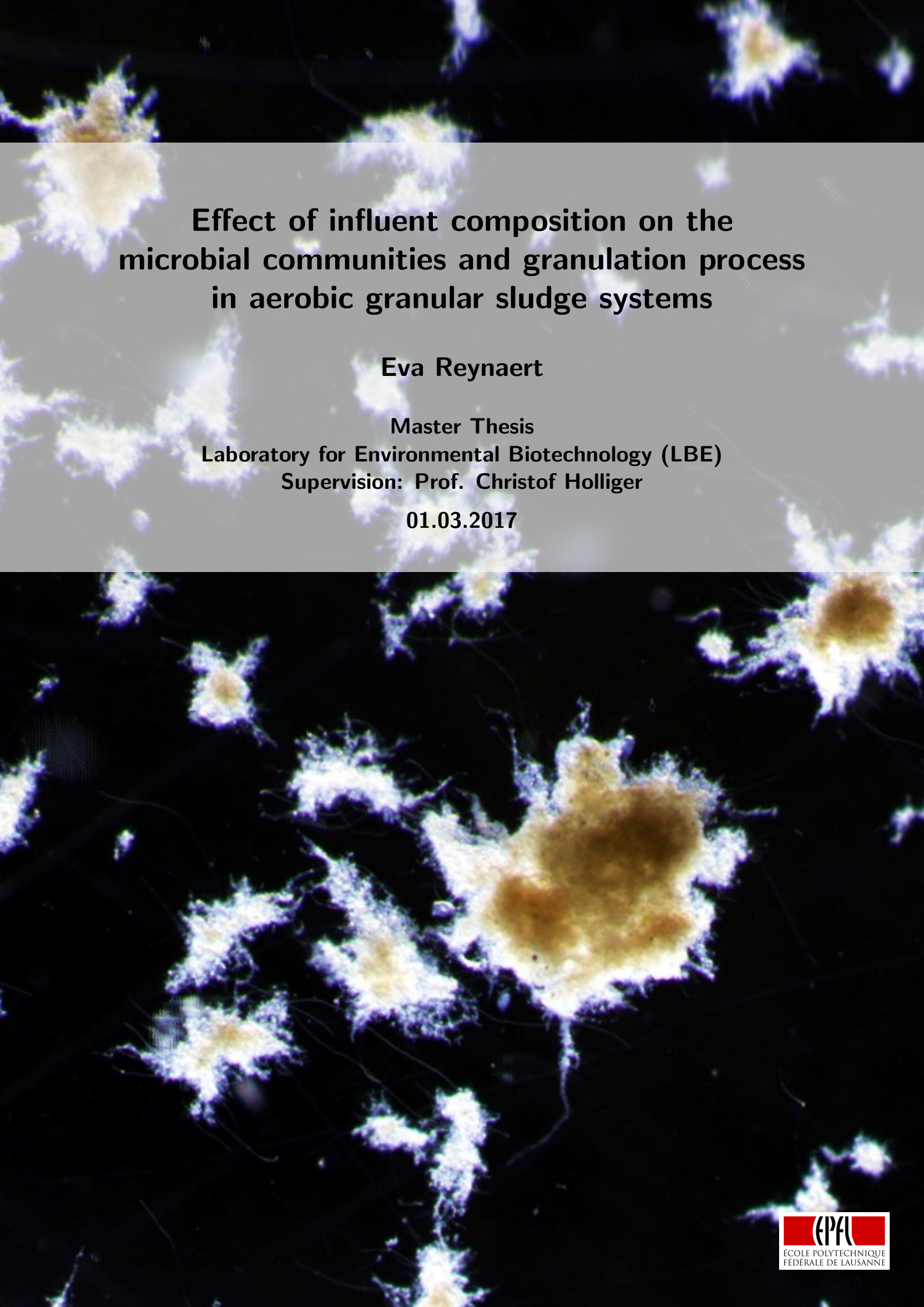

Effect of influent composition on the microbial communities and granulation process in aerobic granular sludge systems

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Abstract

Aerobic granular sludge (AGS) is a promising technology for biological wastewater treatment. However, most research so far has been obtained in laboratory-scale studies with simple synthetic wastewaters that do not represent the complexity of municipal wastewaters. The present study aimed at improving the understanding of the effect of influent composition on the microbial communities and granulation process during the start-up of AGS sequencing batch reactors (SBR). Four column AGS SBRs were started up in parallel with increasingly complex influent compositions and operated under feast and famine conditions. The four influents were (i) a mixture of volatile fatty acids (VFA) (acetate, propionate), (ii) a mixture of VFAs (acetate, propionate), fermentable substrate (glucose, amino acids) and particulate organic matter (starch, peptone), (iii) primary effluent municipal wastewater, and (iv) raw municipal wastewater.

Successful start-up of the AGS SBRs was defined in terms of settling properties (sludge volume index after 30 minutes $SVI_{30} < 70\,mL\,g^{-1}$ and $SVI_{30}/SVI_5 > 0.7$), fraction of granules (biomass with diameter above $0.25\,mm > 80\,\%$) and nutrient removal performances (requirements for the discharge of polluted wastewater from the Swiss Water Protection Ordinance WPO for phosphorus, ammonium and chemical oxygen demand).

Influent composition affected the microbial community composition by providing distinct ecological niches. Shannon's diversity index H' was significantly higher in the AGS systems fed by municipal wastewaters (2.99 ± 0.20) than in those fed by synthetic wastewaters (2.71 ± 0.14). Similarly, Pielou's evenness J was significantly higher in the AGS systems fed by municipal wastewaters (0.71 ± 0.05 compared to 0.66 ± 0.04 in the AGS systems fed by synthetic wastewaters). In contrast, diversity was not higher in the AGS system run with the synthetic wastewater containing fermentable and hydrolysable substrate compared to the AGS system run with VFAs only, in spite of the more complex wastewater composition. It was hypothesized that the more rapid growth of granules in the AGS SBR fed by VFAs only created new ecological niches within the granules, thus increasing the microbial diversity.

K-means clustering of the microbial communities showed that the communities in the AGS SBR fed exclusively by VFAs were clearly distinct from those fed by municipal wastewaters, while those in the AGS SBR fed by the synthetic wastewater containing fermentable and hydrolysable substrate presented characteristics from both groups. Due to high proportions of fermenting organisms (espe-

cially *Tetrasphaera* and *Micropruina*) in the latter, the microbial communities were more similar to the communities fed by municipal wastewaters. In contrast, the microbial communities fed by only VFAs were dominated by polyhydroxylalkanoate- and glycogen-storing organisms like *Zoogloea* and *Candidatus Contendobacter*.

In spite of these major differences in microbial composition, both AGS SBRs fed by synthetic wastewaters had excellent settling properties ($SVI_{30} < 50\,mL\,g^{-1}$), the fraction of granules was between 80 % and 100 % and nutrient removal performances in compliance with the WPO. The AGS SBRs fed by municipal wastewaters significantly lagged behind the former two. The SVI_{30} reached $75\,mL\,g^{-1}$ in the AGS SBR fed by primary effluent wastewater and $50\,mL\,g^{-1}$ in the one fed by raw wastewater. However, the fractions of granules stabilised below 80 % and both SBRs did not reach the required removal performances for phosphorus.

Overall, start-up time was significantly longer in the AGS SBRs fed by municipal wastewaters (>14 weeks, start-up not achieved at the end of the project) than in those fed by synthetic wastewaters (around 7 weeks). However, the physical AGS characteristics and nutrient removal performances in the AGS SBR fed by raw wastewater indicated a shift towards the properties of the AGS fed by synthetic wastewaters. Influent complexity was thus not per se an issue for growing AGS with good settling properties and nutrient removal performances but significantly slowed down granulation kinetics.

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AGS	Aerobic granular sludge
AND	Alternating nitrification-denitrification
AS	Activated sludge
AOO	Ammonium-oxidizing organism
BNR	Biological nutrient removal
COD	Chemical oxygen demand
DO	Dissolved oxygen
dPAO	Denitrifying polyphosphate-accumulating organism
GAO	Glycogen-accumulating organism
Eawag	Swiss Federal Institute of Aquatic Science and Technology
EBPR	Enhanced biological phosphorus removal
EPFL	Swiss Federal Institute of Technology in Lausanne
NOO	Nitrite-oxidizing organism
OHO	Ordinary heterotrophic organism
PAO	Polyphosphate-accumulating organism
PCA	Principal component analysis
PCR	Polymerase chain reaction

PHA	Poly-β-hydroxylalcanoate
PLC	Programmable logic controller
qFISH	Quantitative fluorescence in-situ hybridization
SBR	Sequencing batch reactor
SCADA	Supervisory control and data acquisition
sCOD	Soluble chemical oxygen demand
SND	Simultaneous nitrification and denitrification
SNSF	Swiss National Science Foundation
SRT	Solid retention time
SND	Simultaneous nitrification-denitrification
SVI	Sludge volume index
tCOD	Total chemical oxygen demand
TN	Total nitrogen
TP	Total phosphorus
TSS	Total suspended solids
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant
WPO	Water Protection Ordinance

1 Introduction

1.1 Context

The importance of drinking water supply and safely treated wastewater return cannot be overemphasized. Before the large-scale expansion of wastewater treatment plants (WWTP) in the second half of the 20th century, the pollution of freshwater systems by the discharge of untreated wastewater and the related effects on aquatic life were major concerns in Switzerland [32]. Main problems were the depletion of dissolved oxygen (DO) in rivers and lakes due the decomposition of organic matter and the oxidation of ammonium, and the eutrophication of freshwaters due to the over-fertilization by phosphorus and sometimes nitrogen. Today, more than 95 % of the population is connected to one of more than 800 WWTP in the country that remove the organic matter, oxidise the ammonium, and often also treat the phosphorus and nitrogen in the wastewater. The advancement of technology, however, has also led to increased requirements with respect to the quality and cost of the treatment. With the ageing of facilities, scarce land availability in dense urban settlements, high operating costs of small WWTP and increasingly stringent effluent standards, major restructuring is necessary [29]. It is an exciting time for the development of novel wastewater treatment technologies.

In most WWTP, the biological treatment is accomplished using conventional activated sludge (AS) systems, in which the organic load and the ammonium are oxidised by a consortium of bacteria and protozoa in aerated treatment tanks. If the removal of nitrogenous matter is required, the implementation of additional anoxic denitrification tanks is necessary. Similarly, the removal of phosphorus requires additional anaerobic treatment tanks or the use of chemical reactants. Often, large areas of land are necessary for the separation of the slow-settling flocculent biomass from the treated water.

The subject of current research, aerobic granular sludge (AGS) offers solutions for many of the challenges currently faced by conventional AS WWTP. Aerobic granules are compact spherical aggregates consisting mainly of bacteria, extracellular polymeric substances (EPS),

protozoa, and, sometimes, fungi [70] that settle significantly faster than conventional AS flocs [13]. The process allows stable biological nutrient removal at high conversion rates (Box 1) using less space (Box 2) and energy (Box 3) than conventional AS systems.

Box 1: Stable and simultaneous nutrient removal

Simultaneous removal of the chemical oxygen demand (COD), nitrogen (N) and phosphorus (P) in the wastewater is possible in a single reactor [16]. Thus the implementation of aerobic-anoxic-anaerobic treatment lines for N- and P-removal or the addition of chemical compounds for P-removal are not necessary. Additionally, granules are protected by a matrix of EPS, providing resilience to shock and toxins [70].

Box 2: Reduction of the land footprint

Through its compact structure and increased size, AGS offers excellent settling properties, reducing the need for a settler after the biological treatment of the wastewater. As the increased settleability also allows for higher biomass concentrations, higher substrate loads can be treated, further reducing the required land footprint. Hence, AGS plants can save up to 75 % of the land surface compared to conventional AS plants [11].

Box 3: Reduction of the energy consumption

Due to the lack of equipment necessary in conventional AS plants (mixers, recycle pumps, settlers and sludge return pumps), energy consumption is considerably lower. Part of the organic matter is removed anaerobically, thus reducing the oxygen demand in the aerobic part of the treatment. Energy savings of around 60 % have been reported for a full-scale AGS plant [58].

1.2 Research gap

Given its numerous advantages, AGS has been studied since its discovery in 1997 [49]. Several full-scale WWTP using AGS have already been successfully implemented [24]. But even though AGS is not a new technology, many processes are not yet fully understood. Moreover, most experiences with AGS so far have been performed in well-controlled laboratory set-ups, using different types of synthetic influents. Often, these influents were composed of readily biodegradable substrates like acetate or glucose [12, 14]. Other experiences focused on the treatment of high-strength industrial wastewaters, or sometimes mixtures of industrial and municipal wastewaters [24, 35].

Such studies provided a basic understanding of many factors influencing granulation. However, these influents do not reflect the complex composition of municipal wastewaters. The latter is generally characterized by its low strength and a high diversity of carbon sources.

The proportion of particulate matter usually accounts for 40-60% of the total organic matter after primary settling [67].

As a consequence, the knowledge gained from laboratory-scale studies performed with synthetic influents cannot be directly transposed to the case of municipal wastewaters. **In order to evaluate the feasibility of treating municipal wastewater using AGS, a better understanding of the effect of the influent composition on the process is necessary.**

1.3 Scope of the report and research questions

The present Master Thesis was conducted at the Swiss Federal Institute of Technology (EPFL) and the Swiss Federal Institute of Aquatic Science and Technology (Eawag) from 1.9.2016 to 1.3.2017. The study aimed at gaining a better understanding of the effect of influent composition on the microbial communities and granulation process in AGS systems. Three main research questions were addressed:

1. **What is the effect of influent composition on the microbial community structure and dynamics?** The first research question aimed at studying whether the influent composition affected the microbial composition in terms of presence and absence of organisms, their relative abundances, and their diversity.
2. **How are the microbial communities linked to the physical sludge characteristics and nutrient removal performances?** The second research questions aimed at understanding whether differences in physical sludge characteristics and nutrient performances could be explained by the differences in microbial community structure or whether different microbial communities achieved similar performances.
3. **What is the start-up time for AGS systems based on the physical sludge characteristics and nutrient removal performances?** The third research question aimed at estimating the effect of influent composition on the start-up time of AGS systems.

Due to the limited duration of the thesis compared to the start-up times of AGS systems, not all research questions could be answered conclusively. However, the experiment is ongoing and more extensive results are expected in the future.

2

Theoretical foundations

The present chapter provides the theoretical foundations this study is based on. Section 2.1 introduces the key processes and microorganisms involved in biological nutrient removal. Section 2.2 lists the the main considerations related to the start-up of AGS reactors.

2.1 Microorganisms involved in biological nutrient removal

The alternation between aerobic, anoxic and anaerobic conditions in AGS systems is the base for the biological removal of COD, nitrogen (N) and phosphorus (P) from the wastewater.

2.1.1 Chemical oxygen demand

Anaerobic degradation of complex organic matter is a multi-step process in which several hydrolysing and fermenting bacterial groups take part. When the substrate is in particulate form, hydrolysis is often the rate limiting process [64]. Important hydrolysers in AS systems are filamentous *Microthrix*, *Candidatus Epiflobacter* and numerous organisms belonging to the phylum *Chloroflexi*. The soluble products from hydrolysis or from the wastewater can be metabolised by fermenters or taken up for storage. Fermenters like *Streptococcus* or *Tetrasphaera* consume the soluble compounds and produce simpler products, predominantly in the form of volatile fatty acids (VFA). Various microorganisms like the polyphoshate-accumulating organism (PAO) *Candidatus Accumulibacter* or glycogen-accumulating organism (GAO) *Candidatus Competibacter* are able to store these VFA as polyhydroxyalcanoates (PHA) and glycogen [52]. Finally, a wide range of ordinary heterotrophic organisms (OHO) can catalyse the oxidation of organic matter into carbon dioxide (CO_2) under aerobic conditions.

2.1.2 Nitrogen

Nitrogen removal is the transformation of ammonium (NH_4^+) to nitrogen gas (N_2). It generally consists of two distinct processes: nitrification and denitrification (Figure 2.1).

Nitrification is the oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) by aerobic autotrophic organisms. First, ammonium-oxidizing organisms (AOO) catalyse the oxidation of ammonium into nitrite (NO_2). In a second step, nitrite-oxidizing organisms (NOO) catalyse the oxidation of nitrite into nitrate. *Nitrosospira* and *Nitrosomonas* were the most abundant AOO detected in WWTP designed for the biological removal of phosphorus, while *Nitrospira* was found to be the main NOO [52]. Denitrification is the reduction of nitrate into nitrogen gas by anoxic heterotrophic organisms [52]. Denitrifying organisms are phylogenetically diverse and belong to various physiological groups. A large range of microorganisms found in AS is capable of denitrification, many belonging to the class *Betaproteobacteria*: among others *Thauera*, *Azoarcus*, *Zoogloea*, *Curvibacter* and *Accumulibacter* [52].

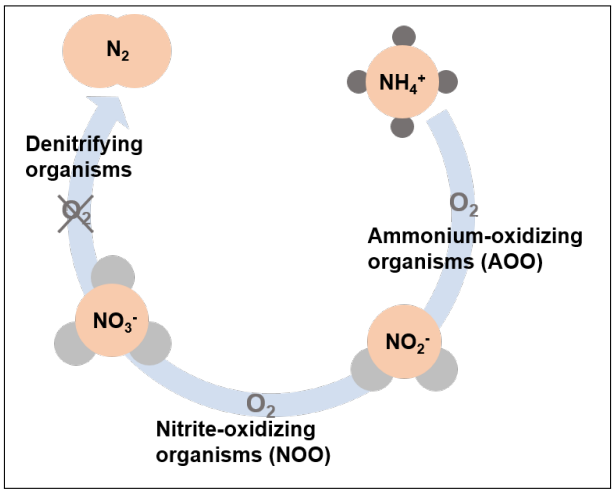


Figure 2.1: Three groups of microorganisms involved in biological nitrogen removal: ammonium-oxidizing organisms (AOO), nitrite-oxidizing organisms (NOO) and denitrifying organisms.

Simultaneous nitrification-denitrification (SND) has been observed in AGS. For larger granules, there is only limited diffusion of dissolved oxygen (DO) into the granules and different redox zones can exist within one granule. Nitrification can then take place in the outer aerobic layer, while the inner anoxic layers are favourable for denitrification [16].

2.1.3 Phosphorus

In enhanced biological phosphorus removal (EBPR), phosphorus from the wastewater is accumulated by heterotrophic polyphosphate-accumulating organisms (PAO) and removed from the water with the excess sludge.

Under aerobic conditions, the PAO *Accumulibacter* is able to constitute intracellular polyphosphate (PolyP) and glycogen energy stocks (Figure 2.2). Using this stored energy, it can take up volatile fatty acids (VFA) such as acetate or propionate and store them as poly-β-hydroxyalkanoates (PHA) during the anaerobic stage. When subject to alternating anaerobic feeding periods and aerobic starving periods, this gives *Accumulibacter* an advantage over OHO not able to consume the VFA anaerobically. The physiology of *Tetrasphaera*, the second important PAO detected in EBPR systems, is different to that displayed by *Accumulibacter*. *Tetrasphaera* can store a variety of substrates like glucose and amino acids as glycogen using energy from PolyP stocks, but is also able to carry out fermentation to supply energy for glycogen synthesis. *Tetrasphaera* and *Accumulibacter* hence do not share exactly the same ecological niches and are believed to co-exist in EBPR process, thus contributing to a more stable phosphorus-removal process [50]. Under anoxic conditions denitrifying PAOs (dPAO) can also use NO_3^- instead of oxygen as an electron acceptor.

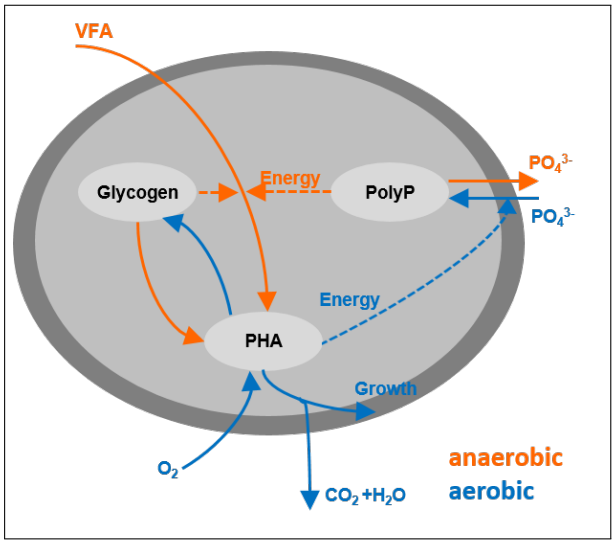


Figure 2.2: Metabolism of the polyphosphate-accumulating organism (PAO) *Accumulibacter*. PO_4^{3-} is taken up during the aerobic stage (blue) and released during the anaerobic stage (orange). Adapted from [44].

Glycogen-accumulating organisms (GAO) can compete with PAO for the uptake of VFA under anaerobic conditions. They have a similar metabolism to PAO, but store only glycogen. As they have no phosphorus-removing abilities, they are not desired in too high abundance in AGS systems. In EBPR systems, GAO microorganisms belonging to the classes *Gammaproteobacteria* (*Competibacter*) and *Alphaproteobacteria* (*Defluviicoccus*) were detected [52].

2.2 General considerations for the start-up of AGS systems

Understanding granule formation mechanisms is a key prerequisite for the start-up of AGS systems, as this allows for the selection of an adequate type of reactor and operation mode.

2.2.1 Granule formation

Generally, three main conditions for the formation of AGS are described in literature [17]:

1. The transformation of rapidly biodegradable substrate into stored substrate by applying feast and famine regimes (anaerobic COD removal)

2. The selection of fast-settling biomass by applying short settling times before removing the residual biomass

3. High shear stress during the aeration

During the start-up of AGS systems, however, the selection pressure on the slow-settling biomass (condition 2) should not be excessive in order to keep a high concentration of biomass in the system during the granulation process. Wash-out conditions have been shown to cause an overgrowth of filamentous microorganisms resulting in the deterioration of the settling properties and nutrient removal performances of the sludge [73]. The washout of small, flocculent biomass (called flocs) should be avoided for their presumed role in granule formation. Two main mechanisms of granulation have been hypothesized in Barr et al. and are illustrated in Figure 2.3 [5]:

1. Microcolony outgrowth: one bacterial type is selected for and grows outward

2. Microcolony aggregation: numerous small colonies (e.g. flocs) consisting of various types of microorganisms aggregate and grow

Both processes have been observed simultaneously in AGS, leading to the formation of two distinct types of granules. By applying less stringent settling times, granule formation by floc densification could be enhanced.

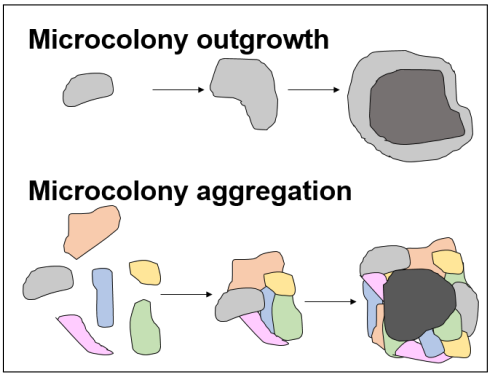


Figure 2.3: Two hypothesized mechanisms of granulation: microcolony outgrowth and microcolony aggregation. Adapted from [5].

These considerations are in accordance with results from Derlon et al. who found that settling properties of the AGS were best when applying a selective utilization of the organic carbon by the fast-settling biomass, in combination with a low selective pressure on the slow-settling biomass [18].

Finally, a recent study indicates that the importance of shear stress (condition 3) might be overrated, as successful granulation of activated sludge has also been observed under low hydrodynamic shear [19].

2.2.2 Sequencing batch reactors

A key prerequisite for BNR is the ability to create niches that favour the desired microorganisms over those without the required metabolic or physical characteristics. In continuous-flow AS plants, these niches are created by a series of aerobic, anoxic and anaerobic treatment tanks. In contrast, sequencing batch reactors (SBR) are a technology in which the wastewater is treated in a single tank. Contrarily to AS continuous-flow reactors, SBRs are filled and emptied discontinuously. The processes are thus separated in time, instead of relying on a spatial separation of the biological treatment and sedimentation. The SBR technology has been reported as favourable for biofilm and AGS processes [75]. SBR cycles designed for AGS usually include five main stages: fill - idle - react - settle - draw. Figure 2.4 presents a SBR cycle together with the main processes from BNR introduced above.

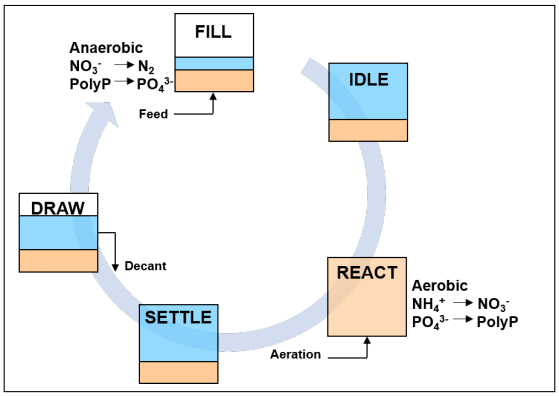


Figure 2.4: Five steps of the sequencing batch reactor (SBR) technology.

2.2.3 Optimized operation parameters

The main reactor operation parameters for the formation of AGS are related to the aeration, settling and feeding strategies.

Aeration strategy

The aeration strategy determines which process of nitrification-denitrification is predominant:

- Alternating aeration describes the alternation between high and low concentrations of dissolved oxygen (DO). These temporal variation of aerobic and anoxic phases results in alternating phases of nitrification-denitrification (AND).
- For larger granules, there is only limited DO diffusion into the granules. Aerobic and anoxic zones can then occur simultaneously within one granule, offering favourable conditions for simultaneous nitrification-denitrification (SND).

AND was shown to be more effective at improving N-removal efficiency than SND [43].

Settling strategy

The settling strategy determines how long the sludge is allowed to settle between the end of the aeration stage and the start of the drawing stage (Figure 2.4). The settling time defines the minimum settling velocity the biomass needs to have in order to remain in the system, as the slow-settling biomass is eliminated via the sludge withdrawal. This minimum settling

velocity is usually called critical settling velocity v_{crit}. As explained above, the settling strategy is a compromise between keeping sufficiently high biomass concentrations and selecting for the fast-settling biomass.

Feeding strategy

The feeding strategy comprises the moment of feeding (aerobic or anaerobic), the type of feeding (plug-flow or fully mixed) and the pollutant load.

Formation of AGS has been observed under both pulse-feeding during the aerobic stage and slow anaerobic feeding. However, AGS cultivated under pulse-feeding was not stable at low DO concentrations. Moreover, long feeding periods are economically more advantageous in full-scale applications [16].

Plug-flow feeding from the bottom creates a high substrate gradient, thus increasing the rate of diffusion of substrate into the granules. It presumably also favours granules over flocs, as the sludge bed is stratified and the largest granules are located at the bottom.

The COD should be low during the aerobic stage, as nitrifiers usually lose the competition for oxygen to OHO [48]. Moreover, the proliferation of filamentous OHO can be avoided by ensuring that the COD is used up anaerobically, thus favouring the enrichment of PAO and avoiding sludge bulking [72].

Lochmatter et al. proposed an optimised set of parameters for the start-up of AGS SBRs [42]. The start-up strategy was based on a stepwise decrease of the settling time in combination with a stepwise increase of the pollution load.

Main conditions for a rapid start-up of an AGS-SBR with good nutrient removal performances from Lochmatter et al. [42].

1. The alternation of high and low dissolved oxygen phases during aeration
2. A settling strategy avoiding too high biomass washout
3. The adaptation of the pollution load in the early stage of the start-up in order to ensure that all soluble COD was consumed before the aeration phase

3

Materials and methods

The present chapter lists the materials and methods used to study the effect of influent composition on the microbial communities and granulation process in AGS systems. Section 3.1 describes the experimental set-up. The detailed start-up strategy adopted for a rapid formation of granules is introduced in Section 3.2. Finally, Section 3.3 lists the analytical methods applied to investigate the microbial communities, physical sludge characteristics and nutrient removal performances.

3.1 Experimental set-up

3.1.1 Reactor set-up

Four identical column sequencing batch reactors (SBR) were run in parallel with four different influent compositions. As presented in Figure 3.1, the internal diameter of the columns was 0.125 m and the height to the effluent outlet 1.05 m for a total working volume of 13 L. A second outlet was placed at 0.8 m for the withdrawal of slow-settling sludge. The reactors were fed from the bottom with peristaltic pumps (Heidolph, Germany). The alimentation with air from the bottom was controlled by a mass flow controller.

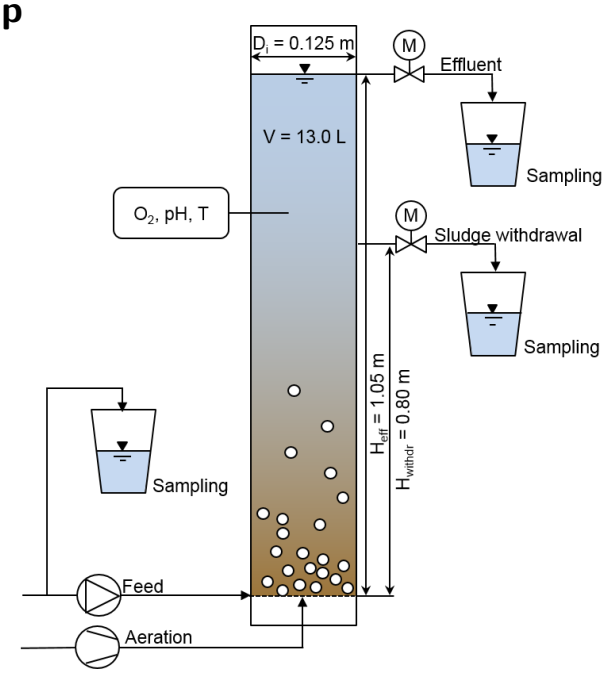


Figure 3.1: Schematic representation of the column sequencing batch reactors (SBR) used in the study.

3.1.2 Reactor control

Each reactor was equipped with an oxygen sensor (Endress & Hauser, Switzerland) as well as a temperature and pH sensor (Endress & Hauser, Switzerland). The two sensors were connected to a programmable logic controller (PLC), which was connected to a supervisory control and data acquisition (SCADA) system. The reactors were operated as SBRs, as detailed in Section 4.3 below. Temperature was not controlled.

3.1.3 Feed composition

The four reactors (called R1-R4) were run in parallel with increasingly complex influent compositions: R1 and R2 were fed by synthetic substrates and R3 and R4 by municipal wastewaters.

Synthetic substrates

R1 was fed by a substrate containing only VFAs (acetate, propionate) as carbon sources. It was referred to as the simple synthetic substrate. R2 was fed by a substrate containing a mixture of VFAs (acetate, propionate), non-fermented soluble substrate (glucose, amino acids) and particulate organic substrate (peptone, starch). It was referred to as the complex synthetic substrate.

The ratio of COD/N/P for the synthetic substrates was fixed at 100/7/1. The total COD was set to $600\,mg_{COD}\,L^{-1}$ in order to allow a comparison with experiments conducted by Aline Adler at EPFL.[1] The corresponding nitrogen in the influent was $42\,mg_{N}\,L^{-1}$ and was added either as ammonium chloride, or as a mixture of ammonium chloride and organic nitrogen contained in the amino acids and peptone. The influent phosphorus concentration was $6\,mg_{P}\,L^{-1}$ and consisted of equal parts of dipotassium hydrogen phosphate and potassium dihydrogen phosphate. The contributions of each carbon, nitrogen and phosphorus source for the two synthetic substrates are presented in Table 3.1.

[1] Aline Adler studies the evolution of microbial communities and nutrient removal performances in AGS SBRs during changes of substrate. When progressively passing from a simple influent composition with acetate and propionate to a more complex substrate composed of VFAs, non-fermented soluble substrate and particulate organic substrate, one of the intermediary steps should be similar to the complex substrate used in this study.

Table 3.1: Composition of the two synthetic substrates used in the study. The carbon and nitrogen sources and the additional compounds were concentrated 20 fold. The phoshorus sources were added directly to the dilution water tank.

Compound	Molecular formula	R1: simple synth. substrate	R2: complex synth. substrate
Carbon source ($mg_{COD}\,L^{-1}$)			
Sodium acetate	$C_2H_3O_2Na{\cdot}3\,H_2O$	300	100
Sodium propionate	$C_3H_5O_2Na$	300	100
Glucose	$C_6H_{12}O_6$	-	100
Amino acids	mix[a]	-	100
Peptone	n.a.	-	100
Starch	n.a.	-	100
Nitrogen source ($mg_N\,L^{-1}$)			
Ammonium chloride	NH_4Cl	42	14.5[b]
Phosphorus source ($mg_P\,L^{-1}$)			
Dipotassium hydrogen phosphate	K_2HPO_4	3	3
Potassium dihydrogen phosphate	KH_2PO_4	3	3
Additional compounds ($mg\,L^{-1}$)			
Magnesium sulfate	$MgSO_4{\cdot}7\,H_2O$	0.8	0.8
Potassium chloride	KCl	1.6	1.6
Calcium chloride	$CaCl_2{\cdot}2\,H_2O$	0.8	0.8
Trace element solution ($mL\,L^{-1}$)			
Trace element solution[c]		0.5	0.5

[a] The amino acids mix for the complex synthetic substrate was composed of equal amounts of COD of the following substances: L-alanine ($C_3H_7NO_2$), L-arginine ($C_6H_{14}N_4O_2$), L-aspartic acid ($C_4H_7NO_4$), L-glutamic acid ($C_5H_9NO_4$), glycine ($C_2H_5NO_2$), L-leucine ($C_6H_{13}NO_2$) and L-proline ($C_5H_9NO_2$).
[b] The remaining $27.5\,mg_N\,L^{-1}$ are already accounted for with the amino acids mix and the peptone
[c] The composition of the trace element solution can be found in Appendix A.

Table 3.2: Average composition of the two synthetic substrates (SS) used in the study (after an initial stage of pump adjustments and change in the experimental set-up). sCOD: soluble COD. TN: total nitrogen. TP: total phosphorus.

Parameter	Unit	R1: simple SS	R2: complex SS
sCOD	$mg_{COD}\,L^{-1}$	604 ± 10	513 ± 22
TN	$mg_N\,L^{-1}$	44 ± 1	38 ± 2
TP	$mg_P\,L^{-1}$	6.0 ± 0.2	5.7 ± 0.2

To avoid biofilm growth in the storage bottles and connection tubes, the concentrated media containing the carbon and nitrogen sources were kept refrigerated. The phosphorus source was added directly to the dilution water tank. The dilution water additionally contained $200\,mg\,L^{-1}$ of sodium hydrogen carbonate to buffer the pH. The concentrated media and the dilution water were mixed together in intermediary storage tanks before being pumped into the reactors. However, an important loss of particulate organic matter in the tubes leading from the refrigerator to the storage tank was observed for the complex synthetic substrate. As a consequence, the medium was stored as close to the storage tank as possible instead of being refrigerated. This change in set-up significantly

decreased the loss of substrate, however, the objective concentration of 600 $mg_{COD}\,L^{-1}$ could not be reached (Table 3.2).

Municipal wastewater

R3 and R4 were fed by real low-strength municipal wastewater (raw wastewater and primary effluent wastewater) originating from the combined sewer system of the city of Dübendorf (ZH, Switzerland). The average compositions of the two wastewaters are presented in Table 3.3. The primary effluent wastewater was pumped directly into the reactor R3. The raw wastewater was continuously pumped into a stirred storage tank from where it was pumped into the reactor R4. This set-up guaranteed a sufficient water flow in the pipe, thus minimising the deposition of solids and subsequent clogging.

Table 3.3: Average composition of the two municipal wastewaters (WW) used in the study. tCOD and sCOD: total and soluble COD. TN: total nitrogen. TP: total phosphorus.

Parameter	Unit	R3: primary effluent WW	R4: raw WW
tCOD	$mg_{COD}\,L^{-1}$	372 ± 110	808 ± 427
sCOD	$mg_{COD}\,L^{-1}$	208 ± 75	271 ± 108
Filtered TN	$mg_{N}\,L^{-1}$	28 ± 5	30 ± 7
Filtered TP	$mg_{P}\,L^{-1}$	2.8 ± 0.5	3.2 ± 0.5
Total susp. solids	$mg_{TSS}\,L^{-1}$	41 ± 24	176 ± 140

3.2 Reactor start-up strategy

3.2.1 Inoculation sludge

The reactors were inoculated with activated sludge taken from an aeration tank of WWTP Thunersee (Uetendorf, Switzerland). Besides of COD- and N-removal, the wastewater treatment plant is also operated for EBPR along an anaerobic-anoxic-aerobic treatment line. Hence, all required organisms for AGS systems with simultaneous COD-, N- and P-removal were present.

The average concentrations of COD, TN and TP in the influent wastewater at WWTP Thunersee were respectively 370 $mg_{COD}\,L^{-1}$, 37 $mg_{N}\,L^{-1}$ and 6 $mg_{P}\,L^{-1}$ during the period 2013-2015. With these influent concentrations, the WWTP achieved removal rates of 94.4 % for the COD, 78.9 % for nitrogen and 94.3 % for phosphorus [3].

The inoculum had a total suspended solids concentration (TSS) of 2.14 $g_{TSS}\,L^{-1}$, with a ratio of volatile suspended solids (VSS) to TSS of 0.69. The sludge volume index after 30 minutes (SVI_{30}) was 103 $mL\,g^{-1}$. Spontaneous granulation occurs at WWTP Thunersee and the inoculum contained already around 13 % of biomass with a diameter above 0.25 mm.

3.2.2 Reactor operation

The DO concentrations were fixed at 2 $mg\,L^{-1}$ with a two-point controller. With the exception of intermittent aeration, the recommendations for an optimised start-up presented in

Section 2.2 were adopted. This included in particular:

1. A stepwise increase of the critical settling velocity v_{crit} from 1 m h^{-1} to 6 m h^{-1} (goal)
2. A stepwise increase of the VER from 0.3 to 0.5 (goal)

The operation strategy was adaptive, implying that the operation parameters were changed as a function of reactor behaviour. Two conditions limited the increase of the critical settling velocity and the VER, respectively:

1. Selection of slow-growing organisms: the increase in critical settling velocity should not decrease the sludge retention time (SRT) below 20 days.
2. Selection of organisms that can take up COD anaerobically: the increase of the VER should not yield an increase of the soluble COD (sCOD) available at the beginning of the aerobic stage.

Table 3.4 below summarizes how the critical settling velocity and the VER were linked to the reactor settings.

Table 3.4: Link between critical settling velocity (v_{crit}) and volume exchange ratio (VER) and reactor settings.

	Reactor settings	**Reactor dimensions**	**Relationship**
Critical settling velocity v_{crit}	Settling time t_{sett}	Height to eff. outlet H_{eff} Height to sludge withdrawal H_{withdr}	$v_{crit} = \frac{H_{eff} - H_{withdr}}{t_{sett}}$
Volume exchange ratio VER	Feeding velocity v_{feed} Feeding duration t_{feed}	Reactor cross section A_r	$VER = v_{feed} \cdot t_{feed} \cdot A_r$

The reactors were operated as SBRs as schematically depicted in Figure 3.2. The cycles consisted of six distinct steps with a total duration of six hours. The stratification step comprised three pulses of aerobic mixing and subsequent settling.

3.2.3 Success criteria

A successful start-up of an AGS SBR meets two conditions: the biomass should be present in the form of granules and nutrient removal performance should be high. The selected success criteria are listed in Table 3.5 and explained in the following paragraphs.

Successful start-up of an AGS-SBR

- Fast-settling biomass present in granular form
- Good nutrient removal performances

The SVI was used to evaluate the settling properties of the sludge. De Kreuk et al. suggest to use the ratio between the SVI after different time intervals to assess granule formation

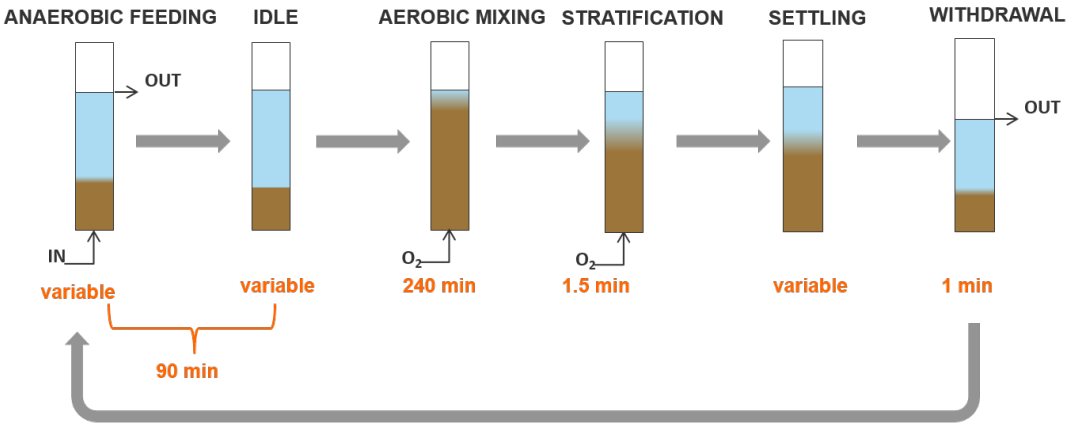


Figure 3.2: Schematic representation of the sequencing batch operation mode. The SBR cycles consisted of 6 stages with a total duration of around 6 hours. The VER (function of feeding velocity and feeding time) and the critical settling velocity (function of settling time) were adjusted in function of reactor behaviour.

[13]. Studies about aerobic granulation using municipal wastewater report ratios between the SVI taken after 5 minutes and after 30 minutes of settling ($SVI_{30/5}$) between 0.66 and 1 [24, 40, 51].

The minimum size of granules is generally set between 0.2 mm to 0.25 mm. In the present study, a threshold value of 0.25 mm was used to distinguish between granules and flocs in accordance with Derlon et al. [18]. Studies on AGS fed by municipal wastewaters report fractions of granules between 80 and 90% [24, 40, 51].

If the AGS technology is to be implemented to treat municipal wastewater in Switzerland, the requirements stated in the Water Protection Ordinance (WPO) need to be met. The requirements for the discharge of polluted wastewater from annex 3.1 (version 02.02.2016, wastewater from plants of more than 10'000 people-equivalent) were used as a base for the assessment of reactor performances in terms of COD-, N- and P-removal.

The start-up was considered successful once all criteria were reached and the start-up time was calculated as the number of weeks to meet all criteria.

3.3 Analytical methods

The following sections introduce the analytical methods used to determine the microbial communities (Subsection 3.3.1), sludge characteristics (Subsection 3.3.2) nutrient removal performances (Subsection 3.3.3) and the reactor monitoring program (Subsection 3.3.4).

Table 3.5: Criteria defining a successful start-up of the AGS-SBR in terms of settling properties, fraction of granules and removal performances.

Settling properties		
SVI_{30}	$< 70\,mL\,g^{-1}$	
$SVI_{30/5}$	> 0.7	
Fraction of granules		
Fraction of biomass $> 0.25\,mm$	$>80\,\%$	
Removal performances[a]	Removal efficiency	Discharge concentration
Total chemical oxygen demand (COD)	$>85\,\%$	$<45\,mg_{COD}\,L^{-1}$
Ammonium (NH_4^+)	$>90\,\%$	$<2\,mg_N\,L^{-1}$
Nitrite (NO_2)		$<0.3\,mg_N\,L^{-1}$
Total phosphorus (TP)[b]	$>80\,\%$	$<0.8\,mg_P\,L^{-1}$

[a] Requirements as stated in annex 3.1 of the Water Protection Ordinance (02.02.2016) for plants treating the wastewater of more than 10'000 people-equivalents. The ordinance includes requirements related to parameters that were not measured in this study (dissolved organic carbon, transparency, adsorbable organic halogen, organic trace substances and biochemical oxygen demand). The requirement on total suspended solids (TSS) was not taken into account, as most TSS in the effluent were due to biomass growth in the tubes (scaling effect).
[b] Additional requirement for plants discharging into sensitive waters.

3.3.1 Microbial communities

Amplicon sequencing is a high-throughput molecular method that determines the succession of nucleotides of amplicons (pieces of DNA that are the product of amplification). The phylogenetic affiliation of the amplicons sequences was determined by comparing the sequences to a database. The different steps involved are introduced in Figure 3.3 and described in the subsequent paragraphs. Gray boxes give additional information supporting the selection of the methods.

Biomass sampling and homogenization

Representativeness of AGS samples

Even within one reactor, AGS presents different compositions on two levels:

1. Intragranular: The granular composition changes along substrate and microhabitat gradients, generating a layered structure. Heterotrophic organisms dominate the outer layers while PAO are usually found inside the granules [16].
2. Intergranular: Different mechanisms of granulation lead to the co-occurrence of microbially distinct granules in the same reactor [5].

In order to obtain an average composition of the AGS, it is thus essential to homogenize a sufficient amount of well-mixed biomass from the reactor.

Sludge samples were taken in the beginning of the aerobic period. Separate 1 mL-samples of granules and flocs were obtained by sieving and washed two times in 5 mL phosphate-buffered

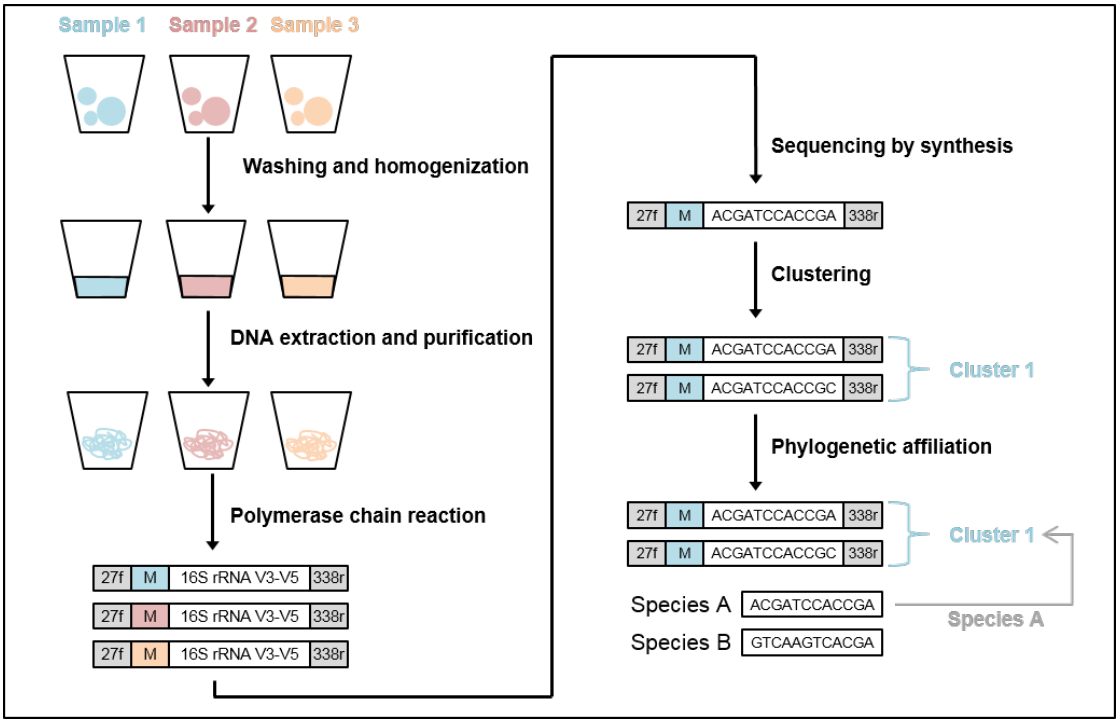


Figure 3.3: Six steps from sludge samples to microbial composition: washing and homogenization, DNA extraction, polymerase chain reaction, sequencing by synthesis, clustering and phylogenetic affiliation.

saline (PBS) solution.[2] The biomass samples were re-suspended in 3 mL PBS solution and homogenized with a glass tissue grinder.

DNA extraction and purification

DNA from 200 µL of biomass was extracted with the automated Maxwell 16 Tissue DNA Purification System (Promega, Dübendorf, Switzerland) according to manufacturer's instructions. The concentration of the extracted DNA was measured with the spectrometer NanoDrop ND1000 (Witec, Switzerland).

Polymerase chain reaction (PCR) amplification

Phylogenetic affiliation based on the 16S rRNA gene

The 16S ribosomal RNA (16S rRNA) gene is frequently used for the identification of bacteria, as it contains highly conserved domains (that can be used as primer binding sites) interspersed with very variable regions that can provide taxon-specific signature

[2] This is less than the 3-4 mL suggested by experiences performed at the LBE [C. Holliger, personal communication]. As only small granules were present during the start-up, the samples still contained a sufficient number of granules. The amount of sampled sludge should, however, be increased in the future.

sequences [55].

The 16S rRNA genes were amplified using polymerase chain reaction (PCR). A PCR mix consisting of of 5 µL DNA ($5\,\mathrm{ng}\,\mu\mathrm{L}^{-1}$), 2.5 µL primers (10 µM), 25 µL polymerase and 15 µL nuclease-free H_2O was used. All PCRs were carried out with the forward primer 27f, barcoded reverse primer 338r and Q5 High-fidelity 2X Master Mix polymerase (New England Biolabs). The PCRs were run by the T3000 Thermocycler (Biometra, Germany) with the following program: (i) the samples were heated to 98 °C for $2\,\mathrm{min}$, (ii) denaturation occured at 98 °C (45 s), (iii) hybridization followed at 50 °C (45 s), and (iv) finally elongation at 72 °C (60 s). The last three steps were repeated 30 times. After a resting phase at 72 °C ($5\,\mathrm{min}$), the samples were cooled down to 4 °C.

Sequencing by synthesis

Determine the succession of nucleotides using sequencing by synthesis

In sequencing by synthesis (SBS), primers attach to the forward strands of the amplicons. Then, fluorescently-labelled dNTP are added base by base. Every nucleotide has a reversible terminator to prevent multiple additions in one round. After each round, the sequencing instrument records which base was added [33].

The amplicon sequences were determined using the NextSeq500 (Illumina, San Diego, USA). The analysis was carried out by the Laboratory of Intestinal Immunology at EPFL.

Clustering

After merging the pairs of sequences, the sequences were demultiplexed and filtered to ensure good quality (minimum and maximum length of sequences, maximum number of ambiguities and homopolymers). The amplicon sequences were clustered based on a similarity of $97\,\%$ using the program Cd-hit [74]. Clusters that had less than 5 individuals in average over all samples were removed from the analysis.

Phylogenetic affiliation

The cluster heads were aligned with the reference database MiDAS [47] and the phylogenetic affiliation was assigned using the program BLASTN based on $95\,\%$ similarity between the cluster head and the database [9].

Amplicon sequencing shares problems inherent to any PCR-based method. The results are only indicative of relative abundances for four main reasons:

1. DNA extraction procedures significantly bias the view of microbial composition. Insufficient or preferential lysis of cells during DNA extraction imply that some species will not contribute to the final analysis of diversity [78].

2. PCR reactions are affected by several parameters, for instance the primer choice, GC content of the targeted region, the PCR cycles and DNA concentrations used, as well as the relative abundances of the target sequences [57].

3. The number of 16S rRNA gene copies in a bacterial cell can vary between 1 and 15, and the number of amplicons is thus only indicative of the number of cells [71].

4. Contaminations and errors are also amplified during the PCR amplification [22].

Many studies believe that the quantitative differences between different methods are mostly due to the DNA extraction bias. For instance, the proportion of *Actinobacteria* (including the PAO *Tetrasphaera*) detected by amplicon sequencing in EBPR systems was only 3 %, when quantitative fluorescence in-situ hybridization (qFISH) revealed a proportion of 30 %. The underrepresentation of *Actinobacteria* can presumably be attributed to a bias in the DNA extraction, as lysing gram positive bacteria (to which *Actinobacteria* belong) is more difficult compared with gram negative bacteria [1].

Overall, results from amplicon sequencing must be interpreted with caution, as they are only semi-quantitative. The temporal evolution of the detected microbial communities should, however, remain unaffected by the method used.

3.3.2 Sludge characteristics

Sludge morphology

The morphology of the granules and flocs was observed using a stereomicroscope (Olympus SZX10, Japan). Images of the different granule size fractions were taken sporadically. The analysis of the images was done qualitatively.[3]

Total suspended solids (TSS) and volatile suspended solids (VSS)

VSS are only indicators of active biomass

Volatile suspended solids (VSS) are commonly used as a rough estimation of the total biomass. This procedure does, however, not allow the distinction between living biomass and dead organic material. When monitoring the VSS over time, this can induce a distortion of the results, as the proportion of active biomass can vary. For instance, the viable fraction of VSS generally decreases with increasing SRT [8].

Total suspended solids (TSS) and volatile suspended solids (VSS) were analysed according to Standard Methods [2], with the modification that the filters were not pre-washed. TSS

[3] Image analysis could provide valuable information on granule morphology, for example through the computation of shape factors. However, granule morphology was extremely variable in the reactors fed by municipal wastewater. Ensuring representative results would have required a considerable effort that would have exceeded the scope of this report.

measures the weight of the residue after filtering a defined volume of sample at 45 µm and drying at 105 °C (Equation 3.1). VSS measures the volatile part of the TSS after igniting the sample at 550 °C (Equation 3.2). VSS were used as an estimation of the biomass.

$$TSS = \frac{A - B}{V} \tag{3.1}$$

$$VSS = \frac{A - B + C - D}{V} \tag{3.2}$$

where:

A: weight of filter and residue after drying at 105 °C during 2 h

B: weight of filter after drying at 105 °C during 5 min

C: weight of dish after drying at 105 °C during 5 min

D: weight of residue and dish after ignition at 550 °C during 2 h

V: volume of sample

The residual ash of the filters (MN 640 dd , Macherey-Nagel, Germany) used for the analysis could, however, represent up to 1 $\%$ of the filter weight and contribute to the mineral suspended solids. For low TSS contents, the VSS were thus underestimated.

Sludge volume index (SVI)

Assessing the extent of granulation based on the SVI

Flocculent sludge usually shows a continuous change of SVI over the conventional measuring-duration of 30 minutes. In contrast, dense granular sludge reaches its maximum compactness within the first five minutes of settling. The granulation process can thus be described in terms of change in SVI over the settling time [62].

The SVIs were determined according to Standard Methods [2]. The biomass volume of a 1 L sample was measured after settling periods of 5, 10 and 30 min and normalized by the TSS (Equation 3.3):

$$SVI_t = \frac{V_t}{TSS}, \tag{3.3}$$

where V_t is the volume occupied by the sludge after time t. The ratio of the SVIs taken after 5 and 30 minutes were used to assess granule formation.

Particle size distribution

Distinguishing between granules and flocs

Literature diverges about the minimum size of granules, the threshold is sometimes set at 0.2 mm, e.g. [13], or at 0.25 mm, e.g. [18].

Particle size distribution was determined according to the method described in [6]. The sludge samples were sieved at 1 mm, 0.63 mm and 0.25 mm and the corresponding TSS of each size fraction measured as explained above. Biomass with a diameter smaller than 0.25 mm was considered as flocs. In order to avoid the formation of a cake layer, the sludge samples were diluted in five parts of tap water before sieving.

Solid retention time (SRT)

Calculating a meaningful SRT for AGS systems is challenging

To maintain slow-growing organisms in the system, the sludge retention time (SRT) should be above the growth rate of the organisms. However, the concept of SRT is not truly applicable in AGS systems for two reasons:

- There can be important variations in SRT based on punctual measurements, which do not reflect what happens in the system. Especially for low TSS and VSS values in the effluent and sludge withdrawal (and hence large SRT values), changes in SRT due to minor measurement uncertainties can be significant. Calculating a dynamic SRT that is influenced by previously measured SRTs could contribute to solving this issue. However, it might be more difficult to use the SRT as a control mechanism in this case.
- The SRT assumes a uniform distribution of the microbial communities. This is a problem in AGS systems, where the microbial composition of flocs and granules differs. When calculating a mean SRT for the entire system, the retention time of organisms present in the flocs is overestimated, while the retention time of organisms present in the granules is underestimated. This issue can be partially solved by calculating a SRT per size fraction.

The mean SRT was calculated as suggested by [28] and did not take the influent biomass into account:

$$SRT_{TSS} = \frac{TSS \cdot V_r}{TSS_{eff} \cdot Q_{eff} + TSS_{withdr} \cdot Q_{withdr}} \tag{3.4}$$

where:

TSS: TSS concentration in the reactor

V_r: reactor volume

TSS_{eff}: TSS concentration in the effluent

Q_{eff}: effluent flow rate

TSS_{withdr}: TSS concentration in the withdrawal sludge

Q_{withdr}: flow rate of sludge withdrawn

The main focus of this study laid on the organic fraction. The SRT was therefore also calculated similarly based on the VSS, as this approach seemed more adequate from a process point of view. The VSS in the effluent and sludge withdrawal (low TSS values) was, however, often underestimated (as explained above) and this second approach resulted in an overestimation of the SRT.

3.3.3 Reactor performance

COD, total nitrogen (TN) and total phosphorus (TP) were determined spectrophotometrically by using standard test kits (Hach, Germany). NO_3^-, NO_2, NH_4^+ and PO_4^{3-} were determined by ion chromatography (881 Compact IC pro, Metrohm, Switzerland). For R3 and R4 (reactors fed by municipal wastewater), the influent and effluent were collected over three cycles (approximately 18 h) to obtain averaged concentrations.

The removal efficiencies r were calculated as defined in the WPO:[4]

$$r_{COD} = \frac{COD_{tot,in} - COD_{tot,out}}{COD_{tot,in}} \tag{3.5}$$

$$r_{TN} = \frac{TN_{filt,in} - TN_{filt,out}}{TN_{filt,in}} \tag{3.6}$$

$$r_{TP} = \frac{TP_{filt,in} - TP_{filt,out}}{TP_{filt,in}} \tag{3.7}$$

$$r_{\mathrm{NH_4}^+} = \frac{\mathrm{NH_4}^+_{filt,in} - \mathrm{NH_4}^+_{filt,out}}{\mathrm{NH_4}^+_{filt,in}} \tag{3.8}$$

Finally, SND corresponds to the ratio between nitrate denitrified and ammonium nitrified during the aerobic stage:

$$SND = \frac{\mathrm{NO_3}^-_{denitrified}}{\mathrm{NH_4}^+_{nitrified}}. \tag{3.9}$$

[4] These calculations are not entirely correct. To be truly comparable with the WPO requirements, r_{TP} and r_{TN} should be based on measurements of unfiltered samples. While this error has no effect on the results for R1 and R2 (synthetic influents), the removal efficiencies for R3 and R4 (municipal wastewater) are underestimated, as the error on the influent concentrations is higher (higher TSS content) than on the effluent (lower TSS content).

3.3.4 Reactor monitoring program

As described in Section 3.1, DO, pH and temperature were continuously monitored and saved through a SCADA system. The monitoring program for the other measurements is presented in Table 3.6 below.

Table 3.6: Reactor monitoring program: parameter, type of measure, sampling location and frequency.

Parameter	Measure/sample	Sampling location	Frequency
Physical properties			
Biomass content	TSS/VSS	reactor	weekly
		influent[a]	weekly
		effluent	weekly
		sludge withdrawal	weekly
Settling properties	SVI	reactor	weekly
Particle size distribution	Sieving	reactor	weekly
		effluent	weekly, once granules present
		sludge withdrawal	weekly, once granules present
System performance			
COD and nutrient removal	COD, N- and P-species	influent	weekly[b]
		reactor[c]	weekly
		effluent	weekly
Microbial communities			
Relative abundances	Biomass sample	reactor	weekly
	Fixed biomass sample[d]	reactor	bi-weekly

[a] Only for the reactors fed by municipal wastewater
[b] COD was measured only bi-weekly once the pumps were correctly adjusted and COD removal consistently high
[c] Start aerobic stage
[d] Biomass samples fixed in paraformaldehyde were taken for qFISH analyses, allowing a comparison with the results from amplicon sequencing. For time constraints, these analyses were not performed during the project.

3.4 Numerical ecology methods

Hierarchical clustering, the computation of biodiversity indices and multivariate statistical analyses were computed in the *R* software (R Development Core Team).

3.4.1 Transformation of the microbial data

The microbial composition was only known in terms of relative abundances. Moreover, biomass samples were taken separately for granules and flocs. The mean composition in g_{VSS} per L of mixed liquid for taxon i was thus calculated as a weighted average of granule and floc VSS according to Equation 3.10.

$$a_{i,mean} = VSS(f_{flocs} \cdot a_{i,flocs} + f_{granules} \cdot a_{i,granules}), \tag{3.10}$$

where a_i is the abundance of taxon i and f is the fraction of granules and flocs determined by sieving.

3.4.2 Cluster analysis

Data transformation

A cluster analysis detects subsets of the data that are similar enough to be grouped together and can help in the identification of the reasons behind these distinct groups. Most clustering methods are computed from association matrices (e.g. distance matrices) that require prior data transformation.

In the Hellinger transformation, the values are square rooted, thus reducing the weight of the highest values. This transformation is particularly recommended for the analysis of species abundance data [7]. The Hellinger distance is an Euclidean distance matrix computed on a Hellinger transformed species matrix.

For data where there is no problems of interpretation of double zeros, the Euclidean distance is usually applied. However, the Euclidean distance is strongly influenced by the scale of the data, and its use thus restricted to dimensionally homogeneous data. Dimensionally not homogeneous data needs to be standardized before calculating the distance.

Two clustering methods were used to assess whether there is a higher similarity in the data from a single reactor than between the four reactors. The K-means clustering algorithm is a flat clustering method procedure in which the data is partitioned into k clusters. The sum of squares from points to the assigned cluster centres is minimized. The optimal number of clusters was defined *a priori* using the Calinski-Harabasz clustering index from the R *vegan* package [53]. The second clustering method, Ward's minimum variance from the R *stats* package, is a hierarchical clustering procedure that minimizes the variance within each group of data [60]. The optimal number of clusters was determined *a posteriori* using Spearman's rank correlation.

Both clustering algorithms were applied separately on the Hellinger-transformed microbial community data and on the standardized sludge characteristics and nutrient removal performances.

3.4.3 Dissimilarities

The double zero problem

The double zero problem deals with the fact that the presence of an organism in two samples does not mean the same as the absence of an organism. While the presence of a organism in two samples usually implies that a minimum set of conditions allowing the organism to survive was provided, there can be various reasons for the absence of an organism in two samples. In most cases, the double absence (double zero) cannot be interpreted as a similarity. Association matrices that treat the double absence as a resemblance are called symmetrical, the others, asymmetrical [7]. When analysing

microbial abundance data, the use of asymmetrical measures is usually preferable.

Dissimilarity indices were used to compare the microbial communities in granules and flocs. In order not to count the simultaneous absence of a species in two samples as a similarity, the analysis was based on asymmetric indices. The Jaccard dissimilarity index d_J (Equation 3.11) was used to compare the presence/absence of species and the Ružička dissimilarity d_R (Equation 3.12) was applied to compare the relative abundances [56]. For both indices, the measures range from 0 (identical microbial composition) to 1 (completely different microbial composition).

$$d_J(j,k) = 1 - \frac{\sum_{k=1}^{S} \min(p_{k,i}, p_{k,j})}{\sum_{k=1}^{S} \max(p_{k,i}, p_{k,j})}, \tag{3.11}$$

$$d_R(j,k) = 1 - \frac{\sum_{k=1}^{S} \min(a_{k,i}, a_{k,j})}{\sum_{k=1}^{S} \max(a_{k,i}, a_{k,j})}, \tag{3.12}$$

where S is the total number of taxons, $a_{k,i}$ the relative abundance of taxon k in sample i and $p_{k,j}$ is the presence (1) or absence (0) of taxon k in sample j.

3.4.4 Diversity, richness and evenness

Importance of community diversity, evenness and richness in AGS systems

The stability of nutrient removal performances in biological wastewater treatment is believed to be positively linked to species diversity, richness and evenness. In most ecological systems, independent species can degrade similar compounds. Diversity is thought to be important for the stability of the systems, because less abundant species can act as buffers when a disturbance affects the dominant species. Besides this buffering effect, a performance enhancing-effect is expected for more diverse ecosystems, as the most productive species will be selected [79]. It is not clear whether the granulation process increases or decreases the microbial diversity in AGS compared to conventional AS [76].

Most diversity measurements are based on the identification of taxa (most often species). An important limitation of these traditional statistics is that diversity within a taxon is not accounted for. In fact, taxa are counted equivalently, when they may harbour significantly different phylogenetic groups. When a microbial community is known in the form of gene sequences, phylogenetic approaches for comparing the diversity might be more adequate than the traditional measures [45].

Due to time constraints, microbial diversity was only assessed based on the taxonomic affiliation of the microbial community. The analysis was performed at genus-level.

Microbial diversity consists of two components, the number of genera (richness) and the distribution of their relative abundances (evenness). Shannon's H' was used to measure

diversity (Equation 3.13). Shannon's H' quantifies the uncertainty in predicting the genus of an individual taken randomly from the dataset. A higher uncertainty corresponds to a higher diversity [27]. Richness was simply calculated as the number of genera detected in a sample.[5] Finally, Pielou's evenness (J) is the ratio between Shannon's H' and the observed richness (Equation 3.14). It is 0 in the case of one dominant genus and 1 if all genera are present in equal abundances.

$$H' = -\sum_{i=1}^{S_{obs}} a_i \ln a_i, \tag{3.13}$$

$$J = \frac{H'}{S_{obs}}, \tag{3.14}$$

where S_{obs} is the total number of species observed across all samples and a_i is the relative abundance of species i.

Pairwise t-tests (R package *stats*) were used to test for statistical differences in mean diversity, richness and evenness between the four AGS systems. The mean indices were said to be significantly different if the computed p-value was smaller than the significance level of 0.05.

3.4.5 Principal component analysis

Objective of a principal component analysis

With large numbers of variables (for instance a large number of detected taxa), there would be too many pairwise correlations between the variables to consider. To interpret the data in a more meaningful form, it is therefore helpful to reduce the number of variables to a few, interpretable linear combinations of the data.

A principal component analysis (PCA) is a data reduction technique that can be used on variables that show a multinormal distribution (each variable is normally distributed). The PCA transform the original set of variables to a new set of variables (so-called principal components) that are linearly uncorrelated. The first principal component explains most of the variance in the data, while the following principal components explain successively less of the variances.

A principal component analysis (PCA) was computed with the R *stats* package to detect the main patterns in the data. It was conducted separately on the Hellinger-transformed microbial community data, the standardized physical sludge characteristics nutrient removal performances.

[5] This method can underestimate genus richness. If a sample contains many genera at very low abundance, it is probable that undetected genera exist. In such cases, some estimators like the $Chao1$ estimator will estimate a greater richness than for a sample without rare genera.

3.4.6 Spearman's rank correlation and heatmap

Interpreting the correlation between variables

Many correlation coefficients (like the much-used Pearson's correlation coefficient) are a measure of the linear relationship between two variables. In contrast, rank correlation coefficients identify whether the variables are in a monotonic relation (e.g., when the first increases, so does to other, but not necessarily linearly). When interpreting the correlations, it is important to keep in mind that not all of these relationships are causal or direct, as they may well depend on an external variable. Especially in AGS systems, where most variables depend one from another, the interpretation of the correlations can be challenging.

Spearman's rank correlations were used to test for monotonic relationships between the microbial communities and the associated characteristics of the AGS (R package *psych* [61]). The pairwise correlations between each microbial taxon and each physical sludge characteristic and nutrient removal performance parameter were graphed in heatmaps (R package *gplots* [69]) where the individual values of the correlations were represented as colors.

4

Results and discussion

Section 4.1 analyses the effect of influent composition on the microbial communities. Section 4.2 investigates the the physical sludge characteristics and nutrient removal performances are linked to the microbial communities. Finally, Section 4.3 assesses the effect of influent composition on the start-up time of the AGS systems. A discussion of the results is given at the end of each subsection.

4.1 Microbial communities

The relative abundances of the genera detected in granules and flocs are presented in Figure 4.1. The first data point is set two days after the start-up of the reactors. The microbial composition of the inoculum was not included in the analysis, as the results provided from a different amplicon sequencing run with a higher number of sequences, and thus with significantly more detected genera. Moreover, the reference database was changed between the runs. The composition of the inoculum is therefore described separately in Appendix B along with an analysis of the main differences between the runs.

Overall, three main observations can already be made from Figure 4.1 and will be quantified and discussed in more detail in the following subsections:

1. **Microbial composition**: the microbial composition of R1 (simple synthetic substrate) was significantly different from R3 (primary effluent wastewater) and R4 (raw wastewater). R2 (complex synthetic substrate) showed characteristics from both groups.
2. **Diversity**: there was no trend in microbial diversity (richness and evenness) over time in the granules in all four systems. However, diversity decreased in the flocs in R1 and R2.
3. **Dissimilarity between flocs and granules**: granules and flocs in R1 and R2 were increasingly dissimilar. In contrast, the dissimilarity remained constant in R3 and R4.

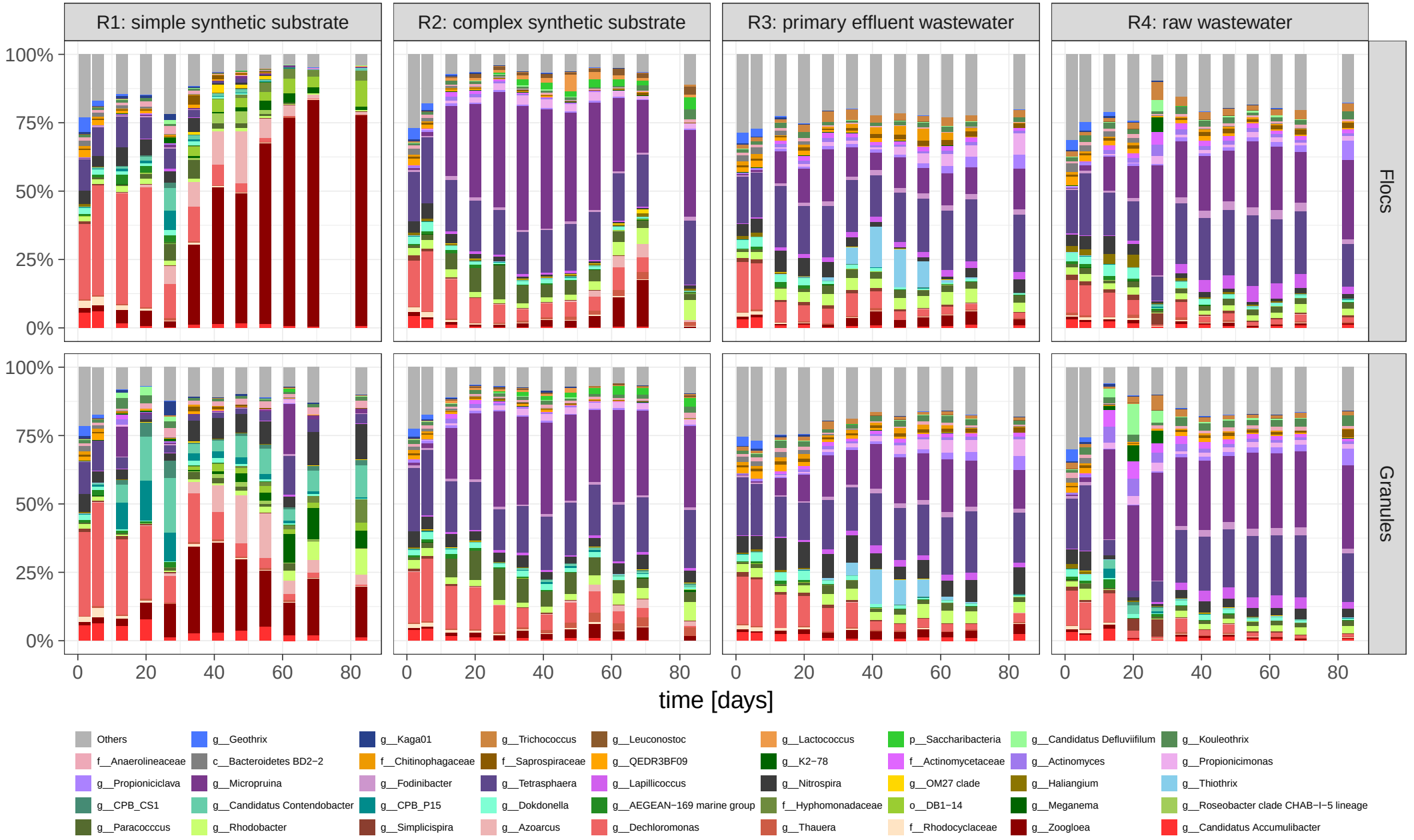


Figure 4.1: Evolution of the relative abundances of genera (or lowest known taxonomic order) in flocs and granules. Only genera with a relative abundance above 3 % in at least one of the samples are displayed separately.

4.1.1 Microbial composition

VSS increased over the experiment (detailed results in Appendix C). Hence the evolution of the relative abundances of taxa in flocs and granules gave no direct information about the evolution of their total abundances. The relative abundances were transformed into mean total abundances as explained in Subsection 3.4.1, thus providing information on the evolution of each taxon over time or on the differences between the AGS systems. As amplicon sequencing is a semi-quantitative method (Subsection 3.3.1), the relative proportions of the different taxa were only indicative and could not be compared.

If not noted otherwise, information on metabolism (PAO, GAO, nitrifiers, denitrifiers and fermenters) or cell properties (filamentous organisms) was provided from the MiDAS database [47]. It is, however, important to note that the database is by no means complete, as the metabolisms of the majority of genera detected in AS remain unknown. In the following paragraphs the total abundances of the main representatives of these groups are presented at genus-level. The reasons for the differences between the four AGS systems are discussed for each group or organisms.

PAO

The total abundances of the PAOs *Accumulibacter*, *Tetrasphaera* and *Dechloromonas*, are presented in Figure 4.2. The fourth organisms for which a PAO metabolism has been observed *in situ*, *Candidatus Accumulimonas*, was not detected.

Accumulibacter remained comparatively high and stable in R1 (simple synthetic substrate) with an average abundance of $0.08\,g_{VSS}\,L^{-1}$. In contrast, the abundance of *Accumulibacter* dropped and remained low throughout the experiment in R2 (complex synthetic substrate), R3 (primary effluent wastewater) and R4 (raw wastewater).

Tetrasphaera dropped rapidly in R1 and remained mostly below $0.1\,g_{VSS}\,L^{-1}$. In contrast, *Tetrasphaera* increased in the other three reactors. At the end of the project, total abundances were around $0.8\,g_{VSS}\,L^{-1}$ in R2 and R4 and $0.6\,g_{VSS}\,L^{-1}$ in R3.

Dechloromonas dropped in all four reactors and stabilized below $0.1\,g_{VSS}\,L^{-1}$.

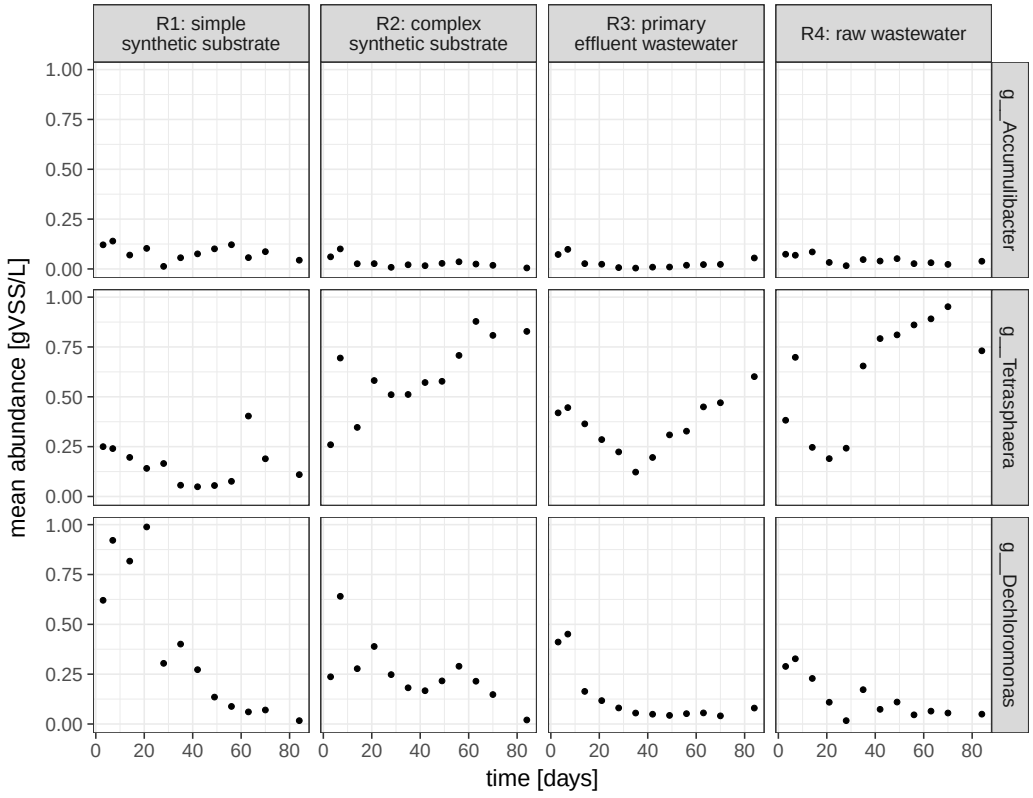


Figure 4.2: Evolution of the polyphosphate-accumulating organisms (PAO) *Accumulibacter*, *Tetrasphaera* and *Dechloromonas* during the start-up of four AGS SBRs fed by different influents. The total abundances were calculated as weighted averages of the microbial populations in the granules and flocs.

Accumulibacter persevered in the systems fed by municipal wastewaters, which were supposed to contain only low VFA contents. The VFAs necessary to sustain *Accumulibacter* were probably produced by hydrolysis and fermentation during the anaerobic stage (see discussion about fermenters below). It was not predictable that the abundance of *Accumulibacter* in R1 would be higher than in R2. The minimum COD/P ratios reported for complete P-removal by *Accumulibacter* are $8.24\,g_{COD}\,g_P^{-1}$ for acetate and $11.4\,g_{COD}\,g_P^{-1}$ for propionate [34]. The VFA/P ratio of $33\,mg_{COD}\,mg_P^{-1}$ ($17\,mg_{COD}\,mg_P^{-1}$ for each, acetate and propionate) in R2 exceeded by far these reported minimum ratios and complete P-removal by *Accumulibacter* would have been conceivable.

The presence of glucose and amino acids in R2, however, also created favourable conditions for *Tetrasphaera* that can take up these compounds directly [50]. Only little information is available about the competition between *Accumulibacter* and *Tetrapshaera* for phosphorus uptake in EBPR systems. Under the imposed conditions in R2, *Tetrasphaera* and *Accumulibacter* probably competed for the influent phosphorus. At first glance, it might seem unexpected that *Tetrasphaera* persevered in R1, although fed exclusively by VFAs. However,

some clades of *Tetrasphaera* are also capable of storing acetate [50]. It is also possible that fermentable substrate, for instance the products of decaying biomass, was produced directly in the reactor and utilized by *Tetrasphaera*.

The strong decrease of the putative PAO *Dechloromonas* can best be explained by the evolution of PAOs in R1. In R2, R3 and R4, the decrease of *Dechloromonas* was accompanied by a simultaneous decrease of *Accumulibacter* and increase of *Tetrasphaera*. It is thus not clear which PAOs were responsible for P-removal in WWTP Thunersee. In contrast, the loss of *Dechloromonas* in R1 was not balanced by an increase of *Accumulibacter* and *Tetrasphaera*. One possible explanation is that *Dechloromonas* was not involved in P-removal in WWTP Thunersee, and its decrease thus did not affect P-removal. As *Dechloromonas* can degrade a wide range of compounds, a more detailed characterization of the wastewater composition would be necessary to determine whether differences in the wastewater compositions in WWTP Thunersee and Dübendorf could be the cause of the decrease. Another possibility is that *Accumulibacter* performed better when fed by the simple synthetic substrate than by the wastewater in WWTP Thunersee, thus outcompeting *Dechloromonas* for the limited influent phosphorus. Such an improvement of the P-removal efficiency was observed in another Master Thesis on AGS conducted at EPFL after an increase of the COD concentrations in the influent of an AGS SBR. The abundance of PAO populations (in particular *Accumulibacter*) were not affected by the increase of the influent COD but apparently they performed better [30].

GAO

The total abundances of the three GAO *Contendobacter*, *CPB_P15* and *CPB_CS1* are presented in Figure 4.3. A fourth microorganisms with a GAO metabolism, *Micropruina*, is discussed with the fermenting organisms below. No other GAO reported in the MiDAS database was detected.

Contendobacter strongly increased in R1, where it was present with an abundance of up to $0.4\,g_{VSS}\,L^{-1}$. It also consistently increased in R2, but its abundance remained below $0.05\,g_{VSS}\,L^{-1}$. Except of a short period of proliferation in R4 around day 20, *Contendobacter* was not present in R3 and R4.

The two other GAO, *CPB_P15* and *CPB_CS1*, were present at considerably lower abundances. At the end of the project, they were below $0.1\,g_{VSS}\,L^{-1}$ in R1 and below $0.03\,g_{VSS}\,L^{-1}$ in R2. Except of a short period of proliferation in R4 around day 20, they were not present in R3 and R4.

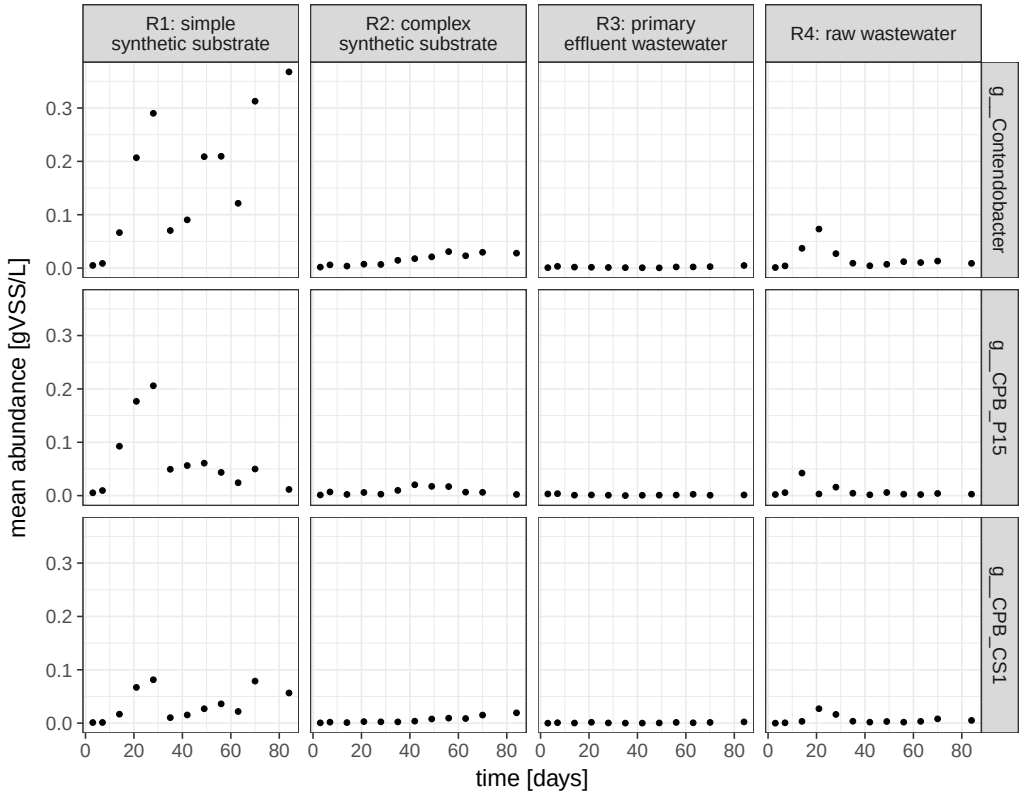


Figure 4.3: Evolution of the glycogen-accumulating organisms (GAO) *Contendobacter*, *CPB_P15* and *CPB_CS1* during the start-up of four AGS SBRs fed by different influents. The total abundances were calculated as weighted averages of the microbial populations in the granules and flocs.

Due to the high COD/P ratio in the influent, it was expected that GAO would become dominant in R1, as there was not sufficient phosphorus for *Accumulibacter* to take up all incoming VFAs. With an influent P-concentration of $6\,mg_P\,L^{-1}$, only around $50\,mg_{COD}\,L^{-1}$ of acetate or $70\,mg_{COD}\,L^{-1}$ of propionate could be taken up by *Accumulibacter*, leaving around $90\,\%$ of the COD available for other organisms.

GAO abundances were surprisingly low in R2. As the abundance of *Accumulibacter* was also low and VFA uptake by *Accumulibacter* thus limited, it is unclear which other organisms could utilize the VFA anaerobically. It is possible that *Tetrasphaera* stored a part of the VFAs (some studies report it can take up acetate, see above) or that other GAO were present in the system.

It is not clear why the three GAO had a short period of proliferation in R4 around day 20. Interestingly, the period coincided with high abundances of filamentous organisms (see below).

Nitrifiers

The abundances of the nitrifiers *Nitrospira* and *Nitrosomonas* are presented in Figure 4.4. None of the other AOO (*Candidatus Brocadia* and *Nitrosospira*) and NOO (*Candidatus Brocadia*, *Candidatus Nitrotoga* and *Nitrobacter*) reported in the MiDAS database were detected.

After an initial stable or even decreasing phase, the NOO *Nitrospira* increased in all four AGS systems. It was highest in R1 (up to $0.5\,g_{VSS}\,L^{-1}$) and lowest in R4 ($0.12\,g_{VSS}\,L^{-1}$).

The AOO *Nitrosomonas* was consistently present at very low abundances in all four reactors. It was apparently not affected by the granulation process.

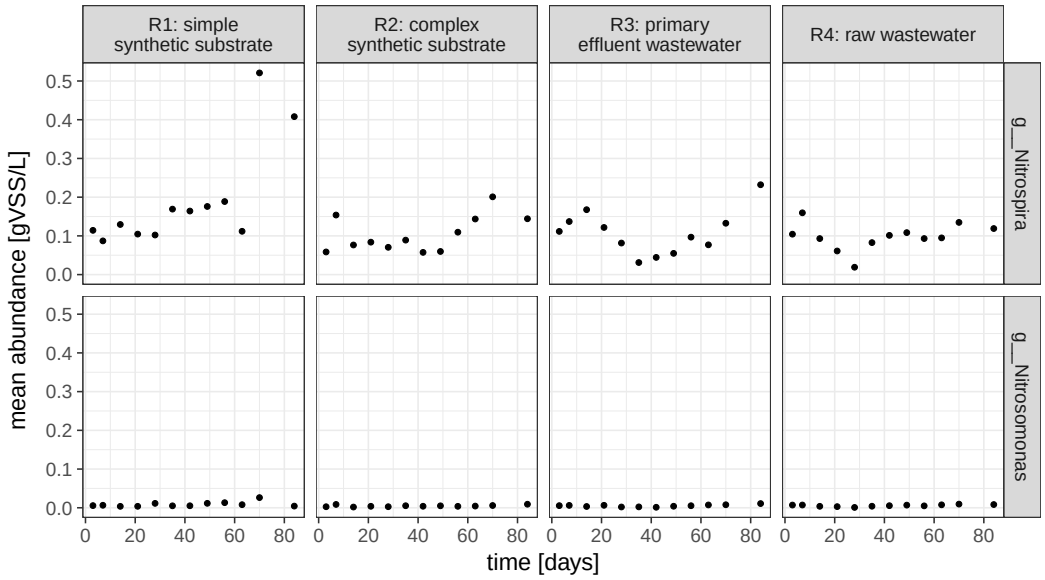


Figure 4.4: Evolution of the nitrifiers *Nitrospira* and *Nitrosomonas* during the start-up of four AGS SBRs fed by different influents. The total abundances were calculated as weighted averages of the microbial populations in the granules and flocs.

The MiDAS database reports only few microorganisms known to be capable of nitrification. It is possible that the absence of systematic differences between the four reactors is due to a limited functional redundancy of nitrifiers in WWTP.

The NOO *Nitrospira* was found in considerably larger abundances than the AOO *Nitrosomonas*. Activity measurements of AOOs and NOOs in AGS systems showed that the ammonium oxidizing capacity was around three times higher than the nitrite oxidizing capacity [77]. NOO abundances were hence expected to be higher than AOO abundances. This difference in oxidizing capacities can, however, only explain a part of the imbalance between *Nitrosomonas* and *Nitrospira*. As *Nitrospira* has been reported to also have an AOO

metabolism, it might be capable of achieving complete nitrification by its own [10]. Finally, amplicon sequencing is only semi-quantitative, and the differences might also be due to the method (e.g. differences in DNA extraction or PCR).

Denitrifiers

The main denitrifiers usually found in EBPR systems are *Azoarcus*, *Thauera* and *Zoogloea*. [72]. Their abundances are presented in Figure 4.5.

Azoarcus rapidly increased in R1 with abundances of up to $0.5\,g_{VSS}\,L^{-1}$ but seemed to decrease again towards the end of the project. It slowly but steadily increased in R2 with final abundances around $0.15\,g_{VSS}\,L^{-1}$. In R3 and R4, *Azoarcus* was not present above the level of detection.

Thauera only showed an increase in R2, where it reached abundances of up to $0.15\,g_{VSS}\,L^{-1}$.

Zoogloea proliferated in R1, becoming one of the main genera with a total abundance of up to $1.1\,g_{VSS}\,L^{-1}$. It also showed a slight increase in R2 (up to $0.25\,g_{VSS}\,L^{-1}$) and R3 (up to $0.1\,g_{VSS}\,L^{-1}$). In contrast, no increase was detected in R4.

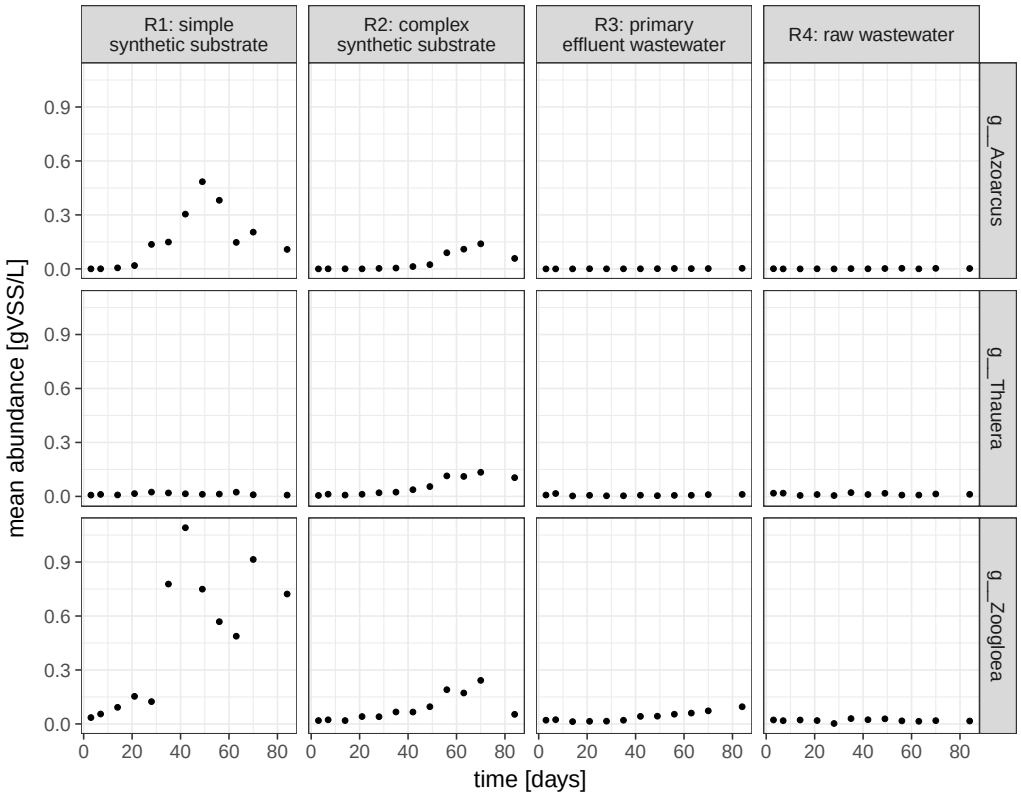


Figure 4.5: Evolution of the main denitrifiers *Azoarcus*, *Thauera* and *Zoogloea* during the start-up of four AGS SBRs fed by different influents. The total abundances were calculated as weighted averages of the microbial populations in the granules and flocs.

The increase of *Azoarcus*, *Thauera* and *Zoogloea* was expected during the granulation process. All three organisms have been reported to produce amyloid adhesins likely to be of key importance to biofilm formation [39]. Hence they were probably favoured by the selection of fast-settling biomass as a means of enhancing granule formation.

A second characteristic shared by the three organisms is their capacity to store PHA [54, 65]. When operating systems with alternating anaerobic feast and aerobic famine regimes, the main groups of PHA-storing organisms favoured in EBPR systems are usually PAOs and GAOs as explained in Subsection 2.1.3. In the case of aerobic feast and famine regimes, however, a different PHA-storing process has been proposed. Long famine periods reportedly lead to a decrease in the amount of intracellular components like RNA or enzymes necessary for cell growth. When COD concentrations suddenly peak during the feast period, the amount of enzymes available is not sufficient to reach the maximum growth rate and the storage of PHA becomes a dominant process. As a consequence, the selection of floc-formers with enhanced storage capacity has been reported in WWTP operated with aerobic feast and famine regimes [66].

It is hypothesized that PHA-storage by organisms like *Azoarcus*, *Thauera* and *Zoogloea* gained importance in R1 and, to a lesser extent, in R2 when the sCOD available at the beginning of the aerobic stage increased as a consequence of the fast granulation process.[1] PHA-storing organisms were supposed to be favoured over OHO in these aerobic feast and famine regimes.

There are various possible reasons why this increase of *Azoarcus*, *Thauera* and *Zoogloea* was not observed in R3 and R4. For instance, the critical settling velocity in the two reactors was considerably lower, as the sludge settled more slowly.[2] Fast-settling EPS-forming organisms were hence less favoured than in the reactors fed by synthetic substrate. Also, a major part of the COD leaking into the aerobic stage was supposed to be in particulate form. The substrate thus first needed to undergo hydrolysis, giving the OHO more time to adapt to the new conditions.

Fermenters

The main fermenters were *Tetrasphaera* (Figure 4.2), *Micropruina*, *Propioniciclava* and *Propionicimonas* (Figure 4.6). Other fermenters like *Actinomyces*, *Trichococcus* and *Geothrix* were also present, but at considerably lower abundances.

Micropruina was the most abundant genus in the reactors containing hydrolysable and fer-

[1] This increase in sCOD was supposed to be due to the decrease of the sludge bed height during the granulation process. The volume of injected influent suddenly exceeded the volume of the sludge bed, sCOD thus leaking into the aerobic stage. Up to 50 % of the influent COD leaked into the aerobic stage in R1 and up to 40 % in R2

[2] As explained in Subsection 3.2.2, the reactor start-up strategy was adaptive and the critical settling velocity changed in function of the settling velocity of the main sludge bed.

mentable substrate, with up to 1.3 $g_{VSS}\,L^{-1}$ in R2 and R4 and 0.5 $g_{VSS}\,L^{-1}$ in R3. Except of a few isolated peaks, it was usually not present in significant abundances in R1.

Propionicimonas increased in all three reactors, but remained considerably lower than *Micropruina* (0.08 $g_{VSS}\,L^{-1}$ in R2 and R3, 0.2 $g_{VSS}\,L^{-1}$ in R4).

Propioniciclava also increased in R3 and R4 with final abundances of around 0.2 $g_{VSS}\,L^{-1}$.

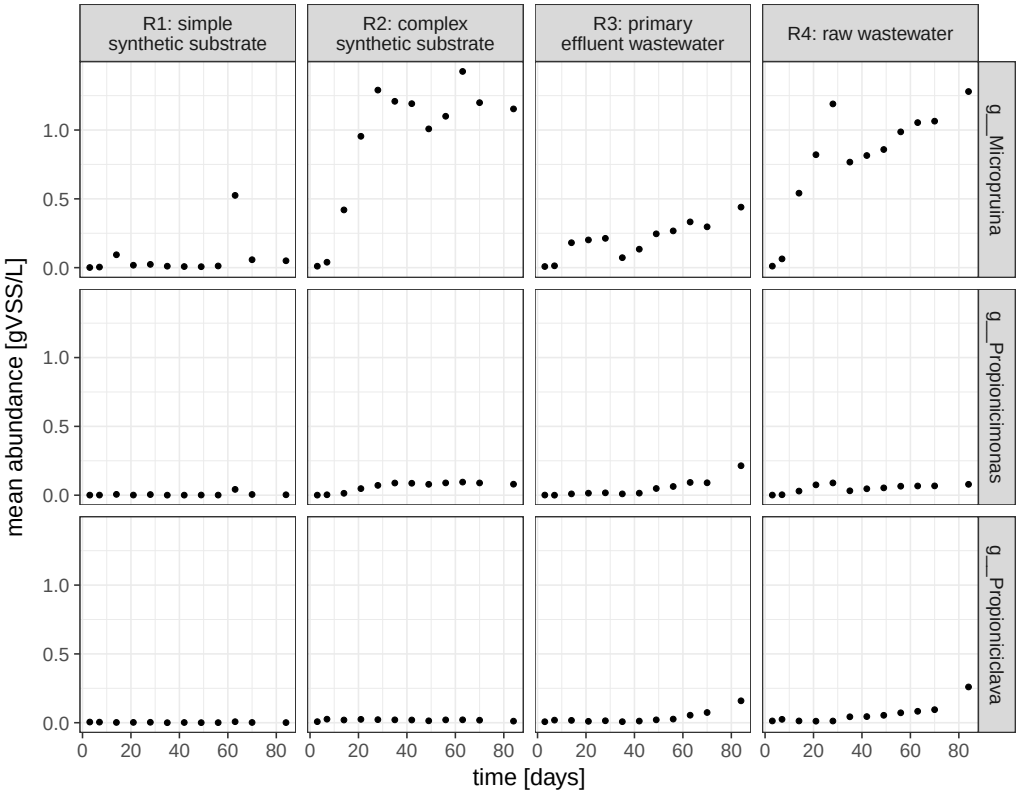


Figure 4.6: Evolution of the most abundant fermenters *Micropruina*, *Propioniciclava* and *Propionicimonas* during the start-up of four AGS SBRs fed by different influents. The total abundances were calculated as weighted averages of the microbial populations in the granules and flocs.

Fermenters were not expected to be present in R1, as the influent contained no fermentable substrate. The presence of *Micropruina* in R1 might seem contradictory to this expectation. However, it is possible that fermentable substrate was produced in the reactor, for instance through the decay of dead biomass. Moreover, *Micropuina* has been reported to have a putative GAO metabolism and is capable of storing acetate and glucose anaerobically [36]. This could also explain why other GAO populations were so low in R2 (and the abundance of *Micropruina* so high).

Propioniciclava and *Propionicimonas* produce acetate and propionate from a large range of carbohydrates [4, 63]. This strengthens the hypothesis that *Accumulibacter* persevered in R3 and R4 due to VFA produced during the anaerobic stage.

Filamentous organisms

The abundances of the three filamentous organisms *Thiothrix*, *Defluviifilum* and *Meganema* are presented in Figure 4.7.

Filamentous organisms proliferated for short periods in R1, R3 and R4 but dropped again rapidly (Figure 4.7). An important peak in *Thiothrix* was observed in R3 around day 59 with concentrations of up to $0.2\,g_{VSS}\,L^{-1}$. Short periods of proliferation of *Defluviifilum* were observed in R1 and R4 around day 20 (concentrations of $0.05\,g_{VSS}\,L^{-1}$ and $0.25\,g_{VSS}\,L^{-1}$). Finally, *Meganema* showed a major increase in R1 around day 60 but seemed to decrease again towards the end of the project.

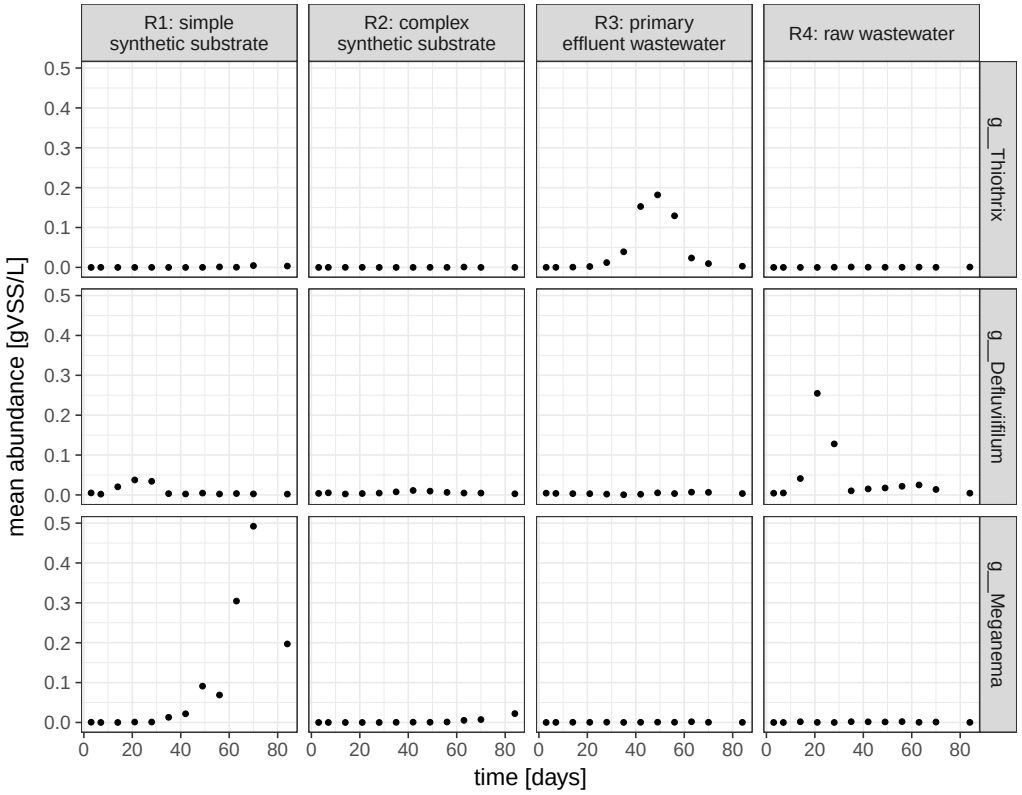


Figure 4.7: Evolution of the filamentous organisms *Thiothrix*, *Defluviifilum* and *Meganema* during the start-up of four AGS SBRs fed by different influents. The total abundances were calculated as weighted averages of the microbial populations in the granules and flocs.

The proliferation of *Thiothrix* and *Defluviifilm* correlated with losses of VSS. Almost one

third of the biomass was lost in R3 on day 23 when a valve was accidentally opened during the aeration period. The decrease of biomass continued after this date, until the reactor was operated at constant volume on day 43 (Appendix C). As a consequence, more COD leaked into the aerobic stage and was available for filamentous organisms. The increase of *Thiothrix* initiated when VSS were lowest. The filamentous outgrowths were clearly visible on the stereomicroscopic images of the sludge (Appendix G). Similarly, the periods of increasing *Defluviifilum* abundances in R1 and R4 around day 20 correlated with a temporary decreases of the VSS, but the reasons behind these biomass losses remain unknown.

Identically to *Azoarcus*, *Thauera* and *Zoogloea*, the filamentous organism *Meganema* is capable of storing acetate and propionate as PHA [38]. The reason for its strong increase in R1 was probably also the increase of the sCOD at the beginning of the aerobic stage.

From selected genera to the complete microbial populations

Results so far show that the microbial populations in R1 were clearly distinct from R3 and R4, while the microbial populations in R2 presented characteristics of both groups. As yet, the evolution of only few genera with known metabolisms or cell properties have been compared. A data reduction method like a PCA extends these limited possibilities and allows the comparison of the complete microbial populations. The visual detection of distinct microbial communities depending on the influent was confirmed by a PCA and cluster analysis on the microbial genera with a total abundance above $0.35\,g_{VSS}\,L^{-1}$ in at least one of the samples.[3] Biomass samples taken during the first week after start-up (day 2 and day 6) were removed from the analysis, as the original microbial communities were identical. The first two principal components explained 51 % of the total variance. From the Calinski-Harabsz clustering criterion, the optimal number of clusters was 3 (Appendix E). The following microbial communities were grouped together (Figure 4.8):

1. The microbial communities in R1 formed a well-defined group of observations. There was a strong and regular shift away from the original microbial community that continued throughout the experiment.

2. The microbial communities in R3 and R4 formed a second group of observations. The communities from the two reactors overlapped and could not be separated when increasing the number of clusters. The communities made a first jump away from their initial composition, but evolved rather slowly though consistently in the same direction afterwards.

3. The microbial communities in R2 formed a third group of observations located between the communities from the group R3/R4 and R1. Though a consistent shift could also be observed in R2, the original and final microbial communities remained more similar than in R1.

[3] This threshold corresponded to 1 % of the average VSS among the four reactors at the end of the study.

These clusters depended on the data transformation and varying the threshold value between $0\,g_{VSS}\,L^{-1}$ (no threshold) and $0.07\,g_{VSS}\,L^{-1}$ (2 % of the final VSS) provided additional information on the microbial community composition (detailed results in Appendix D). For low thresholds, R1 and R2 were grouped together. For high thresholds, the microbial communities in R1 formed a distinct group from the microbial communities in the three other reactors. Finally, a cluster of the same data using Ward's minimum variance method combined with Spearman's optimal number of clusters confirmed the results from the K-means clustering, as the microbial communities from R1 formed a distinct group from the ones in the other reactors (detailed results in Appendix E). The microbial communities in R2 were more similar to the communities in the reactors fed by municipal wastewater, but formed a well-defined separate group.

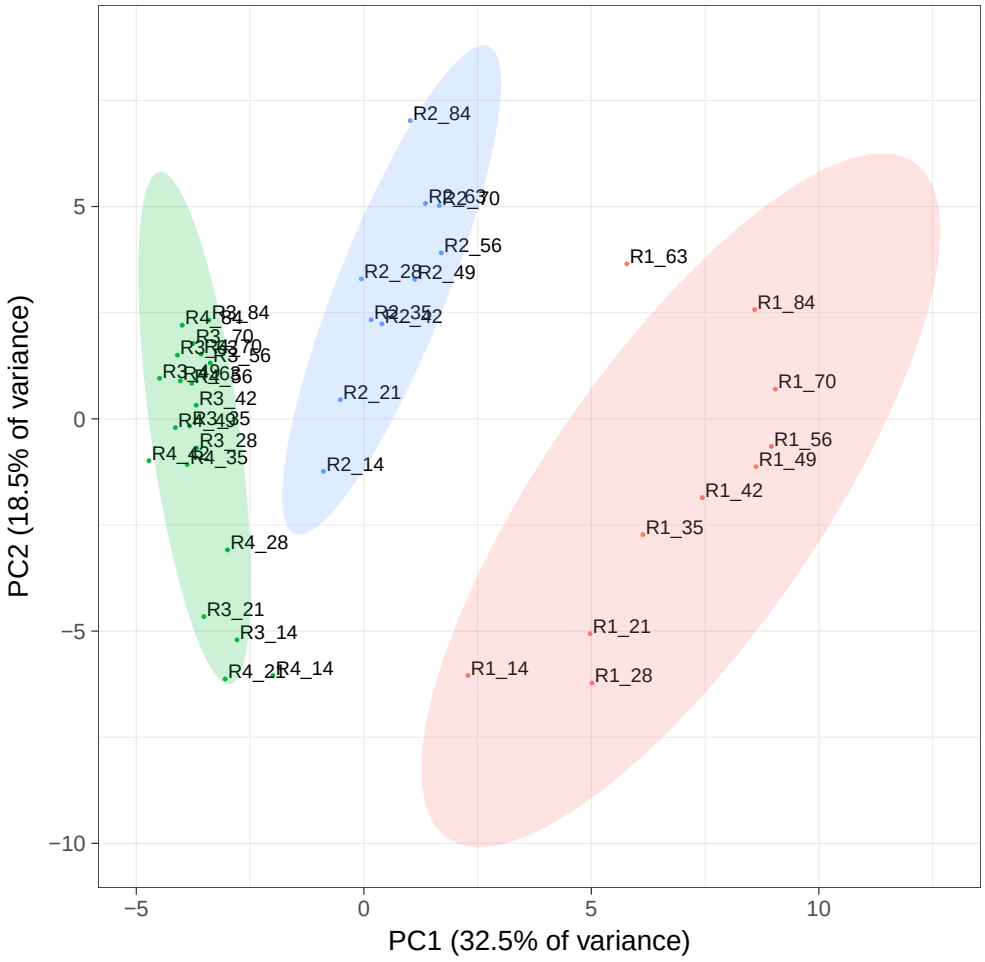


Figure 4.8: Principal component analysis (PCA) of the Hellinger-transformed mean microbial community from day 14 to day 84. Only genera with a total abundance above 0.035 $g_{VSS}\,L^{-1}$ in at least one of the samples were included in the analysis. Coloured areas represent 95 % confidence ellipses of a K-means clustering (optimal number of groups obtained with the Calinski-Harabasz clustering index). The text labels combine the reactor names and the number of days after start-up. R1: simple synthetic substrate. R2: complex synthetic substrate. R3: primary effluent wastewater. R4: raw wastewater. PC: principal component.

Overall, influent composition strongly affected microbial composition as well as the rate of change of the communities. After only two weeks of operation, there were already two distinct groups: R1 on the one side and R3 and R4 on the other side. R2 presented characteristics from both groups. For high thresholds (only genera present at high abundances included in the analysis), R2 was more similar to the reactors fed by municipal wastewater. This high similarity was due to the importance of fermenters (in particular *Micropruina* and *Tetrasphaera*) in the systems with hydrolysable and fermentable substrate. As there were no fermenters in R1, it formed a distinct group of observations. For lower thresholds (genera present at low abundance included in the analysis), R1 and R2 were clustered together.

When considering microorganisms present at low abundances, R2 was thus more different from R3 and R4 than from R1. It is hypothesized that this was due to the constant inflow of new biomass present in the influent wastewater in R3 and R4, which was absent in R2.

The rate of change of the communities was highest in R1, intermediary in R2 and low in R3 and R4. There are two explanations for these differences. Firstly, the difference between the substrates used to feed the four reactors and the wastewater in WWTP Thunersee. The simple synthetic wastewater was presumably most different from the wastewater in WWTP Thunersee and the primary effluent wastewater most similar. The original microbial community in the inoculum was thus furthest from the equilibrium community in R1. Secondly, the granulation process promoted by the reactor operation also induced a shift in the microbial communities. The continued shift in microbial communities in R1 even for the most recent observations indicates that the assemblage of the microbial community was not completed at the end of the project and the granulation process was still ongoing.

4.1.2 Dissimilarity between granules and flocs

The evolution of the dissimilarity between flocs and granules can give information on the role of the flocs in the granulation process. For all influents, the Jaccard dissimilarity index was considerably lower than the Ružička dissimilarity index (Figure 4.9). Hence the same genera were present in granules and flocs, but in different proportions. The influent composition affected the temporal evolution of the two dissimilarity indices. Three main patterns were detected:

1. R1 (simple synthetic substrate) presented a strong and consistent increase in dissimilarity between granules and flocs for both indices.

2. The Jaccard dissimilarity index remained low throughout the experiment in R2 (complex synthetic substrate), but the Ružička dissimilarity increased.

3. Both dissimilarity indices remained constant in R3 (primary effluent wastewater) and R4 (raw wastewater).

Microorganisms that were present with a relative abundance below a threshold of 1 % in all samples were discarded from the analysis. The influence of the choice of this threshold on the interpretation is assessed in detail in Appendix D. Though the exact values of the indices were affected, the main trends were detachable for thresholds between 0 % and 5 %.

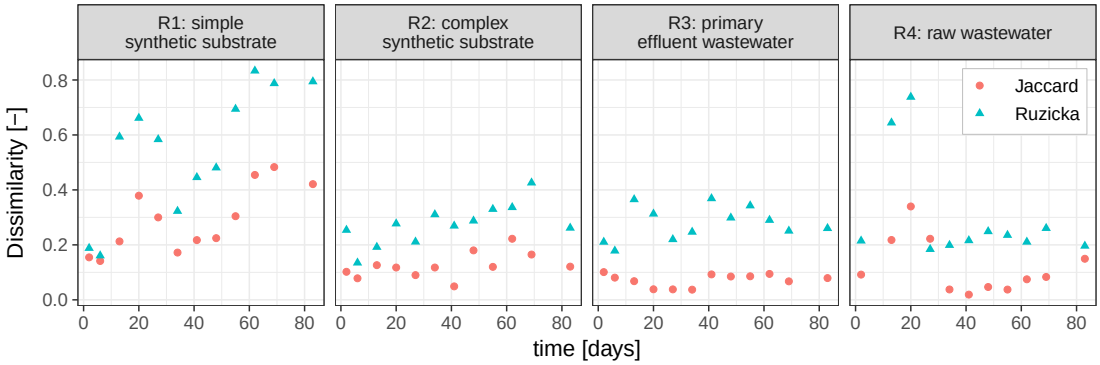


Figure 4.9: Evolution of two dissimilarity indices between granules and flocs during the start-up of four AGS SBRs fed by different influents. The Ružička dissimilarity index compares the relative abundances of the microbial genera in the flocs and granules. The Jaccard dissimilarity compares the presence/absence of the genera. Only genera with a relative abundance above 1 % in at least one of the samples were included in the analysis.

Based on these results, granule formation mechanisms can be discussed. Barr et al. suggested two granule formation mechanisms: microcolony outgrowth and microcolony aggregation ([5], Subsection 2.2.1). If microcolony outgrowth were an important process, only organisms capable of granule formation would be present in the granules, resulting in a high dissimilarity between granules and flocs.[4] The results show that microcolony outgrowth was not a significant process in R3 and R4, as granules and flocs remained similar throughout the study. This observation is also valid for R2, where granules and flocs consisted of the same genera, though with different relative abundances. The increasing dissimilarity between granules and flocs could evidence that microcolony outgrowth was a major process in R1. However, stereomicroscopic images of the sludge indicated that flocs in R1 were probably composed of wastage from the outer layers of the granules (Appendix G, picture i). As the outer layers of the granules presumably contain different microorganisms than the inner layers, this could explain the high dissimilarity between granules (for which the outer layers only make up a small part of the microbial communities) and the flocs (composed exclusively of the outer layers).[5] No conclusion on the granulation process could be drawn for R1, and both processes could occur simultaneously.

Finally, the high similarity between granules and flocs in R3 and R4 indicates that the threshold of 0.25 mm used to distinguish between granules and flocs was arbitrary from a microbial point of view in these two reactors.

[4] For instance, granules that were hypothesized to form via microcolony outgrowth in Barr et al. were composed of 97.5 % of *Accumulibacter*, while granules hypothesized to form via microcolony aggregation were microbiologically more diverse [5].

[5] The dominant organism in the flocs, *Zoogloea*, is an aerobic heterotroph and it is well possible that it was located in the outermost layers of the granules.

4.1.3 Microbial diversity

Microbial diversity is thought to be important for the stability of the nutrient removal performances. Influent composition affected the temporal evolution of the diversity and evenness (Figure 4.10). Three main patterns were detected:

1. In R1 (simple synthetic substrate), diversity and evenness remained constant in the granules and strongly decreased in the flocs.
2. In R2 (complex synthetic substrate) and R4 (raw wastewater), diversity and evenness decreased in the granules and flocs.
3. In R3 (primary effluent wastewater), diversity and evenness remained constant in the granules and the flocs.

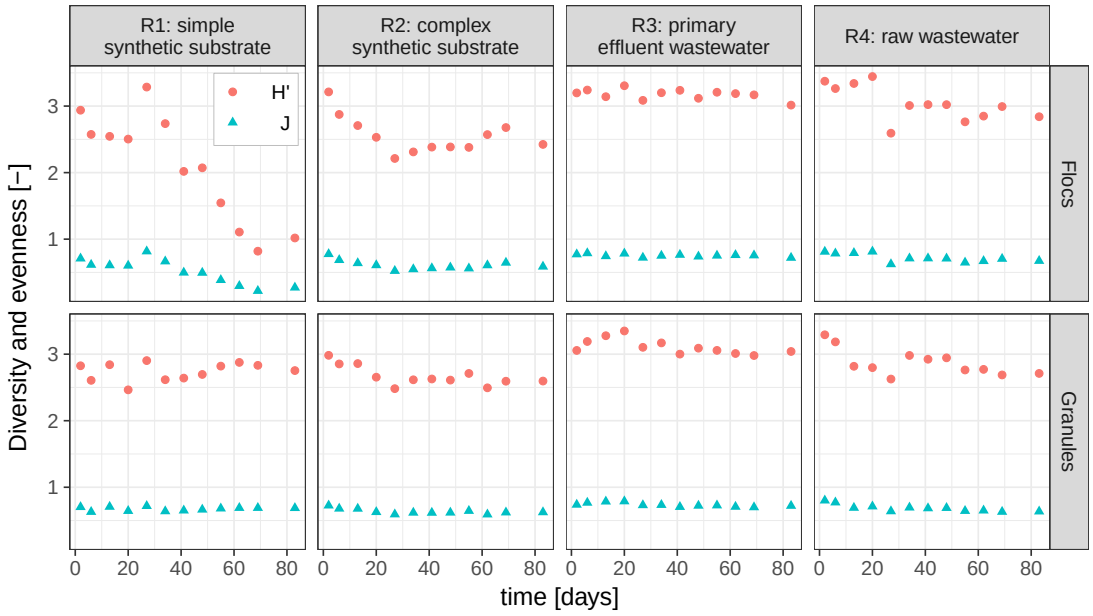


Figure 4.10: Evolution of Shannon's H' and Pielou's evenness J during the start-up of four AGS SBRs fed by different influents. Only genera with a relative abundance above 1 % in at least one of the samples were included in the analysis.

It is difficult to visually compare the mean diversity values between the four reactors, which is why they are presented separately in Table 4.1b for granules and Table 4.1a for flocs. Only genera with a relative abundance above $1\,\%$ in at least one of the samples were included in the calculations. The mean diversity, evenness and richness were not significantly different between granules and flocs from one reactor (all p-values for the pairwise t-tests are given in Appendix F). In contrast, the mean diversity, evenness and richness were significantly higher in R3 than in the reactors fed by synthetic substrates. The three indices were also higher in R4, but the differences were not (yet) statistically significant. When directly comparing the mean diversities from the granules fed by municipal wastewaters and by synthetic substrates, the difference in diversity was highly significant.[6] In contrast, results indicated a higher diversity and evenness in R1 than in R2, and similarly, in R3 than in R4, though the differences were statistically not (yet) significant. These main trends were verified for thresholds between $0\,\%$ and $5\,\%$ (details in Appendix D).

Table 4.1: Mean values (with standard deviation) for three diversity indices: Shannon's H', Pielou evenness J and total richness R. Only genera with a relative abundance above $1\,\%$ in at least one of the samples were considered in the analysis. SS: synthetic substrate. WW: wastewater.

(a) Flocs

Index	R1: simple SS	R2: complex SS	R3: primary eff. WW	R4: raw WW
H'	2.10 ± 0.81	2.56 ± 0.28	3.18 ± 0.08	3.04 ± 0.26
J	0.51 ± 0.18	0.61 ± 0.07	0.75 ± 0.02	0.72 ± 0.06
R	78 ± 18	92 ± 6	99 ± 4	100 ± 5

(b) Granules

Index	R1: simple SS	R2: complex SS	R3: prim. eff. WW	R4: raw WW
H'	2.74 ± 0.14	2.67 ± 0.15	3.11 ± 0.11	2.87 ± 0.20
J	0.66 ± 0.03	0.64 ± 0.04	0.74 ± 0.03	0.68 ± 0.05
R	80 ± 9	94 ± 4	101 ± 4	96 ± 12

Diversity was expected to be positively linked to influent complexity, thus lowest in R1 and highest in R4. Results confirmed the hypothesis that influent composition affects microbial diversity. The mean microbial diversity in the granules fed by municipal wastewater was indeed significantly higher than in the granules fed by synthetic substrates. Municipal wastewater presumably provided a wider range of ecological niches than the system fed by synthetic substrate, due to the complex composition of the wastewater in combination with important temporal and seasonal variations. Moreover, the reactors fed by municipal wastewater received a constant input of new biomass, also contributing to the diversity.

In contrast, the differences in microbial diversity in R1 and R2 and, similarly, in R3 and R4, indicated that the relation between influent complexity and microbial diversity might be inverse from expected. Even with a much simpler substrate composition in R1 (hence fewer ecological niches), diversity in R1 stabilised at a higher value than in R2. It is not clear if these differences will become significant with more data. If so, it would not necessarily contradict the initial hypothesis of higher diversity for more complex substrates. A possible explanation would be that the effect of substrate composition was balanced by a faster gran-

[6] The mean Shannon's H' for the granules fed by municipal wastewaters (3.0) was higher than for the granules fed by synthetic wastewaters (2.7) with $p < 0.001$.

ulation process in R1. The rapid growth of the granules presumably created new ecological niches for genera that were not present in abundances above the detection level before. The same observations and interpretations also apply to the differences between R3 and R4, as the granulation process was more advanced in R4.

Finally, there were no differences in diversity between granules and flocs in the reactors fed by municipal wastewater. This result is well in accordance with the observations made on the high similarity between granules and flocs in these two reactors and strengthens the hypothesis that the use of a threshold of 0.25 mm did not reflect differences at the microbial level.

4.2 Linking the microbial composition to the physical composition of the sludge and nutrient removal performances

4.2.1 Physical sludge characteristics

The particle size distribution was strongly affected by the type of influent (Figure 4.11). No data was available for R4 after day 83 when the sludge was lost. Three main patterns were detected:

1. In R1 (simple synthetic substrate), almost all biomass was in the form of granules. The succession of size fractions was clearest. First, small granules (0.25 mm $<$ d $<$ 0.63 mm) appeared. As the granules grew at similar speed, there was a sudden breakthrough of medium-sized granules (0.63 mm $<$ d $<$ 1 mm) starting from day 55. The granules continued growing and the proportion of large granules (d $>$ 1 mm) finally represented the largest fraction of TSS. Flocs concentrations dropped below the detection level.

2. The shift in particle size distribution in R2 (complex synthetic substrate) started similarly. Granules represented more than half of the TSS starting from day 34, However, no succession of size fractions as in R1 was observed. Most granules remained small, even when the fraction of granules stabilized and a constant fraction of flocs of around 20 % remained in the system.

3. The evolution of the particle size distribution in R3 and R4 were similar, however, with a stronger shift towards granules in R4.[7] The fractions of granules stabilised at around 75 % in R4 respectively 55 % in R3.

[7] From Figure 4.11, R2 and R4 could also be grouped together. Due to high amounts of filamentous materials in R4 (presumably cellulosic materials), the formation of a cake layer during the sieving could not be completely avoided. The fraction of granules might thus be overestimated. It is also not clear whether larger particles in R4 were mostly granules grown in the reactor or influent particulate organic matter.

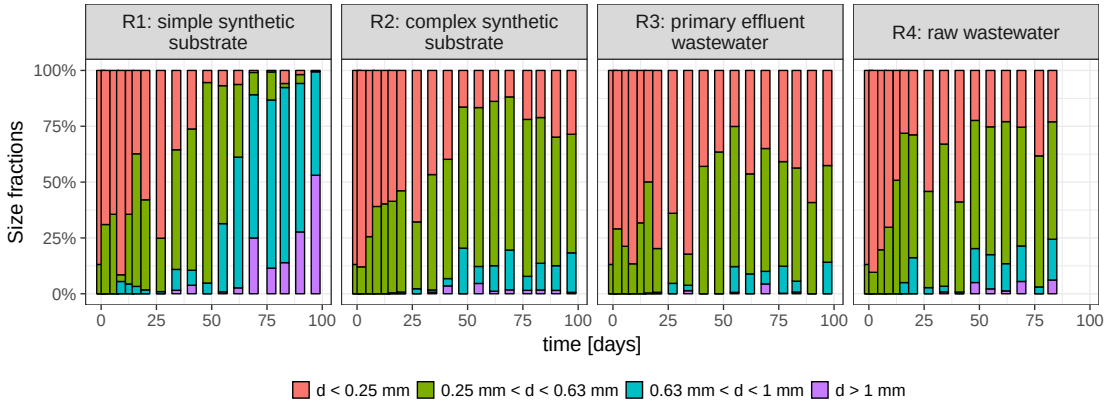


Figure 4.11: Evolution of the particle size distribution during the start-up of four AGS SBRs fed by different influents.

Similarly, the SVIs and ratios of SVI were strongly affected by the type of influent (Figure 4.12). Three main patterns were detected:

1. In R1 and R2 the decrease of SVIs was consistent and the SVI_{30} dropped below $50\,mL\,g^{-1}$ after only a week of operation, while the ratios of SVI were close to 1 after less than a month of operation. The SVI_{30} stabilized around $25\,mL\,g^{-1}$ in R1 and $50\,mL\,g^{-1}$ in R2.

2. After an initial decrease of the SVIs in R3, they started re-increasing after a loss of TSS around day 30. Interestingly, the SVIs decreased again from day 48 on, even though the reactor was operated at constant volume during that period. The SVIs remained relatively constant when a low critical settling velocity was imposed again after day 56. At the end of the experiment, the SVI_{30} was around $75\,mL\,g^{-1}$, but the ratios of SVI remained low.

3. In R4, the SVIs steadily decreased and the SVI_{30} stabilised at around $50\,mL\,g^{-1}$. However, no clear trend for the ratios of SVI could indicate a strong granulation process.

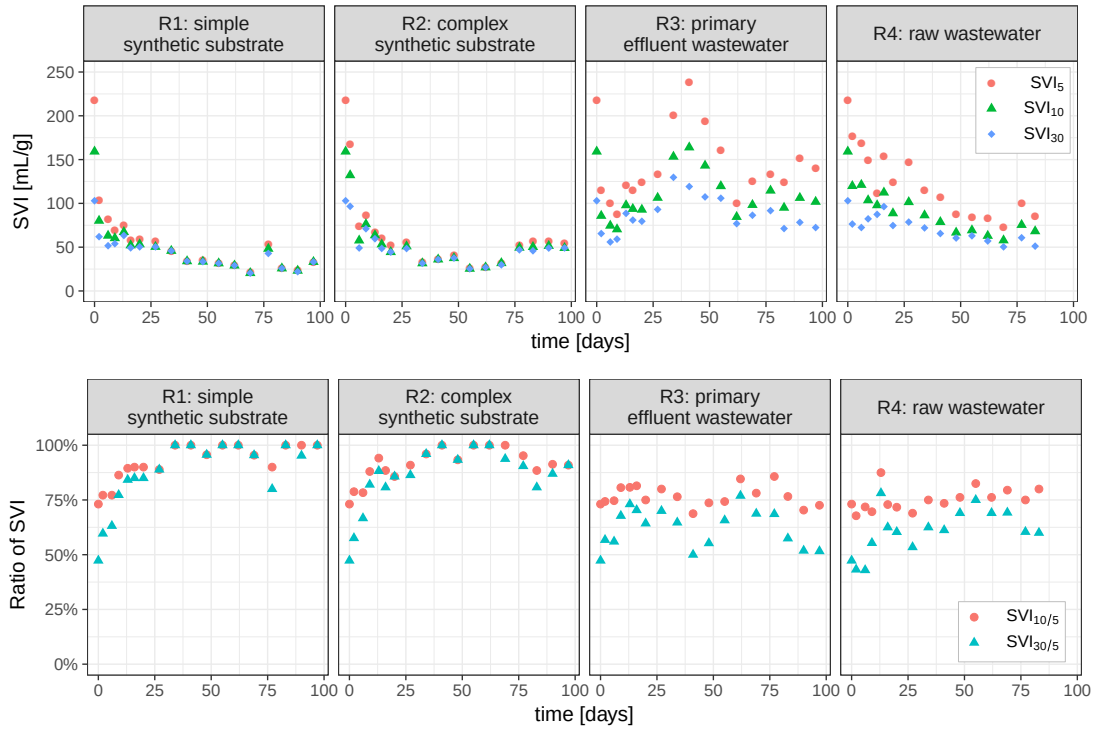


Figure 4.12: Evolution of the sludge volume indices (SVI) after 5, 10 and 30 minutes and ratios of SVI during the start-up of four AGS SBRs fed by different influents.

Overall, the influent composition strongly affected all physical sludge characteristics. Stereomicroscopic images of the sludge in R2, R3 and R4 (Appendix G) and their particle size distributions showed that the biomass was always a mixture of granules and flocs. This co-existence of both types of biomass is often reported for systems treating real wastewaters or synthetic wastewaters containing particulate substrate [67]. In R2, biomass with a diameter below 0.25 mm was still present at the end project. However, these flocs already formed rounded structures (Appendix G, picture j). Overall, they resembled the morphology of granules, but at smaller scale. This observation is well in accordance with the good settling properties of the sludge in R2 and the highly similar microbial communities in the granules and flocs. The observations support the hypothesis that the threshold of 0.25 mm to distinguish granules and flocs was arbitrary and not adequate to describe the AGS systems, not only from a microbial point of view (high similarity and similar diversity between granules and flocs) but also in terms of settling properties. The choice of the threshold was not determining for the AGS system fed only by VFAs, as virtually all biomass had much larger diameters than 0.25 mm. However, in the systems where fermentable hydrolysable material was present, granules remained much smaller in size and the choice of the threshold influenced the evaluation of the state of granulation.

Studies on anaerobic granules concluded that no clear relationship between granule size and granule density could be established. Some reported that density was independent of size,

while others even observed decreasing density for increasing size [31]. These observations might also be applicable to aerobic granules. In the future, it might be advisable to focus rather on density separation than on size separation to distinguish granules and flocs. In fact, linear relationships between EPS contents and density have been reported [41]. Density measurements might thus better reflect EPS contents (and hence the state of granulation) in the biomass.

4.2.2 Nutrient removal performances

COD removal efficiency increased rapidly and remained high for all reactors throughout the experiment (Figure 4.13).

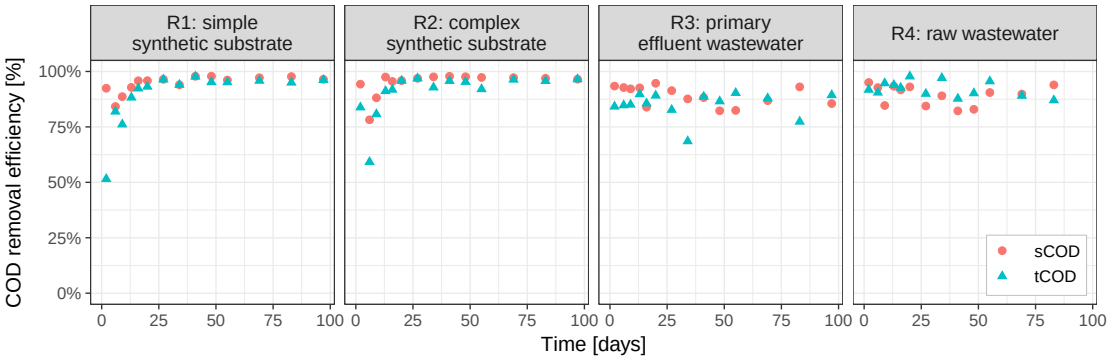


Figure 4.13: Evolution of the soluble COD (sCOD) and total COD (tCOD) removal efficiencies during the start-up of four AGS SBRs fed by different influents.

Removal of phosphorus, ammonium and nitrogen depended on the influent composition (Figure 4.14).

P-removal efficiency decreased rapidly in the AGS systems fed by municipal wastewaters (R3 and R4), but showed an increasing trend towards the end of the experiment. In the AGS systems fed by synthetic substrates, P-removal was generally high throughout the experiment, especially in R2 (complex synthetic substrate). In R1 (simple synthetic substrate), occasional drops in P-removal efficiency were observed.

Nitrification could be maintained in all four reactors and was not affected by influent composition. In contrast, N-removal was dependent on influent composition. It was highest in R1. Intermediary N-removal was observed in R2 (complex synthetic substrate). As no alternating aeration was implemented, all denitrification could be attributed to SND. Differences in N-removal could hence be explained by differences in SND. This was confirmed by a detailed monitoring of the aerobic stage on day 88, where 65 % SND was detected in R1, while there was no SND in R2 (detailed results in Appendix I). No clear trends in N-removal could be detached for the reactors fed by municipal wastewaters. Almost no SND was detected in R3

and 20 % SND in R4.

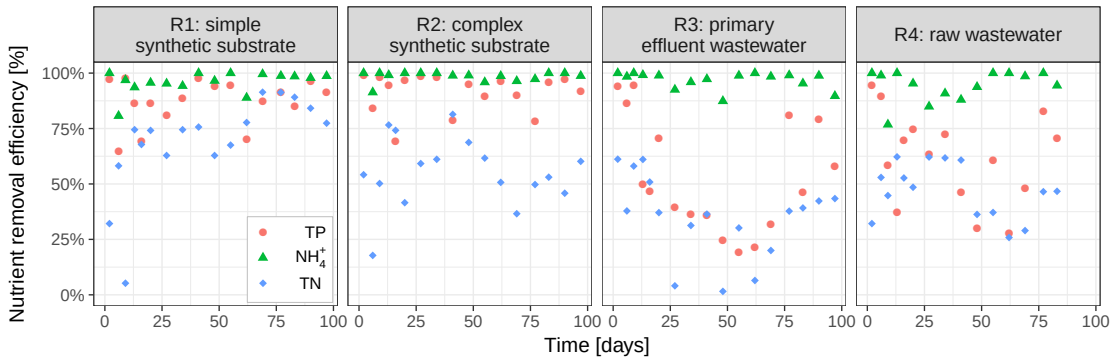


Figure 4.14: Evolution of the total phosphorus (TP), ammonium (NH_4^+) and total nitrogen (TN) removal efficiencies during the start-up of four AGS SBRs fed by different influents.

Removal performances were consistently high in R1 and R2. This is surprising, as other studies reported losses in ammonium, nitrogen and phosphorus removal during the granulation process [72]. However, these studies also reported an initial loss of VSS or constant VSS contents, as wash-out conditions on flocs were applied. In this study, the critical settling velocity was adapted as a function of the settling velocity of the main sludge bed. The selection pressure on the slow-settling biomass was thus relatively low throughout the study. Apparently, this allowed nutrient removal performances to be maintained.

It is unclear why P-removal was first lost in R3 and R4 and recovered only later. One possibility is that the composition of the wastewater arriving in WWTP Thunersee was significantly different from the wastewater used in the study and the PAOs responsible for P-removal in WWTP Thunersee were not adapted to the new wastewater. For instance, decreasing *Accumulibacter* populations after start-up could hint at the possibility that VFA concentrations in WWTP Thunersee were higher. However, detailed information on the wastewater compositions is currently not available.

Differences in N-removal between R1 and R2 could be due to differences in granule size. A mathematical model developed by de Kreuk et al. showed that smaller granules had larger area to biomass ratios, resulting in a deeper penetration of DO and hence less denitrification. The optimum granule diameter for maximum N- and P-removal for a DO of $2\,mg\,L^{-1}$ was between 1.2 mm and 1.4 mm [15]. More than 90 % of the biomass had a diameter above 0.63 mm in R1 during the monitoring of the aerobic stage (Figure 4.11). In contrast, less than 10 % of the biomass had a diameter above that value in R2. It is thus hypothesized that granules in R2 were so small that they were fully aerobic during the aerobic stage, while anoxic zones persevered in R2.

4.2.3 Link to the microbial composition

In a first attempt to assess whether distinct microbial communities led to distinct physical sludge characteristics and nutrient removal performances, PCAs with K-means clustering were computed. In a second step, pairwise correlations were graphed in heatmaps in order to detect how the characteristics could be linked to specific genera.

PCAs

Two separate PCAs were computed for the physical sludge characteristics and the nutrient removal performances.[8]

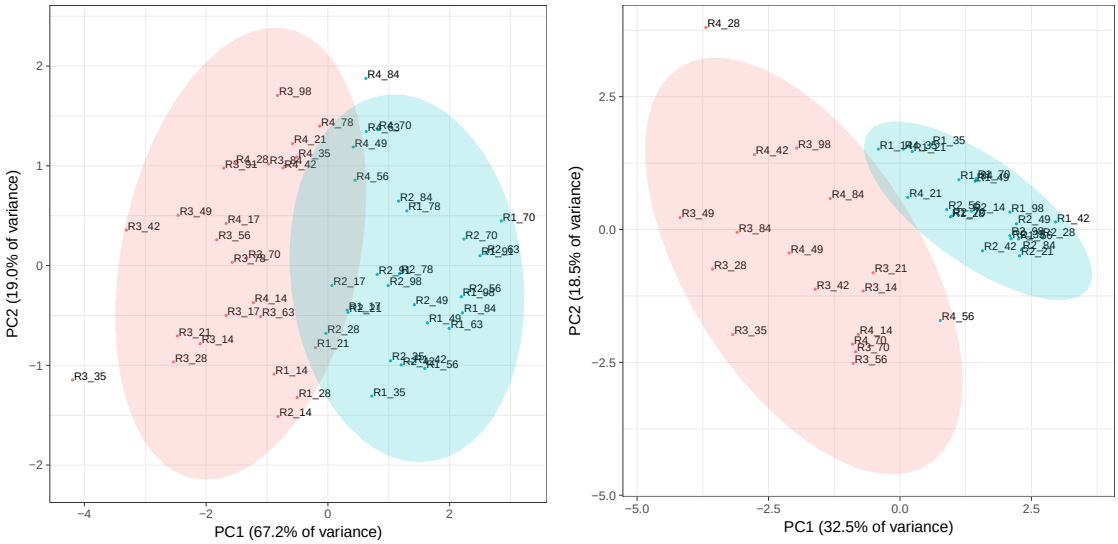
For the PCA of the physical sludge characteristics, the first two principal components explained 86 % of the variance (Figure 4.15a). Two main groups of observations were detected (Calinski-Harabasz clustering index, Appendix E). The first cluster consisted mainly of observations from R1 (simple synthetic substrate) and R2 (complex synthetic substrate). The most recent observations from R4 also fell into this group, indicating that the physical characteristics were becoming more similar to those in R1 and R2.

For the PCA of the nutrient removal performances, the first two principal components explained 51 % of the variance (Figure 4.15b). Again, two main clusters were detected for the nutrient removal performances (Calinski-Harabasz clustering index, Appendix E). The first group consisted primarily of observations from R1 and R2, which showed very similar nutrient removal performances. Sporadically, observations from R4 also fell into this first group. However, the shift towards the group of R1 and R2 was not as apparent as was the shift for the physical sludge characteristics.

Recalling the clustering of the microbial communities, there were three main groups (Figure 4.8). Communities in R1 and R2 formed distinct clusters from the communities in R3 and R4. Despite major differences in microbial composition between R1 and R2, they had comparable physical characteristics and achieved similar nutrient removal performances. This result illustrates the functional redundancy of microbial communities present in AS and shows that the presence of fermentable and particulate organic matter is not per se an issue for the formation of AGS.

Similarly, the microbial communities in R4 did not converge towards those in R1 and R2, while the physical sludge characteristics and nutrient removal performances did get closer towards the end of the project. This means that it is possible to form AGS without high abundances of the microorganisms typically present in AGS systems fed by VFA-based substrates, however, the kinetics of granulation are considerably slowed down by the presence of fermentable substrate and particulate organic matter.

[8] Physical sludge characteristics: VSS, fraction of granules, SVI_{30}, $SVI_{30/5}$. Nutrient removal performances: parameters for which quality criteria are defined in the WPO (COD, ammonium and phosphorus removal efficiencies and discharge concentrations; nitrate discharge concentrations)



(a) Physical sludge characteristics **(b)** Nutrient removal performances

Figure 4.15: Principal component analysis (PCA) of the standardized physical sludge characteristics and nutrient removal performances from day 14 to 84. Coloured areas represent 95 % confidence ellipses of a K-means clustering. The text labels combine the reactor names and the number of days after start-up. R1: simple synthetic substrate. R2: complex synthetic substrate. R3: primary effluent wastewater. R4: raw wastewater. PC: principal component.

Interestingly, this evolution of the physical sludge characteristics and nutrient removal performances towards those in R1 and R2 was not (yet) visible in R3, despite a similar microbial composition to R4. Hence kinetics of AGS formation in R3 were further slowed down compared to R4. The lower influent COD concentration might be the cause of these slower kinetics. In this case, R3 should move towards a more granular state in the future, as microbial communities potentially capable of forming AGS with good nutrient removal were present. Another possibility is that COD and nutrient concentrations in R3 were too low for good removal performances (and not just slowing down the kinetics). For instance, a previous Master Thesis on AGS reported an improvement of the phosphorus removal efficiency in an AGS system after increasing the COD concentrations, without an effect on the abundances of the PAO populations [30]. Possibly sufficient COD and nutrient concentrations are necessary for the formation of AGS with good nutrient removal performances.

Pair-wise correlations

Based on the PCAs, the relation of the entire microbial communities with the physical sludge characteristics and nutrient removal performances was discussed. In order to determine how the individual microbial genera might affect the physical characteristics and the removal performances of the AGS, pair-wise correlations between each genus and each physical sludge

characteristics and nutrient removal performance parameter were graphed in heatmaps.

First results indicated that the signs and intensities of the correlations strongly depended on the AGS system. For instance, *Tetrasphaera* was negatively correlated with P-removal in R1 (where P-removal was presumably achieved by *Accumulibacter*) but positively correlated with P-removal in R3 (where it supposedly was the main PAO). When considering the entire data set at once, these differences would not have been visible and the conclusions drawn on the functions of the microbial genera distorted. The correlations were thus calculated separately for each AGS system. This however affected the significance of the tests, as the number of data points for each analysis was reduced to 10.[9]

Only the few correlations which were statistically significant (p-value < 0.05) are presented in Table 4.2. A positive correlation between a genus and an AGS characteristic indicated that the abundance of the genus and the value of the AGS characteristic increased or decreased simultaneously. Negative correlation indicated that when one variable increased, the other decreased. These results should be viewed as preliminary examples of the type of relationships that may be observed once more data is available. Complete heatmaps can be found in Appendix J.

Table 4.2: Positive (green) and negative (red) pairwise Spearman's rank correlations between the microbial communities genera and the physical sludge characteristics and nutrient removal performances of the AGS. Annotations correspond to p-values with the following significance levels. 0.001: ***, 0.01: ** and 0.05: *.

	Frac. granules	**SVI_{30}**	**VSS**	**COD removal**
R1 (simple synthetic substrate)				
Rhodobacteraceae (genus unknown)	*	***		
Meganema	**	***		
Dechloromonas		**		
AEGEAN169 marine group		***		
R2 (complex synthetic substrate)				
Azoarcus	*			
Thauera	*			
Zoogloea	**	*		
R3 (primary effluent wastewater)				
Kouleothrix				*
Paracocccus				*
R4 (raw wastewater)				
Actinomycetaceae (genus unknown)		*	**	
Actinomyces		**	***	
Chloroflexi (genus unknown)		**	***	
Propioniciclava			*	

[9]Number of biomass samples sequenced for each reactor. There were 12 biomass samples per reactor in total, however, the first week of data was discarded (similarly to the data treatment for the PCA of the microbial communities).

4.2. Link to physical composition and removal performances

The correlations of the microbial genera with the fraction of granules and the SVI_{30} are discussed in the paragraphs below, shedding light on why the interpretation of these correlations is challenging and should be analysed in detail only once more data is available.

As mentioned above, *Azoarcus*, *Thauera* and *Zoogloea* have been reported to produce amyloid adhesins likely to be of key importance to biofilm formation. Therefore, they were expected to be positively correlated with the fraction of granules, as they presumably played a crucial role in their formation. *Meganema* is a filamentous aerobic heterotroph. The organism might be directly or indirectly linked to the fraction of granules (or both). On the one hand, its presence possibly improved granule structure (direct link), acting as a backbone for the attachment of other organisms and thus contributing to the growth of the granules [46]. On the other hand, *Meganema* was probably favoured by the increase of sCOD at the beginning of the aerobic stage during the granulation process (indirect link), as the organisms has the ability to assimilate PHA from VFAs in aerobic feast and famine conditions [47].

Zoogloea presumably contributed to the good settling properties of the AGS through the production of EPS. It was thus expected that the organism would be negatively linked to the SVI_{30}. In contrast, *Meganema* was expected to be positively correlated to the SVI_{30}, as it is has filamentous morphology. This turned out not be the case. As mentioned above, the organism possibly also acted as a backbone for the attachment of other organisms. Moreover, *Meganema* was favoured by similar conditions than *Azoarcus*, *Thauera* and *Zoogloea*. The reduction of the settling properties caused by *Meganema* might thus have been balanced by an increase of the settling properties occasioned by the other three organisms.

Finally, the negative correlation between *Dechloromonas* and the VSS is most probably due to external reasons. *Dechloromonas* presumably decreased due to the change of substrate when starting up the reactors, while the VSS started increasing at the same time due to the new SBR operation mode. If more data was available, the first weeks after the start-up would lose importance and the correlation between *Dechloromonas* and the VSS might change.

These results shed light on four difficulties that arose when interpreting the correlations:

1. The sign and strength of the correlations between the microbial genera and the related physical characteristics and nutrient removal performances of the AGS depended on the influent composition. Correlations thus had to be computed separately for each AGS system.

2. Even when significant correlations between the microbial communities and nutrient removal performances were detected, there was only sparse information on the metabolisms and cell properties of most detected genera that might explain the links.

3. The pairwise correlations were not necessarily due to causal relationships between the variables but sometimes depended on indirect effects or, presumably, microbial interactions. Moreover, the effect of the change of substrate and reactor operation mode during the start-up of the reactors still had an impact on the correlations.

4. Almost all statistically significant correlations were made between microbial genera and the physical sludge characteristics. Presumably, it the functional redundancy of the microbial communities made it challenging to find clear relationships between the microbial genera and the nutrient removal performances.[10] Moreover, the presence of an organism did not allow direct conclusions about its activity in the AGS systems, further impeding the interpretation of the correlations.

Despite these challenges, it will be interesting to use the correlations to make hypotheses on yet unknown functions of the microorganisms in AGS and determine which clusters of microorganisms should be favoured once more data will allow to confirm the preliminary results in Appendix J.

4.3 Start-up and granulation time

Successful start-up as defined in Subsection 3.2.3 was observed after less than 2 months for the reactors fed by synthetic substrate (Table 4.3). The SVI_{30} were below $70\,mL\,g^{-1}$ after less than one week of operation and the criteria for nutrient removal performances were also reached after two weeks in R2 (complex synthetic substrate) to four weeks in R1 (simple synthetic substrate). The slowest process was the formation of granules. However, these results indicate that the presence of biomass with a diameter larger than 0.25 mm is not a necessary condition for AGS with a good settleability and nutrient removal performances.

By the end of the project, none of the three conditions were met by the reactors fed by municipal wastewaters. While the SVI_{30} was below $70\,mL\,g^{-1}$ in R4 (raw wastewater) and slightly above in R3 (primary effluent wastewater), the condition on the $SVI_{30/5}$ was not met by either. This means that the sludge was compact, but nevertheless settled relatively slowly. The fraction of granules remained below 80 % on both reactors. Especially in R4, however, this fraction stabilised somewhat above this threshold, and it is not clear if the criterion will be met in the future. Removal performances in terms of COD, ammonium and nitrite were met by both reactors with municipal wastewater. Phosphorus removal remained poor throughout the study but started increasing towards the end of the project. The criterion on nutrient removal is thus expected to be met in the future. The detailed results for each criterion can be found in Appendix H.

Results show that the influent composition strongly affected the start-up time of the AGS systems. The different kinetics of granulation could presumably be attributed to differences in the influent COD and the presence and absence of particulate substrate. It is hypothesized that increased VFA contents result in faster granulation, as PHA-storing organisms - many of which produce EPS - were favoured. However, the presence of VFA is not indispensable, as a variety of organisms storing more complex carbon sources like glucose or amino acids have been discovered recently. The presence of particulate matter slowed down granulation

[10] This observation is well in accordance with the PCAs on the physical sludge characteristics and nutrient removal performances. Different microbial communities led to distinct physical sludge characteristics. In contrast, the nutrient removal performances were similar even for very different microbial communities.

Table 4.3: Time to reach success criteria (in weeks) for the start-up of four AGS SBRs fed by different influents. SS: synthetic substrate. WW: wastewater. Colours correspond to criterion met (green), mostly met (orange), criterion not met (red).

Criterion	**R1: simple SS**	**R2: complex SS**	**R3: primary effluent WW**	**R4: raw WW**
Settling properties	1	1	not met	not met
Fraction of granules	7	7	not met	not met
Removal performances	4	2	not met	not met
Start-up time	7 weeks	7 weeks	$>$ 14 weeks	$>$ 14 weeks

kinetics. Studies showed that particulate matter can not be fully degraded and stored in anaerobic conditions and leaks into the aerobic stage, where it is available for OHO. This leaked particulate matter is hypothesized to be captured by flocs during the aerobic stage, thus explaining the higher proportion of flocs in AGS systems fed by municipal wastewater [67].

Overall, reactors fed by municipal wastewater with low VFA concentrations and high particulate substrate contents required significantly longer start-up times than reactors fed by synthetic substrates containing high proportions of VFA. However, the experimental set-up did not allow to distinguish between the different effects of the influent (VFAs, fermentable substrate, particulate organic substrate), as the relative proportions of the carbon sources were not varied during the experiment.

5

Conclusions and outlook

5.1 Effect of influent composition on the microbial communities and granulation process

The present study aimed at improving the understanding of the effect of influent composition on the microbial communities and granulation process in AGS systems. Four AGS SBRs were started up in parallel with increasingly complex influent compositions. The four influents were (i) a mixture of VFAs (acetate, propionate), (ii) a mixture of VFAs (acetate, propionate), fermentable substrate (glucose, amino acids) and particulate organic matter (starch, peptone), (iii) primary effluent municipal wastewater, and (iv) raw municipal wastewater. The knowledge gained on the effect of influent composition led to the following main findings:

1. **Microbial composition.** Influent composition significantly affected the microbial composition by providing distinct ecological niches. Due to a high proportion of fermenters, the microbial communities fed by the synthetic substrate containing fermentable and particulate substrate were more similar to the communities fed by municipal wastewater than to the communities fed only by VFAs.

2. **Differences between granules and flocs.** Granules and flocs were largely composed of the same genera, but with different relative abundances. In the SBRs fed by synthetic wastewaters, the differences between granules and flocs increased over time. This evolution was not observed in the SBRs fed by municipal wastewater. This could be either due to slower granulation kinetics in the reactors fed by municipal wastewater (and hence a slower increase of the dissimilarity) or to persistent differences in granule sizes, with small granules being more similar to flocs.

3. **Microbial diversity.** The diversity of the microbial communities was believed to be positively linked to substrate complexity. While the diversity of the microbial community was indeed higher in the SBRs fed by municipal wastewater than in the ones fed by synthetic substrate, this pattern was not observed within the group of synthetic substrates. It was hypothesized that the effect of substrate complexity was balanced

by a faster granulation process in the SBR fed by only VFAs, resulting in the creation of new ecological niches within the granules and thus in a higher diversity in spite of the simpler substrate composition.

4. **Physical sludge characteristics and nutrient removal performances.** Even with distinct microbial compositions, the AGS in both SBRs fed by synthetic substrates had excellent settling properties and achieved good nutrient removal performances. The reactor fed by raw wastewater gradually shifted towards this first group, though none of the success criteria was reached at the end of this project. In spite of a similar microbial composition, the AGS fed by primary effluent wastewater did not show this shift and it is unclear if it will do so in the future. Influent complexity was thus not per se an issue for growing AGS with increasingly good settling properties and removal performances, but low COD and nutrient concentrations could be.

5. **Start-up time.** Influent composition strongly affected the kinetics of granulation. Start-up time in terms of settling properties, fraction of granules and nutrient removal performances was significantly longer in the SBRs fed by municipal wastewater. As good settling properties and nutrient removal performances could be achieved with sludge that was predominantly in flocculent form, the criterion on the fraction of granules should possibly be dropped. This is supported by the fact that the use of a minimum diameter to distinguish between granules and flocs did not always reflect differences in the microbial composition. It might be more adequate to focus on density measurements instead.

5.2 Recommendations for the start-up of AGS SBRs

The study lead to the following recommendations for the start-up of AGS SBRs:

1. **Washout conditions for AGS SBRs fed by synthetic substrates.** The exertion of washout conditions to enhance the granulation process is often promulgated. Contrarily to results from other studies, COD and nutrient removal performances could be maintained during the granulation process in the reactors fed by synthetic substrates. It is hypothesized that this was due to less stringent wash-out conditions allowing for an increase of the VSS. The quantity of sCOD leaking into the aerobic stage was thus reduced, avoiding the proliferation of OHO detrimental to the granulation process.

2. **Washout conditions for AGS SBRs fed by municipal wastewater.** Avoiding the complete washout of flocs was even more important in the reactors fed by municipal wastewater, where the microbial compositions of granules and flocs were highly similar. Removing flocs from the system would thus strongly have decreased the potential of granule formation by flocs densification. Especially the results observed in the reactor fed by raw wastewater indicate that the development of increasingly fast-settling and dense sludge with improving nutrient removal performances is also possible with an important proportion of flocs.

5.3 Limitations

The conclusions drawn in the present report were limited by three main challenges:

1. **Limited duration and data.** (i) The granulation process in the SBRs fed by municipal wastewaters was not completed by the end of the project and it was not clear how the systems would evolve in the future. (ii) As the main analyses were performed separately for each of the four systems, they were based on only 12 data points each (number of sludge samples analysed by amplicon sequencing). This was generally not sufficient to find statistically significant correlations between the microbial communities and their physical characteristics and nutrient removal performances or to apply other numerical ecology methods testing for causal relationships. (iii) Many of the hypotheses trying to explain the findings could not be validated due to the lack of data. For instance, the municipal wastewaters were not characterized in detail, no density measurements were taken on the sludge and no separate activity tests for granules and flocs were performed.

2. **Measurement and method uncertainties.** Method uncertainty in particular affected the interpretation of the results from amplicon sequencing. (i) A rudimentary comparison of the results between two amplicon sequencing runs already indicated that results were significantly different but the lack of time did not allow for a detailed assessment of the differences. The main analyses were based on a run in which many taxa were not detected due to a low number of determined sequences. (ii) Relative abundances of taxa from amplicon sequencing were not compared to methods not involving DNA extraction and PCR amplification, which are known to significantly bias the results.

3. **Lack of control SBRs.** Reactor operation was not always optimal (clogged pumps, injection of air during the anaerobic stage, accidental losses of biomass, etc.). This particularly affected the reactor fed by primary effluent wastewater. It is not clear if its poor performances were due to the loss of biomass or to the influent composition. Similarly, the sludge from the reactor fed by raw wastewater was lost before the end of the experiment, and therefore it will not be possible to assess the continuation of the first trends observed in the present study.

Many of these limitations are addressed in the proposed follow-up of the experiment.

5.4 Follow-up

Due to the major limitations of the present report, it should be viewed as a preliminary study to be used as a base in order to establish an improved experimental setting and answer more detailed research questions in the future:

1. **Effect of influent composition on the microbial communities and granulation in AGS.** A more detailed characterization of the municipal wastewaters (for instance in terms of VFA contents) is necessary if a deeper insight into the effect of the influent

composition on the microbial communities is desired. The current experimental set-up does not allow for a distinction between the effects of the different types of compounds (VFA, fermentable substrate, particulate substrate). To understand the effect of each, it would be necessary to systematically vary the relative proportion of the compounds in the synthetic substrates.

2. **Difference between granules and flocs in AGS.** Density measurements and separate activity tests for granules and flocs could contribute to the elucidatation of the differences between granules and flocs.

3. **Linking microbial communities with the physical sludge characteristics and nutrient removal performances.** More data on the microbial communities and their related physical characteristics and nutrient removal performances will allow to confirm the correlations between the microbial community and their physical characteristics and nutrient removal performances only rudimentary approached in the present study. qFISH could contribute to a better understanding of how the microbial community data provided from amplicon sequencing can be interpreted.

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A

Composition of the trace element solution

Table A.1: Composition of the trace element solution. The solution was diluted 1/2000 in the influent.

Compound	Molecular formula	Mass concentration ($g\,L^{-1}$)
EDTA disodium salt dihydrate	$C_{10}H_{14}N_2Na_2O_8{\cdot}2\,H_2O$	16.215
Zinc sulfate	$ZnSO_4{\cdot}7\,H_2O$	0.44
Manganese(II) chloride	$MnCl_2{\cdot}6\,H_2O$	1.012
Ammonium iron(II)	$(NH_4)_2Fe(SO_4)_2{\cdot}6\,H_2O$	7.049
Ammonium molybdate	$(NH_4)_6Mo_7O_{24}{\cdot}4\,H_2O$	0.328
Copper(II) sulfate	$CuSO_4{\cdot}5\,H_2O$	0.314
Cobalt chloride	$CoCl_2{\cdot}6\,H_2O$	0.322

B

Results of the preliminary amplicon sequencing run

A preliminary amplicon sequencing run was performed with samples from R1 (simple synthetic substrates) and R4 (raw wastewater). The focus was set on the granules to assess the evolution of the microbial communities over time. The first and last floc samples were also sequenced to assess whether an increase of the dissimilarity between granules and flocs could already be observed over a short time span of 27 days. The results of the first run are presented in Figure B.1. As the inoculum was not sieved it is presented as the initial state of both, granules and flocs.

Initially, the data providing from this first run was meant to be included in the global analysis of the microbial communities. However, the average number of sequences was 114'374 in the first run against only 6'040 in the second run. As a consequence, 457 different genera were detected in the first run but only 111 genera in the second. This difference was partly due to a higher number of samples sequenced in the second run (96) compared to the first run (60), though this difference is not sufficient to explain the imbalance between the two runs.

The inoculum was not sequenced in the second run and is thus described separately here. Only six genera had a relative abundance above 2 percent: *Dechloromonas* (8.9 %), *Tetrasphaera* (5.0 %) and *Nitrospira* (3.5 %), a member of the family *Chitinophagaceae* (2.7 %), *Propionivibrio* (2.3 %), and a member of the family *Saprospiraceae*, (2.1 %). In total, 25 genera presented had a relative abundance above 1 %, representing around half of the total abundance.

Appendix B. Results of the preliminary amplicon sequencing run

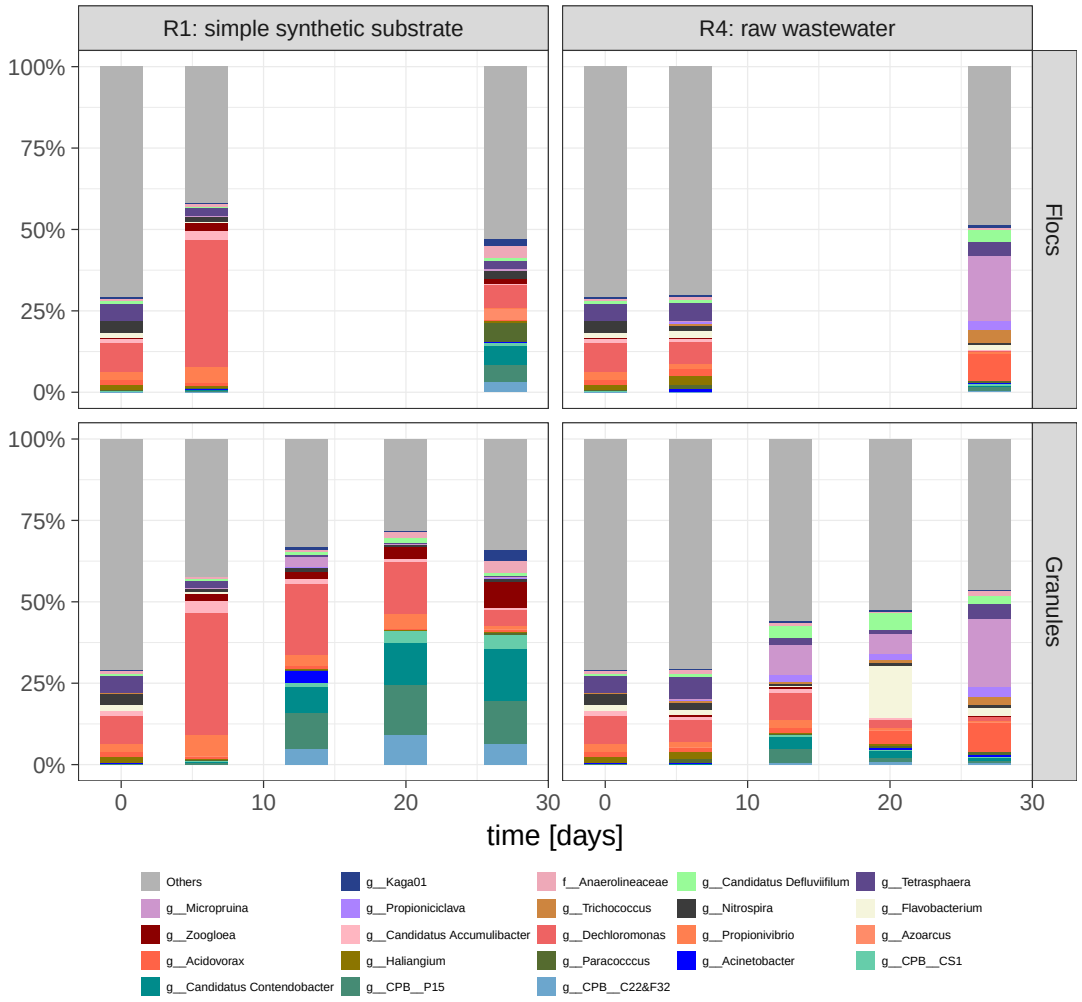


Figure B.1: Evolution of the relative abundances of genera (or lowest known taxonomic order) in flocs and granules providing from a preliminary amplicon sequencing run. Only genera with a relative abundance above 3 % in at least one of the samples are displayed separately.

C

Evolution of total suspended solids (TSS) and volatile suspended solids (VSS)

Figure C.1 presents the evolution of the TSS and VSS. R1 (simple synthetic substrate) and R2 (complex synthetic substrate) both showed a constant increase of TSS from $2\,g_{TSS}\,L^{-1}$ to $4\,g_{TSS}\,L^{-1}$. R3 (primary effluent wastewater) exhibited a sudden decrease of TSS on day 27, when TSS dropped as low as $1.5\,g_{TSS}\,L^{-1}$. This decrease was due to the loss of 3 L of biomass due to inappropriate handling of the reactor on day 23. However, the decrease continued until the reactor was operated at constant volume from day 41 on and TSS augment again. On day 56, a low critical settling velocity was applied again, but the increase of TSS continued. The increase of TSS was strongest for R4 (raw wastewater), where TSS exceeded $5\,g_{TSS}\,L^{-1}$. R4 was also the reactor with the highest total COD in the influent. It is not clear whether the higher TSS were partially due to the accumulation of inorganic particles, as the ratio of VSS/TSS was not significantly lower than in the other reactors.

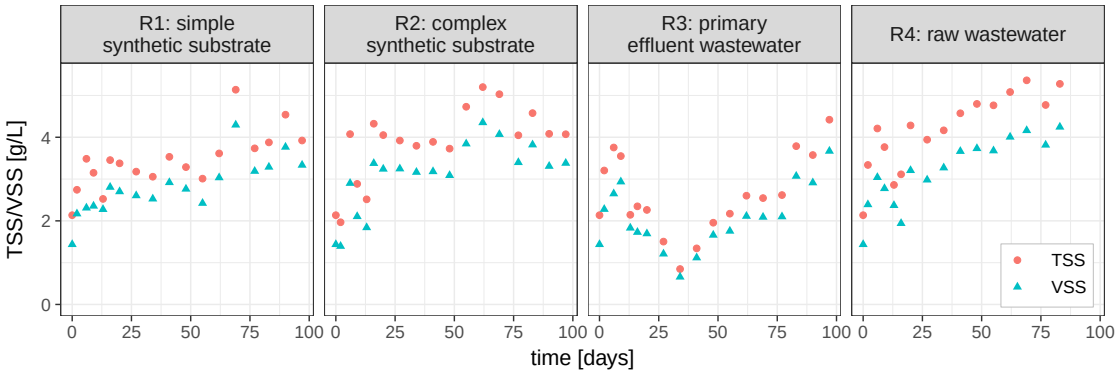


Figure C.1: Evolution of the total suspended solids (TSS) and volatile suspended solids (VSS) during the start-up of four AGS SBRs fed by different influents.

D

Effect of data treatment

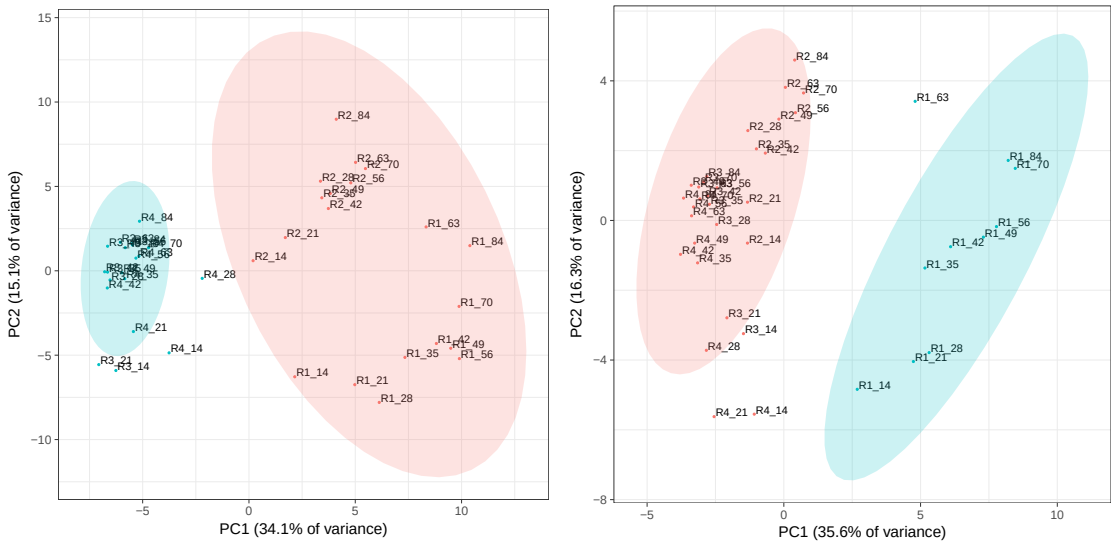
D.1 Microbial composition

In the main analysis, only genera with relative abundances above $0.035\,g_{VSS}\,L^{-1}$ (around $1\,\%$ of the average VSS at the end of the study) in at least one of the samples were considered in the computation of the PCA and K-means clustering. Figure D.1 presents the PCA and K-means clustering of the raw data (no threshold) and with a threshold of $0.07\,g_{VSS}\,L^{-1}$ (around $2\,\%$ of the average VSS at the end of the study). For both cases, two main clusters were detected with the Calinski-Harabasz clustering index (Appendix E):

1. For low thresholds, R1 and R2 were grouped together (Figure D.1a). When considering microorganisms present at low abundances, R2 was thus more different from R3 and R4 than from R1. It is hypothesized that this was due to the inflow of new biomass present in the influent wastewaters in R3 and R4. This constant supply of various low-abundance microorganisms was absent in R2.

2. For high thresholds, R1 formed a distinct group from the three other reactors (Figure D.1b). Microorganisms present at high abundances were hence more different between R1 and R2, than between R2 and the reactors fed by municipal wastewaters. This was due to the importance of fermenters like *Tetrapshaera* and *Micropruina* that were present in high abundances in R1, R2 and R3 but almost absent in R1.

No threshold value performs better or worse: the adequate data treatment depends on the specific research interest and gives complementary information.



(a) no threshold **(b)** threshold 0.07 $g_{VSS}\,L^{-1}$

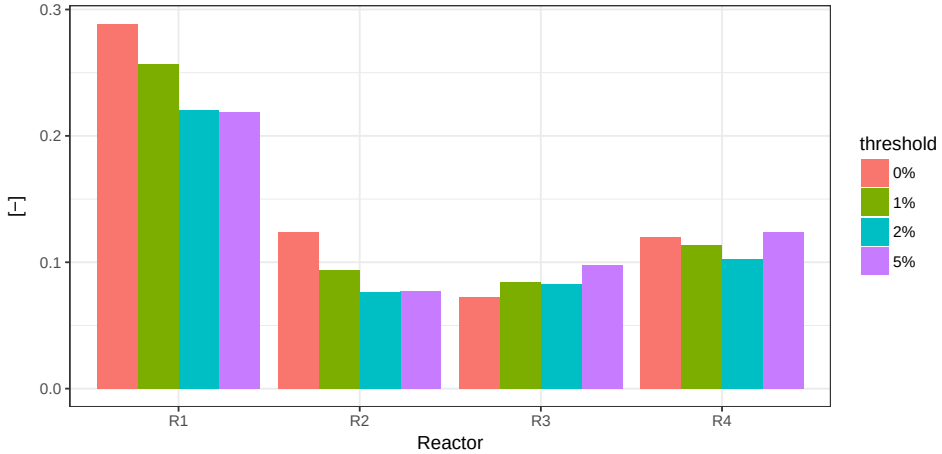
Figure D.1: Principal component analysis (PCA) of the Hellinger-transformed mean microbial community from day 14 to 84. Only genera with a total abundance above 0.035 $g_{VSS}\,L^{-1}$ in at least one of the samples were included in the analysis. Coloured areas represent 95 % confidence ellipses of a K-means clustering. The text labels combine the reactor names and the number of days after start-up. R1: simple synthetic substrate. R2: complex synthetic substrate. R3: primary effluent wastewater. R4: raw wastewater. PC: principal component.

D.2 Dissimilarity between granules and flocs

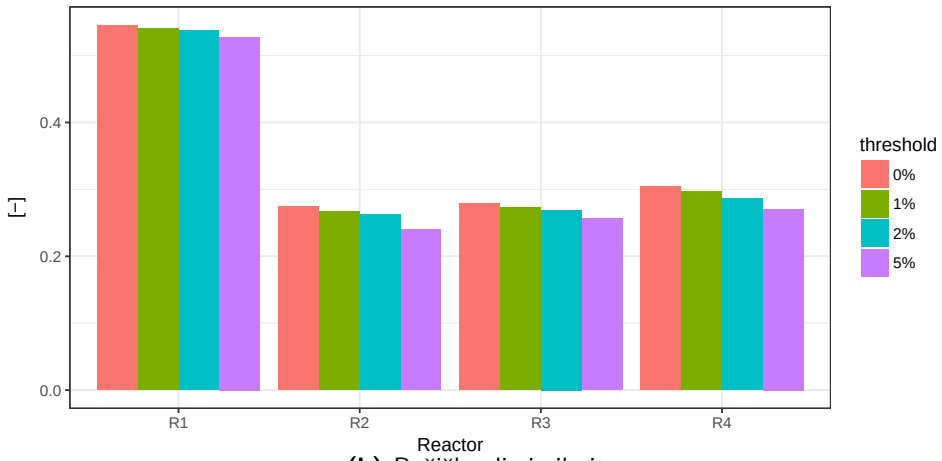
In the main analysis, only genera with a relative abundance above 1 % in at least one of the samples were considered in the calculation of the dissimilarities between granules and flocs. The effect of thresholds between 0 % (raw data) and 5 % were evaluated (Figure D.2).

Data treatment had a major effect on the Jaccard dissimilarity, as the index is based on binary presence/absence data (Equation 3.11). For low thresholds, the presence of different low-abundance species in granules and flocs increased the dissimilarity, though the general trends remained the same. For high thresholds, the dissimilarity generally decreased in the reactors fed by synthetic substrates, as the main genera in flocs and genera were the same throughout the experiment. For thresholds above 5 %, the variance of the index strongly increased, as the passing of a single genus below or above the threshold strongly affected the overall dissimilarity. In this case, no trends could be detached for the Jaccard dissimilarity.
The Ružička dissimilarity indices were only slightly affected by the value of the threshold, as the index is based on relative abundances (Equation 3.12). For low thresholds, the additional genera included in the calculation were present at very low abundances and thus did not affect the index significantly. For high thresholds, only the genera that had also previously dominated the index were included in the calculation and the index remained stable. Overall, the dissimilarity decreased for higher thresholds. The distribution of microorganisms present

at low abundances was more different between flocs and granules than the distribution of the microorganisms present at higher abundances.



(a) Jaccard dissimilarity



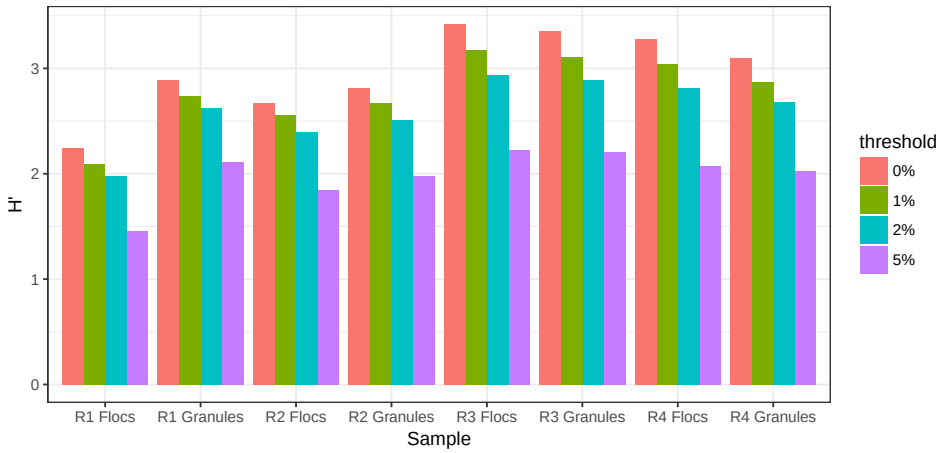
(b) Ružička dissimilarity

Figure D.2: Effect of data treatment on the mean values of the Jaccard and Ružička dissimilarity indices. Genera with a relative abundance above respectively 0 %, 1 %, 2 % and 5 % in at least one of the samples were taken into account in the calculations.

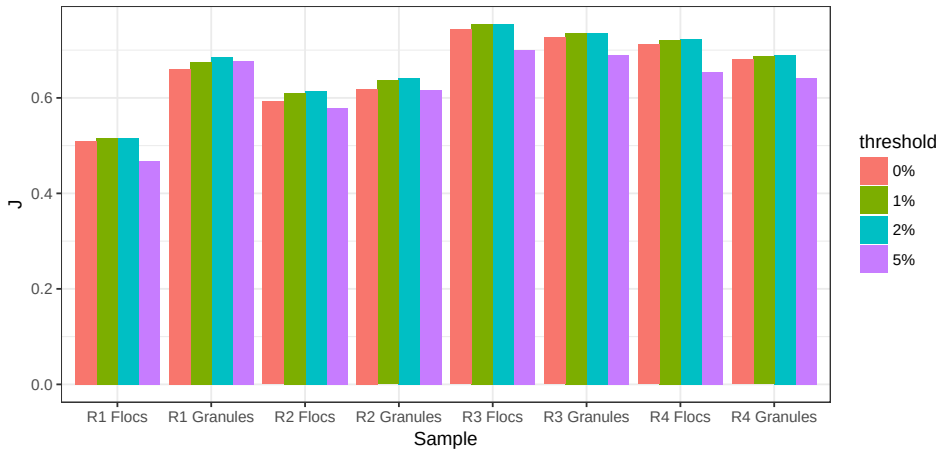
D.3 Microbial diversity

In the main analysis, only genera with a relative abundance above 1 % in at least one of the samples were considered in the calculation of the diversity indices. To evaluate the effect of data treatment, thresholds of 0 % (raw data) and 5 % were applied (Figure D.3). Though Shannon's H' was expectedly higher when considering the raw data and lower when applying a higher threshold, the general trends didn't vary. Similarly, Pielou's evenness J decreased

when considering the raw data and increased when applying a higher threshold, but the main trend remained the same.



(a) Shannon's H'



(b) Pielou's evenness J

Figure D.3: Effect of data treatment on the mean values of Shannon's H' and Pielou's evenness J. Genera with a relative abundance above respectively 0 %, 1 %, 2 % and 5 % in at least one of the samples were taken into account in the calculations.

E

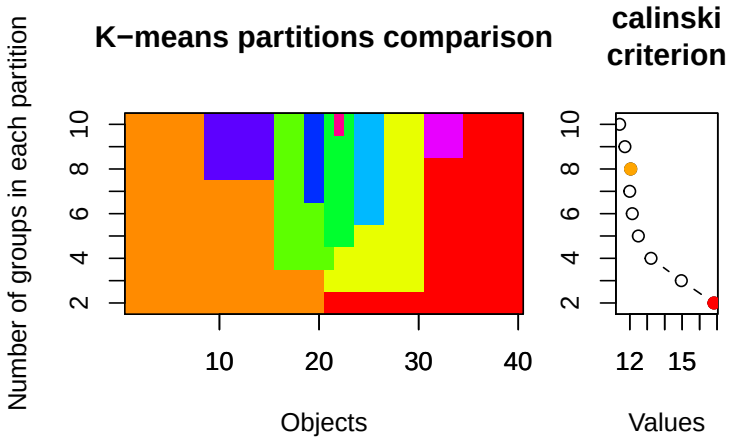
Clustering indices and clustering methods

Figure E.1 shows the K-means cascade plots (function cascadeKM from the R package *vegan*) used to determine the optimal number of clusters using the Calinski-Harabasz criterion. Figure E.1a will be used as an example of how to read the graphs. The plot on the left shows which objects (i.e. which microbial communities) would be grouped together for a certain number of groups. For two groups (y-axis), the first 30 microbial communities would form one group and the remaining 10 microbial communities a second group (x-axis). The first 30 microbial communities correspond to the communities from R2, R3 and R4. The remaining 10 microbial communities correspond to the communities from R1. If the data is partitioned into two groups, the microbial communities from R2 are thus more similar to R3 and R4 than to R1. The calinski criterion plot on the right allows to choose the number of cluster that minimizes the variance within a cluster and maximises the variance between clusters. The Calinski-Harabasz criterion is maximum for the optimal number of clusters. The optimal number of clusters for a threshold of $0.035\,g_{VSS}\,L^{-1}$ is thus 3 (marked in red).

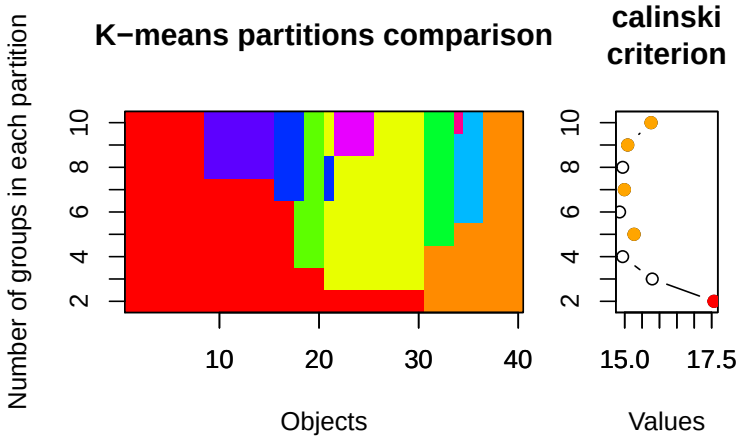
Figure E.2 shows the clustering of the microbial community using Ward's minimum variance method and Spearman's rank correlation to determine the optimal number of clusters. Spearman's rank correlation is maximum for the optimal number of clusters (2). Although this optimal number of groups is not identical with the number with the Calinski-Harabasz criterion, these results are in accordance with the K-means clustering, as the microbial communities from R1 forms a distinct group from the other reactors (Figure E.2 in Appendix E). R2 is more similar to the reactors fed by municipal wastewater, but forms a well-defined separate group.

Appendix E. Clustering indices and clustering methods

(a) threshold $0.035\,g_{VSS}\,L^{-1}$

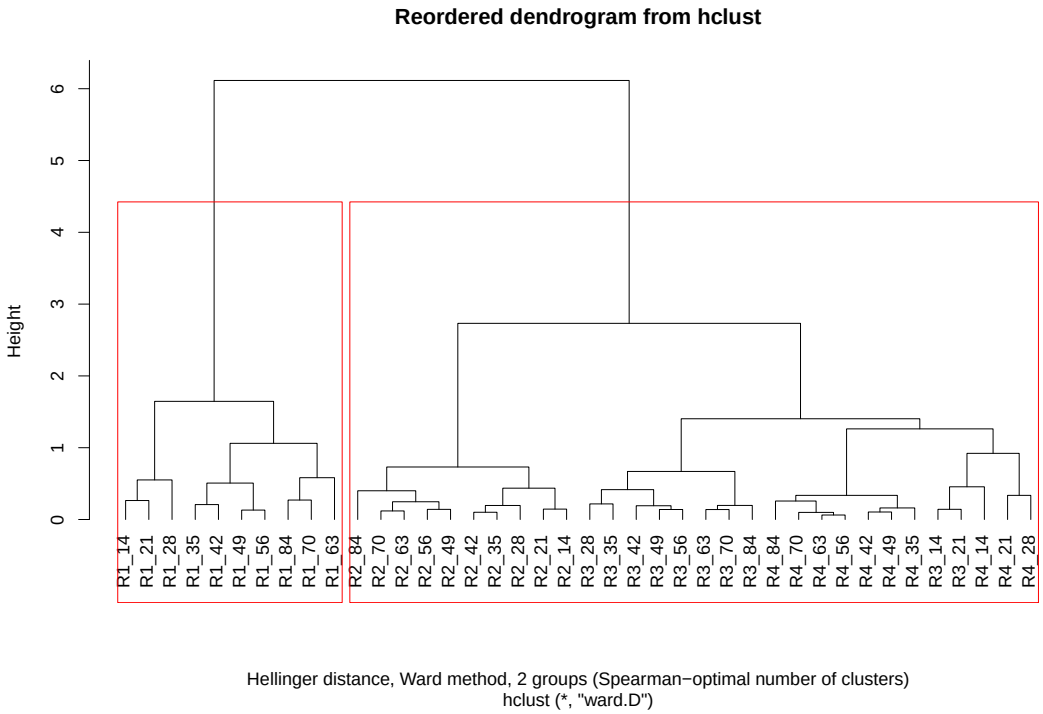


(b) no threshold

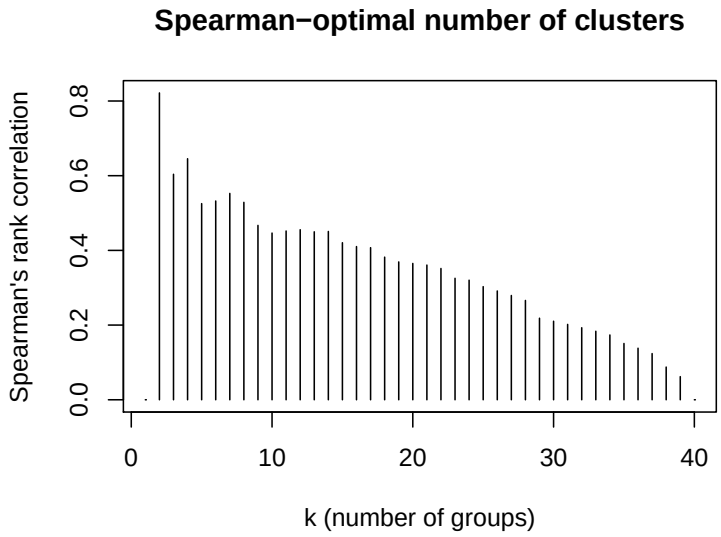


(c) threshold $0.07\,g_{VSS}\,L^{-1}$

Figure E.1: K-means cascade plot showing the partitioning of the Hellinger-transformed microbial communities with different thresholds. The Calinski Harabasz criterion points to an optimum of three groups for a threshold of $0.035\,g_{VSS}\,L^{-1}$ and two groups for no threshold or a threshold of $0.07\,g_{VSS}\,L^{-1}$ (red dots on the right plot).



(a) Ward's minimum variance method clustering



(b) Spearman's rank correlation

Figure E.2: Clustering of the Hellinger-transformed microbial communities from day 14 to 84 using Ward's minimum variance methods and Spearman's rank correlation to determine the optimal number of groups (maximum correlation for two groups).

F

p-values and effect of granulation on diversity

Pairwise t-tests (function pairwise.t.test from the R package *stats*) were used to compare the mean diversity, evenness and richness between granules and flocs from one reactor and between the granules from the four reactors. Table F.1 reports the p-values of these pairwise comparisons computed with the function pairwise.t.test from the R package *stats*. The mean values were considered different if the p-value was below a significance level of 0.05.

Table F.1a will be used to illustrate the differences between granules and flocs from one reactor. For instance, the p-value for the pairwise t-test between the granules and flocs from R2 for Shannon's H' was 0.656. Their mean diversities were thus statistically not different.

Table F.1b will be used to illustrate the differences between granules from two reactors. For instance, the p-value for the pairwise t-test between the granules from R1 and R3 for Pielou's evenness J was 0.012. Their mean evennesses were thus statistically different.

Figure 4.10 also provided information on the effect of granulation on microbial diversity that was not discussed in the main body of text. No prior hypothesis was made on how the granulation process would affect diversity in AGS compared to diversity in AS. Both, an increase or a decrease, would have been arguable. On the one hand, AGS provides a wide range of ecological niches due to substrate and DO gradients. Additionally, granules can harbour slow-growing organisms due to their high SRT. On the other hand, one might argue that only organisms capable of forming granules are enriched [76]. In the present study, diversity in the granules slightly decreased during the experiment, with the exception of the microbial communities in R1 where diversity remained stable. It is, however, possible that the decrease in R1 occured before day 2 (first biomass sample), as the initial diversity indices are lower than in the other reactors. The results indicate that both processes (loss of ecological niches through selection of fast-settling biomass and gain of ecological niches within the granules) could occur simultaneously with comparable effects (though in opposite directions). The difference in evolution between R1 and R2 supports this hypothesis. Even with a much simpler substrate composition in R1 (hence fewer ecological niches), diversity

in R1 stabilised at a higher value, due to the rapid growth of granules, harbouring genera that were present in abundances below the detection level before. In R2, in contrast, even with a more complex substrate composition, diversity in granules decreased, as the granules remained significantly smaller and could not provide the same range of ecological niches.

Table F.1: p-values of the pairwise t-tests on the mean diversity values between groups (separate by granules and flocs from the four AGS SBRs) with corrections for multiple testing using the Holm method.

(a) Shannon's H'

	R1 Flocs	R1 Granules	R2 Flocs	R2 Granules	R3 Flocs	R3 Granules	R4 Flocs
R1 Granules	0.213	NA	NA	NA	NA	NA	NA
R2 Flocs	0.596	0.512	NA	NA	NA	NA	NA
R2 Granules	0.325	0.656	0.656	NA	NA	NA	NA
R3 Flocs	0.014	0	0	0	NA	NA	NA
R3 Granules	0.021	0	0	0	0.598	NA	NA
R4 Flocs	0.033	0.04	0.005	0.012	0.598	0.656	NA
R4 Granules	0.091	0.54	0.061	0.139	0.005	0.04	0.596

(b) Pielou's evenness J

	R1 Flocs	R1 Granules	R2 Flocs	R2 Granules	R3 Flocs	R3 Granules	R4 Flocs
R1 Granules	0.112	NA	NA	NA	NA	NA	NA
R2 Flocs	0.696	0.065	NA	NA	NA	NA	NA
R2 Granules	0.381	0.063	0.724	NA	NA	NA	NA
R3 Flocs	0.021	0	0	0	NA	NA	NA
R3 Granules	0.032	0.012	0.001	0	0.696	NA	NA
R4 Flocs	0.049	0.696	0.012	0.021	0.696	1	NA
R4 Granules	0.11	1	0.078	0.152	0.025	0.152	0.723

(c) Richness

	R1 Flocs	R1 Granules	R2 Flocs	R2 Granules	R3 Flocs	R3 Granules	R4 Flocs
R1 Granules	1	NA	NA	NA	NA	NA	NA
R2 Flocs	0.272	0.022	NA	NA	NA	NA	NA
R2 Granules	0.12	0.002	1	NA	NA	NA	NA
R3 Flocs	0.028	0	0.035	0.093	NA	NA	NA
R3 Granules	0.02	0	0.011	0.021	1	NA	NA
R4 Flocs	0.027	0	0.032	0.093	1	1	NA
R4 Granules	0.12	0.027	1	1	1	1	1

G

Granule morphology

Figure G.1 presents granule morphology for three selected days (7, 34 and 72 days after start-up). The influent composition had a strong impact on the morphology of the granules. Granules in R1 (simple synthetic substrate) were spherical, comparatively large and had a uniform size distribution. Quite differently, various sizes of spherical granules coexisted in R2 (complex synthetic substrate) and a significant proportion of flocs remained in the system. In R3 (primary effluent wastewater) and R4 (raw wastewater), various types and sizes of granules coexisted with flocs. In both reactors, smooth and fluffy and spherical and elongated granules occured at the same time. A more extensive selection of stereomicroscopic images from day 7 to day 93 after the start-up can be found in the next pages.

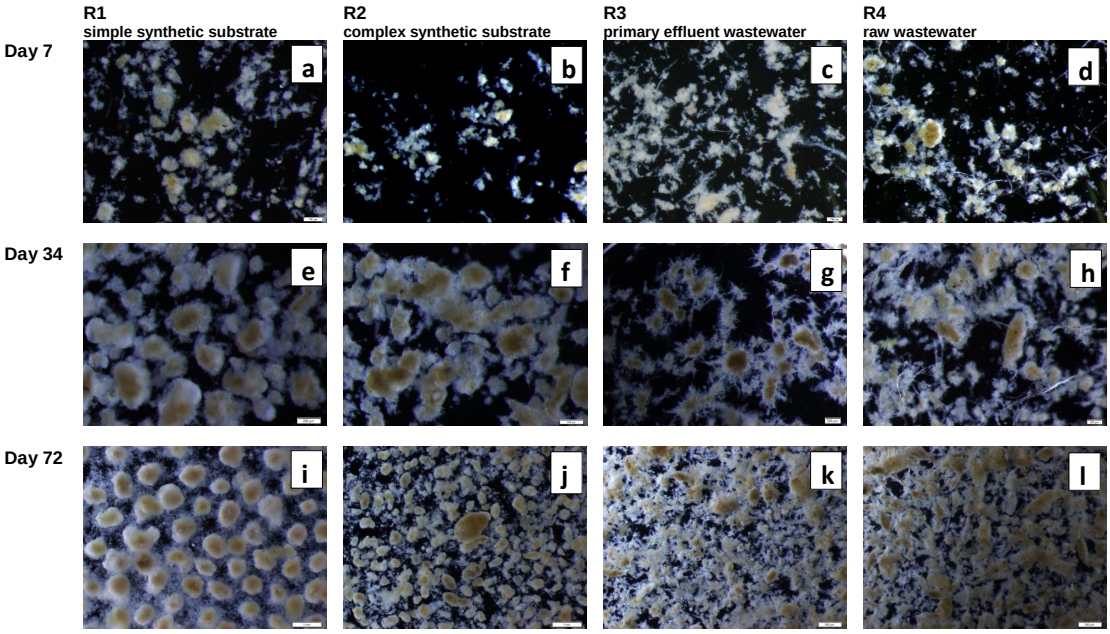
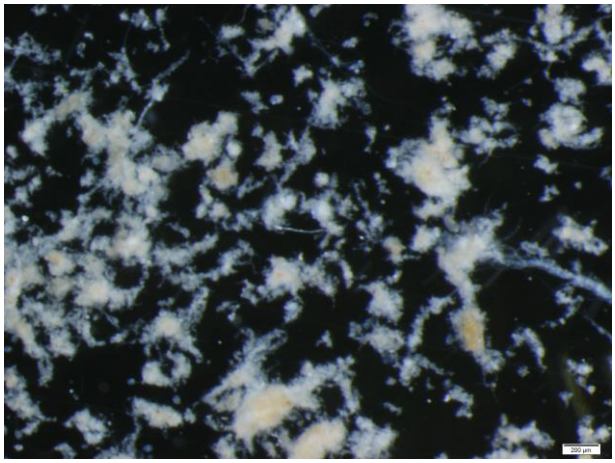
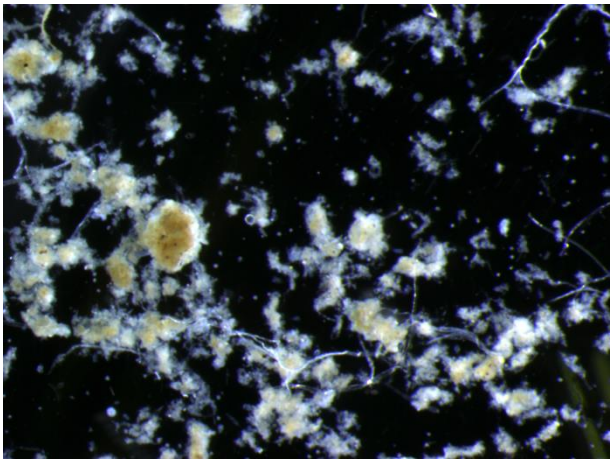
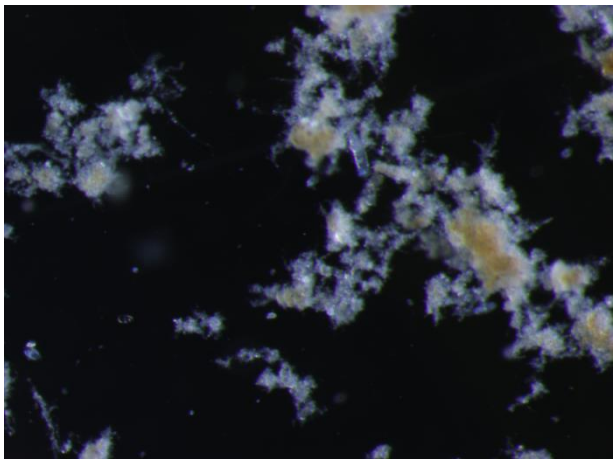
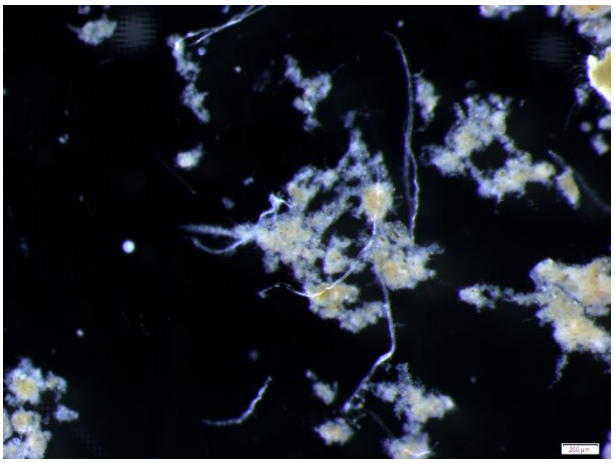
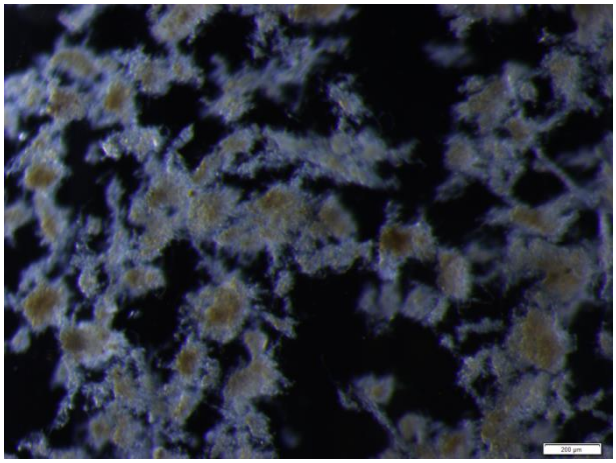
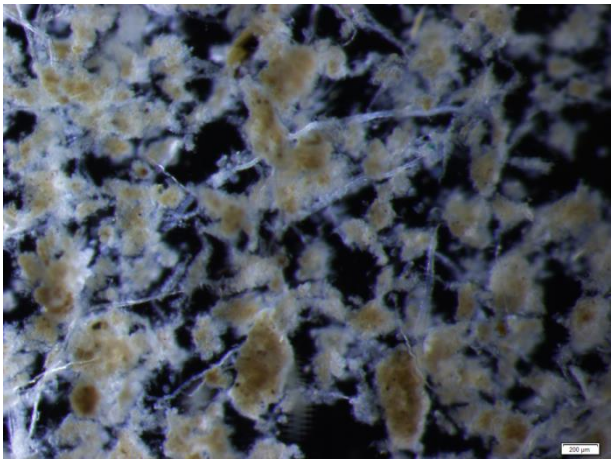
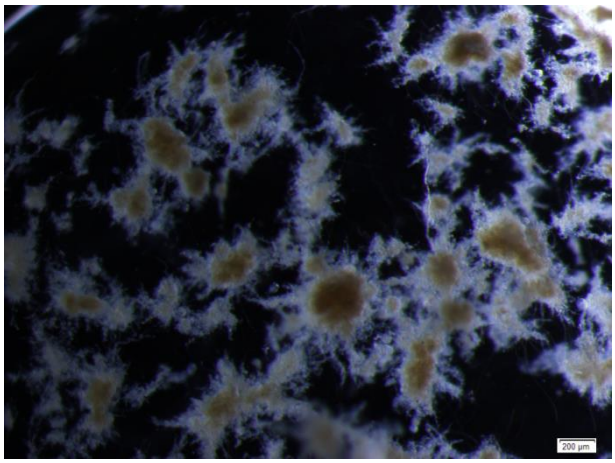
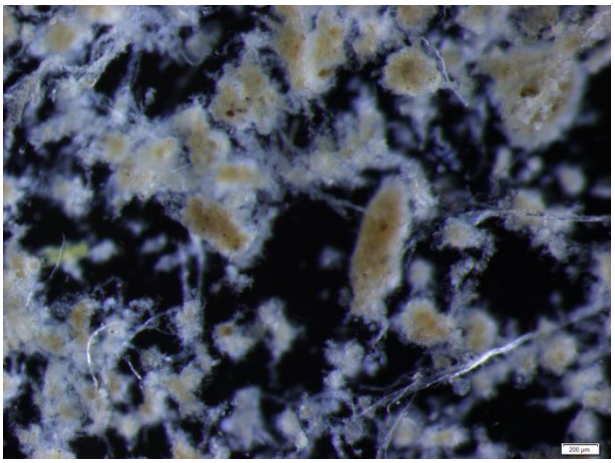
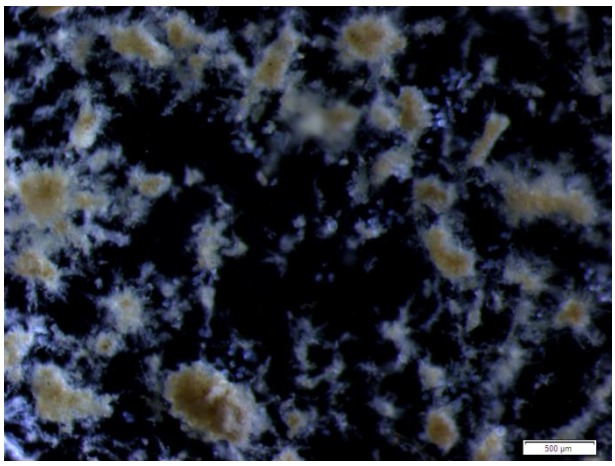
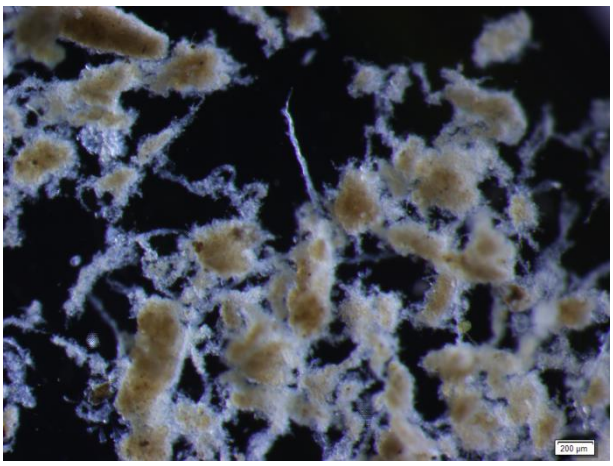


Figure G.1: Stereomicroscopic images of the granular sludge taken 7, 34 and 72 days after the start-up of four AGS SBRs fed by different influents. Scale bars represent 200 µm for images a-h, 1 mm for images i and j, and 500 µm for images k and l.

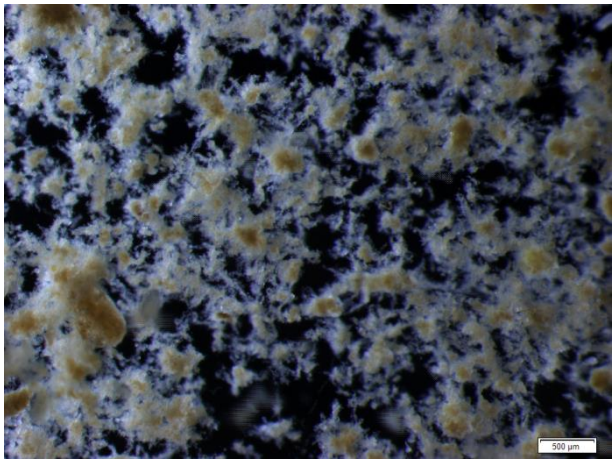
	R3: Primary effluent wastewater	R4: Raw wastewater
Day 7		
Day 12		
Day 22		
Day 34		

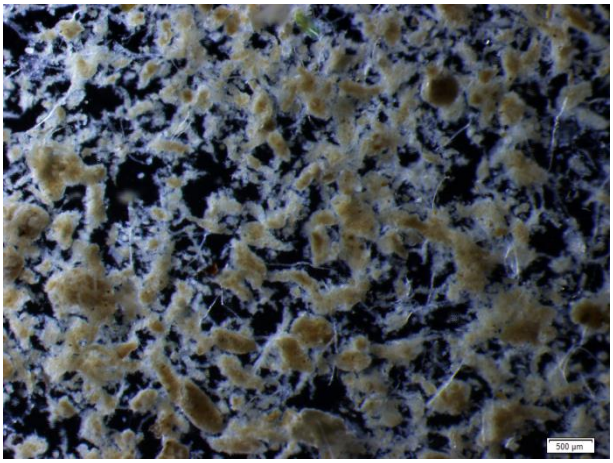
Day 43



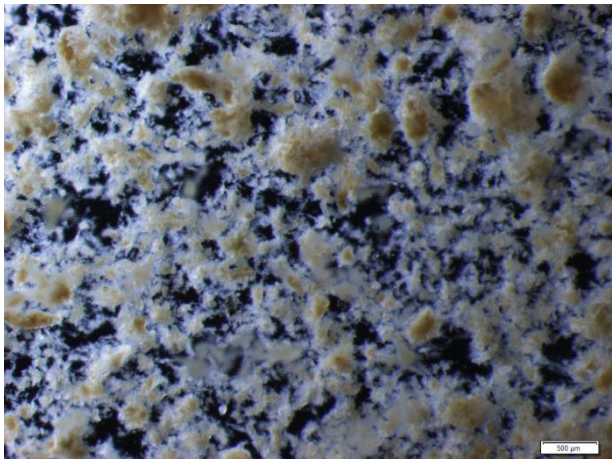


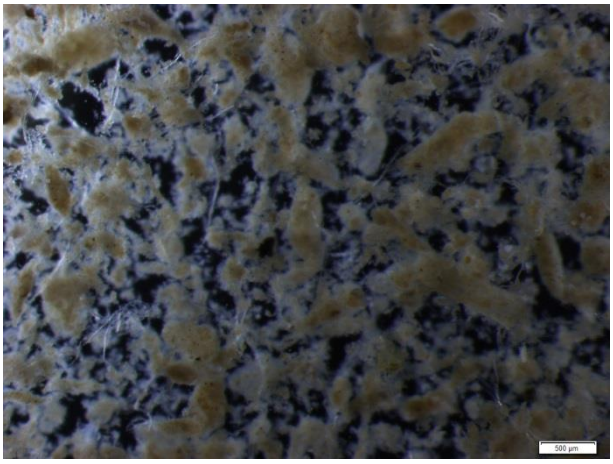
Day 57



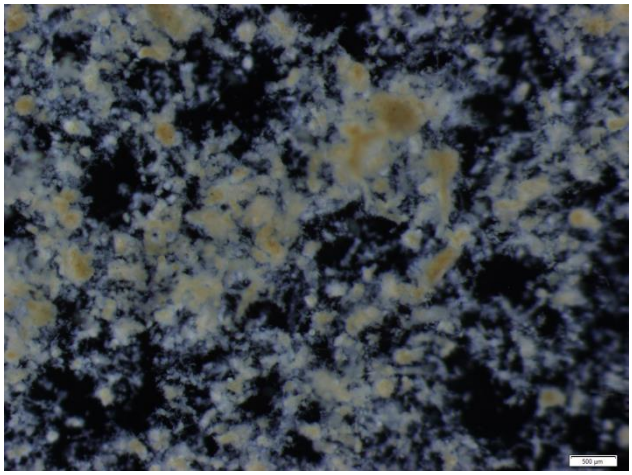


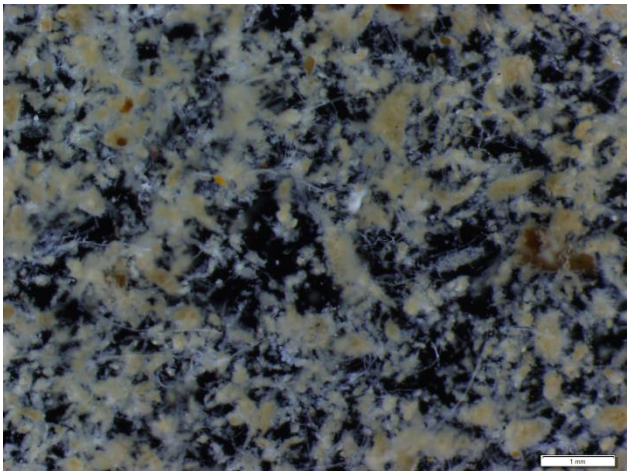
Day 72

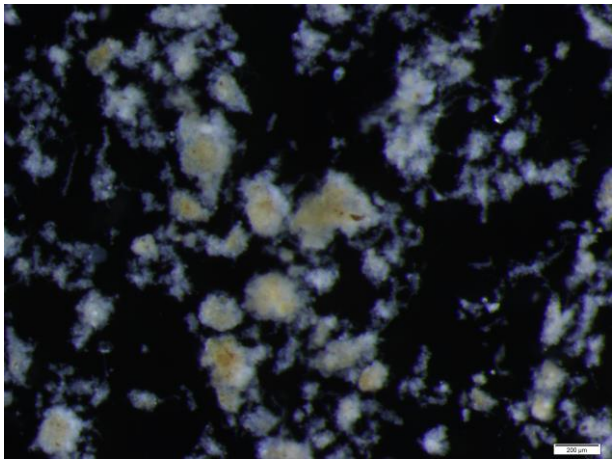
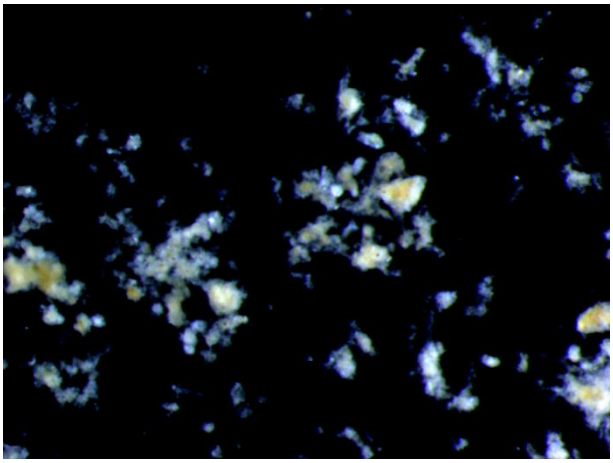
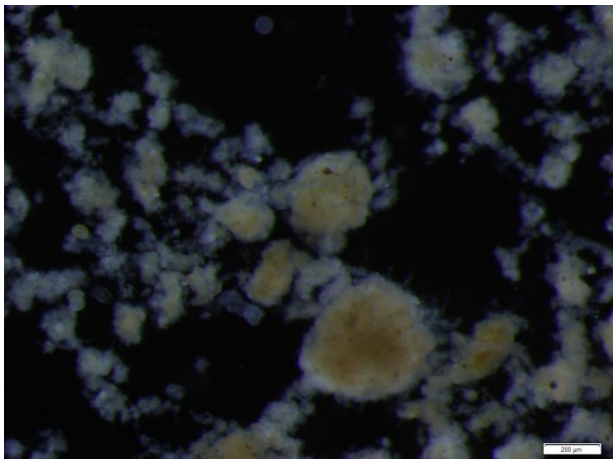
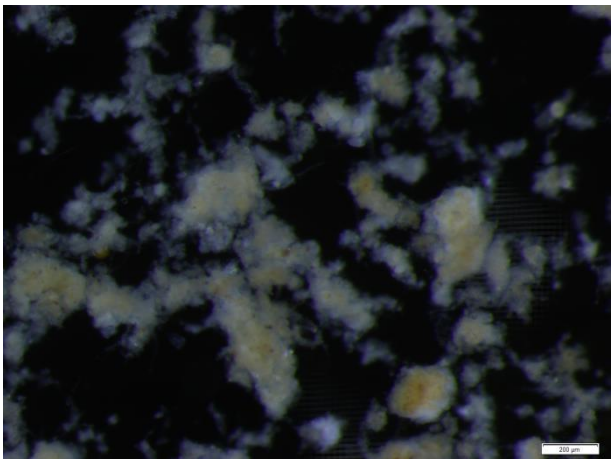
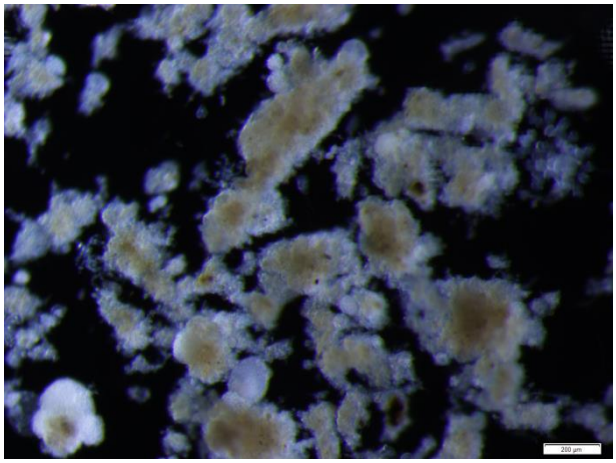
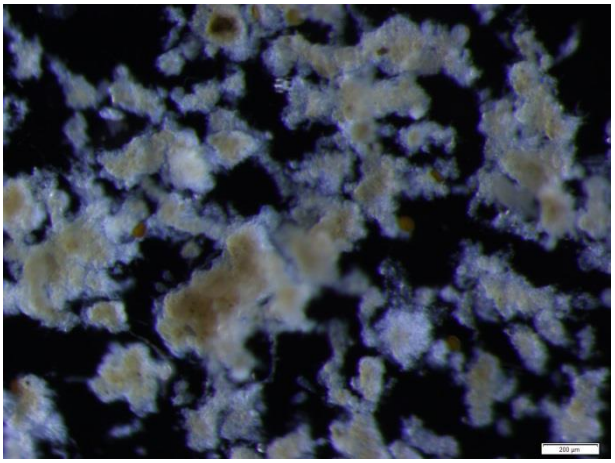
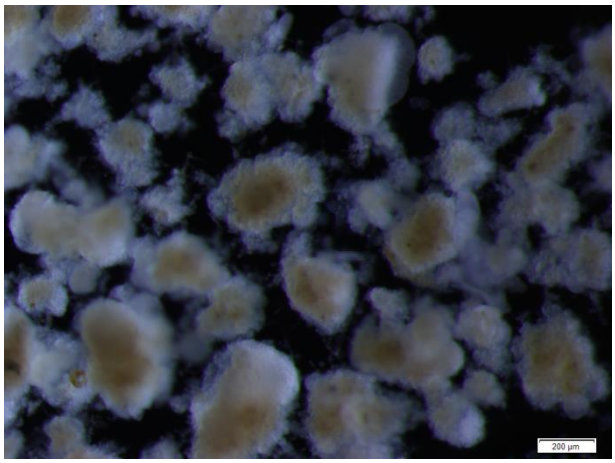
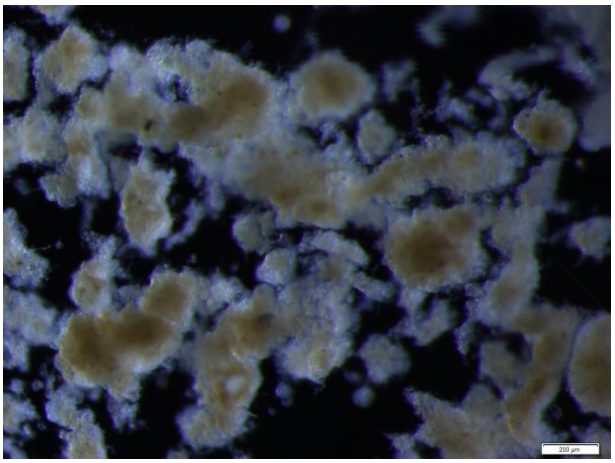




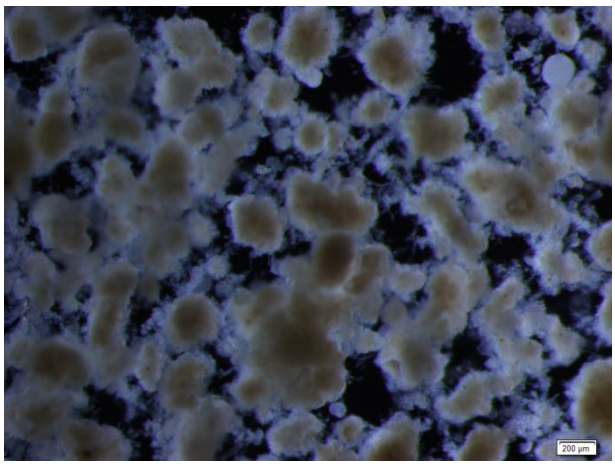
Day 93

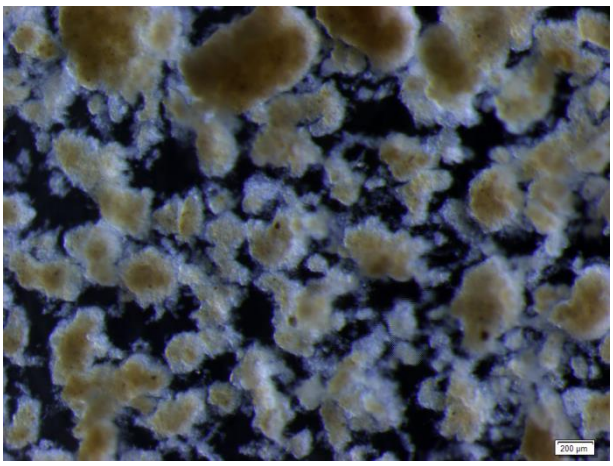




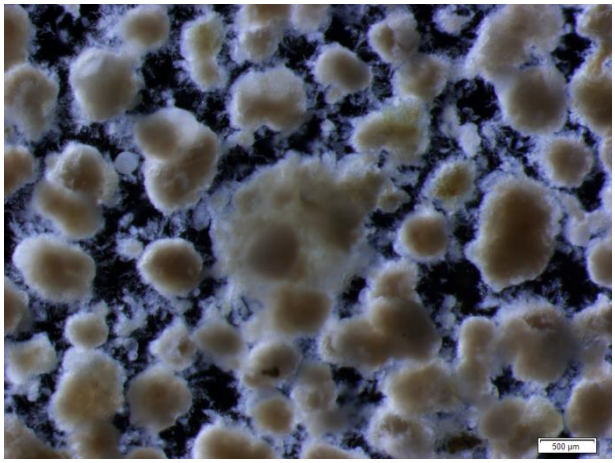
	R1: Simple synthetic substrate	R2: Complex synthetic substrate
Day 7		
Day 12		
Day 22		
Day 34		

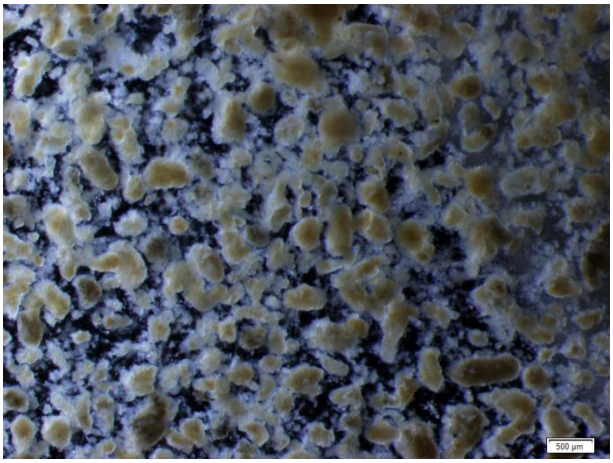
Day 43



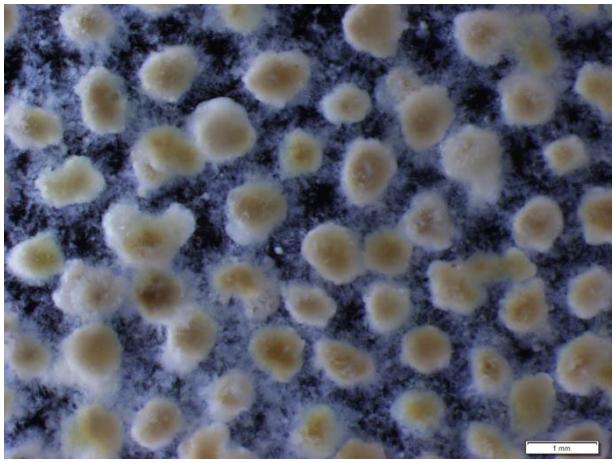


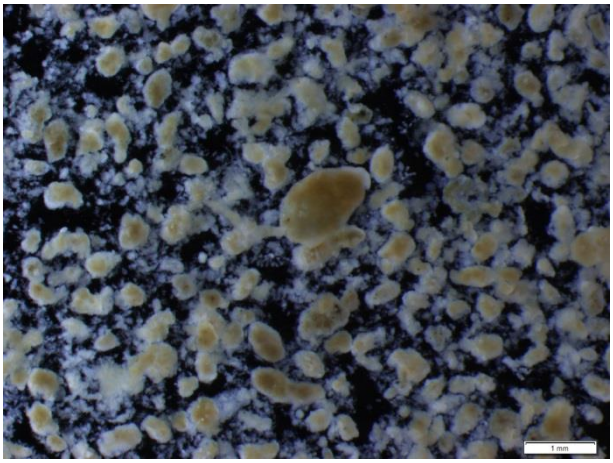
Day 57



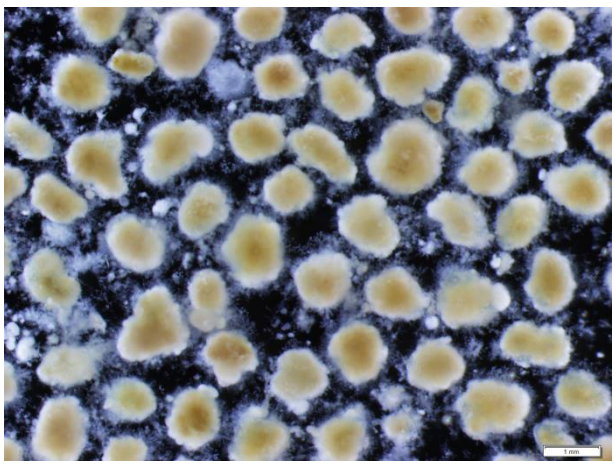


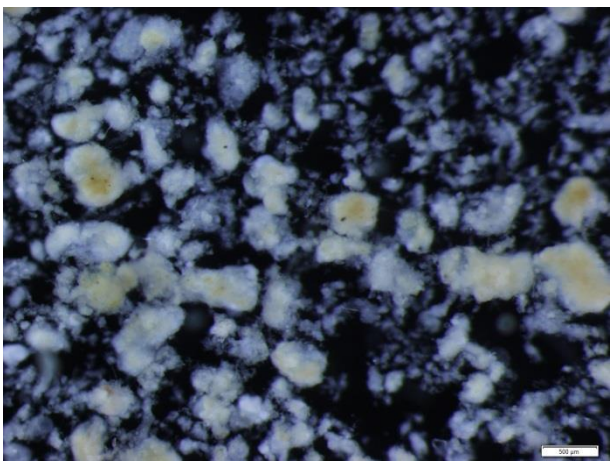
Day 72





Day 93





H

Start-up time: detailed results

Comparison of the removal effiencies and discharge concentrations of COD (Figure H.1), NH_4^+ (Figure H.2), TP (Figure H.3) and discharge concentration of NO_2 (Figure H.4) with the requirements stated in the WPO. The number of weeks necessary to meet the requirements for each reactor is listed in Table H.1.

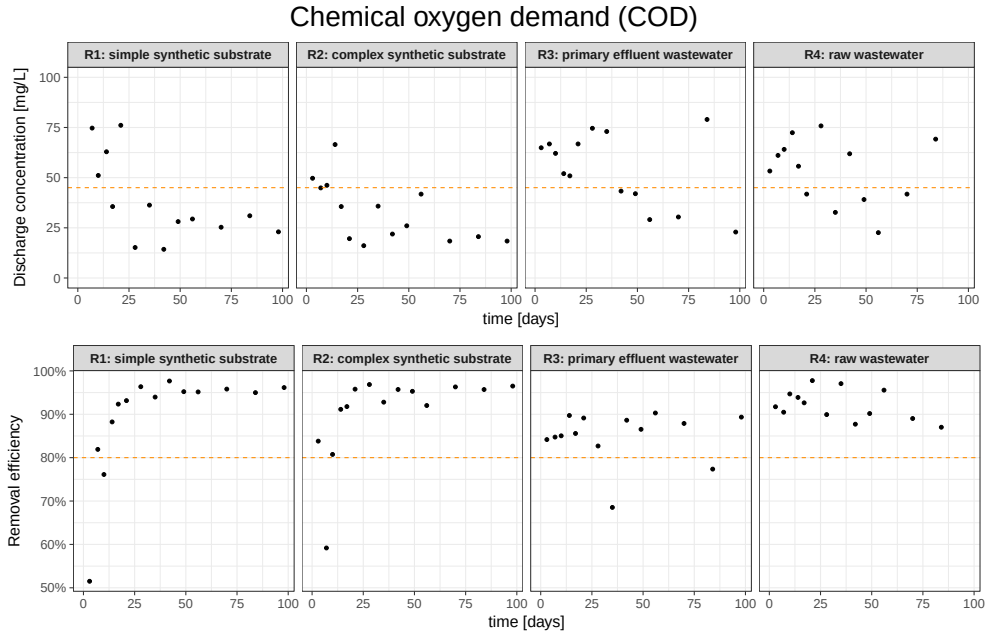


Figure H.1: Comparison of COD discharge concentrations and removal efficiencies (black dots) with the requirements defined in the Water Protection Ordinance (orange line) during the start-up of four AGS SBRs fed by different influents.

Appendix H. Start-up time: detailed results

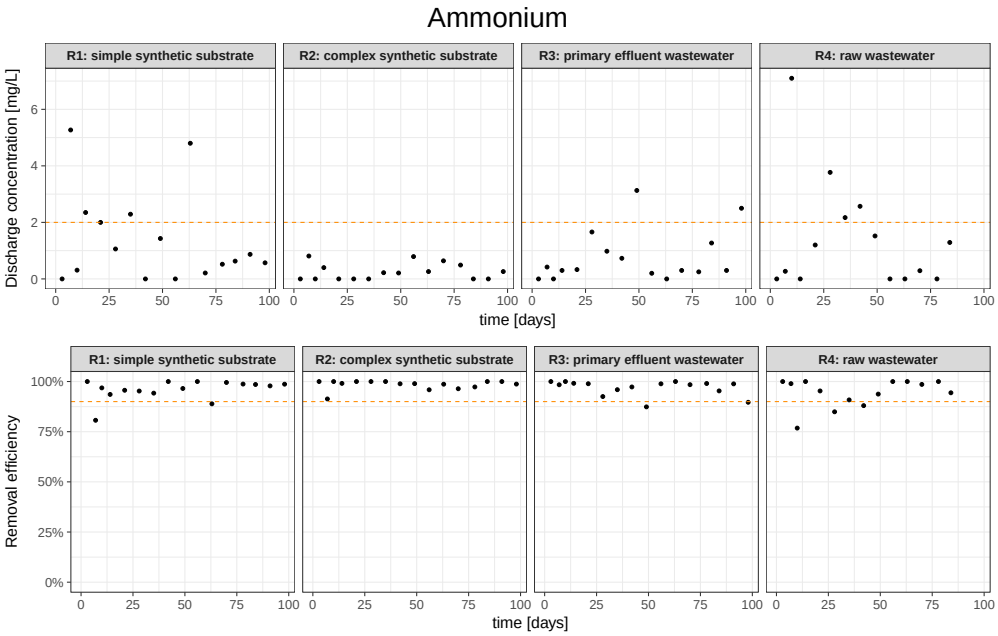


Figure H.2: Comparison of NH_4^+ discharge concentrations and removal efficiencies (black dots) with the requirements defined in the Water Protection Ordinance (orange line) during the start-up of four AGS SBRs fed by different influents.

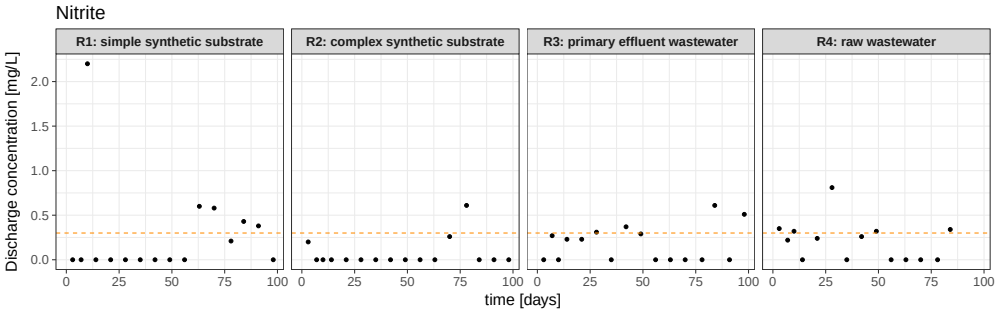


Figure H.4: Comparison of NO_2 discharge concentrations (black dots) with the requirements defined in the Water Protection Ordinance (orange line) during the start-up of four AGS SBRs fed by different influents.

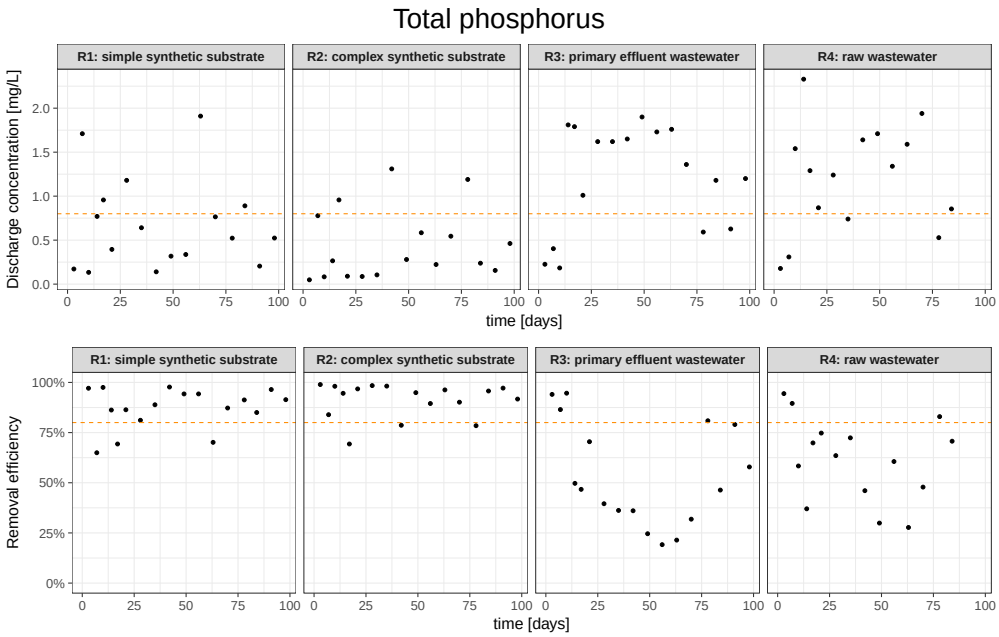


Figure H.3: Comparison of TP discharge concentrations and removal efficiencies (black dots) with the requirements defined in the Water Protection Ordinance (orange line) during the start-up of four AGS SBRs fed by different influents.

Table H.1: Time to reach success criteria (in weeks) for the start-up for four AGS SBRs fed by different influents. SS: synthetic substrate. WW: wastewater. (+): criterion not met in isolated cases. (++): criterion repeatedly not met.

Criterion	Value	R1: simple SS	R2: complex SS	R3: primary effluent WW	R4: raw WW
Settling properties					
SVI_{30}	$<70\,mL\,g^{-1}$	1	1	not met	5
$SVI_{30/5}$	>0.7	1	1	not met	not met
Fraction of granules					
Biomass >0.25 mm	>80 %	7	7 (+)	not met	not met
Removal performances					
COD: discharge concentration	$<45\,mg_{COD}\,L^{-1}$	3	2	6 (+)	5 (++)
COD: removal efficiency	>85 %	2	2	0 (+)	0
NH_4^+: discharge concentration	$<2\,mg_N\,L^{-1}$	0 (++)	0	0 (++)	0 (++)
NH_4^+: removal efficiency	>90 %	0 (+)	0	0 (+)	0 (++)
NO_2: discharge concentration	$<0.3\,mg_N\,L^{-1}$	0 (++)	0 (+)	0 (++)	0 (++)
TP: discharge concentration	$<0.8\,mg_P\,L^{-1}$	0 (++)	0 (++)	not met	not met
TP: removal efficiency	>80 %	0 (++)	0 (++)	not met	not met

I

Detailed monitoring of the aerobic stage

The monitoring of the aerobic stage (Figure I.1) allowed to calculate the proportions of SND presented in Table I.1.

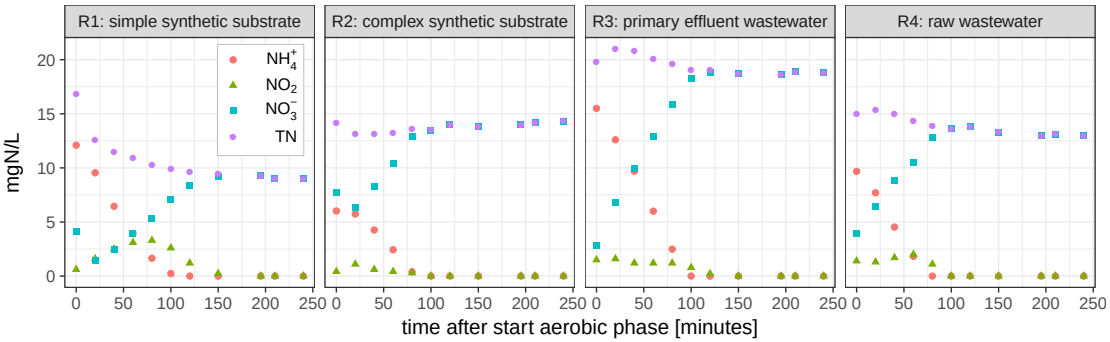


Figure I.1: Ammonium (NH_4^+), nitrite (NO_2), nitrate (NO_3^-) and total nitrogen (TN) concentrations during the aerobic stage of four AGS SBRs fed by different influents (day 88).

Table I.1: Simultaneous nitrification-denitrification (SND) in four AGS SBRs fed by different influents (day 88).

Parameter	Unit	R1	R2	R3	R4
$NH_{4_{nitrified}}^+$	$mg_N\ L^{-1}$	12.1	6.0	15.5	9.7
$NO_{x,denitrified}$	$mg_N\ L^{-1}$	7.8	0	1	2
SND	[-]	0.65	0	0.07	0.21

J

Heatmaps of the pairwise Spearman's rank correlations

The following figures present the heatmaps of the pairwise Spearman's rank correlations between the microbial communities and the physical sludge characteristics and nutrient removal performances separately for each AGS SBR.

- R1 (simple synthetic substrate): Figure J.1
- R2 (complex synthetic substrate): Figure J.2
- R3 (primary effluent wastewater): Figure J.3
- R4 (raw wastewater): Figure J.4

Only genera with a relative abundance above $0.035\,g_{VSS}\,L^{-1}$ (around $1\,\%$ of the average final VSS) in at least one of the samples were included in the analysis. Data from the first week after start-up was discarded. The correlations and associated p-values were calculated with the function *corr.p* from the R package *psych* (with Holm's correction for multiple comparisons). The heatmaps were generated with the R function *heatmap.2* from the R package *gplots* [69]. The top dendrogram defines clusters of physical sludge characteristics and nutrient removal performances correlating with each other. The left dendrogram defines clusters of microorganisms sharing similar correlations with the physical sludge characteristics and nutrient removal performances. Annotations correspond to p-values with the following significance levels. 0.001: ***, 0.01: **, 0.05: * and 0.01: . .

Appendix J. Heatmaps of the pairwise Spearman's rank correlations

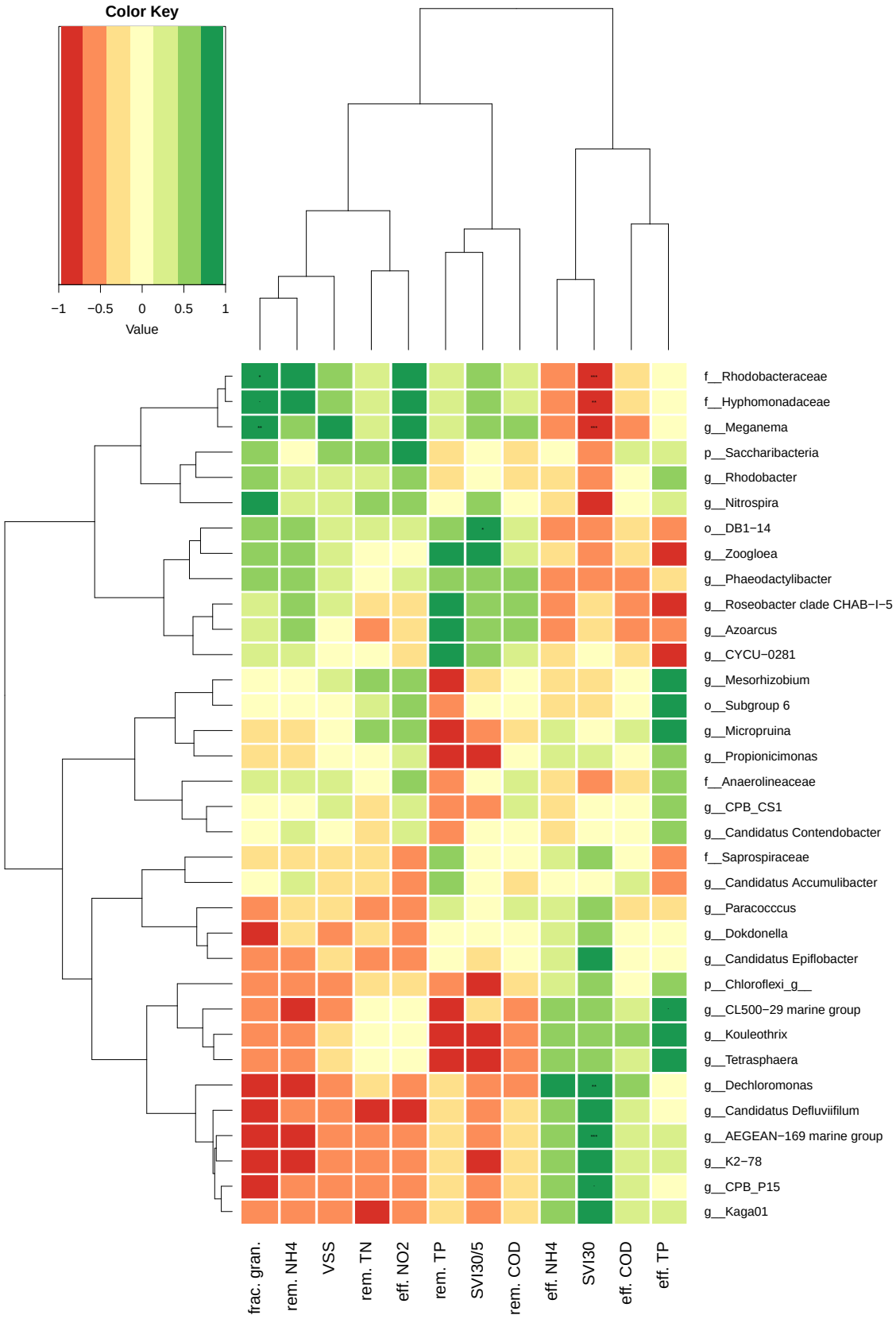


Figure J.1: Heatmap of the pairwise Spearman's rank correlations between the microbial populations and the related physical sludge characteristics and nutrient removal performances in R1 (simple synthetic substrate). rem: removal efficiency. eff: concentration in effluent.

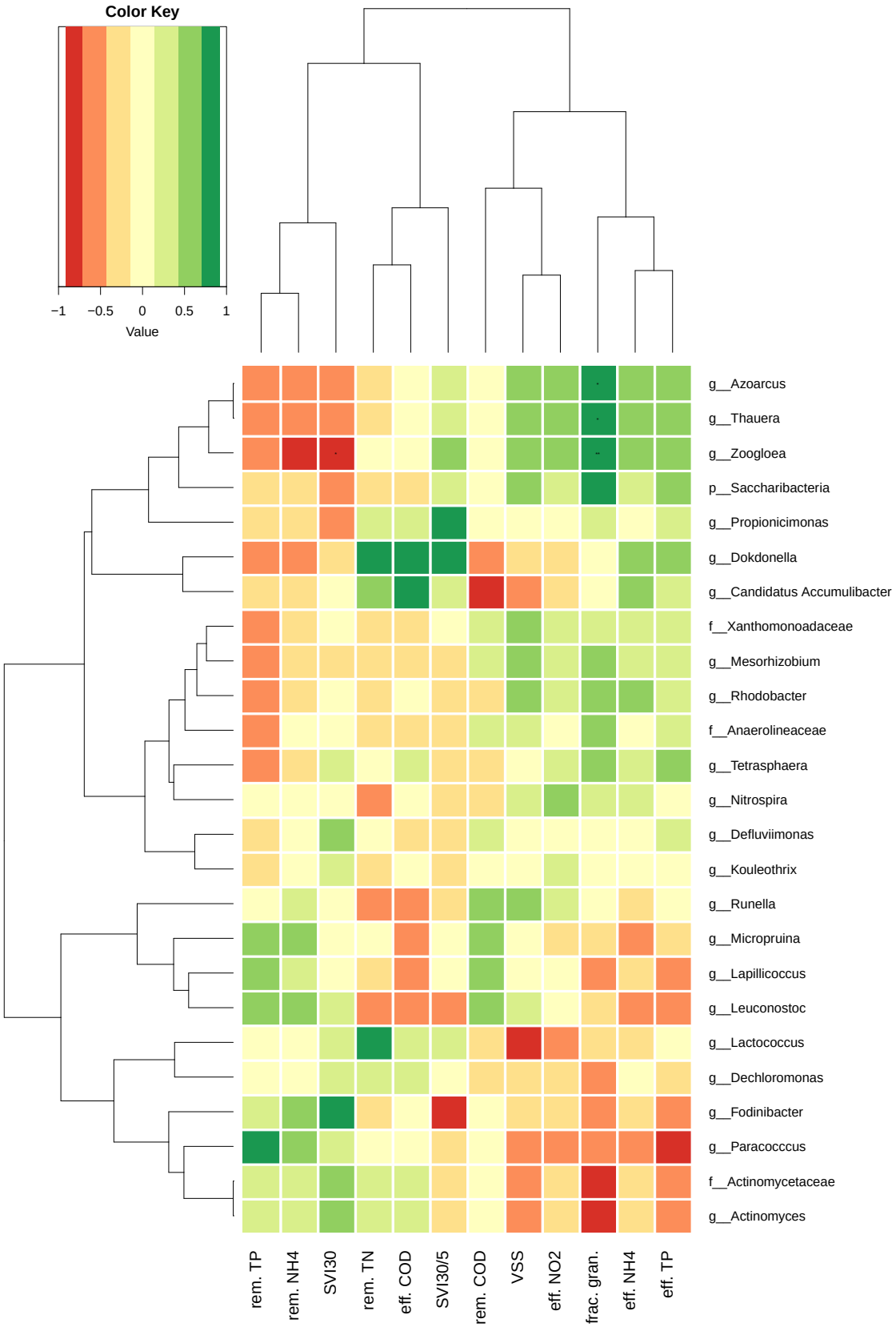


Figure J.2: Heatmap of the pairwise Spearman's rank correlations between the microbial populations and the related physical sludge characteristics and nutrient removal performances in R2 (complex synthetic substrate). rem: removal efficiency. eff: concentration in effluent.

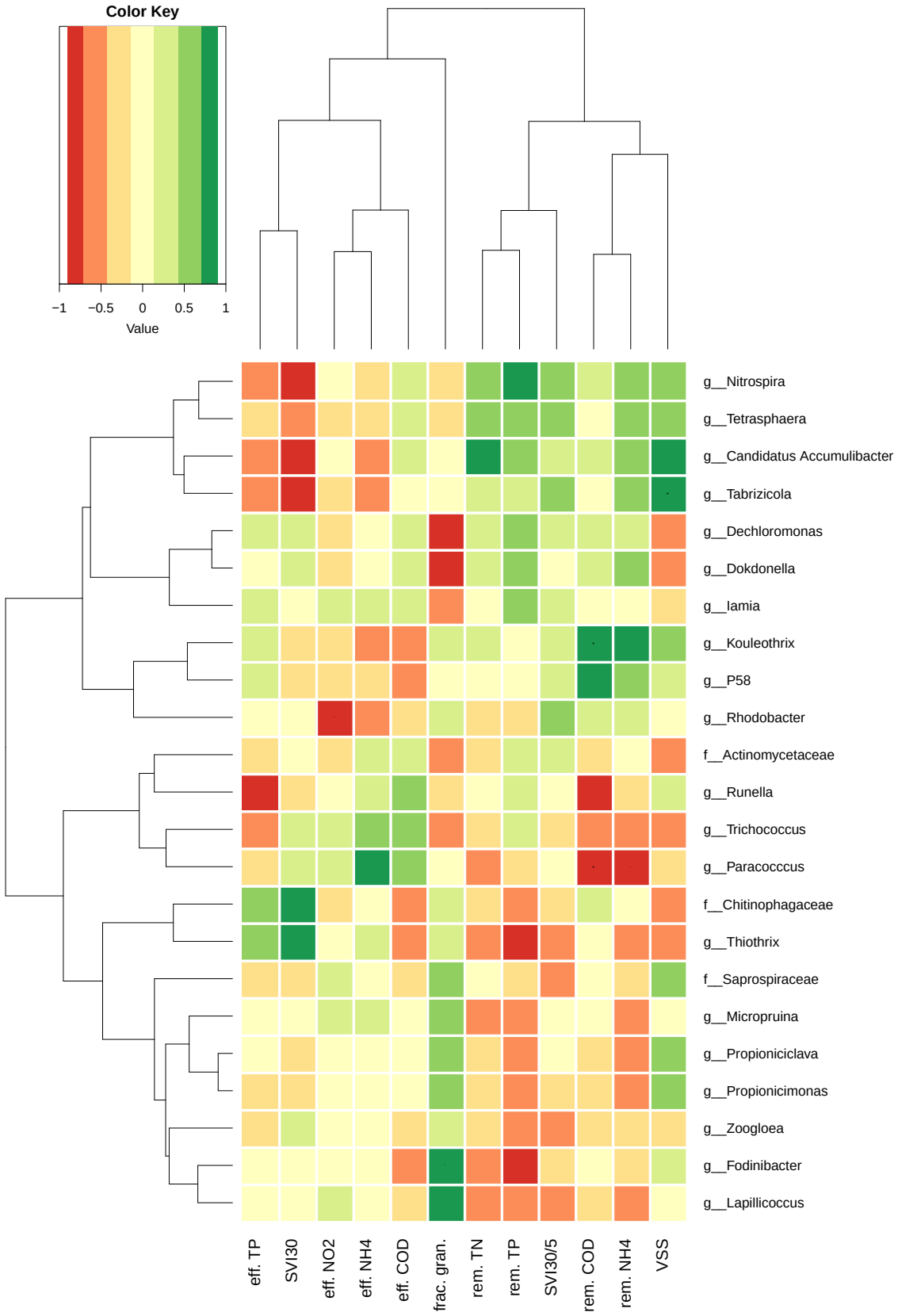


Figure J.3: Heatmap of the pairwise Spearman's rank correlations between the microbial populations and the related physical sludge characteristics and nutrient removal performances in R3 (primary effluent wastewater). rem: removal efficiency. eff: concentration in effluent.

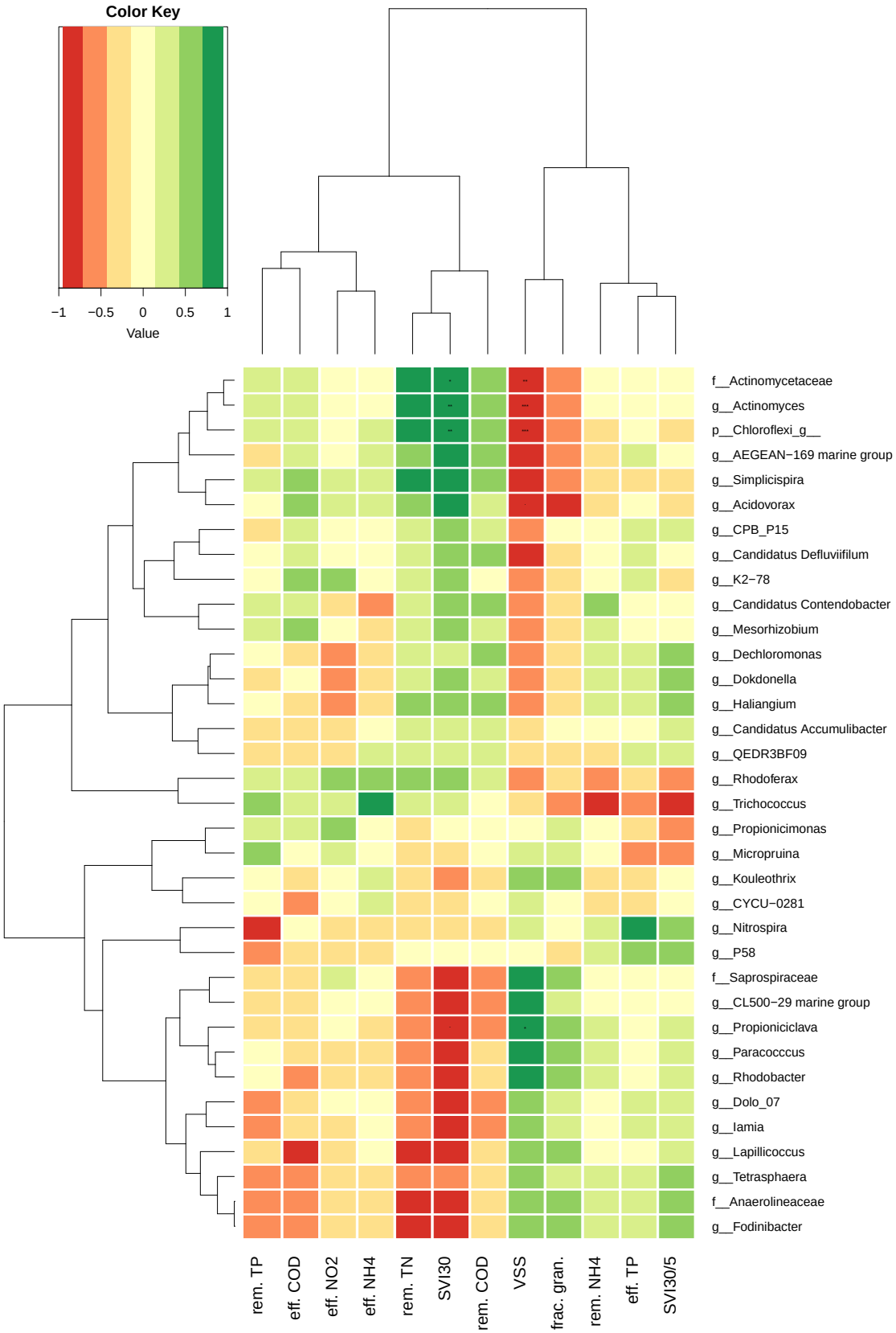


Figure J.4: Heatmap of the pairwise Spearman's rank correlations between the microbial populations and the related physical sludge characteristics and nutrient removal performances in R4 (raw watewater). rem: removal efficiency. eff: concentration in effluent.