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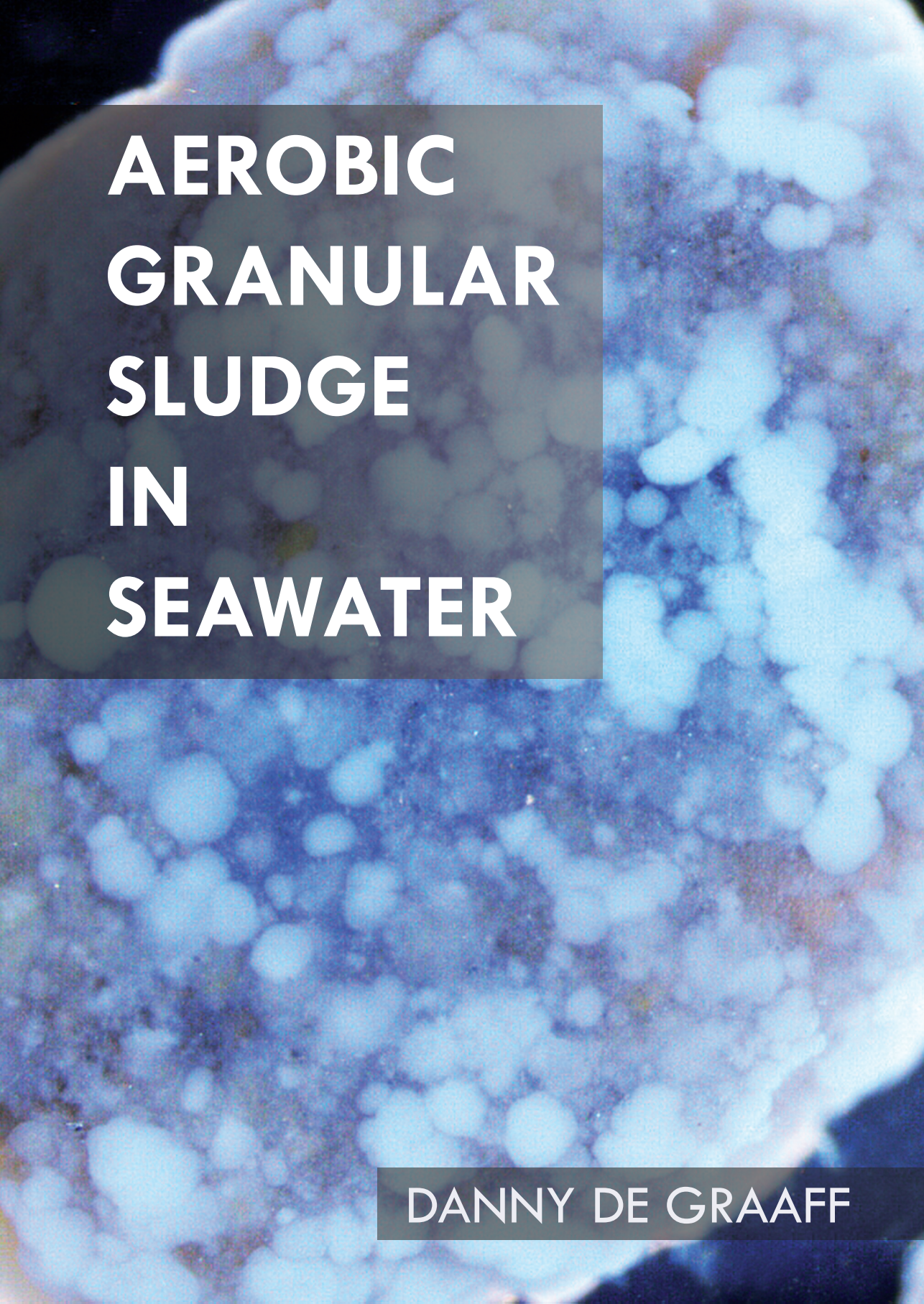
DOI
10.4233/uuid:eb3e337a-972c-498c-9bb9-e9d8bed7f006

Publication date
2020

Document Version
Final published version

Citation (APA)
de Graaff, D. (2020). *Aerobic Granular Sludge in Seawater*. [Dissertation (TU Delft), Delft University of Technology]. https://doi.org/10.4233/uuid:eb3e337a-972c-498c-9bb9-e9d8bed7f006


AEROBIC
GRANULAR
SLUDGE
IN
SEAWATER
DANNY DE GRAAFF

Aerobic Granular Sludge in Seawater

Aerobic Granular Sludge in Seawater

Proefschrift

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus prof. dr. ir. T.H.J.J. van der Hagen,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen op
maandag 10 februari 2020 om 15:00 uur

door

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Dit proefschrift is goedgekeurd door de

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Samenstelling promotiecommissie:

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Prof. dr. ir. J.B. van Lier	Technische Universiteit Delft, reservelid

Keywords: Wastewater treatment, Aerobic granular sludge, EBPR, Nereda, Seawater

Printed by: Proefschriftmaken, De Bilt

Front: Aerobic granular sludge with bright colonies of *Ca.* Accumulibacter phosphatis bacteria.

ISBN 978-94-6384-104-7

An electronic version of this dissertation is available at
http://repository.tudelft.nl/.

Contents

Summary

Increase in sea level will lead to an increase in salinity in domestic wastewater systems. In order to anticipate its effects on biological wastewater treatment, the impact has to be assessed with lab-scale experiments. Aerobic granular sludge (AGS) is a successful technology for simultaneous removal of organic carbon (COD), nitrogen, and phosphorus in a single process step. The impact of seawater on AGS has not yet been reported. The effect of salinity on AGS can roughly be divided in three aspects: biological activity, physical stability, and change in extracellular polymeric substances (EPS). Based on these aspects, the research from this PhD thesis has been structured. The overall question is: "How does long-term exposure of seawater affect the AGS process?"

In order to answer this question, a lab-scale AGS reactor has been operated with artificial seawater in the synthetic wastewater influent. Chapter 2 describes the effect of long-term exposure of AGS to seawater on enhanced biological phosphorus removal (EBPR). Stable granules were formed, consisting of a dominant fraction of polyphosphate accumulating organisms (PAO), and the absence of glycogen accumulating organisms (GAO). Anaerobic acetate uptake was complete within 60 minutes of plug-flow feeding, and aerobic phosphate uptake occurred completely within 170 minutes of aeration. Simulation of a rainwater situation, by introducing demineralized water as influent for a single cycle, led to a decrease in anaerobic acetate uptake and phosphate uptake. The subsequent cycle with reconnected seawater directly led to recovery of complete anaerobic acetate uptake and improved aerobic phosphate uptake. During this osmotic downshock release of COD was measured, which was ascribed to release of osmolytes, and was further investigated in chapter 6.

The effect of short-term exposure of full-scale Nereda® granules to NaCl-containing water was investigated in chapter 3. A strength characterization method was evaluated, based on abrasion of granules at high shear in a stirred tank reactor. Abrasion times up to 90 minutes showed a stable abrasion coefficient (K). Larger granules showed higher abrasion than smaller granules. Granules from four different Nereda® plants showed a difference in K-values. Exposure of these granules to 25 g/L NaCl solutions led to a decrease in K. Interestingly, exposure to 50 g/L NaCl led to a further decrease in K for only two out of four granule samples, while the other two showed a relative increase in K. Release of calcium and magnesium ions were measured in the liquid. Lab-scale acetate-fed seawater-adapted granules had a lower K than freshwater-adapted lab-scale granules.

Adaptation of EPS to saline wastewater is commonly described in literature, but a qualitative approach had so-far not been taken. In chapter 4 fluorescence lectin bar-coding (FLBC) was used to discover the presence of sialic acids in the EPS of seawater-adapted AGS. Sialic acids are a group of monosaccharides with a nine-carbon backbone, and neuraminic acid (Neu5Ac) is one of the most frequently observed ones. Sialic acids are frequently described to protect EPS molecules and cells from attack by proteases or glycosidases. This protective role was also found in AGS; galac-

tose residues in the sugar chains from EPS could be enzymatically cleaved with prior removal of sialic acids, but the presence inhibited this removal. Genome analysis showed that *Candidatus* Accumulibacter phosphatis contains genes that are responsible for the production of neuraminic acid.

Seawater contains large amounts of sulfates, which can enter wastewater treatment systems in the reduced form of sulfide. This can cause toxicity or the formation of filamentous bulking sludge, often containing *Thiothrix* bacteria. However, recent research has shown that *Thiothrix caldifontis* has a similar metabolism as *Ca.* Accumulibacter phosphatis, with anaerobic acetate uptake and aerobic phosphate uptake, next to storage of sulfur in intracellular pools. This resemblance led to the question whether stable granules can be formed with the presence of filamentous *Thiothrix* bacteria. Chapter 5 describes the granulation with the presence of *Thiothrix* bacteria. Thiosulfate was added to the influent to increase the *Thiothrix* fraction, which led to well-settling AGS with good phosphorus removal with up to 18% COD supplied in the form of thiosulfate. Based on these results, it was concluded that granule morphology is determined by reactor operation rather than bacterial morphology.

In chapter 6 the release of COD from seawater-adapted AGS after osmotic downshock (chapter 2) was further investigated, using an enrichment culture of *Ca.* Accumulibacter phosphatis. Cycles of hyperosmotic and hypo-osmotic shocks were applied to both freshwater-adapted and seawater-adapted cultures, which led to the release of trehalose from the cells. Genome analysis showed that a metabolic link between glycogen and trehalose can be possible in *Ca.* Accumulibacter. Long-term adaptation to 30% seawater-based medium led to successful EBPR operation with complete anaerobic uptake of acetate and priopionate, anaerobic phosphate release and complete aerobic phosphate uptake. Consumption of externally supplied trehalose was measured in aerobic and anaerobic conditions in the enrichment reactor.

The future prospects of this field of research are discussed in chapter 7. Quantification of trehalose and glycogen pools in *Ca.* Accumulibacter can shed light on the exact mechanism of osmoadaptation. Higher-resolution methods for granule size measurement can help explain the counterintuitive increase in granule strength after exposure to salts. Also for EPS research, more in-depth methods should be used to differentiate between different types of sialic acids in the EPS matrix. The hydrodynamic effect of plug-flow feeding in an AGS system should have more focus in the future. This aspect plays a large role in process performance, especially in line with osmotic downshocks and change in granule morphology due to filamentous *Thiothrix* bacteria.

This PhD thesis shows the feasibility of the aerobic granular sludge process with seawater-based wastewater. The remarkable flexibility of *Ca.* Accumulibacter to salinity variations is fascinating, and gives a solid basis for further research. Full-scale AGS plants will benefit from these results, expanding the horizon of applications.

Samenvatting

Stijging van de zeespiegel zal leiden tot een toename in zoutgehalte van watersystemen. Om hierop te anticiperen is het noodzakelijk om het effect hiervan op biologische afvalwaterzuivering te bepalen. Aeroob korrelslib (AGS) is een succesvolle technologie waarin organisch koolstof (CZV), stikstof, en fosfor in een enkele processtap verwijderd kan worden. De invloed van zeewater op AGS is nog niet helder en gedetailleerd beschreven in literatuur. Het effect van zout kan ruwweg ingedeeld worden in drie aspecten: biologische activiteit, fysieke stabiliteit en een verandering in de extracellulaire polymerische substanties (EPS). Het onderzoek in dit proefschrift is gestructureerd aan de hand van deze aspecten. De algemene onderzoeksvraag is: "Hoe beïnvloedt lange termijn blootstelling van zeewater het AGS-proces?"

Om deze vraag te kunnen beantwoorden is een labschaal AGS-reactor gedraaid met kunstmatig zeewater en synthetisch afvalwater. Hoofdstuk 2 beschrijft het effect van zeewater op biologische fosfaatverwijdering (EBPR) in AGS na langdurige blootstelling en adaptatie aan zeewater. Stabiele korrels waren gevormd, die bestonden uit een dominante groep aan fosfaat accumulerende organismen (PAO) en de afwezigheid van glycogeen accumulerende organismen (GAO). Acetaat was volledig opgenomen tijdens 60 minuten anaerobe propstroomvoeding. Fosfaat was vervolgens aeroob compleet opgenomen tijdens 170 minuten beluchting. De invloed van een regenwatersituatie was vervolgens onderzocht door het inkomende zeewater te vervangen door gedemineraliseerd water. Een enkele downshock leidde tot onvolledige anaerobe opname van acetaat en verminderde fosfaatopnamesnelheid. Deze effecten waren direct hersteld bij een volgende cyclus met zeewater in het influent. Tijdens de downshock was ook vrijgekomen CZV gemeten, wat verder onderzocht is in hoofdstuk 6.

Het effect van zout op de fysieke stabiliteit van volle schaal Nereda® korrelslib is onderzocht in hoofdstuk 3. Een methode voor de bepaling van korrelsterkte was geëvalueerd, gebaseerd op afschuring van korrels in een geroerde reactor met hoge afschuifkrachten. Testtijden tot 90 minuten leidden tot een stabiele afschuivingscoëfficient (K). Grotere korrels hadden meer schuring dan kleinere korrels. Korrels van vier verschillende Nereda® installaties hadden een verschil in K-waarde. Blootstelling van korrels aan een oplossing van 25 g/L NaCl voor 1 uur leidde tot een afname in afschuring. Verhoging van deze zoutconcentratie naar 50 g/L leidde tot een verdere afname in slechts 2 van de 4 gevallen; de andere 2 slibmonsters lieten juist weer een relatieve toename zien. Tegelijkertijd was een toename in calcium- en magnesiumconcentratie gemeten in de vloeistof. Hiernaast bleek dat labschaal korrelslib na adaptatie aan zeewater sterker was dan zoetwaterkorrels.

Aanpassing van EPS in zeewater is vaak beschreven in literatuur, maar een kwalitatieve benadering was nog niet genomen. In hoofdstuk 4 is hierom fluorescente lectine bar-coding (FLBC) gebruikt met zeewatergeadapteerd korrelslib, en hiermee is de aanwezigheid van siaalzuren ontdekt. Siaalzuren zijn een groep monosacchariden met een keten van negen koolstofatomen, waar-

van neuraminic acid (Neu5Ac) een van de meest beschreven zuren is. De functie van siaalzuren in EPS wordt regelmatig beschreven in lijn met bescherming tegen afbraak door proteases en glycosidases. Deze beschermende rol was ook gevonden in AGS; galactosegroepen in de suikerketen van EPS konden enzymatisch afgebroken worden nadat siaalzuur verwijderd was. Genoomanalyse liet zien dat *Candidatus* Accumulibacter phosphatis genen bevat die coderen voor neuraminic acid-producerende enzymen.

Zeewater bevat een hoog gehalte aan sulfaten, die afvalwaterzuiveringen binnen kunnen komen in de vorm van sulfide. Dit kan toxiciteit veroorzaken en de vorming van bulkend slib veroorzaken, o.a. door de aanwezigheid van filamenteuze *Thiothrix* bacteriën. Recent onderzoek heeft echter aangetoond dat *Thiothrix caldifontis* een vergelijkbaar metabolisme heeft als *Ca.* Accumulibacter phosphatis. Zo kunnen ze ook acetaat anaeroob opnemen en aeroob fosfaat opnemen, naast hun eigenschap om elementair zwavel intracellulair op te kunnen slaan. Deze overeenkomst leidde tot de vraag of stabiele korrels gevormd kunnen worden ondanks de aanwezigheid van deze filamenteuze *Thiothrix* bacteriën. Hoofdstuk 5 beschrijft korreling met de aanwezigheid van *Thiothrix*. Thiosulfaat was toegevoegd om de *Thiothrix* fractie te vergroten, wat leidde tot goed bezinkend korrelslib en een goede fosfaatverwijdering met 18% CZV uit thiosulfaat. Aan de hand van deze resultaten was geconcludeerd dat de korrelmorfologie bepaald wordt door de reactoroperatie in plaats van de morfologie van individuele bacteriën.

In hoofdstuk 6 is de afscheiding van CZV uit zeewater-geadapteerd AGS na osmotische downshock verder onderzocht (hoofdstuk 2), aan de hand van een verrijkingscultuur van *Ca.* Accumulibacter phosphatis. De release van trehalose was gemeten na het blootstellen van zowel zoetwater- als zeewater-geadapteerde culturen aan cycli van hyper- en hypo-osmotische shock. Genoomanalyse liet zien dat er een metabolisch verband kan zijn tussen glycogeen en trehalose in *Ca.* Accumulibacter. Lange termijn adaptatie aan 30% zeewater-gebaseerd medium leidde tot succesvolle EBPR-activiteit met complete anaerobe opname van acetaat en propionaat, anaerobe fosfaatrelease en volledige aerobe opname van fosfaat. Consumptie van extern toegevoegde trehalose was gemeten in zowel aerobe als anaerobe omstandigheden in de verrijkingsreactor.

De conclusie van dit proefschrift en toekomstperspectieven in dit onderzoeksveld zijn beschreven in hoofdstuk 7. Kwantificatie van trehalose en glycogeen in *Ca.* Accumulibacter kan het exacte mechanisme van zoutadaptatie ontrafelen. Analytische methodes met hogere resolutie voor bepaling van korrelgrootte kunnen de contra-intuïtieve toename in korrelsterkte na NaCl-blootstelling helpen uitleggen. Ook voor EPS-onderzoek zijn diepgaandere methodes nodig voor o.a. differentiatie tussen verschillende soorten siaalzuren. Het hydrodynamische effect van propstroomvoeding in een AGS-systeem zou in de toekomst ook een grotere aandacht moeten krijgen. Dit aspect speelt een belangrijke rol in procesvoering, vooral in lijn met osmotische downshocks en verandering in korrelmorfologie door filamenteuze *Thiothrix* bacteriën.

Dit proefschrift laat de toepasbaarheid van aeroob korrelslib in zeewater-gebaseerd afvalwater zien. De opmerkelijke flexibiliteit van *Ca.* Accumulibacter in zoutfluctuaties is fascinerend en geeft een goede basis voor toekomstig onderzoek. Volle schaal AGS installaties zullen baat hebben van deze resultaten, en de breedte van toepassingen kan hierdoor worden uitgebreid.

1. Introduction

Prospective salinization: Challenge or opportunity?
La Guajira Desert, Colombia

Introduction

Increase in salinity of water bodies is one of the most crucial issues for the coming future. Due to climate change and melting of ice masses, sea level is expected to increase by more than 1 meter by the year 2100 [1, 2]. This will have a massive impact on currently existing freshwater systems, and will most probably lead to water stress for industry and drinking water production [3, 4]. Water management solutions are required for sustainable use of freshwater after increased salinization [5].

A paradigm shift for water usage is required to properly manage the prospective salinization [6]. There are already examples for use of seawater, such as seawater-based cooling towers [7, 8], irrigation with brackish water [9] and toilet flushing with seawater in Hong Kong [10–12]. However, these solutions usually only have an impact on a small scale (cooling towers), or require major changes in infrastructure (toilet flushing). Many water systems will therefore still receive saline water, to which they need to adapt for sustainable water management.

The prospective changes in freshwater availability will have to be addressed by means of both technological and governmental implementations. In order to achieve a sustainable transition, the Water Nexus program has been initiated in the Netherlands. This research project involves a consortium of universities, companies, and research institutes, and gives a common platform for achieving a change on multiple levels. "Fresh water when needed, salt water when possible" is the underlying principle by which fundamental research is translated into technological advancements and governance integration. The research that is described in this PhD thesis is part of this program, as part of the work package that is dedicated to advancements in water technologies at high salinity. In close collaboration with Wageningen University & Research and a multitude of technology providers and consulting engineering companies, the output from this research gives guidelines and boundary conditions for system integration.

Saline wastewater treatment

An important part of a healthy water system is wastewater treatment, in order to minimize spreading of pathogens and prevent environmental damage through oxygen depletion and eutrophication [13–15]. Biological treatment with activated sludge is the most frequently used method for removal of pollutants from wastewater. This technology uses bacteria for removal of organic carbon (COD), nitrogen, and phosphorus [16]. Commonly, multiple tanks are used for creating optimal environments for specific removal of these compounds with separated aerobic, anoxic, and anaerobic stages. After the biological treatment step, the activated sludge flocs are separated from the cleaned water in a secondary clarifier. The clean water is usually discharged into surface waters, while the settled sludge is recycled into the system or discarded as excess sludge. This technology has been around since 1913, but newer technologies have since been developed to improve process performance [17].

One of the most promising developments in this field is the aerobic granular sludge (AGS) process. This technology combines the different process stages of the activated sludge in a single granule through an oxidation gradient, thereby removing COD, nitrogen, and phosphorus in a single process step [18, 19]. The improved settleability of granular sludge allows for the settling to be integrated in the treatment tank and thereby also circumvents the necessity of a secondary clarifier. This results in a decrease in energy and space requirements compared to conventional activated sludge plants [20]. The feasibility of this process has led to scaling up and commercialization of the Nereda® process, which is a successful technology that is operational in full-scale installations around the world [21–23].

Basis for the complete removal of COD, nitrogen, and phosphorus in one process step is the presence of an oxygen gradient inside the granule (figure 1.1), and cycles of anaerobic feeding through the settled granular sludge bed and aerobic mixing (figures 1.2 and 1.3). Due to the oxygen gradient inside the granule during aeration, an aerobic outer layer is present in which ammonium gets converted into nitrite and nitrate by nitrifying bacteria. The inner core remains anoxic, where these compounds simultaneously get converted into nitrogen gas by denitrifying bacteria. Polyphosphate accumulating bacteria (PAO) are responsible for enhanced biological phosphorus removal (EBPR) by anaerobic uptake of volatile fatty acids (VFA) followed by aerobic or anoxic uptake of phosphate.

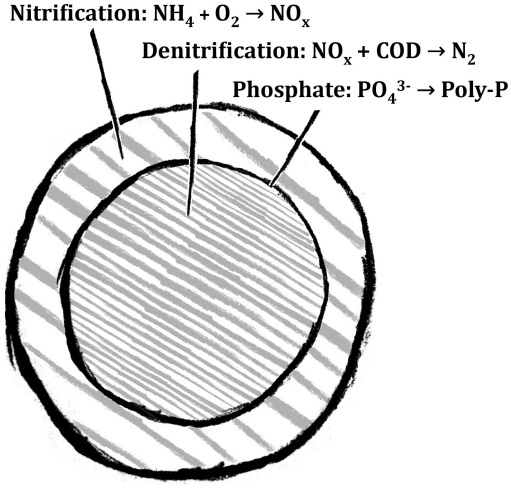


Figure 1.1: Conversions that occur in aerobic granular sludge. Nitrification can occur in the aerobic outer layer, and simultaneous denitrification can occur in the anoxic inner core. Phosphate removal occurs through cycles of anaerobic and aerobic phases, leading to the removal of both COD and phosphate.

The lab-scale AGS process is operated in a sequencing batch reactor (SBR) with cycles typically ranging from 3 to 4 hours (figure 1.2). During this cycle, aerobic and anaerobic periods are alternating which favors PAO metabolism as described in figure 1.3. Every cycle consists of 4 phases:

- *Anaerobic feeding:* The settled granular sludge bed is fed from the bottom of the reactor with fresh influent, containing acetate, phosphate, and ammonium. In this phase, the VFA are consumed and intracellularly stored as polyhydroxyalkanoates (PHA) by PAO bacteria. ATP that is required for this uptake is supplied by intracellular consumption of glycogen and hydrolysis of polyphosphate pools, resulting in release of phosphate into the liquid.
- *Mixed aeration:* Compressed air is pumped through the reactor, which causes both mixing and aeration of the reactor. The released phosphate is taken up again by the PAO to replenish their polyphosphate pools. PHA is consumed to produce glycogen and biomass. Simultaneously, nitrifying bacteria in the outer layer of the granules convert the ammonium into nitrite and nitrate. Denitrifying bacteria in the anoxic core convert this nitrite and nitrate into nitrogen gas, thereby completely removing nitrogen compounds from the liquid phase.
- *Settling:* The mixed liquor is allowed to settle for a period of typically 5-10 minutes. Well-settling granules accumulate at the bottom, while poorly settling particles remain in the top half.
- *Effluent:* The top half of the reactor is pumped out of the reactor. This removes poorly settling sludge, and discharges the effluent with low concentrations of COD, nitrogen, and phosphorus. The well-settling sludge has accumulated at the bottom to be the first ones to receive fresh influent in the subsequent cycle.

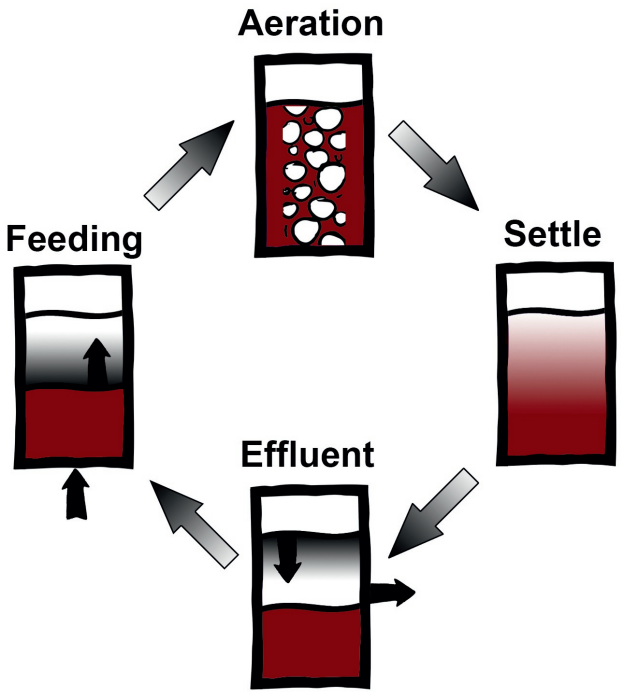


Figure 1.2: Sequencing batch reactor cycle during the aerobic granular sludge process, consisting of anaerobic feeding through the settled granule bed, mixing of granules during aeration, settling of the reactor content, and effluent of the top half of the reactor content.

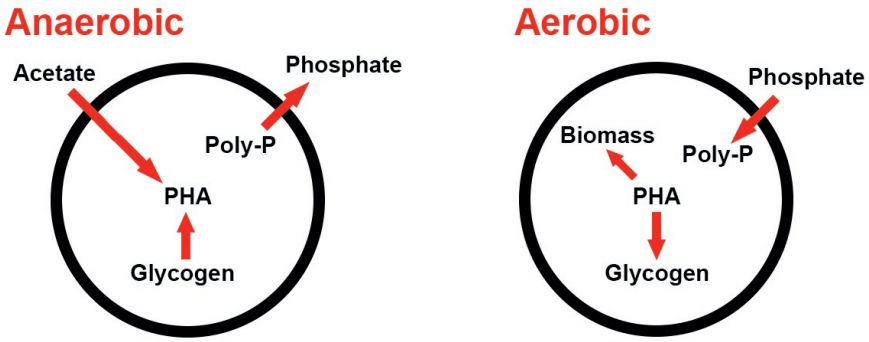


Figure 1.3: Simplified metabolism of *Candidatus* Accumulibacter phosphatis, showing the most important conversions during anaerobic and anoxic or aerobic conditions.

Seawater in the aerobic granular sludge process

The introduction of saline water can pose major problems for biological wastewater treatment [24]. A change in osmotic pressure lead to a decrease in bacterial activity, especially when this is supplied as a shock load [25–27]. The flocculation and thereby the settling properties of sludge flocs can change as the result of salinity [28, 29]. Moreover, fluctuations in density leads to density currents in secondary clarifiers, also negatively impacting the separation of sludge and water.

The magnitude of sensitivity of the overall process to salinity is very much dependent on the influent characteristics and the degree of adaptation of the microbial community. The results from activated sludge studies cannot directly be translated to the aerobic granular sludge process. More information is therefore required for understanding the sensitivity of AGS to seawater.

Common effects of salinity on granular sludge can roughly be divided in three aspects:

1. Biological activity for pollutant removal
2. Physical strength of granules
3. Adaptation of extracellular polymeric substances (EPS)

Based on this division, the research of this thesis has been structured. A short overview on the current state of research is described below, with emphasis on research gaps in existing literature.

Biological effect of seawater on nitrification and phosphorus removal

In aerobic granular sludge, the focus of the impact of saline wastewater on process performance has mainly been in NaCl-based wastewaters. Nitrite accumulation was a common observation, which is frequently observed for nitrifying sludge [30, 31]. Phosphorus removal suffered from nitrite toxicity, and decreased at salinities of 14 g Cl^-/l and higher [32]. Suppression of nitrification and thereby prevention of nitrite accumulation led to good phosphorus removal up to salinity levels of 20 g Cl^-/l [31]. *Ca.* Accumulibacter enrichment studies with NaCl-based medium showed a decrease in anaerobic VFA uptake, aerobic phosphate uptake rate and an increase in both aerobic and anaerobic maintenance coefficients [33, 34].

The effect of the presence of a more complex salt mixture like seawater on AGS has however not been studied. Nitrogen removal has received a lot of attention, and nitrification has been shown to work in seawater [35–37]. On the other hand, biological phosphorus removal in seawater is still not described. EBPR was unsuccessful in a moving bed biofilm reactor, and shown to be problematic in high-saline domestic wastewater [38, 39]. There is a research gap in this important factor for understanding the effect of seawater on PAO activity.

Another important difference is the presence of sulfate in seawater compared to NaCl-based saline wastewaters. Sulfate can be converted into sulphide under anaerobic conditions (e.g. during sewer transport), which can cause toxicity and lead to the presence of filamentous bacteria.

Thiothrix sp. is one of the major filamentous species that thrives under these conditions [40]. Recent research has shown that it can coexist in an EBPR system next to *Ca.* Accumulibacter [41]. *Thiothrix* can anaerobically consume acetate, store it as PHA, and simultaneously release phosphate. Aerobically it takes up phosphate again, by consuming the PHA, similar to the metabolism in figure 1.3. This organism gets its competitive advantage by accumulating the sulfide at the start of the aerobic phase as sulfur. This is used throughout the aerobic growth phase as extra energy source resulting in a higher biomass yield, thereby effectively competing with *Ca.* Accumulibacter sp.

A key aspect in this metabolism is that it allows for slow growth, which is the main proposed reason why a stable biofilm can be formed in a granular form [42]. The similarity between *Thiothrix* and *Ca.* Accumulibacter should allow for stable granulation, despite the presence of filamentous *Thiothrix* bacteria. Coexistence has been shown in a suspended culture, but the effect of filamentous *Thiothrix* bacteria on granular sludge morphology is still unknown.

Physical strength of granules

Next to impacting bacterial activity salts can influence the physical structure of granules. The exact impact is contradicting in literature though, where some studies observed increased settling times [28, 43], while others described a negligible effect or even improved settleability [29, 30, 44, 45].

There are major differences between floc structure and granule structure. Studies in anaerobic granular sludge have shown a decrease in granule strength after exposure to NaCl [46]. Displacement of Ca^{2+} by Na^{+} was discussed to be the main factor for this observation [47]. The effect of salts on the strength of aerobic granular sludge not yet been described in literature.

Extracellular polymeric substances (EPS)

Physical properties of granular sludge are for a large part determined by the properties of the extracellular polymeric substances (EPS). Bacteria in the granules are embedded in EPS, which is a mixture of polysaccharides, proteins, lipids, and DNA [48, 49]. These EPS provide a structural matrix that protects bacteria against adverse conditions [50, 51]. The properties of EPS are determined by reactor conditions, influent characteristics and the microbial community [52, 53]. A change in salinity can therefore have a significant impact on the EPS. Higher protein fractions have been reported [54], higher EPS concentrations [55], and an increase in hydrophobicity [56]. However, most of these studies have focused on quantification, while extraction bias and analytical interference can cause serious difficulties in proper interpretation of data [57]. More qualitative lectin-based methods (FITC) have been able to elucidate the presence of specific glycoconjugates in EPS [58, 59]. Based on this FITC method, the EPS of seawater-adapted AGS can be analyzed without using extractive methods.

Scope of this thesis

The aim of this PhD thesis was to study the effect of seawater on the aerobic granular sludge process. Based on the research gaps that were described in the previous paragraphs, this thesis has been divided in the following chapters:

In **chapter 2**, the effect of seawater on phosphate removal in aerobic granular sludge after long-term adaptation is described. The process performance was evaluated, and the microbial community was analyzed. Osmotic downshocks were introduced in the reactor to mimic rainfall situations, and assess the sensitivity of the seawater-adapted granules to variations in salinity.

Chapter 3 describes the effect of NaCl salts on the physical strength of full-scale aerobic granular sludge. A strength quantification method was developed and evaluated, based on high shear in a stirred tank reactor. Using this method, the effect of short-term salt exposure on granule strength was quantified.

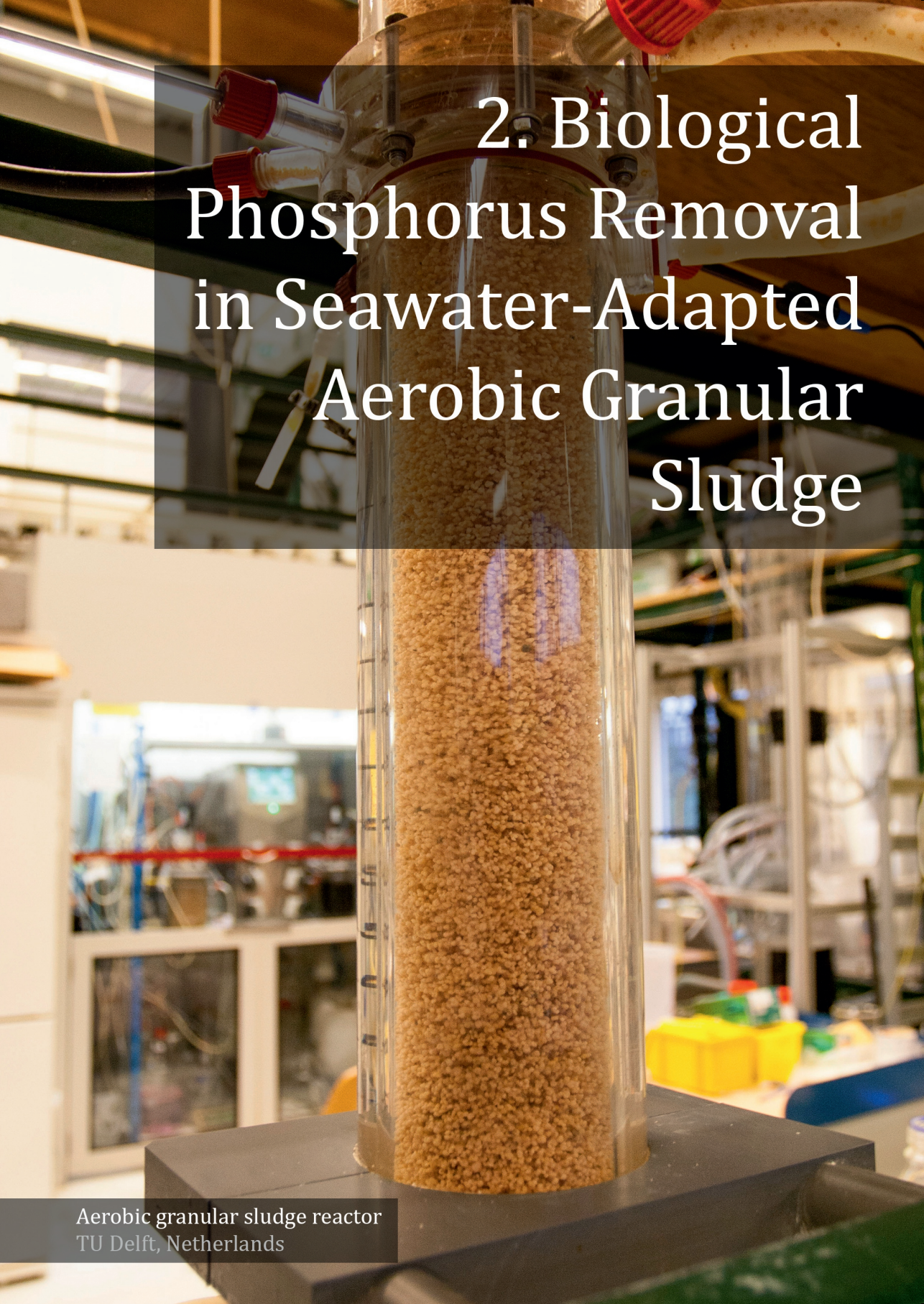
Chapter 4 focuses on the change in EPS of seawater-adapted aerobic granular sludge. Properties of wastewater determine the EPS properties, and seawater can have a similar effect. Quantitative methods for sugar and protein measurement can give misleading results, so a qualitative approach was taken. Using lectin-staining methods, sialic acids were found in these granules. The protective role of these compounds has been evaluated, and a genome analysis was added for determining the feasibility of sialic acid production by *Ca.* Accumulibacter.

In **chapter 5**, the effect of filamentous *Thiothrix* bacteria on granular sludge morphology was investigated. The resemblance between their metabolism and that of *Ca.* Accumulibacter led to the question whether granule morphology is dominated by reactor operation rather than the morphology of individual cells. A reactor was operated with a thiosulfate containing medium to stimulate the growth of *Thiothrix* bacteria. The granule morphology and microbial community were studied to determine the effect of filamentous bacteria on granule properties.

In **chapter 6**, an enrichment culture of *Ca.* Accumulibacter phosphatis was used to study the role of trehalose as an osmolyte. Intracellular production of trehalose at hyperosmotic conditions and subsequent release at hypo-osmotic shock were analysed in both freshwater- and seawater-adapted cultures. Genome analysis was performed to make a link between glycogen and trehalose metabolism in *Ca.* Accumulibacter. Also the consumption of externally supplied trehalose was measured by addition into the reactor during anaerobic and aerobic phases.

Chapter 7 gives concluding remarks on the thesis, and describes the outlook for this field of research.

2. Biological Phosphorus Removal in Seawater-Adapted Aerobic Granular Sludge



Aerobic granular sludge reactor
TU Delft, Netherlands

Biological Phosphorus Removal in Seawater-Adapted Aerobic Granular Sludge

Abstract

Seawater can be introduced or intrude in sewer systems and can thereby negatively influence biological wastewater treatment processes. Here we studied the impact of seawater on the enhanced biological phosphate removal (EBPR) process performance by aerobic granular sludge (AGS). Process performance, granule stability and characteristics as well as microbial community of a seawater-adapted AGS system were observed. Under seawater conditions strong and stable granules formed with an SVI_5 of 20 mL/g and a lower abrasion coefficient than freshwater-adapted granules. Complete anaerobic uptake of acetate, anaerobic phosphate release of 59.5 ± 4.0 mg/L PO_4^{3-}-P (0.35 mg P/mg HAc), and an aerobic P-uptake rate of 3.1 ± 0.2 mg P/g VSS/h were achieved. The dominant phosphate accumulating organisms were the same as for freshwater-based aerobic granular sludge systems with a very high enrichment of *Ca.* Accumulibacter clade I, and complete absence of glycogen accumulating organisms. The effect of osmotic downshocks was tested by replacing influent seawater-based medium by demineralized water-based medium. A temporary decrease of the salinity in the reactor led to a decreased phosphate removal activity, while it also induced a rapid release of COD by the sludge, up to 45.5 ± 1.7 mg COD/g VSS. This is most likely attributed to the release of osmolytes by the cells. Recovery of activity was immediately after restoring the seawater feeding. This work shows that functioning of aerobic granular sludge under seawater conditions is as stable as under freshwater conditions, while past research has shown a negative effect on operation characteristics with NaCl-based wastewater at the same salinity as seawater.

Highlights

- Successful biological phosphorus removal was achieved in seawater-adapted aerobic granular sludge
- *Ca.* Accumulibacter clade I was present and glycogen accumulating organisms were absent in seawater-adapted aerobic granular sludge
- Anaerobic acetate uptake recovered immediately after an osmotic downshock during one reactor cycle
- Osmotic downshocks lead to rapid release of COD from seawater-adapted aerobic granules, which is likely to be ascribed to osmolytes

2.1. Introduction

Aerobic granular sludge (AGS) is a technology for wastewater treatment in which COD, nitrogen, and phosphate can be removed in a single process step [19, 60, 61]. This technology has successfully been applied in full-scale for domestic wastewater, which predominantly consists of freshwater [22]. Municipal wastewater can also contain high fractions of seawater due to intrusion of seawater or saline groundwater intrusion in the sewer system, industrial activity or use of seawater for toilet flushing [4, 24, 62].

Crucial aspects for a stable AGS process are nitrification and enhanced biological phosphate removal (EBPR). There is already a substantial body of literature on the effect of salinity on nitrogen conversions [35–37, 63]. However, literature on the effect of seawater on EBPR is lacking. The effect of saline wastewater has been studied in either NaCl-supplemented wastewater processes or in NaCl-based enrichment cultures of *Ca.* Accumulibacter [34, 64, 65]. Problem with NaCl in AGS is nitrite accumulation and toxicity to polyphosphate accumulating organisms (PAO) [32]. Suppression of nitrification still led to a decrease in PAO activity at concentrations of 33 g/L NaCl, which is similar salinity to seawater [31]. Studies on *Ca.* Accumulibacter enrichments observed an increase in maintenance requirements and a decrease in anaerobic COD uptake and aerobic phosphate uptake [33, 34].

EBPR in seawater was unsuccessful in a moving bed biofilm reactor (MBBR), and also shown to be problematic for AGS in high-saline domestic wastewater [38, 39]. The reasons for failure have not been reported. Literature about successful EBPR in an AGS system under seawater conditions is not available yet. Therefore, we focused on the stability and effect of seawater-based biological phosphorus removal in aerobic granular sludge.

Salinity levels in full-scale wastewater treatment plants are prone to fluctuations, due to e.g. rainfall situations. Rainwater has low concentrations of chlorides (<1.0 mg/L) and low conductivity (20-30 µS/cm), which is even in range of demineralized water [66, 67]. The effect of sudden exposure of seawater-adapted aerobic granules to salinity variations is therefore of great importance for maintaining a stable process operation.

In this study, we characterized the EBPR process performance, stability, granule characteristics, and microbial community of a seawater-adapted AGS system. We tested the effect of salinity variations on the biological removal of phosphorus.

2.2. Materials & Methods

Reactor operation

A 3.0 L bubble column (5.6 cm diameter) was operated as a sequencing batch reactor (SBR). The inoculation source was Nereda® sludge from municipal wwtp Utrecht, the Netherlands. Temperature was controlled at 20 °C. pH was controlled at 7.0 ± 0.1 by dosing either 1M NaOH or 1M HCl. Dissolved oxygen (DO) was controlled at 3.7 mg/L O_2 (50% saturation). The average sludge retention time (SRT) was 20 days.

Reactor cycles had a length of 240 minutes, consisting of 60 minutes anaerobic feeding, 170 minutes aeration, 5 minutes settling, and 5 minutes effluent withdrawal. The feed of 1500 mL consisted of 1200 mL artificial seawater (Instant Ocean®, final concentration 35 g/L), 150 mL of medium A, and 150 mL of medium B. Medium A contained 57.2 mM sodium acetate trihydrate. Medium B contained 42.8 mM NH_4Cl, 4.2 mM K_2HPO_4, 2.1 mM KH_2PO_4, and 10 mL/L trace element solution similar to Vishniac and Santer (1957) [68], but using 2.2 mg/L $ZnSO_4.7H_2O$ instead of 22 mg/L. The combination of these feed streams led to influent concentrations of 366 mg/L COD, 60 mg/L NH_4^+-N and 9.3 mg/L PO_4^{3-}-P. Electrical conductivity of the influent was equal to 40 mS/cm.

Osmotic shocks in reactor

Osmotic shocks were introduced into the reactor by replacing the 1200 mL of influent artificial seawater by demineralized water. After one cycle, the influent demineralized water was replaced again by artificial seawater. The 150 mL of medium A and medium B remained the same during each cycle, giving the same concentrations of nutrients in the influent.

Due to the anaerobic plug-flow feeding, followed by aerobic mixing, there was a difference in the salinity that was experienced by the aerobic granules. This led to the following cycles of conductivity, as experienced by the granules:

Table 2.1: Conductivity (mS/cm) as experienced by the aerobic granular sludge during anaerobic feeding and aerobic mixing in a regular cycle, osmotic downshock, and recovery cycle.

Phase	Regular cycle	Osmotic downshock	Recovery cycle
Anaerobic feeding	40	1.1	40
Aerobic mixing	40	~20	~30

Acetate uptake rate batch test

Granules were taken from the reactor at the end of aeration. Equal amounts of granules were divided over flasks with 200 mL working volume, filled with either filtered effluent or demineralized water. All of the flasks were buffered at pH 7.0 ± 0.1 with a 4.0 mM HEPES buffer, and sparged with N2 gas prior to adding the granular biomass. A spike of acetate was added up to a final concentration of 200 mg/L, after which samples were taken for a period of 60 minutes. All samples were filtered through a 0.45 µm PVDF filter. Their respective masses were registered to compensate for mass decrease in calculations. The amount of biomass was determined by filter-

ing the granules at the end of the test, washing with demineralized water to remove salts, drying for 24 hours at 105 °C, and incinerating for 2 hours at 550 °C. All tests were done in duplicate.

COD release batch test

Granules were taken from the reactor at the start of aeration. Equal amounts of granules were divided over four flasks with 100 mL working volume, filled with either filtered effluent or demineralized water. All of these flasks were sparged with compressed air prior to adding the granular biomass. Samples were taken for a period of 60 minutes, and filtered through a 0.45 µm PVDF filter. Their respective masses were registered to compensate for mass decrease in calculations. The amount of biomass was determined by filtering the granules at the end of the test, washing with demineralized water to remove salts, drying for 24 hours at 105 °C, and incinerating for 2 hours at 550 °C. All tests were done in duplicate.

Analytical methods

Concentrations of phosphate were measured on a Thermo Fisher Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, USA). Chemical oxygen demand (COD) was measured using Hach Lange kits on a DR2800 spectrophotometer. Samples were diluted accordingly prior to measurement, to prevent chlorine interference. Acetate was measured by HPLC with an Aminex HPX-87H column from Biorad, coupled to an RI and UV detector, using 0.01M phosphoric acid as eluent. Strength characterization was carried out as described in chapter 3.

Granule morphology

Pictures of whole granules were taken with a stereo zoom microscope (Leica Microsystems Ltd, M205 FA, Germany), and processed with Leica Microsystems Qwin (V3.5.1) image analysis software.

Fluorescent in-situ hybridization (FISH)

The handling, fixation and staining of FISH samples was performed as described in Bassin et al. (2011) [32]. A mixture of PAO462, PAO651, and PAO846 probes (PAOmix) were used for visualizing polyphosphate accumulating organisms (PAO) [69]. A mixture of GAOQ431 and GAOQ989 probes (GAOmix) were used for visualizing glycogen accumulating organisms (GAO) [70]. *Ca.* Accumulibacter clade I was visualized by Acc-I-444, and *Ca.* Accumulibacter clade II was visualized by Acc-II-444 [71]. A mixture of EUB338, EUB338-II and EUB338-III probes were used for staining all bacteria [72, 73]. Images were taken with a Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 26 (bp 575e625/FT645/bp 660e710), 20 (bp 546/12/ FT560/bp 575e640), 17 (bp 485/20/FT 510/bp 5515e565) for Cy5, Cy3 and fluos respectively.

2.3. Results

Reactor operation

Seawater-adapted aerobic granular sludge was cultivated in a 3.0 L lab-scale sequencing batch operated bubble column reactor at 20 days solid retention time (SRT) and 20 °C, under the same conditions as usually applied in our laboratory [31]. Complete anaerobic acetate consumption was achieved, along with an average anaerobic release of 59.5 ± 4.0 mg/L PO_4^{3-}-P (0.35 mg P/mg HAc), and effluent concentrations of 5.9 mg/L PO_4^{3-}-P (figure 2.1) over a period of 12 months. A typical reactor cycle is shown in figure 2.2, from which a biomass-specific phosphorus removal rate of 3.1 ± 0.2 mg P/g VSS/h was measured. An average concentration of 11.5 ± 1.3 g TSS/L or 8.1 ± 0.8 g VSS/L was present in the reactor (i.e. 28% ash content). The effluent TSS was equal to 0.02 g/L. No nitrite nor nitrate production was measured in the reactor.

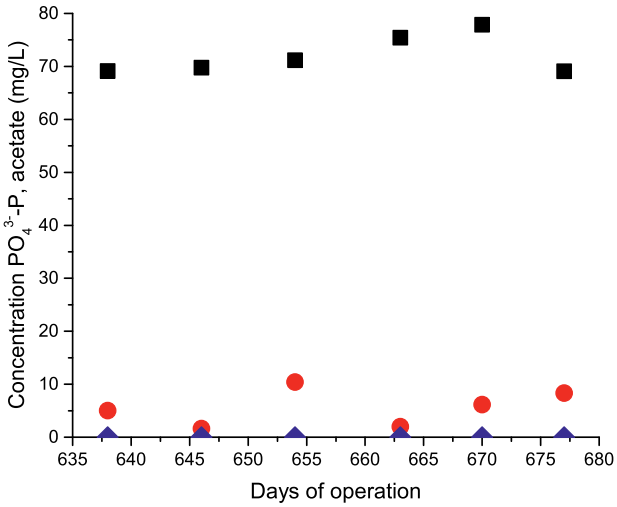


Figure 2.1: Phosphate concentrations at the start of aeration (black squares), end of aeration (red circles), and acetate concentrations at the end of anaerobic feeding (blue diamonds) during several weeks of consecutive operation of a seawater-adapted aerobic granular sludge reactor. The reactor was effectively operated from day 525 in stable mode.

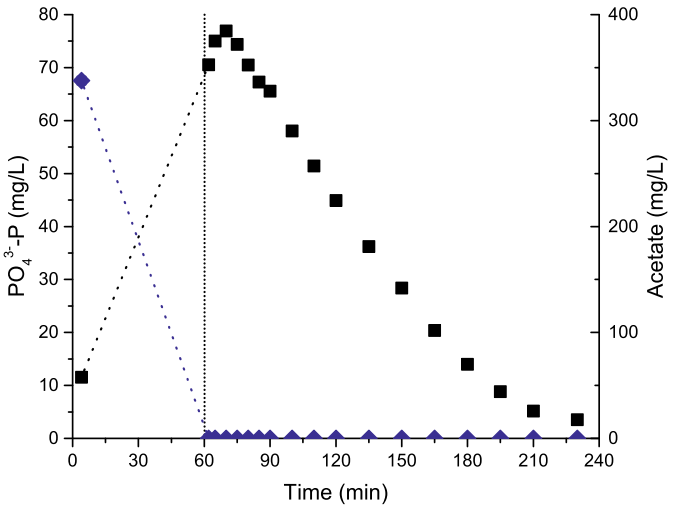


Figure 2.2: Typical reactor cycle of a seawater-adapted aerobic granular sludge reactor, showing concentrations of phosphate (black squares), and acetate (blue diamonds), with 60 minutes of anaerobic plug-flow feeding, 170 minutes of aeration, 5 minutes of settling, and 5 minutes of effluent withdrawal.

Granule characteristics

Successful granulation was achieved in seawater conditions (figure 2.3). No filamentous outgrowth was observed, and dense well-settling granules were formed. The sludge volume index after 5 minutes (SVI_5) equalled 20 mL/g for these seawater-adapted granules. The average granule size was 1.4 mm. An abrasion coefficient of $1.17 \pm 0.01 \cdot 10^{-5}$ s^{-1} was measured for these granules, compared to a value of $1.78 \pm 0.20 \cdot 10^{-5}$ s^{-1} for granules grown in the same system on freshwater [74].

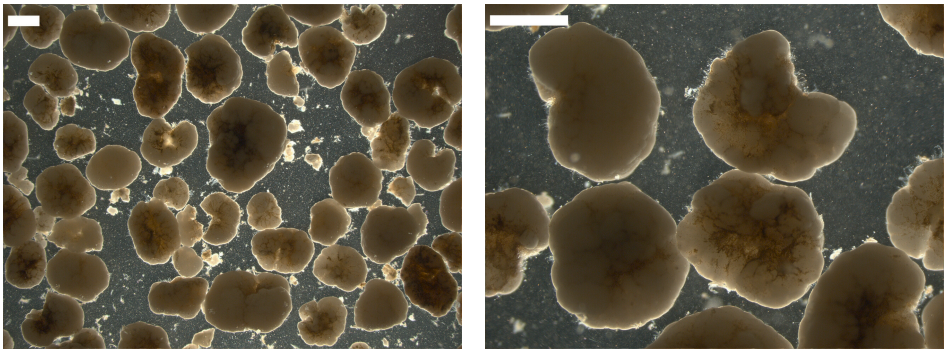
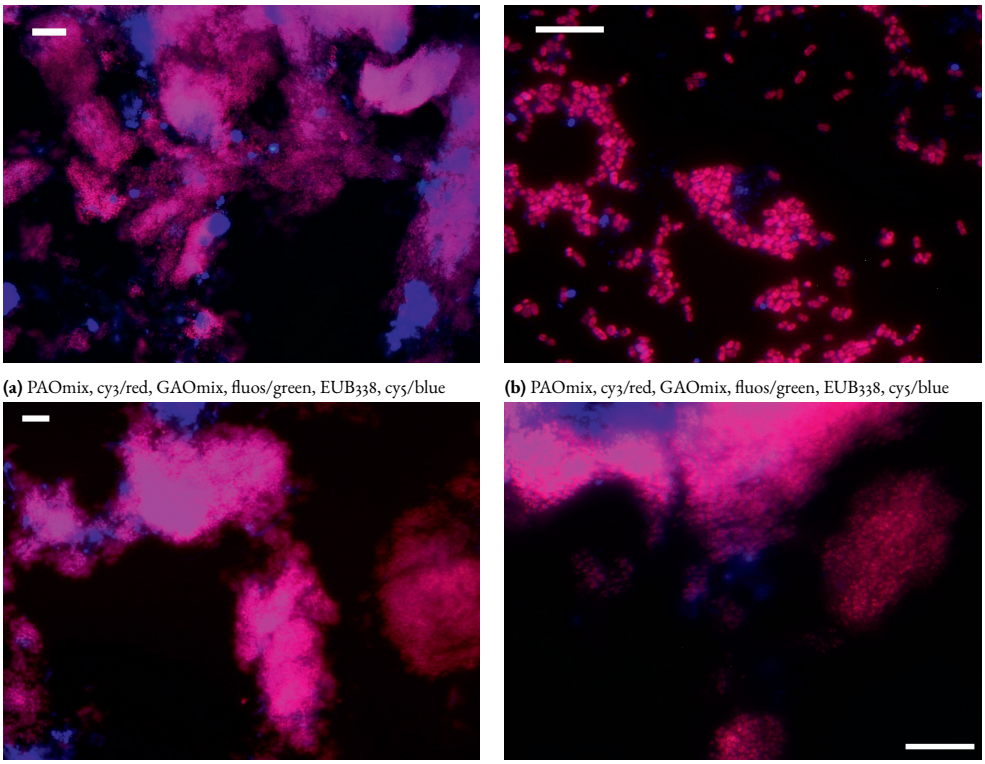


Figure 2.3: Image analyser pictures of seawater-adapted aerobic granular sludge. Scale bar equals 1 mm.

Microbial community

Fluorescence in situ hybridization (FISH) analysis showed the presence of polyphosphate accumulating organisms (PAO), and the absence of glycogen accumulating organisms (GAO) in seawater-adapted aerobic granular sludge (figures 2.4a, 2.4b). A high fraction of PAO was observed from all bacteria that were stained with the general eubacteria probe (EUB338). *Ca.* Accumulibacter clade I was observed in the seawater-adapted granules, whereas clade II was not observed (figures 2.4c, 2.4d).



(a) PAOmix, cy3/red, GAOmix, fluos/green, EUB338, cy5/blue

(b) PAOmix, cy3/red, GAOmix, fluos/green, EUB338, cy5/blue

(c) *Ca.* Accumulibacter I, cy3/red, *Ca.* Accumulibacter II, fluos/green, EUB338, cy5/blue

(d) *Ca.* Accumulibacter I, cy3/red, *Ca.* Accumulibacter II, fluos/green, EUB338, cy5/blue

Figure 2.4: Fluorescence in situ hybridization (FISH) images of seawater-adapted aerobic granular sludge stained with respective probes as indicated below the pictures. Magenta colour is an overlap between eubacteria (blue) and either PAOmix or *Ca.* Accumulibacter I bacteria (red). Scale bar equals 20 μm.

Osmotic shock

The effect of an osmotic downshock on the reactor performance was assessed by replacing the influent seawater by demineralized water. This resulted in a salinity decrease from 40 mS/cm to 1.1 mS/cm for the granular sludge during plug-flow feeding and a conductivity of approximately 20 mS/cm during the aerated mixed reactor period, due to the 50% volume exchange ratio. Recovery after an osmotic shock was studied by reconnecting influent seawater after this cycle at low saline content. This resulted in a salinity of 40 mS/cm for the granular sludge during plug-flow feeding and approximately 30 mS/cm during the mixed reactor period. Concentrations of acetate and phosphate were measured during a regular cycle, the osmotic downshock cycle, and the subsequent recovery cycle. These cycles of salinity shocks were performed in duplicate; a representative graph is shown in figure 2.5.

During plug-flow feeding in a regular cycle at 100% salinity (40 mS/cm), acetate was completely anaerobically consumed. After an osmotic downshock (1.1 mS/cm in feed), anaerobic acetate consumption was incomplete, and acetate became available at the start of the aeration phase. During the mixed reactor phase (approx. 50% salinity, approx. 20 mS/cm), the remaining acetate was completely consumed aerobically while simultaneously a limited amount of phosphate was released during the exact same time. The maximal aerobic phosphate uptake decreased to 72.0 ± 1.2 % of the preceding regular cycle. During the subsequent recovery cycle (100% salinity during feeding, 40 mS/cm), acetate was completely anaerobically consumed again. During the mixed reactor phase (approx. 75% salinity, approx. 30 mS/cm), the maximal aerobic phosphate uptake rate increased to 114.1 ± 9.4 % of the preceding regular cycle.

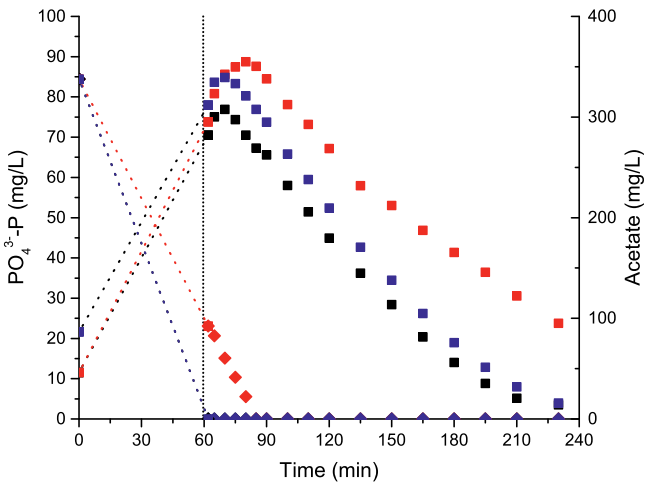


Figure 2.5: Concentrations of phosphate (squares) and acetate (diamonds) during a regular cycle (black, 40 mS/cm during anaerobic feeding, 40 mS/cm during aerobic mixing), downshock cycle (red, 1.1 mS/cm during feeding, approx. 20 mS/cm during mixing), and recovery cycle (blue, approx. 40 mS/cm during feeding, approx. 30 mS/cm during mixing). The first 60 minutes are anaerobic feeding phase, followed by 170 minutes of aeration, 5 minutes of settling, and 5 minutes of effluent withdrawal.

Uncoupling biological effect from hydrodynamic effect

During feeding of freshwater in a seawater-based reactor, there are two effects occurring simultaneously: a biological effect and a hydrodynamic effect. Due to introduction of influent with a lower density channelling occurred, resulting in a fraction of the influent bypassing the sludge blanket. This was visually observed by streams of influent water appearing on top of the sludge bed (figure 2.6). In order to separate the biological effect from this hydrodynamic effect, batch tests were performed to specifically measure the change in biological performance.

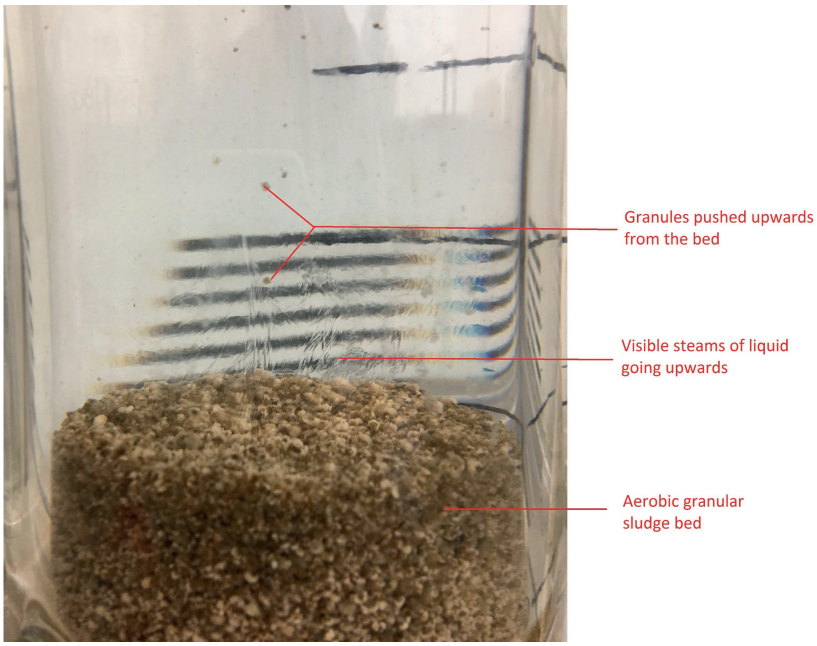


Figure 2.6: Visualization of hydrodynamic effect of introducing a medium with a much lower density in the granular sludge reactor during upflow mode feeding.

Acetate uptake rate after osmotic downshock

Granules were taken from the reactor at the end of aeration, and transferred to an anaerobic flask containing acetate in either saline reactor effluent or demineralized water. Samples were taken during 60 minutes and analysed for acetate and phosphate concentrations over time. Results are shown in figure 2.7.

The biomass-specific acetate uptake rates in reactor effluent and demineralized water were similar (18.9 ± 2.3 and 17.7 ± 1.8 mg acetate/g VSS/h, respectively). The anaerobic phosphate release rate increased by 12.3 ± 0.6 % in demineralized water. The total release of phosphate after 60 minutes increased by 32.1 ± 0.8 % in freshwater compared to seawater-based effluent. This equals a P-mol/C-mol ratio of 0.47 ± 0.01 in saline reactor effluent and a P-mol/C-mol ratio of 0.72 ± 0.02 after an osmotic downshock in demineralized water.

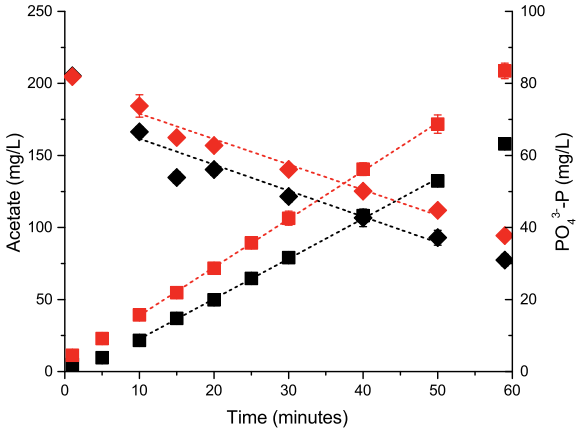


Figure 2.7: Concentrations of acetate (diamonds) and phosphate (squares) during an anaerobic batch test in either seawater-based reactor effluent (black) or demineralized water (red). Lines indicate the points with which the biomass-specific rates have been determined.

COD release during osmotic downshock

Anaerobic incubation of seawater-adapted granules in demineralized water, without addition of acetate, led to release of COD (figure 2.8). Concentration increase of 45.5 ± 1.7 mg COD/g VSS was measured after only 5 minutes in demineralized water, and this decreased slightly over time down to 34.3 ± 4.4 mg COD/g VSS after 60 minutes. Incubation in reactor effluent led to much lower release up to a low concentration of 12.1 ± 9.4 mg COD/g VSS after 60 minutes.

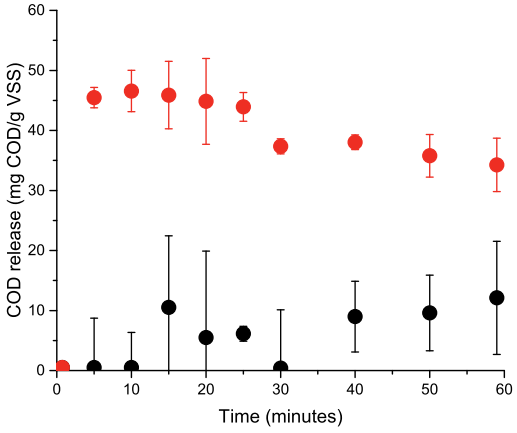


Figure 2.8: Concentrations of COD during incubation of seawater-adapted aerobic granular sludge in reactor effluent (black) or demineralized water (red)

2.4. Discussion

Successful biological phosphorus removal in seawater

This chapter shows that biological phosphorus removal and granulation can occur successfully in seawater conditions. Neither granular sludge nor conventional activated sludge have previously been shown to give stable biological removal of phosphorus from seawater-based wastewater streams. The results from this study demonstrate the remarkable flexibility of *Ca.* Accumulibacter phosphatis to adapt to a wide range of salinities. It shows its exceptional resistance to osmotic shocks, and thereby also greatly contributes to increasing its range of full-scale applications.

Stable phosphate removal was measured with an aerobic removal rate of 3.1 ± 0.2 mg P/g VSS/h and a phosphate release of 59.5 ± 4.0 mg P/L. These values are similar to freshwater-based AGS systems [19]. The amount of phosphate release is higher than AGS in an NaCl-based influent, in which a deterioration of P-release was observed at 20 g/L Cl^- with the same *Ca.* Accumulibacter phosphatis clade I as this study [31]. Similarly, in a *Ca.* Accumulibacter enrichment NaCl caused a decrease in phosphate uptake at 0.18% salinity until a complete inhibition at 3.5 g/L [34].

Complete anaerobic acetate uptake was achieved in our system, with the stable presence of a PAO culture and the absence of GAO as determined by FISH. In AGS with pure NaCl, acetate was still completely consumed up to 33 g/L NaCl, but this occurred simultaneously with a shift in microbial community from PAO to GAO [32]. PAO enrichment studies have also shown that PAO continues to anaerobically consume acetate while having a near-zero P-release at 2% salinity [33]. These results indicate that *Ca.* Accumulibacter phosphatis is more adaptable to seawater salinity than NaCl-based salinity.

Physical properties of seawater-adapted granules

Stable granulation was achieved in seawater-based conditions, resulting in low SVI_5 of 20 mL/g and the absence of filamentous outgrowth (figure 2.3). An essential factor was the complete anaerobic uptake of acetate. The cause of filamentous outgrowth has frequently been discussed in literature [52, 75, 76]. A major reason for their proliferation is the availability of easily degradable COD under aerobic conditions [42, 77]. These conditions are indeed fulfilled in our system, due to the complete anaerobic consumption of acetate.

Strength characterization even indicated that seawater-adapted granules have a lower abrasion coefficient than freshwater-adapted granules ($1.17 \pm 0.01 \cdot 10^{-5}$ s^{-1} and $1.78 \pm 0.20 \cdot 10^{-5}$ s^{-1}, respectively) [74]. These results are supported by Li et al. (2017) [37], who observed that a high-shear cohesion test led to less abrasion in granules that were grown in seawater-based wastewater than those that were grown in less saline wastewater. Moreover, they reported an increased concentration of Ca^{2+} and Mg^{2+} cations in the sludge, which could help bridging of negatively charged functional groups in the polysaccharide fraction of the biofilm matrix.

Interestingly, changes in the composition of extracellular polymeric substances (EPS) can also be the result of adaptation to seawater. An increase in protein content, hydrophobicity, and EPS concentration has been reported when AGS was grown in higher salinity wastewater, which could

be linked to the increase in biofilm strength [54–56]. Adaptation of sugar residues in the EPS was also reported in response to higher salinity, in both anaerobic and aerobic granular sludge [59, 78]. With better EPS analysis techniques, the interaction between salinity and EPS composition could be clarified in the future [79].

Difference between NaCl and seawater

The results from this study signify the importance of distinguishing salinity from NaCl and from seawater. This distinction is often not clearly made in literature discussions. Activated sludge studies on EBPR yielded decreased phosphate uptake rates when NaCl was added to the medium [34, 64, 80]. Decrease in phosphate removal was described in aerobic granular sludge at an NaCl concentration of 33 g/L as well [31, 32]. The observed shift in microbial community from PAO to GAO in NaCl salinity was also not observed in the seawater-based reactor. It is clear that the effect of NaCl salinity should not be used as predictive for seawater salinity.

The reason good phosphorus removal was obtained in our seawater system be due to the higher Na^+/K^+ ratio in seawater [81]. Presence of sufficient amounts of potassium cations is required for the functioning of Na^+/K^+ pumps required for osmotic stabilization of the cytoplasm [82]. Moreover, availability of potassium plays a crucial role in the metabolism of PAOs [83].

Release of osmolytes during osmotic downshock

During osmotic downshock of seawater-adapted granules, a remarkably high COD concentration (45.5 ± 1.7 mg COD/g VSS) was measured in the liquid phase (figure 2.8). This COD is likely to be ascribed to the release of osmolytes [84–86]. Osmolytes are accumulated intracellularly to overcome high osmotic pressure, and protect DNA and proteins from denaturation [87, 88]. During a hypo-osmotic shock, these organic molecules can be released, which has been used as a bioprocess for the production of ectoine [89].

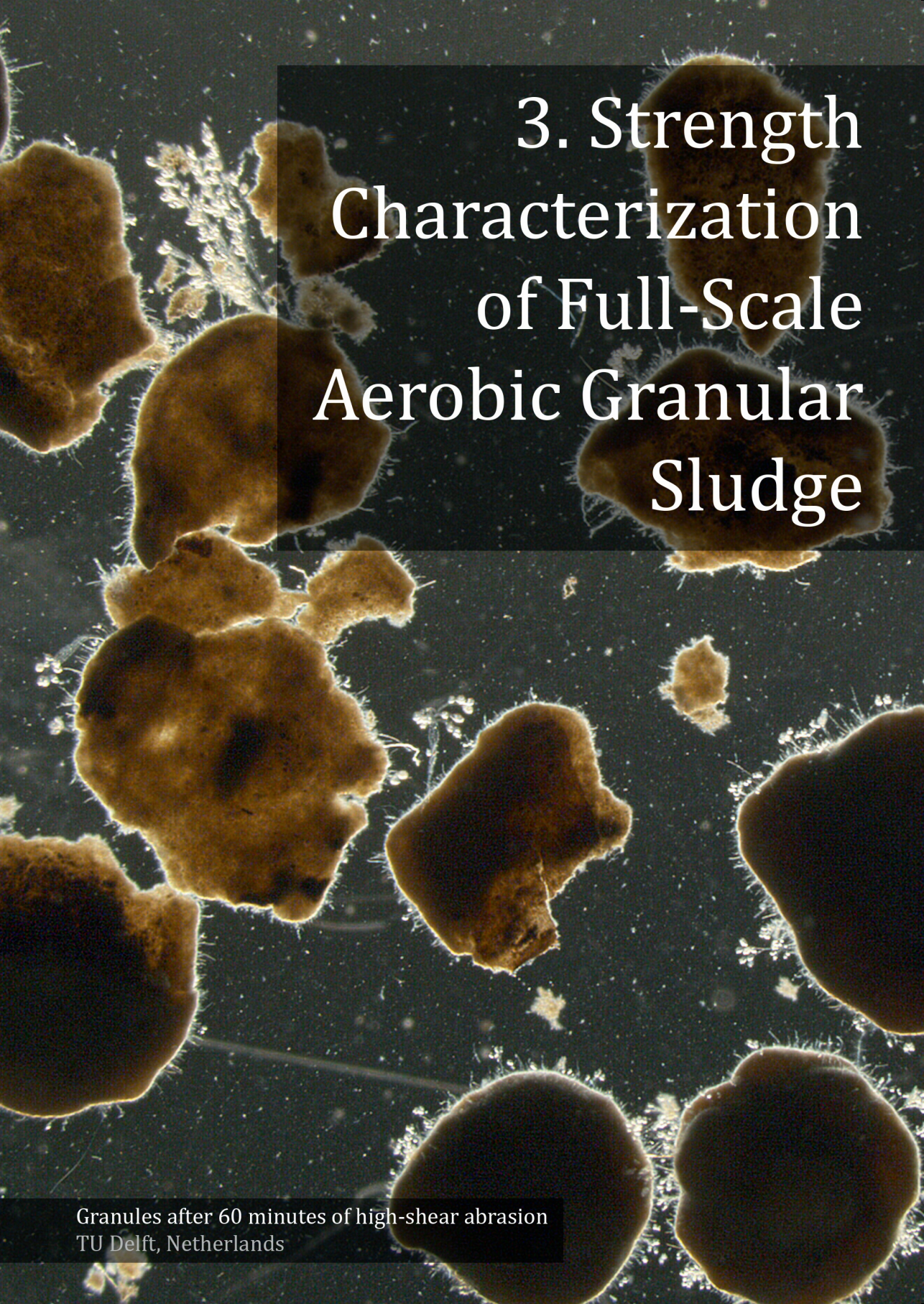
Interestingly, *Ca.* Accumulibacter phosphatis is mainly reported in nature in estuaries which led to the suggestion that they contain osmolytes [90, 91]. These environments have a natural change from saline to brackish water due to tidal variations, along with a variation in anaerobic nutrient supply and aerobic starvation periods [92]. However, the link between adaptation to variable salinity and type of osmolytes has not yet been made in neither environmental science literature nor wastewater engineering literature. The advantages of osmolytes to alleviate osmotic stress have frequently been studied for other technologies such as anaerobic digestion. For example, addition of glycine betaine, α-glutamate, and β-glutamate to anaerobic biomass decreases inhibition of methanogenesis by salinity [93] . It was also found that addition of glutamic acid, aspartic acid, gelatine, and tryptone decreases osmotic stress in anaerobic granular sludge [94].

However, due to the wide variety of osmolytes, the positive effect of specific osmolyte dosing in anaerobic sludge cannot directly be copied to EBPR sludge. Understanding the role of osmolytes in the EBPR process is of greatest importance for improving this technology. Basis has been laid in this study, but further research is required to completely comprehend the fascinating flexibility of *Ca.* Accumulibacter metabolism to variations in salinity.

2.5. Conclusion

- Stable granulation and biological phosphorus removal can occur in seawater-based aerobic granular sludge
- *Ca.* Accumulibacter clade I is adaptable to both freshwater and seawater
- Seawater-adapted AGS releases COD during a salinity downshock, which is likely ascribed to release of osmolytes
- Salinity impact due to NaCl or seawater is very different on EBPR processes

3. Strength Characterization of Full-Scale Aerobic Granular Sludge



Granules after 60 minutes of high-shear abrasion
TU Delft, Netherlands

Strength Characterization of Full-scale Aerobic Granular Sludge

Abstract

For a stable operation the aerobic granular sludge process requires mechanically strong granules in balance with the shear forces in the reactor. Despite a wide general interest in granular stability, the mechanical strength of both anaerobic and aerobic granular sludge received very little attention. In this study, a high-shear method for strength characterization has been evaluated for full-scale aerobic granular sludge (AGS). Abrasion times up to 90 minutes showed a stable abrasion rate coefficient (K), while prolonged periods of abrasion up to 24 hours resulted in a decrease in abrasion rate. Larger granules have higher abrasion rate than smaller granules. No abrasion was observed at low shear rates, indicating a threshold shear rate for abrasion. Lab-scale AGS showed a lower abrasion rate than full-scale AGS. Incubation of full-scale granules in NaCl led to a decrease in abrasion rate at 25 g/L NaCl, but incubation in 50 g/L NaCl led to a further decrease for only half of the tested granular sludge samples.

Highlights

- A strength characterization method based on high shear in a stirred tank has been evaluated
- Aerobic granular sludge show resilience to low shear, indicating a minimal breaking shear rate in line with their hydrogel properties
- Larger granules are weaker than smaller granules
- Short-term exposure of full-scale Nereda® granules to NaCl leads to a decrease in abrasion

This chapter has been published as "De Graaff, D. R., Van Dijk, E. J. H., Van Loosdrecht, M. C. M., & Pronk, M. (2018). Strength characterization of full-scale aerobic granular sludge. Environmental Technology (United Kingdom)." https://doi.org/10.1080/09593330.2018.1543357 [74].

3.1. Introduction

Aerobic granular sludge is a new technology for wastewater treatment with reduced footprint and energy requirements and a good removal rate in comparison to conventional activated sludge processes [20, 22, 95–97]. Granular sludge consists of compact microbial aggregates with excellent settleability in comparison to conventional activated sludge systems [98–100]. For long-term stable operation and sludge characterization the physical strength of the sludge granules is an important factor.

The strength of granular sludge is a result of both the physical and chemical conditions in the treatment reactor. Detachment forces are key factors for the smoothness, density and porosity of biofilm. Higher shear stress leads to formation of a denser biofilm [101–104]. Shear forces control the outgrowth of the granules and are increasingly important with increasing growth rate of the microbial population [42, 76]. A proper balance between shear and growth is therefore important for promoting a dense and stable granule formation [101, 105, 106]. External shear forces that are imposed on the granule depend on the type of reactor and their process conditions. The combination of specific forces in the reactor, the wastewater composition, and the resulting composition of extracellular polymeric substances (EPS) add up to the strength of the granules [103, 107].

Several methods have been proposed for quantifying and comparing the physical strength of granular sludge, such as measurement in a stirred flask [108] or in a bubble column [109]. However, these methods only reach low average shear rates. These low numbers would result in either low abrasion rate of granules, or a duration of experiments in the order of days. The method that was used in the current study is based on agitation in a stirred tank reactor with standard reactor geometry [110]. The rate of abrasion of granules gives a measure for the strength of the granular sludge, at shear rates in the order of 700~2000 s^{-1} (at 800~1600 rpm). A standardized protocol for this short-term high-shear method was not yet available. Moreover, the exact mechanism of the breaking-up or abrasion of granules during the strength test is of great importance for proper interpretation of data, and has not been reported in previous studies. Studies on the strength of aerobic granular sludge are very limited and have been performed with either lab-scale or pilot-scale granules [111]. Granules from these smaller scale reactor systems generally receive a more stable and well-defined influent, but these optimal situations and resulting stability are not representative for a granular sludge from a full-scale installation.

For this study a method was evaluated to quantify the influence of variations in bulk liquid composition, more specific salinity, on the physical strength of granular sludge. Previous studies have shown a negative effect of NaCl on the strength of anaerobic granular sludge [46]. Displacement of Ca^{2+} ions by Na^{+} was suggested to be the major cause for this observation [47]. Also in activated sludge, high sodium concentrations can lead to a deterioration in settling properties [28]. Addition of divalent cations such as calcium and magnesium in turn was found to yield higher floc strength and better settleability [112]. For aerobic granular sludge, it was found that the start-up of a reactor with high salinity wastewater can cause problems, although long-term adaptation can lead to stable granules [31, 113]. The effect of changing NaCl concentrations on the strength of full-scale aerobic granular sludge has not yet been described in literature.

The aim of this research was to quantify the strength of aerobic granular sludge from 4 full-scale aerobic granular sludge (Nereda®) plants. In order to reach this goal, an improved strength characterization method has been evaluated, and the abrasion mechanism of the aerobic granular sludge during this test has been visualized. A comparison is made between lab-scale and full-scale aerobic granular sludge. Finally, the effect of granule size, and the short-term effect of high salinity on full-scale granule strength has been quantified. A detailed protocol is added to allow for future comparative studies.

3.2. Materials & Methods

Granule Collection

Aerobic granular sludge was collected from full-scale Nereda® plants in Utrecht (prototype), Garmerwolde, Vroomshoop and Dinxperlo, all located in the Netherlands. An overview of their influent concentrations is given in table 3.1. The average solids retention time (SRT) in the system was 20~50 days. The aeration tanks are aerated with a gas flowrate of 1 – 2 $m^3/m^3/h$, resulting in a dissolved oxygen concentration of 1 – 2 mg/L.

Table 3.1: Average influent concentrations of Utrecht, Garmerwolde, Vroomshoop, and Dinxperlo Nereda® plants.

Location	COD mg/L	N-tot mg/L	NH_4^+-N mg/L	N-Kj mg/L	P-tot mg/L	PO_4^{3-}-P mg/L	Suspended solids mg/L
Utrecht	707		46.1	64	8.9	5.6	230
Garmerwolde	506	49.4	39		6.7	4.4	236
Vroomshoop	797	55.5		55.5	7.92		
Dinxperlo	635	59		59	8.06		

Lab-scale aerobic granular sludge was taken from a 3.0L bubble column (5.6 cm diameter), operated as a sequencing batch reactor (SBR), inoculated with Nereda® sludge from Utrecht, Netherlands. The temperature was set at 20 °C. The pH was controlled at 7.0 ± 0.1 by dosing either 1M NaOH or 1M HCl. The dissolved oxygen (DO) concentration was controlled at 50% saturation. The average sludge retention time (SRT) was 20 days. Reactor cycles consisted of 60 minutes of anaerobic feeding, 110 minutes aeration, 5 minutes settling and 5 minutes effluent withdrawal. The feed of 1.5L consisted of 1200 mL demineralized water, 150 mL of medium A, and 150 mL of medium B. Medium A contained 7.785 g/L sodium acetate trihydrate (3.66 g/L COD), 0.88 g/L $MgSO_4 \cdot 7H_2O$, and 0.35 g/L KCl. Medium B contained 2.289 g/L NH_4Cl (600 mg/L $NH4^+$-N), 349 mg/L K_2HPO_4, and 136 mg/L KH_2PO_4. The combination of these feed streams led to influent concentrations of 366 mg/L COD, 60 mg/L NH_4^+-N and 9.3 mg/L PO_4^{3-}-P.
Lab-scale seawater-adapted aerobic granular sludge was taken from a reactor with similar conditions as the above-described reactor. The only differences were the absence of $MgSO_4 \cdot 7H_2O$ and KCl in medium A, and a complete replacement of demineralized water in the influent with artificial seawater (35 g/L Instant Ocean® sea salts).

Strength Characterization

Granules were sieved and washed on a 1.6 mm sieve. The granules were dried (wet weight, WW) by soaking up water from the bottom of the sieve with a tissue. These granules were used for (1) determining the total suspended solids per wet weight (TSS/WW), and (2) the strength characterization test.

(1) A known mass of WW granules was transferred to pre-dried 50 mL Falcon tubes. These were either lyophilized, or dried at 60 °C for a minimum of 24 hours until completely dry. The mass was measured after drying (TSS), taking into account weight loss of the tubes itself without granules. Division of the TSS by WW gives the amount of TSS/WW.
(2) 10 g of WW granules was transferred to a measuring cylinder, and filled up to 500 mL with tap water. These contents were transferred to a stirred tank reactor with standard geometry (500 mL volume: 840 mm diameter, 4 baffles of 8.4 mm, and a Rushton impeller with 28 mm diameter), and stirred at 800 rpm for 60 minutes. Afterwards, the reactor contents were sieved over a 200 μm sieve. This filtrate was weighed and divided over 4 50 mL Falcon tubes. These tubes were centrifuged for 10 minutes at 4200 rpm at 4 °C, after which the supernatant was discarded. In case of saline liquid, the pellet was washed and centrifuged several times with demineralized water to prevent precipitation during drying. The centrifuged pellet was either lyophilized, or dried at 60 °C for a minimum of 24 hours until completely dry.

Quantification of strength is derived from the initial amount of TSS and the amount of fine particles (<200 μm) after agitation. Decay of granules into fine particles (X_F) is a function of the remaining amount of granules (X_{NF}). This gives a first-order correlation, which introduces an abrasion rate coefficient (K) in equation 3.1.

$$\frac{dX_F}{dt} = K \cdot X_{NF} \tag{3.1}$$

By solving this differential equation with boundary conditions t = 0, $X_F(0) = 0$, and $X_{NF} = X_0$:

$$\frac{dX_F}{dt} = K \cdot (X_0 - X_F) \rightarrow \ln\left(\frac{X_0 - X_F(t)}{X_0 - X_F(0)}\right) = -K \cdot t$$

$$\ln\left(\frac{X_0 - X_F}{X_0}\right) = -K \cdot t \tag{3.2}$$

X_o is the initial biomass concentration (g TSS/L), X_F is the concentration of fine particles after the shear experiment (g TSS/L), t is the duration of the shear exposure (s).

A detailed protocol for strength characterization is described in Appendix A.

Shear rate calculation

Calculations of the average shear rates are based on derivations by Sanchez-Perez et al. [114]. The equations for a stirred tank reactor (3.3) and a bubble column (3.4) are shown below. N_p is the power number, equal to 6.1 in turbulent flows [115], ρ is the liquid density (kg/m³), d_i is the impeller diameter (m), μ is the dynamic viscosity (Pa·s), N is the rotation speed (revolutions per

second). For the bubble column equation (3.4), ρ is the liquid density (kg/m^3), ϵ is the energy dissipation rate (W/kg), K is the consistency index, equal to the dynamic viscosity (μ) in a Newtonian fluid (Pa·s), n is the flow index, equal to 1 in a Newtonian fluid, g is the gravitational acceleration (9.81 m/s^2[22]), and u_G is the superficial gas velocity (m/s).

$$\gamma = \left(\frac{4 N_p \rho d_i^2}{\pi 3^3 \mu} \right)^{\frac{1}{2}} N^{\frac{3}{2}} = \omega N^{\frac{3}{2}} \tag{3.3}$$

$$\gamma = \left(\frac{\rho \epsilon}{K} \right)^{\frac{1}{n+1}} \quad \text{where} \quad \epsilon = g U_g \tag{3.4}$$

The shear stress profile in a bubble column is rather uniform, so the maximal shear rate is not calculated. For a stirred tank reactor however, there is a major difference between the shear at the impeller tip and the average shear rate. Calculation of this maximal shear rate is done by using an equation by Robertson and Ulbrecht [116].

$$\gamma_{max} = 3.3 N^{1.5} d_i \left(\frac{\rho}{\mu} \right)^{0.5} \tag{3.5}$$

For a full-scale Nereda® plant, the equation for a bubble column is used. The shear rate in a full-scale Nereda® plant is much lower than in a lab-scale bubble column, due to the size and air flow rate. The lab-scale reactor has an airflow of 6 L min^{-1}, and a surface area of 24.6 cm^2, resulting in a superficial gas velocity of 0.04 m/s. A typical Nereda® reactor has a superficial gas velocity in the range of 0.0015 – 0.0040 m/s, which is an order of magnitude lower than the typical lab reactor.

Microscopy and particle size distribution

The particle size distributions of fresh aerobic granules and the remaining, filtered, granules after the strength test (sieve mesh 200 μm) were obtained using Image Analysis with an Olympus reverse microscope with magnification of 7.78x and a Leica Digital Camera, along with its software QWin Pro, version 3.1. An average of 4 representative pictures were taken of each sample.

Analytical methods

Cation concentrations were measured with ICP-OES (OPTIMA 5300DV Optimal Emission Spectrometer, Perkin Elmer). All samples are diluted 10x with 2% HNO_3, and measured against 3 standard samples.

3.3. Results

Influence of stirring time, shear rate, and concentration of granules

The influence of the stirring time on the granular sludge abrasion has been determined for granules from the Nereda® prototype installation in Utrecht, Netherlands (figure 3.1). During the first 90 minutes with an average shear rate of 731.1 s^{-1} (800 rpm), a linear relation can be seen between the natural logarithm of eroded mass and stirring time. However, during prolonged exposure up to 1440 minutes (24h), a decrease in the rate of abrasion was measured after 90 minutes of shearing time.

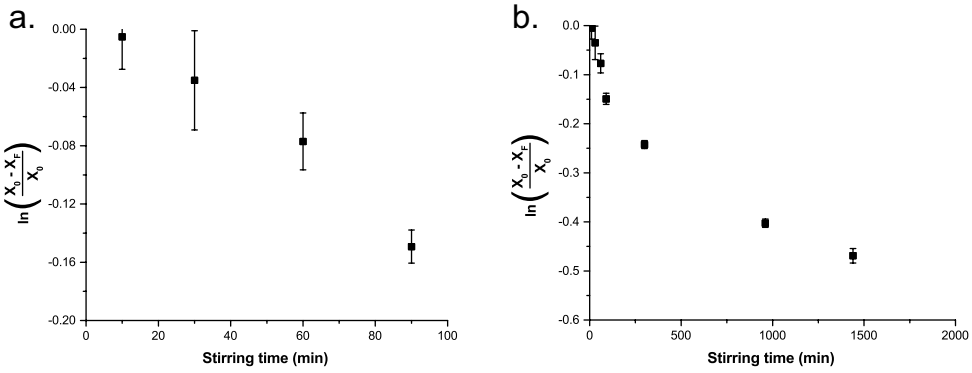


Figure 3.1: Influence of stirring time on abrasion during the strength characterization test, (a) during the initial 90 min, and (b) during a prolonged period up to 1440 min (24 h).

The abrasion rate coefficient K correlates linearly with the average shear rate ($\dot{\gamma}$) (figure 3.2a). At an average shear rate of 285.5 s^{-1} (400 rpm) a negligible amount of eroded material was observed. For comparison, an overview of average and maximal shear rates in a stirred tank reactor, bubble column, and full-scale Nereda® plants is given in table 3.2. The concentration of granules that are added to the strength characterization test have negligible effect on the value for K (figure 3.2b).

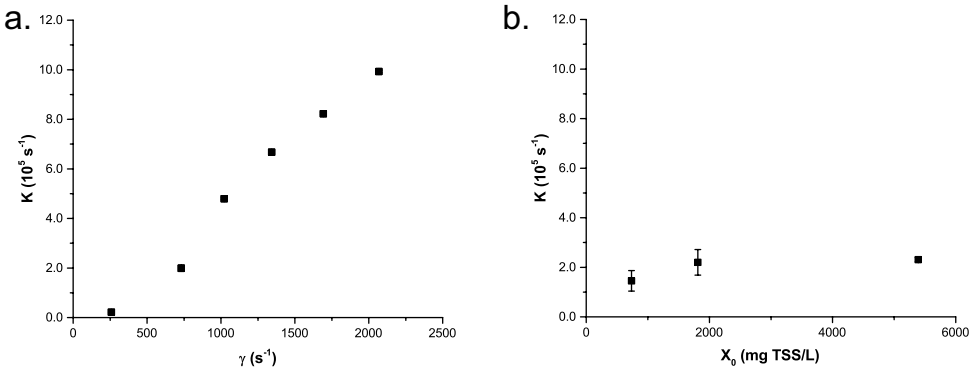


Figure 3.2: Influence of (a) average shear rate ($\dot{\gamma}$) and (b) initial amount of granules in the strength characterization test (X_o), on the abrasion rate coefficient (K) after shear exposure to full-scale Nereda® aerobic granular sludge

Table 3.2: Overview of calculated average shear rates ($\dot{\gamma}$) and maximal shear rates (γ_{max}) in a stirred tank reactor, bubble column, and full-scale Nereda® plant at 20 °C. Shear rates are calculated for a variety of stirrer speed for a standard geometry stirred tank reactor (rpm), or superficial gas velocity for a bubble column and Nereda (mm/s), with its respective gas flow between brackets.

Operation	Conditions	$\dot{\gamma}$ (s^{-1})	γ_{max} (s^{-1})
Stirred tank reactor	200 rpm	91.4	562.3
	800 rpm	731.1	4,498.6
	1600 rpm	2,068.0	12,742.0
Bubble column	6.8 mm/s (1 L/min)	257.6	
	40.6 mm/s (6 L/min)	631.1	
	67.7 mm/s (10 L/min)	814.8	
Nereda plant	1.4 mm/s (500 m^3/h)	116.7	
	2.8 mm/s (1000 m^3/h)	165.1	
	4.2 mm/s (1500 m^3/h)	202.2	

Abrasion mechanism

The abrasion mechanism of granular sludge during the shear experiment was tested by exposing a total of 67 granules to an average shear rate of 731 s^{-1} (800 rpm) during 60 minutes. Upon abrasion of granules during the experiment, the physical structure remains similar as can be seen in figure 3.3. Small chips of granules were observed after 15 minutes already, and the fraction of these chips increased after 60 minutes of shear exposure (figure 3.3e and 3.3f).

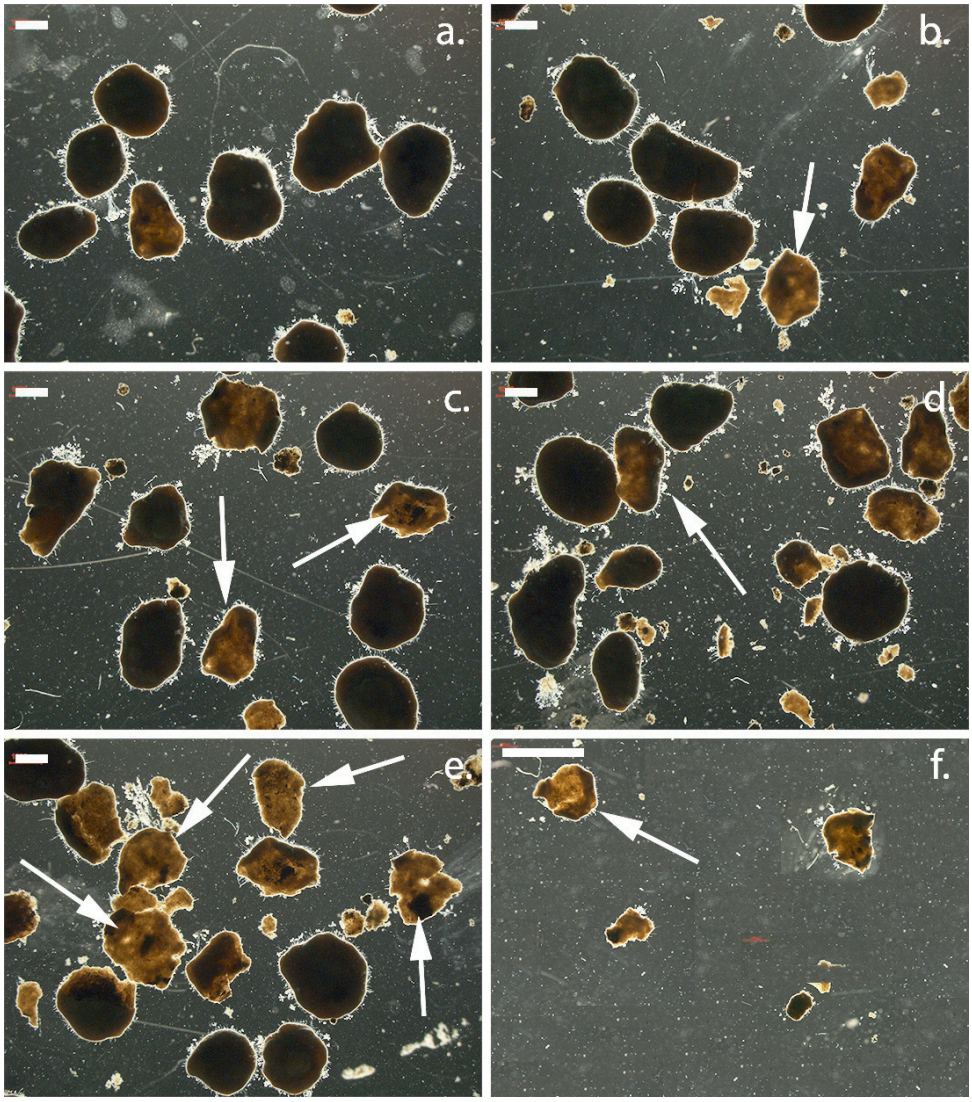


Figure 3.3: Abrasion of full-scale Nereda granular sludge at 7.78× magnification after (a) 0 min, (b) 15 min, (c) 30 min, (d) 45 min, (e) 60 min, and (f) at 25× magnification after 60 min. Scale bars indicate 1 mm. Arrows indicate hollow granules.

This visual observation was quantified by means of size distribution analysis before and after the strength characterization test (figure 3.4). These results confirm that the majority of particles have a size that is just above this sieving threshold. These small fragments were visually observed to be the shells that were sheared off of the granules, as seen in figure 3.3e and 3.3f.

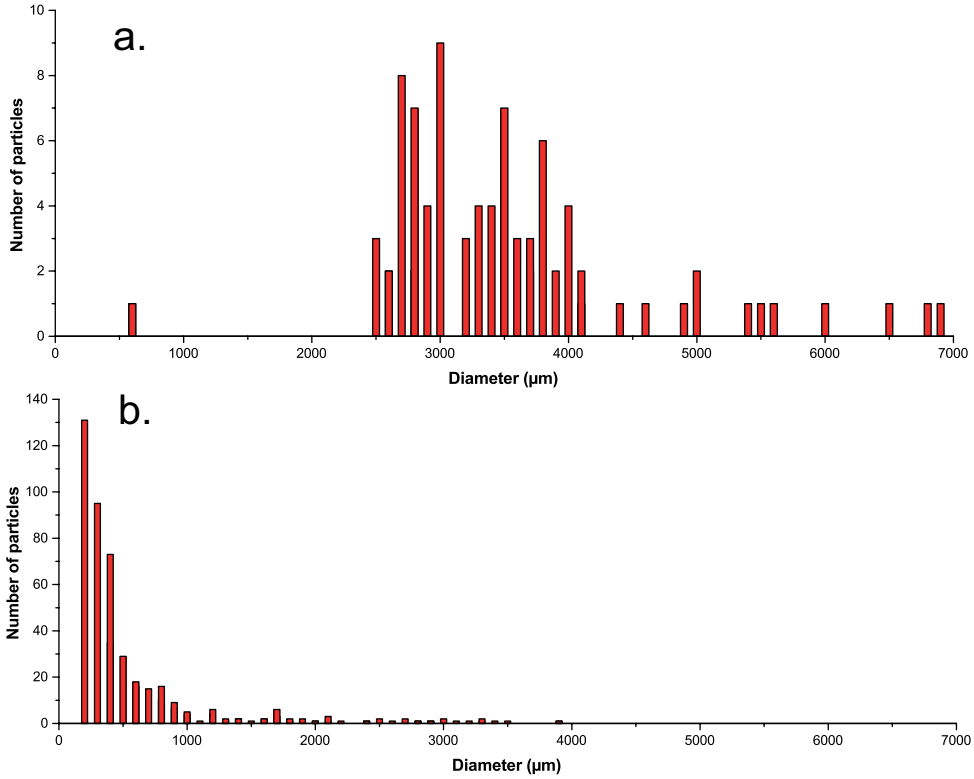


Figure 3.4: Size distribution of (a) the initial granular biomass before the strength test, with a standard amount of 12.5 g wet weight granules, and (b) the particulate fraction after the strength characterization test of 60 min at 800 rpm, after sieving with a 200 µm sieve.

Variability of full scale Nereda® granular sludge

Aerobic granular sludge from 4 different full-scale Nereda® plants was tested on their strength with the standard test conditions (800 rpm for 60 minutes). Results of this study and references of other studies are shown in table 3. The abrasion rate coefficient (K) of the different Nereda® sludge granules ranged from $(2.68 \pm 0.17) \cdot 10^{-5}$ s^{-1} to $(7.69 \pm 0.90) \cdot 10^{-5}$ s^{-1}. Freshwater lab-scale AGS had a lower value for K of $(1.78 \pm 0.20) \cdot 10^{-5}$ s^{-1}, and seawater-adapted lab-scale AGS had an even lower value of $(1.17 \pm 0.01) \cdot 10^{-5}$ s^{-1}.

Table 3.3: Overview of abrasion rate coefficients *K* from different granular sludge systems (lower number equals higher strength).

Description	K (s^{-1})	Reference
Utrecht Nereda® plant	2.85 ± 0.10	This study
Garmerwolde Nereda® plant	5.47 ± 0.14	This study
Vroomshoop Nereda® plant	7.69 ± 0.90	This study
Dinxperlo Nereda® plant	2.68 ± 0.17	This study
Lab-scale acetate-fed aerobic granular sludge	1.78 ± 0.20	This study
Lab-scale acetate-fed saline aerobic granular sludge	1.17 ± 0.01	This study
Anaerobic lab-scale butyric acid producing granules	2451.66	[110]
Aerobic lab-scale nitrifying granules	101.32	[110]
Anaerobic lab-scale methanogenic granules	20.08	[110]
Low-load full-scale methanogenic granules	1.31	[110]
High-load full-scale methanogenic granules	2.16	[110]
Full-scale cannery wastewater-fed anaerobic granules	1.71	[117]
Full-scale abattoir wastewater-fed anaerobic granules	15.23	[117]
Full-scale brewery wastewater-fed anaerobic granules (1)	4.19	[117]
Full-scale brewery wastewater-fed anaerobic granules (2)	3.88	[117]

Effect of granule size on abrasion of full-scale aerobic granular sludge

Granules with smaller size showed a lower abrasion rate than bigger granules, and this correlation was found to be non-linear (figure 3.5). Granules that have a size of >2000 μm show a significantly lower strength than granules that have smaller sizes, whereas the smaller granule fractions did not have a large difference in observed strength.

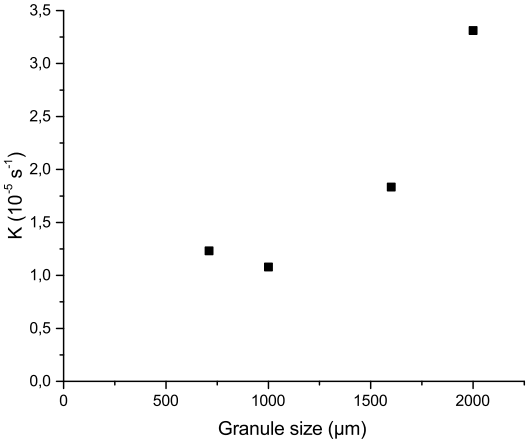


Figure 3.5: Abrasion rate coefficient (*K*) for aerobic granular sludge sieved over 710, 1000, 1600, and 2000 μm sieves.

Effect of NaCl on abrasion of full-scale aerobic granular sludge

The short-term effect of NaCl exposure to freshwater-adapted full-scale aerobic granular sludge was determined after incubation of 1 hour in NaCl solution, prior to the strength characterization test. Results are shown in figure 3.6. At an NaCl concentration of 25 g/L, the abrasion decreased for all of the full-scale granules. At 50 g/L NaCl, granules from Utrecht and Dinxperlo shows even less abrasion, but an increase (relative to 25 g/L NaCl) in abrasion was measured for granules from Garmerwolde and Vroomshoop.

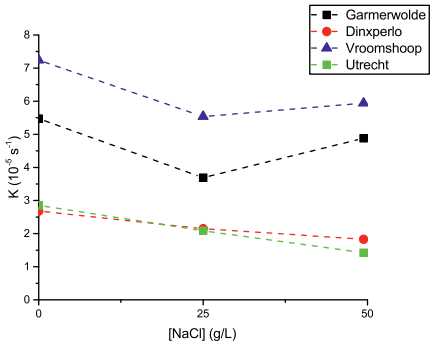


Figure 3.6: Abrasion rate coefficients (K) of aerobic granular sludge from four full-scale Nereda® plants after incubation of 1 h at NaCl concentrations of 0, 25, or 50 g/L.

Exchange of ions in the liquid phase after 1 hour of incubation of Garmerwolde granular sludge was quantified by ICP-OES (figure 3.7). Sodium was taken up from the liquid by 347 mg/g TSS at 25 g/L NaCl, and 449 mg/g TSS at 50 g/L NaCl. Calcium and magnesium both had the same low amount of release at 25 and 50 g/L NaCl, at 4.0 mg/g TSS and 1.1 mg/g TSS, respectively. Changes in concentrations of tin, strontium, lead, manganese, iron and zinc were below the detection limit of 1 mg/L (data not shown). Phosphate release during incubation was negligible (<1 mg/L).

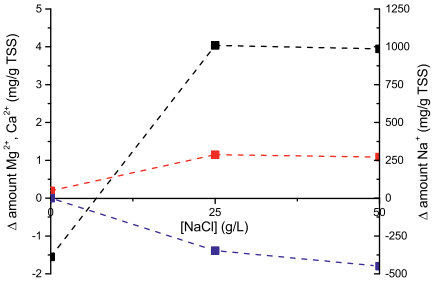


Figure 3.7: Change in amount of Na^+ (blue), Mg^{2+} (black), and Ca^{2+} (red) after 1 h of incubation in 0, 25, or 50 g/L NaCl of full-scale aerobic granular sludge from Garmerwolde, Netherlands.

3.4. Discussion

Method evaluation

Current literature on strength characterization of granular sludge is based on experiments that are either undefined in terms of shear rate [108] or based on experiments that require a long duration [109]. Comparison of quantified strength from different studies is complicated or not possible, because different methods are used for acquiring strength numbers. Some studies have used a bubble column for strength quantification [109, 117]. Using this type of reactor, rather than a stirred tank reactor causes different shearing behavior. A highly turbulent region in a stirred tank reactor induces a maximal shear rate around the impeller, whereas a bubble column does not have this local maximum. Even though a bubble column resembles full-scale practice, the purpose of this strength test is to have a separate and not necessarily similar method of shear exposure.

The results that were obtained in the evaluation of the strength measurement are in line with previous studies, especially Pereboom [110], who used methanogenic granular sludge. A linear abrasion rate over time indicates negligible influence of detached particles and a linear increase in abrasion rate with increasing shear rate. The increasing biomass concentration did not lead to an increasing abrasion rate, matching the results of Pereboom [110] as well.

The linear abrasion rate during the first 90 minutes of abrasion and no effect of granular sludge concentration indicates that the particle-particle collisions do not play a role in this test. This can be explained by looking at energy transfer in a stirred tank reactor. Kinetic energy that is put into the system through the stirrer gets dissipated in the bulk liquid throughout a series of eddies. The smallest scale is in the range of Kolmogorov microscale. When the particle size is smaller than these microscales, viscous forces determine the maximum hydrodynamic force, while these are negligible when the particle size is larger, and thereby in the inertial sub-range of turbulence [118]. The size of the Kolmogorov microscales can be calculated according to equation 3.6, and depends on the kinematic viscosity (ν in m^2/s) and the average dissipation rate (ϵ in W/kg) [119]. Equation 3.7 describes the calculation of ϵ, where P_0 is the power number (= 6.1 for turbulent regions), N is rotational speed (s^{-1}), D_i is impeller diameter (m), and V is the volume (m^3).

$$\eta = \left(\frac{\nu^3}{\epsilon}\right)^{\frac{1}{4}} \tag{3.6}$$

$$\epsilon = \frac{P_0 N^3 D_i^5}{V} \tag{3.7}$$

In a strength measurement test at 800 rpm in tap water ($\nu = 1.00 \cdot 10^{-6}$ m^2/s), the Kolmogorov length equals $4.1 \cdot 10^{-5}$ m (= 41 μm). This number is several orders of magnitude smaller than the size of the granules that were used in this experiment of around 10^{-3} ~ 10^{-4} m, indicating a negligible effect of viscous forces on the granule abrasion. The abraded particles will have a diameter in range of the Kolmogorov length. However, this size will cause them to follow turbulent flow, and thereby cause less collisions [120].

After the first 90 minutes in the abrasion test, no linear abrasion was observed anymore in the test. This could be due to the fact that a relatively high amount of small particles with sizes in

the Kolmogorov length are formed. Since these particles will have smaller physical impact upon collision, the overall abrasion rate decreases. However, since the test protocol uses 60 minutes for quantification, this effect should be negligible.

Comparison of strength of aerobic granular sludge to other kinds of granular sludge

Between different aerobic granular sludge samples in this study, there is a difference in measured strength. Granular sludge from Utrecht and Dinxperlo Nereda® plants have lower abrasion (approx. $2.7 \cdot 10^{-5}$ s^{-1}) than Garmerwolde and Vroomshoop plants ($5.5 - 7.7 \cdot 10^{-5}$ s^{-1}). Contributing factors in the Dinxperlo plant can be dosing of iron, and a higher average SRT than other plants. The former leads to higher amounts of phosphate precipitates in the granular sludge, and potentially an increase in granular strength. The lab-scale acetate-fed aerobic granular sludge has higher strength than all full-scale granules ($1.78 \cdot 10^{-5}$ s^{-1}). This is likely due to having a more defined and easily degradable substrate, leading to selection of a more homogenous microbial population that forms a strong granule (polyphosphate accumulating bacteria in this case).

Strength characterization has previously been carried out on different types of granular sludge. The aerobic granules from all tested Nereda® plants are weaker than anaerobic methanogenic granules [47, 110]. These observations are in line with biofilm being more stable when consisting of bacteria with lower growth rate [101, 121]. However, it was found that slow-growing anammox granules are weaker than the aerobic granular sludge, with no described heterotrophic growth was described in their system [122]. This could be due to differences in ionic composition of the EPS and inorganic compounds within the granules. Batstone and Keller [117] tested shear resistance of anaerobic granules from different full-scale plants. They found that cannery-fed granules had a low abrasion rate, which was lower than the tested Nereda® sludge from this study. Granules that were fed with protein-rich pig abattoir wastewater exhibited high abrasion in comparison to aerobic Nereda® granules. Anaerobic granules that were fed with brewery wastewater showed an abrasion rate that was similar to the aerobic Nereda® granules. The cannery wastewater has likely more easily degradable substrate than abattoir or brewery wastewater. The higher reported percentage of calcium in this water could theoretically increase the granular strength, but its impact on the overall strength was found to be small in some studies [122, 123].

Heterogeneity of density throughout granules

Exposure to intensive shear in the stirred reactor during longer time periods than 90 minutes led to a non-linear abrasion rate (figure 3.1b). The first-order abrasion rate that was described in equation 1 therefore only holds for the first 90 minutes of abrasion. This observation could be the result of a heterogeneity of density throughout the granules. Granules will have a layered structure, conversion of slowly degradable polymeric compounds will be mainly performed by bacteria in the outer shell of the granules. This might have a lower strength then the consolidated (and likely relatively older) inner core of the granules. Measurement of the density and extracellular polymers (EPS) within single granules could give more information on density and EPS differences and their impact on the granular strength.

Minimal breakage shear rate

As can be seen in figure 3.2a, at a shear rate of 258 s^{-1} no abrasion takes place from the granular sludge of the Utrecht Nereda® prototype. This indicates resistance of granules to a certain amount of shear before abrasion [47, 110]. Exposure of granules to shear in a full-scale operation also requires them to resist these forces to prevent washout (table 2). Properties of the EPS can play a major role in this observation. The alginate-like exopolysaccharides (ALE) that were found in the EPS of the granules are a hydrogel, exhibiting behavior of a viscoelastic solid [124]. These properties support the idea that a minimal force is required for breaking the granules, due to their elasticity. This elasticity gives granules some resilience for shear stress without breaking or abrading [125, 126].

Granule size

Larger granules had lower strength than smaller ones (figure 3.5). This could be due to the smaller granules colliding less frequently than their bigger counterparts. The derivation of the previously described Kolmogorov scales can be applied for the effect of granule size on the observed strength. Moreover, the collision impact of bigger granules will be larger since they have higher mass and momentum than the smaller granules [120].

Furthermore, as granules grow larger, the inner core could starve due to diffusion limitation of substrate. Hollow cores have frequently been observed in granules. The life cycle of granular sludge is still a topic under discussion, but breakage of larger granules into smaller granules is one hypothesis that is in line with our observations [127].

Effect of NaCl on strength

Addition of NaCl to full-scale aerobic granular sludge led to an increase in the granular strength. Addition of calcium and magnesium ions to activated sludge decreases the sludge volume index (SVI) and increases the floc density [112]. An increase in sodium led to the opposite effects in this study, presumably by replacement of calcium which is stabilizing the floc EPS. Leaching of these divalent cations has been discussed to be also detrimental to granule stability [47, 110]. ICP-MS analysis indicated that added sodium diffused into the granular sludge but did not lead to a significant release of calcium or magnesium. Therefore the stabilizing effect of these ions on the EPS will not be decreased [124]. In other experiments the use of EDTA, which actively binds Ca^{2+} ions, indeed led to a decreased strength of granular sludge [111].

The effect of granule size can be of importance in explaining the positive effect of NaCl addition on granular strength [48]. Due to the higher osmotic pressure of saline water than freshwater, an exchange of salts into the granules and water out of the granules can occur. The resulting lower amount of water in the granules can cause shrinking, and an increase in granule density. Smaller granules show a lower abrasion rate than bigger granules (figure 3.5). Granules with higher density can similarly have higher strength [111]. This hypothesis needs further analysis, since in the complex heterogeneous non-spherical granular sludge samples we used it was not possible to reliably measure change in granule volume due to salt addition.

The strength of lab-scale artificial seawater-adapted aerobic granular sludge is higher than of lab-scale freshwater-adapted sludge. Difference in density and adaptation of the EPS likely plays

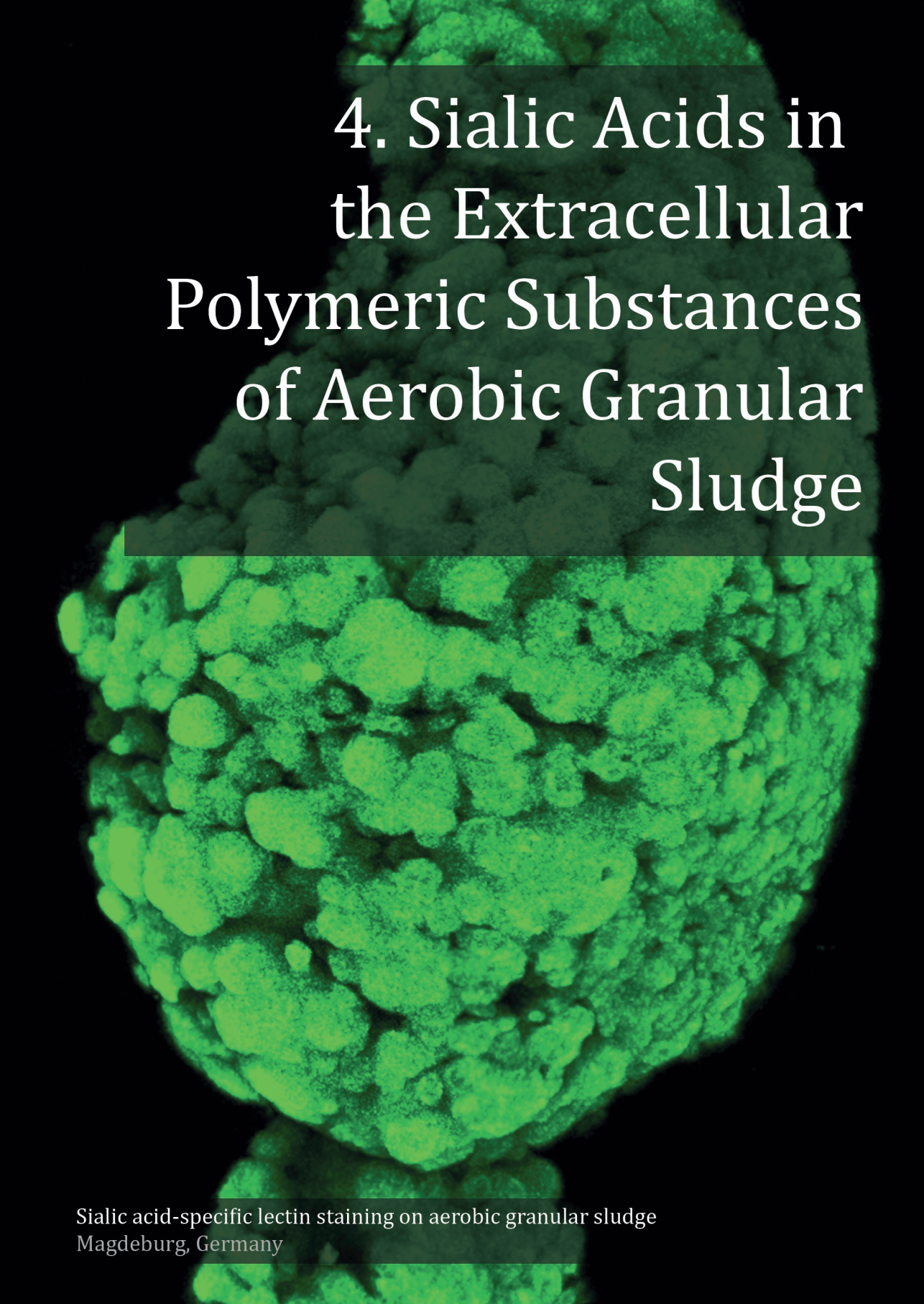
a role in this observation. Higher concentrations of extracted EPS and higher protein contents have for instance been observed in other studies with seawater biofilms [54, 56]. Characterization of alginate-like exopolymers from aerobic granular sludge has been described in literature, but the adaptation of the EPS to seawater should be a major focus for linking this to its strength properties [49].

3.5. Conclusion

This study has described a method for determining the strength of aerobic granular sludge. This method was tested for granular sludge from 4 full-scale Nereda® plants, granule size, salinity, lab-grown acetate-fed aerobic granular sludge, both on freshwater and seawater.

Abrasion times up to 90 minutes showed a stable abrasion rate coefficient (K). Prolonged periods of abrasion up to 24 hours resulted in a decrease in abrasion rate. The amount of granules does not impact the value for K. Higher shear rates lead to higher values for K, but a minimal shear rate was required for the start of abrasion. Lab-scale granules exhibited a lower abrasion rate than full-scale Nereda® granules. Incubation of full-scale granules in NaCl led to a decrease in abrasion rate at 25 g/L NaCl, but incubation in 50 g/L NaCl led to a further decrease for only 2 out of 4 tested granular sludge samples.

4. Sialic Acids in the Extracellular Polymeric Substances of Aerobic Granular Sludge



Sialic acid-specific lectin staining on aerobic granular sludge
Magdeburg, Germany

Sialic Acids in the Extracellular Polymeric Substances of Seawater-Adapted Aerobic Granular Sludge

Abstract

Sialic acids have been discovered in the extracellular polymeric substances (EPS) of seawater-adapted aerobic granular sludge (AGS). Sialic acids are a group of monosaccharides with a nine-carbon backbone, commonly found in mammalian cells and pathogenic bacteria, and frequently described to protect EPS molecules and cells from attack by proteases or glycosidases. In order to further understand the role of these compounds in AGS, lectin staining, genome analysis of the dominant bacterial species, and shielding tests were done. Fluorescence lectin bar-coding (FLBC) analysis showed an overlap with protein staining, indicating presence of sialoglycoproteins in the EPS matrix. Genome analysis gives a positive indication for putative production of sialic acids by the dominant bacteria *Candidatus* Accumulibacter. FTIR analysis shows upon selective removal of sialic acids a decrease in carbohydrates, extension of the protein side chain, and exposure of penultimate sugars. Enzymatic removal of sialic acids results in the removal of galactose residues from the EPS upon subsequent treatment with β-galactosidase, indicating a linkage between galactose and sialic acid at the terminus of glycan chains. This work indicates the importance of sialic acids in the protection of penultimate sugar residues of glycoproteins in EPS, and provides basis for future research in the composition of EPS from biofilms and granular sludge.

Highlights

- Sialic acids are present in the EPS of seawater-adapted aerobic granular sludge
- Sialoglycoproteins in the EPS contain sialic acids as the terminal sugar residue
- *Ca.* Accumulibacter are indicated to be capable of sialic acid production
- Terminal sialic acids protect galactose residues in the granule EPS

This chapter has been published as "De Graaff, D. R., Felz, S., Neu, T. R., Pronk, M., Van Loosdrecht, M. C. M., & Lin, Y. (2019). Sialic acids in the extracellular polymeric substances of seawater-adapted aerobic granular sludge. Water Research, 155, 343–351." https://doi.org/10.1016/j.watres.2019.02.040 [78].

4.1. Introduction

Aerobic granular sludge (AGS) is an upcoming technology for wastewater treatment, capable of simultaneously removing organic carbon, nitrogen, and phosphorus in a single process unit [19, 22, 95]. The sludge granules consist of bacteria embedded in a matrix of extracellular polymeric substances (EPS) [48, 49]. Besides providing a structural matrix in which cells can grow, EPS also serve as a protection against adverse conditions in the bulk liquid [50, 51]. EPS are found in all kinds of granular sludge, and a multitude of properties and compositions have been described as a result of different operating conditions [52, 53].

The application of AGS technology in saline wastewater treatment has been reported before [22, 36, 113]. Saline wastewater originates from many sources such as sea water (either due to intrusion in sewer networks or due to application of seawater flushing), industrial wastewaters such as from fish canning industries, effluent from mining and mineral processing industries [24]. Adaptation of AGS to high salinity leads to a change in EPS composition, e.g. higher protein fractions have been reported [54], as well as an increase in hydrophobicity [56]. Higher concentrations of EPS have been found as a response to growth in saline conditions [55]. These studies have mainly focused on quantification of carbohydrates and proteins in the EPS by using colorimetric methods. It is noted that not all carbohydrates can be measured with those most commonly used methods. For instance, sialic acids have a low response factor for colorimetric methods such as anthrone assay and phenol sulfuric acid assay, so these sugars cannot be detected and quantified by these methods [128, 129].

Sialic acids are a group of special carbohydrates with a nine-carbon backbone. The most common member is N-acetylneuraminic acid (Neu5Ac), which has been observed on the surface of both mammalian cells and pathogenic bacteria [130]. Sialic acids play an important role in the stabilization of molecules and membranes, as well as in modulating interactions with the environment. They can also protect molecules and cells from attack by proteases or glycosidases, extending their lifetime and function [131]. Information of sialic acids' presence in the EPS of aerobic granules and their function in AGS is significantly important to understand their role in maintaining the stability of granular sludge.

Since sialic acids might be overlooked due to the detection limitation of commonly used colorimetric methods, more effective methods to study EPS components and their specific function are required to provide greater insight into the nature of AGS.

One of the proven effective methods for EPS glycoconjugates analysis is fluorescence lectin bar-coding (FLBC) [58] These lectins can bind to specific carbohydrate regions, allowing for screening of glycoconjugates in a hydrated biofilm matrix. This method has been successfully applied for analysis of saline anaerobic granular sludge [59]. Carbohydrates were found to be present as glycoconjugates, with sugar residues including mannose and N-acetyl-galactosamine. Other studies described mannosyl, glucosyl, and N-acetyl-glucosamine residues in anaerobic granular sludge [132]. Interestingly, these sugars are often in conjunction with sialic acids, which is similar as commonly found glycoconjugates in the mucin matrix in animals [133, 134]. Lectin staining studies for sialic acids have been described in biofilms before, but a detailed study on their position and function has not yet been conducted [135].

In this study, glycoconjugates within EPS from seawater-adapted AGS were analyzed using lectin staining methods. A genome analysis was performed on the major species that were found in this sludge, to screen for putative production pathways for sialic acids. Selective removal of sugar residues from whole granules have been performed to give insight into the function of sialic acids in granular sludge. A discussion is added about the evolutionary benefit for producing sialic acids in a granular sludge system.

4.2. Materials & Methods

Reactor operation

Seawater-adapted aerobic granular sludge was taken from the lab-scale bubble column reactor, which was operated as described in chapter 2.

Dominant microorganisms visualization by fluorescence in situ hybridization (FISH)

The handling, fixation and staining of FISH samples was performed as described in Bassin et al. (2011) [32]. A mixture of PAO462, PAO651, and PAO846 probes (PAOmix) were used for visualizing polyphosphate accumulating organisms (PAO) [69]. A mixture of GAOQ431 and GAOQ989 probes (GAOmix) were used for visualizing glycogen accumulating organisms (GAO) [70]. A mixture of EUB338, EUB338-II and EUB338-III probes were used for staining all bacteria [72, 73]. Images were taken with a Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 26 (bp 575e625/FT645/bp 660e710), 20 (bp 546/12/ FT560/bp 575e640), 17 (bp 485/20/FT 510/bp 5515e565) for Cy5, Cy3 and fluos respectively.

Sialic acid visualization and quantification

The granules were stained and mounted in coverwell chambers with a 1 mm spacer in order to avoid squeezing of the samples. Glycoconjugates of the granules were examined by means of fluorescence lectin bar-coding. Thus all commercially available lectins (FITC or Alexa488) were applied as an individual probe to one granule. After this glycoconjugate screening, granules were stained specifically for proteins and subsequently with sialic acid specific lectins. Protein stain solution (Sypro red) was directly put on top of the granules. After 3 hours' incubation, the sample was washed two times with tap water, and stained with sialic acid specific lectin solution for 1 hour. A total of 77 lectins were used for screening of glycoconjugates [136]. The binding sites of the lectins that gave the strongest signal are listed in table 4.1. Then the sample was washed with tap water for three times. For 3D imaging a TCS SP5X confocal laser scanning microscope (Leica, Germany) was employed. The upright microscope was equipped with a super continuum light source and controlled by the software LAS AF 2.4.1. The confocal datasets were recorded by using 25x NA 0.95 and 63x NA 1.2 water immersion lenses. Excitation was at 490 nm and 550 nm. Emission signals were detected simultaneously or sequentially with two photomultipliers from 505-600 nm (lectins) and 600-700 nm (Sypro red). Image data sets were projected using Imaris version 9.1.2 (Bitplane, Switzerland). For deconvolution of the image dataset in figure 4.3 the program Huygens version 18.4 (SVI, The Netherlands) was used.

Table 4.1: Lectins used in the analysis of sialic acids in granular sludge

Lectins	Abbreviation	Specificity	References
Homarus americanus agglutinin	HMA	Sia, Neu5Ac	[137]
Maackia amurensis lectin	MAA	Siaα(2–3)βGal(1–4)βGlcNAc, Siaα(2–3)βGal(1–3)[Siaα(2–6)] αGalNAc	[138]
Limax flavus lectin	LFA	Terminal Sia	[139]
Polyporus squamosus lectin	PSL	Neu5Ac α(2-6) Gal	[140]
Wheat germ agglutinin	WGA	GlcNAcβ(1-4)GlcNAcβ(1-4)GlcNAc, Neu5Ac	[135]

Sialic acid quantification

Quantification of sialic acids (N-acetylneuraminic acid, Neu5Ac) in the seawater-adapted AGS was performed with a Sialic Acid Quantitation Kit (Sigma-Aldrich, USA). Granules were physically crushed and homogenized without disrupting cells, similar to sludge handling for FISH fixation as described in Bassin et al., 2011 [32]. Afterwards the crushed granules were washed with Tris-HCl buffer (pH 7.5) and resuspended in demineralized water (15 mg volatile solids granules per 5 mL final volume). The protocol was followed as described in the manual supplied with the quantitation kit for a whole cell assay. 80 µL of homogenized cells were mixed with 20 µL sialidase buffer and 1 µL of α(2→3,6,8,9)-neuraminidase, and incubated overnight at 37 °C. Afterwards, 20 µL 0.01M β-NADH solution, 1 µL of N-acetylneuraminic acid aldolase and 1 µL of lactic dehydrogenase were added, and incubated at 37 °C for 1 hour. Absorbance at 340 nm was measured prior and after addition of the last enzymes, and used for calculation of the Neu5Ac concentration. Standards were prepared with Neu5Ac from the same quantitation kit.

Genome analysis of enzymes for sialic acid synthesis

Forty-eight available metagenome sequences of *Candidatus* Accumulibacter enrichments were obtained from JGI IMG database (listed in table 4.2 results). These metagenomes were compared with protein sequences of enzymes from known sialic acid synthesis pathways, obtained from the NCBI protein database. The species from which the reference protein sequences were taken were a range of pathogenic bacteria (*Neisseria meningitides*, *Campylobacter jejuni*, *Helicobacter cetorum*, *Photobacterium damselae*), extremophiles (*Chitinivibrio alkaliphilus*, *Psychrobacter arcticus*, *Salinibacter ruber*, *Halanaerobium praevalens*), and the common fruit fly (*Drosophila melanogaster*). BLASTp was performed using the on-line BLASTp tool by JGI IMG. Alignment was performed according to the algorithm as described in Altschul et al., 1997 [141] and Schäffer et al., 2001 [142]. Lower E-values indicate a lower uncertainty in the presence of certain sequences. Values lower than 5E-25 were set as threshold for positive results [143]

Function of sialic acids in seawater-adapted aerobic granular sludge

Galactose removal tests

Granules were taken directly from the lab-scale seawater-fed reactor, and washed by placing them in distilled water for 2 hours. Equal numbers of granules were distributed over 2 Eppendorf tubes, and mixed with 400 µl distilled water and 100 µl sialidase buffer. To the first tube, 5 µl neuraminidase enzyme was added; to the second tube, 5 µl distilled water was added. Both Eppendorf tubes were incubated overnight at 37 °C. Afterwards, the supernatant from both tubes was discarded, and replaced with 450 µL 1x PBS buffer (pH 7.4) and 50 µl β-galactosidase (Roche Diagnostics GmbH, Mannheim, Germany), and incubated overnight at 37 °C. Subsequently, the supernatant was collected from both tubes, and their galactose content was measured with High-Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection (HPAEC-PAD).

FT-IR analysis

The Fourier transform infra-red (FT-IR) spectrum of granular sludge was recorded on an FT-IR spectrometer (Perkin Elmer, Shelton, USA) at room temperature, with a wavenumber range from 750 cm^{-1} to 4000 cm^{-1}. The spectra of granules with and without neuraminidase treatment were normalized at 1630 cm^{-1}.

4.3. Results

Reactor operation and dominant microorganisms

Seawater-adapted aerobic granular sludge was taken from a labscale reactor, performing complete removal of COD (acetate) and phosphate. The reactor was inoculated with Nereda® sludge and it took 8 weeks to reach a stable state as observed from an identical cycle-to-cycle behavior in the online (pH and off-gas) measurements. The reactor was sampled for EPS analysis after operating in stable conditions for three SRTs. The performance of a typical reactor cycle is shown in Fig. 1. Acetate was completely consumed anaerobically within the first 60 min of the cycle, while phosphate was released up to 75 mg PO_4^{3-}-P/L (5.9 net P-mol release). This corresponds to 0.34 P-mol/C-mol of anaerobic phosphate release per carbon uptake, which is in range of values for freshwater labscale AGS and only slightly lower than reported for enriched cultures of *Ca.* Accumulibacter phosphatis [19, 34, 144].

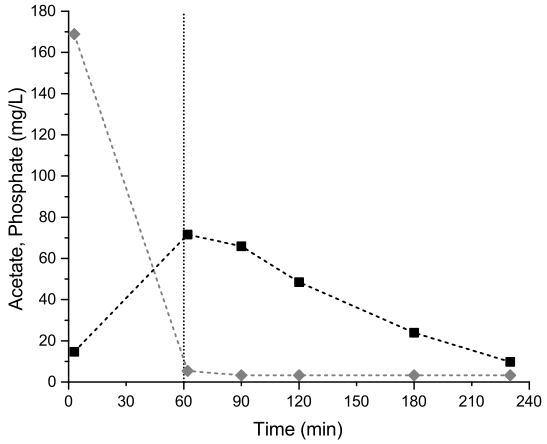


Figure 4.1: Concentrations of acetate (diamonds) and phosphate (squares) during a typical reactor cycle with 60 minutes of anaerobic feeding, followed by 170 minutes of aeration. Acetate and phosphate values at time interval zero indicate influent plus residual amounts left over from the previous cycle.

Fluorescence in situ hybridization (FISH) analysis was performed for analysing the relative amounts of polyphosphate accumulating organisms (PAO) and glycogen accumulating organisms (GAO). Large numbers of PAO were observed in the seawater-adapted aerobic granular sludge, while GAO-specific probes gave no fluorescent signal in the sludge (figure 4.2a). Distinct and uniform PAO cells were distinguished, and seen in clusters of cells (figure 4.2b). These results indicate dominance of PAO over GAO in the system.

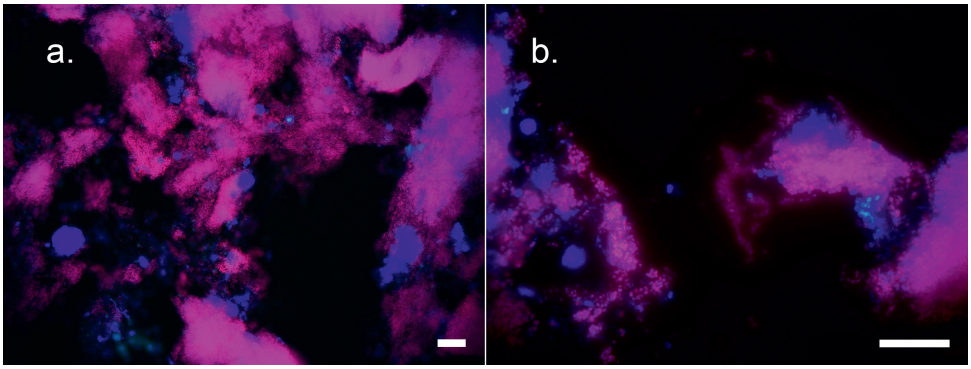


Figure 4.2: Fluorescence in-situ hybridization (FISH) images of seawater-adapted aerobic granular sludge, with PAOmix probes (red), GAOmix probes (green), and eubacteria probes (blue) (scale bars equal 10 μm). Magenta colour is an overlap between eubacteria (blue) and PAO bacteria (red).

Sialic acid visualization and quantification

Sialic acid lectin staining

In granular sludge, microorganisms are organized as microcolonies embedded in the EPS. EPS consists of proteins, polysaccharides, glycoproteins and other substances such as lipids and DNA [145]. Fluorescently labelled lectins have been widely used to stain extracellular glycoconjugates in various biofilm and sludge [135]. In the current research, to visualize the glycoconjugates in granular sludge 77 different lectins were used for screening [136]. From these lectins the LFA lectin gave the strongest signal. A layer of glycoconjugates containing sialic acids on the surface of granular sludge was visualized (figure 4.3). As LFA binds to sialic acids at the termini of the glycan chains of glycoproteins [139], this is an indication that sialic acids are present in the granular sludge associated to glycoproteins (sialoglycoproteins).

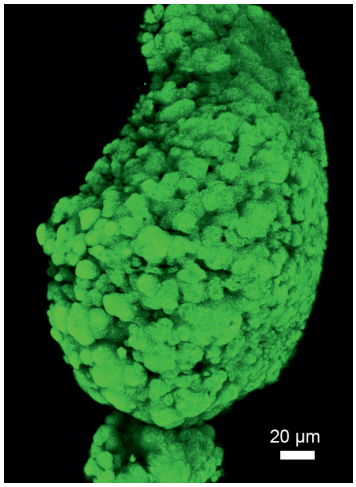


Figure 4.3: Lectin staining of sialoglycoconjugates by LFA lectin.

To study the distribution of sialic acids inside a granule and investigate if they are associated to glycoproteins, granules were sliced and double stained. The granules were first stained by a protein-specific fluorochrome and then with one of four different sialic acid specific binding lectins (figure 4.4). All four lectins (HMA, MAA, PSL and WGA) gave a strong signal, implying sialic acids are widely distributed in granular sludge. Sialic acid signals originate from both the bacterial cell surface and the extracellular matrix. Interestingly, the locations where lectins for sialic acid stained were overlapping with areas where proteins were visualized. This strongly suggests that the glycan chains containing sialic acids bond to proteins as glycoproteins. This is also in line with the specificity of the lectins MAA and PSL: sialic acids binding to those two lectins are positioned as the terminal sugar residue of glycan chains of glycoproteins. In addition, the fact that binding with both MAA and PSL lectins means the link between sialic acids and the penultimate sugar might be α(2→3) linkage and/or α(2→6) linkage [138, 140]. This information of the linkage is important to choose enzymes for sialic acids quantification.

Sialic acid quantification

The result of lectin staining showed that sialic acids are abundantly distributed in granular sludge, and the linkage between sialic acids and the penultimate sugar could be an α(2→3) and/or α(2→6) linkage. Thus, neuraminidase, which cleaves N-acetylneuraminic acid (Neu5Ac), the most widespread form of sialic acids, was applied for sialic acid quantification. This enzyme specifically cleaves Neu5Ac that is α(2→3,6,8,9) linked to the penultimate sugar in the glycan chain. Subsequent quantification yields an amount of 11.33 ± 3.80 mg N-acetylneuraminic acid (Neu5Ac) per gram of volatile solids (VS) (1.1%). Although the presence of sialic acids has been indicated by lectin staining in biofilms [135], information of their amount is hardly found. In comparison, the normal range for sialic acids in human serum is 0.9-1.4% (weight percentage) [146].

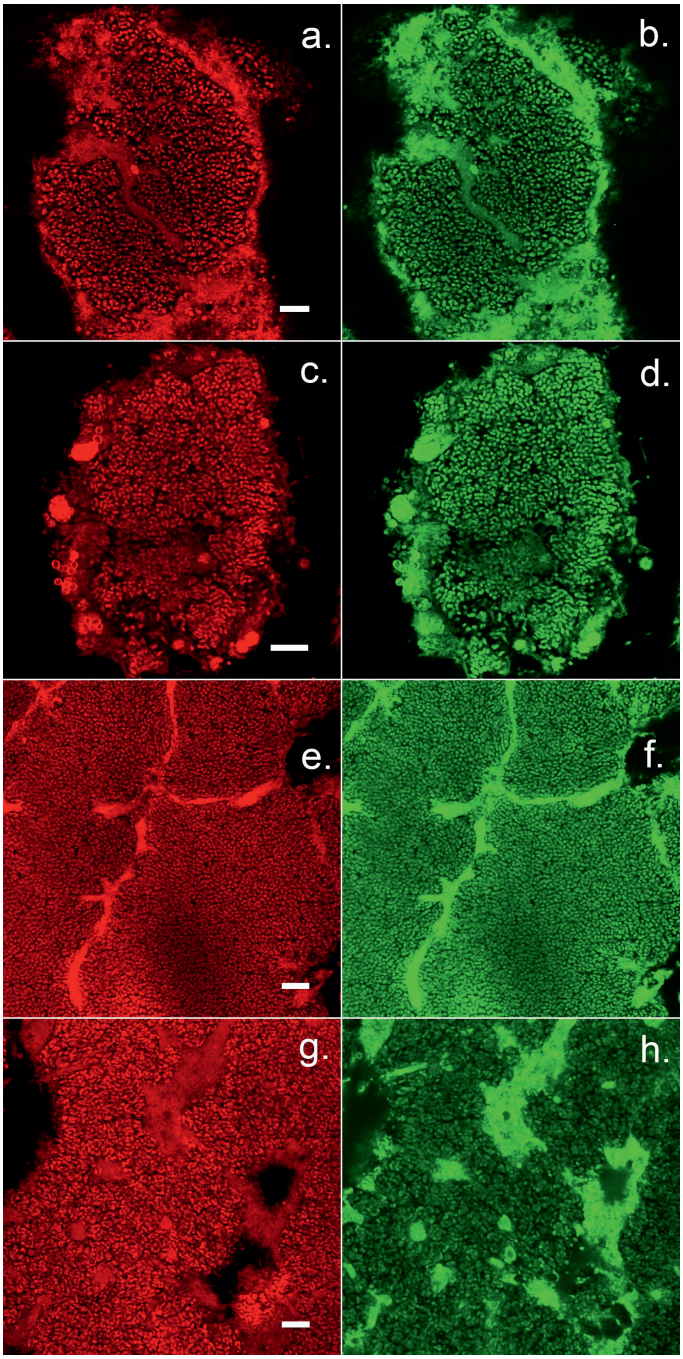


Figure 4.4: Lectin staining of granular sludge with SyproRed protein staining (in red: a., c., e., g.) and different sialic acid specific lectins (in green: b. HMA, d. MAA, f. PSL, h. WGA).

Genome analysis

The successful cleavage of Neu5Ac raises the question which microorganisms in granular sludge could produce sialic acids. From FISH analysis, PAO are the dominant microorganisms in the granular sludge (figure 4.2). Therefore, the potential of sialic acid (Neu5Ac) synthesis by PAO was assessed by means of *in silico* genome analysis.

As shown in figure 4.5, in the metabolic pathway of Neu5Ac, GlcNAc-6-phosphate is epimerized to ManNAc-6-phosphate by GlcNAc-6-P 2-epimerase. The phosphate groups gets removed to form ManNAc, and Neu5Ac is formed by using phosphoenolpyruvate (PEP), catalysed by Neu5Ac synthetase. The active form CMP-Neu5Ac is catalyzed by CMP-Neu5Ac synthetase. This form is finally added to acceptor substrates by sialyl transferase. These key enzymes for Neu5Ac production were analysed with BLASTp on metagenomes of *Ca.* Accumulibacter, which is the dominant PAO in the system. Since *Ca.* Accumulibacter has not yet been isolated in a pure culture, 48 available metagenomes were used in the analysis, which increases accuracy by covering a broad range of clades.

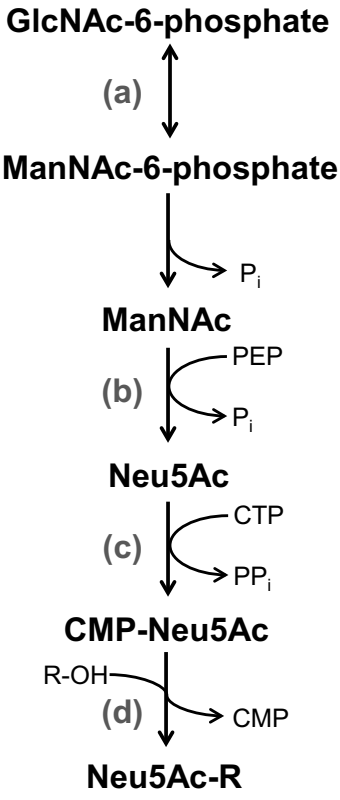


Figure 4.5: Pathway of bacterial Neu5Ac metabolism with its respective key enzymes: a. GlcNAc-6-phosphate 2-epimerase, b. Neu5Ac synthetase, c. CMP-Neu5Ac synthetase, d. Sialyl transferase. Adapted from Angata and Varki, 2002 [147]

Very low E-values (<1E-100) were found for GlcNAc-6-P 2-epimerase and Neu5Ac synthetase from enzymes from all tested reference species (table 4.2). Part of the analysed *Ca.* Accumulibacter metagenomes contained genes that were annotated for Neu5Ac synthetase from *C. alkaliphilus*, *D. melanogaster*, *S. ruber*. CMP Neu5Ac synthetase from *C. jejuni* and *H. cetorum* were also annotated in some *Ca.* Accumulibacter metagenomes.

CMP Neu5Ac synthetase from both *C. jejuni* and *H. cetorum* gave higher E-values than GlcNAc-6-P 2-epimerase and Neu5Ac synthetase, but were still in good range of 1E-40 1E-50. Sialyl transferase from *Halanaerobium praevalens* gave two very low E-values of <1E-100 and three E-values that are around 1E-20. The sialyl transferase from *P. damselae* gave E-values at around 1E-40 ~1E-50.

Overall, the enzymes from the Neu5Ac production pathway show low E-values (<1E-40) during *in silico* genome analysis on *Ca.* Accumulibacter. These results indicate that there is high probability that *Ca.* Accumulibacter possesses genes that can be transcribed into enzymes that are involved in the synthesis of Neu5Ac.

Table 4.2: Genome analysis of enzymes in the Neu5Ac synthesis pathway in multiple *Ca.* Accumulibacter metagenomes (5 highest hits per enzyme shown)

Enzyme *Accession number* *Reference species*	E-value	Metagenome name *
GlcNAc-6-P 2-epimerase	8.00E-125	a
ABW08136.1	2.00E-105	b
Neisseria meningitidis	5.00E-100	c
	6.00E-100	c
	1.00E-98	d
Neu5Ac synthetase	4.00E-118	a
ERP39285.1	5.00E-112	a
Chitinivibrio alkaliphilus	4.00E-112	e
	4.00E-112	f
	4.00E-112	g
Neu5Ac synthetase	4.00E-149	f
WP_011279946.1	1.00E-130	h
Psychrobacter arcticus	1.00E-130	i
	1.00E-130	j
	2.00E-130	k
Neu5Ac synthetase	9.00E-81	f
NP_650195.1	9.00E-81	g
Drosophila melanogaster	1.00E-80	l
	2.00E-79	a
	2.00E-71	m
Neu5Ac synthetase	2.00E-131	l
CBH23620.1	2.00E-82	a
Salinibacter ruber	2.00E-82	e
	2.00E-82	f
	2.00E-82	m
CMP Neu5Ac synthetase	6.00E-44	a
AOW97441.1	2.00E-41	n
Campylobacter jejuni	7.00E-42	n
	2.00E-41	n
	2.00E-42	o
CMP Neu5Ac synthetase	6.00E-50	p
AFI04478.1	1.00E-49	q
Helicobacter cetorum	6.00E-46	p
	1.00E-41	a
	1.00E-41	g
Sialyl transferase	1.00E-115	r
ADO76488.1	2.00E-104	a
Halanaerobium praevalens	6.00E-30	r
	3.00E-23	n
	3.00E-21	n
Sialyl transferase	6.00E-54	e
BAA25316.1	6.00E-54	f
Photobacterium damselae	7.00E-54	a
	6.00E-51	f
	3.00E-51	s

* Metagenome names:

(a) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor_6/25/2014_ DNA

(b) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_6/14/2005_ DNA

(c) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_1/10/2011_ DNA

(d) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_9/17/2007_ DNA

(e) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time F- 52min-Aerobic_ RNA (Metagenome Metatranscriptome)

(f) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time E -22min-Aerobic_ RNA (Metagenome Metatranscriptome)

(g) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time D- omin-Aerobic_ RNA (Metagenome Metatranscriptome)

(h) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI - F_92min_Anaerobic (Metagenome Metatranscriptome)

(i) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI - J_51min_Aerobic (Metagenome Metatranscriptome)

(j) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 2_5/28/2013_ DNA (Hybrid Assembly)

(k) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 2_5/28/2013_ DNA (Illumina Assembly)

(l) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time B -10min-Anaerobic_ RNA (Metagenome Metatranscriptome)

(m) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time C-32min-Anaerobic_ RNA (Metagenome Metatranscriptome)

(n) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI - Type I

(o) Unknown Accumulibacter genome from EPBR bioreactor metagenome

(p) Candidatus Accumulibacter sp. BA-91

(q) Candidatus Accumulibacter sp. SK-01

(r) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor_6/25/2014_ DNA (SPAdes)

(s) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time I- 292min-Aaerobic_ RNA (Metagenome Metatranscriptome)

Function of sialic acids in seawater-adapted aerobic granular sludge

Galactose removal tests

Sialic acids are commonly found in chains of sugars as the terminal residue, rather than directly bound to proteins [148]. In line with the specificity of MFA, LFA, and PSL lectins for linkage between sialic acid and galactose (figure 4.4), the shielding effect of sialic acids to underlying galactose residues was analysed.

Whole granules were treated first with neuraminidase and subsequently with β-galactosidase, to selectively cleave off sialic acids and galactose residues, respectively. After this procedure, a galactose peak was measured by HPAEC-PAD analysis, corresponding to a total of 0.37 mg/L galactose (figure 4.6). Granules that were treated with only β-galactosidase, and thereby still have sialic acids in the outer layer of their EPS, did not result in a galactose peak in the chromatogram. There was a slight shift in retention time between pure galactose reference samples (7.5 minutes) and the observed peaks in our samples (7.8 minutes), but enzyme-treated samples that were spiked with galactose showed an increased peak height at 7.8 minutes. Along with the specificity of used β-galactosidase, this indicates that the measured peaks indeed correspond to galactose. These results imply that sialic acids in the outer layer of granular sludge are bound to galactose, and can play a role in protecting galactose from enzymatic hydrolysis.

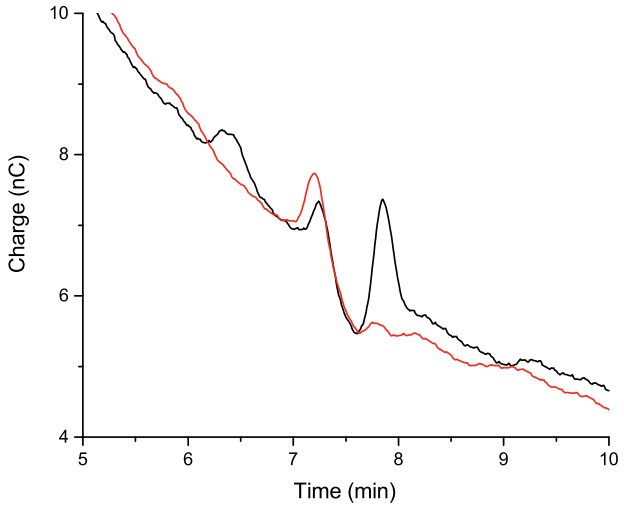


Figure 4.6: HPAEC-PAD spectrum of supernatant of aerobic granular sludge after β-galactosidase treatment, with prior neuraminidase treatment (black) and without prior neuraminidase treatment (red)

Cleavage of sialic acids from the granules was verified by FT-IR analysis (figure 4.7). After neuraminidase treatment, the sharp peak at 1730 cm^{-1}, which is assigned to –COOH groups changed to a shoulder peak. Also the intensity of the peak at 1000–1200 cm^{-1} (C-O-C bond in carbohydrates) was decreased. The change of the spectrum is due to the removal of sialic acids, which have –COOH groups and are linked with the penultimate sugar by C-O-C bond [49, 149, 150]. Due to the fact that neuraminidase only removed part of the sialic acids, the remaining part is still present as a shoulder peak at 1730 cm^{-1}. Moreover, the removal of sialic acids resulted in the extension of protein side chain (increase of the peak intensity at 1560 cm-1), and the exposure of the penultimate sugar (-CH_2 from C6 sugar with the peak intensity increase at 1450 cm^{-1} and 1370 cm^{-1}), which is in agreement with the masking function of sialic acids to the molecules and glycoproteins [151].

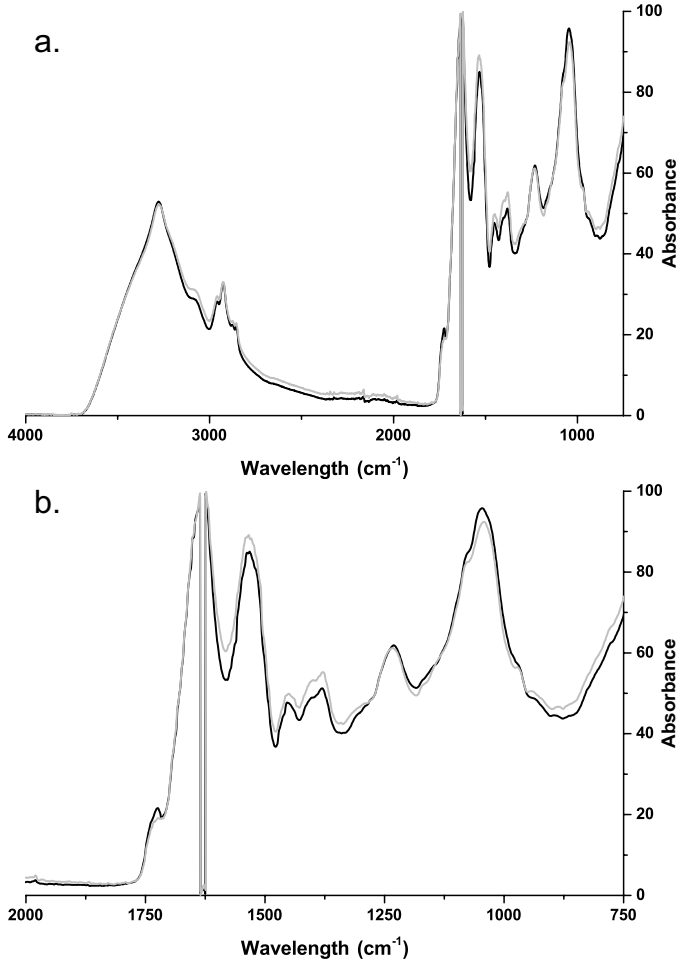


Figure 4.7: FT-IR spectrum of aerobic granular sludge after neuraminidase treatment (grey line) and without neuraminidase treatment (black line). a) Full spectrum from 4000 cm^{-1} to 750 cm^{-1}; b) Part of the same FT-IR spectrum as a., but zoomed in on the region between 2000 cm^{-1} and 750 cm^{-1}

4.4. Discussion

Sialoglycoproteins found in the extracellular polymeric substances in seawater-adapted aerobic granular sludge

Sialic acids are unique nine-carbon sugars. They play significant roles in many biological processes of animals and humans, both in the state of healthy cells and tissue (e.g. recognition, ion transport, differentiation) and in many important diseases (e.g. cancer, autoimmune diseases) [152]. The presence of sialic acids in biofilms has been reported; however, information concerning their function and binding site in biofilms is hardly found.

This chapter provides the first evidence for the presence of sialoglycoproteins in aerobic granular sludge. Lectin staining of sialic acids and protein staining have significant overlapping in the locations of their respective fluorescent signal (figure 4.2). Binding with specific lectins (MAA, HMA, PSL and WGA) provides indications that sialic acids locate at the termini of carbohydrate chains of glycoproteins. The penultimate sugar might be galactose or N-acetyl galactosamine.

The presence of glycoproteins in biofilms has occasionally been described, such as in marine biofilms [153] and full-scale anaerobic granular sludge [132]. Interestingly, presence of glycoconjugates has also been found in saline lab-scale anaerobic granular sludge [59]. The major sugar monomers reported were mannose and N-acetyl galactosamine (GalNAc), which is commonly found in conjunction with sialic acids in mammalian glycans and glycolipids [134, 154].

Most bacteria are not described to produce sialic acids [147]. When present, sialic acids are found mostly in capsular polysaccharides and lipopolysaccharides, instead of in glycoproteins. Unlike the situation in animal glycoconjugates, the sialic acids in these bacterial polysaccharides mostly exist as internal residues, rather than terminal residues [147]. However, in the current research, sialic acids are present as sialoglycoproteins, similar to animals. Therefore, the wide distribution of sialoglycoproteins over the granular structure implies not only the importance of glycoproteins, but also a specific function of sialic acids.

Function of sialic acids

Functionality of sialic acids has frequently been discussed in line of pathogenic bacteria [130]. Sialylation of the cell surface can modify the interaction with the host, and increase resistance against normal human serum [155, 156]. In the current research, sialic acids have now also been found in non-pathogenic bacteria, which would imply a wider range of functions.

Sialic acids show remarkable structural diversity, with the family currently comprising over 50 naturally occurring members. The diversity of sialic acids is reflected by its involvement in a variety of biological functions, many stemming from its unique physical and chemical properties, such as charge and size. The carboxylate group of sialic acid can give a net negative charge, allowing for binding of calcium cations, which is known to be contributing to the stability of aerobic granular sludge [124, 157]. In addition, sialic acids are nine-carbon sugars, they have a much bigger size than fine-carbon sugars (e.g. xylose) and six carbon sugars (e.g. glucose) [131]. Staying on the terminal position in carbohydrate chains, sialic acids can mask penultimate galactose residue and shield it from recognition by β-galactosidases [152]. It has been found that after the loss of sialic

acids, galactose molecules and cells can be bound, or can even be taken up and degraded [131]. This phenomenon has been most extensively studied with serum glycoproteins and blood cells [158].

In the current research, it is observed that removal of sialic acids from the outer layer of granules results to the hydrolysis of galactose by β-galactosidase, while galactose was not cleaved off without removing sialic acids This not only shows that sialic acids are linked to galactose, but most importantly indicates that sialic acids play an important role in maintaining the stability of the sugar chain in the EPS of granulesby masking penultimate galactose residues. Once sialic acids are removed, the sugar chain is prone to degradation, resulting to the instability of the sialoglycoproteins and the EPS matrix in the end. In comparison, in mammalian tissue, sialic acids involve in the stability, turnover und function of glycoproteins [159].Therefore, the function of sialic acids in granules might resemble that in mammalian tissue.

Evolutionary importance of sialic acids in granular sludge

Resemblance between bacterial and mammalian sialic acid structures and function can also give great insight into the evolutionary benefits. Presence of sialic acids is usually found in pathogenic bacteria, where it is proposed to make the cell walls of the pathogens resemble more the glycocalyx of its host. This would make these cells less detectable for the immune system of the host [130, 157, 160]. Granular sludge generally does not contain a significant fraction of pathogens [161]. An important similarity between these types of bacterial communities is the necessity of biofilm formation for their survival. Granular sludge is cultivated in an environment that simulates aggregation, while pathogenic bacteria depend on adhesion to their host environment for successful infection. The presence of sialic acids could therefore be a result of requirement for stable adhesion and recognition, not only for pathogens, but also for bacterial aggregates in general.

Production pathways of sialic acids in bacteria have been frequently studied [147, 162]. A link with the granular sludge microbiome has never been made in literature. The genome analysis that was performed in this study gives a positive indication for a putative production of sialic acids by *Ca.* Accumulibacter in aerobic granular sludge. A general evolutionary benefit for sialic acid production for adhering bacteria is proposed. Similarly to both *Ca.* Accumulibacter in aerobic granular sludge and pathogenic bacteria, the presence of sialic acids would be expected for other types of adhering bacteria as well. Sialic acids could be more widely present than was previously described in literature.

In the current research, the presence of sialic acids is only studied in saline aerobic granular sludge. It is interesting to examine a broad range of biofilm sample under different conditions (e.g. flocculent sludge, granular sludge, biofilm on carriers under saline and non-saline conditions) to explore if sialic acids present in different environmental samples and what function do they involve in. To this end, it is possible to understand comprehensively the biological roles of sialic acids in prokaryotes.

Challenge of sialic acids identification and quantification in environmental samples

The sialic acid family comprises more than 50 natural derivatives of neuraminic acid. Unsubstituted neuraminic acid does not occur in nature. The amino group of neuraminic acid is substituted either by an acetyl or glycolyl residue, and the hydroxyl groups may be methylated or esterfied with sulfate, phosphate, acetyl or lactyl groups. Sometimes several of these substituents are present in one sialic acid molecule. Sialic acids are the only natural sugars to show this great variety [148].

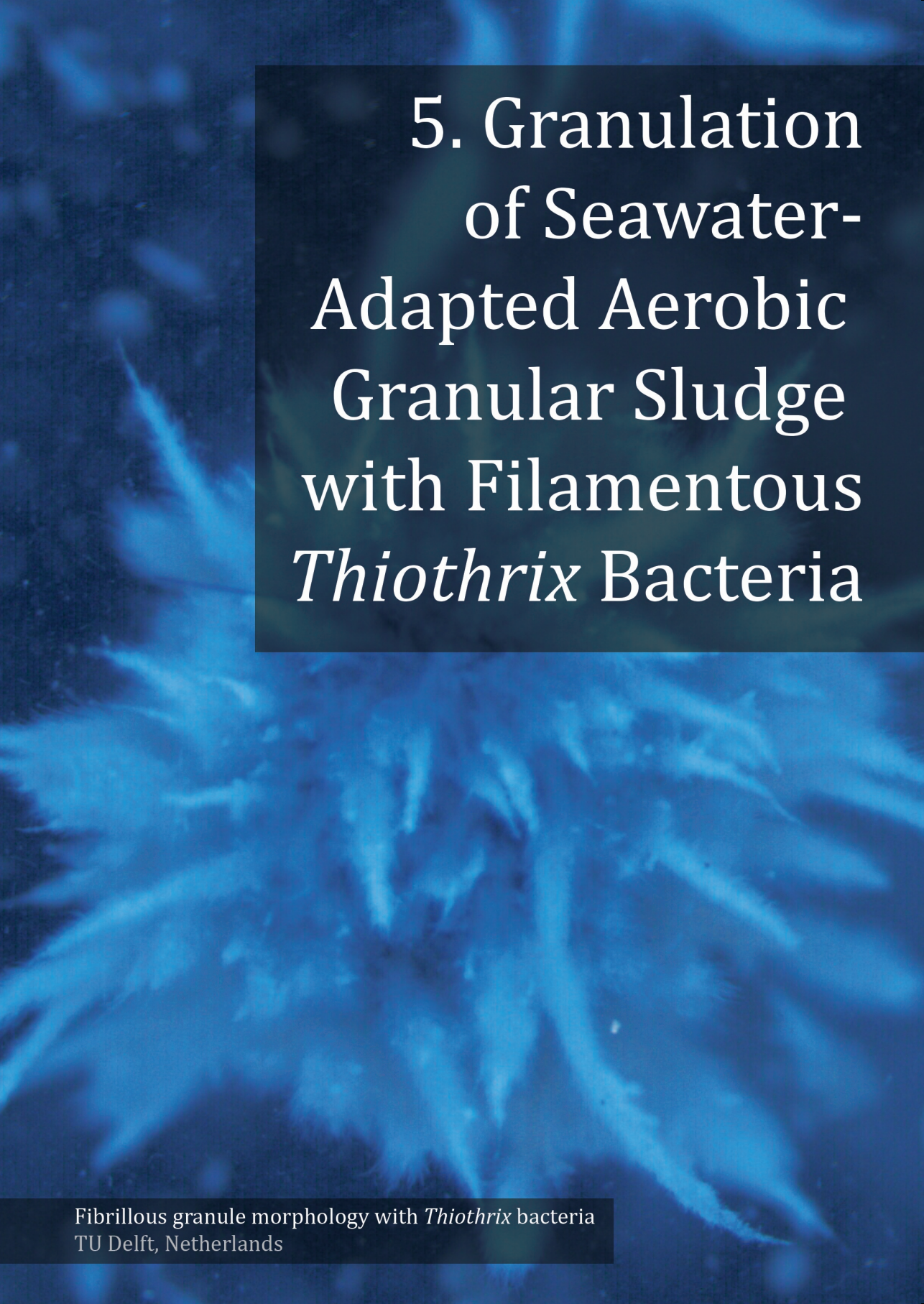
In addition, a number of other nonulosonic acids are described in proteobacteria among which the 5,7-diamino-3,5,7,9-tetradeoxy-D-glycero-D-galacto-nonulosonic acid (legionaminic acid (Leg)) and the 5,7-diamino-3,5,7,9-tetradeoxy-L-glycero-L-manno-nonulosonic acid (pseudaminic acid (Pse)), which show structural, biosynthetic and functional similarities to sialic acids [163, 164]. Therefore, besides natural derivatives of sialic acids, the existence of bacterial sialic acids (e.g. Leg and Pse) contributes to the complexity of sialic acid family identification in environmental samples.

Enzymatic sialic acid quantification method is limited by the recognition of sialidase. If due to the existence of substituents in bacterial sialic acids, the sialidase could not recognize the structure, the result of enzymatic quantification will underestimate the amount of sialic acid in granular sludge or other biofilm systems. Therefore, more specific analytical methods (e.g. mass spectroscopy) which could identify different sialic acid species need to be adjusted towards bacterial sialic acids [165].

4.5. Conclusion

- Sialic acids are discovered and widely distributed in seawater-adapted aerobic granular sludge, both on the bacterial cell surface and the extracellular matrix
- Sialoglycoproteins, with sialic acids as the terminal sugar residue of glycoproteins, are components of the extracellular matrix
- *Ca.* Accumulibacter in seawater-adapted aerobic granular sludge likely contains genes that encode for enzymes that are responsible for sialic acid metabolism
- Sialic acids that are located in the outer layer of the extracellular matrix function as a shield protecting the underlying sugar chain from degradation

5. Granulation of Seawater-Adapted Aerobic Granular Sludge with Filamentous *Thiothrix* Bacteria



Fibrillous granule morphology with *Thiothrix* bacteria
TU Delft, Netherlands

Granulation of Seawater-Adapted Aerobic Granular Sludge with Filamentous *Thiothrix* Bacteria

Abstract

Many sources of wastewater contain sulfides, which can cause filamentous bulking sludge, with *Thiothrix* as one of the major organisms involved in bulking sludge formation. Granular sludge formation could potentially suffer from the growth of filamentous bacteria. Uptake of easily degradable COD by the relative slow growing *Ca.* Accumulibacter bacteria and the absence of strong diffusion gradients due to plug flow feeding through the settled granular sludge bed are assumed to be the dominant factors for successful granulation. Recently it was shown that the filamentous bacterium *Thiothrix caldifontis* is capable of a similar metabolism as *Ca.* Accumulibacter when there is reduced sulfur present in the feed to the reactor. Since this is the case for many wastewater treatment plants connected to anaerobic sewers we evaluated the occurrence of *Thiothrix* and the sludge morphology when in lab-scale aerobic granular sludge reactors a fraction of the incoming acetate COD is replaced by reduced sulfur with 18% of COD originating from thiosulfate *Thiothrix* proliferated forming 51.4 ± 8.3% of the microbial biomass as established by FISH analysis. Despite the strong presence of these filamentous bacteria a well settling sludge was maintained (SVI_{10} equal to 13.3 mL/g). These results confirm that sludge morphology is not necessarily a reflection of the cell morphology of the bacteria.

Highlights

- Presence of *Thiothrix* bacteria leads to stable aerobic granular sludge, despite their filamentous growth
- Granule morphology is determined by reactor operation, rather than by cell morphology
- The absence of strong substrate diffusion gradients during feeding coupled with the relatively low growth rate of *Thiothrix* contributed to the compact biofilm formation
- 18% COD in the form of reduced sulfur results in 51.4 ± 8.3% *Thiothrix* in the microbial community, stable granulation, and complete removal of acetate and phosphate

5.1. Introduction

Aerobic granular sludge (AGS) is an upcoming technology for simultaneous removal of organic carbon (COD), nitrogen, and phosphorus in a single process step [19, 22]. Stability of the process depends on anaerobic uptake and intracellular storage of easily biodegradable COD. The stored substrate is used for energy generation and growth during the aeration phase [18, 166, 167]. This results in a low growth rate, which is favorable for obtaining a smooth biofilm [101, 103]. In general polyphosphate accumulating organisms (PAO) thrive under the applied reactor conditions [32, 161].

Many sources of wastewater contain sulfides. This can originate from iron sulfate usage in drinking water production, from industrial discharges, or after seawater intrusion into sewer systems [24, 168–170]. Commonly sulfide presence is associated in wastewater treatment with proliferation of filamentous organisms, especially *Thiothrix* species, leading to bulking sludge [171]. Many studies report on prevention and handling of filamentous bulking [172–174]. Multiple engineering solutions have been developed for removing filamentous microorganisms from activated sludge systems, such as addition of a selector or intermittent feeding [175–177]. There is limited information on the impact of proliferation of filamentous bacteria on granule morphology in an aerobic granular sludge system.

One particularly interesting group of filamentous microorganisms in regard to its elemental cycling and sulfide oxidation is *Thiothrix spp.* [40, 178]. These bacteria naturally occur in sulfide-containing waters, in both freshwater and saltwater environments [178–183]. *Thiothrix* are capable of forming intracellular sulfur globules, which they can couple to sulfur respiration [184]. Interestingly, *Thiothrix* also have the capacity to store both polyphosphate and poly-β-hydroxybutyrate (PHB) [184–186]. There are multiple species of *Thiothrix* that are capable of a variety of metabolisms, ranging from heterotrophic growth of *T. eikelboomii* [187, 188] to chemolithotrophic growth of *T. ramosa* using energy from thiosulfate oxidation [189–191].

The most fascinating strain is *T. caldifontis*, because of its unique properties with similarity to PAO species [41, 192]. It is a typical filamentous organism with a cell length up to 6.5 μm, growing in multicellular filaments that are protected by polysaccharide sheaths. This strain can anaerobically consume acetate as a carbon source for chemoorganoheterotrophic growth, and store it as PHA [41]. *T. caldifontis* also encodes the *ppk2* gene, which is a widely conserved gene that is responsible for the synthesis of poly-P from GTP or ATP [193]. Simultaneously, this strain can oxidize both sulfide and thiosulfate for the formation of its intracellular pools of elemental sulfur (poly-S). This process occurs rapidly under aerobic conditions, and afterwards these poly-S pools are further oxidized and excreted as sulfate. This oxidation of poly-S serves as another energy source next to PHA oxidation, which leads to an increased growth yield. These properties of *T. caldifontis* have led to its co-occurrence with other PAO species in EBPR systems with sulfide in the influent [41, 184, 194].

The metabolic similarity between *T. caldifontis* and *Ca.* Accumulibacter led to the question whether stable granulation can be achieved when this filamentous microorganism proliferates in sulfide containing wastewater. Formation of compact and non-filamentous granules is based on diffusion-based selection, which prescribes that compact biofilms are formed when the substrate consumption rate is lower than the substrate transport rate [101, 195]. Based on this theory, our hypothesis is that granule morphology is determined by reactor operation, rather than by bacterial morphology [42, 101, 196].

In order to verify this hypothesis, a lab-scale reactor has been operated with up to 25% COD in the form of thiosulfate, and the remaining COD from acetate. The choice for thiosulfate over sulfide has been made to purely study the increase in filamentous *Thiothrix*, and to prevent a potential toxicity of sulfide to the microbial community. Granule morphology, changes in microbial community, and phosphate removal rates have been monitored over time to identify the presence of filamentous bacteria and successful stable granulation.

5.2. Materials & Methods

Reactor operation

Aerobic granular sludge was cultivated in a 3.0 L bubble column (5.6 cm diameter), operated as a sequencing batch reactor (SBR). The reactor was seeded with biomass from a lab-scale seawater-adapted AGS reactor. The temperature was controlled at 20 °C. The pH was controlled at 7.0 ± 0.1 by dosing either 1M NaOH or 1M HCl. The dissolved oxygen (DO) concentration was controlled at 3.7 mg/L O_2 (50% saturation). The average sludge retention time (SRT) was 20 days.

The influent was 1.5 L per cycle, consisting of 1200 mL artificial seawater (Instant Ocean®, final concentration 35 g/L), 150 mL of medium A, and 150 mL of medium B. Medium A contained 57.2 mM sodium acetate trihydrate (3.66 g/L COD). Medium B contained 42.8 mM NH_4Cl (600 mg/L $NH4^+$-N), 4.2 mM K_2HPO_4, 2.1 mM KH_2PO_4, and 10 mL/L trace element solution similar to Vishniac and Santer (1957) [68], but using 2.2 mg/L $ZnSO_4.7H_2O$ instead of 22 mg/L. The combination of these feed streams led to influent concentrations of 366 mg/L COD, 60 mg/L NH_4^+-N and 9.3 mg/L PO_4^{3-}-P.

Thiosulfate was added to the influent by addition to the artificial seawater vessel. Its concentration was gradually increased up to 80 mg S-COD/L influent concentration (equal to 18% COD from thiosulfate) over a period of 58 days. Afterwards the thiosulfate concentration was increased to 120 mg S-COD/L (equal to 25% COD from thiosulfate) in a single step, and operated for a period of 53 days. Reactor cycles with 80 mg S-COD/L (18% S-COD) consisted of 60 minutes of anaerobic feeding, 195 minutes aeration, 10 minutes settling and 5 minutes effluent withdrawal. Reactor cycles under 120 mg S-COD/L (25% S-COD) consisted of 90 minutes of anaerobic feeding, 200 minutes aeration, 5 minutes settling and 5 minutes effluent withdrawal.

Analytical methods

Concentrations of phosphate were measured on a Thermo Fisher Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, USA). Acetate was measured by HPLC with an Aminex HPX-87H column from Biorad, coupled to an RI and UV detector, using 0.01M phosphoric acid as eluent.

Granule morphology

Pictures of whole granules were taken with a stereo zoom microscope (Leica Microsystems Ltd, M205 FA, Germany), and processed with Leica Microsystems Qwin (V3.5.1) image analysis software.

Fluorescence in situ hybridization (FISH)

The handling, fixation and staining of FISH samples was performed as described in Bassin et al. (2011) [32]. A mixture of PAO462, PAO651, and PAO846 probes (PAOmix) were used for visualizing polyphosphate accumulating organisms (PAO) [69]. A mixture of GAOQ431 and GAOQ989 probes (GAOmix) were used for visualizing glycogen accumulating organisms (GAO) [70]. G123T probes were used for staining *Thiothrix* bacteria [197]. A mixture of EUB338, EUB338-II and EUB338-III probes were used for staining all bacteria [72, 73]. Images were taken with a Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 26 (bp 575e625/FT645/bp 660e710), 20 (bp 546/12/ FT560/bp 575e640), 17 (bp 485/20/FT 510/bp 5515e565) for Cy5, Cy3 and fluos respectively. Quantification of FISH images was done by counting the relative amounts of pixels per fluorescence signal of cy3, fluos, and cy5 signals, using Leica Microsystems Qwin (V3.5.1) image analysis software.

5.3. Results

Reactor operation

An aerobic granular sludge sequencing batch reactor was operated with addition of thiosulfate in the influent up to 80 mg S-COD/L in combination with addition of 366 mg HAc-COD/L, equaling 18% of COD derived from thiosulfate. A representative cycle measurement for operation is shown in figure 5.1. Acetate was completely removed by the biomass during the 60 minutes anaerobic feeding phase, resulting in a net release of phosphate up to 65.4 mg/L. Aerobic uptake of phosphate was linear over time, with a rate of 3.2 mgP/gVSS/h. Biomass concentration was stable with a concentration of 7.9 ± 0.7 g VSS/L and a sludge bed density of 77.0 ± 3.2 g TSS/L sludge bed. After increasing the influent concentration to 120 mg S-COD/L (25% COD from thiosulfate, from day 58 onwards), anaerobic acetate uptake and anaerobic phosphate release began to decrease over a period of 36 days. Due to an accidental washout of around 50% of biomass from the reactor at day 104, acetate started leaking into the aeration phase and phosphate removal collapsed. The sludge bed density decreased to 13.4 g TSS/L, and the effluent started containing filaments with a solids concentration of 0.11 g/L (which was near zero before); at this stage reactor operation was stopped.

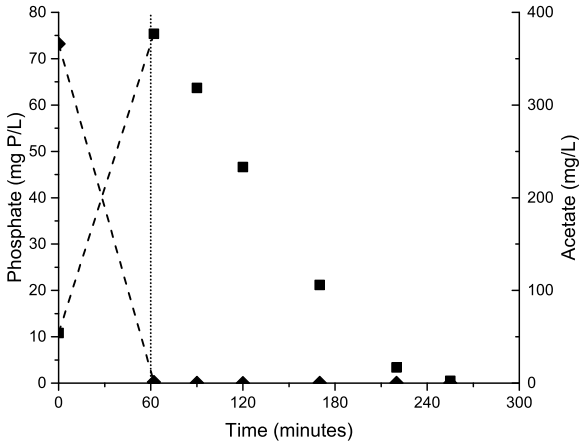


Figure 5.1: Concentrations of phosphate (squares) and acetate (diamonds), during a reactor cycle with the addition of 80 mg S-COD/L in the influent (18% of total COD). The dotted vertical line indicates the switch between anaerobic feeding and aerobic mixing.

Granule morphology

Stable granules were obtained when the reactor was fed with 18% COD from thiosulfate. These thiosulfate-based granules had a similarly dense structure as the thiosulfate-free grown granules used as inoculation source (figures 5.2a, 5.2b). Minor amounts of filamentous outgrowth from the granule core are visible, still resulting in proper settling behavior with a sludge volume index after 10 minutes (SVI_{10}) of 13.3 mL/g. After a sudden drop in SRT after 104 days of operation, the granule morphology changed markedly into a more fibrillous structure (figures 5.2c, 5.2d).

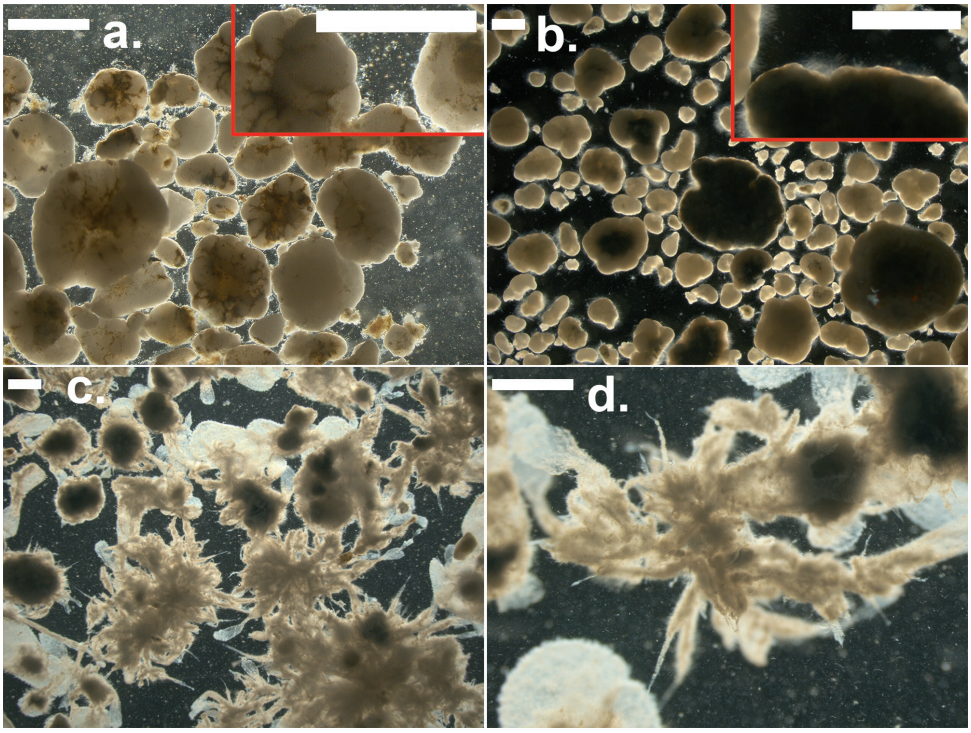


Figure 5.2: Morphology of aerobic granular sludge: (a) Granular biomass prior to crushing them for inoculating the thiosulfate-added reactor; (b) Granules grown with 18% S-COD in seawater conditions after 58 days of operation; (c, d) Granules after a sudden drop in SRT after 104 days of operation, with 25% S-COD in the influent. Scale bar indicates 1 mm.

Microbial community

Robust granules were formed with thiosulfate in the feed, despite the presence of filamentous *Thiothrix* bacteria. Both the inoculum and the granules that were grown with 18% S-COD were analyzed using fluorescence in situ hybridization (FISH), using probes that were specific for PAO, GAO, *Thiothrix sp.*, and eubacteria.

The inoculum contained a large fraction of PAO, and there where no GAO detected (figure 5.4a). No filamentous organisms were detected (figure 5.4b). Granules from the thiosulfate-added reactor showed a high abundance of *Thiothrix* bacteria (figure 5.4c). Based on quantification of FISH images, the fraction of *Thiothrix* was estimated as 51.4 ± 8.3% of the total amount of biomass. The filamentous morphology of *Thiothrix* was clearly visible and these bacteria coexisted with PAO in the granule microbial community (figure 5.4d, 5.4e). Besides these two groups also a smaller amount of GAO was present (figure 5.4f).

Inspection of the filamentous bacteria with bright field microscopy showed the presence of a large number of intracellular globules. These were morphologically similar to storage polymers which are commonly found in *Thiothrix* bacteria (figure 5.3) [41, 198].

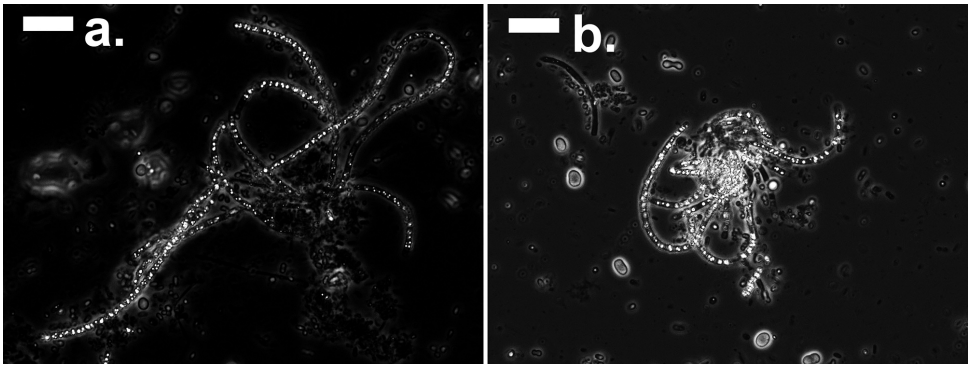


Figure 5.3: Brightfield microscopy images of crushed 120 mg S/L thiosulfate-adapted granular sludge after 104 days of operation (corresponding to figures 5.2c, 5.2d). Bright storage polymers are visible inside the bacteria. Scale bar equals 10 μm.

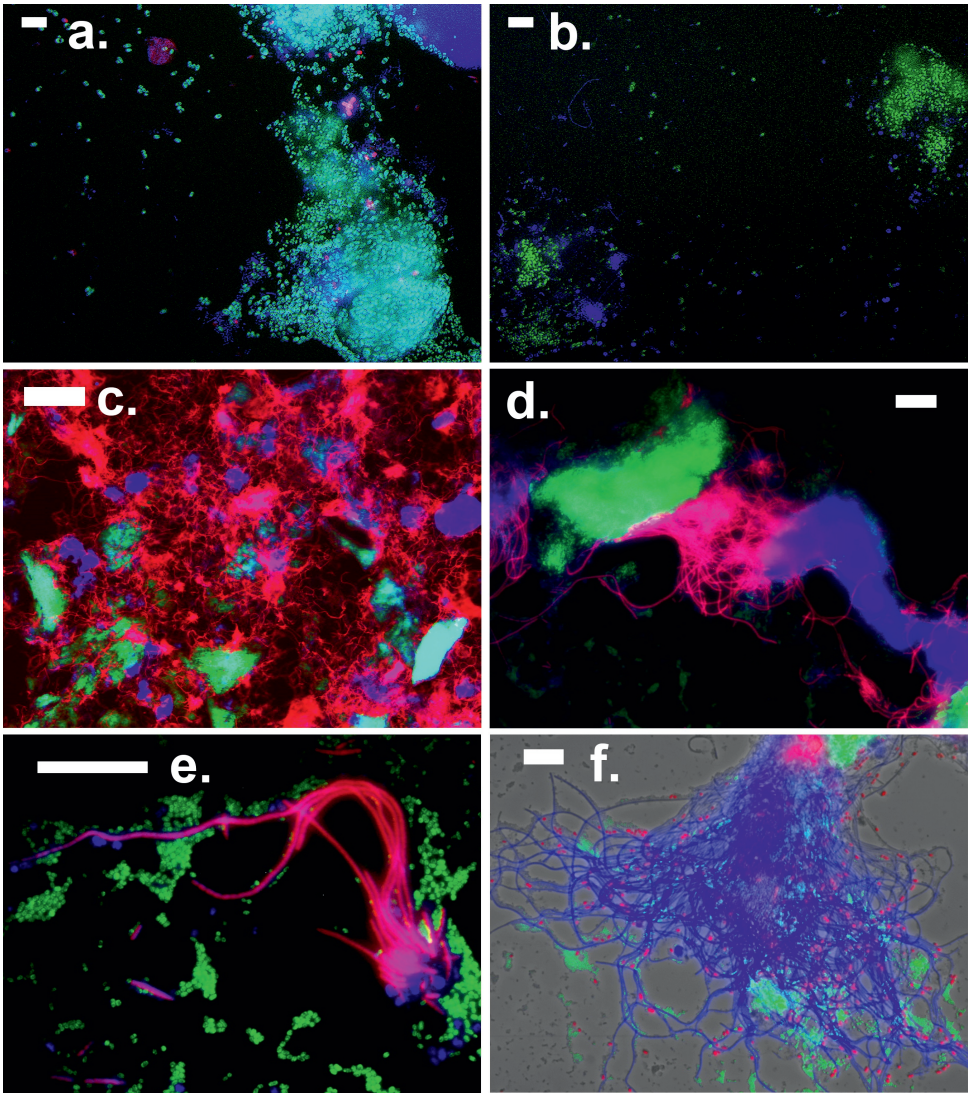


Figure 5.4: Fluorescence in-situ hybridization (FISH) analysis (a, b) crushed granules that were used for inoculation of the thiosulfate-based reactor (corresponding to figure 5.2a) with (a) GAOmix (cy3, red), PAOmix (fluos, green), EUB338 (cy5, blue), and (b) gamma proteobacteria (cy3, red), PAOmix (fluos, green), EUB338 (cy5, blue); (c, d, e, f) 18% S-COD seawater-adapted aerobic granular sludge after 58 days of operation (corresponding to figure 5.2b). Probes that were used are (c, d, e) *Thiothrix*-specific G123T (cy3, red), PAOmix (fluos, green), EUB338 (cy5, blue); (f) GAOmix (cy3, red), PAOmix (fluos, green), EUB338 (cy5, blue). Magenta color indicates overlap between blue cy5 and red cy3 signals. Scale bar (a, b, d, e, f) 20 µm, and (c) equals 100 µm.

5.4. Discussion

Successful granulation with the presence of filamentous bacteria

Stable aerobic granules were obtained despite the dominant presence of filamentous *Thiothrix* bacteria. Bulking sludge with filamentous organisms is a common problem in biological wastewater treatment processes [173, 174], but the results in this study show that these filamentous bacteria can actually be contained in a rather compact granular morphology. These results increase our understanding about which mechanisms lead to granule formation, even with filamentous organisms.

The results from this study show that aerobic granular sludge is not necessarily sensitive for the growth of filamentous bacteria. The standard theory states that bulking sludge occurs when there is a low substrate concentration, due to the low affinity constant (K_S) of filamentous organisms, combined with a maximal growth rate (μ^{max}) [175]. The generally higher μ^{max} of floc-forming bacteria than filamentous bacteria is an important factor in their relative proliferation when substrate is present in higher amounts [76, 172, 199]. This principle has led to the development of aerobic selectors with high spatial substrate gradients [172, 176], and explains why SBR systems suffer less from filamentous outgrowth than CSTR systems [196].

A key factor for stable granulation in our system is the temporal separation of diffusion and consumption of COD and growth. Compact granules are obtained when the biofilm has a higher substrate diffusive transport than substrate consumption rate [195]. The SBR operation in our study consists of anaerobic plug-flow feeding, during which the high substrate concentration allows for complete diffusion of acetate into the granules. This acetate is stored as PHA inside the granule, which get consumed for growth only during the subsequent aeration. If the acetate was supplied aerobically the diffusive flux of oxygen or acetate would be rate limiting compared to the metabolic capacity.

Filamentous outgrowth does occur in SBR granular sludge systems, but commonly when easily biodegradable COD is incompletely consumed during anaerobic feeding. This leads to leakage of this readily available COD into the aeration phase, where it decreases in concentration due to mixed aeration. This is combined with a diffusion limitation of oxygen into granules compared to the substrate consumption rate, which leads to filamentous outgrowth and deterioration of settling properties [200]. On the other hand, stable granules were also obtained when COD sources such as methanol are aerobically oxidized. Substrates like methanol allow only for a low growth and substrate uptake rate and thereby limit the negative impact of diffusion limitations [201]. Also, in an earlier experiment by Martins et al. (2011) it was shown that proliferation of filamentous *Nostocoida* bacteria can lead to compact flocs or bulking sludge depending on the presence of substrate gradients [202].

Proliferation of *Thiothrix* in an EBPR system

The maximal growth rate (μ^{max}) is, in the absence of substrate storage, directly proportional to the maximal substrate uptake rate ($q_S{}^{max}$) times the yield of biomass on substrate ($Y_{SX}{}^{max}$). Rubio-Rincón et al. (2017) observed an increase in $Y_{SX}{}^{max}$ with a higher fraction of *Thiothrix* over *Ca.* Accumulibacter in a suspended EBPR system [41]. This was linked to the extra energy that becomes available by oxidation of poly-S pools, allowing them to achieve mixotrophic metabolism [192].

The choice of thiosulfate instead of sulfide in our study led to a difference in terms of toxicity to *Ca.* Accumulibacter. Sulfide affects its anaerobic metabolism more severely than its aerobic metabolism [203]. Complete anaerobic uptake of acetate is essential for stable granulation, so issues with filamentous outgrowth due to acetate overshoot into aeration were prevented by taking this strategy. Thiosulfate can be disproportionated to sulfide and sulfite or sulfate prior to being taken up by the cells [204]. This sulfide gets taken up rapidly by *Thiothrix* under aerobic conditions, in order to replenish their poly-S pools [41]. The potentially toxic effect of sulfide production from thiosulfate is therefore minor with this small exposure time.

Biological phosphate removal with *Thiothrix* bacteria

EBPR activity was high at a remarkably high sulfur content of 18% COD next to acetate. This amount of sulfur was higher than usual in domestic wastewaters, which is usually up to 10 mg S/L in gravity-driven sewer systems [205]. These results therefore imply that presence of sulfides in wastewater with subsequent growth of filamentous *Thiothrix* bacteria, is not a problem for development of good settling aerobic granular sludge.

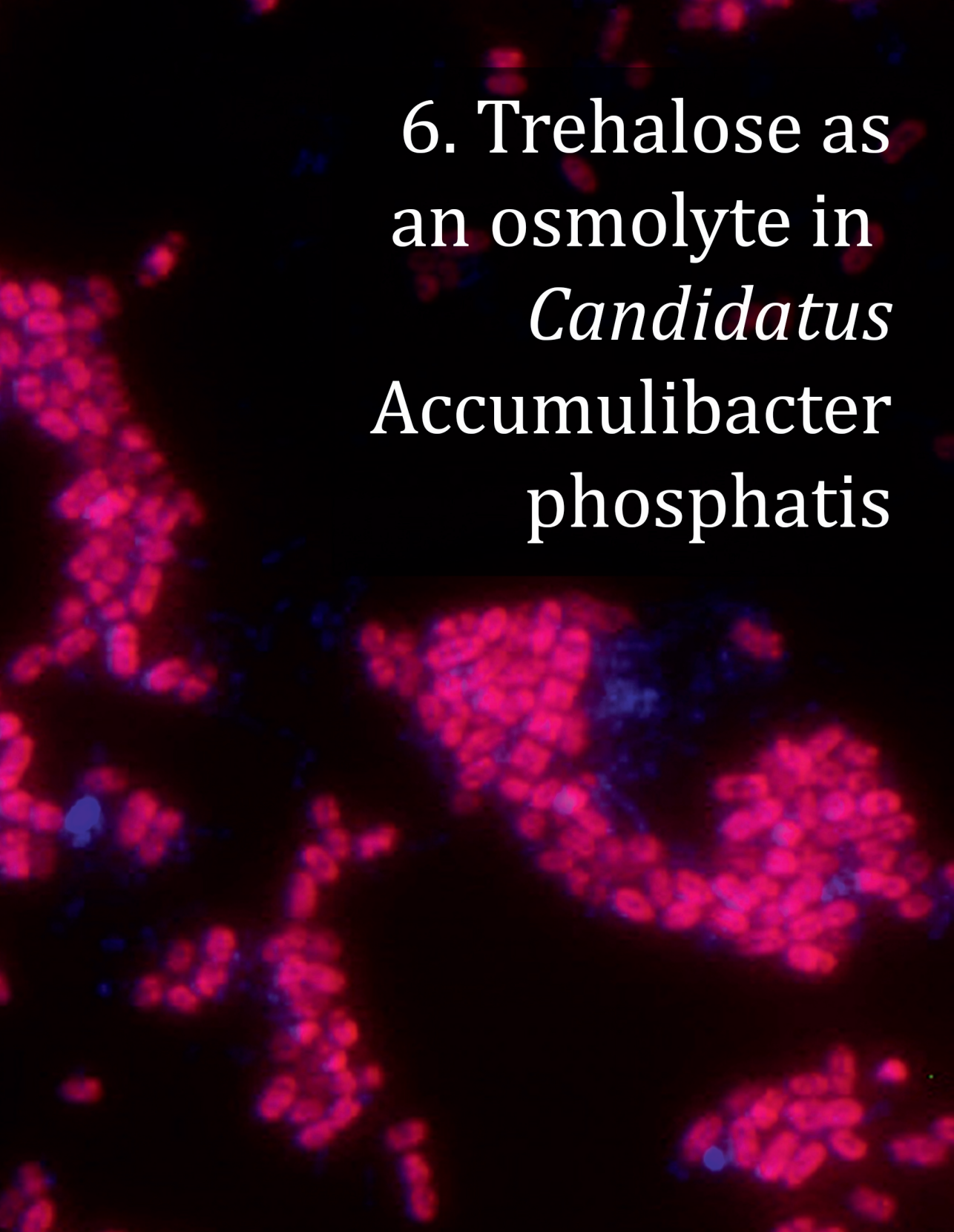
Removal of phosphate and anaerobic acetate uptake started decreasing when the reactor was fed with 120 mg S-COD/L and 366 mg HAc-COD/L (25% COD from thiosulfate). Rubio-Rincón et al. (2017) observed steady phosphate removal with the presence of 65 ± 3% filamentous *T. caldifontis* bacteria after feeding with an influent concentration of 100 mg S-COD/L, 295 mg HAc-COD/L, and 100 mg HPr-COD/L (20% COD from sulfide) [41]. The VFA uptake rate decreased by 65% compared to sulfide-free influent, but their anaerobic time was long enough for complete consumption.

The remarkable change in granule morphology after an SRT drop at 25% S-COD (figures 5.2c, 5.2d) is likely due to the overshoot of acetate into the aeration phase [200]. The granules from our study showed a more fibrous structure. The lower uptake rate of VFA by the *Thiothrix* community, combined with a system more prone to preferential flow in the granular sludge bed with fibrous outgrowth, resulting in acetate presence in the aerated phase. Where limited sulfide in the influent (as in municipal wastewater) seems not to give problems, higher sulfide loadings might need extra attention for the influent distribution and flow rate through the granular sludge bed.

5.5. Conclusion

- Reduced sulfur compounds select for *Thiothrix* type of phosphate accumulating bacteria
- Presence of *Thiothrix* bacteria leads to stable aerobic granular sludge, despite their filamentous cell morphology
- Granule morphology is determined by reactor operation, and in a much lower extent by morphology of the individual cells
- When easy degradable COD is still present when aeration starts in an aerobic granular sludge system the sludge morphology and P-removal efficiency can rapidly deteriorate

6. Trehalose as an osmolyte in *Candidatus* Accumulibacter phosphatis



FISH-stained *Ca.* Accumulibacter phosphatis clade I
TU Delft, Netherlands

Trehalose as an osmolyte in *Candidatus* Accumulibacter phosphatis

Abstract

Candidatus Accumulibacter phosphatis is an important microorganism for enhanced biological phosphorus removal (EBPR). In chapter 2, we found a remarkable flexibility regarding salinity, but the mechanism for its tolerance remained unknown. In this chapter, we identify and describe the role of trehalose as an osmolyte in *Ca.* Accumulibacter. A freshwater-adapted culture was exposed to a cycle of hyperosmotic incubation and hypo-osmotic shock, which led to the release of trehalose up to 5.34 mg trehalose/g VSS. Long-term adaptation to 30% seawater-based medium gave a stable operation with complete anaerobic uptake of acetate and propionate along with phosphate release of 0.73 Pmol/Cmol, and complete aerobic uptake of phosphate. Microbial analysis showed *Ca.* Accumulibacter phosphatis clade I as the dominant organism in both the freshwater- and seawater-adapted cultures (>90% presence). Exposure of the seawater-adapted culture to a cycle of hyperosmotic incubation and hypo-osmotic shock led to an increase in trehalose release upon hypo-osmotic shock when higher salinity is used for the hyperosmotic incubation. Maximum trehalose release upon hypo-osmotic shock was achieved after hyperosmotic incubation at 3x salinity increase, resulting in the release of 11.9 mg trehalose/g VSS. Genome analysis shows the possibility of *Ca.* Accumulibacter to convert glycogen into trehalose by the presence of *treX*, *treY*, and *treZ* genes. Addition of trehalose to the reactor led to its consumption, both during anaerobic and aerobic phases. These results indicate the flexibility of the metabolism of *Ca.* Accumulibacter phosphatis towards variations in salinity variations.

Highlights

- Trehalose is identified as an osmolyte and previously unidentified carbon pool in *Candidatus* Accumulibacter phosphatis
- *Ca.* Accumulibacter phosphatis can convert glycogen into trehalose upon hyperosmotic incubation
- *Ca.* Accumulibacter phosphatis clade I is present and active in both seawater and freshwater
- Trehalose can be consumed both anaerobically and aerobically

6.1. Introduction

Enhanced biological phosphorus removal (EBPR) is an important technology for the removal of phosphate from wastewater streams [206–208]. Anaerobic uptake of volatile fatty acids (VFA) and aerobic uptake of phosphate by phosphate accumulating organisms (PAO) leads to the complete removal of both COD and phosphate in one process step [209, 210]. This process has been extensively studied under freshwater conditions. Recently it has been shown that also seawater-based influents can lead to good EBPR activity (chapter 2). Most interestingly, short-term exposure of this seawater-adapted culture to freshwater led to release of COD, which was proposed to be related to osmolytes.

Intracellular accumulation of osmolytes is a common mechanism for halophilic organisms to survive in saline environments [87, 88]. Osmolytes, sometimes also called compatible solutes or osmoprotectants, are small organic molecules with a net neutral charge, that are used to balance internal and external osmotic pressure without interfering with cellular processes [86, 211]. There is a large range of osmolytes that are widely conserved among organisms ranging from plants to bacteria and fungi [212–214]. Production of osmolytes can be induced by an increase in external osmotic pressure, and release is triggered by a decrease in osmotic pressure [89, 215, 216].

This behavior signifies the importance of understanding osmolyte metabolism in wastewater systems that might undergo dynamic changes in salt content of the influent, as can occur due to industrial discharges or seawater intrusion in the sewer system. The role of osmolytes in saline wastewater treatment has regularly been reported for nitrifying sludge and methanogenic sludge [36, 93, 94, 217]. The most detailed effect described for *Candidatus* Accumulibacter phosphatis is an increase in maintenance coefficients after adaptation to NaCl-amended influent [33]. Identification and production of osmolytes or their function within *Ca.* Accumulibacter under saline conditions has not been described in literature.

In this chapter we identified and described the role of trehalose as an osmolyte in a *Ca.* Accumulibacter phosphatis enrichment. A freshwater-adapted enrichment culture was adapted to a salinity level of 30% seawater. The impact on EBPR activity and microbial community composition was analyzed. the enrichment cultures where exposed to hyperosmotic incubation followed by hypo-osmotic shock, to study the effect on trehalose production and release. The aerobic and anaerobic uptake of trehalose was evaluated. The link between glycogen storage pool and trehalose metabolism is discussed. The impact of this so-far unknown carbon pool on EBPR processes is evaluated.

6.2. Materials & Methods

Reactor operation

A freshwater-adapted *Ca.* Accumulibacter phosphatis enrichment reactor was operated as described in Guedes da Silva et al. (2018) [218]. This reactor was subsequently adapted to a salinity level of 30% seawater with the following operational parameters.

A 1.5L stirred tank reactor was operated as a sequencing batch reactor (SBR) with a 6 hour cycle. Each cycle consisted of 20 minutes settling, 15 minutes effluent withdrawal, 5 minutes N_2 sparging, 5 minutes feeding, 135 minutes anaerobic phase, and 180 minutes aerobic phase. The sludge retention time (SRT) was maintained at 10 days through a short cyclical effluent removal during the mixed aerobic phase. The hydraulic retention time (HRT) was equal to 12 hours (50% exchange ratio). pH was controlled at 7.0 ± 0.1 by dosing either 0.4 M HCl or 0.4 M NaOH. Temperature was controlled at 20 ± 1 °C. Conductivity in the bulk liquid was used to follow phosphate release and uptake profiles. The on-line profiles were used to verify steady operation of the reactor.

The feed of 750 mL was combined from two separate media: a concentrated COD medium and a concentrated mineral medium, which were both diluted with artificial seawater (final concentration of 10 g/L Instant Ocean® sea salts) prior to feeding into the reactor. The combination of these solutions led to influent concentrations of 400 mg COD/L (260 mg COD/L from $NaAc{\cdot}3H_2O$ and 140 mg COD/L from NaPr), 50 mg PO_4^{3-}-P/L (from 222 mg/L $NaH_2PO_4{\cdot}3H_2O$), 40 mg NH_4^+-N/L (from 152.5 mg/L NH_4Cl), 158.6 mg/L $MgSO_4{\cdot}7H_2O$, 48 mg/L KCl, 40 mg/L $CaCl_2{\cdot}2H_2O$, 4 mg/L N-allylthiourea, 4 mg/L yeast extract, and 0.6 mL/L trace element solution (Vishniac and Santer, 1957).

Batch tests

Sludge was taken from the reactor at the end of the aerobic reactor cycle, and transferred to flasks with 100 mL working volume. These flasks contained either demineralized water (buffered at pH 7.0 ± 0.1 with 4.0 mM HEPES buffer) or a solution of Instant Ocean® sea salts, and were sparged with nitrogen gas prior to adding the sludge. Samples were taken over time, and filtered through a 0.45 µm PVDF filter. After the anaerobic phase, the sludge was allowed to settle in the flask, and liquid was decanted and replaced by 100 mL demineralized water, and aerated for a duration of 60 minutes.

The respective masses of all samples were registered to compensate for mass decrease during calculations. The amount of biomass was determined by filtering the granules at the end of the test, washing with demineralized water to remove salts, drying for 24 hours at 105 °C, and burning for 2 hours at 550 °C. All tests were done in duplicate.

Analytical methods

Concentrations of phosphate were measured on a Thermo Fisher Gallery Discrete Analyzer (Thermo Fisher Scientific, Waltham, USA). Acetate and propionate were measured by HPLC with an Aminex HPX-87H column from Biorad, coupled to an RI and UV detector, using 0.01M phosphoric acid as eluent.

Fluorescence in situ hybridization (FISH)

The handling, fixation and staining of FISH samples was performed as described in Bassin et al. (2011) [32]. A mixture of PAO462, PAO651, and PAO846 probes (PAOmix) were used for visualizing polyphosphate accumulating organisms (PAO) [69]. A mixture of GAOQ431 and GAOQ989 probes (GAOmix) were used for visualizing glycogen accumulating organisms (GAO) [70]. *Ca.* Accumulibacter clade I was visualized by Acc-I-444, and *Ca.* Accumulibacter clade II was visualized by Acc-II-444 [71]. A mixture of EUB338, EUB338-II and EUB338-III probes were used for staining all bacteria [72, 73]. Images were taken with a Zeiss Axioplan 2 epifluorescence microscope equipped with filter set 26 (bp 575e625/FT645/bp 660e710), 20 (bp 546/12/ FT560/bp 575e640), 17 (bp 485/20/FT 510/bp 5515e565) for Cy5, Cy3 and fluos respectively.

Amplicon sequencing

DNA was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, The Netherlands). Approximately 250 mg wet biomass was treated according to the standard protocol except an alternative lysis was implemented. This included a combination of 5 minutes of heat (65 °C) followed by 5 minutes of bead-beating for cell disruption on a Mini-Beadbeater-24 (Biospec, U.S.A.). After extraction the DNA was checked for quality by gel electrophorese and quantified using a Qubit 4 (Thermo Fisher Scientific, U.S.A.).

After quality control, samples were sent to Novogene Ltd. (Hong Kong, China) for Amplicon sequencing of the V3-4 region of the 16S-rRNA gene (position 341-806) on a Illumina paired-end platform. After sequencing, the raw read were quality filtered, chimeric sequences were removed and OTUs were generated on the base of ≥ 97% identity. Subsequently microbial community analysis was performed by Novogene using Mothur & Qiime software (V1.7.0). For phylogenetical determination a most recent SSURef database from SILVA (http://www.arb-silva.de/) was used.

Genome analysis

Fifty-nine available metagenome sequences of *Candidatus* Accumulibacter enrichment cultures were obtained from JGI IMG database. These metagenomes were compared with protein sequences of TreX, TreY, and TreZ proteins, obtained from the NCBI protein database. BLASTp was performed using the on-line BLASTp tool by JGI IMG. Alignment was performed according to the algorithm as described in Altschul et al., 1997 [141] and Schäffer et al., 2001 [142]. Lower E-values indicate a lower uncertainty in the presence of certain sequences. Values lower than 1E-40 were set as threshold for positive results.

6.3. Results

Trehalose release from a freshwater-adapted *Ca.* Accumulibacter phosphatis enrichment culture

A freshwater-adapted enrichment culture of *Ca.* Accumulibacter phosphatis was operated as described in Guedes da Silva et al. (2018) [218]. This reactor had a *Ca.* Accumulibacter phosphatis fraction of >90% of the biovolume as determined by fluorescence in-situ hybridization (FISH) analysis. No trehalose was measured in the reactor during a normal cycle of anaerobic COD uptake and phosphate release, and aerobic phosphate uptake.

This freshwater-adapted sludge was used in a batch test to assess the potential for intracellular trehalose production. A sludge sample was anaerobically incubated in 10 g/L artificial seawater (30% of seawater) without presence of a COD source, after which it was transferred to aerated demineralized water. Trehalose concentrations were measured in the liquid phase over time (figure 6.1). After the incubation of freshwater-adapted *Ca.* Accumulibacter for 1 hour in 10 g/L saline medium and subsequent transfer to a fresh water medium resulted in the release of 5.34 mg trehalose/g VSS within only 10 minutes after the osmotic downshock. The control experiment with anaerobic incubation in demineralized water yielded negligible release of trehalose after osmotic downshock.

These results signify the potential for freshwater-adapted *Ca.* Accumulibacter phosphatis sludge to adapt to a short-term sudden increase in salinity by producing trehalose. The next question is whether this enrichment culture can adapt to prolonged exposure of increased seawater salinity, and how this influences trehalose production.

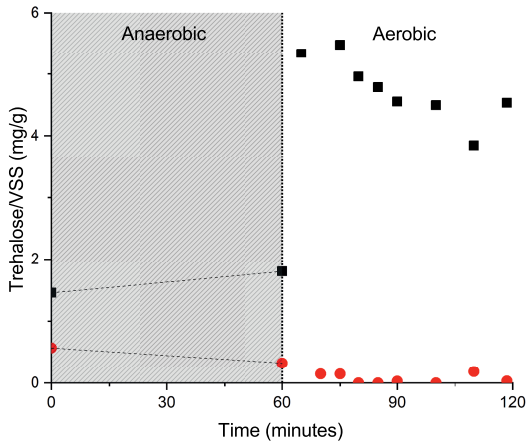


Figure 6.1: Trehalose release of freshwater-adapted *Ca.* Accumulibacter phosphatis enrichment culture after 60 minutes anaerobic incubation in either 30% seawater (squares) or demineralized water (circles), followed by exposure to aerobic demineralized water. The grey area denotes the anaerobic period, and the white area denotes the aerobic period.

Long-term adaptation of *Ca.* Accumulibacter phosphatis to seawater

A *Ca.* Accumulibacter phosphatis enrichment reactor was operated with 30% seawater (10 g/L total dissolved salts). Acetate and propionate were added anaerobically, and completely consumed within 30 minutes after feeding. Phosphate was released up to 167 mg PO_4^{3-}-P/L (0.73 Pmol/Cmol), and completely taken up during aeration. Stable on-line measurements of pO_2, pH, and conductivity confirmed the occurrence of a pseudo steady state during 126 days of stable operation.

Microbial community analysis

The microbial community of the seawater-adapted culture was analyzed by means of fluorescence in-situ hybridization (FISH) and next-generation amplicon sequencing (NGS). FISH analysis with PAO-specific probes and GAO-specific probes showed abundance of PAO and complete absence of GAO (figure 6.3a). The biovolume of the 30% seawater-adapted PAO community as deduced from the FISH microscopy was >90%. Clade differentiation by means of *Ca.* Accumulibacter clade I- and clade II-specific probes showed the presence of clade I, while clade II was not observed (figures 6.3b, 6.3c). The typical morphology of *Ca.* Accumulibacter clade I was verified with brightfield microscopy imaging 6.3d.

NGS analysis showed only a 33% relative abundance of Ca. Accumulibacter (figure 6.2). The relative OTU count of Ca. Accumulibacter is much lower than the fraction that was observed with FISH microscopy, which could be due to bias in extraction and quantification of PAO cultures [219].

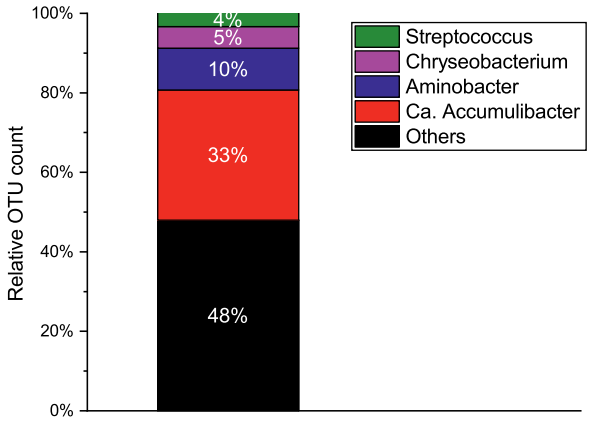


Figure 6.2: Relative OTU count of the most abundant genera of a 30% seawater-adapted *Ca.* Accumulibacter phosphatis enrichment culture

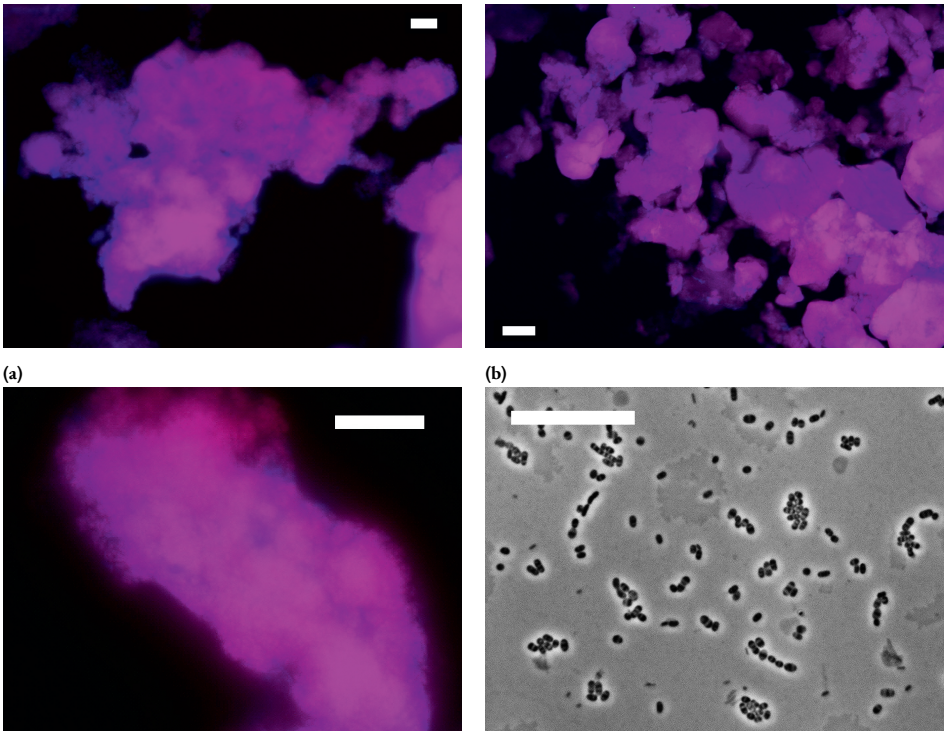


Figure 6.3: Fluorescence in situ hybridization (FISH) analysis of 30% seawater-adapted *Ca.* Accumulibacter phosphatis enrichment culture, with a) PAOmix (cy3, red), GAOmix (fluos, green), EUB338 (cy5, blue); b, c) *Ca.* Accumulibacter clade I (cy3, red), *Ca.* Accumulibacter clade II (fluos, green), EUB338 (cy5, blue); d) brightfield microscopy image showing *Ca.* Accumulibacter clade I morphology. Scale bar equals a, c, d) 20 µm or b) 100 µm.

Trehalose release from seawater-adapted *Ca.* Accumulibacter phosphatis sludge

A single cycle of hyperosmotic incubation and hypo-osmotic shock in a freshwater-adapted *Ca.* Accumulibacter phosphatis enrichment culture caused the release of 5.34 mg trehalose/g VSS (figure 6.1). This led to the question whether seawater-adapted *Ca.* Accumulibacter phosphatis will release more trehalose, and whether exposure to even higher osmotic pressures will lead to even higher intracellular trehalose production. Therefore, the seawater-adapted enrichment culture was anaerobically incubated in different levels of increased salt concentrations. Subsequently they were exposed to demineralized water in order to release their trehalose, similar to the experiment in figure 6.1. Trehalose was measured in the liquid phase during the aerated downshock (figure 6.4).

The control sample of 30% seawater (1x relative salinity) led to release of 1.0 mg trehalose/g VSS after hypo-osmotic shock in demineralized water. This amount increased to 9.2 mg trehalose/g VSS and 11.9 mg trehalose/g VSS after anaerobic incubation in 2x and 3x relative salinity, respectively. After anaerobic incubation in 4x this salinity the amount of released trehalose decreased to 4.9 mg trehalose/g VSS. Negligible amounts of trehalose uptake over time were measured during 60 minutes of aeration. The amount of phosphate release was similar in all samples (between 8.4 and 9.1 mg/L within 10 minutes of hypo-osmotic shock).

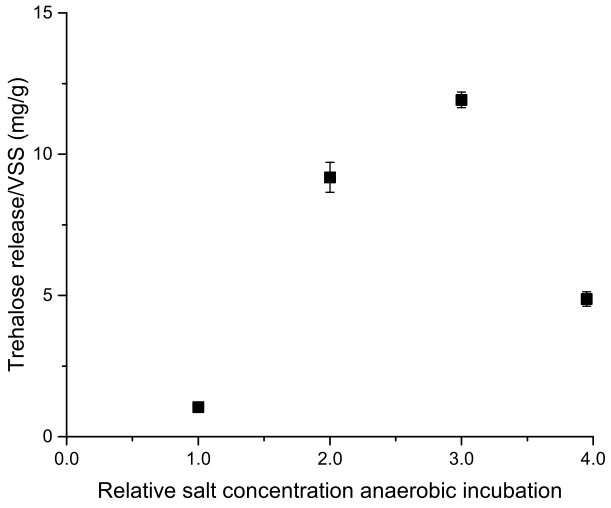


Figure 6.4: Concentrations of released trehalose by the 30% seawater-adapted *Ca.* Accumulibacter phosphatis enrichment culture, during aerated downshock in demineralized water, after 60 minutes anaerobic incubation in either 1x, 2x, 3x, or 4x relative salt concentration compared to 30% seawater.

Trehalose release from seawater-adapted *Ca.* Accumulibacter phosphatis sludge

Our previous results showed the capacity of intracellular trehalose production, but uptake of external trehalose can give great insight into a potential new carbon source from culturing *Ca.* Accumulibacter. Therefore, trehalose was added to the reactor both during aeration (figure 6.5) and during anaerobic feeding, replacing acetate and propionate as COD source (figure 6.6). Characteristic values are shown in table 6.1.

Trehalose was taken up both aerobically and anaerobically. After aerobic addition, phosphorus uptake continued with a similar rate compared to the regular phosphorus uptake rate. Not all trehalose was taken up aerobically, so it leaked into the subsequent anaerobic cycle during which fresh VFA (acetate and propionate) were added to the reactor sludge. Presence of trehalose did not interfere with VFA uptake, but the secondary phosphorus release after VFA uptake increased from 6.3 to 9.3 mg P/g VSS/h. Complete replacement of VFA by trehalose in the anaerobic influent gave a similar phosphorus release rate of 9.5 mg P/g VSS/h.

Table 6.1: Characteristic values of the 30% seawater-adapted *Ca.* Accumulibacter phosphatis enrichment culture after addition of trehalose to the enrichment reactor. Values are shown for both the aerobic and anaerobic phase during which trehalose was added, and for the subsequent anaerobic or aerobic phase, respectively.

	Regular cycle	Aerobic trehalose addition	Subsequent anaerobic cycle	Anaerobic trehalose addition	Subsequent aerobic cycle
Trehalose present	-	+	+	+	+
Acetate & Propionate present	+	-	+	-	-
Aerobic trehalose uptake rate *(mg trehalose/g VSS/h)*		8.0			11.2
Anaerobic trehalose uptake rate *(mg trehalose/g VSS/h)*			2.8	9.7	
Maximal aerobic P-uptake rate *(mg P/g VSS/h)*	53.3	47.8			18.5
Maximal anaerobic P-release rate *(mg P/g VSS/h)*	262.0		208.8	9.5	
Anaerobic P-release after COD uptake *(mg P/g VSS/h)*	6.3		9.3		
Net anaerobic phosphate release *(mg PO_4^{3-}-P/L)*	161.2		166.4	56.9	

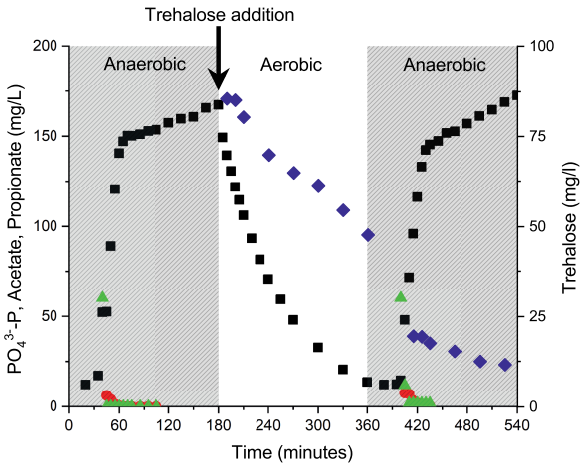


Figure 6.5: Aerobic addition of trehalose in the 30% seawater-adapted *Ca.* Accumulibacter phosphatis enrichment reactor. Concentrations of phosphate (squares), acetate (circles), propionate (triangles), and trehalose (diamonds) are measured over time. The grey areas denote the anaerobic periods, and the white area denotes the aerobic period.

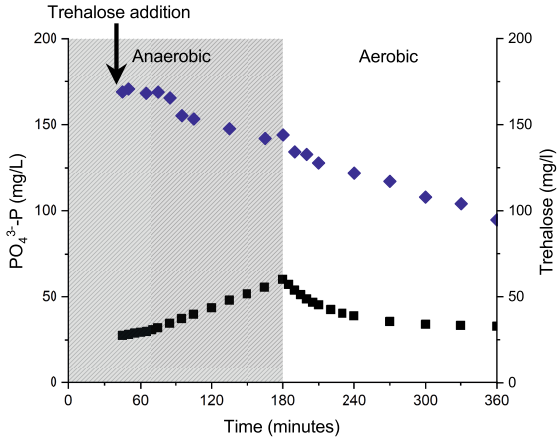


Figure 6.6: Anaerobic addition of trehalose by replacement of acetate and propionate during feeding in the 30% seawater-adapted *Ca.* Accumulibacter phosphatis enrichment reactor. Concentrations of phosphate (squares) and trehalose (diamonds) are measured over time. The grey area denotes the anaerobic period, and the white area denotes the aerobic period.

Metabolic pathway analysis for trehalose production and uptake in *Ca.* Accumulibacter phosphatis

An increase in osmotic pressure led to an increase in trehalose production (figures 6.1 and 6.4). This raises the question through which metabolic pathway this production occurs. Conversion of glycogen into trehalose is a commonly described pathway [220, 221]. The presence of glycogen pools in *Ca.* Accumulibacter makes this pathway a suitable candidate for analysis.

Conversion of glycogen into trehalose commonly occurs in a two-step TreYZ pathway [222, 223]. TreY converts the α-1,4 bonds in glycogen into α-1,1 bonds, from which trehalose disaccharides are cleaved by TreZ. This set of reactions can be aided by the presence of TreX, which is capable of debranching glycogen chains [224].

Uptake of trehalose by bacteria commonly involves breakdown of trehalose into glucose monomers by trehalose [225]. In *Escherichia coli* for example, trehalose is broken down into glucose monomers by periplasmic trehalose, and subsequently taken up by the phosphotransferase system as glucose-6-phosphate [226]. Presence of the *treA* gene that encode for trehalase can indicate whether *Ca.* Accumulibacter has a similar mechanism.

A total of 59 *Ca.* Accumulibacter metagenomes have been analyzed for the presence of genes that encode for TreY, TreZ, TreX, and TreA (table 6.2). Proteins sequences have been used from other bacterial species, and these have been aligned to the metagenome of *Ca.* Accumulibacter cultures. Lower E-values indicate a higher certainty of the gene being present in the genome. All genes for TreY, TreZ, TreX, and TreA have very low E-values, close to or equal to zero. This indicates that the probability for conversion of glycogen to trehalose through the TreYZ pathway is highly likely. Uptake of trehalose can similarly occur by hydrolysis of trehalose into glucose monomers.

Table 6.2: Genome analysis of enzymes that are involved in conversion of glycogen to trehalose (TreY, TreZ, TreX), and involved in the hydrolysis of trehalose for import (TreA).

Enzyme *Accession number* *Reference species*	E-value	Metagenome name *
TreY	1.00E-173	a
CCE37077.1	3.00E-163	a
Mycobacterium tuberculosis	7.00E-156	b
	6.00E-156	c
	7.00E-156	d
TreY	0	a
ABV26725.1	0	a
Actinoplanes sp.	0	e
	3.00E-179	c
	2.00E-179	d
TreZ	0	a
CCE37076.1	0	a
Mycobacterium tuberculosis	0	f
	0	g
	0	h
TreZ	0	g
ABV26726.1	0	h
Actinoplanes sp.	0	i
	0	j
	0	k
TreX	0	f
CCE37078.1	0	a
Mycobacterium tuberculosis	0	k
	0	h
	0	i
TreX	0	a
ABV26724.1	0	a
Actinoplanes sp.	0	l
	0	m
	0	m
TreA	8.00E-170	n
EGT67795.1	2.00E-169	o
Escherichia coli	1.00E-169	p
	3.00E-169	q
	8.00E-170	r
TreA	0	q
CDO15159.1	0	n
Klebsiella pneumoniae	0	o
	0	p
	0	s

* Metagenome names:

(a) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 2_5/28/2013_ DNA (Illumina Assembly)

(b) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_1/23/2012_ DNA

(c) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 2_5/13/2013_ DNA

(d) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_10/4/2010_ DNA

(e) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_2/2/2009_ DNA (SPAdes)

(f) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_1/10/2011_ DNA

(g) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_7/15/2010_ DNA

(h) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_2/2/2009_ DNA

(i) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_7/15/2010_ DNA (SPAdes)

(j) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_4/24/2008_ DNA (SPAdes)

(k) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 1_1/10/2011_ DNA (SPAdes)

(l) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 2_5/28/2013_ DNA (Hybrid Assembly)

(m) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - Reactor 2_5/28/2013_ DNA (PacBio error correction)

(n) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time C-32min-Anaerobic_ RNA (Metagenome Metatranscriptome)

(o) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time B -10min-Anaerobic_ RNA (Metagenome Metatranscriptome)

(p) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor_6/25/2014_ DNA

(q) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time F- 52min-Aerobic_ RNA (Metagenome Metatranscriptome)

(r) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time I- 292min-Aaerobic_ RNA (Metagenome Metatranscriptome)

(s) Wastewater treatment Type I Accumulibacter community from EBPR Bioreactor in Madison, WI, USA - TNR Reactor, Time E -22min-Aerobic_ RNA (Metagenome Metatranscriptome)

6.4. Discussion

Trehalose in *Candidatus* Accumulibacter phosphatis

This chapter shows the presence and role of trehalose in *Candidatus* Accumulibacter phosphatis. Excretion of trehalose after a hypo-osmotic shock reveals the presence of intracellular trehalose pools. Both freshwater-adapted and seawater-adapted cultures produce trehalose after a hyperosmotic incubation, which indicates the remarkable flexibility of *Ca.* Accumulibacter towards salinity variations. These results signify the importance of trehalose in salt adaptation, and can explain the flexibility of the EBPR process towards increased salinity (chapter 2).

The presence of trehalose is of major importance for accurately describing PAO metabolism. Models of PAO metabolism have so far not included the presence of this compound. Quantification of intracellular trehalose pools will be essential for further research. However, common methods for glycogen quantification by extraction and analysis of its hydrolyzed glucose monomers could be interfering, since trehalose is also composed of glucose monomers [227]. Previous studies could potentially even have overestimated their glycogen pools, if trehalose would have been present in significant amounts.

Metabolic link between glycogen and trehalose

A metabolic link between glycogen and trehalose in *Candidatus* Accumulibacter phosphatis is proposed. This link is similar to *Saccharomyces cerevisiae*, where glycogen and trehalose have commonly been described in conjunction [228]. Trehalose can be produced from glycogen through a simple 2-step enzymatic reaction by TreY and TreZ enzymes [222]. TreY can convert α(1-4) glucose polymers such as glycogen into maltooligosyl-trehalose, which TreZ can subsequently hydrolyze into trehalose sugars.

Genome analysis shows that *treX*, *treY*, and *treZ* genes are present in the genome of *Ca.* Accumulibacter (table 6.2). The results presented here indicate that trehalose can be produced very rapidly (figures 6.1 and 6.4), likely enabled by these enzymes. A deeper understanding on the exact regulation of conversion of glycogen into trehalose will lead to improvement of metabolic models. Stoichiometric and kinetic quantification of intracellular metabolite concentrations will be key for filling the gaps in this pathway.

Excreted trehalose is also taken up again albeit at a relative low rate. *Ca.* Accumulibacter phosphatis is described as not using glucose, but trehalose seems to be a sugar substrate that is metabolized by this species. This can occur through hydrolysis of trehalose by a trehalase enzyme and subsequent use of the produced glucose. There is however also a direct pathway described for conversion of trehalose into glycogen, but this seems limited to mycobacteria [229].

Optimum in trehalose release after short-term incubation in higher salinities

Hyperosmotic incubation has frequently been described to be a trigger for intracellular osmolyte production [215, 216, 230, 231]. The amount of trehalose released after hypo-osmotic shock increased with the salinity level during the preceding anaerobic hyperosmotic incubation. Trehalose

release per VSS increased from 2.1 mg trehalose/g VSS at the adapted level of 30% seawater, to 12 mg trehalose/g VSS at three times this salinity. Incubation in four times the adapted salinity gives a much lower release of 4.9 mg trehalose/g VSS (figure 6.4).

A common response to hyperosmotic incubation is the downregulation of the metabolic rate in both eukaryotic and prokaryotic cells [232, 233]. Shrinking of bacterial cells leads to an increase in metabolite and proteins concentrations, also known as molecular crowding [234]. This can result in a decrease in diffusion of proteins, and a change in occurrence of metabolic reactions [235–238].

Intracellular conversion of glycogen into trehalose can therefore have a lower rate at higher salinity. The 1-hour hyperosmotic incubation could have been too short to reach maximal trehalose concentrations. Longer-term salt adaptation would still be a viable option, due to both the longer time for trehalose production, and the decrease in macromolecular crowing after longer duration of hyperosmosis [239].

Clade I present in both freshwater-adapted and seawater-adapted *Ca.* Accumulibacter sludge

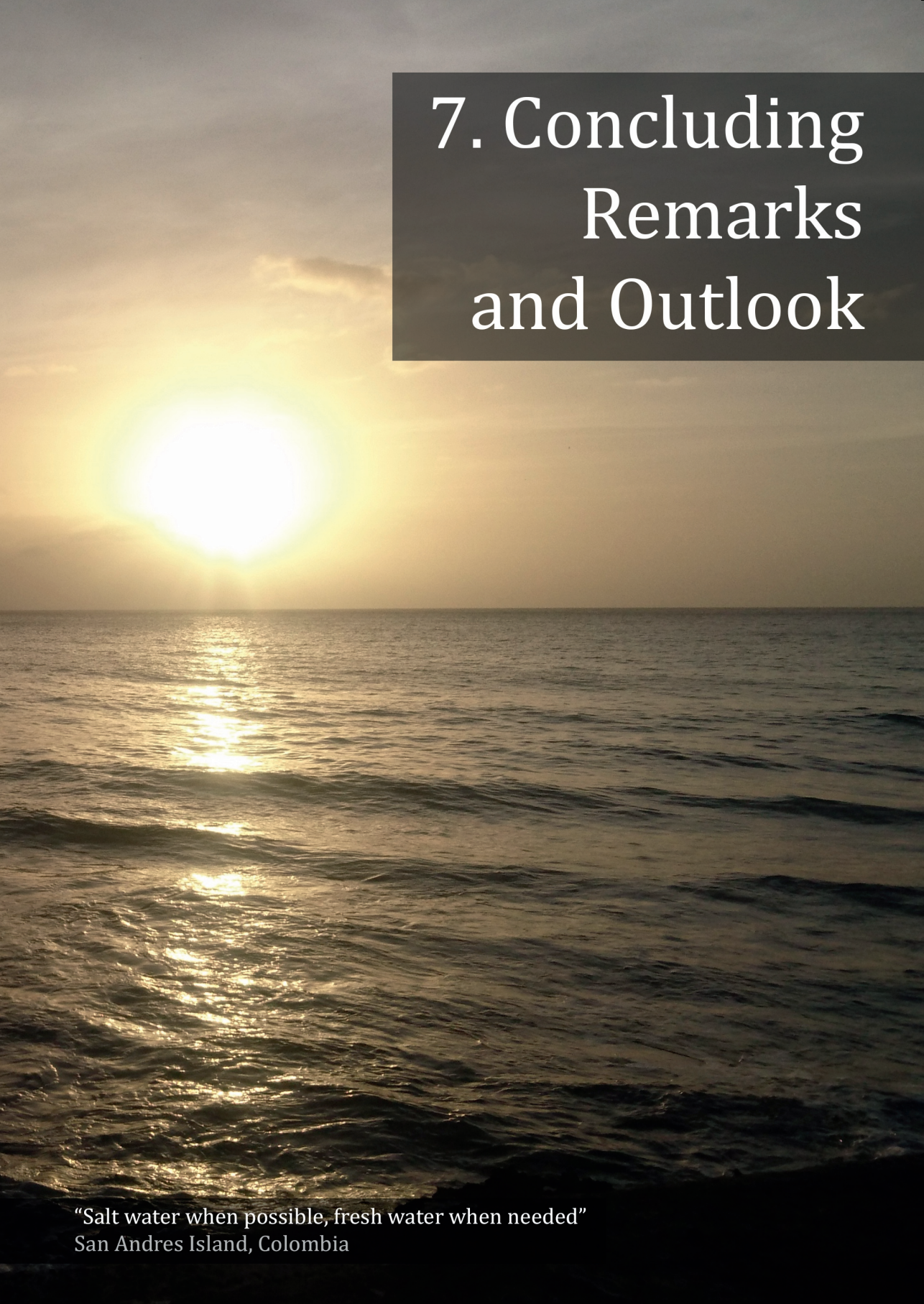
Ca. Accumulibacter clade I was the dominant clade in cultures that were adapted to freshwater and cultures that were adapted to 30% seawater (figure 6.3). This flexibility of a single organism to thrive in different levels of salinity is remarkable, especially compared to nitrifying bacteria and Anammox bacteria [32, 35, 240–242].

The adaptability of *Ca.* Accumulibacter phosphatis to a range of salinities is in line with the estuarine environments in which they were found in nature. This habitat is prone to cyclical variations in salinity, aerobicity, and nutrient availability, due to tidal fluctuations and mixing of seawater and freshwater [243, 244]. *Ca.* Accumulibacter has been found in the sediment-water interface of these dynamic environments [92, 245, 246]. Interestingly, clade I has been observed more frequently than clade II [91]. The new-found presence of trehalose as an osmolyte can add to the understanding of the adaptation strategy of *Ca.* Accumulibacter to these environments.

6.5. Conclusion

- *Ca.* Accumulibacter phosphatis is capable of completely anaerobically removing COD and aerobically removing phosphate in 30% seawater-based medium
- *Ca.* Accumulibacter phosphatis is capable of intracellularly producing trehalose, which is excreted upon hypo-osmotic shock
- *Ca.* Accumulibacter phosphatis can convert intracellular glycogen pools into trehalose
- *Ca.* Accumulibacter phosphatis clade I is present in both freshwater- and seawater-based environments
- A seawater-adapted *Ca.* Accumulibacter phosphatis enrichment culture is capable of consuming externally supplied trehalose

7. Concluding Remarks and Outlook



"Salt water when possible, fresh water when needed"
San Andres Island, Colombia

Concluding Remarks & Outlook

The research described in this thesis demonstrates the feasibility of the application of aerobic granular sludge with seawater-based wastewater. Long-term adaptation of AGS to seawater was successful for biological phosphate removal. With seawater-based influent, polyphosphate accumulating organisms (PAO) remained dominant while glycogen accumulating organisms (GAO) did not proliferate. PAO easily recovered in uptake of acetate and phosphate after an osmotic downshock (chapter 2). The physical strength of granules increased after exposure to salt (chapter 3). The extracellular polymeric substances (EPS) showed a remarkable presence of sialic acids (chapter 4). A sudden decrease in salinity led to the release of trehalose from seawater-adapted *Ca.* Accumulibacter phosphatis (chapter 6). Also the presence of filamentous *Thiothrix* bacteria did not interfere with phosphate removal and granule morphology (chapter 5).

The results from this thesis give a solid basis for future research and application of the technology, so recommendations for future directions will be discussed in this chapter.

7.1. Seawater-based biological phosphorus removal

Biological phosphorus removal was successful in seawater, while NaCl-based wastewater deteriorated at similar salinity [31, 32]. PAO generally showed a higher sensitivity to NaCl than GAO [33], but no GAO were observed in seawater-based AGS (chapter 2). The exact cause for this difference is still unclear. The balance of sodium and potassium can be involved in proper functioning of Na^+/K^+ pumps, which are required for osmotic stabilization [82]. However, these are usually linked to P-type ATPase, which have so-far not been reported in *Ca.* Accumulibacter phosphatis [193, 247, 248].

Osmotic downshock in a seawater-based AGS reactor led to acetate overshoot, which was not caused by biological inhibition but by hydrodynamic behavior. Liquid with a lower density made channels through the sludge blanket, through which influent water bypassed the granules. This effect should get more focus in future research, especially in line with full-scale operation. Lab-scale and full-scale systems have different distribution of feed lines, height/diameter ratios, and size distribution of the granular and floccular sludge. Successful operation of AGS in seawater-based systems is already shown in the Nereda® pilot plant in Sha Tin (Hong Kong), where brackish wastewater is supplied due to toilet flushing with seawater. The impact of varying density of the incoming water in the flow regime during feeding will help in better understanding the stability of granular sludge systems.

7.2. Granule strength

The results from chapter 3 show that full-scale Nereda® aerobic granular sludge has a decreased abrasion coefficient when exposed to NaCl solution. The exact reason behind this observation is still unclear. It likely involves shrinking of the granules due to the increase in salt concentration, which is a common response for hydrogels [249]. Granules with higher density can similarly have higher strength [111]. High-resolution method such as MRI would have to be used to verify this hypothesis, since the used image analysis techniques are not sensitive enough to these changes.

Release of calcium and magnesium ions was measured after incubation of full-scale AGS in NaCl solutions. The importance of divalent cations for alginate-based hydrogel stabilization has frequently been described in literature [49, 250, 251]. The ion exchange effect should cause a decrease in strength in alginate-based gels [252, 253]. The inverse effect demonstrates the difference in the EPS matrix from alginate, since it also contains a large fraction of proteins [57]. This seemingly contradictive effect of calcium decrease in the gel and increased strength would need further attention, but likely in order to come to a good analysis it is first needed to better understand the exact chemical composition of the EPS.

7.3. Extracellular polymeric substances

The adaptability of microorganisms to increased salt concentrations is still a growing field of research. There are indications that one of the adaptations is the change in extracellular polymeric substances (EPS) composition upon seawater adaptation. We qualitatively measured the presence of sialic acids in seawater-adapted aerobic granules using specific binding of lectins. Based on this discovery, the protective properties of sialic acids were investigated, rather than a bulk analysis of proteins and polysaccharides in the EPS.

There are over 50 types of sialic acids, so determination of the exact types that are present in granular sludge should be the next step for research. Higher-resolution methods such as mass spectrometry are required for differentiation between these similar molecules. The remarkable widespread appearance of sialic acids in both bacteria and higher organisms demonstrates evolutionary preservation. Actually, it is generally considered that sialic acids are a specific feature of multicellular organisms. The finding of these acids in acetate-grown granular sludge indicates that they have an origin in the early evolution of life and might have other functions than those related to functioning of multicellular organisms.

Overall characterization of EPS will benefit from a shift from quantitative black-box analytical methods to qualitative methods. The described effects of saline wastewater on the EPS composition has therefore also suffered from contradicting results. Corsino et al. (2017) [54] found an increase in protein/polysaccharide ratio at higher salinity, while Wang et al. (2013) [254] and Campo et al. (2018) [55] described a decrease in this ratio. There are many factors that influence the composition and characterization of EPS:

1. **Reactor operation and microbial community**
   Design of the reactor operation will have a major influence on the resulting microbial community and EPS. Factors such as the sludge retention time and medium composition have an impact on proliferation of certain groups of bacteria. The difference between seawater and NaCl-based salinity can therefore also change EPS properties. Good EBPR activity in seawater was linked to a steady presence on PAO, while GAO was absent (chapter 2). Comparative studies in NaCl-based medium resulted in a shift in microbial community from PAO to GAO. Different microorganisms can produce different EPS, so shifts in bacterial composition can result in significant shifts in EPS properties [145].

2. **Extraction of EPS**
   Many methods for EPS extraction are described in literature [255–258], but different extraction methods will lead to different results. For example, the commonly used alkaline extraction for AGS has given good results for EPS from full-scale Nereda® granules, but acid-soluble EPS cannot be accurately characterized with this extraction method [259]. Also a solid residue is always present after extraction, which means that part of the granule will not be used for further analysis.

3. **Analytical biases**
   The EPS matrix consists of a variety of components, and these components can cause interference in analytical methods. For example, humic substances can interfere with the Lowry method for protein quantification [260–262], the Folin-Ciocalteu reagent for sugar quantification is sensitive to a range of compounds [263, 264], and also the selection of glucose as a sugar standard can lead to inaccurate results [57]. Moreover, the presence of glycoproteins can cause biases when they are quantified in both sugar and protein measurements [145, 265].

7.4. Trehalose in *Candidatus* Accumulibacter phosphatis

The presence of trehalose as an osmolyte in *Candidatus* Accumulibacter phosphatis is another factor of importance to explaining osmoadaptation in these bacteria. This previously undescribed compound gives a solid basis for further research.

Trehalose production and uptake can be closely linked to the well-described glycogen pools in *Ca.* Accumulibacter phosphatis, but this requires verification. The metabolic link between these two compounds is suggested based on the results in chapter 6, but direct measurement would give the most conclusive data. Quantification of these compounds should not solely be based on hydrolysis of glucose monomers. This is a common step for glycogen measurement, but it can cause false positives since both trehalose and glycogen are glucose polymers. Direct measurement of trehalose with HPAEC-PAD is a good alternative. Draining the cells of trehalose, and subsequently hydrolysing leftover glycogen might also give a proper differentiation.

7.5. Stable granulation with filamentous *Thiothrix* bacteria

The results from chapter 5 showed that biological phosphorus removal can work with 18% of COD supplied in the form of thiosulfate. This amount is significantly higher than commonly found in full-scale wastewater treatment plant influents. The applicability of these results is therefore high for capturing filamentous *Thiothrix* bacteria in granular sludge.

One important aspect of this research is that we showed that due to the reduced sulfur in the influent a high fraction of the population became *Thiothrix* bacteria. These filamentous bacteria normally result in bulking sludge. Although there was a slight decrease in settling of the formed sludge it remained of a granular nature. This supports that microbial morphology is not necessarily related to the morphology of formed aggregates.

The absence of strong gradients in the granular sludge during the substrate uptake phase (due to the plug flow feeding) is the main reason for obtaining compact aggregates. Nevertheless, the research pointed also to the weakness of granular sludge relying on this plug flow feeding phase. When the granules got fibrillar extensions, the plug flow of the influent through the settled sludge blanket was disturbed with a fraction shortcutting through the bed. The result is that substrate becomes available under aerobic conditions which results in deterioration of the granule formation. Likely this aspect is more present in the relatively shallow granular sludge bed in lab-scale reactors than in full scale reactors. The hydraulic behavior of granular sludge systems and designs that prevent or minimize short cut flows would need more attention in the future.

8. Acknowledgements

"If I have seen further, it is by standing on the shoulders of giants"
– Sir Isaac Newton

Acknowledgements

I couldn't have finished this thesis all by myself, so I'd like to extend my warmest gratitude to everyone who was involved during the process.

First of all, **Mario**. The combination of your theoretical knowledge and your practical mindset helped me a lot during the research project. Whenever I didn't see the points in my data, you could see where our stories should be going, and how it should be told. Your never-ending enthusiasm motivated me. Even though this exact enthusiasm caused you to be out of the running for a while. This made me realize personally that there is a subtle balance between personal life and work…

Mark, thank you for all the discussions and input during the project. I'm always amazed by the connections you can make between different fields of research, and I've never left a meeting without a new insight. I also like how you're the only full professor I know who regularly joins for beers on a Friday afternoon – that clearly shows your involvement with the group, not only on a scientific basis, but also on a personal level.

Yuemei, thank you for your enthusiasm about EPS research. You constantly kept coming back to our sialic acid research, walking into the office with new insights we can draw from the results. That was very motivating for me! Next to your research inputs I really valued the 'motherly' mindset, even though I was only part of the extended EPS family.

Simon, thanks for all the good times we had. We stuck together in both the office and graduate school courses, and this was for a good reason. Both inside of the lab and outside of it we had great times, that we don't always remembered so clearly afterwards.. but it really helped in you speaking Dutch and me speaking German, or Spanish, of French, or Chinese, or any combination of these. **Marissa**/Maurice, secretly younger than me, but never mentioning it when Simon made jokes about my age. I'm happy you're my paranymph! **Jure**, thanks for your great knowledge about both polymers and cooking. You're one of the most enthusiastic people I know, so make sure to not lose that! **Edward**, thanks for the more hydrodynamic input into my project. Your contributions to the strength paper we wrote were very valuable, and your full-scale knowledge added a lot. **Viktor**, always busy cleaning pipes or helping people. Make sure you take a rest sometimes!

My students, **Sanne**, **Vincent**, **Shwetha**, **Koen**, **Les**, **Nick**, **Carolina**, thank you all for contributing to the project. Sometimes I felt I was learning more from you than the other way around, but that's part of being a manager for you. I'm sure you'll all find your way.

I'd like to thank everyone at the Water Nexus project. **Dainis**, **Hardy**, **Cristina**, **Caroline**, **Grietje**, **Huub**, thank you for all discussions we had during the project meetings. **Arjen**, **Paul**, **Jaap**, **Nadine**, **Frank**, **Georg**, **Dennis**, **Perry**, thanks for the inputs from the company side of the project. Everyone else who I've met during the General Assemblies, you're with too many to mention everyone individually, but I have good memories of especially the evening programs. At Royal HaskoningDHV I'm grateful for the interactions we had over the years. **Edward**, **Andreas**, **Robin**, **Paul**, **Bart**, **Jimmy**, I'm always happy to hear about the full-scale implications of saline Nereda. Also **Pascal**, thank you for your help in getting granules from Garmerwolde, and I was happy to show you the lab in Delft, for which you drove all the way. At UNESCO-IHE, thank you **Pancho**, **Welles**, **Carlos**, **Tineke** for the discussions we had. **Mary Luz**, thanks for the great times in the EBT lab, and showing me around IHE. **Stephen**, **Martin**, **Carlos**, thank you for being part of the thesis committees and your input to the research. **Paula**, **Huub**, **Helle**, **Damir**, **Merle**, **Jules**, thank you for taking part in my PhD defense committee.

All of the technicians, thank you all for the work that you did during the project. Your contributions are immensely helpful – and often forgotten during the process when all goes well. **Udo**, you were actually the one who led me to this PhD project. I'd like to acknowledge our shared musical interest, because it was during a concert that you mentioned that Mario was looking for a candidate for his project. Our musical connection was further exemplified in your office, where we spent hours playing music, up to the point where Ben fled to the lab. **Ben**, at least now you got rid of the music in the new building. I'm happy to have talked to you about the ups and downs at work, from both sides. **Mitchell**, it was such a pity you had to leave EBT at some point. I loved your commitment at work, and you definitely performed well on a research level. **Dirk**, **Rob**, **Song**, **Alexandre**, without you the reactors would've probably exploded at some point. I generated most of my data based on the infrastructure that you are maintaining, so I'd like to express my gratitude. **Cor**, thanks for analyzing all trehalose samples Nick gave to you. You're really one of the most dedicated technicians I know. **Stef**, thank you for your help with the image analyzer. **Apilena**, **Astrid**, **Janny**, thanks for the gezelligheid in the lab. **John**, thank you for the ICP measurements you did for us at Civil Engineering. **Ben N.**, thank you for your help with the hydration tests at ChemE. **Roel**, your music taste, beardedness, and FISH skills really kept the balance up after Udo left. **Rhody**, you were only at EBT for one year, but thanks for all input and structure you added. **Zita**, you arrived at EBT when I finished my contract, but I'm happy about the organization you're adding to the chaos that's called EBT.

Merle, **David**, **Sara**, **Lenno**, **Hongxiao**, **Simon**, **Viktor**, **Marta**, **Pieter**, it was great organizing the Granular Sludge Conference with all of you. The transition from relaxed meetings to stressful preparation was overwhelming, but definitely worth it. **Merle**, also thanks for your input in my project during the times we spoke over coffee.

Ingrid, thank you for being there over the years. We had great times together, and I hope that we will keep having those moments. **Hugo**, synthesizer man. Whatever is not obscure or alternative is not your cup of tea. Your eclectic taste in music brought us to some good festivals, so let's keep that up! **Bart**, forever part of EBT if you'd ask me. Thanks for always being there, especially when I had a small breakdown during my 2nd year. Also our shared interest in music brought us to some good concerts. **Leonor**, all the karaoke nights were great. Thank you for being a friend,

and available through good and bad times. **Robin**, thanks for letting me bake poffertjes at your party because I was the only Dutch person there. Luckily we went to the gym often, so at least we could sport it away with Leonor. We'll forget the fact that we always had a burger afterwards though… **Maaike**, thanks for all discussions about the water world and your help by sending job openings. **Eveline**, both chaotic and social, you were always taking charge in the social sides of EBT. Without you there would've been a different vibe! All office mates I had over the years, **Bart, Eveline, Matthijs, Emmanuelle, Laura, Simon, Ana Maria, Pascalle, Annie**. Thank you for the livability of the place we spent most time of our days.

Jules, thank you for the personal talks we had at times. **Morez**, thanks for nice talks and some amazing Persian cooking! **Gerben, Jelmer, Michel, Robbert,** I enjoyed joining occassionally for lunch and having both funny and insightful conversations! **Peter**, thank you for having introduced me to the research world. **Geert-Jan**, **Dimitry**, **David**, **Gijs**, **Cristian**, thank you for the scientific input on this project. **Aina**, **Emma**, **Monica**, **Michele**, **Stefan**, **Chris**, thank you for the nice times we had! **Miranda**, thank you for all of the secretarial work during the project! Also people who were at EBT temporarily, **Andrea**, **Celia**, **Clara**, **Lorena**, **Julio**, **Cuijie**, **Janis**, **Felipe**, **Shengle**, **Mahsa**, **Vojtech**, **Dana**, **Rubén**, thank you for the company.

Florence, it was nice to have paranymphed together and let's keep making music! **David, Sergio, Marta, Alexandra, Maria-Paula, Fabio, Georg, Daniel, Cansel,** I don't always remember that clearly what we did, but I think we had great times! **Mario B.**, it was great talking with you about music production. **Philipp**, we had some good coffee talks with our shared interested in water and resource recovery!

Pilar, **Koen**, **Luuk**, **Stefan**, **Pablo**, **Silvia**, **Debby**, **Jasmine**, **Suellen**, **Marijn**, **Johan**, **Nayyar**, **Robert**, **Bradley**, **Duncan**, **Ranko**, **Miguel**, thank you for being around and the good times we had.

Sassen, **Berben**, **Daniel**, thanks for your friendship, and sharing your lab experiences which made me realize that my PhD project was not too bad after all! All evenings together, full of board games and the occasional whisky, were great. Even though we moved away from each other for a while, to the US, Ireland, and Belgium, I'm happy that we are still so much in touch. My study friends **Els**, **Eleonoor**, **Esmee**, **Laura**, **Loes**, **Maaike**, **Maureen**, **Oscar**, **Xavier**, **Tom**, I'm happy we still manage to see each other multiple times per year. The weekendje weg, and Sinterkerst are definitely here to stay! **Harm**, **Jeroen**, **Kenny**, let's keep up our mountainbiking efforts. Everyone at the Music Production Academy, thank you for improving my creative musical side. **Michaël**, **Zazala**, **Marijn**, **Jelle**, **Kenneth**, **Hardstjel**, thanks for all the jam sessions and production classes. I learned a lot and really enjoy the vibe!

Mom, **dad**, **Jennifer**, **Jordy**, and the rest of my family, I'm really happy about the relationship that we have. I'm happy I can always openly talk to you about my ups and downs, and I hope this will remain like this forever. You know the little details in my life, which is very important to me.

Appendix A - Granule Strength Characterization Protocol

Sample preparation

- Take a mixed sample of granular sludge
- Let the sludge settle and decant the supernatant
- Wash and filter the granules on a 1600 µm sieve
- Dry the granules on the sieve by adding paper to the bottom of the sieve, and refreshing this piece until no more water gets soaked up

Granule weighing

- Tare the balance with an empty cup
- Add granules to this cup, and note down their mass ('$WW_{reactor}$')
- Weigh 2 empty 50 mL Falcon tubes for X_0 samples ('Empty')
  - NB: Make sure to remove the gas that new tubes initially contain, by blowing compressed air into the tubes
- Add granules to the 2 X_0 tubes, and note down their mass ('Sample')
- Weigh 4 empty 50 mL Falcon tubes for X_F samples ('Empty')

Starting the operation

- Transfer the $WW_{reactor}$ granules to a 250 mL glass measuring cylinder
- Add tap water up to a total volume of 250 mL
  - NB: When working with different salinity, this water should contain the correct salt concentrations
- Pour this mixture into the reactor
- Add another 250 mL of the same liquid to the reactor
- Stir for 60 minutes at 800 rpm

Processing after strength characterization test

- Weigh an empty bucket ('Empty bucket')
- Flush the reactor contents through a 200 µm sieve into this bucket
- Let the fine particles in the permeate settle, and use the supernatant twice to remove remaining granules from the reactor, and to wash the biomass on the sieve
- Weigh the bucket with the permeate and fines ('Bucket + permeate')
- Transfer 50 mL of this liquid into each empty X_F tube, and note down their mass ('Sample')
- Centrifuge these tubes for 10 minutes at 4200 rpm (4 °C)
- Decant the supernatant
  - NB: When working with high salinity, wash the pellet twice with distilled water
- Dry the samples by either freeze drying, or by drying at 60 °C for at least 24 hours until completely dry
  - NB: Dry a small number of empty reference tubes, to be able to compensate for mass loss of the tubes themselves
- Note down the mass of all dried samples ('Dry')

Table 8.1: TSS measurement

	Empty (g)	Sample (g)	Dry (g)
X_o-a			
X_o-b			
X_F-a			
X_F-b			
X_F-c			
X_F-d			

Table 8.2: Process conditions

$WW_{reactor}$ (g)	
Empty bucket (g)	
Bucket + Permeate (g)	

$m_{permeate}$ (g) = (Bucket + Permeate) - (Empty bucket)

Calculation of abrasion rate coefficient (*K*)

The equation for the abrasion rate coefficient (K, in s^{-1}) is as indicated in equation 8.1.

$$K = \ln\left(\frac{X_0 - X_F}{X_0}\right) \cdot t \tag{8.1}$$

X_o is the initial biomass concentration (mg TSS/g), X_F is the concentration of abraded particles after the strength characterization test (mg TSS/g). t is the duration of the strength test (should be 3600 s). Values for X_o and X_F can be calculated as indicated in equations 8.2 and 8.3, respectively.

$$X_0 = \left(\frac{\text{dry} - \text{empty}}{\text{sample} - \text{empty}}\right) \cdot 1000 \frac{\text{mg}}{\text{g}} \cdot \left(\frac{WW_{reactor}}{m_{reactor}}\right) \tag{8.2}$$

$$X_F = \left(\frac{\text{dry} - \text{empty}}{\text{sample} - \text{empty}}\right) \cdot 1000 \frac{\text{mg}}{\text{g}} \cdot \left(\frac{m_{permeate}}{m_{reactor}}\right) \tag{8.3}$$

Values for 'dry', 'empty', and 'sample' (g) are their respective masses that were weighed before and after the strength characterization test. $WW_{reactor}$ (g) is equal to the mass of wet weight granules that were used in the strength characterization test. $m_{permeate}$ (g) is the mass of the liquid that was sieved through the 200 μm sieve after the 60 minutes of agitation. $m_{reactor}$ (g) is the mass of liquid in the reactor during the strength test (taking into account the liquid density). The value of 1000 mg/g gives an easier order of magnitude for calculations.

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Curriculum Vitæ

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List of Publications

3. **D.R. De Graaff**, S. Felz, T.R. Neu, M. Pronk, M.C.M. van Loosdrecht, & Y. Lin, *Sialic acids in the extracellular polymeric substances of seawater-adapted aerobic granular sludge*, Water Research **155**, 343–351 (2019).
2. **D.R. De Graaff**, E.J.H. van Dijk, M.C.M. van Loosdrecht & M. Pronk, *Strength characterization of full-scale aerobic granular sludge*, Environmental Technology (2018).
1. P.R. Mooij, **D.R. de Graaff**, M.C.M. van Loosdrecht, & R. Kleerebezem (2015). *Starch productivity in cyclically operated photobioreactors with marine microalgae—effect of ammonium addition regime and volume exchange ratio*, Journal of Applied Phycology, 27(3)

List of Oral Presentations

4. Granular Sludge Technologies for Biological Wastewater Treatment of Saline Wastewater (joint presentation with Dainis Sudmalis, WUR). *Water Science for Impact Conference*, Wageningen, Netherlands (2018)
3. Effect of Salinity on the Physical Strength of Full-Scale Aerobic Granular Sludge. *IWA ecoSTP Conference*, London, Ontario, Canada (2018)
2. Overview of Environmental Biotechnology Research, *BT symposium*, Schipluiden, Netherlands (2018)
1. Salinity Effect on Physical and Biochemical Stability of the Aerobic Granular Sludge Process (flash). *Young Water Professionals*, Ghent, Belgium (2017)

List of Poster Presentations

3. Effect of salinity on the strength of aerobic granular sludge. *IWA Biofilm Specialists Conference*, Dublin, Ireland (2017)
2. Salinity Effect on Physical and Biochemical Stability of the Aerobic Granular Sludge Process. *MMWWRR Symposium*, Delft, Netherlands (2017)
1. Wastewater treatment with aerobic granular sludge in saline water. *Young Water Professionals Conference*, Leeuwarden, Netherlands (2015)

List of Awards

2. Best Flash Presentation Award. *Young Water Professionals Conference*, Ghent, Belgium (2017)
1. Best Poster and Pitch Award. *MMWWRR Symposium*, Delft, Netherlands (2017)