Moreover, owing to increased water efficiency in industrial processes and developments towards loop closure, the resulting wastewaters are more concentrated and increasingly characterised by harsh environmental conditions for biological treatment, such as high salinity and high temperatures. At present, granular sludge based anaerobic high-rate reactors, such as the upflow anaerobic sludge blanket (UASB) and the expanded granular sludge bed (EGSB) are most commonly applied to treat concentrated, organically polluted wastewaters (van Lier, 2003). High-rate anaerobic treatment offers the possibility of efficient wastewater treatment with low operational cost, high removal of organic carbon and small footprint size. However, the success of the anaerobic high-rate systems and particularly the expended bed reactors is dependent upon a successful sludge granulation process. The excellent settling properties and high strength of the granular configuration minimize biomass wash-out, resulting in high solids retention times and concomitant high loading capacities reaching up to 40 kg COD/m^3.d (van Lier, 2008). Also, the dense cell packing in granular aggregates optimizes the interspecies exchange of metabolites and hence the overall activity (Lettinga et al., 1980, Quarmby and Forster, 1995, Ghangrekar et al., 1996, Britz et al., 2002).

The presence of high Na^+ concentrations is negatively impacting the anaerobic treatment process, affecting the physiology of the microorganisms as well as the morphology of the required granular consortia. It has already been reported that a sodium concentration exceeding 10 g/L strongly inhibits methanogenesis (Gourdon et al., 1989, Kugelman and McCarty, 1965). Rinzema et al. (1988) found that at neutral pH, sodium concentration exceeding 5 g/L inhibited aceticlastic methanogenic activity of granular sludge, reflecting the sensitivity of *Methanosaeta* spp. towards sodium. So far, no researches were conducted on the impact of high Na^+ concentrations possibly affecting the stabilization of anaerobic granular sludge.

The main objective of our present study is to elucidate the effects of high sodium concentrations on the biological and physical properties of anaerobic granular sludge in continuously fed UASB reactors. The biological properties of the obtained sludge were determined by assessing the specific methanogenic activities (SMA) on defined substrate. Characterization of the physical properties was done by determining the granule strength.

2.2 Materials and Methods

2.2.1 Inoculum characteristics

The reactors were inoculated with sludge from a full-scale UASB reactor treating wastewater from a styrene and propene-oxide production plant of Shell, Moerdijk, the Netherlands. The industrial wastewater contains a sodium concentration in the range 10 – 15 g/L, with acetic acids and benzoic acids as main sources of COD. The sludge was adapted to such high salinity wastewater for more than 10 years (Biothane-Veolia, personal communication). This

inoculum had a VSS/TSS ratio of 0.57 and was used for both the UASB experiments and the batch tests.

2.2.2 Continuous UASB reactor experiments

The experiments were performed in a temperature controlled room at 30 ±2 ^{0}C using three glass UASB reactor with a volume of 3 litres. The UASB reactors were equipped with a reversed funnel phase separator. After passing the gas through a concentrated sodium hydroxide solution for removing carbon dioxide and through a column filled with soda lime pellets with indicator (Merck Art. no. 6839, Darmstadt, FRG), methane production was monitored by a wet-test gas meter (Schlumberger, Dordrecht, The Netherlands). All reactors (R1, R2 and R3) were inoculated with 333 g/L of inoculum with 96 g VSS/L, giving a final concentration of 32 g TSS/L reactor and were fed with 70 % of acetate, 20 % of gelatine and 10 % of ethanol as substrate. The media were prepared in distilled water. The influent parameters for the reactors are given in Table 1.

Table 1 | Influent parameters for the three reactors; R1, R2 and R3.

	Reactor		
	R1	R2	R3
COD (g/L)	5	50	50
Na^+ (g/L)	5	5	15

The upflow liquid velocity was controlled by liquid recirculation and was fixed at 0.8 m/h. The initial organic loading rate (OLR) was 2.5 kg COD/m^{3}d, gradually increasing to 18 kg COD/m^{3}d. Reactor R1 was operated at a hydraulic retention time (HRT) of 12 h, which was 10 times shorter than the HRT of reactors R2 and R3. Higher loading rate were imposed on the system by increasing the flow rate of a separated peristaltic pump (Watson Marlow 202, UK) for the concentrated feed stock solution. Basal medium consisted of (g/L): NH_4Cl (7.5), K_2HPO_4 (2.10), $MgSO_4.7H_2O$ (1.5), $CaCl_2.2H_2O$ (0.3), yeast extract (0.5) and a trace element solution, adding 4.5 mL per litre of basal medium consisting of (mg/L): $FeCl_2.4H_2O$ (2000), H_3BO_3 (50), $ZnCl_2$ (50), $CuCl.3H_2O$ (38), $MnCl_2.4H_2O$ (500), $(NH_4)6MoO_{24}.4H_2O$ (50), $AlCl_3.6H_2O$ (90), $CoCl.6H_2O$ (2000), $NiCl_2.6H_2O$ (92), $Na_2SeO_3.5H_2O$ (194), EDTA (1000), resazurine (200) and HCl 36 % (1 mL).

2.2.3 Strength measurement

For granule strength tests 250 mL of UASB reactor sludge was used, sampled with a tap at a height of 22 cm (liquid height was 67 cm). Granule strength was determined as the production of "fines" in a gas sparging column of 1.2 L, according to a procedure described by Pereboom (1997). The column dimensions were 5.6 cm diameter and 50 cm height. Shear on the granules was provided by a nitrogen gas flow of 27 m/h. Production of fines in g volatile suspended solids (VSS)/L, defined as particles which did not settle within 1 minute after stopping the nitrogen gas flow, was measured regularly for a period of 5 – 7 hours.

2.2.4 Specific methanogenic activity (SMA) assessments

All SMA tests were performed in duplicate in 120 ml glass serum bottles at a sodium con-

centration 5 g Na^+/L (reactors R1 and R2); 15 g Na^+/L (reactor R3) and biomass concentration was 1 g VSS/L. Anaerobic conditions were obtained by flushing the headspace for 10 seconds with N_2 gas. Acetate, propionate and butyrate were used as sole substrates at an initial concentration of 1.5 g COD/L. 360 ml/L macro nutrients solution were used (170 g/L NH_4Cl, 8 g/L $CaCl_2.2H_2O$ and 9 g/L $MgSO_4.7H_2O$) and 36 mL/L trace elements solution (2 g/L $FeCl_3.4H_2O$, 2 g/L $CoCl_2.6H_2O$, 0.5 g/L $MnCl_2.4H_2O$, 30 mg/L $CuCl_2.2H_2O$, 50 mg/L $ZnCl_2$, 50 mg/L HBO_3, 90 mg/L $(NH_4)6Mo_7O_{24}.4H_2O$, 100 mg/L $Na_2SeO_3.5H_2O$, 50 mg/L $NiCl_2.6H_2O$, 1 g/L EDTA, 1mL/L 36 % HCl and 0.5 g/L resazurin). The serum bottles were incubated at 30 ± 2 ^{0}C on a platform shaker operated at 120 rpm (C10 Edison, NJ, USA). The pressure was measured manually at regular intervals with a Greisinger GMH 3150 Digital Pressure Meter (Greisinger Electronic, Germany).

2.2.5 Analytical techniques

VFA was analysed in a Hewlett Packard 5890A gas chromatograph equipped with a 2 m × 6 mm × 2 mm glass column packed with Supelco port, 100 – 120 mesh, coated with 10 % Fluorad FC 431. The flow rate of the carrier gas, i.e. nitrogen saturated with formic acid, was 40 ml/min, and the column pressure was 3 bar. The temperatures of the column, the injector port, and the flame ionization detector were 130, 200, and 280 ^{0}C, respectively. Biogas composition was analysed in a Fisions Instrument GC 8340 gas chromatogram equipped with a 30 m × 0.53 mm × 25 μm Molsieve column (Alltech 13940), and a 2 × 25 m × 0.53 mm × 10 μm PoraBond Q column (Varian 7354). The columns were connected in parallel. Helium was the carrier gas and its flow rate was 42.5 ml/min. The temperatures of the oven, the injection port, the thermal conductivity detector and the filament were 40, 110, 100 and 140 ^{0}C, respectively. Biomass yield analysis was done by measuring the amount of biomass produced related to the amount of substrate consumed in a specific period. Biomass production was assessed by measuring the biomass amount in the effluent and the biomass increase in the sludge bed during reactor operation. The biomass yield was subsequently expressed by g VSS produced/g COD converted. All other analysis were carried out according to Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

2.3 Results and Discussion

2.3.1 UASB experiments

Figure 1 presents the effluent VFA concentration and applied loading rate during the operation of the UASB reactors, fed with the partly acidified substrate. OLR was increased gradually from 7 until 18 kg COD/m^3.d. The VFA concentrations in the effluents dropped to a lower level as the operation proceeded except immediately after increasing the OLR. The decrease in effluent VFA concentrations shows that the reactors performed well respective to the OLR increments. The single-day peaks sometimes occurring prior to the OLR increase can be ascribed to technical disturbances. Reactor performance, however, was restored before loading rate increments were imposed to the system. The remnant fractions of VFA in the effluents were mainly composed of propionate. At the end of the operation, propionate concentration in the effluent was about 80 % in reactor R3, whereas reactors R1 and R2 had only 25 %.

Considerable sludge wash-out was observed in the effluent of reactor R1, operated at 5 g

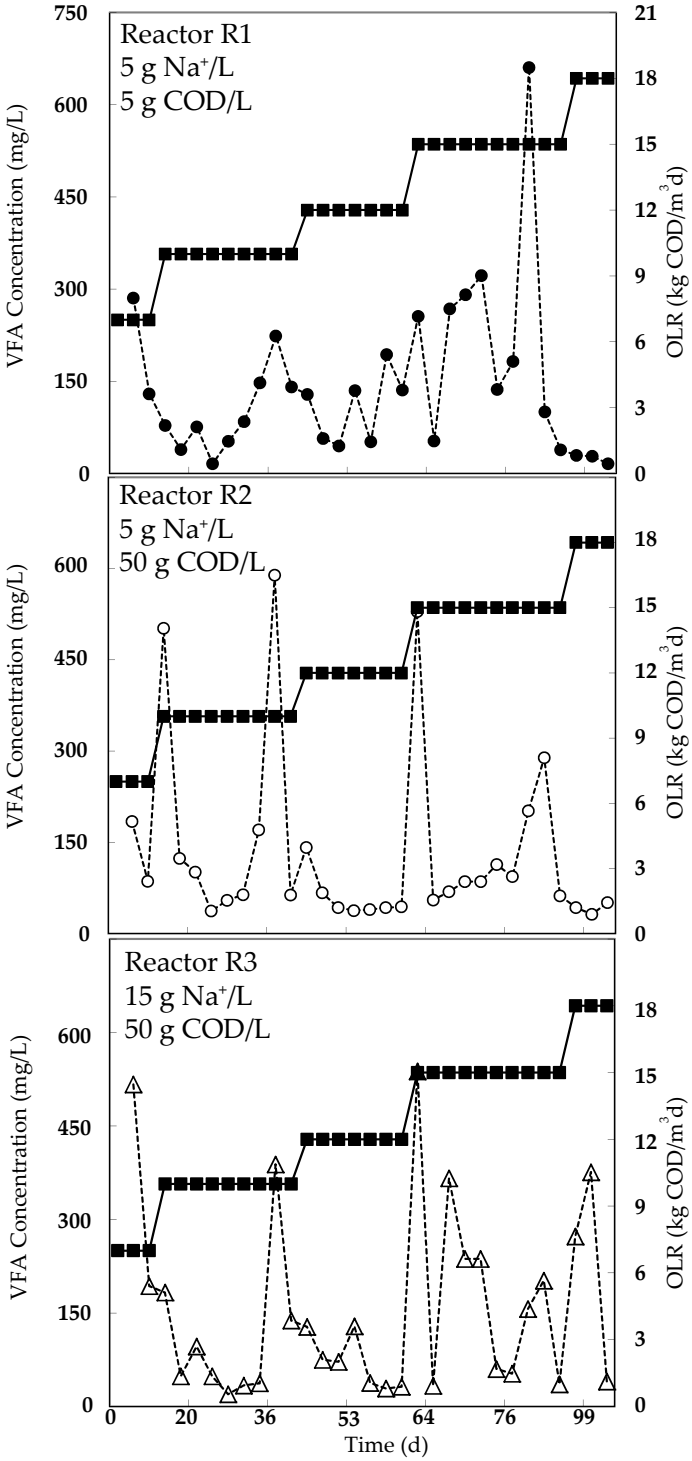

Figure 1 | Effluent VFA concentration and applied OLR during the operation of the UASB reactors. ■ Organic Loading Rate (kg COD/m³d); ● R1-5 g Na^+/L; ○ R2 - 5 g Na^+/L; Δ R3 - 15 g Na^+/L.

Na^+/L and an influent COD concentration of 5 g/L. At the end of the reactor operations about 45 g of sludge were washed out from reactor R1. In contrast, only about 15 g of sludge were washed-out from reactors R2 and R3. This difference can be attributed to the different HRTs at which the reactors were operated. The applied low HRT apparently introduces a high selection pressure into the reactor R1, resulting in the carry-over of poor settling sludge and possible abrasion of weak granular conglomerates.

2.3.2 Biological properties

The SMA was measured of the washed-out sludge from each reactor after 25, 50 and 75 days of operation. The washed-out sludge exerted an extremely high SMA especially for reactor R1, as depicted in Figure 2. Very likely, the high loading rate and concomitant high biomass growth led to effluent suspended solids consisting predominantly of methanogenic biomass. Notably, the influent COD concentration in reactor R1 was 5 g COD/L, whereas in reactors R2 and R3 this was 50 g COD/L, resulting in relatively long HRTs in reactors R2 and R3.

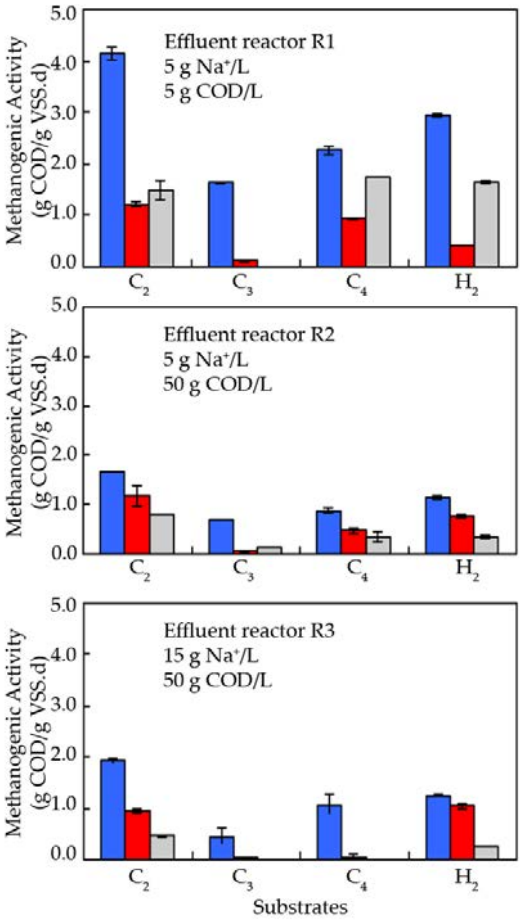

Figure 2 | SMA of washed-out sludge measured with acetate (C_2), propionate (C_3), butyrate (C_4) and hydrogen (H_2) as the substrates. 25 day of operations; 50 day of operations; 75 day of operations. Error bars represent the variance between duplicates.

Figure 3 presents the SMA of the sludge measured at the end of the experimental period, when the reactors were characterized by a loading rate of 18 kg COD/m^3.d and 95 % of COD removal. The used inoculum of all reactors showed a relatively low SMA on all substrates tested. Interestingly, with regard to the inoculum, the measured SMA does not give any clear differences comparing the various Na^+ concentrations, while applying the same substrate. Apparently, the inoculum can tolerate high Na^+ concentration, a finding which agrees with the previously published Na^+ response curve performed with inoculum sludge coming from the same full scale reactor (Jeison et al., 2008).

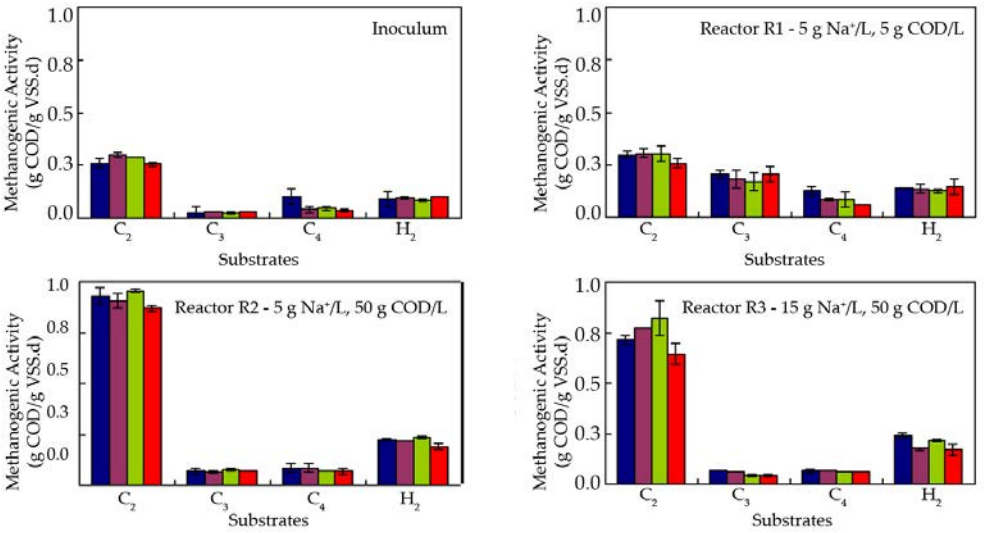

Figure 3 | SMA of the inoculum and reactor sludges at the end of operation at different added Na^+ concentrations, using acetate (C_2), propionate (C_3), butyrate (C_4) and hydrogen (H_2) as the sole substrates; background Na^+ concentrations was 17 mg/L. 0 g Na^+/L; 5 g Na^+/L; 10 g Na^+/L; 15 g Na^+/L. Error bars represent the variance between duplicates.

The observed high acetate-SMA values of the sludges from reactors R2 and R3 at the end of the UASB experiments indicate that growth and retention of methanogenic biomass was considerable, despite the high Na^+ concentration applied. The calculated yield values were 0.04 ± 0.01 and 0.05 ± 0.01 g VSS-COD/$COD_{converted}$ for 5 and 15 g Na^+/L, respectively. The obtained biomass yields are within the range reported in the anaerobic digestion model no.1, i.e. about 0.04 – 0.06 g VSS-COD/$COD_{converted}$ (Batstone et al., 2002). In agreement with Figure 2, sludge retention was much poorer in reactor R1, subjected to a short HRT, likely resulting in the observed relatively low SMA on acetate at the end of the experiment. Based on substrate composition, it is assumed that acetate by far was the main intermediate VFA. Interestingly and surprisingly, the SMA on propionate and, to a lesser extent, butyrate did increase in the reactor R1 sludge, whereas increase in acetate SMA was only minimal. Results indicate a selective wash-out of acetate consuming methanogens, whereas the syntrophic consortia degrading propionate and butyrate were apparently kept in the sludge bed.

2.3.3 Physical properties

Abrasion experiments were performed in a bubble column to measure the granule strength of the inoculum and the sludge from each reactor at the end of the operational period. The

strength of the granules was plotted in function of the production of the fines measured as volatile suspended solid after 1 minute of settling. All reactors presented lower strength granules in comparison with the inoculum. However, with the sludge from reactor R3, a higher concentration of fines was found. Apparently, the granule strength of this sludge was lowest (Figure 4). This finding supports our hypothesis that a high amount of sodium reduces the granule strength, producing a weak dispersed sludge.

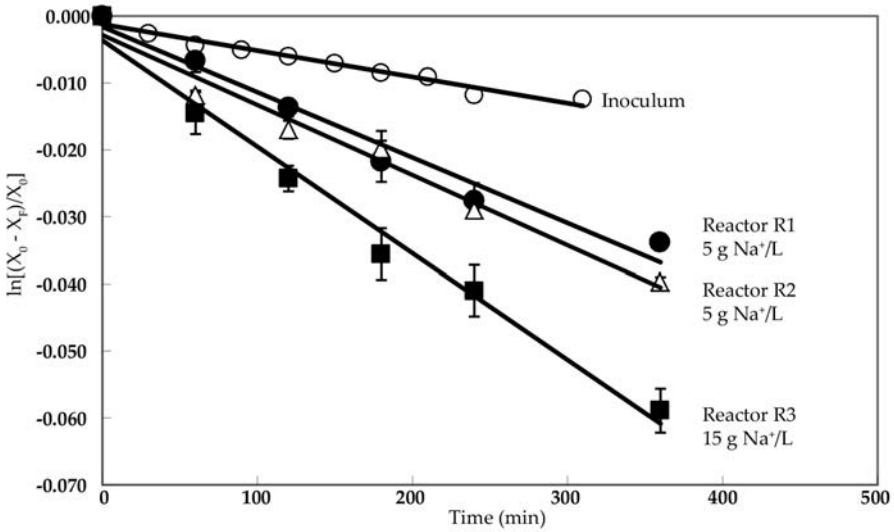

Figure 4 | Determination of granule strength by assessing the production of fines versus time of assay (time of bubbling) Shell Sludge - ○ Inoculum; • R1-5 g Na^+/L; Δ R2-5 g Na^+/L; ■ R3-15 g Na^+/L. Error bars represent the variance between duplicates. (X_0 = granular sludge concentration at t = 0; X_F = granular sludge concentration at t = F).

2.4 Conclusions

In summary, the SMA was not affected by high salinity concentrations, but a significant drop in granule strength was observed under conditions of long-term exposure to high Na^+ concentrations. The performance of the granular sludge system shows the appropriateness of methanogenic inocula, pre-grown under saline conditions, for the anaerobic treatment of high salinity wastewater. The later conditions, however, cause a decrease in granule strength and washout of bacterial mass.

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Chapter 3

This chapter has been adapted from the published version: S.B. Ismail, C.J. de La Parra, H. Temmink and J.B. van Lier – "Extracellular Polymeric Substances (EPS) in Up-flow Anaerobic Sludge Blanket (UASB) Reactors Operated under High Salinity Conditions", *Water Research*, vol. 12, no. 7, pp. 729–739, Jan 2010.

Extracellular Polymeric Substances (EPS) in Up-flow Anaerobic Sludge Blanket (UASB) Reactors Operated under High Salinity Conditions

"Waar de dijk het laagst is, loop het eerst het water over," Where the dyke is lowest, water runs over it first.
Dutch proverb

Abstract - Considering the importance of stable and well–functioning granular sludge in anaerobic high rate reactors, a series of experiments were conducted to determine the production and composition of EPS in high sodium concentrations wastewaters pertaining to anaerobic granule properties. The UASB reactors were fed with either fully acidified substrate (FAS) consisting of an acetate medium (reactor R1) or partially acidified substrate (PAS) consisting of acetate, gelatine and starch medium (reactors R2, R3, and R4). For EPS extraction, the cation exchange resin (CER) method was used. Strength and particle size distribution were determined by assessing the formation of fines sludge under conditions of high shear rate and by laser diffraction, respectively. Batch tests were performed in 0.25 L bottles to study Ca^{2+} leaching from anaerobic granular sludge when incubated in 20 g Na^{+}/L in absence of feeding for 30 days. Results show a steady increase in the bulk liquid Ca^{2+} concentration during the incubation period. UASB reactor results show that the amounts of extracted proteins were higher from reactors R2 and R3, fed with PAS compared to the sludge samples from reactor R1, fed with FAS. Strikingly, the amount of extracted proteins also increased for all reactor sludges, irrespective the Na^{+} concentration applied in the feed, i.e. 10 or 20 g Na^{+}/L. PAS–grown granular sludges showed an important increase in particle size during the operation of the UASB reactors. Results of reactor R4 showed that, addition of 1 g Ca^{2+}/L to the high salinity wastewater increases the granules strength.

Keywords: Anaerobic granules, extracellular polymeric substances (EPS), granule strength, high salinity, particle size distribution.

3.1 Introduction

Cleaner production strategies and the need for improved water efficiencies in industries have led to a more extensive closure of the (internal) water loops. The resulting wastewaters, therefore, are more concentrated and often characterised by harsh environmental conditions, such as high salinity, extreme pH, and high temperatures. Consequently, biological wastewater treatment systems that are part of the internal water loop, or that need to treat the wastewaters to discharge criteria, also need to deal with such conditions. In addition, harsh environmental conditions also occur in specific waste streams such as fish & seafood processing wastewater. For example, Lefebvre et al. (2007) reported that the treatment of saline and hypersaline wastewater may represent as much as 5 % of worldwide effluent treatment requirements. High salinity is regarded as a limiting factor for applying biological wastewater treatment systems.

High sodium concentrations in wastewaters induce salt stress to microbial species, resulting in the inhibition of many enzymes, a decrease in cell activity and eventually leading to plasmolysis (Uygur, 2006, Rene et al., 2008). Salt stress seriously hampers the treatment performance of anaerobic systems (Rinzema et al., 1988). To counteract these problems, several approaches were investigated. In some of them halophilic inocula were used (Aspe et al., 2001, Lefebvre et al., 2006, Mosquera-Corral et al., 2001), whereas in others salt-adapted microorganisms were used capable of degrading pollutants, while withstanding high salinities (Omil et al., 1995, Kargi and Uygur, 1997).

Nowadays, granular sludge based anaerobic high-rate reactors, such as the upflow anaerobic sludge blanket (UASB) and the expanded granular sludge bed (EGSB) are most commonly applied to treat concentrated, organically polluted wastewaters (van Lier, 2003). However, the success of these treatment systems, particularly the expanded bed reactors, is dependent upon a successful (bio-)granulation process. The excellent settling properties and high strength of the granules minimizes biomass washout, resulting in long solids retention times and concomitant high loading capacities up to 40 kg COD/m^3d (van Lier, 2008). Also, the dense cell packing in granular aggregates optimizes the interspecies exchange of metabolites and hence the overall activity (Lettinga et al., 1980, Britz et al., 2002).

Liu et al. (2004) found that extracellular polymeric substances (EPS) contribute to the matrix structure and stability of anaerobic granules. EPS are sticky materials excreted by cells and contain variable proportions of proteins, polysaccharides, nucleic acids, humic–like substances, lipids, and heteropolymers such as glycoproteins (Frølund et al., 1996). In addition, anaerobic sludge granules have been found to have a net negative charge, which results from the EPS functional groups. Dignac et al. (1998) did a study by characterizing the extracellular proteins, demonstrating that the proteins, which are rich in amino acids, such as glutamic and aspartic acid, containing carboxylic groups will contribute to the negative charge of the flocs. A number of researchers have studied the role of cations in biogranulation and/or bioflocculation processes (Sobeck and Higgins, 2002, Pevere et al., 2007).

Divalent metal ions, such as Ca^{2+}, are widely recognized to play an important role in the bacterial self-immobilization to microbial aggregates (Yu et al., 2001). Recent experimental evidence shows that extensive accumulation of Ca^{2+} occurs in microbial aggregates (Wang et al., 2007). Ca^{2+} binding with proteins is often involved in bacterial adhesion to a surface, and is

essential for cell–cell aggregation (Rose, 2000). Ca^{2+} also is an important ionic cross–bridging molecule for the negatively charged bacterial polysaccharides (Patrauchan et al., 2005b). Yu et al. (2001) found that Ca^{2+} at concentrations from 150 to 300 mg/L enhanced the anaerobic granulation process and Jiang et al. (2003) reduced the granulation time of activated sludge by dosing 100 mg/L of Ca^{2+}. Specific removal of Ca^{2+} resulted in disintegration of anaerobic sludge granules (Grotenhuis et al., 1991). Nonetheless, most information regarding the impact of Ca^{2+} on microbial flocs applies to aerobic conditions, whereas the effects of high salinity (by elevated sodium concentrations) on EPS production and the potential stabilizing role of Ca^{2+} in anaerobic granular sludges still are largely unknown.

Considering the importance of stable and well-functioning anaerobic granular sludge, even under harsh environmental conditions, our specific objective in this study was to determine the production and function of EPS in high sodium concentration wastewaters pertaining to anaerobic granule properties.

3.2 Material and Methods

3.2.1 Source of inocula

The reactors were inoculated with granular sludge from a full-scale UASB reactor treating wastewaters from a styrene and propene-oxide production plant of Shell, Moerdijk, the Netherlands. This wastewater contained a sodium concentration in the range 10 – 15 g/L, with acetic acids and benzoic acids as the main sources of COD. The sludge was already adapted to a high salinity of 10 – 15 g Na^+/l for more than 10 years (Biothane-Veolia, personal communication). The inoculum with a VSS/TSS ratio of 0.68 was used for the UASB experiments and batch tests.

3.2.2 Experimental set-up

Continuous UASB reactors operation

The experiments were performed in a temperature-controlled room at 30 ± 2 ^{0}C using four glass UASB reactors with a volume of 3 L each. The UASB reactors were inoculated with 400 g/L of inoculum with 158 g TSS/L, giving a final concentration of 63.3 g TSS/L reactor. The top part of each reactor was equipped with a reversed funnel phase separator. After passing the produced biogas through a concentrated sodium hydroxide solution to remove carbon dioxide and through a column filled with soda lime pellets with indicator (Merck Art. no. 6839, Darmstadt, Germany), methane production was monitored by a wet-test gas meter (Schlumberger, Dordrecht, the Netherlands). The media were prepared in demineralised water. The nutrient solution consisted of macro and micronutrients and was prepared as described in Chapter 2. The synthetic wastewater was further enriched with sodium chloride reaching a final concentration of 10 or 20 g Na^+/L, dependent on the reactor served (Table 1). Operational and influent parameters for the reactors are given in Table 1.

The upflow liquid velocity was controlled by liquid recirculation and was fixed at 0.8 m/h with a peristaltic pump (Watson Marlow 202, UK). The initial organic loading rate (OLR) was 12 kg COD/m^{3}d, but was gradually increased to 22 kg COD/m^{3}d by increasing the flow rate

of another peristaltic pump (Watson Marlow 202, UK) with concentrated feed stock solution.

Batch tests

Serum bottles (0.25 L) batch tests were conducted to study Ca^{2+} leaching from anaerobic granular inoculum. A total amount of 2.77 g of wet sludge was incubated at a sodium concentration of 20 g Na^+/L in the absence of feeding for 30 days. Bottles without Na^+ addition served as blanks. The bottles were placed on a reciprocal shaker at 30 ^{0}C. Before sampling, the serum bottles were vigorously shaken and allowed to settle for 2 minutes. Liquid samples were collected using a 10 – 15 ml syringe. Meanwhile, granular sludge was collected from the serum bottles and dried on filter paper prior to the calcium analyses. All leaching tests were performed in duplicate.

Table 1 | Operational and influent parameters for the reactors fed with fully acidified substrates (FAS) (reactor R1) and partially acidified substrates (PAS)(reactors R2, R3 and R4).

	Reactor			
	R1	R2	R3	R4
OLR[1] (kgCOD/m^3d)	22	22	22	22
HRT[2] (/day)	1.34	1.34	0.34	1.34
COD (g/L)	16	16	16	16
Ac:Gel:Sta[3] ratio	1:0:0	1:0.2:0.1	1:0.2:0.1	1:0.2:0.1
	FAS	PAS	PAS	PAS
Sodium influent (g/L)	20	20	10	20
Calcium influent (g/L)	0	0	0	1
M/D[4] ratio (g/L)	1540	1540	770	20

[1]OLR = organic loading rate; [2]HRT = hydraulic retention time; [3]Ac: Gel: Sta = acetate : gelatine : starch (w:w, based on COD equaivalent); [4]M/D = monovalent (Na^+) / divalent (Ca^{2+})

3.2.3 Granule properties determination

Scanning electron microscopic (SEM)

Granules were fixed in culture medium by adding glutaraldehyde in a growth medium, resulting in a 2.5 % glutaraldehyde concentration. After 1 hour of fixation, the granules were rinsed in water and subsequently dehydrated in a graded series of acetone (10, 30, 50, 70, and two times 100 %, 15 minutes per step). The granules were critical point dried with carbon dioxide (CPD 030 BalTec, Liechtenstein) and glued on a sample holder by carbon adhesive tabs (EMS, Washington, USA) or with carbon glue (Leit-C, Neubauer Chemicalien, Germany). The samples were sputter coated with 10 nm platinum in a dedicated preparation chamber (CT 1500 HF, Oxford Instruments, Oxford, UK) and analyzed with a field emission scanning electron microscope (JEOL 6300 F, Tokyo, Japan) at 5 kV at room temperature. Images were digitally recorded (Orion 6 PCI, E.L.I. sprl. Belgium) and optimized and resized with Adobe Photoshop CS.

Strength measurement

Granule strength was evaluated by fines production in a gas–sparging column (Pereboom,

1997). A 1.2 L effective volume column was used. Column dimensions were 5.6 cm diameter and 50 cm height. Tests were performed with 250 mL of settled granular sludge and at an up-flow velocity of 27 m/h of nitrogen gas. The concentration of fines was measured every hour, for a period of 5 hours. Fines production in time, expressed as solids percentage of the original amount of the sludge, was used to indicate the granule strength.

Particle size distribution

Particle size distribution was measured using a Mastersizer 2000 (Malvern, UK). This is a light scattering instrument, which operates on the principle of the Fraunhofer diffraction theory. The same sample was measured in triplicate and each size distribution was calculated by instrument software. The size distribution is based on volume and the average size is quoted as the 'median' based on volume equivalent diameter.

Extracellular polymeric substances (EPS) extraction

EPS extraction was carried out by cation exchange resin (CER) (Dowex Marathon C, 20 – 50 mesh, sodium form, Fluka 91973) proposed by Frølund et al. (1996). Sludge samples were harvested by centrifugation at 4500 rpm for 15 min (IEC Microlite, USA) and then the pellets were washed twice with 100 mM NaCl solution. Later, the sludge pellets were re-suspended in NaCl solution and the solution was transferred to an extraction bottle, followed by the CER addition with a dosage of 70 g/g suspended solids. These suspensions were stirred at 450 rpm and 4 ^{0}C, and were stopped when the COD value was constant after 107 hours. After removing settled CER, the solutions were centrifuged at 10,000 rpm and 4 ^{0}C for 30 min to remove remaining sludge components. The supernatants were then filtered through 0.45 μm cellulose membranes and used as the EPS fraction for protein and carbohydrate analysis. The total EPS content was measured as the sum of these two substances.

3.2.4 Analysis

Calcium analyses

Calcium content of the sludge per gram of total solids was determined after destruction with Agua regia (mixture of 2.5 ml 65 % HNO3 and 7.5 ml 37 % HCl) added to 1 g (wet weight) of granular sludge. After microwave destruction (Milestone ETHOS E temperate controlled; Milestone INC., Monroe, CT, USA), the samples were paper-filtered (Schleider Schuell 589, Germany) and diluted to 0.1 L with demineralised water. The calcium concentration was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES; Varian, Australia) as described in Chapter 2. Ultra-pure water (Milli-Ro System, Millipore, Bedford, MA, USA) was used to prepare standard solutions of all reagents, which were of suprapur quality (Merck, Darmstadt, Germany) and were checked for possible trace metal contamination. All glassware and plastic material used was treated for 12 h with 10 % (v/v) HNO_3 and rinsed vigorously with demineralised water.

Carbohydrate measurement

Carbohydrate concentrations contained in the fluid after CER extractions were measured according to Dubois et al. (1956). Samples of 0.4 mL were added to test tubes and then mixed

with 0.4 mL of 5 % (w/w) phenol (Sigma, UK). 2 mL of H_2SO_4 was added to each test tube and left at room temperature (18 – 26 ^{0}C) for 10 minutes. The content of each tube was transferred to cuvettes and the samples were analysed on a Jenway UV/VIS spectrophotometer (Model 6505 S) against the blank at a wavelength of 480 nm. Carbohydrate concentration (in mg/L) was determined from the calibration curve obtained with glucose standards. This concentration was normalised against the VS concentration (mg of carbohydrate per g of VS).

Protein measurement

Protein was measured with a diagnostic kit purchased from Sigma, England (Kit no. 690). Samples were diluted with sodium chloride solution (0.85 %) such that the final protein concentration was between 150 and 1000 mg/L. Diluted samples of 0.2 mL with 2.2 mL Biuret reagent were added to each tube and mixed well. Samples were kept at room temperature (18 – 26 ^{0}C) for 10 minutes. 0.1 mL of Folin and Ciocalteu's Phenol reagent was added to each tube and mixed each tube well after addition. These samples were left at room temperature for 30 minutes. The content of each tube was transferred to cuvettes and the samples were analysed on a Jenway UV/VIS spectrophotometer (Model 6505 S) against the blank at a wavelength of 595 nm. Protein concentrations were determined from a calibration curve obtained with protein standards, and normalized against VS concentration.

Other analysis

Volatile fatty acids (VFAs) and biogas composition were determined by gas chromatography as described in Chapter 2. All other analyses were carried out according to Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

3.3 Results

3.3.1 Reactor performance

Figure 1 presents the percentage of COD removal and the applied loading rate during the operation of the UASB reactors, fed with two different substrates, i.e. partially acidified (PAS) and fully acidified substrates (FAS), and at different sodium and calcium concentrations. The percentage of COD removal remained relatively stable, between 87 and 98 % for all reactors as the operation proceeded, except for a sudden decrease after day 130 resulting from a technical disturbance. Reactor performance, however, slowly restored after this drop. From day 160 onwards, the increase in the OLR up to 22 kg COD/m^{3}d lead to unstable COD removal, which was, however, partly masked by preceding technical failures. A dramatic decrease in COD removal of reactors R1 and R3 was observed on days 171 and 173, caused by failure of the control equipment, which led to involuntary sludge wash out.

The stable COD removal before day 130 shows that the reactors performed well, at the imposed OLR of 18 kg COD/m3d, irrespective of the type of C-source. This shows that the anaerobic inocula, pre-grown under saline conditions (10 – 15 g Na^+/L), were even capable to also deal with a salinity level of 20 g Na^+/L in the wastewater, as shown previously for 15 g Na^+/L in Chapter 2.

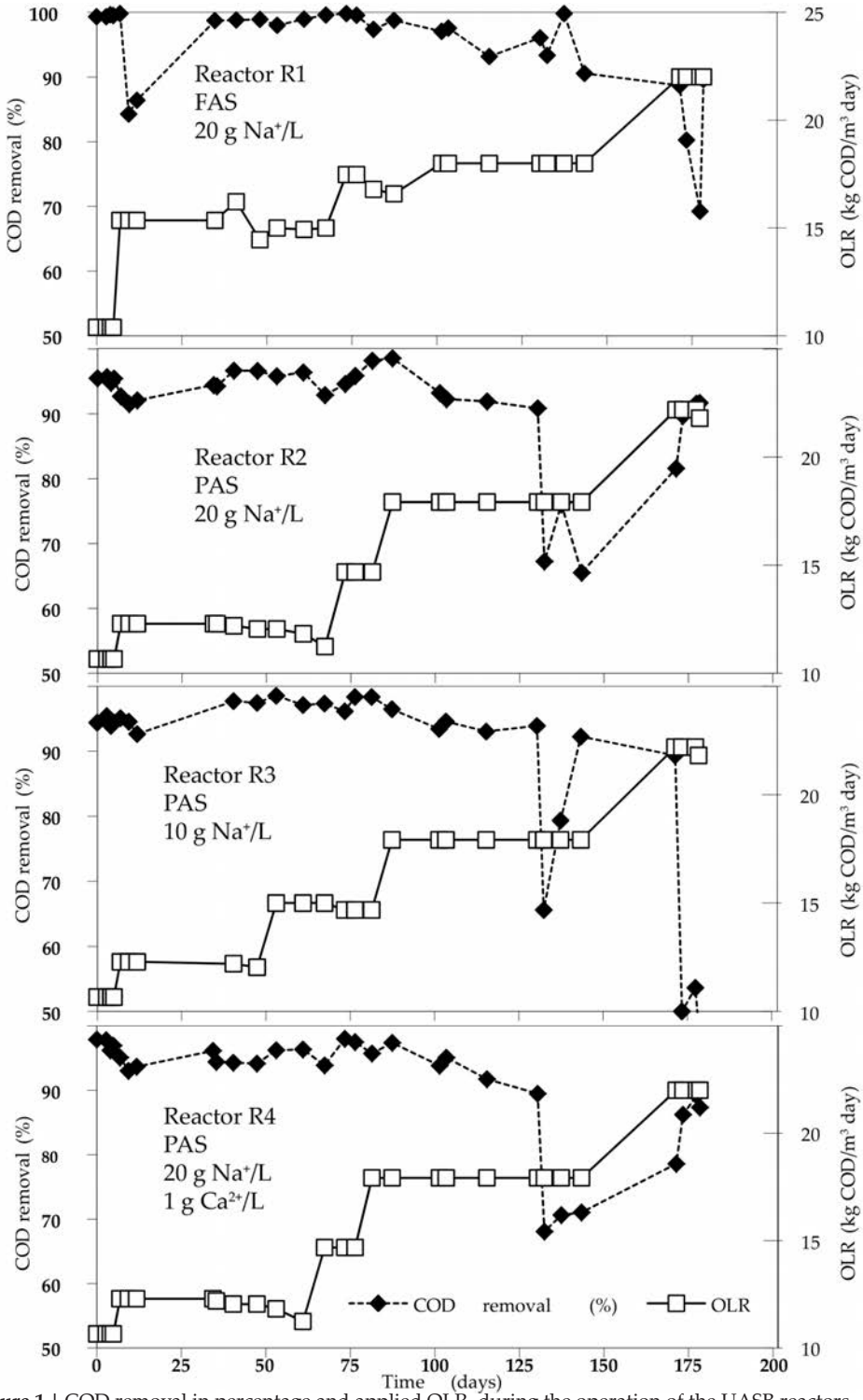

Figure 1 | COD removal in percentage and applied OLR, during the operation of the UASB reactors.

3.3.2 Extracellular polymeric substances (EPS) production

The extracted proteins and polysaccharides contents of the sludges were analysed at the end of the operational period of 180 days and are given in Table 2. Proteins and carbohydrates were considered to represent the extracted EPS, assuming that these are likely to be the dominant components in extracted EPS (Yu et al., 2006). The protein fraction of the EPS was much higher (around 87 and 94 %) than the polysaccharide fraction in all reactors sludges, and increased in all reactor sludges compared to the inoculum. The inoculum and FAS grown granular sludge from reactor R1 showed a lower fraction of proteins and polysaccharides. Extracted proteins fraction from inoculum and FAS grown granular sludge from reactor R1 were 32 and 18 % lower than PAS grown granular sludge from reactor R2, respectively.

The applied Na^+ concentration apparently did not affect the EPS content as no significant differences in total EPS were found in the sludges from reactor R3 (10 g Na^+/L) and reactor R2 (20 g Na^+/L). However, the extracted polysaccharides concentration from reactor R3 sludge was considerably higher than that for reactor R2 sludge. The increase in the amount of proteins was similar for all reactor sludges, irrespective of the Na^+ concentration applied in the feed, i.e. 10 or 20 g Na^+/L.

Addition of 1 g Ca^{2+}/L in reactor R4 resulted in an increase of 5 and 47 % of extracted poly-proteins and poly-saccharides, respectively, compared to the reactor R2 sludge. The total COD concentrations in the extraction of the reactor R4 sludge samples increased by 16.5 % compared to the reactor R2 sludge extraction (Table 2).

Table 2 | Composition of extracellular polymeric substances (EPS) extracted from reactor sludges. Inoculum sludge is presented for comparison. Values between brackets represent variance between duplicate analyses.

Sludge	Type of substrates	Na^+ (g/L)	Ca^{2+} (g/L)	EPS		Total COD (g $COD_{extracted}$/g VS)
				Polypeptides (mg Protein/g VS)	Polysaccharides (mg Sugar/g VS)	
Inoculum				154(± 60)	11(± 2)	620(± 50)
Reactor R1	FAS	20	0	187(± 50)	12(± 2)	660(± 10)
Reactor R2	PAS	20	0	228(± 120)	17(± 5)	790(± 40)
Reactor R3	PAS	10	0	239(± 60)	30(± 9)	920(± 1)
Reactor R4	PAS	20	1	239(± 150)	32(± 5)	920(± 50)

SEM images of the granules obtained at the end of reactor operations are presented in Figure 2. The texture (surface topography) of methanogenic PAS grown granules in reactor R2 were strikingly different from FAS grown granule from reactor R1. The reactor R2 granule was more rigid and stable with a densely packed outer layer, while granules from reactor R1 were less rigid with a loos exterior surface (Figure 2). This is verified at higher magnification of the FAS grown granule surface (Figures 2A and B). EPS were observed within the entire granule surface of the PAS grown granules and a larger number of bacteria cells were surrounded by EPS (Figure 2C). A further close-up of this structure shows that bacterial cells are inter-woven by a network of fibrous strands analogous to a cobweb, which appears to hold the pellets intact (Figure 2D). Granules from reactor R3 were quite similar to reactor R2 granules while reactor R4 granules exhibited a fissured surface, probably due to excessive amount of calcium (Figure 3).

Figure 2 | SEM images of a fully (left) and partially (right) acidified substrate fed granule, respectively from reactor R1 and reactor R2. Figure 2A and 2B show aggregates of bacterial cells on the surface of FAS grown-granule. Figure 2C shows arrangement of bacterial cells on the surface of PAS grown-granule surrounded by extracellular polymeric substances (EPS). Figure 2D shows a x1000 magnification of 2C.

The production of EPS is expected to enhance the granulation process, whereby the physical properties of the anaerobic granules would be changed. How this EPS production influences the bio-granulation in high salinity wastewaters, complemented with different substrates, is discussed in a later section.

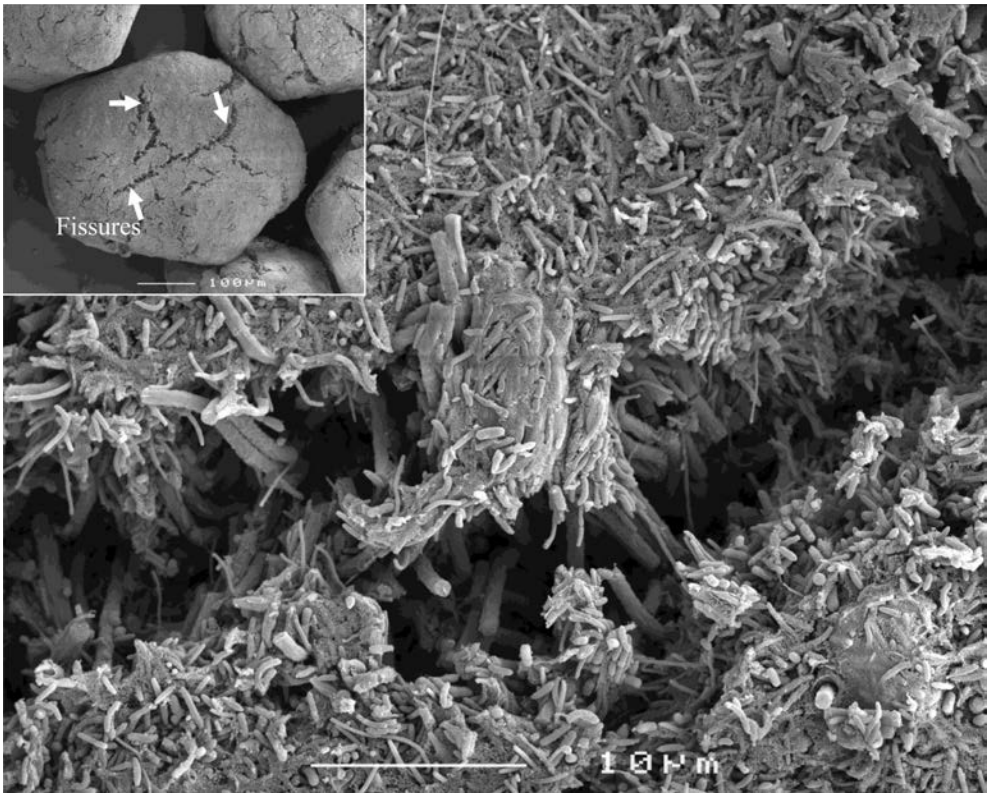

Figure 3 | Scanning electron micrographs showing large cracks in the anaerobic granule grown in the UASB reactor continuously fed with partly acidified substrate and 1.0 g Ca^{2+}/L (reactor R4). Inset: Micrograph showing the numerous cracks (arrows) on the granule surface.

3.3.3 Granules development

Particle size distribution

The particle size distribution of the sludge samples expressed as a volume fraction and determined with laser diffraction analysis is shown in Figure 4. Results show a binomial distribution of particle sizes in the range from 0 to 2000 µm. The median particle diameter ($d_{v,0.5}$) is used to compare the granule sizes of the different reactors. After 180 days of operation, granular sludge from all reactors presented an increase in particle size compared to the inoculum. The particle size distribution of the FAS grown granules show only a slight increase, with a $d_{v,0.5}$ of about 510 µm, compared to the inoculum sludge, having a $d_{v,0.5}$ of 502 µm. The $d_{v,0.5}$

of PAS grown granules was about 1.5 times bigger than that of the inoculum sludge and the FAS grown sludge from reactor R1, i.e. about 750 μm (Figure 4). Interestingly, the particle size distribution measurements also show a clear impact of the Na^+ concentration on the granule size; the $d_{v,0.5}$ of granular sludge from reactor R3, operated at 10 g Na^+/L, was about 630 μm, which was considerably lower than the 720 μm of the R2 granules, grown at 20 g Na^+/L.

The effect of Ca^{2+} addition on particle size becomes apparent when comparing the size distributions of the sludges from reactors R2 and R4. Strikingly, the addition of 1 g Ca^{2+}/L results in a significant decrease in $d_{v,0.5}$ from 720 μm to 460 μm.

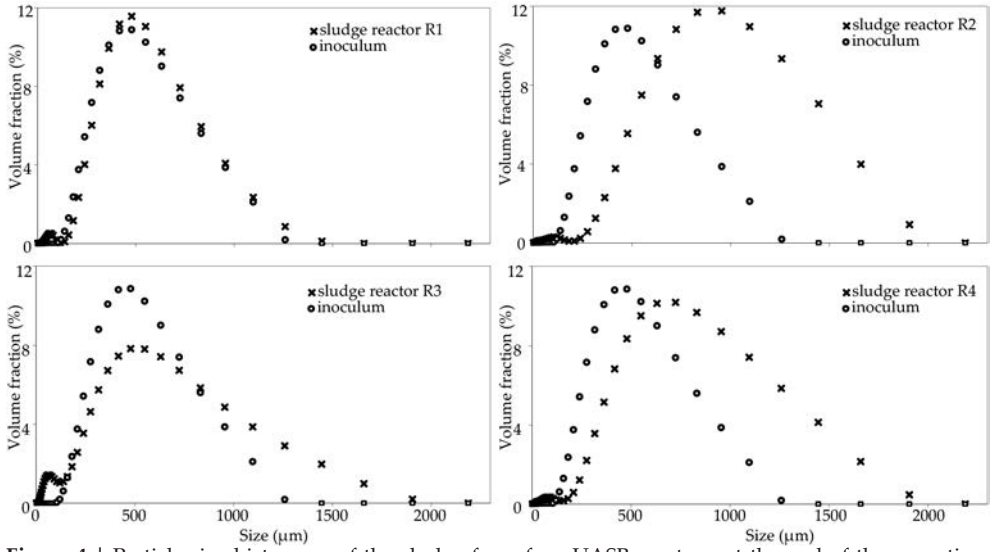

Figure 4 | Particle size histogram of the sludge from four UASB reactors, at the end of the operation. Inoculum sludge is presented for comparison.

Granule strength

Shear strength resistance is commonly used to characterise the "surface strength" of microbial aggregates such as flocs and granules (Mikkelsen and Keiding, 2002). Therefore, abrasion experiments were performed in a bubble column to measure the granules' strength of the sludge from each reactor at the end of the operational period. The strength of the granules is derived from the slope of the residual granular sludge amount as a function of bubbling time. During bubbling, part of the granule sludge is dissolved into fines, which are measured as volatile suspended solids after 1 minute of settling (Figure 5). The results clearly show that PAS grown granules from reactor R4 in the presence of calcium are characterised by the highest granule strength in comparison to the other granules. In contrast, the FAS grown granules are the weakest. In addition, granules grown at 20 g Na^+/L of reactor R2 were more fragile than the 10 g Na^+/L grown granules of reactor R3. This finding supports the hypothesis that a high amount of Na^+ reduces the granule strength, producing a weak dispersed sludge, which is also discussed in Chapter 2.

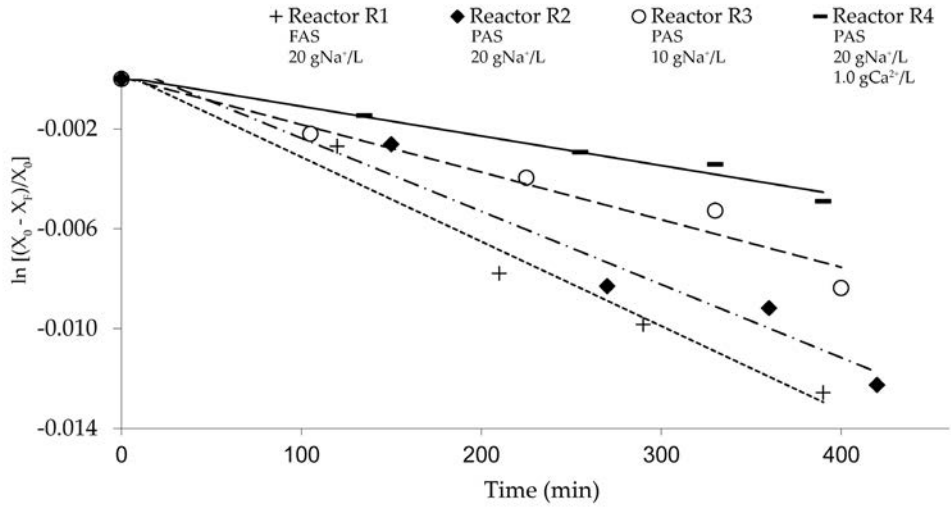

Figure 5 | Determination of the granule shear strength by assessing the production of fines versus time of assay (time of bubbling). Lines were calculated by linear regression. (X_0 = granular sludge concentration at t=0; X_F = granular sludge concentration at t=F).

Calcium accumulation and leaching

Figure 6 presents the Ca^{2+} content of the granular sludge coming from each reactor, measured at the end of reactor operation. As was expected, the Ca^{2+} content in reactor R4 sludge was higher than in the inoculum, i.e., 150 and 60 mg Ca^{2+}/g TSS, respectively.

Batch tests were carried out to determine calcium leaching from the inoculum granules, grown at 10 – 15 g Na^+/L, in response to a high Na^+ concentration of 20 g/L. The calcium content of the sludge was determined at the beginning, middle and last day of incubation. The Ca^{2+} concentration in the liquid was determined eight times during incubation. Results from the experiments clearly show an increase in the bulk liquid Ca^{2+} concentration from 4 to 20 mg Ca^{2+}/g TSS added at Na^+ concentration of 20 g/L (Figure 7). The calcium content of the granular sludge concomitantly decreased from 84 to 52 mg Ca^{2+}/g TSS added, at the end of the incubation period. Apparently, about 50 % of the leached Ca^{2+} is present as colloidal matter as this fraction was not covered in both measurements.

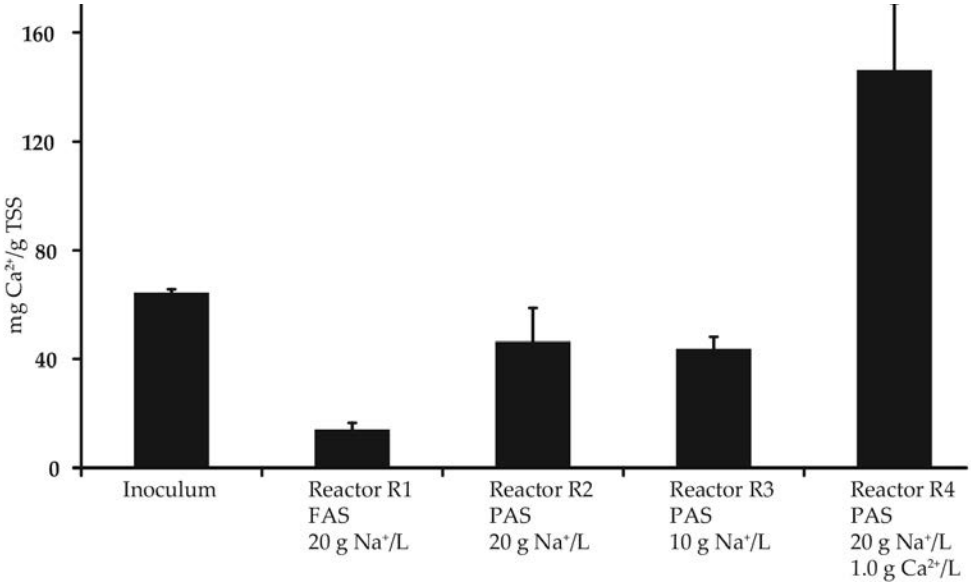

Figure 6 | Calcium content of the sludge from each UASB reactor, at the end of the operation. Inoculum sludge is presented for comparison. Error bars represent the variance between duplicates.

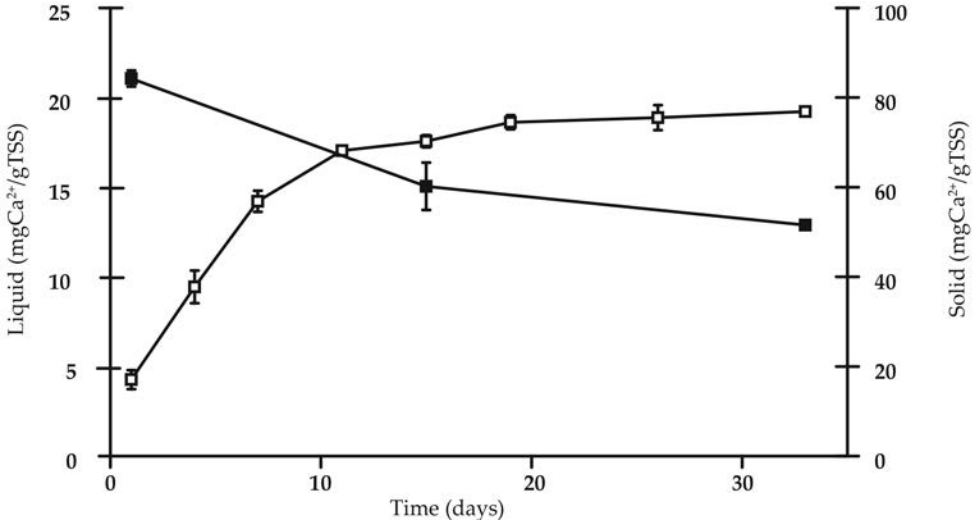

Figure 7 | Evolution of the Ca^{2+} content in the sludge (■, right y-axis) and in the liquid (□, left y-axis) in the batch experiments at 30 ^{0}C, incubated at a Na^{+} concentration of 20 g /L. Error bars represent the variance between duplicates.

3.4 Discussion

Our present results clearly show the potential of high salinity anaerobic wastewater treatment using sludge bed systems. Apparently, the required granule formation for these types of systems depends on the applied substrate composition, the salinity strength, and the presence of divalent cat-ions in the influent. Results listed in Table 2 shows that granules fed with partially acidified substrate have distinct higher proteins and polysaccharides contents than granules growing on fully acidified substrate. Fukuzaki et al. (1995) also reported that starch- and sucrose-grown granules produced more abundant EPS than ethanol– or fatty acids–grown granules. Interestingly, reactors fed with PAS were characterized with a higher granules' size compared to the FAS grown granules at high salinity.

The higher concentrations of polysaccharides in reactor R4 compared to reactor R2 might be attributed to the presence of additional Ca^{2+} in the feed. Morgan et al. (1990) found that Ca^{2+} has a high affinity for EPS and that Ca^{2+} was involved in the adhesion process of anaerobic sludge. Free Ca^{2+} is essential to the bacterial consortium integrity because it maintains the tertiary structure of EPS. As a result, the interactions between the adjacent sugars on different chain are promoted (Yu et al., 2001).

Although still speculatively, we postulate that under strong saline conditions the deterioration of granule strength can be attributed to the replacement of Ca^{2+} in the matrix by the abundantly available Na^{+}, resulting in a weaker granule structure. Our results (Figure 7) indeed indicate that such Ca^{2+} displacement can be brought foreword by high Na^{+} concentration, subsequently affecting the resulting granule strength, as researched in more details in Chapter 4. Bruss et al. (1992), who observed Ca^{2+} displacement in aerobic activated sludge flocs when exposing the flocs to high Na^{+} concentrations, made a similar finding. This displacement resulted in deterioration in floc properties measured by an increased supernatant turbidity and higher specific resistance to filtration (SRF).

The granules grown at 20 g Na^{+}/L were distinctly bigger than the granules of reactor R3 sludge, grown at 10 g Na^{+}/L. This may be due to presence of excessive amounts of monovalent cations (Na^{+}) at 20 g/L, which might lose the binding of EPS and result in a swollen granule structure owing to repulsive electrostatic forces. This may also explain why the observed bigger granules at 20 g Na^{+}/L become weaker.

Granular sludge fed with Ca^{2+}– rich influent (reactor R4) produced higher amounts of polysaccharides compared to the other sludges. Increasing polysaccharide levels also have been reported for aerobic granules (Jiang et al., 2003) and anaerobic granules, in which the carbon utilization shifts towards polysaccharide production in the presence of excess divalent ions such as Fe^{2+} and Mg^{2+} (Shen et al., 1993, Veiga et al., 1997). Polysaccharides can form a strong and sticky matrix in which the bacteria are embedded (Sutherland, 2001). Very likely, such matrix enhances the formation and stabilizes the stable granular structure in both aerobic and anaerobic granules. Moreover, a positive correlation has been observed for the sludge polysaccharide content and the density and strength of attached biofilms (Ohashi and Harada, 1994), which is consistent with the data presented in the current study. The secondary functional groups in the polysaccharides, i.e. OH^{-}, could also interact with Ca^{2+} to form a rigid, non–deformable polymeric gel–like matrix (Sutherland, 2001) and further enhance the

structural stability of the aerobic granules. On the other hand, specific removal of calcium weakens the granule, ultimately leading to complete deterioration (Grotenhuis et al., 1991). In additions, Urbain et al. (1993) found the specificity of components in the EPS related to the available divalent cations, where Ca^{2+} has a higher affinity for proteins in the EPS. The proteins - polysaccharides ratio is generally used to evaluate the granule's stability and strength (Franco et al., 2006, Wu et al., 2009). As indicated by other researchers, a low protein - polysaccharides ratio in microbial granules indicates a high strength and settleability (Batstone and Keller, 2001, Martínez et al., 2004). In our reactors, the highest granule strength was observed in sludge from reactor R4 followed by reactors R3, R2 and R1 with proteins - polysaccharides ratios of 7.5, 8.0, 13.4, and 15.6, respectively.

The amount of extracted proteins in our sludge was about 10 fold higher than the extracted polysaccharides and was similar in all PAS fed reactors. It must be noted, however, that the observed accuracy of the protein measurement was rather low. Although not very common for anaerobic granular sludge, the very high measured protein - polysaccharide ratio is in agreement with the results recently published by d'Abzac et al. (2010). The authors used the same extraction method for extracellular polymers as in our work and found for specific sludge types also a much higher protein content compared to polysaccharides, i.e. granules grown on alcohol production wastewater showed 409 mg proteins and 31 mg polysaccharides per g volatile dry weight, respectively. While comparing various extraction procedures, they concluded that the proportion of each biochemical EPS compound varies with the type of extraction protocol used. Nonetheless, the huge differences found in our work suggest that high salinity conditions possibly induce higher amounts of proteins in EPS. Large variations in dependence to sludge origin were also found by d'Abzac et al. (2013).

Ca^{2+} supplemented substrates improved the anaerobic granules' strength but did not enlarge the granules' size. Strength improvement can be explained by the fact that Ca^{2+} binds to negatively charged groups present on bacterial surfaces and EPS, and acts as a bridge to interconnect these components (Sobeck and Higgins, 2002), promoting bacterial aggregation. On the other hand, the presence of too much calcium in the granules could damage the environment required for maintenance of the granular structure and the bacterial activity, as previously shown by van Langerak et al. (2000). In our research, addition of 1 g Ca^{2+}/L to the influent resulted in relatively small granules with a high granule strength. This finding agrees with previous research that clearly demonstrates that high Ca^{2+} concentration (>0.6 g Ca^{2+}/L) had a negative influence on the granules size (Yu et al., 2001).

3.5 Conclusions

The following conclusions can be drawn from the present research:

- PAS grown granules are characterized by a larger size, a higher strength, and the presence of more EPS than FAS grown granules.
- The production of extracted proteins and polysaccharides of sludge grown at 20 g Na^{+}/L was 5 and 43 % higher, respectively, compared to sludge grown at 10 g Na^{+}/L.
- Problems related to granule structure and strength due to high salinity can be overcome by addition of divalent cations to the influent.

- Size distribution of granules fed with 20 g Na^+/L were increased compa red to granules fed with 10 g Na^+/L but shear strength results show that the granules fed with 10 g Na^+/L are distinctly stronger.
- Granular sludge fed with 1 g Ca^{2+}/L has a relatively high shear–force resistance but the average diameter is smaller than the control granules, not exposed to calcium in the feed.

Acknowledgement

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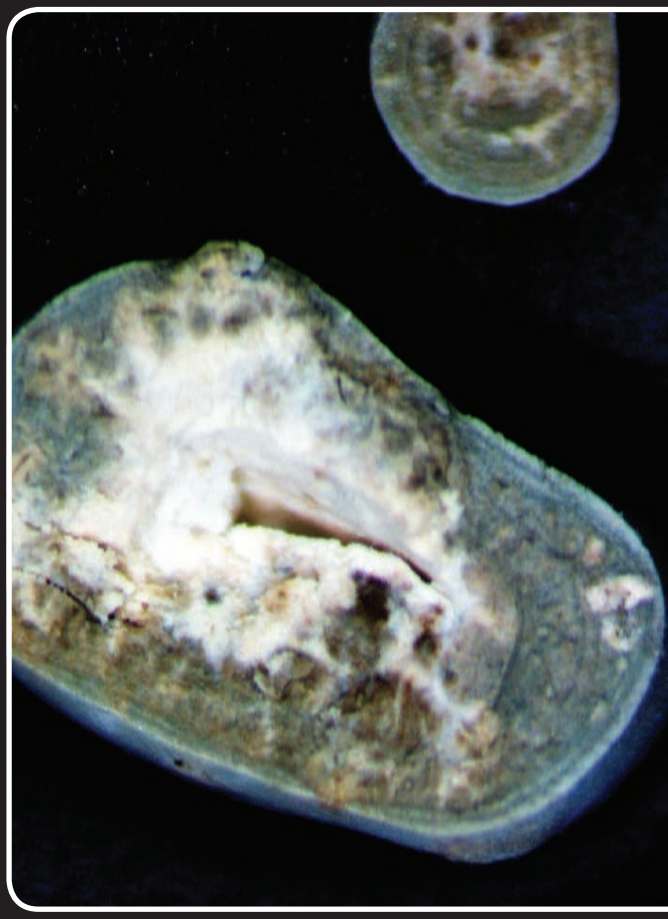
Chapter 4



Effect of calcium augmentation on anaerobic granular sludge under saline conditions

"Ada hujan ada panas, ada hari boleh balas" With the passing of time comes the opportunity to pay back.
Malay proverb

Abstract – Batch incubations with anaerobic granular sludge were carried out at a sodium concentration of 20 g/L to investigate the effect of calcium augmentation. Calcium measurements, supported by scanning electron microscopic tools, showed that in the presence of 20 g Na^+/L anaerobic granules start leaching calcium, and that the calcium ions in granule structure were being replaced by sodium ions. Calcium leaching was largely prevented in the presence of 0.3 g Ca^{2+}/L (10.5 mg Ca^{2+}/g of granule solids). Because calcium depleted granules are more prone to shear, calcium augmentation can be a useful tool to enhance anaerobic granulation and biomass retention under saline conditions.
Keywords: Anaerobic granule, Ca^{2+} augmentation, high salinity wastewaters, Scanning electron microscope – energy dispersive X-ray (SEM-EDX) analysis.

4.1 Introduction

Anaerobic granular sludge treatment is an established technology for treatment of fresh, warm and highly concentrated wastewaters (Chong et al., 2012, van Lier, 2008). However, for saline wastewaters, anaerobic treatment in granular sludge bed reactors is not a proven technology (Lefebvre and Moletta, 2006). High concentrations of sodium, the dominant cation in most saline wastewaters, have a toxic effect on the anaerobic conversion processes (Rene et al., 2008, Feijoo et al., 1995, Gebauer and Eikebrokk, 2006) and may cause poor granulation (e.g. Mendez et al., 1995 and Chapter 2). Both effects of sodium will result in lower volumetric conversion rates and thus large reactor systems that may not be economically feasible.

It is well known that divalent (earth)alkali ions, such as calcium, play an important role in microbial aggregation (Flemming and Wingender, 2010, Yu et al., 2001, Ahmad et al., 2011, Adav et al., 2008). For example, Liu et al. (2009) showed that the resistance of calcium rich granules against shear was much higher than that of granules with a lower calcium content. Similarly, an extensive accumulation of calcium was demonstrated in microbial aggregates (Batstone et al., 2002, Wang et al., 2007) and selective removal of calcium from anaerobic granules by EGTA resulted in their disintegration (Grotenhuis et al., 1991).

Chapter 3 showed that high sodium concentrations cause calcium leaching from anaerobic granules, probably because the calcium ions in the (granular) polymeric matrix were replaced by the abundant sodium ions. As a result, the granules became less resistant to shear and granule density and size decreased. From this it was hypothesized that an increased divalent to monovalent cation ratio may prevent calcium leaching from granules under these conditions. In Chapter 3 we described the performance of a calcium augmented (1 g Ca^{2+}/L or 9 mg Ca^{2+}/g of total suspended solids [TSS]) anaerobic granular sludge bed reactor treating synthetic saline (20 g Na^{+}/L), partially acidified wastewater. It was demonstrated that the calcium augmented granules indeed were more resistant against shear than granules in a similar reactor without calcium augmentation.

To study the effect of calcium augmentation in more detail, granules were incubated for a period of 33 days at a sodium concentration of 20 g Na^{+}/L, with and without additional calcium (0.3 g Ca^{2+}/L or 10.5 mg Ca^{2+}/g TSS), without any additional carbon source. Calcium measurements and scanning electron microscopy (SEM) were used to evaluate the effect of this calcium augmentation.

4.2 Material and methods

4.2.1 Inoculum

The granular sludge inoculum was taken from a full–scale UASB reactor treating wastewaters from a styrene and propene-oxide production plant of Shell, Moerdijk, the Netherlands. This reactor is operated to treat wastewater containing a sodium concentration in the range of 10 – 15 g/L, with acetic acids and benzoic acids as the main sources of COD. The sludge, with a volatile to total suspended solids ratio of 0.32, was already adapted to a high salinity for a period exceeding 10 years (Biothane-Veolia, personal communication).

4.2.2 Batch experiments

Batch experiments were performed in parallel in two duplicate sets of 100 mL serum bottles (sets A and B). Set A consisted of 2 x 10 bottles of which a duplicate bottle was sequentially sacrificed for liquid sampling. Set B consisted of 2 x 3 bottles and was used for periodic sludge sampling. In the batch experiments 28.7 g of total suspended solids (TSS)/L inoculum sludge was exposed to 20 g Na^+/L in the absence of an organic growth substrate. Except for the duplicate blank, in both Set A and B the bottles were augmented with $CaCl_2.2H_2O$, reaching a final calcium concentration of 0.3 g Ca^{2+}/L liquid. The bottles were placed on a reciprocal shaker at 30 ^{0}C for a period of 33 days. All chemicals were supplied by Merck (Darmstadt, Germany).

4.2.3 Scanning electron microscopy

After sampling, the wet granules were fixed by adding glutaraldehyde up to a 2.5 % (w/v) glutaraldehyde concentration. After 1 hour of fixation, the granules were rinsed in water and subsequently dehydrated in a graded series of acetone (10, 30, 50, 70, and two times 100 %, 15 minutes per step). The granules were critical point dried with carbon dioxide (CPD 030 BalTec, Liechtenstein). Granules were glued on a sample holder by carbon adhesive tabs (EMS, Washington, USA) or with carbon glue (Leit-C, Neubauer Chemicalien, Germany). The samples were sputter coated with 10 nm platinum in a dedicated preparation chamber (CT 1500 HF, Oxford Instruments, Oxford, UK) and structurally analysed with a field emission scanning electron microscope (JEOL 6300 F, Tokyo, Japan) at 5 kV and at room temperature. Images were digitally recorded (Orion 6 PCI, E.L.I. sprl. Belgium). The uncoated samples were analysed and recorded with backscattered electron (BSE) detection at 12 kV with a 15 mm working distance to the objective pole piece and with 9 mm detection distance to the BSE detector. The BSE recording was done by a retractable Autrata YAG crystal detector (AutraDet, Brno, Czech Republic). The optimum BSE and SE signal-to-noise ratio was detected empirically at 12 kV. INCA X-ray analyser (Oxford Instruments Analytical, High Wycombe, England) at an acceleration voltage of 15 and 20 kV and a working distance of 15 mm accomplished EDX analyses. The images were optimised and resized with Adobe Photoshop CS.

4.2.4 Analyses

Calcium content of the sludge per gram of total solids was determined after destruction with Agua regia (mixture of 2.5 ml 65 % HNO_3 and 7.5 ml 37 % HCl), added to 1 g (wet weight) of granular sludge. After microwave destruction (Milestone ETHOS E temperate controlled; Milestone INC., Monroe, CT, USA), the samples were paper-filtered (Schleider Schuell 589, Germany) and diluted to 0.1 L with demineralised water. Liquid samples were collected using a 10 – 15 ml syringe and were centrifuged at 4500 rpm for 15 min (IEC Microlite, USA). Supernatants were filtered through 0.45 µm cellulose membranes (Schleicher & Schuell ME 25, Germany). The calcium concentration was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES; Varian, Australia) as described by van Hullebusch et al. (2005). Ultra-pure water (Milli-Ro System, Millipore, Bedford, MA, USA) was used to prepare standard solutions of all reagents, which were of suprapur quality (Merck, Darmstadt, Germany) and were checked for possible trace metal contamination. All glassware and plastic materials used were treated for 12 h with 10 % (v/v) HNO_3 and rinsed vigorously with demineralised water.

All other analyses were carried out according to Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

4.3 Results

4.3.1 Calcium behaviour

The results of an earlier batch experiment described in Chapter 3, are presented in Figure 1. Anaerobic granules started leaching considerable amounts of calcium in the presence of 20 g Na^+/L within 33 days granule associated calcium concentrations (referred to a as solids bound calcium) decreased from 84 to 52 mg Ca^{2+}/g TSS, while dissolved calcium concentrations (normalized against TSS) increased from 0 to 20 mg Ca^{2+}/g TSS.

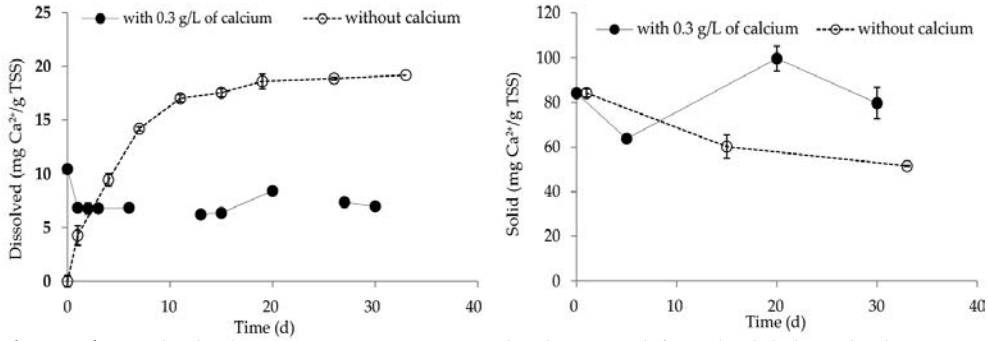


Figure 1 | Dissolved calcium concentrations (normalized to TSS) (left) and solids bound calcium concentrations (right) during batch incubations at 30 ^{0}C at a sodium concentration of 20 g Na^+/L, and at a sodium concentration of 20 g/L with 0.3 g/L calcium addition. Error bars represent differences between duplicates.

Figure 1 also shows the behaviour of dissolved and solids bound calcium, when the granules were simultaneously exposed to 20 g Na^+/L and 0.3 g dissolved Ca^{2+}/L, which is equivalent to 10.5 mg Ca^{2+}/g TSS. Already after one day the calcium concentration in the bulk liquid had decreased from 10.5 to approximately 6.5 mg Ca^{2+}/g TSS. Afterwards, with the exception of a higher concentration on day 20, the dissolved calcium concentration remained relatively stable. Calcium concentrations in the granules exhibited considerable variation (60 to 100 mg Ca^{2+}/g TSS) and do not seem very reliable. Mass balance calculations based on the measurements showed that 5 – 25 % of the initial amount of calcium was missing. Nevertheless, in particular the bulk liquid calcium concentrations show that calcium leaching did take place at a sodium concentration of 20 g Na^+/L, but that this leaching could be prevented by calcium augmentation. Furthermore, stabilisation of the dissolved calcium concentrations at 6.5 mg Ca^{2+}/g TSS in the calcium augmented experiment suggested that for this purpose, and for this particular sludge, 10.5 – 6.5 = 4.0 mg Ca^{2+}/g TSS of granular sludge would be sufficient to achieve a new equilibrium without (net) calcium leaching.

4.3.2 SEM observations

To study the distribution of sodium and calcium in the granules, randomly selected granules from the inoculum and from the serum bottles after 30 days of incubation with and without

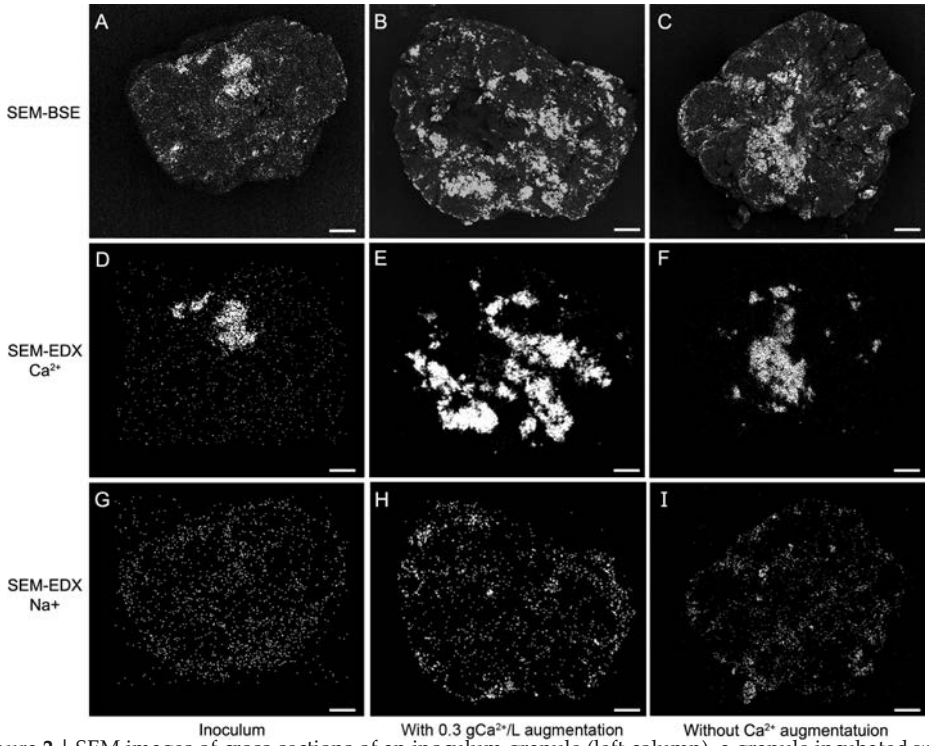


Figure 2 | SEM images of cross-sections of an inoculum granule (left column), a granule incubated with 20 g Na^+/L (middle column) and a granule incubated with 20 g Na^+/L + 0.3 g Ca^{2+}/L (right column). In the SEM-BSE images (top row) dark areas represent organic rich zones and light areas metal rich zones. SEM-EDX images in the middle and bottom row show the distribution of calcium (bright spots) and sodium (bright spots), respectively. White bar = 100 μm.

0.3 g/L calcium augmentation, were further investigated by SEM-BSE and SEM-EDX. The top row of Figure 2 shows SEM-BSE results with organic rich and metal rich zones represented by dark and bright areas, respectively.

SEM-EDX was conducted for qualitative calcium and sodium distributions. The middle row of Figure 2 shows the results for calcium (bright spots) and the bottom row for sodium (also bright spots). A comparison of the middle row images with those of the top and bottom row revealed that the presence of calcium in all granules was more abundant than of sodium. The images for sodium in the bottom row show that sodium was distributed over the entire cross section of the granule. However, particularly for the granule without calcium augmentation, more sodium can be observed in the peripheral part of the granule.

Figure 3, from top to bottom, gives line spectrum analyses of the inoculum granule, the granule that was incubated with 20 g Na^+/L and the granule that was incubated with 20 g Na^+/L plus 0.3 g Ca^{2+}/L, respectively. The lines that were scanned are displayed in the SEM-BSE images left to the line spectra, and were selected to include organic as well as metal rich zones. The scanning started from the core of the granules (line unit 0 on the x-axis) to the periphery of the granules (line unit 40).

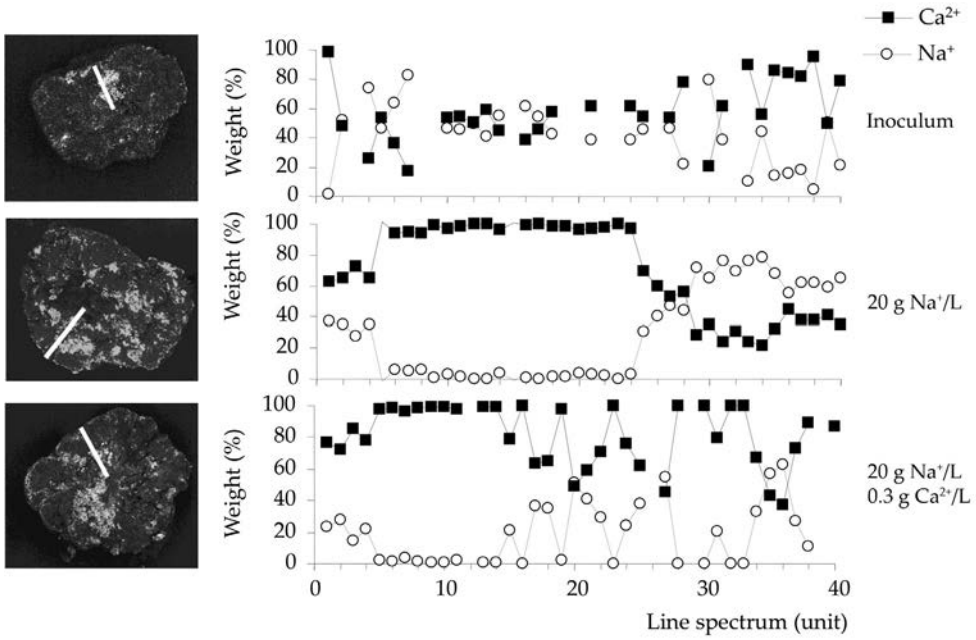


Figure 3 | Plot of an EDX scanned line of the relative weighted presence of calcium and sodium in an inoculum granule, granule incubated with 20 g Na^+/L and granules incubated with 20 g Na^+/L and 0.3. The line scan proceeded from the core of the granule (line unit 0) to the peripheral part (line unit 40). Cumulative EDX counts for calcium and sodium are 100 %.

Although variable along the scan, the contributions of sodium and calcium in the inoculum granule were similar. Only in the periphery of the granule, between line units 32 and 40, the contribution of calcium was much higher than of sodium, i.e. approximately 80 % for calcium compared to 20 % for sodium. Interestingly, in the granule that was exposed to 20 g Na^+/L, sodium dominated the weight distribution towards the periphery of the granule (approximately 70 and 30 % for sodium and calcium between line units 30 and 40, respectively). With the exception of the core (line units 0 to 5), calcium dominated the remaining part of the line scan. This strongly suggests that during the incubation period of 33 days, sodium had penetrated the periphery of the granule and calcium had started leaching from the granule structure to the bulk liquid. The almost complete dominance of calcium between line units 5 and 25 can possibly be attributed to the presence of calcium precipitates in that location of the granule. Calcium dominated all along the line scan in the granule that was exposed to 20 g Na^+/L and 0.3 g Ca^{2+}/L.

Figure 4 shows the results of SEM-EDX spectrum analyses of the whole cross section area for the inoculum granule (left), and for the granule that was incubated with 20 g Na^+ and 0.3 g Ca^{2+}/L. Table 1 gives the relative weighted presence of calcium and sodium, which were calculated from these data. The results are in good agreement with the observations in Figure 3 that the contributions of calcium and sodium in the inoculum were similar, but that calcium dominated the weight distribution in the calcium augmented granule.

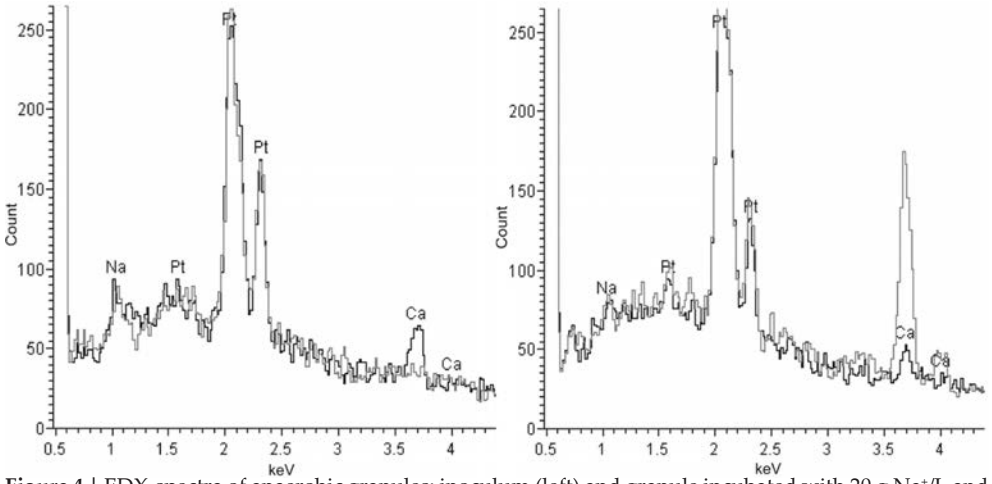


Figure 4 | EDX-spectra of anaerobic granules: inoculum (left) and granule incubated with 20 g Na^+/L and 0.3 g Ca^{2+}/L (right).

Table 1 | The relative weighted presence of sodium and calcium in a cross section of an inoculum granule and of a granules incubated for 35 days with 20 g Na^+/L and 0.3 g Ca^{2+}/L (Na^+ and Ca^{2+} were qualitatively measured by SEM-EDX).

Element	Atomic percentage (%)	
	Inoculum	Granule with 0.3 gCa^{2+}/L augmentation
Na	57.7	29.5
Ca	42.3	70.5
Total	100	100

4.4 Discussion

The results showed that addition of 0.3 g Ca^{2+}/L (10.5 mg Ca^{2+}/g TSS) helps to maintain the calcium content of anaerobic granular sludge when this sludge is exposed to high sodium concentrations of 20 g Na^+/L. Under similar conditions, but in the absence of calcium, anaerobic granules started to leach calcium.

Leaching of divalent cations such as calcium from the granule matrix at high sodium concentrations is in agreement with Bruus et al. (1992) and Higgins and Novak (1997) who observed calcium displacement in aerobic activated sludge flocs when these were exposed to high sodium concentrations. Sobeck et al. (2002) explained this phenomenon with the so called cation bridging theory: calcium cations bind to negatively charged functional groups on bacterial surfaces and to extracellular polymeric substances (EPS), and in this manner act as a bridge to interconnect these components and promote the formation of microbial aggregates (Jiang et al., 2003). The EPS functions like a cation-exchange material, which explains why calcium can be replaced by sodium when the ratio between sodium and calcium in the bulk liquid is high, as was the case in our experiments.

Our results support findings by Liu and Sun (2011) that 50 mg/L of calcium augmentation increased the size and improved the settling properties of denitrifying granules. They attributed this effect to formation of more bonds between calcium and negatively charged extracel-

lular polymers, in particular proteins, causing a stronger three dimensional structure of the granules. Also others have reported the positive effect of calcium on (anaerobic) granulation, granule strength and biomass retention (Batstone and Keller, 2001, Mahoney et al., 1987, De Graaff et al., 2011). Apparently this strategy also works at high sodium levels when sodium can (partially) replace the calcium ions in the polymeric matrix. This also implies that calcium addition under saline conditions can be a useful tool to prevent granules to become more shear sensitive as discussed in Chapter 3, and in this manner avoid a deteriorating biomass retention and process failure.

Batch incubations with 0.3 g/L of calcium (10.5 g/g TSS) suggested that in the presence of 20 g Na^+/L approximately 4 mg Ca^{2+}/g TSS would be sufficient to counteract calcium leaching in the used sludge granules. However, an optimised calcium augmentation probably will differ per sludge type since it likely depends on many factors including the sodium concentration, the presence and type of other mono- and divalent ions, presence and type of EPS, the type of organic substrate, prevailing shear conditions, sludge loading rate, etc. More research therefore is required to establish the relation between calcium and these parameters. This may also explain why in the literature conflicting results are reported about the effect of calcium (Chen et al., 2008). Low calcium concentrations of 0.1 – 0.2 g/L generally were found to stimulate granulation and biofilm formation, whereas concentrations above 0.3 g/L on some occasions were detrimental. The latter can be caused by sludge calcification or even complete cementation (van Langerak et al., 2000).

4.5 Conclusions

Under saline conditions calcium addition helps to maintain the calcium content of granular sludge and in this manner prevents replacement of calcium by sodium. If applied at an appropriate concentration, calcium addition therefore could be beneficial for anaerobic treatment of saline wastewaters because it reduces granule disintegration and wash out of viable biomass.

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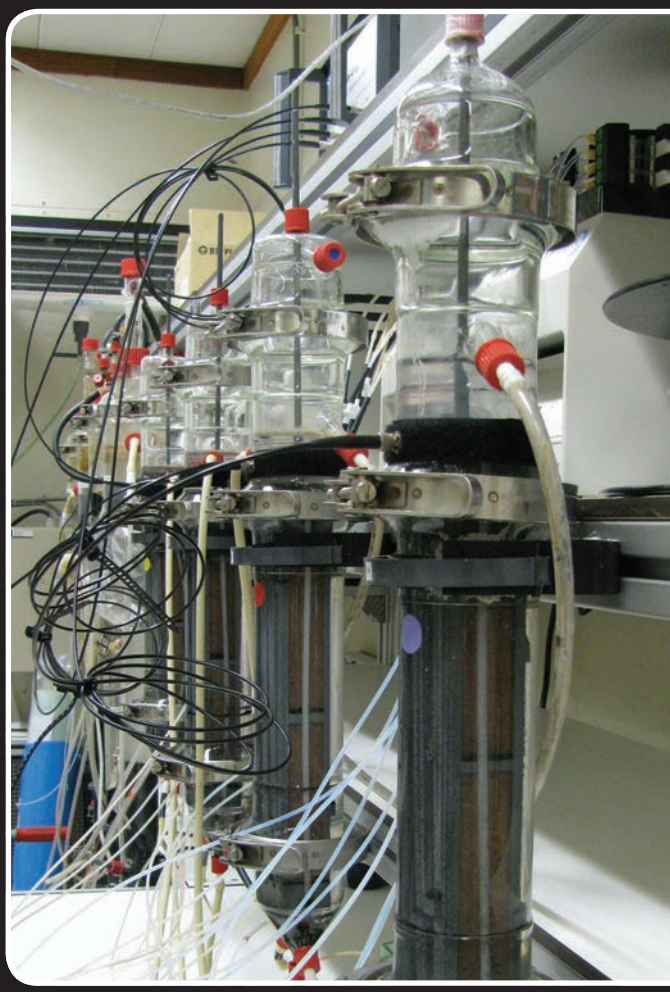
Chapter 5



Anaerobic Biofilm Formation at High Salinity

"Waar de zon schijn, is de maan niet nodig" Who need the moon when the sun rising
Dutch proverb

Abstract - Anaerobic biofilm formation was studied under saline conditions (10 and 20 g Na^+/L) with acetate as the sole substrate. For this purpose four up-flow reactors containing non-woven fabric sheets as support material for biofilms formation were operated in fed batch mode. Biofilm formation was studied based on periodic assessment of acetate removal rates and by using SEM and FISH-CLSM techniques. Development of the microbial population was studied with DGGE, cloning and sequencing. The results demonstrated that it was possible to grow anaerobic biofilms at least up to 20 g Na^+/L, even though acetate conversion rates were considerably lower than at 10 g Na^+/L. The negative effect of sodium on the specific methanogenic activity was alleviated in the presence of 0.7 g K^+/L. Surprisingly, with 0.3 g Ca^{2+}/L both methanogenic activity and biofilm formation were strongly reduced. The acetoclastic methanogen *Methanosaeta harundinicea* dominated the biofilm in all reactors but, also a fraction of the archaeal population consisted of hydrogenotrophic methanogens even though syntrophic acetate oxidizing bacteria were absent.
Keywords: Anaerobic biofilm, biofilms reactor, FISH-CLSM techniques, initial attachment, microbial profiling analysis, *Methanosaeta harundinicea*.

5.1 Introduction

High salinity wastewaters are generated by various food processing industries including fish and seafood processing industries, alcohol distilleries, olive mills and yeast factories (Lefebvre and Moletta, 2006). Also many chemical wastewaters are characterised by extreme salinity levels, mainly caused by efficient water management practices towards process water loop closure, resulting in less fresh water intake.

Granular sludge based anaerobic high-rate reactors, such as the up-flow anaerobic sludge blanket (UASB) reactor and the expanded granular sludge bed (EGSB) reactor, are commonly applied to treat relatively warm and concentrated, organically polluted fresh wastewaters (van Lier, 2008). High salinity levels, generally equivalent to high Na^+ concentrations, not only inhibit anaerobic conversion rates but also can reduce granule strength, causing viable biomass to wash out from the reactor (Chapters 2, 3 and 4). Both phenomena can result in poor reactor performance.

Granule formation still is poorly understood, and the literature is often contradictory (Hulshoff Pol et al., 2004). Probably, several factors contribute to granulation and multiple mechanisms are involved (Ahn et al., 2009). For example, divalent cations such as calcium play an important role in the aggregation of microorganisms (Geesey et al., 2000, Yu et al., 2001). They form positively charged bridges between negatively charged bacterial surfaces and extracellular polymeric substances (EPS) (Patrauchan et al., 2005, Shen et al., 1993, Veiga et al., 1997). In addition, calcium precipitates can provide an (initial) surface for attachment of microorganisms, stimulating the granulation process (Abbasi and Abbasi, 2012). As discussed in Chapter 4, when the ratio between mono- and divalent cations in the bulk water becomes too high, the multivalent cations in the polymeric matrix are (partially) replaced by monovalent cations. This makes granules and biofilms more prone to shear and, as mentioned previously, may result in wash-out of viable biomass and a poor reactor performance (Mendez et al., 1995).

Although methanogenesis, the final step in anaerobic biodegradation of complex organic substrates, is very common in fresh water systems, it has not been demonstrated at extremely high salt concentrations. The record for hydrogenotrophic methanogens is 48 g Na^+/L and the upper limit for acetoclastic methanogens probably is even lower (Oren, 1999). However, some authors claimed to have operated anaerobic reactor systems at salinity levels as high as 40 – 60 g Na^+/L (e.g. Dafale et al., 2008, Guo et al., 2007). There are halophilic methanogens that use methanol, methylamine compounds or dimethylsulfide to generate methane. These substrates yield a similar amount of energy (-78.7 to -191.1 kJ per mol substrate) as H_2/CO_2 conversion (-131 kJ/mol substrate) under standard conditions, but much more than acetate conversion (-31 kJ mol substrate), which likely allows methylotrophic and hydrogenotrophic methanogens to grow at these very high salt concentrations, while offsetting the energy costs of the increased osmo-regulatory burden (Oren, 1999, Waldron et al., 2007). In subsurface environments, with generally lower salinity levels, the Archaea commonly predominantly present in anaerobic treatment reactors, i.e. the acetoclastic methanogens, are more commonly described (Kotelnikova, 2002).

To counteract negative effects of high salinity on their metabolism, i.e. when extracellular Na^+

concentration exceeds that of the cell cytoplasm, microorganisms can apply two strategies. The salt-in strategy based on accumulation of KCl probably is restricted to halophilic bacteria and does not occur with methanogens (Oren, 1999). A more common strategy is osmoregulation, in which halotolerant microorganisms accumulate, or sometimes even produce, low-molecular-weight organic compounds, known as compatible solutes (Lai and Gunsalus, 1992) such as glycinebetaine, carnitine and proline. According to Sleator and Hill (2002) the common response to increased salinity levels is that glutamate and K^+ are taken up as a primary response, which is followed by a secondary response with a dramatic increase in cytoplasmic concentrations of compatible solutes. This also explains why K^+ may act as an antagonistic salt alleviating the impact of high concentrations of Na^+ on the methanogenic conversion rates (e.g. Kugelman and McCarty, 1965). According to Roberts (2005), methanogens apply a similar strategy as other (halotolerant) microorganisms. Below a salinity level of 1 M of NaCl they tend to accumulate glutamate and K^+, but above this salinity they start using zwitterions such as Nε-acetyl-β-lysine as compatible solutes.

Initial attachment of microorganisms is very important for the development of granules and biofilms, but has not been studied for anaerobic microorganism under saline conditions. Therefore, in our present research anaerobic biofilm formation was followed for a period of 175 days at two different Na^+ levels (10 and 20 g/L), with acetate as the methanogenic substrate and with Ca^{2+} or K^+ as additional cations that may stimulate methanogenic activity and biofilm formation. Calcium and potassium concentrations of respectively 0.3 g/L (150 mg Ca^{2+}/g volatile suspended solids [VSS] of crushed granular inoculum) and 0.7 g/L (350 mg K^+/g VSS of inoculum) were selected to resemble seawater concentrations because it is known that anaerobic biofilm formation does take place in marine systems (e.g. Zhang et al., 2003). The biofilms were characterized based on acetate conversion capacity, scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM), fluorescence in-situ hybridization (FISH), Archaeal and Bacterial denaturing gradient gel electrophoresis (DGGE) profiles and cloning and sequencing.

5.2 Material and Methods

5.2.1 Source of inocula

Anaerobic granular sludge from a full-scale UASB reactor treating wastewaters from a styrene and propene-oxide production plant of Shell, Moerdijk, the Netherlands, was used in this study. The sludge already had adapted to a high salinity in the range 10 – 15 g Na^+/L, with acetic acids and benzoic acids as the main sources of chemical oxygen demand (COD) for more than 10 years (Biothane-Veolia, personal communication). The granular sludge, with a volatile to total suspended ratio of 0.32, was crushed prior to the experiments by using a Waring blender (1 L, Model 7012S, New Hartford, USA).

5.2.2 Experimental set-up and biofilm cultivation

The experiments were performed in a temperature controlled room at 30 ± 2 ^{0}C, using four identical up-flow fixed bed glass biofilm column reactors (reactors R1, R2, R3, and R4). The reactors had an inner diameter of 60 mm, a height of 330 mm and a liquid volume of 0.9 L (Figure 1). Non-woven fabric sheets in a rope-like structures materials (38 x 3.0 x 0.05 cm;

Hanotex B.V., Joure, the Netherlands) were used as removable support materials. Each reactor consisted of four sheets, which were placed vertically in PVC plastic frames in the reactor and comprised a total surface area of 0.18 m^2. The reactors were operated in fed batch mode. Operational parameters are given in Table 1. The up-flow liquid velocity was controlled by liquid recirculation and was fixed at 0.8 m/h with a peristaltic pump (Watson Marlow 202, UK). The reactors were seeded with 2 g VSS/L of crushed granules. During the first 40 days of operation, acetate-COD (10 – 30 mL of 200 g/L $CH_3COONa.3H_2O$) was added, based on the results of daily acetate analyses. After this period, the reactors received an additional 2 g of acetate-COD/L reactor for every week until the end of the experiment on day 175. Because sodium acetate was added, the sodium concentration in the reactors increased by 5 – 10 % during the first 40 days, and by approximately 50 % during 175 days. The media were prepared in demineralised water. The nutrient solution consisted of macro and micronutrients with a final pH of 7.5, and was prepared according to Vallero et al. (2002). Background calcium and potassium concentrations in the feed were 5 mg Ca^{2+}/L and 55 mg K^+/L, respectively. Additional calcium ($CaCl_2.2H_2O$) was added to reactor R3 to obtain a concentration of 0.3 g Ca^{2+}/L. The potassium concentration in reactor R4 was set to obtain a Na^+/K^+ ratio of 0.037, similar to seawater, i.e. at a concentration of 0.7 g K^+/L (KCl) (Jeison et al., 2008).

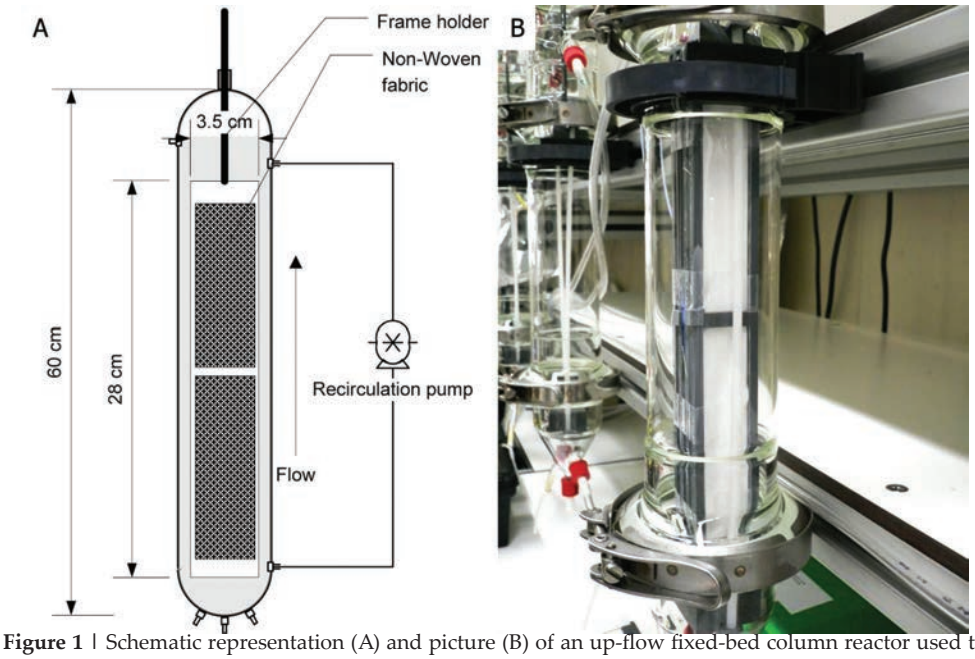


Figure 1 | Schematic representation (A) and picture (B) of an up-flow fixed-bed column reactor used to evaluate bacterial adhesion on fixed materials.

Table 1 | Operational parameters for the biofilm reactors (background calcium and potassium concentrations were 5 mg Ca^{2+}/L and 55 mg K^+/L, respectively).

	Reactors			
	R1	R2	R3	R4
Inoculum concentration (gVSS/L)	2.0	2.0	2.0	2.0
Na^+ concentration (g/L)	10	20	20	20
Ca^{2+} concentration (g/L)	0	0	0.3	0
K^+ concentration (g/L)	0	0	0	0.7

5.2.3 Microbial community analysis

For all microbial community analysis and SEM, biofilm sampling was done periodically, i.e. after 0, 40, 94 and 175 days. A sheet of non–woven fabrics was gently removed from the reactor, cut into small pieces (5 x 5 mm), and directly placed in Eppendorf tube containing 2 mL anoxic reactor medium.

Fluorescent in situ hybridization

Samples were fixed directly with 2 ml of 0.1 M PBS (phosphate buffered saline) containing 4 % (w/v) ice – cold paraformaldehyde at 4 ^{0}C for 2 hours. Paraformaldehyde solution was removed by carefully decanting the liquid. Samples were gently washed in 1800 µl 0.1 M PBS and 200 µl 0.1 % Nonidet P–40 (NP40) for at least 1 min. PBS/NP40 solution was removed by carefully decanting the liquid. Then, samples were stored in 96 % ethanol at –20 ^{0}C.

Two 16S rRNA targeting oligonucleotide probes with different fluorescent dyes, EUB338 (Eubacteria domain probe, 5′ ACT CCT ACG GGA GGC AG 3′) with CY3 (red) and ARC915 (Archaea-domain probe, 5′ GTG CTC CCC CGC CAA TTC CT 3′) with CY5 (yellow-orange) (Eurogentec, Belgium) were used for dual hybridization of the biofilm samples. Fixed samples were then dehydrated with a series of ethanol/water solutions (50, 80, and 96 %, 3 min each). 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl [pH 7.5], 0.1 % [wt/vol] sodium dodecyl sulphate [SDS]) was added to each sample, and 1 µl of each probe (50 ng/µl) was added to the samples and this was followed by incubation at 46 ^{0}C for 2–3 hours. After hybridization, the slides were washed in 50 ml of pre-warmed (48 ^{0}C) washing buffer (0.225 mM NaCl, 20 % Formamide hybridization buffer [v/v], 2250 µl NaCl [5 M], 500 µl EDTA [0.5 M]) with SDS for 10 minutes. After this, the slides were rinsed in the water, immediately dried, mounted in Vectashiled (Vector Labs, Burlingame, USA) and covered with a cover slide (42 x 60 mm, Menzel–Glaser, Germany).

Detection and visualization of the stained cells and biofilm thickness assessment was carried out by using CLSM (Carl Zeiss, LSM 510, Germany) coupled with an Ar-iron laser (488 nm) and HeNe laser (543 nm). Z-sectioning was performed on whole granules and rendered three-dimensionally by using the LSM 510 Viewer software (Zeiss).

DNA extraction, amplification and DGGE

The eppendorf cups with the samples were directly placed in a sonicator (low power output) for 1 min and this was repeated for at least five times, to detach the biomass from the fabric. The samples then were centrifuged for 1 min at 14000 rpm. The cell pellets were stored in RNAlater® preservation solution (Applied Biosystems/Ambion, Austin, USA) at –20 ^{0}C until further analysis. Total genomic DNA was extracted from samples using a FastDNA® SPIN kit for soil (Qbiogene, Calsbad, CA). DNA concentration and integrity were measured with the NanoDrop® spectrophotometer. For denaturing gradient gel electrophoresis (DGGE) analysis, PCR was performed as described by Sousa et al. (2007). Archaeal 16S rRNA fragment were amplified using archaeon–specific primer with primers A109(T)–f (5′-ACT GCT CAG TAA CAC GT-3′) and universal reverse primer 515–r (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC GCG GCT GCT GGC AC-3′).

Bacterial 16S rRNA fragment were amplified using bacterial–specific primer with primers U968–f (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and universal reverse primer L1401–r (5′-CGG TGT GTA CAA GAC CC-3′). All primers used were synthesized commercially by Biolegio (Nijmegen, the Netherlands). Amplicons were subsequently separated by DGGE as described by Zoetendal et al. (2001) using the D–code system (Bio-Rad, Hercules, CA) with 8 % (v/v) polyacrylamide gels having a denaturant gradient of 30 – 60 %.

Cloning and sequencing

For cloning, full-length bacterial and archaea 16S rRNA genes were amplified in a PCR with forwards primers, Bact–27–f (5′-AGA GTT TGG ATC MTG GC TCA G-3′) or A109–f (5′-ACT GCT CAG TAA CAC GT-3′), respectively, and the universal primer Uni1492–r (5′-CGG TTA CCT TGT TAC GAC TT-3′) and the thermocycling conditions as described by Sousa et al. (2007).

16S rRNA gene amplicons integrity and length was checked on an agarosegel and subsequently purified with high pure PCR clean up micro kit (Roche, Mannheim, Germany). Ligation of the PCR products into the vectors was performed with the use of a pGEM®-T Easy Vector System II kit (Promega, Madison, WI, U.S.A.). 5 µl PCR products were added to a mixture of 5 µl 2x Rapid Ligation Buffer, 1 µl pGEM easy vector, 1 µl of T4 DNA ligase and 3 µl PCR water. A positive control was performed by adding 2 µl control insert DNA and 1 µl extra PCR water instead of the PCR product. A negative control was performed by adding 3 µl extra PCR water instead of the PCR product and incubated overnight at 4 ^{0}C. Plasmids were transformed into *E. coli*, 2 µl of each ligation product was added in a sterile tube, together with 50 µl of XL-1 Blue Competent Cells (Stratagene, La Jolla, CA, USA), according to the manufacturers protocol. Cells medium were diluted 10x, 20x, 100x and 500x. 100 µl of the cell suspensions were streaked in duplo on a LB++ agar plate and incubated overnight at 37 ^{0}C.

Visible colonies were placed at 4 ^{0}C for 1 – 2 hours to enhance the blue colour of cells for blue/white screening. White cells likely have taken up a plasmid with PCR products while blue cells have plasmids without PCR product. Two x 96 white cell colonies were randomly selected with a toothpick and transferred to a 96 well Masterblock® plate (Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands), each well filled with 200 µl LB medium + ampicillin for both archaea and bacteria. The plates were covered with adhesive gas permeable seals (ABgene® Ltd., Surrey, UK) and incubated overnight at 37 ^{0}C. Nearly full-length 16S rRNA genes were sequenced by GATC Biotech (Konstanz, Germany). The analyzed 16S rRNA sequences were compared with sequences in the GenBank database using the NCBI Blast search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Zhang et al., 2000). Closest relative and cultured relatives were retrieved from database, with the degree of similarity.

5.2.4 Scanning Electron Microscopy (SEM)

The biofilm samples were fixed by adding glutaraldehyde to the culture medium resulting in a 2.5 % (w/v) glutaraldehyde concentration. After 1 hour of fixation, the biofilms were rinsed in water and subsequently dehydrated in a graded series of acetone (10, 30, 50, 70, 2x 100 %, 15 minutes per step). Biofilms were critically point dried with carbon dioxide (CPD 030

BalTec, Liechtenstein). Biofilms were glued on a sample holder by carbon adhesive tabs (EMS Washington USA) or with carbon glue (Leit- C, Neubauer Chemicalien, Germany) and sputter coated with 2 – 4 nm Tungsten in a MED 020 (Leica, Vienna, Austria). The samples were analysed with a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands) at room temperature at a working distance of 4 mm with SE detection at 2 KV. Images were digitally recorded and were optimized and resized with Adobe Photoshop CS.

5.2.5 Chemical Analyses

Volatile fatty acids (VFAs) analyses were performed as described in Chapter 2. All other analyses were carried out according to Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

5.3 Results

5.3.1 COD removal

Figure 2 shows effluent acetate-COD concentrations in the four reactors during the first 40 days of operation. The initial slope of the acetate-COD concentration in reactor R1, operated at 10 g Na^+/L, was 670 mg COD/L.d, which corresponds to a specific conversion rate of 335 mg COD/g VSS.d. This specific conversion rate matched very well with the specific methanogenic activity (SMA) of the inoculum of 300 mg acetate-COD/g VSS.d as determined for the same inoculum at the same sodium concentration (see Chapter 2). Although varying in time, the acetate conversion rates in reactor R1 were much higher (670 – 1300 mg COD/L.d) than those in reactor R2 operated at 20 g Na^+/L (210 – 320 mg COD/L.d). Apparently the higher Na^+ concentration in reactor R2 had a negative effect on the specific acetate conversion rate of the biomass. Surprisingly, reactor R3, also operated at 20 g Na^+/L but with additional calcium of 0.3 g Ca^{2+}/L, exhibited a very poor acetate removal rate (15 – 40 mg COD/L.d). Acetate removal rates in reactor R4, operated at 20 g Na^+/L with 0.7 g K^+/L, were between 525 and 900 mg COD/L.d (last cycle not included). These rates are somewhat slower than in reactor R1 at 10 g Na^+/L, but considerably faster than in reactor R2 also operated at 20 g Na^+/L. This indicates that, when the anaerobic biomass is exposed to a higher sodium concentration, the presence of potassium alleviates the negative effect of sodium on the acetate conversion rate.

5.3.2 Microbial attachment

As a representative example, Figure 3 shows SEM and CLSM images of the biofilm that was sampled from reactor R4 after 40 days. Both images confirm that cells with different morphologies (coccoidal, rod and filamentous shaped) had attached to the non-woven fibre material.

Figure 4 shows a representative SEM image for each reactor of the biofilms on the non-woven fabric sampled after 40 days. Mainly rods and small coccoids were observed, embedded in an EPS matrix. Surprisingly, surface coverage in reactor R3, operated at 20 g Na^+/L and 0.3 g Ca^{2+}/L, was significantly lower than in the other three reactors, indicating a reduced microbial attachment in the presence of calcium. Surface coverage in the other two reactors operated at 20 g Na^+/L (reactors R2 and R4) was not visibly lower than in reactor R1 at 10 g Na^+/L, suggesting that the higher salinity did not have a negative impact on microbial attachment.

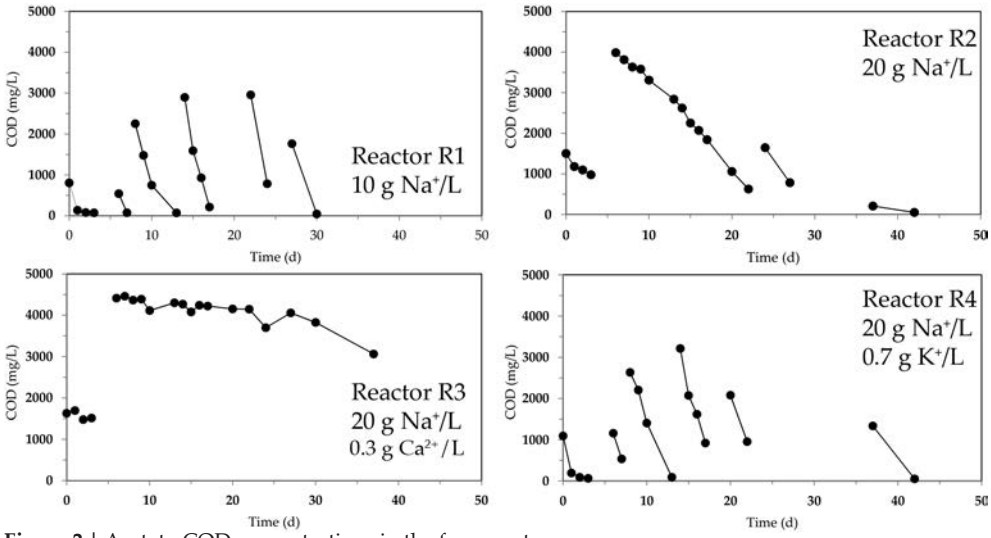


Figure 2 | Acetate-COD concentrations in the four reactors.

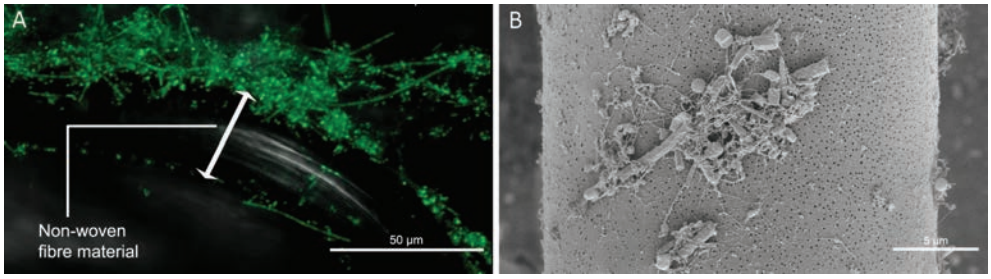


Figure 3 | (A) CLSM image of immobilized microorganisms (SYTO 9 stained- for total cells) and (B) SEM image of the biofilm in reactor R4 on the non-woven fabric after 40 days of operation.

Representative FISH-CLSM images of biofilm samples taken from reactors R1, R3 and R4 after 40 days of operation are shown in Figure 5. A FISH-CLSM image of reactor R2 of sufficiently high quality unfortunately was not available. Two general probes were used: ARC915-Cy5 (red) and EUB338-Cy3 (yellow-orange), complementary to conserved regions within members of Archaea and most Bacteria, respectively. Filaments of archaeal cells were dominant in reactors R1, R2 and R4. In the sample of reactor R3, which received additional calcium, mainly bacterial cells can be seen. These cells probably were remaining from the original inoculum and were not overgrown by Archaea as was the case in the other reactors.

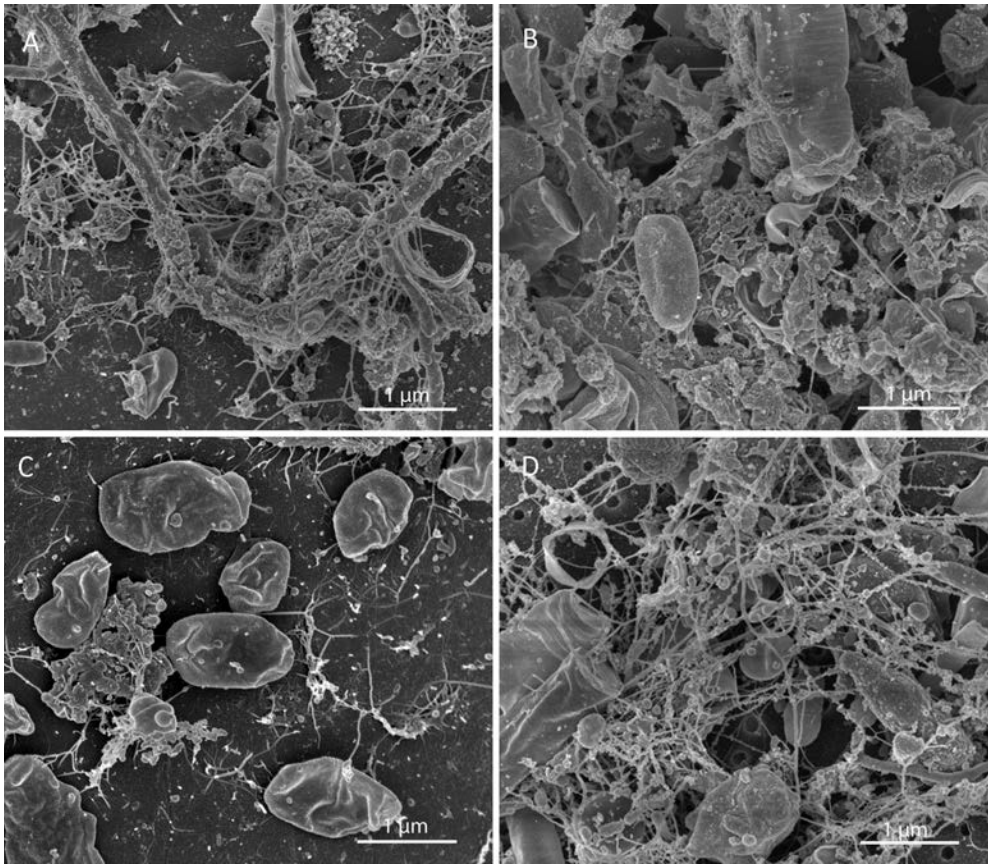


Figure 4 | SEM images of biofilms samples from the four reactors after 40 days of operation.

5.3.3 Microbial community analysis

Samples taken from the biofilms in the four reactors over time were used to characterize the composition and dynamics of the attached microbial community. Extracted amounts of DNA (data not shown) varied in time, but always were in the order reactors R3 < R2 < R4 < R1. The lower amounts of DNA extracted from the non-woven fabric in reactor R3 is in agreement with the SEM images in Figure 4 showing a significantly lower surface coverage in reactor R3 compared to the other reactors. In addition, the order in the extracted amounts of DNA was in line with the acetate removal rates of Figure 2, which probably can be explained by faster microbial growth.

Figure 6 shows the development of the Archaeal population, as determined by DGGE profiling of 16S rRNA gene amplicons. In general, the band patterns revealed no significant differences in the Archaeal community between the reactors or in time. The only differences were that new bands appeared in reactor R1 (10 g Na^+/L) on day 175 and in reactor R3 (20 g Na^+/L and 0.3 g Ca^{2+}/L) on day 94. The latter band however had disappeared towards the end of the experimental period on day 175.

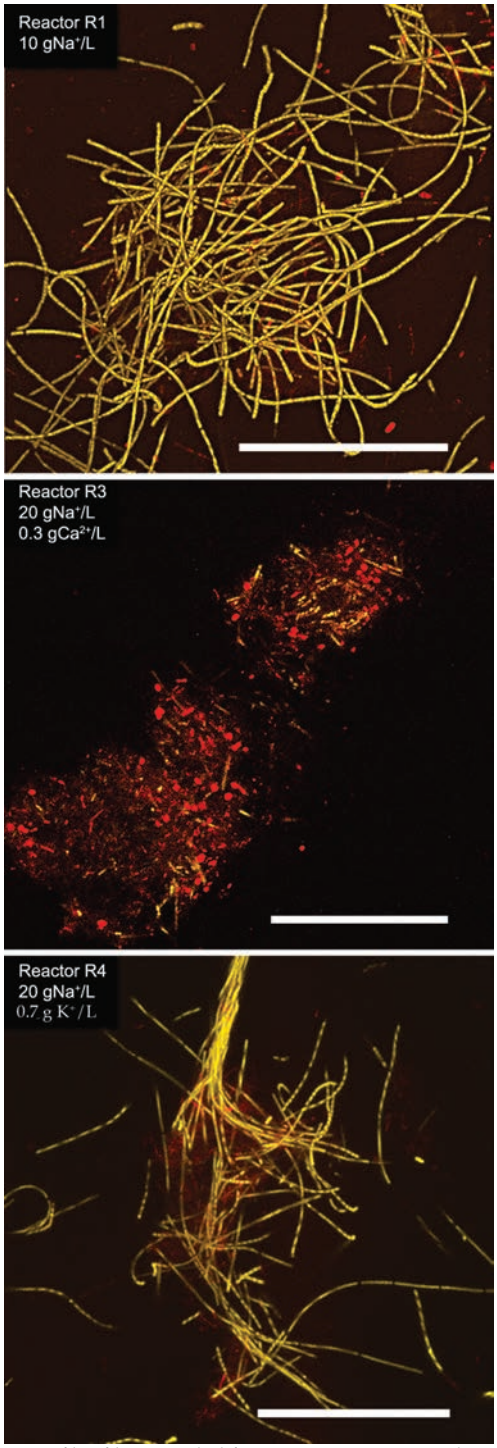


Figure 5 | FISH-CLSM images of biofilm sampled from reactors R1, R3 and R4 after 40 days of operation. The samples were simultaneously hybridized with the universal bacterial probe (EUB338-Cy3, red) and Archaea probe (ARC915-Cy5, yellow-orange). White bar = 50 μm.

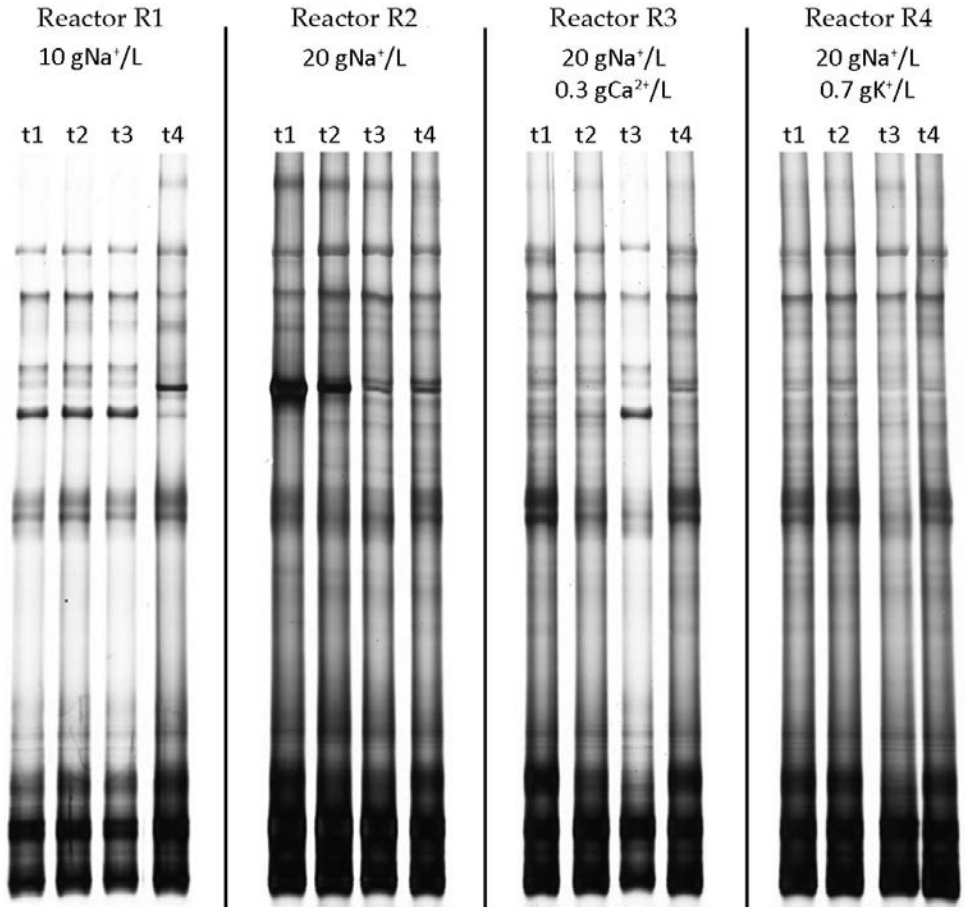


Figure 6 | DGGE band patterns showing changes in the Archaea 16S rRNA gene amplicons in the biofilm sampled from reactor R1 to R4 after 0, 40, 94 and 175 days.

In all four reactors the Bacterial population seemed to be very stable (data not shown), but the DNA yield was very low and the band patterns were too vague to allow for a more detailed interpretation.

To represent all biodiversity observed in the DGGE analyses, samples taken after 40 and 94 days from reactors R1 (10 g Na^+/L) and reactor R3 (20 g Na^+/L and 0.3 g Ca^{2+}/L) were prepared for sequencing. All sequenced clones, with their closest relative and closest cultured relative and GenBank accession number are listed in Table 2. Three types of Archaea were identified, which were closely related to *Methanosaeta harundinacea* (96 – 98 % similarity), *Methanolinea tarda* (96 % similarity), and *Methanobacterium subterraneum* (99 % similarity). Table 2 also shows that approximately 80 % (26/31) of the archaeal clones from reactor R1 after 40 days and 84 % (16/19) from reactor R3 after 94 days were most closely related to *Methanosaeta harundinacea*. *Methanolinea tarda* was found in both samples and *Methanobacterium subterraneum* was only detected in reactor R3 after 94 days.

Sequence clones of the bacterial 16S rRNA showed a very high inconsistency in bacterial di-

versity (data not shown). Interestingly, in none of the four reactors known acetate-oxidizing bacteria were detected.

5.4 Discussion

Our results clearly showed that it is possible to obtain anaerobic biofilms at least up to a salinity of 20 g Na^+/L, even with a simple organic substrate such as acetate. As such, our results are in line with findings that anaerobic biofilms occur in marine ecosystems (Briggs et al., 2011), i.e. typically at sodium concentrations of 10 – 15 g Na^+/L.

Table 2 | Archaeal clones sequenced and their similarity with the closest NCBI Genbank relative (uncultured and cultured). Sample R1T2 was taken from reactor R1 after 40 days and sample R3T3 was taken from reactor R3 after 94 days.

Reactor R1T2

Clone no.	Closest relative	Similarity (%)	Accession No.	Closest cultured relative	Similarity (%)	Accession No.
9	Uncultured *Methanosaeta* clone MOB7-4	97 - 99	DQ841239	*Methanosaeta harundinacea*	96 - 98	NR_102896
7	*Methanosaeta* clone D003011I03	92 - 98	EU721747	*Methanosaeta harundinacea*	92 - 96	NR_102896
3	Uncultured *Methanolinea* clone	99	AB266915	*Methanolinea tarda*	96	NR_028163
Total = 19 clones						

Reactor R3T3

Clone no.	Closest relative	Similarity (%)	Accession No.	Closest cultured relative	Similarity (%)	Accession No.
15	Uncultured *Methanosaeta* clone MOB7-4	98 - 99	DQ841239	*Methanosaeta harundinacea*	98	DQ841239
11	*Methanosaeta* clone D003011I03	96 - 99	EU721747	*Methanosaeta harundinacea*	96 - 98	DQ841239
3	*Methanobacterium subterraneum*	99	NR_028247	*Methanobacterium subterraneum*	99	NR_028247
2	Uncultured *Methanolinea* clone	99	AB266915	*Methanolinea tarda*	96	NR_028163
Total = 31 clones						

5.4.1 Effect of salinity and cations on acetate removal and biofilm formation

Acetate removal at 20 g Na^+/L (reactor R2) was a factor 2 – 4 slower than at 10 g Na^+/L (reactor R1). However, the results in Chapter 2 did not reveal a detrimental effect of sodium up to 15 g Na^+/L on the SMA of the same inoculum as used in this study. Using methanogenic sludge adapted to high salinity from the same full scale reactor, Jeison et al. (2008) reported the highest SMA at 5 g Na^+/L with a steady drop in activity at increased Na^+ concentrations up to 30 g Na^+/L. In their studies, the SMA drop between 10 and 20 g Na^+/L was about 35 %. Possibly, the higher sensitivity for sodium of the biomass in reactor R2 might be attributed to medium composition rather than to the direct inhibitory impact of sodium on the methanogens.

Addition of potassium at a concentration of 0.7 g K^+/L (reactor R4) clearly alleviated the negative effect of sodium at 20 g Na^+/L. Acetate removal rates only were slightly lower compared to 10 g Na^+/L (reactor R1) and were much more in agreement with the SMA/sodium-dose response curves as published by Jeison et al. (2008). This positive effect of potassium agrees with Roberts et al. (2005) who explained that the primary response of methanogens to counteract elevated salinity is to increase their internal levels of glutamate and potassium. For example, within 30 minutes of switching *Methanococcus thermolithotrophicus* cells from 15 to 32 g Na^+/L higher internal potassium and glutamate concentrations were observed whereas

later on, these concentrations decreased while a zwitterion was synthesized as a compatible solute (Martin et al., 2000).

In all three reactors mentioned above (reactors R1, R2 and R4), i.e at 10 as well as at 20 g Na^+/L, biofilm formation was observed by SEM and FISH-CLSM. No significant visible differences were observed in microbial coverage of the non-woven fabric. Although biofilm formation was not further quantified, this implies that 20 g Na^+/L did not prevent (initial) microbial attachment under anaerobic conditions. However, it remains unclear whether this result can be extrapolated to other types of inocula, different (more complex) organic substrates and sodium concentrations exceeding 20 g Na^+/L.

Surprisingly, acetate removal rates were extremely low in the presence of 0.3 g Ca^{2+}/L (reactor R3). SEM and FISH-CLSM images and DNA extraction data indicated that also coverage of the non-woven carrier material with methanogens in this reactor was much lower than in the other reactors. No clear explanation is available for this. In the literature conflicting results about the effect of calcium can be found (Chen et al., 2008). Low calcium concentrations of 0.1 – 0.2 g/L were reported to stimulate granulation and biofilm formation, whereas concentrations above 0.3 g/L on some occasions were reported to be detrimental. However, in this study the calcium to biomass ratio (150 mg Ca^{2+}/g VSS) may have been too high, and precipitated calcium may have "shielded" the methanogens present in the crushed granules that were used as the inoculum, preventing mass transfer leading to low an overall lower acetate conversion rate and preventing attachment to the non-woven carrier material. It must be noted that at micro-level, the catabolic production of bicarbonate during methanogenesis may have resulted in a pH increase in the direct vicinity of the methanogens, and thus, easily trespassing the saturation level for calcium carbonate precipitation.

5.4.2 Microbial community of the biofilms

Acetate was used as the sole organic substrate in this study. Methanogenic acetate degradation can proceed either via direct conversion by acetoclastic methanogens, or via syntrophic conversion performed by an anaerobic acetate-oxidizing bacterium combined with a hydrogenotrophic methanogen (Schnürer and Nordberg, 2008). In this study both acetoclastic and hydrogenotrophic methanogens were identified in the biofilms during the anaerobic conversion of acetate to methane at high salinity levels.

Relatives of *Methanosaeta harundinacea* were identified as the dominant acetoclastic methanogens in the anaerobic biofilms that were formed at 10 and 20 g Na^+/L (reactors R1 and R3) (Table 2). The other acetoclastic methanogen, *Methanosarcina* sp., was not identified. According to Collins et al. (2005) the filamentous structure of *Methanosaeta* sp. is an important factor for anaerobic granulation process and perhaps also for biofilm formation.

Although acetate was the sole organic substrate, a fraction of the clones showed high similarity with *Methanolinea tarda* (16 % of the clones in reactor R1, and 6.4 % in reactor R3, Table 2) and *Methanobacterium subterranum* (0 % in reactor R1 and 10 % in reactor R3, Table 2). Both methanogens are known to utilize H_2/CO_2 or formate as their growth substrates. *Methanolinea tarda* was isolated from anaerobically digested sludge (Imachi et al., 2008) and recently was reported to be present in an anaerobic hybrid reactor (Lykidis et al., 2011). *Methanolinea tarda*

was also found in oil sand tailings ponds as discussed by Ramos-Padron et al. (2011). *Methanobacterium subterranum* was isolated from deep granitic groundwater (Kotelnikova et al., 1998) and is a halotolerant strain tolerating salinity levels of 2 – 28 g Na^+/L. *M. subterraneum* has also been found to be dominantly present in anaerobic methanogenic digesters with high microbial diversity (Leclerc et al., 2004). The usual role of hydrogenotrophic methanogens in anaerobic biodegradation is to scavenge protons and electrons produced during the conversion of complex carbon containing wastes. However, in none of our reactors known acetate-oxidizing bacteria were found. Therefore, it is assumed that H_2 leakage by the acetoclastic methanogens explains their presence in the reactors. Valentine et al. (2000) reported that several methanogens, including *Methanosaeta*, produce excess reducing equivalents during active growth and convert them to H_2, which is excreted. A similar process may have occurred enabling growth of hydrogenotrophic methanogens in our reactors. In addition, H_2 may have been produced by bacteria remaining from the inoculum and sponging on dead biomass.

5.5 Conclusions

The following conclusions can be drawn from this research.

- Anaerobic biofilm formation in the presence of high Na^+ concentrations (i.e. 10 and 20 g Na^+/L) is possible, even with a simple organic substrate such as acetate.
- Potassium, at a concentration of 0.7 g/L (350 mg K^+/g VSS), alleviated the negative effect of 20 g Na^+/L on the specific methanogenic activity.
- Calcium, at a concentration of 0.3 g/L (150 mg Ca^{2+}/g VSS), had a negative effect on biomass activity and on the formation of an anaerobic biofilm under saline conditions.
- The archaeal microbial population was relatively stable in time and no significant differences were observed due to salinity level and the presence of potassium or calcium.
- The acetoclastic methanogen *Methanosaeta harundinicea* dominated the biofilm in all reactors but also a fraction of the archaeal population consisted of hydrogenotrophic methanogens, even though syntrophic acetate oxidizing bacteria were not detected.

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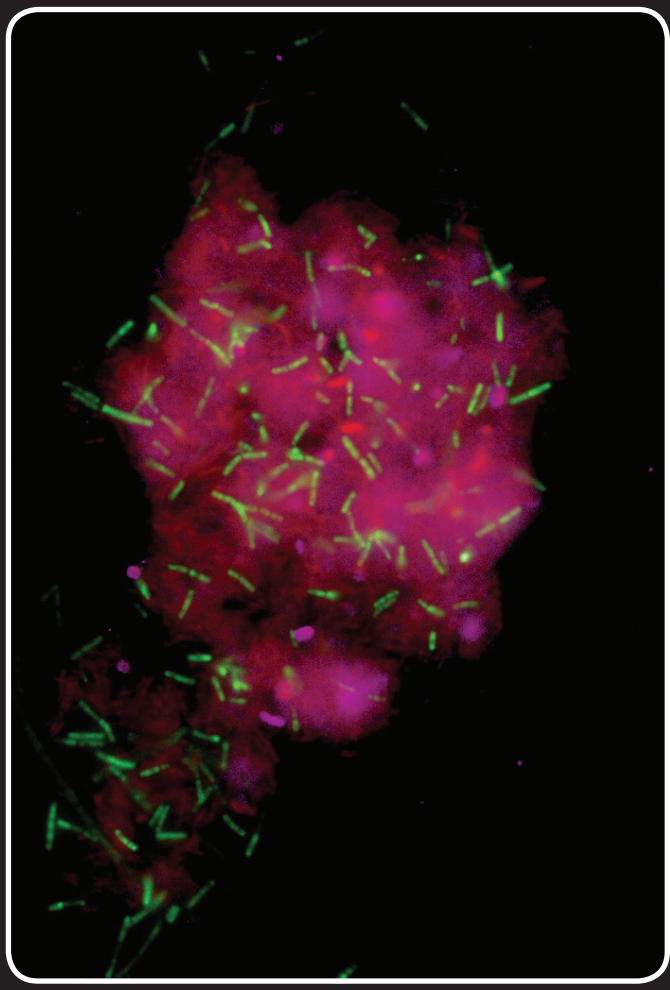
Chapter 6



Anaerobic Granule Properties at High Salinity

"Di mana bumi dipijak, di sana langit di junjung", Where the land is stepped, there the sky is upheld.
Indonesian proverb

Abstract – Four laboratory scale upflow anaerobic sludge blanket (UASB) reactors were operated at a high salinity of 20 g Na^+/L for a period of 120 days and at gradually increasing organic loading rates. The reactors were fed with different types of substrate (fully acidified or partially acidified). Two of the reactors were supplied with calcium at a concentration of 0.3 g/L, and all the reactors received potassium at a concentration of 0.7 g/L. Dissolved chemical oxygen demand (COD) removal efficiency of the reactors, physicochemical granule properties and microbial populations were characterized and compared. The results showed that high COD removal efficiencies are possible at 20 g Na^+/L, up to an organic loading rate of at least 14 g COD/L.d. At a loading rate of 25 g COD/L.d the performance and stability of all reactors deteriorated. There were indications that calcium augmentation had a positive effect on biomass retention, but this could not be further quantified. The archaeal population was dominated by the acetoclastic methanogen, *Methanosaeta harundinacea*. The bacterial population, in particular in the reactors fed with partially acidified substrate, was much more diverse. No effect of calcium on the archaeal and bacterial population was observed.
Keywords: Acetoclastic methanogen, anaerobic granule, denaturing gradient gel electrophoresis (DGGE), upflow anaerobic sludge blanket (UASB) reactor.

6.1 Introduction

Closure of industrial water loops has become a very important issue because it decreases the consumption of scarce fresh water resources. However, this can only be accomplished with wastewater treatment processes that can cope with extreme environmental conditions with respect to pH, temperature and salinity (de Faria et al., 2009). Saline wastewaters containing large amounts of soluble inorganic salts and organic compounds are generated during several production processes, including fish and seafood processing industries, tanning and petrochemical industries. Regulations regarding the discharge of these wastewaters are becoming more stringent and treatment, therefore, is becoming a major concern for regulators, engineers and researchers (Xiao and Roberts, 2010).

For non-saline wastewaters containing high concentrations of organic pollutants anaerobic treatment is an established technology. Major advantages of anaerobic treatment include, the avoidance (or vast reduction) of fossil fuel for treatment, low or negligible amounts of stabilised excess sludge production, conversion of organic pollutants into biogas, from which energy (heat and electricity) can be produced (van Lier, 2008). In particular when it is possible to grow anaerobic granules, modern high rate reactors can be constructed. Practical applications of anaerobic granular sludge technology do not yet exist for wastewaters with a salinity exceeding 10 – 15 g Na^+/L. This study therefore focuses on anaerobic treatment of wastewaters containing high sodium levels (20 g Na^+/L), with emphasis on the granule properties under such conditions.

High sodium concentrations in wastewaters induce salt stress to microbial species, resulting in the inhibition of enzymes, a decrease in cell activity and eventually leading to plasmolysis (Uygur, 2006, Rene et al., 2008). Salt stress therefore can seriously hamper the treatment performance of anaerobic systems (Rinzema et al., 1988). This negative effect of high sodium concentrations on anaerobic conversion rates (partially) can be overcome by adding potassium to the substrate, as was demonstrated in Chapter 5. Other strategies also may be possible, including the use of halotolerant inocula (e.g Riffat and Krongthamchat, 2007), addition of (organic) compatible solutes to the reactor medium (e.g. Vyrides et al, 2010) or the use of bioelectrochemical methods, that combine an electric field with the biological process to enhance COD removal at high salinity (Zhang et al., 2012).

High sodium concentrations not only inhibit the microbiological conversion processes, but also have a negative effect on the formation and stability of anaerobic granular sludge. In previous chapters we reported that at sodium concentrations of 10 – 20 g Na^+/L calcium started leaching from anaerobic granules and these granules became more vulnerable to shear. As a consequence valuable, slow growing biomass can wash out of the bioreactor (Mendez et al., 1995). This, together with sodium toxicity, will cause a deteriorating treatment performance. Calcium augmentation therefore may present a solution to sustain the strength of anaerobic granules when exposed to higher sodium concentrations (Chapters 3 and 4).

Formation of (anaerobic) granules and biofilms is a complex process involving numerous physicochemical and biological interactions. Although many hypotheses are available to explain formation of biofilms and granules, most authors state that the microorganisms are held together by a matrix of extracellular polymers (EPS), predominantly consisting of polysac-

charides, proteins and glycoproteins (e.g. Liu et al., 2011; Jiao et al., 2010). Multivalent cations such as Ca^{2+}, Mg^{2+} and Fe^{3+} form bridges between these negatively charged polymers, and in this manner maintain the structure of the microbial aggregates. With saline wastewaters, containing high monovalent ion concentrations such as Na^{+}, formation of granules or a biofilm is more difficult. At high mono- to multivalent cation ratio's the multivalent cations in the polymeric matrix are replaced by monovalent ions (Bruss et al., 1992). This weakens granule and biofilm structures and makes them more prone to shear. Cammarota and Sant'Anna (1998) and Yang et al. (2004) showed that when microbial EPS production is blocked, microbial aggregation does not occur, demonstrating the crucial role of EPS for granule and biofilm formation. Studies in marine habitats have shown that the role of EPS indeed is very important, but also that anaerobic biofilm formation is possible under these conditions (Decho, 2000; Bhaskar and Bhosle, 2005). Indirectly, the role of EPS production was also demonstrated by several observations that the presence of sufficient amounts of "high-energy" substrates, such as carbohydrates, is essential for EPS production and granule formation (e.g. Thaveesi et al., 1995; Schmidt and Ahring, 1996). This is supported by other studies reporting that with fully acidified substrates granules took much longer to mature and were more fragile and less stable during storage (Grotenhuis et al., 1992; Imai et al., 1997).

In this study four identical up-flow anaerobic sludge blanket (UASB) reactors treating concentrated wastewaters (10 – 30 g COD/L) were operated at 20 g Na^{+}/L. To compare the effect of different organic substrates on granule activity, stability and growth, two reactors were fed with partially acidified substrate (PAS), consisting of a mixture of acetate, gelatine and starch, while the other two reactors were fed with acetate, i.e. a fully acidified substrate (FAS). The effect of calcium augmentation on anaerobic granules properties was studied by feeding two of the reactors with additional calcium at a concentration of 0.3 g Ca^{2+}/L (15.2 mg Ca^{2+}/ g of initial inoculum total suspended solids [TSS] per day). In Chapter 5, a beneficial effect of potassium was demonstrated and it therefore was added to all the reactors, at a concentration of 0.7 g K^{+}/L (16.2 mg/g of initial inoculum TSS) to obtain a K^{+}/Na^{+} ratio similar to seawater. The treatment performance of the reactors was compared during a period of 120 days at increasing volumetric organic loading rates (OLRs). Physicochemical and microbiological properties of the anaerobic granules were determined and will be discussed.

6.2 Material and Methods

6.2.1 Source of inocula

The reactors were inoculated with granular sludge from a full-scale UASB reactor treating wastewater from a styrene and propene-oxide production plant of Shell, Moerdijk, the Netherlands. This wastewater contained a sodium concentration of 10 – 15 g/L, with acetic acid and benzoic acids as the main sources of chemical oxygen demand (COD). The sludge, with a volatile fraction of 32 %, had already adapted to a salinity of 10 – 15 g Na^{+}/L for more than 10 years (Biothane–Veolia, personal communication).

6.2.2 UASB reactor operation

The experiments were performed in a temperature controlled room at 30 ± 2 ^{0}C, using four identical glass UASB reactors (R1, R2, R3 and R4) with a working volume of 3 L each. The re-

actors were equipped with a reversed funnel phase separator. The produced biogas was guided through a water lock and monitored by a wet-test gas meter (Schlumberger, Dordrecht, the Netherlands). Biogas production rate was, however, not recorded during the experiments. The media were prepared in demineralised water. Feed characteristics for the reactors are given in Table 1.

The up-flow liquid velocity was controlled by liquid recirculation and was fixed at 0.8 m/h with a peristaltic pump (Watson Marlow 202, UK). The reactors were inoculated with 130 g of total suspended solids (TSS), with an organic content of 41 g of volatile suspended solids (VSS). The hydraulic retention time (HRT) in the reactors was 1.34 days for the entire experimental period. The OLR was gradually increased from 10 to 24 g COD/L.d by feeding the reactors with synthetic wastewater containing 13 – 32 g COD/L using a peristaltic pump (Watson Marlow 202, UK). The synthetic wastewater consisted of sodium chloride at a concentration of 20 g Na^+/L (Merck, Germany) and macro and micronutrients prepared according to (Vallero et al., 2002). Reactors R1 and R3 were fed with fully acidified substrate (FAS) consisting of acetate ($NaC_2H_3O_2.3H_2O$) as the sole growth substrate. Reactors R2 and R4 were fed with partially acidified substrate (PAS) consisting of acetate, gelatine and starch in a 7:2:1 COD ratio. The background calcium and potassium concentration in the feed were 5 and 55 mg/L, respectively. To stimulate granulation, additional calcium ($CaCl_2.2H_2O$) was added to reactors R3 and R4 at a concentration of 0.3 g Ca^{2+}/L or a load of 15.2 mg Ca^{2+}/ g of inoculum TSS.day. In all the reactors the Na^+/K^+ ratio was adjusted to 0.037, similar to seawater, to alleviate sodium toxicity, i.e. at a concentration of 0.7 g K^+/L or 16.2 mg/g of inoculum TSS (KCl).

Table 1 | Feed characteristics for the UASB reactors (FAS = Fully Acidified Substrate = acetate; PAS = Partially Acidified Substrate = acetate:gelatine:starch in a 7:2:1 COD ratio; background calcium and potassium concentrations were 5 mg Ca^{2+}/L and 55 mg K^+/L, respectively).

	Reactors			
	R1	R2	R3	R4
Sodium concentration (g/L)	20	20	20	20
Additional potassium concentration (g/L)	0.7	0.7	0.7	0.7
Additional calcium concentration (g/L)	0	0	0.3	0.3
Type of organic substrate	FAS	PAS	FAS	PAS

6.2.3 Granule properties

Specific methanogenic activity (SMA)

SMA tests were performed in duplicate with 1.8 g of dry solids of reactor sludge in 120 ml glass serum bottles at sodium and potassium concentrations of 20 g Na^+/L and 0.7 g K^+/L, respectively. Anaerobic conditions were obtained by flushing the headspace for 10 seconds with N_2 gas. Acetate, at a concentration of 1.2 g COD/L, was used as substrate, together with 0.7 g K^+/L and 360 ml/L macro nutrients solution (170 g/L NH_4Cl, 8 g/L $CaCl_2.2H_2O$ and 9 g/L $MgSO_4.7H_2O$) and 36 mL/L trace elements solution (2 g/L $FeCl_3.4H_2O$, 2 g/L $CoCl_2.6H_2O$, 0.5 g/L $MnCl_2.4H_2O$, 30 mg/L $CuCl_2.2H_2O$, 50 mg/L $ZnCl_2$, 50 mg/L HBO_3, 90 mg/L $(NH_4)6Mo_7O_{24}.4H_2O$, 100 mg/L $Na_2SeO_3.5H_2O$, 50 mg/L $NiCl_2.6H_2O$, 1 g/L EDTA, 1mL/L 36 % HCl and 0.5 g/L resazurin). In several SMA tests the pH was measured at the start and at the end of the tests and was in a range of 7.6 – 9.0. The serum bottles were incubated at

30 ± 2 ^{0}C on a platform shaker operated at 120 rpm (C10 Edison, NJ, USA). The pressure was measured manually at regular intervals with a Greisinger GMH 3150 Digital Pressure Meter (Greisinger electronic, Germany).

Sampling of sludge blanket solids and fines

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

Particle size distribution

Particle size distributions of sludge blanket solids and fines (see above) were determined for each reactor. Particle size distribution was determined by light scattering using a Mastersizer 2000 (Malvern, UK). Each sample was measured in triplicate and size distributions based on particle volume were calculated by instrument software.

Extracellular polymeric substances (EPS)

EPS extraction was conducted by cation exchange resin (CER) (Dowex Marathon C, 20 – 50 mesh, sodium form, Fluka 91973) as described by Frølund et al. (1996). UASB sludge samples were harvested by centrifugation at 4500 rpm for 15 min (IEC Microlite, USA). The pellets were washed twice with a 100 mM NaCl solution. The sludge pellets were subsequently re-suspended in NaCl solution and the solution was transferred to extraction bottles, followed by the CER addition with a dosage of 70 g per suspended solids. These suspensions were stirred at 450 rpm and 4 ^{0}C. Extraction was stopped after 107 hours, a period which was previously determined to result in stable EPS concentrations. After removing settled CER, the solutions were centrifuged at 10000 rpm and 4 ^{0}C for 30 min to remove remaining sludge components. The supernatants were filtered through 0.45 μm cellulose membranes and used to determine protein and carbohydrate content.

Carbohydrate concentrations were measured according to Dubois et al. (1956). Samples of 0.4 mL extracted EPS were added to test tubes and then mixed with 0.4 mL of 5 % (w/w) phenol (Sigma, UK). Two mL of H_2SO_4 (reagent grade, 95 %) was added to each test tube and left at room temperature (18 – 26 ^{0}C) for 10 minutes. The content of each tube was transferred to cuvettes and the samples were analysed on a Jenway UV/VIS spectrophotometer (Model 6505 S) against the blank at a wavelength of 480 nm. Carbohydrate concentration (in mg/L) was determined from the calibration curve obtained with glucose standards.

Protein was measured with a diagnostic kit purchased from Sigma, England (Kit no. 690). Samples were diluted with sodium chloride solution (0.85 %) such that the final protein concentration was between 150 and 1000 mg/L. Diluted samples of 0.2 mL EPS extraction fluid with 2.2 mL Biuret reagent were added to each tube and mixed well. Samples were kept at room temperature (18 – 26 ^{0}C) for 10 minutes. 0.1 mL of Folin and Ciocalteu's Phenol reagent was added to each tube and mixed each tube well after addition. These samples were left at room temperature for 30 minutes. The content of each tube was transferred to cuvettes and the samples were analysed on a Jenway UV/VIS spectrophotometer (Model 6505 S) against

the blank at a wavelength of 595 nm. Protein concentrations were determined from a calibration curve obtained with bovine serum albumin (BSA) standards.

Scanning electron microscopy

Granules were examined by scanning electron microscopy (SEM) coupled to either a back-scattered electron (BSE) detector or an energy dispersive X-ray (EDX) detector to determine surface morphology and the element distribution, respectively. Granules were fixed in culture medium by adding glutaraldehyde in growing medium, resulting in a 2.5 % (w/v) glutaraldehyde concentration. After 1 hour of fixation the granules were rinsed with water and subsequently dehydrated in a graded series of acetone (10, 30, 50, 70, and 2x 100 %, 15 minutes per step). The granules were critical point dried with carbon dioxide (CPD 030 BalTec, Liechtenstein). Granules were glued on a sample holder by carbon adhesive tabs (EMS Washington USA) or with carbon glue (Leit- C, Neubauer Chemicalien, Germany) and sputter coated with 2 – 4 nm Tungsten in a MED 020 (Leica, Vienna, Austria). The samples were analysed with a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands) at room temperature at a working distance of 4 mm with SE detection at 2 kV. Images were digitally recorded and were optimized and resized with Adobe Photoshop CS (Adobe Systems Inc., USA).

Fluorescence in-situ hybridization (FISH)

FISH was used to visualize and characterize the presence of Bacteria and Archaea in fines. Two 16S rRNA targeted oligonucleotide probes with different fluorescent dyes, were used for dual hybridization of the fines. For Bacteria EUB338 (Eubacterial domain probe, 5′ ACT CCT ACG GGA GGC AG 3′) with Cy3 (red) and for Archaea ARC915 (Archaea-domain probe, 5′ GTG CTC CCC CGC CAA TTC CT 3′) with Cy5 (yellow-orange) were applied (Eurogentec, Belgium). Samples were fixed for 3 hours at 4 ^{0}C with 4 % (w/v) formaldehyde, centrifuged (12000 rpm, 10 minutes) and washed twice with Phosphate Buffered Saline (PBS) and finally stored in 98 % ethanol at -20 ^{0}C until further processing. Stored samples were thawed and diluted in 1 x PBS and homogenized for 5 minutes in a homogenizer (RW 20 DZM; Janke & Kunkel, Staufen, Germany). Ten-fold dilution series of samples were prepared in order to determine the optimal cell concentration for counting with the different probes. 10 µl of the fixed sample was spotted on the well of a gelatine coated slide (8 mm well, 10 well Multitest slide, MP Biomedicals) and dried for 10 min at 46 ^{0}C. The cells were dehydrated for 2 to 3 min in a graded ethanol series with the ethanol concentration increasing from 50 to 80 % and finally in 96 % ethanol. 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 0.1 % [wt/vol] sodium dodecyl sulfate [SDS]) was added to each well, together with 1 µl of each probe (50 ng/µl). This was followed by incubation at 46 ^{0}C for 2 – 3 h. After hybridization the slides were washed in 50 ml of pre-warmed (48 ^{0}C) washing buffer (0.225 mM NaCl, 20 % formamide hybridization buffer [v/v], 2250 µl NaCl [5 M] and 500 µl EDTA [0.5 M]) with SDS for 10 min. For total counts 4′, 6-diamidino-2-phenylindole (DAPI) was added to the washing buffer at a final concentration of 100 ng/ml. After the slides were rinsed in water, they were immediately air dried, mounted with Vectashield (Vector Labs, Burlingame, USA) and covered with a cover slide (42 x 60mm, Menzel-Glaser, Germany). Finally, slides were examined under a Zeiss Axioplan 2 Imaging Photomicroscope equipped with epifluorescence illumination and filter sets for Cy3, and Cy5. The images were captured by a Photometrics

Sensys 1,305 x 1,024 pixel CCD camera and analysed with the Genus Image Analysis software (Applied Imaging Corporation). The fluorescence images were yellow-orange (Cy5) and red (Cy3), and merged in a multichannel mode. Image optimization was conducted using Adobe Photoshop CS (Adobe Systems Inc., USA).

Lectin staining

To identify glycoconjugates, important carbohydrate groups in EPS, lectin staining with FITC–labelled lectin stain (10 μg/ml for 2 minutes staining time) (Sigma Aldrich, UK) was conducted on fines according to a method by Neu et al. (2001). The samples were subsequently examined with a procedure already described for the FISH analyses (see above).

6.2.4 Microbial analysis

DNA extraction, amplification and DGGE

Granules for microbial population analysis were sampled from each reactor on day 60, 90, and 120. The samples were centrifuged for 1 min at 14 000 g. The cell pellets, containing the biomass, were stored in RNAlater® preservation Solution (Applied Biosystems/Ambion, Austin, USA) at –20 ^{0}C. Total genomic DNA was extracted from samples using a FastDNA® SPIN kit for soil (Qbiogene, Calsbad, CA). DNA concentration and integrity were measured with the NanoDrop® spectrophotometer. For denaturing gradient gel electrophoresis (DGGE) analysis, PCR was performed as described by Sousa et al. (2007). Archaeal 16S rRNA fragments were amplified using archaeon–specific primer with primers A109(T)–f (5′-ACT GCT CAG TAA CAC GT-3′) and universal reverse primer 515–r (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC GCG GCT GCT GGC AC-3′, with GC clamps). Bacterial 16S rRNA fragments were amplified using bacterial–specific primer with primers U968–f (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′, with GC clamps) and universal reverse primer L1401–r (5′-CGG TGT GTA CAA GAC CC-3′). All primers used were synthesized commercially by Biolegio (Nijmegen, the Netherlands). Amplicons were subsequently separated by DGGE as described by Zoetendal et al. (2001) using the D–code system (Bio-Rad, Hercules, CA) with 8 % (v/v) polyacrylamide gels having a denaturant gradient of 30 – 60 %.

Cloning and sequencing

PAS grown granules sampled from reactor R2 after 120 days were selected to make bacterial 16S rRNA and archaeal 16S rRNA gene clone libraries. The selected sample represented the Bacterial and Archaeal population for all reactors. For cloning, full-length bacterial and archaea 16S rRNA genes were amplified in a PCR with forward primers, Bact–27–f (5′-AGA GTT TGG ATC MTG GC TCA G-3′) and A109–f (5′-ACT GCT CAG TAA CAC GT-3′), respectively, and the universal primer Uni1492–r (5′-CGG TTA CCT TGT TAC GAC TT-3′) and the thermocycling conditions as described by Sousa et al. (2007).

16S rRNA gene amplicons integrity and length was checked on an agarosegel and subsequently purified with high pure PCR cleanup micro kit (Roche, Mannheim, Germany). Ligation of the PCR products into the vectors was performed with the use of a pGEM®-T Easy

Vector System II kit (Promega, Madison, WI, U.S.A.). 5 µl PCR products were added to a mixture of 5 µl 2X Rapid Ligation Buffer, 1 µl pGEM easy vector, 1 µl of T4 DNA ligase and 3 µl PCR water. A positive control was performed by adding 2 µl control insert DNA and 1 µl extra PCR water instead of the PCR product. A negative control was performed by adding 3 µl extra PCR water instead of the PCR product and incubated overnight at 4 ^{0}C. Plasmids were transformed into *E. coli*, 2 µl of each ligation product was added in a sterile tube, together with 50 µl of XL-1 Blue Competent Cells (Stratagene, La Jolla, CA, USA) according to the manufacturers protocols. Cells in SOC medium were diluted 10x, 20x, 100x and 500x. 100 µl of the cell suspensions were streaked in duplo on a LB agar plate plus ampicillin (100 mg/L) and incubated overnight at 37 ^{0}C.

Visible colonies were placed at 4 ^{0}C for 1 – 2 hours to enhance the blue colour of cells for blue/white screening. White cells likely have taken up a plasmid with PCR products while blue cells have plasmids without PCR product. Two x 96 white colonies were randomly selected with a toothpick and transferred to a 96 well Masterblock® plate (Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands), each well filled with 200µl LB medium + 100 mg/L ampicillin for both archaea and bacteria. The plates were covered with adhesive gas permeable seals (ABgene® Ltd., Surrey, UK) and incubated overnight at 37 ^{0}C. Nearly full-length 16S rRNA genes were sequenced by GATC Biotech (Konstanz, Germany). The analyzed 16S rRNA sequences were compared with sequences in the GenBank database using the NCBI Blast search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Zhang and Schwartz, 2000). Closest relative and cultured relatives were retrieved from database, with the degree of similarity.

6.2.5 Other analyses

Volatile fatty acids (VFAs) and biogas composition in SMA tests were determined by gas chromatography as described previously (Chapter 5). Granules from each reactor were investigated using a SMZ800 (Nikon, Melville, USA) stereomicroscope, fitted with a Nikon camera (Nikon Coolpix5000).

Dissolved calcium concentrations in reactor effluents were determined in grab samples after 10 minutes centrifugation at 10000 rpm. Calcium concentrations were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES; Varian, Australia), as described in detail (Chapter 2). Ultra-pure water (Milli-Ro System, Millipore, Bedford, MA, USA) was used to prepare standard solutions of all reagents, which were of suprapur quality (Merck, Darmstadt, Germany) and were checked for possible trace metal contamination. All glassware and plastic material used was treated for 12 h with 10 % (v/v) HNO_3 and rinsed vigorously with demineralised water. All other analyses were carried out according to Standard Methods for the Examination of Water and Wastewater (APHA, 1998).

6.3 Results

6.3.1 UASB reactor performance

Figure 1 shows influent dissolved COD, effluent dissolved COD concentrations, calculated from effluent volatile fatty acids (VFA), and the imposed OLR to the various reactors. Until day 60, at an OLR of 14 g COD/L.d, effluent COD concentrations of the FAS fed reactors R1

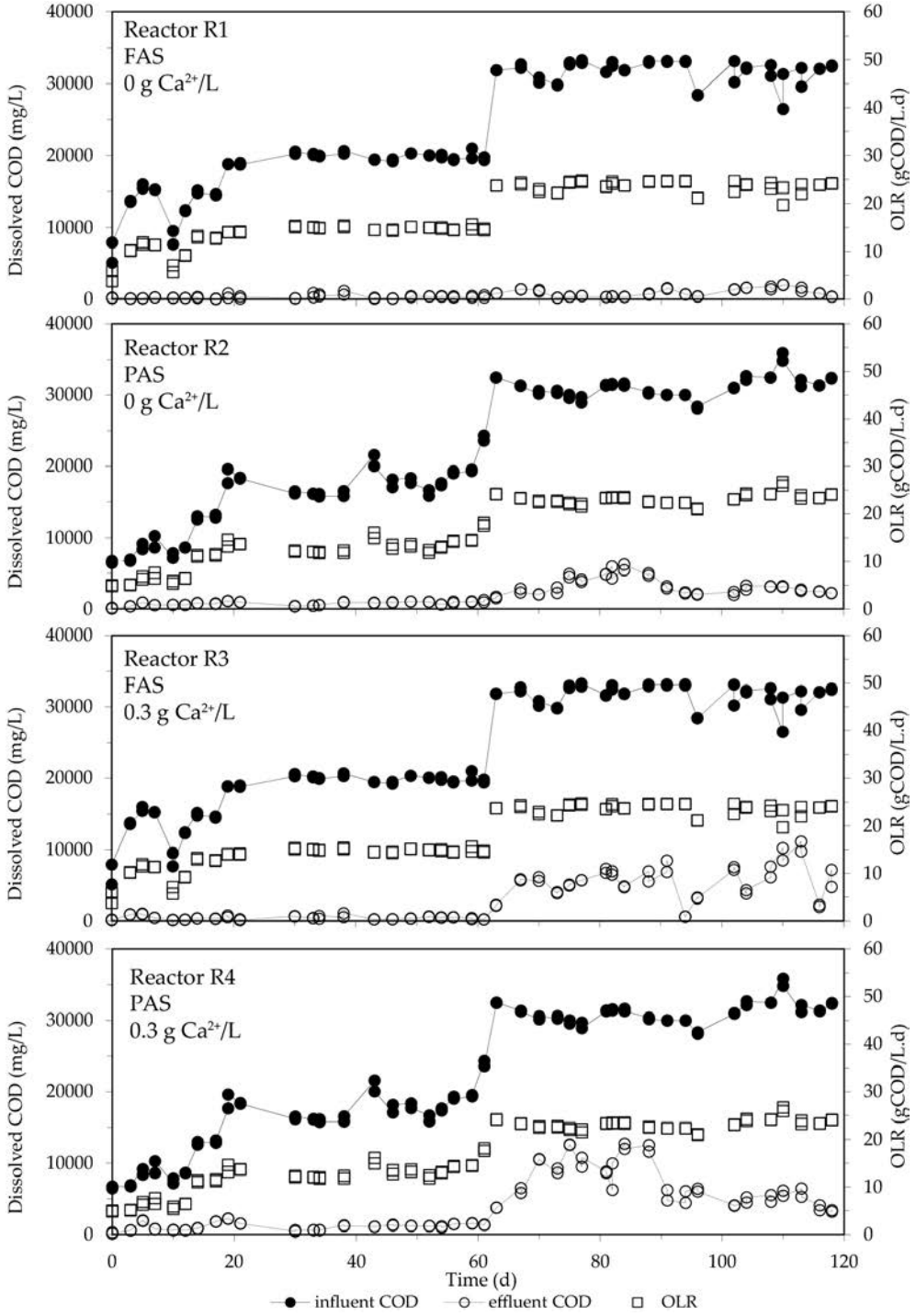


Figure 1 | Influent and effluent dissolved COD concentrations and volumetric organic loading rate (OLR) of the four UASB reactors (FAS = Fully Acidified Substrate, PAS = Partially Acidified Substrate; Influent COD was measured, effluent COD was calculated from VFA concentrations).

and R3 were very low and stable. Average dissolved COD removal efficiencies between days 50 and 60 were 98.2 ± 0.2 and 97.9 ± 0.8 % for reactors R1 and R3, respectively. During the same period, average dissolved COD removal efficiencies of the PAS fed reactors R2 (without extra calcium) and reactor R4 (extra calcium) were somewhat lower, i.e. 95.2 ± 0.9 and 93.2 ± 1.0 %, respectively. This difference in performance between the FAS and the PAS fed reactors could be attributed to the presence of propionate in the effluent of the PAS fed reactors (data not shown).

From day 60 onwards, an increase in the OLR to 25 g COD/L.d resulted in a lower and less stable dissolved COD removal efficiency in all four reactors. In the PAS fed reactors R2 and R4, effluent dissolved COD concentrations initially showed a high peak, but after this peak it gradually decreased towards the end of the operational period of 120 days. However, propionate concentrations in the effluent in both of these reactors (graphs not shown) increased to higher concentrations than at an OLR of 14 g COD/L.d, i.e. 1600 – 2000 mg COD/L at an OLR of 25 g COD/L.d compared to 700 – 1000 mg COD/L at an OLR of 14 g COD/L.d. This resulted in average dissolved COD removal efficiencies between days 110 and 120 of 92.4 ± 1.1 % in reactor R2 (without extra calcium) and 86.8 ± 4.1 % in reactor R4 (extra calcium). Remarkably, in the FAS fed reactor R1 (without extra calcium) the sudden increase in the OLR from 14 to 25 g COD/L.d only gave a small peak in effluent dissolved COD concentration, and the average removal efficiency between days 110 and 120 was very high (96.7 ± 2.4 %) compared to the other reactors. The FAS fed reactor R3 (extra calcium) exhibited the least stable performance of all reactors, with highly fluctuating effluent concentrations and a low average COD removal efficiency (78.5 ± 11.7 %) compared to the other reactors.

SMAs of the inoculum and of sludge blanket solids, with acetate as the sole organic substrate, were determined at 20 g Na^+/L and 0.7 g K^+/L after 60, 90 and 120 days (Figure 2). After 60 days the SMA of all reactor sludges had increased five to seven times compared to the SMA of the inoculum of 0.05 g COD/g VSS.d. Also in Chapter 2 a three times higher SMA was found for reactor sludges compared to the same inoculum. In those experiments the reactors were operated at a slightly lower OLR of 18 g COD/L.d, with a acetate:gelatine:ethanol substrate mixture (7:2:1 on COD basis), and lower sodium concentrations of 5 or 15 g Na^+/L. This huge SMA increase can be explained by enrichment of acetoclastic methanogens compared to other anaerobic microorganisms that were present in the inoculum and/or by long-term adaptation of the biomass to the higher sodium level of 20 g Na^+/L compared to the 10 – 15 g Na^+/L that the inoculum originally was exposed to. The SMAs of the FAS fed reactors R1 and R3 were significantly higher than the SMAs of the PAS fed reactors R2 and R4. This can be explained by the type of substrate that was used. With acetate as the sole carbon source in the FAS fed reactors R1 and R3, the biomass merely consisted of acetoclastic methanogens, whereas in PAS fed reactors R2 and R4 a large fraction of the biomass consisted of fermentative and acidifying bacteria. This was also confirmed by FISH images and microbial population data as will be discussed later on.

With the exception of the SMA of reactor R1 sludge after 90 days, the SMAs in all reactors increased between 60 and 120 days when the OLR was increased from 14 to 25 g COD/L.d. After 120 days, sludge blanket solids from reactors R1, R2 and R3 gave similar SMAs in a range of 0.15 – 0.18 g COD/g VSS.d, while the SMA of reactor R4 sludge (PAS and extra calcium) was considerably lower, i.e. 0.10 g COD/g VSS.d.

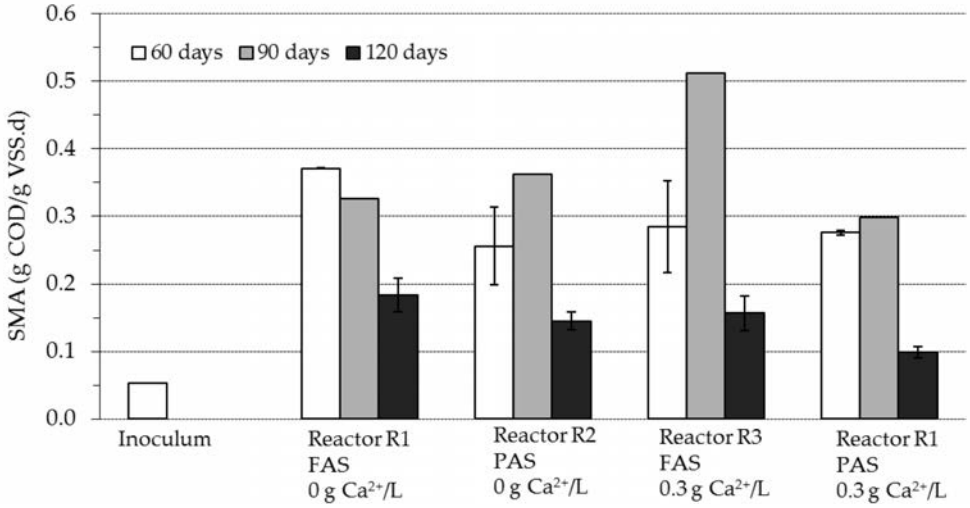


Figure 2 | Specific methanogenic activities (SMA) at 20 g Na^+/L and 0.7 g K^+/L, measured with acetate as substrate, of the inoculum and of sludge blanket solids sampled after 60, 90 and 120 days. Error bars represent differences in duplicate measurements.

6.3.2 Granule and fines properties

After 120 days, reactor solids were sampled at a height of 22 cm (liquid height was 67 cm). So called "fines" were determined as the solids in these samples that after 1 minute had not settled. Table 2 gives total and fines related solids concentrations, and the volatile fraction of these solids to allow a comparison between the different reactors. Since the sludge bed is not well mixed, the concentrations in Table 2 may not be representative for the overall solids concentrations in the reactors.

After 120 days, reactors R3 and R4, both fed with extra calcium but different types of organic substrate, contained the highest TSS concentrations (118 and 162 g/L, respectively). In total, 79.2 g of calcium were dosed to the system, calculated by using a total operational period of 120 days and a calcium load of 660 mg/day. However, the exact calcium retention could not be calculated using the operational data. Nonetheless, despite the imposed calcium dose, the volatile fraction of these solids (38 % for reactor R3 and 48 % for reactor R4) was higher than of the inoculum (32 %). Apparently, calcium was not merely retained as inorganic precipitate, such as $CaCO_3$, but had a role in actively retaining the newly grown biomass. The higher VSS concentration in the PAS fed reactor R4 compared to the FAS fed reactor R3 most likely can be explained by a higher microbial growth yield on PAS than on FAS (e.g. Batstone et al., 2000). Possibly, the lower VSS/TSS ratio in reactor R3 is attributable to increased calcium precipitation in reactor R3 compared to reactor R4. TSS concentration in reactors R1 and R2, which did not receive extra calcium, were much lower than in reactors R3 and R4 (48 and 68 g/L, respectively). Also the volatile fraction of their solids was much lower (22 % in reactor R1 and 15 % in reactor R2) compared to the calcium fed reactors R3 and R4 and compared to the inoculum (32 %).

Table 2 | TSS and the volatile fraction of solids and fines in samples taken after 120 days from the four UASB reactors at 22 cm reactor height (FAS = Fully Acidified Substrate, PAS = Partially Acidified; Differences in duplicate measurements all were below 10 % of average values).

Reactor	Substrate	Calcium (g/l)	Reactor TSS (g/L)	% Volatile	Fines TSS (g/L)	% Volatile	% Fines in sample
R1	FAS	-	48	22	0.77	50	1.62
R2	PAS	-	68	15	0.14	33	0.21
R3	FAS	0.3	118	38	0.18	36	0.15
R4	PAS	0.3	162	48	0.09	38	0.05

In particular in reactor R1, a much higher fraction of the solids consisted of fines (1.62 %), and also in reactor R2 this fraction was somewhat higher (0.21 %) than in the calcium augmented reactors R3 (0.15 %) and R4 (0.05 %). Noteworthy is that the volatile fraction of the fines in reactor R2, and particularly reactor R1, were much higher than the volatile fraction of the sampled solids in these reactors, while in reactors R3 and R4 the volatile fractions of the fines were similar to, or even lower than in the reactor samples. Results indicate that reactors R1 and R2 were prone to selective washout of the volatile sludge fraction.

Effluent dissolved calcium concentration of reactors R1, R2, R3 and R4, measured after 120 days, were 0.17, 0.25, 0.31 and 0.67 mg/L, respectively. For the calcium augmented reactors R3 and R4 this implies that most of the additional calcium in the influent (0.3 g/L) must have precipitated, presumably as (amorphous) calcium carbonate, or must have been taken up by the EPS matrix of the granules. Over the entire experimental period of 120 days this should have resulted in an accumulation of solids bound calcium in the reactors of approximately 80 g calcium (or 200 g $CaCO_3$ when this would be the formed precipitate) in agreement with the calcium dose to the system (see above). However, considering the solids concentrations in Table 2, this seems very unlikely. Effluent concentrations of solids bound calcium were not measured and its wash-out therefore cannot be excluded.

Figure 3 shows microscopic images and particle size distributions of the sludge samples that were taken from the four UASB reactors after 120 days. The same samples were used as for the solids analyses presented in Table 2. Particle size distributions of the inoculum and of the fines are also included. Table 3 shows volume median diameters (50 %) of reactor sludges and fines that were calculated from these distributions.

Except in reactor R3, in all the reactors the granule size had increased compared to the inoculum (412 μm). The smallest granules were found in FAS and calcium fed reactor R3 (376 μm), followed by reactor R1, also fed with FAS but without additional calcium (537 μm). The biggest granules were present in samples taken from the two PAS fed reactors, i.e. in reactor R2 without extra calcium (763 μm) and in reactor R4 with extra calcium (581 μm). PAS clearly resulted in bigger granules than FAS, whereas calcium augmentation seemed to reduce growth of the granule size. A similar trend was also observed in Chapter 3.

The fines that were produced in reactors R2, R3 and R4 had similar 50 % volume median diameters between 70 and 82 μm (Table 3). The particle size of the fines in FAS fed reactor R1 was much bigger (150 μm). Interestingly, the particle size distribution of the fines in reactor R2 was binominal (Figure 3), with particles sizes close to those of the granules as well as particle sizes close to those of the fines in the other reactors. This could indicate that the granules in

reactor R2 not only suffered from abrasion by shear, but also were breaking up into smaller granules having a lower settleability.

Table 4 gives extracted EPS related protein and polysaccharide concentrations per gram of volatile solids (VS) of the inoculum and of solids samples taken on day 120 at 22 cm reactor height. The protein concentrations are in the same order as those reported by Zhang et al. (2012) for anaerobic granules under saline conditions. However, extracted protein concentrations were much lower than those found in Chapter 3. Possibly, the very high Na^+ concentration of 20 g/L negatively impacted EPS-protein production.

Table 3 | Volume median (50 %) particle sizes of granules and fines in samples taken after 120 days from the four UASB reactors at 22 cm reactor height (FAS = fully acidified substrate, PAS = partially acidified substrate).

			Particle size (μm)	
Reactor	Substrate	Calcium (g/l)	Granules	Fines
Inoculum	-	-	412	-
R1	FAS	-	537	150
R2	PAS	-	763	75
R3	FAS	0.3	376	70
R4	PAS	0.3	581	82

The extracted polysaccharide concentrations that were found are somewhat higher than those reported in Chapter 3. In reactors R1 and R2, without extra calcium, protein related EPS concentrations (58 – 61 mg/g VS) did not significantly change compared to the inoculum (61 mg/g VS). In the calcium augmented reactors R3 and R4 these concentrations were significantly lower (40 and 33 mg/g VS, respectively). Polysaccharide related EPS concentrations increased in all reactors compared to the inoculum (15 mg/g VS), with the highest increase for the PAS fed reactors R2 (79 mg/g VS) and R4 (47 mg/g VS). This higher production of extracellular polysaccharides with PAS is in agreement with findings by Fukuzaki et al. (1995). As a result, the protein to polysaccharide EPS ratio in all reactors became much lower than in the inoculum, in particular in the PAS fed reactors. D'Abzac et al. (2010) reported a similar effect of substrates with a different degree of acidification on protein to polysaccharide ratios in UASB reactors, albeit under non-saline conditions.

Representative SEM images of granules sampled on day 120 are presented in Figure 4. Filamentous microorganisms, probably *Methanosaeta*-like Archaea, were distributed on the surface of granules in reactors R1, R2 and R3; whereas the granule surface of calcium augmented PAS grown granules of reactor R4 was characterized by the presence of more cells which were embedded in EPS (insets of SEM images in Figure 4). On the surface of reactor R3 granules (FAS plus calcium) small crystals were observed. The surface texture of PAS fed granules from reactor R2 and of FAS fed granules in reactor R3 was smooth and densely packed. The granules in reactors R1 (FAS) and R4 (PAS with calcium) exhibited smooth as well as less densely packed regions.

SEM-BSE was used to characterize the composition of the inorganic fraction on the granule surface of calcium augmented granules from FAS fed reactor R3 and PAS fed reactor R4 (Figure 5). Inorganic, crystalline precipitation was found on the surfaces of the FAS grown granules. The precipitation on surface of the PAS grown granules had a more amorphous character. A more detailed analysis of the element distribution by SEM-EDX (Figure 5) revealed that the crystals on the FAS grown granules of reactor R3 mainly consisted of calcium, most likely

calcium carbonate. The spectrum for PAS fed granules of reactor R4 indicated the presence of iron and sulphur, suggesting that the amorphous precipitation on the surface of these granules consisted of iron sulphide, possible in combination with calcium carbonate.

Table 4 | Extracted EPS concentrations in mg/g of volatile solids (VS) of the inoculum and of solids from the four UASB reactors (FAS = Fully Acidified Substrate, PAS = Partially Acidified Substrate).

Reactor	Substrate	Additional Ca^{2+} (g/l)	Proteins mg/g VS	Polysaccharides mg/g VS	Proteins/polysaccharides mg/mg
Inoculum	-	-	61	15	4.0
R1	FAS	-	61	35	1.7
R2	PAS	-	58	79	0.7
R3	FAS	0.3	40	31	1.3
R4	PAS	0.3	33	47	0.7

Figure 6 shows representative FISH images of fines, sampled from the UASB reactors after 120 days. As expected, fines of the PAS fed reactors R2 and R4 showed a higher fraction of Bacteria than the fines of the FAS fed reactors R1 and R3. No significant effects of calcium augmentation could be detected. Glycoconjugate groups, visualized by lectin staining and important in EPS, were present in the fines of every reactor but were more abundant in the PAS fed and calcium augmented reactor R4, and particularly in the PAS fed reactor R2. This is in agreement with the higher polysaccharide concentrations that were found in these reactors.

6.3.3 Microbial diversity analysis

To investigate changes in the microbial community, DNA was extracted from the inoculum sludge and from 12 reactor samples at different time intervals (60, 90 and 120 days).

As shown in Figure 7A, a clear difference in banding patterns, qualitatively representing the bacterial community, can be observed between the FAS fed reactors (R1 and R3) and the PAS fed reactors (R2 and R4). Some new bands were detected in all the PAS fed sludges, whereas others disappeared from the FAS fed sludges. Substantial changes also were found between the bacterial community of the PAS fed reactors (R2 and R4) and the inoculum, i.e. a more complex band pattering with new bands emerging in reactors R2 and R4, which can also be expected with a more complex substrate as PAS. No effects of calcium augmentation could be observed on the bacterial community between the FAS fed reactors (R3 versus R1) or for the PAS fed reactors (R4 versus R2).

Figure 7B shows the DGGE gel with 16s rRNA amplicons for Archaea. Generally, no significant changes took place in the Archaeal community, irrespective of the type of substrate that was applied (FAS or PAS) or of calcium augmentation. However, some bands appeared and/or disappeared during reactor operation. For example, a new band was found in reactor R4 after 120 days, and a new band in reactor R3 was found at day 60 but disappeared afterwards.

All sequenced clones of the 16S rRNA of Archaea and Bacteria with their closest relative and the closest cultured relative are grouped in Table 5. All retrieved 88 archaeal 16S rRNA gene sequences were highly similar (96 – 99 %) to those of the acetoclastic methanogen; *Methano saeta harundinacea*. No sequences were retrieved that had a similarity with hydrogenotrophic methanogens. It may well be that due to the dominance of *Methanosaeta*-like phylotypes no clones were present in the library of 88 clones.

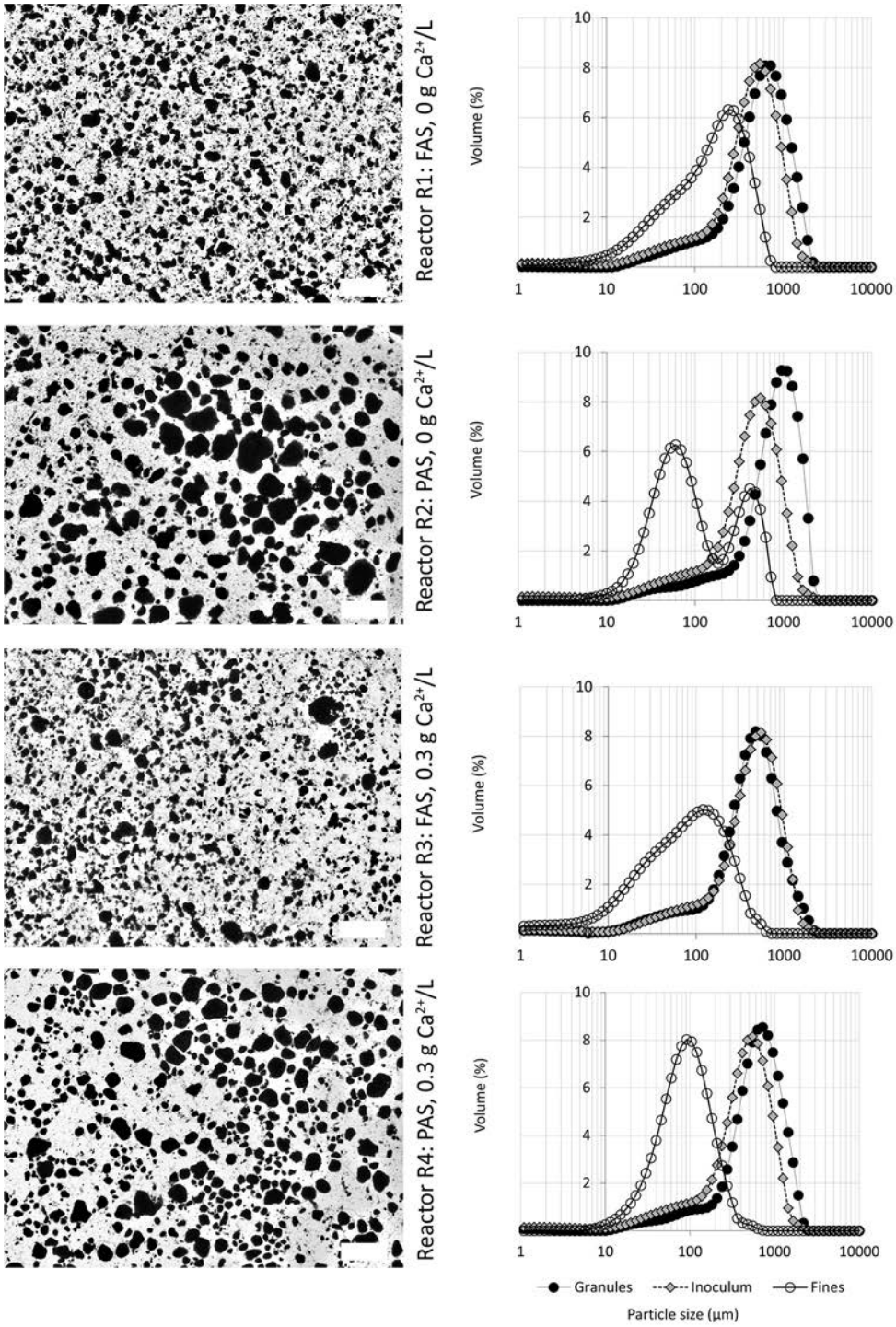


Figure 3 | Microscopic images and particle size distribution for sludge in the four UASB reactors and for fines produced in these reactors. Samples were taken after 120 days. Yellow bars in microscopic images are 2000 µm. (FAS = Fully Acidified Substrate = acetate; PAS = Partially Acidified Substrate).

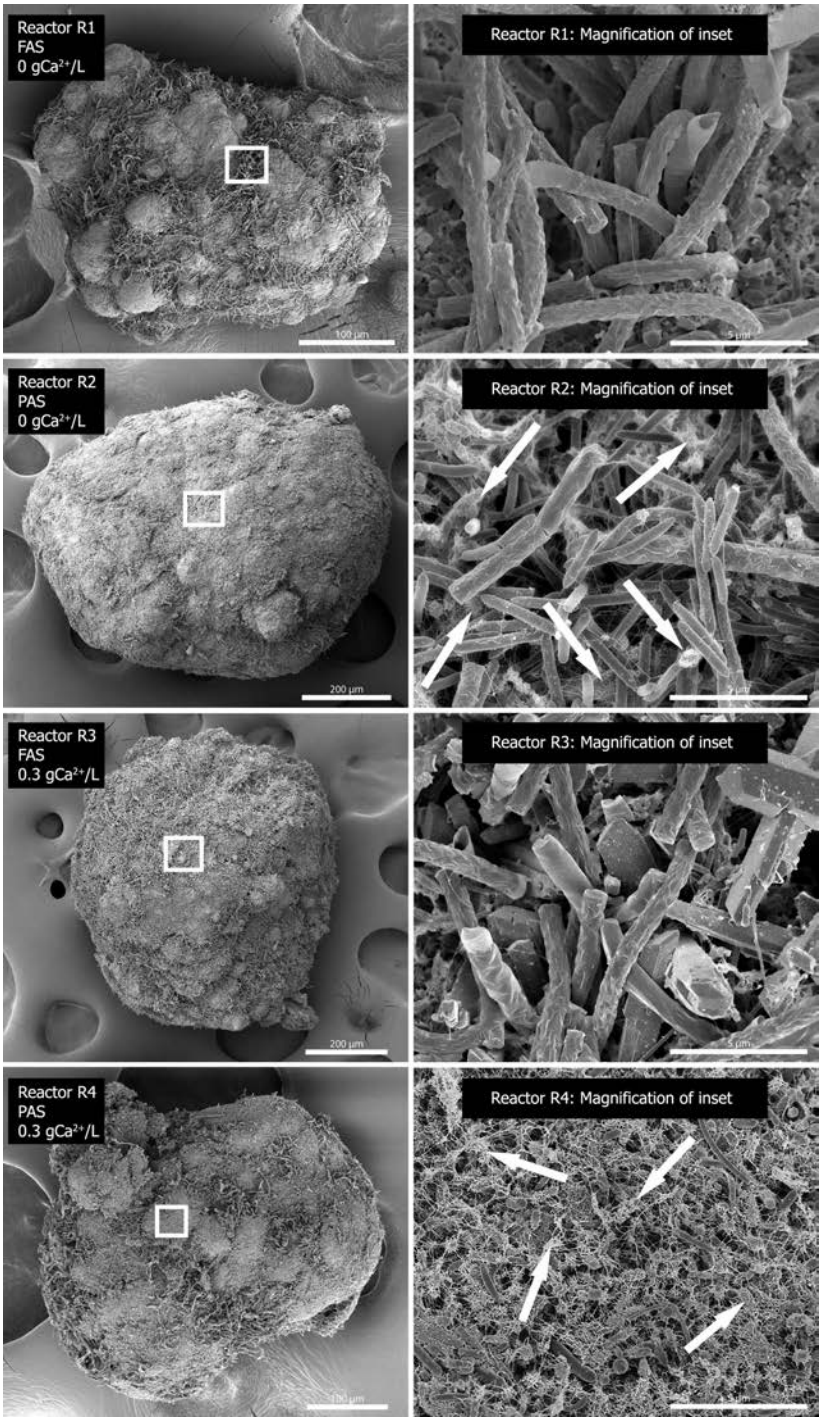


Figure 4 | SEM images of granules sampled from the four UASB reactors after 120 days. Aggregates of microorganisms on the surface of the granules at higher magnification (insets) are shown for each image. Arrows indicate arrangements of microorganisms on the surface of PAS grown granules embedded in an EPS matrix. (FAS = Fully Acidified Substrate, PAS = Partially Acidified Substrate).

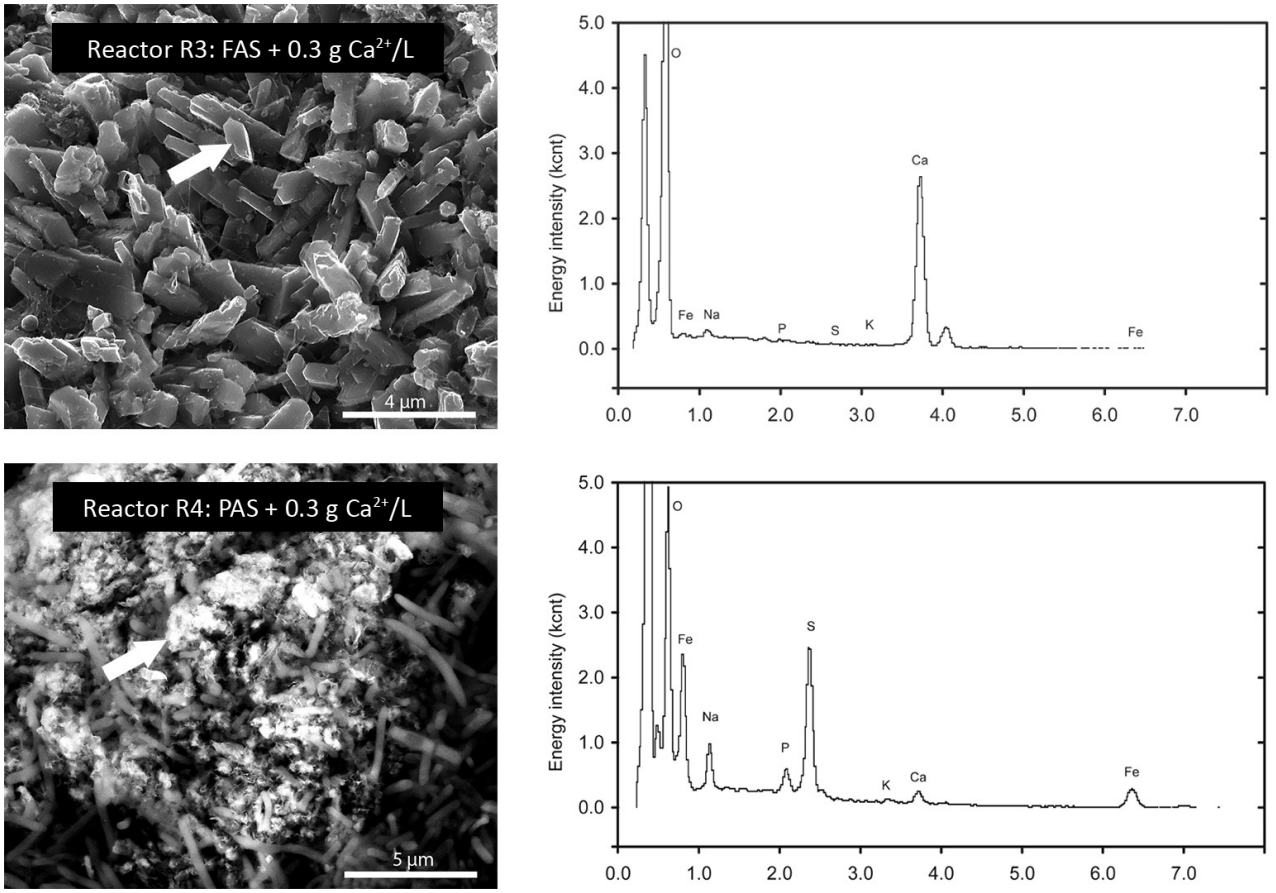


Figure 5 | SEM-BSE (left) images of calcium augmented FAS (reactor R3) and PAS (reactor R4) grown granules. SEM-EDX spectra of major elements (calcium, sulphur, iron, and phosphorus) are presented to the right of the SEM-BSE images. White arrows indicate the x-ray zone for elemental distribution analyses.

Retrieved bacterial clones (Table 5) showed more diversity. Most of the 73 bacterial clones had high similarity (95 – 99 %) with 16S rRNA of uncultured biodiversity from marine or hypersaline environments. The most dominant phylotype (53 from 73 clones) showed 90 - 96 % similarity with the 16S rRNA of *Alkaliflexus imshenetskii* from a soda lake (Zhilina et al., 2004). The other 24 clones were distantly related to: an unclassified *Clostridium*, strain S710-2-1 (10/73) isolated from chinese marine sediment; *Alkaliphilus halophilus* (2/73) from a saline lake (Wu et al., 2010); *Spirochaeta alkalica* (2/73) (Zhilina et al., 1996) from a continental soda lake; an unclassified *Desulfotomaculum* strain Lac2 (2/73) from a hypersaline lake sediment (Kjeldsen et al., 2007); *Prolixibacter bellariivorans* (2/73), a sugar-fermenting, psychrotolerant anaerobe of the phylum *Bacteroidetes*, isolated from a marine-sediment fuel cell (Holmes et al., 2007); *Spirochaeta americana* (1/73) a haloalkaliphilic, obligately anaerobic spirochaete isolated from soda Mono Lake; and *Dethiosulfatibacter aminovorans* (1/73), a thiosulfate-reducing bacterium isolated from coastal marine sediment (Takii et al., 2007).

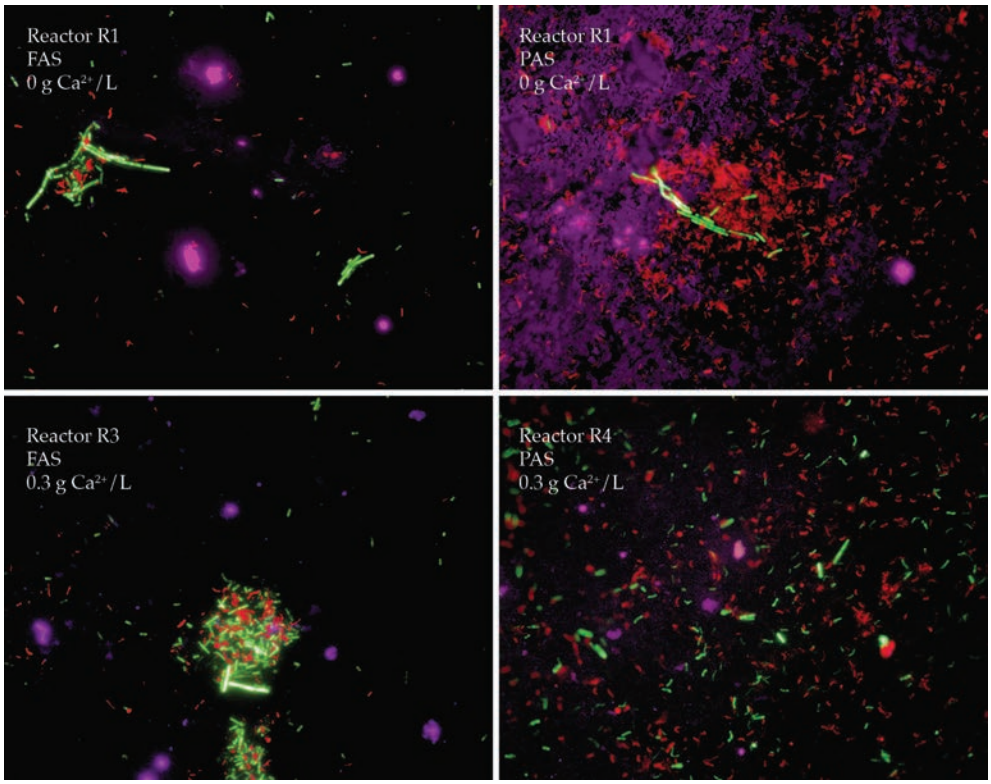


Figure 6 | FISH images of fines sampled from the four UASB reactors after 120 days, hybridized with specific probes for Archaea (ARC915-FITC, green), Bacteria (EUB338-Cy3, red) and glycoconjugate groups (lectin, purple).

6.4 Discussion

6.4.1 Reactor performance

In spite of the high salinity of 20 g Na^+/L, four differently operated UASB reactors all showed a high COD removal efficiency of more than 93 % at an OLR up to 14 g COD/L.d (Figure 1, between 0 and 60 days). This OLR fits very well in the feasibility range for a practical application of UASB reactors (van Lier, 2008). According to data collected by Lefebvre and Moletta (2006), such high removal efficiencies at similar or higher salinities have not been documented before. This, together with the huge SMA increase compared to the inoculum (Figure 2), demonstrates that anaerobic granular sludge technology is feasible at a salinity of 20 g Na^+/L, provided that a sufficient amount of potassium is present to alleviate sodium toxicity (Chapter 5).

In response to the sudden increase by more than 70 % of the OLR from 14 to 25 g COD/L.d, reactors R2, R3 and R4 became less stable with respect to their COD removal performance. Towards the end of the experimental period of 120 days, the PAS fed reactors R2 and R4 seemed to have largely recovered from this increase in OLR, although their removal efficiency was 3 to 6 % lower than at an OLR of 14 g COD/L.d. This was mainly caused by the presence of more propionate in the effluent. Surprisingly, also the effluent of the FAS fed reactors R1 and R3 contained some propionate (< 150 mg COD/L), although at much lower concentrations than in the PAS fed reactors R2 and R4 (1600 – 2000 mg COD/L). Possibly, this was caused by propionate production from decaying biomass or from an unknown reductive carboxylation pathway under saline conditions. This however cannot be further substantiated. The FAS fed and calcium augmented reactor R3 did not fully recover and remained rather unstable until the end of the experimental period, even though no operational problems were encountered.

FAS fed reactor R1 by far showed the best response to the increased OLR in terms of stability and recovery after the increase of the OLR and gave an average dissolved COD removal efficiency of 97 %. The large difference in behavior between the FAS fed reactors R1 and R3 might be attributable to a detrimental effect of localized calcium precipitation on the methanogenic biomass in agreement with the results described in Chapter 5. Unfortunately, no further data are available to substantiate this hypothesis.

Based on the results of Chapters 3 and 4, it was expected that calcium augmentation would reduce the shear sensitivity of anaerobic granules, and in this manner would reduce or even prevent wash-out of biomass from reactors R3 and R4. A comparison of the solids concentration of the calcium augmented reactors R3 and R4 with those of reactors R1 and R2 (Table 2) indeed indicated a positive effect of calcium. Also the higher contribution of fines to the solids in reactors R1 and R2, in combination with the abundant presence of anaerobic microorganisms in these fines (FISH images of Figure 6), could indicate such a positive effect of calcium. Nonetheless, despite the enhanced biomass wash-out in reactors R1 and R2, both reactors, and in particular reactor R1, showed an excellent performance during the increased OLR during days 60 – 120, compared to the other reactors.

In addition, it is highly questionable whether a fair comparison between the solids concentrations of Table 2 is possible at all. As mentioned, the concentrations were determined in

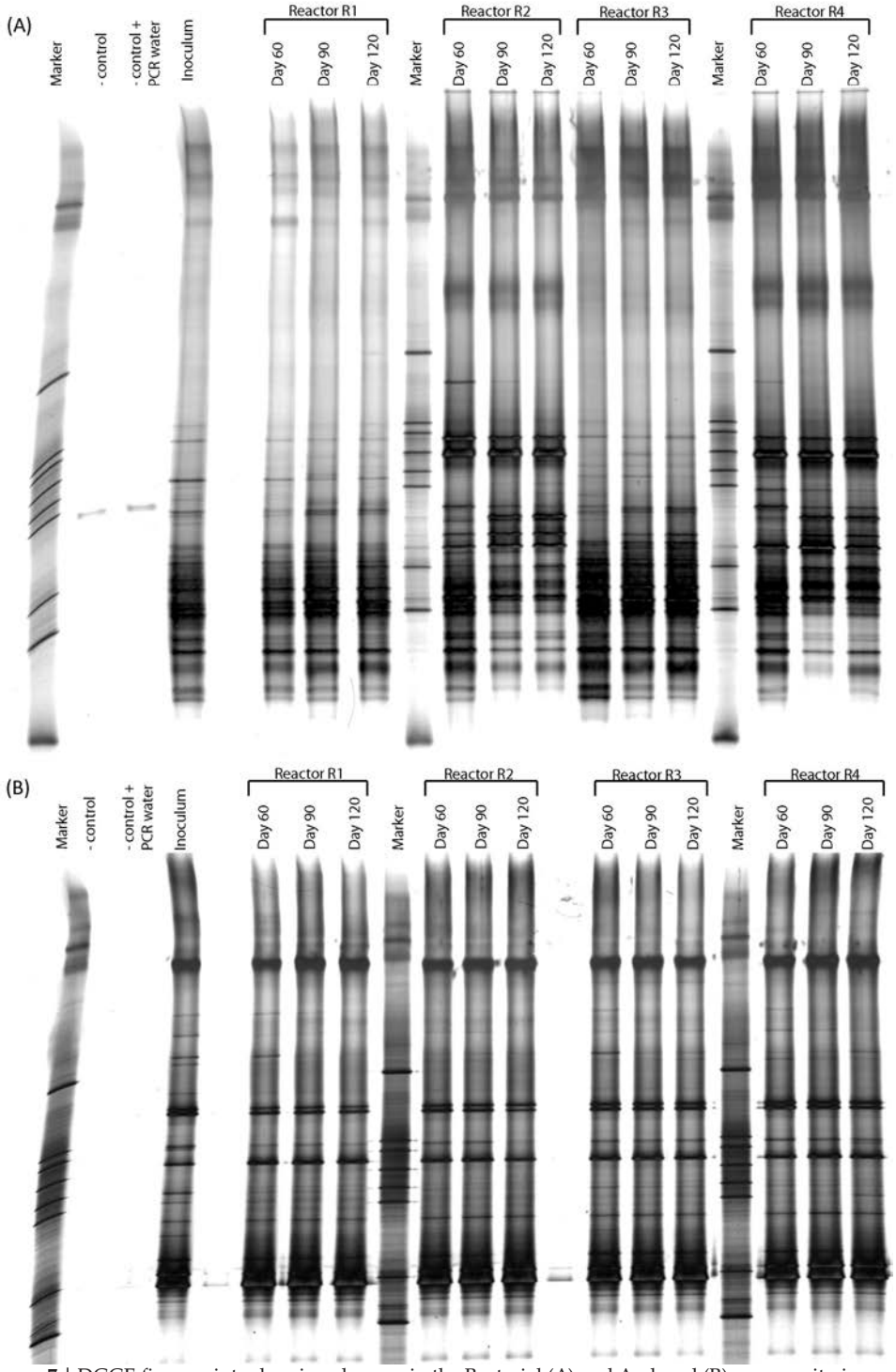


Figure 7 | DGGE fingerprints showing changes in the Bacterial (A) and Archaeal (B) community in reactors R1 to R4 after 60, 90 and 120 days.

Table 5 | Archaeal and Bacterial clones sequenced and similarity with their closest relative (uncultured and cultured) for the sludge sample of reactor R2 after 120 days.

Archaeal

Clone no.	Closest relative	Similarity (%)	Accession no.	Closest cultured relative	Similarity (%)	Accession no.
68	Uncultured archaeon clone AR80A32	97 - 99	AB539923.1	*Methanosaeta harundinacea strain 6Ac*	89 - 98	NR_102896
9	Uncultured archaeon clone GZK75	98 - 99	AJ576240.1	*Methanosaeta harundinacea strain 6Ac*	97 - 98	NR_102896
7	Uncultured *Methanosaeta* sp. clone D003011I03	98 - 99	EU721747.1	*Methanosaeta harundinacea strain 6Ac*	96 - 98	NR_102896
3	Uncultured archaeon clone MOB7-4	98 - 99	DQ841239.1	*Methanosaeta harundinacea strain 6Ac*	97 - 99	NR_102896
1	Uncultured *Methanosaeta* sp. clone D_A03	98	AY454771.1	*Methanosaeta harundinacea strain 6Ac*	97	NR_102896
Total = 88 clones						

Bacterial

Clone no.	Closest relative	Similarity (%)	Accession no.	Closest cultured relative	Similarity (%)	Accession no.
52	Uncultured bacterium clone G35_D8_L_B_H09	94 - 99	EF559148	*Alkaliflexus imshenetskii*	91 - 96	NR_042317
1	Uncultured bacterium clone Asc-w-36	97	EF632714.1	*Alkaliflexus imshenetskii*	89	NR_042317
7	Uncultured *Firmicutes* bacterium clone NRB18	96 - 99	HM041935	*Clostridium* S710(2)-1	94 - 97	GU136590
1	Uncultured *Firmicutes* bacterium clone NRB18	91	HM041937	*Clostridium* S710(2)-1	87	GU136590
1	Uncultured bacterium clone 155_BE1_1	92	FJ825473.1	*Clostridium* S710(2)-1	87	GU136590
1	Uncultured organism clone MAT-CR-H4-C10	89	EU245221.1	*Clostridium* S710(2)-1	89	GU136590
2	Uncultured bacterium clone ARDBACWH19	95	EU869412	*Desulfotomaculum* sp. Lac2	86	DQ386219
2	Uncultured *Spirochaetaceae* bacterium clone ML623J-23	93 - 94	AF507855	*Spirochaeta alkalica* strain Z-7491	92 - 94	NR_026301
2	Uncultured bacterium clone WF16S_44	96 - 97	EU939394	*Alkaliphilus halophilus*	96	EU627628
2	Uncultured bacterium clone 91A_BS1_2	88	FJ825525	*Prolixibacter bellariivorans* strain F2	87	NR_043273
1	Uncultured bacterium clone LGH02-B-149	88	HQ916639	*Spirochaeta americana*	83	NR_028820
1	Uncultured bacterium clone YWB38	95	AB294307	*Dethiosulfatibacter aminovorans*	93	NR_041309
Total = 73 clones						

samples taken from the reactors at a height of 22 cm and differences in settleability may have influenced the results. The measured concentrations also cannot explain the high removal efficiencies that were observed in the reactors. As an example, assuming that the reactors were operated under non-limiting substrate conditions, the required reactor solids concentrations in the FAS fed reactor R1 at an OLR of 25 g COD/L.d can be calculated from the SMAs. Even if the highest SMA in the FAS fed reactors of 0.512 g COD/g VSS.d (reactor R3, day 90 in Figure 2) is used, such calculation would yield a biomass concentration in reactor R1 of 55 g VSS/L. This concentration is much higher than the measured VSS concentration of 11 g VSS/L (Table 2, reactor R1 with 48 g TSS/L and a VSS/TSS ratio of 0.22). Substrate limiting conditions in the reactor, the fact that the VSS concentration at a reactor height of 22 cm probably must have been higher than 55 gVSS/L, and using lower SMA values from Figure 2 for the calculation would even further increase the difference between calculated and measured solids concentrations. Unfortunately, no data are available for effluent solids concentrations, biomass retention, and solids wash-out to further quantify the observed phenomenon. This implies that no clear conclusions can be drawn regarding the impact of calcium augmentation on biomass retention, granule stability and reactor conversion capacity.

The SMAs which were determined with samples taken from the reactors all increased as soon as the OLR was increased from 10 to 14 g COD/L.d, with the exception of the SMA of reactor R1 sludge on day 90. In particular the low SMAs, measured at the end of the reactor operational period on day 120, are questionable because they would imply that impossibly high reactor biomass concentrations are required to take care of the high removal efficiencies that were achieved at an OLR of 25 g COD/L.d (as already outlined above).

6.4.2 Microbial diversity

The DGGE profiles of Figure 8 showed that the addition of calcium did not significantly influence the dominant bacterial and archaeal population in the reactors. Similar findings were also reported by Liu et al. (2010), where calcium augmentation did not affect the microbial diversity in aerobic granules. Also the size of granules can play an important role because larger granules induce more mass transfer limitation for the essential nutrients. This could lead to a decreased microbial diversity and decreased microbial activity due to starvation of the biomass in the centre of the granules (Hulshoff Pol et al., 2004). However, although median volumetric granule sizes were different in reactors (395 – 760 μm), such an effect was not observed.

The complex organic PAS feed resulted in a more complex bacterial population, reflecting the different physiological groups that are needed to convert this complex substrate (starch and gelatine) into methane (Figure 8A) The archaeal population was not significantly affected by the type of feed (Figure 8B) but new bacterial bands appeared with PAS as the substrate, indicating rapid growth of Bacteria in the presence of complex substrates, even at a salinity level of 20 g Na^+/L. Compared to the inoculum, the bacterial diversity in FAS fed reactors R1 and R3 did not change significantly. The bacterial diversity in PAS fed reactors R2 and R4, however, changed considerably compared to the inoculum.

The dominant archaeal species in the reactors was related to *Methanosaeta harundinacea*. *Methanosaeta* spec. has been frequently mentioned as being crucial for a successful granulation

process. Acetoclastic methanogens have been found in high salinity wastewaters, but mostly these were related to *Methanosarcina* species, which are known as salt tolerant methanogens (Oren 2002). Also *Methanosaeta harundinacea* is a halotolerant species which was recently found to grow in high salinity environments (Joye et al., 2009). No known hydrogenotrophic archaea were detected in the reactors. Although it can be hypothesized that breakdown of carbon from PAS at 20 g Na^+/L does not generate hydrogen and/or formate, and methane is exclusively produced by acetoclastic methanogens, this seems highly unlikely. Also, the occurrence of hydrogenotrophic archaea is reported at even higher salinities, i.e. around 47 g Na^+/L (Oren, 2002). Another, perhaps more likely explanation is that the clone library with 88 clones was not large enough to harbour the minor fraction of hydrogenotrophic methanogens, or that due to the dominance of *Methanosaeta*-like phylotypes no clones were present in the library of 88 clones. Further cloning efforts or next generation sequencing are needed to elucidate the presence and role of hydrogenotrophic methanogens.

The bacterial community in the PAS reactors showed the presence of a dominant phylotype, belonging to the family of *Marinilabiliaceae* and specifically *Alkaliflexus imshenetskii*. Most of the clones (74 %) that were identified, related to members of this family with a high similarity (90 – 95 %). Members of *Marinilabiliaceae* are capable of degrading polymeric substances such as starch and gelatine, but also sugars (Zhilina et al., 2004, Sorokin et al., 2011), which are the substrates in the PAS fed reactors. All bacterial clones could be related to cultured or uncultured biodiversity from a variety of origins, but all of them from high salinity environments. The bacterial biodiversity must have originated from the inoculum which was exposed to a sodium concentration of 10 – 15 g/L for more than 10 years. The results demonstrate this biodiversity can easily adapt to higher sodium concentrations of at least 20 g/L.

6.5 Conclusions

The following conclusions can be drawn from this research:

- Even at a Na^+ concentration of 20 g/L anaerobic granular sludge reactors can achieve high COD removal efficiencies at high organic loading rates of 14 g COD/L.d.
- At a higher organic loading rate of 25 g COD/L.d reactor performance generally deteriorated, although the extent of this depended on the type of organic susbtrate (FAS or PAS) and on the presence of calcium.
- There were indications that calcium, at a concentration of 0.3 g/L (20 mg Ca^{2+}/g VSS), had a positive effect on biomass retention, but this could not be further quantified.
- The acetoclastic methanogen *Methanosaeta harundinacea* dominated the Archaeal population in all reactors.
- The archaeal microbial population was relatively stable in time and no significant differences were observed due to calcium augmentation.
- The bacterial population in reactors fed with partially acidified substrate easily acclimated to the high salinity level of 20 g Na^+/L.

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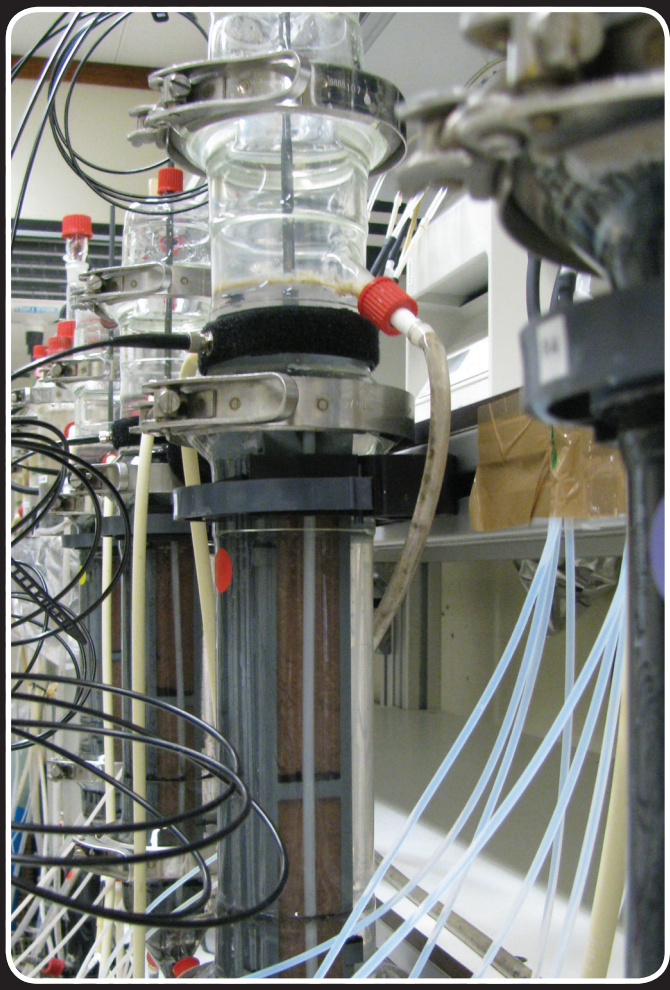
Chapter 7



General Discussion and Outlook

Tomorrow the snow will melt and grass will appear.
Arabic proverb

7.1 Introduction

Nowadays, anaerobic sludge bed reactor technologies are considered the most popular anaerobic high-rate wastewater treatment systems (van Lier, 2008). Sludge bed reactors are currently challenged to maintain their high efficiency, while treating specific types of wastewater under more extreme conditions, such as high temperatures, pH and salinity. With regard to the latter, if wastewaters contain salt concentrations above 3 g Na^+/L, they are characterised as saline wastewaters. The upper limit for known saline wastewaters is about 60 g Na^+/L (Lefebvre and Moletta, 2006). It is known that high Na^+ concentrations would give a negative impact to the anaerobic treatment process (Rinzema et al., 1988). Moreover, high Na^+ concentrations can induce the disintegration of anaerobic sludge (Pevere et al., 2007) and may result in wash-out of viable biomass and a poor reactor performance (Mendez et al., 1995). Therefore, a research focusing on biomass activity and biomass retention at high salinity level was conducted in order to investigate whether the robustness of anaerobic treatment technology can be sustained while treating high-concentrated organic effluents. In this study, the effects of high Na^+ concentrations on physicochemical properties as well as the biological conver-

sion capacity were studied. Experiments on the initial attachment of anaerobic consortia were conducted, in order to study the development of anaerobic biofilms under saline conditions. Although many hypotheses are available to explain the formation of biofilms and granules, based on our results, we found that divalent cations (i.e. Ca^{2+}) are important to form bridges in a matrix of extracellular polymeric substances (EPS), predominantly proteins and polysaccharides (partly negatively charged polymers) and anaerobic microorganisms, which is in agreement with others (e.g. Bhatia et al., 2013). In this manner the structure of the biofilms or granules will be maintained. Another important factor is the availability of a sufficient amount of potassium to alleviate Na^+ toxicity. Finally, an outlook with respect to further laboratory investigations, research assessments and full scale wastewater treatment is presented.

7.2 UASB reactor performance at high Na^+ concentrations

7.2.1 Effects of high Na^+ concentrations on methanogenic activities

A series of experiments (Chapters 2, 3 and 6) were set up to study the effect of high Na^+ concentrations on anaerobic processes in upflow sludge bed reactors. All reactor operations, which were conducted at high Na^+ concentrations in the range between 5 to 20 g Na^+/L, show a high COD removal efficiency. The efficient anaerobic conversion process that immediately started after reactor inoculation, indicates that the used inoculum, which was already adapted to 10 – 15 g Na^+/L, can tolerate the imposed high Na^+ concentrations. The reactors in Chapter 3 showed a good and stable COD removal before day-130, even at an OLR of 18 kg COD/m^3.d, irrespective of the type of organic substrate. Interestingly, with regard to the inoculum, the measured SMA was only limitedly impacted by the actual Na^+ concentrations, while applying the same substrate. Apparently, the inoculum that was used can tolerate high Na^+ concentration, a finding which agrees with a previously published Na^+ response curve for this inoculum (Jeison et al., 2008). In spite of a relative high salinity of 20 g Na^+/L, four differently operated UASB reactors all showed a high COD removal efficiency of more than 92 % at organic loading rates up to 14 – 15 g COD/L.d (Chapter 6). This loading rate would also be feasible for a practical application (van Lier, 2008). According to data collected by Lefebvre and Moletta (2006), such high efficiencies for the applied loading rates, i.e. 14 – 18 kg COD/m^3.d at similar or higher salinities, have not been reported before. This, together with 5 to 8 times higher specific methanogenic activities compared to the inoculum, demonstrates that anaerobic granular sludge technology is feasible at a salinity of at least up to 20 g Na^+/L.

However, during the initial attachments of anaerobic microorganisms, acetate removal at 20 g Na^+/L (reactor R2) was a factor 2 – 4 slower than at 10 g Na^+/L (reactor R1), as described in Chapter 5. This lower reactor performance could be due to the crushed granular sludge that was used as inoculum. However, the negative effect of sodium in this experiment was alleviated in the presence of 0.7 g K^+/L. Apparently, K^+ acts as an antagonistic salt alleviating the impact of high concentrations of Na^+ on the methanogenic conversion rates as previously suggested by Sleator and Hill (2002). Unexpectedly, with the addition of 0.3 g Ca^{2+}/L to the influent, the reactor R3 in Chapter 5 exhibited a very poor performance. Very likely, not merely the calcium concentration but the calcium – sludge mass ratio determines whether calcium stimulates or counteracts biofilm formation and/or granulation. Regarding the Ca^{2+}/VSS ratio, a clear overdosing was experienced applying a ratio of 150 mg Ca^{2+}/g VSS, probably leading to encapsulation of the microorganisms instead of immobilisation to the inert support material.

The conversion capacity and treatment performance of the resulting reactor was extremely low.

The pre-adapted inoculum, which was acclimated to 10 – 15 g Na^+/L, immediately could be used for reactor operation showing a good performance at Na^+ concentrations up to 20 g /L. With regard to this high performance, the upper limit of admissible Na^+ concentrations clearly has not been reached. On the other hand, the presence of an adequate K^+/Na^+ ratio was shown to be indispensable for the observed high performance. An additional boost to the methanogenic conversion rates at high Na^+ concentrations might be expected when using compatible solutes in the medium.

7.2.2 K^+ additions

During anaerobic biofilm reactor operation at 20 g Na^+/L described in Chapter 5, addition of 0.7 g K^+/L showed to be indispensable for a high acetate removal rate. This indicates that, when the anaerobic biomass is exposed to a Na^+ concentration, the presence of K^+ alleviates the negative effects of Na^+ on the acetate conversion rate. According to Sleator and Hill (2002) the common microbial strategy to increased salinity levels is that glutamate and K^+ are taken up as a primary response, which is followed by a secondary response with a dramatic increase in cytoplasmic concentrations of compatible solutes. This also explains why K^+ may act as an antagonistic salt alleviating the impact of high concentrations of Na^+ on the methanogenic conversion rates (e.g. Kugelman and McCarty, 1965). The ideal K^+ concentration in high Na^+ concentrations has not been investigated, but in this study the Na^+/K^+ ratio in seawater was used as reference.

7.2.3 Microbial community

The microbial communities that proliferated in the biofilm reactors at high salinity levels described in Chapters 5 and 6 were further identified focusing on the acetoclastic and hydrogenotrophic methanogens (see Figure 1). Relatives of *Methanosaeta harundinacea* were identified as the dominant acetoclastic methanogens in the anaerobic biofilms that were formed at 10 and 20 g Na^+/L. According to Collins et al. (2005) the filamentous structure of *Methanosaeta* sp. is an important factor for anaerobic granulation process and perhaps also for biofilm formation. Therefore, *Methanosaeta harundinacea* is crucially important for anaerobic granule formation and also tolerates high Na^+ concentrations. Two H_2/CO_2 and/or formate utilizing methanogens were found; *Methanolinea tarda* and *Methanobacterium subterranum*. The absence of hydrogenotrophic methanogens in all reactors is remarkable as high Na^+ concentrations would create the possibility for those microorganisms to grow under stress conditions.

In Chapter 6, four UASB reactors were fed with different types of substrates, i.e. partially acidified substrates (PAS) and fully acidified substrates (FAS). The complex organic PAS feed resulted in a more complex bacterial population, reflecting the different physiological groups that are needed to convert this complex substrate (starch and gelatine) into methane. The archaeal population was not significantly affected by the type of feed but new bacterial bands appeared with PAS as the substrate. Obviously, the more complex PAS substrate selected for those bacteria present in the inoculum that could sustain and proliferate at a salinity level of 20 g Na^+/L. Compared to the inoculum, the bacterial diversity in the purely methanogenic

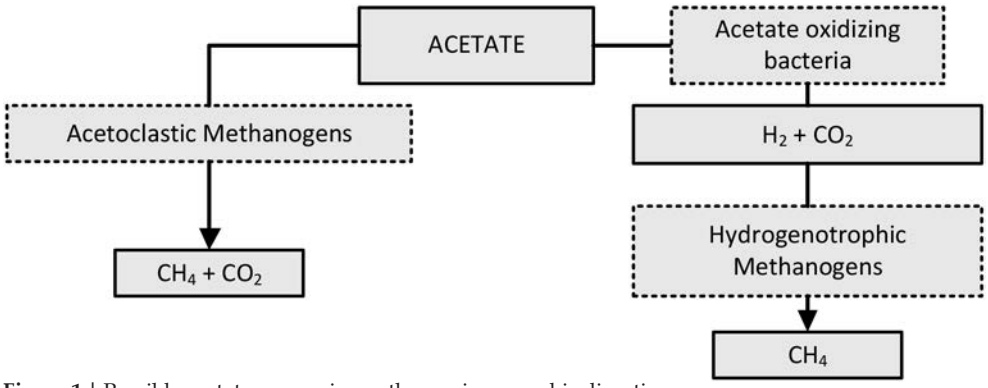


Figure 1 | Possible acetate conversion pathways in anaerobic digestion.

FAS fed reactors did not change significantly.

Interestingly, the presence of an apparently stable bacterial population in the PAS-fed granular sludge shows that the microorganisms can tolerate high Na^+ concentrations. Similar to archaea, no adaptation period to increased salt concentration (20 g Na^+/L) is required for the bacterial population. Therefore, the use of PAS as a substrate at high Na^+ concentrations should not be a problem.

7.3 Anaerobic biomass retention at high Na^+ concentrations

In particular for slow growing anaerobic microorganisms, excellent retention of biomass is crucial in high-rate anaerobic reactors. Depending on the wastewater characteristics, environmental and operational parameters, granules or biofilms will develop. High density granules or a biofilm are the most desired agglomerates to allow a high reactor biomass concentration and therefore smaller reactor volumes. Thus far, most papers of the various researchers reported on the toxicity of Na^+, but very few discuss the effect of Na^+ on biomass retention and/or granulation.

7.3.1 Effect of high Na^+ concentrations on anaerobic granular sludge

The research presented in Chapters 2 and 3, showed that high Na^+ concentrations cause a decrease in granule strength and washout of bacterial mass. It is postulated that under strong saline conditions the deterioration of granule strength can be attributed to the replacement of Ca^{2+} in the matrix by the abundantly available Na^+, resulting a weaker granule structure. From batch test results (Chapter 3) it is shown that such Ca^{2+} displacement can be brought forward by high Na^+ concentration. Leaching of divalent Ca^{2+} from the granule matrix at high Na^+ concentrations is in agreement with Bruus et al. (1992) and Higgins and Novak (1997) who explained this observation as an ion-exchange process. Sobeck et al. (2002) explained this phenomenon with the so called cation bridging theory: Ca^{2+} binds to negatively charged functional groups on bacterial surfaces and to extracellular polymeric substances (EPS), and in this manner acts as a bridge to interconnect these components and promote the formation of microbial aggregates (Jian et al. 2003). Nonetheless, biofilm studies in Chapter 5 showed effective anaerobic biofilm formation at salinities to at least 20 g Na^+/L, even with a simple organic substrate such as acetate. Apparently, the back ground divalent cation concentration in

our medium was sufficient for the initial attachments and biofilm developments. As such, our findings are in line with findings that anaerobic biofilms occur in marine ecosystems (Briggs et al., 2011), i.e. typically at sodium concentration of 10 – 15 g Na^+/L.

7.3.2 Ca^{2+} additions

As mentioned, Ca^{2+} is a key factor in the granulation process, as a necessary agent for polymer binding in anaerobic granular sludge (Pevere et al., 2007). At higher Na^+ concentrations, anaerobic granular sludge was previously reported to become more shear sensitive. Our hypothesis is the Ca^{2+} leaching could be a reason for granule stability loss. In Chapter 3, series of batch tests were carried out to determine Ca^{2+} leaching from granular sludge as a result of high Na^+ concentrations. The results clearly show an increase in the bulk liquid Ca^{2+} concentration at a Na^+ concentration of 20 g/L. Taking the observed calcium dynamics into account, Ca^{2+} addition to anaerobic granular sludge at high Na^+ concentrations may very well prevent Ca^{2+} leaching, as demonstrated in Chapter 4. We found that 0.3 g Ca^{2+}/L (10.5 mg Ca^{2+}/g TSS) helps to maintain the calcium content of anaerobic granular sludge when this sludge is exposed to high Na^+ concentrations of 20 g/L. Under similar conditions, but in the absence of Ca^{2+}, anaerobic granules started to leach calcium. In Chapter 3, addition of 1 g Ca^{2+}/L to the influent resulted in relatively small granules with high granule strength. This finding agrees with previous research that clearly demonstrates that high Ca^{2+} concentration (> 0.6 g Ca^{2+}/L) had a negative influence on the granular size done by Yu et al. (2001). Apparently, Ca^{2+} exerts a dual effect that can turn out either positive or negative for the sludge to be cultivated treating saline wastewaters. Depending on the type of organic substrate and the pH, care much be taken that Ca^{2+} is added at an appropriate Ca^{2+} to biomass ratio in order to prevent sludge calcification and even complete cementation (Van Langerak et al., 2000). For instance, in our biofilm formation experiments a calcium addition of 150 mg Ca^{2+}/g VSS was applied, and this resulted in a strong negative effect on biomass activity and biofilm formation (Chapter 5). Too high calcium additions resulted to the cementation processes. In addition, (local) super-saturation of Ca^{2+} and CO_3^{2-} ions may result in intensive nucleation forming $CaCO_3$ fines that may act as support for new bacterial mass. The very low density of such fines may subsequently result in significant sludge washout. Likely, because of these reasons, very low acetate conversion rates and very limited biofilm growth on the fixed substratum was found in the Ca^{2+} added reactor R3 in Chapter 5.

In Chapter 6, a much lower Ca^{2+} addition of about 20 mg Ca^{2+}/g VSS was used, which shows the need for appropriate calcium doses under high salinity conditions. The optimum Ca^{2+} addition may be determined by several factors including the organic loading rate, Na^+ concentration and the type of organic substrates. Therefore the ideal Ca^{2+} concentrations can manipulate the Ca^{2+} roles in anaerobic granule formation either as bridging agents between extracellular polymers or precipitate formation. In this study, Ca^{2+} additions in 20 g Na^+/L at 10 – 20 mg Ca^{2+}/g TSS gave a positive effect to the granulation processes, meanwhile 150 mg Ca^{2+}/g TSS additions showed detrimental effects.

7.3.3 Extracellular polymeric substances (EPS)

The EPS functions as a kind of cation-exchange material, which explains why Ca^{2+} can be replaced by sodium when the ratio between Na^+ and Ca^{2+} in the bulk liquid is high, as was

the case in our experiment in Chapter 3. PAS-fed granular sludges have much higher proteins and polysaccharides contents than FAS-fed granular sludges. Fukuzaki et al. (1991) also reported that starch- and sucrose-grown granules produced more EPS than ethanol- or fatty acids-grown granules. Interestingly, reactors fed with PAS were characterized with a higher granules size compared to the FAS grown granules at high salinity. In addition, with addition of Ca^{2+} concentrations at optimum concentration, PAS-fed granules have better physical properties as evidenced by the measured higher granule strength.

7.4 Outlook

This research clearly showed the perspectives of using high-rate anaerobic sludge bed systems for the treatment of high salinity wastewater. The grown anaerobic granular sludge showed distinctly bigger sizes when reactors were fed with partially acidified substrates. In addition, methanogenic consortia could adapt to high Na^+ concentrations as much as 20 g Na^+/L. In many publications and literature findings it is reported that high Na^+ concentrations affects the anaerobic biomass activity and sludge retention. However, based on our present results, satisfactory operation of anaerobic sludge bed reactors treating saline wastewater can be expected considering the following aspects:

- Microbial adaptation is a pre-requisite for the success of any bio-reactor system working under extreme conditions. Likely, such adaptation means enrichment of specific bacteria and Archaea that are suitable to resist the more harsh conditions or that even show their optimal growth under such conditions. In our studies, the excellent reactor performances could be ascribed to the presence of halo-tolerant and/or halophilic organisms. Considering the expected (extreme) low growth rates of such organisms, the start-up and adaptation time will take considerable amounts of time. Therefore, the application of inoculum grown under the conditions similar to the target conditions should be pursued. The inoculum used in this research showed great potential to be used at Na^+ concentrations of at least up to 20 g/L. Considering the SMA values at higher Na^+ concentrations, even higher concentrations are likely possible. Most suitable seed materials for saline reactors are pre-adapted sludge from comparable systems, such as those treating sea-food processing wastewater, chemical wastewaters, or very concentrated wastes such as distilleries slops. Saline mud sludge from estuaries receiving organic waste streams can be considered as an alternative 'low grade' inoculum.

- Improvement of in-reactor activity and/or acceleration of the first start-up can be accomplished by adding osmoregulatory compounds such as glycine-betaine to the system. The uptake of such compounds costs much less energy for the bacteria than bio-synthesis. On the other hand, costs-wise, continuous addition of such compounds will likely not be feasible. Moreover, eventually a fully adapted culture is pursued being independent from continuous addition of osmo-regulators. Nonetheless, during the first (batch-wise) start-up, the use of such compounds could be a strategy of interest.

- A complete imbalance in the K^+/Na^+ ratio results in reactor failure. Our results clearly showed the need for a proper K^+/Na^+ ratio, e.g. similar to sea water, in the feed of the reactor. Rapid immobilisation and biofilm development was observed at a K^+/Na^+ ratio of 0.035. It is strongly advised to raise the K^+ level in the feed water to at least this ratio when Na^+ salinity levels are high. Further research is required if even higher ratios are needed when the Na^+ concentration will exceed 20 g/l.

- The physical properties of anaerobic granule such as morphological surface structure, density, granule strength, etc., are severely affected by high Na^+ concentrations. Our results showed a clear leaching of Ca^{2+} from the granule matrix when exposed to high Na^+ salinity conditions, lowering to granule strength. As a result, the granules become non-resistant to the high shear forces that are generally experienced in anaerobic reactors. Addition of low concentrations of Ca^{2+} to the reactor influent can help to sustain the granule structure and strength of the anaerobic sludge. Based on our results we hypothesise that an appropriate monovalent – divalent cation ratio is crucial for proper sludge development. An imbalance in this ratio must be restored by adding the divalent cation to the feed. The stable mono- and divalent cation ratio in marine ecosystem is apparently suitable for the formation of marine biofilms. Taking into account both the proper K^+/Na^+ ratio and the monovalent/divalent cation ratio it is postulated that sea water beneficially can be used as water matrix in the feed to the saline sludge bed reactor, especially during the start-up period.

- Additions of too high Ca^{2+} concentrations to the feed may have severe adverse effects in the sludge development. Our results showed retardation in the increase in bio-activity, leading to very poor conversion capacities when Ca^{2+} additions were too high. In addition, sludge bed development in upflow reactors was also severely hampered by the high Ca^{2+} loadings, although the strength of the conglomerates was significantly increased. In our work the exact Ca^{2+}/Na^+ ratio could not be assessed and will very likely depend on the entire water matrix as well as the reactor operational conditions, such as the applied loading rate and the substrate composition. It is therefore advised to start-up a saline reactor with low amounts of Ca^{2+}, e.g. similar to sea water conditions, after which any increase should be carefully followed based on calcium mass balances and monitoring the sludge morphological properties.

- From the scientific point of view many questions remained unanswered. The surprising absence of hydrogenotrophic methanogens is very remarkable in our methanogenic population analyses. Likely, the applied methodology was not adequate to detect all the present (sub) populations since the 'non-existence' of these methanogens in the grown sludges cannot be explained. Nonetheless, the observed low abundance of these methanogens in relation to the assessed methanogenic activities at high Na^+ concentrations is puzzling and certainly deserves further studies.

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Summary
Samenvatting

Summary

Anaerobic sludge bed reactor systems like the upflow anaerobic sludge blanket (UASB) and expended granular sludge bed (EGSB) reactors are currently the mostly applied high-rate reactor systems for anaerobic wastewater treatment. The success of both systems has changed the world conception of wastewater treatment with energy recovery being an intrinsic part of the treatment process, avoidance of excess sludge problems and extremely low space requirement. Nevertheless, while broadening the UASB reactor application to a diverse type of wastewaters, high salinity wastewaters were found to give an adverse effect to the granulation processes. Accumulation of Na^+ at high concentrations produced weaker and fluffy granules endangering the applicability of the mentioned sludge bed systems. In this thesis, research was conducted to investigate the mechanisms of destabilization of the granules at high Na^+ concentrations, while trying to improve the granules' properties. **Chapter 1** gives an overview of high salinity wastewaters, the application and the bottlenecks of anaerobic wastewater treatment (AnWT) technology under extreme conditions. Focus is given to the granulation process as a key factor in the operation of high rate anaerobic reactors. Indeed, it is a complex process that involves physicochemical as well as biological mechanisms. A short overview of the previous research on anaerobic wastewater treatment processes for high salinity wastewaters is discussed, followed by granulation theories and processes. Referring to the imbalance in the monovalent to divalent cation ratio, the Ca^{2+} augmentation approach is discussed in this chapter as a tool to establish a favourable ratio for the required granulation process. The chapter also discusses the need for extracellular polymeric substances (EPS) production, depending on the types of substrates, as a major factor for a successful granulation process. Specifically for high Na^+ concentrations, also the importance of K^+ and more specifically the K^+/Na^+ ratio is discussed as a control regulator to alleviate the negative effects of Na^+. Finally, the review discusses microbiological aspects related to the anaerobic treatment of high salinity wastewaters such as the adaptation of sludge to high Na^+ concentrations and the presence of halophilic/halotolerent microorganisms and their application in anaerobic wastewater treatment.

In **Chapter 2**, the effects of high Na^+ concentrations on methanogenic sludge bed reactor systems were investigated. In three different UASB reactors a rapid acclimation to 5 or 15 g Na^+/L was observed, showing satisfactory results for a period of 100 days, when the reactors were fed with a mixture of acetate, gelatine and ethanol. Loading rates up to at least 18 kg COD/m^3.d gave a good COD removal performance and the cultivated sludge showed high specific methanogenic activities (SMA) on acetate, propionate, butyrate and H_2 compared to the inoculated granules. Remarkably, only the reactor which was operated at a lower COD (5 instead of 50 g/L) exhibited severe washout, probably due to the much shorter HRT that was applied, i.e. 12 hours compared to 120 hours. Interestingly, the SMA of the biomass that washed out from this reactor showed considerably higher SMAs than the washed-out biomass from the other reactors. The performance of the granular sludge systems in this study shows the appropriateness of anaerobic inocula, pre-grown under saline conditions for the anaerobic treatment of high salinity wastewater.

In **Chapter 3** the production of EPS in UASB reactor systems that were operated under high salinity conditions was investigated. Four different UASB reactors were operated at loading rates up to 22 kg COD/m^3.d with different acetate:gelatine:starch ratios as the substrate. Re-

actors were fed with partially acidified and fully acidified substrates, i.e. PAS and FAS respectively, and Na^+ concentrations of either 10 or 20 g Na^+/L. One of the reactors additionally received 1 g of Ca^{2+}/L. All four reactors showed a good performance with COD removal efficiencies exceeding 90 %. Proteins were the dominant EPS and the PAS-fed granules gave much higher EPS concentrations than FAS-fed granules. However, the proteins concentration was found not dependent on the Na^+ concentration in the feed. Interestingly, the granules from the reactors which were operated at a Na^+/Ca^{2+} ratio of 770 contained more polysaccharides than granules from reactors operated at a high Na^+/Ca^{2+} ratio of 1540. SEM images showed that that PAS-fed granule had a smoother granule surfaces than FAS-fed granule. PAS-fed granules also were considerably larger than FAS-fed granules. When the influent contained additional Ca^{2+}, clear cracks or fissures could be observed on the surface of the granules. Na^+ concentrations of 10 g/L seemed to increase the granule size, which may have been caused by swelling of the EPS matrix. Shear tests indicated that PAS-fed granules were stronger than the FAS fed granules and that Ca^{2+} addition had a positive effect on granule strength. The calcium content of these granules also was higher with 150 mg/g TSS compared to 60 mg/g TSS in the other reactors. Batch tests at high Na^+ concentrations confirmed calcium leaching from the granules. When granular sludge was exposed to 20 g Na^+/L in batches, leaching of Ca^{2+} from granules took place with a maximum obtained after 10 days. The calcium content of the granules decreased from approximately 85 to 52 mg/g TSS. Therefore, the highest Na^+ concentrations resulted in the weakest granules.

Chapter 4 describes the results of batch incubation studies in which anaerobic granular sludge was augmented with 0.3 g Ca^{2+}/L at Na^+ concentrations of 20 g/L. Experiments followed the previously described results of Chapter 3 showing that high Na^+ concentrations caused Ca^{2+} leaching from anaerobic granules. Extensive SEM-EDX and SEM-BSE measurements confirm leaching of calcium from the granules when these are exposed to higher Na^+ concentrations. Moreover Ca^{2+} additions seemed to indeed maintain the Ca^{2+} content of granular sludge.

Initial attachment of microorganism is very important for the development of granules and biofilms. Therefore, biofilm studies were conducted and explained in **Chapter 5**. Four different biofilm reactors with a non-woven carrier material were exposed to different Na^+ concentrations (10 or 20 g/L) and inoculated with crushed granules. Acetate was used as the substrate. One of the reactors received 0.3 g Ca^{2+}/L and another one 0.7 g K^+/L. The reactors were operated as sequencing batches. The reactor fed with K^+ gave the best performance. Lower salinity (10 compared to 20 g Na^+/L) improved the performance and the reactor which received calcium gave the worst results. This finding contradicts with previous experiments in Chapter 3. However, the situation in the biofilm reactors is completely different because the biofilms had to form from scratch. FISH-CLSM images revealed no significant visible differences in microbial coverage (i.e. bacteria and archea) of the non-woven fabric, except for the calcium enriched reactor. After 40 days of operation, it was clearly observed that 20 g Na^+/L does not prevent the initial microbial attachment under anaerobic conditions. From 16 sRNA DGGE measurements it was shown that the biofilms had a similar population and that this population did not change very much in time. The Archea were related to *Methanosaeta harundinacae* (acetoclastic), *Methanolinea tarda* and *Methanobacterium subteraaneum* (both hydrogenotrophic). Apparently these species can easily adapt to high salinity. However, the biofilms in the reactors did not show any of the known acetate-oxidizing bacteria that are expectedly needed for the production of H_2 from acetate as source for the growth of the found

hydrogenotrophic Archea. Therefore, it is assumed that H_2 leakage by acetoclastic methanogen explain their presence in the reactor.

Four identical UASB reactors treating concentrated wastewaters (10 – 30 g COD/L) were operated at 20 g Na^+/L and in detail described in **Chapter 6**. PAS and FAS substrates were fed to different reactors to compare the effect of different organic substrates on granule activity, stability and growth. The effect of calcium augmentation on anaerobic granules' properties was studied by feeding two of the reactors with additional calcium at a concentration of 0.3 g Ca^{2+}/L. A beneficial effect of potassium was demonstrated in Chapter 5 and it therefore was added to all the reactors, at a concentration of 0.7 g K^+/L. The treatment performance of the reactors was compared during a period of 120 days at increasing volumetric organic loading rates (OLRs). Physicochemical and microbiological properties of the anaerobic granules were determined and discussed. The results showed that high COD removal efficiencies are possible at 20 g Na^+/L, up to an organic loading rate of at least 14 g COD/L.d. At a loading rate of 25 g COD/L.d the performance and stability of all reactors deteriorated. There were indications that calcium augmentation had a positive effect on biomass retention, but this could not be further quantified. The microbial assays gave similar results as in Chapter 5. Compared to the inoculum, bacterial diversity in FAS-fed granules did not change significantly but was changed considerably in PAS-fed granules. Complex organic PAS feed resulted in more complex bacterial populations that were not related to archea. The bacterial presence of a dominant phylotype, belonging to the family of *Marinilabiliaceae* and specifically *Alkaliflexus imshenetskii* was evidenced. Members of *Marinilabiliaceae* are capable of degrading polymeric substances such as starch and gelatine. The dominant archeal species in the reactors was related to *Methanosaeta harundinacea*. *Methanosaeta* have been found to play a major role in granulation.

In **Chapter 7** the results of this research are discussed in a general context. Directions for further research are presented focussing on the increase in biomass activity and biomass retention in high salinity wastewaters by improving the adaptability of microbes and the anaerobic sludge granulation process.

Samenvatting

Anaerobe slibbed reactor systemen, zoals de UASB ('Upflow Anaerobic Sludge Bed') en EGSB ('Expanded Granular Sludge Bed') reactoren zijn de meest toegepaste hoogbelaste reactor systemen voor anaerobe behandeling van afvalwater. Het succes van beide systemen heeft de houding ten aanzien van afvalwaterbehandeling veranderd, met als intrinsieke onderdelen het terugwinnen van energie, verminderen van de spuislibproductie en het lage ruimtebeslag. Echter, als gevolg van het uitbreiden van het toepassingsgebied van UASB reactoren, bleek de hoge zoutconcentratie van sommige afvalwaters een negatief effect te hebben op de korrelvorming. Accumulatie van natrium (Na^+) tot hoge concentraties resulteerde in zwakkere en minder compacte korrels, waardoor de toepasbaarheid van de slibbed systemen belemmerd werd. Dit proefschrift beschrijft het onderzoek naar de mechanismen achter de destabilisatie van de korrels bij hoge natrium concentraties en de pogingen die gedaan werden om de eigenschappen van de korrels te verbeteren.

Hoofdstuk 1 geeft een overzicht van afvalwaters met een hoge zoutconcentratie en toepassingen en belemmeringen van anaerobe afvalwaterbehandeling technologieën onder extreme omstandigheden. De nadruk ligt op het korrelvormingsproces als de sleutelfactor voor het bedrijven van hoogbelaste anaerobe reactoren. Korrelvorming is een complex proces met zowel fysisch-chemische als biologische mechanismen. Een kort overzicht van voorgaand onderzoek naar anaerobe zuiveringsprocessen voor hoog zoute afvalwaters wordt bediscussieerd, gevolgd door theorieën over korrelvorming en de processen die hierbij een rol spelen. Met betrekking tot het uit balans zijn van de ratio van monovalente en bivalente kationen, wordt de toevoeging van Ca^{2+} in dit hoofdstuk besproken als een mogelijkheid om de juiste ratio te bereiken voor het korrelvormingsproces. Tevens wordt in dit hoofdstuk de noodzaak van EPS ('Extracellular Polymeric Substances') besproken, afhankelijk van het type substraat, als een bepalende factor voor succesvolle korrelvorming. Specifiek voor hoge Na^+ concentraties, wordt ook het belang van K^+ besproken en in het bijzonder de K^+/Na^+ verhouding als een parameter voor het ongedaan maken van de negatieve effecten van Na^+. Tenslotte gaat het overzicht in op de microbiologische aspecten die gerelateerd zijn aan anaerobe behandeling van hoog zoute afvalwaters waaronder de aanpassing van slib aan de hoge Na^+ concentratie, de aanwezigheid van halofiele/halotolerante micro-organismen en de toepassing hiervan in anaerobe afvalwater behandeling.

In **Hoofdstuk 2** worden de effecten onderzocht van hoge Na^+ concentraties in een methanogene slibbed reactor. In drie verschillende UASB reactoren werd een snelle aanpassing aan 5 of 15 g Na^+/L waargenomen, met een acceptabel resultaat over een periode van 100 dagen, waarbij de reactoren gevoed werden met een mengsel van acetaat, gelatine en ethanol. Een volumetrische belasting tot minstens 18 kg COD/m^3d gaf een goede COD verwijdering en het slib had een hoge specifieke methanogene activiteit (SMA) op acetaat, propionaat, butyraat en H_2, vergeleken met het originele entslib. Opmerkelijk was dat alleen de reactor met een lage influent COD concentratie (5 in plaats van 50 g/L) een fors verlies van slib met het effluent liet zien, mogelijk als gevolg van de veel kortere hydraulische verblijftijd (HRT) van 12 uur in plaats van 120 uur. De SMA van deze uitgespoelde biomassa had een aanzienlijk hogere SMA dan de uitgespoelde biomassa uit andere reactoren. De werking van de korrelslib systemen in deze studie lieten zien anaeroob entslib dat gegroeid is onder zoute condities, geschikt is voor de behandeling van hoog zout afvalwater.

In **Hoofdstuk 3** is de productie van EPS onderzocht in UASB reactor systemen onder hoge zoutconcentraties. Vier verschillende UASB reactoren werden bedreven met belastingen tot 22 kg COD/m^3d, met verschillende ratios acetaat:gelatine:zetmeel als substraat. De reactoren werden gevoed met partieel (PAS) of volledig (FAS) voorverzuurd substraat en bij een zoutconcentratie van 10 of 20 g Na^+/L. Aan één van de reactoren werd ook 1 g Ca^{2+}/L toegevoegd. Alle vier de reactoren behaalden een goede COD verwijderingefficiëntie van meer dan 90%. Eiwitten waren de dominante component van het EPS en de korrels gevoed met PAS substraat lieten een hogere EPS productie zien vergeleken met korrels gevoed met FAS substraat. De eiwit concentratie bleek echter niet afhankelijk te zijn van de Na^+ concentratie in het influent. Opmerkelijk was de hogere fractie polysacchariden in de korrels uit de reactoren die bedreven werden bij een Na^+/Ca^{2+} ratio van 770, vergeleken met de korrels uit de reactoren met een hoge Na^+/Ca^{2+} ratio van 1540. SEM ('Scanning Electron Microscopy') beelden lieten zien dat de PAS-gevoede korrels een gladder oppervlak hadden dan de FAS-gevoede korrels. Met extra Ca^{2+} in het influent werden duidelijke breuken en spleten gezien aan het korreloppervlak. Na^+ concentraties van 10 g/L leken tot een toename van de korrelgrootte te leiden, mogelijk veroorzaakt door het opzwellen van de EPS matrix. Afschuifspanningstesten gaven aan dat PAS-gevoede korrels sterker waren dan FAS-gevoede korrels en dat toevoeging van Ca^{2+} een positief effect had op de sterkte van korrel. Het calcium gehalte van deze korrels was hoger met 150 mg/g TSS, vergeleken met 60 mg/g TSS in de andere reactoren. Batch testen bij een hoge Na^+ concentratie bevestigden dat calcium uit de korrels geloogd werd. Bij blootstelling van korrelslib aan 20 g Na^+/L in de batch testen, vond het maximum in het uitlogen van Ca^{2+} plaats na 10 dagen. Het calciumgehalte van de korrels nam af van ongeveer 85 tot 52 mg/g TSS. Hierdoor resulteerde de hoogste Na^+ concentraties in de zwakste korrels.

Hoofdstuk 4 beschrijft de resultaten van batch incubatie experimenten waarbij 0.3 g Ca^{2+}/L aan anaeroob korrelslib werd toegevoegd bij een Na^+ concentratie van 20 g/L. De resultaten bevestigden de resultaten uit Hoofdstuk 3, namelijk dat een hoge Na^+ concentratie leid tot uitlogen van Ca^{2+} uit de anaerobe korrels. Uitgebreide SEM-EDX ('Energy-Dispersive X-ray spectroscopy') en SEM-BSE ('Elastic (non-Rutherford) Backscattering Spectrometry') metingen bevestigden het uitlogen van calcium uit de korrels na blootstelling aan hogere Na^+ concentraties. Bovendien leek de toevoeging van Ca^{2+} inderdaad het Ca^{2+} gehalte van de korrels te behouden.

De eerste aanhechting van micro-organismen is heel belangrijk in de vorming van korrels en biofilmen. Om deze reden werden experimenten met biofilmen uitgevoerd en uitgelegd in **Hoofdstuk 5**. Vier verschillende biofilm reactoren met een non-woven dragermateriaal werden bedreven bij verschillende zoutconcentraties (10 of 20 g/L) en geënt met opgebroken korrelslib. Acetaat was het substraat voor alle reactoren. Aan één van de reactoren werd 0.3 g Ca^{2+}/L toegevoegd en aan een tweede reactor 0.7 g K^+/L. De reactoren werden bedreven als sequencing batch reactoren. De reactor met toegevoegd K^+ behaalde het beste resultaat. Een laag zoutgehalte (10 vergeleken met 20 g Na^+/L) verbeterde het zuiveringsrendement en de reactor met toegevoegd calcium behaalde het slechtste resultaat. Deze bevinding is in tegenstelling tot de eerdere resultaten uit Hoofdstuk 3. De omstandigheden in de biofilm reactoren zijn echter compleet anders, aangezien de biofilm vanuit het niets gevormd moest worden. FISH-CLSM ('Fluorescence *In Situ* Hybridization - Confocal Laser Scanning Microscopy') beelden toonden geen significante verschillen in de microbiële begroeiing (bacteriën en archea) van het non-woven materiaal, behalve in de reactor met toegevoegd calcium. Na 40 dagen be-

drijven van de reactoren, bleek duidelijk dat 20 g Na^+/L de initiële aanhechting van micro-organismen onder anaerobe condities niet verhinderde. Uit 16 sRNA DGGE ('Denaturing Gradient Gel Electrophoresis') metingen bleek dat de biofilmen een vergelijkbare populatie bevatten en dat deze populatie niet veel veranderde in de tijd. De archea waren verwant aan *Methanosaeta harundinacae* (acetoclastisch), *Methanolinea tarda* en *Methanobacterium subteraaneum* (beide hydrogenotroof). Blijkbaar kunnen deze soorten zich eenvoudig aanpassen aan een hoge zoutconcentratie. De biofilmen in de reactoren bleken echter geen van de bekende acetaat oxiderende bacteriën te bevatten, waarvan verwacht is dat ze nodig zijn voor de productie van H_2 uit acetaat, en daarmee het substraat voor de gevonden hydrogenotrofe archea. Er wordt daarom aangenomen dat een lekkage van H_2 uit de acetoclastische methanogenen de aanwezigheid van de hydrogenotrofe methanogenen in de reactor verklaard.

Vier identieke UASB reactoren die geconcentreerd afvalwater (10-30 g COD/L) behandelden bij een zoutconcentratie van 20 g Na^+/L worden in detail beschreven in **Hoofdstuk 6**. PAS en FAS substraat werd gevoed aan de verschillende reactoren om het effect van diverse organische substraten op de korrelactiviteit, -stabiliteit en –groei te bestuderen. Het effect van calcium toevoeging op de eigenschappen van anaerobe korrels werd bestudeerd door twee reactoren met 0.3 g Ca^{2+}/L te voeden. Een positief effect van kalium was aangetoond in Hoofdstuk 5 en daarom werd er 0.7 g K^+/L toegevoegd aan alle reactoren. Het verwijderings rendement van de reactoren werd vergeleken gedurende een periode van 120 dagen bij een toenemende organische belasting. Fysisch-chemische en microbiologische eigenschappen van de anaerobe korrels werden bepaald en bediscussieerd. De resultaten lieten zien dat hoge COD verwijderingspercentages mogelijk zijn bij 20 g Na^+/L, tot een organische belasting van ten minste 14 g COD/Ld. Bij een belasting van 25 g COD/Ld verslechterde het verwijderingspercentage en de stabiliteit van alle reactoren. Er werden aanwijzingen gevonden dat een toevoeging van calcium een positief effect had op retentie van biomassa, maar dit kon niet gekwantificeerd worden. De microbiologische testen resulteerden in vergelijkbare resultaten als in Hoofdstuk 5. Vergeleken met het inoculum, veranderde de microbiële diversiteit in de FAS-gevoede korrel niet significant, maar werd een aanzienlijke verandering waargenomen in PAS-gevoede korrels. Complex organisch PAS substraat resulteerde in een complexere microbiële populatie die niet gerelateerd was aan archea. De aanwezigheid van een dominant bacterieel phylotype werd aangetoond, behoord tot de familie *Marinilabiliaceae* en in het bijzonder *Alkaliflexus imshenetskii*. Leden van *Marinilabiliaceae* zijn in staat polymeerverbindingen af te breken, zoals zetmeel en gelatine. *Methanosaeta* bleken een centrale rol te spelen in korrelvorming.

In **Hoofdstuk 7** worden de resultaten uit dit proefschrift in een breder perspectief bediscussieerd. Richtingen voor verder onderzoek worden gepresenteerd, waarbij de nadruk ligt op activiteit van de biomassa en de retentie van biomassa in hoog zoute afvalwaters door het verbeteren van de aanpassingsmogelijkheden van de micro-organismen en het korrelvormingsproces van anaerobe biomassa.

Nomenclatures

Ca^{2+}	calcium
$CaCO_3$	calcium carbonate
CH_4	methane
K^+	potassium
N_2	nitrogen gas
Na^+	sodium
t	time
V	volume

Abbreviations

BSE	back scattered electron
CLSM	confocal laser scanning electron microscope
COD	chemical oxygen demand
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EDX	energy dispersive x-ray
EPS	extracelllar polymeric substance
FAS	fully acidified substrate
FISH	fluorescence *in situ* hybridization
HRT	hydraulic retention time
ICP-OES	inductively coupled plasma - optical emission spectrometry
OLR	organic loading rate
PAS	partly acidified substrate
PCR	polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscope
SMA	specific methanogenic activity
TSS	total suspended solids
UASB	upflow anaerobic sludge blanket
VFA	volatile fatty acids
VSS	volatile suspended solids

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Wageningen…,
turhutangnya aku tentang budi, tentang janji,
yang masih aku belum sudahi

Shahrul Ismail
Wageningen, December 2013.

About the author

Shahrul Ismail was born on 9th May 1980 in Kelantan, Malaysia. He finished the secondary school in 1997 from MARA Junior Science College. In 2001, he accomplished the Environmental Technology degree at University Science of Malaysia (USM) with Honors. The final year thesis was about, upgrading efficiency study of sedimentation tank at Kelantan water treatment plant. He continued his master of engineering (MEng.) from University Technology of Malaysia (UTM). In 2002, he obtained his master degree. MSc thesis research was conducted on treating domestic wastewaters by using constructed wetlands. In 2004, directly after obtained master degree, he was admitted to joining University Malaysia Terengganu (UMT) as a lecturer. In November 2006, he was granted a SLAB scholarship by the Ministry of Higher Education (MOHE), Malaysia to carry out his PhD research at the Sub-Department of Environmental Technology, Wageningen University, which resulted in this thesis. From November 2011, Shahrul proceed along his academician career at the Department of Engineering, Faculty of Science and Technology, University Malaysia Terengganu. His main task in the future is to anticipate on the anaerobic technology as a sustainable approach, especially in treating extreme condition wastewaters.

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Management and Didactic Skills Training

- Thesis supervision of four MSc students
- Co-organizing Environmental Technology Department trip to Turkey

Oral Presentations

- *Effects of high salinity wastewater on methanogenic sludge bed systems.* IWA International Conference on Biofilm Technologies, 8-10 January 2008, NTU, Singapore
- *EPS in high salinity wastewater with defined substrate properties.* SENSE Symposium Novel and Cost Effective Technology Symposium, 4 September 2008, Wageningen
- *Attachment and growth of anaerobic consortia in high salinity wastewaters.* International Congress - Microbial Biotechnology for Development, 2-5 Nov 2009, Marrakech , Morocco
- *Effects of high salinity on extracellular polymeric substances production and function in methanogenic granular sludge.* SENSE symposium Innovative Technique for a Sustainable Environment, 19-20 February 2009, Wageningen
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- *Anaerobic granular sludge properties at high salinity conditions.* 11th International Annual Symposium on Sustainability Science and Management (UMTAS 2012), 9-11 July 2012, Terengganu, Malaysia
- *Attachment of anaerobic consortia in high salinity wastewaters.* Asia Water 2012 Conference, 27-29 March 2012, Kuala Lumpur, Malaysia
- *Initial immobilization of anaerobic consortia under high salinity conditions.* Anaerobic Digestion Asia, 25 April 2012, Bangkok , Thailand
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Front cover thesis: Scanning electron microscopis picture of anaerobic granule