THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Composition and dynamics of the bacterial community in aerobic granular sludge reactors

ENIKŐ SZABÓ

Department of Architecture and Civil Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2017

Composition and dynamics of the bacterial community in aerobic granular sludge reactors

ENIKŐ SZABÓ
ISBN 978-91-7597585-6

Doktorsavhandlingar vid Chalmers tekniska högskola. Ny serie nr 4266
ISSN 0346-718X

Department of Architecture and Civil Engineering
Chalmers University of Technology
SE-412 96 Gothenburg
Sweden
Telephone + 46 (0)31-772 1000

Chalmers Reproservice
Gothenburg, Sweden 2017

Composition and dynamics of the bacterial community in aerobic granular sludge reactors

ENIKŐ SZABÓ
Division of Water Environment Technology
Department of Architecture and Civil Engineering
Chalmers University of Technology

Abstract

The aerobic granular sludge (AGS) technology is probably the future standard for wastewater treatment, due to its low footprint and low energy consumption. Although achieving granulation is usually not a challenge anymore, our understanding of the community assembly during start-up, and of the microbial ecology of these reactors in general, is incomplete. Earlier studies have shown that high removal efficiency and stable process performance in bioreactors are dependent on the microbial community composition. High functional redundancy, which is a result of high evenness and phylogenetic variability, was found to be the key to a resilient bioreactor.

The research presented in this thesis aimed to expand on the current knowledge about the composition and dynamics of the bacterial community in AGS reactors, using molecular biology techniques including qPCR, T-RFLP and Illumina MiSeq. The harsh wash-out conditions, typical for the start-up of AGS reactors, were found to drastically decrease the abundance of nitrifiers. A stepwise decrease of settling time enabled better retention of nitrifying organisms, but seemed to have no long term effects on the general composition of the community. The community assembly was affected mainly by deterministic factors – e.g. short settling time – during start-up, and the stochastic components became evident only when the strong selection pressure decreased. Reactors with different operational parameters were found to be dominated by different taxa, but high functional redundancy was observed within all key guilds in all reactors. Certain functional groups – denitrifiers, EPS and PHA producers – were over-represented in granular sludge compared to the flocculated seed sludge. The typical AGS reactor operation seems to favor these traits, but whether these are necessary for successful granulation and reactor operation depends on the intended function of the reactor.

At present, microbial ecology studies – including our experiments – on AGS processes are predominantly of descriptive nature. However, it was suggested that in the future microbial ecology may provide a tool to predict or even design diversity and thus process stability. The increasing availability of next generation sequencing methods allows us to study the rare biosphere in AGS reactors and gain insights in how we can integrate ecology in biotechnology.

Keywords: wastewater; aerobic granular sludge; sequencing batch reactors; nitrogen removal; microbial community composition; population dynamics; functional groups; qPCR; T-RFLP; Illumina MiSeq

LIST OF PUBLICATIONS

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

I. Szabó E, Hermansson M, Modin O, Persson F, Wilén B-M (2016) Effects of Wash-Out Dynamics on Nitrifying Bacteria in Aerobic Granular Sludge During Start-Up at Gradually Decreased Settling Time. *Water* 8:172. doi: 10.3390/w8050172

II. Szabó, E., Liébana, R., Hermansson, M., Modin, O., Persson, F., and Wilén, B.-M. (2017). Microbial population dynamics and ecosystem functions of anoxic/aerobic granular sludge in sequencing batch reactors operated at different organic loading rates. *Front. Microbiol.* 8, 770. doi:10.3389/fmicb.2017.00770.

III. 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. *Manuscript*

IV. Liébana R, Szabó E, Persson F, Modin O, Hermansson M, Wilén B-M (2017) Microbial community dynamics in replicate sequencing batch reactors during aerobic granulation. *Manuscript*

CONTRIBUTION REPORT

The author of this thesis has made the following contributions:

I. First author. Contributed to the data collection by molecular methods, and the analysis and interpretation of the results. Wrote the paper for the most part and made adjustments after consultation with the co-authors.

II. First author. Contributed to the design of the experiment, the data collection (reactor operation, sample collection, chemical analysis, bioinformatic processing and statistical analysis), and the analysis and interpretation of the results. Wrote the paper for the most part and made adjustments after consultation with the co-authors.

III. First author. Contributed to the design of the experiment, the data collection (reactor operation, sample collection, chemical analysis, bioinformatic processing and statistical analysis), and the analysis and interpretation of the results. Wrote the paper for the most part and made adjustments after consultation with the co-authors.

IV. Co-author. Contributed to the design of the experiment, the data collection (bioinformatic processing and statistical analysis), and the analysis and interpretation of the results.

ABBREVIATIONS

AGS aerobic granular sludge

CAP constrained analysis of proximities

CAS conventional activated sludge

COD chemical oxygen demand

DO dissolved oxygen

FISH fluorescence in situ hybridization

MANOVA multivariate analysis of variance

NGS next generation sequencing

NMDS non-metric multidimensional scaling

OLR organic loading rate

OTU operational taxonomic unit

PHA polyhydroxyalkanoate

qPCR quantitative polymerase chain reaction

SBR sequencing batch reactor

SRT sludge retention time

T-RFLP terminal restriction fragment length polymorphism

VER volume exchange ratio

WWTP wastewater treatment plant

Contents

1 Preface

Sustainability by definition means that the needs of today are met without compromising the needs of tomorrow. When it comes to the water cycle, it is not only the needs of the future generations that are of concern, it is our own present needs that are at stakes. Water is one of the most important resources, used in households (as drinking water, for cooking, washing, etc.), by the industry (e.g. as a solvent, or for food processing), by agriculture (for irrigation), for recreation (e.g. swimming, diving) and many other purposes. But the utilization of water can be directly or indirectly hampered by pollution from human activities. It is not only water (the substance itself) that we need to protect, we also benefit from the services of aquatic ecosystems, which are at risk when untreated wastewater is released into the environment.

The composition of wastewater varies depending on where it originates from. Municipal wastewater usually contains high concentrations of organic matter, nitrogen and phosphorus, which, if released untreated, results in eutrophication and oxygen depletion. It also contains pathogens that can be a health risk for downstream users. Therefore, municipal wastewater must be treated, and the most widespread method is to collect and transfer the wastewater to a wastewater treatment plant (WWTP). For the last century, the conventional activated sludge (CAS) process was the most common technology applied at WWTPs. As a result of urbanization, increasing pollutant loads, and limited availability to expand, the CAS process can not always meet the effluent limits. The aerobic granular sludge technology is probably the future standard for wastewater treatment due to its low footprint and low energy consumption.

Granular sludge can be considered as a special case of biofilm, where self-immobilized bacterial cells form strong, dense and well-settling aggregates. Although achieving granulation is usually not a challenge anymore, our understanding of the microbial ecology of these reactors is incomplete. Earlier studies have shown that the performance and efficiency of bioreactors are dependent on the microbial community composition. Studying the microbiome of these ecosystems will allow us to better understand, predict, and design granular sludge processes.

1.1 Aim and objectives

The aim of this thesis was to expand on the current knowledge about microbial community composition and population dynamics in aerobic granular sludge reactors during start-up and steady-state conditions.

In Paper I, the abundance and robustness of the nitrifying population was assessed in reactors operated under different wash-out conditions. In Paper II, the diversity of functional groups was studied, and the core community was identified in reactors operated at different organic loading rates (which in turn resulted in different food-to-microorganism and carbon-to-nitrogen ratios). The reproducibility of the bacterial community was tested among three parallel reactors in Paper IV, and the deterministic and stochastic factors governing community assembly were discussed. By comparing the community composition in the granular and suspended phase of AGS reactors in Paper III, we hope to have gained new

insights on the granulation process. The core community members' relative abundance in the two phases was reported, and the observed differences between the two phases were linked to spatial location and successional patterns.

1.2 Outline of the thesis

Chapter 2 provides a short summary of the relevant literature within the research field, focusing on the engineering aspects (reactor design, operational conditions, etc.) of the granular sludge technology in general, and of the granulation process in particular. Studies published about full-scale processes are also reviewed shortly.

Chapter 3 summarizes the more detailed background of each appended paper, discusses the findings published earlier, and outlines the findings presented in our own studies.

Chapter 4 gives an overview of the methods applied during this research project, and evaluates the different techniques used for microbial community analysis.

Chapter 5 concludes the thesis by presenting the main findings of this research project and highlighting once more the importance and possible role of microbial ecology studies in biotechnology.

2 Introduction to wastewater treatment by aerobic granular sludge

Wastewater treatment by means of aerobic granular sludge is an emerging high-rate technology first reported in the 1990s (Mishima and Nakamura, 1991; Morgenroth et al., 1997). Granular sludge can be considered as a special case of biofilm, where self-immobilized bacterial cells form strong, dense and well-settling aggregates (Show et al., 2012). The main advantage provided by this technology is low footprint due to good settleability and effective pollutant removal even at high loading rates (Beun et al., 1999; de Bruin et al., 2004). Urbanization results in increasing pollutant loads and often limited availability to expand existing treatment plants, which makes this effective, low-footprint process an attractive alternative (Khan et al., 2013).

Although our understanding of the granulation process is incomplete (Bindhu and Madhu, 2014), achieving granulation in practice is usually not a challenge any more. The time needed for granulation is dependent on the reactor configuration, the wastewater composition and the operational conditions (Morales et al., 2013). Current selection pressure theories consider hydraulic selection pressure (the wash-out of slow settling particles) and hydrodynamic shear force as the most important operational parameters affecting the granulation process (Bindhu and Madhu, 2014; Zhu et al., 2013). The most common reactor configuration used for granulation is the sequencing batch reactor, and high hydraulic selection pressure is usually achieved either by short settling time (variable volume operation mode) or by high upflow velocity (constant volume operation /fill-draw mode) (Derlon et al., 2016; Qin et al., 2004b).

The majority of the granular sludge experiments use synthetic wastewater, with acetate or glucose as the main carbon source. Phenol, starch, ethanol, molasses, sucrose and other synthetic wastewater components have also been reported in successful granulation experiments (Adav et al., 2008; Bindhu and Madhu, 2014). Extensive research has revealed that aerobic granules can be effective in removing nutrients and organic compounds from municipal, industrial, and toxic wastewaters (Abdullah et al., 2013; Duque et al., 2015; Khan et al., 2013; Su et al., 2012; Val del Río et al., 2012a; Weber et al., 2007).

The dense structure of the granular sludge creates substrate gradients in the granule, which in turn results in different niches for different functional groups (Winkler et al., 2013). Studies have shown that aerobic and anaerobic metabolic activities can co-exist in granules, like the simultaneous removal of organic compounds, nitrogen and phosphorus (de Kreuk et al., 2005; Li et al., 2014b), simultaneous nitrification and denitrification (Third et al., 2003; Yang et al., 2003), or simultaneous nitritation and anammox (Nielsen et al., 2005; Vlaeminck et al., 2010)

2.1 The granulation process

Aerobic granules are achieved by encouraging the self-immobilization of bacterial cells with appropriate operational conditions. The most common reactor configuration used for aerobic granulation is the sequencing batch reactor (bubble column or airlift), where the solids retention time can be uncoupled from the hydraulic retention time (HRT). Under the last decades, a number of theories have been proposed as an explanation of the granulation

mechanisms, and numerous reports discussed the importance of the different process parameters (including low minimal settling velocity; high shear force; short hydraulic retention time; suitable organic loading rate; feast-famine conditions; and the presence of bacteria with granulation propensity).

It was established already in the 1990s that the primary design criterion is to provide conditions where granules have an advantage over flocs. This was achieved by high hydraulic selection pressure, i.e. biomass with too low settling velocity was washed out by applying short sedimentation time and a short draw phase (Beun et al., 1999; Morgenroth et al., 1997; Qin et al., 2004a). Today, it is still the most common method to obtain granulation, although some studies were published showing that sludge can be granulated with long settling times too (Chen et al., 2015; Wan et al., 2011; Zhou et al., 2014). To achieve high hydraulic selection pressure, the SBR can be operated either in variable volume mode, or in constant volume mode (fill-draw mode). The former means that the reactor is partially emptied (after a short sedimentation phase) through a withdrawal port at the mid-height of the reactor, and then refilled; while the latter means that the reactor is filled from the bottom and the effluent is withdrawn from the top simultaneously (Derlon et al., 2016; Giesen et al., 2013; Ni et al., 2009; Wagner and da Costa, 2013).

In studies where long settling time was applied, high shear force (provided by high superficial upflow gas velocity) was usually found necessary to achieve granulation (Chen et al., 2015; Zhou et al., 2014). High superficial gas velocity was found beneficial even in SBRs operated with short settling times, resulting in smoother, more compact, and more stable granules (Liu and Tay, 2002; Tay et al., 2004b). Granules were successfully developed at superficial gas velocities between 1.2 and 4.1 cm/s, while granulation failed in reactors operated with superficial velocities between 0.3 and 2.0 cm/s (Beun et al., 1999; Chen et al., 2007; Tay et al., 2001b). The overlap between the successful and unsuccessful range of superficial gas velocity is attributed to the fact that granule stability depends also on other operational parameters, e.g. dissolved oxygen concentration or organic loading rate (Chen et al., 2008; Liu and Tay, 2007; McSwain Sturm and Irvine, 2008).

Short HRT was also found to be beneficial for granule growth (Beun et al., 1999; Morgenroth et al., 1997), but more recent research revealed that this is mainly because a change in HRT directly influences the organic (and nutrient) loading rate, unless the concentration of the wastewater is adjusted (Muda et al., 2011). Adjusting the influent concentration is not a feasible method to control the volumetric loading rates in full scale bioreactors that treat real wastewater, it is more realistic to control the load by adjusting the HRT.

Successful granulation has been reported at volumetric organic loading rates between 1.05 and 15.0 kg COD/m^3/d (Chen et al., 2008; Liu et al., 2003; Wang et al., 2009). In some cases, high organic loading rates (> 8.0 kg COD/m^3/d) were reported to result in granule deterioration (Moy et al., 2002; Tay et al., 2004a), presumably due to insufficient shear forces (Chen et al., 2008). Low OLR (1.0 kg COD/m3/d) was found to result in biomass wash-out during the start-up phase, due to insufficient biomass growth (de Kreuk and van Loosdrecht,

2006). By applying stepwise decreased settling time, granules can be formed at low OLR while the biomass is retained (Su et al., 2013; Szabó et al., 2016).

Feast and famine periods were also considered to play an important role in granulation, as aggregation was hypothesized to be a strategy against starvation (Liu et al., 2005; Tay et al., 2001a). Later, contradictory results were published by the same research group, where starvation was declared not to be the main factor affecting granulation (Liu and Tay, 2008). Nonetheless, feast/famine conditions play an important role in aerobic granular sludge reactors operated for nitrogen removal: during the feast period, readily available carbon sources can be converted to storage polymers (PHAs), and during the famine period, the storage polymers can be used for denitrification (Beun et al., 2001; de Kreuk and van Loosdrecht, 2004). Introducing an anaerobic (anoxic) phase at the beginning of the SBR cycle can enhance the formation of storage polymers (de Kreuk and van Loosdrecht, 2004), but even if the feast period is aerobic, storage polymers can be formed in the anoxic core of the granules (Beun et al., 2001).

2.2 Full-scale AGS reactors

Although the aerobic granular sludge process was first applied in full-scale plants more than a decade ago, only a limited number of studies were published about the operation of these reactors. The first full-scale process treating a mixture of municipal and industrial wastewater (Epe, Netherlands) was briefly described by Giesen et al. (2013). The plant was designed to treat up to 1500 m^3 wastewater per hour, and met the effluent requirements of TN <5 mg/L and TP <0.3 mg/L. The reactor configuration and the operational parameters were not discussed in detail.

Pronk et al. (2015) analyzed the performance and design of a full-scale plant (Garmerwolde, Netherlands) treating municipal wastewater, operated for COD, nitrogen and phosphorus removal. The plant was operated in constant volume (fill-draw) mode, the volume exchange ratio (VER) varied depending on the weather (30-40% under dry weather flow, 65% under rain weather operation). The SBR cycle consisted of anaerobic feeding/withdrawal (60 min), reaction/aeration (5 h), and settling (30 min). Under periods with heavy rainfall, the cycle time was shortened to 3 h to treat the increased flow; if this resulted in increased effluent concentrations, phosphorus was removed chemically at the end of the cycle. To allow simultaneous nitrification and denitrification the dissolved oxygen (DO) was controlled at 1.9 mg/L during aeration. After the ammonium concentration in the reactor reached the desired set-point the DO was lowered (<0.5 mg/L) to allow post-denitrification of nitrate. If the nitrate concentration was still too high at the end of the cycle, bulk liquid from the top of the reactor was recycled to the bottom and mixed with the fresh influent (average recycle ratio 0.3). The effluent quality met the limits of 1 mg/L for phosphorus and 7 mg/L for total nitrogen, even during the start-up period. This was achieved by stepwise increased loading, which resulted in a somewhat longer start-up time of three month. The high removal rates of the granular process resulted in a volume reduction of 33% compared to the existing treatment plant at Garmerwolde. Due to the lack of mixers, settlers, recycle and sludge return pumps, the energy consumption of the granular process was 51% lower compared to the existing

treatment plant. The microbial community composition of the reactors was not compared in this study.

Li et al. (2014a) reported about the operation and microbial community composition of a full-scale AGS reactor treating a mixture of domestic (30%) and industrial (70%) wastewater (Yancang WWTP, China). The SBR was operated in variable volume mode, with a cycle of 40 min filling, 4 h aeration, 50 min settling and 30 min effluent withdrawal. The VER varied between 50% and 70%. The COD, ammonium, TN and TP removal rates were 85%, 95.8%, 59.6% and 48%, respectively. To meet the effluent requirements, phosphorus was removed via physical and chemical treatment after the SBR. Compared to the A/O plug flow process of the already existing plant, the AGS reactor had 4-times higher capacity (4 m^3/d wastewater treated per m^3 reactor volume), similar COD and ammonium removal efficiency, but higher TN removal and lower TP removal efficiency. The microbial community analysis showed that *Flavobacterium*, *Thauera* and an uncultured *Aquabacterium* sp. were the dominant genera in the AGS reactor, while the A/O and OD reactors were dominated by *Nitrospira*, *Thauera* and an uncultured *Bacteroidetes*-related genus.

3 Microbial ecology in AGS reactors

High removal efficiency and a stable, resilient process performance in bioreactors are dependent on the microbial community composition (Briones and Raskin, 2003; Werner et al., 2011). The correlation between diversity and process stability has been shown in laboratory- and full-scale reactors treating wastewater (von Canstein et al., 2002; Yang et al., 2011). Moreover, it was shown that it is functional redundancy, rather than species diversity, that results in stable process performance (Briones and Raskin, 2003). Werner et al. (2011) showed in a study about anaerobic digesters that evenness and phylogenetic variability are more appropriate measures of functional diversity than species richness or species diversity. At present, microbial ecology studies on AGS reactors usually focus on the core community of the reactors. In our experiments, we focused on different functional groups: the temporal variation in the abundance of nitrifiers under wash-out conditions (Paper I), and the diversity of all major functional groups in three different AGS ecosystems (Paper II). We studied the effect of putative deterministic and stochastic factors on community assembly during start-up (Paper IV), and discussed the preferential retention of certain taxa during start-up (Paper III).

3.1 Effect of biomass wash-out on the nitrifying population

Granulation is usually achieved by high hydraulic selection pressure, i.e. the sequencing batch reactor is operated either with short settling time (variable volume operation mode) or with high upflow velocity (constant volume operation / fill-draw mode), both resulting in the wash-out of slow settling particles (Derlon et al., 2016; Qin et al., 2004b).

The harsh wash-out conditions can affect the microbial community composition considerably. McSwain et al. (2004) operated two parallel laboratory-scale AGS reactors with different settling times (2 and 10 min), and observed entirely different microbial consortia in the samples taken after 160 and 220 days of operation. The microbial community of the reactor with shorter settling time was less diverse, but more stable. The difference in the community composition was not reflected in the measured process parameters as the removal efficiency of organic matter and the oxygen uptake rate were similar in the two reactors. The only reported difference in process performance was the suspended solids concentration in the effluent (290±170 and 170±100 mg/L at 10 and 2 minutes, respectively). Adav et al. (2009) made similar observations in reactors operated at 10, 7 and 5 min settling time. The diversity of the microbial community was lowest at 5 min settling time, and after 75 days of operation the community composition showed only 60-69% similarity between the reactors, but the process performance was alike in the three reactors. (Nitrogen removal was not investigated in these studies.)

In early studies, short settling time was applied from the first cycle of operation (Beun et al., 1999; McSwain et al., 2004; Moy et al., 2002). Later, it was suggested that a stepwise increased hydraulic selection pressure may increase the stability and performance of granular sludge (Cassidy and Belia, 2005; Lochmatter and Holliger, 2014; Ni et al., 2009; Wang et al., 2007b). The underlying ecological reasons were, however, not discussed in these studies.

Rapid changes and decreased diversity in the microbial community have been documented during the start-up of AGS reactors (Li et al., 2008; Liu et al., 2010; Lv et al., 2014; Weissbrodt et al., 2012a, 2013). The harsh wash-out conditions during start-up were hypothesized to result in reduced nutrient removal efficiency (Weissbrodt et al., 2012a). Excessive wash-out dynamics results in decreased sludge retention time (SRT) that is disadvantageous for slow growing bacterial guilds, such as ammonium or nitrite oxidizers (Val del Río et al., 2012b). This can lead to deteriorated nitrogen removal efficiency.

In Paper I, we studied the abundance and dynamics of the nitrifying population during granulation, and found that the stepwise decrease of settling time enables good retention of nitrifying organisms and thus a rapid start-up of nitrification in the reactors. The rate of the stepwise decrease in settling time strongly affected the abundance of nitrifying organisms, but not the general composition of the bacterial community.

3.2 Effect of the organic loading rate on the bacterial community

Most studies investigate the effects of operational parameters by analyzing the community composition in samples obtained at a single sampling date during steady-state operation (e.g. Cydzik-Kwiatkowska, 2015; Ebrahimi et al., 2010; Gonzalez-Gil and Holliger, 2011). However, long-term studies of full-scale CAS treatment plants revealed strong variation in the microbial community and a gradual succession away from initial conditions, in spite of steady-state operation (Fredriksson, 2013; Kim et al., 2013; Wells et al., 2011). The temporal variations of dominant and rare taxa were reported to show different patterns, the dominant taxa being more persistent (Kim et al., 2013). Nonetheless, dominant taxa were strongly affected by the availability of organic carbon in the influent.

Li et al. (2008) applied loading rates of 1.5, 3.0 and 4.5 kg COD/m^3/d, and found that at different organic loading rates the bacterial communities changed at different rates. The highest loading rate resulted in the lowest diversity, but the same dominant genera (*Zoogloea*, *Thauera*, *Pseudomonas*, *Flavobacterium*, and a *Comamonadaceae* related genus) were found in all three reactors, although in different proportions.

In Paper II, we analyzed the evolution of the microbiome organic loading rates of 0.9, 1.9 and 3.7 kg/m^3/d, and found that the bacterial communities at different loading rates diverged rapidly after start-up. The microbiomes showed less than 50% similarity after 6 days, and below 40% similarity by the end of the experiment. Species diversity decreased in all three reactors during the start-up period, presumably due to the lack of complex substrates in the influent. By the end of the experiment all three reactors showed similar richness and evenness, around 37% and 16% lower than that of the seed sludge. The different reactors were dominated by different genera (mainly *Meganema*, *Thauera*, *Paracoccus* and *Zoogloea*), but these genera have similar ecosystem functions of EPS production, denitrification and PHA storage. Many less abundant but persistent taxa were detected within these functional groups. At steady state operation of the reactors, the identity of the core community members was rather stable, but with considerable changes over time in their relative abundances. Furthermore, nitrifying bacteria were low in relative abundance and diversity in all reactors,

despite their large contribution to nitrogen turnover. The results suggest that the organic loading rate has considerable impact on the formation of the aerobic granular sludge communities, but also that the granule communities can be dynamic even at steady state reactor operation, which can be explained by high functional redundancy of several key guilds.

3.3 Stochastic and deterministic factors affecting the community assembly

Although the aerobic granular sludge process was first reported in the early 1990s (Mishima and Nakamura, 1991; Morgenroth et al., 1997), we still have an incomplete understanding of the parameters that drive the evolution of the microbial community composition of aerobic granules (Bindhu and Madhu, 2014; Weissbrodt et al., 2012a). As mentioned before, functional stability of the process is often accompanied by a dynamically changing microbial community composition (Fredriksson, 2013; Kim et al., 2013; Wells et al., 2011). The community assembly is thought to be driven by a combination of stochastic (birth, death, and dispersal rates) and deterministic (niche differentiation, interspecies interaction) factors (Ayarza and Erijman, 2011; Van Der Gast et al., 2008; Ofiteru et al., 2010; Pholchan et al., 2013)

In engineered ecosystems, like reactors treating wastewater, certain functional groups are selected for in order to achieve the desired process performance, e.g. aerobic or anaerobic ammonia oxidizing bacteria (AOB and anammox), nitrite oxidizing bacteria (NOB), denitrifiers, or phosphate accumulating organisms (PAO). Other microorganisms are selected because of their life style, such as EPS producing, floc-forming bacteria (Larsen et al., 2008; Zhang et al., 2016). Despite the fact that the same functional groups of microorganisms are present in granular and floccular sludge, differences in proportions between phylogenetic groups at phylum- or class level are found between the two types of sludge (Guo et al., 2011; Winkler et al., 2013).

The effect of settling time, one of the most important deterministic factors in granular sludge reactors, was studied by Adav et al. (2009). The reactors were started up with different settling times (10, 7 and 5 minutes), thus different microbial communities developed, but when a settling time of 5 minutes was applied to all reactors, the similarity increased between the reactors with initially longer settling time. The third reactor, with 5 min settling time from the beginning, was still different from the other two. It is possible, that after a longer period, the similarity would have increased, but it is also possible that the short settling time resulted in the definitive wash-out of certain bacterial species, and the communities would have remained different due to the differences during the start-up period.

In Paper IV, we studied the putative deterministic and stochastic factors during granulation in three identically operated parallel reactors. We found that during the start-up, the community structure of the reactors converged, indicating that the community assembly was mainly affected by deterministic factors – the strong hydraulic selection pressure and the shear force. Diversity decreased during the transformation of floccular sludge into aerobic granules as a consequence of habitat specialization (feast-famine operation, less complex feed). The effect

of stochastic processes became more evident when the strong selection pressure, caused by the wash-out conditions, decreased. The microbial community in one of the reactors diverged from the other two, the differences were mainly found in intermediate and rare taxa. It was reported before that the temporal variation of dominant and rare taxa show different patterns in a full-scale CAS reactor, the dominant taxa being more persistent, presumably because dominant taxa are less likely to disappear due to stochastic processes (Kim et al., 2013).

3.4 Effect of the granulation process on the microbial ecology

In Paper II, we found that certain functional groups are over-represented in granular sludge compared to the flocculated seed sludge. EPS producers were approx. 13% of the community in the flocculated sludge, and approx. 40% in the granular sludge; denitrifiers had a cumulative relative read abundance of 25% in the seed and 45-60% in the granules (note that many of the EPS producing genera found are also considered denitrifiers). The role and importance of EPS production on the formation and stability of granular sludge has been extensively studied (Ding et al., 2015; Lemaire et al., 2008; Liu et al., 2004; Weber et al., 2007), and denitrification has been reported to accelerate granule formation (Suja et al., 2015; Wan and Sperandio, 2009).

In Paper III, we followed the relative read abundance of the most abundant genera in the effluent of three granular sludge reactors during start-up and steady-state conditions. Our results show that EPS producers were not preferentially retained during harsh wash-out conditions; all examined genera appeared to be washed out proportionally to its relative abundance in the reactor during start-up. Thus, while the strong hydraulic selection pressure is beneficial for the physical selection of fast-settling aggregates, it is almost certain that this operational condition does not directly select for EPS producing bacteria. This is supported by the studies where granules were formed with long settling times (Chen et al., 2015; Wan et al., 2011; Zhou et al., 2014). Short settling time does, however, negatively affect the abundance of nitrifiers, as it was shown in Paper I (this was not studied in Paper III due to the low relative read abundance of nitrifiers).

Other stressful culture conditions (e.g. high shear force, feast-famine regimes, alternating anaerobic-aerobic operation) have been reported to enhance EPS production (Liu et al., 2004), and are more likely to be the reason behind the increased relative abundance of EPS producers. Moreover, the feast-famine regimes and the anaerobic-aerobic conditions favor mixotrophic genera and genera with the ability to produce storage polymers (de Kreuk and van Loosdrecht, 2004). However, it is estimated that 75% of the activated sludge bacteria are capable of producing storage polymers (Inoue et al., 2016), therefore it is difficult to assess whether storage-polymer-producers are overrepresented in granules. The functional group of EPS producers is also very versatile, consists of phylogenetically unrelated genera from the phyla *Bacteria*, but presumably also from *Archaea*, *Fungi*, and *Protista* (Weber et al., 2007).

While these traits are apparently favored by the typical granular sludge SBR operation, it can be hypothesized that not all of them are necessary for granulation. Mixotrophy and storage

polymers are beneficial for nutrient removal, but aerobic granular sludge for the removal of COD only can, theoretically, be formed and operated successfully without these traits.

4 Overview and discussion of methods applied during this research project

Three laboratory-scale sequencing batch reactors were used during the studies, the reactor set-up and the feed is described in every paper. The main carbon source was acetate in every experiment, while the nitrogen source was NH_4Cl (Paper I), reject water (Paper II-III), or ammonium acetate (Paper IV). The cycle length was 4 hours, consisting of anaerobic feeding, anoxic phase (except in Paper I), aerobic phase, settling, withdrawal and idle phase (Table 1). The settling time was gradually decreased to avoid extensive biomass wash-out, as it is described in Paper I. The aerobic cycle was adjusted to permit an even 4 hour cycle length.

Table 1. Length of each phase in the SBR cycle.

	Feeding	Anoxic phase	Aeration	Settling	Withdrawal	Idle
Paper I	5 min	0 min	198-226 min	2-30 min	5 min	2 min
Paper II-IV	5 min	55 min	143-171 min	2-30 min	5 min	2 min

When a SBR reactor is operated for complete nitrogen removal, the length of the anoxic phase plays an important role in whether denitrification is achieved, since acetate may leak into the aerobic phase if the anoxic phase is too short, thus the simultaneous nitrification-denitrification process will be hampered (de Kreuk et al., 2010; Weissbrodt et al., 2012a). Cycle studies were performed during the experiments (except Paper IV), to monitor the consumption of acetate during the cycle (Fig. 1 and 2).

The reactors were sampled three times per week: the height of the settled sludge bed was measured, biomass samples were taken for DNA analysis, and effluent samples were taken for the analysis of the effluent quality (the used methods are described in every paper). Intact granules were harvested and fixed for fluorescence in situ hybridization (FISH) analysis that was used to study the structure of the granules and to assess the spatial location of species. Details about the FISH protocol can be found in Paper II and III. The community composition and the population dynamics were studied either by NGS or by T-RFLP supplemented with qPCR. Details about the qPCR protocol can be found in Paper I, while the techniques used for assessing community dynamics are compared and discussed below.

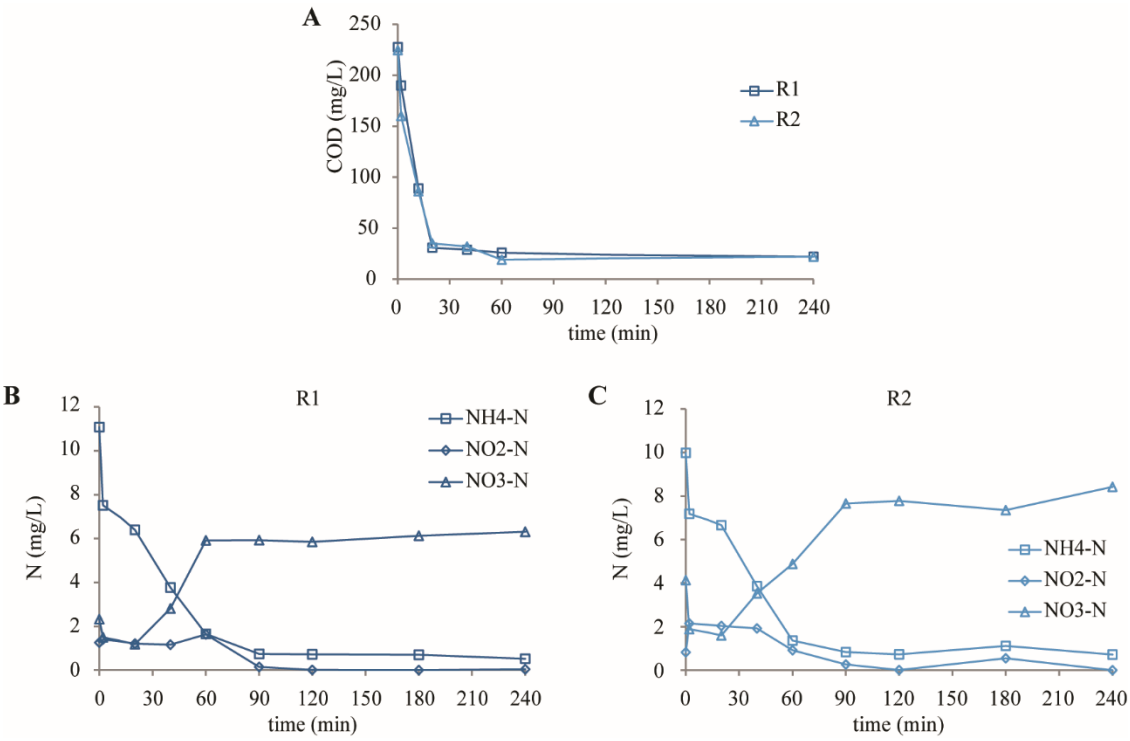

Figure 1. COD (A) and N concentration profiles during the cycle study in R1 (B) and R2 (C) in Paper I.

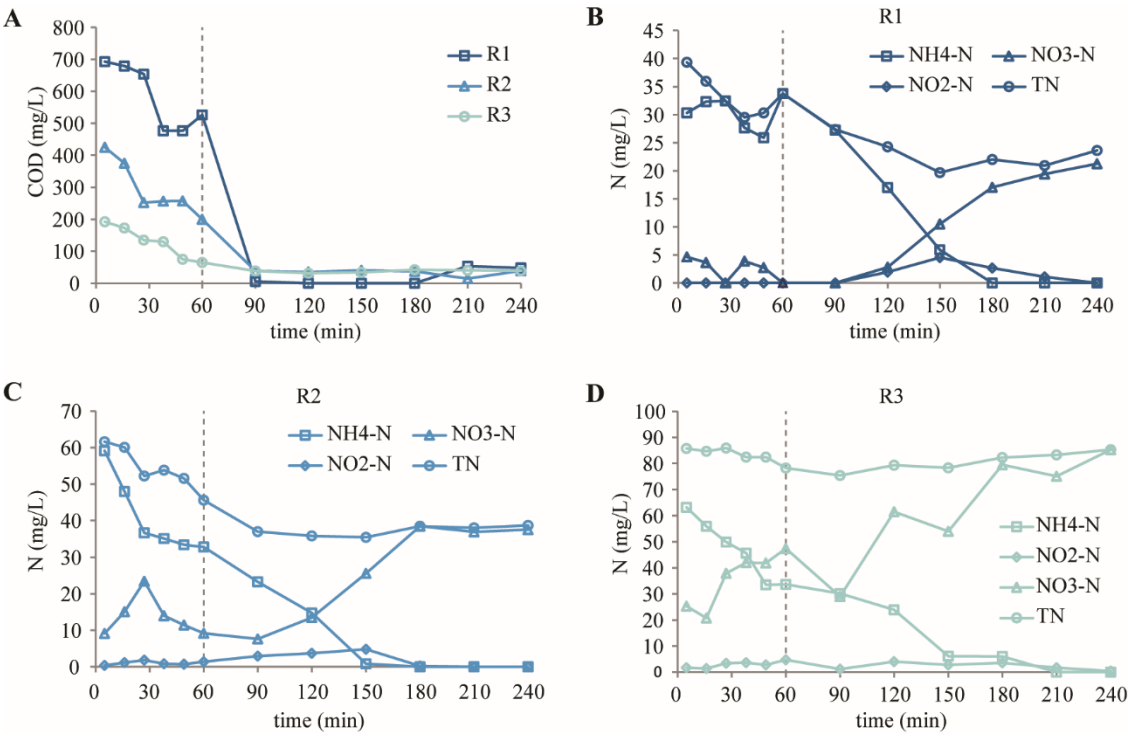

Figure 2. COD (A) and N concentration profiles during the cycle study in R1 (B), R2 (C) and R3 (D) in Paper II. The dashed vertical line shows the transition between the anoxic and aerobic phase.

4.1 Microbial community analysis

Microbial community analysis was done either by T-RFLP or high throughput sequencing (Illumina MiSeq). In both protocols, DNA was extracted using the same protocol. In the T-RFLP analysis, the 16S rRNA gene was amplified using the forward primer 27F (6-FAM labeled) and the reverse primer 1492R (Lane, 1991). In case of the MiSeq, the V4 hyper-variable region of the 16S rRNA gene was targeted with the primers 515F and 806R, dual indexed according to Kozich et al. (2013).

The T-RFLP protocol continued with the purification of the PCR products, then the samples were digested with mung bean nuclease, purified again, and digested using the restriction enzymes RsaI and MspI. The digested products were purified, and finally analyzed with a 3730 fragment analyzer (Applied BioSystem). The results were quantified with the software GeneMapper (Applied Biosystems, Foster City, MA, USA), analyzed based on the relative peak area using the Microsoft Excel template "Tools for T-RFLP data analysis" (Fredriksson et al., 2014), and the OTU table was subsequently analyzed in R (R Core Team, 2016).

In the MiSeq analysis, the DNA concentration of the PCR products was normalized, then the samples were purified, multiplexed and the concentration was normalized again, and finally analyzed with a MiSeq Illumina sequencer. The sequence reads were processed and assigned to taxonomy as published in Albertsen et al. (2015), and the OTU table was subsequently analyzed in R (R Core Team).

Although the popularity and availability of next generation sequencing techniques has increased during the last few years, community fingerprinting is still a commonly used and suitable approach to assess microbial community dynamics (van Dorst et al., 2014). It was found that fingerprinting methods had a similar capacity to capture significant biological patterns and correlate environmental variables as pyrosequencing, although pyrosequencing did that with far greater resolution. But the biological patterns were found to be mainly dependent on the most abundant OTUs (top 0.1%) in the NGS dataset, which are also detected by the fingerprinting technologies (van Dorst et al., 2014). Rare OTUs might contribute more to the separation of samples if the rarefaction coverage curves were saturated, but this usually can not be achieved, not even with NGS techniques (Gilbert et al 2009).

The possibility to examine rare taxa in microbiomes is unarguably a great advantage of sequencing techniques. NGS provides a much better basis for diversity estimates simply due to the lower detection limits and thus higher resolution (Bent et al., 2007). The fingerprinting methods generated about 2- to 10-times less OTUs than the NGS method (van Dorst et al., 2014). Thus, fingerprinting methods severely underestimate alpha diversity and are inappropriate for comparison across studies (but not within studies). However, NGS techniques are not exempt of the PCR biases already known from fingerprinting techniques (e.g. selection and drift), and are further burdened with some biases unique for massively parallel sequencing (e.g. sequencing errors, chimeras, and PCR biases due to barcoding) (Alon et al., 2011; Berry et al., 2011; Kennedy et al., 2014; Kunin et al., 2010; Pinto and Raskin, 2012). Although high throughput sequencing is cheaper than ever, scientists still compromise on replicates, which results in findings with little statistical support.

Genotypic fingerprinting does not provide taxonomic information, but the results can be further analyzed to identify sequences (Pilloni et al., 2012; Weissbrodt et al., 2012b). Moreover, taxonomic information is not always more informative (van Dorst et al., 2014). When studying microbial communities in engineered systems, the population dynamics and the ecosystem functions of the community are often of more interest than the actual taxonomic identity of the community members (Paper I-II). Not only are the NGS results severely biased to known species, but it is also more tedious and labor intensive to extract this information from an NGS dataset (Paper II) than from T-RFLP complemented with qPCR analysis (Paper I). Therefore, community fingerprinting is still a commonly used and suitable approach to assess microbial community dynamics.

4.2 Processing of Illumina MiSeq results

The sequence reads of the MiSeq analyses were processed and assigned to taxonomy in Paper II, III and IV as published in Albertsen et al. (2015). However, prior to this bioinformatic analysis, the MiSeq data of Paper II and III were also analyzed in the mothur software package (v.1.33.3.)(Schloss et al., 2009) as published in Kozich et al. (2013), using the Greengenes database (v.13.8.99.)(DeSantis et al., 2006). This allowed us to compare the results obtained with these two procedures.

In the mothur standard operating procedure (Kozich et al., 2013), the paired-end reads for each sample were joined into contigs, then too long sequences (>275 bp) or sequences with ambiguous bases were removed. The remaining sequences were aligned to the reference database (those sequences that did not align to the V4 region were removed), and then pre-clustered (allowing up to 2 nt differences) to further denoise sequences. Putative chimeras were removed using the UCHIME algorithm, then the sequences were classified against the Greengenes database using a naive Bayesian classifier (Wang et al., 2007a) with a confidence threshold of 0.8, and those sequences that were not classified as Bacteria (or were not classified at all) were removed. Finally, sequences were split into groups according to which taxonomic order they belonged to, and then assigned to operational taxonomic units at 97% similarity.

In the second procedure, the UPARSE workflow was used (Albertsen et al., 2015; Edgar, 2013). The paired-end reads were merged (sequences with ambiguous bases were removed), the too long (>275 bp) or too short (<225 bp) sequences were removed, reads were dereplicated and singletons removed. The remaining sequences were clustered into OTUs using default settings (97% identity), while chimeras were removed simultaneously (using the UPARSE-OTU algorithm). Then the sequences were classified against the MiDAS database (v.1.20) (McIlroy et al., 2015) using the same classifier as in the mothur workflow (Wang et al., 2007a) with a confidence threshold of 0.5.

As it can be seen, the two approaches use different taxonomy databases. Moreover, the mothur workflow first classifies the sequences, and clusters them into OTUs later, while the UPARSE workflow clusters the sequences in OTUs first and classifies them later. The same classifier is used, but different confidence thresholds are applied.

As a consequences of these differences, the results obtained from the two procedures differ considerably. This is demonstrated in Fig. 3, a stacked column chart showing the relative read abundance of families belonging to the phyla *Proteobacteria* and *Bacteroidetes*. The family level was chosen for demonstrating the differences because at this level the figure is still readable (although rare families, <0.1%, had to be removed). Similar issues prevail on lower taxonomic levels.

Since the sequences were classified against different databases, some taxa were present only in one OTU table, but not in the other. Many of these are taxa without valid published or candidate names, *NS9Marinegroup* in MiDAS, or suggested taxonomic changes (McDonald et al., 2012), indicated with square brackets, e.g. *[Weeksellaceae]* in the Greengenes database. In total, 15 such families were found by the mothur-procedure, and 32 by UPARSE. The MiDAS taxonomy deliberately proposes putative names for abundant and important taxa found in wastewater treatment plants, to be used as a common vocabulary by researchers within the field. It is also manually curated, and therefore includes e.g. Meganema, which is missing from the version of the Greengenes database we used. Another important difference is the confidence threshold used for classification. Due to the lower threshold used in the UPARSE-workflow, some sequences/OTUs were binned together in the same family (e.g. *Comamonadaceae*) which were separated by the mothur procedure, resulting in classifications like *Comamonadaceae(100)*, *Comamonadaceae(86)*, *Comamonadaceae(66)*, etc. Depending on the research hypothesis and the intended usage of the OTU table, a more or less strict confidence threshold can be used, but in order to obtain comparable results from multiple studies, the same threshold should be used. With the procedures described above, the mothur workflow resulted in more unclassified families (Fig. 4A-C) compared to the UPARSE-workflow (Fig. 3A-C).

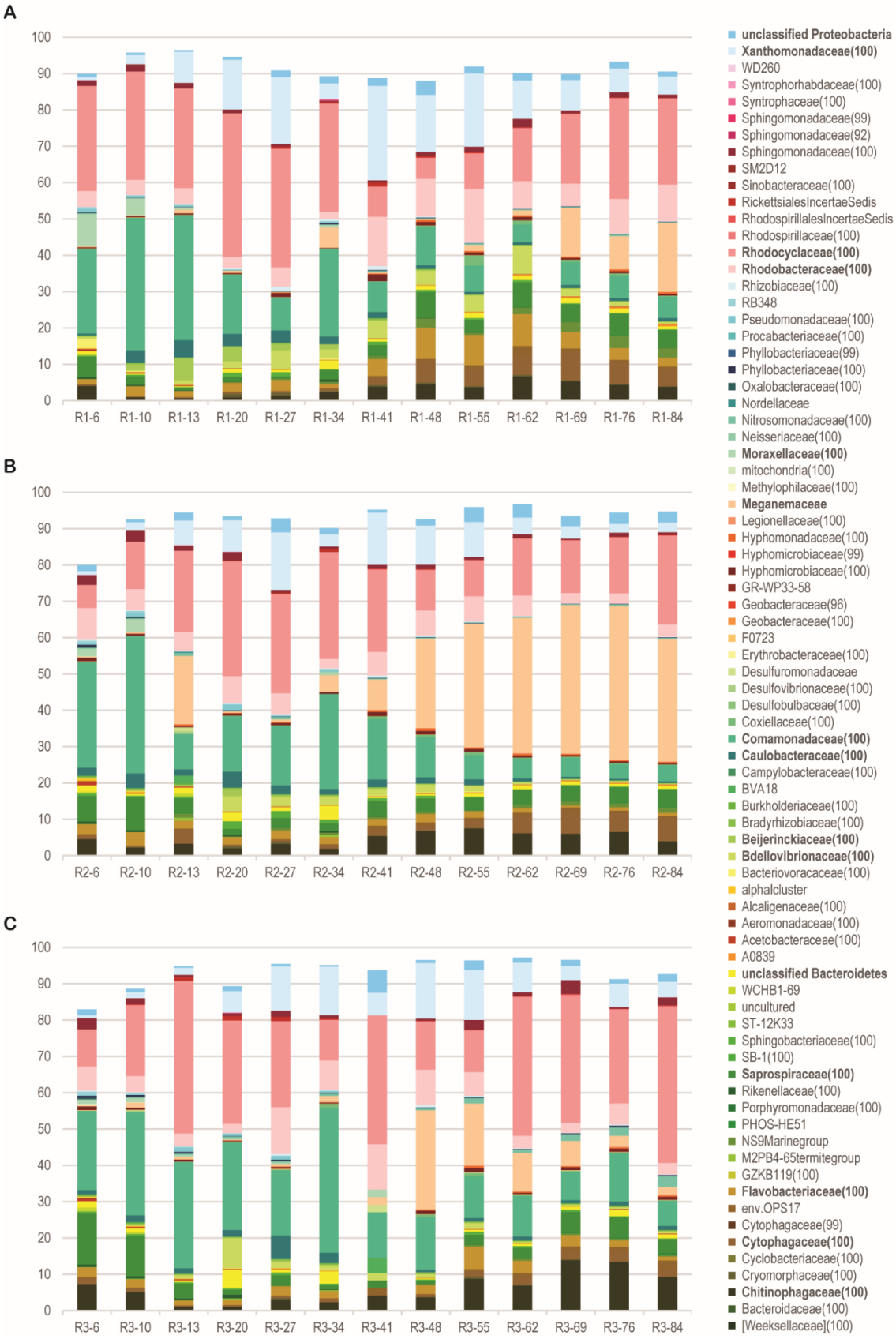

Figure 3. Relative read abundance of families belonging to the phyla *Proteobacteria* and *Bacteroidetes* in R1 (A), R2 (B) and R3 (C). Results obtained from the UPARSE-workflow. Rare families, <0.1%, were removed from the dataset. The most abundant taxa are highlighted in bold font.

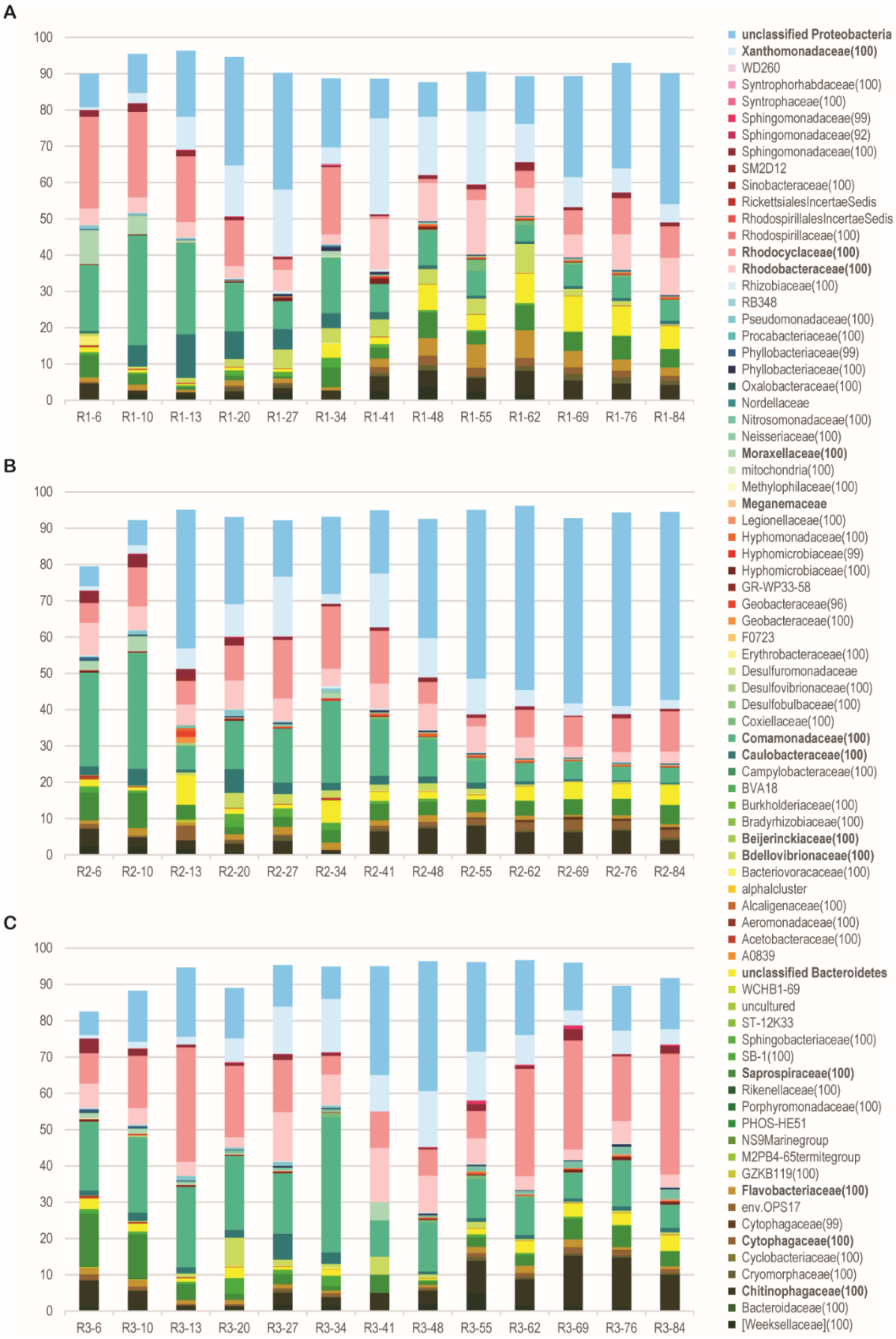

Figure 4. Relative read abundance of families belonging to the phyla *Proteobacteria* and *Bacteroidetes* in R1 (A), R2 (B) and R3 (C). Results obtained from the mothur-workflow. Rare families, <0.1%, were removed from the dataset. The most abundant taxa are highlighted in bold font.

4.3 Statistical analysis

The statistical analysis of the OTU tables obtained from the fingerprinting (T-RFLP) or sequencing (Illumina MiSeq) techniques was performed using Excel and R. Different alpha diversity indices were calculated: richness (observed richness or Margalef richness), evenness (Pielou's or Simpson's), and diversity (Shannon's or Simpson's). The beta diversity was assessed with Bray-Curtis dissimilarity index and non-metric multidimensional scaling (NMDS) was used to visualize the community dynamics.

In Paper IV, three replicate reactors were run with the same operational conditions, and one of the reactors was found to deviate from the other two. R1 showed lower richness and higher evenness than R2 and R3 (Fig. 5), but similar diversity (Simpson's diversity index). The decreased richness in R1 was assumed to be a result of the uneven library size, but upon statistical testing it was found that the library size of the samples from R1 (Fig. 5D) was not significantly different ($p>0.6$). The NMDS ordination also showed that R1 was different from R2 and R3 (Fig. 6A).

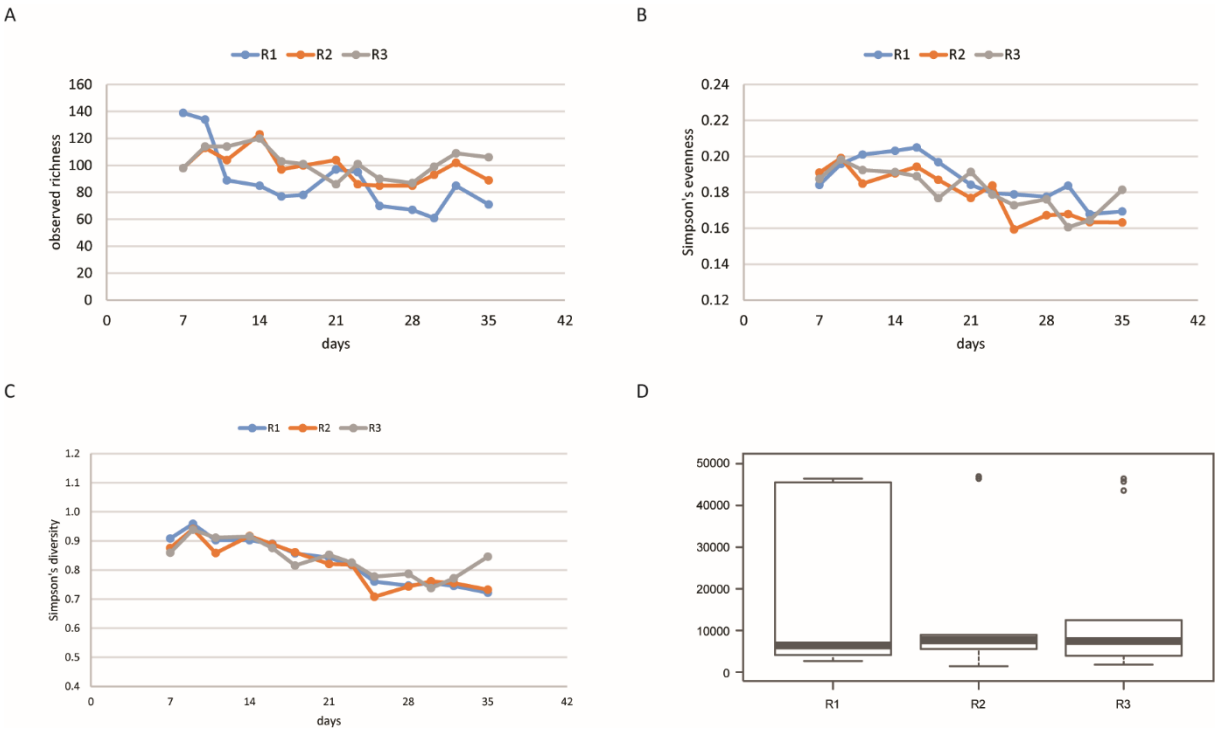

Figure 5. Richness (A), evenness (B) and diversity (C) of the microbial communities in the replicate reactors; boxplot showing the library sizes of the samples (D).

In order to further examine the reason behind the deviation, constrained analysis of proximities (CAP) was performed on the dataset. For this analysis the non-rarefied dataset was used, in order to take into account all the rare OTUs that would be disregarded otherwise. The results confirmed R1 to be different to the other two reactors. 47 OTUs were found to significantly contribute to the separation between R1 and the other two reactors. The majority of these OTUs were rare or intermediate OTUs (Lawson et al., 2015), with a cumulative relative read abundance of 4.9±2.0%.

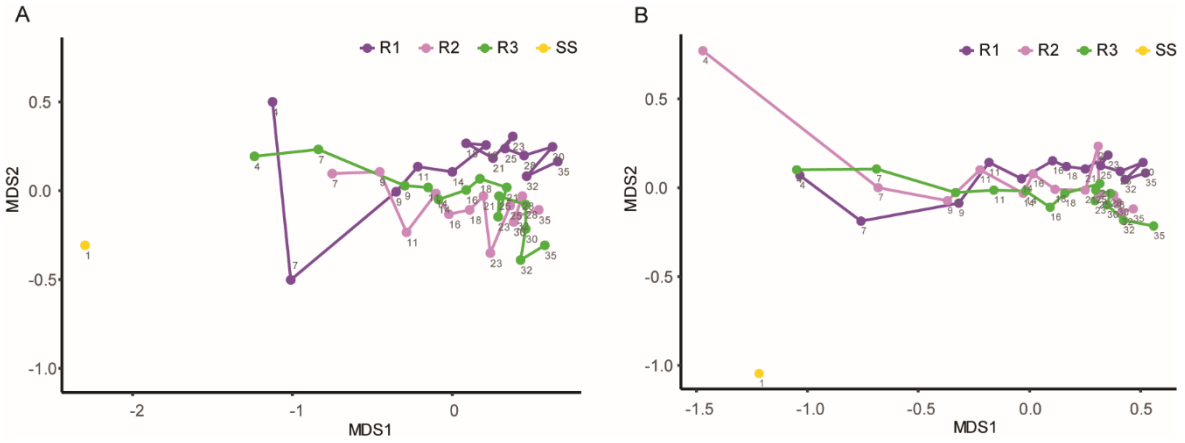

Figure 6. NMDS ordination plot based on Bray-Curtis dissimilarities of the rarefied (A) and downscaled (B) dataset. Each line represents the community structure evolution over time of the replicate reactors (R1, R2, and R3) and the seed sludge (AS, day 1) during the experiment.

In a study by McMurdie and Holmes (2014), it was found that rarefying resulted in decreased clustering accuracy due to omitted samples (with low read numbers), omitted read counts, and added noise from the random sampling step. Upon the analysis of very similar reactors (as in Paper IV), the decreased clustering accuracy might lead to incorrect conclusions. Based on the results published by McMurdie and Holmes (2014), the clustering accuracy of our analysis should be approx. 85%, depending on the "effect size" (expected difference), but irrespective of the mean library size. It was suggested that instead of *random* subsampling, it is better to *downscale* the read counts (McMurdie and Holmes, 2014). Therefore, we examined the results of NMDS based on relative read abundances (instead of rarefied read numbers). Although the difference seems less pronounced, R1 is still diverging from the other two reactors (Fig. 6B).

As the results of the CAP analysis showed, the differences in richness, evenness, and community dynamics (NMDS) between R1 and R2-R3 are presumably due to rare and intermediate species. To verify the *identity* of the OTUs that have significantly different relative abundances in the different reactors, multivariate ANOVA test was used. The MANOVA test requires replicate measurements, therefore the last 4 sampling dates of the experiment were considered replicates. This can be justified by the fact that the process performance was stable, although real replicate measurements would be more desirable. Out of the 47 OTUs identified by the CAP analysis, only 24 were found to have significantly different relative abundance. (Note that the CAP analysis considers all 14 sampling dates, not just the last 4.) The usage of the R packages edgeR, DESeq, or DESeq2 was suggested for the analysis of differential abundance (McMurdie and Holmes, 2014). Since the dataset is a timeseries, multivariate timeseries analysis would also be an option. However, testing and evaluating these different statistical methods is outside the scope of this thesis.

5 Summary and outlook

In the research presented in this thesis, the bacterial community composition and the population dynamics in laboratory-scale aerobic granular sludge reactors were analyzed, with the help of molecular biology techniques including qPCR, T-RFLP and Illumina MiSeq. The abundance and dynamics of the nitrifying population was studied during granulation under wash-out conditions, and it was found that a stepwise decrease of settling time enables good retention of nitrifying organisms. The rate of the stepwise decrease in settling time strongly affected the abundance of nitrifiers, but not the general composition of the bacterial community. The diversity of major functional groups was assessed in three different AGS ecosystems, operated at different organic loading rates. The different operational parameters were found to affect considerably the composition of the bacterial communities, but high functional redundancy was observed within the key guilds in all three reactors. The bacterial communities were highly dynamic even at steady-state operation.

The effect of putative deterministic and stochastic factors on community assembly were studied in three parallel reactors. During start-up, the community dynamics was found to be affected mainly by deterministic factors – the strong hydraulic selection pressure and the high shear force. The effect of stochastic factors became evident when the strong selection pressure decreased, but it seemed to affect primarily the rare and intermediate taxa.

The effect of the granulation process itself was explored by assessing preferential retention of certain taxa and discussing advantageous traits in AGS reactors. During start-up, all taxa appeared to be washed out with the same probability. After granules started to emerge, certain taxa were found to be more abundant in the effluent, but were, in spite of that, among the most abundant members of the community. Other taxa seemed to be well retained, since they had higher abundance in the granules than in the effluent, but their relative abundance still decreased with time. This suggests that the preferred spatial location of a taxon seems to be the primary reason behind its high abundance in the effluent, while the growth rate or other advantageous traits of a taxon have a greater impact on its temporal variation. Indeed, certain functional groups – EPS producers, denitrifiers, PHA producers – were found to be over-represented in granular sludge compared to the flocculated seed sludge. The typical SBR operation seems to favor these traits, but whether these are necessary for successful granulation and reactor operation depends on the intended function of the reactor.

At present, microbial ecology studies – including our experiments – on the AGS process are predominantly of descriptive nature. However, it was suggested that in the future microbial ecology may provide a tool to predict or even design diversity and thus process stability (Curtis and Sloan, 2006; McMahon et al., 2007). The importance of functional redundancy, as well as of rare and intermediate taxa was demonstrated earlier (Power et al., 1996; Shade and Handelsman, 2012). The increasing availability of next generation sequencing methods allows us to study the rare biosphere in AGS reactors and to gain insights in how ecology can be integrated in biotechnology.

References

Abdullah, N., Yuzir, A., Curtis, T. P., Yahya, A., and Ujang, Z. (2013). Characterization of aerobic granular sludge treating high strength agro-based wastewater at different volumetric loadings. *Bioresour. Technol.* 127, 181–187. doi:10.1016/j.biortech.2012.09.047.

Adav, S. S., Lee, D.-J., and Lai, J.-Y. (2009). Aerobic granulation in sequencing batch reactors at different settling times. *Bioresour. Technol.* 100, 5359–5361. doi:10.1016/j.biortech.2009.05.058.

Adav, S. S., Lee, D.-J., Show, K.-Y., and Tay, J. H. (2008). Aerobic granular sludge: recent advances. *Biotechnol. Adv.* 26, 411–23. doi:10.1016/j.biotechadv.2008.05.002.

Albertsen, M., Karst, S. M., Ziegler, A. S., Kirkegaard, R. H., and Nielsen, P. H. (2015). Back to Basics – The Influence of DNA Extraction and Primer Choice on Phylogenetic Analysis of Activated Sludge Communities. *PLoS One* 10, 1–15. doi:10.1371/journal.pone.0132783.

Alon, S., Vigneault, F., Eminaga, S., Christodoulou, D. C., Seidman, J. G., Church, G. M., et al. (2011). Barcoding bias in high-throughput multiplex sequencing of miRNA. *Genome Res.* 21, 1506–1511. doi:10.1101/gr.121715.111.

Ayarza, J. M., and Erijman, L. (2011). Balance of Neutral and Deterministic Components in the Dynamics of Activated Sludge Floc Assembly. *Microb. Ecol.* 61, 486–495. doi:10.1007/s00248-010-9762-y.

Bent, S. J., Pierson, J. D., Forney, L. J., Danovaro, R., Luna, G. M., Dell'Anno, A., et al. (2007). Measuring Species Richness Based on Microbial Community Fingerprints: the Emperor Has No Clothes. *Appl. Environ. Microbiol.* 73, 2399–2401. doi:10.1128/AEM.02383-06.

Berry, D., Mahfoudh, K. Ben, Wagner, M., and Loy, A. (2011). Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. *Appl. Environ. Microbiol.* 77, 7846–7849. doi:10.1128/AEM.05220-11.

Beun, J. J., Heijnen, J. J., and van Loosdrecht, M. C. M. (2001). N-Removal in a granular sludge sequencing batch airlift reactor. *Biotechnol. Bioeng.* 75, 82–92. doi:10.1002/bit.1167.

Beun, J. J., Hendriks, A., van Loosdrecht, M. C. M., Morgenroth, E., Wilderer, P. A., and Heijnen, J. J. (1999). Aerobic granulation in a sequencing batch reactor. *Water Res.* 33, 2283–2290.

Bindhu, B. K., and Madhu, G. (2014). Selection pressure theory for aerobic granulation – an overview. *Int. J. Environ. Waste Manag.* 13, 317–329.

Briones, A., and Raskin, L. (2003). Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. *Curr. Opin. Biotechnol.* 14, 270–276. doi:10.1016/S0958-1669(03)00065-X.

de Bruin, L. M. M., de Kreuk, M. K., van der Roest, H. F. R., Uijterlinde, C., and van

Loosdrecht, M. C. M. (2004). Aerobic granular sludge technology: an alternative to activated sludge? *Water Sci. Technol.* 49, 1–7.

von Canstein, H., Kelly, S., Li, Y., and Wagner-Dobler, I. (2002). Species Diversity Improves the Efficiency of Mercury-Reducing Biofilms under Changing Environmental Conditions. *Appl. Environ. Microbiol.* 68, 2829–2837. doi:10.1128/AEM.68.6.2829-2837.2002.

Cassidy, D. P., and Belia, E. (2005). Nitrogen and phosphorus removal from an abattoir wastewater in a SBR with aerobic granular sludge. *Water Res.* 39, 4817–4823. doi:10.1016/j.watres.2005.09.025.

Chen, X., Yuan, L., Lu, W., Li, Y., Liu, P., and Nie, K. (2015). Cultivation of aerobic granular sludge in a conventional, continuous flow, completely mixed activated sludge system. *Front. Environ. Sci. Eng.* 9, 324–333. doi:10.1007/s11783-014-0627-3.

Chen, Y., Jiang, W., Liang, D. T., and Tay, J. H. (2007). Structure and stability of aerobic granules cultivated under different shear force in sequencing batch reactors. *Appl. Microbiol. Biotechnol.* 76, 1199–1208. doi:10.1007/s00253-007-1085-7.

Chen, Y., Jiang, W., Liang, D. T., and Tay, J. H. (2008). Aerobic granulation under the combined hydraulic and loading selection pressures. *Bioresour. Technol.* 99, 7444–7449. doi:10.1016/j.biortech.2008.02.028.

Curtis, T. P., and Sloan, W. T. (2006). Towards the design of diversity: Stochastic models for community assembly in wastewater treatment plants. *Water Sci. Technol.* 54, 227–236. doi:10.2166/wst.2006.391.

Cydzik-Kwiatkowska, A. (2015). Bacterial structure of aerobic granules is determined by aeration mode and nitrogen load in the reactor cycle. *Bioresour. Technol.* 181, 312–320. doi:10.1016/j.biortech.2015.01.101.

Derlon, N., Wagner, J., da Costa, R. H. R., and Morgenroth, E. (2016). Formation of aerobic granules for the treatment of real and low-strength municipal wastewater using a sequencing batch reactor operated at constant volume. *Water Res.* 105, 341–350. doi:10.1016/j.watres.2016.09.007.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi:10.1128/AEM.03006-05.

Ding, Z., Bourven, I., Guibaud, G., van Hullebusch, E. D., Panico, A., Pirozzi, F., et al. (2015). Role of extracellular polymeric substances (EPS) production in bioaggregation: application to wastewater treatment. *Appl. Microbiol. Biotechnol.* 99, 9883–9905. doi:10.1007/s00253-015-6964-8.

van Dorst, J., Bissett, A., Palmer, A. S., Brown, M., Snape, I., Stark, J. S., et al. (2014). Community fingerprinting in a sequencing world. *FEMS Microbiol. Ecol.* 89, 316–330. doi:10.1111/1574-6941.12308.

Duque, A. F., Bessa, V. S., and Castro, P. M. L. (2015). Characterization of the bacterial communities of aerobic granules in a 2-fluorophenol degrading process. *Biotechnol. Reports* 5, 98–104. doi:10.1016/j.btre.2014.12.007.

Ebrahimi, S., Gabus, S., Rohrbach-Brandt, E., Hosseini, M., Rossi, P., Maillard, J., et al. (2010). Performance and microbial community composition dynamics of aerobic granular sludge from sequencing batch bubble column reactors operated at 20 degrees C, 30 degrees C, and 35 degrees C. *Appl. Microbiol. Biotechnol.* 87, 1555–1568. doi:10.1007/s00253-010-2621-4.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–8. doi:10.1038/nmeth.2604.

Fredriksson, N. J. (2013). *Investigations of the microbial diversity and dynamics in activated sludge using molecular methods*. Göteborg: Department of Civil and Environmental Engineering, Water Environment Technology, Chalmers University of Technology.

Fredriksson, N. J., Hermansson, M., and Wilén, B. (2014). Tools for T-RFLP data analysis using Excel. *BMC Bioinformatics* 15, 1–18. doi:10.1186/s12859-014-0361-7.

Van Der Gast, C. J., Ager, D., and Lilley, A. K. (2008). Temporal scaling of bacterial taxa is influenced by both stochastic and deterministic ecological factors. *Environ. Microbiol.* 10, 1411–1418. doi:10.1111/j.1462-2920.2007.01550.x.

Giesen, A., de Bruin, L. M. M., Niermans, R. P., and van der Roest, H. F. (2013). Advancements in the application of aerobic granular biomass technology for sustainable treatment of wastewater. *Water Pract. Technol.* 8, 47–54. doi:10.2166/wpt.2013.007.

Gonzalez-Gil, G., and Holliger, C. (2011). Dynamics of microbial community structure of and enhanced biological phosphorus removal by aerobic granules cultivated on propionate or acetate. *Appl. Environ. Microbiol.* 77, 8041–51. doi:10.1128/AEM.05738-11.

Guo, F., Zhang, S. H., Yu, X., and Wei, B. (2011). Variations of both bacterial community and extracellular polymers: The inducements of increase of cell hydrophobicity from biofloc to aerobic granule sludge. *Bioresour. Technol.* 102, 6421–6428. doi:10.1016/j.biortech.2011.03.046.

Inoue, D., Suzuki, Y., Uchida, T., Morohoshi, J., and Sei, K. (2016). Polyhydroxyalkanoate production potential of heterotrophic bacteria in activated sludge. *J. Biosci. Bioeng.* 121, 47–51. doi:10.1016/j.jbiosc.2015.04.022.

Kennedy, K., Hall, M. W., Lynch, M. D. J., Moreno-Hagelsieb, G., and Neufeld, J. D. (2014). Evaluating bias of Illumina-based bacterial 16S rRNA gene profiles. *Appl. Environ. Microbiol.* 80, 5717–5722. doi:10.1128/AEM.01451-14.

Khan, M. Z., Mondal, P. K., and Sabir, S. (2013). Aerobic granulation for wastewater bioremediation: A review. *Can. J. Chem. Eng.* 91, 1045–1058. doi:10.1002/cjce.21729.

Kim, T.-S., Jeong, J.-Y., Wells, G. F., and Park, H.-D. (2013). General and rare bacterial taxa demonstrating different temporal dynamic patterns in an activated sludge bioreactor. *Appl. Microbiol. Biotechnol.* 97, 1755–1765. doi:10.1007/s00253-012-4002-7.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl. Environ. Microbiol.* 79, 5112–5120. doi:10.1128/AEM.01043-13.

de Kreuk, M. K., Heijnen, J. J., and van Loosdrecht, M. C. M. (2005). Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. *Biotechnol. Bioeng.* 90, 761–9. doi:10.1002/bit.20470.

de Kreuk, M. K., Kishida, N., Tsuneda, S., and van Loosdrecht, M. C. M. (2010). Behavior of polymeric substrates in an aerobic granular sludge system. *Water Res.* 44, 5929–5938. doi:10.1016/j.watres.2010.07.033.

de Kreuk, M. K., and van Loosdrecht, M. C. M. (2004). Selection of slow growing organisms as a means for improving aerobic granular sludge stability. *Water Sci. Technol.* 49, 9–17.

de Kreuk, M. K., and van Loosdrecht, M. C. M. (2006). Formation of Aerobic Granules with Domestic Sewage. *J. Environ. Eng.* 132, 694–697. doi:10.1061/(ASCE)0733-9372(2006)132:6(694).

Kunin, V., Engelbrektson, A., Ochman, H., and Hugenholtz, P. (2010). Wrinkles in the rare biosphere: Pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environ. Microbiol.* 12, 118–123. doi:10.1111/j.1462-2920.2009.02051.x.

Lane, D. J. (1991). “16S/23S rRNA sequencing,” in *Nucleic Acid Techniques in Bacterial Systematics*, eds. E. Stackebrandt and E. Goodfellow (Chichester: John Wiley & Sons Ltd), 115–175.

Larsen, P., Nielsen, J. L., Otzen, D., and Nielsen, P. H. (2008). Amyloid-like adhesins produced by floc-forming and filamentous bacteria in activated sludge. *Appl. Environ. Microbiol.* 74, 1517–1526. doi:10.1128/AEM.02274-07.

Lawson, C. E., Strachan, B. J., Hanson, N. W., Hahn, A. S., Hall, E. R., Rabinowitz, B., et al. (2015). Rare taxa have potential to make metabolic contributions in enhanced biological phosphorus removal ecosystems. *Environ. Microbiol.* 17, 4979–4993. doi:10.1111/1462-2920.12875.

Lemaire, R., Webb, R. I., and Yuan, Z. (2008). Micro-scale observations of the structure of aerobic microbial granules used for the treatment of nutrient-rich industrial wastewater. *ISME J.* 2, 528–541. doi:10.1038/ismej.2008.12.

Li, A., Yang, S., Li, X., and Gu, J. (2008). Microbial population dynamics during aerobic sludge granulation at different organic loading rates. *Water Res.* 42, 3552–3560. doi:10.1016/j.watres.2008.05.005.

Li, J., Ding, L.-B., Cai, A., Huang, G.-X., and Horn, H. (2014a). Aerobic Sludge Granulation in a Full-Scale Sequencing Batch Reactor. *Biomed Res. Int.* 2014, 1–12. doi:10.1155/2014/268789.

Li, Y.-M., Zou, J., Zhang, L., and Sun, J. (2014b). Aerobic granular sludge for simultaneous accumulation of mineral phosphorus and removal of nitrogen via nitrite in wastewater. *Bioresour. Technol.* 154, 178–184. doi:10.1016/j.biortech.2013.12.033.

Liu, Q. S., Tay, J. H., and Liu, Y. (2003). Substrate concentration-independent aerobic granulation in sequential aerobic sludge blanket reactor. *Environ. Technol.* 24, 1235–1242. doi:10.1080/09593330309385665.

Liu, Y.-Q., and Tay, J.-H. (2007). Cultivation of aerobic granules in a bubble column and an airlift reactor with divided draft tubes at low aeration rate. *Biochem. Eng. J.* 34, 1–7. doi:10.1016/j.bej.2006.11.009.

Liu, Y. Q., Kong, Y. H., Zhang, R., Zhang, X., Wong, F. S., Tay, J. H., et al. (2010). Microbial population dynamics of granular aerobic sequencing batch reactors during start-up and steady state periods. *Water Sci. Technol.* 62, 1281–1287. doi:10.2166/wst.2010.408.

Liu, Y. Q., Liu, Y., and Tay, J. H. (2004). The effects of extracellular polymeric substances on the formation and stability of biogranules. *Appl. Microbiol. Biotechnol.* 65, 143–148. doi:10.1007/s00253-004-1657-8.

Liu, Y. Q., and Tay, J. H. (2008). Influence of starvation time on formation and stability of aerobic granules in sequencing batch reactors. *Bioresour. Technol.* 99, 980–985. doi:10.1016/j.biortech.2007.03.011.

Liu, Y., and Tay, J.-H. (2002). The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge. *Water Res.* 36, 1653–1665.

Liu, Y., Wang, Z.-W., Qin, L., Liu, Y.-Q., and Tay, J.-H. (2005). Selection pressure-driven aerobic granulation in a sequencing batch reactor. *Appl. Microbiol. Biotechnol.* 67, 26–32. doi:10.1007/s00253-004-1820-2.

Lochmatter, S., and Holliger, C. (2014). Optimization of operation conditions for the startup of aerobic granular sludge reactors biologically removing carbon, nitrogen, and phosphorous. *Water Res.* 59, 58–70. doi:10.1016/j.watres.2014.04.011.

Lv, Y., Wan, C., Lee, D.-J., Liu, X., and Tay, J.-H. (2014). Microbial Communities of Aerobic Granules: Granulation Mechanisms. *Bioresour. Technol.* 169, 344–351. doi:10.1016/j.biortech.2014.07.005.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., et al. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6, 610–8. doi:10.1038/ismej.2011.139.

McIlroy, S. J., Saunders, A. M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A. A., et al.

(2015). MiDAS: the field guide to the microbes of activated sludge. *Database* 2015, bav062. doi:10.1093/database/bav062.

McMahon, K. D., Martin, H. G., and Hugenholtz, P. (2007). Integrating ecology into biotechnology. *Curr. Opin. Biotechnol.* 18, 287–292. doi:10.1016/j.copbio.2007.04.007.

McMurdie, P. J., and Holmes, S. (2014). Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. *PLoS Comput. Biol.* 10, e1003531. doi:10.1371/journal.pcbi.1003531.

McSwain, B. S., Irvine, R. L., and Wilderer, P. A. (2004). The influence of settling time on the formation of aerobic granules. *Water Sci. Technol.* 50, 195–202.

McSwain Sturm, B. S., and Irvine, R. L. (2008). Dissolved oxygen as a key parameter to aerobic granule formation. *Water Sci. Technol.* 58, 781–787. doi:10.2166/wst.2008.393.

Mishima, K., and Nakamura, M. (1991). Self-Immobilization of aerobic activated sludge - a pilot study of the aerobic upflow municipal sewage treatment. *Water Sci. Technol.* 23, 981–990.

Morales, N., Figueroa, M., Fra-Vázquez, A., Val del Río, Á., Campos, J. L., Mosquera-Corral, A., et al. (2013). Operation of an aerobic granular pilot scale SBR plant to treat swine slurry. *Process Biochem.* 48, 1216–1221. doi:10.1016/j.procbio.2013.06.004.

Morgenroth, E., Sherden, T., van Loosdrecht, M. C. M., Heijnen, J. J., and Wilderer, P. A. (1997). Aerobic granular sludge in a sequencing batch reactor. *Water Res.* 31, 3191–3194.

Moy, B. Y.-P., Tay, J.-H., Toh, S.-K., Liu, Y., and Tay, S. T. L. (2002). High organic loading influences the physical characteristics of aerobic sludge granules. *Lett. Appl. Microbiol.* 34, 407–12.

Muda, K., Aris, A., Salim, M. R., Ibrahim, Z., van Loosdrecht, M. C. M., Ahmad, A., et al. (2011). The effect of hydraulic retention time on granular sludge biomass in treating textile wastewater. *Water Res.* 45, 4711–4721. doi:10.1016/j.watres.2011.05.012.

Ni, B. J., Xie, W. M., Liu, S. G., Yu, H. Q., Wang, Y. Z., Wang, G., et al. (2009). Granulation of activated sludge in a pilot-scale sequencing batch reactor for the treatment of low-strength municipal wastewater. *Water Res.* 43, 751–761. doi:10.1016/j.watres.2008.11.009.

Nielsen, M., Bollmann, A., Sliekers, O., Jetten, M., Schmid, M., Strous, M., et al. (2005). Kinetics, diffusional limitation and microscale distribution of chemistry and organisms in a CANON reactor. *FEMS Microbiol. Ecol.* 51, 247–256. doi:10.1016/j.femsec.2004.09.003.

Ofiteru, I. D., Lunn, M., Curtis, T. P., Wells, G. F., Criddle, C. S., Francis, C. A., et al. (2010). Combined niche and neutral effects in a microbial wastewater treatment community. *Proc. Natl. Acad. Sci.* 107, 15345–15350. doi:10.1073/pnas.1000604107.

Pholchan, M. K., de Baptista, J. C., Davenport, R. J., Sloan, W. T., and Curtis, T. P. (2013). Microbial community assembly, theory and rare functions. *Front. Microbiol.* 4, 1–9. doi:10.3389/fmicb.2013.00068.

Pilloni, G., Granitsiotis, M. S., Engel, M., and Lueders, T. (2012). Testing the limits of 454 pyrotag sequencing: Reproducibility, quantitative assessment and comparison to T-RFLP fingerprinting of aquifer microbes. *PLoS One* 7. doi:10.1371/journal.pone.0040467.

Pinto, A. J., and Raskin, L. (2012). PCR biases distort bacterial and archaeal community structure in pyrosequencing datasets. *PLoS One* 7. doi:10.1371/journal.pone.0043093.

Power, M. E., Tilman, D., Estes, J. A., Menge, B. A., Bond, W. J., Mills, S., et al. (1996). Challenges in the quest for keystones. *Bioscience* 46, 609–620. doi:10.2307/1312990.

Pronk, M., de Kreuk, M. K., de Bruin, B., Kamminga, P., Kleerebezem, R., and van Loosdrecht, M. C. M. (2015). Full scale performance of the aerobic granular sludge process for sewage treatment. *Water Res.* 84, 207–217. doi:10.1016/j.watres.2015.07.011.

Qin, L., Liu, Y., and Tay, J. H. (2004a). Effect of settling time on aerobic granulation in sequencing batch reactor. *Biochem. Eng. J.* 21, 47–52. doi:10.1016/j.bej.2004.03.005.

Qin, L., Tay, J.-H., and Liu, Y. (2004b). Selection pressure is a driving force of aerobic granulation in sequencing batch reactors. *Process Biochem.* 39, 579–584. doi:10.1016/S0032-9592(03)00125-0.

R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available: http://www.R-project.org/.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi:10.1128/AEM.01541-09.

Shade, A., and Handelsman, J. (2012). Beyond the Venn diagram: the hunt for a core microbiome. *Environ. Microbiol.* 14, 4–12. doi:10.1111/j.1462-2920.2011.02585.x.

Show, K.-Y., Lee, D.-J., and Tay, J.-H. H. (2012). Aerobic granulation: advances and challenges. *Appl. Biochem. Biotechnol.* 167, 1622–40. doi:10.1007/s12010-012-9609-8.

Su, B., Cui, X., and Zhu, J. (2012). Optimal cultivation and characteristics of aerobic granules with typical domestic sewage in an alternating anaerobic/aerobic sequencing batch reactor. *Bioresour. Technol.* 110, 125–129. doi:10.1016/j.biortech.2012.01.127.

Su, K.-Z., Ni, B.-J., and Yu, H.-Q. (2013). Modeling and optimization of granulation process of activated sludge in sequencing batch reactors. *Biotechnol. Bioeng.* 110, 1312–1322. doi:10.1002/bit.24812.

Suja, E., Nancharaiah, Y. V., Krishna Mohan, T. V., and Venugopalan, V. P. (2015). Denitrification accelerates granular sludge formation in sequencing batch reactors.

Bioresour. Technol. 196, 28–34. doi:10.1016/j.biortech.2015.07.045.

Szabó, E., Hermansson, M., Modin, O., Persson, F., and Wilén, B.-M. (2016). Effects of Wash-Out Dynamics on Nitrifying Bacteria in Aerobic Granular Sludge During Start-Up at Gradually Decreased Settling Time. *Water* 8, 172. doi:10.3390/w8050172.

Tay, J.-H., Pan, S., He, Y., and Tay, S. T. L. (2004a). Effect of organic loading rate on aerobic granulation. II: Characteristics of aerobic granules. *J. Environ. Eng.* 130, 1102–1109. doi:10.1061/(ASCE)0733-9372(2004)130.

Tay, J. H., Liu, Q. S., and Liu, Y. (2001a). Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor. *J. Appl. Microbiol.* 91, 168–175.

Tay, J. H., Liu, Q. S., and Liu, Y. (2001b). The effects of shear force on the formation, structure and metabolism of aerobic granules. *Appl. Microbiol. Biotechnol.* 57, 227–233. doi:10.1007/s002530100766.

Tay, J. H., Liu, Q. S., and Liu, Y. (2004b). The effect of upflow air velocity on the structure of aerobic granules cultivated in a sequencing batch reactor. *Water Sci. Technol.* 49, 35–40.

Third, K. A., Burnett, N., and Cord-Ruwisch, R. (2003). Simultaneous nitrification and denitrification using stored substrate (PHB) as the electron donor in an SBR. *Biotechnol. Bioeng.* 83, 706–720. doi:10.1002/bit.10708.

Val del Río, Á., Figueroa, M., Arrojo, B., Mosquera-Corral, A., Campos, J. L., García-Torriello, G., et al. (2012a). Aerobic granular SBR systems applied to the treatment of industrial effluents. *J. Environ. Manage.* 95, S88–S92. doi:10.1016/j.jenvman.2011.03.019.

Val del Río, Á., Morales, N., Figueroa, M., Mosquera-Corral, A., Campos, J. L., and Méndez, R. (2012b). Effect of coagulant-flocculant reagents on aerobic granular biomass. *J. Chem. Technol. Biotechnol.* 87, 908–913. doi:10.1002/jctb.3698.

Vlaeminck, S. E., Terada, A., Smets, B. F., De Clippeleir, H., Schaubroeck, T., Bolea, S., et al. (2010). Aggregate size and architecture determine microbial activity balance for one-stage partial nitritation and anammox. *Appl. Environ. Microbiol.* 76, 900–909. doi:10.1128/AEM.02337-09.

Wagner, J., and da Costa, R. H. R. (2013). Aerobic Granulation in a Sequencing Batch Reactor Using Real Domestic Wastewater. *J. Environ. Eng.* 139, 1391–1396. doi:10.1061/(ASCE)EE.1943-7870.0000760.

Wan, J., Mozo, I., Filali, A., Liné, A., Bessière, Y., and Spérandio, M. (2011). Evolution of bioaggregate strength during aerobic granular sludge formation. *Biochem. Eng. J.* 58–59, 69–78. doi:10.1016/j.bej.2011.08.015.

Wan, J., and Sperandio, M. (2009). Possible role of denitrification on aerobic granular sludge formation in sequencing batch reactor. *Chemosphere* 75, 220–227. doi:10.1016/j.chemosphere.2008.11.069.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007a). Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi:10.1128/AEM.00062-07.

Wang, S.-G., Gai, L.-H., Zhao, L.-J., Fan, M.-H., Gong, W.-X., Gao, B.-Y., et al. (2009). Aerobic granules for low-strength wastewater treatment: formation, structure, and microbial community. *J. Chem. Technol. Biotechnol.* 84, 1015–1020. doi:10.1002/jctb.2127.

Wang, X. H., Zhang, H. M., Yang, F. L., Xia, L. P., and Gao, M.-M. (2007b). Improved stability and performance of aerobic granules under stepwise increased selection pressure. *Enzyme Microb. Technol.* 41, 205–211. doi:10.1016/j.enzmictec.2007.01.005.

Weber, S. D., Ludwig, W., Schleifer, K.-H., and Fried, J. (2007). Microbial composition and structure of aerobic granular sewage biofilms. *Appl. Environ. Microbiol.* 73, 6233–40. doi:10.1128/AEM.01002-07.

Weissbrodt, D. G., Lochmatter, S., Ebrahimi, S., Rossi, P., Maillard, J., and Holliger, C. (2012a). Bacterial Selection during the Formation of Early-Stage Aerobic Granules in Wastewater Treatment Systems Operated Under Wash-Out Dynamics. *Front. Microbiol.* 3, 332. doi:10.3389/fmicb.2012.00332.

Weissbrodt, D. G., Neu, T. R., Kuhlicke, U., Rappaz, Y., and Holliger, C. (2013). Assessment of bacterial and structural dynamics in aerobic granular biofilms. *Front. Microbiol.* 4, 175. doi:10.3389/fmicb.2013.00175.

Weissbrodt, D. G., Shani, N., Sinclair, L., Lefebvre, G., Rossi, P., Maillard, J., et al. (2012b). PyroTRF-ID: a novel bioinformatics methodology for the affiliation of terminal-restriction fragments using 16S rRNA gene pyrosequencing data. *BMC Microbiol.* 12, 306. doi:10.1186/1471-2180-12-306.

Wells, G. F., Park, H.-D., Eggleston, B., Francis, C. a, and Criddle, C. S. (2011). Fine-scale bacterial community dynamics and the taxa-time relationship within a full-scale activated sludge bioreactor. *Water Res.* 45, 5476–88. doi:10.1016/j.watres.2011.08.006.

Werner, J. J., Knights, D., Garcia, M. L., Scalfone, N. B., Smith, S., Yarasheski, K., et al. (2011). Bacterial community structures are unique and resilient in full-scale bioenergy systems. *Proc. Natl. Acad. Sci.* 108, 4158–4163. doi:10.1073/pnas.1015676108.

Winkler, M. K. H., Kleerebezem, R., de Bruin, L. M. M., Verheijen, P. J. T., Abbas, B., Habermacher, J., et al. (2013). Microbial diversity differences within aerobic granular sludge and activated sludge flocs. *Appl. Microbiol. Biotechnol.* 97, 7447–7458. doi:10.1007/s00253-012-4472-7.

Yang, C., Zhang, W., Liu, R., Li, Q., Li, B., Wang, S., et al. (2011). Phylogenetic Diversity and Metabolic Potential of Activated Sludge Microbial Communities in Full-Scale Wastewater Treatment Plants. *Environ. Sci. Technol.* 45, 7408–7415. doi:10.1021/es2010545.

Yang, S.-F., Tay, J. H., and Liu, Y. (2003). A novel granular sludge sequencing batch reactor for removal of organic and nitrogen from wastewater. *J. Biotechnol.* 106, 77–86. doi:10.1016/j.jbiotec.2003.07.007.

Zhang, Q., Hu, J., and Lee, D.-J. (2016). Aerobic granular processes: Current research trends. *Bioresour. Technol.* 210, 1–7. doi:10.1016/j.biortech.2016.01.098.

Zhou, D., Niu, S., Xiong, Y., Yang, Y., and Dong, S. (2014). Microbial selection pressure is not a prerequisite for granulation: Dynamic granulation and microbial community study in a complete mixing bioreactor. *Bioresour. Technol.* 161, 102–108. doi:10.1016/j.biortech.2014.03.001.

Zhu, L., Dai, X., Lv, M., and Xu, X. (2013). Correlation analysis of major control factors for the formation and stabilization of aerobic granule. *Environ. Sci. Pollut. Res. Int.* 20, 3165–3175. doi:10.1007/s11356-012-1202-8.