THESIS FOR THE DEGREE OF DOCTOR PHILOSPPHY

Application of aerobic granular sludge for municipal wastewater treatment

Process performance and microbial community dynamics under fluctuating conditions

JENNIFER EKHOLM

Department of Architecture and Civil Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

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

Cover: Microscope image of a full-scale granule from the Österröd WWTP, and figures describing different characteristics of aerobic granular sludge.

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ABSTRACT

Pressures of growing cities, competition for use of urban areas and higher influent loads, are pushing for innovative technologies for wastewater treatment with low demands for land footprint and costs. Furthermore, wastewater treatment is needed to move towards a circular economy by harvest of valuable resources such as nutrients and energy. Aerobic granular sludge (AGS) is a biofilm process without a carrier material for wastewater treatment, exhibiting efficient treatment performance, excellent settleability, high biomass retention, tolerance to toxicity and high loads of organic matter. In this thesis, the first implementation of the AGS process in the Nordic countries was studied to assess the treatment performance, microbial community structure, energy usage, land footprint, and volume needs. The results in this project suggested that selective sludge withdrawal, retaining long solids retention time, sufficient substrate availability, and operational flexibility are important factors for granulation. Both the AGS and parallel conventional activated sludge (CAS) process achieved stable organic matter, nitrogen, and phosphorus removal with low average effluent concentrations. Seasonal variations and environmental factors were identified as important for microbial community succession. The granular biofilm demonstrated higher biomass concentration, diversity, and lower seasonal fluctuations in community composition than the flocculent sludge. A one-year energy comparison resulted in lower specific energy usages (kWh m^{-3} and kWh reduced P.E.$^{-1}$) and land footprint for the AGS compared to the CAS process. However, a potential for decreased energy usage was recognised for both systems, leading to the conclusion that operational optimisation and process design might be as important as the type of technology. Additionally, the influence of decreasing temperature on AGS was studied in lab-scale reactors, revealing different responses of the functional groups in the microbial community, and even various response of ASVs at the genus level. In conclusion, the AGS technology for municipal wastewater treatment under fluctuating conditions achieved low average effluent concentrations, was more compact and energy efficient compared to the CAS.

Keywords: municipal wastewater treatment, aerobic granular sludge, granulation, sequencing batch reactors, activated sludge, microbial community dynamics, nutrient removal, low temperature, full-scale operation, start-up, energy usage

SAMMANFATTNING

Nya krav från växande städer, så som konkurrens om användning av stadsområden, ett växande behov av resurshushållning och ökande belastning, driver fram innovativa tekniker för avloppsrening som är kompakta och kostnadseffektiva. Avloppsvattenrening kan även stödja en cirkulär ekonomi genom återvinning av värdefulla resurser såsom näringsämnen och energi. Aerobt granulärt slam (AGS) är en biofilmprocess med effektiv reningsprestanda, utmärkta sjunkegenskaper, hög slamhalt, tolerans mot toxicitet och hög organisk belastning. Den här avhandlingen beskriver studier av den första implementeringen av AGS-processen i de nordiska länderna vid Österröds avloppsreningsverk i Strömstad, där reningsprestandan, mikrobiella samhällsstrukturen, energianvändningen, samt yt- och volymbehovet har utvärderats. Resultaten i detta projekt tyder på att selektivt slamuttag, lång slamålder, tillräcklig substrattillgång och flexibilitet är viktiga faktorer för granulering. Både AGS- och aktivslamprocessen uppnådde stabil avskiljning av organiska ämnen, kväve och fosfor, med låga utgående koncentrationer. Säsongsvariationer och miljöfaktorer identifierades som viktiga för dynamiken i det mikrobiella samhället. AGS visade sig ha högre slamhalt, diversitet och lägre säsongsvariationer i biomassans sammansättning, än flockarna i aktivslamprocessen. En studie av energianvändning under ett år resulterade i lägre specifik energianvändning (kWh m^{-3} och kWh $P.E.^{-1}$) och lägre fotavtryck för AGS-processen jämfört med aktivslamprocessen. Dock fanns en potential för minskad energianvändning för båda systemen, vilket leder till slutsatsen att driftsoptimering och processdesign kan vara lika viktiga faktorer som typen av teknologi. Dessutom studerades effekterna av sjunkande temperaturer på AGS i labb-skala, som visade på olika respons hos funktionella grupperna av mikroorganismer, och även på varierande respons hos olika unika ”arter” (ASVs) på genusnivå. Sammanfattningsvis visades att AGS-processen kan uppnå låga utgående koncentrationer under varierande förhållanden, och även är mer energieffektiv och mer kompakt jämfört med aktivslamprocessen.

LIST OF PUBLICATIONS

This thesis is based on the following publications, referred to by Roman numerals:

Paper I: Ekholm, J., Persson, F., de Blois, M., Modin, O., Pronk, M., van Loosdrecht, M.C., Suarez, C., Gustavsson, D.J. and Wilén, B.M., 2022. Full-scale aerobic granular sludge for municipal wastewater treatment–granule formation, microbial succession, and process performance. Environmental Science: Water Research & Technology, 8(12), pp.3138-3154.

Paper II: Ekholm, J., de Blois, M., Persson, F., Gustavsson, D.J. Bengtsson, S., van Erp, T., and Wilén, B.M. Case study of aerobic granular sludge and activated sludge – energy, footprint and nutrient removal (submitted).

Paper III: Ekholm, J., Burzio, C., Saeid Mohammadi, A., Modin, O., Persson, F., Gustavsson, D.J., de Blois, M., and Wilén, B.M. Influence of decreasing temperature on aerobic granular sludge: microbial community dynamics and treatment performance (manuscript).

Paper IV: Ekholm, J., Persson, F., de Blois, M., Modin, O., Gustavsson, D.J., Pronk, M., van Loosdrecht, M.C., and Wilén, B.M. Comparison of microbial community structure and function in parallel full-scale granular sludge and activated sludge processes (submitted).

The author made the following contributions:

Paper I: Conceptualisation of research goals and aims; Sample collection at the treatment plant; Planning and performing sample analysis; Data collection; Result analysis and visualisation; Formal analysis; Writing the original draft, editing, and revising the draft after co-author and external reviewer feedback.

Paper II: Conceptualisation of research goals and aims; Sample collection at the treatment plant; Planning and performing sample analysis; Data collection; Result analysis and visualisation; Formal analysis; Writing the original draft, editing, and revising.

Paper III: Conceptualisation of research goals and aims; Designing the operation of the reactor; Reactor maintenance; Sample collection; Planning and performing experiments; Data collection; Result analysis and visualisation; Formal analysis; Writing the original draft, editing, and revising.

Paper IV: Conceptualisation of research goals and aims; Sample collection at the treatment plant; Planning and performing sample analysis; Data collection; Result analysis and visualisation; Formal analysis; Writing the original draft, editing, and revising.

Publications completed during this PhD, but not included in the thesis:

C. Burzio, **J. Ekholm**, O. Modin, P. Falås, O. Svahn, F. Persson, T. van Erp, D. J. I. Gustavsson and B.-M. Wilén, 2022. Removal of organic micropollutants from municipal wastewater by aerobic granular sludge and conventional activated sludge. *Journal of Hazardous Materials*, 438, 129528.

B.-M. Wilén, C. Burzio, **J. Ekholm**, O. Svahn, F. Persson, O. Modin, M. de Blois and D. Gustavsson. 2022. Biologisk rening av organiska mikroföroreningar. Svenskt Vatten, Rapport Nr 2022-8.

M. de Blois, S. Bengtsson, K. Gunnarsson, S. Engström, **J. Ekholm**, F. Persson, B.-M. Wilén, T.van Erp, and D. Gustavsson. 2022. Tillämpning av aerobt granulärt slam i Sverige: En fullskalestudie. (Application of aerobic granular sludge in Sweden: A full-scale study) Svenskt Vatten, Rapport Nr 2022-13.

LIST OF ACRONYMS AND ABBREVIATIONS

AOA: ammonia-oxidizing archaea
AOB: ammonia-oxidizing bacteria
AGS: aerobic granular sludge
CAS: conventional activated sludge
COD: chemical oxygen demand
DNA: deoxyribonucleic acid
DOC: dissolved organic carbon
EBPR: enhanced biological phosphorus removal
EPS: extracellular polymeric substances
FISH: fluorescence in-situ hybridization
F/M: food-to-microorganism
GAO: glycogen-accumulating organism
GHG: green-house gas
GRTD: granule residence time distribution
IFAS: integrated fixed-film activated sludge
MABR: membrane aeration biofilm reactor
MBBR: moving bed biofilm reactor
MBR: membrane bioreactor
MLSS: mixed liquor suspended solids
NOB: nitrite-oxidizing bacteria
N: nitrogen
P: phosphorus
PAO: polyphosphate-accumulating organism
PCR: polymerase chain reaction
q: order of Hill-based diversity index
RNA: ribonucleic acid
SBR: sequencing batch reactor
SRT: solids retention time
TN: total nitrogen
TP: total phosphorus
TSS: total suspended solids
VSS: volatile suspended solids
WRRF: water resource recovery facilities
WWTP: wastewater treatment plant

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TABLE OF CONTENTS

1. Background

Wastewater treatment is a critical process for preserving the environment and safeguarding public health. Ever since humans started to live in settlements, we have disrupted the environment by concentrating the presence of certain compounds in space and time. High concentrations of carbon, nitrogen and phosphorus lead to environmental impacts, and often one disturbance leads to a chain reaction of impacts in the ecosystems. Eutrophication, algal blooms, dead sea bottoms and loss of biodiversity are commonly known impacts of water pollution (Wurtsbaugh et al., 2019). Furthermore, the pressures of growing cities; competition for use of urban areas and higher influent loads, are pushing for innovative technologies for treatment with low demands for land footprint and costs (Winkler and van Loosdrecht, 2022).

The conventional activated sludge (CAS) process is widely known and one of the most commonly used wastewater treatment processes around the world. It has been used for over a century and has undergone numerous refinements and improvements to become an efficient and effective method for treating wastewater (Jenkins and Wanner, 2014). Aerobic Granular Sludge (AGS) is a fairly new technology for the treatment of municipal wastewater, operated at about 80 full-scale installations worldwide (Hamza et al., 2022). AGS is more compact compared to activated sludge (Pronk et al., 2015), and theoretically more energy efficient than other compact technologies (Bengtsson et al., 2019). The operational results which have been reported from reactors in Poland, the Netherlands, and South Africa, show well-functioning process performances (Giesen et al., 2013; Pronk et al., 2015; Pronk et al., 2017; Świątczak and Cydzik-Kwiatkowska, 2018; Toja Ortega et al., 2021b). However, the present knowledge is still limited, and more studies are needed at varying operational conditions. One main question is if the start-up and operation of AGS are successful under fluctuating conditions, including large fluctuations in both temperature and flow, as well as diluted influent wastewater due to combined sewer networks and seawater intrusion. Furthermore, little is yet known about the dynamics of microbial community structure during full-scale granulation.

To make an informed, weighted choice of how to increase the capacity of existing wastewater treatment plants (WWTPs) or build new processes, the stakeholders need scientific knowledge, comparisons and applied research. The Swedish Water & Wastewater Association´s Research Fund (SVU) project "Aerobic granules, a new technology for municipal wastewater treatment – the state-of-the-art (AGNES I)" was a knowledge collection about AGS, with a particular focus on the applicability under Swedish conditions. "AGNES" is an acronym for Aerobic Granular sludge – Nutrient removal and recovery Efficiency in Sweden. "AGNES I" found potential for AGS in Sweden, but the project also identified challenges such as the delivery of very low effluent concentrations, and treatment performance under low temperatures and peak flows. The work in this thesis is part of the following SVU project called "Implementation of aerobic granular sludge in Sweden – a full-scale study (AGNES II)". This project focused on the Österröd WWTP in the municipality of Strömstad, where the first AGS process in the Nordic countries was implemented. The aim of the SVU project "AGNES II" was to give Swedish water and wastewater organizations a deeper understanding and more comprehensive knowledge of AGS, and to provide a strong foundation for decision-making as to whether AGS is a suitable technology for their organizations.

2. Research aims and objectives

The overall aim of this thesis is to provide insights into the application aerobic granular sludge under fluctuating conditions. Full-scale studies of the implementation and operation of AGS under various conditions are few, and the available knowledge and references are limited (Bengtsson et al., 2019; Hamza et al., 2022). The implementation of AGS on a full scale was anticipated to encounter challenges related to the conditions in South-West Sweden, such as cold and long winters and large variations in load (Bengtsson et al., 2018). Furthermore, the microbial community dynamics of AGS in full-scale reactors has not previously been studied covering several seasons repeatedly.

More specifically, the objective was to answer the following research questions:

1. Which factors are important for full-scale start-up and operation of aerobic granular sludge under fluctuating conditions?
2. What factors influence the process performances of AGS?
3. What factors govern the microbial community structure in full-scale AGS exposed to seasonal variability and how does it differ from the microbial community in CAS?
4. How is the treatment performance related to the key functional groups in the AGS microbial community?
5. What are the differences in energy usage, footprint, volume, and process performance between the AGS and CAS at the Österröd WWTP?

Process parameters such as sludge concentration, settleability, and removal performances were examined at the full-scale AGS plant. The study period included the start-up (**Paper I**) and the following 2.5 years of operation (**Paper II** and **Paper IV**), which was characterised by seasonal fluctuations of nutrient concentrations, flow and temperature. Factors that shaped the AGS process in terms of sludge characteristics, microbial succession, and spatial localisation of key functional groups were assessed and connected to treatment performance. To isolate the influence of the decreasing temperature on process performance and microbial community structure, AGS was studied in lab-scale reactors (**Paper III**). In order to elucidate AGS process performance from the aspect of microbial ecology, the diversity, community composition, and relative abundance of functional groups were assessed in **Paper I, Paper III,** and **Paper IV**. DNA sequence data over a period of 1,269 days from the two AGS reactors at the Österröd WWTP was analysed. The AGS microbial community dynamics were also compared with the full-scale CAS community over a period of 1.5 years (**Paper IV**). Since energy efficiency and small footprint are two of the key arguments for AGS, a study of energy usage and land footprint in comparison with CAS was conducted (**Paper II**). Additionally, treatment performance and volume requirements were compared between the two processes.

3. Introduction

Wastewater treatment is connected to the United Nations' Sustainable Development Goals (SDG) in a number of ways (United Nations, 2022). The goals of clean water and sanitation (SDG 6), sustainable cities and communities (SDG 11) and climate action (SDG 13) are of high concern for WWTPs. Firstly, improving access to safe and reliable wastewater treatment services is essential for achieving the UN's goal of providing universal access to adequate and equitable sanitation for all. This includes providing access to safely managed wastewater treatment facilities, which are essential for reducing water pollution, preventing the spread of water-borne diseases, and protecting freshwater resources. Furthermore, wastewater treatment is necessary for protecting the environment. By reducing water pollution, wastewater treatment helps to maintain the health of ecosystems and prevents further environmental degradation. Finally, wastewater treatment can be used to generate energy, which can help to meet the UN's goal of providing affordable and clean energy (SDG 7). Furthermore, resource recovery is essential for a sustainable future. It is predicted that the supply of phosphorus, which is sourced from mines and used in chemical fertilizers for agriculture, will be depleted by 2170 if the population growth rate remains similar to what it is today (Daneshgar et al., 2018). Research in resource recovery from wastewater is a high priority, recognizing the value of energy, nutrients, and other resources (Bohra et al., 2022).

3.1 Municipal wastewater treatment

The simplest WWTPs perform removal of organic matter, but extended treatment for removal of various pollutants to low concentrations can be applied in communities with enough resources and when receiving waters demand so. A WWTP with the design to reach low effluent concentrations can consist of three treatment steps. First, the mechanical (primary) treatment of the coarse matter, often screens, sand trap, fat trap, grit removal and sometimes primary settler. Secondly, the biological treatment of suspended pollutants utilizes microbiology. Then might follow a polishing step (tertiary treatment), which can be filtration, disinfection, and advanced oxidation. WWTPs are designed for the site-specific conditions regarding capacity-, and effluent quality requirements, available space, and the composition of the influent wastewater. Some plants receive wastewater from both municipal- and industrial sources. The fraction of industrial wastewater is often small but can lead to specific characteristics. Municipal wastewater is typically diluted and contains a complex mixture of pollutants. The composition can vary over time and is a combination of microorganisms such as pathogens, bacteria, and viruses, organic compounds, macro- and micronutrients (nitrogen and phosphorus), and metals and other inorganic compounds (for example Al, Fe, acids) (Henze et al., 2008).

3.2 Biological wastewater treatment

Today, the majority of WWTPs apply the CAS process as the biological wastewater treatment step. The first reference to the activated sludge process was made by the British scientist Edward Ardern and his colleague W.T. Lockett in 1914 (Jenkins and Wanner, 2014). They observed that the activated sludge, which is the mixture of microorganisms and wastewater used in the process, was able to effectively remove organic matter from wastewater. Their work led to the development of the activated sludge process as a method for treating wastewater, which has since become one of the most widely used wastewater treatment processes in the world. Since then, the activated sludge process has undergone numerous refinements and improvements (Jenkins and Wanner, 2014).

In biological wastewater treatment, the pollutants (carbon, nitrogen and phosphorus) are converted into biomass, carbon dioxide, nitrogen gas, or other by-products. Different technologies have been developed where the microorganisms are employed in various reactor configurations. Irrespective of technology, the removal of organic matter, nitrogen and phosphorus is performed by functional groups of microorganisms. The key is to provide various microbial niches to enrich diverse functional groups of microorganisms. These functional groups are typically the ammonia-oxidizing bacteria (AOB), the nitrite-oxidizing bacteria (NOB), polyphosphate-accumulating organisms (PAOs), the glycogen-accumulating organisms (GAOs), denitrifiers, and ordinary heterotrophic organisms (OHOs). It has previously been shown that among activated sludge processes around the world, a small group of microorganisms was found in more than 200 WWTPs (Wu et al., 2019). This "core community" was evidently coupled with the functions of the treatment process.

Biological removal processes

The function of organic matter removal is done by aerobic heterotrophs, including denitrifiers, PAOs, GAOs, and denitrifying PAOs and GAOs (de Kreuk and Van Loosdrecht, 2004). PAOs accumulate phosphorus in the form of polyphosphate after anaerobic uptake of biodegradable organic matter. This feast-famine operation (anaerobic feeding followed by aeration) enables enhanced biological phosphorus removal (EBPR). GAOs are also taking up organic substrate during anaerobic conditions, but they do not accumulate phosphate. Therefore, the amount of organic substrate (and for PAOs also phosphate) in the influent wastewater is governing the abundances of PAOs and GAOs (Wei et al., 2020), and hence will the phosphorus removal partly depend on the competition between PAOs and GAOs for the available organic carbon substrate. Nitrogen is removed in several steps via nitrification by AOB and NOB, called nitrifiers as they perform nitrification which is the oxidation of ammonium to nitrate. Ammonium can also be oxidised by ammonia-oxidising-archaea (AOA), or by comammox bacteria which can oxidise ammonium to nitrate. Nitrate produced in nitrification is further converted to nitrogen gas (N_2) in denitrification by denitrifiers and denitrifying PAOs and GAOs. The nitrogen gas is released into the atmosphere. Denitrification involves four reactions in which each an enzyme is needed. Some bacteria can produce all enzymes and perform full denitrification, and many can perform truncated denitrification (Srinandan et al., 2011).

Conventional activated sludge

In CAS systems, the sludge is composed of filamentous bacteria, microcolonies of bacteria, extracellular polymeric substances (EPS), and also organic fibres and inorganic particles providing small surfaces for attached growth (biofilm-like). The microorganisms aggregate to flocs to varying extent, dependent on environmental and operational conditions. An activated sludge reactor can have different configurations, such as continuous-flow systems (Fig. 1) or sequencing batch reactors (SBRs). Depending on the redox conditions (aerobic, anoxic, anaerobic), different functional groups in the activated sludge can remove organic matter, nitrogen, and phosphorus in different reactor tanks, or at different times in an SBR. Organic matter removal and nitrification can be achieved by supplying oxygen via aeration, while denitrification can either be conducted before (pre-denitrification) or after (post-denitrification) the aeration, by mixing the biomass with the nitrate-rich wastewater produced in nitrification. After the reaction process, the sludge needs to be separated from the treated wastewater, usually by settling. The reaction and settling processes can be separated either spatially (in a separate settler tank, Fig. 1) or temporally (in the settling phase of an SBR cycle). To maintain the biomass in the continuous-flow system, the activated sludge is recirculated back to the reaction reactor after settling. If denitrification is applied prior to nitrification, the nitrate-rich wastewater leaving the nitrification process must also be circulated back to the denitrification process. Anaerobic conditions for phosphorus removal are typically created by mixing the return sludge with influent wastewater (Henze et al., 2008).

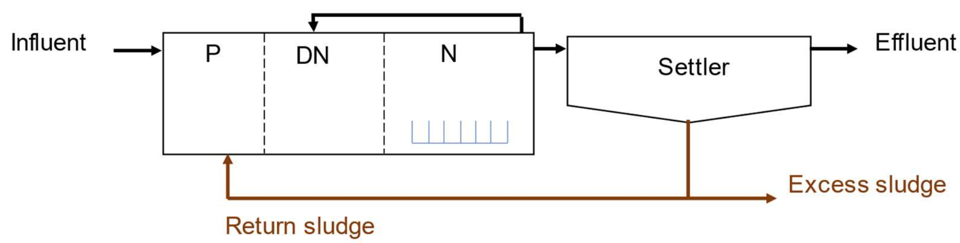

Figure 1. Schematics of a conventional activated sludge process with biological phosphorus removal (P), pre-denitrification (DN), and nitrification (N).

New technologies have been developed where the microorganisms grow in biofilms using membranes and carriers, such as the moving bed biofilm reactor (MBBR), the membrane biofilm reactor (MBR), and the membrane aerated biofilm reactor (MABR). Biofilm systems can be combined with flocculent sludge in hybrid systems, for example, integrated fixed-film activated sludge (IFAS). AGS is a biofilm technology without carrier material, in which granules as well as a smaller fraction of flocs co-exist (Ali et al., 2019; Pronk et al., 2015).

Aerobic granular sludge

AGS is a biofilm process where the microorganisms form dense and fast-settling aggregates, called granules (Fig. 2). Granules were defined by de Kreuk et al. (2007) as biofilm aggregates of “microbial origin, which do not coagulate under reduced hydrodynamic shear, and which settle significantly faster than conventional [activated] sludge” (de Kreuk et al., 2007). Physical, chemical and cellular mechanisms are involved in the formation of granules from activated sludge: cell-to-cell contact and attraction, microbe-to-microbe attachment, formation of EPS which enhance microbial adhesion, and hydrodynamic shear force that stabilise the granule structure (Wilén et al., 2018). The EPS are essential to granulation, as they give the granules structural stability and interconnect the bacterial cells. The EPS are mainly constituted of polysaccharides, proteins, humic acid, uronic acids, and phospholipids. The formation of EPS is a stress response and is a protection from stressors such as starvation, desiccation, toxic compounds (e.g., antibiotics, metals), ultraviolet radiation, and predation from some protozoan grazers (Flemming and Wingender, 2010). Also, fungi have been suggested to form the backbone of granules (Weber et al., 2007).

The granules typically have a spherical shape with sizes ranging from 0.2 to > 4 mm in diameter (Fig. 2). However, in AGS reactors treating municipal wastewater, the biomass is a mixture of both granules and flocculent sludge. AGS is normally applied in SBR operation mode, where batches of wastewater are treated sequentially. In the typical AGS, the removal of organic matter and nutrients, as well as the separation of the treated wastewater and the sludge takes place in the same reactor. The phases of the SBR cycle are typically simultaneous feeding and decanting (anaerobic), reaction (aeration and pulse-aeration), settling, and selective sludge discharge. Fast formation of granules in lab-scale reactors has been accomplished using synthetic wastewater and has been linked to high wash-out stress of slow-settling biomass (short settling), high shear forces, and microbial selection through anaerobic-feast-aerobic-famine SBR operation (Adav et al., 2008). The abundance of PAOs and GAOs is a key component for the granulation to occur (de Kreuk and Van Loosdrecht, 2004), and the cultivation of these groups can be enhanced by operational measures such as feast-famine operation and a feeding time long enough for full uptake of organic substrate. The relatively long anaerobic feeding phase benefits the PAOs and GAOs in the competition with aerobic heterotrophs and limits the growth of aerobic heterotrophs by limiting the available organic substrate in the subsequent aerobic phase. Furthermore, bottom feeding (Fig. 2) favours the most fast-settling aggregates, which promotes the growth of dense, large granules (Haaksman et al., 2023).

In full-scale, the content of the so-called granule-forming substrate in the influent, and partly formed in the reactor, determines if granulation can occur, and the selection pressures associated with the SBR mode govern the rate of granulation. Granule-forming substrates are the substrates that can lead to growth of aerobic granules, such as volatile fatty acids, as well as readily biodegradable substrates which can be anaerobically converted into storage polymers (van Dijk, 2022). The selection pressures are, first, the bottom plug-flow feeding which allows for high substrate gradients and promotes the diffusion of substrates into the granules. Second, selective feeding, means that the fastest settled granules located at the bottom of the reactor due to the in-reactor short settling time have priority during the feeding. Third, the selective discharge of slow-settling sludge ensures that granules are preferentially maintained (van Dijk et al., 2022). Although the SBR mode is advantageous, recent studies have succeeded to grow AGS in continuous-flow reactors (Xu et al., 2022a).

In activated sludge, the growth occurs in the form of activated sludge flocs. The flocs are not as dense but settle slower than the granular biofilm. To separate the flocs from the treated wastewater in CAS, often large settler tanks are needed. Low settleability results in long sedimentation times, which limit the treatment capacity of the WWTP. The surplus growth of sludge is removed from the reactor and the rest is re-circulated. CAS is a reliable method that can produce very low effluent concentrations if the reactor volumes are large, and the treatment times are long enough. The possible benefits of the AGS process compared to the CAS process are several: (1) higher biomass concentration; a granule residence time distribution (GRTD) instead of one SRT (van Dijk, 2022); denser biomass structure with excellent settling capacity; high microbial diversity due to the presence of different microhabitats; capability of simultaneous removal of organic carbon, nitrogen, and phosphorus; and the ability to withstand shock loads (Show et al., 2012). Another potential advantage is the production of biopolymers from AGS, due to its special EPS properties (Pronk et al., 2017; van Dijk, 2022). Furthermore, recovery of energy, biopolymers and struvite (phosphorus) from AGS has a promising outlook (Kehrein et al., 2020).

Due to the selective feeding and sludge discharge in the AGS operation, the largest or fastest settling granules have a higher probability to stay in the reactor, thus, the small granules and flocs are more likely removed. This leads to different SRT of the different aggregate size fractions. The SRT of full-scale granular sludge can vary from 6 days for the flocculent fraction (<0.2 mm), to 142 days for large granules (>1 mm) (Ali et al., 2019). Granules also have a gradient of SRTs along the radius (Winkler et al., 2012c), as the outer part of the granules are subjected to shear forces and is more likely to end up in the effluent (Szabó et al., 2017a). Thus, new growth probably occurs in the outer parts of the granule. The GRTD allows for microorganisms with different preferences in SRT.

The dense granular biomass leads to high biomass concentration and causes limitations in the mass transfer which creates concentration gradients in the granule (Fig. 2). Large compounds or compounds in low concentrations will not be able to diffuse into the granule core, which form different microenvironments at different depth of the granule. The microbial community composition will differ over the radius of the granule, due to these concentration gradients of substrates (Picioreanu et al., 1998). The diversity of microenvironments inside granules and flocs found in a full-scale AGS reactor is assumed to result in higher microbial diversity than in CAS. Two examples of concentration gradients are dissolved oxygen (DO) and organic substrate. The concentration gradient of DO in the granular biofilm allows for anoxic conditions during aeration in the core of the granule, making it possible to nitrify and denitrify simultaneously. Due to the diffusion limitations, organic substrate availability is different in AGS and CAS. Only a fraction of the organic substrate will be available inside the granule, due to the thickness of the granular biofilm (Layer et al., 2019; van Dijk et al., 2022). The diffusible substrate is often found in low concentrations but can also be formed from non-diffusible substrates by hydrolysation on the granule surface or by the flocculent fraction of biomass often present in AGS systems fed with non-diffusible substrates (Layer et al., 2019; Toja Ortega et al., 2021a).

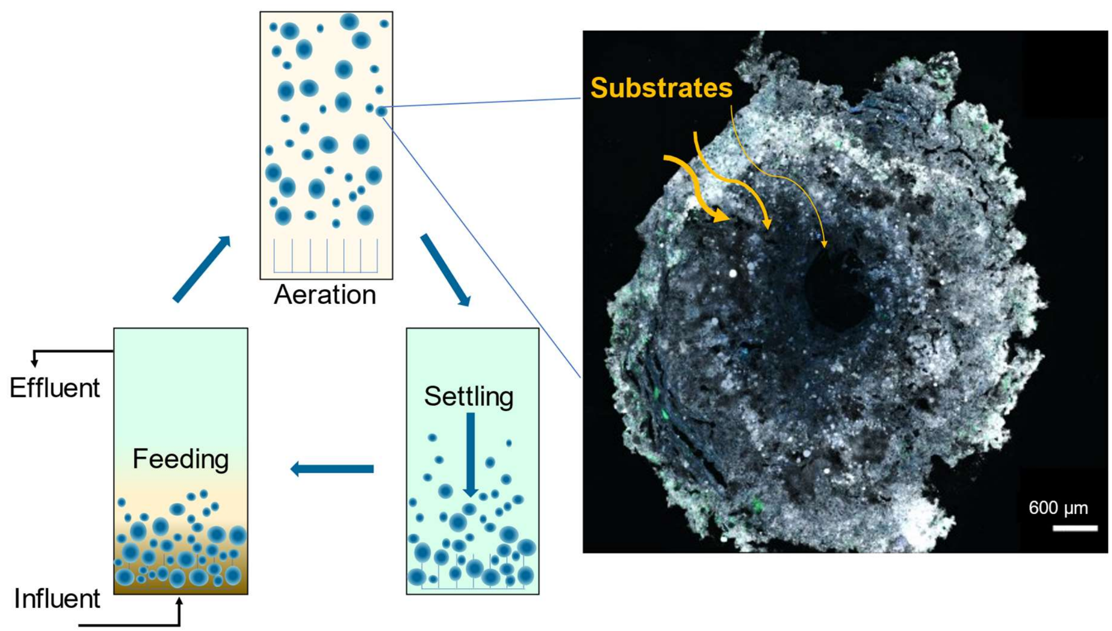

Figure 2. Schematics of a simplified SBR-AGS cycle, and different diffusion depths of substrates along the radius of a granule (in a picture of full-scale AGS), creating concentration gradients with higher concentration in the granule surface layers. The picture is a full-scale granule from the Österröd WWTP where AOB (green) and NOB (blue) are visualised with fluorescence in situ hybridization (FISH). The biomass DNA/RNA is displayed in white.

3.3 Microbial community structure

Microbial community ecology has been widely studied in numerous environments regarding how communities are assembled and shaped by different mechanisms. The dynamics of microbial communities is important to understand to improve the functional performance and stability by strategic operation of the WWTPs. Previous studies suggest that microbial communities in wastewater treatment are shaped by both deterministic and stochastic mechanisms (Ofiţeru et al., 2010). Deterministic mechanisms refer to those that are predictable, including species traits, interspecies interactions (for example competition and predation), and environmental conditions (such as pH, temperature, and salinity). Species sorting is a deterministic factor which occurs when certain species become more prevalent in a given environment due to their ability to outcompete other species for resources (Langenheder and Székely, 2011). Stochastic mechanisms are, on the other hand, random and based on chance events, for example birth, death, and immigration (Zhou and Ning, 2017). Activated sludge has been suggested to be largely shaped by mass immigration (Dottorini et al., 2021), but also deterministic processes, such as selection pressures and competition (de Celis et al., 2022). In a global study of activated sludge, it was suggested that stochastic factors (dispersal and drift), as well as deterministic factors (temperature and organic input) played important roles in the regulation of the microbial community composition (Wu et al., 2019). On a regional scale (Griffin and Wells, 2017) and for a single WWTP (de Celis et al., 2020), the temperature was proposed as a major environmental factor shaping the activated sludge community.

Survival strategies are an important concept in microbial community ecology, as they can affect the composition of microbial communities and the functioning of these systems. Two common strategies observed in microbial systems are r-strategy and K-strategy (Ho et al., 2017). A rapid growth rate at conditions with extensive substrate availability is referred to as r-strategy, while a slow growth rate and high substrate affinity is designated as K-strategy. K-strategists thus have greater competitive advantage in substrate-limited conditions. Since both high and low substrate concentrations are present in the wastewater treatment process, both strategists can coexist. Suspended flocs may have a higher abundance of fast-growing microorganisms (r-strategists), whereas biofilm systems may promote the survival of slow growers (K-strategists) (Wu and Yin, 2020). This is an essential difference between activated sludge and AGS, which likely influences the microbial community assembly. The microbial community composition was found to be different, with comparable diversity, in AGS and activated sludge systems, studied in a full-scale activated sludge reactor and a AGS pilot reactor at the same WWTP (Winkler et al., 2013b).

Activated sludge is often used as inoculum for the cultivation of AGS (Sengar et al., 2018). When a full-scale activated sludge reactor was upgraded to AGS, the microbial community structure shifted from filamentous bacteria to EPS-producing bacteria (Świątczak and Cydzik-Kwiatkowska, 2018), demonstrating a difference in the microbial community composition between AGS and activated sludge. During the granulation process in AGS reactors, three distinct successional phases were suggested (Liébana et al., 2019). The communities were shaped firstly by high turnover and selection based on influent composition and aggregate formation, in the second phase by selection based on settling velocity and increased stochasticity, and the third phase was characterized by low turnover and stable performance, but also drift affected the community members of low abundance (Liébana et al., 2019). Interestingly, variations in the community composition between individual granules within the same reactor indicated that interspecies interactions played an important role in the microbial community assembly (Leventhal et al., 2018). Currently, there are few studies investigating the microbial community structure of full-scale AGS, however, species sorting (deterministic factors), as opposed to immigration from the microbial community in the influent, was suggested to have larger importance in the assembly of full-scale AGS (Ali et al., 2019). The floc fraction and granules in the full-scale AGS reactor were suggested to be shaped by different mechanisms, as the flocs and small granules were influenced by immigration from the influent wastewater to a higher extent compared to large granules. The relative abundance of key functional groups (for example *Nitrospira*, *Tetrasphaera*, and *Ca*. Accumulibacter) was enriched in the large granules but was rare in the influent wastewater community (Ali et al., 2019).

Temperature is a key factor affecting microbial metabolism in aquatic environments, and temperature variability impacts community dynamics (Adams et al., 2010). Low temperatures in lab-scale studies of AGS have caused washout of biomass, disintegration of granules, worsening settling properties, and deteriorating nutrient removal (de Kreuk et al., 2005; Gonzalez-Martinez et al., 2018; Winkler et al., 2012a). Recent studies of AGS have mainly focused on constant temperatures, but seasonal dynamics can also affect the microbial community and reactor performance (Johnston and Behrens, 2020). Previously, the influence of temperature decrease has been studied in different wastewater treatment processes, but AGS is relatively unexplored and further research of AGS under low temperature is needed (Zhou et al., 2018).

3.4 Sustainability aspects and footprint

WWTPs are facing ever-increasing requirements, such as reaching high treatment performance in terms of low effluent concentrations of organic matter, nitrogen (N) and phosphorus (P) compounds, and at the same time minimising the land footprint (space requirements), energy usage and greenhouse gas (GHG) emissions. The goals in wastewater treatment can however be contradictory, for example, increasing the effluent quality can lead to increased use of resources. Additionally, the recovery of resources (e.g., N, P) from wastewater should be part of a circular economy. A new name has been proposed, water resource recovery facilities (WRRF), instead of WWTP, to promote the required shift (Mannina et al., 2022). Water and waste processing and treatment are within the primary sectors which cause or increase GHG emissions (United Nations EPA, 2022). Other primary sectors include energy production and land use systems including industry, agriculture, transportation, urbanisation/deforestation and the built environment. Recognition, interest, and action to reduce GHG emissions in the water and wastewater treatment sector are becoming widespread, which is fortunate, since water is essential to all aspects of life. In addition, increased energy efficiency in the water sector is an important component of the SDG.

The energy efficiency of a WWTP can vary significantly depending on its configuration and mechanical equipment. Low energy usage is often a high priority of WWTPs (Neth et al., 2022). Some of the newer wastewater treatment technologies on the market are able to produce very good effluent qualities in a short time but at a high energy cost. For example, the MBBR and the membrane bioreactor (MBR) require high demands on aeration, mixing, and requires pumping of sludge for recirculation (Bengtsson et al., 2019). A study by Pronk et al. (2015) demonstrated that AGS systems can achieve up to 50% lower energy usage compared to a parallel activated sludge plant. This comparison was conducted at a single installation in the Netherlands (Garmerwolde WWTP) and was only conducted over a period of ten months, including three months of start-up, which may not accurately reflect the energy usage over an entire year. Furthermore, the process performance of AGS systems is highly dependent on the availability of organic substrates (Henze et al., 2019), with higher concentrations of COD, BOD, and BOD/N-ratio observed in the Dutch AGS plant (506 mg L^{-1}, 224 mg L^{-1}, and 4.5 respectively (Pronk et al., 2015)) than in the full-scale AGS plant in Sweden (277 mg L^{-1}, 110 mg L^{-1}, and 3.2 respectively, **Paper I**). Thus, the location might influence the energy usage of the plant.

In comparison, CAS processes have been widely studied in relation to energy usage, biogas production, and environmental impacts (Foladori et al., 2015; Hao et al., 2018; Llácer-Iglesias et al., 2021; Silva and Rosa, 2022; Yang et al., 2010). For example, aeration efficiency has been extensively studied for CAS systems (Baquero-Rodríguez et al., 2018) but much less so for AGS processes (Strubbe et al., 2023). Similarly, biogas production from activated sludge has been thoroughly researched in terms of methods and improvements (Uthirakrishnan et al., 2022), while studies of the biogas potential of AGS waste are much fewer (Bernat et al., 2017; Cydzik-Kwiatkowska et al., 2022a; Guo et al., 2020; Jahn et al., 2019). Anaerobic digestion of excess sludge can provide a positive energy balance (Gude, 2015) and consequently contribute to the sustainability of the WWTP. The biodegradability of AGS waste sludge has been found to be lower than that of CAS (Guo et al., 2020), but in another study, the methane production from both processes was similar (Jahn et al., 2019). The amount of sludge produced by AGS and CAS processes has yet to be studied.

Phosphorus was traditionally removed chemically, but the use of phosphorus precipitation chemicals is associated with increased costs and environmental impact. EBPR, also called bio-P, is now implemented worldwide (Oehmen et al., 2007). EBPR requires no or low consumption of precipitation chemicals, which also decreases the use of resources compared to the chemical treatment of phosphorus and contributes to sustainable development (Seviour and Nielsen, 2010).

Concerning the climate impact of the WWTP, nitrous oxide (N_2O) can be produced in the nitrogen removal process, both in AGS and CAS, and is a greenhouse gas with a global warming effect about 300 times greater than carbon dioxide (United Nations, 2023). Nitrous oxide is an intermediate product in denitrification and a side-product in nitrification and can be produced under aerobic and anoxic conditions (Song et al., 2020). While several lab-scale studies have been conducted on nitrous oxide emissions from AGS, only one has been done at a full-scale AGS plant (van Dijk et al., 2021). The results from this study showed an average (7 months) emission factor of 0.33% of total nitrogen in the influent, which was in the same range or lower compared to the activated sludge systems. The nitrous oxide concentration in the AGS reactor was significantly reduced in the post-denitrification, but denitrification was also found to be a source of nitrous oxide, and it was also likely produced in the nitrification. The nitrous oxide emissions were found to be higher during dry weather flow than rain weather flow, and a stable aeration set-point led to lower emissions. The effect of temperature was uncertain but showed higher variability at low temperatures, and the data showed vague diurnal and seasonal patterns (van Dijk et al., 2021). However, higher emissions at low temperatures were found in lab-scale studies (Massara et al., 2017). Further, the influent carbon source and load were identified as factors influencing the nitrous oxide emissions in lab-scale studies (Massara et al., 2017). In conclusion, the dynamics of nitrous oxide formation is not yet clearly understood, hence leading to high uncertainty regarding the estimated emissions.

4. Materials and Methods

The materials and methods used to answer the research questions in this thesis are presented briefly, more detailed information can be found in the appended papers.

4.1 Description of the full-scale plant

The Österröd WWTP (Fig. 3) is located in the municipality of Strömstad, Sweden, and designed for 30,000 population equivalents (P.E.), and a flow of 300 $m^3\ h^{-1}$. The influent wastewater is mainly domestic, typically with higher concentrations of organic matter and nutrients during the summer due to tourism. The combined sewer network leads to higher flows during heavy rainfalls and snowmelts (Fig. 4). In 2021, the effluent quality demands at the Österröd WWTP were 10 mg L^{-1} of BOD_7, 70 mg L^{-1} of COD, 0.30 mg L^{-1} of phosphorus, and 15 mg L^{-1} of nitrogen.

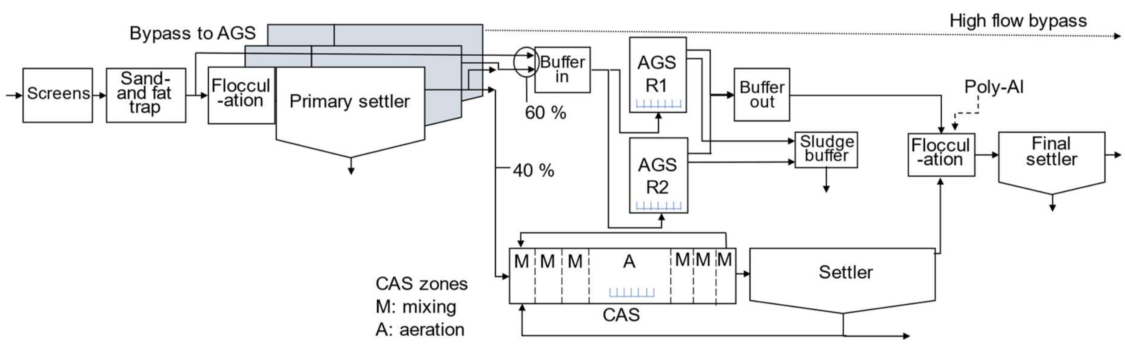

Figure 3. Simplified schematic of the Österröd WWTP (not to scale), presented in **Paper II**.

The biological treatment consists of an AGS plant in parallel with a CAS process. A more detailed description of the treatment line is described in **Paper II**. Primary-, biological-, and chemical sludge is treated and transported away to be spread on agricultural land. Reject water from the sludge treatment was led to the pre-settlers, the CAS, or to the flocculation after the biological treatment. The AGS plant consists of two AGS reactors (758 m^3 each), called Nereda® reactors which is a trademark owned by Royal HaskoningDHV and are designed to treat 60% of the flow, and the CAS the remaining 40%. The reactors are 7 m deep and have sensors for temperature, redox potential, DO, pH, suspended solids, and nitrate concentrations, and analysers sampling every ten minutes for measurements of ammonium and phosphate concentrations (Endress + Hauser). Each of the two aeration systems contains 360 disc diffusers with 20% degree of coverage (Sulzer PIK S D88,9) and they are supplied by compressed air from four rotary lobe blowers (Kaeser DB166C, 30 kW).

The CAS process consists of a reactor (1,300 m^3, 3.6 m deep) divided into seven compartments which are mixed and/or aerated and a secondary settler. The aerated volume is 520 m^3 (zone 4), the pre-denitrification volume is 470 m^3 (zone 1-3) and the post-denitrification volume (zone 5-7) is in total 310 m^3. In the CAS reactor, DO, ammonium, suspended solids, and nitrate concentrations are monitored. The number of zones which were aerated was adapted automatically depending on the ammonium content in the effluent, but normally only zone 4 was aerated. The CAS line has a secondary settler with surface area of 380 m^2 and a volume of 1,290 m^3, equipped with a sensor measuring suspended solids. The aeration system contains 339 disc diffusers (170 in zone 4, with 20% degree of coverage) (IFU 520 ABK/IFU diffuser 02-GIGANT) and the air is supplied from three rotary lobe blowers (Kaeser DB166C, 18.5 kW).

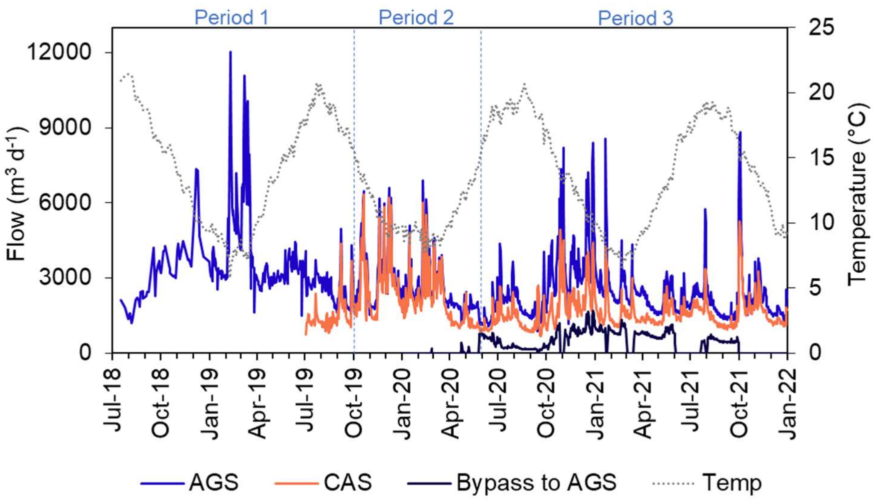

Figure 4. Flow to the AGS, CAS, the bypassed flow to the AGS, and the temperature. Period 1, 2 and 3 are explained in Chapter 4.1.

Influent characteristics and loads

The plant receives mainly domestic wastewater, which varies vastly due to increased inflow at rain- and snow-melt events. The average influent concentrations for the period when both the AGS and CAS were studied are presented in Table 1. During the years of this study, two measures were implemented to increase the concentration of organic matter in the influent to the AGS; hydrolysation in the pre-settler (from August 2019) and partly bypass of the pre-settler (from June 2020). The hydrolysation was implemented by letting a sludge bed accumulate, and circulate the sludge by pumping, in the pre-settler feeding the AGS. These measures resulted in significantly increased ($p<0.05$) influent concentrations of BOD_7, COD and SS to the AGS compared with the CAS which received only pre-settled wastewater. When the AGS was started-up and during the first year of operation (June 2018 – July 2019), the influent concentrations were similar to those entering the CAS, as the hydrolysis and bypass had not been implemented.

Table 1. Influent wastewater composition average ± standard deviation from October 2019 to September 2021, n is the number of samples. [a] Filtered through 0.45 µm.

Parameter	AGS Sep-Jun	AGS Jul-Aug	CAS Sep-Jun	CAS Jul-Aug
BOD_7	97 ± 45	160 ± 40	69 ± 27	104 ± 13
$BOD_{7,\ soluble}$ [a]	30 ± 17	70 ± 20	26 ± 15	53 ± 6
COD	250 ± 100	380 ± 90	187 ± 76	270 ± 44
TP	2.9 ± 1.2	5.0 ± 1.0	2.7 ± 1.2	4.3 ± 0.6
$TP,_{\ soluble}$ [a]	1.4 ± 0.6	2.6 ± 0.8	1.6 ± 1.0	3.1 ± 0.5
TN	29 ± 13	51 ± 12	26 ± 10	46 ± 6
NH_4^+-N	24 ± 9	45 ± 12	23 ± 9	37 ± 4
SS	130 ± 70	160 ± 60	80 ± 20	80 ± 20
BOD_7 / TN	3.3 ± 1.0	3.3 ± 0.9	2.7 ± 0.7	2.3 ± 0.3
BOD_7 / TP	34 ± 9	32 ± 7	27 ± 6	25 ± 3
n	103	41	28	13

The flow was typically divided according to the design, i.e., 60/40% (± 4%) to the AGS and CAS respectively (Fig. 4, period 3 (see chapter 4.1)). The volumetric loads of total nitrogen (TN) and total phosphorus (TP) were clearly increasing during the summers, due to tourism (Fig. 5). The volumetric loads of BOD_7 were fluctuating, probably depending on variability in the removal efficiency of the pre-settler, and also due to hydrolysis and bypassed flow to the

AGS. The volumetric loads on the CAS (not including the secondary settler) were generally lower than on the AGS, due to the flow division. The biomass specific load of BOD_7 was rather similar in the two systems, however, those of TN and TP were often higher in the CAS compared with the AGS (Fig. 5).

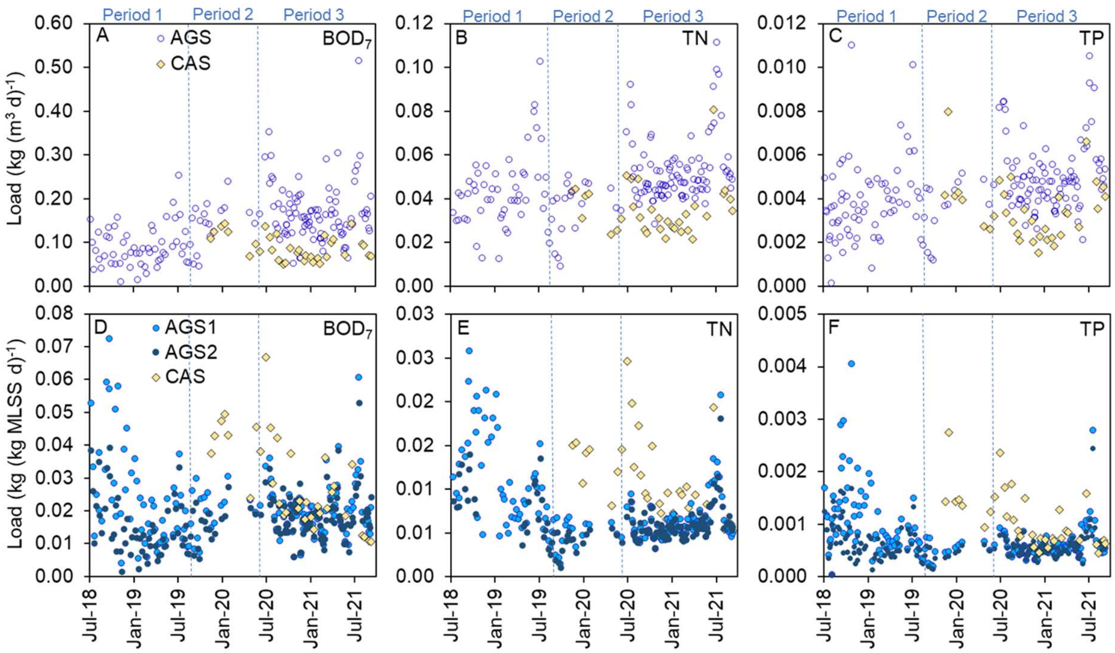

Figure 5. Volumetric load of A) BOD_7, B) TN and C) TP in the AGS and the CAS. Biomass specific load of D) BOD_7, E) TN and F) TP in AGS1, AGS2 and the CAS. Period 1, 2 and 3 are explained in chapter 4.1.

Operation of the AGS

In the AGS, an SBR cycle starts with a fixed time for simultaneous filling of influent wastewater and decanting of effluent followed by sludge discharge. Then follows a reaction phase consisting of a main aeration phase and sometimes a pre-and/or post-denitrification phase. The cycle is terminated by a settling phase with a fixed time. More details on phase times can be found in **Paper II**. In the post-denitrification phase, aeration in short pulses is applied to mix the reactor content, and hence the Nereda reactors operate without requirements for mechanical mixing equipment. With a fixed filling time, the exchange ratio is dictated by the inflow. Typical exchange ratios were 35-50%. The total cycle time is decided by a model (Aquasuite® Nereda controller) based on the predicted inflow. The total cycle time, and as result, the hydraulic retention time (HRT), is hence, shorter during wet weather flow than during dry weather flow. The time of the aerated phase is determined by the control system with input from the online measurement of ammonium (the aeration phase ended when 3-3.5 mg L^{-1} was reached). During the studied period, the DO concentration was controlled by a set-point, typically set at a higher value during the colder months (2.5-3 mg L^{-1}) than during the warmer months (1-2 mg L^{-1}) to maintain nitrification.

Operation of the CAS

The CAS process consists of seven zones and one well for return sludge. The influent wastewater is led by gravity to the first zone. Normally zone 1-3 were mixed, zone 4 aerated and mixed, and zone 5-7 mixed. Zone 1-3 and 5 can be aerated, depending on the load. The concentration of NH_4^+-N regulates which zones are aerated, and the DO set-point. The nitrate recirculation is controlled by the influent flow to the CAS and was pumped from zone 7 (the last mixed zone) to zone 1, and from February 19th, 2021 (period 3, see chapter 4.1), the nitrate recirculation flow was pumped to zone 2. The return sludge flow is controlled by the influent flow. The DO set points are normally 1.5-2 mg L^{-1} during summer, and 2-3 mg L^{-1} during winter.

Table 2. Operational parameters of the AGS- and CAS reactors, average ± standard deviation in October 2020 - September 2021 (Period 3, see Chapter 4.1).

Parameter	Unit	AGS1	AGS2	CAS
Solids retention time (SRT)[a]	d	28-49	28-66	26-43
Sludge concentration[a]	g MLSS L^{-1}	8.8 ± 1	9.1 ± 1	3.1 ± 0.8
Biomass ratio [a]	VSS/MLSS	0.86 ± 0.02	0.86 ± 0.02	0.86 ± 0.04
SVI_{30} [a]	mL g^{-1}	47 ± 6	46 ± 4	263 ± 102
SVI_{10} / SVI_{30} [a]	ratio	1.0 ± 0.05	1.0 ± 0.04	1.6 ± 0.35
Flow	m^3 d^{-1}	1,210 ± 560	1,220 ± 570	1,625 ± 680
Influent pH	-	8.2 ± 1.1	8.2 ± 1.1	-
F/M-ratio[b]	kg BOD_7 (kg TSS d)$^{-1}$	0.018 ± 0.008	0.018 ± 0.007	0.024 ± 0.007
P.E. load[c]	P.E.	1,650	1,650	1,500
Return sludge flow	m^3 d^{-1}	-	-	2,174 ± 1,277
Exchange ratio	-	0.46 ± 0.06	0.45 ± 0.06	-
Feed velocity	m h^{-1}	3.44 ± 0.02	3.44 ± 0.05	-
Hydraulic retention time	h^{-1}	17 ± 6	17 ± 6	43 ± 13 (22 ± 7) [d]

[a] SRT based on averages ± Std. For the AGS the different aggregate sizes have different SRT. Sampling from June 2020 – December 2021.
[b] Based on kg BOD_7 divided by the total amount of sludge in the reactor.
[c] Based on average BOD_7-load and 70 g BOD_7 per person and day.
[d] Values in parenthesis do not include the secondary settler after the CAS.

Sampling and analyses of water and sludge

Flow proportional water samples were collected from the influent and effluent, from July 2018 to October 2021. The sampling of influent and effluent from the AGS was intensely done from October 2020 to September 2021 due to a process guarantee period stating maximum effluent concentrations from the AGS of 8 mg L^{-1} of BOD_7, 10 mg L^{-1} of TN, and 1 mg L^{-1} of TP (yearly average and average May-August). The rest of the year 2021 were very few samples collected (two in October). The influent and effluent water samples were analysed for BOD_7, COD, ammonium, nitrate, nitrite, total-Kjeldahl nitrogen (TKN), TN, phosphate and TP according to standard methods (APHA 1992). Analysis of soluble BOD_7, COD and phosphate phosphorus was conducted with 0.45 µm pore size filtration. Analyses of mixed liquor suspended solids (MLSS), volatile suspended solids (VSS), total solids (TS), volatile solids (VS) and sludge volume index (SVI) after 10 and 30 minutes were measured according to standard methods (APHA 1992). The granule size distribution was analysed by sieving 1 L of sludge through sieves with pore sizes of 2, 1.4, 0.6, 0.4 and 0.2 mm. The sample remaining on each sieve was washed, collected, and dried at 105 °C until dry. The sludge morphology was observed by light microscopy (Olympus BX53) with micrographs taken by a digital camera (Olympus DP11).

Biomass samples from the AGS reactors covering a time series of 1,269 days from June 2018 to December 2021 were analysed. The period for the CAS was chosen to cover stable operation, why the first year after the start-up was excluded, and the sampling was conducted from June 2020 to December 2021, covering 547 days. Monthly AGS samples were taken at the depth of 1.5 and 5 m with a Ruttner sampler during the main aeration phase, whereafter the sludges from the two depths were mixed, and a biomass sample of 15 mL was collected. Grab samples were collected from the sludge discharge, influent and effluent of the AGS. Activated sludge samples of 15 mL were taken from the aerated zone on monthly basis. The average SRT was calculated to be 28-49 days in AGS1, 28-66 days in AGS2, and 26-43 days in the CAS reactor.

4.2 Description of lab-reactors

AGS was studied in two lab-scale SBRs for the temperature decrease study (**Paper III**), located in a temperature-controlled room (21 ± 0.5 °C) with no sunlight. The reactor design had a diameter of 5.6 cm and a total height of 160 cm. The double-walled glass reactor enabled temperature control by means of cold water, cooled in thermostat baths, which recirculated in the jacket of the reactors. Each reactor was operated at a volume of 3.2 L, with the effluent port located at 1.6 L, hence resulting in an exchange ratio of 50%. Sampling ports were located in the middle of the volume and at the bottom of the reactor. The reactor was operated in a closed system where the gas was recirculated, and air or nitrogen gas was sparged to control the DO concentration in the aeration phase at a constant gas flow. The pH was controlled by a slow delivery of acid and base solutions (HCl and NaOH) to a value of 7.5 ± 0.3. During stable operation, the SBRs cycles were 6 hours; 90 minutes of feeding, 257 minutes of aeration, 3 minutes of settling and 10 minutes of decanting. During the start-up, the settling time was initially 90 minutes and was stepwise decreased, and the aeration time was consequently adjusted to keep the total cycle time constant. The composition of the synthetic wastewater was chosen to mimic municipal wastewater, why it was composed of several carbon sources to give complexity and stimulate a diverse microbial community (Layer et al., 2019). The carbon sources were acetate, propionate, glucose, and peptone from enzymatic digest, which were added in equal amounts of COD equivalents. The concentrations in the influent were 400 mg L^{-1} COD, 50 mg L^{-1} ammonium nitrogen and 6 mg L^{-1} phosphate phosphorus. A detailed recipe, as well as the micronutrient solution are described in **Paper III**.

Granules from the full-scale AGS (the Österröd WWTP) were used as inoculum, which were crushed through a 1 mm sieve and added to the reactors to a concentration of 7 g L^{-1}. The study consists of two phases, an adaption period where re-granulation took place for 40 days, and an experimental period of 110 days. Two temperature decrease rates were applied, 0.5 °C and 1 °C per week, and all other operational parameters were kept the same during the experimental period. In R1, the temperature was decreased from 20 to 6 °C, and in R2 from 20 to 13 °C. To study the specific nitrification and phosphorus uptake rates, triplicate cycle studies were performed at temperatures of 20, 17, 15, 9 and 6 °C. To control the SRT and remove biologically bound phosphorus from the system, excess sludge was withdrawn from the middle and bottom ports at the end of aeration so that the SRT was approximately 30 days, using Eq. 1 (see below).

Sampling and analyses of water and sludge
Effluent and samples collected one minute into aeration were analysed twice a week, and the influent was analysed once per month. The water samples were filtered through 0.2 μm pore size. The dissolved organic carbon (DOC) and TN were analysed with a TOC/total N analyser (Shimadzu). The ion concentrations (NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P) were analysed with an ion chromatograph (Dionex ICS-900). The sludge characteristics MLSS, effluent SS, VSS, and SVI were measured according to standard methods (APHA, 2005) on a bi-weekly basis. Biomass samples for DNA analysis were collected every seven days when also the morphology of the sludge and granules were observed by light microscopy (Olympus BX53) equipped with a digital camera (Olympus DP11). To analyse the granule sizes, 50 images of granules diluted with tap water (to avoid overlapping aggregates) were taken. The images were calibrated by distance and processed by ImageJ software (https://imagej.nih.gov/ij). The granules were assumed to be spherical with a diameter calculated from the projected area, and the diameter was calculated from the projected area.

4.3 Estimation of the SRT

Eq. 1 was used to estimate the SRT for both the lab-scale and full-scale reactors:

$$\mathrm{SRT} = \frac{V \cdot X}{Q_{rem} \cdot X_{rem} + Q_{eff} \cdot X_{eff}} \quad (1)$$

V is the reactor volume (L), X is the mixed liquor suspended solids (MLSS) concentration in the reactor (g L^{-1}), Q_{rem} is the average flow rate of removed sludge (L d^{-1}), X_{rem} is the MLSS concentration of the removed sludge (g L^{-1}), Q_{eff} is the flow rate of effluent (L d^{-1}), and X_{eff} is the SS concentration in the effluent (g L^{-1}). Weekly averages were used in Eq. 1 in **Paper III**.

In **Paper II**, and **Paper IV**, a slightly different approach was used in the calculations for the full-scale reactors. For the CAS, the estimation of the SRT was calculated based on yearly averages over the period October 2020 to September 2021, with the exception of the return sludge (X_{rem}), which was an average from three measurements in November 2020 as well as a calculated value from a mass balance. For the AGS reactors, the estimation of the SRT was calculated based on averages over the period of October 2020 to September 2021. However, the concentration of SS in the discharged sludge showed a large variation, and visual inspection indicated that the concentration was very low after a few days of increased flow (resulting in many sludge discharges). The flow rate of the removed sludge was calculated based on two sludge removal methods: (1) sludge discharge which occurred every cycle and, (2) mixed granule spill (about 30 m^3 removed during the aeration phase), which occurred on average two times per month (ranging from every 10 to 21 days).

How long solids are retained in a full-scale AGS reactor can better be described as a GRTD (van Dijk, 2022). This means that even though the average SRT in the CAS and the AGS at the Österröd WWTP was in a similar range, the large granules and core fractions in the AGS reactors likely had much longer residence times. The calculated value from equation 1 will only present a rough estimate of an "average SRT", but for simplicity, both the terms SRT and GRTD will be used in this thesis.

4.4 Microbial community analysis

The microbial communities at the full-scale (biomass from AGS, CAS, influent, effluent and discharged sludge) and the lab reactors' biomass were analyzed by 16S amplicon sequencing.

For the full-scale biomass samples, the DNA extraction, sequencing and bioinformatic pipeline were performed as described in **Paper I** and **Paper IV**. Briefly, sludge biomass was centrifuged (Setting 3,000 × g, for 5 min), while influent and effluent biomass was filtered through sterile membranes (0.2 µm, Sartorius Stedim Biotech) and the remaining biomass was analysed. For biomass samples in **Paper III**, the biomass samples were collected from the middle and bottom ports of the reactor, combined and mixed with a blender. Sludge precipitates, membranes with biomass and mixed sludge were stored at -20 °C. The full-scale reactor sludge samples were thawed, resuspended in sterile pure water, and homogenised using a BagMixer 100 Minimix (Interscience), whereafter DNA was extracted from 350 µL of homogenised sludge and membranes using the FastDNA spin kit for soil (MP Biomedicals).

The V4 region of the 16S rRNA gene was amplified and sequencing was conducted on a MiSeq (2×300) using reagent kit V3 (Illumina). For details on DNA extraction, PCR, purification, quality control and sequencing see the supplementary material in **Paper I**. In **Paper I**, 89,649 to 688,416 sequence reads were obtained per sample, and 104,476 to 526,555 sequence reads in **Paper IV**, and for the lab-reactors in **Paper III,** 63,148 to 192,742 reads were obtained. Two bioinformatics pipelines (sequence processing and generation of count tables) were used, DADA2 v.1.16 (Callahan et al., 2016) and VSEARCH v.2.13.1 (Rognes et al., 2016), resulting in one consensus table created in qdiv (Modin et al., 2020). The taxonomic assignment was done using the Silva database v.132 (Quast et al., 2013) in **Paper I**, and with Midas 4 (Dueholm et al., 2022) in **Paper III** and **Paper IV**. The consensus ASV table was rarefied to the lowest number of reads by subsampling without replacement. Multivariate statistics and visualisations were generated in qdiv. Raw sequence reads are deposited at the NCBI sequence read archives (SRA), and accession numbers can be found in the respective paper.

In **Paper I**, FISH on cryosections of granules was carried out as previously described (Szabó et al., 2017b). Briefly, granules fixed in 4% paraformaldehyde (Gram-negative bacteria), or ethanol (Gram-positive bacteria) were embedded in O.C.T compound (VWR) and sliced in 20 µm cryosections. FISH was conducted with FAM-, Cy3- and Cy5-labelled probes, SYTO40 counterstaining, and DAPI was used to simultaneously target poly-P granules. Probes for *Nitrosomonas*, *Ca*. Nitrotoga, *Nitrospira*, *Tetrasphaera*, *Ca*. Accumulibacter, *Dechloromonas*, *Ca*. Competibacter and *Trichococcus* were used. Confocal microscopy was carried out with a Zeiss LSM700 (Carl Zeiss, Germany), and a 40×/1.3 apochromatic objective using 405, 488, 555 and 639 nm laser lines in frame mode with averaging =4 and pinhole size equivalent to 1AU at 639 nm. Large pictures were acquired with the tile function of the ZEN software and were reconstructed with Fiji (Schindelin et al., 2012) using the Grid/Collection Stitching plugin (Preibisch et al., 2009).

Method considerations in microbial community analyses

In the studies of this thesis, the main method to investigate the microbial community structure over time and between sample types was amplicon sequencing. The method enables in-depth diversity measurements and thorough statistical analysis and is feasible for analyses of datasets comprising millions of sequences (Hugerth et al., 2014). Amplicon sequencing is expected to result in a well assessed microbial community composition and relative abundances (Tan et al., 2015), and is a common method in studies of water and wastewater (Garner et al., 2021). However, all analysis methods of microbial communities suffer from biases and limitations (Kleikamp et al., 2022), and the relative abundance of some taxa was likely underestimated at the expense of others. The abundance of *Ca.* Accumulibacter may be underestimated with 16S amplicon sequencing (Kleikamp et al., 2022). However, the temporal dynamics and dissimilarities between sample types were likely captured with high precision.

4.5 Data analysis

To analyse significant differences between the same type of samples, t-test for paired samples was used and the two-tail p-value was calculated. Relationships between factors were analysed with linear regression, Spearman (non-normal distribution) and Pearson (normal distribution) correlation tests to assess for potential correlations. The add-in RealStats in Microsoft Excel was used, and a statistically significant correlation was considered for a p value ≤ 0.05. However, full-scale operation includes many variables, which makes a complex case for the assessment of causation.

Alpha- and beta diversity indices, the principal coordinates analysis (PCoA) and heatmaps were calculated with qdiv (Modin et al., 2020). Network analysis was performed in **Paper III**. The

ASVs detected in at least 50% of the samples and correlations with a p-value below 0.05 and a correlation coefficient above 0.8 were included in the network. The correlation matrix was established with fastspar v1.0.0 (Watts et al., 2018), an application of the SparCC algorithm (Friedman and Alm, 2012). For visualization, NetworkX v2.8.4 was used (Hagberg et al., 2008).

In **Paper II**, the land footprint (area) and volume requirements were calculated for the AGS and the CAS. The flocculation tanks, pre-settlers, and final settler are shared components of the two lines. Components included for the AGS are the two buffers, the two SBRs and the sludge buffer, and for the CAS, the reactor tank and the secondary settler are included. The flow from the pre-settlers was on average divided as 55% to the AGS, and 45% to the CAS. Hence, the pre-settlers' volume and footprint of the pre-settlers were calculated accordingly for each process. As the AGS received an additional flow not passing the pre-settlers, the total flow was divided 60/40, and 60% of the volume and footprint for the shared flocculation and final settler was considered for the AGS, and 40% for the CAS.

For both systems, the data on energy usage (kWh day^{-1}) was collected from the SCADA system, in the period from 2020-10-01 to 2021-09-30. The blowers, mixers, pumps, and sludge scrapers were included in the energy comparison. The energy usage was grouped into the categories of aeration, mixing, sludge pumping, feeding (AGS) and sedimentation (CAS). The sludge treatment process (dewatering and thickening) received sludge from both the CAS and the AGS, which are mixed in the sludge storage, thus, determining the contribution of each sludge (CAS, AGS) to the total energy usage was therefore not possible. The sludge treatment and additional components that were not directly linked to the processes (the heating system, dehumidifier, fan system, analysers, hydrolysis pump and compressor) were excluded from the energy comparison. The energy usage per treated m^3 of wastewater was based on average daily flow, and energy usage per reduced load of P.E. was calculated based on a BOD_7-load of 70 g BOD_7 per P.E. and day.

5. Results and discussion

In **Papers I** and **IV**, the process performance and microbial community dynamics were investigated at the Österröd WWTP. The operational phases are described in chapter 4.1. **Paper I** covered the start-up and tuning-in period, and **Paper IV** contained a more stable phase where the AGS was compared with the parallel CAS. The AGS and CAS systems were studied in **Paper II**, which assessed the energy usage, volume requirement and land footprint. In **Paper III**, granule characteristics, process performance, and microbial community composition and dynamics were studied in two lab-scale reactors during decreasing temperatures. In this chapter, the main focus lies on the full-scale AGS plant, starting with a description of the different operational periods.

5.1 Operational periods of the full-scale AGS and CAS

The operation of the biological treatment at the Österröd WWTP can be divided into three periods (Fig. 6), namely (1) the start-up of the AGS and the following year with fluctuating performances, (2) more stable operation which was still under development, and the CAS was started up after a reconstruction, and (3), the following period when the operation was more stable, and the comparison studies were conducted.

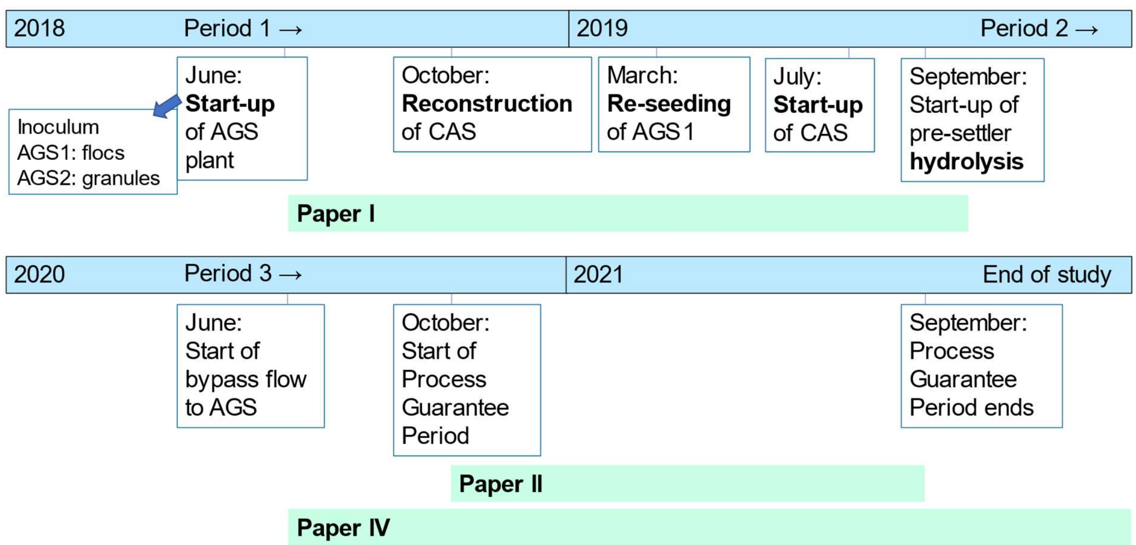

Figure 6. Timeline covering events at the Österröd WWTP, the operational periods, and the periods covered by Paper I, II and IV.

Period 1 (June 2018 – September 2019) covered the start-up and the first year

The AGS plant was started up in June 2018, where AGS1 (called R1 in **Paper I**) was seeded with activated sludge, and AGS2 (called R2 in **Paper I**) was seeded with granules, both from plants treating municipal wastewater with enhanced biological nitrogen- and phosphorus removal. The seeding was done in June when the temperature was high (about 20 °C) and resulted in similar sludge concentrations in the two reactors, 3.4 - 3.6 g L^{-1}. The granular seed came from the AGS at the Simpelveld WWTP in the Netherlands (RoyalHaskoningDHV, 2022). The activated sludge seed was taken from the neighbouring Bodalen WWTP in the municipality of Tanum, an SBR plant with compact and well-settling sludge (SVI_{30} of 67 mL g^{-1} as average during January - May 2018). During the first three months, the strategy was to gradually increase the up-flow velocity during the filling and simultaneous decanting, to promote fast-settling aggregates but avoid excessive washout of biomass. From October 2018 the parallel CAS plant was under reconstruction, why the AGS was treating the total flow to the plant. The design flow division was 60/40 to the AGS and CAS, respectively. The first year was challenging with fluctuating effluent concentrations from the AGS. Due to the low sludge concentration (about 2.5 g L^{-1}) and lack of nitrification, AGS1 was re-seeded with granules from AGS2 in March 2019. The reasons for this are discussed in chapter 4.2.

Period 2 (October 2019 – May 2020) covered the tuning in of the AGS and the start-up of the CAS
During this period, the CAS was started up and the total flow could be divided according to the design of 60% to the AGS and 40% to the CAS. The in-line hydrolysation/fermentation was implemented in the pre-settler feeding to the AGS at the end of August 2019. This correlated in time with increased BOD/N-ratio and presumably also increased bioavailability of the organic matter, even though no substantial difference in concentrations of dissolved COD could be detected. The plant also started to feed the AGS with a fraction of incoming wastewater (after screens sand- and fat-trap) by bypassing the pre-settlers in June 2020. The impact of the amount of bypassed flow was evaluated during this period. The control strategies were developed to fit the local conditions, for example, the sludge discharge, mixed granule outtake, and the possibility to control the aeration phase based on pH. During this time regular sampling was conducted in the AGS reactors for long-term microbial community analysis.

Period 3 (June 2020 – December 2021) covered more stable operation of the AGS and a comparison with the CAS
In June 2020 the AGS had been operated for two years, the CAS had been operated for one year, and sampling of biomass was started also in the CAS. In October 2020 started one year of close follow-up of the AGS, as this period was a process guarantee period (Fig. 6) when the effluent quality parameters were guaranteed by the supplier, RoyalHaskoningDHV. The guaranteed effluent quality from the AGS was a yearly average, and average in the high loaded season May-August, of 8 mg L^{-1} of BOD_7, 10 mg L^{-1} of TN, and 1 mg L^{-1} of TP.

5.2 Important factors in the start-up of full-scale AGS

Which factors are important for full-scale start-up and operation of aerobic granular sludge under fluctuating conditions?

Successful granulation
In **Paper I**, the granulation by seeding from either activated sludge (AGS1) or from mature granules (AGS2) was investigated. In AGS2, the adaption of mature granular sludge and the continuous maintenance of granules were successful, whereas the inoculum in AGS1 did not reach satisfactory granulation. The biomass in AGS2 showed a gradual increase in concentration to 6-8 g L^{-1} and granule sizes (Fig. 7). The fast-settling biomass in AGS2 enabled settling below the sludge discharge outtake (Fig. 8), which selected for dense granular sludge early on.

In the middle of March 2019 AGS1 was re-seeded with biomass from AGS2, as the biomass concentration was critically low, and the nitrification performance was almost completely lost. A few weeks after the re-seeding, the MLSS concentration was similar to AGS2, the SVI_{30} was around 50 g mL^{-1} and the fraction of larger granules (>1.4 mm) had increased (Fig. 7). Granulation with real influent wastewater from activated sludge has been observed at pilot-scale, and from seeding with surplus granular sludge (almost without granules) at full-scale

(Giesen et al., 2013; Ni et al., 2009; Pronk et al., 2015). At the full-scale AGS seeded with surplus sludge the biomass concentration reached 8-10 g L^{-1} after approximately nine months, and the flow- and treatment capacity were reached after a much shorter time (approximately three months) (Pronk et al., 2015). These previous results indicate that granulation from activated sludge could have been possible also at the Österröd WWTP, with an operational strategy centred around granulation conditions.

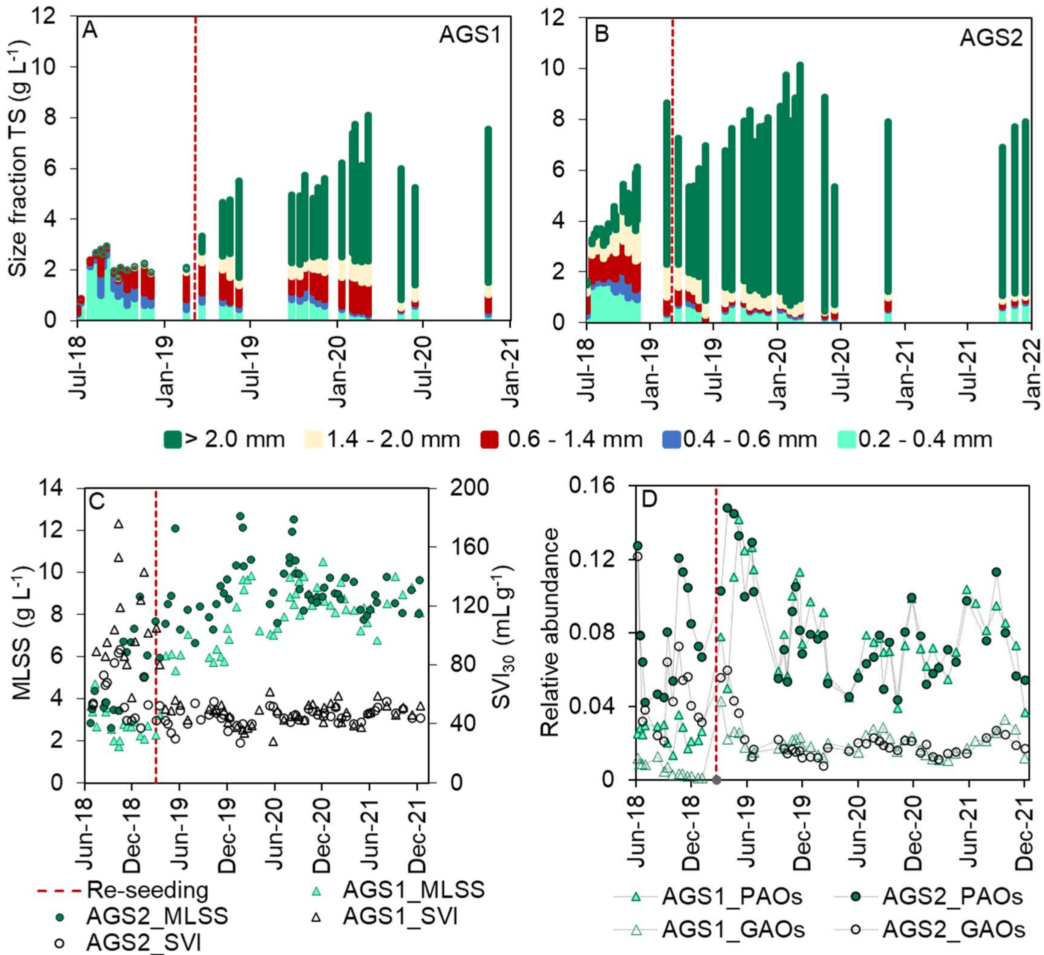

Figure 7. The size distribution of the biomass (TS) in A) AGS1 and B) AGS2. C) The concentration of MLSS and SVI_{30}. D) Relative abundance of PAOs and GAOs. The dashed line marks the re-seeding of AGS1 (March 2019).

From the lack of granulation in AGS1, the following factors to determine the success of granulation in a full-scale AGS reactor are suggested (**Paper I**); (1) selective sludge withdrawal, (2) retaining a long enough SRT (GRTD), (3) relatively high abundance of key functional groups, (4) substrate availability, and (5) operational flexibility.

Selective sludge withdrawal and SRT

The selective removal of the flocculent fraction is a major factor to achieve granulation (Rollemberg et al., 2019; van Dijk et al., 2022), among other environmental factors influencing the granulation (Bengtsson et al., 2018; Hamza et al., 2022). AGS1 contained large fractions of flocculent sludge and small granules, and the sludge discharge resulted in incomplete removal of the flocculent fraction as well as the removal of small granules since the sludge bed was settling too slowly. Samplings of the sludge discharge indicated that even smaller aggregates (top fraction in Fig. 8) could be present in the reactor after the sludge discharge.

The feeding velocity, and hence the simultaneous decanting velocity, was lowered in AGS1 to avoid excessive biomass loss with the effluent, which additionally decreased the selection for fast-settling aggregates and prevented the removal of slow-settling aggregates with the effluent. Indeed, the effluent suspended solids concentration from AGS1 was generally low. The low sludge concentration, a leaking valve for sludge discharge, and suboptimal sludge discharge operation probably resulted in a low average SRT. A substantial growth of slow-growing microorganisms needed for the granulation (de Kreuk and Van Loosdrecht, 2004) was probably limited by the lack of residence time.

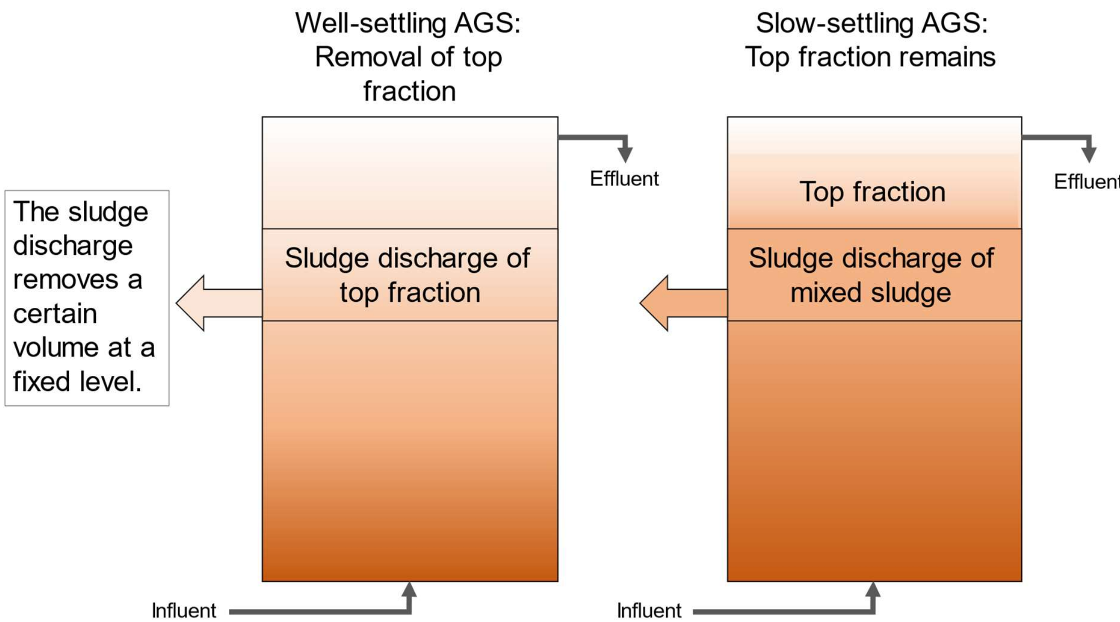

Figure 8. Illustration of sludge discharge in an SBR AGS reactor with well-settling and slow-settling sludge. In the case of well-settling AGS, all the smallest aggregates i.e., the top fraction, is removed. In the case of slow-settling AGS, the top fraction remains.

The filamentous bacteria *Trichococcus* and *Ca*. Microthrix (Dueholm et al., 2022), observed especially during the winter and spring in high abundance (AGS1), were probably related to the flocs that were allowed to stay in the reactor. Furthermore, this suggests that organic matter was available during the aerobic phase. The lack of selective sludge discharge of the slow-settling biomass, and the availability of organic substrate in the aeration phase probably resulted in the growth of flocculent filamentous bacteria, which probably led to the loose and porous aggregates. *Trichococcus* was indeed located all over the cross-section of a small granule from AGS1, suggesting that organic carbon could diffuse to the inner parts of the granules.

Key functional groups

After the re-seeding of AGS1, the relative abundances of *Trichococcus* and *Ca*. Microthrix decreased, and the abundance of PAOs and GAOs increased (Fig. 7). At the same time, the settling properties improved, and the larger granules started to increase (Fig. 7). This confirms that the groups performing anaerobic storage of COD are key microorganisms for the granulation (de Kreuk and Van Loosdrecht, 2004). PAOs form dense, compact and fast-settling aggregates and contribute to granule formation (Winkler et al., 2013a). The abundances of nutrient removal (nitrogen and phosphorus) related groups observed in full-scale AGS have previously been positively correlated with the microbial aggregate (granular) size (Ali et al., 2019). Abundances of PAOs and GAOs were also different in the AGS and the CAS (**Paper**

IV), in comparison was the average relative abundance of PAOs 7.3% in the AGS, and 3.9% in the CAS, and the relative abundance of GAOs was 2.2% in the AGS and 0.6% in the CAS. This means that the PAOs and GAOs were enriched in the AGS and were probably contributing to the granular biofilm growth. Furthermore, EPS-producing bacteria are contributing to sludge aggregation (Liu et al., 2010). For example, the GAO *Ca*. Competibacter is an EPS-producer (Seviour et al., 2011) and was high in abundance in AGS2, compared to the early stages of AGS1 and in the CAS (**Paper IV**).

Substrate availability
Low temperatures were proposed as a factor involved in the failure of granulation in AGS1, not alone but in combination with other factors. Lab-scale studies have shown that granules can form even at temperatures as low as 7 °C (Gonzalez-Martinez et al., 2018). Furthermore, the granules cultivated in the laboratory (**Paper III**) were growing throughout the study, despite the decreasing temperature down to 6 °C. Even at the lowest temperature, the TSS concentrations reached about 10-12 g L^{-1}, probably due to the comparably high load of the organic substrate (400 mg COD L^{-1}). This suggests that the biomass growth at the full-scale during the start-up of AGS1 was likely not hindered by low temperatures alone. In **Paper I**, studying the full-scale start-up, the content of granule-forming substrate was probably critically low during the late autumn and winter following the start-up, resulting in a small substrate penetration depth, and hence limited growth of large granules (> 2 mm).

Flexibility
Another important aspect of the Österröd start-up was the limited flexibility. Since the CAS was being refurbished three months after the start-up and was hence out of operation for nine months, there was no possibility to adjust the flow. During the start-up, it is desirable to be able to adjust the load depending on the current treatment performance, which was not possible. Furthermore, the operation of the sludge discharge had very little flexibility. When high flows had to pass through the AGS plant, the number of treatment cycles per day increased. As the sludge discharge occurred every cycle, this resulted in a large sludge removal rate during high flows. These operational issues probably contributed to the very slow granulation in AGS1.

Flocs and granules coexist in full-scale AGS
From February 2019 and onwards, a majority of the biomass in AGS2 was composed of large granules. The large granules in AGS1 increased fast after the re-seeding in spring 2019, and from October 2019 onwards both AGS reactors constituted mainly large granules, >2 mm in diameter (Fig. 7). The reactors also contained fractions of flocculent biomass, 21 ± 7% in AGS1, and 17 ± 8% in AGS2, which probably originated from eroded granular fragments and suspended growth (Layer et al., 2022; Szabó et al., 2017a). Some of the granules always had finger-like outgrowths, previously suggested to originate from aerobic competition for substrate uptake with ordinary heterotrophs (Haaksman et al., 2020). Outgrowths can affect the settling properties (Layer et al., 2019; Wagner et al., 2015), however, in the studied systems the extent of outgrowths was small and did not deteriorate settling properties. The flocculent fraction and the outgrowths suggest that complex organic matter in the influent was probably only partly hydrolysed (Layer et al., 2019), and leaked into the aerobic phase.

After the initial build-up of granule sizes, the fraction of large granules was dominating and remained in both reactors (Fig. 7). In 2020, the plant started with a new strategy for excess sludge removal. Removal of sludge during the aeration phase, called granule spill, was implemented, as well as an extra sludge discharge phase in the cycle operation, if the cycle had enough time. The granule spill was operated at first about twice per month, and later once per month. These were measures to control the sludge concentration and SRT and to remove accumulated phosphorus in the biomass (especially in the PAOs).

5.3 Process performance under various conditions

What factors influence the process performances of AGS?

The conditions at the WWTP were characterised by high variations in flow rates (1,200-18,200 m^3 day^{-1}) and temperature (5-22 °C). In **Paper I**, the process performance was assessed during the start-up of the AGS at the Österröd WWTP. Fluctuating effluent concentrations of ammonium, nitrate and phosphorus were observed in both reactors, and were suggested to relate to short cycle times, low temperatures, low concentrations of readily biodegradable organic matter and high nitrogen loads. In period 3, organic matter removal, assimilation, nitrification, denitrification and biological phosphorus uptake produced low average effluent concentrations in the AGS (Table 3) (**Paper II** and **Paper IV**). Both AGS reactors had stable sludge concentration and SVI (Fig. 7, Table 2) during this period. The dynamics of the influent loads of nitrogen and phosphorus followed a seasonal trend with increased loads in the summer months in both systems.

The effluent concentrations from the AGS were in the same range as other full-scale AGS plants (Pronk et al., 2015; Świątczak and Cydzik-Kwiatkowska, 2018). However, fluctuating performances were observed (Fig. 9). Elevated effluent concentrations of phosphate and nitrate in the AGS were probably caused by a lack of organic substrate (Table 1). During winter, low influent concentrations of readily biodegradable organic matter were likely caused by limited hydrolysis in the sewer system and pre-settler. In 2021, the plant implemented an anaerobic waiting phase after feeding in the cycle operation (if the cycle time is long enough), to allow for further hydrolysis.

Table 3. Effluent wastewater concentrations (mg L^{-1}) from the AGS- and CAS reactors, and from the final effluent sampling point of the plant, as average ± standard deviation. The data covers the operational period 3, and n is the number of samples.

Parameter	CAS	AGS	Plant
BOD_7	3 ± 0.5	5 ± 3	3 ± 0
COD	35 ± 4	45 ± 16	30 ± 5
COD soluble	32 ± 4	34 ± 5	*
TN	9.5 ± 3	7.9 ± 3	7.6 ± 2
NH_4^+-N	0.3 ± 0.4	0.6 ± 0.9	0.4 ± 0.6
NO_3^--N	8.4 ± 3	6.2 ± 3	6.4 ± 2
TP	1.2 ± 1	0.5 ± 0.5	0.2 ± 0.2
PO_4^{3-}-P soluble	1.0 ± 1.0	0.3 ± 0.4	*
SS	7 ± 3	14 ± 9	8 ± 4
n	31	96	84

* Not measured.

The effluent BOD_7 concentrations were low compared to other full-scale AGS plants. For example, at two AGS plants, one in the Netherlands, and one in Poland, the effluent concentrations of BOD_5 were approximately 10-20 mg L^{-1} (Pronk et al., 2015; Świątczak and Cydzik-Kwiatkowska, 2018). However, lower effluent concentrations of BOD (in the range 4-10 mg BOD L^{-1}) have been achieved in other full-scale AGS plants (personal communication with RoyalHaskoningDHV). The pre-settling before the AGS at the Österröd WWTP might explain the lower values since pre-settling was not applied at those other AGS plants. At the Österröd AGS, the effluent concentration of SS was on average 14 ± 9 mg L^{-1}, which is in the normal range of 5 - 20 mg L^{-1} (Pronk et al., 2015). Nitrogen stripping and scum baffles to decrease the rising of lighter biomass (van Dijk et al., 2018) were implemented at the Österröd

AGS plant. On a few occasions in June and July 2020, remarkably higher concentrations of SS in the effluent were observed (50 - 60 mg L^{-1}), which coincided in time with a slight increase in the relative abundance of the filamentous bacteria *Ca*. Microthrix in AGS1 ($p<0.05$), which was previously associated with bulking sludge in activated sludge (Nierychlo et al., 2021).

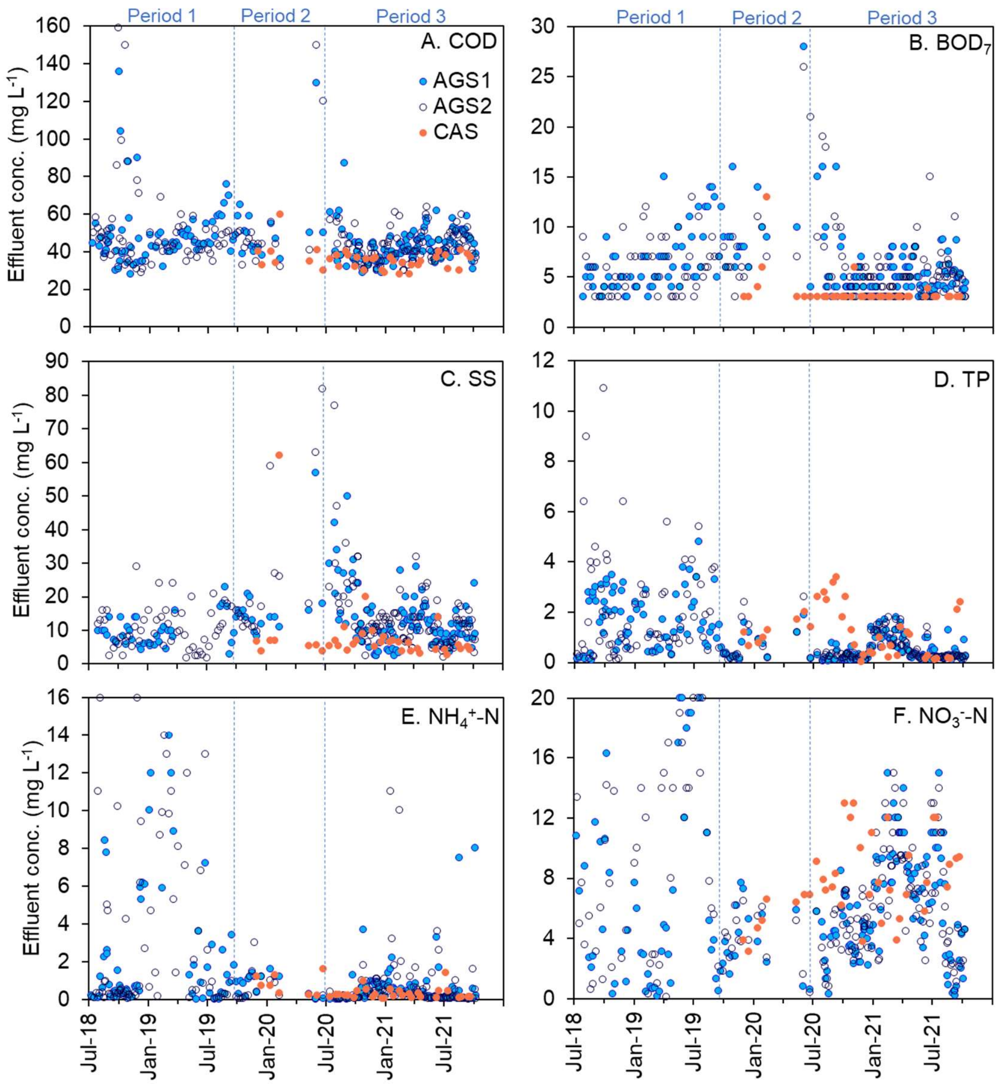

Figure 9. Effluent concentrations of A) COD, B) BOD_7, C) SS, D), TP, E) NH_4^+-N, and F) NO_3^--N from AGS1, AGS2, and the CAS. The dashed lines mark the operational period 1, 2, and 3 (defined in Chapter 4.1). Note that the influent concentrations were different in the two lines (Table 1).

Process performance of the CAS during the comparison period

In **Paper II** and **Paper IV**, the AGS was compared with the CAS operated in parallel (Fig. 3). As the flow ratio was kept constant around 60/40 to the AGS/CAS, both systems experienced similar dynamics in flow rates. The removal processes in the CAS resulted in low average effluent concentrations of organic matter, nitrogen, and phosphorus, comparable to those of the AGS (Table 3, Fig. 9). For the period assessed in **Paper II**, similar effluent concentrations of TN, PO_4^{3-}-P and TP were observed from the CAS and the AGS ($p>0.05$). Higher effluent concentrations of NO_3^--N were observed from the CAS, and from the AGS, the concentrations of BOD_7, COD, NH_4^+-N and TSS were higher ($p<0.05$). The higher effluent concentration of NH_4^+-N from the AGS compared to the CAS depended on the set-point of NH_4^+-N (1 – 3.5 mg

L^{-1}) at which the aeration was terminated. The higher effluent concentrations of BOD_7, COD, and SS from the AGS compared to the CAS (Fig. 9), likely linked to the higher influent concentrations to the AGS (Table 1). The difference in the effluent COD concentrations appears to be mainly due to the SS in the effluent. The higher effluent concentrations of SS could be attributed to the AGS technology, and/or may also reflect the higher SS content of the AGS influent (approximately 40% higher). The biomass specific removal rates were comparable in the AGS and the CAS, with higher removal rates at higher loads, which was observed in the summer months.

The TP removal in the CAS was assessed regarding phosphorus assimilation, which showed that biological P-uptake was occurring along with the assimilation, which was also supported by the detected PAOs. In the CAS, the EBPR was estimated to 18% of the TP removal (difference between total removal and assimilation), and 29% in the AGS. The effluent phosphorus concentration from the CAS increased during the spring and peaked in September 2020. Limited anaerobic volumes due to the recirculation of nitrate-rich wastewater to the same zone as the influent feeding could be a part of the explanation. Elevated phosphate concentrations in the effluent were also observed in March 2021 and September 2021 from the CAS, likely caused by low BOD/N and BOD/P ratios.

Higher effluent nitrate concentrations from the CAS coincided with low concentrations of organic matter in the influent (October 2020), low temperatures (February 2021), and particularly high influent TN concentration (July 2021). The incoming wastewater was not pumped to the zones after the aeration (due to technical problems), which implies that the post-denitrification (the zones after the aeration, normally zone 5-7) could only utilise internally stored carbon as an electron donor.

5.4 Microbial community structure in AGS

What factors govern the microbial community structure in full-scale AGS exposed to seasonal variability and how does it differ from the microbial community in CAS?

The microbial community structure of full-scale AGS was studied in **Paper I** and further investigated in **Paper IV**, wherein the microbial community in the CAS operated in parallel was also analysed. The AGS system was based on two SBRs, whereas the CAS system was constructed as tanks in series with continuous flow. The two systems had very different biomass configurations (Fig. 10). **Paper III** assessed how full-scale granular sludge adapted to the laboratory environment, and how the microbial community was influenced by decreasing temperatures.

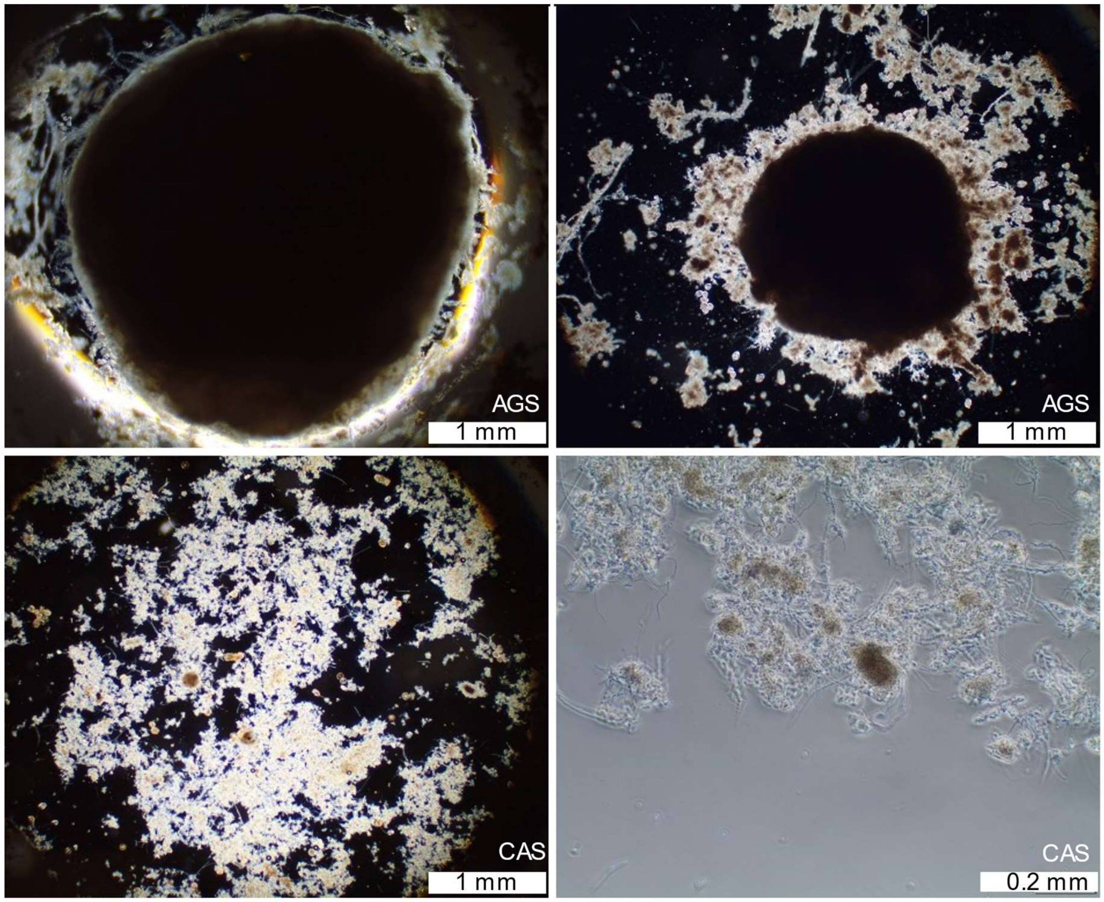

Figure 10. Microscopic images of the full-scale granular sludge and activated sludge.

Measurements of diversity and dissimilarity

In this thesis, alpha- and beta diversity were used to study the microbial community structure. Alpha diversity measures diversity within a single habitat or location, while beta diversity measures dissimilarity between different habitats, locations, or points of time. The taxonomic diversity was calculated as Hill numbers (Jost, 2006). Two different diversity orders were used, q=0 and q=1. For q=0 all ASVs are equally weighted, resulting in a measurement of the species richness. For q=1, the abundance of the ASVs is weighted in the diversity, meaning that ASVs with very few reads are disregarded, and this diversity order reflects the number of ASVs in the community of "common ASVs". The dissimilarity measurements show a relation among the microbial population at different points in time or between reactors. Comparisons of community data of one sampling day compared with the population on the following sampling dates were calculated as a value between 1 and 0 (Chao et al., 2014). If two populations are identical, the dissimilarity is 0, and if two populations have nothing in common, the dissimilarity is 1.

Alpha diversity was different in the systems

In **Paper IV**, the alpha diversities at q=0 and q=1 were similar between the AGS reactors (Fig. 11A), with an average diversity for the common community members (q=1) of 320 ASVs, while the average diversity for the common community members (q=1) in the CAS was 250 ASVs. Thus, the diversity was slightly higher in the AGS compared with the CAS (Fig. 11A) ($p<0.05$). The higher diversity in the granular sludge might be attributed to several factors; more micro niches (Flemming et al., 2016) and biomass containing both flocs and granules, a spectrum of SRTs (van Dijk, 2022), substrate concentration gradients created by the biofilm, feast-famine operation, and spatial segregation of biomass during feeding. The dense biofilm structure (Fig. 10) facilitates concentration gradients of substrates and DO within a single granule. Furthermore, the feast-famine conditions in the SBR operation and plug-flow bottom-feeding in the AGS created temporal and spatial variability in substrate availability. These mechanisms probably favoured both r- and K-strategic microbes (Wu and Yin, 2020). Both the AGS and the CAS had higher diversity than the influent microbial community (Fig. 11A), which may be explained by the larger variety of microhabitats in the reactors compared to the sewer system. The microbial community composition was different in the AGS and CAS (**Paper IV**), and the average dissimilarity between the AGS and CAS (q=1) was approximately 0.45 (Fig. 11A). The bypass led to different influent characteristics with higher concentrations of BOD_7, COD, and SS to the AGS, which likely contributed to some extent to the dissimilarity of the microbial community in comparison with the CAS. However, previous studies of AGS and flocs have suggested that the community composition (Winkler et al., 2013b), and functional diversity (Burzio et al., 2022) differ between the two systems. In chapter 5.5 (p. 48), differences in relative abundances of functional groups are discussed.

In the AGS, the diversity was increasing with higher temperatures ($p<0.05$). This is supported by the results in **Paper III**, where the microbial communities in the lab reactor exposed to decreasing temperatures showed decreasing diversity, and the reactor that reached 6 °C had lower diversity than the reactor that reached 13 °C at the end of the experiment. In the full-scale, as well as in the lab scale, the temperature ranged from 20 to 6 °C, and probably, fewer members of the microbial community were adapted to the lower temperatures, leading to lower diversities. It should be mentioned that at the full-scale AGS plant (**Paper IV**), other parameters were likely co-varying with the temperature, for example, the nutrient loads (Fig. 5). The diversity of the full-scale AGS and CAS showed different responses to temperature, which might be related to the difference in the microbial community composition. One possible explanation for the increased alpha diversity at lower temperatures could be the reduced metabolic activity, leading to the inhibition of competitive exclusion, allowing the coexistence of a larger variety of species.

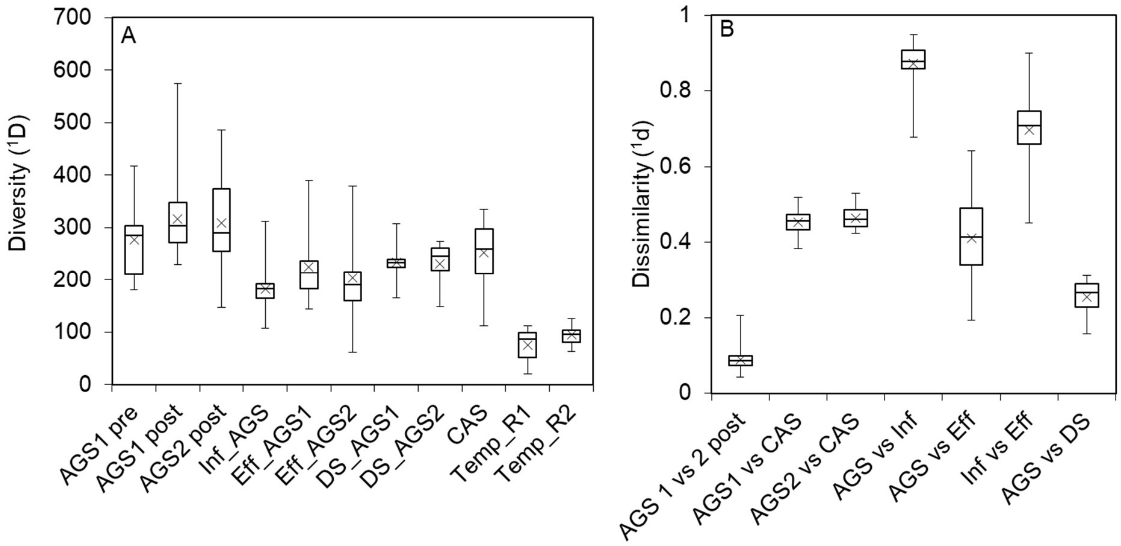

Figure 11. A) Alpha diversity (q=1) in the granular sludge; AGS1 before re-seeding (AGS1 pre), AGS1 after re-seeding (AGS1 post), AGS2 after re-seeding of AGS1 (AGS2 post), AGS influent (Inf_AGS), effluents from AGS1 and AGS2 (Eff_AGS1 and Eff_AGS2), and discharged sludge from AGS1 and AGS2 (DS_AGS1 and DS_AGS2), the CAS, and the lab-scale reactors R1 and R2 (Temp_R1 and Temp_R2) from that the temperature started to decrease until the end of the experiment. B) Dissimilarity (q=1) between samples from AGS1, AGS2 (after re-seeding), the CAS, AGS influent, AGS effluent and AGS discharged sludge.

Microbial community structure in AGS

In **Paper I**, a large dissimilarity in community composition was observed for the influent wastewater and the reactor communities (Fig. 11B), suggesting that mass flow immigration from the influent wastewater community was likely not shaping the AGS communities to a large extent. This is in agreement with previous research, where species sorting (selection based on environmental conditions) was proposed to have a higher impact on the microbial community assembly of large granules, as opposed to immigration (Ali et al., 2019). Furthermore, the study suggested that the floc fraction and small granules were more influenced by immigration compared to large granules. Previous studies of activated sludge suggested that mass immigration from the influent microbial community was important for the assembly of the reactor microbial communities (Dottorini et al., 2021). Reactor conditions, biofilm growth mode and generally longer SRT of large granules and core fractions of the biomass are probable factors influencing the granular microbial community structure, and why AGS might be less shaped by the influent community compared to activated sludge.

The microbial communities of the effluent and discharged sludge were different from the reactor community and had slightly lower diversity (Fig. 11), which indicates that a sub-fraction of the rich AGS community was removed with the effluent and sludge discharge. Due to biomass segregation and selective sludge discharge, this community fraction was likely composed of flocculent sludge and perhaps small less well-settling granules (Fig. 8). The biomass in the effluent was probably emerging from granule erosion, granule breakage and suspended growth. It can be assumed that these two sub-fractions of the AGS community will have shorter SRT compared with the majority of the reactor content, which is composed of large granules (>2 mm) (Fig. 7). The microbial communities in the effluent and the discharged sludge contained lower relative abundances of the functional groups AOB, NOB, PAOs, and GAOs compared to the reactor communities, with one exception. Observations of the relative abundance in the effluent showed higher relative abundance of AOB compared to the reactors during the first year after start-up.

In **Paper I** and **Paper IV**, the dissimilarity was observed to vary with the time gap between samples, showing increasing dissimilarity with increasing time gaps with local maxima and minima (Fig. 12). The seasonal periodicity in dissimilarity was assessed for AGS1, AGS2 and the CAS. Local maximums were found between samples taken around half a year apart and local minimums for samples taken one year apart. In both processes, samples with one year of time gap were more similar than samples taken half a year apart. However, a higher magnitude of the seasonal periodicity was observed for the CAS compared to the AGS (Fig. 12A), suggesting that the microbial community in the CAS had a stronger periodicity. Probably, the seasonal fluctuations in environmental conditions affect the flocculent sludge and granules to varying extents. Previous observations suggest that environmental conditions might influence flocs more than granules: when the particulate load in the influent was increased to a full-scale AGS reactor, the microbial community in the flocculent fraction in the AGS reactor showed a larger shift in community composition than the large granules (Toja Ortega et al., 2021b).

A period covering three years could be analysed for AGS2, not subjected to re-seeding. Several local maximums and minimums were observed at different time gaps, but no clear re-occurrence of dissimilarity was observed on a yearly basis (Fig. 12B). The reasons for this are likely due to differences from year to year in nutrient loads, temperature variability, operational parameters and stochasticity. Yet, these fluctuations indicate a periodicity in the microbial community compositions and suggest that the seasonal environmental conditions were influencing the microbial community structure. Seasonal periodicity in dissimilarity has previously been observed in activated sludge reactors (de Celis et al., 2022; Griffin and Wells, 2017; Jiang et al., 2018; Johnston and Behrens, 2020; Sun et al., 2021), which supports our findings. Seasonal fluctuations at the Österröd WWTP are characterised by high variations in temperature, increased loads of nutrients during the summers, and high flows in autumn and winter. These factors likely contributed to the observed periodicity. Previous studies of lab-scale AGS have found that nutrient concentrations and temperature influenced the microbial community structure (Muñoz-Palazon et al., 2020a; Xu et al., 2022b).

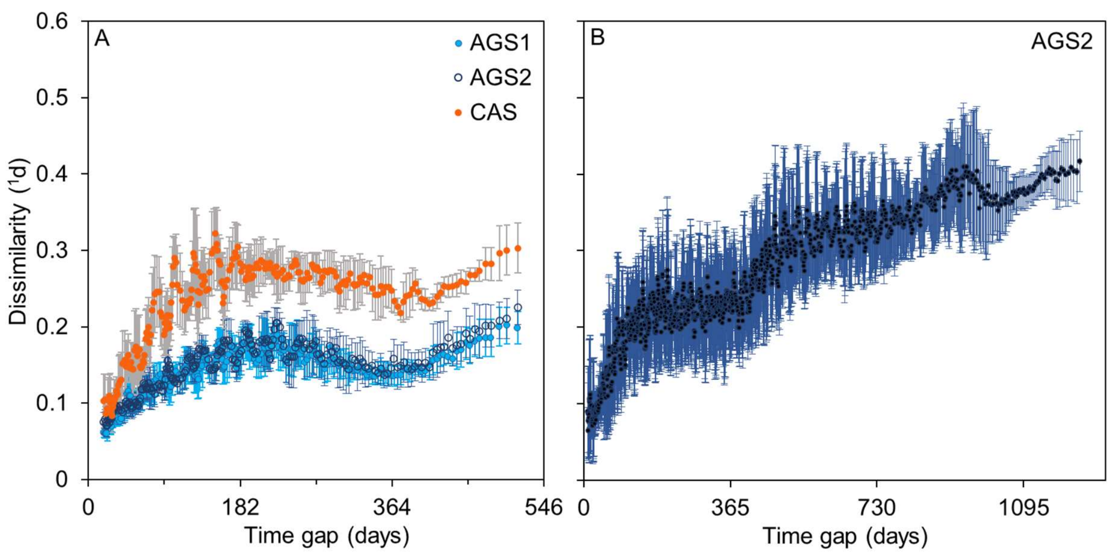

Figure 12. Dissimilarity (q=1) as a function of the time gap between biomass microbial communities in A) the CAS, AGS1, and AGS2 for the period June 2020 - December 2021, and B) AGS2 from the start-up in June 2018 to December 2021. Each point represents a moving window average of 10 samples, error bars show the standard deviation.

The rate of change was calculated as the dissimilarity between two sampling days divided by the number of days between them. Expressed as dissimilarity per day, the rate of change shows how quickly the population composition changed over time. The rate of change in the AGS reactors were higher in the initial phase of the start-up and decreased during the first months of operation, and AGS2 reached a stable rate of change more quickly than AGS1 (Fig. 13A) (**Paper I**). The fast changes in AGS1 in the initial phase indicate that the seed of activated sludge was rapidly changing due to granulation and the new environmental and operational conditions, and also the seed of granular sludge (AGS2) was changing in composition at a high rate as it was adapting to the local environment. In **Paper III**, the rate of change in the lab-scale reactors were also initially high, when the full-scale granules adapted to the laboratory conditions, and interestingly, later on, the rate of change was similar between the two lab-scale reactors despite the temperature differences (Fig. 13B). The communities in both lab-scale reactors were subjected to large changes in environmental- and operational conditions which might overshadow the shift induced by the temperature alteration. In **Paper IV**, the rate of change (q=1) was slightly higher in the CAS compared to the AGS ($p<0.05$). A higher rate of change of common ASVs (q=1) could be expected in flocculent sludge due to the relatively shorter SRT, which also agrees with the higher magnitude of seasonal periodicity compared to the AGS (Fig. 12A).

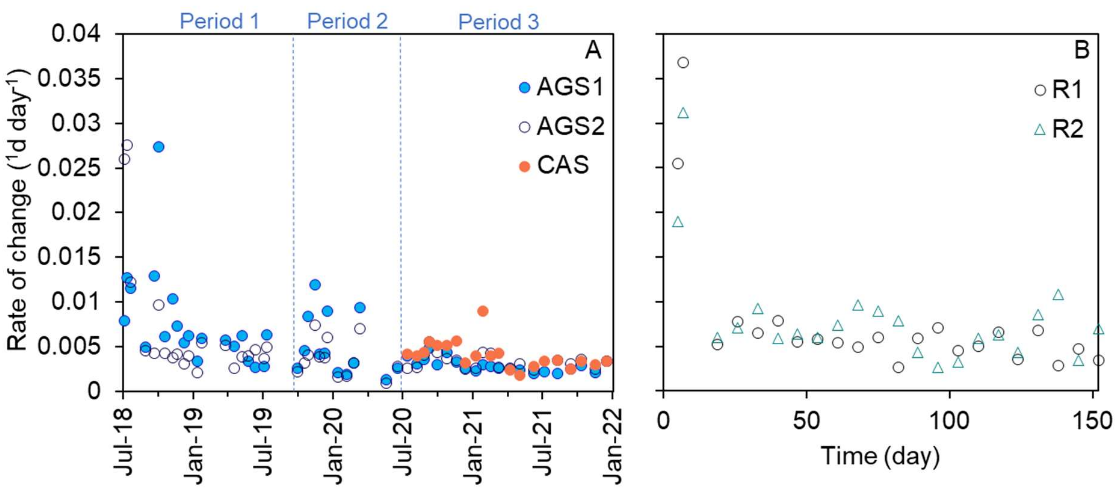

Figure 13. Rate of change (q=1) in A) the full-scale reactors AGS1, AGS2 and the CAS, and B) in the lab-scale reactors R1 and R2.

In operational period 3 (**Paper IV**), the full-scale AGS communities followed comparable trajectories in terms of community composition (Fig. 14), which would indicate that deterministic factors were involved in shaping the microbial communities (Liébana et al., 2019), however, no null-models were included in this study which would be a proper tool to analyse deterministic versus stochastic mechanisms.

Microbial community structure is different in full-scale and lab-scale

Dynamics in microbial communities in full-scale reactors are influenced by several, and often intercorrelated, environmental- and operational conditions, as well as ecosystem interactions such as competition and cooperation. Hence, coinciding variables complicate the cause-and-effect evaluation of full-scale studies. Lab-scale studies can be designed for parameter testing, where controlled conditions and isolation of one variable enable the evaluation of significant impact from the tested parameter. However, controlled laboratory conditions often poorly mimic real-life conditions, and the complexity of full-scale operational and environmental conditions selects for a larger variety of microorganisms, and probably a higher diversity of functional traits. In **Paper III**, the full-scale granules sampled from the Österröd WWTP in January 2021 were used as inoculum for the lab-scale reactors, and during the first 40 days in which the temperature had not yet started to decrease, the species richness and alpha diversity decreased. Furthermore, the community composition shifted away from the seed. In the PCoA (Fig. 14), both the full-scale- and the lab reactors were subjected to decreasing temperatures from 20 to 6 °C. The diverging trajectories show the differences in microbial community evolution in a lab-scale system and a full-scale system. Simplified, the y-axis describes temperature and seasonal conditions, and the x-axis describes differences between the conditions related to the laboratory and the full-scale. The lower diversity (Fig. 11) and change in community composition in the lab-scale reactors can be explained by several factors. Probably, a lack of immigration of microorganisms from real influent wastewater, lower complexity of the composition of the synthetic wastewater, and the constant temperature (20 C°) were factors inducing the shift. Irrespective of the reasons, this illustrates the difference between communities growing at full-scale (**Paper IV**) and those cultivated in the laboratory (**Paper III**). Substantial knowledge has been obtained from lab-scale studies, however, the responses of microbial communities in lab-scale might be different from those in full-scale.

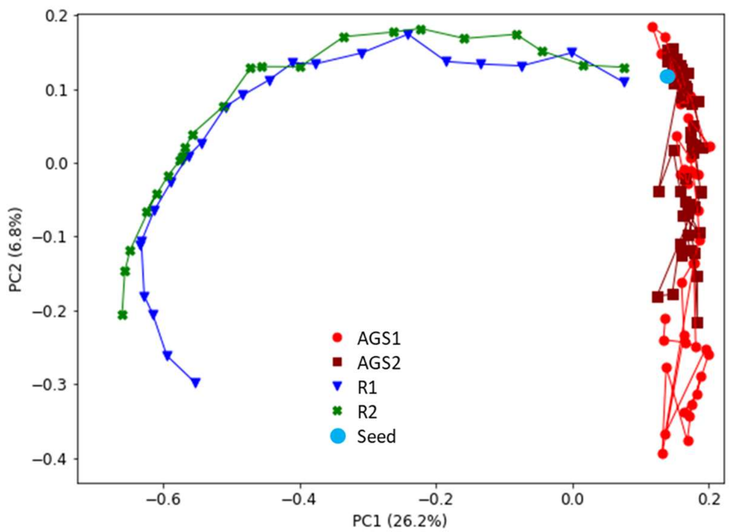

Figure 14. PCoA of the microbial community dissimilarity (q=1) in samples from AGS1 and AGS2 at the full-scale plant, the lab-scale reactors R1 and R2, and the seed taken from the full-scale AGS to the lab. The full-scale samples cover 3.5 years, whereas the lab-scale samples cover 150 days.

5.5 Removal processes and population dynamics of key functional groups

How is the treatment performance related to the key functional groups in the AGS microbial community?

Functional groups and their localisation in granular sludge

AGS1 and AGS2 at the full-scale AGS plant, in operational period 3 (**Paper IV**), had similar abundances of AOB, NOB, PAOs and GAOs ($p>0.05$). The relative abundances of AOB (*Nitrosomonas*) in the AGS reactors were low (0.1-0.4%) and highly dynamic (Fig. 15). However, low abundances of AOB seem to be common in AGS (Ali et al., 2019; Świątczak and Cydzik-Kwiatkowska, 2018), and previous studies observed full nitrification in AGS and activated sludge despite low abundances of AOB (Zhang et al., 2011). The relative abundance of NOB (*Ca*. Nitrotoga and *Nitrospira*) was representing 1-2% and showed less pronounced fluctuations (Fig. 15). *Nitrosomonas* and *Nitrospira* are commonly detected in municipal wastewater treatment reactors (Daims et al., 2006), and *Ca*. Nitrotoga at colder conditions (Lücker et al., 2015; Spieck et al., 2021). Interestingly, in **Paper III**, the relative abundance of the NOB *Ca*. Nitrotoga drastically decreased during the adaption period at the relatively higher temperature of 20 °C (compared to 7.5 °C in the full-scale reactors at the time of sampling of the seed), suggesting a preference for lower temperatures and/or real municipal wastewater. It is not clear whether the genus *Nitrospira* ought to be identified as NOB or comammox, as some species within *Nitrospira* may be able to carry out full nitrification (Daims et al., 2015; van Kessel et al., 2015). The group of putative PAOs was highly abundant (4-12%), which was also observed in other full-scale AGS reactors (Ali et al., 2019; Świątczak and Cydzik-Kwiatkowska, 2018). Detected PAOs were *Ca*. Accumulibacter, *Tetrasphaera* and *Dechloromonas*. GAOs showed dynamics in relative abundances from 1-3% and belonged mainly to *Ca*. Competibacter and *Propionivibrio* (Fig. 15). The differences in relative abundance of the genera presented in Fig. 15 between the AGS and CAS are discussed in a section further below (p. 48).

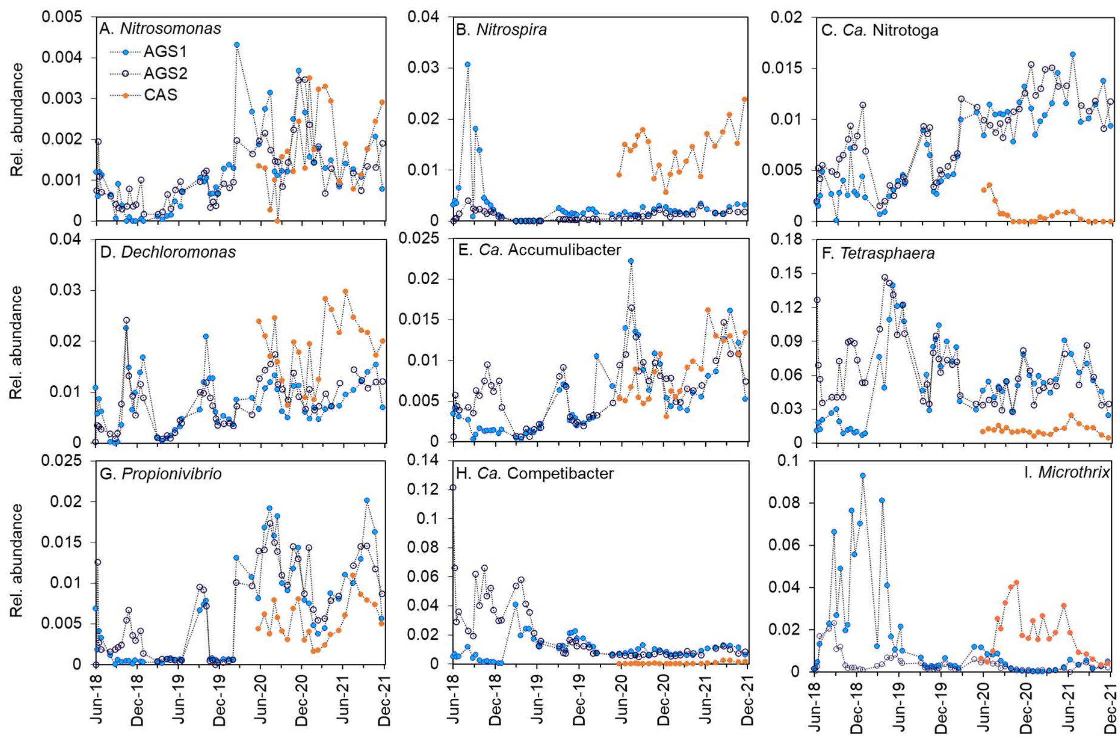

Figure 15. Temporal dynamics in relative abundance of *Nitrosomonas* (AOB), *Nitrospira* (NOB/Comammox), *Ca*. Nitrotoga (NOB), *Dechloromonas* (PAO), *Ca*. Accumulibacter (PAO), *Tetrasphaera* (PAO), *Propionivibrio* (GAO), and *Ca*. Competibacter (GAO) in the full-scale reactors AGS1, AGS2 and CAS.

In **Paper I**, the spatial localisation of key functional genera was assessed. In accordance with previous research, AOB and NOB (*Nitrosomonas* and *Ca*. Nitrotoga) were located closer to the surface (Winkler et al., 2012b), with some colonies detected deeper inside the granule (Szabó et al., 2017b). This reflects that the outer layer of a granule has higher concentrations of DO, and hence better conditions for the growth of AOB and NOB (Fig. 16). PAOs within *Tetrasphaera* and *Ca*. Accumulibacter were detected throughout the entire granule (AGS2). In contrast, GAOs (*Ca.* Competibacter) were mainly located in the inner granule parts. Voids empty of cells were observed in the core of many of the larger granules (Fig. 17), probably due to diffusion limitations. It was also observed that the granules had a porous outer layer with channels.

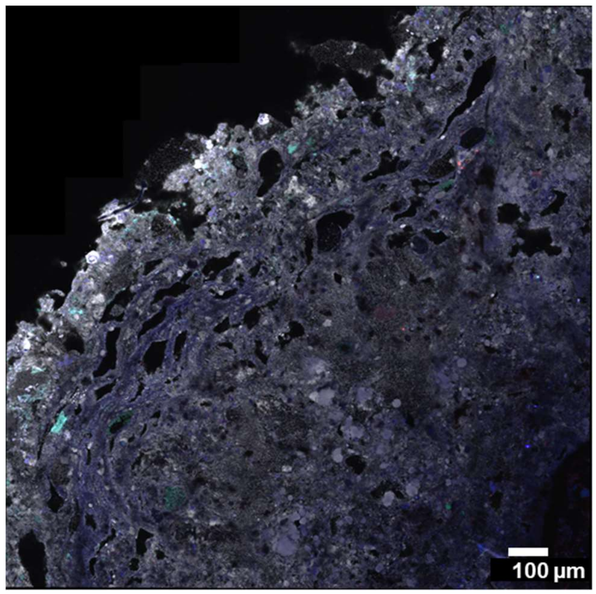

Figure 16. Fluorescence in situ hybridization of granule cryosection. Granules from AGS2 (October 2019), showing *Nitrosomonas* (cyan/green), *Ca.* Nitrotoga (blue), *Nitrospira* (red), and biomass (DNA and RNA, displayed in white).

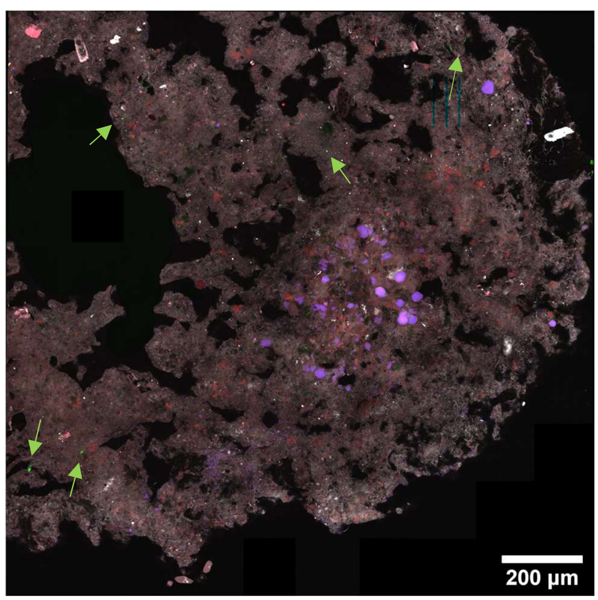

Figure 17. Fluorescence in situ hybridization of granule cryosection. Granule from AGS2 (October 2019), showing *Tetrasphaera* (red), *Ca*. Competibacter (purple) and *Dechloromonas* (green, some pointed out with arrows), and biomass (DNA and RNA, displayed in grey).

The composition within the functional groups AOB, NOB, PAOs and GAOs were similar to other studies of full-scale AGS reactors in the Netherlands (Ali et al., 2019; Toja Ortega et al., 2021b), indicating high similarity among these guilds across AGS plants. The NOB *Ca*. Nitrotoga was, however, more abundant at the Österröd WWTP, probably reflecting the colder water temperatures during a large part of the year (Lücker et al., 2015; Spieck et al., 2021). *Tetrasphaera* was the most abundant genus at the Österröd WWTP, whereas both *Tetrasphaera* and *Ca*. Accumulibacter were both highly abundant in the Netherlands. The difference might reflect the influent composition, as the concentrations of VFAs at the Österröd WWTP were assumed to be low or even very low (around 20 mg L^{-1}). *Tetrasphaera* is commonly detected in municipal wastewater treatment (Stokholm-Bjerregaard et al., 2017), a fermenting PAO able to utilize complex substrates such as amino acids and sugars, besides VFA (Nielsen et al., 2019). *Ca*. Accumulibacter, which prefers volatile fatty acids (VFAs) (Dueholm et al., 2022), was detected in lower abundances compared to *Tetrasphaera*.

Several studies have shown indications of core communities in activated sludge, at a single WWTP (de Celis et al., 2020), regional scale (Begmatov et al., 2022), national scale (Saunders et al., 2016; Zhang et al., 2018), and worldwide scale (Wu et al., 2019). Of the global core community, the NOB/Commamox *Nitrospira*, the PAOs *Dechloromonas* and *Ca*. Accumulibacter, the denitrifiers *Haliangium*, *Zoogloea*, *Rhodoferax*, and *Sulfuritalea*, and the aerobic heterotrophs *Dokdonella*, *Cloacibacterium*, *Turneriella*, and *Acinetobacter*, and the fermenter *Arcobacter* (Dueholm et al., 2022) were all detected at the Österröd WWTP.

Influence of decreasing temperature on the microbial community and process performance in lab-scale AGS

The removal processes during temperature alterations in two AGS lab-scale reactors were assessed in **Paper III**. One reactor (R1) was exposed to a faster temperature decrease rate (-1 °C $week^{-1}$), and the other reactor (R2) to a slower rate (-0.5 °C $week^{-1}$) (Fig. 18). The biomass had adapted to the new conditions after 40 days, when the SVI_{15} decreased to <40 mL g^{-1} and the ratio of SVI_5/SVI_{15} drastically dropped. The granules shifted from a mix of small granules and floccular sludge in the inoculum to larger granules in both reactors and the MLSS concentration decreased and then increased in both reactors; from 4 to 9 g L^{-1} in R1, and from 4 to 12 g L^{-1} in R2. Overall showed the size distribution by volume slightly larger granules in R2 than in R1. At the end of the study, the largest granules in R1 were 6.5 mm and 7 mm in R2. The average concentrations of effluent SS were 0.06 ± 0.03 g L^{-1} in R1, and 0.05 ± 0.02 g L^{-1} in R2, with no correlation with the temperature ($p>0.05$).

The performances of organic matter removal were steady in both reactors, at all temperatures ranging from 20 to 6 °C. The average removal of DOC was 96 ± 3% for R1 and 96 ± 2% for R2. This agrees with a previous study of AGS treating saline wastewater, where the COD removal of > 95% was observed during a temperature decrease from 25 to 13 °C (Han et al., 2022). In R1, the aerobic removal rate of DOC was linearly increasing with decreasing temperature ($p<0.05$, Fig. 19), and the concentration of removed DOC during the aerobic phase was increasing at lower temperatures at the end of the study, suggesting an increased activity of aerobic heterotrophs. Towards the end of the study, the concentration of removed DOC in the feeding phase was reduced, likely linked to the decrease of PAOs and GAOs. Both reactors removed phosphate-phosphorus to 99 ± 3% during the whole experiment (Fig. 18). Phosphorus removal has previously been observed in constant temperature lab-scale AGS studies, at 13 °C to 97% removal (Bao et al., 2009), and at 7 °C to 50-60% (Gonzalez-Martinez et al., 2017). In this study, the removal of organic matter and phosphorus was stable despite the decreasing temperatures to 13 °C (R2) and 6 °C (R1). However, the activity measurements within the cycles showed that the P-uptake rate (Fig. 19) was slowing down along with the decreasing temperature in R1 ($p<0.05$). The reduced P-uptake rates might be caused by the temperature decrease, or the vast decrease of the relative abundance of *Tetrasphaera*, which likely decreased due to the composition of the synthetic wastewater, as *Tetrasphaera* is a fermenting PAO which prefers more complex carbon sources (Barnard et al., 2017).

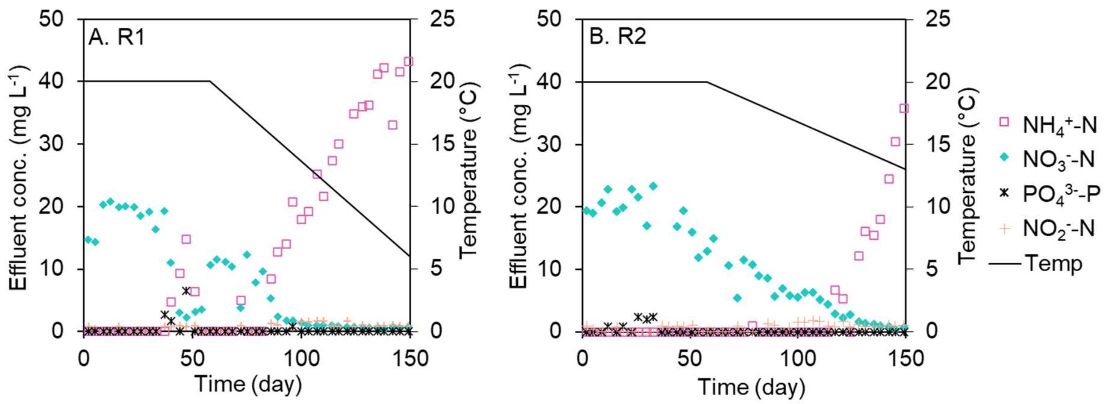

Figure 18. Effluent concentrations of NH_4^+-N, NO_3^--N, NO_2^--N and PO_4^{3-}-P and temperature in A) R1 and B) R2 (**Paper III**).

The nitrification started to deteriorate when each lab reactor had reached around 15 °C, observed as ammonium concentrations no longer reaching zero in the effluent (Fig. 18). Previous studies have found deteriorating ammonium aerobic oxidation in lab-scale AGS at low and constant temperatures (3, 5, 7, 8, 10 and 15) (Bao et al., 2009; Gonzalez-Martinez et al., 2018; Muñoz-Palazon et al., 2020b), but also full nitrification at 8 °C (Muñoz-Palazon et al., 2020a). The nitrification rate (Fig. 19) was correlated with the temperature in both reactors ($p<0.05$), but not with the relative abundance of the nitrifiers ($p>0.05$).

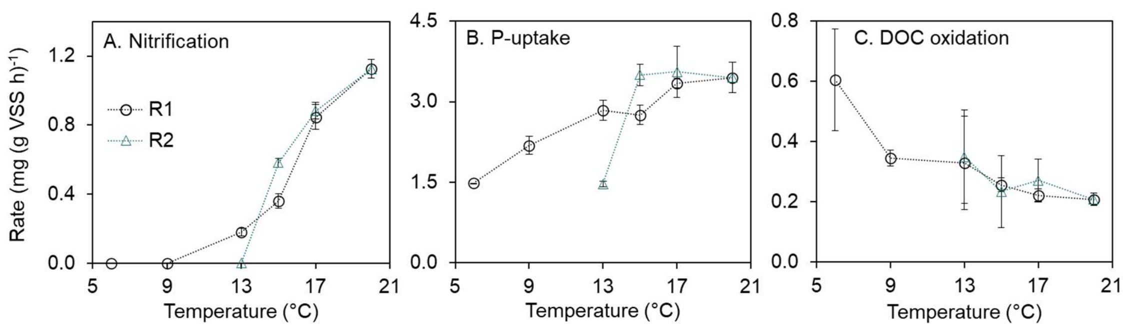

Figure 19. Measurements from lab-scale reactors exposed to decreasing temperatures. A) Nitrification rate, B) P-uptake rate, and C) DOC oxidation rate in R1 and R2 as a function of temperature. Error bars indicate the standard deviation for each mean value (rate) from three cycle studies at each temperature.

Constant abundances of nitrifiers were previously observed in activated sludge during low temperatures and failure of nitrification (Johnston et al., 2019). A possible explanation to the decreasing nitrification rates (Fig. 19) may be an increasing competition for oxygen, as more organic matter was consumed during aeration at lower temperatures. The denitrification was not straightforward to assess, as the production of nitrate varied with the nitrification performance and assimilation. However, the effluent concentrations of nitrate were decreasing from approximately 20 mg L^{-1} at 20 °C to 8 m L^{-1} in R1 (16 °C) and 4 mg L^{-1} in R2 (15.5 °C), during the period when ammonium was fully removed. In this temperature range, from 20 to 16 °C, the relative abundance of *Ca*. Competibacter increased, previously suggested to be a denitrifying GAO, which might indicate that *Ca*. Competibacter played a large role in the denitrification.

Significant positive co-occurrence patterns in a network analysis of the microbial community were studied in **Paper III** (Fig. 20). Network analyses can reveal potential ecological relationships and functional roles of the microbial species. For example, some community members may act as keystone species, which have a critical influence on the overall community structure and function. In the network analysis of the microbial communities in the lab-scale reactors, three dominant clusters with 191, 162 and 64 nodes respectively, five minor clusters, and approximately 7,000 links were identified (further details can be found in **Paper III**). The relative abundance of the members in cluster one was decreasing with the same slope in both reactors, despite the temperature differences (Fig. 20). The similarity between R1 and R2 for cluster one suggests that the relative abundance of those ASVs decreased due to other factors than the temperature. Clusters two and three showed a temperature-related dynamic in the relative abundances, where cluster two decreased and was replaced by cluster three (Fig. 20). Thus, the network suggests that the microbial communities were subject to two major processes: a temperature induced shift, and a shift related to the change of environmental conditions between full-scale and the laboratory. Those conditions were for example the stable temperature of 20 °C during the adaption period, synthetic wastewater, no or very small fluctuations of flow, organic matter, nutrients and DO, and controlled pH at 7.5 ± 0.3.

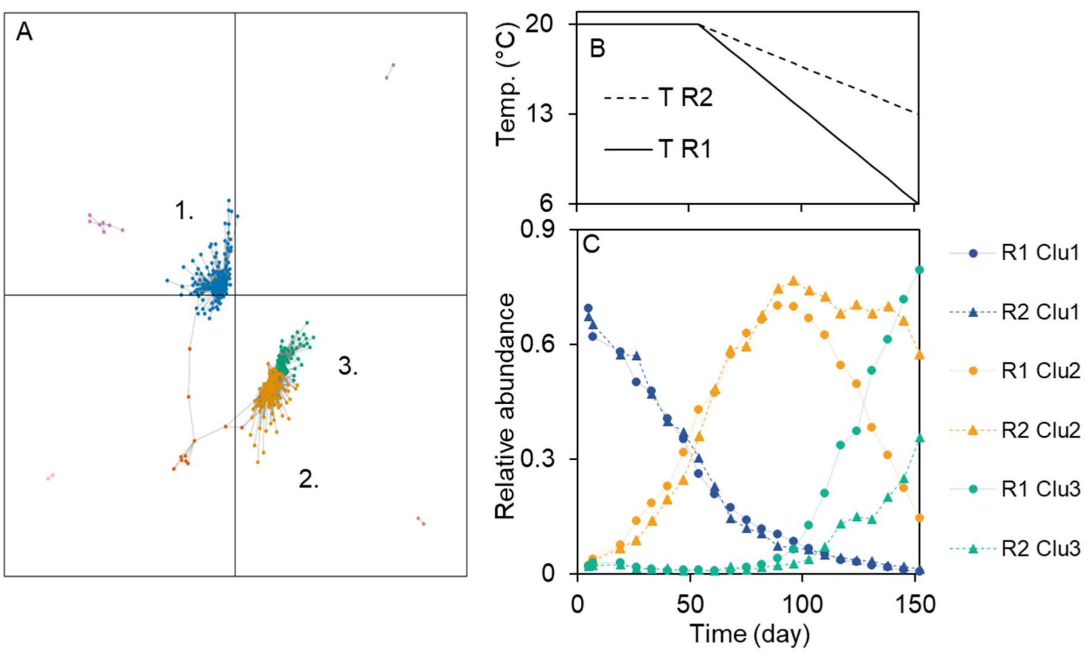

Figure 20. A) Network positive correlations in both reactors microbiome, B) the temperature alteration over time, and C) the relative abundance of the sum of the ASVs in cluster 1 (blue), 2 (yellow) and 3 (green) (**Paper III**).

ASVs within the genera *Tetrasphaera*, *Terrimonas*, and *Ferruginibacter*, were grouped to cluster one, and have been associated with hydrolysis of complex compounds (Dueholm et al., 2022). Most likely, these genera were outcompeted by genera with a higher affinity for simple carbon sources. Furthermore, *Dechloromonas* and *Ca*. Nitrotoga were decreasing rapidly at the same rate in both reactors and were found in cluster one. Cluster two harboured *Ca*. Competibacter and *Nitrospira*, which initially increased in relative abundance, but decreased at around 15-17 °C and below. *Ca*. Competibacter was previously found to cope with temperatures 15-35 °C, but at 10 °C, lower activity (Lopez-Vazquez et al., 2009) and relative abundance (Yuan et al., 2019) were observed. Members in cluster three were positively influenced by the temperatures below 15-17 °C, those were for example *Chryseobacterium*, *Cloacibacterium* (aerobic heterotrophs), *Acidaminobacter* (anaerobic heterotroph), *Ca*. Accumulibacter and *Nitrosomonas*. ASVs within *Nitrosomonas* were grouped to both cluster two and three, showing that different ASVs had various responses to the decreasing temperatures. Cluster two and three were closely located, suggesting positive correlations between the clusters. The results showed the temperature had an influence on the diversity and abundance of different genera, as well as the nutrient removal performances.

Removal of organic matter in full-scale AGS
Microbial community dynamics of genera performing organic matter removal in the full-scale AGS reactors were examined in **Paper IV**. The organic matter was removed by PAOs, GAOs (Fig. 15 and 17) and aerobic heterotrophs to low effluent concentrations. Several aerobic heterotrophs were observed in varying abundance, for example, *Rhodoferax*, *Thiothrix*, *Lewinella*, *Limnohabitans*, *Sulfuritalea*, *Rhizobacter*, *Rhodobacter*, *Nannocytis* and *Methylorosula* (Dueholm et al., 2022). The presence of aerobic heterotrophs reveals that organic substrate was available in the aeration phase and can originate from complex organic matter which was hydrolysed at the granule surface and in the flocculent sludge (Toja Ortega et al., 2021a; Toja Ortega et al., 2022). Functional groups related to hydrolysis and fermentation have not been studied as extensively as PAOs, GAOs and nitrifiers, possibly due to the large collection of organisms performing these functions (Toja Ortega et al., 2021b). However, in studies of microbial communities in full-scale AGS treating municipal wastewater (Toja Ortega et al., 2021b), as well as in lab-scale AGS reactors fed with complex wastewaters (Layer et al., 2019) several fermenting microorganisms were detected, such as *Rhodoferax, Terrimonas, Geothrix*. *Lautropia*, *Zoogloea* and *Sphaerothilus*. *Rhodoferax* was observed in the Österröd AGS, and was previously characterised as a core denitrifier in activated sludge, are putative fermenters and capable of acetate utilisation under anoxic conditions (McIlroy et al., 2016). Also, the genera *Terrimonas* and *Geothrix* were detected in the Österröd AGS, which are putative hydrolysers (Toja Ortega et al., 2021b), as well as the putative fermenters *Lautropia*, *Zoogloea* and *Sphaerothilus* (Dueholm et al., 2022).

Organic substrate availability was important for full phosphorus removal and denitrification in full-scale AGS
Fluctuating phosphate and nitrate concentrations in the effluent from both AGS reactors (Fig. 9) were observed during the first year (**Paper I**). The P-uptake rates decreased well before the aeration phase finished (observed in July 2019), despite considerable phosphorus concentrations in the water, which suggested a lack of internally stored organic carbon in the PAOs (**Paper I**). In the following operational periods, occasionally high phosphate and nitrate concentrations in the effluent were observed (**Paper II** and **IV**). For example, effluent concentrations of phosphate increased in period 3 (**Paper IV**) from December 2020 to January 2021 and correlated with a small decrease in the relative abundance of *Ca*. Accumulibacter ($p<0.05$). The influent concentration of soluble BOD_7 was very low in the same period (17 ± 0 mg L^{-1}). The decrease of EBPR and denitrification was likely caused by a lack of organic carbon that could diffuse into the granules, and high flows leading to short cycle times. Low temperatures during winters presumably lead to low hydrolysis rates in the sewer system and in the pre-settler, which likely contributed to the lack of easily biodegradable organic substrate. The organic matter in the bypass likely improved the EBPR and denitrification; when the bypass flow was turned off in the summer of 2021, this coincided with a peak of phosphate and nitrate concentrations in the effluent. The denitrifying- PAOs and GAOs can utilize nitrate or nitrite as an alternative to oxygen as an electron acceptor (Carvalho et al., 2007), and possibly contributed to the removal of nitrate via denitrification performed with stored carbon.

In the temperature study (**Paper III**), the specific P-uptake rate (Fig. 19) was significantly correlated with the temperature ($p<0.05$). Though, this might be a reflection of the decrease in the relative abundance of *Tetrasphaera*, which decreased apparently regardless of the temperature alteration. Nevertheless, the lab reactors performed 100% P-removal at 6 °C at a higher load (0.01 kg P (kg m^3 $d)^{-1}$) than in the full-scale plant (average 0.005 ± 0.002 kg P (kg m^3 $d)^{-1}$). These results suggest that the deteriorating EBPR during winter was more likely caused by the lack of substrate, than by decreased conversion rates caused by low temperatures.

GAOs are often presented as a competitor for bioavailable carbon during anaerobic feeding. However, the competition between PAOs and GAOs for substrates is uncertain, given the limited proof of the detrimental effects of GAOs on phosphorus removal in full-scale WWTPs (Nielsen et al., 2019). This is supported by the observations at the Österröd WWTP; when the hydrolysis was introduced the abundance of *Propionivibrio* (GAO) increased at the same time as the phosphorus removal improved (**Paper I**).

Denitrification probably relied on internally stored organic endogenic carbon
Nitrate was reduced in SND in the aeration phase, and in the previous and/or following pulse-aeration (pre- and post-denitrification operation when the cycle time was long enough). The SND was possibly performed by denitrifying PAOs and GAOs using stored organic carbon. The online measurements within a cycle showed accumulation of nitrate during the aeration phase and that complete reduction of nitrate was often delayed until the post-denitrification, suggesting limited SND (**Paper I**). Previously, SND was suggested to be limited by small anoxic volumes and low availability of electron donors (Layer et al., 2020), where the inadequate availability of electron donors was probably caused by insufficient concentrations of VFAs in the influent. The Österröd AGS reactors performed denitrification with an average nitrate effluent concentration of 6.0 ± 3.2 mg L^{-1} in operational period 3 (**Paper IV**). The detected presumed denitrifiers were *Thaurea*, *Zoogloea*, *Ca*. Accumulibacter, *Ca*. Competibacter, *Flavobacterium*, *Rhodoferax*, *Dechloromonas*, and *Sulfuritalea*, but also other bacteria may carry the genes for full or partial denitrification (Harter et al., 2016; McIlroy et al., 2016; Nielsen et al., 2010). Since the soluble COD was mainly removed at the beginning of the cycle (**Paper I**), the denitrification primarily relied on internally stored organic carbon and endogenous denitrification (Layer et al., 2020).

Stable nitrification was realised after the tuning-in period at the Österröd WWTP
The relative abundances of AOB were low throughout the study period in both reactors (Fig. 15), and particularly low abundances were observed in AGS1 in the winter of 2018/2019. At that time, the effluent concentrations of ammonium were elevated from both reactors. The nitrification rates measured in **Paper III** were significantly correlated with temperature (Fig. 19), suggesting that low temperature was one of the factors causing the low nitrification rates. Probably, the combination of very short cycle times, high nutrient loads (above the designed capacity), and low temperatures caused the periodically elevated effluent ammonium concentrations at the full-scale AGS (Fig. 9, period 1). The nitrification performance improved from May 2019 onward, when the cycle times were longer and the temperature was higher (13 °C), providing better conditions for complete nitrification and growth of AOB and NOB. Nitrite was not accumulated in the full-scale, or lab-scale, AGS reactors, suggesting that the NOB activity could always meet up the activity of the AOB. Metabolic flexibility may complicate the correlation between abundance and activity, since actively growing populations of AOB and NOB (*Nitrosomonas*, *Ca*. Nitrotoga and *Nitrobacter*) have been observed during seasonal nitrification failure (Johnston et al., 2019). The relative abundance of heterotrophic bacteria may additionally have contributed to the limited nitrification, by the competition for space and

DO. *Ca*. Microthrix (Fig. 15) and *Trichococcus* increased in AGS1 in winter 2018/2019 (**Paper I**) and *Trichococcus* was observed over the whole granule cross-section of a small granule.

Microbial composition was different in full-scale AGS and CAS within the functional groups
In **Paper IV**, both the AGS and the CAS harboured similar members within the PAOs, namely *Tetrasphaera*, *Ca*. Accumulibacter, and *Dechloromonas*, as well as the GAOs; *Ca*. Competibacter and *Propionivibrio*, the AOB *Nitrosomonas* and the NOB; *Nitrospira* and *Ca*. Nitrotoga (Fig. 15). The AGS and the CAS harboured similar relative abundances of AOB and NOB ($p>0.05$), suggesting that the similar influent nitrogen loads and long SRT/GRTD (Table 2) were creating similar conditions for growth of the nitrifiers. However, the composition of AOB and NOB were different, with a higher relative abundance of Ca. *Nitrotoga* in the AGS, while *Nitrospira* was detected in higher relative abundance in the CAS. Previous studies suggest a preference for *Ca*. Nitrotoga of the core of the granule (Cydzik-Kwiatkowska et al., 2022b) and in large granules (Liu et al., 2017), whereas *Nitrospira* was proliferated in the smaller fraction <200 µm (Liu et al., 2017). It appears as though *Ca*. Nitrotoga is favoured in the granular biofilm. Previous studies off genomic profiling suggested that some species of *Ca*. Nitrotoga has the ability to grow in microaerophilic environment (Boddicker and Mosier, 2018). Other studies showed contrasting results, as *Nitrospira* was detected in higher abundance in the granules compared to the flocs in a lab-scale AGS system fed with municipal wastewater (Layer et al., 2019). *Nitrospira* can grow in in a wide range of DO and might be adapted to microoxic conditions (Latocheski et al., 2022). Furthermore, *Nitrosomonas* was observed in higher diversity in the CAS (four ASVs) compared to AGS (two ASVs) and an indication of species replacement was observed in the CAS.

The higher abundances of PAOs and GAOs in the AGS compared to the CAS ($p<0.05$) probably reflect the selection pressures applied in the AGS for these slow-growing organisms, such as the long anaerobic feeding and selective sludge discharge. Interestingly, the overarching dynamics of PAOs and GAOs were generally similar in the AGS and the CAS, suggesting that environmental conditions in both processes influenced the PAOs and GAOs growing in granular biofilm and flocculent sludge in a similar way. However, the genera within the groups differed in relative abundance (Fig. 15). *Dechloromonas* was more common in the CAS compared to the AGS. Abundances of *Ca*. Accumulibacter were similar in both systems, whereas *Tetrasphaera* was higher in relative abundance in the AGS, on average 5% in the AGS, and 1% in the CAS. This difference might reflect the influent composition of the bypassed flow (increased complexity) as *Tetrasphaera* is a fermenting PAO. *Ca*. Competibacter, which was the most common GAO in the AGS, was detected in very low abundances in the CAS, whereas *Propionivibrio* was the most common GAO in the CAS and was detected in more comparable abundances in the two systems (Fig. 15). The composition and dynamics of both PAOs and GAOs are closely related due to their similar preferences for substrates, and the relative abundance of one genus likely depend upon the abundance of the other genera.

Another difference between the AGS and CAS processes was the abundance of the filamentous bacteria *Ca*. Microthrix (Fig. 15), a known aerobic heterotroph associated with troublesome floating and bulking sludge in activated sludge processes (Nierychlo et al., 2021). *Ca*. Microthrix was detected in higher abundances in the CAS reactor, corresponding to the low settleability of the activated sludge flocs (**Paper IV**). Previous studies have suggested that *Ca*. Microthrix is a PAO, but evidence for the typical anaerobic P-release followed by aerobic P-uptake has not yet been observed (Nierychlo et al., 2021). Nevertheless, the P-accumulating capability and high relative abundance suggest that *Ca*. Microthrix was taking part in the P-removal in the CAS. The inoculum and the type of wastewater were previously suggested as factors influencing the presence of filamentous bacteria in AGS (Figueroa et al., 2015). The difference in the two processes suggests that *Ca*. Microthrix did not thrive or was outcompeted in the granular microbial community. Furthermore, *Trichococcus* can be filamentous and was previously found in higher abundance in flocs compared to granules (Ali et al., 2019). At the Österröd WWTP, *Trichococcus* was detected at high abundances (17%) in AGS1 during the time of poor granulation. In the operational period 3, *Trichococcus* was observed in low relative abundance in the AGS, on average 0.5%. The relative abundance of *Trichococcus* was also low in the CAS, on average 0.8%.

5.6 Energy usage and footprint

What are the differences in energy usage, land footprint, volumes, and process performance between the AGS and CAS at the Österröd WWTP?

In **Paper II**, the energy usage, volumes, land footprint, and effluent quality of a wastewater treatment process based on AGS were compared to a parallel process based on CAS. The effluent quality from the two processes was comparably low and under the discharge limits (Table 3), which facilitates the comparison, as this is the overall aim of wastewater treatment.

Design and operation were important factors for energy usage in this case-study

The average volumetric energy usage for the AGS and CAS was 0.22 ± 0.08 kWh m^{-3} and 0.26 ± 0.07 kWh m^{-3}, respectively, resulting in a 15% reduction in energy usage per m^3 of wastewater treated by the AGS. Since wastewaters vary in organic (and nutrient) loads, measuring energy usage per P.E. takes into account the treatment loading capacity and has been used in several studies of energy usage in municipal WWTPs, and was proposed to be more meaningful than kWh m^{-3} (Vaccari et al., 2018). At the Österröd WWTP, the energy usage per reduced P.E. was 38% lower for the AGS, on average 0.19 ± 0.08 kWh $P.E.^{-1}$, compared to 0.30 ± 0.08 kWh $P.E.^{-1}$, for the CAS. The P.E. was calculated as 70 g BOD_7 per person and day. The increased organic matter load via the bypassed flow to the AGS decreased the energy usage per reduced P.E. However, this bypassed flow was theoretically leading to a decrease of primary sludge and from a plant-wide perspective, the primary sludge is a potential energy source via biogas production (Ghimire et al., 2021). On one hand, the reduced amount of primary sludge leads to less potential for energy recovery, but on the other hand, the reduction of primary sludge in the AGS line can decrease the cost of sludge handling. Before the implementation of the AGS, the specific sludge production of the whole WWTP was 0.12 ± 0.03 kg TS $P.E.^{-1}$, and with AGS in operation (2020-2021), it was similar and on average 0.13 kg TS $P.E.^{-1}$. Thus, the type of technology had likely limited influence on the potential biogas production and/or handling costs. Not only the sludge amount is important for biogas production, but also the biodegradability of the waste sludge. Previously it was observed that the biodegradability of AGS waste sludge was lower than that of CAS (Guo et al., 2020), but contrasting results have also been observed, where the methane production for AGS and CAS was in the same range (Jahn et al., 2019).

The volumetric energy usage was lower for the AGS compared with the CAS ($p<0.05$), which is in agreement with a previous full-scale study where observations showed that the AB-plant used 51% more energy per m^3 treated wastewater compared to the parallel AGS plant (Pronk et al., 2015). This difference in energy savings likely depends on the age of the plants, design, and operational strategies. Energy usage of both AGS and CAS plants can vary widely, depending on process configuration, operating- and environmental conditions, and machine equipment. At the Österröd WWTP, the energy usage was different in the two processes and varied over the year, mostly due to fluctuations in aeration and mixing demands (Fig. 21). On average most of the energy usage in the AGS was consumed in aeration, including pulse aeration and mixing (71%). For the CAS, 51 and 42% of the energy usage were consumed in mixing, and aeration, respectively (together 93%), which is in line with other recent findings (Silva and Rosa, 2022).

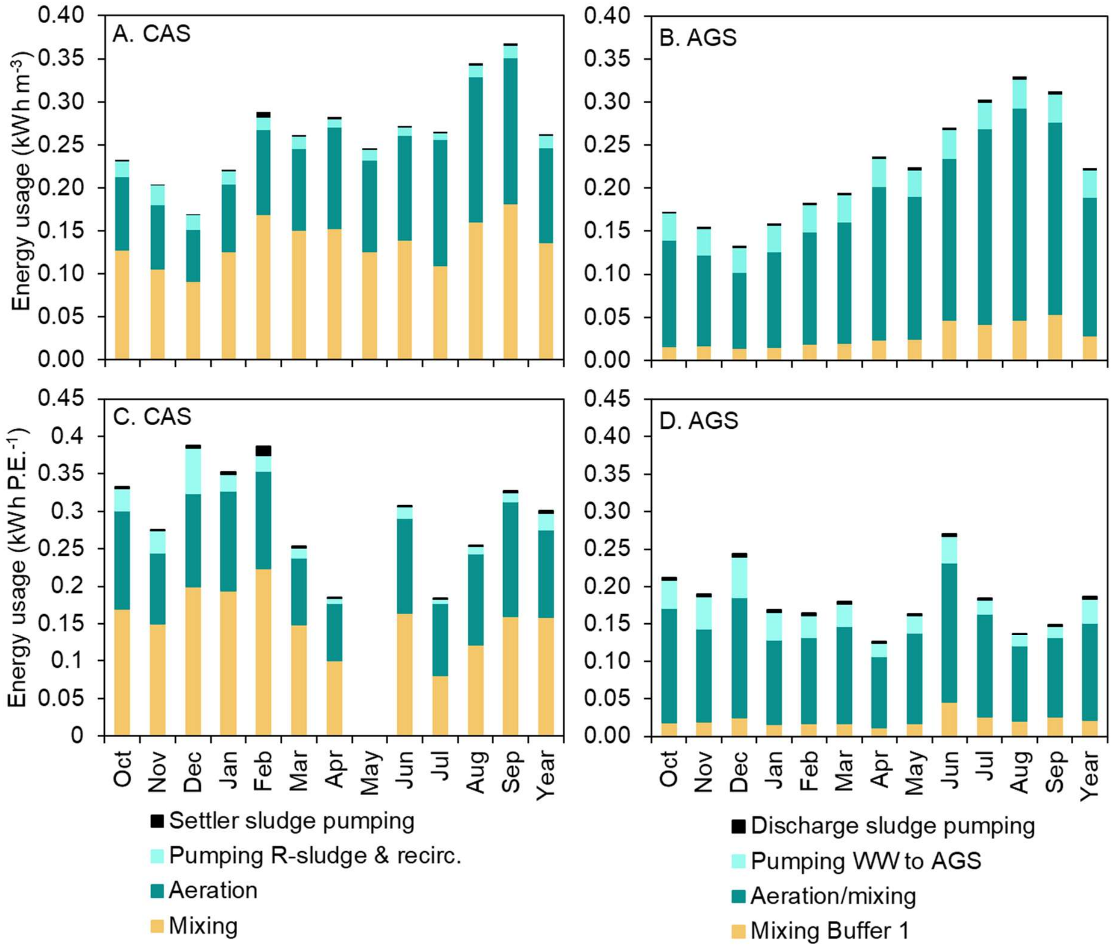

Figure 21. Monthly averages of volumetric energy usage for A) the CAS and B) the AGS, and energy usage per reduced P.E. for C) the CAS, and D) the AGS, divided into the energy categories of pumping, mixing and aeration. (**Paper II**).

The volumetric energy usage for aeration in the CAS and AGS reactors were found to be correlated ($p<0.05$), suggesting that conditions the systems had in common were influencing the aeration energy usage in a similar manner. Aeration energy usage depends on a number of factors, including the influent concentration, aerator type, diffuser type, temperature, DO concentration and oxygen transfer coefficient (α) (Silva and Rosa, 2022). The volumetric aeration energy usage for the AGS was about 30% higher than in the CAS, caused by several factors. About 20% of the aeration energy was used for mixing purposes during pulse-aeration and nitrogen gas stripping, and hence not directly related to the oxygenation of the water. Additionally, the higher influent concentrations of organic matter and nitrogen were possibly resulting in a small contribution to a higher demand of air. The average DO concentrations in the two systems were similar, on average 2 mg L^{-1}, thus not a reason for the higher aeration energy usage in the AGS. The higher depth of the AGS reactors is probably one reason behind the higher aeration energy usage, as the energy usage increases sharply with the counterpressure for this type of blower, namely rotary lobe blowers (AtlasCopco, 2015; van Leuven, 2010).

The volumetric aeration energy usage in the AGS was observed to vary with the temperature and the DO concentration, and increased energy usage was observed at higher temperatures and lower DO concentrations. This might depend on that during the summer season, when the temperature and loads were higher, a lower concentration of DO was applied. The energy usage increased with the TN removal, which showed the best linear relation to energy usage, compared to the removal of organic matter. This is in agreement with the higher stoichiometric oxygen demand for nitrification, in comparison with the stoichiometric oxygen demand of organic matter removal (Henze et al., 2019). Hence, the increased energy usage during summer was likely linked mainly to an increased extent of nitrification. The volumetric aeration energy usage was generally higher at higher temperatures in both systems. As the oxygen saturation concentration in water decreases with increasing temperature, leading to higher energy usage at higher temperatures. Even though the mass transfer of oxygen increases with temperature, the oxygen saturation concentration dependency of the temperature is the governing mechanism. Additionally, the concentration of oxygen in the air is lower at higher temperatures due to the lower density, leading to higher energy requirements of aeration at higher temperatures.

In this case-study, the design and operation of AGS and CAS were not optimised for energy efficiency, however, studies have shown that efficient design and operation can reduce the energy usage (Foladori et al., 2015; Yang et al., 2010). The AGS as installed at the Österröd WWTP could potentially decrease its energy usage with blowers which are better suited for the depth of the reactors (7 m) (AtlasCopco, 2015; van Leuven, 2010). The CAS had the potential to decrease the energy used in mixing by a more energy-strategic operation. Previous studies conducted at other wastewater treatment plants (WWTPs) revealed considerably lower values for mixing energy usage (Füreder et al., 2017). WWTPs can have energy usage varying on broad spans depending on process configuration, operating and environmental conditions as well as machine equipment (Cardoso et al., 2021; Maktabifard et al., 2018). Factors identified influencing the energy usage in the AGS and the CAS were temperature, DO, influent concentrations, the water depth, and properties and operation of aeration and mixing equipment. Hence, the choice of technology might not be the most important factor governing energy usage, but rather the design and operational strategy of the chosen technology. Processes based on both AGS and activated sludge can avoid mixing and internal recirculation.

Granular biofilm characteristics led to low footprint

The AGS had higher sludge concentration, lower SVI, and considerably more stable SVI compared to the CAS (Fig. 7). However, the AGS received higher loads (kg d^{-1}) of organic matter and nutrients, which partly can explain the higher sludge concentration. These characteristics resulted in the small land footprint and volume of the AGS plant (Table 4). The main components of the AGS line had 70% lower land footprint per treated volume of wastewater compared to the CAS (area excluding flocculation, pre-settler and final sedimentation), and 50% lower considering the total area of all related process components. The granule size fraction >2 mm constituted the majority of the TSS in the reactors, and the granules grew as large size as 5 mm in diameter. Due to the compact granular biofilm, the time required for settling was short, and as high biomass concentrations denote more microorganisms performing the removal processes, it led to higher volumetric removal rates compared to the CAS (Fig. 22). In this case study, the process based on the AGS technology was found to be compact, and with lower energy usage compared to the process based on CAS technology. Other compact technologies such as MBR and IFAS have typically higher energy usage than CAS (Bengtsson et al., 2019).

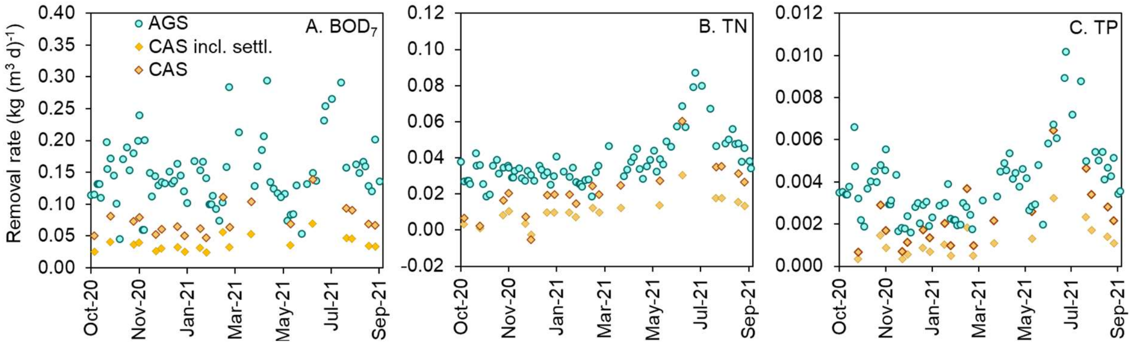

Figure 22. Volumetric removal rates of A) BOD_7, B) TN and C) TP in the AGS (average of AGS1 and AGS2) and the CAS including the secondary settler, and the CAS reactor.

Table 4. Footprint and volume for AGS and CAS (**Paper II**).

AGS	Footprint (m^2)	Volume (m^3)	Specific footprint ($m^2\ m^{-3}\ d^{-1}$)	Specific volume ($m^3\ m^{-3}\ d^{-1}$)
Flocculation	20	70	0.0076	0.027
Pre-settler (together with CAS) [a]	110	440	0.042	0.17
Buffer 1	70	340	0.027	0.13
AGS reactors	220	1510	0.083	0.57
Buffer 2	70	450	0.027	0.17
Sludge buffer	10	30	0.038	0.011
Flocculation (together with CAS) [b]	20	80	0.0076	0.019
Final settler (together with CAS) [b]	270	550	0.10	0.21
Total	790	3440	0.30	1.3
Total without floccul. pre-settl. and final settl.	370	2330	0.14	0.88
CAS				
Flocculation	20	70	0.012	0.042
Pre-settler (together with AGS) [a]	90	360	0.054	0.21
Activated sludge reactor	360	1300	0.21	0.77
Settler for activated sludge	380	1290	0.23	0.77
Flocculation (together with AGS) [b]	10	80	0.0059	0.018
Final settler (together with AGS) [b]	180	360	0.11	0.21
Total	1040	3410	0.62	2.0
Total without floccul. pre-settl. and final settl.	740	2590	0.44	1.5

[a] Divided as 55/45% for the AGS and CAS respectively.
[b] Divided as 60/40% for the AGS and CAS respectively.

The CAS was refurbished in an existing volume. If it would have been equally deep as the AGS reactors (7 m), the land footprint per treated m^3 of wastewater for the AGS would still be 40% lower than for the CAS (total area). Furthermore, the specific volume (reactor volume per treated flow of wastewater, $m^3\ m^{-3}\ d^{-1}$) was 40% lower for the AGS compared to the CAS (excluding flocculation, pre-settler and final sedimentation), which agrees with a previous comparison where the volume needed for AGS was 33% lower than for CAS (Pronk et al., 2015).

6. Summary and conclusions

The goal of this project was to increase the knowledge of application of AGS under fluctuating conditions, typical for temperate climate with cold winter temperatures, diluted wastewater and large variations in flow and load. Granulation and community succession were studied in two full-scale AGS reactors for more than three years. Granulation and reactor performance were highly influenced by the seeding sludge and start-up conditions. In addition to selective bottom feeding and feast-famine operation, factors suggested as important for successful granulation include selective sludge withdrawal, maintenance of a long SRT (GRTD), abundance of functional groups, adequate substrate availability, and operational flexibility.

The AGS and CAS systems were applied in SBRs and in a series of tank reactors with continuous flow, respectively, with differing influent concentrations; yet, comparable effluent quality was achieved throughout the operation. Primary settler hydrolysis and flow bypassed the pre-settling were successfully applied to increase the organic load to the AGS. However, both processes had fluctuating effluent concentrations of phosphorus and nitrate. In the AGS, short cycle times, high nutrient loads, low temperatures, and periodically limited organic substrate had negative impacts on the removal performances. In the CAS, periodic limitations of anaerobic volumes and availability of organic matter were probably causing the elevated effluent concentrations of phosphorus and nitrate.

In the AGS, the microbial community structure was influenced by the operating parameters and environmental conditions. Immigration of microorganisms from the influent wastewater probably had a minor influence on the microbial community in the AGS reactors, as indicated by the high dissimilarity between the influent- and reactor communities. Seasonally fluctuating environmental conditions and reactor operation were likely the main drivers in shaping the microbial community structure, as seasonal periodicity and selection of key functional groups were observed. The feast-famine operation, selective feeding and -discharge, and long SRT, probably selected for a microbial community adapted to the reactor conditions. The functional group members were similar to those in other full-scale AGS plants with high abundances of PAOs and GAOs.

A higher diversity in the AGS compared to the CAS was explained by differences in SRT, temporal and spatial substrate gradients, created by reactor operation mode (SBR versus continuous flow) and biomass growth mode (biofilm versus flocculent). The members of the functional groups PAOs, GAOs, AOB, and NOB were similar in the two systems, but the abundances within the groups as well as among the ASVs were different. *Ca*. Nitrotoga was detected in higher relative abundance in the AGS compared to the CAS, whereas the opposite was observed for *Nitrospira*. Both processes had similar relative abundance of *Ca*. Accumulibacter, while the relative abundance of *Tetrasphaera* was higher in the AGS, likely reflecting the difference in the influent composition.

The temperature showed a large influence the microbial community in lab-scale AGS reactors. In a network analysis of positive correlations, the microbial community could be clustered into three major clusters expressing two major drivers for the shift in the microbiome: a temperature-dependent replacing dynamic of two clusters (cluster two and three), and "washing out" of bacteria in cluster one, more likely to be caused by the composition of the synthetic wastewater. For example, *Tetrasphaera* and *Terrimonas* were grouped to cluster one, and were more likely influenced by the composition of the synthetic influent wastewater. *Tetrasphaera* was replaced by *Ca*. Accumulibacter, which was grouped to cluster three which increased in relative abundance as the temperature dropped. *Nitrospira* and *Ca*. Competibacter were identified in cluster two, which had a dynamic temperature response. The decreasing temperatures were found to influence on the removal performances and microbial community structure and composition.

Data from a full year of energy usage at the Österröd WWTP showed that the AGS had 15% lower volumetric energy usage (kWh m^{-3}), and 38% lower in energy usage per load (kWh $P.E.^{-1}$), compared to the CAS. Seasonally fluctuating energy usage was linked to higher temperatures and more extensive nitrification during the summer. However, these results reflect the site specific conditions of the Österröd WWTP, which included possibilities to reduce the energy usage both in the CAS and the AGS. Therefore, the differences in energy usage between the technologies would likely be different at other plants. Factors such as the reactor depth, properties and operation of the aeration system and mixers were found to be equally important for reducing energy usage as the treatment technology. For the AGS, the specific land footprint was 70% lower and the specific volume was 40% lower than the CAS (area and volume excluding flocculation, pre-settler and final sedimentation per treated m^3 of wastewater). The higher biomass concentration and removal rates in the AGS were leading to the compact treatment process.

7. Future work

Recommendations and suggestions for future work are presented in this chapter.

- Deeper understanding of microbial community ecology of AGS can help in the optimisation of treatment performance, troubleshooting treatment failures, and in the development of new treatment technologies. To conduct the microbial community analysis with a combination of methods, such as 16S gene sequencing and metagenomics, would provide a higher resolution of the community, since amplicon sequencing of the 16S rRNA gene under- and overestimates different members in the microbiome. Furthermore, metagenomics can capture functional gene variations, and hence contribute to a deeper understanding of the correlation between microbial community composition and functional traits.
- Analyses of EPS content and composition in the AGS and CAS from full-scale reactors could help to deepen the understanding of the differences in biomass configuration and how the granules are formed.
- In this thesis, the influence of decreasing temperature was studied for AGS treating a semi-complex synthetic wastewater. The synthetic wastewater was applied to isolate the variable parameter, which was the temperature. However, the synthetic wastewater had a major impact on the microbial community. Therefore, it is suggested to study the effects of cold temperatures on AGS treating real municipal wastewater. Furthermore, it is suggested to sample the seed when the temperature at the WWTP is similar to the starting temperature of the experiment, to decrease the effect of a sudden temperature change which might cause a shift in the microbial population. This was not possible in this study due to collaborations and time restrains.
- For full-scale WWTPs in regions such as Sweden, low temperatures often coincide with diluted wastewater and a decrease in concentrations of easily biodegradable substrates due to less hydrolysis in the sewer network. In this work, we saw that the phosphorus removal worked well down to 6 °C in the lab-scale system, however, that was with supply of easily biodegradable substrates. Therefore, the performance of phosphorus removal in AGS during cold temperature conditions in combination with low-strength wastewater is suggested to be studied.
- For WWTPs with varying loads of organic matter, nutrients and flow, it is interesting to study measures to improve the organic substrate availability (quality and C/N and C/P ratios). For example, studies focusing on effects of different exchange ratios and anaerobic waiting time after the feeding. This could possibly make the EBPR and denitrification more reliable.
- The energy usage associated with aeration is typically one of the largest categories of energy consumption in WWTPs, why optimisation of the aeration is beneficial. In this research, we showed that most of the energy was used in the aeration and pulse-aeration of the AGS. Studies of AGS applying different aeration systems (for example blower- and disc types) could give us insight into the optimal aeration design and operation.
- Studies of energy usage are highly valuable in the work towards fulfilling the SDGs. The energy comparison between AGS and CAS in this work is a case study, and the results might look different for another WWTP. One question is whether larger WWTP with less seasonal fluctuations in flow and loads would have a lower energy usage per treated m^3 of wastewater. Also, the energy usage of AGS in comparison with other compact treatment methods such as MBR, MBBR, MABR and IFAS could verify theoretical studies showing lower energy usage of AGS.

- Measurements of nitrous oxide gas emissions are highly recommended to be studied at full-scale AGS for a period of one year, in order to account for seasonal changes in the emissions. As the results of this study point out, the microbial community in AGS changes seasonally and might impact the emissions. Furthermore, mitigation methods to reduce emissions should be outlined and tested, with a focus on an optimum between the best effluent quality and the least greenhouse gas emission. van Dijk et al (2018), found that the emissions were reduced by denitrification, which is recommended to be investigated at other full-scale AGS plants.
- Sludge production and biogas potential from waste granular sludge are interesting factors to investigate, as these factors with positive impact on the energy balance of WWTPs applying anaerobic digestion. For a comprehensive approach of comparison of wastewater treatment technologies, also the production of valuable by-products should be considered.
- For a comprehensive comparison of wastewater treatment technologies, the removal of micropollutants and the production of valuable by-products should also be taken into account. While studies have been conducted on this topic, more research is needed.

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