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Microbial Ecology of Granular Sludge

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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.
Photo: Raquel Liébana García

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ABSTRACT

Granular sludge is an efficient and compact biofilm process for wastewater treatment. Despite the well-established methods for granule cultivation, the ecological processes underpinning the microbial community assembly during granulation are poorly understood. Unveiling fundamental aspects of the microbial ecology of granular sludge will contribute to the improvement of the granulation methods and to an upgrade in the technology. In this thesis, reviews of the available literature were undertaken to assess critical points of current knowledge about the combination of aerobic granular sludge and membrane filtration, and to gain further knowledge on the ecology of the granular sludge and the granular structure. In parallel, three sequencing batch reactors were employed in different experiments and molecular biology techniques, such as high-throughput DNA sequencing, fluorescence in-situ hybridization and confocal laser scanning microscopy, were used. The reproducibility of the reactors was tested, showing the reactors to be generally reproducible for the abundant community members and for the reactor functions when constant conditions were applied. However, when subjected to periodic disturbances, the replicate reactors did not display a high degree in reproducibility in microbial community. Granulation responded to deterministic factors driven by the reactor conditions. During the start-up of the reactors, microorganisms were washed-out randomly and the granulation started as a response to the shear forces applied in the reactor. Simultaneously, there was a deterministic selection of microorganisms involved in aggregate development and for those that were well adapted to grow at the specific reactor conditions. It was also observed that stochastic processes, i.e. drift, had considerable effect on the less abundant community members. Moreover, stochasticity seemed to be important when the community was subjected to periodical disturbances. Also, bacterial predators appeared as part of the core community and they were found to predate on bacteria that were exerting important reactor functions. Ammonia-oxidizing bacteria were observed in the inner locations of the granules, which did not follow the commonly accepted multilayer model of stratification of different functional groups. The granules were able to withstand high pressures showing a high stability and strength when submitted to different water fluxes. In a separate study, it was shown that the choice of bioinformatics pipelines and dissimilarity indices affects the conclusions drawn from experimental data and the use of Hill-based indices was proposed for robust data analysis.

Keywords: aerobic granular sludge, sequencing batch reactors, granulation, reproducibility, microbial community dynamics, wash-out dynamics, disturbance, granular structure, fluorescence in-situ hybridization, high-throughput DNA analysis.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Liébana, R.,** Modin, O., Persson, F., Wilén, B.M., (2018). Integration of aerobic granular sludge and membrane bioreactors for wastewater treatment. Critical Reviews in Biotechnology, 38(6), p 801-816.

II. Wilén, B.M., **Liébana, R.,** Persson, F., Modin, O., Hermansson, M. (2018). The mechanisms of granulation of activated sludge in wastewater treatment, its optimization and impact on effluent quality. Applied Microbiology and Biotechnology, 102(12), 5005-5020.

III. **Liébana, R.**, Modin, O., Persson, F., Szabó, E., Hermansson, M., Wilén, B.M. (2019). Combined deterministic and stochastic processes control microbial succession in replicate granular biofilm reactors. Environmental Science and Technology, DOI: 10.1021/acs.est.8b06669.

IV. Szabó, E., **Liébana, R.**, Hermansson, M., Modin, O., Persson, F., Wilén, BM. (2017). Microbial population dynamics and ecosystem functions of anoxic/aerobic granular sludge in sequencing batch reactors operated at different organic loading rates. Frontiers in Microbiology, 8, 770.

V. Szabó, E., **Liébana, R.**, Hermansson, M., Modin, O., Persson, F., Wilén, B.M. (2017). Comparison of the bacterial community composition in the granular and the suspended phase of sequencing batch reactors. AMB Express, 7(1), 168.

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

VII. **Liébana, R.**, Modin, O., Persson, F., Hermansson, M., Wilén, B.M. Microbial community dynamics in response to periodical disturbances in granular sludge reactors. *Manuscript.*

VIII. Modin, O., **Liébana, R.**, Saheb Alam, S., Persson, F., Wilén, B.M., Suarez, C., Hermansson, M. Similar or dissimilar dissimilarities? Hill-based indices allow robust analysis of marker-gene amplicon sequencing data. *Manuscript.*

The author of this thesis has made the following contributions:

Paper I. First author. Contributed to the conception of the manuscript, drafted and critically reviewed the manuscript.

Paper II. Co-author. Contributed to drafting and critically reviewed the manuscript.

Paper III. First author. Contributed to the design of the experiment, the data analysis and interpretation (reactor operation, sample collection, chemical analysis, microbial community analysis and bioinformatics, statistical analysis), drafted and critically reviewed the manuscript.

Paper IV. Co-author. Contributed to the data collection and interpretation (FISH-CLSM analysis) and critically reviewed the manuscript.

Paper V. Co-author. Contributed to the data collection and interpretation (FISH-CLSM analysis) and critically reviewed the manuscript.

Paper VI. First author. Contributed to the conception and design of the experiment, the data analysis and interpretation (granule strength assessment, granule staining, CLSM analysis, statistical analysis), drafted and critically reviewed the manuscript.

Paper VII. First author. Contributed to the conception and design of the experiment, the data analysis and interpretation (reactor operation, sample collection, chemical analysis, microbial community analysis and bioinformatics, statistical analysis), drafted and critically reviewed the manuscript.

Paper VIII. Co-author. Contributed to the conception of the study, the data analysis and interpretation (granule reactor operation, sample collection, DNA extraction and PCR), and critically reviewed the manuscript.

Other publications completed during the realization of this PhD:

Liébana, R., Szabó, E., Tabesh, N., Persson, F., Modin, O., Hermansson, M., Wilén, B.M. (2018). Selection forces behind sludge granulation. IWA World Water Congress & Exhibition, Tokyo, Japan.

Liébana, R., Szabó, E., Persson, F., Modin, O., Hermansson, M., Wilén, B.M. (2017). *Bdellovibrio* sp. predation on aerobic granular sludge. FEMS 7th Congress of European Microbiologists, Valencia, Spain.

Liébana, R., Szabó, E., Persson, F., Suarez, C., Modin, O., Hermansson, M., Wilén, B.M. (2016). Complex architecture allows aerobic metabolism at depth in granules. IWA Microbial Ecology in Water Engineering & Biofilms, Copenhagen, Denmark.

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.

Liébana, R., Arregui, L., Belda, I., Gamella, L., Santos, A., Marquina, D., Serrano, S. (2015). Membrane bioreactor wastewater treatment plants reveal diverse yeast and protist communities of potential significance in biofouling. *Biofouling*, *31*(1), 71-82.

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Xabier, mila esker denbora guzti honetan nire bide-lagun izateagatik eta zure sostengu guztiagatik, une zailetan bereziki. Zu nire ondoan edukita, dena errezago bihurtzen da.

"From the paramecium to the human race, all life forms are meticulously organized, sophisticated aggregates of evolving microbial life."

Lynn Margulis

Margulis, L., & Sagan, D. (1986). Microcosmos: Four Billion Years of Evolution from our Microbial Ancestors

LIST OF ACRONYMS AND ABBREVIATIONS

AGMBR: aerobic granular membrane bioreactor
AOA: ammonia-oxidizing archaea
AOB: ammonia-oxidizing bacteria
βdis: β-diversity
βNTI: β-nearest taxon index
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
F/M: food-to-microbe
GAO: glycogen-accumulating organism
IFAS: fixed-film activated sludge
MBR: membrane bioreactor
NMDS: non-metric multi-dimensional scaling
NOB: nitrite-oxidizing bacteria
OLR: organic loading rate
OTU: operational taxonomic units
PAO: polyphosphate-accumulating organism
PCR: polymerase chain reaction
PERMANOVA: permutational multivariate analysis of variance
PD: phylogenetic diversity
q: order of Hill-based diversity index
RC_{bray}: Raup-Crick measures based on Bray-Curtis dissimilarities
RNA: ribonucleic acid
SBR: sequencing batch reactor
TD: taxonomic diversity
TN: total nitrogen
TOC: total organic carbon
WWTP: wastewater treatment plant

TABLE OF CONTENTS

1. Preface

Human activities generate urban and industrial wastewater that, when discharged untreated, causes the deterioration of aquatic environments due to the release of pollutants, such as organic matter and nutrients. 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. Large efforts have been done to improve wastewater treatment systems, especially for the removal of nutrients. The release of nutrients (nitrogen and phosphorous) to the environment is causing eutrophication of the water bodies on a global scale. Eutrophication has been a major concern during the past decades and wastewater effluents are one of the main sources of nitrogen and phosphorous. For instance, the Baltic Sea is very sensitive to nutrient enrichment which has resulted in large scale eutrophication of the sea. Baltic Sea countries face important challenges to meet the restrictive limits for nutrient discharge that have been set (Andersen *et al.*, 2009). Therefore, extensive and continued efforts in wastewater treatment research and the implementation of new processes are a priority.

Wastewater is treated in wastewater treatment plants (WWTPs) where the pollutants are reduced to below the regulated discharge limits. The biological treatment of wastewater, based on natural microbiological processes, is the main step in a WWTP where specific environmental conditions are applied in different bioreactors to select certain microorganisms which will remove the targeted contaminants (Henze & Knovel, 2008). Organic matter is removed through aerobic and anaerobic biological processes. Nitrogen is mainly removed in a two-step process: nitrification (aerobic) and denitrification (anoxic). Phosphorous is removed by chemical precipitation and/or by biological processes through the alternation of aerobic and anaerobic conditions. Therefore, during the biological treatment of wastewater, the microorganisms (sludge) and the wastewater are driven through several tanks with differences in their aeration. By providing alternation of aerobic, anoxic and anaerobic conditions in the different tanks, the targeted microorganisms grow and are able to metabolize these compounds (Bitton, 2011). Subsequently, the treated water is separated from the sludge by different methods. The most common method is through sedimentation in clarifiers allocated after the biological reactor (Sheik *et al.*, 2014). The wastewater treatment process has evolved and experienced many operational- and design changes to improve its effectiveness and flexibility and also to reduce the footprint of WWTPs (Jenkins *et al.*, 2014). Despite the substantial improvements that have been accomplished, the conventional process for wastewater treatment still faces important limitations: 1) high area requirements needed for the separation of the activated sludge and the treated water and, 2) multiple biological tanks needed for the removal of the pollutants.

Wastewater treatment using biofilm processes is becoming increasingly common. This is due to the existence of different habitats within the biofilm which favours the coexistence of different microbial groups, allowing the removal of organic matter and nutrients in the same reactor (Sperling & Knovel, 2007). Also, biofilms have long retention times and slow growing

bacteria (for instance those involved in nitrogen removal) can be kept in the WWTP by growing in a biofilm. Aerobic granular sludge is a biofilm process that has received much attention in the last decades due to the competitive advantages compared to the conventional process. Aerobic granular sludge is obtained by the granulation of the activated sludge into suspended biofilms with spherical shape. The physical features of the sludge substantially improve the sedimentation process. Besides their high settleability, aerobic granular sludge also displays high microbial densities and activities, ability to treat wastewater with high concentrations of organic matter and nutrients, and tolerance to toxicity. One of the most important features of aerobic granular sludge is the synchronization of nitrification, denitrification and biological phosphorus removal while degrading the organic carbon. This feature renders a highly efficient process for nutrient removal. Typically the granular sludge is separated from the treated water using sedimentation; however, membrane filtration is an alternative method that could be used (Iorhemen *et al.*, 2016). Integrating aerobic granular sludge with membrane filtration in an aerobic granular sludge membrane bioreactor (AGMBR) would be highly attractive due to the high-quality effluent that could be obtained using a much smaller space than a conventional activated sludge WWTP. However, the design and improvement of wastewater treatment systems, and granular sludge technology specifically, is hindered by the lack of understanding of the ecology of the microbial community on which they rely (McMahon *et al.*, 2007). Detailed studies on the microbial mechanisms involved in the granulation of the sludge into aerobic granules and the microbial community composition and population dynamics in aerobic granular reactors are imperative to our understanding of the factors involved in this process. A deeper knowledge will allow the development of improved granulation methods and the combination of aerobic granular sludge with membrane filtration.

1.1. Research motivation and scope of the thesis

The overall objective of this thesis is to unravel the ecological mechanisms behind the granulation process and the fundamental aspects of the microbial community succession in aerobic granular reactors, aiming to contribute to the development of AGMBRs.

The specific aims of this thesis were to:

- Assess the critical points of current knowledge in the combination of aerobic granular sludge and membrane filtration (Paper I).
- Assess microbial dynamics during sludge granulation (Papers II, III and IV).
- Examine the granular sludge structure and functions (Papers II, IV, V and VI).
- Assess the effects of perturbations on the granular structure and microbial community dynamics (Papers VI and VII).
- Assess the ecological processes and selection pressures affecting the sludge granulation and the microbial community dynamics in granular sludge reactors (Papers II, III, V and VII).
- Assess the impact of bioinformatics methods and choice of dissimilarity index on the results from high-throughput sequencing experiments (Papers III and VIII).

1.2. Scientific approach

An extensive literature study was conducted to assess the critical points of current knowledge in the combination of aerobic granular sludge and membrane filtration (Paper I). This literature review was undertaken as an opening in the PhD research to provide the background and scientific rationale for the laboratory studies. Results obtained by other researchers showed that there are still major challenges that have to be addressed: how to achieve granulation in these reactors and how to maintain the granular stability. The AGMBR technology is young and at this stage, its development is directly dependent on fundamental research performed on the ecology of the granular sludge and the granular structure. Aiming to gain further knowledge in these aspects, the available literature was assessed with special focus on the mechanisms of aerobic granulation and the microbial interactions involved (Paper II). In parallel, several experiments were carried out to investigate the research objectives.

Three identical sequencing batch reactors (SBRs) were used to perform the experiments described in this thesis. In paper III, the reproducibility of the microbial community structure and dynamics and fundamental aspects of the microbial community assembly during granulation were tested when the reactors were operated identically as replicates. In paper IV, the diversity of functional groups was evaluated, and the core community was identified when the reactors were operated at different organic loading rates (OLRs). In paper V, the impact of the washout dynamics on the bacterial community in the granular and suspended phases were assessed to understand why some bacteria grow in the granules and other are washed out. In paper VI, the strength and stability of aerobic granules when submitted to different water fluxes, and the granular matrix structure were studied. In paper VII, the microbial community structure and dynamics of the granular sludge and the reactor performance were examined when periodic disturbances were applied to the reactors, operated as replicates. Finally, since during the research it was observed that the bioinformatic methods and the choice of dissimilarity index can affect conclusions drawn from experimental data, the impact of different computational pipelines and input parameter choices on the results from high-throughput amplicon sequencing experiments was assessed in paper VIII.

1.3. Limitations

The performed experiments described in this thesis used lab-scale SBRs. These reactors were not optimized for nutrient removal since the performance was not the main focus. Therefore, due to the cycle parameters (4 hours cycle with anaerobic feeding, anoxic phase, aerobic phase, settling, withdrawal and idle phase), the lack of dissolved oxygen control and type of mixing (no mixing during the anoxic phase), complete nitrogen removal was not achievable. Also, changing the cycle parameters (i.e. increasing the cycle length and introducing an anaerobic phase) would have increased phosphorous removal. However, the optimization of the reactors is out of the scope of this thesis.

Due to practical reasons, working with lab-scale reactors limits the number of replicates to be used. This has important implications in further statistical analysis. The data is collected in time series and, ideally, several replicates from each data point should be used for robust statistical

tests. In some cases, samples from consecutive days in the time series were used as replicates when stable performance and/or microbial community dynamics were observed. However, measurements from replicate reactors would be more desirable. Due to this reason, the reproducibility of the system was assessed, and the reactors were operated as replicates in two of the studies.

2. Wastewater treatment

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. During the 19th century, several wastewater treatment methods were developed and during the 20th century, water quality standards were set and wastewater treatment facilities were constructed in the main cities of Europe. During the 20th century, eutrophication of water bodies became a great problem and research showed that excess nitrogen and phosphorus discharge to recipients from various sources, including wastewater treatment, was the cause. Since then, the biological elimination of nutrients from wastewater has been developed and there are today numerous processes and different configurations for wastewater treatment to achieve better efficiency in nitrogen and phosphorus removal (Lofrano & Brown, 2010, Jenkins *et al.*, 2014).

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

Wastewater is collected and then treated in WWTP where the pollutants are reduced to below the limits set by national regulations and guidelines. The most important pollutants in wastewater are biodegradable 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 pre-treatment where coarse materials and debris are eliminated. Then, settleable 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 polished 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).

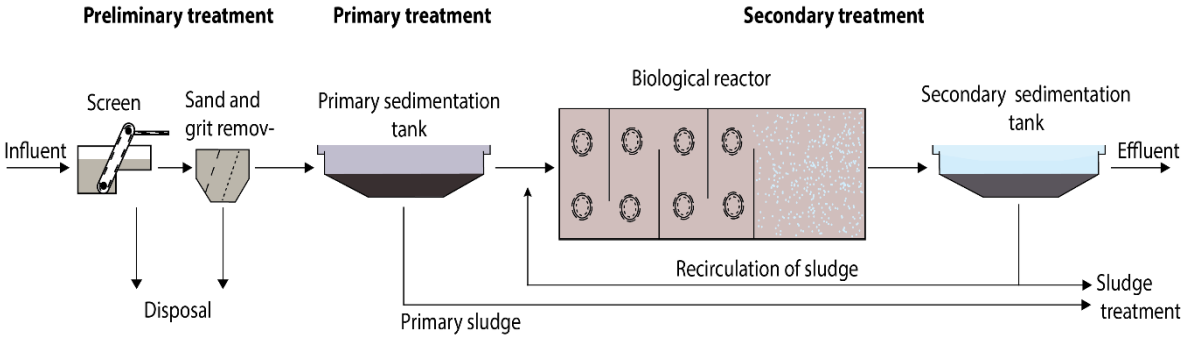

Figure 1. Schematic representation of a conventional wastewater treatment plant.

2.2. Biological treatment of wastewater

The biological treatment of wastewater is based on microbiological processes. Various environmental 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 film 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 and membrane bioreactors (MBRs) are examples of suspended growth processes. Fixed film processes use an inert support material inside the biological reactor on which 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).

2.2.1. 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 Ardern and William T. Lockett in 1914 (Ardern & 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 prevalent 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 pollutants available in wastewater. These pollutants are oxidized by the microbial metabolic activity and transformed into microbial biomass and other by-products (i.e. CO_2, N_2) (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 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).

2.2.2. Microbiology of wastewater treatment

The contaminants of wastewater are removed through a food chain 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).

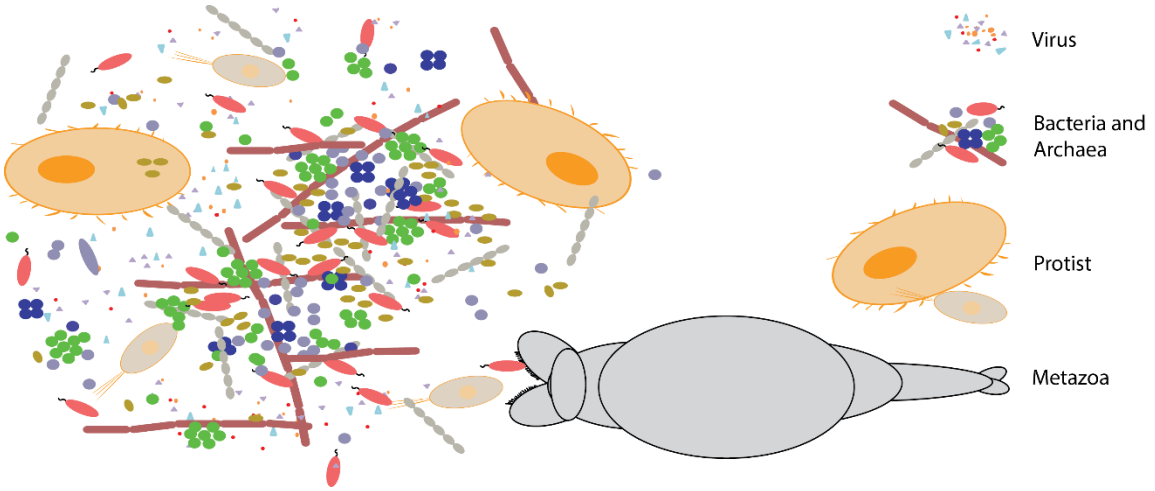

Figure 2. Most representative groups of microorganisms in the wastewater ecosystem.

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 extracellular polymeric substances (EPS) 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 *Nitrosospira* sp. are the main AOB representatives and members of the genera *Nitrobacter* 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). Since the ability for denitrification is spread among many different bacterial lineages and the populations change depending on the treatment plant and wastewater process, it is not clear which populations are dominant. The genera *Aquaspirillum*, *Azoarcus*, *Thauera* 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 and *Tetrasphaera* sp. have been reported to be the main organism responsible of phosphate accumulation. Other PAOs that have been reported are *Pseudomonas* sp., *Microlunatus phosphovorus*, *Ca.* Accumulimonas, *Dechloromonas* sp., *Ca.* Obscuribacter, *Thiothrix caldifontis* and *Comamonadaceae* members (Sheik *et al.*, 2014, Stokholm-Bjerregaard *et al.*, 2017, Qiu *et al.*, 2019).

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 WWTPs have to be upgraded to achieve compact, efficient and less energy consuming treatment processes to meet the increasing 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.

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 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). These features make the aerobic granular sludge process compact and energy efficient. For comparison, the aerobic granular process has been reported to be 50% and 13% more compact than the activated sludge process with biological P removal and the fixed-film activated sludge (IFAS) process respectively, displaying also a lower estimated energy demand (23% lower than activated sludge process, 35% lower than IFAS, or 50-70% lower than MBR) (Bengtsson *et al.*, 2018).

Aerobic granules are considered as suspended biofilms of microorganisms embedded in a matrix of 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 that can occur while degrading the organic carbon. This is possible due to the establishment of substrate gradients inside the developed granules. 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 3) (Morgenroth & Wilderer, 1998, Singh & Srivastava, 2011). Several reactor conditions are important for sludge granulation. When short setting times are applied, bacteria that lack the ability to aggregate will be washed out of the reactor whereas those forming aggregates that settles fast enough will remain. High hydrodynamic shear forces 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).

The aerobic granular sludge technology also displays some problems. Reactor cycle times need to be shorter during high flow conditions (i.e. rain events), which results in poorer treatment performance (Pronk *et al.*, 2015). Moreover, the suspended biomass in granular reactors needs to be washed out every cycle and generally do not attach to aggregates as easily as in activated sludge (Wilén *et al.*, 2003, Schwarzenbeck *et al.*, 2005). Therefore, regardless of the type of reactor, a post-treatment process is generally required in order to fulfil the effluent standards (Sanchez *et al.*, 2010, Morales *et al.*, 2013, Vijayalayan *et al.*, 2014). However, suspended solid concentrations below 50 mg L^{-1} have been reported in granules developed in a pilot reactor with low-strength domestic wastewater (Derlon *et al.*, 2016) and after the start-up period in full-scale plants treating domestic wastewater once granules were developed (Giesen *et al.*, 2013, Pronk *et al.*, 2015).

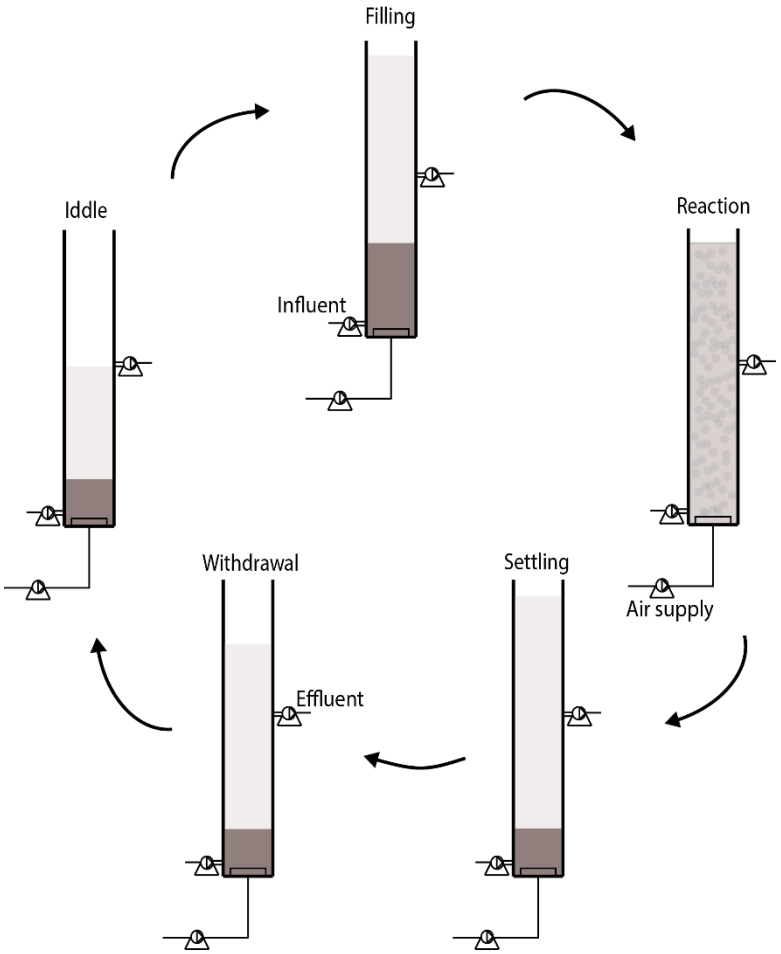

Figure 3. Stages of the SBR cycle.

3.2. Combining granular sludge with membrane filtration

AGMBRs can be considered a hybrid system where the biomass consists of aerobic granules and where the water is subsequently treated by membrane filtration. Integrating aerobic granular sludge with membrane filtration technology would be a way to combine the advantages and address the main problems associated with both processes. The main advantage of membrane filtration is the high-quality effluent obtained, being 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 MBRs 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 results from the physical, chemical and biological interactions of the foulants with the membrane surface. 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).

AGMBRs emerged from the need of decreasing fouling of membranes and the hypothesis that an increase in the density and particle diameter of the biomass in the reactor would decrease fouling of membranes. Therefore, early studies analysed the filterability of the mixed liquor and the treatment performance when aerobic granular sludge was used as seed in MBRs. After the first AGMBRs were developed, the interest in combining aerobic granular technology with membrane filtration technology increased as AGMBRs showed substantially better filtration performances than conventional MBRs (Li *et al.*, 2005, Tay *et al.*, 2007, Thanh *et al.*, 2008, Jing-Feng *et al.*, 2012, Li *et al.*, 2012, Thanh *et al.*, 2013, Sajjad & Kim, 2015). In general terms, membrane filtration improves when aerobic granular sludge is used as biomass, with a delayed transmembrane pressure rise (Juang *et al.*, 2008, Yu *et al.*, 2009, Tu *et al.*, 2010, Juang *et al.*, 2011, Wang *et al.*, 2013) even at high fluxes (Wang *et al.*, 2013). Most research is now directed towards the creation of specific AGMBR systems, either as continuous reactors or as sequencing batch systems coupled to an MBR. In a full-scale WWTP, AGMBRs would be highly attractive due to the high-quality effluent obtained using a much smaller space than with conventional biological treatment. With granular sludge, organic carbon and nutrients can be removed simultaneously, and consequently the different biological tanks could be replaced by one reactor.

The development of the AGMBR technology is expected to contribute to more efficient, compact and less energy demanding processes for wastewater treatment, which would enable increased treatment capacity and water reuse. The research on this topic could also have an impact on a global scale since many countries face severe water scarcity which is likely to increase with climate change. The interest in the AGMBR technology is manifested by an increasing number of research papers during the last decade. Nevertheless, despite the many advantages shown by this technology, many challenges remain to be solved before full-scale

application is reached, summarized in Figure 4. Research on granulation of the sludge under different conditions than those found in SBRs and the enhancement of granular stability is of vital importance for AGMBRs. Therefore, this technology will benefit from research performed on fundamental ecological aspects of aerobic granular sludge. Oxygen and substrate gradients inside the aerobic granules (Wilén *et al.*, 2004) allow different biochemical processes to take place inside, which is determined by the granular structure and diameter, and the reactor operational conditions (Winkler *et al.*, 2013, Weissbrodt *et al.*, 2014). Therefore, it is important to preserve stable granules in the reactor.

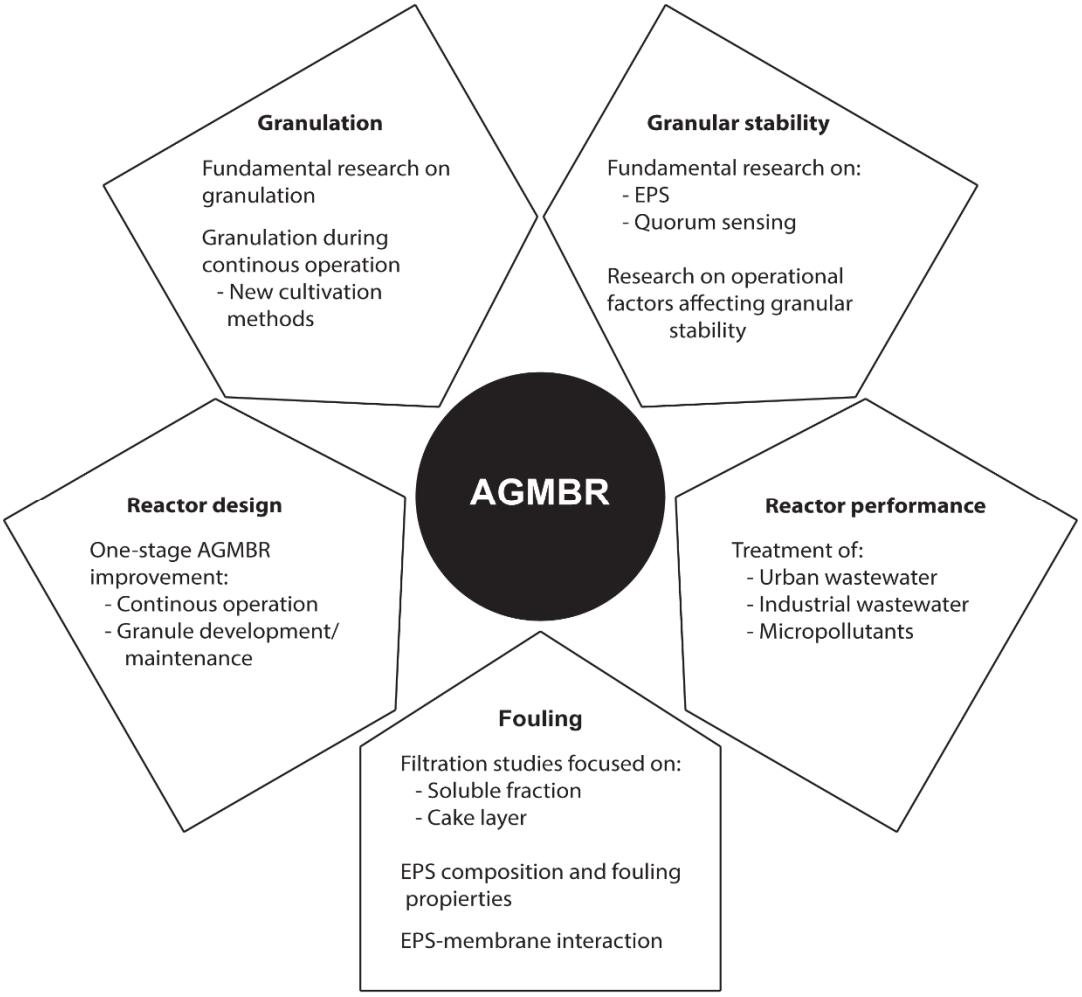

Figure 4. Most important future research needs for AGMBR development and scale-up. Paper I.

4. Granulation of activated sludge under aerobic conditions

4.1. Micro-aggregation and granular size increase during the granulation process

Granulation has been described to occur in several steps (Liu & Tay, 2002) including (1) cell-to-cell contact; (2) attractive forces between cells causing them to aggregate; (3) maturation of the microbial aggregates by forming a matrix of EPS onto which cells can attach and multiply; and (4) formation of a three-dimensional structure shaped by hydrodynamic forces and the microorganisms involved. Filamentous fungi and stalked protozoa have been reported to be important for the granular structure conformation, increasing the surface where the bacteria can attach (Beun *et al.*, 1999, Weber *et al.*, 2007). Granulation has also been described as a consequence of a dynamic floc/particle aggregation and breakage (Verawaty *et al.*, 2012, Zhou *et al.*, 2014) or microcolony outgrowth (Barr *et al.*, 2010). However, each step in the formation of granules is complex and is influenced by different physical, chemical and cellular mechanisms.

The initial stages of the sludge granulation are controlled by different forces and properties of the biomass (Liu & Tay, 2002, Liu *et al.*, 2009). Cell-to-cell contact and aggregate formation are caused by cellular mechanisms and physical and chemical interactions, influenced by cell-surface characteristics (Liu & Tay, 2002). For example, cell surface hydrophobicity is an important factor for granule development initiation. During granulation the protein/polysaccharide ratio tends to increase, which will cause increased cell surface hydrophobicity and a decreased negative surface charge (Liu *et al.*, 2003, Show *et al.*, 2012). This would lead to hydrophobic interactions and reduced electrostatic repulsion between bacterial cells facilitating aggregation and granulation (Liu & Tay, 2002, Zhang *et al.*, 2007, Gao *et al.*, 2011). The increase in protein/polysaccharide ratio is caused by changes both in EPS and bacterial community composition (McSwain *et al.*, 2005, Guo *et al.*, 2011). It has been shown that the EPS chemical composition changes during the transformation of floccular sludge into granules. Exopolysaccharides or glycosides have been found to be gelling agents in aerobic granules, distinctly more adhesive than EPS in activated sludge (Seviour *et al.*, 2009). Once the microbial aggregates have developed, they grow in size and the reactor conditions shape the young granules and select for regular, round, dense and compact aggregates (Liu & Tay, 2004). The development in granule size is dependent on a complex interaction of different environmental parameters and is relatively uncontrollable. Granules seem to reach a certain more or less stable granule size determined by the balance between granule growth, attrition and breakage, which is a consequence of the process conditions such as shear (Verawaty *et al.*, 2012).

In Paper III, we observed that during the early transformation from flocs to granule-like particles, previously reported primary colonizers and bridging bacteria in aggregate/biofilm development, such as *Acinetobacter* sp., were detected (Katharios-Lanwermeyer *et al.*, 2014, Liébana *et al.*, 2016). *Acinetobacter* sp., which have previously been observed during initial

stages of granulation (Weissbrodt *et al.*, 2012), is a hydrophobic bacterium and produces EPS, and this likely explains their frequent occurrence during the initial phase. The microbial communities shifted as the granules started to increase in size and bacteria within *Comamonadaceae*, *Rhodocyclaceae*, *Flavobacteriaceae*, *Xanthomonadaceae* and *Caulobacteraceae* became more abundant. Many of these bacteria are associated with granulation (Weissbrodt *et al.*, 2012). *Thauera* sp., which was particularly abundant in this period, is an important EPS producer commonly found in acetate-fed aerobic granules (Xia *et al.*, 2018), which was also one of the most abundant taxa during granulation in Paper IV.

4.2. Microbial community dynamics during sludge granulation

Aerobic granular sludge has a higher microbial diversity than floccular sludge since more ecological niches are available due to the substrate gradients created within the aggregate. Microbial selection is triggered by operational parameters such as type of substrate, OLR, food-to-microbe (F/M) ratio, chemical oxygen demand (COD) to-nitrogen ratio, solids retention time, settling time and redox conditions. The same functional groups of microorganisms are present in granular and floccular sludge, but with differences in the proportions between phylogenetic groups at a phylum or class level (Guo *et al.*, 2011, Winkler *et al.*, 2013). He *et al.* (2016) analysed the microbial community from an aerobic granular sludge reactor treating synthetic wastewater with low OLR and observed a fast change in microbial diversity and richness during granulation with *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi* and *Actinobacteria* as the most abundant phylum. Aqeel *et al.* (2016) showed, in laboratory scale reactors fed with synthetic wastewater, that the genera *Rhodanobacter* dominated at the maturation stage of granulation which coincided with an increase in protein content of the EPS. Fan *et al.* (2018) observed in identical reactors operated either with domestic wastewater or synthetic wastewater that the microbial community was similar for the two reactors where minor genera in the seed sludge, *Arcobacter*, *Aeromonas, Flavobacterium* and *Acinetobacter*, became dominant in the granules and ammonium-oxidizing archaea (AOA) were gradually washed out whereas AOB and NOB were retained. The microbial community during granulation has also been studied in full-scale reactors. Świątczak & Cydzik-Kwiatkowska (2018) showed that the abundance of *β-Proteobacteria*, δ-*Proteobacteria*, *Flavobacteria* and *Cytophaga* increased in abundance when converting activated sludge into granules in a full-scale plant performing COD, nitrogen and phosphorus removal. Liu *et al.* (2016) compared the microbial community of flocs and granules belonging to the same reactor, in a full-scale treatment plant designed for COD and nitrogen removal, receiving both industrial and domestic wastewater and observed that granules contained mainly *Planctomycetes, Proteobacteria*, *Bacteroidetes* and *Euryarchaeota*, whereas the flocs were dominated by *Proteobacteria, Bacteroidetes, Planctomycetes* and *Methanosaeta.*

The *Comamonadaceae* and *Rhodocyclaceae* families have been related with granulation and genera within these families are important EPS producers (Li *et al.*, 2008, Weissbrodt *et al.*, 2012, Xia *et al.*, 2018). Indeed, in Papers III and IV these families were the most abundant ones (Figure 5B). In paper III, the microbial community composition was highly dynamic during the transition from floccular to granular sludge when an OLR of 3 kg COD $m^{-3}d^{-1}$ was used in the reactors, operated as replicates. The seed sludge, mainly composed of *Betaproteobacteria*,

Alphaproteobacteria, *Sphingobacteria* and *Clostridia* at class level, changed rapidly after inoculation to a community dominated by *Gammaproteobacteria.* Thereafter, *Betaproteobacteria* dominated in the three reactors, with high abundances of the genera *Thauera*, *Acidovorax*, *Simplicispira*, *Comamonas*, *Curvibacter* and *Alicycliphilus*. At the end of the experiment at day 35, *Actinobacteria* were dominant, with *Corynebacterium* sp. as only representative of this class. Many taxa that were rare or undetected in the seed sludge became abundant during the granulation, e.g. *Corynebacterium* sp., *Brevundimonas* sp., *Pedobacter* sp., *Fluviicola* sp. and *Bacteriovorax* sp., while other taxa that were common in the seed sludge decreased drastically in relative abundance, e.g. *Rhodoferax* sp., *Rhodobacter* sp., *Pseudorhodobacter* sp. and *Hydrogenophaga* sp. In paper IV, *Proteobacteria* and *Bacteroidetes* were the most abundant phyla in all three reactors. By the end of the experiment, *Meganema*, *Thauera* and *Paracoccus* dominated R1, the reactor with the highest OLR (3.7 kg COD m^{-3} d^{-1}). The genera *Meganema*, *Thauera* and *Zoogloea* were the most abundant in R2, the reactor with the intermediate OLR (1.9 COD m^{-3} d^{-1}). In R3, the reactor with the lowest OLR (0.9 kg COD m^{-3} d^{-1}), *Zoogloea* and *Thauera* were most abundant genera. *Thauera* sp. and *Zoogloea* sp. have been found at both low and high organic loading rates, between 1 and 15 kg COD m^{-3} d^{-1} (Ebrahimi *et al.*, 2010, Zhao *et al.*, 2013, Lv *et al.*, 2014). *Meganema* sp. has been found to be abundant in reactors operated at 1.5-3 kg COD m^{-3} d^{-1}, treating industrial or synthetic wastewater (Kong *et al.*, 2014, Figueroa *et al.*, 2015). *Paracoccus* sp. has been reported at loading rates of 1.5-3.3 kg COD m^{-3} d^{-1}, in reactors treating synthetic wastewater (Lv *et al.*, 2014, Cydzik-Kwiatkowska, 2015). In paper V, we observed that, besides some differences when the reactors reached steady-state, the composition of the washed-out population followed closely the community composition of the reactor regardless of the applied OLRs. Some taxa (e.g. *Acidovorax* in reactors R2 and R3, *Brevundimonas* in reactor R1) were increasingly retained as the experiment progressed. Other genera became progressively more abundant in the effluent (e.g. *Meganema* in R1, *Leptothrix* in R2), although this was a less common phenomenon.

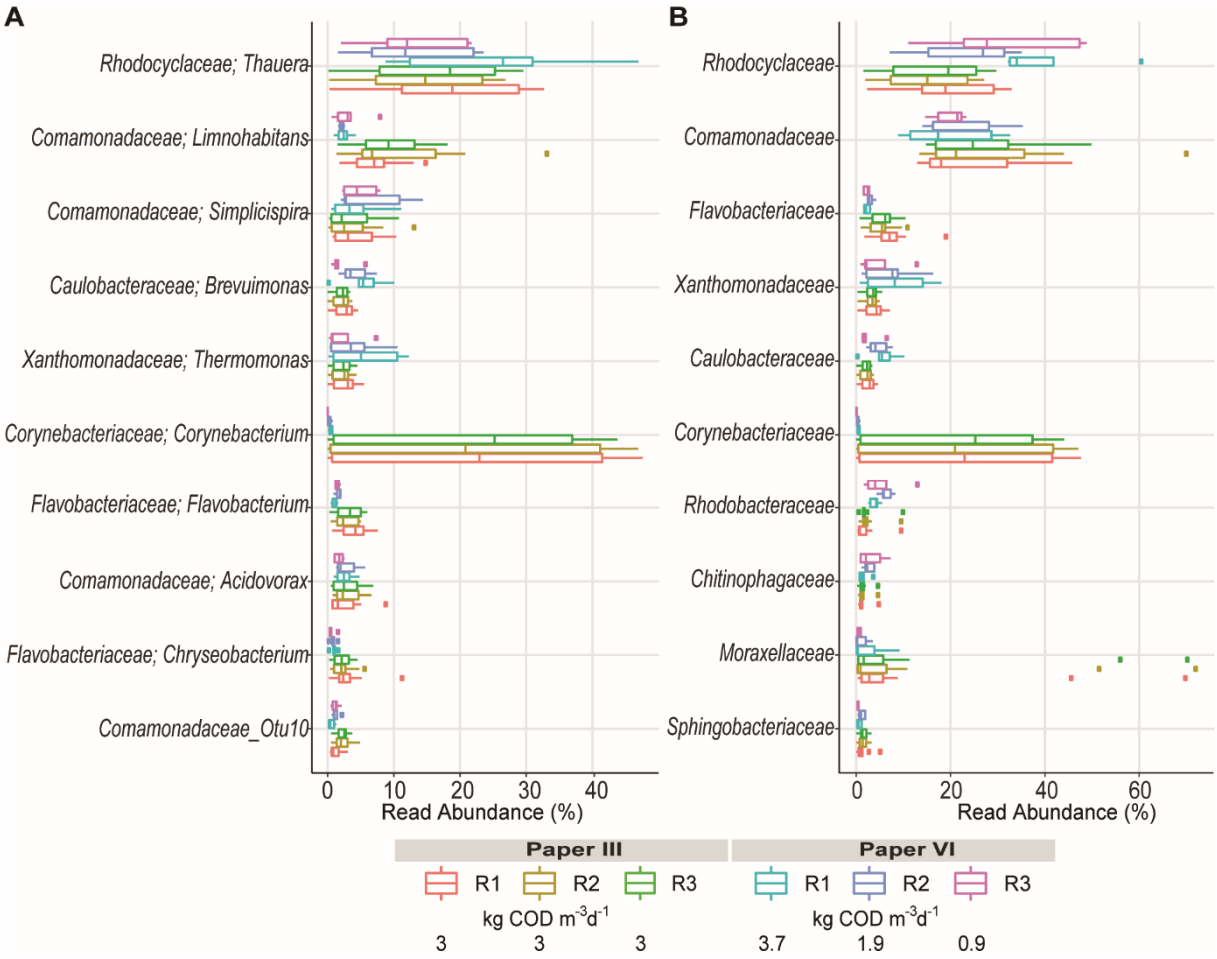

Figure 5. Relative abundance of the 10 most abundant genera (A) and families (B) in the reactors (R1, R2 and R3). In Paper III, the reactors were operated as replicates with synthetic wastewater and an OLR of 3 kg COD $m^{-3}d^{-1}$. In Paper IV, the reactors were operated with a mixture of real and synthetic wastewater (1:1) with an OLR of 3.7, 1.9 and 0.9 kg COD $m^{-3}d^{-1}$ in R1, R2 and R3 respectively.

4.3. Selection forces applied in the reactors for sludge granulation

Granules are generally thought to be obtained by 1) applying high hydrodynamic shear forces; 2) feast-famine alternation; and 3) washing-out of the non-granulated biomass (de Kreuk & van Loosdrecht, 2004, Liu & Tay, 2004, Adav *et al.*, 2008, Lee *et al.*, 2010, Show *et al.*, 2012). Generally speaking, bacteria can exist both in a planktonic or attached mode and biofilm/granule development is a key for retention in flowing environments that develop under shear forces (Boltz *et al.*, 2017). Thus, granulation is a response to specific selection pressures applied in the reactors.

Washing out the non-granulated biomass is considered an important selection force for sludge granulation. But according to the results from various studies, high wash-out rates is not a prerequisite for granulation to occur, although the process is accelerated considerably. Indeed, in our laboratory, and in others, granulation has been observed even at long settling times with a low degree of wash-out of suspended matter (Dangcong *et al.*, 1999, Barr *et al.*, 2010, Weissbrodt *et al.*, 2013), but as expected, much longer reactor run times were needed to obtain

aerobic granules under these circumstances. Higher shear forces have been found necessary to achieve granulation when long settling times are applied (Zhou *et al.*, 2014, Chen & Lee, 2015). High shear forces increase the production of extracellular polymers in aerobic granular sludge, with a higher polysaccharide/protein ratio, increasing the hydrophobicity of the biomass (Tay *et al.*, 2001, Xia *et al.*, 2018). Therefore, high shear force assists the formation of compact and denser aerobic granules shaping the granules into rounded aggregates by removing outgrowing structures. Feast-famine alternation and anaerobic feeding increases bacterial cell surface hydrophobicity, accelerate the microbial aggregation and selects for slow growers (Liu *et al.*, 2004, Adav *et al.*, 2008).

In Paper V it was observed that the community composition of the effluent and the granules was similar, but not identical. 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. During the initial stages of granulation, most genera were washed out proportionally to their relative abundance on the floc-particles. Therefore, the biomass was proportionally washed out until granules emerged. Once granules emerged, microorganisms located on the granular surface where preferentially washed out from the reactors due to erosion of the granules while those growing in the granular interior were retained in the reactor. Some bacteria retained in the reactors still displayed a decreasing trend of relative abundance, indicating that they were retained during the physical particle selection but were thereafter outcompeted by better adapted ones. Zhou *et al.* (2014) observed that when flocs and crushed granules were differently labelled with fluorescent microspheres and mixed in a reactor, flocs detach and re-attach to granules in a random manner. This indicates that floccular sludge is not washed out from the reactor due to the inability of certain microorganisms to form granules, instead microorganisms move between granular and floccular sludge randomly (Verawaty *et al.*, 2012, Zhou *et al.*, 2014). These results together reinforce the notion that high wash-out dynamics is not a requisite for granulation but, instead, it acts as an accelerant.

4.4. Predation and granulation

Predation is one of the most important interactions between living organisms, and is a major cause of bacterial mortality with direct implications on the genetic and functional structure of communities (Jousset, 2012). Bacteriophages (virus), protists and predatory bacteria are the most important microbial predators (Johnke *et al.*, 2014). Predation has been reported to have important implications for the process performance in WWTP.

Bacteriophages constitute a highly diverse group of organisms, having 10-100-fold higher diversity than bacteria in aquatic ecosystems. They are highly important for regulating the bacterial community and can be responsible for up to 71% of the bacterial mortality (Johnke *et al.*, 2014). Shapiro *et al.* (2009) observed phage predation to affect the microbial community composition in a full-scale MBR treating industrial wastewater. Barr *et al.* (2010) operated a laboratory-scale SBR for enhanced biological phosphorous removal. They associated an unexpected drop in phosphate-removal performance with bacteriophages infection of the key phosphate-accumulating bacterium in the reactor due to the presence of elevated levels of virus-like particles in the reactor.

Stalked ciliates have been observed in higher numbers growing on granule surfaces (Lemaire *et al.*, 2008). Winkler *et al.* (2012) reported *Vorticella*-like protist actively grazing on bacteria in aerobic granules. In Paper VII, the granules were broadly colonized by Peritrich ciliates throughout the whole experimental period. The *Epistylis* genus was found to predominate and some individuals of the genus *Carchesium* were also detected. Colonies with a varying number of zooids (indicating a probable coexistence of different species within the genus) were distributed throughout the entire granular surface. Protistan grazing activity induces different phenotypes of bacteria, such as biofilm development, as a survival strategy (Matz & Kjelleberg, 2005). A higher biofilm production and aggregation has been reported due to the grazing activity of protists (Matz *et al.*, 2004, Liébana *et al.*, 2016). Predation by protists can also cause a reduction of the biofilm bacteria (Huws *et al.*, 2005) and even extend to deep biofilm layers (Suarez *et al.*, 2015).

Predatory bacteria feed on other microbial cells and they have been found in a variety of environments (Martin, 2002). They prey on gram negative bacteria, with some being generalist, some specialists, and some versatile in their prey preferences (Chen *et al.*, 2011, Johnke *et al.*, 2014). The presence of predatory bacteria in aerobic granules and its persistence during granulation has been reported (Li *et al.*, 2014, Wan *et al.*, 2014, Weissbrodt *et al.*, 2014). Predatory bacteria were also detected in the experiments presented in this thesis, displaying high relative abundances, as high as at 10% relative abundance in Paper III, and was also one of the most abundant taxa in Papers IV and V. Predatory bacteria and their effect on microbial community is poorly understood. Evidence suggest that they have an important influence on microbial community structure, function and dynamics. For instance, it has been reported the predation of *Nitrospira* sp. by *Micavibrio*-like bacteria can have a direct impact on the nitrification process (Dolinšek *et al.*, 2013). Interestingly, we have observed, with fluorescence in-situ hybridization (FISH) and confocal laser scanning microscopy (CLSM), *Bdellovibrio* sp. to actively predate inside the granules, preferentially on AOB, to which they are co-located (Figure 6).

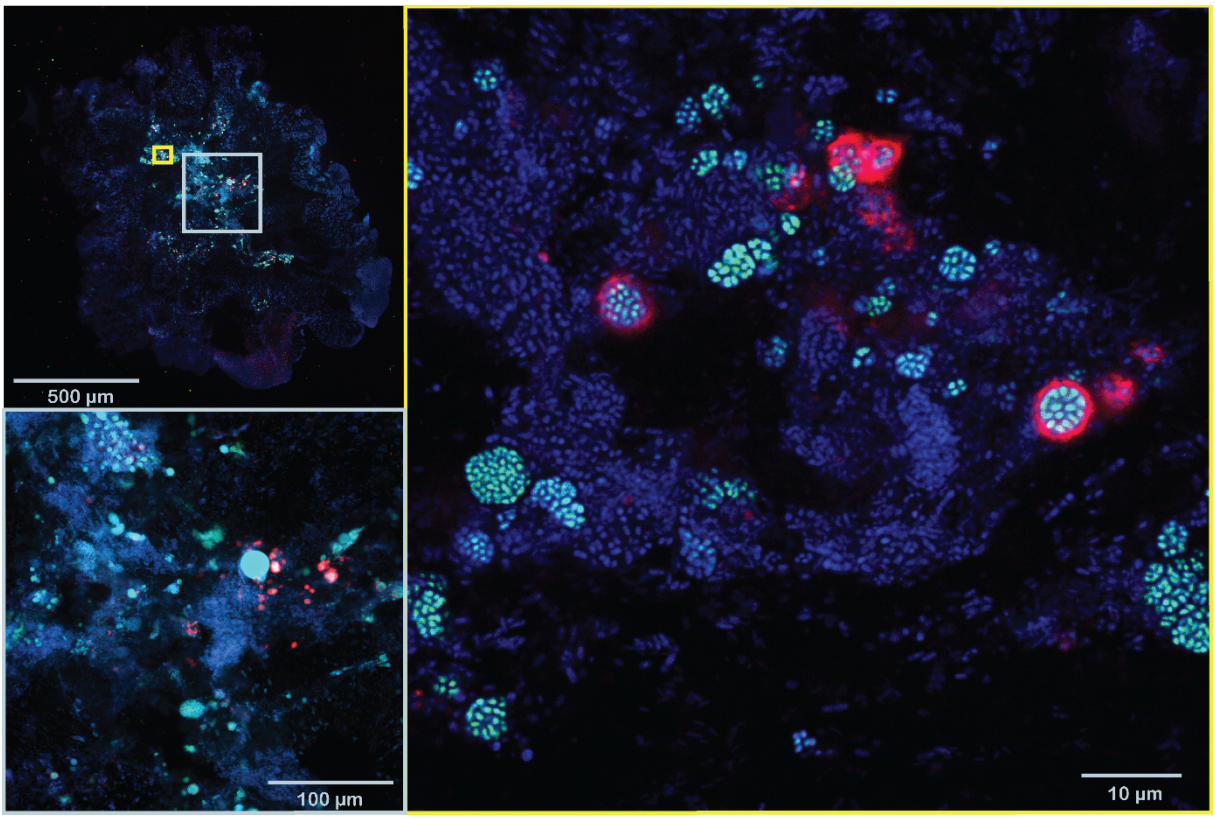

Figure 6. FISH-CLSM images from cryosections of aerobic granules from R1 at 200 × magnification and detailed sections at 400 × magnification. Blue: total bacteria (EUBmix); green: AOB (NEU654, Nse1472, and Cluster6a192); red: genus *Bdellovibrio* (BDE525).

5. Microbial stratification within the granules

5.1. Granular structure and microbial functions

The reactor conditions, together with substrate gradients in the granule, create ecological niches allowing the coexistence of a diverse community with multiple functions, which are utilized in wastewater treatment (Winkler *et al.*, 2013). These different guilds of microorganisms are not randomly distributed within the granules, instead, their distribution is generally determined by oxygen and substrate gradients inside the granule where heterotrophic-, nitrifying-, denitrifying-, phosphorous accumulating- and glycogen-accumulating organisms (GAOs) can coexist (Adav *et al.*, 2008, Lee *et al.*, 2010, Gao *et al.*, 2011). Although, there are studies that indicate that organisms are not always sorted according to a "redox tower" (e.g .Chen *et al.* (2017), Suarez *et al.* (2018)), nitrifiers are generally located in the oxygen penetrated outer layers whereas denitrifiers and PAOs have been shown to be located in the inner layers of granules (Xavier *et al.*, 2007, Winkler *et al.*, 2013). Therefore, the granular stability is very important for the processes since granular structure and diameter are linked to the function and activity. If granules lose their structure or if their diameter change, mass transfer and oxygen diffusion gradients are affected. This has a negative impact on the simultaneous nitrification/denitrification and biological phosphorus removal. Thus, studying granular stability under different stresses is important. In paper VI, the differences in compressibility and breakage dynamics of aerobic granules submitted to different water fluxes (from 116 to 4720 m^3 m^{-2} h^{-1}) through a nylon mesh membrane were evaluated. The results showed that the tested granules displayed a high stability and strength and could withstand high pressures ranging from about 0.2 to 4.5 kN m^{-2} and fluxes of 1100-1600 m^3 m^{-2} h^{-1} before breakage.

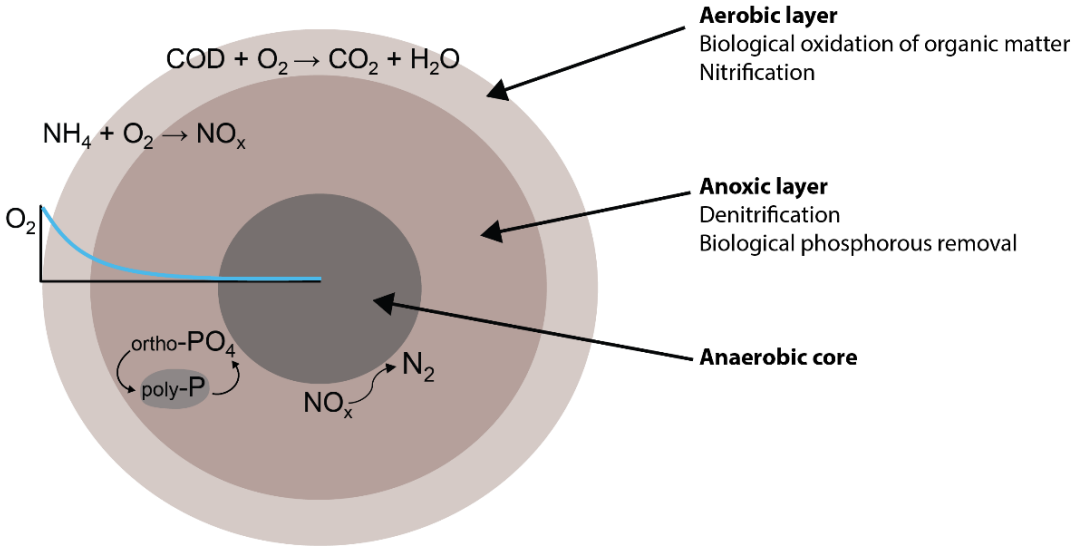

Figure 7. Structural representation of an aerobic granule and biological processes occurring inside.

Conceptual and mathematical models often simplify the granular structure by considering granules as a multilayer sphere with decreasing oxygen and substrate gradients from the outside to the core of the granule (Figure 7). Guimarães *et al.* (2017) showed that a microbial stratification existed in granules: AOBs dominated the outer layer, whereas NOB and

denitrifiers were located in the inner parts. However, in paper IV, FISH-CLSM performed on granule cryosections revealed nitrifiers growing both at the oxygen rich surface and inside the granule in channels and voids (Figure 8). Both oxygen and ammonia were transported across the granule through the channels where AOB grew. These results were observed for three different OLRs and are in contradiction to the conceptual and mathematical models commonly employed. Gonzalez-Gil & Holliger (2014) also observed the presence of channels and voids in mature granules where the substrate penetrated. It is well known that biofilms are heterogeneous structures and in most of the cases they contain pores, channels, mushroom-like structures and water-filled voids (Wimpenny *et al.*, 2000, Flemming & Wingender, 2010) and, therefore, it is no surprising that aerobic granules would also show high structural heterogeneity.

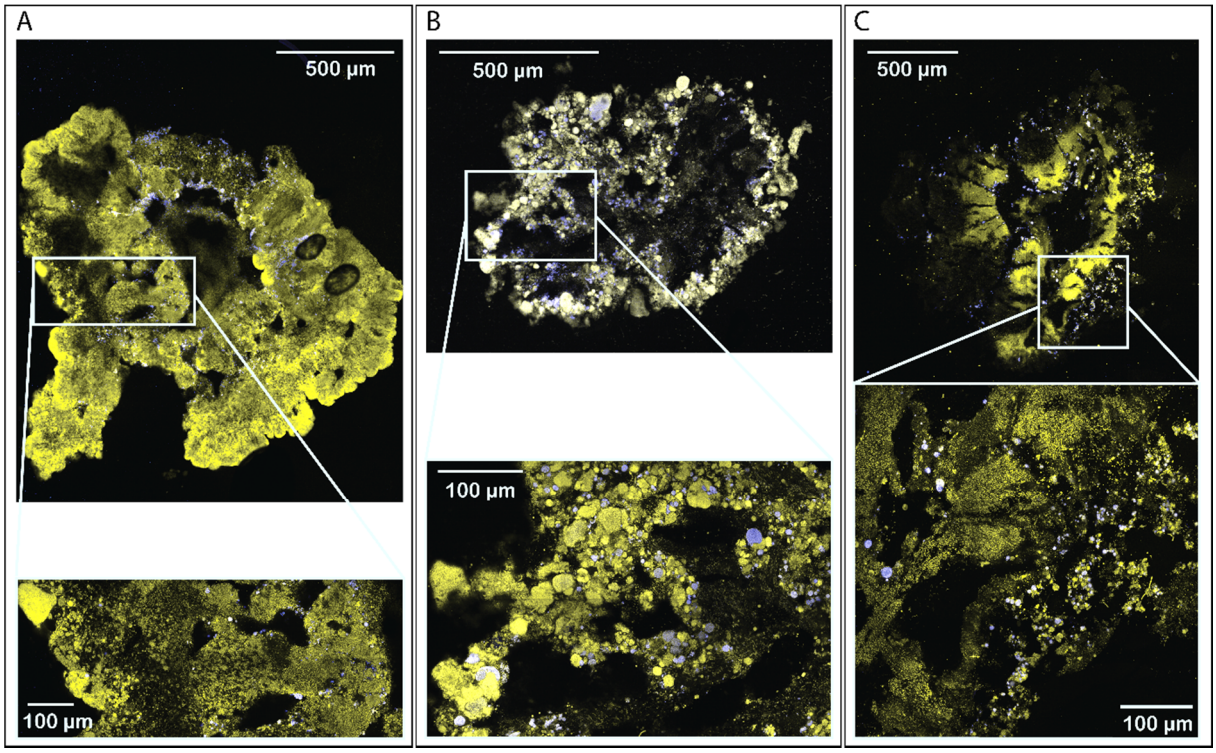

Figure 8. FISH-CLSM images from cryosections of aerobic granules from reactors R1 (A), R2 (B), and R3 (C) at 200 × magnification (upper images) and detailed sections at 400 × magnification (lower images). Yellow: total cells (Syto 40); blue: AOB (NEU654, Nse1472, and Cluster6a192). Paper IV.

The EPS granular matrix, where the microorganisms are embedded, is an important component of the aerobic granular sludge. They exert a major role during the granulation of the sludge and for the stability of the granules (Nancharaiah & Kiran Kumar Reddy, 2018). The distribution, proportion and chemical composition of polysaccharides, proteins, humic acids, nucleic acids and lipids will determine the physical characteristics of granules (Adav *et al.*, 2008, Seviour *et al.*, 2010). Differences in the distribution of EPS have been found during the process of granulation. Several authors reported proteins as the major EPS component in granules and being responsible for the structural strength (Gao *et al.*, 2011). McSwain *et al.* (2005) found proteins to be more abundant in the core, while in the outer layers, where active cells are located, polysaccharides were actively secreted in high amounts. Chen *et al.* (2007) showed that β-

polysaccharides and proteins were located in the core and cells accumulated in the outer layers of acetate-fed granules while only proteins were accumulated in the core of phenol-fed granules. In Paper VI, it was observed that β-polysaccharides and proteins were in high concentrations in the vicinity of the cells, distributed in higher abundance in the outer layers of the granule. These molecules were also detected in the inner layers surrounding the core, which appeared to be void of cells (Figure 9). In agreement with McSwain *et al.* (2005), β-polysaccharides appeared to embed the cells. Calcium ions had the widest distribution, being abundant not only in the outer layers, but also in the inner layers of the granule, even in the deepest parts. It has been proposed that calcium ions bind to the EPS increasing the granular strength by forming an EPS-Ca^{2+}-EPS complex (Liu & Sun, 2011).

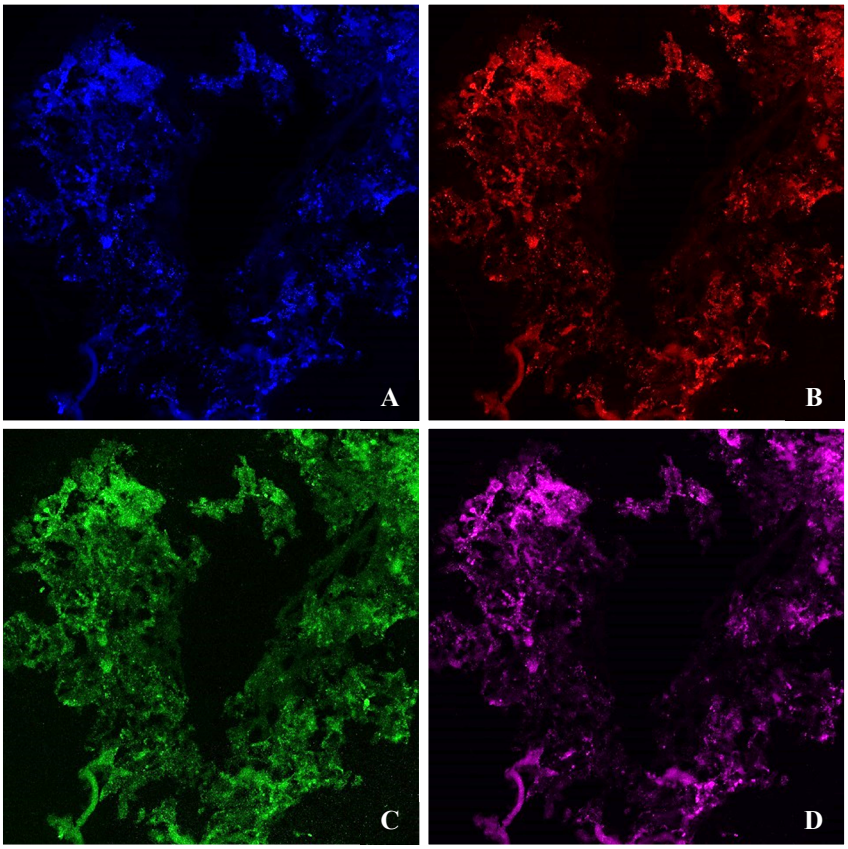

Figure 9. CLSM images of the cryosectioned granule stained with dyes for some of the key EPS components of the granular matrix. A: β-polysaccharides (Calcofluor White); B: proteins (SYPRO Ruby); C: calcium ions (Calcium Green-1); D: total cells (SYTO 62). Paper VI.

5.2. Location of selected taxa in granules

Bacteria are not equally distributed within the granules as some are more abundant at the outer layers of the granule and some in the interior (de Kreuk *et al.*, 2005). Lv *et al.* (2014) observed that mature granules had a core with *Rhodocyclaceae* covered by an outer shell containing both aerobic and anaerobic strains. It is assumed that bacteria growing at the surface of granules are more easily detached due to shear forces compared to the ones further in. Winkler *et al.* (2012) observed nitrifiers in the outer oxic layers, which were retained in the reactor for shorter times,

than PAOs and GAOs, which were found both at the outer parts and inner parts, or Archaea which were only present in the inner part.

In Paper V, FISH-CLSM 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 (ratio between abundance in the granules and abundance in the effluent). *Meganema* sp. and *Zoogloea* sp. were located on the granular surface (Figure 10). Both genera had retention ratios significantly lower than one, meaning they had higher relative abundance among suspended cells than in the granular biomass. These bacteria grew in loosely packed layers around the granules and were likely subjected to erosion. *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). 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. *Bdellovibrio* sp. and *Flavobacterium* sp. were 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). Despite being obligate aerobic, *Bdellovibrio* sp. was located in the inner parts of the granule predating 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-CLSM analysis targeting AOB in Paper IV revealed that oxygen penetrated to deeper regions of the granules.

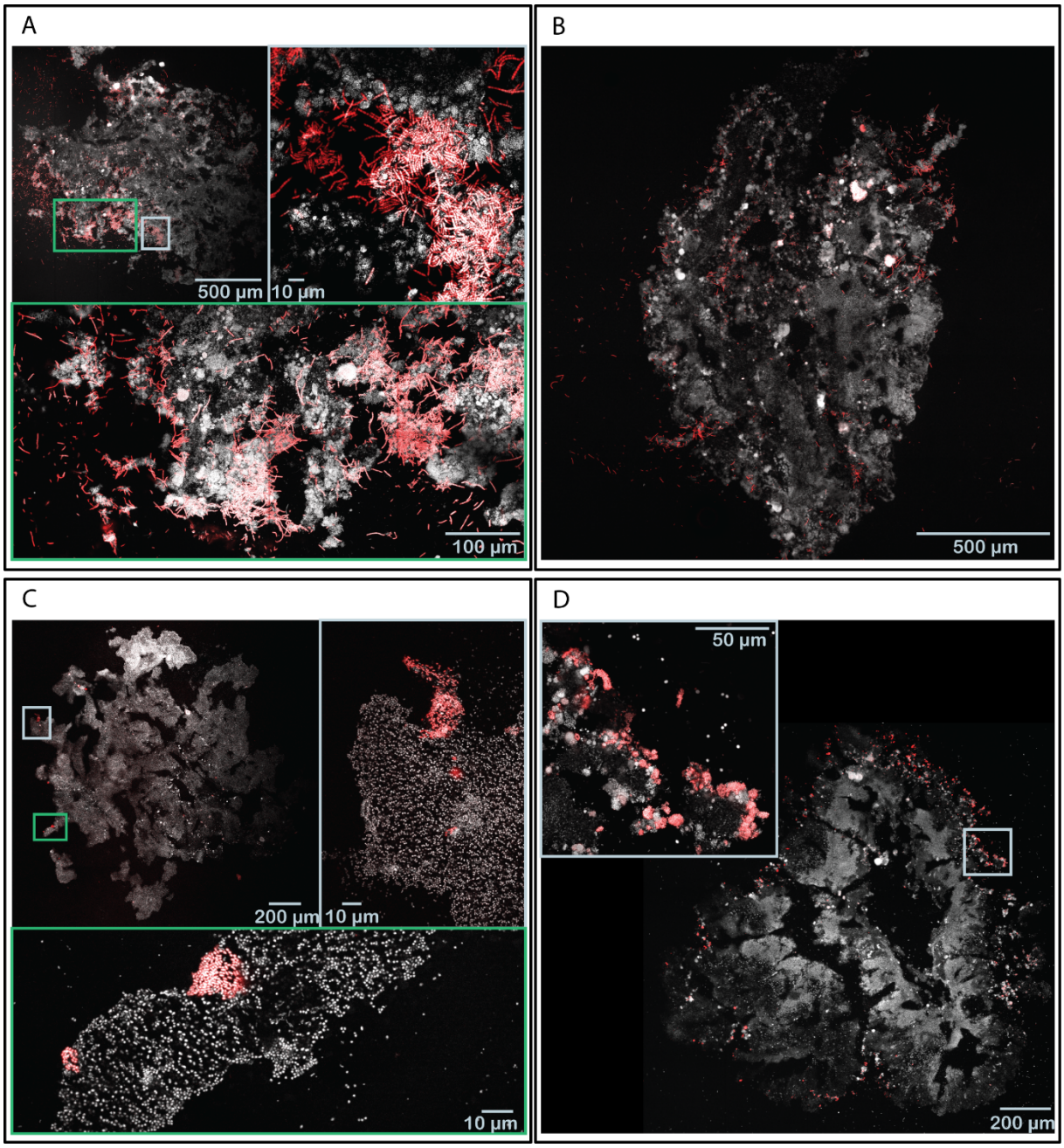

Figure 10. FISH-CLSM images from cryosections of aerobic granules of the selected bacteria with retention ratios significantly lower than one at 200 × magnification and detailed sections at 400 × magnification. A and B: *Meganema perideroedes* in aerobic granules from reactor R2; C: *Zoogloea* spp. in aerobic granules from reactor R2; D: *Zoogloea spp.* in aerobic granules from reactor 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). Paper V.

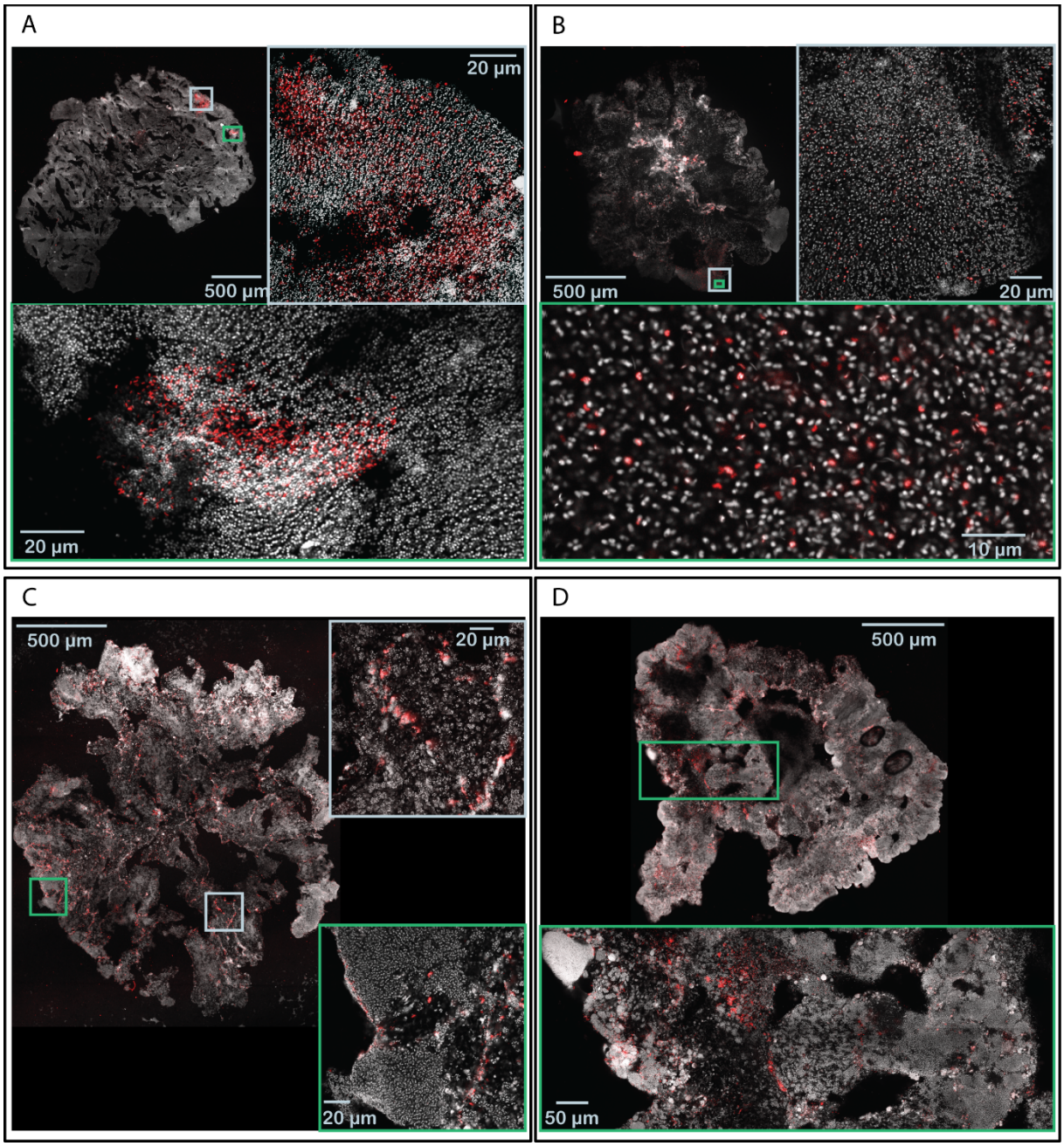

Figure 11. FISH-CLSM images from cryosections of aerobic granules of the selected bacteria with retention ratios significantly higher than one at 200 × magnification and detailed sections at 400 × magnification. A and B: genus *Bdellovibrio* in aerobic granules from reactor R1; C and D: *Flavobacteria*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

6. Ecological processes affecting microbial community assembly during granulation

The structure of microbial communities results from complex and dynamic ecological mechanisms (Widder *et al.*, 2016), which traditionally have been grouped into deterministic and stochastic factors (Zhou & Ning, 2017). Environmental conditions, species interactions and species traits are considered deterministic (Leibold, 1995, Chase & Leibold, 2003), whereas random events, such as birth and death, are considered stochastic (Hubbell, 2001). These deterministic and stochastic factors can be framed into four fundamental processes: selection, dispersal, diversification and drift. Selection refers to deterministic factors that modify the community structure according to the environmental conditions, differences in fitness between individuals and microbial interactions. Dispersal refers to the movement and establishment of microorganisms among communities, which can be both deterministic and stochastic. Diversification refers mainly to stochastic factors, which generate genetic variation. Drift refers to stochastic changes as a result of birth, death and reproduction (Vellend, 2010, Nemergut *et al.*, 2013). These four processes act simultaneously in natural ecosystems and their influences vary in time and space (Nemergut *et al.*, 2013, Zhou & Ning, 2017), therefore it is challenging to determine their contribution to microbial community assembly. The study of the biodiversity within and between microbial communities can help us to understand the underlying ecological processes (Nemergut *et al.*, 2013). Studying the compositional differences (turnover) between two different communities (β-diversity) provides a link between biodiversity at the local scale (within-sample or α-diversity) and at the regional scale (overall diversity or γ-diversity) (Anderson *et al.*, 2011).

Disturbances occurring to a microbial community can cause deterministic changes, affecting specific taxa or affecting the availability of resources creating new niches, which could be exploited by other taxa. Disturbances can also cause stochastic changes, randomly affecting interactions between members of the community or due to the stochastic colonization of new created niches (Herren *et al.*, 2016, Shade, 2016). Disturbances can be defined as events that can directly affect the community or can create a change in the environment that will eventually affect the community. The effects on the community include the death of some members, which can affect the phylogenetic diversity, or changes in the relative abundance, affecting the taxonomic diversity (Shade *et al.*, 2012). The extent of community changes following a disturbance and possible changes in the ecosystem functions will be determined by the stability of the ecosystem. This stability will depend on the resistance, resilience and the functional redundancy of the community. Resistance can be defined as the ability of an ecosystem to remain unchanged after a disturbance. Resilience is defined as the rate at which the community returns to a pre-disturbance state. Functional redundancy is the presence of numerous taxa performing the same function in a given community, so changes in the community composition do not affect the ecosystem functions (Shade *et al.*, 2012).

6.1. Influence of deterministic and stochastic processes on microbial community assembly during aerobic granulation

In Paper III, the three reactors were used as replicates, operated identically for 35 days with synthetic wastewater, to investigate microbial succession during the formation of granular sludge. It was observed that the relative importance of deterministic and stochastic factors varied during the granulation process. The experimental design excluded dispersal as a major process because synthetic feed was used and the reactors were not hydraulically connected. Diversification can maybe also be neglected as a major process because of the short time frame of the study. This leaves selection as the major deterministic process and drift as the major stochastic process affecting community assembly. The community assembly during granulation could be divided into three successional phases and the diversity and turnover was variable during these phases (Figure 12).

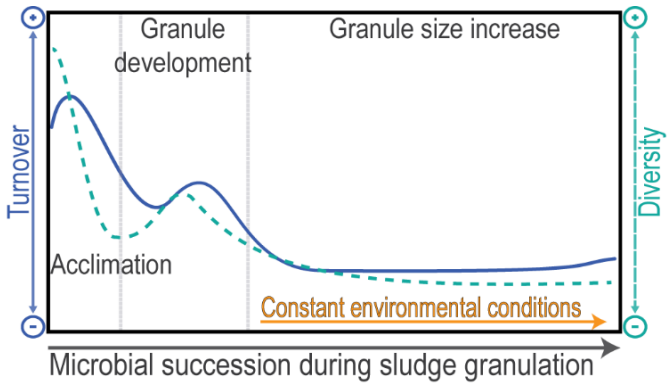

Figure 12. Conceptual model showing the turnover and diversity during the different stages of the temporal succession during the granulation of the sludge. Paper III.

During the early start-up, the settling time for the biomass was long and, therefore, there was no physical particle selection. The microbial communities shifted rapidly from the inoculum in a similar manner in all reactors (Figure 13A). Despite these temporal changes, the reactors were highly similar during this period, around 70% (Figure 14G). As a comparison, in Paper IV similarities were below 50% between reactor communities after 6 days of operation when the reactors were operated with different OLR. The switch from a complex- to a simple and easily biodegradable substrate selected for specific taxa in Paper III and, consequently, α-diversity decreased (Figure 14A-C), which was particularly evident for highly abundant taxa, and β-diversity increased between successive sample points (Figure 14D-F). This was also observed in Paper IV during the initial stages of granulation. Since α-, β- and γ-diversity are interconnected so that e.g. a difference in richness between samples may in itself cause a difference in β-diversity between the samples (Chase *et al.*, 2011, Zhou *et al.*, 2013), null model analysis was used to disentangle turnover due to succession from changes in α- and γ-diversity. We used two different types of null models for this: Raup-Crick measures based on Bray-Curtis dissimilarities (RC_{bray}), which quantifies the taxonomic turnover, and β-nearest taxon index (βNTI), which quantifies phylogenetic turnover (see Appendix, section A.3.4). The results showed that the phylogenetic- and taxonomic turnover was relatively high (Figure 15), especially the phylogenetic successional turnover in reactor R1. Altogether, the observations suggest that selection was the main ecological process influencing the microbial community assembly during this initial successional phase, with synthetic wastewater as the major selecting factor.

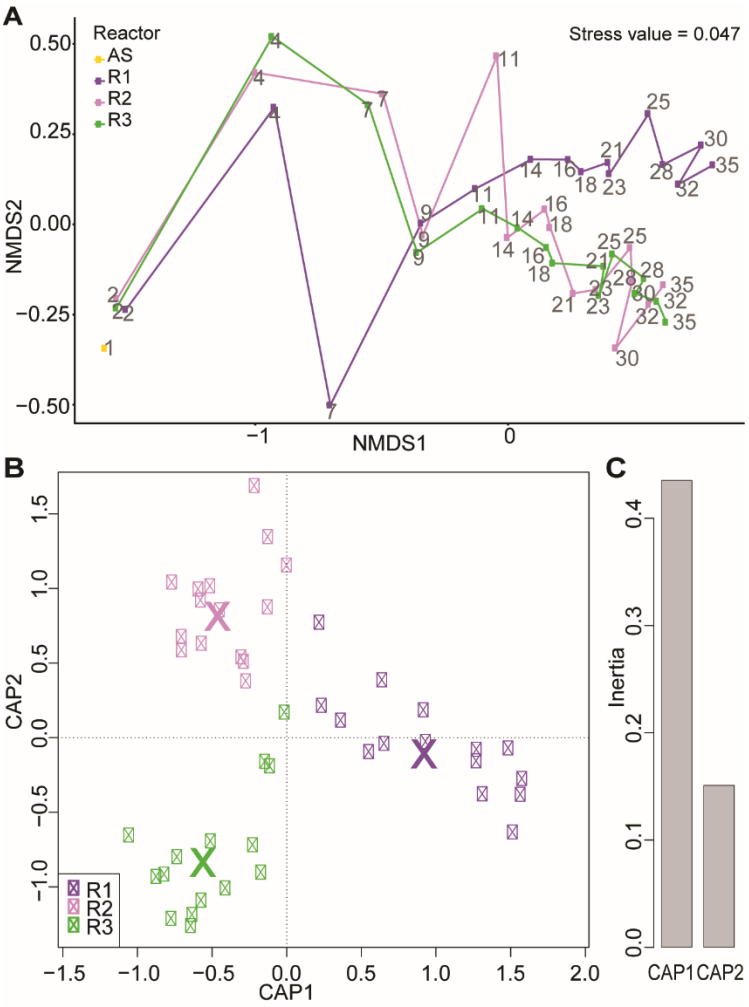

Figure 13. Ordination plots of the microbial community in the replicate reactors (R1, R2 and R3). A: Non-metric multi-dimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities after square root transformation (each line represents the community succession of each reactor starting from the seed sludge, AS, and numbers refer to days after startup); B: Contrained analysis of proximities ordination (CAP) showing the centroids (large crosses) of the constraining variable (factor with 3 levels: R1, R2 and R3); C, scree plot of the variances explained by each CAP axis. Paper III.

A second successional phase occurred when the settling time was drastically reduced and granules emerged. The communities changed rapidly over time, especially at day 7 (Figure 13A). The null models indicated that stochastic factors, here mainly drift, were important for the succession and the turnover between reactors (Figure 15A, B, E, F). When the settling time was decreased, only the microorganisms belonging to larger and denser aggregates were retained in the reactors, increasing the stochasticity. At the same time, the stress applied in the reactors promoted the production of EPS causing a granular size increase. The increase in granule size likely caused new niches to form as a result of the substrate gradients created in the incipient granules (Veach *et al.*, 2016, Xia *et al.*, 2018), enabling some taxa to increase in relative abundance, resulting in temporarily increased α-diversity (Figure 14A-C). Altogether, in this second phase granules emerged as the settling time was drastically reduced, resulting in a transition from high to low successional turnover during which both stochasticity and determinism played an important role.

A third phase occurred when the settling time was decreased less dramatically. During this phase, the microbial community change was slower, with similarity time-decay rates of about 0.02-0.03 d^{-1} in the three reactors between days 21 and 35, and turnover was generally lower than predicted by chance (Figure 15A, B, E, F). The reactor R1 diverged from day 14 (Figure 13A) and was different from R2 and R3 as indicated by constrained analysis of proximities (CAP) (Figure 13B, C). This was confirmed by pairwise permutational multivariate analysis of

variance (PERMANOVA) using Bray-Curtis distance metrics performed for this phase. The divergence of R1 was mainly caused by rare and intermediate taxa as indicated by β-diversity measurements (Figure 14G-I), by Kendall's rank correlation analysis between α-diversity time series, and analysis by Pearson correlation based on CAP. For taxa with a significant difference in relative abundance between reactors, null models showed drift to have a larger influence on the successional turnover in R2 and R3, (Figure 15C) and on the turnover between R2-R3 (Figure 15D). Within the same microbial community, selection and drift can affect different subpopulations differently (Jiao *et al.*, 2017, Zhou & Ning, 2017). Less abundant members are likely more susceptible to drift, as a small decrease in their abundance could result in extinction (Nemergut *et al.*, 2013). Moreover, drift has been observed to be more important when selection forces are weaker, which together with dispersal limitation and low α-diversity can lead to an increased turnover between initially similar communities (Rosindell *et al.*, 2011, Nemergut *et al.*, 2013). Indeed, R1 diverged from the other two reactors when the settling time was only slightly decreased and no other changes occurred in the reactors.

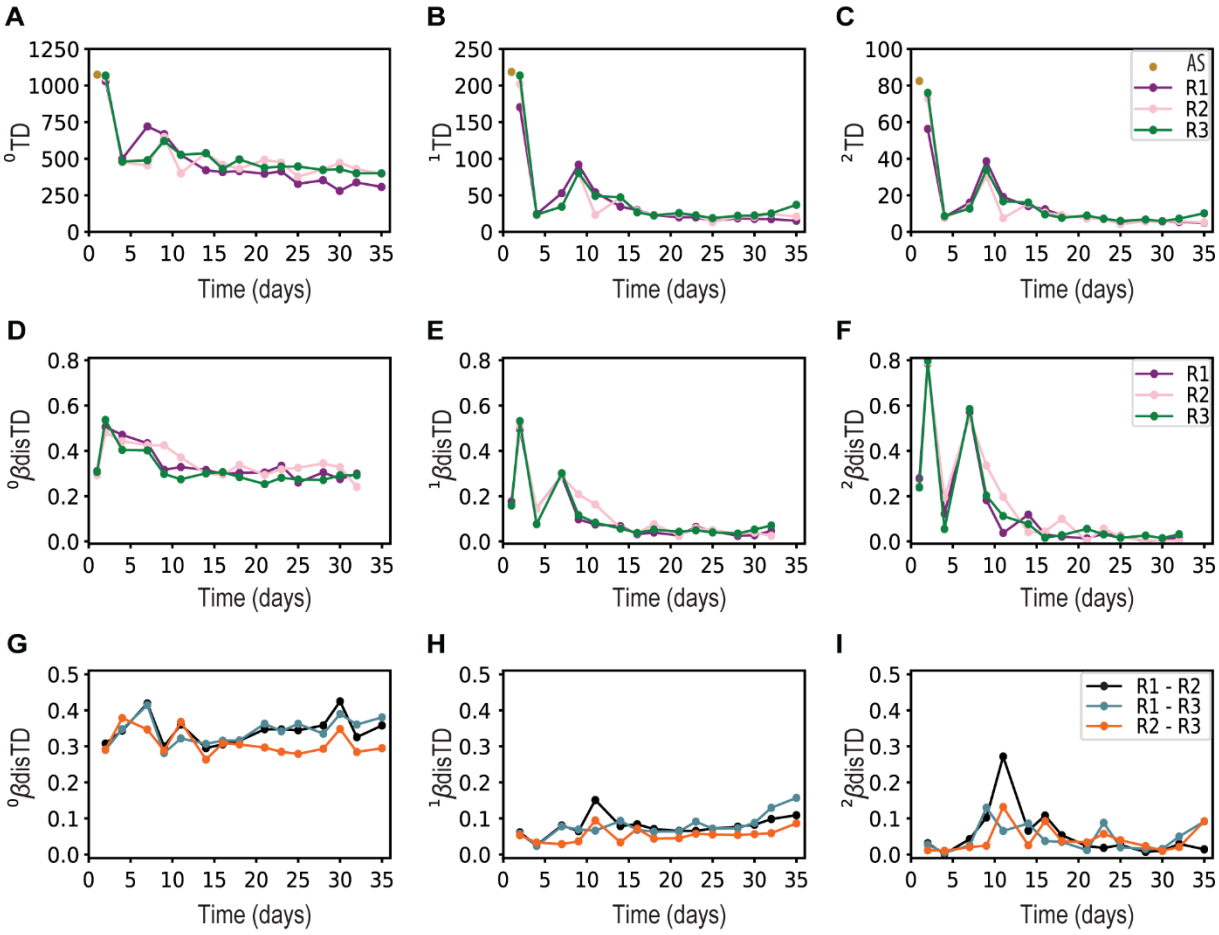

Figure 14. Dynamics of the taxonomic diversity (TD) for the replicate reactors. A, B and C: α-diversity (qTD); D, E and F: β-diversity (qβdisTD) between two successive sample points over time; G, H and I: β-diversity between the reactors over time. Diversity was calculated as Hill numbers for which diversity is a function of order q. At a q of 0, all OTUs are considered equally important; at a q of 1, OTUs are weighted according to the relative abundance; at a q of 2, abundant OTUs are given a larger weight. The β-diversities were converted into dissimilarity indices constrained between 0 (two identical samples) and 1 (two samples with no shared OTUs). Paper III.

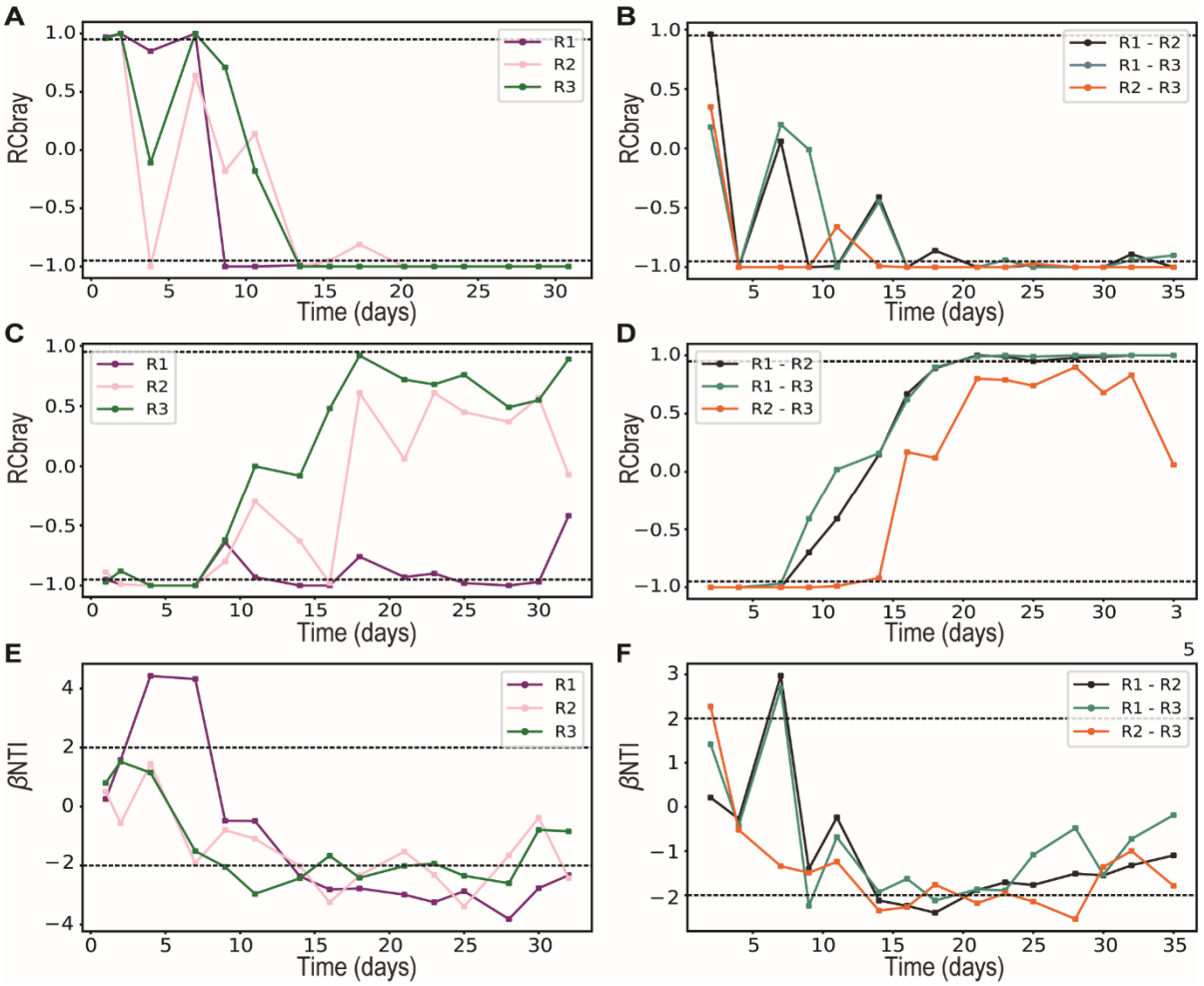

Figure 15. Null model analysis results for RC_{bray}, based on taxonomic turnover, and for βNTI, based on phylogenetic turnover. A: RC_{bray} between two successive sample points; B: RC_{bray} between the replicate reactors; C: RC_{bray} between two successive sample points of the subset of OTUs having significant correlation for the CAP1 axis; D: RC_{bray} in community composition between replicate reactors of the subset of OTUs having significant correlation for the CAP1 axis; E: βNTI between two successive sample points; F: βNTI between replicate reactors. Horizontal dotted lines indicate thresholds for significant deviations from the null expectation, -0.95 and +0.95 for RC_{bray} (A-D) and -2 and +2 for βNTI (E-F). Paper III.

6.2. Effect of disturbances on the microbial community dynamics

In Paper VII, reactors R2 and R3 were inoculated with granular sludge acclimatized to the reactor conditions for 369 days and operated as replicates, and the microbial community and the reactor performance were studied for 149 days, when periodic disturbances were applied. Three disturbances were applied, consisting of the removal of half of the biomass. Differences in the response of the microbial community diversity were observed between reactors and between the most and least abundant taxa. Abundant taxa showed higher dynamics in both α- and β-diversity (Figure 16, 18). An increasing trend in dissimilarity between the seed and successive sample points was observed for all diversity orders, which was fairly marked for higher diversity orders (Figure 18E-H), especially for the phylogenetical β-diversity. It seems that there was a bigger change for the most abundant taxa and not only in relative abundance but also in the taxonomic affiliation. The rare biosphere remained more stable during the disturbances.

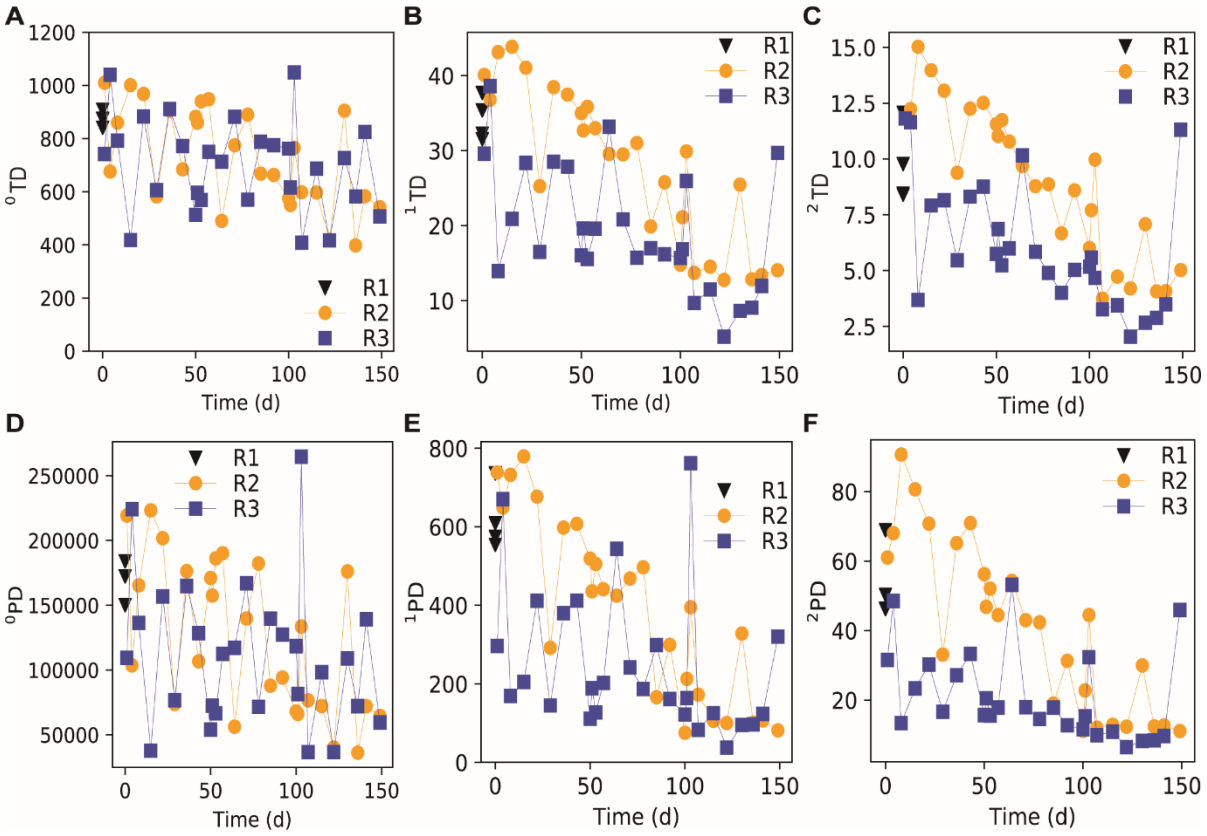

Figure 16. Dynamics of the taxonomic (TD) α-diversity (A-C) and phylogenetic (PD) α-diversity (D-F) during the experiment in reactor R1 (seed sludge), reactor R2 and reactor R3. At a q of 0, all OTUs are considered equally important; at a q of 1, OTUs are weighted according to the relative abundance; at a q of 2, abundant OTUs are given a larger weight. Disturbances were applied to the reactors on days 0, 50 and 100. Paper VII.

The time-decay rates show how the logarithm of the similarity between two samples collected from the same reactor change with the time interval between the sampling occasions (Shade *et al.*, 2013). Reactor R2 showed a significant community change with time in the abundant biosphere for disturbances 1 and 2 (Figure 17), but this reactor seemed to be somewhat more resistant to the disturbances than the reactor R3 in terms of diversity stability (Figure 18 A-D). The reactor R3 experienced more changes in microbial composition over time, as indicated by measurements of α- and β-diversity and by the higher dispersion of the decay-rates (Figure 17). The α-diversity in reactor R3 was lower than in reactor R2, especially for higher diversity orders, displaying R3 a lower evenness (^{2}TD, Figure 16C). Also, the third disturbance had a larger effect on the microbial diversity in reactor R3 (Figure 18 B, D), when α-diversity was lower. High levels of evenness in a microbial community have been shown to be related to a higher functional stability (Shade *et al.*, 2012). Feng *et al.* (2017) evaluated the link between biodiversity and stability in response to a pH disturbance in microbial electrolysis cells and observed that the biofilms with higher diversity could recover faster to a stable performance. Communities showing higher levels of diversity are likely more functionally redundant, however contradictory results have been obtained in this regard (Shade *et al.*, 2012).

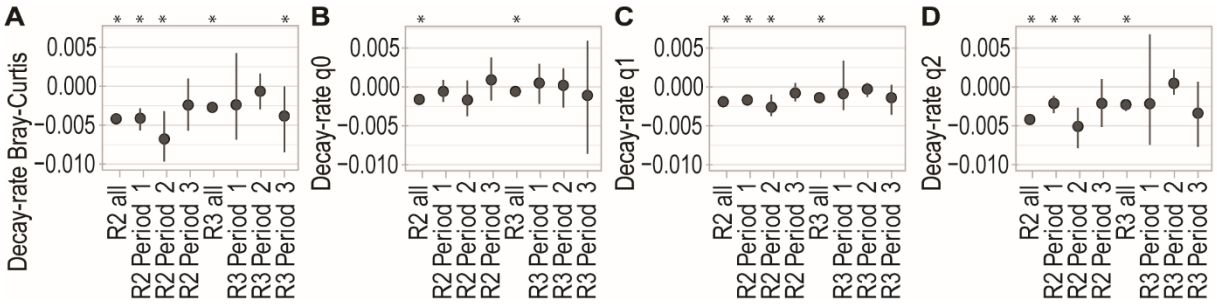

Figure 17. Time decay-rates for the Bray-Curtis dissimilarity index (A), for Hill numbers q0 diversity order (B), for Hill numbers q1 diversity order (C) and for Hill numbers q2 diversity order (D) in reactors R2 and R3 calculated for the whole experiment and the periods between each disturbance. Asterisk (*) indicate time-decay rates being significantly different from zero ($p<0.05$, based on bootstrapping and 999 randomizations).

It is, however, difficult to disentangle differences in diversity due to successional patterns in the microbial community structure from changes in diversity due to the disturbances applied to the reactor. One would expect diversity to remain unchanged if the community was resistant, or to recover to a pre-disturbance state if it was resilient (Vuono *et al.*, 2015). Here, the community in both reactors experienced a decreasing trend in α-diversity (Figure 16), an increasing trend in β-diversity between the seed sludge and the successive sample points (Figures 18E-H) and, although close to 0, statistically significant decay-rates (Figure 17). This is consistent with what was observed in Paper III and IV: the conditions applied in the reactors selects for the microbial communities adapted to those conditions and biodiversity is reduced as a result of competitive exclusion. However, it is important to bear in mind that the granulated sludge was pre-adapted to the reactor conditions when it was used to inoculate R2 and R3, and the settling time was the only parameter that changed (reduced from 30 to 2 min).

The replicate reactors did not display a high degree in reproducibility (Figure 18I, J). Between the reactors, β-diversity was higher for q0 diversity order, with a median of 0.38 (SD=0.07) and 0.31 (SD=0.07) in taxonomic and phylogenetic β-diversity respectively, showing that 62% and 69% of the OTUs were shared among the reactors. As a comparison, in Paper III, the non-acclimatized microbial community displayed a median taxonomic β-diversity of 0.29 (SD=0.03) between reactors R2 and R3, the ones showing the highest similarity, sharing 71% of the OTUs. The divergence of the microbial community in replicate bioreactors due to disturbances has been observed before. de Jonge *et al.* (2017) observed three anaerobic digesters with similar community dynamics to diverge after a starvation period due to differences in the adaptation to the disturbance applied. When null models where used to study the taxonomic and phylogenetic turnover, both βNTI and RC_{bray} indexes indicated that, overall, the turnover between successive samples points (community succession), between reactors (community reproducibility) and between the inoculum and successive sample points (community change from initial conditions) was not statistically different from the null expectation. This result indicates that, in addition to the deterministic factors affecting the community structure, stochastic processes affected the community dynamics after disturbances. This might explain the observed differences in diversity between the reactors and the increase in β-diversity between reactors after each disturbance (Figure 18I, J).

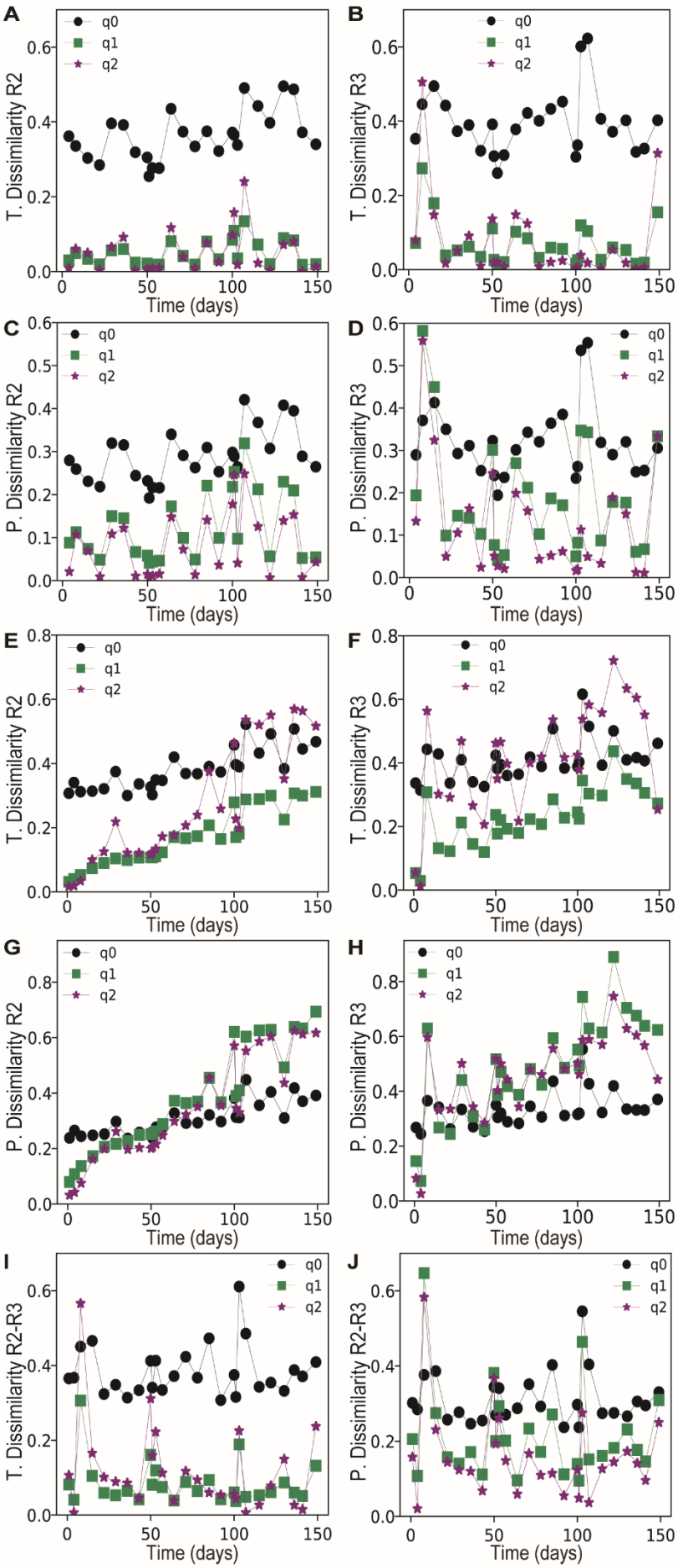

Figure 18. A and B: taxonomic β-diversity between successive samples over time for R2 and R3, respectively. C and D: phylogenetic β-diversity successive samples over time for R2 and R3, respectively; E and F: taxonomic β-diversity between a given sample and the inoculum (R1, day 369) for R2 and R3, respectively; G and H: phylogenetic β-diversity between a given sample and the inoculum sample (R1, day 369) for R2 and R3, respectively; I: taxonomic β-diversity between reactors over time; J: phylogenetic β-diversity between reactors over time. At a q of 0, all OTUs are considered equally important; at a q of 1, OTUs are weighted according to the relative abundance; at a q of 2, abundant OTUs are given a larger weight. The β-diversities were converted into dissimilarity indices constrained between 0 (two identical samples) and 1 (two samples with no shared OTUs). Disturbances were applied to the reactors on days 0, 50 and 100. Paper VII.

6.3. Ecological processes and wastewater treatment

Over the last decade, many descriptive studies have been conducted to analyse the microbial dynamics of granular sludge. However, there is a need to understand the factors shaping the granular microbial community. For this, laboratory scale studies that enable controlled environmental conditions and tests of reproducibility are valuable test-benches (Widder *et al.*, 2016). Laboratory experiments in granular sludge reactors, and generally in the field of wastewater treatment, are typically performed in one reactor because of practical reasons. Hence, conclusions regarding reactor performance and microbial community structure are drawn from single reactors operated at different conditions (Weissbrodt *et al.*, 2014, Fan *et al.*, 2018, Gonzalez-Martinez *et al.*, 2018). It is, however, unclear how reliable such conclusions are, especially for the complex processes underlying microbial community assembly. It is therefore necessary to assess the reproducibility of these systems. In previous studies on other parallel wastewater treatment bioreactors the results are inconsistent. Some studies report similar microbial communities in replicate reactors, such as membrane bioreactors (Falk *et al.*, 2009), anaerobic digesters (Vanwonterghem *et al.*, 2014, Lucas *et al.*, 2015, Luo *et al.*, 2015), microbial fuel cells (El-Chakhtoura *et al.*, 2014), biofilters (McGuinness *et al.*, 2006, Cabrol *et al.*, 2016) and sequencing batch reactors (Wittebolle *et al.*, 2009, Ayarza *et al.*, 2010), due to selection caused by the reactor environmental conditions. On the contrary, diverging communities and functions have been reported in replicate microbial electrolysis cell reactors (Zhou *et al.*, 2013), sequencing batch reactors (Akarsubasi *et al.*, 2009) and anaerobic digesters (Solli *et al.*, 2014, Han *et al.*, 2016), due to the roles of stochastic factors.

In Paper III, the three reactors were generally reproducible for the abundant community members. Deterministic factors were important during sludge granulation. The reactor conditions selected for bacteria involved in aggregate development and for those that were well adapted to grow on acetate. From an engineering perspective, this is important as it suggests that it is possible to select for certain dominating taxa by manipulating the reactors conditions, even for a process as common as acetate oxidation. For instance, as observed in Paper IV, a considerable divergence of the microbial communities in the reactors was observed as a consequence of feeding at different carbon concentrations. Altogether, this means that conclusions about reactor performance and microbial community dynamics (at least for abundant community members) can be drawn from experiments with single aerobic granular sludge reactors. Differences in the abundance of a small fraction of the community as a result of drift did not have major impact on the reactor functions. It is important to bear in mind, however, that for certain complex processes, such as nitrogen and phosphorus removal, anaerobic digestion or microbial electrolysis, stochastic variations in community composition have resulted in altered microbial functions (Graham *et al.*, 2007, Zhou *et al.*, 2013, Goux *et al.*, 2015). These processes are often carried out by taxa with a low relative abundance requiring, in many cases, multispecies cooperation. Drift could have a major impact on reactor functions when affecting low-abundant members of the community. For instance, we have observed nitrifiers with relative abundances lower than 0.1% to be the ones mainly responsible for nitrification (Szabó *et al.*, 2016). Furthermore, predatory bacteria were found predating on AOB, thus, predation could have a major impact on nitrification. In this context, it is important to study how perturbations affect the microbial community and the resilience of these systems.

In Paper VII, it was observed that the microbial community dynamics were not stable and some effects in the community structure were observed due to the disturbances. The removal of half of the sludge and the subsequent increase in F/M ratio did not generally affect the performance of the reactors. It has been previously reported that a stable performance of bioreactors treating wastewater is not necessarily associated to the stability of the microbial community (Ayarza & Erijman, 2011, Bagchi *et al.*, 2015). This is due to the high degree of functional redundancy often found for general functions such as carbon oxidation. Functions that are carried out by few taxa highly depends on the abundances of those microbes (Griffiths & Philippot, 2013), being more susceptible to changes in the reactor. Indeed, in Paper VII, nitrogen concentration in the effluent showed a higher variability compared with total organic carbon, but no statistical differences were observed between disturbance periods, and overall it was not affected. However, a higher variability of the concentration of phosphorus in the effluent was observed, which was statistically significant, possibly related to the fluctuations observed for Accumulibacter concentrations.

7. Assessment of the effect of sequencing, bioinformatics methods and choice of dissimilarity index in the results

High-throughput sequencing technologies for DNA analysis are currently essential when studying the mechanisms governing microbial community assembly. The main advantages of high-throughput methods are 1) fast analysis of many samples and 2) increased sequencing depth. However, there are still challenges due to bias and limitations of the technique that needs to be considered and addressed in biodiversity studies (Porter & Hajibabaei, 2018).

Obtaining representative DNA samples is a major issue in microbial ecology studies. The DNA extraction method and the primer choice have an impact on the obtained results. Different taxa require different extraction methods, also, the primers have a better coverage for some taxa compared to others (Albertsen *et al.*, 2015). For instance, in Paper IV it was observed that, despite the relative abundance of AOB was low, as indicated with MiSeq sequencing analysis, AOB appeared to be abundant, as observed by FISH-CLSM analysis. Moreover, when studying the ecological mechanisms involved in microbial community assembly, studying the rare biosphere is important. A high sequence depth is, therefore, necessary when sampling rare taxa, requiring millions of reads per sample (Zhou *et al.*, 2015). In Paper III, to ensure that the variability between reactors among less abundant OTUs was not due to limited precision of sequencing for low-abundant taxa and artificial methodological biases (Porter & Hajibabaei, 2018), the samples were sequenced twice, in order to increase the sequencing depth in the second sequencing run. Despite differences in the sequence depth, the two sequencing runs were statistically similar, as shown by Mantel tests and Procrustes test on Bray-Curtis dissimilarity matrices for both datasets, confirmed also by CAP analysis. Also, the impact of the sequencing processing was assessed comparing Bray-Curtis dissimilarity matrices obtained with the UNOISE (Edgar, 2010, Edgar, 2016) and the DADA2 (Callahan *et al.*, 2016) pipelines. The two dissimilarity matrices were statistically similar, as shown by Mantel tests and Procrustes test. Sequence subsampling was also assessed by comparisons of rarefied and non-rarefied sequence data and no effect of sequence subsampling was observed.

In Paper VIII, the impact of the choice of different computational pipelines and input parameter choices on the results from high-throughput sequencing was assessed. For this, granular sludge from the experiment described in Paper VII was used and DNA extractions of the seed sludge and of the last day of each reactor (R2 and R3) were performed (18 samples in total, six replicates per sample). The sequence reads were processed using DADA2, Deblur (Amir *et al.*, 2017), USEARCH (UPARSE and UNOISE), and Mothur (Schloss *et al.*, 2009). DADA2 and Deblur generate SVs whereas Mothur generate OTUs. USEARCH can either generate SVs using UNOISE or OTUs using UPARSE (Edgar, 2013). Several frequency tables were generated: the sequence reads were processed sample-by-sample or by pooling all reads and compared for the DADA2, UNOISE, and UPARSE pipelines, and relaxed quality filtering thresholds and stringent settings were also compared for the DADA2 and UNOISE pipelines. A consensus frequency table, consisting of sequence variants found with all three pipelines, was also generated. The results showed that there was a large span in the number of inferred

sequence variants/OTU for different pipelines, with Deblur having the lowest number and Mothur having the highest. In general, the frequency tables generated using the different bioinformatics pipelines captured the same trends in the data. Pairwise differences in microbial community composition between samples were quantified using the Jaccard, Bray-Curtis, and Hill-based dissimilarity indices. The bioinformatics pipelines had a strong effect on the dissimilarity between replicates, particularly for incidence-based indices and low values of q, (Figure 19). The magnitude of this dissimilarity varied depending on the bioinformatics pipelines. In general, for Hill-based indices, the dissimilarity between replicates decreased as q increased from 0 to 1 (Figure 19). For low values of q, there is a large difference between the dissimilarity from different pipelines. The consensus tables always resulted in the lowest dissimilarity probably due to the removal of low abundant sequence variants. In DADA2 and USEARCH, processing using stringent filtering thresholds and pooled samples resulted in lower dissimilarity between replicates.

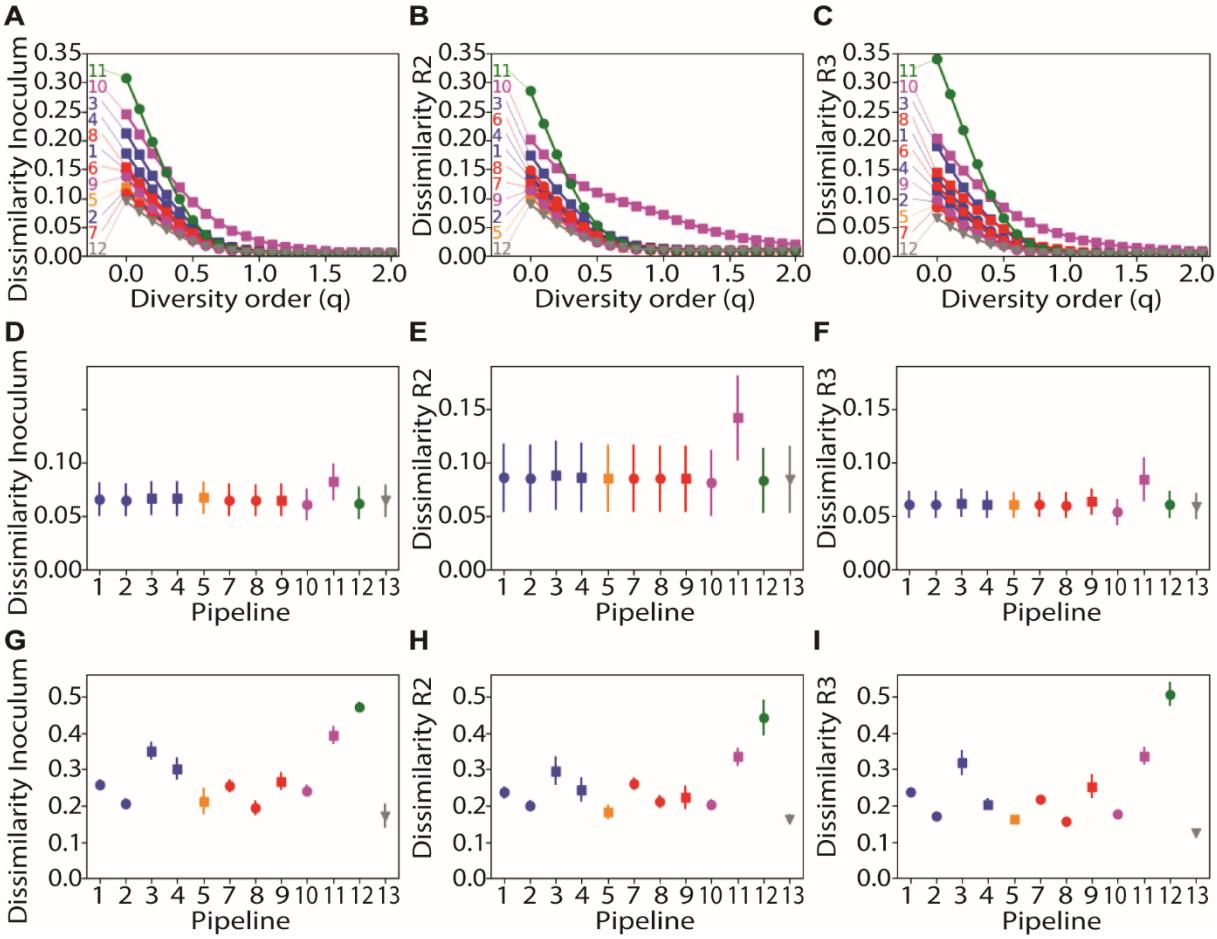

Figure 19. Hill-based dissimilarity (A-C), Bray-Curtis dissimilarity (D-F) and Jaccard dissimilarity (G-I) between technical replicates (DNA extractions) from granules belonging to the experiment described in Paper VII, from inoculum (A, D and G) and of the last day of the experiment for reactor R2 (B, E and H) and reactor R3 (C, F and I). The numbers indicate bioinformatics pipeline. 1-DADA2, pooled together, stringent settings. 2-DADA2, pooled together, relaxed settings. 3-DADA2, pooled separately, stringent settings. 4-DADA2, pooled separately, relaxed settings. 5-Deblur, pooled separately. 6-UNOISE, pooled together, stringent settings. 7-UNOISE, pooled together, relaxed settings. 8-UNOISE, pooled separately, relaxed settings. 9-UPARSE, pooled together, relaxed settings. 10-UPARSE, pooled separately, relaxed settings. 11-Mothur, pooled together. 12-Consensus.

Both the bioinformatics methods and the choice of dissimilarity index influence the results as observed in Paper VIII. The use of a single dissimilarity index would have given misleading information for the data set investigated. All frequency tables showed that the inoculum had significantly higher dissimilarity with R3 than with R2 for Hill $q \geq 0.6$ diversity order (Figure 20) and for Bray-Curtis dissimilarity (Figure 21). However, for Jaccard index and Hill-based dissimilarity for $q < 0.6$, some frequency tables showed that the dissimilarity is larger between R3 and the inoculum, some showed it is larger between R2 and the inoculum, and some did not show a statistically significant difference. Therefore, the Jaccard and Bray-Curtis indices, which often are used in microbial ecology studies, are not necessarily the most logical choices. Instead, Hill-based dissimilarity indices, which make it possible to systematically investigate the impact of relative abundance on dissimilarity values, should be used for robust interpretation of data.

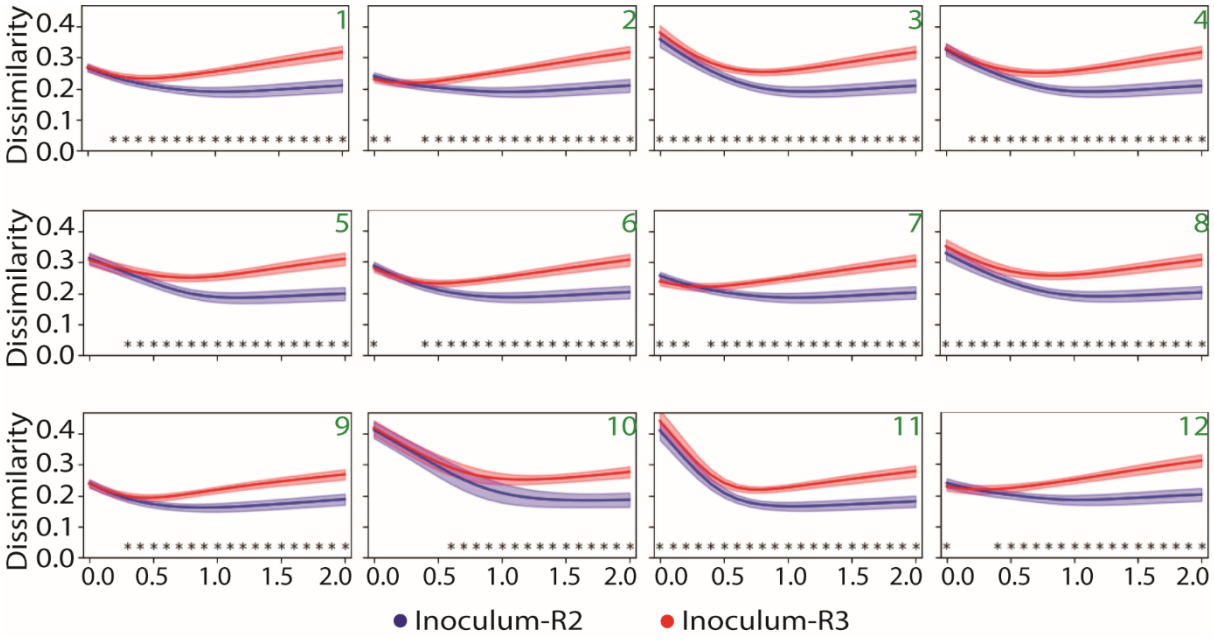

Figure 20. Hill-based dissimilarity between the inoculum and R1, and between the inoculum and R2 for frequency tables generated by different pipelines (indicated with the numbers). The asterisks indicate statistically significant difference between the dissimilarities ($p < 0.05$, Welch's ANOVA). 1-DADA2, pooled together, stringent settings. 2-DADA2, pooled together, relaxed settings. 3-DADA2, pooled separately, stringent settings. 4-DADA2, pooled separately, relaxed settings. 5-Deblur, pooled separately. 6-UNOISE, pooled together, stringent settings. 7-UNOISE, pooled together, relaxed settings. 8-UNOISE, pooled separately, relaxed settings. 9-UPARSE, pooled together, relaxed settings. 10-UPARSE, pooled separately, relaxed settings. 11-Mothur, pooled together. 12-Consensus.

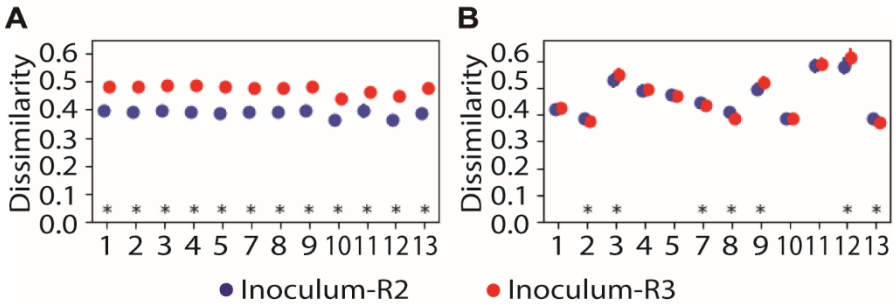

Figure 21. Bray-Curtis (A) and Jaccard (B) dissimilarity between the inoculum and R1, and between the inoculum and R2 for frequency tables generated by different pipelines (indicated with the numbers). The asterisks indicate statistically significant difference between the dissimilarities ($p<0.05$, Welch's ANOVA). 1-DADA2, pooled together, stringent settings. 2-DADA2, pooled together, relaxed settings. 3-DADA2, pooled separately, stringent settings. 4-DADA2, pooled separately, relaxed settings. 5-Deblur, pooled separately. 6-UNOISE, pooled together, stringent settings. 7-UNOISE, pooled together, relaxed settings. 8-UNOISE, pooled separately, relaxed settings. 9-UPARSE, pooled together, relaxed settings. 10-UPARSE, pooled separately, relaxed settings. 11-Mothur, pooled together. 12-Consensus.

8. Summary and further research

The remarkable diversity and the high community dynamics, together with the high functional flexibility and redundancy of microbial communities in wastewater bioreactors renders the perfect scenario for fundamental research on microbial ecology. A higher level of understanding of the ecology of the microbial communities found in these engineered systems is necessary to improve their design and their development (McMahon *et al.*, 2007, Cabrol *et al.*, 2016, Xia *et al.*, 2018). Research dealing with fundamental ecological processes involved in sludge granulation is needed to improve the reactor start-up and to understand the microbial community dynamics in full-scale reactors, where important parameters such as OLR or temperature are not constant. Furthermore, there are important research questions related to the reproducibility of the granular sludge process. The results presented in this thesis suggest that it is possible to draw conclusions about the reactor performance and for the abundant biosphere dynamics when the reactors experience constant environmental conditions. It was observed, however, that when the reactors experience disturbances, the microbial communities are not reproducible between reactors, but the performance is overall similar. It would be interesting to assess the reproducibility in meta-studies where the microbial community assembly during sludge granulation is studied for different conditions and reactor set-ups.

Granular sludge is a technology showing superior features compared to conventional activated sludge processes and is currently applied worldwide. Despite the well-established methods for granule cultivation, the ecological processes underpinning the microbial community assembly during granulation are poorly understood. The results obtained in this thesis might help us to understand the complex processes behind granulation. Altogether, the results show that granulation responds to deterministic factors driven by the reactor conditions. It was observed that during the start-up of the reactors, microorganisms are washed-out randomly and the granulation starts as a response of the shear forces applied in the reactor which produces the switch of bacterial growth from a planktonic to an aggregate mode. The high washout dynamics act as an accelerant of granulation by selecting particles of larger size in the reactor. Simultaneously, there is a deterministic selection of microorganisms better adapted to the conditions found in the reactor. It was also observed that stochastic processes, i.e. drift, had a considerable effect on the less abundant community. Moreover, stochasticity appeared to be important when the community was subjected to periodical disturbances. It would be desirable to understand how stochasticity affects the microbial community dynamics and the granulation process in granular reactors, especially at full-scale, where the community experiences a constantly changing environment often submitted to disturbances. The methodologies employed and developed in this thesis could be applied for this purpose.

Stochasticity seemed to mainly affect rare taxa. These results should be taken into consideration, especially when the reactor functions depend of rare taxa. The interest of microbial ecologists for rare taxa is increasing. With the improvement of high-throughput sequencing technologies, the study of the rare biosphere dynamics is becoming more accessible. Results so far are slowly revealing an important role of this fraction of the community on maintenance of diversity and ecosystems functions. It is therefore necessary to perform studies

targeting rare taxa. However, biases caused by the sequencing technique and the bioinformatics and biostatistics methods affect the results, especially when focusing on rare taxa. Consequently, more studies dealing with this aspect are necessary.

In this thesis, the AOB were observed in the inner locations of the granules, which do not follow the commonly accepted multilayer model of different functional groups in different layers of the granules. This added complexity of granule architecture needs to be considered to better understand and model the aerobic granular sludge processes. Also, bacterial predation should be more thoroughly investigated in granular sludge as they are part of the core community and they were found predating on AOB, which exert an important reactor function and are, additionally, more susceptible to drift. Predation has an important role in bacterial ecology with direct impact on the microbial community and, ultimately, on the microbial functions. Research dealing with the interaction between predator and prey is necessary to understand the effect of predation on the microbial community structure and dynamics. Protistan grazing and bacteriophage attack are well recognized processes that exert a control in the microbial populations. Very little is understood about the roles of bacteriophages in wastewater bioreactors, despite being found at high concentrations in these environments. Moreover, the effects of predatory bacteria on the microbial community remain largely unknown. The results in this thesis suggest that predatory bacteria increased stochasticity during community assembly. It would be very interesting to assess the link between predation and the fundamental ecological factors governing the microbial community assembly.

Research on granular stability is imperative to develop strategies to improve the start-up and maintenance of granules in full-scale granular reactors. Also, detailed studies on granule architecture will allow us to understand, and more easily overcome the loss of granular structure observed in AGMBRs. If granules lose their structure or if their diameter change during the reactor operation, the diffusion properties and the mass and oxygen gradients will be affected which will impact the nutrient removal. However, it was observed that granules were able to withstand high pressures showing a high stability and strength.

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APPENDIX: materials and methods

A.1. Experimental set-up

A.1.1. Reactor design

Three identical lab-scale reactors were employed for the experiments presented in this thesis (Figure A1). The reactors consisted of four main units: an SBR with a working volume of 3 L, a 2 L storage bottle 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 air was provided at the bottom of the reactor with porous diffusers (pore size 1 μm).

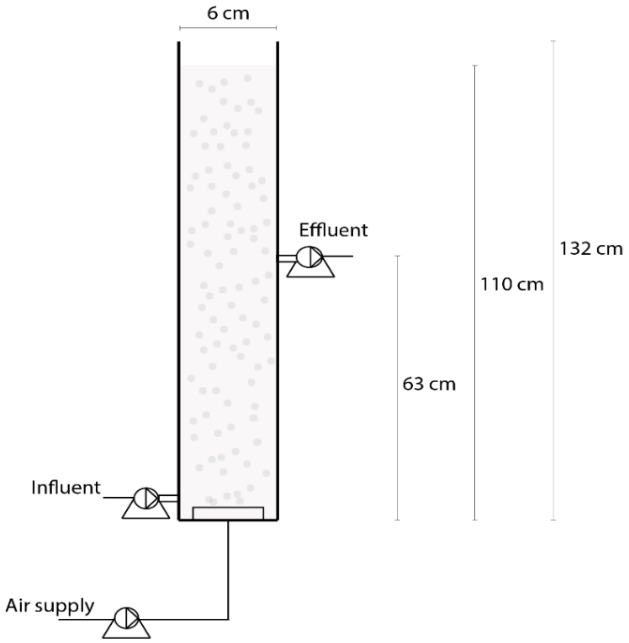

Figure A1. Schematic representation of the reactor design (non-scaled image). The shape of the employed SBRs, the height to diameter ratio and the volumetric exchange ratio ensure washout of non-granulated biomass and apply appropriate hydrodynamic shear forces to develop more regular, rounder and compact granules.

A.1.2. Operational conditions

The SBRs were operated at room temperature (20-22 ˚C) in a 4-hour cycle (5 min filling, 55 min anaerobic/anoxic, 173 min aerobic, 2 min settling, 5 min 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 (1). Short cycle times ensure the suppression of suspended growth, but too short cycles will hinder the microbial growth and accumulation (2). The influent was pumped from the bottom of the reactors 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. 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, see Table A1. 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.

Table A1 Wastewater organic loading rate (OLR) and nitrogen loading rate (NLR) used in the experiments in lab scale SBRs.

	Paper III			**Papers IV and V**			**Paper VI**	**Paper VII**		
Wastewater	Synthetic			Real and synthetic (1:1)			Real and synthetic (1:1)	Synthetic		
Reactor	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**		**R1**	**R2**	**R3**
OLR (kg COD/ m^3/d)	3	3	3	3.71	1.87	0.91	2.5	2	2	2
NLR (kg NH_4-N/ m^3/d)	0.7	0.7	0.7	0.2	0.2	0.2	0.2	0.3	0.3	0.3

A.1.3. Sludge inoculum

The reactors were inoculated with aerobic/anoxic activated sludge from two full-scale WWTPs: Gryaab AB (Gothenburg, Sweden) in Papers III, IV, and V; and Hammargården (Kungsbacka, Sweden) in Paper VII. For this purpose, fresh sludge collected from the WWTP was directly introduced into the reactors by allowing the first batch of sludge to settle, removing the supernatant and refilling the reactors with a second batch of sludge.

A.2. Analytical methods

The reactor performance 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 (3). Microscopic observations were performed using an Olympus BX60 light microscope and particle size was assessed with CellSens (Olympus) and ImageJ software. (4).

A.3. Microbial community analysis

A.3.1. 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) or Qubit 3.0 fluorometer (Invitrogen), using the dsDNA HS assay kit (Invitrogen).

A.3.2. DNA amplification and sequencing

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 (5, 6) were employed indexed with sequences published by Kozich et al. (7). 515F was used in papers IV and V, and a modified 515'F, with a better coverage among Archaea, was used in paper III and VII. 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 (8). 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 thermocyler (papers III, IV and V) or a Bio-Rad T100 thermocyler (papers VI and VIII) 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. PCR products were purified with the MagJET NGS Cleanup and Size Selection Kit (Thermo Scientific) in papers III, VII and VIII or using the Agencourt AMPure system (Beckman Coulter) in papers IV and V, and concentration was measured by NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and Qubit 2.0 fluorometer (Life Technologies), using the dsDNA HS assay kit (Invitrogen). The PCR products were pooled in equimolar amounts, the concentration and size were confirmed by TapeStation 2200 (Agilent Technologies) and sequencing was performed with a MiSeq using reagent kit v3 (Illumina) in Papers III and VIII and reagent kit v2 (Illumina) in Papers IV and V. PhiX control library was spiked at 7.5%.

A.3.3. DNA sequencing processing

Raw sequence reads were processed following the UNOISE pipeline (9, 10) with USEARCH v.10 and USEARCH v.11 (11) in Papers III and VII respectively. The DADA2 (12) pipeline was also used in Paper III for result comparison. In papers IV and V, the UPARSE pipeline (13) was followed with USEARCH v.10. In Paper VIII, DADA2, Deblur (14), USEARCH (UPARSE and UNOISE), and Mothur (15) pipelines were used. For detailed information on pipeline setting, see the appended papers.

The OTUs were taxonomically classified with the SINTAX algorithm (16) based on the MiDAS database, v.2.1 in Papers III and VII and v.1.20 in papers IV and V (17). In papers III and VII, the sequences were aligned with the R package DECIPHER (18) and an approximately maximum-likelihood tree was generated with FastTree 2 software (19) using the GTR+CAT (General Time Reversible with per-site rate CATegories) model of approximation for site rate variation and computation of Gamma20-based likelihood.

A.3.4. DNA sequencing data analysis

Basic R functions were used to perform Wilcoxon signed-rank tests and to calculate Pearson correlation coefficients. NMDS ordination and heatmaps were created using the R package ampvis (20). CAP, ANOVA like permutation test using anova.cca function (999 permutations), Mantel tests (999 permutations) and Procrustes tests using the protest function (999 permutations) were performed using the R package vegan (21). Analysis of PERMANOVA was conducted using adonis from the package pairwiseAdonis (22). Time-decay rates were calculated as in Shade et al. (23). Welch's ANOVA was calculated with the Scipy package in Python (24). Dissimilarities between samples were converted to similarities by subtracting from one. The time-decay rate was the linear slope of the log-transformed similarities plotted against the time difference between samples.

Taxonomic Hill numbers were used to calculate α-diversity (25). The parameter q is the diversity order. At a q of 0, all OTUs are considered equally important and, hence, q=0 is the sample richness (i.e. the number of OTUs in a sample). For higher values of q, more weight is put on abundant OTUs. Phylogenetic diversity, which take the sequence dissimilarity into account, was also calculated (26). The same calculation framework was used to calculate β-diversity and was converted into dissimilarity indices constrained between 0 (two identical samples) and 1 (two samples with no shared OTUs) (26, 27). The dissimilarity indices are based on the taxonomic and the phylogenetic β-diversity values. The Hill-based α- and β diversities were calculated using qDiv (github.com/omvatten/qDiv). Correlations between series of α-diversity data were investigated using Kendall's rank correlation coefficient (tau), which was calculated with the Scipy package in Python.

Taxonomic turnover was estimated with RC_{bray}, calculated with the R package vegan as by Stegen et al. (28). A null distribution (999 randomizations) of expected Bray-Curtis dissimilarities was created for each pair of communities, which was compared to the empirically observed Bray-Curtis dissimilarities. The RC_{bray} index values range between -1 and 1. A negative value means that the two communities are more similar than expected by chance whereas a positive value means that they are more dissimilar. Values > |0.95| were considered statistically significant. Values < |0.95| indicates that the taxonomic turnover between the community pair are not different from the null expectation and therefore, influenced by stochastic factors (29). Phylogenetic turnover was estimated with βNTI, calculated with the R package PICANTE (30) as previously described (28). For this, βMNTD, which measures the mean phylogenetic distance between the most closely related OTUs in two communities, was first calculated based on relative abundance data. In each iteration (999 randomizations) the OTUs were moved randomly across the tips of the regional pool phylogeny and the resulting phylogenetic relationships were used to calculate the $\beta MNTD_{null}$. The βNTI was calculated as the difference in standard deviation units between the observed βMNTD and the mean of $\beta MNTD_{null}$ in the pairwise sample comparisons. A negative βNTI value means that the samples are more phylogenetically similar than expected by chance and a positive value means that they are more phylogenetically distant from each other. Pairwise comparisons with βNTI > |2| were considered statistically significant. Values < |2| were not significantly different from the null expectation, which indicate that stochastic factors influenced the phylogenetic turnover (29).

A.4. Fluorescence in situ hybridisation analysis and confocal laser scanning microscopy analysis

FISH was employed in combination with CLSM to study the spatial distribution of the microbial communities in the granular sludge. CLSM was also used to assess the distribution of EPS within the granules, using specific stains.

A.4.1. Cryosectioning

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 (31).

A.4.2. 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 (32) using the probes and applying the hybridization conditions shown in Table A2. 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 A2. Probes and hybridization conditions for FISH.

Probe	Target organism	FA[a] (%)	Reference
BDE525	Genus *Bdellovibrio*	35	Mahmoud et al. (33)
CFB563	Most *Flavobacteria*	20	Weller et al. (34)
Cluster6a192[b]	*Nitrosomonas. oligotropha*	35	Adamczyk et al. (35)
EUB338 (I-V)	Most bacteria	35	*
Meg983	*Meganema perideroedes*	35	Thomsen et al. (36)
Meg1028	*Meganema perideroedes*	45	Thomsen et al. (36)
NEU[b]	*Nitrosomonas europaea/eutropha/halophila*	35	Wagner et al. (37)
Nse1472	*Nitrosomonas europaea/eutropha*	50	Juretschko et al. (38)
ZRA23a	*Zoogloea* lineage, not *Z. resiniphila*	35	Rosselló-Mora et al. (39)
ZOGLO-1416	*Zoogloea spp.*	35	Loy et al. (40)

[a] FA = formamide concentration in hybridization buffer.
[b] Probe applied with unlabeled competitor probe according to the reference.
* EUB338 I, Amann et al. (41); EUB338 II, Daims et al. (42); EUB338 III, Daims et al. (42); EUB338 IV, Schmid et al. (43).

A.4.3. EPS staining

The cryosectioned granule samples were stained with the following dyes: SYTO 62 (Life Technologies, Carlsbad, CA, USA), which is a cell-permeant red fluorescent nucleic acid stain, was used to stain total cells; FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain (Life Technologies), which labels most classes of proteins, was used to stain the matrix of the granules; Calcofluor White Stain (Sigma Aldrich), which is a non-specific fluorochrome that binds to cellulose and chitin, was used to stain β-D-glucopyranose polysaccharides; and Calcium Green™-1 Hexapotassium Salt cell impermeant (Life Technologies), which is a visible light-excitable Ca^{2+} indicator, was used to stain calcium ions in the granules.

The staining protocol applied to the cryosections was as follows: SYTO 62 (20 μM, 50 μL) was first added to cryosections for 30 min, then SYPRO (1x, 50 μL) was added and incubated for 30 min, followed by Calcofluor White (1mg/mL, 50 μL) for 30 min, and finally, Calcium green (10 μM, 50 μL). After each staining step, the sample was washed with PBS buffer to remove the excess stain. Prior to the staining procedure, a hydrophobic barrier frame was applied to the glass slides around the regions containing the cryosections by using a Liquid Blocker Mini PAP Pen (Life Technologies).

A.4.4. 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 acquired 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.

A.5. Granule strength assessment

In total 78 granules were investigated to test differences in the compressibility and breakage of the granules by introducing the granule in a 5 mm diameter tube capped with a nylon mesh. The granules were subjected to increasing flux (from 116 to 4720 m^3 m^{-2} h^{-1}) and the flux at which the granules broke (denoted as critical flux) was recorded to determine the maximum flux that granules were able to withstand. Measurements of the pressure applied at the different fluxes were performed by a vertical column manometer. A regression analysis was performed to study correlations between the strength of granules and their properties using the software SPSS 22.

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