# Fermentative Substrates in Aerobic Granular Sludge

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# Fermentative Substrates
# in
# Aerobic Granular Sludge

## Dissertation

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by

**Ali ELAHINIK**

**This dissertation has been approved by the promotors.**

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Keywords: Wastewater treatment, Aerobic granular sludge, EBPR, Nereda, Extracellular polymeric substances, Salinity

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# Contents

**The supplementary material of all chapters can be found in the online version of the publications.**

# Summary

Industrial wastewaters often have unique properties and contain impurities that pose a significant challenge to their treatment. Lab-scale experiments were performed to provide answers on the feasibility of aerobic granular sludge (AGS) technology for the treatment of organically polluted industrial wastewater.

Glycerol is found in a variety of industrial effluents such as biodiesel and epoxy resin production facilities. However, little is known about the conversion and the impact of glycerol on AGS processes. **Chapter 2** describes glycerol conversion in AGS capable of enhanced biological phosphorus removal (EBPR). Robust granules with good phosphorus removal capabilities were formed in an AGS sequencing batch reactor fed with glycerol as the sole carbon source. The interaction between the fermentative conversion of glycerol and product uptake by polyphosphate accumulating organisms (PAO) was studied using stoichiometric and microbial community analysis. The analysis of the biomass identified a community dominated by Actinobacteria (*Tessaracoccus and Micropruina*) and a typical PAO known as *Ca.* Accumulibacter. Glycerol uptake facilitator (glpF) and glycerol kinase (glpK), two proteins involved in the transport of glycerol into the cellular metabolism, were only observed in the genome of the Actinobacteria. The anaerobic conversion appeared to be a combination of substrate fermentation and product uptake-type reaction. Initially, glycerol fermentation led mainly to the production of 1,3-propanediol (1,3-PDO) which was not taken up under anaerobic conditions. Despite the aerobic conversion of 1,3-PDO, stable granulation was observed. Over time, 1,3-PDO production decreased and complete anaerobic COD uptake was observed. Overall, the results demonstrate that glycerol-containing effluents can effectively be treated by the AGS process via a collaboration between fermentative and polyphosphate accumulating organisms.

The sugar production industries generate a significant amount of wastewater rich in sugars such as glucose. In **Chapter 3,** glucose conversion by AGS and its impact on phosphate removal is studied. Long-term stable phosphate removal and successful granulation were observed. Glucose was rapidly taken up with a rate of 273 mg/gVSS/h at the start of the anaerobic phase, while phosphate was released during the full anaerobic phase. Lactate was produced as the main product during glucose consumption, which was anaerobically consumed once glucose was depleted. Other products such as propionate, acetate, and formate were also detected in minor quantities. The phosphate release appeared to be directly proportional to the uptake of lactate. The ratio of phosphorus released to glucose carbon taken up over the full anaerobic phase was 0.25 Pmol/Cmol. Along with glucose and lactate uptake in the anaerobic phase, polyhydroxyalkanoates and glycogen storage were observed. Quantitative fluorescence in-situ hybridization (qFISH) revealed that PAOs accounted for the majority of the total biovolume. Anaerobic conversions were evaluated based on theoretical ATP balances to provide the substrate distribution among the dominant genera. In conclusion, this research

shows that AGS can be applied for the treatment of glucose-containing effluents and it is a suitable substrate for achieving phosphate removal.

Industrial wastewaters often have high levels of salt, either due to seawater or e.g. sodium chloride (NaCl) usage in the processing. In **Chapter 4,** the impact of NaCl concentration gradient and seawater on the granulation and conversion processes of AGS was investigated. Glycerol was used as the carbon source since it is regularly present in industrial wastewaters, and to allow the evaluation of microbial interactions that reflect industrial effluents. Smooth and stable granules as well as EBPR were achieved up to 20 g/L NaCl or when using seawater. However, at NaCl levels comparable to seawater strength (30 g/L) incomplete anaerobic glycerol uptake and aerobic phosphate uptake were observed, the effluent turbidity increased, and filamentous granules began to appear. The latter was likely due to the direct aerobic growth on the leftover substrate after the anaerobic feeding period. In all reactor conditions, except the reactor with 30 g/L NaCl, *Ca*. Accumulibacter was the dominant microorganism. In the reactor with 30 g/L NaCl, an increase in the genus *Zoogloea* was observed. Throughout all reactor conditions, *Tessaracoccus* and *Micropruina*, both actinobacteria, were present which were likely responsible for the anaerobic conversion of glycerol into volatile fatty acids. None of the glycerol metabolizing proteins were detected in *Ca*. Accumulibacter which supports previous findings that glycerol can not be directly utilized by *Ca*. Accumulibacter. The exposure of salt-adapted biomass to hypo-osmotic conditions led to significant trehalose and $PO_4^{3-}$-P release which can be related to the osmoregulation of the cells. The findings provide insights into the effect of salt on the operation and stability of the AGS processes and suggest that maintaining a balanced cation ratio is likely to be more important for the operational stability of the system than absolute salt concentrations.

Extracellular polymeric substances (EPS) are important constituents of biofilms with promising application potential. The properties of EPS vary depending on environmental conditions and microbial communities which also entails inconsistencies in the material. In **Chapter 5**, we investigated the EPS of AGS grown under varying salinities induced by NaCl concentration gradient and seawater conditions. Fourier transform infrared (FTIR) spectroscopy revealed the likely presence of polysaccharides, phosphates, proteins, carboxylic esters, and lipids in all extracted EPS. Further analysis with 2-D correlation spectroscopy identified notable differences in various regions corresponding particularly to phosphate and glycan functional groups. Sugar monomer analysis of acid-hydrolysed EPS identified eight monosaccharides, with glucose dominant in saltwater conditions and glucosamine in freshwater. We further evaluated the potential of the extracted EPS as a bio-based flame retardant, via burning tests on EPS-coated cellulose fibres. The tests indicated a linear correlation between increased residual mass and the condensed phosphate content in the EPS, suggesting that higher condensed phosphate levels enhance the flame-retardant properties of the EPS. The EPS from saline conditions had higher condensed phosphate content in contrast to the freshwater EPS with higher orthophosphate fraction. In conclusion, the findings highlighted the potential of wastewater-derived EPS as a bio-based flame retardant and the impact of salt on EPS properties.

Finally, the thesis is concluded with **Chapter 6** providing an outlook on the future research, economics, and application of AGS technology. Overall, the findings suggest that AGS technology can be applied for the treatment of industrial wastewater containing salts (pure NaCl or sea salt crystals) as well as glycerol and glucose as organic pollutants, with the added benefit of recovering valuable resources.

# Samenvatting

Industriële afvalwaters hebben vaak unieke eigenschappen en bevatten onzuiverheden die een aanzienlijke uitdaging vormen voor de behandeling ervan. Laboratoriumexperimenten zijn uitgevoerd om antwoorden te geven over de haalbaarheid van aerobe korrelslibtechnologie (AGS) voor de behandeling van organisch vervuild industrieel afvalwater.

Glycerol komt voor in verschillende industriële afvalstromen, zoals in biodiesel- en epoxyharsproductiefaciliteiten. Er is echter weinig bekend over de omzetting en de impact van glycerol op AGS-processen. **Hoofdstuk 2** beschrijft de omzetting van glycerol in AGS dat in staat is tot verbeterde biologische fosforverwijdering (EBPR). Robuuste korrels met goede fosforverwijderingscapaciteiten werden gevormd in een AGS sequencing batch reactor gevoed met glycerol als enige koolstofbron. De interactie tussen de fermentatieve omzetting van glycerol en de opname van producten door polyfosfaat-accumulerende organismen (PAO) werd bestudeerd met behulp van een stoichiometrische en microbiële gemeenschapsanalyse. De analyse van de biomassa identificeerde een gemeenschap die werd gedomineerd door Actinobacteria (Tessaracoccus en Micropruina) en een typische PAO, bekend als Ca. Accumulibacter. Glycerol-opnamefacilitator (glpF) en glycerolkinase (glpK), twee eiwitten die betrokken zijn bij het transport van glycerol naar de cellulaire stofwisseling, werden alleen waargenomen in het genoom van de Actinobacteria. De anaerobe omzetting bleek een combinatie van substraatfermentatie en productopnamereacties te zijn. Aanvankelijk leidde de fermentatie van glycerol voornamelijk tot de productie van 1,3-propaandiol (1,3-PDO), dat niet werd opgenomen onder anaerobe omstandigheden. Ondanks de aerobe omzetting van 1,3-PDO werd stabiele granulatie waargenomen. In de loop van de tijd nam de productie van 1,3-PDO af en werd volledige anaerobe CZV-opname waargenomen. Over het algemeen tonen de resultaten aan dat glycerol-bevattende afvalstromen effectief kunnen worden behandeld met het AGS-proces via een samenwerking tussen fermentatieve en polyfosfaat-accumulerende organismen.

De suikerindustrieën genereren een aanzienlijke hoeveelheid afvalwater dat rijk is aan suikers zoals glucose. In **Hoofdstuk 3** wordt de omzetting van glucose door AGS en de impact ervan op fosfaatverwijdering bestudeerd. Langdurige stabiele fosfaatverwijdering en succesvolle granulatie werden waargenomen. Glucose werd snel opgenomen met een snelheid van 273 mg/gVSS/u aan het begin van de anaerobe fase, terwijl fosfaat werd vrijgegeven gedurende de volledige anaerobe fase. Lactaat werd geproduceerd als het belangrijkste product tijdens het glucoseverbruik, dat anaeroob werd geconsumeerd zodra glucose was uitgeput. Andere producten zoals propionaat, acetaat en formiaat werden ook in kleine hoeveelheden gedetecteerd. De fosfaatvrijgave bleek direct evenredig te zijn met de opname van lactaat. De verhouding van fosfaatvrijgave tot opgenomen glucosekoolstof gedurende de volledige anaerobe fase was 0,25 Pmol/Cmol. Naast de opname van glucose en lactaat in de anaerobe fase werden polyhydroxyalkanoaten en glycogeenopslag waargenomen. Kwantitatieve

fluorescentie in-situ hybridisatie (qFISH) toonde aan dat PAO's het grootste deel van het totale biovolume uitmaakten. Anaerobe omzettingen werden geëvalueerd op basis van theoretische ATP-balansen om de substraatverdeling onder de dominante geslachten te bepalen. Concluderend toont dit onderzoek aan dat AGS kan worden toegepast voor de behandeling van glucose-bevattende afvalstromen en dat het een geschikt substraat is voor het bereiken van fosfaatverwijdering.

Industriële afvalwaters bevatten vaak hoge zoutconcentraties, hetzij door zeewater of bijvoorbeeld het gebruik van natriumchloride (NaCl) in de verwerking. In **Hoofdstuk 4** werd de impact van het NaCl-concentratiegradiënt en zeewater op de granulatie- en omzettingsprocessen van AGS onderzocht. Glycerol werd gebruikt als koolstofbron omdat het regelmatig aanwezig is in industrieel afvalwater, en om de evaluatie van microbiële interacties die industriële afvalstromen weerspiegelen mogelijk te maken. Gladde en stabiele korrels evenals EBPR werden bereikt tot 20 g/L NaCl of bij gebruik van zeewater. Echter, bij NaCl-niveaus die vergelijkbaar zijn met de sterkte van zeewater (30 g/L) werd onvolledige anaerobe glycerolopname en aerobe fosfaatopname waargenomen, nam de troebelheid van het effluent toe en begonnen filamentachtige korrels te verschijnen. Dit laatste was waarschijnlijk het gevolg van directe aerobe groei op het resterende substraat na de anaerobe voedingsperiode. In alle reactorcondities, behalve de reactor met 30 g/L NaCl, was Ca. Accumulibacter het dominante micro-organisme. In de reactor met 30 g/L NaCl werd een toename van het geslacht Zoogloea waargenomen. In alle reactorcondities waren Tessaracoccus en Micropruina, beide actinobacteriën, aanwezig, die waarschijnlijk verantwoordelijk waren voor de anaerobe omzetting van glycerol in vluchtige vetzuren. Geen van de glycerolmetaboliserende eiwitten werd gedetecteerd in Ca. Accumulibacter, wat eerdere bevindingen ondersteunt dat glycerol niet direct door Ca. Accumulibacter kan worden gebruikt. De blootstelling van zout-aangepaste biomassa aan hypo-osmotische omstandigheden leidde tot significante trehalose- en PO43--P-vrijgave, wat kan worden gerelateerd aan de osmoregulatie van de cellen. De bevindingen geven inzicht in het effect van zout op de werking en stabiliteit van de AGS-processen en suggereren dat het handhaven van een gebalanceerde kationenverhouding waarschijnlijk belangrijker is voor de operationele stabiliteit van het systeem dan absolute zoutconcentraties.

Extracellulaire polymere stoffen (EPS) zijn belangrijke bestanddelen van biofilms met veelbelovende toepassingsmogelijkheden. De eigenschappen van EPS variëren afhankelijk van de omgevingsomstandigheden en microbiële gemeenschappen, wat ook inconsistenties in het materiaal met zich meebrengt. In **Hoofdstuk 5** onderzochten we de EPS van AGS die werd gekweekt onder verschillende zoutgehaltes, geïnduceerd door een NaCl-concentratiegradiënt en zeewateromstandigheden. Fourier-transform infraroodspectroscopie (FTIR) onthulde de waarschijnlijke aanwezigheid van polysachariden, fosfaten, eiwitten, carbonzuuresters en lipiden in alle geëxtraheerde EPS. Verdere analyse met 2-D correlatiespectroscopie identificeerde opvallende verschillen in verschillende gebieden die met name overeenkwamen met fosfaat- en glycanfunctionele groepen. Suikermonomeeranalyse van zuur-gehydrolyseerde EPS identificeerde acht monosachariden, met glucose dominant onder zoutwateromstandigheden en glucosamine in zoetwater. We evalueerden verder het potentieel van de geëxtraheerde EPS als een bio-gebaseerde

brandvertrager, via brandtesten op met EPS-gecoate cellulosevezels. De tests toonden een lineaire correlatie aan tussen de toegenomen residuele massa en het gecondenseerde fosfaatgehalte in de EPS, wat suggereert dat hogere gecondenseerde fosfaatniveaus de brandvertragende eigenschappen van de EPS verbeteren. De EPS uit zoute omstandigheden hadden een hoger gecondenseerd fosfaatgehalte, in tegenstelling tot de zoetwater-EPS met een hoger orthofosfaatgehalte. Concluderend benadrukten de bevindingen het potentieel van uit afvalwater verkregen EPS als een bio-gebaseerde brandvertrager en de impact van zout op de eigenschappen van EPS.

Tot slot wordt in **Hoofdstuk 6** het proefschrift afgesloten met een vooruitblik op toekomstig onderzoek, economie en toepassing van AGS-technologie. Over het algemeen suggereren de bevindingen dat AGS-technologie kan worden toegepast voor de behandeling van industrieel afvalwater dat zouten bevat (puur NaCl of zeezoutkristallen), evenals glycerol en glucose als organische verontreinigingen, met het bijkomende voordeel van het terugwinnen van waardevolle hulpbronnen.

۳۰ گرم در لیتر NaCL آزمایش شد، Ca. Accumulibacterمیکروارگانیسم غالب بود. در راکتور با ۳۰ گرم در لیتر NaCl، افزایش در ژن Zoogloea مشاهده شد. در تمام شرایط راکتور، TessaracoccusوMicropruina ، هر دو از اکتینوباکترها، حضور داشتند که احتمالاً عامل تبدیل بی‌هوازی گلیسرول به اسیدهای چرب فرار بودند. هیچ‌کدام از پروتئین‌های متابولیزه‌کننده گلیسرول در Ca. Accumulibacter شناسایی نشدند که یافته‌های قبلی را تایید میکند که گلیسرول نمی‌تواند مستقیماً توسط Ca. Accumulibacter استفاده شود. توسعه زیست توده های سازگار با نمک در شرایط هیپواسموتیک منجر به آزادسازی قابل‌توجه تریهالوز و PO43--P شد که می‌تواند به تنظیم اسمزی سلول‌ها مربوط باشد. این یافته‌ها شناخت اولیه ای درباره اثر نمک بر عملکرد و پایداری فرآیندهای AGS ارائه می‌دهند و پیشنهاد می‌کنند که حفظ نسبت متوازن کاتیون‌ها احتمالاً برای پایداری عملیاتی سیستم از غلظت‌های مطلق نمک مهم‌تر است.

مواد پلیمری خارج‌سلولی (EPS) اجزای مهم بیوفیلم‌ها با کاربردهای بالقوه جذاب هستند. خواص EPS بسته به شرایط محیطی و جوامع میکروبی متفاوت است که این امر به ناهماهنگی‌هایی در مواد منجر می‌شود. در **فصل ۵**، EPS زیست توده گرانولی توسعه یافته تحت شوری‌های مختلف القا شده توسط گرادیان غلظت NaCl و شرایط آب دریا مورد بررسی قرار می گیرد. تبدیل فوریه طیف‌سنجی مادون قرمز (FTIR)نشان داد که احتمالاً پلی‌ساکاریدها، فسفات‌ها، پروتئین‌ها، استرهای کربوکسیلیک و لیپیدها در تمامEPS های استخراج‌شده حضور دارند. تحلیل بیشتر با طیف‌سنجی همبستگی دوبعدی تفاوت‌های قابل‌توجهی در نواحی مختلف به‌ویژه مربوط به گروه‌های عاملی فسفات و گلیکان شناسایی کرد. تحلیل مونوساکاریدهایEPS های هیدرولیزشده اسیدی هشت مونوساکارید را شناسایی کرد که گلوکز در شرایط آب شور و گلوکزآمین در آب شیرین غالب بود. ما همچنین پتانسیلEPS های استخراج‌شده را به عنوان یک بازدارنده آتش زیستی، از طریق آزمون‌های سوزاندن روی الیاف سلولزی پوشش‌داده‌شده با EPS ارزیابی کردیم. آزمون‌ها نشان دادند که همبستگی خطی بین افزایش جرم باقیمانده و محتوای فسفات متراکم در EPS وجود دارد، که نشان می‌دهد میزان بالاتر فسفات متراکم خواص بازدارندگی آتش EPS را بهبود می‌بخشدEPS های شرایط شور محتوای فسفات متراکم بالاتری در مقابلEPS های آب شیرین با نسبت بالاتر ارتوفسفات داشتند. در نتیجه، یافته‌ها پتانسیلEPS های مشتق شده از فاضلاب به عنوان یک بازدارنده آتش زیستی و تأثیر نمک بر خواص EPS را برجسته می‌کنند.

در نهایت، پایان‌نامه با **فصل ۶** به ارائه چشم‌اندازی بر پژوهش‌های آینده، اقتصاد و کاربرد فناوری AGS می پردازد. به طور کلی، نتایج نشان می‌دهند که فناوری AGS می‌تواند برای تصفیه فاضلاب‌های صنعتی حاوی نمک NaCl خالص یا کریستال‌های نمک دریایی و همچنین گلیسرول و گلوکز به عنوان آلاینده‌های آلی به کار گرفته شود و در ضمن مزیت اضافی بازیافت منابع ارزشمند را نیز داشته باشد.

# خلاصه


فاضلاب‌های صنعتی اغلب دارای ویژگی‌های منحصربه‌فردی هستند و حاوی ناخالصی‌هایی می‌باشند که به چالش‌های بزرگی در فرآیند تصفیه آن‌ها منجر می‌شود. در این تحقیق، آزمایش‌هایی در مقیاس آزمایشگاهی برای ارزیابی امکان‌پذیری فناوری زیست توده گرانولی (AGS) در تصفیه فاضلاب های صنعتی آلوده به مواد آلی انجام شد .گلیسرول درفاضلاب صنعتی مختلفی مانند کارخانجات تولید بیودیزل و رزین اپوکسی یافت می‌شود. با این حال، اطلاعات کمی در مورد تبدیل گلیسرول و تأثیر آن بر فرآیندهای AGS وجود دارد.

در **فصل ۲**، تبدیل گلیسرول در AGS که قادر به حذف زیستی پیشرفته فسفر (EBPR) است، توضیح داده شده است. گرانول های پایدار با قابلیت حذف خوب فسفر در یک راکتور ناپوسته تناوبی AGS که با گلیسرول به عنوان تنها منبع کربن تغذیه شده بود، تشکیل شدند. تعامل بین تبدیل تخمیری گلیسرول و برداشت محصولات تولیدی توسط میکروارگانیسم‌های ذخیره کننده پلی‌فسفات (PAO) با استفاده از تحلیل‌های استوکیومتری و جامعه میکروبی مورد بررسی قرار گرفت. تحلیل توزیع جمعیت میکروبی زیست توده نشان داد که جمعیت غالب باکتریایی، باکتری‌های اکتینوباکتر Tessaracoccusو Micropruina و یک PAO معمولی به نام Ca. Accumulibacter می باشند. دو پروتئین تسهیل‌کننده برداشت گلیسرول (glpF) و گلیسرول کیناز (glpK) که در انتقال گلیسرول به متابولیسم سلولی دخیل هستند، تنها در ژنوم اکتینوباکترها مشاهده شدند. تبدیل بی‌هوازی گلیسرول به نظر می‌رسید ترکیبی از تخمیر سوبسترا و واکنش برداشت محصول باشد. در ابتدا، تخمیر گلیسرول عمدتاً به تولید 1,3-پروپان‌دیول (1,3-PDO) منجر شد که تحت شرایط بی‌هوازی برداشت نمی‌شد. با وجود تبدیل هوازی 1,3-پروپان‌دیول، تشکیل گرانول های پایدار مشاهده شد. با گذشت زمان، تولید 1,3-پروپان‌دیول کاهش یافت و حذف کامل COD بی‌هوازی رخ داد. به طور کلی، نتایج نشان می‌دهند که پساب‌های حاوی گلیسرول می‌توانند به‌طور مؤثری از طریق فرآیند AGS با تعامل بین ارگانیسم‌های تخمیری و ذخیره کننده پلی‌فسفات تصفیه شوند.

صنایع تولید شکر مقدار قابل توجهی فاضلاب تولید می‌کنند که غنی از قندهایی مانند گلوکز است. در **فصل ۳**، تبدیل گلوکز توسط AGS و تأثیر آن بر حذف فسفر بررسی شده است. حذف پایدار و طولانی‌مدت فسفر و تشکیل موفقیت‌آمیز زیست توده گرانولی مشاهده شد. گلوکز با نرخ ۲۷۳ میلی‌گرم بر گرم VSS در ساعت در ابتدای فاز بی‌هوازی برداشت شد، در حالی که فسفر در طول فاز کامل بی‌هوازی آزاد شد. لاکتات به عنوان محصول اصلی در طی مصرف گلوکز تولید شد که به‌طور بی‌هوازی پس از اتمام گلوکز مصرف شد. محصولات دیگری مانند پروپیونات، استات و فرمات نیز در مقادیر کم مشاهده شدند. به نظر می‌رسید که آزادسازی فسفر مستقیماً متناسب با جذب لاکتات باشد. نسبت فسفر آزاد شده به کربن گلوکز برداشت شده در طول فاز بی‌هوازی کامل ۰٫۲۵ مول P به ازای هر مول C بود. همراه با برداشت گلوکز و لاکتات در فاز بی‌هوازی، ذخیره پلی‌هیدروکسی‌آلکانوات‌ها و گلیکوژن نیز مشاهده شد. هیبریداسیون فلورسانس در محل (qFISH) نشان داد کهPAO ها اکثریت بیوولوم کل را تشکیل می‌دهند. تبدیل‌های بی‌هوازی بر اساس موازنه‌های تئوری ATP ارزیابی شدند تا توزیع سوبسترا بین ژن های غالب مشخص شود. نتایج این تحقیق نشان می‌دهد که AGS می‌تواند برای تصفیه پساب‌های حاوی گلوکز به کار گرفته شود و گلوکز یک سوبسترای مناسب برای امکان حذف فسفر است.

در فرآوری، اغلب دارای میزان فاضلاب‌های صنعتی به دلیل استفاده از آب دریا یا استفاده مستقیم از سدیم کلراید و آب دریا بر فرآیند تشکیل زیست توده گرانولی و تبدیل NaCl بالایی از نمک هستند. در **فصل ۴**، تأثیر گرادیان غلظت بررسی شد. گلیسرول به عنوان منبع کربن استفاده شد زیرا به‌طور متداول در فاضلاب‌های صنعتی وجود دارد و AGS با هنگام ، 20 گرم در لیتر NaClبرای ارزیابی تعاملات میکروبی که منعکس‌کننده فاضلاب‌های صنعتی هستند. با میزان خوبی حاصل شد. با این حال، EBPR استفاده از آب دریا، زیست توده گرانولی صاف و پایداری توسعه یافت و همچنین مشابه با قدرت یونی آب دریا (۳۰ گرم در لیتر) برداشت ناقص گلیسرول بی‌هوازی و فسفات هوازی NaCl در میزان مشاهده شد، شفافیت پساب افزایش یافت و گرانول های رشته‌ای شروع به ظاهر شدن کردند. احتمالاً این امر به دلیل رشد هوازی مستقیم روی باقیمانده سوبسترا پس از دوره تغذیه بی‌هوازی بود. در تمام شرایط راکتور، به جز راکتوری که

# Preface

*The Inception of WATER-MINING*

This PhD research is part of the overarching WATER-MINING project, which aims to provide real-world implementations of the Water Framework Directive and other water-related legislation. Additionally, it supports the Circular Economy and EU Green Deal packages by showcasing and validating innovative next-generation water resource solutions at a pre-commercial demonstration scale (www.watermining.eu). The WATER-MINING project is an EU initiative, involving 38 partners from 12 countries over four years, with a budget of 19.1 million euros. These partners include universities, industries, and research institutes. The project encompasses urban and industrial wastewater treatment, resource recovery, and seawater desalination projects (Figure 1). This PhD project falls under the Industrial-Mining group.


***Figure 1**. The concept of Water-Mining.*

The WATER-MINING project was initiated by Dr. Dimitris Xevgenos in 2017 to address the high organic loads in brine streams. Brine, defined as a solution of water with high concentrations of salts, primarily sodium chloride (NaCl), is a byproduct of various industrial activities such as seawater desalination and chemical production. The project drew inspiration from the work of Prof. Mark van Loosdrecht, a scientist in the field of environmental biotechnology who holds the 2014 Spinoza and the 2018 Stockholm Water Prize for revolutionising wastewater treatment. Due to the importance of innovation in bio-based technologies and societal challenges, Prof. Patricia Osseweijer took the lead in addressing the challenges of building a water-smart economy and society.

*Case Studies in INDUSTRIAL-MINING*

To capture the full potential of the circular water economy, the WATER-MINING project involved 6 sector-specific case studies. The INDUSTRIAL-MINING group included 2 case studies.

Case Study 1 – **The epoxy resin production facility in the Netherlands**.

The epoxy resin producer, Westlake Corporation (formerly Hexion), is situated within the industrial cluster of the Port of Rotterdam in the Netherlands. The wastewater generated from this facility contains a significant amount of salt (~12% w/v NaCl) and organics, primarily due to the presence of glycerol (~95% of the total chemical oxygen demand (COD)). In close proximity to Westlake Corporation is Nobian (formerly Nouryon), a major chlorine producer, responsible for nearly 90% of the total chlorine produced in the Netherlands. Since 2006, with the termination of train transportation in the Netherlands, almost all chlorine has been supplied via pipelines. This proximity creates a strong dependency between the producers and users. The close vicinity of the chlorine producer (Nobian) and the epoxy resin producer (Westlake Corporation) presents substantial circular economy opportunities. For instance, Nobian can utilize the NaCl-containing effluent (brine) from Westlake's epoxy resin production as input material for their chloralkali process to produce chlorine and other chemicals. This integration not only promotes resource efficiency but also enhances sustainability within the industrial cluster. To use the brine in the chloralkali process, the organic impurities must be removed as they can reduce system's efficiency.

Case Study 2 – **The sugar production facility in India**

The Vasantdada Sugar Institute, formerly known as Deccan Sugar Institute, was established in 1975 by a group of sugarcane growers in India. The institute is dedicated to producing sugar and conducting various scientific, technical, and educational activities related to the sugar and alcohol industry. The wastewater generated from the sugar production process contains a significant amount of COD and has a dark brown color. It undergoes a series of treatments before discharge. Initially, the raw wastewater from the sugar mill plant, which has a significant COD concentration of 130 – 160 $gO_2$/L and a pH of 4-5, is treated via anaerobic digestion. This process results in a waste byproduct known as fermentation spent wash, which exits the anaerobic digestion tanks with a reduced COD of 50 – 60 $gO_2$/L and a pH of 7-8. This fermentation spent wash is then utilised for the production of bioethanol. These two steps are common strategies applied for high-strength wastewater treatment in industries, producing biogas and bioethanol as products. The effluent from the distillery, after bioethanol production, has a much lower COD of 3 – 7 $gO_2$/L and a neutral pH of 7, indicating that the combined treatments reduce the COD load by 95 – 98%. This final effluent contains volatile fatty acids (VFA) and sugars such as glucose. However, it is still not suitable for discharge based on India's effluent limits, which has a COD limit of 250 $mgO_2$/L (www.cpcb.nic.in). To

achieve this effluent quality, an additional treatment step is required to oxidize the remaining COD.

*Challenges in Treating Sugar- and Salt-Rich Industrial Wastewaters*

Industrial wastewaters rich in sugars or salts present distinct challenges during treatment due to their unique characteristics. High concentrations of sugars, as seen in Case Studies 1 and 2, contribute to elevated COD levels, while salts increase the osmolarity of the wastewater. Both factors pose significant difficulties in conventional treatment processes. When these wastewaters are subjected to aerobic granular sludge treatment, their specific characteristics may impact the granular structures and their settling properties. The excessive organic load from sugars and the osmotic stress caused by salts can hinder the stability and functionality of the aerobic granules, potentially compromising the efficiency of the treatment process. Addressing these challenges requires thorough investigation and tailored strategies to optimise aerobic granular sludge process for the effective treatment of industrial wastewater containing sugars and salts. One of the proposed solutions was the application of aerobic granular sludge as a biological wastewater treatment technology in Case Studies 1 and 2. However, information about the impact of sugars such as glycerol and glucose and high NaCl concentrations on granulation, conversions, microbial community, and operational stability was limited.

# 1. General Introduction



### History of Biological Wastewater Treatment

The importance of wastewater treatment for civilisation is immense. It enables the sustainable management of water resources, prevents disease proliferation, and helps conserve the ecosystem by minimising environmental pollution. From ancient civilizations up until the early 1900s, wastewater was typically transported out of cities and either discharged into the environment untreated or used as fertiliser, commonly known as "nightsoil." In regions without sewer systems, human excreta, or nightsoil, was collected by workers, usually at night, and transported out of towns to be sold as fertiliser for agricultural use (Wikipedia/Nightsoil). After this period, municipal and industrial wastewater treatment gained traction mainly in the US and the UK due to rising health concerns, increasing population, and rapid industrialisation (Jacob Gardner, 2022). When it comes to modern biological wastewater treatment, it is generally believed that Edward Ardern and William Lockett (**Figure 2**), two chemists at Davyhulme laboratories in Manchester, marked the discovery of the activated sludge process, a biological method for treating wastewater, with their work entitled: "*Experiments on the oxidation of sewage without the aid of filters*" (Ardern & Lockett, 1914). Biological wastewater treatment, unlike physicochemical processes, harnesses the power of the microbial world to treat wastewater. Microbial communities, consisting of bacteria, archaea, fungi, and protozoa, act as natural purifiers, breaking down organic pollutants present in wastewater.

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ammonio silver nitrate in a similar manner to the others gave on analysis:—silver, 36·18 and 36·35; nitrogen, 25·65 and 25·63 per cent. Calculated for $(C_3NO_3)_3NHAg_3NH_3$, Ag 35·9, N, 25·7 per cent.

Molecular weight determination (boiling point) in acetone gave results distinctly confirmatory of the above formula, but ammonia seems to be split off from these compounds by long heating in acetone or alcohol. All these substances explode or burn rapidly when heated and are also sensitive when dry to percussion or friction.

Beyond the analyses and ebullioscopic molecular weight determination, no proof of the above formula is at present put forward.

## Manchester Section.

*Meeting held at the Grand Hotel on Friday, April 3rd, 1914.*

MR. J. H. HOSEASON IN THE CHAIR.

### EXPERIMENTS ON THE OXIDATION OF SEWAGE WITHOUT THE AID OF FILTERS.

BY EDWARD ARDERN, M.SC., AND WILLIAM T. LOCKETT, M.SC.

It has long been known that if sewage be exposed to the air for a sufficient period of time, the organic contents are gradually oxidised, with the formation of a deposit of so-called "humus" and the final production of nitrate from the ammonium salts and the nitrogenous organic matter.

This purification change of which the course of the reaction has been so carefully studied and thoroughly worked out by Adeney in his researches on behalf of the Royal Commission on Sewage Disposal, takes place, however, comparatively slowly, and even if aided by direct aeration, by no means becomes a practical method of sewage purification.

Numerous investigators have from time to time endeavoured to utilise aeration methods in the practical solution of the sewage problem, but until quite recently without any reasonable amount of success.

Among the earlier investigations may be mentioned those of Dupré and Dibdin[1] on the aeration of London Sewage, and those of the Massachusetts State Board of Health relating to the use of aeration in the filtration of sewage through gravel and sand filters. Dr. Drown,[2] chemist to this Board, concluded from the results of a series of experiments, that the oxidation of organic matter in water was not hastened by vigorous agitation with air.

Waring[3] of the United States was one of the first to apply aeration methods in the purification of sewage on a working scale.

In 1880 Hartland patented an aeration chamber for the purification of sewage or tank effluent, which Kaye Parry[4] employed in experiments undertaken in 1887, while in 1892 Lowcock conducted experiments on the aeration of filter beds by a forced air supply.

In the latter year Mason and Hine[5] published the results of a research on the aeration of mixture of sewage and water in which they concluded that aeration had but little oxidation effect on the sewage.

In 1897 Fowler[6] investigated the effect of aeration on the effluent resulting from the chemical precipitation of Manchester sewage, without any very tangible results being obtained, at any rate so far as the total amount of oxidation was concerned.

It would thus appear that the results of the earlier investigations on the subject of aeration of sewage, indicated that aeration *per se* could not be considered as a practicable adjunct in the process of sewage purification.

Recently, however, the subject has been reopened by the work of Black and Phelps, Clark, Gage and Adams, and Fowler and Mumford.

In dealing with the question of the pollution of the New York Harbour, Black and Phelps[7] studied the possibilities of the application of aeration to the treatment of sewage.

Their experiments dealt with the aeration of both fresh and partially septicised sewage, in various types of tanks and it was shown that under certain conditions it was possible by means of a reasonable amount of aeration to remove the more readily putrescible matters from the sewage and thereby to a certain extent increase its stability.

Black and Phelps were so far convinced of the practicability of such methods of treatment of sewage, as to recommend that the sewage from a certain section of the New York area should be dealt with on these lines, prior to discharge into the waters of the harbour.

In the Annual Report of the Massachusetts State Board of Health for the year 1912, published at the end of 1913, is described an investigation by Clark and De M. Gage on the possibilities of the use of aeration for preliminary treatment of sewage prior to filtration. They found that simple aeration of sewage for 24 hours reduced the free and albuminoid ammonia to some extent and that with sewage which was both aerated and seeded with green growths—*Protococcus* and *Scenedesmus*—the albuminoid ammonia was even more noticeably reduced. Later it was found that appreciable nitrification was obtained within 24 hours in the aerated sewage containing the green growths.

Subsequently Clark and Gage found that aeration for a much shorter period, in a tank containing slabs of slate about one inch apart, covered with a compact brown growth of sewage matters, was sufficient to coagulate the sewage colloids, and thus to produce a well clarified non-nitrified effluent capable of satisfactory filtration at several times the normal rate.

Later Fowler and Mumford[8] carried out experiments on the action in the presence of air, of an organism designated "M7" (isolated by Mumford from ponds receiving water discharged from a colliery) on sewage containing a certain proportion of iron salts.

This organism has the property of precipitating the iron as ferric hydroxide from solutions of iron salts.

In the paper referred to, it is stated that sewage (in presence of a certain quantity of iron salts) inoculated with this organism, can be thoroughly clarified by six hours aeration. The resultant effluent after settlement of the separated organic colloids, was quite clear and practically free from colloids. It is further stated that although the ordinary methods of analysis failed to reveal the extent of the change effected by the above treatment, the effluent after aeration was always non-putrefactive on incubation, and could be readily oxidised and nitrified by filtration at a high rate.

In quite a recent publication Clark and Adams[9] give the results obtained during 12 months operation of the specially constructed tank used in their earlier experiments previously referred to.

It is shown that a rather better coagulation of the organic colloids and purification of the sewage generally, can be obtained by means of aeration for a period of five hours, under the conditions of experiment, than is obtained by efficient chemical precipitation, and at a considerably reduced cost.

The effluent resulting from the aeration treatment was applied to trickling filters 10 feet deep, at rates varying from 8 to 10 million gallons per acre per day, with the production of a well-nitrified and thoroughly stable filtrate.

In connection with the experiments carried out by Clark and his colleagues, it should be mentioned that the sewage treated was considerably more dilute than the

[1] Report to Royal Commission on the Metropolitan Sewage Disposal, 1884, Vol. 2.
[2] Clark and Adams, Engineering Record, February 7th, 1914, p. 158.
[3] Rafter and Baker, 1894, Sewage Disposal in the United States, p. 535.
[4] Trans. Inst. C.E. Ireland, Vol. XX., 1888.
[5] Journ. Amer. Chem. Soc., Vol. 14, p. 7.
[6] Annual Report, 1897, Rivers Dept., Manchester Corporation.
[7] Mass Inst. of Technology, Contributions from the Sanitary Research Laboratory, Vol. VII., Boston, Massachusetts, 1911.
[8] Journal of Roy. San. Inst., November, 1913.
[9] Engineering Record, February 7th, 1914, p. 158.

***Figure 2**. Left image: Edward Ardern (standing far left) and William Lockett (standing far right), likely the first two who demonstrated floc formation from sewage oxidation and their corresponding publication (right image). Courtesy of Historia Sanitaria.*

During the treatment process, microorganisms present in wastewater treatment plants (WWTP) form biofilms, attaching to surfaces or forming suspended aggregates. Biological WWTPs have anaerobic (oxygen-depleted) and aerobic (oxygen-rich) zones. In the anaerobic zone, bacteria consume the organic matter (i.e. pollutants), converting it into simpler compounds and storage material in the absence of oxygen. In the aerobic phase, the storage material and the remaining pollutants are oxidised into $CO_2$ generating energy for microbial processes. During the treatment process not only pollutants are removed, but they can also be transformed into harmless valuable byproducts, that might in future be sold or used at the WWTP, offsetting some of the costs of the wastewater treatment process. In a municipal WWTP, three main biochemical conversions involving carbon, phosphorus, and nitrogen are crucial for reducing pollutants.

**Carbon oxidation** (glucose in this example): This process involves the aerobic conversion of organic compounds by microorganisms into carbon dioxide, water, and biomass.

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + Biomass$$

**Nitrogen removal** (nitrification-denitrification): Nitrogen removal is a two-step process where ammonium is oxidised into nitrate by nitrifying bacteria and then nitrate is converted into nitrogen gas by denitrifying bacteria under anoxic conditions.

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$$

$$2NO_3^- + 10H^+ + 10e^- \rightarrow N_2 + 5H_2O$$

**Phosphorus removal** (enhanced biological phosphorus removal): Phosphorus is taken up by the phosphorus-accumulating organisms which store it as polyphosphate and phosphorus removal is achieved by discharging the polyphosphate-enriched biomass.



$$PO_4^{3-} + Biomass + Energy \rightarrow Polyphosphate\ in\ biomass$$

### Chronological Development of Biological Wastewater Treatment Processes

The evolution of biological wastewater treatment processes over the past century reflects ongoing efforts to enhance treatment efficiency, reliability, and sustainability. Each advancement has contributed to improved water quality and environmental protection, from the foundational activated sludge process to modern membrane bioreactors and aerobic granular sludge. These processes continue to evolve, incorporating new materials, control systems, and energy-efficient technologies to address the growing challenges of wastewater management. Below are some of the examples of widely applied technologies around the world.

*Trickling Filters (1865 - 1900s)*

The patent of a biologically related purification system that describes similar principles to trickling filters dates back to 1865 when a German chemist demonstrated that sewage can be purified by living organisms in a filter column (Alleman, 1982). However, the first *reported* use of trickling filters was in 1901 in the United States (Alleman, 1982). It is unclear whether earlier or similar applications were reported in non-English literature. Regardless, trickling filters can be regarded as the earliest fixed-film biological treatment technologies. In this process, wastewater is distributed over a media, such as stones or plastics, where biofilm develops. As the wastewater "trickles" down through the media, the biofilm degrades the organic pollutants and purifies the influent. Trickling filters are known for their simplicity, reliability, and low operational costs. They are particularly effective for treating smaller wastewater flows and are still widely used around the world in various forms, including modern adaptations like rotating biological contactors.

| Pros | Cons |
|---|---|
| **Low OPEX:** low energy requirement due to passive aeration | **Large footprint**: requires a large area |
| **Robust:** can handle flow and composition fluctuations | **Odorous**: can produce odours if not regularly maintained |
| **Simple operation:** requires low maintenance | **Biofilm shedding**: detachment of biofilm can increase effluent suspended solids |

*Activated Sludge Process (1920s)*

The activated sludge process, developed in the early 1900s by Edward Ardern and W.T. Lockett, revolutionised the biological wastewater treatment process (Ardern & Lockett, 1914). This process involves aerating wastewater to promote the growth of microorganisms that degrade organic pollutants. The term "activated" refers to the active biological matter, i.e. microorganisms, suspended in the wastewater. The process effectively reduces biochemical oxygen demand making it a cornerstone of modern wastewater treatment. The process has been continually refined over the decades through scientific exploration, incorporating advancements in aeration technology, understanding biochemical conversions, and system design to enhance efficiency and scalability.

| Pros | Cons |
|---|---|
| **High treatment efficiency:** can achieve high-quality effluent | **Energy intensive**: requires energy input for aeration |
| **Flexibility:** can be adapted for nutrient removal | **Sludge production**: generates large volumes of sludge |
| **Scalability:** suitable for a wide range of sizes | **Large footprint**: requires a large area |

*Anaerobic Digestion (1930s-1940s)*

Anaerobic digestion gained traction in the 1930s and 1940s as a method for treating high-strength industrial wastewater, sludge, and manure (Wilkinson & Wilkinson, 2011). However, the earliest *reported* application dates back to 1859 when methane was produced from cattle manure in India. This process involves the breakdown of organic matter in the absence of oxygen, i.e. anaerobically, producing biogas (a mixture of methane and carbon dioxide) and a nutrient-rich liquid called digestate. Anaerobic digestion is particularly suited for treating high-strength organic-rich waste streams from agricultural and food processing facilities for instance. The biogas produced can be used as a renewable energy source, to partially cover the operational costs of the treatment plant.

| Pros | Cons |
|---|---|
| **Energy production:** produces biomethane that can be used as a source of energy | **Long star-up time**: requires a long start-up time to establish an anaerobic microbial community |
| **High organic load:** suitable for manure and high-strength wastewater treatment | **Temperature sensitive:** performance significantly affected by temperature fluctuations |
| **Resource recovery:** generates a nutrient-rich digestate that can be applied as fertiliser | **Effluent quality:** requires tertiary treatment to reduce the remaining load |

*Sequencing Batch Reactors (1950s)*

Sequencing batch reactors (SBRs) started to gain major developments in the 1950s as an innovative variation of the activated sludge process (Cabrera Jr. et al., 2011). SBRs operate in a repeated batch mode, with a single reactor alternating between periods of filling, aeration, settling, and decanting. This flexibility allows for precise control of reaction conditions and makes SBRs suitable for treating a wide range of wastewater types, including municipal and industrial discharges. SBRs are known for their ability to handle varying flow rates and loadings, as well as their effectiveness in removing nutrients such as nitrogen and phosphorus.

| Pros | Cons |
|---|---|
| **Flexibility:** operation can be adjusted based on flow and load fluctuations | **Batch operation**: intermittent effluent flow requires equalisation |
| **Effluent quality:** can achieve a high effluent quality | **Automation**: requires automated control systems |
| **Nutrient removal:** can be adapted for nutrient removal | **Complex operation**: requires skilled operators to control mechanical and electrical components |

*Membrane Bioreactors (1970s)*

Membrane Bioreactors (MBRs) represent a significant advancement in biological wastewater treatment, gaining popularity since the 1970s. The first use of MBRs was introduced by Smith et al. in 1969 through the Dorr-Oliver research program (Al-Asheh et al., 2021). MBRs combine traditional biological treatment with membrane filtration, providing a high level of treatment efficiency and producing excellent-quality effluent. The membranes act as a physical barrier, retaining biomass and suspended solids, which allows for higher mixed liquor suspended solids concentrations and improved biological activity. MBRs are particularly known for their compact footprint, ability to handle high organic loads, and high effluent quality.

| Pros | Cons |
|---|---|
| **Effluent quality:** can achieve a very high effluent quality with low suspended solids | **High OPEX:** requires membrane replacement and aeration that increases the operational costs |
| **Small footprint:** requires less space due to high biomass concentration and absence of clarifiers | **Membrane fouling**: regular cleaning and replacement increases the maintenance requirement |
| **Scalability:** suitable for a wide range of sizes | **Complex operation**: requires skilled workers to control membrane system performance and operational stability |

*Aerobic Granular Sludge Technology*

Aerobic granular sludge (AGS) is a relatively modern biotechnology for wastewater treatment, offering important advantages over conventional activated sludge, such as a smaller footprint and enhanced energy efficiency (Bengtsson et al., 2018; Etterer & Wilderer, 2001). One of the main advantages of AGS is related to the co-existence of aerobic, anoxic, and anaerobic zones

within the granules which enables the simultaneous occurrence of different biological processes such as phosphate removal, nitrification, and denitrification (Figure 3). Some of the drawbacks of AGS technology include a long start-up period if readily formed granules are not available for inoculation; it's a batch process, making it challenging to retrofit into existing continuous-flow plants; and its heavy reliance on automation for consistent performance. The first proof of concept for aerobic sludge granulation dates back to 1998 when granules were cultivated in fully aerated sequencing batch reactors with molasses as substrate (Heijnen & Van Loosdrecht, 1998; Morgenroth et al., 1997). The AGS process is commercially branded as the Nereda® technology and is a product of Royal HaskoningDHV, an independent engineering consulting company in the Netherlands https://nereda.royalhaskoningdhv.com/. The principles behind granulation and the benefits of AGS have already been explained in detail by my predecessors (de Kreuk et al., 2005; Pronk et al., 2015; van Dijk et al., 2022). In summary, successful granulation depends, among others, on:

- **Anaerobic feeding of the substrate** – This approach selects organisms that can store the substrate as storage polymers anaerobically and utilise them under aerobic conditions. The separation of substrate uptake from the growth process is also called the feast-famine regime. The organisms capable of separating the two processes are typically slow-growing such as polyphosphate accumulating organisms (PAO) that form dense and smooth granules. Generally, granulation is achieved when the substrate uptake rate is slower than the substrate transport rate into the granules (Picioreanu et al., 1997). The anaerobic feeding of the substrates separates these two processes.
- **The type of substrate** – A suitable substrate for granulation is taken up and stored under anaerobic conditions such as volatile fatty acids (VFA). Substrates that are not anaerobically stored are utilised aerobically by fast-growing heterotrophs resulting in floc formation or filamentous growth (van Loosdrecht et al., 1997). Substrates such as some sugars or alcohols (as studied in this thesis) can also be suitable for granulation. These substrates typically select for a more diverse microbial community as they need to be first converted into VFAs by the fermentative communities. PAOs or other substrate-storing organisms then take up the VFAs.
- **Physical selection pressure** – Biofilms with poor settling properties are almost always present. These can be flocs that are formed from the aerobic oxidation of organic matter that is anaerobically not stored. Or fragments of old granules that are broken due to decay. By applying a short settling time, granules that settle quickly are given a competitive advantage and the biofilm with poor settling properties is washed out of the system (Liu & Tay, 2015).

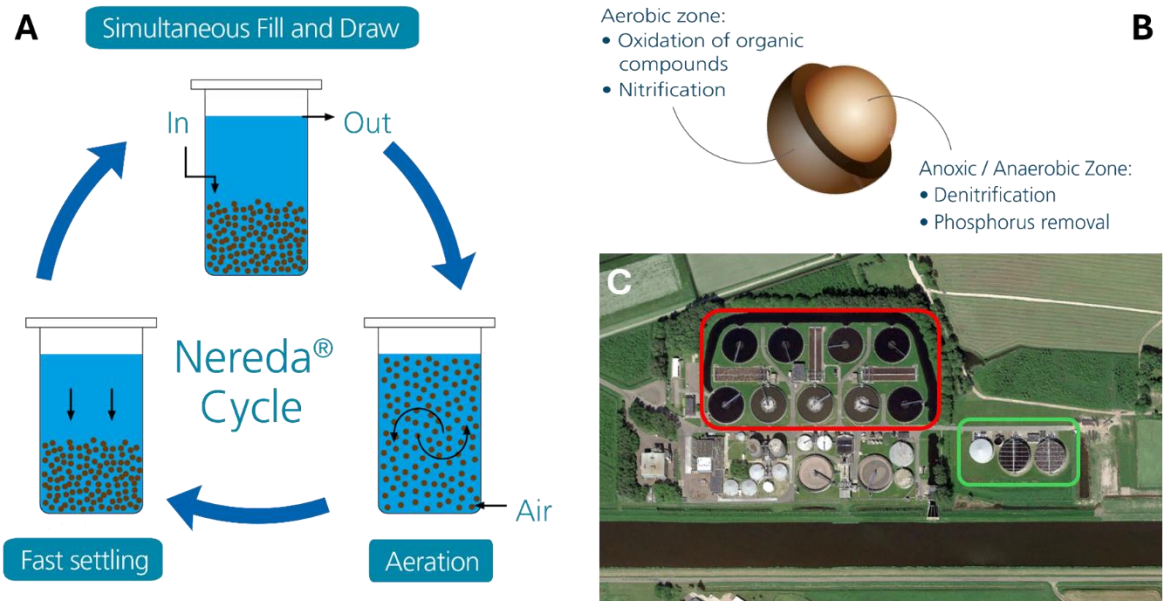


*Figure 3. Schematic representation of the Nereda® process cycle (A) and spatial zones with their respective conversions within granules (B). Picture (C) shows the Nereda® reactor footprint (green box) in comparison to the conventional activated sludge plant (red box). Courtesy of Royal HaskoningDHV.*

| Pros | Cons |
|---|---|
| **Resource recovery:** the granules contain large quantities of high-value organic material | **Long star-up time**: requires a long start-up time to establish a granular sludge blanket |
| **Small footprint:** requires less space due to high biomass concentration, simultaneous conversions, and fast-settling granules | **Batch process**: challenging to retrofit into existing continuous-flow plants and the effluent requires flow equalisation |
| **Energy efficiency:** simultaneous conversions eliminate the need for separate tanks, and fast-settling biomass requires smaller tank volumes decreasing associated energy costs | **Automation**: requires automated control systems |

### Enhanced Biological Phosphorus Removal

Enhanced Biological Phosphorus Removal (EBPR) is a cost-effective and sustainable method for phosphate removal that leverages the cyclical anaerobic-aerobic phases in wastewater treatment process (Ketchum et al., 1987). This cyclical environment is a key operational parameter that links AGS to the EBPR process. Central to this process is the microorganism *Candidatus* Accumulibacter Phosphatis, a typical PAO, which plays a crucial role in phosphate removal within WWTPs. These microorganisms are naturally found in aquatic sediments, where they adapt to cycles of nutrient and oxygen availability caused by seasonal and tidal mixing (He & Mcmahon, 2011). Although their precise ecological function is not fully understood, their metabolic capabilities suggest a significant role in the natural phosphorus cycle. To survive periodic substrate availability, these microorganisms rapidly take up and

store substrates as polyhydroxyalkanoates (PHA) under anaerobic conditions, using energy from the breakdown of their polyphosphate reserves. When oxygen becomes available and substrates are limited, they utilise PHA for growth and replenish their polyphosphate pools. This unique metabolic strategy provides *Candidatus* Accumulibacter Phosphatis with a competitive advantage in WWTPs that perform EBPR. In EBPR systems, the phosphorus is removed by extracting the excess sludge, which is enriched with phosphorus stored as polyphosphate, at the end of the treatment cycle (**Figure 4**).

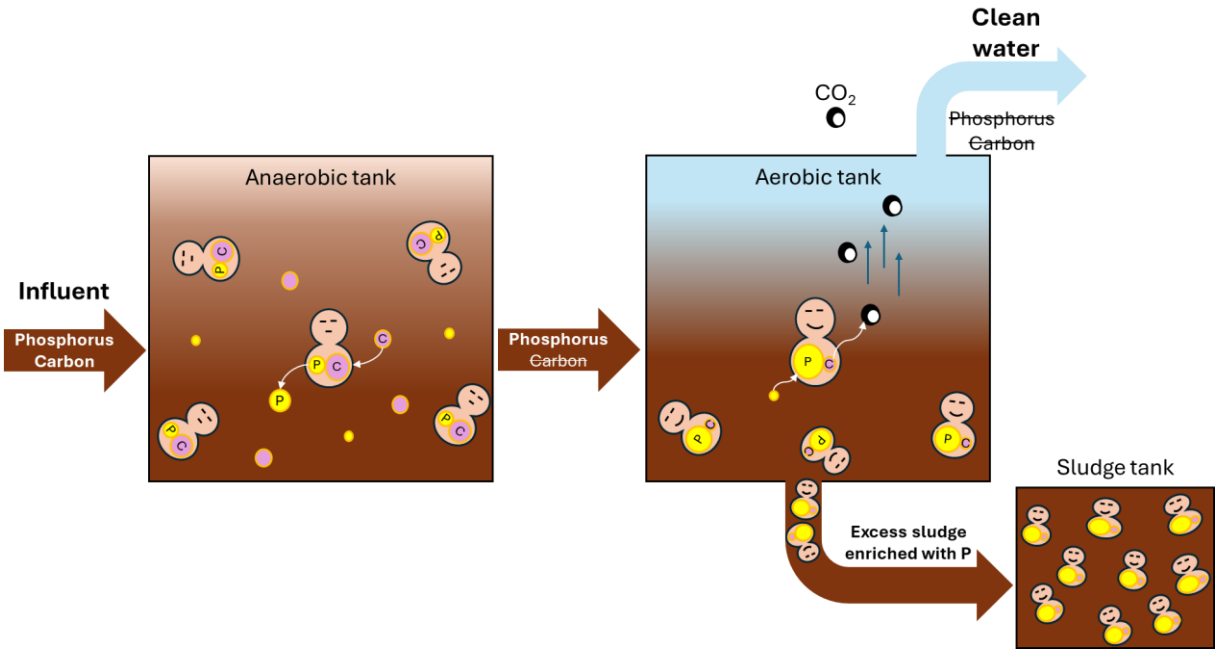


***Figure 4**. Graphical representation of phosphorus removal in WWTPs.*

### Effect of Substrate on Granulation and Phosphate Removal

When substrates like acetate or propionate are present, PAOs dominate due to their competitive advantage. However, PAOs cannot directly utilise all substrates which may require the help of other microorganisms, such as fermentative glycogen-accumulating organisms (fGAOs). These fermentative organisms can compete with PAOs for substrate, reducing EBPR efficiency. Despite their prevalence in WWTPs around the world, the role of these fGAOs is not well understood, making further study crucial to improving EBPR processes (Seviour et al., 2000). Glycerol is a common “waste” product in industrial processes like biodiesel and epoxy resin production, where it is generated in significant quantities (Pinto & De Araujo Mota, 2014). Its abundance and low cost have made glycerol an attractive carbon source for various applications, including wastewater treatment, where it can be dosed as an external carbon source when WWTPs face carbon shortage. However, the use of glycerol for granulation and phosphate removal has not been thoroughly investigated and can lead to deleterious effects. In mixed microbial cultures, glycerol can be fermented into a variety of products such as propionate or 1,3-propanediol under anaerobic conditions (Barbirato et al., 1997; Clomburg & Gonzalez, 2013) though the specific fermentation products depend on factors like the SRT, pH, the duration of anaerobic period, etc. For achieving phosphate

removal, it is important that glycerol is either directly utilised by PAOs or converted into PAO-utilisable products, such as VFAs, in the anaerobic phase. For granulation alone, the presence of PAOs is not a necessity as other slow-growing heterotrophs can also form compact and smooth granules. Glycerol can also be oxidised aerobically by fast-growing heterotrophs which can potentially harm the granulation process. Similar to glycerol, glucose is a compound that can be found in municipal wastewater from cellulose and starch polymers and in industrial effluents such as sugar production facilities. However, it has received limited attention regarding its role in granule formation. In EBPR studies, glucose has often been used with other substrates, making it difficult to isolate its specific effects.

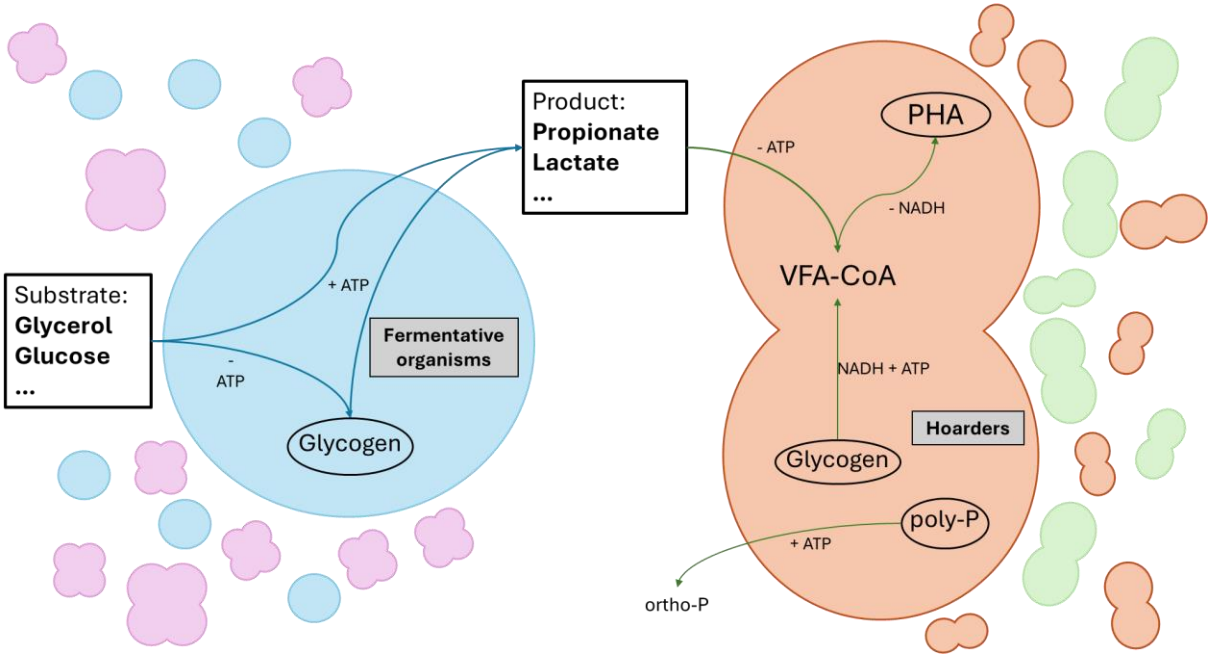


***Figure 5**. Graphical overview of substrate conversion in aerobic granular sludge. PHA: polyhydroxyalkanoates, Poly-P: polyphosphate, VFA-CoA: the combination of acetyl and propionyl-CoA.*

### Extracellular Polymeric Substances

In biofilm systems like AGS, microbial cells are embedded in extracellular polymeric substances (EPS), a hydrogel-like matrix that constitutes a significant portion of the sludge (**Figure 6**). EPS consists of primarily proteins, carbohydrates, lipids, and nucleic acids. It plays a crucial role in biofilm formation and functionality, making its extraction and characterisation a major research focus. With the rise of resource recovery ambitions in WWTPs, EPS has attracted the attention of various communities for its potential applications. Studies have highlighted its use in protein binding (Chen et al., 2024), as a biobased flame retardant (Kim et al., 2023), and as a bioflocculant (Ajao et al., 2021). However, despite its potential for various applications, the practical utilisation of EPS on commercial scales remains challenging. The challenge lies primarily in the supply chain but also in our lack of understanding of the exact properties and the influence of the extraction process on the final product properties.

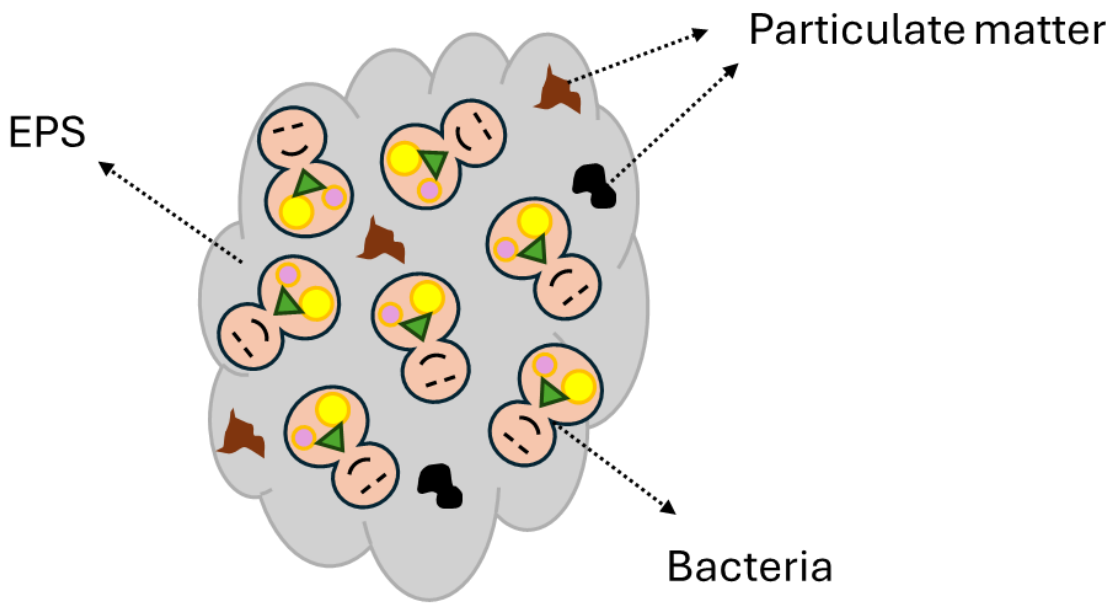


*Figure 6. Illustration of extracellular polymeric substances: The protective slime around microorganisms.*

**EPS as a flame-retardant**

The novel and complex composition of EPS makes it a suitable product for a variety of applications. One important component that makes EPS a good candidate for flame retardancy, is the organic phosphate esters. The organic phosphorus in EPS helps form a stable char layer during combustion, preventing heat exposure to the underlying material. Additionally, nitrogenous compounds, such as proteins found in EPS, release nitrogen gas when burned, diluting flammable gases. Such properties of EPS make it a suitable flame retardant that is non-toxic and biodegradable. In general, flame retardants can either be additive, applied after polymerization, or reactive, integrated during polymerization to become part of the material (Stockholm Convention, 2009). EPS offers versatility as both a reactive flame retardant resin for composite production and an additive for surface coating applications. The flame retarding properties of EPS-containing composites have been validated in the laboratory and meet the flame-retardancy requirements of US Federal Aviation Regulation standards (Kim et al., 2020). Moreover, the role of EPS in determining the flame-resistant and mechanical properties of composites made from natural fibres such as flax and cellulose was also studied and validated (Kim et al., 2023).

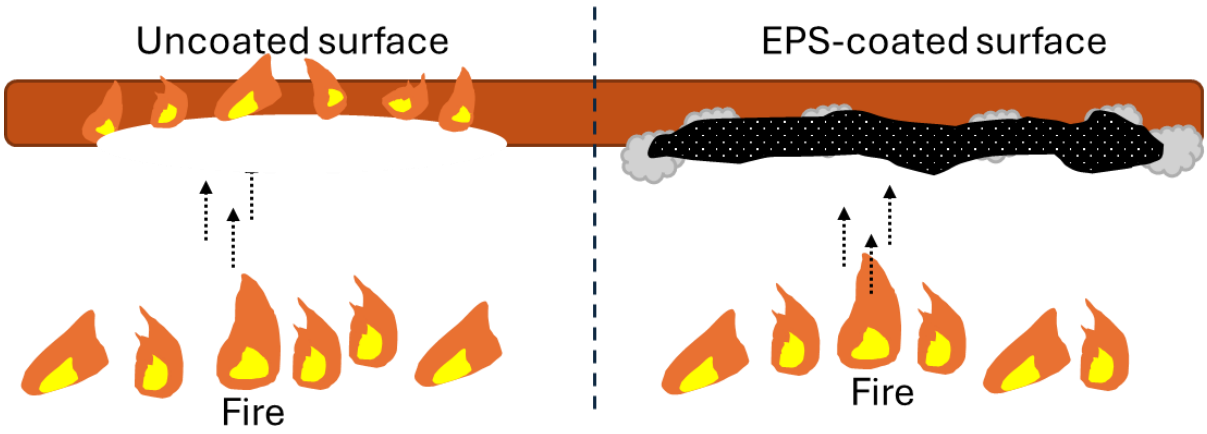


*Figure 7. Illustration of EPS as a flame retardant.*

### Saline Wastewater Treatment

Salt can enter WWTPs through industrial discharges and seawater intrusions, among other sources. Salinity affects various biological processes in WWTPs, with the degree of impact depending on factors such as salt concentration and composition. High salt concentrations can create osmotic pressure, inhibiting crucial microbial activities. While moderate salinity may enhance conversion rates up to a certain threshold, beyond this tipping point, salinity begins to hinder metabolic processes. This tipping point varies across microbial groups; for example, extremophiles thrive in conditions with over 15% NaCl, moderate halophiles perform best at 5–15% NaCl, and most conventional WWTP bacteria experience inhibition when salt concentrations exceed 2–3% NaCl (wt/v) (Sleator & Hill, 2001). There is evidence suggesting that salinity from pure sodium chloride impacts microbial processes differently than salinity from mixed salts, such as those found in seawater (de Graaff et al., 2020). Salinity induced by pure sodium chloride can disrupt cellular functions by interfering with intracellular sodium balance. In WWTPs, high salinity can alter the microbial community, influence the conversions, and destabilise the EPS matrix leading to poor effluent quality and solid-liquid separation.

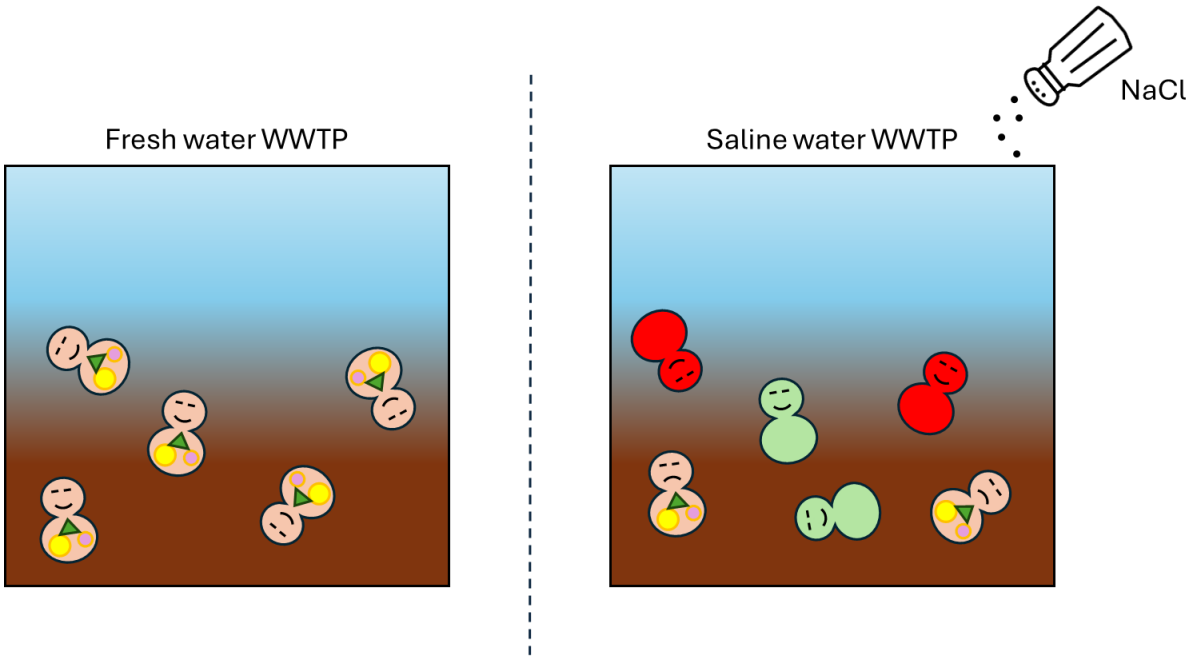


*Figure 8. Graphical overview of how salt influences the microbial community of WWTPs.*

## Thesis Outline

This thesis aimed to address key questions related to Case Studies 1 and 2 of the WATER-MINING project described in the preface section and the research gaps in AGS literature. This involved conducting a literature review to identify knowledge gaps and performing experimental research to address some of the gaps. In short, studying the conversions of fermentative substrates has received limited attention in the aerobic granular sludge literature. However, it is an important research topic for several key reasons:

1- **Microbial community dynamics**: the presence of fermentable substrates influences the composition and the behaviour of the microbial community in AGS. For instance, certain fermentative communities can compete with PAOs, reducing phosphate removal efficiency.
2- **Granule formation and stability**: substrates not utilised anaerobically are oxidised aerobically, negatively influencing the granulation process and ultimately the solid-liquid separation.
3- **Industrial relevance**: many industrial wastewaters contain fermentable substrates. Investigating how different substrates are converted allows for the optimisation of AGS processes.

Chapter 2

Chapter 2 investigated the conversions of glycerol in a lab-scale AGS reactor. A synthetic glycerol-containing medium was used to simulate the industrial effluent from the epoxy resin production facility described in Case Study 1. A bubble column sequencing batch reactor was

employed to cultivate granules capable of carbon and phosphorus removal. The study evaluated granulation, anaerobic conversions, nutrient removal, and microbial community dynamics.



Chapter 3

Chapter 3 investigated the use of glucose as a substrate for phosphate removal using AGS. A synthetic glucose-containing medium, simulating the effluent from the sugar industry described in Case Study 2, was prepared. The study aimed to understand the impact of glucose on phosphate removal and granule formation. A series of batch tests and cycle measurements were conducted to evaluate the anaerobic conversions. The microbial community was analyzed using metaproteomics and fluorescence in-situ hybridization to elucidate the relationship between fermentative microorganisms and polyphosphate-accumulating organisms and their contributions to AGS processes.

Chapter 4

Chapter 4 evaluated the impact of varying salinity levels on AGS processes. The investigation involved the long-term adaptation of the granules to increasing NaCl concentrations, comparing the effects of salinity induced by NaCl with those of seawater using aquatic salt crystals. The study provided detailed information on kinetic and anaerobic-aerobic conversions, the microbial community, and granulation. Additionally, the enzymatic machinery of the dominant taxa and key processes such as osmoregulation, substrate storage, and fermentation under saline conditions were assessed via metaproteomics.

Chapter 5

Chapter 5 was built on previous studies that investigated glycerol conversion in AGS reactors and the long-term performance of AGS under various salinities. We employed chemical analyses and Fourier-transform infrared spectroscopy as rapid screening tools to characterise lyophilised EPS from the samples obtained in Chapters 2 and 4. The results were analysed for correlations and differences using both 2-D correlation spectroscopy and qualitative analysis of IR peaks. Furthermore, we explored the potential application of EPS as a bio-based flame retardant, qualitatively assessing its effectiveness.

Chapter 6

Chapter 6 concludes the findings of this dissertation and provides an outlook for future research activities. The proposed research directions are divided into two parts: those based on scientific questions arising directly from this work, and those based on the economic evaluation encompassing OPEX, CAPEX, and profitability of a proposed treatment solution applicable to WP6 of the Water-Mining project. To support this, a simple model for AGS

treatment with EPS and biogas recovery was created using data derived from this thesis, literature, and expert knowledge.

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# 2. Glycerol Conversion by Aerobic Granular Sludge

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# Abstract

Glycerol is abundantly present in wastewater from industries such as biodiesel production facilities. Glycerol is also a potential carbon source for microbes that are involved in wastewater nutrient removal processes. The conversion of glycerol in biological phosphorus removal of aerobic granular sludge processes has not been explored to date. The current study describes glycerol utilization by aerobic granular sludge and enhanced biological phosphorus removal (EBPR). Robust granules with good phosphorus removal capabilities were formed in an aerobic granular sludge sequencing batch reactor fed with glycerol. The interaction between the fermentative conversion of glycerol and product uptake by polyphosphate accumulating organisms (PAO) was studied using stoichiometric and microbial community analysis. Metagenomic, metaproteomic and microscopic analysis identified a community dominated by *Actinobacteria* (*Tessaracoccus* and *Micropruina)* and a typical PAO known as *Ca*. Accumulibacter. Glycerol uptake facilitator (glpF) and glycerol kinase (glpK), two proteins involved in the transport of glycerol into the cellular metabolism, were only observed in the genome of the *Actinobacteria.* The anaerobic conversion appeared to be a combination of a substrate fermentation and product uptake-type reaction. Initially, glycerol fermentation led mainly to the production of 1,3-propanediol (1,3-PDO) which was not taken up under anaerobic conditions. Despite the aerobic conversion of 1,3-PDO stable granulation was observed. Over time, 1,3-PDO production decreased and complete anaerobic COD uptake was observed. The results demonstrate that glycerol-containing wastewater can effectively be treated by the aerobic granular sludge process and that fermentative and polyphosphate accumulating organisms can form a food chain in glycerol-based EBPR processes.


# Introduction

Aerobic Granular Sludge (AGS) is a state-of-the-art biological wastewater treatment technology that performs simultaneous carbon, nitrogen, and phosphorus removal (de Kreuk et al., 2007; Pronk et al., 2015b). Several factors distinguish AGS from conventional activated sludge systems. For instance, due to the compact structure and smooth surface of the granules, settling occurs rapidly and the redox gradient across the biofilm accommodates simultaneous conversions to take place within the granules (Bengtsson et al., 2018; de Bruin et al., 2004; de Kreuk and van Loosdrecht, 2006). These features reduce the treatment time significantly, enhance the solid-liquid separation and eliminate the need for multiple reactor compartments (Pronk et al., 2015a). The formation of aerobic granules was first demonstrated in 1998 and applying certain environmental pressures was reported to be key in achieving granules (Heijnen and Van Loosdrecht, 1998). The underlying mechanisms for granulation and achieving a system with stable performance are extensively studied and described in the literature (Etterer and Wilderer, 2001; Liu et al., 2005; Morgenroth et al., 1997; Picioreanu et al., 1997; van Dijk et al., 2022; Winkler et al., 2013).

Several parameters are important for obtaining stable and smooth granules. Of prime importance is the selection of slow-growing microorganisms such as Polyphosphate

Accumulating Organisms (PAO) and Glycogen Accumulating Organisms (GAO) that form dense smooth granules (de Kreuk and van Loosdrecht, 2004). When biodegradable organic carbon, usually Volatile Fatty Acids (VFA), is supplied to the system under anaerobic conditions, it is converted by certain microorganisms (PAOs for instance) into storage polymers known as Poly-Hydroxy-Alkanoates (PHA) (Oehmen et al., 2005b). Microorganisms growing on storage polymers have a slower growth rate than heterotrophic bacteria growing on readily biodegradable organic carbon in the presence of an electron acceptor (van Loosdrecht et al., 1997). Generally, slow-growing microorganisms (such as methanogens, nitrifiers, anammox, PAOs, GAOs) form a more compact structure and smoother granular biomass than fast-growing microorganisms. In the subsequent aerobic phase, the stored organic carbon is oxidized to provide energy for the formation of biomass and replenishment of the intracellular polymer pool (Oehmen et al., 2007; Tarayre et al., 2016). Similarity in operational parameters such as anaerobic-aerobic cycles in both AGS and Enhanced Biological Phosphorus Removal (EBPR) is also a common link between the two systems which selects organisms that are important for the successful application of these technologies.

EBPR is an environmentally sustainable and economically competitive technology (Bunce et al., 2018; Ketchum et al., 1987). The technology is based on the enrichment of the sludge with PAOs that can excessively accumulate phosphorus (P) in the form of poly-phosphate (poly-P) after which P removal is achieved through excess sludge discharge (Smolders et al., 1994a). However, the stability of these systems depends on various factors such as the carbon source which act as the electron donor (Guerrero et al., 2011; Wei et al., 2014). In literature, an overwhelming amount of information exists on EBPR with VFAs mainly acetate and propionate as the carbon source (Bunce et al., 2018; Oehmen et al., 2007, 2005a, 2005b; Pijuan et al., 2005, 2004; Smolders et al., 1994a). However, when other sources of carbon such as alcohol or sugars are used, these systems manifest different behaviours. For instance, a period of adaptation is required by PAOs when ethanol is used (Puig et al., 2008) whereas methanol can be used via a syntrophic consortium between acetogens and PAOs (Tayà et al., 2013). In a study by (Begum and Batista, 2012), the authors reported EBPR deterioration due to the proliferation of GAOs over PAOs with glucose as substrate. In addition to traditional PAOs, the presence of fermentative microorganisms in dynamic feast-famine conditions that can perform fermentation and anaerobically accumulate storage compounds without cycling polyphosphate has been reported (Carucci et al., 1999; McIlroy et al., 2018). These fermentative GAOs (fGAO) play a potentially important role in EBPR systems since they can convert the substrate into PAO-utilizable product enabling PAO proliferation or they can compete for substrate uptake compromising the phosphorus removal efficiency of the system. Therefore, the carbon source in the wastewater plays a decisive role in the success of the EBPR process.

Glycerol is a compound that can be dominant in a variety of industrial wastewaters such as biodiesel and epoxy resin production (Pinto and De Araujo Mota, 2014). For instance, the production of every 10 kilograms of biodiesel generates about 1 kilogram of glycerol (Plácido and Capareda, 2016), and the wastewater generated from the epoxy resin production facilities contain a significant amount of glycerol. Due to its abundance and relatively low price, the use of glycerol has attracted the attention of different engineering communities for

various applications (Lima et al., 2021). In wastewater treatment, glycerol has been reported to be a suitable source of carbon for the removal of nitrogen and phosphorus (Coats et al., 2015; Smyk and Ignatowicz, 2017; Yuan et al., 2010). However, the conversion of glycerol in AGS-EBPR processes or the formation of AGS using glycerol has not been studied in detail. The anaerobic conversion of glycerol by both mixed and pure microbial cultures has been extensively studied over the years (Barbirato et al., 1995; Gupta et al., 2009; Katarzyna Leja et al., 2011; Temudo et al., 2008) and 1,3-propanediol (1,3-PDO) is the most common reported product among the products of glycerol fermentation in literature (Clomburg and Gonzalez, 2013; Moscoviz et al., 2016). Several studies have also reported the production of propionate from the fermentation of glycerol using mixed microbial cultures (Chen et al., 2016; Yuan et al., 2010). In EBPR systems, glycerol could directly be taken up by PAOs, but also potentially fermented to a variety of products that can subsequently be utilized by the PAOs or only be converted under aerobic conditions. The latter process could potentially harm the granulation process as fast aerobic growth would lead to less favourable sludge morphologies (Pronk et al., 2015a). Given that 1,3-PDO is a more reduced compound than glycerol, microorganisms are required to generate more oxidized compounds (e.g. $CO_2$) to maintain their redox balance. Propionate synthesis from glycerol is both redox neutral (**Table** 1) and energy positive with 1 or more mol ATP produced per mol glycerol fermented (Barbirato et al., 1997; Gonzalez-Garcia et al., 2017; Liu et al., 2011) versus no ATP generated from 1,3-PDO production (Temudo et al., 2008).

$$Y_{ATP,1,3-PDO} \qquad Glycerol + H^+ + NADH \rightarrow 1{,}3\ propanediol + H_2O \qquad (1)$$

$$Y_{ATP,Propionate} \qquad Glycerol \rightarrow Propionate + H_2O + 1\ ATP \quad (Wood - Werkman\ cycle) \qquad (2)$$

Information on the effect and conversion of glycerol in AGS and EBPR processes is lacking which is important for a wider application of the technology. Here, we study the potential of glycerol as an electron donor to promote aerobic granular sludge formation and evaluate the carbon and phosphorus removal of the system. We further elucidate the anaerobic-aerobic conversions by linking process stoichiometry to microbial community analysis.

# Material and Methods

### Experimental setup and reactor operation

The experiments were conducted in a bubble column reactor and operated in a Sequencing Batch Reactor (SBR) configuration. The working volume of the reactor was 2.8 L with an internal diameter of 5.6 cm and a total height of 90 cm. After effluent withdrawal, 1.3 L remained in the reactor after each cycle representing a volumetric exchange ratio of 54%. The pH was controlled at 7.1 ± 0.1 by either doing 1M NaOH or 1M HCl. The Dissolved Oxygen (DO) was controlled at 0% and 50% (3.5 mg/L) saturation during the anaerobic and aerobic

phases, respectively, by a controlled mixture of nitrogen gas and air. The temperature was not controlled. The room temperature was controlled at 20 °C. The reactor was seeded with aerobic granular sludge from a pilot-scale municipal wastewater treatment reactor performing EBPR located in Harnaschpolder, the Netherlands.

The influent was 1,500 mL consisting of 1,200 mL of demineralized water, 150 mL of medium A and 150 mL of medium B. Medium A contained 35.7 mM glycerol, 3.6 mM $MgSO_4.7H_2O$, and 4.7 mM KCl. Medium B contained 41.1 mM $NH_4Cl$, 1.95 mM $K_2HPO_4$, 1.98 mM $KH_2PO_4$, 0.6 mM Allythiourea (ATU) to inhibit nitrification and 10 mL/L of trace element solution. The trace element solution contained 4.99 g/L $FeSO_4.7H_2O$, 2.2 g/L $Zn.SO_4.7H_2O$, 7.33 g/L $CaCl_2.2H_2O$, 4.32 g/L $MnSO_4.H_2O$, 2.18 g/L $Na_2MoO_4.2H_2O$, 1.57 g/L $CuSO_4.5H_2O$, 1.61 g/L $CoCl_2.6H_2O$ and 50 g/L EDTA. The combination of these feed streams resulted in an influent concentration of 400 mg/L COD, 57.6 mg/L $NH_4^+$-N, and 12.2 mg/L of $PO_4^{-3}$-P.



The reactor cycles consisted of 5 min of nitrogen sparging to ensure an anaerobic condition before feeding followed by 5 min of feeding, 60 min of nitrogen sparging, 120 min of aeration, 5 min of settling, and 5 min of effluent withdrawal. The off-gas was recirculated with a flow of 5 L/min to keep a steady DO concentration.

### Analytical methods

Concentrations of phosphate and ammonium were measured using a Gallery Discrete Analyser (ThermoFisher Scientific, USA). Chemical Oxygen Demand was measured with a spectrophotometer cuvette system (DR2800, Hach Lange, USA). Volatile fatty acids and sugars were measured using an HPLC (Vanquish, ThermoFisher Scientific, USA) equipped with an RI and UV detector, Aminex HPX-87H column (BioRad, USA) using 0.0015M phosphoric acid as eluent.

### PHA and Glycogen determination

Before both glycogen and PHA analysis, the biomass sample from the reactor was fixed with the addition of 4% w/v paraformaldehyde, washed with demineralized water, freeze-dried, and pottered into a fine powder. The extraction and measurement of PHA were performed according to the protocol described by (Riis and Mai, 1988) with minor adjustments. To summarize, the PHA in the freeze-dried biomass (30 mg) was hydrolysed and esterified in an HCL, Propanol, and Dichlorethane solution with a ratio of 1:4:5 v/v/v for 3h (Poly-Hydroxy-Butyrate & Poly-Hydroxy-Valerate) and 20h (Poly-Hydroxy-2-Methyl-Valerate) at 100°C with frequent manual vortexing. After cooling to room temperature, 3 mL of ultrapure water was added to the sample, vortexed, and centrifuged to separate the two phases. The formed propyl esters in the organic phase were then filtered and analysed by Gas Chromatography (6890N, Agilent, USA). Benzoic acid (50 µL) was used as an internal standard. Quantification of PHB, PHV, and PH2MV was done using commercial 3-hydroxybutyrate, R-3-hydroxyvalerate, and 2-hydroxyhexanoate as standards (Sigma-Aldrich, USA).

The glycogen content of the biomass was extracted according to the method described by (Smolders et al., 1994b) but with the addition of 0.9 M HCl to the sample and heating it for 5 h at 100 °C with frequent manual vortexing. The digestate was then filtered using 0.45 µm Millipore filters and the poly-glucose was analysed using an HPLC.

### Batch experiments

A batch test was performed by addition of excess glycerol (14 mM) and prolonging the anaerobic phase to 29 hours to determine the fermentation product. The propionate batch test was performed with a similar concentration in a normal cycle as described above. The pH was controlled at 7.1 ± 0.1 by either dosing 1M NaOH or 1M HCl. The Dissolved Oxygen (DO) was controlled at 0% and 50% saturation during the anaerobic and aerobic phases, respectively, by a controlled mixture of nitrogen gas and air. The temperature was not controlled. The room temperature was controlled at 20 °C. Liquid and biomass samples were collected for bulk liquid analysis and PHA and glycogen content determination.

### Biomass measurements

Granules were taken from the reactor and washed with demineralized water to wash off the impurities and salts. The granules were then placed in an oven at 105 °C for 24 hours to determine the Total Suspended Solids (TSS). The dried biomass was then incinerated at 550 °C for 3 hours to determine the ash content and Volatile Suspended Solids (VSS).

### Microscopy

A stereo zoom microscope (M205 FA, Leica Microsystems, Germany) was used to capture the images of the granules equipped with Qwin image analysis software (V3.5.1, Leica Microsystems, Germany).

Safranin and crystal violet were used to stain Gram-positive and -negative bacteria, respectively. Iodine was added to fix the safranin to the peptidoglycan molecules of Gram-positive bacteria. Microscopic images were captured with Axio Imager M2 (Zeiss, Germany) equipped with ZEN (blue edition) software.

Biomass samples were handled and stained for FISH according to the protocol described by (Bassin et al., 2011). A mixture of probes EUB338, EUB338-II, and EUB338-III (EUBmix) was used to stain all bacteria (Amann et al., 1990; Daims et al., 1999). To visualize PAOs, probe PAO651 was used (Crocetti et al., 2000). Probe Actino658 was used to visualize Actinobacteria (Kong et al., 2005). List of FISH probes tested are available in the supplementary material in the online publication. The images were captured with an epifluorescence microscope equipped with filter set Cy3 (ET545/25x ET605/70m T565LPXR), Cy5 (ET640/30x ET690/50m T660LPXR), and FITC (ET470/40x ET525/50m T495LPXR) (Axio Imager M2, Zeiss, Germany).

### Microbial community analysis by metagenomics and metaproteomics

DNA from fresh biomass was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to the protocol from the manufacturer. DNA concentration was determined with Qubit fluorometer (ThermoFisher Scientific, USA). Metagenome sequencing and raw data processing (reads assembly and identification of genes) were performed by Novogene (Novogene Co., China). Protein extraction and shotgun proteomic analysis were performed according to (Kleikamp et al., 2022). Briefly, approx. 250 ng of the proteolytic digest was analysed using a nano-liquid-chromatography system consisting of an EASY nano-LC 1200 equipped with an Acclaim PepMap RSLC RP C18 separation column and a QE plus Orbitrap mass spectrometer (ThermoFisher Scientific, Germany). The Orbitrap was operating in data-dependent acquisition (DDA) mode, where the top 10 signals were isolated at a window of 2.0 m/z and fragmented using an NCE of 28. Fragments were acquired at 17 K resolution with a max IT of 75 ms and an AGC target of 2e5. Unassigned, singly and >6 times charged mass peaks were excluded from fragmentation. The analysis of the mass spectrometric raw data was performed by database searching using the metagenomics constructed reference database (or alternatively a focused database containing only Uniprot genomes of the 3 main genera) and PEAKS X (Bioinformatics Solutions Inc., Canada). Thereby, the search allowed for 20 ppm parent ion and 0.02 m/z fragment ion mass error, 2 missed cleavages, carbamidomethylation as fixed, and methionine oxidation and N/Q deamidation as variable modifications. Peptide-spectrum matches were filtered for a 1% False Discovery Rate (FDR) and protein identifications with 2 or more unique peptides were considered significant. Annotation of functions of the proteins identified using the metagenomics reference sequence database was performed using the KEGG database using GhostKoala: www.kegg.jp/ghostkoala.

### ATP & NADH balances

To evaluate the substrate distribution between the fermentative organisms and the PAO community, ATP and NADH balances were made for each organism. The balance was made based on the assumption 1- that glycerol is anaerobically converted to propionate and glycogen and that the produced propionate is subsequently taken up by the PAO community to synthesize PHA (verified via mass balances) and 2- the majority of the community was dominated by PAOs (using FISH and proteomics) indicating minor contribution to substrate distribution by other potential communities (see discussions for further elaborations). The parameters used in the calculations to estimate propionate production are listed through equations 3 – 6 derived from Principles of biochemistry (Nelson et al., 2013). Note that the degree of reduction of glycerol and propionate are the same and thus, the NADH balance is a conserved moiety in this reaction.

$$-Glycerol - ATP + DHAP + NADH \quad (3)$$

$$-2\ DHAP - ATP + \ Glycogen \quad (4)$$

$$-DHAP + Pyruvate + 2\ ATP + NADH \quad (5)$$

$$-Pyruvate - 2\ NADH + Propionate \quad (6)$$

The ATP and NADH balance over the PAOs was performed according to the model describing the anaerobic metabolism of propionate by PAOs in EBPR systems by (Oehmen et al., 2005c)). In short, PAOs derive the required energy for propionate uptake and PHA synthesis through poly-P and glycogen hydrolysis. The latter process also generates reducing equivalents, i.e. NADH, required for the maintenance of redox balance within the cell.

***Table 1**. List of reactions showing complete oxidation of each compound to $CO_2$. k represents mole electrons per mole substrate.*

| **Compounds** | **Reactions** | **k** |
|---|---|---|
| *Glycerol* | $C_3H_8O_3 + 3H_2O \rightarrow 3CO_2 + 14H^+ + 14e^-$ | 14 |
| *Propionate* | $C_3H_6O_2 + 4H_2O \rightarrow 3CO_2 + 14H^+ + 14e^-$ | 14 |
| *1,3-Propanediol* | $C_3H_8O_2 + 4H_2O \rightarrow 3CO_2 + 16H^+ + 16e^-$ | 16 |

# Results

## Reactor operation

Granular sludge from a pilot municipal Wastewater Treatment Plant (WWTP) in the Netherlands performing EBPR was used to inoculate the reactor. After a few weeks of reactor operation and the formation of new biomass, the sludge colour changed from dark brown to light brown/orange. The settling time was step-wise decreased from 20 to 5 minutes within 4 weeks of operation. With the development of granular sludge, the effluent became clearer and the flocculent biomass disappeared from the system. Once granular biomass formation stabilized (from day 65 onwards), a Solids Retention Time (SRT) of 12 days was maintained by manual sludge removal and solids washout via the effluent. After the reactor reached a pseudo-steady state, an average Sludge Volume Index ($SVI_5$) of 33.4 mL/g was obtained from the sludge settled bed in the reactor after 5 minutes of settling. The TSS and VSS concentration in the reactor stabilized at an average of 7.3 and 5.5 g/L (i.e. 24% ash content), respectively.

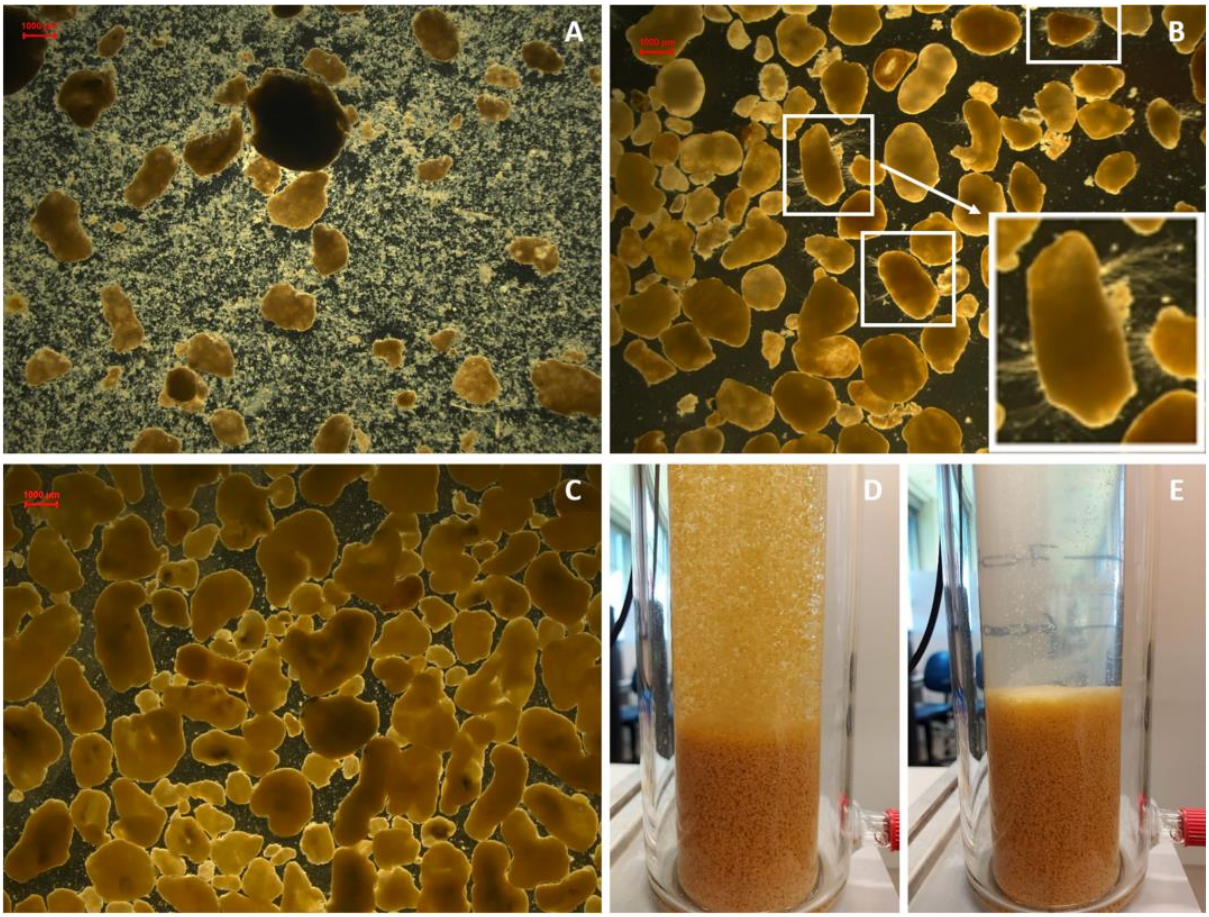


***Figure 9**. Stereoscopic overview of the aerobic granular sludge grown on glycerol. Picture (A) was taken during the initial days of reactor operation, (B) day 37, (C) end of the experimental period day 226, (D & E) shows biomass settling and the settled bed day 226, respectively. The pictures show the washout of the flocculent biomass and the formation of dense smooth granules over time. Scale bar equals 1000 µM. Colour.*

Once a pseudo-steady-state was achieved (day 126 onwards) an average P-release of 64.3 mg/L was obtained with a specific P-release and -uptake rate of 10.9 and 16.3 mgP/gVSS/h, respectively. Pseudo-steady-state was defined on the basis of stable conversion of substrates and phosphate throughout the cycles. The anaerobic glycerol uptake rate was 2 C-mmol/gVSS/h (76 mgCOD/gVSS/h). The anaerobic P-release to complete carbon uptake (P:C) ratio during a typical cycle was 0.23 P-mol/C-mol with an aerobic ammonium consumption rate of 0.8 mgN/gVSS/h for biomass growth (no nitrification).

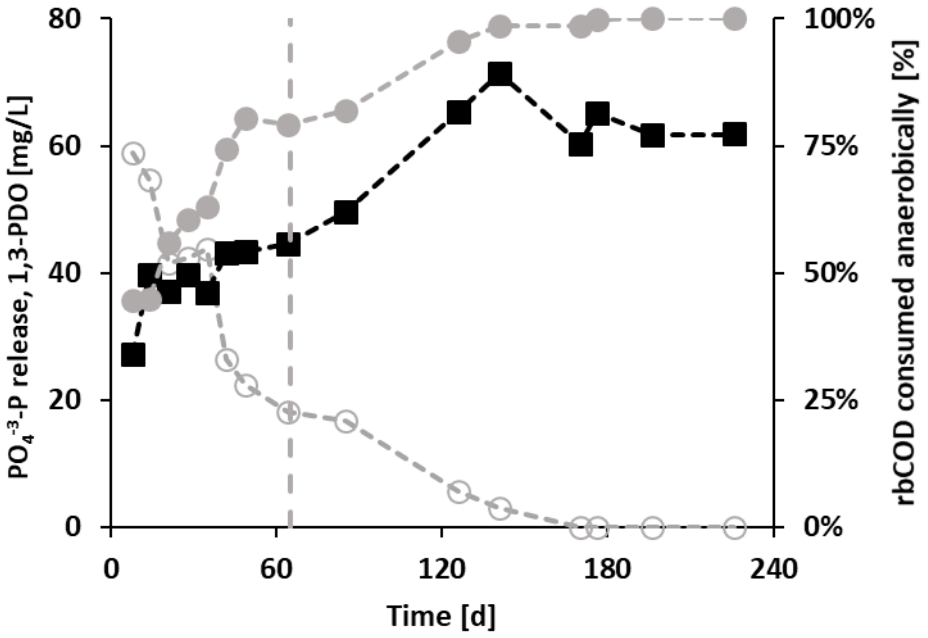


***Figure 10**. Concentrations of 1,3-PDO (hollow circles), percentage of rbCOD removed (closed circles), and orthophosphate (squares) at the end of the anaerobic phase over time. rbCOD represents glycerol in COD equivalents. The dashed vertical line indicates the start of SRT control.*

**Figure 10** shows the concentrations of 1,3-PDO and orthophosphate in the bulk liquid at the end of the anaerobic phase versus the percentage of rbCOD (representing glycerol) removed anaerobically. Coupled with increased anaerobic glycerol removal and the reduction of 1,3-PDO production, P release was increased. Initially, the leftover COD from the anaerobic phase was oxidized in the subsequent aerobic phase. The aerobic conversion caused floc formation that was washed out once the settling time decreased to 5 minutes. As the anaerobic production of 1,3- PDO decreased, a sludge bed with smooth granules was formed (**Figure 9**). Anaerobic 1,3-PDO formation decreased over time, indicating formation of other fermentation products (such as propionate). The formation of a different fermentation product was also reflected in increasing P release, likely due to uptake of formed propionate by PAOs.

### Chemical transformations in a typical cycle

**Figure 11** shows the anaerobic-aerobic chemical transformations during a cycle. In the anaerobic phase, glycerol uptake, P release, PHA accumulation, and minor glycogen degradation occurred concurrently. In the aerobic phase, PHA oxidation, P uptake, and glycogen replenishment were observed.

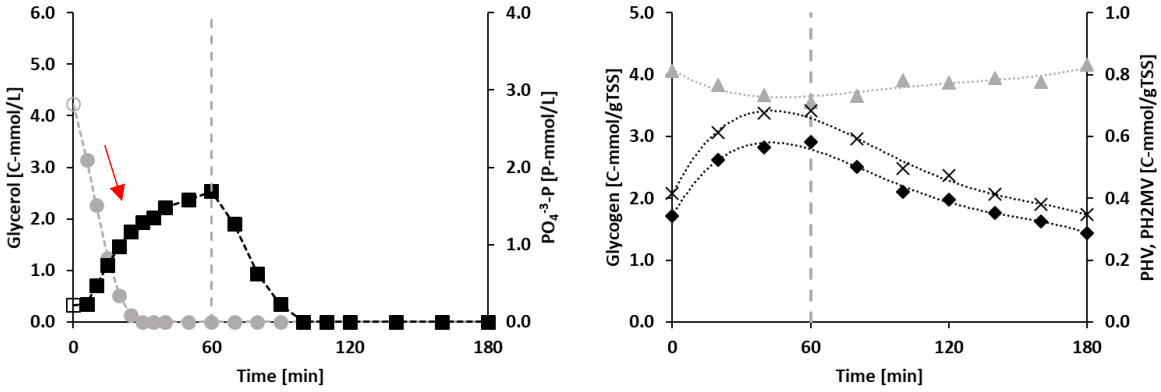


***Figure 11**. Left- Glycerol (circles), $PO_4^{-3}$-P (squares) and right- PHV (crosses), $PH_2MV$ (diamonds), and glycogen (triangles) profiles during a typical cycle. The dashed vertical line indicates the switch between the anaerobic and aerobic phases. The first hollow points in the graph (left) are calculated based on dilution and influent concentration. The arrow indicates the start of secondary P release which occurs after complete glycerol uptake.*

The ratio of glycogen degraded and PHA accumulated to glycerol consumed in the anaerobic phase was 0.27 C-mol$_{glycogen}$/C-mol$_{glycerol}$ and 0.97 C-mol$_{PHA}$/C-mol$_{glycerol}$. The average distribution of the PHA content in the biomass at the end of the anaerobic phase was 54% PHV and 46% PH2MV. No PHB was detected. The mass balance performed over the anaerobic glycerol uptake, PHA production, and glycogen degradation closed with a recovery of 77% and 81% for carbon and electron (COD), respectively. The incomplete carbon and electron recovery was assumed to be due to $CO_2$ losses and anaerobic growth on the stored glycogen. The anaerobic growth was calculated using the theoretical glycogen production of 0.5 mol$_{glycogen}$/mol$_{glycerol}$ based on ATP calculations and the anaerobic biomass yield of 0.2 gCOD/gCOD for *Propionibacterium* (Sykes, 1975). See discussion for the explanation of anaerobic glycogen production and growth.

## Batch experiments

To identify the products formed from glycerol fermentation in the anaerobic phase, a batch test with excess glycerol (14 mmol/L) and an extended anaerobic period of 23 hours was conducted. Once glycerol was completely removed, the formation of mainly propionate (74% molar basis) along with minor amounts of lactate, formate, acetate, valerate, iso-butyrate, and 1,3- PDO was observed. The mass balance performed over the entire anaerobic phase with glycerol uptake, product formation, PHA production, and glycogen degradation closed with a recovery of 98% and 106% for carbon and electron balance, respectively. The distribution of the PHA at the end of the anaerobic phase was 9% PHB, 53% PHV, and 38% PH2MV. Further information regarding the batch experiment can be found in the supplementary material of the electronic version.

*Table 2. Comparison of anaerobic stoichiometries with glycerol and propionate as substrate. $P_{rel}$: Phosphorus release, $PHA_{pro}$: PHA production, $C_{up}$: Carbon uptake, $Gly_{deg}$: Glycogen degradation*

| Conversions | Glycerol | Glycerol | Propionate | Propionate | Units |
|---|---|---|---|---|---|
| | This study | Guerrero et al., 2012 | This study | Oehmen et al., 2005 | |
| $P_{rel}/C_{up}$ | 0.23 | 0.20 | 0.47 | 0.42 | P-mol/C-mol |
| $PHA_{pro}/C_{up}$ | 0.97 | 0.31 | 1.13 | 1.23 | C-mol/C-mol |
| $PHB_{pro}/C_{up}$ | 0.00 | 0.08 | 0.00 | 0.04 | C-mol/C-mol |
| $PHV_{pro}/C_{up}$ | 0.53 | 0.14 | 0.68 | 0.55 | C-mol/C-mol |
| $PH2MV_{pro}/C_{up}$ | 0.44 | 0.09 | 0.45 | 0.65 | C-mol/C-mol |
| $Gly_{deg}/C_{up}$ | 0.27 | 0.25 | 0.30 | 0.33 | C-mol/C-mol |

To compare the results obtained from the conversion of glycerol, a batch test with propionate (similar initial concentration) was conducted (**Figure 12**). Similar to the glycerol reactor, propionate uptake, P release, PHA accumulation, and glycogen degradation occurred in the anaerobic phase. In the aerobic phase, PHA oxidation, P uptake, and glycogen replenishment were observed. The anaerobic balance performed over propionate uptake, PHA production, and glycogen degradation closed with a recovery of 87% and 92% for carbon and electron (COD) balance. The incomplete carbon and electron recovery was assumed to be due to $CO_2$ losses and possibly anaerobic growth. The distribution of the PHA content in the biomass at the end of the anaerobic phase was 60% PHV and 40% PH2MV with no PHB detection.

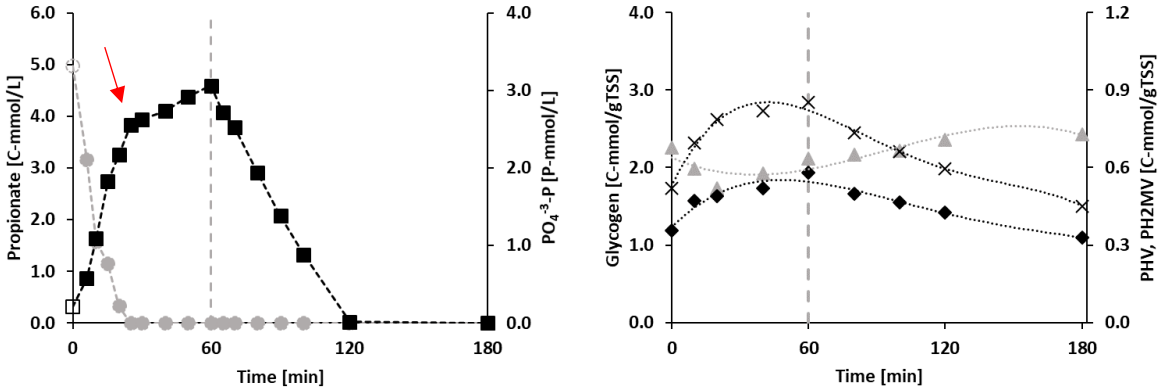


***Figure 12**. Propionate (circles), PO4-3-P (squares), PHV (crosses), PH2MV (diamonds), and glycogen (triangles) profiles during the batch test. The dashed vertical line indicates the switch between the anaerobic and aerobic phases. The first hollow points in the graph (left) are calculated based on dilution and influent concentration. The arrow indicates the start of secondary P release which occurs after complete glycerol uptake.*

## ATP & NADH balances

The ATP & NADH balances were made over the anaerobic phase to estimate substrate distribution among the fGAOs and the PAOs. We assumed a two-step process, fermentation of glycerol to propionate and glycogen by fGAOs and propionate uptake by PAOs. First, an ATP balance was made over the fGAOs by assuming that the ATP generated via propionate

formation from glycerol is used for substrate uptake and possibly storage processes (**Table** 3). Second, the amount of glycogen hydrolyzed by the PAOs was estimated based on the NADH required for the synthesis of PHA produced in the anaerobic phase (**Table** 4). Third, by taking the theoretical propionate yield and the estimated hydrolyzed glycogen into account, an ATP balance was made over the PAOs (**Table** 5).



***Table 3**. ATP balance over the fermentative organisms.*

| Fermentative organisms | | | | |
|---|---|---|---|---|
| **ATP required** | **ATP produced** | **ATP balance** | **Estimated** | |
| Glycerol uptake and storage | Propionate synthesis | | Propionate yield | Glycogen Yield |
| mol/C-mol | mol/C-mol | % | mol/mol | |
| -0.33 | 0.33 | 100 | 0.5 | 0.5 |

***Table 4**. NADH balance over the PAOs.*

| PAO | | | | |
|---|---|---|---|---|
| **NADH required** | | **NADH produced** | **NADH balance** | **Estimated** |
| PHV | PH2MV | Glycogen hydrolysed | | Glycogen hydrolysed |
| mol/C-mol | mol/C-mol | mol/C-mol | % | C-mmol |
| -1.31 | -0.88 | 2.13 | 97 | 3.28 |

***Table 5**. ATP balance over the PAOs. VFA-CoA is the combination of acetyl- and propionyl-CoA.*

| PAO | | | | |
|---|---|---|---|---|
| **ATP required** | | **ATP produced** | | **ATP balance** |
| VFA-CoA activation | Transport | Poly-P hydrolysis | Glycogen hydrolysis | |
| mol/C-mol | mol/C-mol | mol/C-mol | mol/C-mol | % |
| -0.33 | -0.11 | 0.35 | 0.09 | 101 |

## Microbial community

Biomass samples from the reactor were frequently observed under the microscope to track the evolution of the microbial community during the experimental period. Visually, two morphologically distinct types of microorganisms were observed (**Figure 13**). Gram staining was performed as the first identification technique and for FISH protocol adjustment. Tetrad-shaped microorganisms were identified as Gram-positive and the microorganisms growing in pairs were Gram-negative. The presence of storage compounds (dark spots) was only observed in the latter morphotype (**Figure 13**.A).

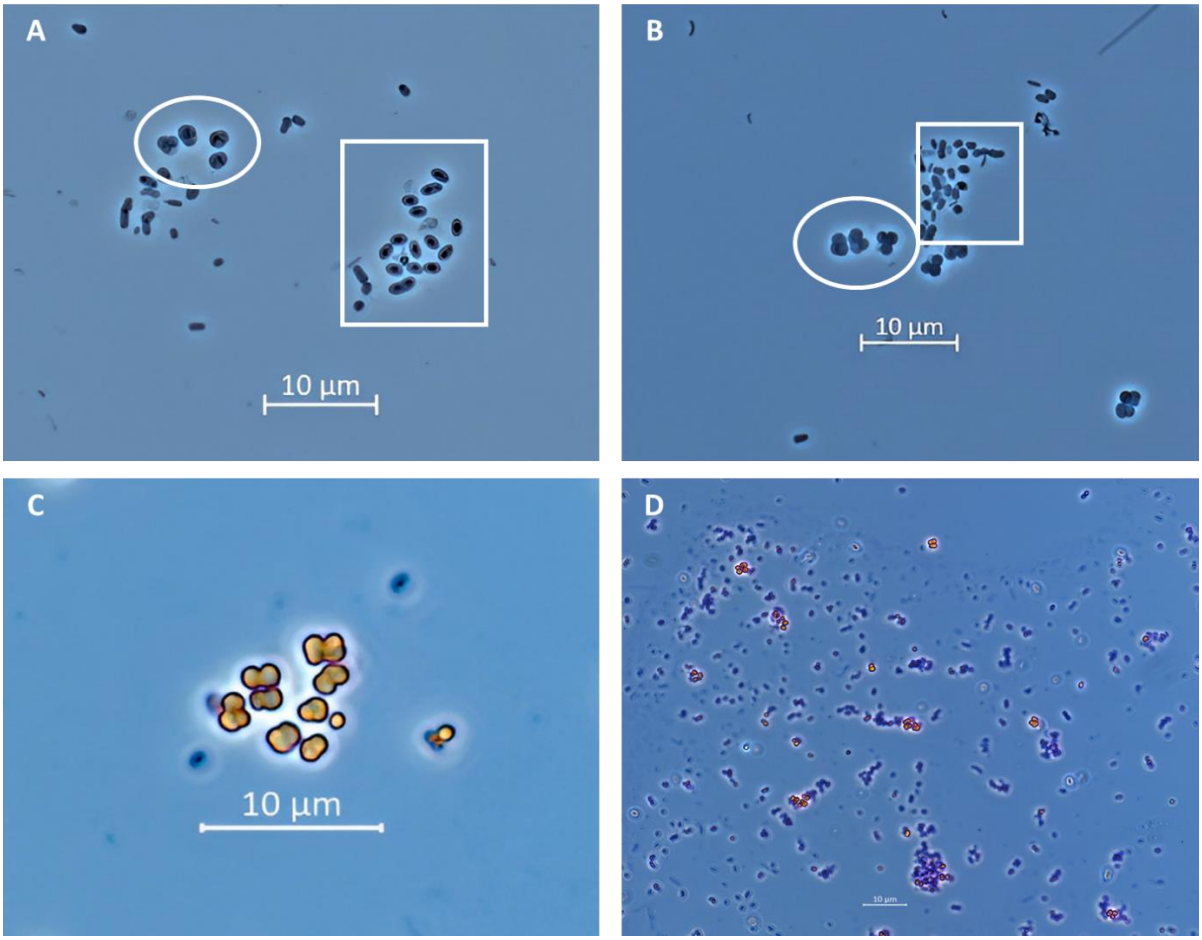


***Figure 13**. Microscopic image of glycerol-cultivated biomass sample taken with 1000x magnification. Picture (A & B) cocci-shaped microorganisms arranged in tetrads (circles), rod-shaped cocci arranged in pairs or single cells (squares), (C) close-up of Gram-positive cells stained with Safranin, (D) Gram-positive cells (orange) and Gram-negative cells (violet). Colour.*

Gram staining showed a sparse presence of the tetrad-shaped microorganisms (**Figure 13**.D). FISH analysis of the biomass indicated the dominant presence of PAOs along with tetrad-shaped microorganisms in the sludge sample (**Figure 14**). The presence of both Gram-positive and –negative made hybridization difficult. Thus, to visualize both types of microorganisms in one sample, the protocol to hybridize the cells was adjusted and several strategies were tested. To prevent Gram-negative cells disintegration and ensuring hybridization of Gram-positive cells, permeabilization was not performed instead the hybridization duration was increased to 3 days. Other probes have also been tested to visualize the tetrad-shaped microorganisms but no signal was observed.

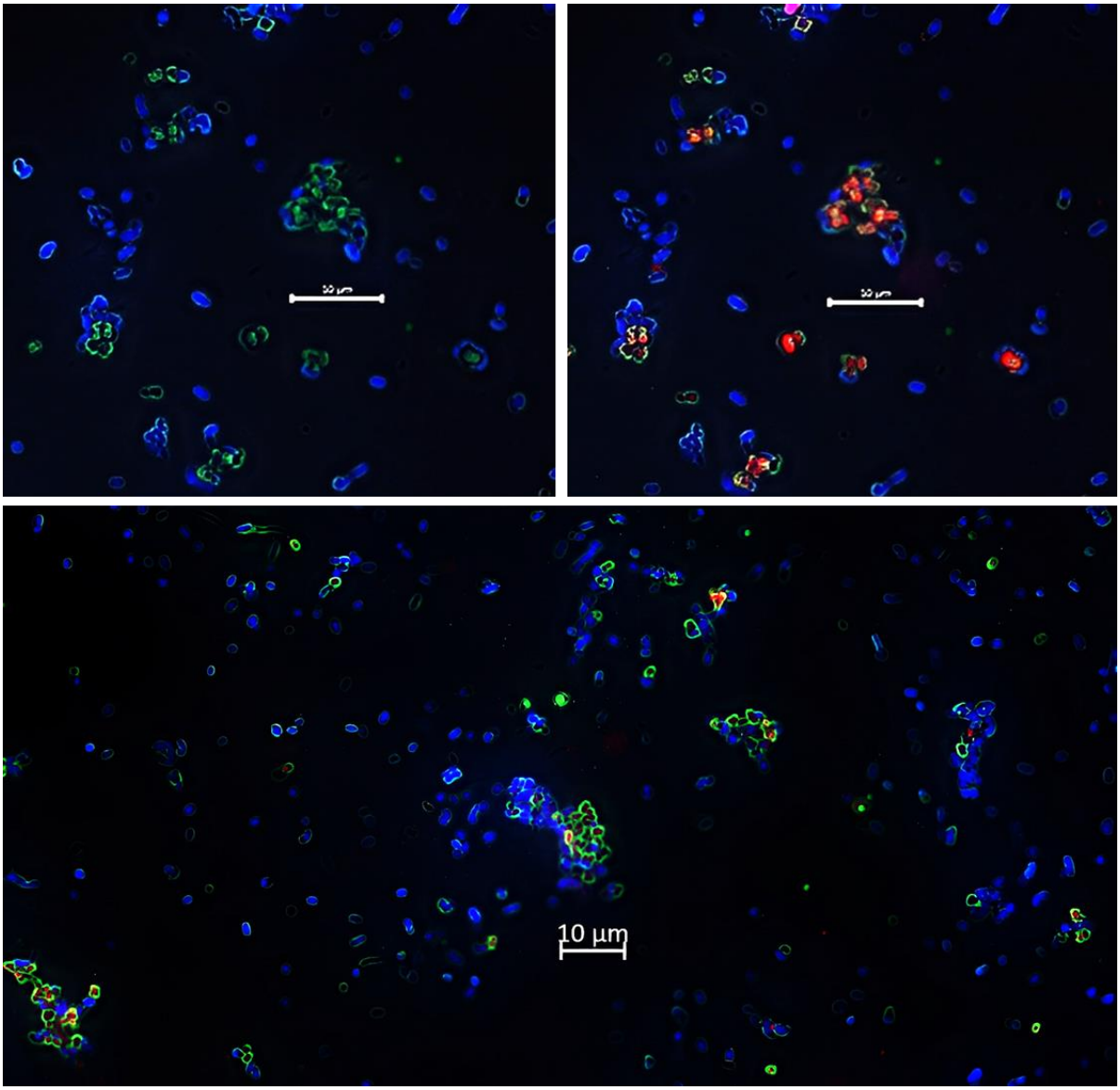


***Figure 14.*** *FISH images of glycerol-cultivated biomass sample taken with 630x magnification. Probes EUBmix was used to stain all bacteria (fluos-green), PAO651 to stain PAOs (Cy3-blue), and Actino658 to stain Tetrad-shaped cells (Cy5-red). Both images were overlaid with phase-contrast pictures to show that all cells were stained. Scale bar equals 10 µm. Colour.*

To determine the composition of the microbial community a whole metagenome sequencing was performed. Genome information of the functional bins has been submitted to the NCBI database under the accession number PRJNA891235. Among all identified taxonomies (data not shown – see supplementary material available online) 2 of the most abundant genera matched microscopic observations; namely *Tessaracoccus* and *Ca.* Accumulibacter (Figure 13). Subsequently, metaproteomics was performed that provided the expressed pathways. These furthermore enabled to track the biochemical conversions of the respective genera. First, the metaproteome raw data were analysed using the complete metagenome-constructed sequence database. The 3 most abundant genera, based on spectral counts (and with 2 or >2 unique peptides per protein) were *Ca.* Accumulibacter*, Tessaracoccus,* and *Micropruina* with approx. 55%, 7%, and 5%, respectively. However, other >30% were

extremely low abundant taxa with only a few protein matches and very low protein sequence coverage. Therefore, the database searching was repeated with (Uniprot) genomes from only the 3 most abundant genera. This (as expected) increased the coverage towards those taxa significantly. Considering this result, the fact that most other taxonomies showed only a very small number of significant protein matches as well as the microscopy data, we hypothesised that 90% of the protein biomass may originate from the 3 main microorganisms. Among this protein mass, over 90% of the expressed proteins are associated with *Ca.* Accumulibacter. The remaining fraction was approximately 9% and 1% for *Tessaracoccus* and *Micropruina* (collectively referred to as fGAOs), respectively. The key anaerobic functioning metabolic pathways such as substrate uptake, fermentation, PHA synthesis, and polyphosphate metabolism in the genome of *Ca.* Accumulibacter, *Tessaracoccus,* and *Micropruina* were analysed based on the expressed proteins involved in the reactions summarized in **Table** 6. Central metabolic pathways such as the TCA cycle, glycolysis, and gluconeogenesis were not tracked as they are shared by the majority of microorganisms.

***Table 6**. Key metabolic pathways and the expressed proteins involved in the reaction by organisms with over 90% of the protein mass in the reactor.*

| Pathway | Protein | Symbol | *Ca. Accumulibacter* | *Tessaracoccus* | *Micropruina* |
|---|---|---|---|---|---|
| Glycerol uptake | Glycerol kinase | *glpK* | - | + | + |
| | Glycerol uptake facilitator | *glpF* | - | + | + |
| | Glycerol dehydrogenase | *glpD* | - | + | + |
| Fermentation | Pyruvate kinase | *pyk* | + | + | + |
| | Alcohol dehydrogenase | *adh* | + | - | - |
| | Pyruvate decarboxylase | *pdc* | + | - | - |
| | D-lactate dehydrogenase | *dld* | + | - | - |
| Storage polymers | PHA synthase | *phaC* | + | - | - |
| | PHA polymerase | *phaE* | + | - | - |
| | Glucokinase | *glk* | + | - | - |
| | Starch synthase | *glgE* | + | + | + |
| Polyphosphate metabolism | Polyphosphate glucokinase | *ppgk* | - | + | - |
| | Polyphosphate kinase | *ppk* | + | + | + |
| | Polyphosphate AMP phosphotransferase | *pap* | + | - | - |
| VFA transporter | Propionyl-CoA synthetase | *prpE* | + | - | - |
| | Propionyl-CoA carboxylase | *pccB* | + | + | - |
| | Propionyl-CoA transferase | *pct* | + | - | - |
| | Acetyl-CoA synthetase | *acs* | + | - | + |
| | Acetyl-CoA carboxylase | *accC/B* | + | + | - |
| | Phosphate acetyl transferase | *pta* | + | - | - |
| Trans-carboxylation | Methylmalonyl-CoA mutase | *mut* | + | + | + |
| | Methylmalonyl-CoA epimerase | *mcee* | - | + | - |
| | Methylmalonyl-CoA carboxyltransferase | *YgfG* | - | + | - |

# Discussions

Overall, the experimental results have indicated that glycerol can be a good substrate for biological phosphorus removal and that good granulation can be achieved. The observed microbial community is more complex when compared to traditional studied laboratory EBPR communities with acetate or propionate as substrate.

## Anaerobic conversions

As shown in **Figure 10**, the system achieved a pseudo-steady-state after 4 months of operation which was likely due to a small competitive difference between different fermentative organisms leading to 1,3-PDO production in the initial months. The conversion seemed to be a combination of a substrate fermentation and product uptake type of reaction (**Figure 15**). In stable conditions, the main fermentation product was likely propionate since PHV & PH2MV were the synthesized storage polymers that corresponded to the uptake of propionate (Oehmen et al., 2005c). These results are also in agreement with similar studies that used glycerol as the carbon source for EBPR (Guerrero et al., 2012; Yang et al., 2018; Yuan et al., 2010) where propionate was also identified as the main glycerol fermentation product. Additionally, two batch tests indicated that propionate was likely the main intermediate product. First, in a batch test where an excess amount of glycerol was fed to the system (to saturate the product-utilizing organisms, such as the PAOs) propionate accumulation was observed. Unlike a normal cycle, a minor amount of PHB formation (0.3 C-mmol/gTSS) was observed towards the end of the anaerobic phase which might have been the result of redox imbalances. PHB is formed from the condensation of 2 acetyl-CoA molecules which can be derived from acetate. Part of the glycerol in the batch test was converted into acetate (7% mol fraction), thus PHB formation can be expected. However, in a normal cycle with lower glycerol concentration less acetate is produced which can explain absence of PHB detection. Similarly, a small amount of PHB was also observed at the end of the anaerobic phase in a batch test with excess propionate (data not shown – see supplementary material available electronically). This observation indicates that the duration of the anaerobic phase and/or the initial concentration of carbon substrate may affect PHA distribution. Second, the direct uptake of propionate in a separate batch test (**Figure 12**) supports the argument that the microbial community was able to take up propionate as substrate.

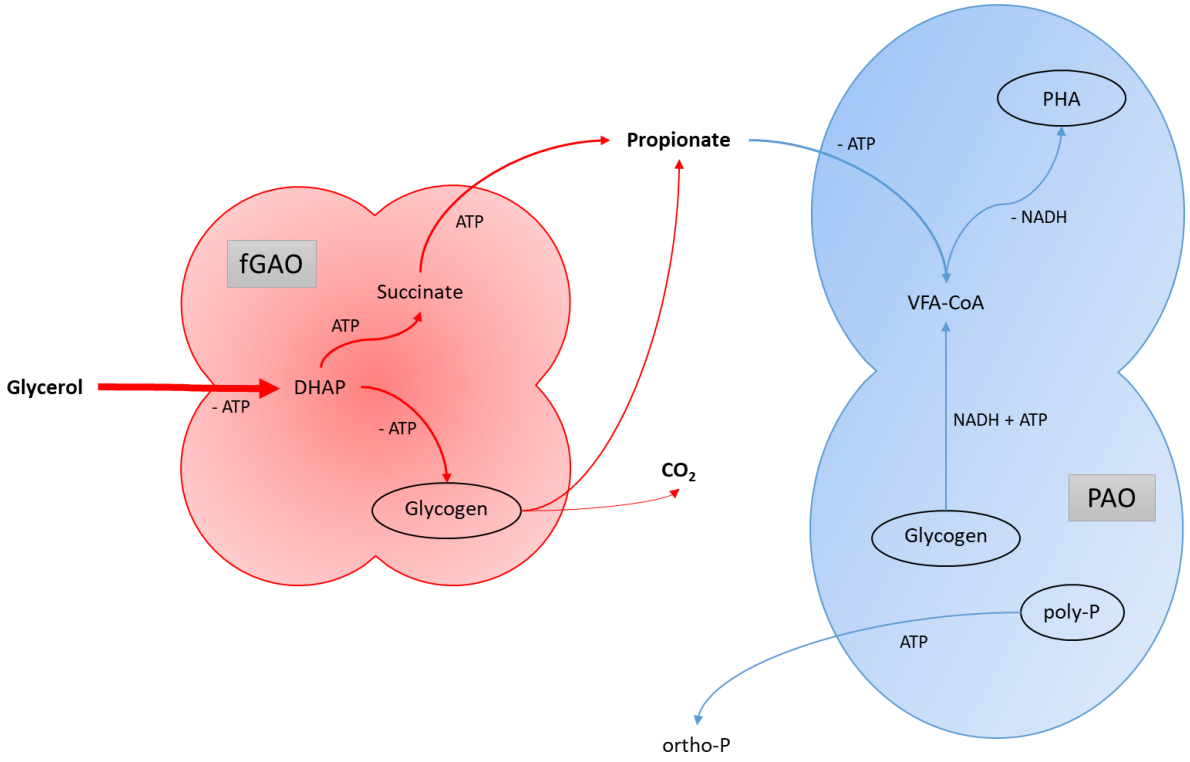


***Figure 15.*** *Graphical representation of anaerobic glycerol conversion by aerobic granular sludge. DHAP: dihydroxyacetone phosphate, PHA: polyhydroxyalkanoates, Poly-P: polyphosphate, VFA-CoA: the combination of acetyl and propionyl-CoA. Colour.*

The fraction of glycerol ending up as propionate was estimated based on an ATP balance over the fGAOs. By inferring the balance, half of the consumed glycerol is directly converted into propionate (0.5 $mol_{propionate}/mol_{glycerol}$) similar to the yield reported by (Guerrero et al., 2012). The other half is assumed to be used for the accumulation of storage compounds such as glycogen. Both *Tessaracoccus* and *Micropruina* are facultative anaerobes that can metabolize glycerol and accumulate glycogen (Maszenan et al., 1999). Therefore, due to this potential occurrence of simultaneous accumulation and consumption of glycogen in the biomass, the hydrolysed glycogen by the PAO was estimated based on the amount of PHA synthesized, assuming that PHA was only synthesised by the PAOs. The estimated hydrolysed glycogen (3.3 C-mmol) and the measured value (3.2 C-mmol) did not differ significantly which supports the arguments for both simultaneous accumulation and consumption of glycogen during the anaerobic period and PHA synthesis by the PAOs. Using the estimated amount of hydrolysed glycogen by the PAOs and the theoretical propionate yield, an ATP balance was made over the PAOs with 101% recovery. As shown in **Table** 5, the PAOs produced most of their ATP requirement through poly-P hydrolysis, manifesting a typical PAO metabolism. The amount of ATP required for VFA-CoA activation and transport of substrate into the cell was calculated based on PHA measurements. The amount of ATP produced was calculated using the total P-release (assuming that P-release was only due to PAO activity) and glycogen hydrolysis. Estimation of substrate utilization between PAOs and fGAOs from the fermentation of anaerobically stored glycogen is further discussed below.

### Propionate vs glycerol as substrate

As shown in **Table** 2, the P:C ratio increased from 0.23 to 0.47 P-mol/C-mol (51%) when propionate was used as the substrate which is likely due to the direct uptake of propionate by the PAOs. The PHA yields, however, only increased from 0.97 C-mol/C-mol$_{glycerol}$ to 1.13 C-mol/C-mol$_{propionate}$ (14%) which is likely the result of higher glycogen degradation. The extra P-release during the batch experiment could be explained by the "uncoupling effect" of higher propionate concentration in the bulk liquid than the propionate generated from the fermentation of glycerol in a normal cycle. The increased propionate concentration likely induces a shift in the energy metabolism of PAOs also known as the overflow metabolism which leads to higher substrate uptake rates that is reflected in higher poly-P hydrolysis (Verhagen et al., 2020). The extra ATP generation was also observed when a balance was made over the PAOs during the propionate batch experiment with an excess of 49%, corresponding well to the increased P:C ratio (data not shown). Moreover, during cycle measurements, a considerable "secondary" P-release with a rate of 4.6 mgP/gVSS/h was observed after complete glycerol uptake. A secondary P-release was also observed during the batch test with propionate at a similar rate of 4.8 mgP/gVSS/h. In literature, the reported secondary P-release rates vary between 0.6 – 3.5 mgP/gVSS/h (Oehmen et al., 2005c; Puig et al., 2008; Smolders et al., 1995) and seem to be related to the maintenance needs of the microorganisms. The higher rate observed in this study is an indication for the uptake of an extra substrate by the PAOs after all carbon substrate has been sequestered. We postulate that fGAOs have initially stored glycogen, which was fermented after the depletion of glycerol, with the released energy used for growth. Thus, in the anaerobic phase, a fraction of stored glycogen is fermented into products and used by PAOs which can be calculated using the secondary P-release. Combining the propionate yield with the fraction of glycogen fermented into PAO-utilizable products indicated that 90% of the substrate was available for PAOs for the production of PHA while the remaining fraction was used by the fGAOs for biomass synthesis. However, the anaerobic biomass formation by fGAOs is based on our postulation and thus, requires further investigations.

### Glycerol-driven EBPR

In the majority of EBPR studies, simple substrates such as acetate and propionate have been used as carbon sources. Using VFAs as substrate results in the selection of one group of microorganisms performing carbon uptake and storage with the energy and reducing power derived from poly-P and glycogen hydrolysis, respectively. Such an approach is based on a simple system that is convenient for metabolic studies but it fails to fully describe more complicated EBPR narratives. In EBPR studies where a different substrate such as glycerol is used, the fermentative organisms are traditionally thought to anaerobically generate the acetate and propionate for the PAO population. This consortium of fermentative organisms and substrate hoarders might also be a feature shared by similar EBPR systems where substrates other than glycerol are used. For example, (Tayà et al., 2013) reported a successful methanol-driven EBPR that was achieved by a syntrophic consortium of methanol fermenters and PAOs. In our study, a glycerol-driven EBPR system was achieved with a microbial consortium of glycerol fermenters and PAOs.

Over time, P-release and -uptake improved as the anaerobic COD removal increased which could be due to a shift in the microbial population and the formation of a stable granular sludge bed with VFA-producing microorganisms. Incomplete anaerobic COD uptake in the initial days of reactor operation was likely because a larger fraction of glycerol was fermented into 1,3-PDO that was not taken up by the PAOs (**Figure 10**). The produced 1,3-PDO was oxidized immediately in the aerobic phase which lead to some filamentous growth (**Figure 9**.B). Generally, rapid substrate uptake in the presence of an electron acceptor (e.g. oxygen) could lead to filamentous growth on the outer fraction of granules (Beun et al., 1999). The slow replacement of the 1,3-PDO fermentative bacteria by propionate fermentative bacteria indicates that the competitive difference between the both is small. It seemed that lowering the SRT control to 12 days after day 65 helped to outcompete the 1,3-PDO fermentation process. This SRT control could be a potentially practical way to select for good conditions in full-scale reactors where glycerol is an important substrate for EBPR processes.



Substrate storing fermentative anaerobes

Many microorganisms are capable of accumulating internal storage compounds (van Loosdrecht et al., 1997). The storage in aerobic feast-famine processes and in anaerobic-aerobic processes have been well described. Anaerobic feast-famine conditions and substrate storage got only limited attention in the literature. For instance, (Shimada et al., 2007) showed the accumulation and degradation of trehalose by a mixed microbial culture growing in a sequencing batch reactor converting glucose into methane. Recently, in a similar study where sucrose-rich synthetic wastewater was used, anaerobic accumulation of glycogen and glycogen-like compounds was reported (Ni et al., 2015). In this study, we observed the fermentation of glycerol and accumulation of glycogen at the end of the anaerobic phase presumably by the fGAOs. The anaerobic uptake of glycerol could lead to the accumulation of storage compounds (not PHA) by *Tessaracoccus* (Maszenan et al., 1999). The degree of this accumulation can partly be explained by the mechanism by which microorganisms derive their energy from the fermentation process. For instance, several catabolic pathways exist in the conversion of glycerol into propionate. A common pathway found predominantly in *propionibacterium* is the Wood-Werkman cycle which involves a transcarboxylase transferring a carboxyl group from methylmalonyl-CoA to pyruvate to generate propionyl-CoA (Gonzalez-Garcia et al., 2017). As shown in **Table** 6, the transcarboxylase enzymes are also expressed in the genome of *Tessaracoccus*. This cycle conserves an ATP which is otherwise lost to the fixation of $CO_2$ to oxaloacetate and thus the energy can be used for growth and storage processes. Likely half of the glycerol is fermented to propionate while the other half is stored as glycogen or poly-glucose. When glycerol is depleted, the fGAOs start to grow fermentative on the stored compound, resulting in the production of VFAs such as propionate in the bulk liquid that is subsequently taken up by the PAOs. In line with the accumulation of propionate observed in a batch experiment after glycerol depletion, the formation of propionate from glucose fermentation using granular sludge was also reported by (Shimada et al., 2007). Thus, it is likely that the stored glycogen was also fermented into propionate.

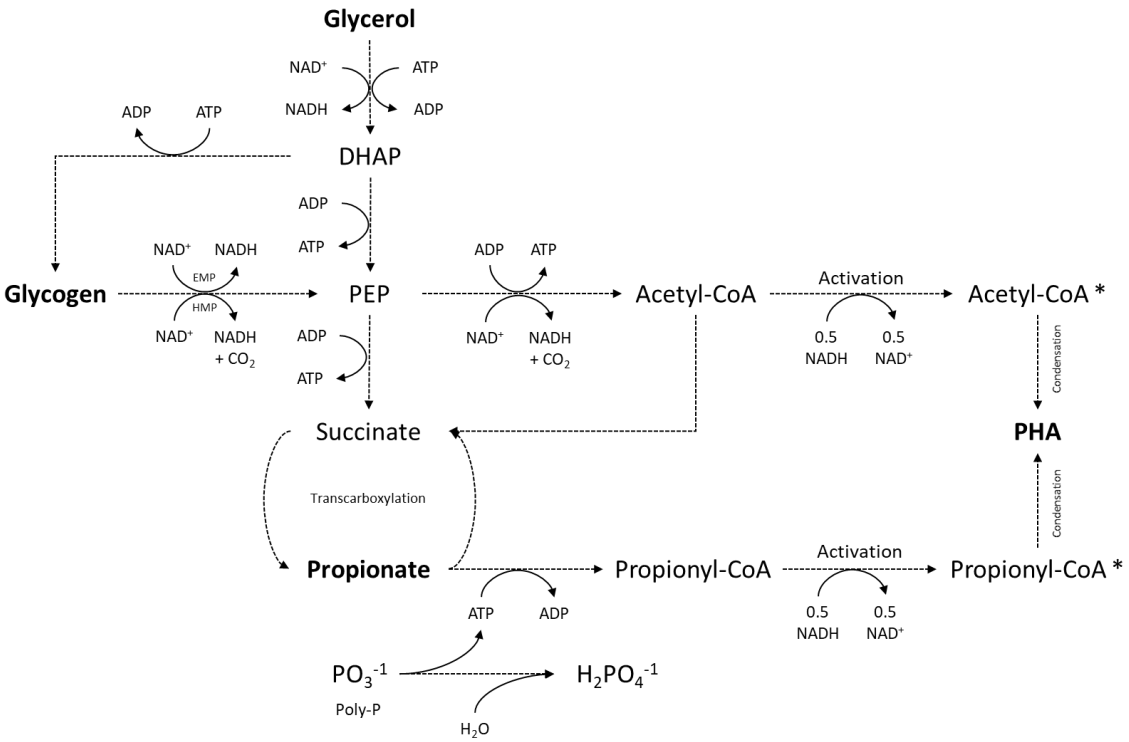


***Figure 16**. Overview of proposed anaerobic glycerol conversion pathway by aerobic granular sludge. DHAP: Dihydroxyacetone phosphate, PEP: Phosphoenol pyruvate, PHA: Poly-hydroxy-alkanoates. Conversion of glycerol into propionate follows the Wood-Werkman cycle.*

## Microbial community

A metabolic pathway for glycerol conversion was hypothesised based on the metaproteome data obtained for the microbial community (**Figure 16**). Although *Ca.* Accumulibacter possesses a diverse metabolic potential, these microorganisms seem to lack the enzymatic machinery required for uptake and conversion of glycerol (**Table** 6). Glycerol uptake facilitator (glpF) and glycerol kinase (glpK), two proteins involved in the transport of glycerol into cellular metabolism (Voegele et al., 1993), were observed in the genome of *Tessaracoccus* and *Micropruina.* Both of these organisms belong to the *propionibactericeae* family which is one of the suborders of the class *Actinobacteria.* As the name suggests, the production of propionic acid is the hallmark of *propionibacteria* (Stackebrandt et al., 2006) and illustrated in a study by (Barbirato et al., 1997) where propionate production from glycerol fermentation by different strains of *Propionibacterium* was reported. Interestingly, these microorganisms have been also commonly observed in activated sludge samples with phosphorus removal capabilities around the world (Nielsen et al., 2019). Genomic analysis of *Tessaracoccus* and *Micropruina* showed that these organisms lack PHA synthase (phaC), a key enzyme encoding for PHA synthesis (Zher Neoh et al., 2022). In an earlier study by (Maszenan et al., 1999) absence of poly-P and PHA granules in *Tessaracoccus* was also reported as these compounds were not observed when stained by Methylene blue and Nile blue, respectively. However, staining methods are prone to error due to false positive results and provide limited

information about the potential of enzymatic machinery of the organisms. Here, through proteomics, we have investigated the presence or absence of storage compounds by searching for the existence of key enzymatic reactions in each organism. *Tessaracoccus* have the capacity to produce poly-glucose, but PHA production proteins were not identified. Although poly-P granules were not observed in *Tessaracoccus* and *Micropruina* they seem to possess some of the enzymes involved in poly-P metabolism. Indicated by the presence of polyphosphate kinase (ppk), these microorganisms can likely accumulate poly-P but are not able to utilize it for energy generation since AMP phosphotransferase (pap) is absent. Further study on the capabilities of these fGAOs in the future could elucidate their full potential.



Practical implications

- Stable granulation by PAO-dominated microbial community can be achieved by steering glycerol fermentation to propionate over 1,3-Propanediol. Potentially the SRT can be a control parameter.
- The dominance of polyphosphate accumulating organisms in the culture indicates that the extracellular matrix is likely similar to those produced using municipal wastewater indicating that glycerol-based sludge might also be used to extract the Kaumera bio-polymers.
- Glycerol as a relatively cheap carbon source could be dosed to wastewater treatment plants to overcome carbon deficiency.

## Conclusions

- Successful and stable granulation with glycerol as the sole carbon source was shown.
- Glycerol-driven EBPR was achieved by a microbial consortium of glycerol fermenters and PAOs.
- Lack of *glpF*, *glpD,* and *glpK* in the genome of *Ca.* Accumulibacter implied that these organisms are likely not able to directly utilize glycerol.
- Over 90% of the expressed proteins in glycerol-based aerobic granular sludge belonged to *Ca*. Accumulibacter.

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# 3. Aerobic Granular Sludge Phosphate Removal Using Glucose

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## Abstract

Enhanced biological phosphate removal and aerobic sludge granulation are commonly studied with fatty acids as substrate. Fermentative substrates such as glucose have received limited attention. In this work, glucose conversion by aerobic granular sludge and its impact on phosphate removal was studied. Long-term stable phosphate removal and successful granulation were achieved. Glucose was rapidly taken up (273 mg/gVSS/h) at the start of the anaerobic phase, while phosphate was released during the full anaerobic phase. Some lactate was produced during glucose consumption, which was anaerobically consumed once glucose was depleted. The phosphate release appeared to be directly proportional to the uptake of lactate. The ratio of phosphorus released to glucose carbon taken up over the full anaerobic phase was 0.25 Pmol/Cmol. Along with glucose and lactate uptake in the anaerobic phase, poly-hydroxy-alkanoates and glycogen storage were observed. There was a linear correlation between glucose consumption and lactate formation. While lactate accounted for approximately 89% of the observed products in the bulk liquid, minor quantities of formate (5%), propionate (4%), and acetate (3%) were also detected (mass fraction). Formate was not consumed anaerobically. Quantitative fluorescence in-situ hybridization (qFISH) revealed that polyphosphate accumulating organisms (PAO) accounted for 61 ± 15% of the total biovolume. Metagenome evaluation of the biomass indicated a high abundance of *Micropruina* and *Ca*. Accumulibacter in the system, which was in accordance with the microscopic observations and the protein mass fraction from metaproteome analysis. Anaerobic conversions were evaluated based on theoretical ATP balances to provide the substrate distribution among the dominant genera. This research shows that aerobic granular sludge technology can be applied to glucose-containing effluents and that glucose is a suitable substrate for achieving phosphate removal. The results also show that for fermentable substrates a microbial community consisting of fermentative organisms and PAO develop.


## Introduction

Aerobic Granular Sludge (AGS) technology is a biological wastewater treatment technique that offers significant advantages over conventional activated sludge systems, including a smaller footprint and improved energy efficiency (Bengtsson et al., 2018; de Bruin et al., 2004; de Kreuk et al., 2005). To ensure the formation of AGS and maintain stable reactor performance, various parameters have been identified as crucial (van Dijk et al., 2022). Among these parameters, the type of substrate plays a key role. A suitable substrate for granulation is one that is taken up and stored under anaerobic conditions by the microorganisms, and subsequently oxidized during an aerobic phase to provide energy for microbial growth processes (Pronk et al., 2015). This scenario selects for slow-growing organisms which are associated with the formation of smooth and stable granules (de Kreuk and van Loosdrecht, 2004). Substrates that do not undergo anaerobic sequestration are utilized aerobically by fast-growing heterotrophic organisms causing floc formation or filamentous growth and negatively influencing stable granulation (van Loosdrecht et al., 1997). Organisms that grow on storage polymers such as polyhydroxy-butyrate have a lower

growth rate (< 2 $d^{-1}$; Beun et al., 2002) than normal heterotrophs directly growing on the available substrate (e.g. *E. Coli* <2 $h^{-1}$; Buchanan & Klawitter, 1992).

Enhanced biological phosphorus removal (EBPR) is an efficient and sustainable technique for phosphate removal from water bodies (Ketchum et al., 1987). A common link between the AGS and EBPR process is the operational parameter, i.e. the induction of cyclical anaerobic-aerobic phases, which selects for a slow-growing organism known as polyphosphate accumulating organism (PAO). When acetate or propionate is the available substrate, PAOs tend to dominate the system, ensuring compact granule formation and effective phosphate removal (Oehmen et al., 2005a; Pronk et al., 2015). Acetate and propionate can also be utilized by glycogen accumulation organisms (GAO) that can compete with PAOs, negatively affecting the EBPR efficiency. Despite their metabolic flexibility, PAOs are unable to directly utilize substrates such as certain sugars (Elahinik et al., 2022). Presence of substrates that cannot be directly sequestered into storage polymers in the anaerobic phase, either result in aerobic oxidation which is unfavourable for both the AGS and EBPR process or are fermented by fermentative organisms. The fermentation products can often be sequestered by PAO and similar organisms such as glycogen accumulating organisms (GAO). The fermentative organisms often also store a fraction of the substrate and are denoted as “G-bacteria” (Cech and Hartman, 1993) or fermentative GAO (fGAO) (McIlroy et al., 2018). They are observed in wastewater treatment plants performing EBPR around the world but understanding their role has had limited attention (Seviour et al., 2000). It is important to study these organisms as they can compete for substrate, decreasing EBPR efficiency, or form non-granular and fragile biofilms, impacting solid-liquid separation.



Glucose, a substrate found in polymers in municipal wastewater (e.g. cellulose and starch) and in the effluent of various industries, such as food and sugar production facilities, has been marginally studied concerning granule formation. Regarding EBPR studies, glucose has been used in combination with fatty acids (e.g. glucose and acetate) in the past which makes it difficult to distinguish the effect of glucose independently (Gebremariam et al., 2012; Ziliani et al., 2023). In a study by Jeon & Park (2000), the long-term effect of glucose as the sole carbon source on the EBPR process using an activated sludge system was evaluated. The authors reported a probable synthesis of a lactate polymer which has not been reported in non-modified microorganisms in the literature. Therefore, in this research, an AGS reactor operating in sequencing batch mode was studied with glucose as the sole substrate to evaluate its potential for granulation and study the microbial community in relation to process stoichiometry and EBPR efficiency.

## Material and Methods

### Experimental setup and reactor operation

A bubble column reactor with a working volume of 2.8 L, an internal diameter of 5.6 cm and a total height of 90 cm was operated in Sequencing Batch Reactor (SBR) mode. After 1.5 L effluent withdrawal at the end of each cycle, a volumetric exchange ratio of 54% was

maintained. The pH was controlled at 7.0 ± 0.1 by automatic dosing of 0.5 M NaOH or HCl. The Dissolved Oxygen (DO) concentration was controlled at 0% and 50% (3.5 mg/L) saturation during the anaerobic and aerobic phase, respectively, by a mixture of nitrogen gas and air. The off-gas was recirculated with a flow of 5 L/min to maintain the DO concentration. The temperature of the reactor was not directly controlled but the room temperature was controlled at 20 °C. The reactor was inoculated with a mixture of aerobic granular sludge from a full-scale municipal wastewater treatment reactor performing EBPR located in Harnaschpolder, the Netherlands and glycerol-adapted sludge performing EBPR from previous experiments. Each cycle consisted of 5 min of nitrogen sparging to ensure anaerobic condition before feeding followed by 5 min of feeding, 60 min of nitrogen sparging (anaerobic phase), 120 min of aeration (aerobic phase), 5 min of settling, and 5 min of effluent withdrawal, equivalent to a total duration of 200 min. The sludge retention time (SRT) was controlled at 10 days via manual sludge removal. The SRT was calculated based on the solids in effluent and solids manually removed.

The synthetic influent fed at the beginning of each cycle with a total volume of 1,500 mL consisted of 1,200 mL of demineralized water, 150 mL of nutrient medium and 150 mL of carbon medium. Carbon medium contained 20.8 mM glucose, 3.6 mM $MgSO_4.7H_2O$, and 4.7 mM KCl. The nutrient medium contained 41.1 mM $NH_4Cl$, 1.95 mM $K_2HPO_4$, 1.98 mM $KH_2PO_4$, 0.6 mM Allythiourea (ATU) to inhibit nitrification and 10 mL/L of trace element solution. The trace element solution contained 4.99 g/L $FeSO_4.7H_2O$, 2.2 g/L $Zn.SO_4.7H_2O$, 7.33 g/L $CaCl_2.2H_2O$, 4.32 g/L $MnSO_4.H_2O$, 2.18 g/L $Na_2MoO_4.2H_2O$, 1.57 g/L $CuSO_4.5H_2O$, 1.61 g/L $CoCl_2.6H_2O$ and 50 g/L EDTA. The combination of these feed sources resulted in a final influent concentration of 400 mg/L COD, 58 mg/L $NH_4^+$-N, and 12 mg/L of $PO_4^{3-}$-P.

Batch experiments were performed in 250 mL Schott bottles, sparged with nitrogen to ensure an anaerobic environment, and controlled pH at 7 ± 0.1 by manual dosing of 0.5 M HCl or NaOH. Biomass at the end of the aerobic phase (end of the cycle) was taken from the reactor and used as inoculum (20 mL) for each test.

## Analytical methods

Concentrations of $PO_4^{3-}$-P and $NH_4^+$-N were measured using a Gallery Discrete Analyser (ThermoFisher Scientific, USA). Chemical Oxygen Demand was measured with a spectrophotometer cuvette system (DR2800, Hach Lange, USA). Volatile fatty acids and sugars were measured using an HPLC (Vanquish, ThermoFisher Scientific, USA) equipped with an RI and UV detector, Aminex HPX-87H column (BioRad, USA) using 0.0015M phosphoric acid as eluent.

## PHA and glycogen determination

Biomass samples from the reactor were fixed with 4% w/v paraformaldehyde, washed with demineralized water, freeze-dried overnight, and crushed into fine powder. For extraction, approximately 30 mg of the powered biomass was hydrolysed and esterified in a 3% sulphuric acid, methanol, and chloroform solution for 24 hours at 100 °C with frequent manual vortex.

For phase separation, 3 mL of ultrapure water was added to the samples and the formed esters in the organic phase were then filtered and analysed by a GC (6890N, Agilent, USA). Quantifications of PHB, PH2MV and PHV were done using commercial 3-hydroxybutyrate, 2-hydroxyhexanoate and a synthetic copolymer of (R)-3-hydroxybutyrate-(R)-3-hydroxyvalerate (Sigma-Aldrich, USA) as standards, respectively. Benzoic acid was also added to the samples as an internal standard.

### Biomass measurements

To determine the Total Suspended Solids (TSS) in the reactor, granules at the end of the cycle were taken and filtered through 0.45 µm glass fibre filters and placed in an oven at 105 °C for 24 hours. The dried biomass sample was then incinerated at 550 °C for 3 hours to determine the ash content. The Volatile Suspended Solids (VSS) were then calculated based on the difference between TSS and the ash content.



### Microscopy and FISH

To observe and capture the morphology of the granules, a stereo zoom microscope (M205 FA, Leica Microsystems, Germany) equipped with Qwin image analysis software (V3.5.1, Leica Microsystems, Germany) was used. For Fluorescence in-situ Hybridization (FISH), the handling and staining of the biomass samples were done using the protocol described by (Bassin et al., 2011). The images were captured with an epifluorescence microscope equipped with filter set Cy3 (ET545/25x ET605/70m T565LPXR), Cy5 (ET640/30x ET690/50m T660LPXR), and FITC (ET470/40x ET525/50m T495LPXR) (Axio Imager M2, Zeiss, Germany). Quantitative FISH (qFISH) was done as a percentage of total biovolume which was stained with a general probe (EUBmix) and a specific probe (PAO651) (Daims et al., 1999). Quantification was done over 9 representative pictures using daime software (DOME, Vienna, Austria) (Daims et al., 2006).

### Metagenomics and metaproteomics

The DNA from the biomass samples was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) following the manufacturer's protocol. Metagenome sequencing and raw data processing were conducted by Novogene (Novogene Co., China). For protein extraction and metaproteome analysis, the methodology described by Kleikamp et al. (2022) was followed. Similar to (Elahinik et al., 2022), the functional proteins were annotated using the KEGG and EggNOG databases, and the metaproteome raw data was analysed using the metagenome-constructed dataset. Pathway reconstruction with expressed proteins was performed with GhostKOALA.

### Substrate distribution considerations

To calculate substrate distribution among the dominant genera, ATP balances over metabolic pathways were made assuming glucose conversion in the anaerobic phase followed a three-step process; 1- uptake of glucose and storage as glycogen, 2- fermentation of

glycogen/glucose to lactate by fGAOs, and 3- lactate conversion into PHA by PAOs. To assess the energy balance within the fGAOs, it was assumed that the ATP needed for glucose uptake and storage as glycogen is provided through lactate fermentation *(1)* (Jeon and Park, 2000). The ATP generated from lactate production can be 1 or 2 mol depending on whether the reaction follows the Entner–Doudoroff (ED) or the Embden-Meyerhof-Parnas (EMP) pathway, respectively (Mino et al., 1994). Assuming the EMP pathway, (supported by the expression of proteins involved in the EMP pathway) (**Table** 7), this balance suggests that half of the glucose is utilized for glycogen synthesis, while the other half is converted into lactate.

$$-Glucose - 2\ ATP + Glycogen \quad (1)$$

$$-Glucose + 2\ ATP + 2\ Lactate \quad (2)$$

The ATP balance for the subsequent uptake of lactate and storage as PHA by PAOs was done according to the model described by Mino et al. (1994). The model takes into account the ATP needed for transport (1 $mol_{ATP}/mol_{substrate}$) and acetyl-CoA formation (1.5 $mol_{ATP}/mol_{lactate}$), supplied through poly-P hydrolysis. According to models describing the anaerobic metabolism of PAOs, the ATP needed for substrate uptake and PHA synthesis is generated through poly-P hydrolysis, and glycogen conversion is primarily used for NADH generation (Smolders et al., 1994). When lactate is utilized, the cells can maintain a redox balance through simultaneous conversion of pyruvate into acetyl-CoA and propionyl-CoA as shown in equations (*3*) (Mino et al., 1994). The produced NADH is then used in the condensation of acetyl-CoA and propionyl-CoA into PHA.

$$-2\ Pyruvate - 1\ ATP + \ Propionyl\_CoA + Acetyl\_CoA\ + CO_2 + NADH \quad (3)$$

# Results

## Reactor operation

A combination of full-scale and laboratory-scale aerobic granular sludge performing EBPR was used as inoculum. During the experimental period, the reactor performance was monitored on a daily basis via online pH, dissolved oxygen, and acid/base dosing measurements. Additionally, several cycle measurements were performed when the reactor reached a steady state to ensure reactor stability and that the presented figures are reliable and representative. The reactor reached a pseudo-steady state in terms of stable conversions within 4 weeks and in terms of granular sludge formation after about 2 months of reactor operation. The granules exhibited a smooth and stable structure, devoid of filamentous growth (Figure 17) with excellent settling properties indicated by an average sludge volume index ($SVI_5$) of 24 mL/g. Stereoscopic imaging revealed a granule size range of 0.3 to 1 mm. The sludge bed displayed a light brown-orange colour, which was darker compared to

granules fed with acetate. The TSS in the reactor averaged around 5.7 g/L, with a VSS to TSS ratio of approximately 80%. The effluent was relatively clear, with an average TSS concentration of approximately 90 mg/L.

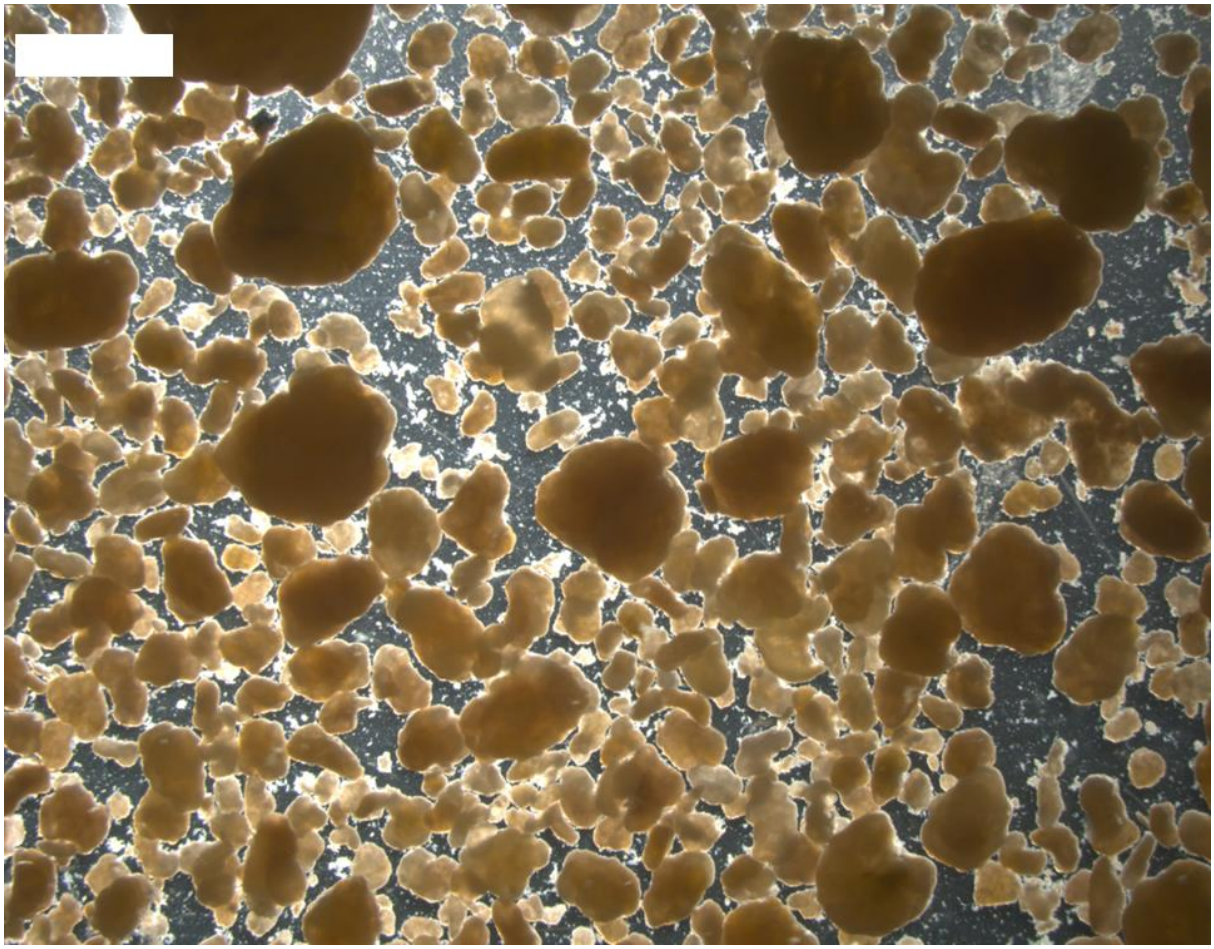

***Figure 17**. Stereoscopic image of aerobic granular sludge fed with glucose after 88 days of reactor operation. The scale bar is equal to 1 mm.*

During stable conditions, the anaerobic conversions were a combination of substrate (glucose) fermentation and product (lactate) uptake type reaction. As shown in Figure 18, glucose was rapidly taken up (273 mg/gVSS/h) and phosphate was released (15.5 mgP/gVSS/h) in the anaerobic phase. Simultaneously, a minor amount of lactate was produced alongside glucose consumption. The lactate was also anaerobically consumed once glucose was depleted. The phosphate release appeared to be directly proportional to the uptake of lactate when glucose was no longer available. The calculated ratio of phosphorus released to lactate carbon uptake was 0.55 Pmol/Cmol based on the ATP balance (half of glucose converted to lactate). When lactate was depleted, the rate of phosphate release also decelerated. The secondary phosphate release can be attributed to cellular maintenance processes and had a rate of 2.3 mgP/gVSS/h. In the aerobic phase, phosphate was seemingly taken up in two stages expressed by the primary (9.3 mgP/gVSS/h) and the secondary (3.9

mgP/gVSS/h) phosphate uptake rates. The observed delay in phosphate uptake within the initial five minutes into the aerobic phase was maybe related to the aerobic utilization of formate that was produced in the anaerobic phase. The aerobic specific ammonium consumption rate was 0.93 mgN/gVSS/h for biomass growth since nitrification was inhibited due to ATU addition.

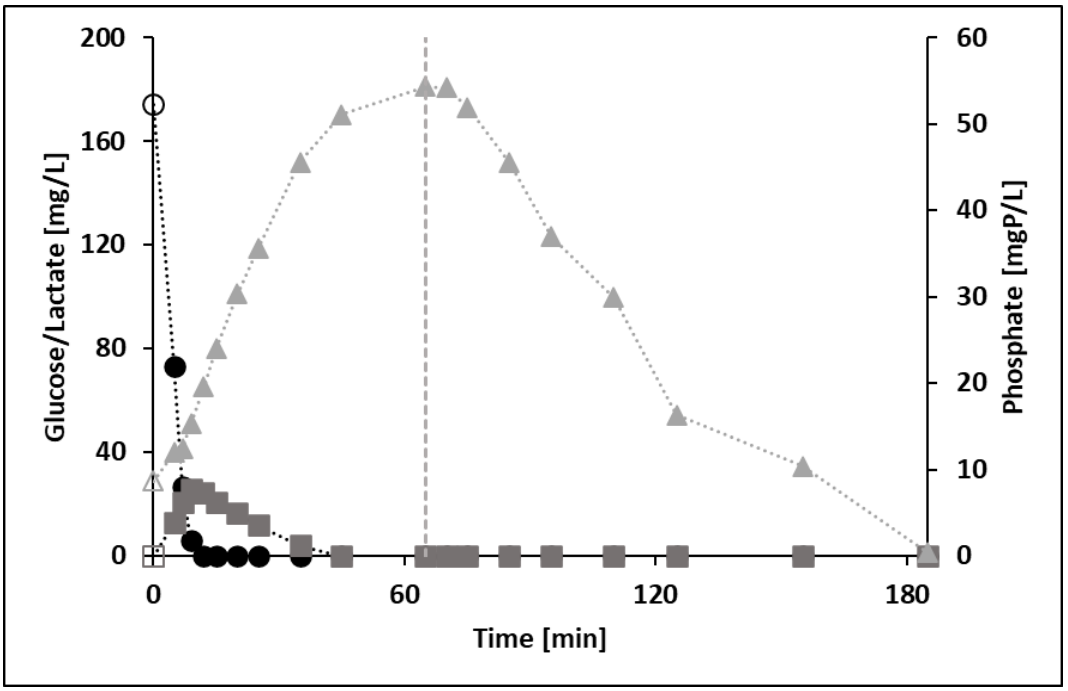


***Figure 18**. Overview of a representative cycle during steady state condition. Glucose (circles), lactate (squares), and phosphate (triangles) transformations are shown. The first hollow makers show the calculated concentrations based on dilution and influent concentration. The dashed-vertical line shows the switch between the anaerobic and aerobic phases.*

The ratio of phosphorus released to glucose carbon taken up in the anaerobic phase was 0.25 Pmol/Cmol. Along with glucose and lactate uptake in the anaerobic phase, PHA and glycogen storage was observed (Figure 19). The electron balance distribution performed over the anaerobic phase shown in Figure 19 closed with a recovery of 94% (molar basis). This glucose-COD was recovered as storage of glycogen (46%) and PHA (29%), as well as growth (18%) estimated based on anaerobic ammonium consumption of 0.7 mgN/gVSS. A minor amount of formate was anaerobically produced (≤ 1%) which was oxidized aerobically.

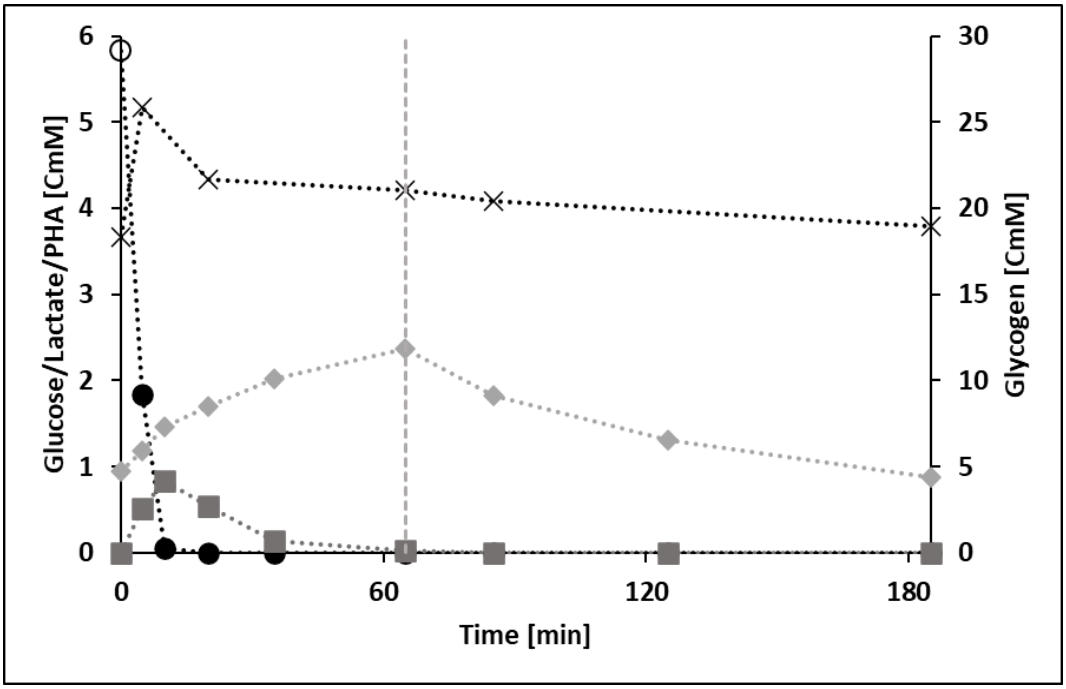


***Figure 19**. Glucose (circles), lactate (squares), PHA (diamonds), and glycogen (crosses) profile during the complete cycle. The first hollow maker representing glucose shows the calculated concentration based on dilution and influent concentration. The dashed-vertical line shows the switch between the anaerobic and aerobic phases.*



## Batch tests

To gain further insights into the conversions occurring within the reactor during the anaerobic phase, a series of batch tests were conducted. The first batch test involved separate bottles containing formate and lactate, where biomass from the reactor (collected at the end of the cycle) was used to assess their anaerobic utilization (Figure 20). Nitrogen gas was continuously sparged to ensure an anaerobic environment in the bottles. As anticipated, formate demonstrated no anaerobic utilization, aligning with the observations made in the reactor. Lactate was anaerobically utilized at a rate of 13.7 mg/gVSS/h and PHV formation was observed. No glycogen accumulation was observed. Electron balancing performed over anaerobic lactate uptake, PHA accumulation, and biomass growth closed with a recovery of 108%. With both substrates P-release was observed, with the assumption that P-release in the presence of formate was attributed to maintenance processes and the higher P-release in the presence of lactate was due to lactate consumption. The P-release rate with lactate was 11.6 mgP/gVSS/h with a P/C ratio of 0.45 Pmol/Cmol.

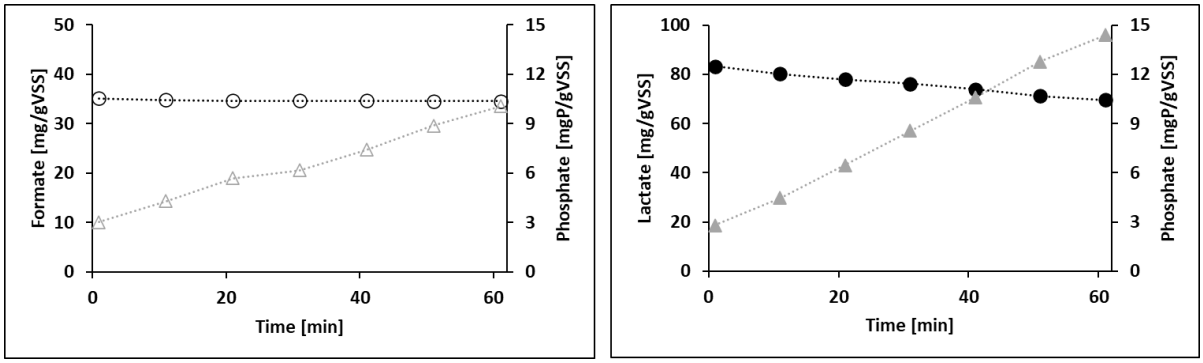


***Figure 20**. Lactate (left) and formate (right) batch tests showing substrate (circles) and phosphate (triangles) anaerobic transformations.*

To identify the product formation during the anaerobic phase fed with glucose a batch test with a high glucose addition (10 times more than in the regular operation) was performed. A linear correlation between glucose consumption and lactate formation was observed (Figure 21). Lactate accounted for approximately 89% of the products in the bulk liquid (by mass fraction) with minor quantities of formate (5%), propionate (4%), and acetate (3%). The rates of glucose uptake and lactate production were determined to be 310 and 83 mg/gVSS/h, respectively. Note that the intrinsic lactate production rate was likely to be higher than the observed rate due to simultaneous consumption. During the test, glycogen accumulation (21.3 mg/gVSS) and PHA production (6.7 mg/gVSS) were also observed (as expected).

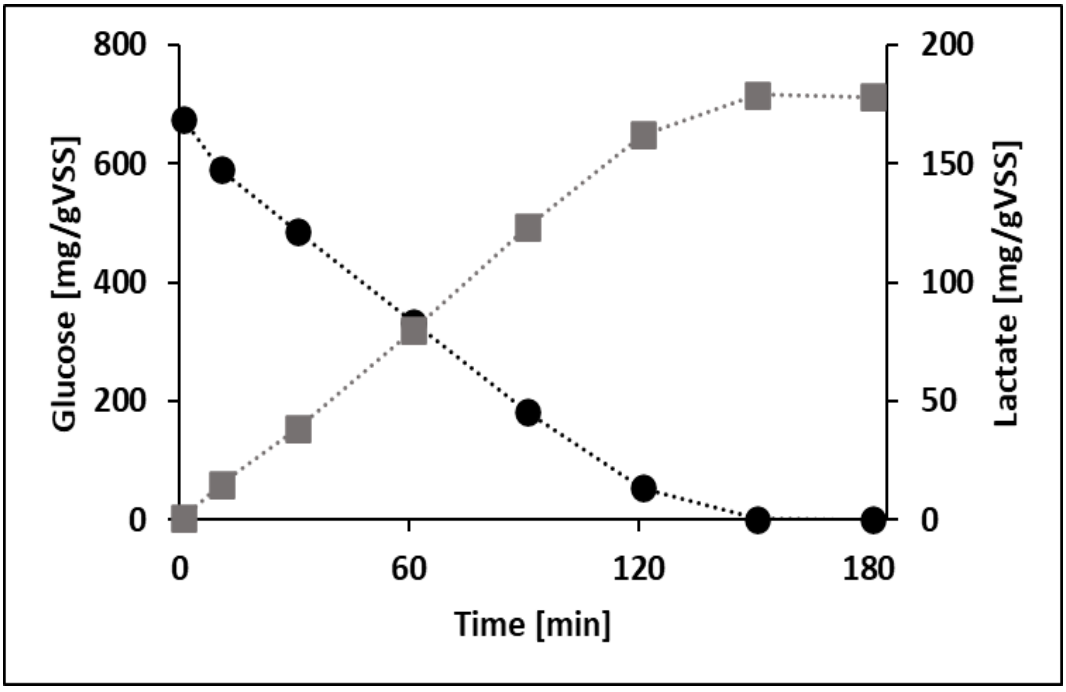


***Figure 21**. Anaerobic glucose consumption (circles) and lactate production (squares) as a function of time.*

## Microbial community

Biomass samples at the end of the experimental period were taken from the reactor for microscopic and meta-omics analysis. Phase contrast images showed that distinct microbial communities with diverse morphologies were present (Figure 22.A). A representative FISH picture of the biomass is shown in Figure 22.B. and qFISH results revealed that the PAOs accounted for 61 ± 15% of the total biovolume.

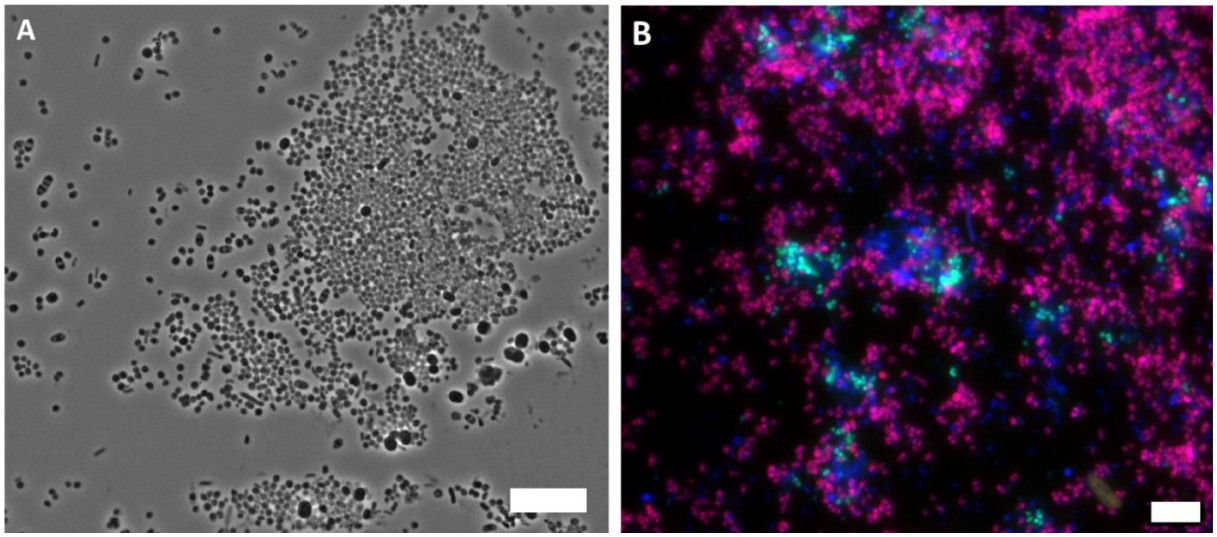


***Figure 22****. Phase contrast and FISH picture of the microbial population. The scale bar is equal to 10 µm. Blue represents all microorganisms stained with probe EUBmix, magenta shows the PAO population stained with probe PAO651, and cyan shows the cluster stained with probe Tess644.*

Extracted DNA and proteins of the biomass were analysed and the genome information of the functional bins was submitted to the NCBI database under the accession number PRJNA1020750. The metaproteome was analysed using the metagenome-constructed database. The relative abundance of the top 7 genera accounting for 21% and 34% coverage according to metagenomics and metaproteome analysis, respectively, is shown in Figure 23. The "other" excluded fraction accounted for extremely low abundant taxa with few protein matches and low protein sequence coverage. The area of the peptide per protein was summed to calculate protein mass and estimate the relative abundance. Based on protein mass fraction and metagenome contigs, *Ca*. Accumulibacter and *Micropruina* were identified as the dominant genera among all of the identified taxa which corresponded to microscopic observations (Figure 22). Based on class distribution, *Actinobacteria* and *Betaproteobacteria* accounted for 19% and 43% of all of the protein mass, respectively. Moreover, the metaproteome analysis provided the expressed proteins that were used to investigate the anaerobic conversions and associate the reactions to the respective class (**Table** 7). Since *Ca*. Accumulibacter and *Micropruina* were the respective dominant members of the *Betaproteobacteria* and *Actinobacteria* classes, and the protein sequence coverage was higher in class distribution (as expected), the expressed proteins are shown as a function of class rather than genus.

***Table 7.*** *Key metabolic pathways and the relevant detected expressed proteins by organisms with 75% of the total protein mass. Enzymes typically involved in EMP (*) and ED (**) pathways.*

| Pathway | Protein | KEGG Symbol | Betaproteobacteria | Actinobacteria |
|---|---|---|---|---|
| Glycogen synthesis | Phosphoglucomutase | *pgm* | + | + |
| | Glucose-1-phosphate adenylyltransferase | *glgC* | + | + |
| | Glycogen synthase | *glgA* | + | - |
| | 1,4-alphaglucan branching enyzme | *glgB* | + | + |
| | Glycogen phosphorylase | *glgP* | + | + |
| | Glycogen debranching enzyme | *TreX* | + | + |
| | D-lactate dehydrogenase | *dld* | + | + |
| | Starch synthase | *glgE* | - | + |
| PHA synthesis | PHA synthase | *phaC* | + | - |
| | PHA polymerase | *phaE* | - | - |
| | Acetyl-CoA transferase | *phaA* | - | - |
| | Acetoacetyl-CoA reductase | *phbB* | + | - |
| | Propionyl-CoA synthetase | *prpE* | + | - |
| | Propionyl-CoA carboxylase alpha | *pccA* | + | - |
| | Propionyl-CoA carboxylase beta | *pccB* | - | - |
| | Acetyl-CoA synthetase | *acs* | + | + |
| | Methylmalonyl-CoA mutase | *mut* | - | + |
| Polyphosphate metabolism | Polyphosphate glucokinase | *ppgk* | - | - |
| | Phosphoglycerate | *pgk* | + | + |
| | Polyphosphate kinase | *ppk* | + | + |
| | Polyphosphate AMP phosphotransferase | *pap* | + | - |
| | Phosphate acetyl transferase | *pta* | + | + |
| | Exopolyphosphatase | *ppx* | + | - |
| Glucose metabolism | Phosphotransferase system | *pts* | - | + |
| | 6-phosphofructokinase* | *pfk* | + | + |
| | Fructose-1,6-bisphosphatase* | *fbp* | + | + |
| | Triosephosphate isomerase* | *tpi* | + | + |
| | Glyceraldehyde 3-phosphate dehydrogenase* | *gapA* | + | + |
| | glucose-6-phosphate 1-dehydrogenase** | zwf | - | - |
| | 6-phosphogluconate dehydrogenase** | gnd | - | - |

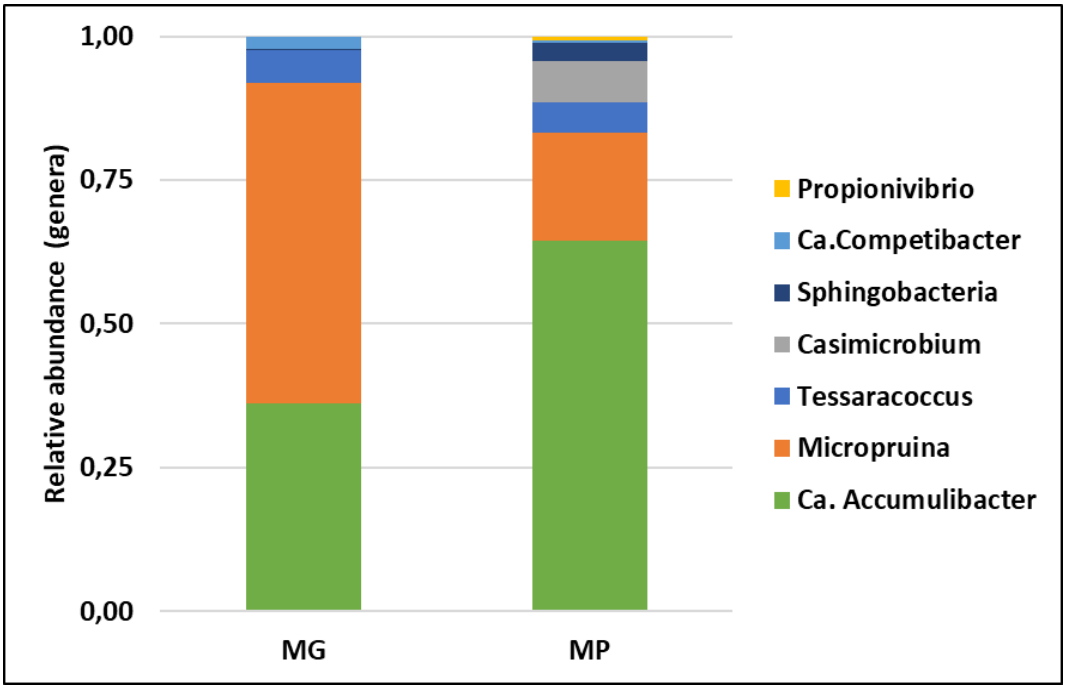


***Figure 23**. Relative abundance of the top genera according to metagenome (MG) contigs and metaproteome (MP) analysis using protein mass fraction.*



ATP balances

To further evaluate the anaerobic conversions and estimate substrate distribution among PAOs and fGAOs, an ATP calculation was performed. By combining the existing information from literature, our experimental results, and the genetic potential of *Micropruina* (fGAOs) and *Ca*. Accumulibacter (PAOs), glucose conversion in the anaerobic phase was assumed to follow a three-step process (Figure 24); 1- uptake of glucose and storage as glycogen, 2- fermentation of glycogen (and glucose) to lactate by fGAOs, and 3- lactate sequestration into PHA by PAOs. According to the energy balance within fGAOs, half of the glucose is utilized for glycogen synthesis, while the other half is converted into lactate (**Table** 8). When comparing theoretical estimates to measured values, it was found that both PHA and glycogen synthesis aligned closely with the theoretical estimates. The amount of glycogen synthesized was determined to be 0.5 Cmmol/Cmmol, which closely matched the measured value of 0.46 Cmmol/Cmmol. Additionally, by extrapolating the theoretical lactate yield, the estimated PHA yield was calculated to be 0.5 Cmmol/Cmmol, which also exhibited a strong correlation with the measured value of 0.47 Cmmol/Cmmol. Finally, an ATP balance was performed for the PAOs and considered the energy required for lactate transport and PHA synthesis was supplied only through polyphosphate hydrolysis (96% recovery, **Table** 9).

*Table 8. Theoretical ATP balance performed over the fermentative organisms assuming ATP required for glycogen synthesis is replenished by lactate fermentation*

| fGAO | | | | |
|---|---|---|---|---|
| **ATP required** | **ATP produced** | **ATP balance** | **Theoretical** | |
| Glycogen synthesis<br>mol/C-mol<br>-0,33 | Lactate synthesis<br>mol/C-mol<br>0,33 | <br>%<br>100 | Glycogen yield<br>mol/mol<br>0,50 | Lactate yield<br>mol/mol<br>0,50 |

*Table 9. ATP balance over the PAOs.*

| PAO | | | |
|---|---|---|---|
| **ATP required** | | **ATP produced** | **ATP balance** |
| VFA-CoA activation<br>mol/C-mol<br>0,12 | Lactate transport<br>mol/C-mol<br>0,17 | Poly-P hydrolysis<br>mol/C-mol<br>0,28 | <br>%<br>96 |

# Discussions

In summary, the experimental findings suggest that glucose is a suitable substrate for the EBPR process and that it can facilitate the formation of stable granules. The anaerobic conversions seemed to be a biotransformation cascade; substrate fermentation (and storage) and product uptake reaction by different microbial communities. Microbial evaluation of the biomass indicated a dominant presence of *Ca*. Accumulibacter and *Micropruina* in the system were mainly responsible for metabolizing glucose.

## Anaerobic conversions and substrate distribution

During the anaerobic phase, several biochemical reactions took place simultaneously. Glucose was rapidly taken up within the first 10 minutes and glycogen concentration increased within the same time frame (Figure 19). In a separate batch with 10 times higher glucose concentration, the direct correlation between glucose conversion and lactate production was verified (**Figure 20**). In this test, the glucose uptake rate was similar as in the regular reactor operation. The batch experiments also indicated that P-release was associated with the uptake of lactate rather than the uptake of glucose. Based on these observations and the analysis of the microbial community, we hypothesized that the anaerobic conversion followed a biotransformation cascade, which was similar to the findings of Jeon & Park. (2000) where glucose was used as the sole electron donor in an EBPR system. However, Jeon & Park. (2000) proposed a probable metabolic pathway which included a “lactate polymer” synthesis based on an undefined peak in their $^{13}$C-NMR spectra. Despite multiple attempts, we were unable to detect any lactate-based storage compounds from the biomass in this study. To the best of the author’s knowledge, lactate-based biopolymers are currently only produced through genetically modified organisms (Taguchi et al., 2008). Moreover, since glucose can be used for extracellular polymer formation (Fuhrer et al., 2005), the undefined NMR peak

spectra could potentially be products like gluconate that are extracellularly produced and constitute the extracellular polymeric substance (EPS).

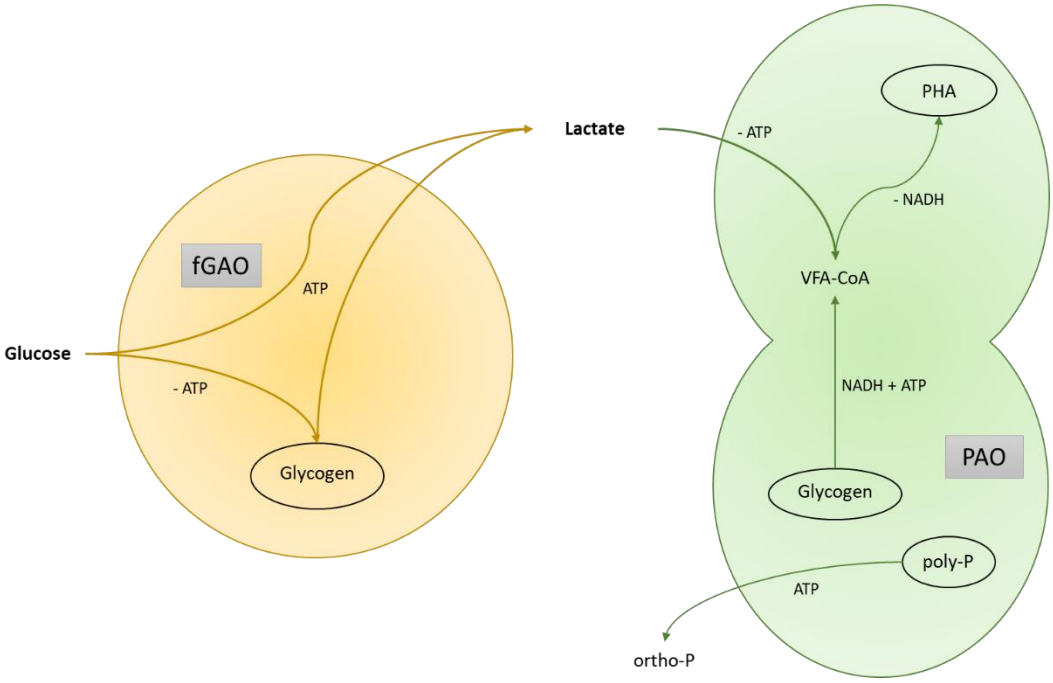


***Figure 24**. Conceptual overview of microbial interaction between fGAOs and PAOs utilizing glucose in the anaerobic phase of AGS-EBPR process.*

We hypothesize that glucose was initially assimilated and stored as glycogen by the fGAOs, with the subsequent conversion into lactate. While it remains unclear whether simultaneous glycogen storage and lactate production occurred, it is still a plausible scenario. Subsequently, the generated lactate served as a substrate for PAOs, contributing to the production of PHA. To validate our hypothesis, we compared the theoretical estimates and the measured values. The measured P/C ratio during a batch test with lactate as the only substrate was 0.45 Pmol/Cmol (Figure 20). This value is comparable to the estimated value of 0.55 Pmol/Cmol during a normal cycle which was calculated with the assumption that half of the glucose was converted into lactate and that the lactate was only utilized by PAOs. In literature, a lactate yield of 0.65 $g/g_{sugar}$ was reported (Tsapekos et al., 2020), which is slightly higher than the estimated lactate yield here but that is likely attributed to the utilization of a pure culture. Regarding the overall P/C ratio, the ratio of 0.25 $Pmol/Cmol_{glucose}$ is similar to the ratio of 0.24 Pmol/Cmol reported by Ziliani et al. (2023) using glucose in a batch test. The P/C ratios obtained with sugar substrates are generally lower ≈ 0.25 Pmol/Cmol (Elahinik et al., 2022; Guerrero et al., 2012; Ziliani et al., 2023) compared to the ratios obtained using VFAs ≈ 0.5 Pmol/Cmol (Oehmen et al., 2005a; Smolders et al., 1994). This difference is likely due to partial consumption of the substrate by other organisms (e.g. fermentative population) when sugars are used as substrate. The partial consumption of substrate decreases the available food for PAOs which results in lower P/C ratios. According to the theoretical lactate yield (0.5

$mol_{lactate}/mol_{glucose}$) and the assumption of polyphosphate hydrolysis by PAOs only (supported by the absence of *pap* in *Actinobacteria*), the ATP balance over PAOs closed with 96% recovery (**Table** 9). Moreover, the balance suggested that the energy required for the uptake of lactate was only provided via polyphosphate hydrolysis and that additional glycogen cleavage was likely not occurring.

Regarding the PHA distribution, PHV was the only detected polymer similar to the findings of Jeon & Park. (2000). Detection of PHV is an indication of acetyl- and propionyl-CoA generation, two precursors of PHV synthesis (Oehmen et al., 2005b). In lactate metabolism, a proposed mechanism suggests the simultaneous oxidation and reduction of pyruvate, leading to the production of acetyl-CoA and propionyl-CoA (Mino et al., 1994). Given that low amounts of acetate and propionate were also detected during the batch test conducted with excess glucose, it is reasonable to anticipate such reactions took place. Furthermore, the NADH produced via the acetyl-CoA pathway can be consumed in the methylmalonyl-CoA pathway (a.k.a. Wood-Werkman cycle) generating propionyl-CoA, maintaining the redox balance within the cell.

### Proteomic insights

The microbial community analysis showed that the two predominant genera in the system were *Ca*. Accumulibacter and *Micropruina,* which are referred to as PAOs and fGAOs, respectively. In an EBPR study by Jeon & Park (2000) where glucose was used as the sole electron donor, the authors hypothesized that EBPR was likely achieved by two groups of microorganisms despite any microbial community analysis; lactic acid-producing organisms and PAOs. In this study, the dominant presence of PAOs (*Ca*. Accumulibacter) and lactic acid producers (*Micropruina* a.k.a. fGAOs) were observed which confirms their hypothesis. Both of these organisms have been observed in EBPR performing WWTPs around the world and information related to their metabolism is available in the literature (Hesselmann et al., 1999; McIlroy et al., 2018). Of particular relevance to this study is the genus *Micropruina*, as they can metabolise glucose, supported by the activity of the phosphotransferase system (*pts*) and their described physiological characteristics in literature (Shintani et al., 2000). *Micropruina* belongs to the *Actinobacteria* class and thrives particularly in carbohydrate-rich environments (Seviour et al., 2000). They are Gram-positive facultative anaerobes that can perform fermentation and glycogen storage (Shintani et al., 2000). By using the combination of FISH and Raman spectroscopy, McIlroy et al. (2018) reported the observation of glycogen in *Micropruina* but neither PHA nor polyphosphate. In this study, the absence of PHA synthesizing proteins in these organisms confirmed that these organisms are unable to produce PHA polymers. Regarding polyphosphate metabolism, the expression of *ppk* enzyme indicates polyphosphate accumulation ability but the absence of *pap* suggests that *Micropruina* do not rely on energy generation from polyphosphate hydrolysis which is in agreement with previous findings (Elahinik et al., 2022). Detection of lactate dehydrogenase and expression of PHA synthesizing proteins in *Ca*. Accumulibacter suggests that these organisms are indeed able to metabolize lactate anaerobically and likely produce PHA using lactate. Moreover, as shown in **Table 7**, glycolysis in both organisms followed the EMP

pathway which is also the commonly reported pathway in EBPR studies (Guedes da Silva et al., 2020; Martín et al., 2006; Ziliani et al., 2023).

### Influence of carbohydrates on the microbiology of EBPR systems

When substrates such as acetate or propionate are used, a highly enriched monoculture is usually achieved that performs anaerobic substrate uptake and PHA synthesis. Alternatively, when non-VFA substrates such as glycerol or glucose are used, a consortium of microorganisms is enriched that performs a biotransformation cascade. The cascade entails the fermentation of substrate into fermentation products by fermentative organisms that use the obtained energy for substrate storage. The fermentation products are subsequently taken up and stored by PAO or GAO-like organisms. During the cascade, storage of glycogen or glycogen-like materials by fermenters can also take place, hence the name "G-bacteria" which was proposed by Cech & Hartman (1993) for these microorganisms. In a minireview by Seviour et al. (2000), the physiology of this group of organisms and why they thrive in certain environments is thoroughly discussed. In short, systems that operate under anaerobic-aerobic conditions with carbohydrates in the influent, allow the proliferation of fGAOs, which also corresponds to the observations in this study. The storage strategy gives these organisms a competitive advantage through uncoupling substrate uptake and growth (Rombouts et al., 2020; Shimada et al., 2007). They can maximize their substrate uptake rate during the feast period and grow in famine conditions using their storage pool. In this study, the observations suggest that a part of the stored glycogen was fermented anaerobically (producing lactate) and likely the other part was utilized aerobically.



### Practical implications

- The application of AGS technology is suitable for the treatment of wastewater with high sugar content since stable granulation can be achieved.
- The dominance of fGAOs may be unfavourable for an efficient EBPR system, however, their complete absence can be detrimental to the overall EBPR process, particularly when sugars like glucose or glycerol are used as the sole electron donor. These organisms compete for substrate but also produce PAO-utilizable products. However, finding ways to provide a selective advantage towards pure fermentative organisms that, unlike fGAOs, do not store substrate might further optimize phosphate removal.
- In situations of carbon scarcity, a potential remedy is to introduce external carbon sources to mitigate the risk of EBPR failures. Glucose can serve as a viable substitute for an external carbon source, considering its cost, availability, and that it is first fermented into PAO-utilizable products.
- In the case of biopolymer recovery, the abundant presence of organisms other than the typical PAOs in the sludge can impact the properties of the extracted Kaumera biopolymer (de Bruin et al., 2022; Tomás-Martínez et al., 2023).

# Conclusions

- Long-term stable reactor performance and successful granulation were shown with glucose as the sole carbon source in an AGS system.
- Phosphate removal was achieved through a collaboration between fGAOs (*Micropruina)* and PAOs (*Ca*. Accumulibacter).
- Lactate was the main product of glucose conversion in the anaerobic phase.
- Absence of PHA synthesizing enzymes in *Micropruina* indicates that these organisms are likely unable to produce PHA which confirms previous findings in the literature.
- Detection of lactate dehydrogenase and expression of PHA synthesizing proteins in *Ca*. Accumulibacter suggests that these organisms are able to metabolize lactate anaerobically and likely produce PHA using lactate.
- Lack of PTS protein components in *Ca*. Accumulibacter suggests that these organisms are not able to directly utilize glucose.

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# 4. Effects of Salinity on Glycerol Conversion and Biological Phosphorus Removal by Aerobic Granular Sludge

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## Abstract

Industrial wastewater often has high levels of salt, either due to seawater or e.g. sodium chloride (NaCl) usage in the processing. Previous work indicated that aerobic granular sludge (AGS) is differently affected by seawater or saline water at similar osmotic strength. Here we investigate in more detail the impact of NaCl concentrations and seawater on the granulation and conversion processes for AGS wastewater treatment. Glycerol was used as the carbon source since it is regularly present in industrial wastewaters, and to allow the evaluation of microbial interactions that better reflect real conditions. Long-term experiments were performed to evaluate and compare the effect of salinity on granulation, anaerobic conversions, phosphate removal, and the microbial community. Smooth and stable granules as well as enhanced biological phosphorus removal (EBPR) were achieved up to 20 g/L NaCl or when using seawater. However, at NaCl levels comparable to seawater strength (30 g/L) incomplete anaerobic glycerol uptake and aerobic phosphate uptake were observed, the effluent turbidity increased, and filamentous granules began to appear. The latter is likely due to the direct aerobic growth on the leftover substrate after the anaerobic feeding period. In all reactor conditions, except the reactor with 30 g/L NaCl, *Ca.* Accumulibacter was the dominant microorganism. In the reactor with 30 g/L NaCl, the relative abundance of *Ca.* Accumulibacter decreased to ≤1% and an increase in the genus *Zoogloea* was observed. Throughout all reactor conditions, *Tessaracoccus* and *Micropruina*, both actinobacteria, were present which were likely responsible for the anaerobic conversion of glycerol into volatile fatty acids. None of the glycerol metabolizing proteins were detected in *Ca*. Accumulibacter which supports previous findings that glycerol can not be directly utilized by *Ca*. Accumulibacter. The proteome profile of the dominant taxa was analysed and the results are further discussed. The exposure of salt-adapted biomass to hypo-osmotic conditions led to significant trehalose and $PO_4^{3-}$-P release which can be related to the osmoregulation of the cells. This study provides insights into the effect of salt on the operation and stability of the EBPR and AGS processes. The findings suggest that maintaining a balanced cation ratio is likely to be more important for the operational stability of EBPR and AGS systems than absolute salt concentrations.




## Introduction

Many wastewaters of industrial origin contain high concentrations of sodium chloride (NaCl), which are often accompanied by high organic content (>1000 mg/L chemical oxygen demand (COD) (Lefebvre and Moletta, 2006). In line with the objectives of circular economy solutions for industrial brines, compounds such as NaCl present in industrial brines can be recovered by applying suitable brine treatment technologies able to capture the circular water value of brine effluent streams (Xevgenos et al., 2024). The reuse of salt entails the removal of organics to achieve high-purity brine to be used in other applications such as the chlor-alkali process. Utilizing reverse osmosis for NaCl recycling similarly demands high-purity brine, as the presence of organics can severely decrease the efficiency of these systems. Moreover,

municipal wastewater with seawater flushing or intrusion also contains high salinity that must be treated before discharge (EU Water Framework Directive, 2000).

To remove organics, aerobic granular sludge (AGS) can be applied as a biological wastewater treatment technology. AGS is a highly efficient method of treating wastewater, offering numerous benefits over traditional activated sludge systems (Bengtsson et al., 2018; de Bruin et al., 2004; Pronk et al., 2015). The granular structure of the biofilm allows for fast settling and the substrate and oxygen gradient across the granules enables simultaneous conversions to take place, leading to a reduced treatment time and improved solid-liquid separation (Bassin et al., 2012). The formation of aerobic granules was first reported in the late 1990s (Beun et al., 1999; Heijnen and Van Loosdrecht, 1998; Morgenroth et al., 1997), and has since been the subject of extensive research to better understand the mechanisms behind their formation and stability (Haaksman et al., 2023; van Dijk et al., 2022). During substrate feeding under anaerobic conditions, polyhydroxyalkanoates (PHA) are stored and later utilized by slow-growing heterotrophs in the subsequent aerobic phase. This leads to the formation of compact granular biofilms by preventing transfer-limited growth (de Kreuk and van Loosdrecht, 2004).

Salt can negatively affect wastewater processes including AGS processes. For example, by altering the microbial community composition and anaerobic-aerobic conversions which can influence the effluent quality and solid-liquid separation due to the proliferation of filamentous organisms and destabilization of the biofilm matrix (Bassin et al., 2011; Pronk et al., 2014; Sivasubramanian et al., 2021; Welles et al., 2014). There are indications that salt water with mainly NaCl will have a different impact than seawater salinity (de Graaff et al., 2020a). In this study, the aim was to evaluate this difference in more detail. All studies describing some effects of salt have been performed with acetate as the carbon source (Bassin et al., 2011; de Graaff et al., 2020b; Figueroa et al., 2008; Pronk et al., 2014; Wang et al., 2017). Here, we chose to use glycerol as the carbon substrate. The use of sugars such as glycerol promotes a more diverse microbial community compared to VFAs (Elahinik et al., 2022), which allows for studying the microbial interactions in such systems. Fermentative microorganisms convert sugars into fermentation products, that can be utilized by e.g. phosphate-accumulating and glycogen-accumulating microorganisms. This creates a microbial cross-feeding relationship, where different species depend on each other for the breakdown and utilization of substrates, fostering diversity (Elahinik et al., 2023, 2022). From the application point of view, glycerol is also a compound found in many industrial waste streams such as the epoxy and biodiesel production facilities, which makes it an interesting substrate to study concerning the AGS processes.

The enhanced biological phosphorus removal (EBPR) process is an energy-efficient and cost-effective technology (Barnard, 1975). This technology is centred on the enrichment of the sludge with phosphorus-accumulating organisms (PAO) that can accumulate excessive amounts of phosphorus (P) in the form of poly-phosphate (poly-P). The removal of phosphorus is then achieved by discharging the excess sludge containing accumulated phosphorus (Smolders et al., 1995). Given the similarity in operational parameters between the EBPR and AGS processes, they can be studied concurrently.

This study aims to investigate the effects of varying concentrations of NaCl (10-30 g/L) in comparison to synthetic seawater (35 g/L) on granulation and EBPR process using glycerol as the sole carbon source. Detailed information on the kinetic and anaerobic-aerobic conversions, the main bacterial species involved, the changes in the microbial community, and the granulation of the AGS system performing EBPR is provided. The study also evaluates the enzymatic machinery of the dominant taxa and the key processes such as osmoregulation, substrate storage, and fermentation under saline conditions via metaproteomics.

## Material and Methods

### Experimental setup and reactor operation

A bubble column reactor with a working volume of 3.1 L, an internal diameter of 5.6 cm, and a total height of 90 cm was operated in sequencing batch reactor (SBR) mode. After 1.5 L effluent withdrawal at the end of each cycle, a volumetric exchange ratio of 48% was maintained. The pH was controlled at 7.1 ± 0.1 by automatic dosing of 0.5 M NaOH or HCl. The dissolved oxygen (DO) concentration was controlled at 0% and 50% (3.5 mg/L) saturation during the anaerobic and aerobic phase, respectively, by a mixture of nitrogen gas and air. The off-gas was recirculated with a flow of 5 L/min to maintain the DO concentration. The DO probes were calibrated for each salt concentration. The temperature of the reactor was not directly controlled but the room temperature was controlled at 20 °C. The reactor was inoculated with ≈ 600 mL of a mixture of aerobic granular sludge from a pilot-scale municipal wastewater treatment reactor performing EBPR located in Harnaschpolder, the Netherlands and glycerol-adapted sludge performing EBPR from previous experiments. Each cycle consisted of 5 min of nitrogen sparging to ensure anaerobic condition before feeding followed by 5 min of feeding, 60 min of nitrogen sparging (anaerobic phase), 120 min of aeration (aerobic phase), 5 min of settling, and 5 min of effluent withdrawal.



The synthetic influent fed at the beginning of each cycle with a total volume of 1,500 mL consisted of 1,200 mL of salt water (10 – 30 g/L NaCl or 35 g/L synthetic sea salt), 150 mL of nutrient medium and 150 mL of carbon medium. Synthetic seawater with a final concentration of 35 g/L was made with Instant Ocean® sea salt (Atkinson et al., 1997). 35 g/L sea salt was used to account for the mass of other elements in the mixture and to achieve a 30 g/L NaCl equivalent. Carbon medium contained 35.7 mM glycerol, 3.6 mM $MgSO_4.7H_2O$, and 4.7 mM KCl. The nutrient medium contained 41.1 mM $NH_4Cl$, 1.95 mM $K_2HPO_4$, 1.98 mM $KH_2PO_4$, 0.6 mM Allythiourea (ATU) to inhibit nitrification and 10 mL/L of trace element solution. The trace element solution contained 4.99 g/L $FeSO_4.7H_2O$, 2.2 g/L $Zn.SO_4.7H_2O$, 7.33 g/L $CaCl_2.2H_2O$, 4.32 g/L $MnSO_4.H_2O$, 2.18 g/L $Na_2MoO_4.2H_2O$, 1.57 g/L $CuSO_4.5H_2O$, 1.61 g/L $CoCl_2.6H_2O$ and 50 g/L EDTA. The combination of these feed sources resulted in a final influent concentration of 400 mg/L COD, 58 mg/L $NH_4^+$-N, and 12 mg/L of $PO_4^{3-}$-P. The ratio of major cations relative to sodium in seawater, synthetic seawater (I.O.), and medium dosed with pure NaCl with different concentrations is presented in **Table** 10.

Batch experiments were performed in 250 mL bottles, sparged with nitrogen to ensure an anaerobic environment, and controlled pH at 7 ± 0.1 by manual dosing of 0.5 M HCl or NaOH. Biomass at the end of the aerobic phase (end of the cycle) was taken from the reactor and used as inoculum (20 mL).

***Table 10**. The ratio of magnesium and potassium relative to sodium in seawater, synthetic sea salt (I.O.), and medium dosed with pure NaCl with different concentrations. The absolute concentrations of the respective elements fed each cycle are shown. The elemental composition of seawater and I.O. is given in* (Atkinson et al., 1997).

| Parameters | Seawater | I.O. | 30 g/L NaCl | 20 g/L NaCl | 10 g/L NaCl |
|---|---|---|---|---|---|
| $Na^+/Mg^{2+}$ | 8 | 8 | 1346 | 897 | 449 |
| $Na^+/K^+$ | 27 | 29 | 643 | 429 | 214 |
| $Na^+$ [g] | 16 | 16 | 18 | 12 | 6 |
| $K^+$ [mg] | 598 | 551 | 28 | 28 | 28 |
| $Mg^{2+}$ [mg] | 1932 | 1895 | 13 | 13 | 13 |
| Osmolarity [atm] | 27 | 26 | 26 | 21 | 17 |

## Analytical methods

Concentrations of $PO_4^{3-}$-P and $NH_4^+$-N were measured using a Gallery Discrete Analyser (ThermoFisher Scientific, USA). Chemical Oxygen Demand was measured with a spectrophotometer cuvette system (DR2800, Hach Lange, USA). Volatile fatty acids and sugars were measured using an HPLC (Vanquish, ThermoFisher Scientific, USA) equipped with an RI and UV detector, Aminex HPX-87H column (BioRad, USA) using 0.0015M phosphoric acid as eluent.

## PHA & glycogen determination

Biomass samples from the reactor were fixed with 4% w/v paraformaldehyde, washed with demineralized water, freeze-dried overnight and crushed into fine powder. For extraction, approximately 30 mg of the powered biomass was hydrolysed and esterified in a 10% sulphuric acid, methanol, and chloroform solution for 24 hours at 100 °C with frequent manual vortex. For phase separation, 3 mL of ultrapure water was added to the samples and the formed esters in the organic phase were then filtered and analysed by a GC (6890N, Agilent, USA). Quantifications of PHB, PHV, and PH2MV were done using commercial 3-hydroxybutyrate, a synthetic copolymer of (R)-3-hydroxybutyrate-(R)-3-hydroxyvalerate (Sigma-Aldrich, USA), and 3-Hydroxy-2-methylvaleric acid (BenChem, USA), respectively. Benzoic acid was also added to the samples as an internal standard.

## Microscopy

To observe and capture the morphology of the granules, a stereo zoom microscope (M205 FA, Leica Microsystems, Germany) equipped with Qwin image analysis software (V3.5.1, Leica Microsystems, Germany) was used. The phase contrast pictures of the microbial population were taken with Axio Imager M2 (Zeiss, Germany).

### Metagenomics and metaproteomics

The DNA from the biomass samples was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) following the manufacturer's protocol. Metagenome sequencing and raw data processing were conducted by Novogene (Novogene Co., China). For protein extraction and metaproteome analysis, the methodology described by Kleikamp et al. (2022) was followed. Similar to (Elahinik et al., 2022), the functional proteins were annotated using the KEGG database, and the metaproteome raw data was analysed using the metagenome-constructed dataset.

## Results

### Reactor operation



A bubble column reactor with aerobic granular sludge was operated in sequencing batch mode for over 300 days. The reactor was operated in four different conditions each characterized by varying salinity (**Table** 10). About 50 mL of crushed granular sludge from a full-scale WWTP located in Harnaschpolder, the Netherlands was used to supplement the biomass in the reactor at the beginning of each condition to ensure proper microbial diversity. The concentrations of orthophosphate and readily biodegradable COD (rbCOD) measured over time are shown in **Figure 25**. The rbCOD represents glycerol and potential products formed in the bulk liquid at the end of the anaerobic phase. The conversion of glycerol in an AGS system under fresh-water conditions was previously studied (Elahinik et al., 2022) where the anaerobic reactions seemed to be a combination of glycerol conversion (to mainly propionate) by the fermentative community and product uptake-type reaction by the PAOs. At 10 and 20 g/L NaCl complete anaerobic glycerol uptake as well as phosphate release and uptake was observed, similar to fresh-water conditions. At 30 g/L NaCl, glycerol was only partly converted into propionate in the anaerobic phase and propionate accumulation in the liquid phase was observed (**Figure 28**). Leftover glycerol and propionate were oxidized in the subsequent aerobic phase. This resulted in reduced anaerobic phosphate release and aerobic uptake until complete EBPR deterioration. In seawater conditions (switching to 35 g/L sea salt), anaerobic glycerol uptake increased and phosphate release was observed (**Figure 25**). Average specific phosphate and ammonium conversions at each salt concentration are presented in **Table 11**. Ammonium consumption is due to biomass growth since ATU was added to inhibit nitrification.

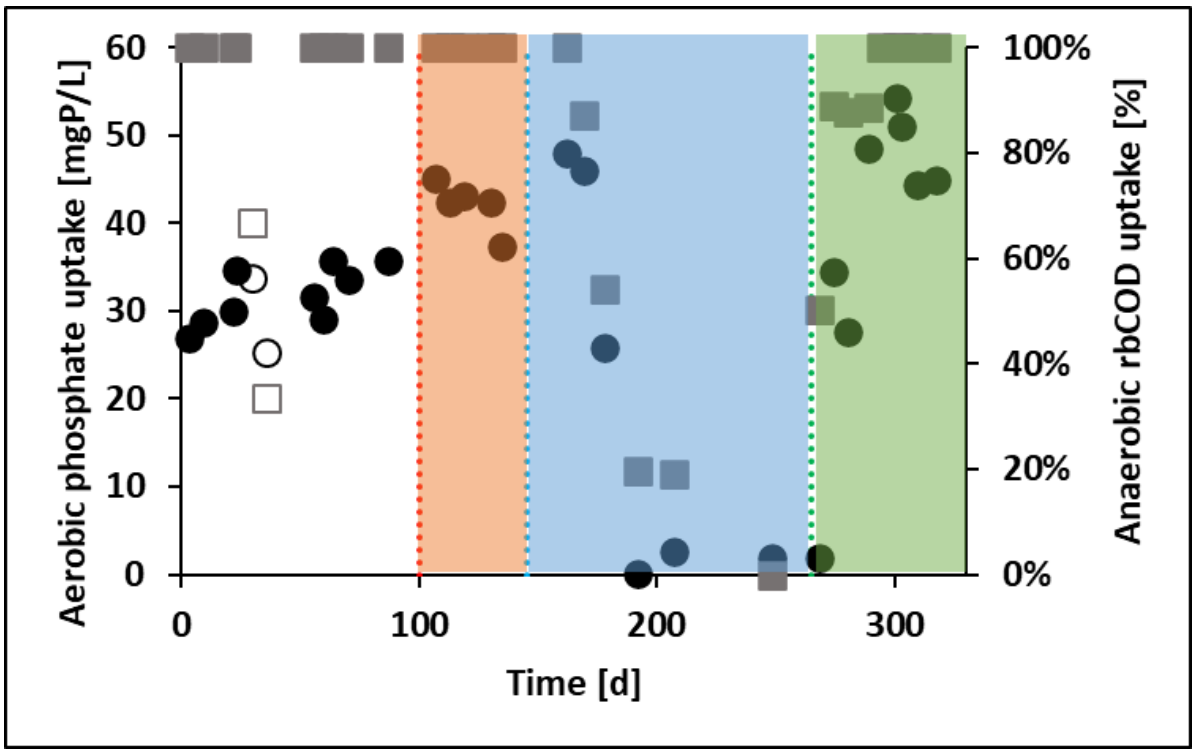


***Figure 25**. Concentrations of orthophosphate (circles) and percentage of rbCOD consumed (squares) at the end of the anaerobic phase over time. rbCOD represents glycerol and potential products formed in the anaerobic phase. Hollow markers show operational failure due to technical issues. The shaded areas indicate the switch from 10 to 20 g/L NaCl (red), 20 to 30 g/L NaCl (blue), and from 30 g/L NaCl to 35 g/L sea salt (green).*

***Table 11**. Average specific phosphate and ammonium conversions during reactor pseudo-steady-state operation. Standard deviations are included. * Values from the freshwater conditions are retrieved from* (Elahinik et al., 2022) *and are provided as a reference.*

| Salt | Freshwater* | NaCl 10 | NaCl 20 | NaCl 30 | Sea salt 35 | g/L |
|---|---|---|---|---|---|---|
| P-release | 11.21 ± 1.0 | 3.54 ± 0.9 | 5.10 ± 1.1 | 0.10 ± 0.1 | 3.84 ± 0.8 | *mgP/gVSS* |
| P-uptake | 8.09 ± 0.8 | 4.17 ± 1.0 | 5.89 ± 1.1 | 0.24 ± 0.2 | 4.35 ± 0.8 | *mgP/gVSS* |
| N-consumption | 1.42 ± 0.1 | 1.02 ± 0.4 | 1.30 ± 0.2 | 1.02 ± 0.2 | 0.78 ± 0.2 | *mgN/gVSS* |

At 30 g/L NaCl, incomplete anaerobic substrate uptake led to aerobic conversion of the carbon source and some filamentous growth was observed (**Figure 26**.C&D). With increasing NaCl concentration, the effluent suspended solids concentration increased threefold (**Table 12**) as can also be seen in the murky background (**Figure 26**.D). Microscopic analysis of the effluent showed the presence of suspended cell aggregates and staining of effluent suspended solids with Alcian blue, showed the presence of cells entrapped in extracellular acidic polysaccharides (**Figure 26**.F). By switching the salt from 30 g/L NaCl to 35 g/L sea salt, the effluent turbidity decreased and the formation of new granular biomass and a compact

settled bed was observed. The average dry weight measurements, solid retention time (SRT), and sludge volume index ($SVI_5$) are presented in **Table** 12.

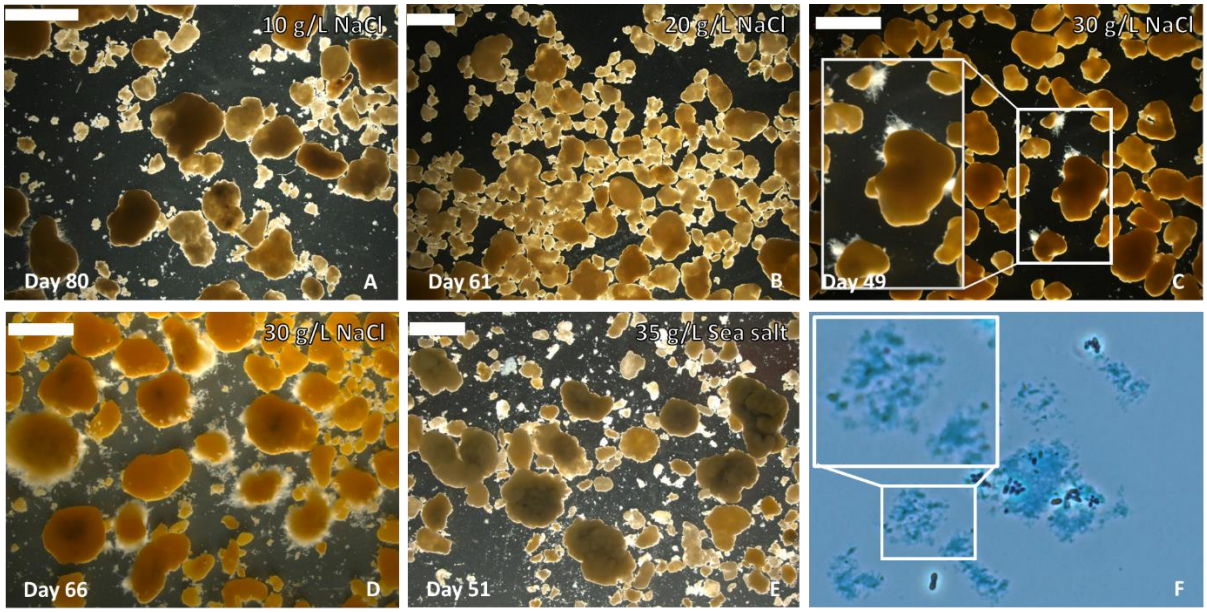


***Figure 26**. Stereoscopic image of aerobic granular sludge cultivated at different salt concentrations. The number of operation days after which the pictures are taken is indicated. Pictures (A) and (B) show the granules at 10 and 20 g/L NaCl, respectively. Pictures (C) and (D) show the appearance and proliferation of filamentous granules at 30 g/L NaCl, respectively. Picture (E) shows the granules at 35 g/L sea salt. Picture (F) shows Alcian blue staining of the effluent suspended solids. Scale bar equals 3 mm (A-D) and 1 mm (E).*

The SRT was controlled between 12 - 17 days through a combination of manual sludge removal and solids washout with the effluent. At 20 and 30 g/L NaCl, manual sludge removal was reduced to preserve a sufficient amount of biomass within the reactor, resulting in an increase in the SRT (**Table 12**). This was done to increase substrate utilization and offset potential disturbances due to higher salinity. The VSS/TSS ratio at 10 and 20 g/L NaCl enrichment were 0.72 & 0.73, respectively, and are comparable to freshwater enrichments with glycerol with a ratio of 0.75. However, the ratio increased to 0.89 as the NaCl concentration increased to 30 g/L. At 35 g/L sea salt, with the restoration of EBPR activity, the VSS/TSS ratio dropped to 0.75.

***Table 12**. Average dry weight measurements, SRT, and $SVI_5$ for each respective salt concentration are shown. Standard deviations are also provided. * Values from the freshwater conditions are retrieved from* (Elahinik et al., 2022) *and are provided as a reference.*

| **Parameters** | **Values** | | | | | **Units** |
|---|---|---|---|---|---|---|
| Salt | Freshwater* | NaCl 10 | NaCl 20 | NaCl 30 | Sea salt 35 | *g/L* |
| SRT | 12 | 12 | 17 | 17 | 12 | *d* |
| $SVI_5$ | 33.4±1.9 | 15.4±3.3 | 16.7±1.7 | 18.1±2.6 | 9,8±2,2 | *mL/g* |
| TSS | 7.3±0.5 | 13.1±0.5 | 10.2±1.1 | 8.8±1.9 | 15.3±2.2 | *g/L* |
| VSS | 5.5±0.3 | 9.5±0.5 | 7.4±0.8 | 7.8±1.8 | 11.47±1.6 | *g/L* |
| VSS/TSS | 0.75±0.02 | 0.73±0.02 | 0.72±0.00 | 0.89±0.02 | 0,75±0,01 | *g/g* |
| Effluent TSS | 0.07±0.02 | 0.12±0.01 | 0.19±0.09 | 0.30±0.16 | 0.10±0.02 | *g/L* |

## Batch tests

Batch tests were performed with glycerol- and propionate-adapted sludge performing EBPR to evaluate whether glycerol can be directly taken up under anaerobic conditions by PAOs. We chose biomass from an existing reactor in the lab that was fed with propionate only as a control. This test aimed to verify the findings of a recent study (Elahinik et al., 2022) which suggested that glycerol conversion in AGS-EBPR systems was a combination of substrate fermentation by fermentative organisms and product uptake reaction by PAOs. As shown in Figure 27, the results revealed that the propionate-adapted biomass was not able to utilize glycerol directly as expected. Nonetheless, both systems displayed a comparable trend in primary phosphorus release with a rate of 2.9 mgP/gVSS/h. This finding prompted us to investigate if glycerol induced an osmotic stress on the propionate-enrichment, which could cause P-release. To address this concern, we conducted a separate test (data not shown) with and without glycerol in the medium but found that the P-release profile was similar. This indicated that P-release occurs regardless of the presence of glycerol in the glycerol-adapted biomass.

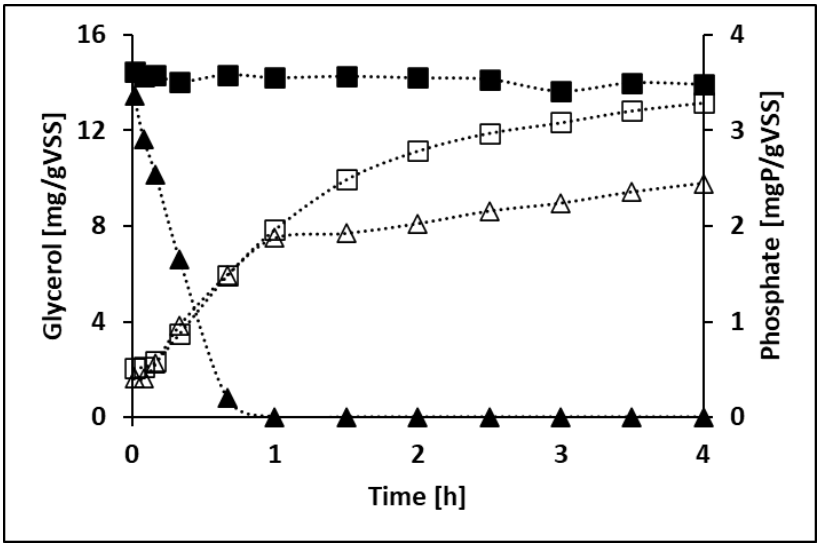


*Figure 27. Glycerol (solid markers) and phosphate (hollow markers) concentrations under anaerobic conditions by glycerol enrichment (triangles) and propionate enrichment (squares).*



Typical cycle behaviour

**Figure 28**. shows the behaviour of a typical cycle for each salinity tested during reactor pseudo-steady-state operation. From this, the rates and stoichiometries are obtained and listed in **Table 13**. The pseudo-steady-state was defined based on stable glycerol and phosphate conversions for more than at least 1 SRT. In the anaerobic phase, glycerol uptake, P-release and PHA accumulation were observed at 10 and 20 g/L NaCl. In the subsequent aerobic phase, P-uptake and PHA oxidation occurred. The only storage polymer detected was PHV. No PHB or PH2MV was detected. Due to the potential occurrence of simultaneous glycogen degradation and accumulation, and trehalose presence, the anaerobic glycogen hydrolysis was estimated based on the type and the amount of PHA produced as described by (Elahinik et al., 2022). Mass balances were performed over anaerobic glycerol uptake, PHV accumulation and theoretical glycogen hydrolysis. The electron (COD) balances closed with a recovery of 105% and 93% for reactor conditions with 10 and 20 g/L NaCl concentration, respectively. At 30 g/L NaCl, only 58% of the glycerol was converted into propionate during the anaerobic phase, leading to the observed accumulation of propionate. The remaining glycerol and the formed propionate were subsequently oxidized during the aerobic phase. In seawater conditions, complete anaerobic glycerol uptake and PHV formation were observed. The COD balance closed with a recovery of 110% and the EBPR process was restored.

***Table 13**. List of anaerobic rates and stoichiometries. $P_{rel}$: Phosphorus release, $PHA_{pro}$: PHA production, $C_{up}$: Carbon uptake, $Gly_{deg}$: Glycogen degradation. * Values from the freshwater conditions are retrieved from* (Elahinik et al., 2022) *and are provided as a reference. ** indicates estimated values which are calculated based on the PHA accumulated.*

| Salt | Freshwater* | NaCl 10 | NaCl 20 | NaCl 30 | Sea salt 35 | *g/L* |
|---|---|---|---|---|---|---|
| Stoichiometries | | | | | | |
| $P_{rel}/C_{up}$ | 0.23 | 0.15 | 0.16 | 0.00 | 0.28 | *Pmol/Cmol* |
| $PHA_{up}/C_{up}$ | 0.97 | 1.98 | 1.66 | 0.85 | 2.66 | *Cmol/Cmol* |
| $Gly_{deg}/C_{up}$** | 0.27 | 0.87 | 0.98 | 0.46 | 1.65 | *Cmol/Cmol* |
| Rates | | | | | | |
| qS | 2.0 | 1.0 | 1.5 | 0.2 | 0.7 | *Cmmol/gVSS/h* |
| $qP_{rel}$ | 11.3 | 2.3 | 6.0 | 0.1 | 6.1 | *mgP/gVSS/h* |
| $qP_{up}$ | 16.3 | 7.9 | 11.8 | 0.0 | 3.6 | *mgP/gVSS/h* |

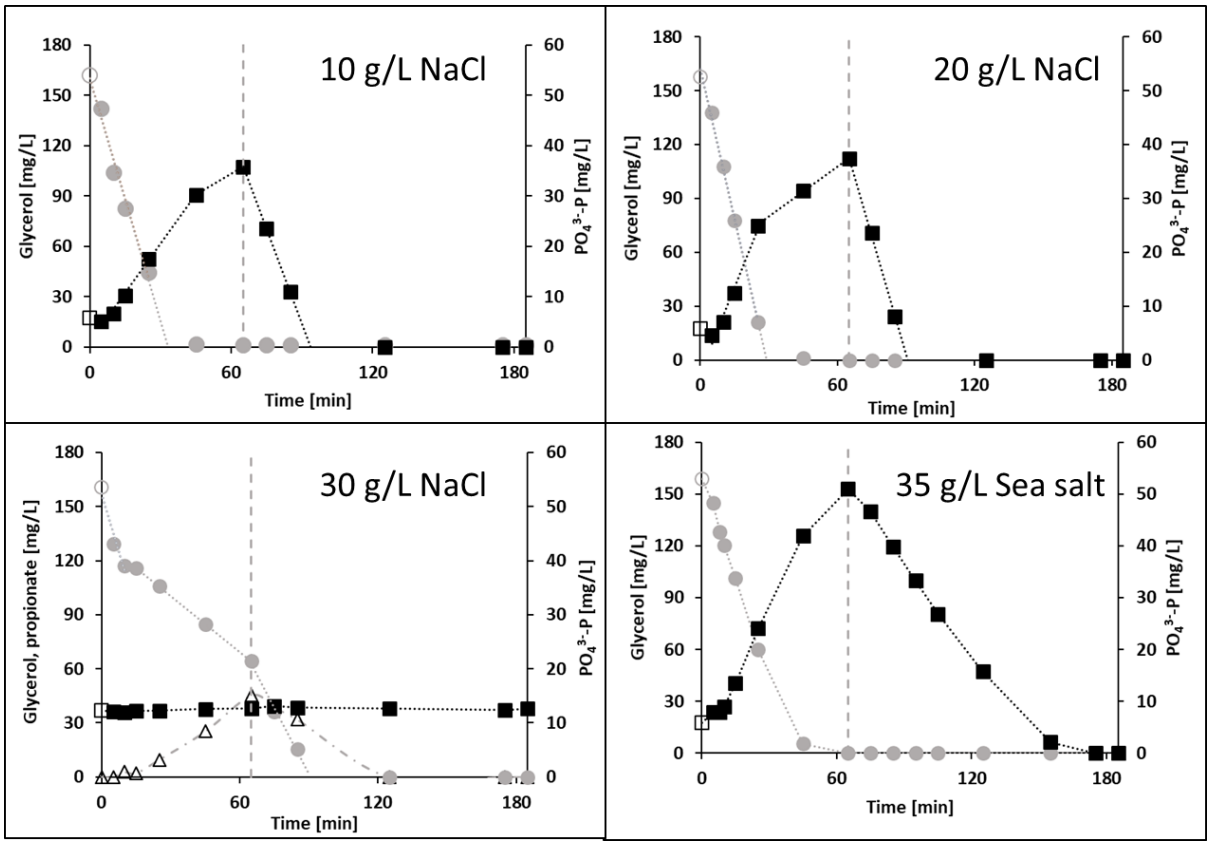


***Figure 28**. Glycerol (circles), and $PO_4^{3-}$-P (squares) profiles during a typical cycle at each salt concentration. Propionate (triangles) accumulation is shown at 30 g/L NaCl. The dashed vertical lines indicate the switch between anaerobic and aerobic phases. The hollow points at time 0 are calculated concentrations based on influent concentration and the dilution.*

## Osmotic shock test

To assess the effect of hypoosmotic conditions on biomass activity, batch tests were performed with biomass taken from the reactor at the end of the aerobic phase and placed in demineralized water sparged with nitrogen. No trehalose was detected in the bulk liquid during the normal cycles of reactor operation. Demineralized water was used to minimise the

presence of cations and anions. Liquid samples were taken and analysed by an HPLC and the results are shown in **Figure 29**.

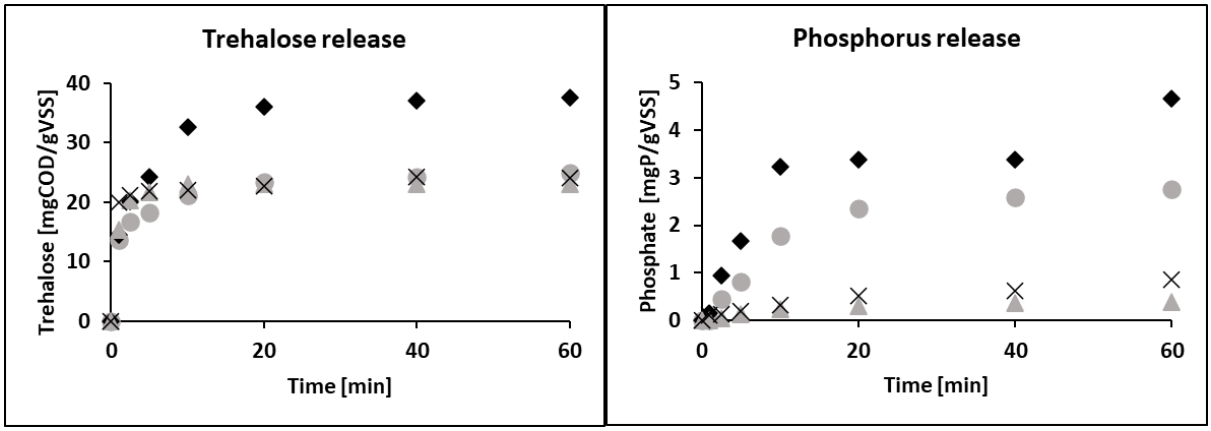


***Figure 29**. Trehalose and phosphate release as a function of time under anaerobic conditions. Biomass cultivated in 10 g/L NaCl (circles), 20 g/L NaCl (diamonds), 30 g/L NaCl (triangles), and 35 g/L sea salt (crosses).*



Incubation of salt-adapted granules in demineralized water showed significant trehalose and accompanied phosphate release (**Table 14**). The maximum release occurred rapidly within minutes and then reached a plateau (**Figure 29**).

***Table 14**. List of trehalose and orthophosphate release of the biomass cultivated in 10-30 g/L NaCl and 35 g/L sea salt.*

| **Parameters** | **Values** | | | | **Units** |
|---|---|---|---|---|---|
| Salt concentration | 10 g/L NaCl | 20 g/L NaCl | 30 g/L NaCl | 35 g/L Seasalt | *g/L* |
| Trehalose release | 20.9 | 32.2 | 20.6 | 21.6 | *mg/gVSS* |
| $PO_4^{-3}$-P release | 2.4 | 3.4 | 0.2 | 0.9 | *mg/gVSS* |

## Microbial community

To visually observe the changes in the microbial community distribution, frequent microscopic analysis was performed. At 10 and 20 g/L NaCl, the microbial community looked similar to the microbial community of a fresh-water system, where two morphologically distinct microorganisms were present (**Figure 30**.A). The microbial community structure of a fresh-water AGS system with glycerol as the carbon source is described in Elahinik et al. (2022). The first group consisted of cocci with white storage material indicating a probable presence of *Ca.* Accumulibacter, a common PAO found in EBPR systems. Meanwhile, the second group exhibited cocci arranged in tetrads, suggesting the likely presence of *Tessaracoccus,* a fermentative glycogen-accumulating organism (fGAO), capable of anaerobic glycerol conversion. At 30 g/L NaCl, while tetrad-like microorganisms were detected, the PAOs could not be detected. Notably, the tetrad-like organisms were not detected at 35 g/L sea salt and large circular cells were observed instead

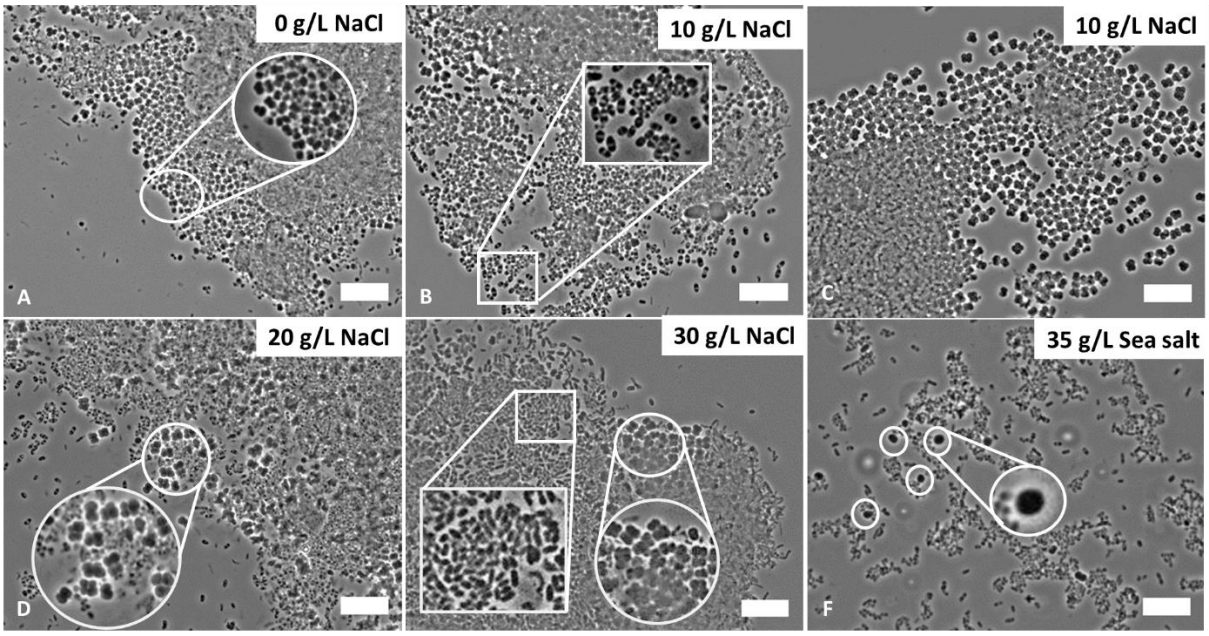


***Figure 30**. Phase contrast images of the microbial community representing each condition. Square delineations show the hoarder population, i.e. PAOs, and circle delineations show the fermentative population, likely responsible for the conversion of glycerol. Picture A shows the microbial community of the reactor in fresh-water conditions as a reference. Picture C shows a cluster of tetrad microorganisms. Pictures B, D, E, & F show the microbial community of each respective salinity.*

To further evaluate the microbial community of the reactor conditions of each salinity, biomass samples from the end of each condition were used for metagenome sequencing. The metagenome data was only used as a constructed dataset for the metaproteome analysis. Metaproteomics were performed in parallel to track the potential changes of protein expression with respect to different salinities as well as estimation of the microbial community distribution based on protein mass fraction. The relative abundance of the top 5 genera in each reactor is shown in **Figure 31**. The fraction labelled as “other” are known but low abundant taxa with very low protein masses and thus are not assigned. In reactor conditions exhibiting stable granulation and efficient EBPR performance, *Ca.* Accumulibacter emerged as the predominant microorganism. At 30 g/L NaCl, where both EBPR efficiency and granulation deteriorated, *Zoogloea* outcompeted *Ca.* Accumulibacter, establishing itself as the dominant taxon. Throughout all reactor conditions, *Tessaracoccus* and *Micropruina*, both actinobacteria, persisted, albeit with a low relative abundance. Similarly, *Stappia*, a motile, Gram-negative aerobe, also maintained a presence across all reactor conditions.

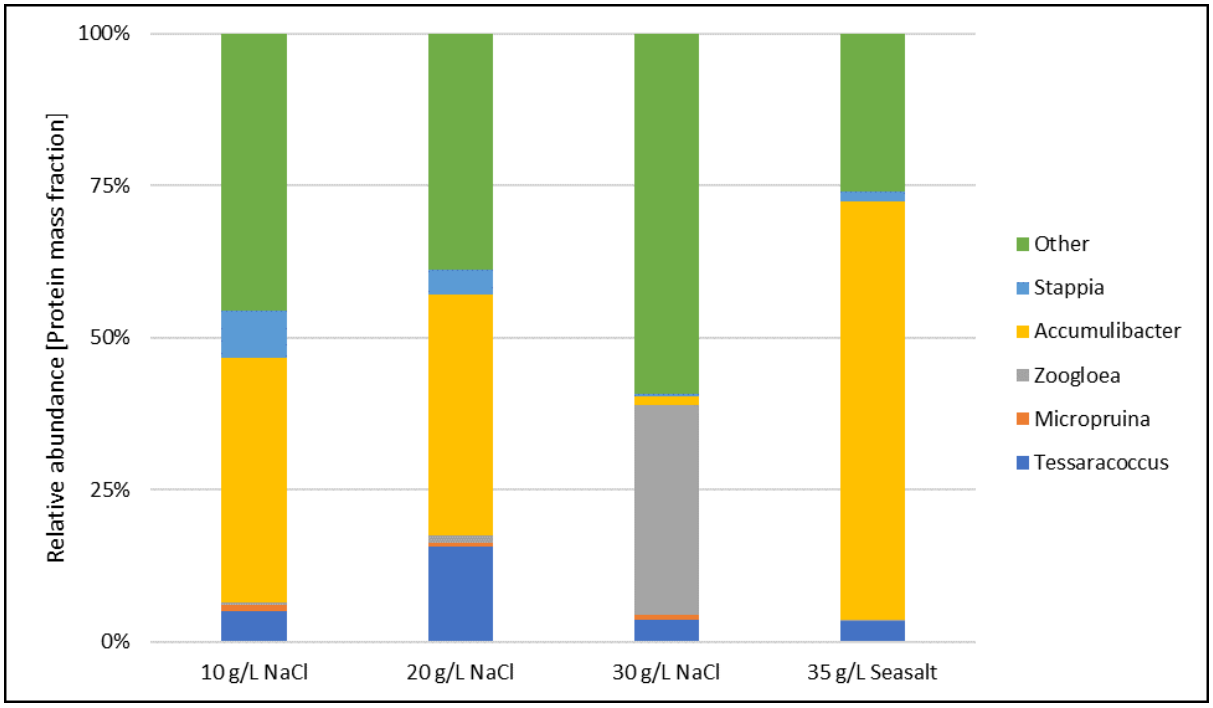


*Figure 31. The relative abundance of the top 5 genera across the reactor conditions with respective salinities based on protein mass fraction.*



The key protein expressions involved in trehalose, polymer storage, polyphosphate, glycerol, potassium, and magnesium metabolism in PAOs (*Ca.* Accumulibacter) and fGAOs (actinobacteria), the two dominant taxa in stable reactor conditions, are listed in **Table 15**. Note that the absence of indicated proteins does not necessarily imply their non-existence within the microorganism as it may be attributed to the limitations of detection methods and extraction protocol.

***Table 15**. List of key metabolisms and respective proteins in PAOs and fGAOs as dominant taxa across all reactor conditions with stable operation.*

| Metabolisms | Proteins | Symbols | PAOs | fGAOs |
|---|---|---|---|---|
| Trehalose | α,α-trehalase | *TreA* | - | - |
| | 1-α-D-glucosylmutase | *TreY/glgY* | + | - |
| | Isoamylase | *TreX* | + | - |
| | Trehalohydrolase | *TreZ/glgZ* | + | - |
| Storage polymer | 1,4-α-glucan branching enzyme | *glgB* | - | + |
| | Glucose-1-phosphate adenylyltransferase | *glgC* | + | - |
| | Starch synthase | *glgE* | - | - |
| | α-maltose-1-phosphate synthase | *glgM* | + | + |
| | Glycogen phosphorylase | *glgP* | - | + |
| | PHA synthase | *phaC* | + | - |
| | PHA polymerase | *phaE* | + | - |
| | Acetoacetyl-CoA reductase | *phbB* | + | + |
| | Acetyl-CoA synthetase | *acs* | + | + |
| | Propionyl-CoA synthetase | *prpE* | + | - |
| Polyphosphate | Phosphotransferase | *pap* | + | - |
| | Exopolyphosphatase | *ppx* | + | - |
| | Polyphosphate glucokinase | *ppgk* | + | - |
| | Polyphosphate kinase | *ppk* | + | - |
| Glycerol | Glycerol-3-phosphate dehydrogenase | *glpA/B/C/D* | - | + |
| | Glycerol uptake facilitator | *glpF* | - | + |
| | Glycerol kinase | *glpk* | - | + |
| Potassium | $K^+$ uptake protein | *trkA* | + | + |
| | $K^+$-dependent mechanosensitive channel | *kefA/aefA* | + | - |
| Magnesium | $Mg^{2+}$ exporter | *tlyC* | + | - |
| | $Mg^{2+}$ transporter | *corA* | + | - |

## Discussions

An AGS reactor was operated in SBR mode to investigate and compare the effect of various concentrations of NaCl and synthetic seawater on granulation, anaerobic-aerobic conversions, phosphate removal, and changes in the microbial community. Glycerol was used as the carbon source since it is regularly present in industrial wastewaters and to allow the evaluation of microbial interactions that better reflect real conditions. The results showed that granulation and phosphate removal deteriorated with 30 g/L NaCl while at similar NaCl

concentrations but under seawater conditions, the reactor operation behaved comparable to fresh-water conditions. The findings and the potential underlying mechanisms are discussed.

## Influence of ionic property differences on AGS and EBPR

Phosphate removal was affected by increasing NaCl concentration, particularly at 30 g/L (**Figure 25**). Both anaerobic P-release and aerobic P-uptake gradually decreased until no more release or uptake was observed. Several reasons for EBPR failure at elevated salt concentrations have been reported in the literature, such as inhibition of PAOs due to nitrite accumulation (Pronk et al., 2014), out competition of PAOs by GAOs (Bassin et al., 2011), enrichment of different type of PAOs with PAO clade I being more tolerant than PAO clade II to salt stress (Wang et al., 2018), or shift of energy generation from poly-P dependent ATP generation to energy generation via glycolysis (Welles et al., 2014). In this study, phosphate removal was restored when synthetic seawater was used instead of NaCl. Using synthetic seawater, de Graaff et al. (2020a) also showed stable granulation and phosphate removal with comparable operation to freshwater AGS systems. The reactor with synthetic seawater contained similar concentrations of NaCl as the reactor with 30 g/L NaCl, yet both reactor conditions manifested a different operational performance. Since other operational parameters were kept constant, we postulate that it is the ionic property of the medium (i.e. the ratio of cations) that affects EBPR in saline environments. The additional salts present in seawater, such as $Mg^{2+}$ and $K^{+}$, appear to counteract the negative effects of $Na^{+}$ on cellular activity. Transport of $Na^{+}$ and $K^{+}$ across the cell membrane plays an important role in several cellular homeostatic mechanisms including osmoregulation (Kakinuma and Harold, 1985). The primary response to increased osmolarity in both Gram-negative and -positive microorganisms is characterized by the quick uptake of potassium ions to neutralize the negative charges on halophilic enzymes (Sleator and Hill, 2001). This neutralization process serves to counterbalance the repulsive forces on the enzymes and prevent protein denaturation during osmotic fluctuations. Potassium is also reported to play an important role in the water activity of the cell, and the ability of some non-halophilic organisms to tolerate high salt concentrations is closely linked to their capacity to accumulate $K^{+}$ (Christian and Waltho, 1961). In a study involving 32 strains of both Gram-negative and -positive microorganisms, an inverse relationship was observed between cellular $K^{+}$ and $Na^{+}$ content. This finding suggests that organisms with a higher $K^{+}$ content, which is often associated with greater salt tolerance, tend to have a lower $Na^{+}$ concentration (Christian and Waltho, 1961). The $Na^{+}$-$K^{+}$ pump works by exporting 3 $Na^{+}$ ions and importing 2 $K^{+}$ ions into the cell which could be interrupted by the high $Na^{+}/K^{+}$ ratio in the medium. The addition of salts, such as $Mg^{2+}$ and $K^{+}$, as a strategy to enhance the biological treatment of organically polluted brines has only received limited attention in the current literature until recently. For instance, studying the anaerobic digestion of an organically polluted brine, Li et al. (2018) found that the addition of potassium salt increased the process's efficiency in terms of COD removal. The authors also reported stimulated dehydrogenase activities and maintenance of the morphology of anaerobic microorganisms at a higher $K^{+}/Na^{+}$ ratio. Moreover, Sudmalis et al. (2018), in their examination of osmolytes and their potential to alleviate osmotic stress in anaerobic granular sludge systems, observed that the introduction of potassium (relative to

sodium) at a ratio equivalent to seawater (27 w/w - **Table 10**) could alleviate the osmotic stress experienced by methanogens during the incubation period. For PAOs in particular, $K^+$ is also an essential element since it is taken up aerobically (along with $Mg^{2+}$ or $Ca^{2+}$) as a counter ion to $PO_4^{3-}$ (van Groenestijn et al., 1988) and thus, lack or insufficient amount of necessary cations in the medium interrupts their activity. Considering the above discussion, the significantly higher $Na^+/K^+$ and $Na^+/Mg^{2+}$ in the reactor with 30 g/L NaCl (**Table 10**), could potentially be the cause of disruption in microbial processes, including phosphate uptake.

### Microbial community dynamics as a function of salinity

A previous study highlighted the intricate process of glycerol conversion in an AGS-EBPR system which involved the combination of substrate fermentation by members of the actinobacteria such as *Tessaracoccous* and *Micropruina* (i.e. fGAO) and product uptake by *Ca.* Accumulibacter (i.e. PAO) (Elahinik et al., 2022). In this study, these microorganisms were also present in the system even at elevated salinities. The fGAOs possess the enzymatic machinery to convert sugars into fatty acids which can be assimilated by the PAOs into PHA under anaerobic conditions. It was shown that glycerol is mainly fermented into propionate by the fGAOs and propionate is taken up by the PAOs. With the assumption that glycerol conversion to propionate was done solely by the fGAOs, it can indicate that these organisms are not inhibited at 30 g/L NaCl since propionate accumulation was observed, although their metabolic rates decreased significantly (**Figure 28**). PAOs on the other hand are completely inhibited at 30 g/L NaCl since both phosphate and propionate uptake were halted. As a consequence of substrate overflow into the aerobic phase, the genus *Zoogloea*, a notorious microorganism known for its excessive extracellular polymeric substance (EPS) production and floc formation replaced PAOs as the dominant taxon. These microorganisms are Gram-negative, facultative aerobes that can utilize a wide range of substrates (Shao et al., 2009). The proliferation of *Zoogloea* in WWTPs has been linked to sludge bulking, scum formation, and increased effluent turbidity (Novak et al., 1994), phenomena that were also observed in the reactor at 30 g/L NaCl. In full-scale granular sludge reactors, the occurrence of *Zoogloea* is observed to be related to insufficient readily biodegradable COD removal under anaerobic feeding conditions.

The absence of glycerol metabolizing enzymes in PAOs was used to indicate their inability to directly utilize glycerol (Elahinik et al., 2022). The batch tests (**Figure 27**) and the metaproteome analysis in this study support the genome-based evaluation. As expected, PAOs possess the proteins involved in polyphosphate, PHA, and glycogen metabolism. The proteins involved in trehalose metabolism such as *TreY*, *TreX*, and *TreZ* were also detected in PAOs. The presence of genes encoding for *TreA, TreY, TreX, and TreZ* in the genome *Ca.* Accumulibacter was previously reported by de Graaff et al. (2021). In short, *TreY* (glucosylmutase) catalyses the conversion between different forms of glucose linkages within a molecule, *TreX* (isoamylase) breaks down glycogen molecules for energy production during periods of increased energy demand, and *TreZ* (trehalase) catalyses the breakdown of trehalose into glucose molecules, a requirement for ATP production. Interestingly, none of these enzymes was found in fGAOs. This lack of detection could be either due to extraction

bias or an indication that these microorganisms might have a different coping mechanism for osmotic stress. Moreover, proteins involved in both potassium and magnesium transport across the cellular membrane, essential for osmoregulation and metabolising polyphosphate, were all detected in PAOs. The lack of *PhaC* and polyphosphate metabolising enzymes in the fGAOs subject to this study confirms the previous findings that they do not contribute to PHA accumulation and phosphate removal in EBPR systems (Elahinik et al., 2023, 2022).

### Cytoplasmic solute and osmoregulation

Transfer of salt-adapted enrichment to demineralized water resulted in a rapid trehalose release (**Figure 29**). Trehalose is a disaccharide, compatible solute accumulated in microorganisms as a response to stress conditions. One of the stress management strategies bacterial species have developed is the ability to accumulate compatible solutes which helps in cell rehydration without interfering with cellular activity (Ruhal et al., 2013). Just like their ability to accumulate, microorganisms have also developed a mechanism in which they can rapidly eject the compatible solutes to regulate their turgor pressure (hydrostatic pressure between the cell interior and exterior) when transitioning from environments of high to low osmolarity (Sleator and Hill, 2001). The biosynthesis of trehalose in bacteria is proposed to follow different pathways depending on the carbon source and the microorganism (Ruhal et al., 2013). Different sugars such as glucose and glycerol for trehalose production have been studied and the metabolic pathways for trehalose biosynthesis in different microorganisms are extensively described in the literature (Cardoso et al., 2004; Dalmasso et al., 2012; Giaever et al., 1988; Ruhal and Choudhury, 2012; Wu et al., 2017). However, information on trehalose transformations in the context of wastewater ecology is scarce although it is a metabolite that plays an important role in saline conditions. In a previous study, trehalose was identified as an osmoprotectant in *Ca*. Accumulibacter, pointing out the metabolic flexibility and the coping mechanism of these microorganisms to salinity (de Graaff et al., 2021). In this study, during the batch test, in addition to the release of trehalose, a considerable amount of P-release was also observed (**Figure 29**). This observation suggests that the ejection of trehalose, which is necessary for osmoregulation, phosphate, and potentially other compounds are released due to the passive efflux of cytoplasmic solutes. Under increased membrane tension (low osmolarity), mechanosensitive channels on the cell membrane open to allow passage of water and cytoplasmic solutes (Buda et al., 2016), including metabolites, ions, and ATP (Berrier et al., 1992; Epstein, 1986).

### Effluent turbidity and granule morphology at elevated NaCl concentration

Compact granular sludge with a low $SVI_5$ of <20 mL/g was achieved at all tested salinities. The formation of granules shows that the production of EPS is not hampered due to salt presence. Increasing NaCl concentration resulted in higher effluent turbidity and altered the morphology of the granules. It is a common observation that effluent turbidity increases at elevated NaCl concentrations (Bassin et al., 2011). At 33 g/L NaCl, Pronk et al. (2014) reported a washout of single-cell bacteria encapsulated in acidic polysaccharides, a constituent of the EPS, via the effluent. They hypothesized that the weakening of the EPS and detachment of

granules (hence higher effluent turbidity) at higher NaCl concentrations might be due to the replacement of $Ca^{2+}$ ions by $Na^{+}$. However, these arguments explaining the underlying mechanisms are speculative and thus require further research. In this study, at 30 g/L NaCl, the change in the microbial community (proliferation of genus *Zoogloea*) likely due to aerobic availability of rbCOD can explain the increase in effluent turbidity. Haaksman et al. (2020) studied the effect of incomplete anaerobic substrate uptake on the morphology and process stability of the AGS systems. It was shown that the aerobic conversion of minor amounts of rbCOD, by granules enriched for anaerobic storage, results in minor filamentous growth on the granule's surface. This phenomenon was observed in the reactor condition with 30 g/L NaCl (**Figure 26**.C) where substrate overflow to the aerobic phase occurred (**Figure 28**). Switching from NaCl to seawater condition, smooth granules were restored (**Figure 26**.E) and the effluent suspended solid concentration decreased by three folds to approximately 100 mg/L (**Table 12**) which is comparable to the concentration of about 70 mg/L in freshwater conditions with glycerol as substrate (Elahinik et al., 2022). These values are slightly higher than the concentration of 20 mg/L reported from a similar system (AGS operated in synthetic seawater) running on acetate as substrate (de Graaff et al., 2020a). Since the effluent turbidity was higher in both fresh and seawater conditions with glycerol compared to acetate as substrate, it can indicate that effluent turbidity is likely to be affected by other factors such as the substrate and not necessarily by the salinity. An effluent turbidity of 90 mg/L was also observed in a fresh-water reactor fed with glucose as substrate (Elahinik et al., 2023), which raises the question of whether using sugars as substrate can lead to a more turbid effluent compared to VFAs such as acetate or propionate. A possible explanation for higher effluent turbidity with sugars could be the different microbial communities, forming an EPS with different properties.

### Practical implications

- In wastewater treatment processes for saline streams, the ratios of cations may play a more significant role than the absolute concentrations. The ionic properties of the influent can affect not only phosphate removal but also the effluent quality in terms of solids concentration. Therefore, it is crucial to consider not only the total salinity but also the ratio of cations for an effective treatment. This can be achieved through mixing the influent with seawater if available or adjusting the ratios of cations in the wastewater influent by the addition of salts. Therefore, identification of the minimum ratio of major cations such as $Mg^{2+}$, $Ca^{2+}$, and $K^{+}$ relative to $Na^{+}$ required for a stable AGS system could help alleviate the limitations of saline wastewater treatment biologically.
- Effluent turbidity can be a concern in full-scale applications but is often neglected in scientific literature. Fluctuating salt concentrations in the influent of WWTPs interrupt the metabolic activity of microorganisms, causing the discharge of cellular molecules such as trehalose that would negatively affect the effluent quality. Moreover, the elevated salt concentrations change the microbial community which in turn affects the effluent quality such as increased turbidity. Maintaining a reactor with a stable microbial population proves effective in treating saline streams and minimizing effluent turbidity.

- Overflow of biodegradable COD to the aerated phase leads to the filamentous growth of heterotrophs in AGS systems such as *Zoogloea* and ultimately causes sludge bulking. Therefore the detection of *Zoogloea* might be a good biomarker for systems with suboptimal operation of the anaerobic phase.
- Research endeavours about the impact of salt require special consideration of experimental procedures due to the interference of high salinity with analytical techniques.

## Conclusions

- Successful granulation and long-term stable reactor operation were shown at 10 and 20 g/L NaCl and seawater condition (35 g/L) using glycerol as the carbon source.
- Incomplete anaerobic substrate uptake at 30 g/L NaCl led to aerobic conversions. This led to filamentous growth and gradual deterioration of granular structure.
- *Ca.* Accumulibacter was the dominant taxon in stable AGS reactors performing EBPR at 10 and 20 g/L NaCl and seawater conditions (35 g/L).
- In the reactor condition with 30 g/L NaCl, characterized by impaired granulation and turbid effluent, *Zoogloea* emerged as the predominant taxon.
- Cellular compounds including trehalose and $PO_4^{3-}$-P were released from salt-adapted biomass during osmotic down shock.
- The ionic composition of wastewater is likely to be more important for the AGS reactor's operational stability and EBPR than absolute salt concentration.

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# 5. Extracellular Polymers Characteristics of Aerobic Granular Sludge Cultivated at High Salinity

## Abstract

Extracellular polymeric substances (EPS) are crucial components of biofilms and have promising application potential. This study investigated the EPS of aerobic granular sludge (AGS) grown under varying salinities induced by NaCl concentration gradient and seawater conditions. Fourier transform infrared spectroscopy revealed the likely presence of polysaccharides, phosphates, proteins, carboxylic esters, and lipids in all extracted EPS. Further analysis with 2-D correlation spectroscopy identified notable differences in the ~860 – 950 $cm^{-1}$ and ~1090 - 1200 $cm^{-1}$ regions. The 860 – 950 $cm^{-1}$ region corresponds to the C-C, C-O, and C-H bonds which can be associated with many functional groups such as alkenes. The ~1090 - 1200 $cm^{-1}$ region corresponds to the vibrations of C-O-P, P-O-P, C-H bonds and O-H stretch which are likely attributed to the glycan and phosphate functional groups. Sugar monomer analysis of acid-hydrolysed EPS identified eight monosaccharides, with glucose dominant in saltwater conditions and glucosamine in freshwater. The polysaccharide-to-protein ratio increased slightly in saltwater EPS, suggesting saline conditions might reduce EPS protein content. The 30 g/L NaCl reactor condition showed poor granulation, phosphate removal, and lower alkaline soluble EPS yield. To evaluate the potential of the extracted EPS as a bio-based flame retardant, burning tests on EPS-coated cellulose fibres were performed. The tests indicated a linear correlation between increased residual mass and the condensed phosphate content in the EPS, suggesting that higher condensed phosphate levels enhance the flame-retardant properties of the EPS. The EPS from saline conditions had higher condensed phosphate content in contrast to the freshwater EPS with higher orthophosphate fraction. These findings highlight the potential of wastewater-derived EPS as a bio-based flame retardant.


## Introduction

About 20 years ago, a collaborative effort between the Dutch private and public sectors catalysed the inception of a biological wastewater treatment process known as aerobic granular sludge (AGS), commercially branded as the Nereda® technology (de Kreuk and van Loosdrecht, 2006; Heijnen and Van Loosdrecht, 1998). The granular sludge settles quickly, allowing for fast solid-liquid separation and high biomass retention, which enhances the overall treatment efficiency (van Dijk et al., 2022). This allows a compact design, energy efficiency, and reduced operational costs in comparison to the conventional activated sludge process, which has resulted in the widespread adoption of AGS technology in both municipal and industrial wastewater treatment across numerous nations (www.nereda.royalhaskoningdhv.com).

In biofilm systems such as AGS, microbial cells are embedded in extracellular polymeric substances (EPS), a hydrogel-like matrix (Lotti et al., 2019). The EPS is produced by the microorganisms and constitutes a major fraction of the sludge volume and mass. Since it's an integral part of biofilms, studying the EPS, from the functionality point of view to its characterisation, and extraction, has been the focal point of many research groups in the past

(Ismail et al., 2010; Lotti et al., 2019; Seviour et al., 2009; Tomás-Martínez et al., 2023). In the context of circular wastewater treatment and resource recovery, EPS research has also gained traction due to its promising application potential (Bahgat et al., 2023). For instance, Chen et al. (2024a) showed that EPS extracted from AGS cultivated in seawater contains anionic polymers such as nonulosonic acids that can be used for binding proteins. In a study by Kim et al. (2020), EPS extracted from both granular and activated sludge was demonstrated as a biobased flame retardant material. Ajao et al. (2021) reported on the potential application of EPS as a bioflocculant. In the Netherlands, 2 demonstration-scale EPS extraction plants from AGS have already been commissioned in 2020 under the name Kaumera® (www.kaumera.com). Despite its potential as a recoverable resource, the practical utilisation of EPS remains challenging due to its inconsistencies and our incomplete understating of its exact properties. These challenges necessitate further research to fully exploit the potential of EPS as a resource.

Salt enters wastewater treatment plants (WWTP) through various pathways, mainly by industrial discharges and seawater intrusion. Industrial effluents from food canning, pickling, and chemical production contain salinity levels exceeding 10 g/L NaCl (Kubo et al., 2001; Srivastava et al., 2021). Additionally, in coastal cities like Hong Kong seawater—approximately 30 g/L in salinity—is used for toilet flushing, leading to its introduction into WWTPs (Li et al., 2018). Elevated salt levels pose significant challenges to biological treatment processes. One critical component impacted by salinity is the EPS, which plays a role in protecting microorganisms from adverse environmental conditions. This study focuses on understanding how varying salinity levels influence the properties of EPS in AGS systems and ultimately how it would affect its application.



This research is complementary to prior research that detailed conversions in an AGS reactor (Elahinik et al., 2022), and the long-term performance of aerobic sludge granules under various salinities (Elahinik et al., 2024). We employed chemical analyses and Fourier-transform infrared spectroscopy (FTIR) spectroscopy as rapid screening tools to characterise the lyophilised EPS samples extracted from the aforementioned studies. The data were analysed for correlations and differences using both 2-D correlation spectroscopy (2D-COS) and qualitative analysis of the IR peaks. 2D-COS is an analytical technique used to enhance the resolution of spectral data and reveal relationships between spectral features (Noda, 1990). It involves correlating spectral changes induced by external perturbations, such as temperature or pH changes, over two dimensions. In this study, the external perturbation was the salinity level.Additionally, we explored the potential application of EPS as a bio-based flame retardant, qualitatively assessing its effectiveness. The results of this study, integrated with previous findings, provide a broader understanding of the properties and potential applications of EPS derived from AGS cultivated with glycerol as a carbon source under varying salinities.

# Material and Methods

## Reactor operation

The details of the reactor operation are explained in (Elahinik et al., 2024). In summary, the granules were cultivated in a bubble column reactor operating in sequencing batch mode. The reactor had a working volume of 3.1 L. After effluent withdrawal at the end of each cycle, a volumetric exchange ratio of about 50% was maintained. The pH was automatically controlled at 7.1 ± 0.1 by dosing 0.5 M NaOH or HCl. The dissolved oxygen (DO) concentration was controlled at 0% and 50% saturation (≈ 0 - 4.5 mg/L) during the anaerobic and aerobic phases by a mixture of nitrogen gas and air. The off-gas was recirculated with a flow of 5 L/min to maintain the DO concentration. The DO probes were calibrated for each salt concentration. The room temperature where the reactor was located was controlled at ≈ 20 °C. The reactor was inoculated with about 600 mL of a mixture of AGS from a municipal WWTP located in Harnaschpolder, the Netherlands and a glycerol-adapted sludge performing phosphate removal from the lab. Each operational cycle consisted of 5 min of nitrogen sparging before feeding, 5 min of feeding, 5 min of settling, 5 min of effluent withdrawal, 60 min of nitrogen sparging (anaerobic phase), and 120 min of aeration.

The synthetic influent fed at the beginning of each cycle with a total volume of 1,500 mL consisted of 1,200 mL of (salt) water, 150 mL of nutrient medium and 150 mL of carbon medium. The synthetic seawater with a final concentration of 35 g/L was made with Instant Ocean® sea salt crystals (Atkinson et al., 1997). 35 g/L sea salt was used to account for the mass of other elements in the mixture and to achieve a 30 g/L NaCl equivalent. Carbon medium contained 35.7 mM glycerol, 3.6 mM $MgSO_4.7H_2O$, and 4.7 mM KCl. No extra $MgSO_4.7H_2O$ and KCl were added when sea salt crystals were used. The nutrient medium contained 41.1 mM $NH_4Cl$, 1.95 mM $K_2HPO_4$, 1.98 mM $KH_2PO_4$, 0.6 mM Allythiourea (ATU) to inhibit nitrification and 10 mL/L of trace element solution. The trace element solution contained 4.99 g/L $FeSO_4.7H_2O$, 2.2 g/L $Zn.SO_4.7H_2O$, 7.33 g/L $CaCl_2.2H_2O$, 4.32 g/L $MnSO_4.H_2O$, 2.18 g/L $Na_2MoO_4.2H_2O$, 1.57 g/L $CuSO_4.5H_2O$, 1.61 g/L $CoCl_2.6H_2O$ and 50 g/L EDTA. These feed streams combined resulted in a final influent concentration of 400 mg/L COD, 58 mg/L $NH_4^+$-N, and 12 mg/L of $PO_4^{3-}$-P. The reactor conditions were distinguished based on their salinity and are referred to as R0 (0 g/L NaCl), R10 (10 g/L NaCl), R20 (20 g/L NaCl), R30 (30 g/L NaCl), and R35x (35 g/L sea salt).

Volatile fatty acids and sugars were measured using an HPLC (Vanquish, ThermoFisher Scientific, USA) equipped with an RI and UV detector, Aminex HPX-87H column (BioRad, USA) using 0.0015M phosphoric acid as eluent. The visualization of the morphology of the granules was done with a stereo zoom microscope (M205 FA, Leica Microsystems, Germany) equipped with Qwin image analysis software (V3.5.1, Leica Microsystems, Germany). The details of the microbial community analysis, including metagenomics and metaproteomics, are explained in (Elahinik et al., 2024, 2022).

### Extracellular polymeric substances extraction

The EPS extraction was performed according to the method described by Chen et al. (2024). In short, freeze-dried granules were placed in 0.1 M NaOH for 30 min at 80 °C while stirring at 400 rpm (1% volatile solids w/v). The solution was centrifuged at 4000 × g for 20 min and cooled down at 4 °C. The collected supernatant was dialysed against demi-water overnight in dialysis bags with a molecular weight cut-off of 3.5 kDa MWCO (Snakeskin™, ThermoFisher Scientific, Landsmeer). The dialysed EPS solution was then freeze-dried for further analysis.

### Sugars, protein, and phosphate measurements

The phenol sulfuric acid method was used to measure total polysaccharides (Dubois et al., 1956). Phenol 5% (w/v) and concentrated sulfuric acid (95%) were added to lyophilised EPS dissolved in water and incubated for 20 min at room temperature. The samples were then measured with a spectrophotometer at 490 nm wavelength. The measurements were done in duplicate.

To measure and quantify the monosaccharides, lyophilised EPS was hydrolysed in 1 M HCl at 105 °C overnight. The hydrolysed sample was centrifuged and the supernatant was neutralised with 1 M NaOH. The neutralised sample was filtered through a 0.45 µm PVDF filter and analysed by high-performance anion-exchange chromatography with pulsed amperometry detection (HPAEC-PAD) on a Dionex CarboPac PA20 column (Thermo Fisher, USA) using NaOH and NaHAc as the eluent.



To quantify total proteins, BCA analysis was performed according to the manufacturer's protocol with the commercially available kit (Pierce BCA protein assay Kit, Thermo Scientific). The absorbance was measured at 562 nm wavelength with a 96-well plate (TECAN Infinite M200 PRO, Männedorf, Switzerland). The measurements were done in duplicate.

Lyophilised EPS was dissolved in ultrapure water (<0.5 mg/mL) and the total and orthophosphate content of the solution was measured using Hach Lange cuvette tests following the instructions of the kit (LCK350 and LCK351, Hach, USA). Hydrolysable phosphate was measured after mild sulfuric acid hydrolysis (EPA, 1978) by skipping the persulfate oxidation step using the LCK350 kit. These measurements were done to differentiate the different forms of phosphate in EPS. The organic phosphate was calculated by subtracting acid hydrolysable phosphate from total phosphate and the condensed phosphate was calculated by subtracting orthophosphate from acid hydrolysable phosphate. The measurements were performed in duplicates.

### Fourier-transform infrared and 2-D correlation spectroscopy

FTIR spectroscopy was performed on a Spectrum 100 spectrometer (PerkinElmer, Shelton, CT). The spectra of the lyophilised samples (extracted EPS and granules) were recorded at room temperature over the range of 600 – 4000 $cm^{-1}$ wavenumber with 8 accumulations and 2 $cm^{-1}$ resolution. Normalisation was done to correct baseline shifts and intensity variations. After baseline correction of the IR spectra, 2D-COS was performed using an in-house MATLAB

script provided in the supplementary material of the electronic version. In short, a series of spectra were collected and laid out in a matrix format where each row represented a spectrum and each column represented a point in the spectrum. Correlation analysis was then performed by comparing intensities at each point in the spectrum change as the conditions change. Finally, a 2D map was created where both axes represented points in the spectra. Each point on the map shows how strongly the corresponding points in the original spectra are correlated.

### Burning test

To qualitatively assess the impact of EPS as a biobased flame-retardant coating, EPS-coated cellulose fibres were prepared as follows (**Figure 37**.F). The preparation of EPS-coated cellulose fibres was done according to Kim et al. (2023) with some modifications. Tissue papers (purchased from a local supermarket) were soaked in water, blended to disintegrate the fibres and make a pulp solution, and dried at 105 °C. The dried tissue pulp (~ 200 mg) was then mixed with an EPS solution of 3% w/w, pressed into a flat plastic container, and let air dry at room temperature for 72 hrs. The EPS solution was made by dissolving ~45 mg of EPS from each sample in 1.5 mL demineralised water. In terms of mass distribution, EPS accounted for approximately 20% of the sample weight. A blow torch was placed 10 cm from the samples and a blue flame was applied for 12 s. A cellulose fibre sample with no added EPS was used as a control. The flame extinguishing and afterglow time, dripping, and burning of the samples were monitored. The residual mass of each sample was measured in triplicates.

## Results

### Reactor performance

Aerobic sludge granules were cultivated under different salinities using glycerol as the sole carbon source at an average sludge retention time (SRT) of 12 ± 4 days. The salinities were obtained by varying the NaCl concentration or using sea salt for medium preparation. The details of the reactor performance are reported in Elahinik et al. (2024). In short, the reactor conditions with 0, 10, 20 g/L NaCl and 35 g/L sea salt (R0, R10, R20, and R35x), showed successful granulation, EBPR performance, and complete anaerobic glycerol removal. In the reactor operated with 30 g/L NaCl (R30), glycerol was only partially converted anaerobically and EBPR performance gradually decreased until completely lost. The incomplete anaerobic substrate uptake led to COD overflow to the aerobic phase and the growth of floccular biomass. Glycerol uptake and phosphate release and uptake during a representative cycle under pseudo-steady-state conditions at each salinity are shown in the supplementary materials of the electronic version. The microscopic and stereoscopic picture of the biomass obtained from each reactor condition at the end of each study period is shown in **Figure 32**.

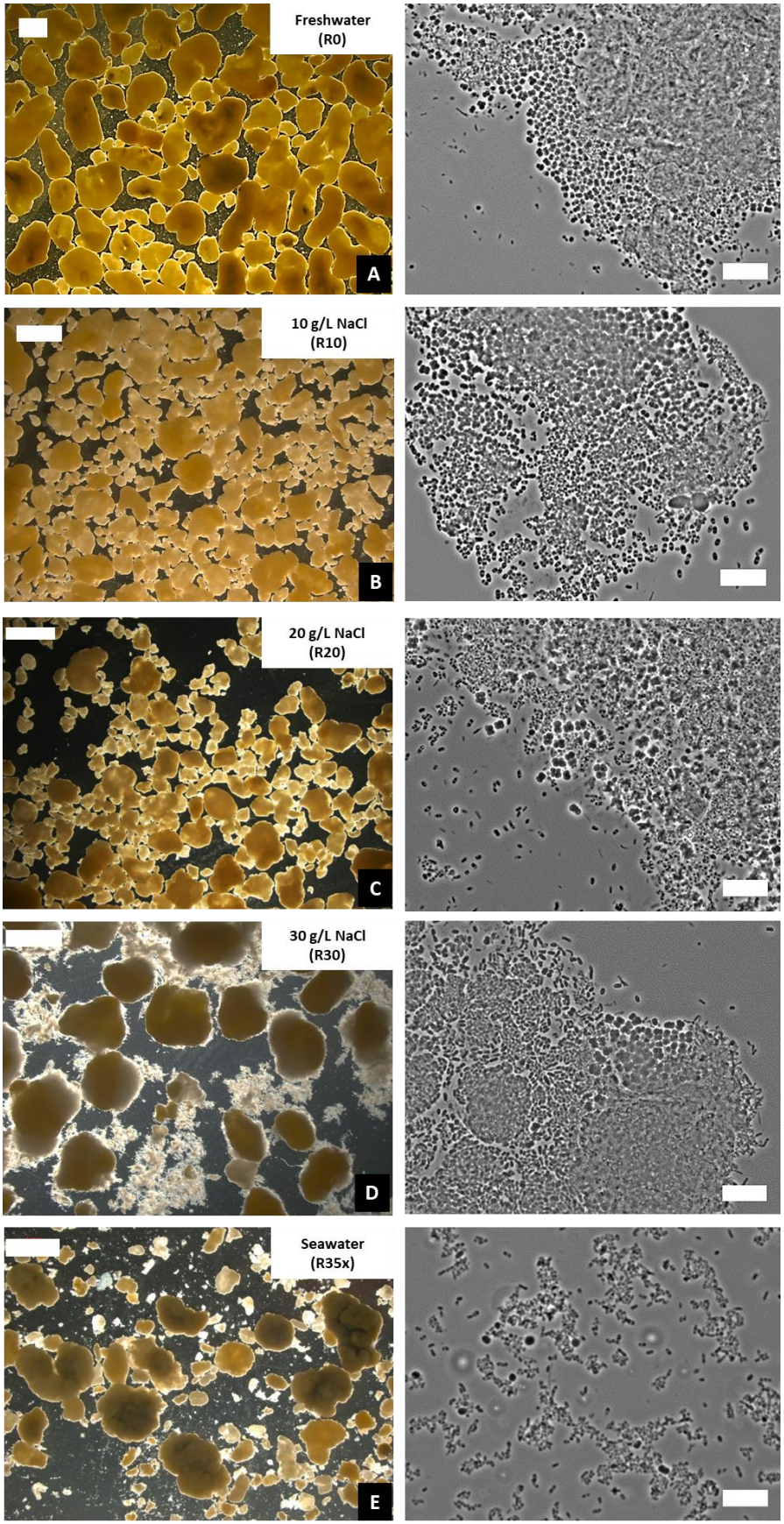


***Figure 32**. Stereozoom picture of the biomass obtained from each reactor condition at the end of each study period (A-E). The scale bars are equal to 1 mm in A, D, E, and 3 mm in B and C. Phase contrast images of the abundant taxon representing the respective granules. The scale bars in phase contrast images are equal to 10 µm.*

Metaproteomic analysis in combination with microscopic observations of the microbial community revealed the dominant presence of *Ca*. Accumulibacter Phosphatis, a well-known polyphosphate accumulating organism (PAO), under stable reactor conditions performing EBPR (R0, R10, R20, R35x). Additionally, the genera *Tessaracoccus* and *Micropruina*, two actinobacteria classified as fermentative glycogen accumulating organisms (fGAO) responsible for glycerol conversion, were detected in the biomass (Elahinik et al., 2022). Both PAOs and fermentative organisms have slower growth rates compared to typical heterotrophs and are often associated with the formation of compact granules (Beun et al., 2002; de Kreuk and van Loosdrecht, 2004; Elahinik et al., 2023). In R30, characterised by poor EBPR performance and granulation, the genus *Zoogloea* was the predominant taxon. Overall, the microbial community composition was similar across all reactor conditions except in R30. Detailed microbial community distribution across the reactor conditions, along with respective salinities, is provided in the supplementary material of the online publication.

### FTIR analysis of the EPS

FTIR spectroscopy was utilised to provide quick and qualitative information about the functional groups and chemical bonds present in the samples (**Figure 33**). This technique offers important insights into the molecular structure and composition of EPS (Chen et al., 2024a; de Graaff et al., 2019; Zhang et al., 2014). The assignment of infrared spectra was based on existing literature (Comte et al., 2006; Li et al., 2021; Zeng et al., 2016). The main peaks, vibrations, and their likely corresponding functional groups are provided in the supplementary material of the electronic version. The manual analysis of the spectra revealed that the samples contain functional groups likely corresponding to lipids (~2930–2965 $cm^{-1}$), polysaccharides and phosphates (~1000 - 1200 $cm^{-1}$), proteins (1535 and 1650 $cm^{-1}$), nucleic acids (975 $cm^{-1}$), and carboxylic esters (~1735 $cm^{-1}$) based on the vibration of the bonds.

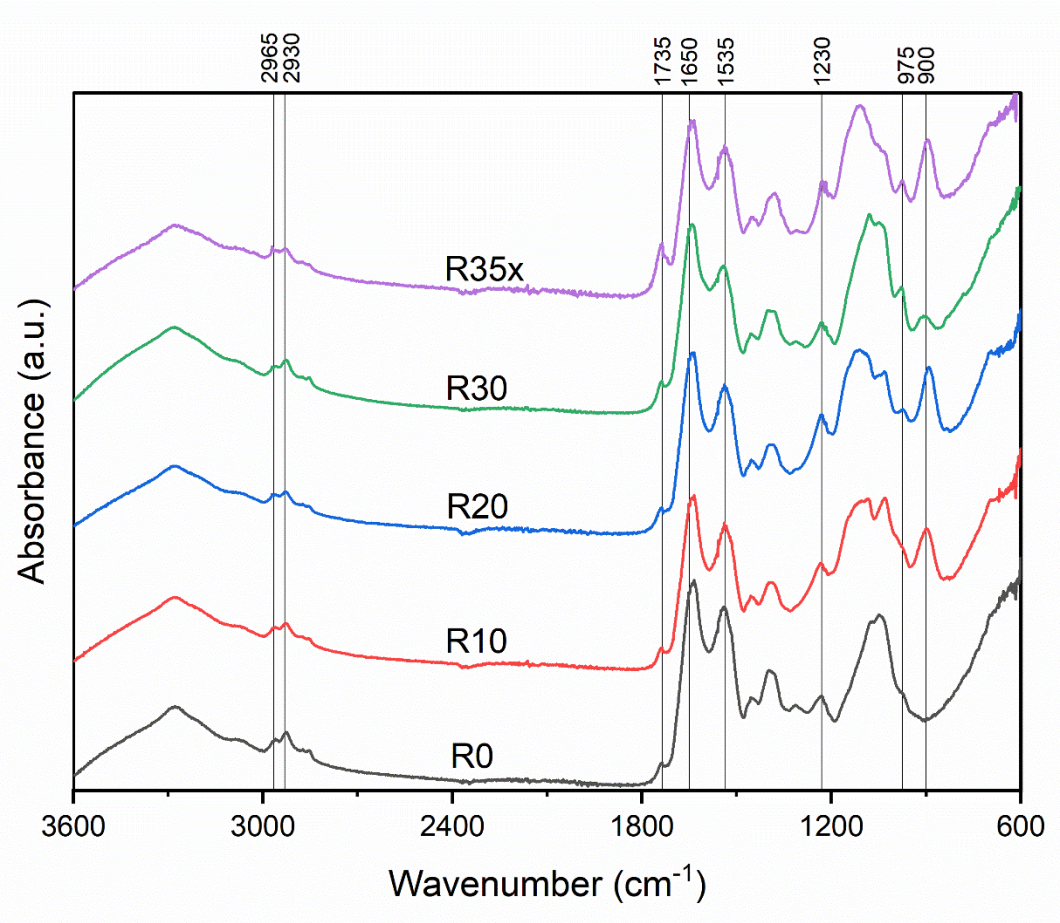


*Figure 33. Normalized FTIR spectra of the EPS at different salinities.*

The normalised IR spectra were analysed for correlations using 2D-COS, a technique used to unveil hidden relationships among IR spectra. A brief explanation of the basic concept of 2D-COS is given below. For instance, the technique has been used by Cai et al. (2018) to explore the interaction mechanism between EPS and goethite. In this study, 2D-COS revealed that the main differences were in the ~860 – 950 $cm^{-1}$ and ~1090 - 1200 $cm^{-1}$ regions based on a change in spectral intensity as a function of external perturbation which was salinity (Figure 34). The 860 – 950 $cm^{-1}$ region corresponds to the C-C, C-O, and C-H bonds which can be associated with many functional groups such as alkenes. It's important to note that 860 – 950 $cm^{-1}$ falls within the fingerprint region of the infrared spectrum, which is known for its complex and often overlapping absorption bands. The ~1090 - 1200 $cm^{-1}$ region corresponds to the vibrations of C-O-P, P-O-P, C-H bonds and O-H stretch (Comte et al., 2006; Zeng et al., 2016) which are likely attributed to the glycan and phosphate functional groups.

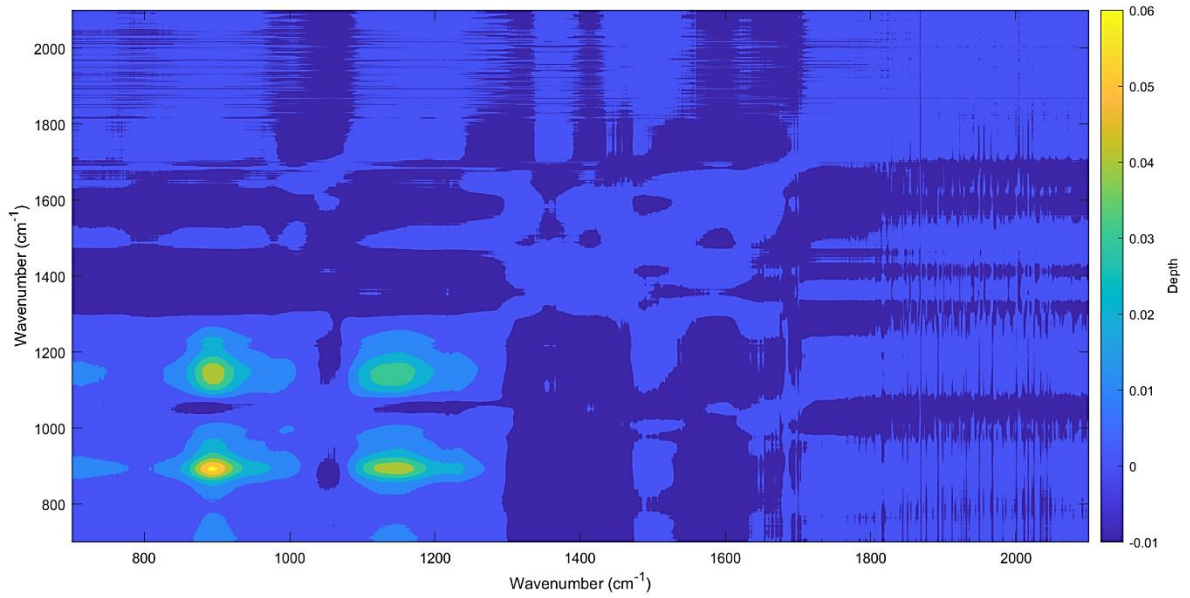


***Figure 34**. Covariance matrix contour plot generated from the normalised FTIR spectra of the samples. The axes represent the wavenumbers. Depth, the magnitude of covariance, is indicated by the colour gradient, where yellow represents a higher deviation from the mean and blue represents a lower deviation from the mean.*

## Total sugar, protein, and phosphate

Lyophilised aerobic sludge granules underwent digestion according to a thermal-alkaline protocol, enabling the extraction of alkaline soluble polymers from the samples. The extracted EPS samples were then analysed for total protein, sugar, and phosphate content, with the results presented in **Table 16**. Notably, sample R0 exhibited the lowest sugar and phosphate content in contrast to having the highest protein concentration compared to the other samples. Moreover, the EPS yield of sample R30 was found to be almost half compared to the rest.

***Table 16**. EPS yield, total sugar, and total protein of the samples. The values are given with their standard deviations.*

| Samples | EPS yield [mg/gVS] | Total sugar [mg glucose eq./gVS-EPS] | Total protein [mg BSA eq./gVS-EPS] |
|---|---|---|---|
| R0 | 368 ± 8 | 142 ± 6 | 480 ± 12 |
| R10 | 499 ± 14 | 171 ± 1 | 392 ± 15 |
| R20 | 382 ± 9 | 185 ± 22 | 409 ± 16 |
| R30 | 214 ± 8 | 186 ± 17 | 384 ± 13 |
| R35x | 395 ± 0 | 145 ± 37 | 377 ± 13 |

Polysaccharides (PS) and proteins (PN), which are among the main components of EPS, were quantified and their respective ratios are displayed in Figure 35. The PS/PN ratio of the samples showed a slight increase in the samples from saline conditions, compared to the freshwater sample. In all samples, the protein content of the EPS was higher than the

polysaccharides, aligning with findings commonly observed in mixed microbial systems and slow-growing communities (Doloman et al., 2024; Pellicer-Nàcher et al., 2013).

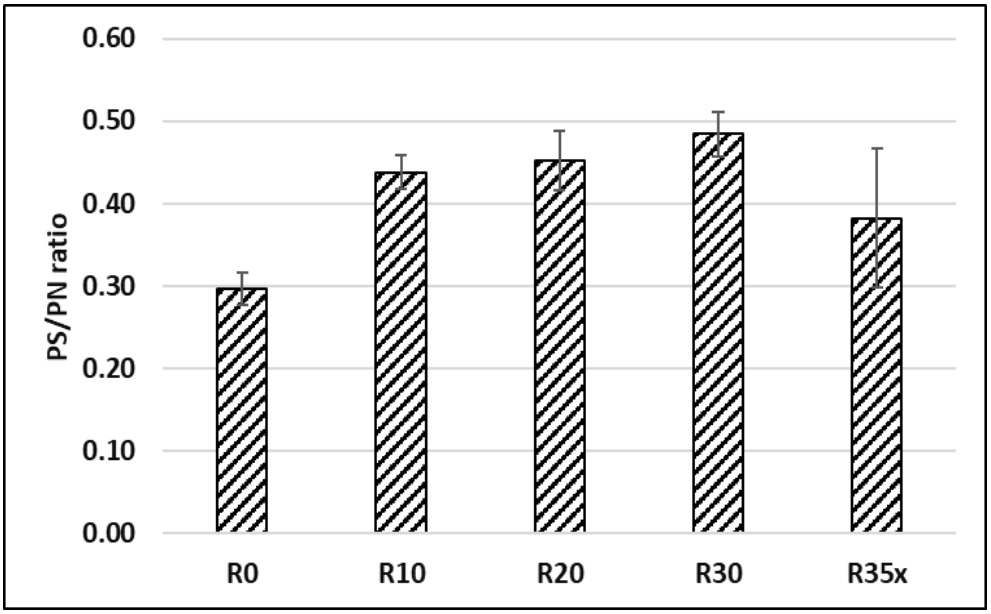


***Figure 35**. Polysaccharide (PS) to protein (PN) ratio of the samples. The PS and PN content are expressed as glucose and BSA equivalents. Error bars indicate standard deviation.*



The qualitative assessment of the IR spectra indicated potential differences among the samples, particularly in regions likely associated with the phosphate functional groups. For further investigations, we measured the total, hydrolysable, and ortho-phosphate of the EPS and the results are given in **Table 17**. The acid hydrolysable fraction accounted for most of the total phosphate content while orthophosphate was only a minor fraction. The condensed and the organic phosphates are calculated and presented in **Table 17**. Notably, the organic phosphate fraction of the total phosphate was higher in samples from saline conditions. Overall, the total phosphate concentration of the samples from saline conditions was higher than the sample from freshwater conditions. Considering that the freshwater sample originated from a reactor characterised by stable granulation, efficient EBPR performance, and an abundant PAO population, the low phosphate content in the EPS is likely influenced by factors beyond typical expectations such as low EBPR efficiency. One potential explanation for the low phosphate content of R0 could be the prolonged storage period (>6 months) of the biomass in the refrigerator before the EPS extraction since our research focus did not initially include EPS-related investigations.

*Table 17. Total, hydrolysable, and orthophosphate of the samples. Organic and condensed phosphates are calculated as described in the material and methods. The values are given in mgP/gVS-EPS with their standard deviations.*

| Samples | Total-P | Hydrolysable-P | Ortho-P | Organic-P | Condensed-P |
|---|---|---|---|---|---|
| R0 | 38 ± 0.0 | 35 ± 0.0 | 24 ± 0.0 | 3 ± 0.0 | 11 ± 0.0 |
| R10 | 96 ± 0.6 | 93 ± 0.1 | 22 ± 0.0 | 3 ± 0.6 | 71 ± 0.1 |
| R20 | 106 ± 0.4 | 101 ± 0.4 | 42 ± 0.2 | 5 ± 0.6 | 59 ± 0.5 |
| R30 | 45 ± 0.1 | 33 ± 0.1 | 8 ± 0.4 | 12 ± 0.1 | 25 ± 0.4 |
| R35x | 67 ± 0.5 | 62 ± 0.1 | 22 ± 0.0 | 4 ± 0.5 | 41 ± 0.1 |

## Monosaccharide distribution

Measuring and quantifying monosaccharides revealed that glucose and glucosamine were the predominant sugars in the samples. Specifically, glucose emerged as the dominant sugar in samples cultivated under saline conditions, whereas glucosamine dominated in the freshwater sample. Another notable difference between the EPS from freshwater and saline conditions was the absence of ribose in the freshwater sample. A total of eight monosaccharides were identified, and their mass distribution across the samples is depicted in Figure 36. Given the similar molecular weights of these monosaccharides, their distribution on a molar basis parallels the mass distribution. Approximately half of the total sugar measured colourimetrically was accounted for by monosaccharide measurement. The presence of unidentified peaks in the chromatograph of the HPAEC-PAD suggests that the remaining mass fraction likely corresponds to unidentified monosaccharides.

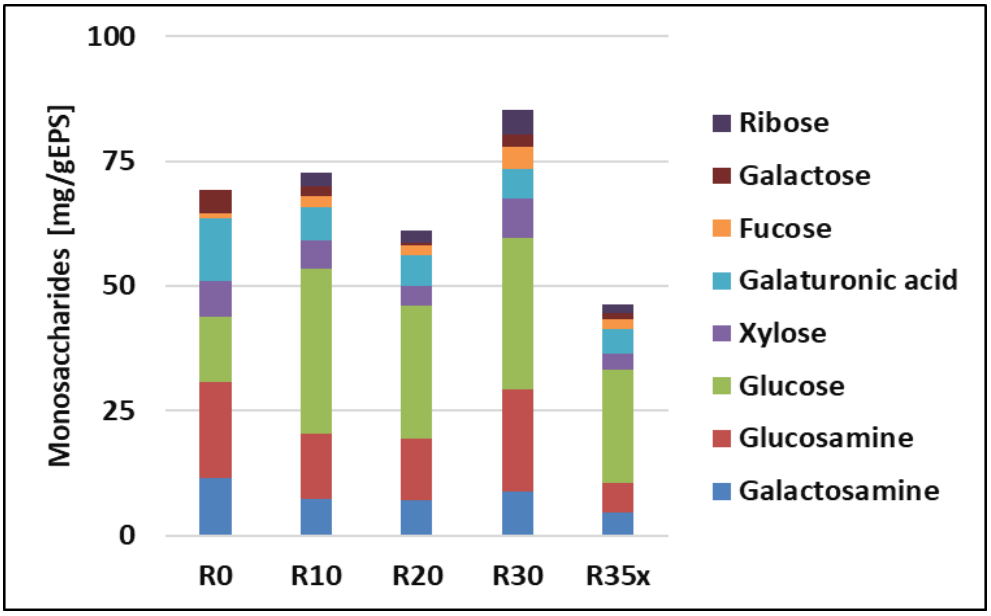


*Figure 36. Distribution of the EPS monosaccharides content measured with HPAEC-PAD.*

## Flame retardancy of the EPS

To understand the impact of EPS composition on its flame retardant properties, a burning test was performed with EPS-coated cellulose fibres. The flame extinguishing and afterglow time

and burning of the samples were assessed. The control sample (cellulose fibres with no EPS coating) was completely burned to ash after exposure to fire with >99% mass loss, as expected. The EPS-coated samples had a higher residual mass than their uncoated counterpart. The samples with higher residual mass (R10, R20, and R35x) had a shorter after-glow and extinguishing time (**Table 18**) with visible char formation (**Figure 37**) compared to R0 and R30. The cellulose fibres coated with R10, R20, and R35x maintained better structural integrity due to char formation compared to R0 and R30 where more ash formation was observed.

***Table 18**. Samples mass residue with standard deviation, afterglow time, and extinguish time.*

| Sample | Mass residue [%] | Afterglow time [s] | Extinguish time [s] |
|---|---|---|---|
| R0 | 4.0 ± 0.4 | 40.0 | 8.5 |
| R10 | 15 ± 0.5 | 24.0 | 4.5 |
| R20 | 14 ± 1.0 | 29.5 | 4.5 |
| R30 | 5.0 ± 0.6 | 43.5 | 5.5 |
| R35x | 11 ± 1.3 | 40.0 | 6.5 |



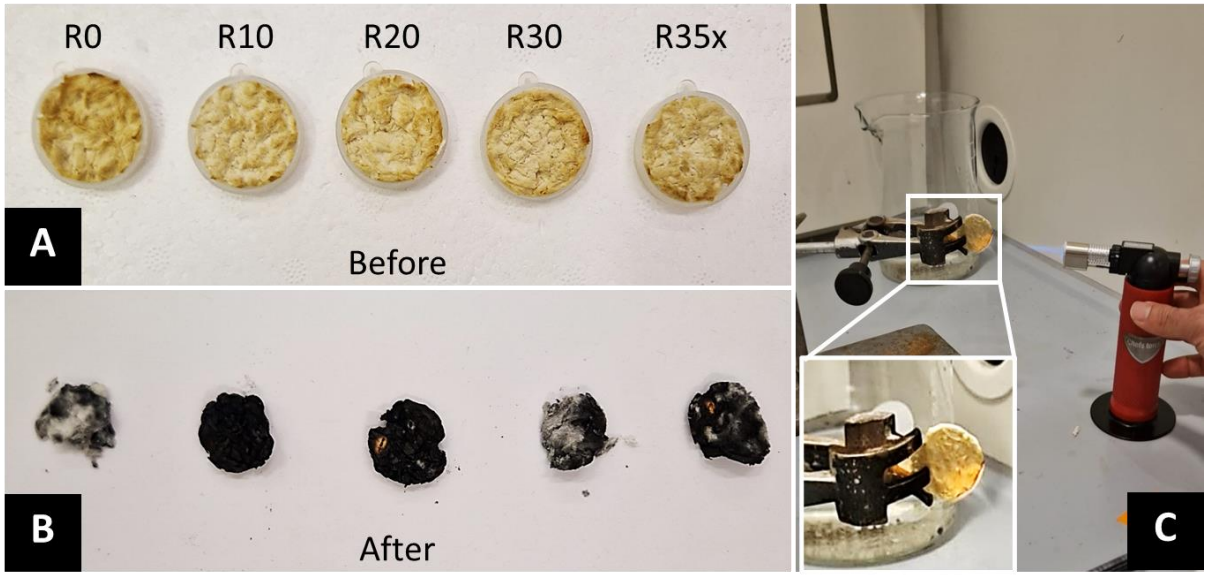


***Figure 37**. Pictures of the EPS-coated cellulose (tissue) fibres before (A) and after flame application (B). Picture C shows the flame application setting.*

To assess the impact of various phosphate moieties on the flame-retardancy of EPS, their effects were analysed by plotting each moiety against the residual mass of the EPS-coated samples. The results indicated a linear correlation between increased residual mass and the condensed phosphate content in the EPS, suggesting that higher condensed phosphate levels enhance the flame-retardant properties of the EPS (**Figure 38**). In contrast, no significant

correlation was observed between the residual mass after burning and other phosphate moieties.

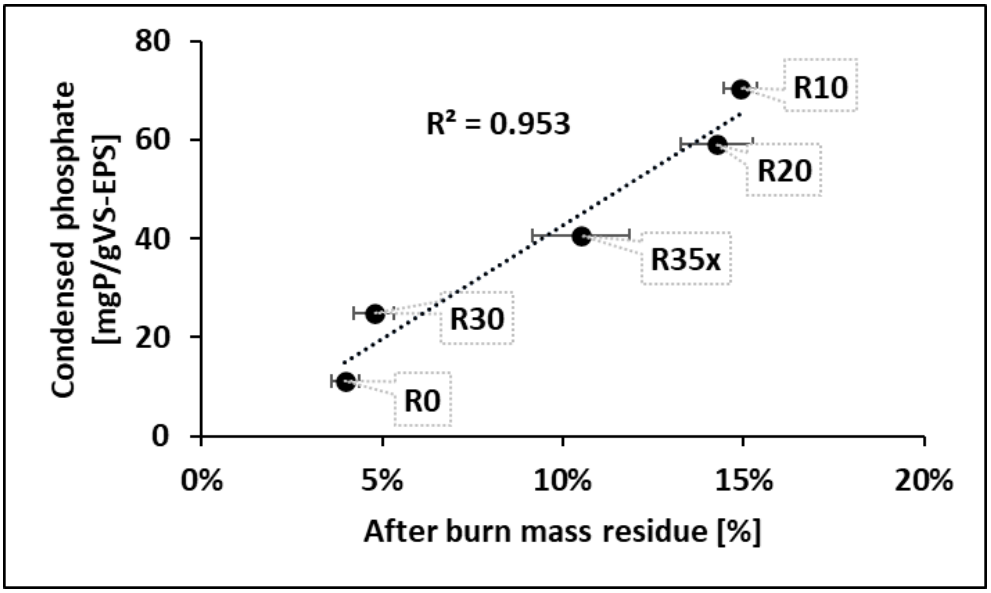


***Figure 38.*** *EPS-coated cellulose fibres residual mass after burning plotted against the EPS condensed content.*

## Discussions

Sludge handling accounts for a considerable fraction of the total wastewater treatment costs (Cicekalan et al., 2023). A large fraction of the sludge mass is EPS and if extracted, it has significant application potential (Kehrein et al., 2020). Extracting EPS reduces the waste sludge mass and volume and improves the dewaterability and digestibility of the remaining fraction (Bahgat et al., 2023). Recovering the extracted EPS would thus decrease the environmental footprint and enhance the economics of WWTPs, aligning with circular economy objectives. Consequently, exploring EPS applications is a research priority, promising immediate benefits and paving the way for increased research funding. In this study, aerobic granular sludge performing phosphate removal was cultivated under different salinities using glycerol as the sole carbon source. Our focus was on understanding how varying salinities influence the properties of EPS in AGS systems and, ultimately, its potential application as a bio-based flame retardant. We employed chemical analyses and FTIR spectroscopy to investigate this. The IR spectra were analysed for correlations and differences using 2D-COS. The results are discussed below.

### EPS yield and salinity

The analysis of the EPS yield revealed that the reactor condition marked by poor granulation (R30) yielded the lowest alkaline soluble EPS. This could be due to the fact that NaCl concentrations of 0.5 M and above can solubilise EPS (Zeng et al., 2020), as the replacement

of divalent ions by $Na^+$ ions can disrupt the electrostatic interactions between charged EPS molecules and the cell surface. From a microbial community perspective, the genus *Zoogloea* was the dominant microorganism in R30. *Zoogloea* is a microorganism commonly associated with operational challenges such as sludge bulking and turbid effluent which are argued to be linked to its ability to produce excessive volumes of EPS (Unz & Farrah, 1976). However, quantitative information related to the EPS produced by *Zoogloea*-dominated sludge or pure cultures is scarce. The relatively low EPS yield in R30 compared to other samples can indicate that the produced EPS was loosely bound or bound stronger with water molecules and flushed out via the supernatant, unlike the entangled EPS in granules. If true, this assumption also explains the highly turbid effluents in *Zoogloea*-dominated systems. Also, this observation suggests that the granule morphology, i.e. smooth and compact granules, is likely a better biomarker for EPS yield than the microbial community composition. From a practical standpoint, lower alkaline soluble EPS yield at high NaCl concentration negatively impacts the economics of WWTPs with resource recovery ambitions. The reduction in yield means higher sludge disposal costs and decreased potential revenue from the sale of EPS.

### EPS profile: Insights from FTIR and 2D-COS Analysis



The proteins and the sugars accounted for a significant portion of the extracted EPS (**Table 16**). High protein and polysaccharide content is a common observation in EPS studies from both activated sludge (Zeng et al., 2016) and granular sludge systems (Chen et al., 2023). The total protein measured colourimetrically remained constant across all samples. The analysis of the IR spectra via 2D-COS suggested some differences among the polysaccharide and phosphate functional groups of the samples (**Figure 34**). To analyse the sugar composition of the EPS, samples were hydrolysed in hydrochloric acid and analysed by HPAEC-PAD. The analysis revealed three main differences between freshwater and saltwater samples. In the freshwater sample, no ribose was detected, glucosamine was the dominant monomer, and galacturonic acid had a relatively higher concentration. Ribose is an important metabolite of the pentose phosphate pathway (PPP), an alternative glucose metabolising. The observed changes in the glycan profile could indicate an adaptive response to environmental conditions, such as the presence of salt. Additionally, the unidentified fraction of the sugar composition may indicate differences between samples that are not currently detected. Alterations in the glycan and glycome profile of EPS from AGS due to salinity have also been demonstrated by (Chen et al., 2024b; de Graaff et al., 2019). Also, their study showed that glucose emerged as the dominant monomer after exposing AGS to saline conditions, and the relative concentration of galacturonic acid decreased.

The presence of organic and inorganic phosphates in the EPS has been reported in numerous studies, specifically from EPS derived from EBPR systems (Li et al., 2015). Extensive literature explaining the transportation and transformation mechanism of phosphates in the EPS already exists (Zhang et al., 2013). In short, the phosphate in the EPS can either originate from the leakage of orthophosphate due to the degradation of intracellular polyphosphate or the adsorption of free phosphate present in the bulk liquid onto the EPS matrix. Regardless, it is an important component as it influences the EPS properties and thus can have an impact on

the application of EPS. For instance, in a study testing the EPS as a bio-based flame-retardant, phosphate was found to be one of the main components in the EPS that contributed to its flame retardancy (Kim et al., 2020).

### EPS as a biobased flame retardant

In this study, the impact of EPS as a biobased flame retardant was evaluated by coating cellulose fibres with EPS and subjecting them to controlled burning tests. Cellulose was chosen as a carrier for conducting burning tests. Cellulose can also be recovered from tissue papers collected in WWTPs in large quantities. It is widely used in the construction industry as an adhesive or building material (Tsivadze et al., 2015). The EPS-coated cellulose fibres can therefore be used as biobased flame retardant fillers in the construction industry. After flame application, the cellulose fibres coated with EPS (R10, R20, and R35x) retained their structural integrity, exhibited char formation, and experienced less mass loss compared to the other samples. These samples had a higher condensed phosphate content, which correlated with the observed char formation. In a study by Kim et al. (2023) where phosphate-containing EPS was used as a coating for cellulose-based fibres, the authors suggested that orthophosphate may play an important role in EPS flame retardancy which is in agreement with the case of freshwater EPS in the current research. In addition, we observed that salinity can induce the production of condensed phosphate in EPS with enhanced flame retardancy compared to freshwater EPS. Therefore, it is likely that EPS flame retardancy is related to its total phosphate content, especially the condensed phosphate fraction. In the case of fire, the reactive phosphates in the EPS transform into phosphoric acid due to thermal degradation and react with carbonaceous material which contributes to char formation (Kim et al., 2023). The phosphorus decomposition dehydrates the carbon source and facilitates char formation through graphitisation. This char layer acts as a barrier that slows the release of flammable gases and insulates the underlying material from heat. Thus, char formation can be regarded as the main mechanism behind EPS flame retardancy.

EPS is believed to possess intrinsic intumescent i.e. flame-retardant properties due to the presence of biomacromolecules such as proteins, carbohydrates, and DNA (Alongi et al., 2014). Intumescence refers to increased volume and decreased density, i.e. swelling process, due to heat exposure. For instance, a factor that contributes to intumescence is the combustion of proteins (amides) and DNA (nitrogen-containing molecules) releasing nitrogenous gases such as ammonia, which help to dilute the flammable gases generated during the decomposition of EPS. Based solely on these parameters, EPS can be regarded as a biobased flame retardant. However, although all EPS samples contained comparable amounts of proteins and sugars (**Table 16**), the charring effect varied (**Figure 37**), raising questions about the role of proteins and carbohydrates in the intumescence of EPS.

In a recent study evaluating the phosphorus-enriched algae as a flame retardant in polylactide by Dudziak et al. (2024), the authors argued that the reactivity of phosphorus is more crucial than its content in the material. In this study, char formation in EPS-coated cellulose fibres was closely related to the organic phosphate content of the EPS. It is assumed that the improved fire retardancy of organic phosphates arises from their ability to form a stable char

layer, absorb heat through endothermic decomposition, release non-flammable gases, and catalyse charring. These mechanisms collectively contribute to their superior fire-resistant properties compared to other phosphate compounds (Scharte, 2010). In other words, other phosphate moieties, such as orthophosphates, likely play a less important role in EPS flame retardancy.

Lastly, since the flame retardant components in the EPS can contribute to char formation, they can interfere with the determination of sample volatile solids. Traditional volatile solid measurement protocols can therefore significantly underestimate biomass concentration in e.g. EBPR systems due to this char formation phenomenon.

## Conclusions

- Salinity induced a change in the sugar monomer and the total phosphate composition of EPS from aerobic granular sludge. Under saline conditions, the condensed phosphate is the dominant phosphate moiety in the EPS total phosphate content.
- The analysis of IR spectra with the 2D-COS method showed differences in regions corresponding to polysaccharides and phosphate functional groups which were experimentally verified.
- The flame-retardancy of EPS-coated cellulose fibres correlated linearly with the condensed phosphate content of the EPS.

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# 6. Outlook

## Scientific Relevance and Proposed Research Questions

This study explored the use of aerobic granular sludge technology for treating industrial effluents containing sugars (glycerol and glucose) and salts, chosen to represent the waste streams from the industries involved in this PhD project. The findings were primarily obtained from laboratory experiments using synthetic media. Given that the results were derived from synthetic media, the logical next step is to conduct experiments with real wastewater to address the challenges in this project. This would help evaluate the impacts on substrate conversions, microbial communities, and nutrient removal under real conditions. The proposed research questions aim to build on the current findings and address gaps in our understanding, ultimately enhancing the applicability and efficiency of aerobic granular sludge technology in treating industrial effluents.

### Collaborative Microbial Communities in EBPR

Our studies indicated that treating industrial effluents containing glycerol and glucose with AGS requires a collaborative effort between fermentative microbial communities and polyphosphate-accumulating organisms (PAOs). Since PAOs lack the enzymatic machinery to uptake certain sugars, they rely on fermentative communities to convert these sugars into PAO-utilisable products, i.e. VFAs. This interaction also likely occurs in municipal wastewater treatment plants (WWTP) due to the presence of complex substrates like proteins, amino acids, and carbohydrates. The fermentative microbial communities are commonly found in WWTPs around the world but understanding their role did not receive much attention in the literature (Seviour et al., 2008, 2000).

Proposed research questions: How do different sugars impact granulation and phosphate removal? Does the fermentative population contribute to phosphate uptake?

### Carbon Availability for PAOs

During the research, we found that the fermentative organisms accumulate some of the substrates as glycogen anaerobically. Substrate storage provides an energy source for growth under anoxic or aerobic conditions. However, substrate storage by organisms other than PAOs can negatively impact phosphate removal by reducing carbon availability for them.

Proposed research question: How can the system be manipulated to minimize substrate storage by the fermentative microbial community or select non-storing fermentative organisms? Do glycogen-storing organisms grow anaerobically or grow on the storage material when oxygen is present?

### Impact of SRT on Glycerol Fermentation Product Spectrum

Using glycerol as the sole carbon source, the system achieved a pseudo-steady state, in terms of glycerol conversions and granulation, after four months of operation. This long stabilization period was likely due to a small competitive difference between various fermentative organisms, initially leading to 1,3-propanediol (1,3-PDO) production. The produced 1,3-PDO was only aerobically oxidised, leading to floc formation. Eventually, 1,3-PDO was replaced with propionate formation. The slow replacement of 1,3-PDO by propionate suggests a minimal competitive difference between the two fermentation pathways. Lowering the SRT from 20 to 12 days appeared to help outcompete the 1,3-PDO fermentation process. Thus, SRT control could be a practical method for selecting favourable conditions in full-scale reactors where glycerol is a substrate. However, the impact of SRT on product formation requires further validation.

Proposed research question: How does SRT affect the product spectrum of glycerol fermentation in AGS reactors? And how would this affect the overall process performance for industrial waste streams?

### Enzymatic Machinery of *Actinobacteria*

Metaproteomic analysis identified the presence of *Tessaracoccus* and *Micropruina*, two actinobacteria likely responsible for glycerol and glucose conversions. Polyphosphate granules have not been observed in these organisms (Maszenan et al., 1999) but they seem to possess some enzymes involved in polyphosphate metabolism. The presence of polyphosphate kinase (ppk) suggests these microorganisms can accumulate polyphosphate but the absence of AMP phosphotransferase (pap) suggests that they cannot use it for energy generation. Actinobacteria including these two genera, are observed in high abundance in WWTPs with EBPR performance around the world (Seviour et al., 2000). They are likely responsible for fermentation processes but their influence on nutrient removal is unclear.

Proposed research question: What are the enzymatic capabilities of these organisms? Can they metabolise sugars other than glycerol and glucose? In what way do they influence EBPR?

### Ratio of Cations

In wastewater treatment processes for saline streams, the ratio of cations may play a more significant role than the absolute salt concentrations. The ionic properties of the influent can affect not only phosphate removal but also the effluent quality in terms of solids concentration. Both $Mg^{2+}$ and $K^{+}$ are essential cations as counterions for poly-P metabolism and $Ca^{2+}$ and $Mg^{2+}$ enable gelling of the EPS which is relevant for granular stability. Therefore, it is crucial to consider not only the total salinity but also the ratio of cations for an effective treatment. This can be achieved through mixing the influent with seawater if available or adjusting the ratio of cations in the wastewater influent by the addition of salts.

Proposed question: What is the minimum ratio of major cations such as $Mg^{2+}$, $Ca^{2+}$, and $K^{+}$ relative to $Na^{+}$ required for a stable granulation?

### EPS as a Profitable Product

Extracellular polymeric substances (EPS) currently lack monetary value because it has no proven positive business case. Although several potential applications have been proposed (Bahgat et al., 2023; Chen et al., 2024; Kim et al., 2020; Tomás-Martínez et al., 2023), downstream processing and product development are essential to commercialise EPS and make it market-ready. For instance, EPS can be used as a biobased flame-retardant material to make composites such as medium-density fibreboards. The global flame retardant market is growing which is driven by increasingly stringent fire safety regulations (GVR, 2023). The building and construction industry currently holds the largest market share, followed by sectors such as textiles and appliances (KSI, 2024). There is a noticeable market shift toward non-halogenated flame retardants due to rising environmental and health concerns. EPS can fill this market need but it requires major product development.

Proposed research question: How much EPS (in weight fraction) is needed to make a flame-resisting composite material? Which flame-retardancy classifications can EPS-based composites potentially achieve? How big is the flame-retardant market compared to the annual EPS production volume? What societal and regulatory challenges exist for the use of wastewater-derived products such as EPS in composite manufacturing?

## Economic Analysis and Project Outlook

Economic analysis measures the overall desirability of the project in financial terms and determines the feasibility of the project. An economic analysis can also highlight bottlenecks and guide the research direction to address the challenges effectively. This economic analysis serves a dual purpose: suggesting a future research direction and providing a concluding economic perspective to this PhD research.

### Background

An epoxy resin producer located within the industrial cluster at the Port of Rotterdam in the Netherlands generates wastewater containing high levels of salt (~12% w/v NaCl) and organics, mainly glycerol (accounting for ~95% of the total COD). To either discharge the effluent into the environment or reuse the brine, the organic impurities must be removed. To achieve this, the application of Nereda® technology combined with Kaumera recovery was proposed as one of the solutions. Some of the technical feasibility aspects have been experimentally addressed, with the results presented throughout the thesis.

AGS is commercially known as Nereda® technology and is already in use globally (https://nereda.royalhaskoningdhv.com/). According to the results of this study, AGS is effective at removing glycerol and tolerating certain levels of salinity. We have also demonstrated that biopolymer recovery, commercially known as Kaumera, from the produced sludge is feasible and has flame-retardant properties. The existence of two large-

scale Kaumera extraction facilities in the Netherlands (https://kaumera.com/) further supports the feasibility of large-scale Kaumera production, indicating that this technology is well-established, market-ready, and can be supplied in large quantities.

### Objectives

Questions that are addressed here are whether Nereda® treatment with Kaumera recovery can be effectively applied under the specified conditions and what are the economic implications. Based on an economic evaluation, we identify the main bottlenecks to address. Based on the identified bottlenecks, we then formulate corresponding research questions.

### Methodology and Assumptions

The economic evaluation is based on a conceptual system including three technologies depicted in **Figure 39**. The influent with the characteristics given in **Table 19** enters the system. The products are effluent, biogas, and Kaumera. The functional unit, serving as a unit of comparison, is defined as 1 $m^3$ of treated influent. The overall treatment cost is compared between the current situation, the proposed system, and its potential outlook.

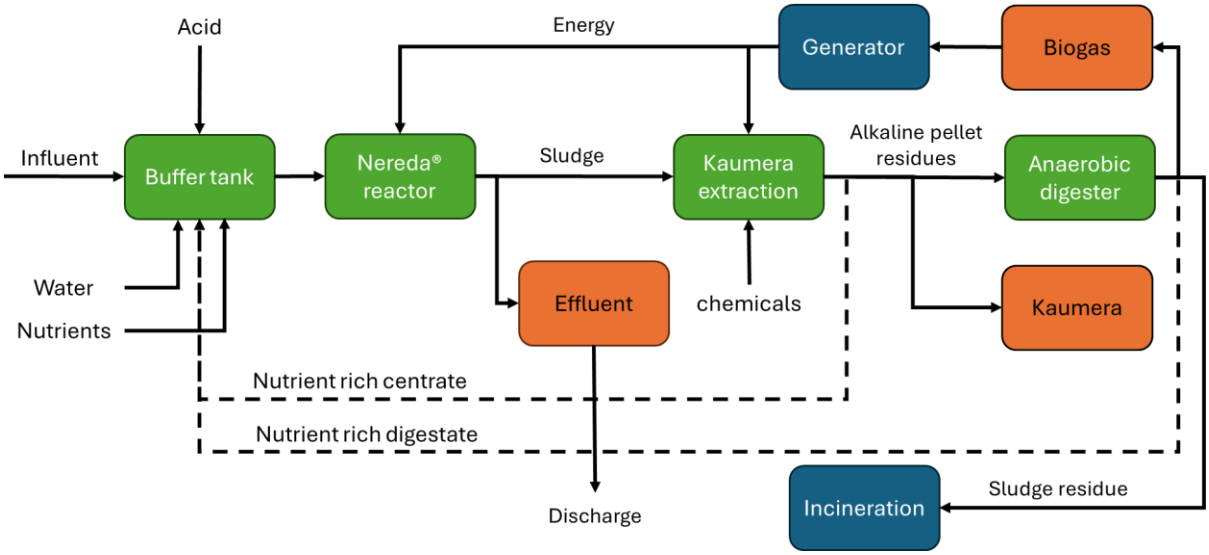


***Figure 39**. Flow scheme of the proposed system. Evaluated processes (green boxes), products (orange boxes) and untouched processes (blue boxes) are depicted. Dashed lines show the nutrient recycling possibilities.*

***Table 19**. Influent characteristics.*

| Parameters | Values | Units |
|---|---|---|
| Flow rate | 1200 | m3/d |
| COD load | 5000 | kg/d |
| Salinity | 12 | % |
| pH | 12.5 | - |

Plant Capacity

The real wastewater has a salinity level too high for the standard WWTP microbial community, necessitating dilution. Diluting with freshwater is an option but it is wasteful and economically unattractive. Using surface waters (e.g. directly from the rivers) is more cost-effective. To dilute the effluent to a salinity of approximately 4% w/v, about 2000 $m^3$/day of water is required. Research indicates that stable granulation and effective carbon removal are achievable at this salinity (data not published yet). This dilution will increase the total flow to 3,200 $m^3$/day. Based on the assumptions listed in **Table 20**, two reactors with a combined volume capacity of approximately 890 $m^3$ are thus needed. The reactor volume is calculated using the equation below (Pronk et al., 2023). If we assume a reactor height of 10 meters, this corresponds to a total required surface area of 89 $m^2$. For flow equalisation, a buffer tank with a volume of ¼ of the reactor's capacity is considered.

$$Reactor\ volume = \frac{Q * COD}{t_{React} * MLSS * SLR * n_R}$$

***Table 20**. List of assumptions and parameters used to calculate Nereda® reactor volume and oxygen requirement for aeration.*

| **Parameters** | **Values** | **Units** |
|---|---|---|
| Flowrate (Q) | 3200 | $m^3$/d |
| COD concentration (COD) | 1.56 | kg/$m^3$ |
| Sludge loading rate (SLR) | 0.5 | kgCOD/kgMLSS.d |
| Cycles per day | 6 | - |
| Reaction time ($t_{React}$) | 18/24 | d |
| Sludge concentration (MLSS) | 15 | kg/$m^3$ |
| Number of reactors ($n_R$) | 2 | - |
| Biomass yield | 0.58 | gBiomassCOD/gCOD $_{glycerol}$ |
| Oxygen requirement | 0.88 | $gO_2$/gCOD $_{glycerol}$ |
| Oxygen transfer efficiency | 1.52 | $kgO_2$/kWh |
| Biomass COD | 1.42 | $gO_2$/g |
| Biomass molecular weight | 113 | g/mol |
| Glycerol COD | 1.22 | $gO_2$/g |
| Glycerol molecular weight | 92 | g/mol |

To estimate the energy required for aeration, the oxygen needed for the oxidation of glycerol is stoichiometrically calculated using the formula below :

**Catabolism (energy giving):**

$$-1\ C_3H_8O_3 - 3.5\ O_2 + 3\ CO_2 + 4\ H_2O$$

**Anabolism (biomass production):**

$$-0.33\ C_3H_8O_3 - 0.12\ O_2 - 0.2\ NH_4^+ + CH_{1.8}O_{0.5}N_{0.2} + 0.73\ H_2O + 0.2\ H^+$$

**Metabolism (energy required for growth coupled to energy production):**

$$-2.46\ C_3H_8O_3 - 7.55\ O_2 - 0.2\ NH_4^+ + 1\ CH_{1.8}O_{0.5}N_{0.2} + 6.37\ CO_2 + 9.23\ H_2O + 0.2\ H^+$$

For full oxidation, about 4381 $kgO_2/d$ is required which means 2.4 $kWh/m^3$ of energy for aeration. With a biomass yield of 0.58 gBiomassCOD/gCOD and a COD load of 5000 kg/d, around 2910 kg of biomass (volatile solids) is produced per day which sets the basis of Kaumera mass balance. The addition of essential elements to sustain microbial growth might be required which is calculated based on the biomass composition ($CH_{1.8}O_{0.5}N_{0.2}P_{0.01}Ca_{0.005}Mg_{0.0025}K_{0.01}$). For simplicity, the 5 main elements required to add are given in **Table 21** in their commercially available moieties.

***Table 21**. List of nutrients for microbial growth. Values are in kg/d.*

| Ammonium chloride | Sodium phosphate | Potassium chloride | Magnesium sulphate | Calcium chloride |
|---|---|---|---|---|
| 995 | 168 | 41 | 110 | 30 |



### Mass Balances

For mass balances, the following assumptions are made. The plant is assumed to operate for 340 days and 24 hours per day. The rest of the days are used for maintenance. For Kaumera mass balance, the basis of calculation is 2910 kg volatile solids per day. The yield of Kaumera is assumed to be 30% via centrifugation.

### Location

The geographical location is an important factor in the project as it can influence various aspects such as resource planning, logistics, etc. The industry is located in the industrial cluster of the port of Rotterdam in the Netherlands, close to the river and the North Sea. This close proximity to the water sources is important considering water availability and piping costs.

### Economics

The main criterion for evaluation in this chapter is based on the economic analysis which includes the operational costs and capital investment of a Nereda® unit, a Kaumera extraction unit, and an anaerobic digester. The conceptual system shown in Figure 39 is compared between the current situation, the proposed system, and its potential outlook.

### Operational Costs

Operational costs encompass expenditures on chemicals, energy, residual sludge disposal, operator and maintenance, and overhead. For overhead costs, 0.5% of the capital investment is considered. Fixed costs were allocated for operator and maintenance. For the Nereda® process, chemical costs include expenses for dosing additional nutrients. The chemical costs associated with Kaumera extraction include KOH for alkaline extraction, polyelectrolytes for sludge thickening, and HCl for precipitation. Energy costs for the Nereda® process are derived from the aeration requirement. For the Kaumera extraction process, an energy consumption rate of 0.2 kWh/kg is applied *(personal communication)*. The costs for residual sludge disposal, which cover transportation and incineration, are estimated at 90 €/ton of wet sludge *(personal communication)*.

***Table 22**. Operational costs of the proposed system.*

| **Parameters** | **Kaumera unit** | **Nereda®** | **Digester** |
|---|---|---|---|
| Chemicals (€/d) | 257 | 1,118 | - |
| Energy (€/d) | 175 | 346 | - |
| Residual sludge disposal (€/d) | 344 | - | - |
| Operator and maintenance (€/d) | 300 | 300 | 200 |
| Overhead (€/d) | 447 | 130 | 34 |
| Total daily cost (€/d) | 1,522 | 1,894 | 234 |
| | 1.74 €/kg $_{\text{Kaumera}}$ | 1.58 €/$m^3$ $_{\text{influent}}$ | 0.16 €/$m^3$ $_{\text{biogas}}$ |

### Capital Investment

To estimate the capital investment, the unit total cost is calculated (**Table 23**). The unit total cost includes the equipment and steel costs for the reactors. To cover the costs of installation, engineering, contractor fees, etc. a Lang factor of 3.5 is used as a multiplier. It is a commonly used value in plants processing solids and liquids *(personal communication)*. The lang factor was developed in the 1940s to estimate the total capital costs at the early stages of project planning based on the equipment costs.

*Table 23. Unit total cost breakdown of the proposed system. Values are in €.*

| Units | Kaumera unit | Nereda® | Buffer tank | Digester |
|---|---|---|---|---|
| Steel cost | 8,640 | 256,000 | 64,000 | 10,080 |
| Pumps | 21,000 | 42,000 | 21,000 | 21,000 |
| Agitators | 70,000 | 70,000 | 35,000 | 35,000 |
| Centrifuges | 1,750,000 | - | - | - |
| Membrane unit | - | - | - | - |
| Decorations (sensors, valves, etc.) | 175,000 | 175,000 | 87,500 | 87,500 |
| Unit total equipment cost | 2,024,640 | 543,000 | 207,500 | 153,580 |

### Financial Overview

Lastly, the financial overview of the proposed system with a potential outlook is provided (**Table 24**). Since an asset depreciates over time, depreciation must be factored into financial calculations. In this case, depreciation is accounted for over a project lifetime of 15 years. Annual revenue is determined by the sale of Kaumera at 2.5 €/kg dry weight *(personal communication)* and biogas at an average price of 0.3 €/m³ (Next Energy, 2024). Gross profit is then calculated by subtracting depreciation and operational costs from the total revenue. Net profit is derived by applying a 25% tax (standard tax value in the Netherlands) to the gross profit. Cash flow is obtained by adding the net profit to the depreciation amount. To calculate the amount of money needed to recover the investment over the project's lifetime, the capital recovery factor (0.1) was calculated with an interest rate of 5% (Cicekalan et al., 2023). Finally, the payback period is determined by dividing the total investment by cash flow.



*Table 24. Financial overview of the proposed system and its corresponding potential outlook.*

| Financial overview | Proposed system | Potential outlook | Units |
|---|---|---|---|
| Depreciation | -184,691 | -138,025 | €/yr |
| Revenue | 837,456 | 1,532,296 | €/yr |
| Operational cost | -1,240,954 | -1,031,788 | €/yr |
| Gross profit | -588,190 | 362,484 | €/yr |
| Gross profit margin | - | 24 | % |
| Net profit | - | 271,863 | €/yr |
| Cashflow | - | 133,838 | €/yr |
| Total investment | 2,770,372 | 2,070,372 | € |
| Investment in annualized form | 266,904 | 199,464 | € |
| Payback period | - | 15 | yr |

## Conclusions and Recommendations

Brine recycling, with the aim of using chlorine in the chlor-alkali process, is not practical due to the need for dilution when using AGS treatment. The dilution poses several challenges: it increases the hydraulic load significantly, necessitating larger treatment units; it involves

costly and wasteful use of fresh water; and it necessitates up concentration after treatment, leading to substantial energy consumption. Consequently, AGS treatment is not a logical solution if brine recycling is the aim.

An alternative approach is to use Nereda® treatment with Kaumera and biogas production, focusing on COD rather than salt recovery. Based on the proposed system, the annual wastewater treatment cost can be reduced by 30% to 3.48 €/$m^3$ in comparison to the reference scenario of 5 €/$m^3$. The extraction of Kaumera and biogas generation can offset some of the wastewater treatment expenses but the system would not yield a return on investment.

However, the proposed system has the potential to be improved by recycling the nutrient-rich streams and increasing the Kaumera yield using ceramic membranes for separation, leading to a further decreased wastewater treatment costs to 2.11 €/$m^3$.

Recommendations for future research:

- **Conduct phosphorus-limiting experiments** to evaluate the effects on the granulation process and Kaumera properties, especially given the minimal phosphorus dosing. Nutrient-limiting experiments are also important, as many industrial discharges lack sufficient nutrients for microbial growth.
- **Investigate nutrient recycling** to minimize the need for additional nutrient dosing, which is a significant cost factor in AGS treatment in pilot studies.
- **Perform thorough market research and validation** on Kaumera, as its price significantly impacts system feasibility and profitability. This can be achieved by conducting surveys in relevant communities of practice, organizing workshops, and engaging industrial stakeholders and focus groups for insights.
- **Account for downstream processing costs** in Kaumera production. Current cost estimates do not factor in the expenses linked with downstream processes. For commercialization, it's essential to determine the production cost, including downstream processing, through lab- and pilot-scale studies.

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# Curriculum vitae

Ali Elahinik was born in Tehran, Iran, on December 23, 1993. After completing primary school, he moved with his family to Dubai, UAE, where he earned his BSc in Environmental Science from Canadian University Dubai. Alongside his studies, Ali gained hands-on experience working part-time as an environmental technician at Scentroid, where he conducted fieldwork in odor sampling for several months.

After his bachelor's degree, Ali took a gap year to explore career options and reflect on his goals. Realizing his passion for science and his desire to contribute to environmental sustainability, he pursued a double-degree MSc in Translational Ecosystem-Based Water Management at the University of Duisburg-Essen (Germany) and Radboud University (Netherlands). This intensive program covered diverse water-related topics, including flood management, hydraulic planning, environmental modelling, wastewater treatment, hydroclimatology, and water quality modelling, among others

During a wastewater treatment course, Ali first encountered the aerobic granular sludge (AGS) process while learning how to design a basic activated sludge biprocesses. Fascinated by the technology, the curiosity led him to a six-month internship at Royal HaskoningDHV, where he worked with the Nereda® team on creating an online dashboard delivering real-time analytics.

For his master's thesis, Ali joined Wetsus, a European water technology hub in Leeuwarden, to work on electrogenic biofilm development in bio-electrochemical systems, applying advanced tools like optical coherence tomography and electrochemical impedance spectroscopy. After completing his MSc, he took on a role as a process engineer at Bluecon International in Arnhem, where he contributed to the R&D and commissioning of modular physico-chemical wastewater treatment units.

During his master's thesis at Wetsus, Ali experienced the joy of curiosity-driven research, where he could fully immerse himself in science without the burden of administrative tasks. In 2021, Ali's interest in the water sector and AGS technology in particular led him to a PhD opportunity at the environmental biotechnology group at TU Delft. Ali started his PhD journey as part of the Water-Mining project, a collaboration between 38 academic and industrial partners across Europe. The PhD project aimed to evaluate the feasibility of AGS for the treatment of organically polluted industrial discharges. Throughout his PhD, Ali gained extensive experience in bioreactor operation, student supervision, and effective communication. He also honed essential project management skills such as decision-making, time management, and fostering partnerships between academia and industry.

# List of patents and publications

**Patent:** Elahinik, A. and Lin, Y. (2025). Extracellular polymeric substances as a biobased flame-retarding binding material. First filing: NL2039514.

**Elahinik, A.**, de Clercq, F., Pabst, M., Xevgenos, D., van Loosdrecht, M. C., & Pronk, M. (2024). Effects of salinity on glycerol conversion and biological phosphorus removal by aerobic granular sludge. *Water Research*, *257*, 121737.

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# Conference contributions

Water Mining, Delft, The Netherlands. (Oct 2024). A green flame-retardant from waste. Elevator pitch.

IWA Large Wastewater Treatment Plants conference, Budapest, Hungary. (Sep 2024). Aerobic sludge granulation, conversion, and phosphate removal using sugar substrates. Poster.

IWA Leading Edge Technologies conference, Essen, Germany. (Jun 2024). Aerobic sludge granulation, conversion, and phosphate removal using sugar substrates. Poster.

Water Mining, Larnaca, Cyprus. (Oct 2023). 1-minute business pitch competition.

Paques. The Netherlands. (May 2023). Business idea pitch.

IWA Biofilms Phuket, Thailand. (Oct 2022) Glycerol conversion in aerobic granular sludge. Oral.

Water Mining, Palermo, Italy. (Sep 2022). Industrial wastewater treatment with Aerobic Granular Sludge. Oral.

IWA Young Water Professional, Aarhus, Denmark. (Jun 2022). Glycerol conversion in aerobic granular sludge. Oral.

Water Mining, Barcelona, Spain. (Oct 2021). Industrial Mining, Bench scale tests. Oral.

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