

THESIS FOR THE DEGREE OF LICENTIATE OF ENGINEERING

Microbial Community Assembly during Aerobic
Granulation

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Cover:

FISH-CLSM image from an aerobic granule cryosection at 400 × magnification. Blue, total bacteria; green: ammonia oxidizing bacteria; red: predatory bacteria *Bdellovibrio* spp.

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ABSTRACT

Environmental deterioration together with the increasingly restrictive legislation of water quality standards have led to a demand for compact, efficient and less energy consuming technologies for wastewater treatment. Aerobic granular membrane bioreactors (AGMBRs) combine the advantages of aerobic granular sludge and membrane filtration. Although recent studies have shown that AGMBRs are promising, research is needed to develop these systems. One important aspect is the development and stability of aerobic granules in the reactor. The objective of this thesis is to unravel the ecological mechanisms behind the granulation process and the resulting granular structure.

The impact of the washout dynamics applied in sequencing batch reactors (SBRs) on the bacterial community, the microbial spatial organization in granules and the reproducibility of the granulation process were analyzed. High throughput sequencing was used to analyze the microbial community structure and dynamics during granulation. Fluorescence in-situ hybridization (FISH) analysis was performed to study the microbial spatial distribution. Results showed that the bacterial community composition of granules and the biomass in the effluent from three parallel SBRs subjected to high washout conditions and different organic loading was very similar but not identical. Retention ratios predicted the spatial location of the taxa in the granules. Bacteria having lower relative abundance in the effluent were located in the granular interior whereas bacteria growing on the granular surface were more susceptible to erosion. Using FISH analyses of intact granules, ammonia oxidising bacteria (AOB) were found in the inner locations of the granules indicating that both oxygen and ammonia were transported across/into the granule allowing aerobic metabolism also in the interior. Interestingly, the predatory bacteria *Bdellovibrio* sp. was found inside the granules and seemed to be preferentially attacking AOB. Moreover, the reproducibility analysis of three replicate SBRs showed some statistical differences in the reactor performance and in the microbial community diversity and structure. Both deterministic and stochastic processes seemed to be involved in the microbial community assemblage during granulation. Biodiversity decreased due to habitat specialization and competitive exclusion during the acclimatization of the sludge to the reactor conditions. When the washout dynamics were stronger the community assemblage was niche-oriented and once the selection pressures decreased, stochastic processes became more evident, especially for intermediate and rare species. *Acinetobacter* sp., *Comamonadaceae* and *Rhodocyclaceae* families together with the polymer producers *Thauera* sp., *Flavobacterium* sp. and the family *Xanthomonadaceae* seemed to have important roles during the first stages of granulation.

Keywords: aerobic granular sludge, membrane bioreactors, sequencing batch reactors, granulation, washout dynamics, reproducibility, microbial community dynamics, fluorescence in-situ hybridization.

PREFACE

This licentiate thesis is based on the research performed in the Division of Water Environment Technology (Chalmers University of Technology) and the Department of Chemistry and Molecular Biology (University of Gothenburg) between May 2014 and February 2017 under the supervision of Britt-Marie Wilén, Frank Persson, Oskar Modin and Malte Hermansson. This research has been funded by the Swedish Research Council FORMAS.

This licentiate thesis is based on the appended manuscripts referred in the text as papers I-V:

- i. **Liébana, R.**, Modin, O., Persson, F., Wilén BM. Integration of aerobic granular sludge and membrane bioreactors for wastewater treatment. *Submitted to Critical Reviews in Biotechnology*.
- ii. Szabó, E., **Liébana, R.**, Hermansson, M., Modin, O., Persson, F., Wilén BM. Comparison of the bacterial community composition in the granular and the suspended phase of sequencing batch reactors. *Manuscript*.
- iii. **Liébana, R.**, Szabó, E., Persson, F., Suárez, C., Modin, O., Hermansson, M., Wilén BM., (2016). Complex architecture allows aerobic metabolism at depth in granules. IWA Microbial Ecology in Water Engineering & Biofilms, Copenhagen, Denmark.
- iv. **Liébana, R.**, Szabó, E., Persson, F., Modin, O., Hermansson, M., Wilén BM., (2017). *Bdellovibrio* sp. predation on aerobic granular sludge. FEMS 7th Congress of European Microbiologists, Valencia, Spain. *Submitted*.
- v. **Liébana, R.**, Szabó, E., Persson, F., Modin, O., Hermansson, M., Wilén BM. Reproducibility of microbial communities in parallel sequencing batch reactors during aerobic granulation. *Manuscript*

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Liébana, R., Szabó, E., Modin, O., Persson, F., Suárez, C., Hermansson, M., Wilén BM., (2015). Stability of nitrifying granules exposed to water flux through a coarse pore mesh. IWA Nutrient Removal and Recovery, Gdansk, Poland.

Liébana, R., Arregui, L., Santos, A., Murciano, A., Marquina, D., Serrano, S. (2016). Unravelling the interactions among microbial populations found in activated sludge during biofilm formation. *FEMS Microbiology Ecology*, 92(9), fiw134.

Szabó, E., **Liébana, R.**, Hermansson, M., Modin, O., Persson, F., Wilén. BM. (2017) Microbial population dynamics and ecosystem functions of aerobic granular sludge in sequencing batch reactors operated at different organic loading rates. *Submitted to Frontiers in Microbiology*.

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Raquel

LIST OF ACRONIMS AND ABBREVIATIONS

AGMBR: aerobic granular membrane bioreactors
AOB: ammonia-oxidizing bacteria
CAP: constrained analysis of proximities
CLSM: confocal laser scanning microscopy
COD: chemical oxygen demand
DNA: deoxyribonucleic acid
EPS: extracellular polymeric substances
FISH: fluorescence in-situ hybridization
MBR: membrane bioreactors
NMDS: non-metric multi-dimensional scaling
NOB: nitrite-oxidizing bacteria
OTU: operational taxonomic units
PAO: polyphosphate-accumulating organism
GAO: glycogen-accumulating organism
PCR: polymerase chain reaction
RNA: ribonucleic acid
SBR: sequencing batch reactor
TN: total nitrogen
TOC: total organic carbon
WWTP: wastewater treatment plant

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1. Introduction

Human activities generate urban and industrial wastewater which causes deterioration of aquatic environments due to the release of pollutants, such as organic matter and nutrients, if discharged un-treated. Moreover, an important proportion of wastewater has a faecal origin, being a potential risk due to the presence of pathogenic microorganisms and viruses, which can lead to epidemic outbreaks and other biological hazards (Seviour & Nielsen, 2010). In this context, one of the most important biotechnological applications in an urban society is wastewater treatment.

1.1. Wastewater treatment

1.1.1. Wastewater treatment historical development

During the last centuries, the rapid social and industrial development along with the rapid population growth, especially in urban areas, has led to the need to treat the generated wastewater. Despite the historical evidence of wastewater collection by Babylonians and the Assyrians, it was not until the industrial revolution that political actions were taken to organize wastewater treatment. As the population grew in the cities during this period, there were important sanitary problems due to the untreated wastewater, mainly due to the introduction of water closets. In 1848, the National Public Health Act in England forced all houses to have some method of wastewater collection. After this enforcement, during the 19th century, several wastewater treatment methods were developed and in 1898 new standards for treated wastewater were set. Since then numerous methods and systems for wastewater treatment have emerged with the goal to treat the largest possible volumes of wastewater in a fast and efficient way (Seviour & Nielsen, 2010, Bitton, 2011, Jenkins *et al.*, 2014).

During the 20th century, eutrophication of water bodies had become a great problem and research showed that excess nitrogen and phosphorus discharge to recipients from various sources, including wastewater treatment, was identified as the cause. Since then, the biological elimination of nutrients from wastewater has been developed and there are numerous processes and different configurations for wastewater treatment to achieve better efficiency in nitrogen and phosphorus removal (Jenkins *et al.*, 2014).

1.1.2. The water purification process in a conventional wastewater treatment plant

Wastewater is collected and then treated in wastewater treatment plants (WWTPs) where the pollutants are reduced to the limits set by national regulations and guidelines. The most important pollutants in wastewater are biodegradable organic compounds, volatile organic compounds, suspended solids, nutrients (nitrogen and phosphorus), pathogens, heavy metals, recalcitrant compounds and xenobiotics (Bitton, 2011).

Wastewater is treated in different steps (Figure 1). First it is subjected to a preliminary treatment where coarse materials and debris are eliminated. Then, sedimentable suspended solids are removed in sedimentation tanks during the primary treatment. Next, in the secondary treatment

the wastewater is introduced into the biological reactor(s) were mainly dissolved organic matter, nutrients and pathogens are removed and the sludge is thereafter separated from the treated water by sedimentation. Finally, as an optional additional step, the wastewater is regenerated during the tertiary treatment by the elimination of pathogens, suspended solids and other compounds that have not been eliminated in the previous stage, by the application of physical and chemical treatments such as membrane filtration or chlorination (Bitton, 2011).

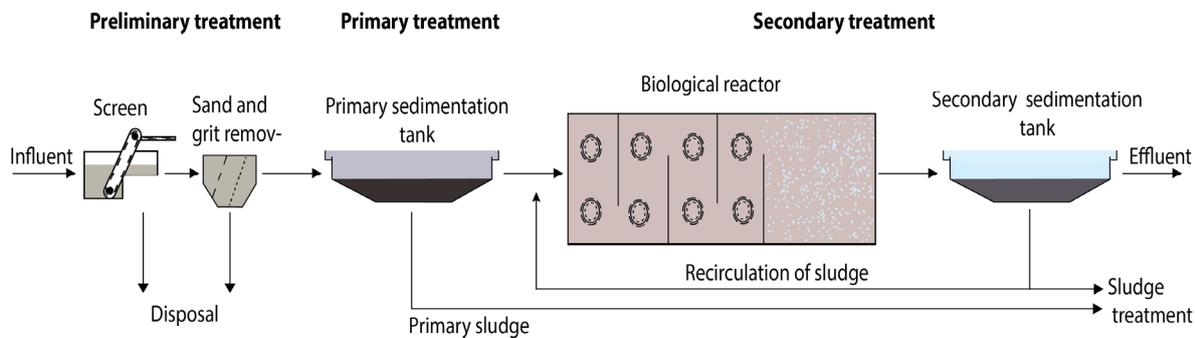


Figure 1. Schematic representation of a conventional wastewater treatment plant.

1.2. Biological treatment of wastewater

The biological treatment of wastewater is based on natural microbiological processes. Various conditions applied in the biological reactors create different selection pressures which in turn select for certain microorganisms whose activity remove the targeted contaminants from wastewater (Henze & Knovel, 2008). Generally, the biological wastewater treatment is classified into suspended growth and fixed films processes and they can be aerobic and/or anaerobic/anoxic. In the suspended growth processes, the microorganisms grow in suspension, in a more or less aggregated state, in direct contact with the wastewater. Activated sludge, oxidation ponds, anaerobic digestion, sequencing batch reactors and membrane bioreactors are examples of suspended growth processes. Fixed film processes use an inert support material inside the biological reactor on which the microorganisms grow as biofilms and the substrates diffuses into the biofilm from the water phase. Trickling filters, rotating biological contactors, biological aerated filters, fluidised beds and moving bed biofilm reactors are fixed films processes. Besides, both types of processes can be combined into a hybrid process where both suspended biomass and biofilms coexist in the reactor (Stuetz & Stephenson, 2009).

1.2.1. The activated sludge process

The first and most widespread biological system for wastewater treatment is the activated sludge process. The activated sludge process was conceived by Edward Arden and William T. Lockett in 1914 (Arden & Lockett, 1914). The main idea was to retain the accumulated suspended solids, containing microorganisms, to “activate” and use these organisms as an inoculum for the subsequent treatment of the wastewater in an aerated bioreactor. With this procedure, biodegradable organics were removed much faster and complete nitrification was achieved. The activated sludge process is now the most widespread system for wastewater treatment in the world (Bitton, 2011, Jenkins *et al.*, 2014).

In the activated sludge process, most of the microorganisms and other solids are organized into discrete units called flocs. The flocs are kept in suspension in an aerated tank to ensure the contact of microorganisms with the dissolved organic matter and nutrients available in wastewater. These pollutants are oxidized by the microbial metabolic activity and transformed into microbial biomass and CO₂ (Bitton, 2011). Subsequently, the treated water is separated from the sludge flocs by different methods. The most common method is through sedimentation in clarifiers allocated after the biological reactor (conventional treatment). A considerable fraction of the settled sludge is then recirculated to the biological reactor (Sheik *et al.*, 2014). The activated sludge process has evolved and experienced many operational and design changes to improve the effectiveness and increase the flexibility of WWTPs (Seviour & Nielsen, 2010, Jenkins *et al.*, 2014). Depending on the process design, organic carbon, but also nutrients (nitrogen and phosphorous) can be biologically removed. To achieve this, biological tanks/reactors with different aeration are alternated in several different configurations. By providing aerobic, anoxic and anaerobic conditions in the different tanks, the ideal environment is created so the targeted microorganisms grow and therefore, metabolize these compounds (Bitton, 2011).

1.2.2. Microbiology of wastewater treatment

The contaminants of wastewater are removed through a food chain which is accelerated in the biological reactor. Bacteria, Archaea and to a minor extent fungi, remove the organic matter and nutrients which are dissolved in wastewater, obtaining energy and carbon for their own growth and maintenance. Then, protists and micrometazoa are the main predators in the food chain. Protists consume organic matter and feed mainly on bacteria and other protists, influencing the microbial population dynamics and contributing to pathogen removal. Metazoa predate on suspended bacteria and protists, hence contributing to the removal of suspended particles in the water. Virus (bacteriophages) and predatory bacteria also play an important role regulating the bacterial population (Figure 2) (Seviour & Nielsen, 2010, Bitton, 2011, Johnke *et al.*, 2014).

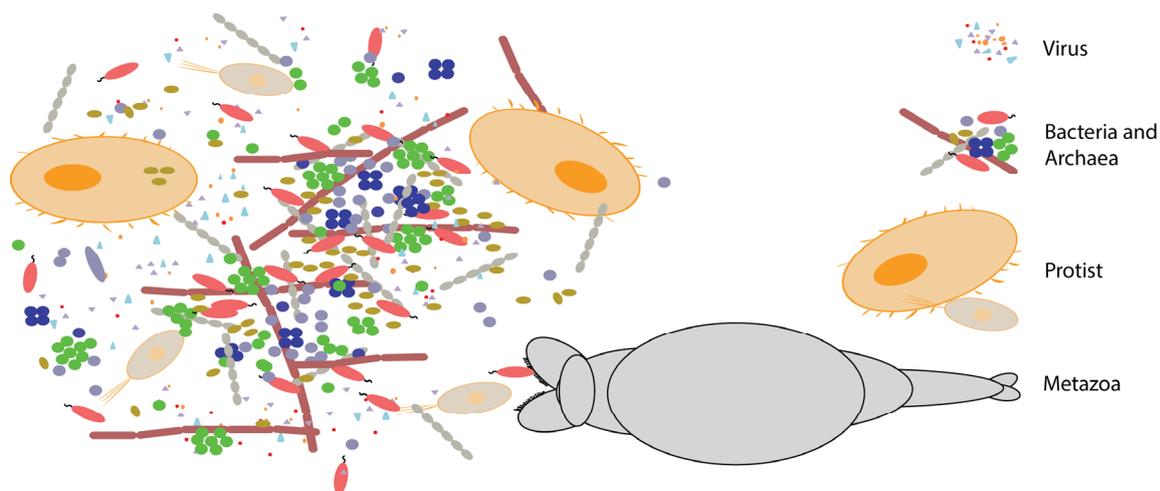


Figure 2. Most representative groups of microorganisms in the wastewater ecosystem.

Bacteria constitute the major fraction of the biomass, being the dominant group within the biological community in wastewater treatment systems. They belong mostly to the *Proteobacteria* phylum, but *Bacteroidetes*, *Chloroflexi*, *Planctomycetes* and *Actinobacteria* are also found in significant numbers (Wagner *et al.*, 2002, Seviour & Nielsen, 2010, Bitton, 2011). The predominant bacteria are chemoorganoheterotrophs responsible for the degradation and mineralization of organic compounds. These bacteria also produce polysaccharides and other polymeric compounds that facilitate the microbial aggregation (Bitton, 2011).

Autotrophic bacteria are also important components for wastewater treatment. Nitrifiers are aerobic chemolithoautotrophs, which are key organisms in the biological removal of nitrogen by oxidizing ammonium to nitrate. This is performed in a two-step process by different bacterial groups: ammonia-oxidizing bacteria (AOB) which oxidize ammonium to nitrite, and nitrite-oxidizing bacteria (NOB) which oxidize nitrite to nitrate (Schmidt *et al.*, 2003, Daims *et al.*, 2006). *Nitrosomonas* sp. and *Nitrospira* sp. are the main AOB representatives and members of the genera *Nitrobacter*, *Nitrococcus* and *Nitrospira* are the most common NOB. However, by the continuous use of molecular approaches, new players such as the NOB *Nitrotoga* sp. and new pathways involved in the nitrification process have been identified, as the complete oxidation of ammonia to nitrate, or comammox, by a single organism (Daims *et al.*, 2006, Sheik *et al.*, 2014, Daims *et al.*, 2015, Lucker *et al.*, 2015, van Kessel *et al.*, 2015). Nitrogen can also be oxidized via anaerobic ammonia oxidation (anammox) by chemolithoautotrophic bacteria belonging to the phylum *Planctomycetes*, which anaerobically oxidize ammonium and reduce nitrite producing dinitrogen gas (Daims *et al.*, 2006).

Denitrifying bacteria are also key players in wastewater treatment since they reduce oxidized nitrogen compounds like nitrite or nitrate to dinitrogen gas (Schmidt *et al.*, 2003). It is not clear which populations are dominant since ability for denitrification is spread among many different bacterial lineages and the populations change depending on the treatment plant and wastewater process. The genera *Aquaspirillum*, *Azoarcus*, *Thaurea* and polyphosphate accumulating organisms (PAOs) have been described as dominant denitrifiers (Sheik *et al.*, 2014). PAOs are also involved in the biological elimination of phosphorus by the intracellular accumulation of polyphosphate. The uncultured and unclassified genus *Candidatus Accumulibacter phosphatis* belonging to the *Betaproteobacteria* class has been reported to be the main organism responsible of phosphate accumulation (Daims *et al.*, 2006, Sheik *et al.*, 2014).

1.3. Wastewater treatment upgrade

The rapid population growth together with the social and industrial development has increased the need for new wastewater technologies. Moreover, the severe water scarcity which many countries face, makes reuse of wastewater a necessity. Therefore, many wastewater treatment plants have to be upgraded to achieve compact, efficient and less energy consuming treatment processes to meet the increasingly high standards of water effluent quality. Despite the substantial improvements accomplished, wastewater treatment still faces important limitations such as the high area requirements needed for the separation of the sludge and the treated water, the multiple biological tanks needed for nutrient removal, the need for more stable and efficient treatment processes, the high energy use and the considerable costs, among others.

Wastewater treatment using biofilm processes is becoming increasingly common. This is due to the high microbial diversity and density found in biofilms. The existence of different microhabitats in biofilms favours the coexistence of different microbial groups and increases the efficiency in the removal of various pollutants (Sperling & Knovel, 2007). Membrane filtration is also an important and increasingly used process that overcome the problems associated with the separation of the sludge from the treated water (van Loosdrecht & Brdjanovic, 2014, Iorhemen *et al.*, 2016). Moreover, the combination of different processes, such as membrane filtration and aerobic granular sludge, are being investigated to take advantage of the benefits of each technology and to overcome possible limitations.

1.3.1. Aerobic granular sludge

Granular sludge has received much attention since it was first reported (Lettinga *et al.*, 1980) and the cultivation process was optimized using sequencing batch reactors (SBRs) (Heijnen & Van Loosdrecht, 1998). Aerobic granular sludge is a technology that has competitive advantages compared to activated sludge processes due to excellent settling properties, compact structure, smooth surface, regular morphology, high microbial densities and activities, ability to withstand high organic and nitrogen loadings, and tolerance to toxic substances (Adav *et al.*, 2008, Show *et al.*, 2012). Aerobic granules are considered as suspended biofilms of microorganisms embedded in a matrix of extracellular polymeric substances (EPS). Polymers of polysaccharides, proteins, humic acids, nucleic acids and lipids constitute the EPS and the distribution, proportion and chemical composition of these polymers determine the physical characteristics of granules (Adav *et al.*, 2008, Seviour *et al.*, 2010). One of the most important features of aerobic granular sludge is the simultaneous nitrification, denitrification and biological phosphorus removal while degrading the organic carbon. This is possible due to the presence of oxygen and substrate gradients inside the granule where heterotrophic-, nitrifying, denitrifying-, PAOs and glycogen-accumulating organisms (GAOs) can coexist (Figure 3) (Adav *et al.*, 2008, Lee *et al.*, 2010, Gao *et al.*, 2011).

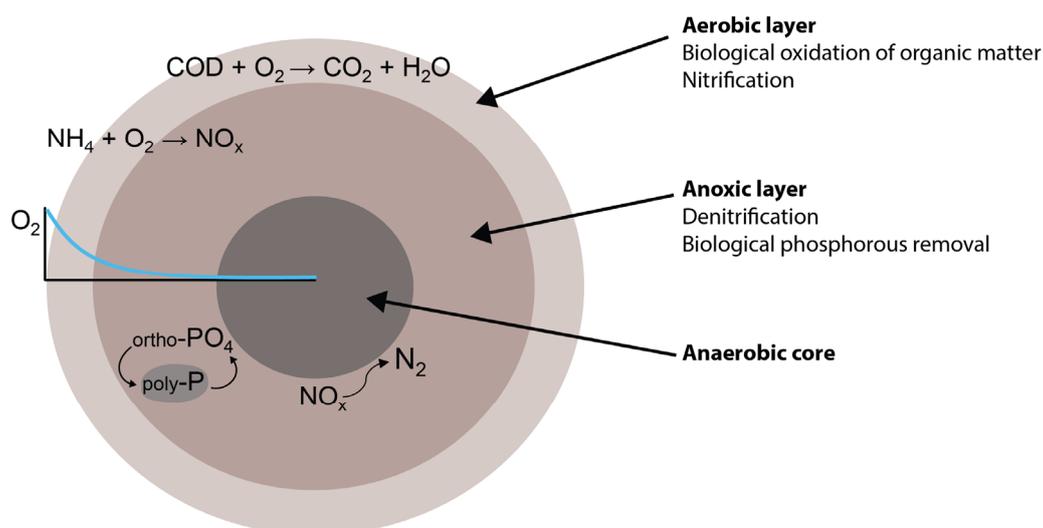


Figure 3. Structural representation of an aerobic granule and biological processes occurring inside.

The required conditions for cultivation of aerobic granules can be obtained in SBRs. In SBRs the reaction- and sedimentation steps take place in the same reactor at different times offering a substantial optimization of the conventional process, minimising the space and energy needs (Morgenroth & Wilderer, 1998, Jenkins *et al.*, 2014). SBRs operate in consecutive cycles that consist of several stages: filling, reaction, settling and withdrawal (Figure 4) (Morgenroth & Wilderer, 1998, Singh & Srivastava, 2011). The short settling time is one of the most important driving forces for granulation. When short setting times are applied, bacteria that lack the ability to aggregate will be washed out of the reactor whereas those which forms aggregates that settles fast enough will remain. High hydrodynamic shear forces are also important since they enhance the development of regular, round, dense and compact aerobic granules. This is provided by aeration rates high enough to erode the surface of the granules and to stimulate the bacterial production of EPS. Also, the shape of the reactor is important. Columns are often used where the aeration creates a circular flow and vortex forces enhance the aggregation of microorganism into round particles. Additionally, high height to diameter ratio and volume exchange ratio also ensure the washout of non-granulated biomass. Furthermore, feast-famine alternation and anaerobic feeding increases bacterial cell hydrophobicity, which accelerates microbial aggregation and creates the appropriate substrate- and oxygen gradients in the granule (de Kreuk & van Loosdrecht, 2004, Liu & Tay, 2004, Adav *et al.*, 2008, Lee *et al.*, 2010, Show *et al.*, 2012).

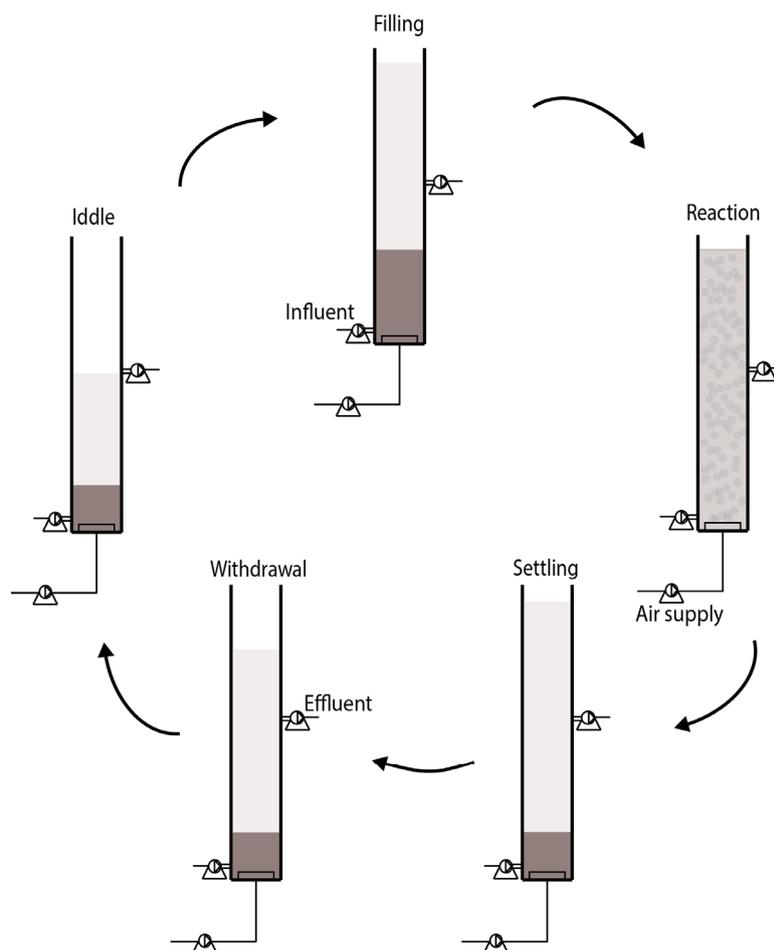


Figure 4. Stages of the SBR cycle.

1.3.2. Membrane bioreactors

Membrane bioreactors (MBR) are an alternative to the conventional activated sludge process, where the separation of the biomass and the treated water is achieved by filtration rather than by sedimentation. This technology therefore combines the biological treatment of wastewater and a physical separation method of the sludge and the effluent (Iorhemen *et al.*, 2016). Consequently, frequent problems displayed by the activated sludge process, such as poor sludge sedimentation, are solved. This makes the treatment of wastewater with high contents of biodegradable organic matter at high flow rates possible. Moreover, MBRs reduce WWTPs area footprint due to the elimination of the secondary sedimentation tanks. Other attractive advantages of the MBR technology are operational flexibility, low excess sludge production, long solid retention times which allow simultaneous nitrification/denitrification, high volumetric loading rates and short hydraulic retention times (Judd, 2008, Iorhemen *et al.*, 2016). But above all, the main advantage of MBRs is the high quality effluents obtained with the membrane system, making the MBR effluent suitable for many water reuse applications. The membrane is a barrier for suspended solids and microorganisms and depending on the pore size, even viruses and some organic- and inorganic components are retained in the reactor. These characteristics make MBR technology particularly suitable for areas of high environmental sensitivity where high quality effluents are needed, and for the treatment of complex industrial effluents (Radjenović *et al.*, 2008, Meng *et al.*, 2009, Neoh *et al.*, 2016).

However, fouling of the membranes is a main challenge of these systems, which decreases the overall efficiency of the process and raises the energy demand (Drews, 2010, Guo *et al.*, 2012). Membrane fouling is complex and depends on many factors. Membrane properties, operational mode, feed composition, physical-chemical characteristics of the mixed liquor and hydrodynamic conditions, are some of the factors that have direct implications on the fouling process (Drews, 2010, Guo *et al.*, 2012). Fouling results from the physical, chemical and biological interactions of the membrane surface with the existing foulants, which are distributed in particulate, colloidal and dissolved fractions. Membrane fouling is due to pore blocking and sludge cake deposition which depends on the foulant size and the pore diameter of the membrane. Fouling is also caused by the development of a biofilm on the membrane surface which is called biofouling (Meng *et al.*, 2009, Iorhemen *et al.*, 2016). Fouling is often classified as reversible and irreversible. In general terms, reversible fouling is defined as fouling which can be removed by physical and chemical cleaning methods such as relaxation, shear force, backflush, acids, bases, oxidants and disinfectants. Irreversible fouling is referred to as fouling which cannot be removed by the typical cleaning methods and results in pore blocking and chemical interactions in the membrane pore wall (Meng *et al.*, 2009, Wang *et al.*, 2014).

1.3.3. Aerobic granular membrane bioreactors

The integration of aerobic granular sludge and membrane bioreactors is a promising technology where a compact and efficient treatment process is obtained. The technology has so far shown many advantages, but at the same time many challenges remain to be solved before full-scale application. A comprehensive review of the available literature on the combination of aerobic granules and membrane filtration is presented in Paper I of this thesis.

2. Research motivation and scope of the thesis

Aerobic granular membrane bioreactors (AGMBRs) display several advantages compared to the aerobic granular sludge and the membrane technology alone. AGMBRs can accomplish a more efficient, compact and less energy demanding wastewater treatment processes with an increased treatment capacity. This technology offers improved filtration characteristics compared to conventional membrane bioreactors since granules reduce membrane fouling. However, the development of this process is challenging and there are several research needs to fulfill in order to scale up this technology. A key aspect is the development and maintenance of granules in AGMBRs. Therefore, detailed studies on the microbial mechanisms involved in the granulation of the activated sludge are imperative to understand the factors involved in this process. This will allow the development of improved granulation methods and to be able to operate granular sludge under continuous conditions.

The overall goal of the research project is to contribute to the development of AGMBRs, however, this particular thesis focuses on the ecology and development of aerobic granules. The objective of this thesis is to unravel the ecological mechanisms behind the granulation process and the resulting granular structure. This research objective was set after an extensive review of the available literature on the combination of aerobic granules and membrane filtration, which is appended as Paper I in this thesis. The AGMBR technology is young and at this stage, its development is directly dependent on fundamental research performed on the granulation mechanisms and the granular structure. In order to accomplish this, research on this topic was conducted and the results are presented as two manuscripts (II, V) and two conference papers (III and IV).

- Paper II. The bacterial community composition in granular and suspended phase during granulation was studied. The effect of the strong washout conditions on the microbial ecosystem applied in SBRs is not fully understood despite the fact that it is a main selective force applied in SBRs for aerobic granulation. The objective of this experiment was to elucidate the impact of the washout dynamics on the bacterial community in the granular and suspended phases to understand why some bacteria grow in the granules and other are washed out. Moreover, this study provides information of potential bacteria which could contribute to membrane fouling in AGMBRs, as washed out bacteria, not able to aggregate or eroded from the granule surface, will accumulate on the membrane surface.
- Paper III. This study examines the internal structure of aerobic granules. Despite being considered as suspended biofilms, granules are assumed to be a multilayer structure in conceptual and mathematical models, and pores and channels are normally disregarded. The objective of this experiment is to examine if oxygen penetrates in granules through channels and pores, allowing aerobic metabolism also in the deeper parts of the granules.
- Paper IV. In this study the predation activity of *Bdellovibrio* sp. on AOB in aerobic granules is presented. Predation is one of the main causes of bacteria mortality, having a big influence on the microbial community structure and functionality and it is poorly understood.

- Paper V. The results of the analysis of the reproducibility of microbial communities during granulation are presented in this manuscript. Despite the high interest in the aerobic granular technology showed by the scientific community, the granulation mechanisms are still poorly understood and the reproducibility of this process has not been tested. The objective of this experiment was to test if the microbial community structure and dynamics during granulation is reproducible between parallel reactors. The results of this study help us to understand the ecological factors involved in the microbial community assemblage during the granulation of activated sludge into granules.

3. Methods

3.1. Experimental design

3.1.1. Retention and washout dynamics of the bacterial community during aerobic granulation in SBRs (Paper II)

To examine the impact of washout dynamics on the bacterial community during aerobic granulation, the microbial composition of three SBRs, R1, R2 and R3, was evaluated for 84 days both in the granular phase and the effluent. The settling time was gradually decreased as shown in Figure 5. The reactors were fed with a 1:1 mixture of real and synthetic wastewater with different organic loading rates (kg COD m⁻³d⁻¹: 3.7 in R1, 1.9 in R2 and 0.9 in R3) and a nitrogen load of 0.22 kg COD m⁻³d⁻¹.

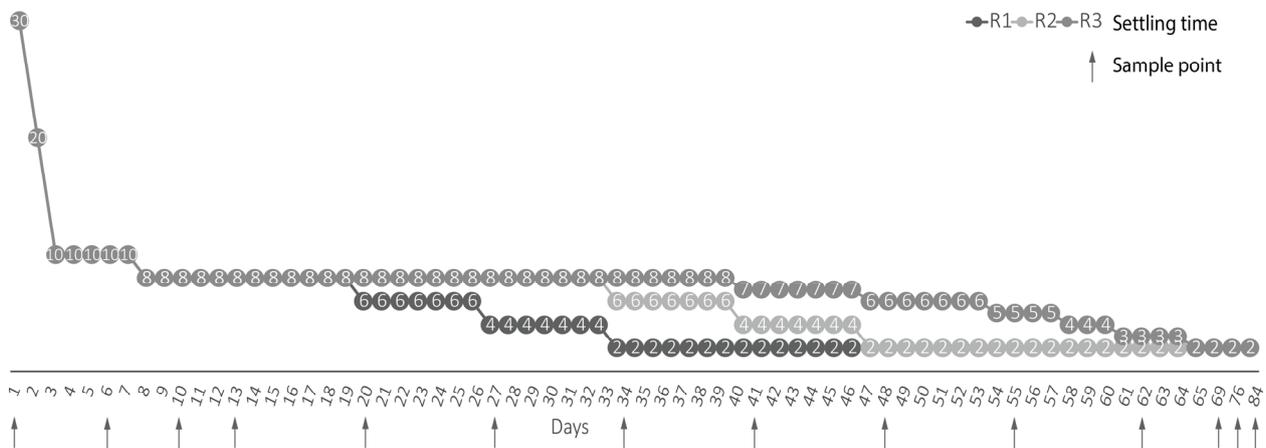


Figure 5. Experimental design scheme (Paper II).

3.1.2. Study of the internal structure of aerobic granules (Paper III)

The internal structure of aerobic granules was examined with special emphasis on channels and voids aiming to find obligate aerobes. For this purpose, fluorescence in-situ hybridization (FISH) was performed on intact granules cultivated for 9 weeks focusing on AOB, which would grow along the channels and voids if oxygen and substrate is transported to the inner regions of the granules. This study was performed in the same experimental set-up as in Paper II.

3.1.3. Study of *Bdellovibrio* sp. predation on aerobic granular sludge (Paper IV)

Cryosections of 9 week granules harvested from sequencing batch reactors were used for FISH analysis targeting the genus *Bdellovibrio* and AOB. This study was performed in the same experimental set-up as in Paper II.

3.1.4. Reproducibility of microbial communities in parallel SBRs during aerobic granulation (Paper V)

Three replicate SBRs were seeded with the same activated sludge and operated at identical conditions for 35 consecutive days treating synthetic wastewater with an organic loading rate of $3 \text{ kg COD m}^{-3}\text{d}^{-1}$ and a nitrogen load of $0.75 \text{ kg NH}_4\text{-N m}^{-3}\text{d}^{-1}$. The settling time was gradually decreased as shown in Figure 6. Reactor performance and the microbial community structure and dynamics were analysed over time during the transition from floccular to granular sludge. For this, samples were collected from the reactor three times per week and disturbances to the reactors and between them were minimized by careful maintenance of the reactors so that reproducible conditions were achieved.

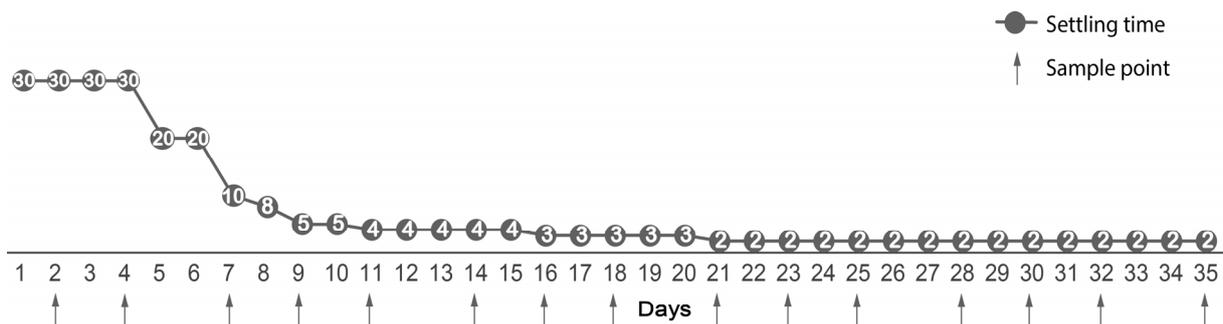


Figure 6. Experimental design scheme (Paper V).

3.2. Experimental set-up

3.2.1. Reactor design

Three identical lab-scale reactors were employed for the experiments presented in this thesis (Figure 7). The reactors consisted of four main units: a SBR with a working volume of 3 L, a 2 L storage bottles containing the carbon source and two 30 L storage tanks where the nutrients and micronutrients sources were kept, respectively. The SBRs consisted on a bubble-column of 6 cm diameter and 132 cm of total height, where the water level was kept at a height of 110 cm. The effluent was discharged 63 cm from the bottom (volumetric exchange ratio of 43%). The shape of the employed SBRs, the height to diameter ratio and the volumetric exchange ratio ensure washout of non-granulated biomass and apply appropriate hydrodynamic shear forces to develop more regular, rounder and compact granules. The wastewater and the air were provided at the bottom of the reactor with porous diffusers (pore size $1 \mu\text{m}$).

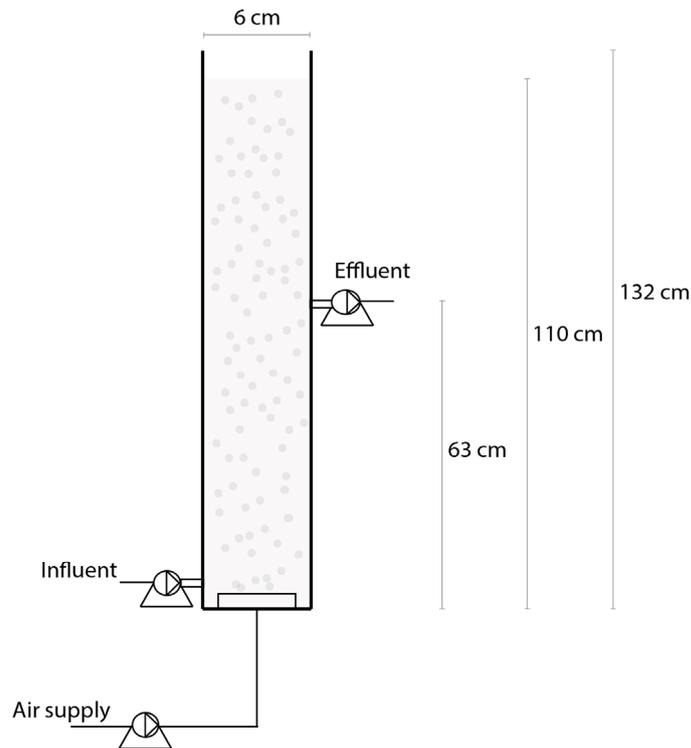


Figure 7. Schematic representation of the reactor design (non-scaled image).

3.2.2. Operational conditions

The SBRs were operated at room temperature (20-22 °C) in a 4 hour cycle (0:05 h filling, 0:55 h anaerobic/anoxic, 2:53 h aerobic, 0:02 h settling, 0:05 h withdrawal). The length of the cycle time, i.e. the frequency of solids discharge, was chosen because compact granules have been obtained with 4 h cycle (Adav *et al.*, 2008). Short cycle times ensure the suppression of suspended growth, but too short cycles will hinder the microbial growth and accumulation (Liu & Tay, 2004). The influent was pumped at a flow rate of 1.33 L cycle⁻¹ without aeration, followed by the anoxic phase to enhance the stability of granular sludge and select for slow growing microorganisms. In addition, the settling time was decreased in a stepwise mode in order to retain slow-growing bacteria and the length of the aerobic phase was adjusted correspondingly to achieve an even 4 hour cycle length. The reactors were fed with synthetic wastewater, or a mixture of synthetic and real wastewater, as described in Papers II and V. The air was set at a flow rate of 2.5 L min⁻¹ and superficial up-flow air velocity of 1.5 cm s⁻¹, high enough to ensure the appropriate hydrodynamic shear force. The pH was not controlled and was measured with a portable pH probe and data was continuously logged.

3.2.3. Sludge inoculum

The reactors were inoculated with aerobic/anoxic activated sludge from a full-scale WWTP (Gryaab AB, Gothenburg). For this purpose, fresh sludge collected from the WWTP was directly introduced into the reactors and was concentrated two-fold by allowing the first batch of sludge to settle, removing the supernatant and refilling the reactors with a second batch of sludge.

3.3. Analytical methods

The reactor performance and efficiency was measured by analysing the total organic carbon (TOC), total nitrogen (TN), NH_4^+ , NO_2^- and NO_3^- . For this purpose, effluent samples were collected and filtered through 0.2 μm pore size filters and analysed in a Shimadzu TOC analyser (TOC, TN) and a Dionex ICS-900 ion chromatograph (NH_4^+ -N, NO_2^- -N, NO_3^- -N). Total suspended solids and volatile suspended solids were measured according to standard methods (APHA, 1995). Microscopic observations were performed using an Olympus BX60 light microscope and particle size was assessed with CellSens (Olympus) software.

3.4. Microbial community analysis

The analysis of the microbial community was performed by DNA analysis. For this purpose, the Illumina/Solexa next-generation sequencing platform was selected, which is able to produce massive amounts of DNA sequence reads (Zhang *et al.*, 2011). The Illumina system utilizes a sequencing-by-synthesis approach. The process requires that the sequences to be sequenced are flanked by adapters which are needed for the bridge amplification process. These adapters also contain tags, or indexes, which are used to identify the samples. This enables simultaneous sequencing of multiple samples, pooled together, in one sequencing run (Mardis, 2008, Kircher & Kelso, 2010, Kozich *et al.*, 2013).

3.4.1. DNA extraction

Samples for the microbial community analysis were collected from the reactor. Prior to DNA extraction, the biomass weight of the samples was standardized. Total genomic DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals) following the manufacturer's protocol. The extracted genomic DNA concentration was quantified by NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and pooled in equimolar amounts.

3.4.2. DNA amplification

The V4 hyper-variable region of the 16S rRNA gene was amplified in duplicates, using a barcode-tagged primer set designed for MiSeq platform. Forward primers 515F and 515'F and the reverse primer 806R (Caporaso *et al.*, 2011, Hugerth *et al.*, 2014) were employed indexed with sequences published by Kozich *et al.* (2013). 515F was used in paper II and a modified 515'F, with a better coverage among *Achaea*, was used in paper V. These primers were chosen to maximize the coverage of Bacteria and Archaea and, as they are standard primers proposed by the Earth Microbiome Project, it is possible to compare results with previous studies (Parada *et al.*, 2016). Duplicate PCR reactions were conducted in a 20 μL reaction volume using 17 μL of the AccuPrime Pfx SuperMix (Life Technologies) kit, 1 μL of genomic DNA (20 ng template), and 1 μL each of the forward and reverse primers (10 μM). The PCR reaction was carried out in a Biometra T3000 Thermocycler with the following thermal cycling parameters: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (95°C, 20 s), annealing (50°C, 15 s) and elongation (68°C, 60 s), and finished by a 10 min final elongation at 68°C. The amplification was confirmed by 1% agarose gel electrophoresis and the DNA quality and concentration was measured by NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and Qubit 2.0 fluorometer (Life Technologies).

3.4.3. DNA sequencing and data analysis

The duplicate PCR products were pooled, the DNA concentrations of the PCR products were normalized and the samples were purified using the Agencourt AMPure system (Beckman Coulter), prior to pooling. The pooled PCR product was diluted with Tris-Cl (pH 8.5, 0.1% Tween20) for a final concentration of 0.6 ng μl^{-1} , as measured by Qubit. The expected concentration and size of the pooled PCR product was confirmed by TapeStation 2200 (Agilent Technologies). PhiX control library was spiked at 7.5%. Sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit v2.

3.4.4. Data analysis

The results were processed as published in Albertsen *et al.* (2015). Briefly, the UPARSE workflow was followed for dereplicating and formatting the reads. The reads were then clustered into operational taxonomic units (OTUs) using the USEARCH algorithms and the OTU abundance was estimated at a cut-off level of 97%. The taxonomical information was assigned using the RDP classifier and the MiDAS 1.20 database, created from the SILVA database. The results were analysed in R (Team, 2013). Richness, Simpson's evenness, Margalef's species richness and Pielou's evenness indices were calculated using the package vegan (Oksanen *et al.*, 2015), heatmaps were created using the package ampvis (Albertsen *et al.*, 2015) which builds on the packages vegan, phyloseq, Biostrings, ggplot2, ggplot2, reshape2, dplyr, data.table, DESeq2, knitr, ggdendro and stringr.

3. 5. Statistical analysis

The statistical analyses were performed using R (Team, 2013). The generic functions were used to calculate Pearson correlation coefficients and create Tukey boxplots, perform Wilcoxon signed-rank tests and constrained analysis of proximities (CAP). Non-metric multi-dimensional scaling (NMDS) ordination plots were created using the package ampvis (Albertsen *et al.*, 2015). Several statistical tests which need further explanation are described below.

3.5.1. Wilcoxon signed-rank tests

The Wilcoxon signed-rank test is a non-parametric dependency test used for the analysis of two paired samples which is based on ranked observations by their distance to the median, with the null hypothesis that the median difference between pairs of observations is zero (Taheri & Hesamian, 2013). The Wilcoxon signed-rank test was used in the analysis of the reproducibility of microbial communities to compare and to test if the results were reproducible in terms of reactor performance and biodiversity. For this, paired samples Wilcoxon signed-rank tests between reactors were performed on mixed liquor suspended solids, filtered TOC, filtered TN, ammonium, nitrite and nitrate and the biodiversity indexes obtained of the microbial community analysis. The Wilcoxon signed-rank test was also used during the analysis of the bacterial community composition in the granular and the suspended phase, to verify the consistently high or low retention ratios (relative read abundance in the granules/relative read abundance in the effluent) of different bacteria.

3.5.2. Non-metric multi-dimensional scaling

NMDS is an ordination method which ranks distances between objects, in this case (OTUs), and use these distances to represent the objects nonlinearly into a reduced ordination space (Ramette, 2007). The ordination is based on a distance or dissimilarity matrix which is built using a dissimilarity coefficient. The Bray-Curtis dissimilarity coefficient was used to create the different NMDS shown in this thesis. The Bray-Curtis dissimilarity coefficient is an asymmetrical measure used to quantify the compositional dissimilarity between two different sites, which treats equally the differences between high and low variables (Buttigieg & Ramette, 2014). The data was square root transformed to reduce the impact of variables with large values on the overall distribution. The objective of transforming the variables in a raw data set is to meet the assumptions of statistical inference and to make variables more comparable by reducing differences in scale (Buttigieg & Ramette, 2014). NMDS was used to identify and compare patterns followed by the microbial community during the transition of floccular sludge to granular sludge, to compare the microbial community evolution of the reactor and effluent samples when studying the effect of the selection pressure applied in the SBRs and to assess if the same trends were followed in the three parallel reactors when studying reproducibility of the microbial community.

3.5.3. Constrained analysis of proximities

CAP analysis was also used to compare patterns followed by the microbial community and to assess the reproducibility between reactors. CAP is a constrained ordination method that allows the use of any dissimilarity coefficient which takes into account the correlation structure between variables. Constrained ordination methods use an initial hypothesis to produce the spatial representation of the distances between the objects (Anderson & Willis, 2003). In this case, the ordination is constrained by reactor and Bray-Curtis was used as dissimilarity coefficient.

3.6. Fluorescence in situ hybridisation analysis

Fluorescence in situ hybridisation (FISH) was employed in combination with confocal laser scanning microscopy (CLSM) to study the spatial distribution of the microbial communities in the granular sludge. FISH is based on the identification of specific microorganisms by the use of DNA probes with a sequence which hybridize specifically with target molecules (often rRNA) of the target cells and a fluorescent dye molecule which is covalently linked at the 5'-end of the probe (Amann *et al.*, 2001). Thereafter, the labelled cells are detected by different fluorescent methods such as CLSM, used in this thesis. CLSM is a fluorescence microscopy technique, which uses two pinholes to limit the signal to a defined, small focal plane. Successive series of sections can be scanned enabling the reconstruction of three-dimensional structures at a very high resolution (Paddock, 2000).

3.6.1. Cryosectioning

To enable FISH and visualization of intact structures, samples have to be sliced in thin sections to enable probe accessibility during FISH and efficient visualization during CLSM. For this, intact granules harvested from the reactors were immersed in 4% paraformaldehyde for 8 h at

4°C and washed twice with PBS. Fixed granules were then stored in PBS/ethanol 50:50 at -20°C until use. For cryosectioning, fixed granules were embedded in O.C.T. compound (VWR, Radnor, PA, USA) and incubated overnight at 4°C in individual plastic containers. Thereafter, each granule was frozen solid in blocks in a dry ice fume chamber and stored at -80°C until use. Granule sections of 10-20 µm thickness were obtained at -20°C using a HM550 microtome cryostat (MICROM International GmbH, Germany), which subsequently were collected on SuperFrost® Plus Gold microscope slides (Menzel GmbH, Germany) and stored at -20°C until use (Persson *et al.*, 2016).

3.6.2. Hybridization

Before FISH, the cryosections on the slides were framed with a hydrophobic barrier using a Liquid Blocker Mini PAP Pen (Life Technologies) and the glass slides were covered with a thin layer of agarose (1%) to preserve the integrity of the cryosections. After dehydration in an ethanol series (50%, 80% and 96% v/v), FISH was performed at 46 °C for 2 h (Manz *et al.*, 1992) using the probes and applying the hybridization conditions shown in Table 1. To visualize several microbial groups simultaneously, multiple probes with different fluorophores and stains were applied on the same cryosection. For this, the probes were 5' labelled with Alexa 488, Cy3 and Cy5 fluorophores and Syto 40 as counterstain. Slices were then washed with water and mounted with Citifluor AF1 (Citifluor Ltd., UK).

Table 1. Probes and hybridization conditions for FISH.

Probe	Target organism	FA ^a (%)	Reference
BDE525	Genus <i>Bdellovibrio</i>	35	Mahmoud <i>et al.</i> (2007)
CFB563	Most <i>Flavobacteria</i>	20	Weller <i>et al.</i> (2000)
Cluster6a192^b	<i>Nitrosomonas. oligotropha</i>	35	Adamczyk <i>et al.</i> (2003)
EUB338 (I-V)	Most bacteria	35	*
Meg983	<i>Meganema perideroedes</i>	35	Thomsen <i>et al.</i> (2006)
Meg1028	<i>Meganema perideroedes</i>	45	Thomsen <i>et al.</i> (2006)
NEU^b	<i>Nitrosomonas europaea/eutropha/halophila</i>	35	Wagner <i>et al.</i> (1995)
Nse1472	<i>Nitrosomonas europaea/eutropha</i>	50	Juretschko <i>et al.</i> (1998)
ZRA23a	<i>Zoogloea</i> lineage, not <i>Z. resiniphila</i>	35	Rosselló-Mora <i>et al.</i> (1995)
ZOGLO-1416	<i>Zoogloea spp.</i>	35	Loy <i>et al.</i> (2005)

^a FA = formamide concentration in hybridization buffer.

^b Probe applied with unlabeled competitor probe according to the reference.

* EUB338 I, Amann *et al.* (1990); EUB338 II, Daims *et al.* (1999); EUB338 III, Daims *et al.* (1999); EUB338 IV, Schmid *et al.* (2003).

3.6.3. Image acquisition

CLSM analysis was performed in a Zeiss LSM700 (Carl Zeiss, Germany) using 10x/0.45 plan-apochromat and a 40x/1.3 plan-apochromat oil objectives and laser diode lines of 405, 488, 555 and 639 nm. Images were acquired at image size of 1024 × 1024 pixels using frame mode and averaging = 4. Large images, covering the entire granules, and large sections were acquire using the tiling functions of Zeiss ZEN2010 software. A pinhole equivalent to 1 AU for the Cy5 channel was used and to reduce the autofluorescence of Cy3, a 600 nm short pass filter was employed.

4. Results and discussion

4.1. Washout dynamics and localization of the microbial community during aerobic granulation in SBRs (Papers II-IV)

4.1.1. Microbial dynamics in the granular and the suspended phase and retention ratios of abundant genera during granulation

The results show that the community composition of the effluent is very similar but not identical to the community composition of the granular sludge in the three reactors throughout the experiment (Figure 8) with an average similarity between contemporaneous granular and effluent samples of $65 \pm 2\%$ in R1, $63 \pm 6\%$ in R2, and $65 \pm 7\%$ in R3. To analyze more thoroughly the differences between effluent and granule samples, the retention ratio of the dominant genera in contemporaneous samples was calculated by dividing the relative read abundance in the granules with the relative read abundance in the effluent. A retention ratio larger than one indicates high degree of retention of the taxa in the reactor. The correlation analysis of retention ratios and number of days since start-up (Table 2) showed that some genera became more abundant in the effluent as the experiment progressed (e.g. *Acidovorax* in R2 and R3, *Brevundimonas* in R1) while other genera were increasingly retained as the floccular sludge granulated (e.g. *Meganema* in R1, *Leptothrix* in R2). In general, no statistically significant correlation was found between the retention ratio and the temporal variation of relative abundance (Table 2). Therefore, the successional patterns can probably be explained better by substrate specificity or other ecophysiological factors.

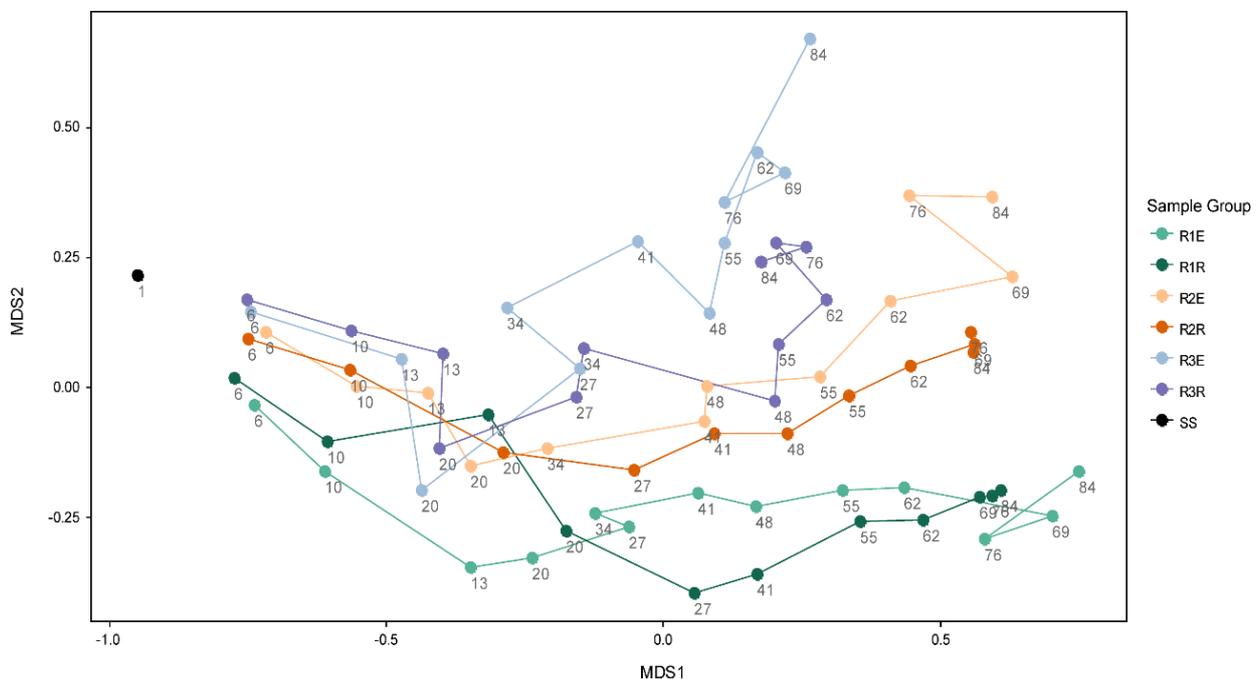


Figure 8. NMDS ordination plot based on Bray-Curtis dissimilarity matrix. Samples are indicated with the number of days since seeding. R1E, R2E, R3E: effluent samples (suspended phase); R1R, R2R, R3R: samples from the reactor content (granular phase), SS: seed sludge (day 1).

Table 2. Correlation analyses of retention ratios and the number of days since start-up. Pearson coefficients and p-values (*<0.05, **<0.01, ***<0.001) are provided.

Genus/OTU	R1		R2		R3	
	p	pearson	p	pearson	p	pearson
<i>Acidovorax</i>	0.8285	-0.0668	0.0099	** 0.6841	0.0065	** 0.7104
<i>Bdellovibrio</i>	0.2792	0.3584	0.0579	. 0.5380	0.0143	* 0.7405
<i>Brevundimonas</i>	0.0084	** 0.6947	0.0953	. 0.4821	0.0542	. 0.5449
<i>Comamonas</i>	0.8125	-0.0731	0.0127	* 0.6915	0.3800	0.2941
<i>Flavobacterium</i>	0.0073	** 0.7033	0.0006	*** 0.8418	0.0596	. 0.5350
<i>Hydrogenophaga</i>	0.0720	. 0.5145	0.6511	0.1388	0.0198	* 0.6346
<i>Leptothrix</i>	0.5783	0.1702	0.0376	* -0.5803	0.6190	0.1525
<i>Meganema</i>	0.0296	* -0.6524	0.1573	-0.4352	0.1005	-0.4756
<i>Paracoccus</i>	0.2592	0.3377	0.5587	0.1789	0.9165	0.0323
<i>Pseudorhodobacter</i>	0.0042	** 0.7350	0.4281	0.2408	0.8144	-0.0723
<i>Rhodobacter</i>	0.1282	0.4443	0.4015	0.2545	0.8502	0.0612
<i>Simplicispira</i>	0.5592	0.1787	0.2985	0.3125	0.1308	0.4417
<i>Taibaiella</i>	0.5992	-0.1610	0.4869	0.3181	0.1255	0.4471
<i>Thauera</i>	0.4142	-0.2479	0.0008	*** 0.8100	0.0258	* 0.6132
<i>Zoogloea</i>	0.4673	-0.2324	0.9779	-0.0090	0.5396	-0.1875
OTU_11 ^a	0.4770	-0.2167	0.4361	0.2368	0.3820	-0.2778
OTU_6 ^a	0.0007	*** 0.8571	0.0105	* 0.7612	0.0185	* 0.6640
OTU_3 ^a	0.2225	0.3632	0.7140	0.1331	0.0049	** 0.7767
CYCU.0281	0.5169	0.1979	0.0103	* 0.6818	0.0677	* 0.5437

^a Closest matches by BLASTn [28] are shown in Table S2 of Paper II.

4.1.2. Retention ratios of abundant microorganisms during start-up

During the start-up (weeks 1-6), most genera had a retention ratio around one, i.e. they were washed out proportionally to their relative abundance in the granular biomass (Paper II, Figure S5). During this period, a large fraction of the biomass is still flocculated, and every floc has similar settleability. Thus the probability of cells being discharged with the effluent is similar for every taxa, irrespective of which floc they are attached to or where in the floc they are situated.

4.1.3. Retention ratios of abundant microorganisms during steady-state

During the steady-state (weeks 7-12), most of the biomass is granulated and dense enough to be retained in the reactor, but the retention ratios of certain genera were significantly different than one indicating that some microorganism were more likely to be washed out from the reactors than others (Figure 9). This could be a consequence of the differences in settleability between microbial aggregates during the granulation process and the spatial location. Retention ratios lower than one indicates that these bacteria were more abundant in the effluent than in the granules, which could be due to their localization in the outer layers of the granule, therefore being more exposed to erosion (Winkler *et al.*, 2012). Genera having retention ratios larger than one had a higher relative abundance in the granules than in the effluent, and were not washed out of the reactor. Those genera that also showed a high or increasing relative abundance (Paper II), such as *Flavobacterium* and *Bdellovibrio*, were probably growing in the core of the granules protected from erosion. However, a high retention ratio did not necessarily mean high or increasing relative abundance in the granules. *Brevundimonas* sp. in R1, *Acidovorax* sp. in R2, or *Comamonas* sp. in R3 had average retention ratios above one, but their relative abundance decreased with time (Paper II, Figure S7). This could indicate that, despite being located in the interior of the granule, they were outnumbered by other species with higher growth rates.

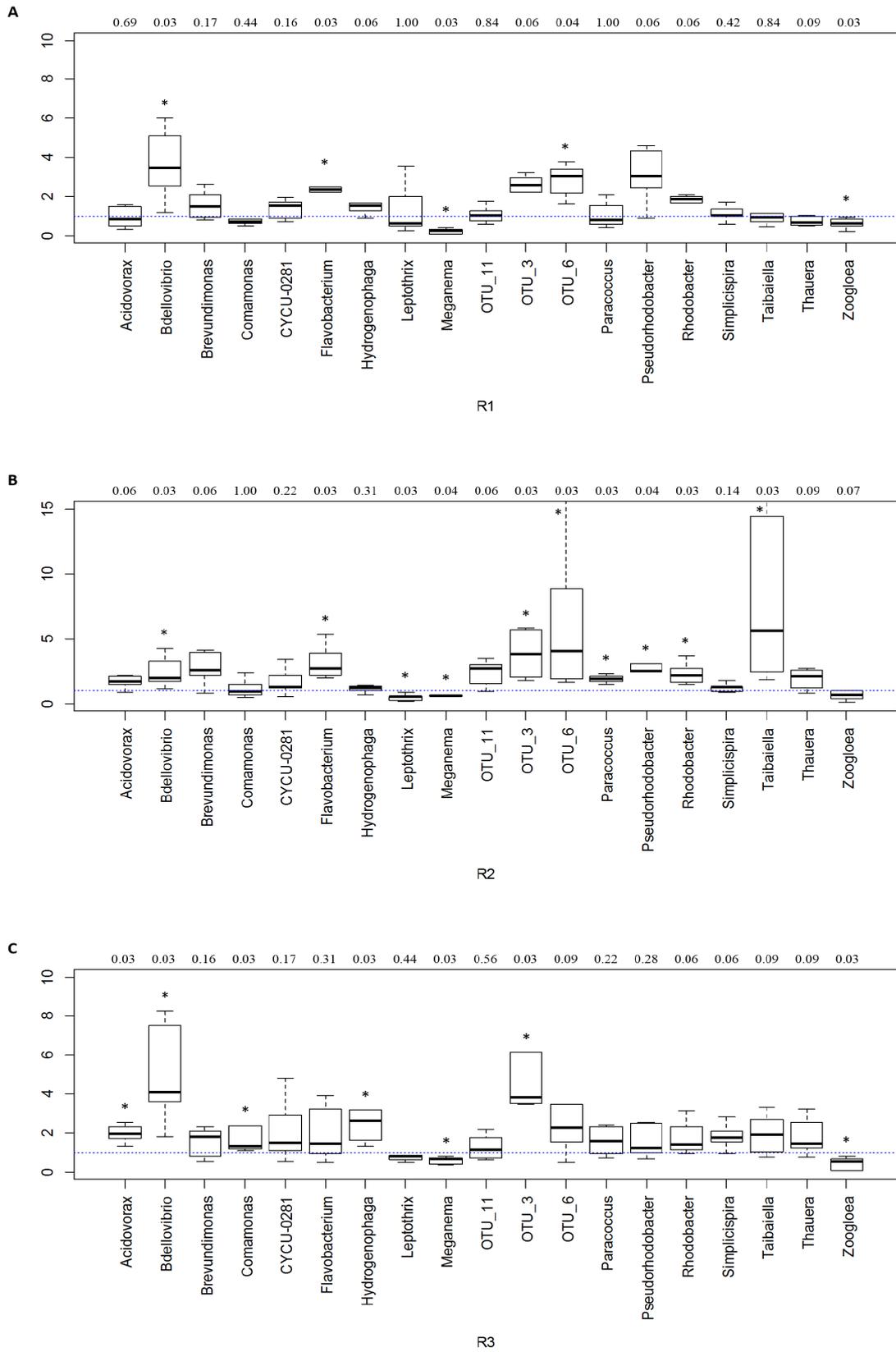


Figure 9. Boxplots of the retention ratios during steady-state operation (weeks 7-12) in R1 (a), R2 (b) and R3 (c). Values significantly different from 1 are marked with asterisk (p-values are shown above the plots).

4.1.4. Granular location of selected genus and functional groups

FISH analysis was performed on cryosectioned slices of granules for some of the most abundant genera (*Meganema*, *Zoogloea*, *Bdellovibrio* and *Flavobacterium*) to assess whether the spatial location of bacterial species in the granule can influence the retention ratio (Figures 10 and 11).

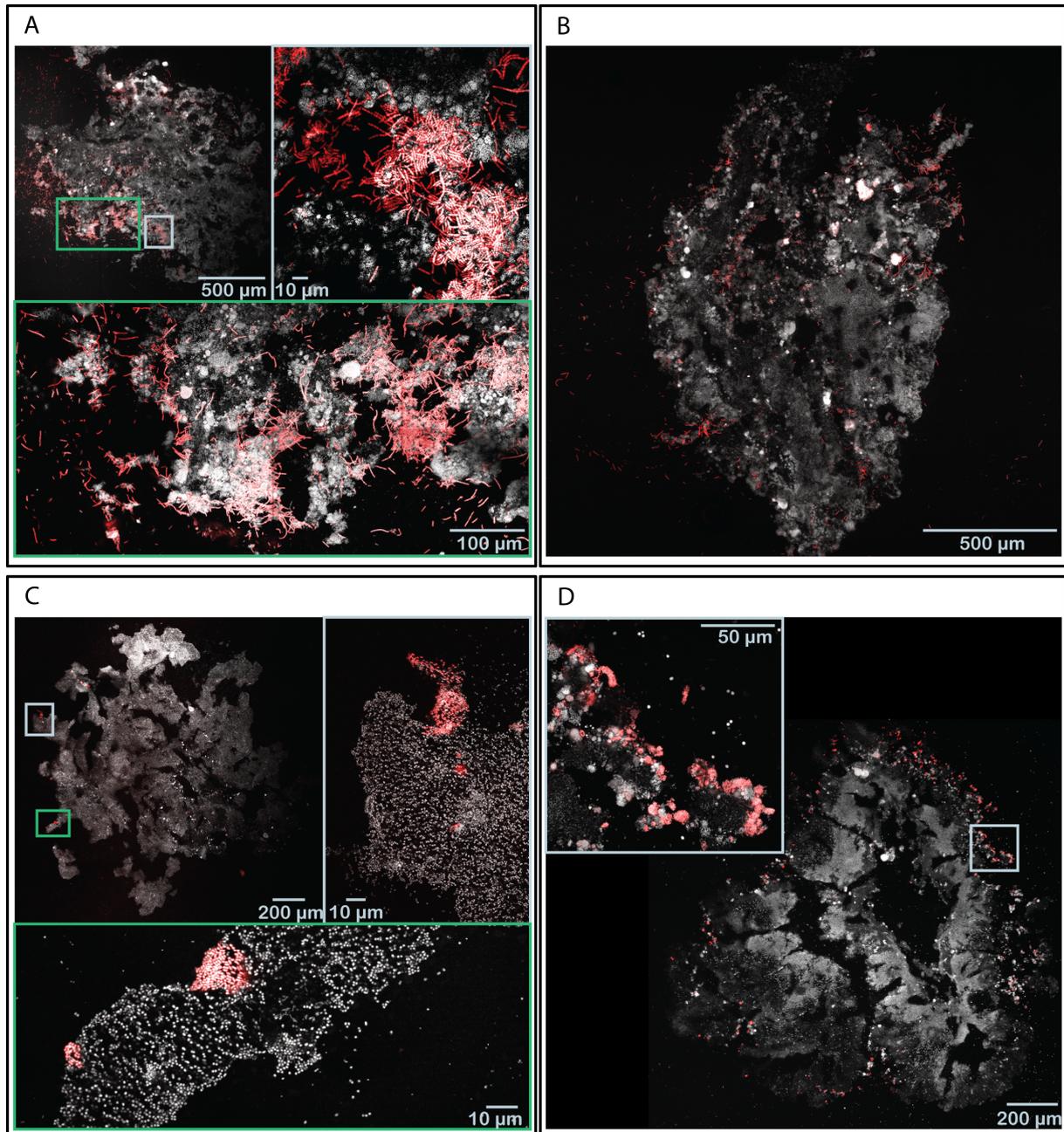


Figure 10. FISH-CLSM images from cryosections of aerobic granules of the selected bacteria with retention ratios significantly lower than one at 200 × magnification and detailed sections at 400 × magnification. A and B, *Meganema perideroedes* in aerobic granules from R2; C, *Zoogloea* spp. in aerobic granules from R2; D, *Zoogloea* spp. in aerobic granules from R3. Grey: total cells (Syto 40); red: A and B, *Meganema perideroedes* (Meg983 and Meg1028) and C and D, *Zoogloea* spp. (ZRA23a and ZOGLO-1416).

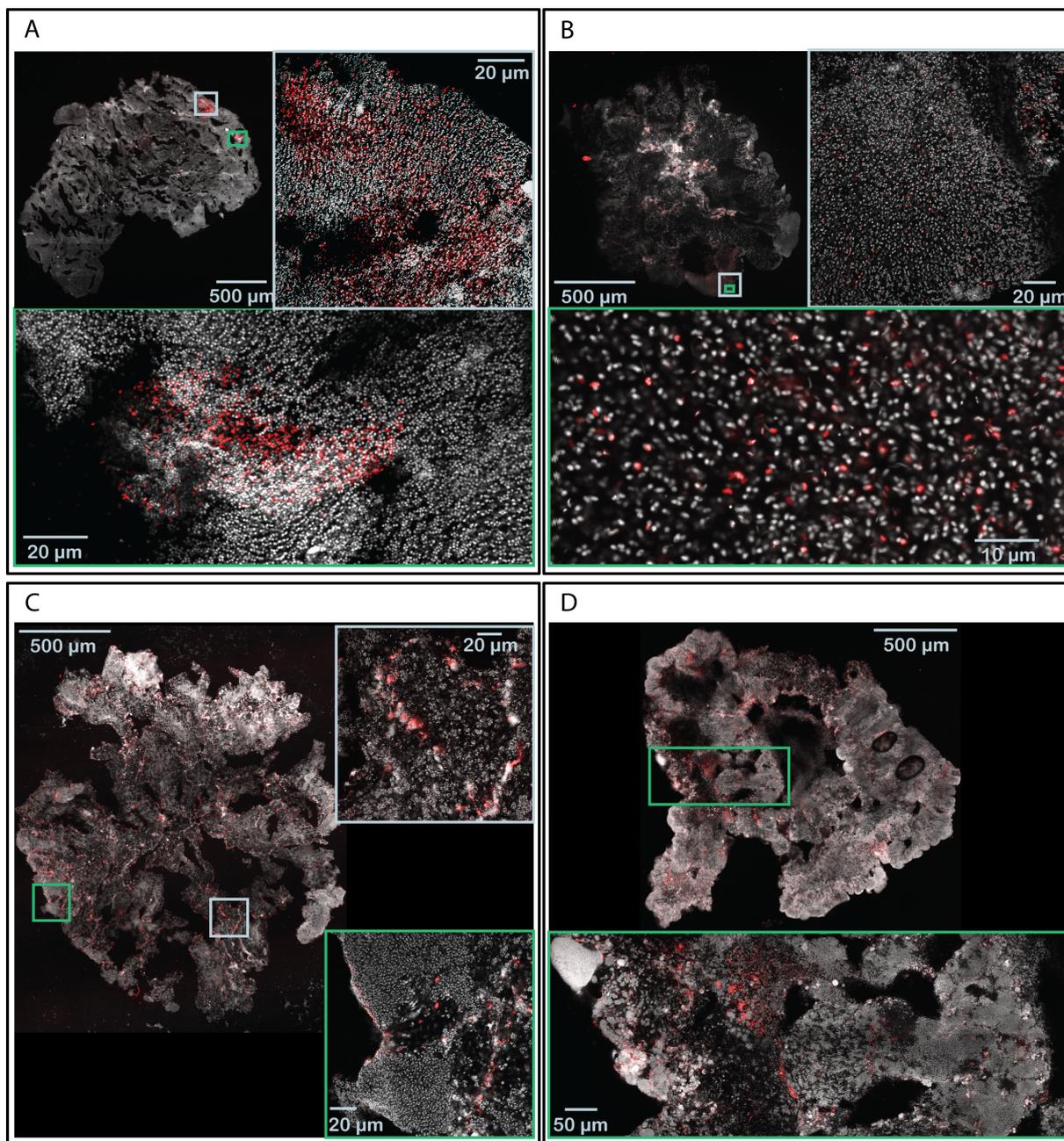


Figure 11. FISH-CLSM images from cryosections of aerobic granules of the selected bacteria with retention ratios significantly higher than one at 200 × magnification and detailed sections at 400 × magnification. A and B, genus *Bdellovibrio* in aerobic granules from R1; C and D, *Flavobacteria spp.* in aerobic granules from R1. Grey: total cells (Syto 40); red: A and B, genus *Bdellovibrio* (BDE525) and C and D, *Flavobacteria spp.* (CFB563).

FISH analysis revealed that *Meganema* sp. and *Zoogloea* sp. were located on the granular surface (Figure 10). Both genera had retention ratios significantly lower than one. These bacteria were washed out from the reactors probably due to granule erosion, as they were growing in the loosely packed outer layer. *Meganema* sp. displays a filamentous growth and is usually found in aerobic environments (Kragelund *et al.*, 2006) and *Zoogloea* sp. grows as finger-like structures and produces EPS containing high amounts of water (Rosselló-Mora *et al.*, 1995, Thomsen *et al.*, 2007, Nielsen *et al.*, 2010). Therefore, both genera are expected to grow in the granular outer layer, which together with their growth mode, makes them more likely to detach when exposed to high shear forces. Moreover, both bacteria could be growing also in the suspended phase as they possess a high substrate uptake rate and growth rate (Roinestad & Yall, 1970, Kragelund *et al.*, 2006).

The core of the granule is protected from erosion and also provides microaerobic and anaerobic niches. Therefore, denitrifying organisms such as *Acidovorax* sp., *Pseudorhodobacter* sp. and *Rhodobacter* sp. can be expected to have a high retention ratio (Figure 9 and Table 2). FISH analysis showed *Bdellovibrio* sp. and *Flavobacterium* sp. located in the deeper regions of the granules (Figure 11). They had retention ratios significantly higher than one during steady-state operation, thus suggesting that genera with high retention ratios are actually growing in deeper parts of the granules. *Flavobacterium* spp. have been reported to hydrolyze soluble microbial products and EPS which can be found in the core of the granule (Bernardet *et al.*, 1996). The presence of predatory bacteria like *Bdellovibrio* sp. has been reported earlier in granular sludge (Li *et al.*, 2014, Wan *et al.*, 2014, Weissbrodt *et al.*, 2014). Despite being obligate aerobic, *Bdellovibrio* sp. was located in the inner parts of the granule predated actively, where oxygen is supposed to be at lower concentrations. The ability of *Bdellovibrio* sp. to predate under anoxic conditions was reported by Monnappa *et al.* (2013). Nevertheless, the FISH analysis targeting AOB revealed that oxygen penetrated to deeper regions of the granules. As observed in Figure 12, AOB were found along the channels and even in voids located in the core of the granules. The presence of AOB in the inner locations of the granules indicates that both oxygen and ammonia are transported across the granule through the channels. These results are in contradiction to the conceptual and mathematical models commonly employed since the aerobic granules analysed in this study did not follow the multilayer model where AOB are located in the aerobic outer layer. Instead, aerobic surfaces appear to be present also in voids and channels throughout the entire granule. Interestingly, *Bdellovibrio* sp. was found attacking AOB (Figure 13). This results suggest that ammonium oxidizers were subjected to predation which might have an impact on the nitrification process in engineered and natural ecosystems. This added complexity of granule architecture needs to be taken into account to better understand the granulation process for future applications.

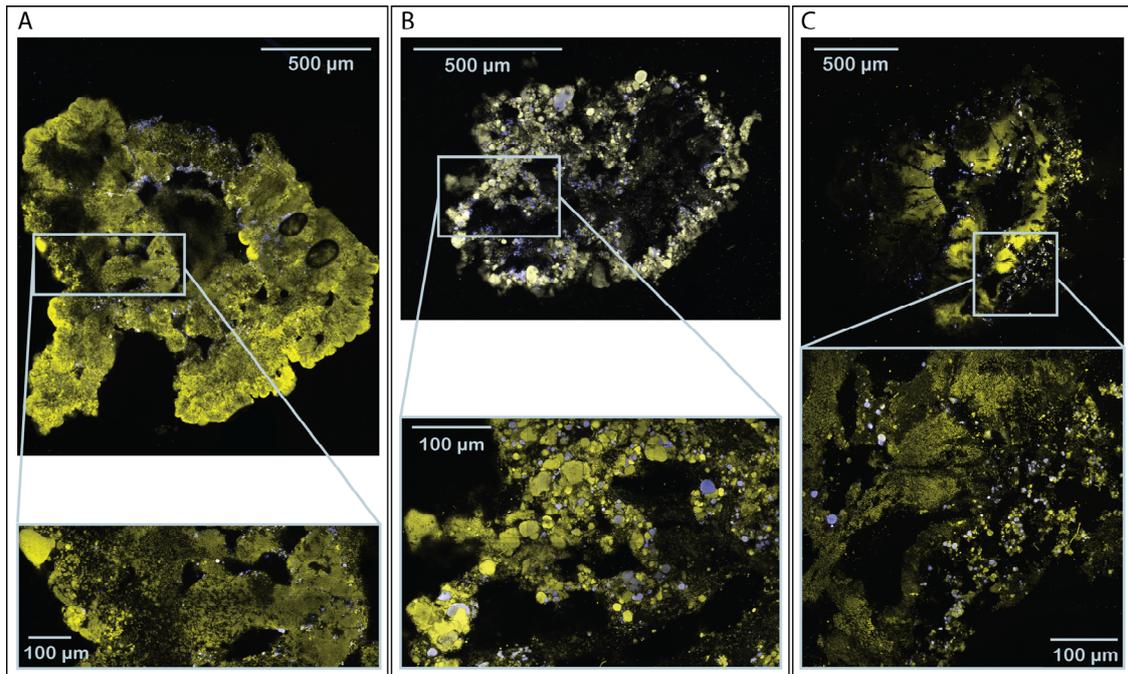


Figure 12. FISH-CLSM images from cryosections of aerobic granules from R1 (A), R2 (B), and R3 (C) at 200 × magnification (upper images) and detailed sections at 400 × magnification (lower images). Yellow: total cells (Syto 40); blue: AOB (NEU654, Nse1472, and Cluster6a192).

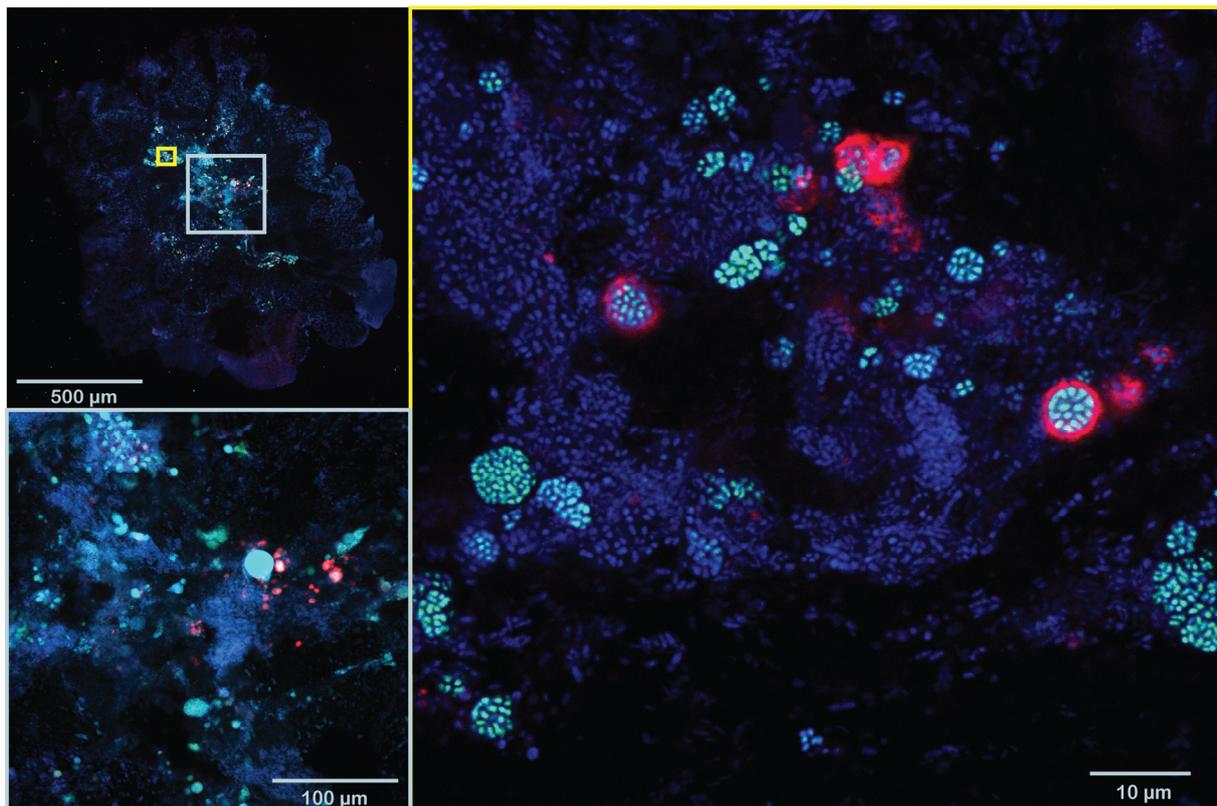


Figure 13. FISH-CLSM images from cryosections of aerobic granules from R1 at 200 × magnification and detailed sections at 400 × magnification. Blue: total bacteria (EUBmix); green: AOB (NEU654, Nse1472, and Cluster6a192); red: genus *Bdellovibrio* (BDE525).

4.2. Microbial community dynamics in replicate SBRs during aerobic granulation

4.2.1. Reactor performance and ecosystem functions

The three replicate reactors followed a similar evolution in performance and sludge properties (Paper V, Figures 2, 3 and S1), however R1 behaved slightly different from R2 and R3 as the suspended solids in the reactor and the effluent ammonium were statistically different in R1 (Paper V, Table S1). Complete carbon removal was achieved within a few days in the three reactors (always above 90% since day 4) and despite not having a complete ammonium removal, nitrification was taking place (up to 40%).

Regarding the functions of the most abundant taxa found in the reactors, most of them were heterotrophic nitrifiers, denitrifiers and polyphosphate-accumulating bacteria (Figure 14). Some of the most abundant genera in the aerobic granules belonged to the families *Comamonadaceae* (e.g. *Simplicispira* sp. and *Comamonas* sp.) *Rhodocyclaceae* (e.g. *Thauera* sp.), *Flavobacteriaceae* (e.g. *Chryseobacterium* sp. and *Flavobacterium* sp.) and *Xanthomonadaceae*. These taxa are well known denitrifiers and in addition some have been reported to be capable of heterotrophic nitrification (Ginige *et al.*, 2005, Chen & Ni, 2011, Abdullah *et al.*, 2013, Cavaillé *et al.*, 2013, Zhao *et al.*, 2013, DeLong *et al.*, 2014, Kundu *et al.*, 2014, Yang *et al.*, 2014, Fitzgerald *et al.*, 2015, Ma *et al.*, 2016). Despite the nitrification-denitrification metabolisms associated with many of the most abundant genera, the total nitrogen removal was incomplete (around 50%), and values above 70% were achieved after day 28 when the actinobacteria *Corynebacterium* sp. were dominating the reactors. The increase in nitrogen removal could be due to the dominance of these bacteria, as this genera has been reported to perform denitrification, heterotrophic nitrification and might use nitrate as an electron acceptor for phosphate accumulation (Zhao *et al.*, 2013, Seenivasagan *et al.*, 2014, Jena *et al.*, 2015). AOB and NOB were detected in very low numbers, nevertheless good nitrification has been reported in reactors recording low relative abundances for these bacteria (Szabó *et al.*, 2016).

4.2.2. Bacterial succession during the granulation process

Granulation occurred fast since granules started to emerge at day 7 with an average diameter of 351 μm , 357 μm and 426 μm in R1, R2 and R3 respectively and at day 14 the granules had more than 0.5 mm in diameter (Paper V, Figures 4 and S2). This rapid granulation of acetate fed granules has been reported before (Liu & Tay, 2004, Ebrahimi *et al.*, 2010, Szabó *et al.*, 2016). The reactors showed a very similar pattern in temporal variation of the microbial community. The microbial composition rapidly changed once the reactors were inoculated and many genera with very low abundance in the seed sludge increase during the experiment (Figure 14). Certain bacteria such as *Corynebacterium* sp., *Chryseobacterium* sp., *Pedobacter* sp. and *Elizabethkingia* sp. and members of the families *Xanthomonadaceae*, *Beijerinckiaceae*, *Flavobacteriaceae* and *Bacteriovoraceae*, were not detected or had a very low relative abundance in the seed sludge but became more abundant during granulation. On the contrary, *Limnohabitans* sp. and *Brachymonas* sp. which were highly abundant in the seed sludge, decreased in relative abundance along the experiment.

During the transformation from flocs to granule-like particles on the first 7 days, when the settling time was 10 minutes, *Acinetobacter* sp. clearly dominated in the three reactors (Figure 14). *Acinetobacter* sp. is a hydrophobic bacterium and an EPS producer, which has been reported as primary colonizer and bridging bacteria in aggregate/biofilm development (Zhu *et al.*, 2012, Faust *et al.*, 2015, Liébana *et al.*, 2016), Therefore, *Acinetobacter* sp. could contribute to granulation by bridging flocs and other sludge particles, thus having an important role during the first stage of granulation. *Acinetobacter* sp. drastically decreased in abundance at day 9 (Figure 6). After that, the *Comamonadaceae* and *Rhodocyclaceae* families, which have been related with granulation (Li *et al.*, 2008, Weissbrodt *et al.*, 2012), dominated the reactors until day 21 (Figure 14) and precisely during this period, the young granules increased in size. EPS producers such as *Thauera* sp., *Flavobacterium* sp. and the family *Xanthomonadaceae* increased in relative abundance during this period (Liu *et al.*, 2010, Yang *et al.*, 2014). Since day 28, *Corynebacterium* sp. became the dominant genus, accounting for the 50% of the total read abundance and the only representative of this class.

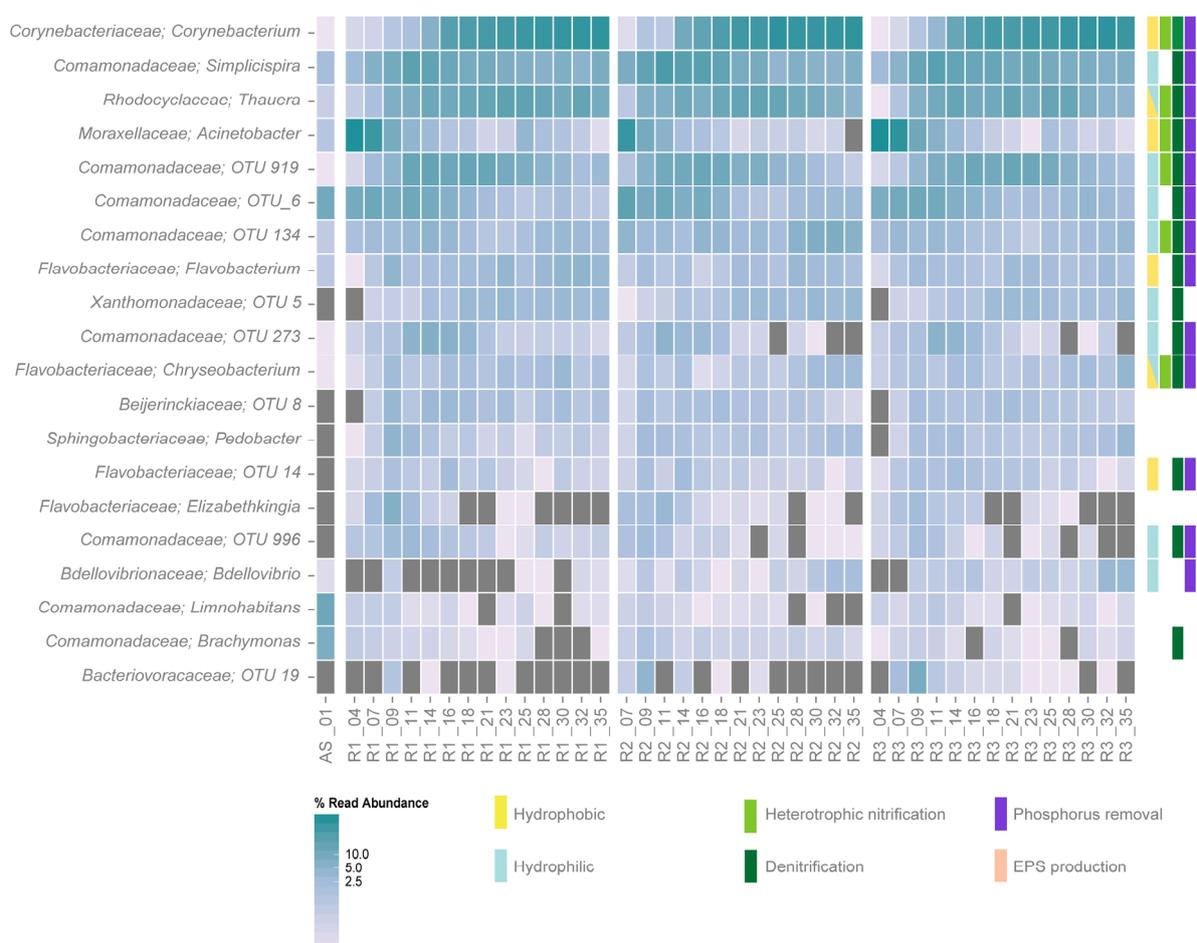


Figure 14. Temporal variation during the experiment of the 20 most abundant genus in the replicate reactors expressed as the percentage of read abundance. R1, R2, R3 denotes reactor; the number separated by an underscore indicates the day of the experiment that the sample was taken. AS_01 is the seed sludge.

4.2.3. Microbial diversity dynamics during the granulation process

Richness (observed OTUs) and evenness (Simpson's evenness index) of the replicate reactors along the experiment described the same trend in the three reactors, but R1 behaved slightly different (Figure 15) with a significantly different evenness than R2 and R3 (Paper V, Table S2). After the inoculation of the reactors, the microbial community structure drastically changed with a drop in richness and evenness at the first sample point followed by a high increase in evenness at day 9 and the slight increase in richness at day 7, 9 and 11 in R1, R2 and R3 respectively. During this period, granules started to emerge and the sludge consisted of a mixture of flocs and granules which might have offered more niches. These rapid changes during the start-up correspond to the adaptation or acclimatization of the microbial community to the reactor conditions (Kaewpipat & Grady, 2002, Curtis & Sloan, 2006, Wittebolle *et al.*, 2009, Cabrol *et al.*, 2012) when habitat specialization and competitive exclusion takes place. This acclimatization period is specially marked in SBRs operated to develop granules due to the high washout dynamics in the reactor (Weissbrodt *et al.*, 2013). The decrease in richness and evenness during the transformation of floccular sludge into aerobic granules has been shown in previous studies (Gonzalez-Gil & Holliger, 2011, Szabó *et al.*, (not published)). Moreover, the change from wastewater to synthetic feed, with an easily biodegradable substrate, could have favoured some taxa at the start of our experiment, and consequently, evenness and richness decreased (Li *et al.*, 2008, Gonzalez-Gil & Holliger, 2011, Weissbrodt *et al.*, 2012, Fra-Vázquez *et al.*, 2016).

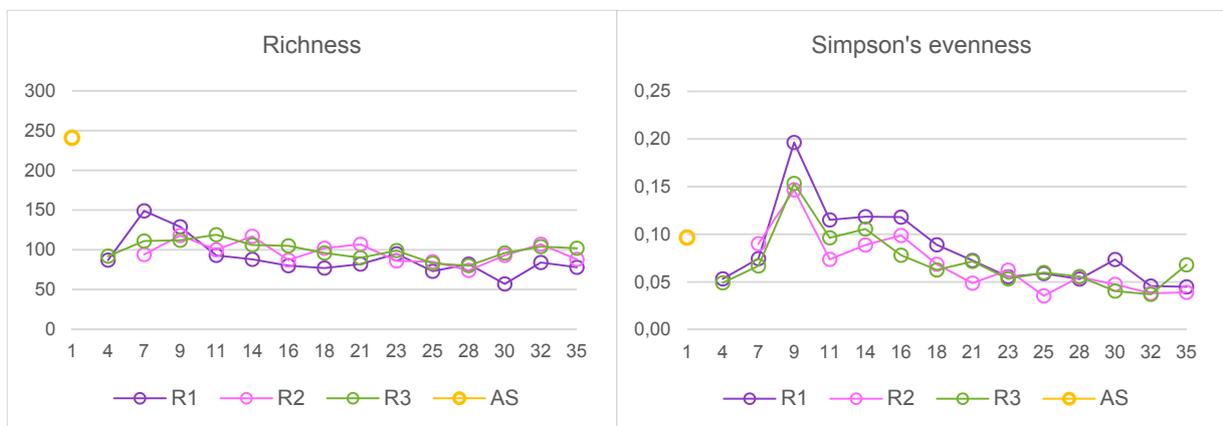


Figure 15. Richness (observed) and evenness (Simpson's evenness index) of the microbial communities of the replicate reactors during the experiment. R1, R2, R3 denotes reactor and AS is the seed sludge.

4.2.4. Ecological factors involved in the microbial community assemblage

The community structure dynamics is shown in Figure 16 using NMDS time series plots. The microbial community structure experienced an abrupt change once the reactors were inoculated, differing considerably from the seed sludge already at the first sample point. During the first week the microbial communities were quite different between reactors but they converged at day 9. Thereafter, R2 and R3 evolved similarly until the end of the experiment but R1 slightly diverged from the other two (Figure 14).

These results suggest that deterministic factors had an important influence on the microbial community assembly in the replicate bioreactors after the inoculation period. The niche-oriented component seemed to dominate as the sludge experienced a change of feed and other environmental conditions, especially under the high selective forces applied in the SBRs. This is denoted by the competitive advantage of some species such as *Acinetobacter* sp. which dominated the reactors during the first week of the experiment in the three reactors (Figure 14). The microbial community assemblage has been reported to be governed by deterministic factors in other studies where niche-oriented component exerts an important influence (McGuinness *et al.*, 2006, Falk *et al.*, 2009, Wittebolle *et al.*, 2009, Cabrol *et al.*, 2012, Vanwonterghem *et al.*, 2014). Not only deterministic factors influenced the microbial community assemblage but also stochastic processes were involved since R1 diverged from R2 and R3 after the initial convergence. The CAP analysis results confirmed R1 to be different to the other two reactors (Paper V, Figure S3), with the intermediate and rare species being responsible for the divergence. This differences in the less abundant members of the microbial community could be a consequence of stochastic processes in which immigration, extinction, speciation and dispersal become more important. The stochastic changes in relative abundance in a microbial community, or ecological drift, have a higher impact in the least abundant community which are more affected by small events (Nemergut *et al.*, 2013). Moreover, it has been proved that drift is more important when selection forces are weaker (Nemergut *et al.*, 2013, Wang *et al.*, 2013). Indeed, the washout dynamics, one of the strongest selective force applied in SBRs for sludge granulation, were more pronounced during the first 11 days of the experiment when the settling time was decreased from 30 to 4 min (Figure 6). Thereafter, the washout dynamics were less harsh which could have made stochastic processes to contribute more. This results indicate that both deterministic and stochastic processes might be involved in the microbial community assemblage during aerobic granulation.

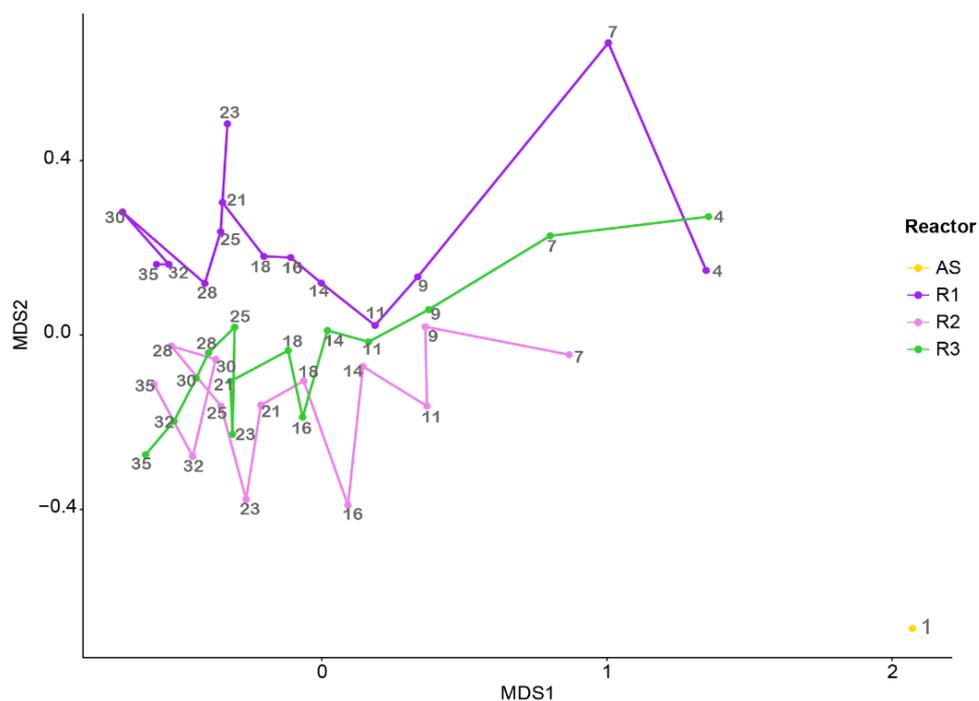


Figure 16. NMDS ordination plot based on Bray-Curtis dissimilarity and square root transformation. R1, R2, R3 denotes reactor and AS is the seed sludge.

5. Conclusions

- The community composition of granules and the washed biomass from three SBRs subjected to high washout conditions and different OLRs is very similar but not identical.
- The ratio between relative abundance in the granular biomass and in the effluent did not predict temporal variation of the taxa in the reactors. Therefore, the successional patterns of the temporal variation of relative abundance can probably be explained by other ecological factors.
- The ratio between relative abundance in the granular biomass and in the effluent appear to predict the spatial location of the taxa in the granules. Functional groups located in the interior of the granules tend to be present in lower numbers in the effluent and they might be gradually outcompeted resulting in a decreasing relative abundance. Bacteria growing on the granular surface are more susceptible to erosion and therefore to be washed out of the reactors. Also, bacteria with high growth rate could contribute more to the washed out due to their growth in the suspended phase.
- The presence of AOB in the inner locations of the granules indicates that both oxygen and ammonia are transported across the granule through channels and pores, allowing aerobic metabolism also in the granular interior as aerobic surfaces appear to be present in deeper parts of the granule.
- *Bdellovibrio* sp. was found actively preying inside the granules and seemed to be preferentially attacking AOB which could have an impact on the nitrification process.
- Three parallel reactors operated identically and inoculated with the same seed sludge showed a similar behaviour but statistical differences were found in the reactor performance and in the microbial community diversity and structure for one of the reactors.
- During granulation, *Acinetobacter* sp. seemed to have an important role during the first stage of granulation by bridging flocs and other sludge particles. During granular maturation, the *Comamonadaceae* and *Rhodocyclaceae* families and important EPS producers such as *Thauera* sp., *Flavobacterium* sp. and the family *Xanthomonadaceae* increased in relative abundance. *Corynebacterium* sp. became the dominant genus during the last part of the experiment.
- Richness and evenness decreased during the transformation of floccular sludge into aerobic granules. Both indexes experienced a steep decrease during the acclimation period at the beginning of the experiment, as a consequence of the habitat specialization and competitive exclusion due to the high selection forces applied in the reactors.
- The microbial community structure followed the same trend during the first part of the experiment when the selection forces were stronger indicating the prevalence of deterministic factors during the first stages of granulation. Once the washout dynamics were less pronounced, stochastic processes became more important, having a bigger impact in microorganisms showing lower relative abundance. These results indicate that both deterministic and stochastic processes might be involved in the microbial community assemblage during aerobic granulation.

6. Implications and further research

Research on the granulation process and granular stability is imperative to develop strategies to obtain aerobic granules in AGMBRs and to solve the problems with loss of granular stability in such reactors. Therefore, detailed studies on the granulation process are of great importance.

The results obtained in this thesis might help us to understand the complex processes behind granulation. The study of the microbial community composition in the granules and the effluent (Paper II) showed that during the first part of the experiment, most genera were washed out proportionally to their relative abundance on the floc-particles. Therefore, the biomass is proportionally washed out until granules emerge. Biofilm development is key for prokaryotic survival in hostile environments (Boltz *et al.*, 2017). Granules are suspended biofilms that develop under high shear forces in column shape bioreactors. Therefore, granulation is a response to stress. Short settling time and high height to diameter ratio apply a physical selection for dense and big particles and thus, microorganisms are retained in the reactor due to the size and density of the floc-aggregate they happen to belong to. The obtained results during the following phase showed that microorganisms located on the granular surface were preferentially washed out from the reactors due to erosion of the granules. Those which were retained in the reactor were protected from erosion. Moreover, those which were retained did not necessarily have an increasing relative abundance. Some bacteria retained in the reactors displayed a decreasing trend in relative abundance indicating that they were retained during the physical particle selection but were outcompeted thereafter. Therefore, the selection of certain genera in the granules was due to competitive exclusion and microcolony outgrowth.

AGMBRs are likely to be operated with a mixture of granules and flocs since the membrane is a barrier for suspended solids. Other processes which combine fixed-film and suspended activated sludge, such as integrated fixed-film activated sludge (IFAS), have been proven to increase the treatment efficiency for nutrient removal (Stricker *et al.*, 2009). It should be taken into account that the microorganisms eroded from the granular surface and those with high growth rates and ability to grow in the suspended phase would accumulate on the membrane surface and are likely contribute to fouling by the development of a biofilm. Therefore, more research on the washed out biomass fraction of SBRs should be carried out in order to determine the bacteria potentially causing fouling.

The presence of oxygen and substrate gradients inside the aerobic granules allows the co-existence of different guilds of microorganisms and therefore, different biochemical processes can take place simultaneously. It is generally assumed that granules consist of an aerobic outer layer where nitrifiers and heterotrophs are located, an anoxic substrate-rich interior layer where denitrifiers, PAOs and GAOs are situated and an anaerobic core containing mainly dead or inactive cells due to the absence of oxygen and substrate. The presence of AOB in the inner locations of the granules do not follow this multilayer model in the location of the different functional group in the aerobic granules. Nevertheless, it should be taken into account that dissolved oxygen in the reactor influences the granular location of autotrophs and oxygen was at saturated levels and therefore it could had penetrated to the inner zones of the granules (Ni

et al., 2008). This added complexity of granule architecture needs to be considered to better understand and model the aerobic granular sludge processes. This will allow us to understand and overcome more easily the granular structure loss observed in AGMBRs. If granules lose their structure or if their diameter change during the operation, the diffusion properties and the mass and oxygen gradients will be affected which will impact the simultaneous nitrification and denitrification.

Bacterial predation should also be more thoroughly investigated in wastewater treatment systems. Predation has an important role in bacterial ecology with direct impact on the microbial community and, ultimately, on the microbial functions. Protists grazing and bacteriophage attack are well recognized processes that exert a control in the microbial populations, but research is needed about predatory bacteria such as *Bdellovibrio* sp. to understand their impact on important microbial functions.

Moreover, the results showed an important influence of deterministic factors on the microbial community assemblage of aerobic granules. Therefore, this could indicate that it might be possible to direct more granulation under continuous operation and/or AGMBRs. More studies dealing with basic ecological processes involved in granulation are needed. For instance, it is important to know how perturbations affect the microbial community and the resilience of the system. This knowledge will help to overcome the granular disaggregation observed after the inoculation of AGMBRs with aerobic granules due to the sudden change of environment (see Paper I).

The results of the studies suggest that granulation is driven by deterministic factors. When short settling times are set during the start-up of the reactors, microorganisms are washed-out randomly. Then, when granulation starts, there is a physical selection of particles based on their settleability and, at the same time, microorganisms that are better adapted to the reactor conditions outgrowth others that were initially retained. The results together suggest that high washout dynamics might not be a requisite for granulation but instead, the selection by short settling times could act as an accelerant. Granulation with long settling times has been observed by other researchers (Dangcong *et al.*, 1999, Dulekgurgen *et al.*, 2003, Barr *et al.*, 2010, Weissbrodt *et al.*, 2013) and in our laboratory (results not published), but much longer reactor run times were needed to obtain aerobic granules. These results should be taken into account for AGMBR technology development as other factors may be more important for aerobic granulation, such as feast-famine regime, which could be applied to obtain aerobic granules in AGMBRs.

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