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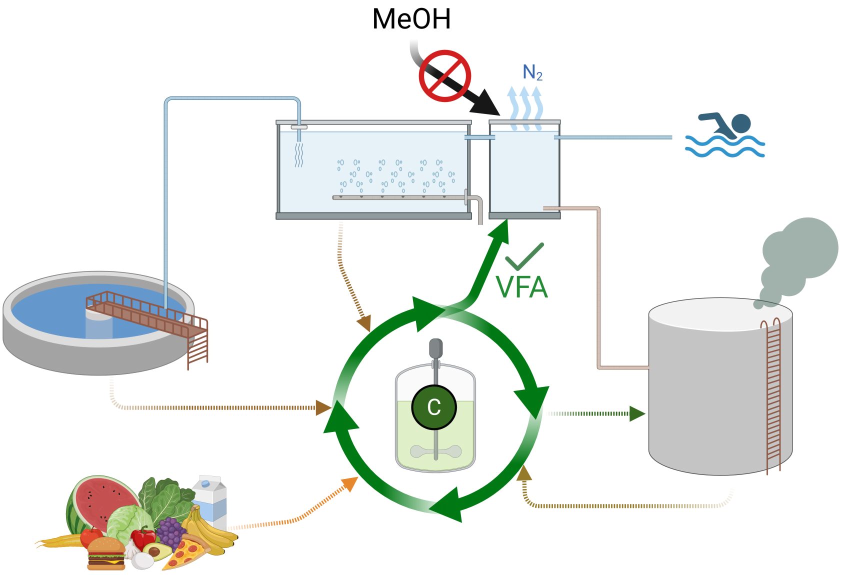
Sustainable carbon sources for nitrogen removal in WWTP

Balancing denitrification performance, biogas potential, and economic feasibility

Andrea Carranza-Muñoz



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Sustainable carbon sources for nitrogen removal in WWTP

Balancing denitrification performance, biogas potential, and economic feasibility

Abstract

Nitrogen removal, emission reduction, and climate neutrality are central goals for modern wastewater treatment plants, reinforced by stricter nutrient limits from the EU and Swedish regulations. Methanol is widely used for nitrogen removal in the denitrification process. However, alternative carbon sources are needed to promote circular resource management and reduce climate impact. This thesis investigated the production and use of volatile fatty acid (VFA)-rich carbon sources through the direct use or fermentation of primary sludge (PS), waste-activated sludge, food waste (FW), and digestate, alongside a full-scale evaluation of their technical and economic feasibility. Fermentation performance varied depending on substrate, with FW and FW:PS mixes achieving higher sCOD (up to 82 g sCOD/L) and VFA concentrations (up to 56 g VFA_{COD}/L) than sludge carbon sources, together with favourable carbon-to-nutrient ratios and low biogas losses. Sludge- and digestate-based fermentates exhibited lower sCOD concentrations (<12 g/L and <20 g/L with thermal hydrolysis pre-treatment) and higher nutrient release, impacting carbon dosing needs and implementation costs. Despite these differences, denitrification was consistent across all fermentate carbon sources (7-9 mg NO_3/gVSS·h), regardless of substrate origin. Replacing methanol with fermentates reduced fossil CO_2 emissions associated with chemical use and operation, but required significant capital investment. A plant-wide economic and environmental analysis confirmed that FW and FW:PS fermentates are the most cost-effective alternatives to methanol, while sludge- and digestate-based options are feasible but associated with higher capital and operational costs. FW-based options provided the lowest treatment costs, reducing costs by up to 0.039 EUR/$m^3_{\text{treated-water}}$, while the digestate-based option increased costs by up to 0.026 EUR/$m^3_{\text{treated-water}}$ compared to using methanol. The results demonstrate a feasible pathway for WWTPs to replace methanol with waste-derived carbon sources, supporting climate targets, stricter nitrogen limits, and circular resource management.

Keywords: Carbon source, Denitrification, Fermentation, Volatile fatty acids, Resource recovery, Wastewater treatment.

Hållbara kolkällor för kväverening på avloppsreningsverk

Optimering av balansen mellan denitrifikationseffektivitet, biogaspotential, och ekonomisk genomförbarhet

Abstract

Kväverening, minskade utsläpp och klimatneutralitet är centrala mål för moderna avloppsreningsverk, förstärkt av striktare utsläppskrav i både EU- och svenska regelverk. Metanol används ofta för denitrifikation men alternativa kolkällor efterfrågas alltmer för att minska reningsverkens CO_2-utsläpp och främja en cirkulär ekonomi. Den här avhandlingen undersökte produktion och användning av flyktiga fettsyror (VFA) som kolkälla för kväverening genom direktanvändning eller fermentering av primärslam (PS), överskottslam, matavfall (FW) samt rötrest (tidigare obeprövat). Studien inkluderade även en utvärdering av den teknoekonomiska genomförbarheten i fullskala för respektive kolkälla. Resultatet av fermenteringen varierade med substraten; FW och FW:PS-blandningar erhöll högre halter sCOD (<82 g COD/L) och VFA (<56 g VFA_{COD}/L) jämfört med övriga kolkällor samt mer fördelaktiga C:N/P-kvoter och mindre biogasförluster. Slam- och rötrestbaserade fermentat visade lägre sCOD-halter (<12 g/L och <20 g/L efter termisk hydrolys) samt högre frisättning av näringsämnen, vilket ger högre doseringsbehov och investeringskostnad. Trots dessa skillnader var denitrifikationshastigheten likvärdig för alla fermentat (7-9 mg NO_3^--N/g VSS·h), oberoende av substrat. De fossila CO_2-utsläppen från drift minskades då metanol ersattes av fermentat, men kostnaden för implementering var hög. En övergripande hållbarhetsanalys visade att de mest kostnadseffektiva alternativen till metanol var fermentat från FW och FW:PS medan slam- och rötslambaserade alternativ också fungerar bra men medför högre investerings- och driftkostnader. FW-baserade alternativ minskade kostnaderna med <0,039 EUR/m^3_{vatten}, medan rötrestbaserade alternativ ökade kostnaderna med <0,026 EUR/m^3_{vatten} jämfört med metanol. Resultaten visar en möjlig väg för reningsverk att ersätta metanol med restproduktsbaserade kolkällor, vilket stödjer klimatmål, skärpta kvävekrav och cirkulär resurshantering.

Nyckelord: Kolkälla, Denitrifikation, Fermentering, Flyktiga fettsyror, Resursåtervinning, Avloppsrening.

Producción y aplicación de fuentes de carbono sostenibles para la eliminación de nitrógeno en PTAR’s

Análisis del rendimiento en desnitrificación, el potencial energético y la factibilidad económica

Resumen

La eliminación de nitrógeno, la reducción de emisiones y la neutralidad climática son objetivos centrales de las plantas de tratamiento de aguas residuales (PTAR’s), reforzados por los límites de nutrientes más estrictos de la Unión Europea y la normativa sueca. El metanol se usa ampliamente en la desnitrificación, pero ahora se requieren fuentes de carbono alternativas para reducir emisiones y promover la gestión circular de recursos. Esta tesis evaluó la producción y aplicación de fuentes de carbono ricas en ácidos grasos volátiles (AGVs) mediante fermentación o uso directo de lodos primarios (LP), lodos activados, residuos alimentarios (RA) y, de forma novedosa, digestato, junto con un análisis técnico y económico a escala real. La fermentación mostró mejores resultados con RA y mezclas LP:RA, alcanzando hasta 82 g DQO/L y 56 g AGV_{DQO}/L, con relaciones carbono-nutrientes favorables y bajas pérdidas de biogás. Los fermentados de lodos y digestato presentaron menores concentraciones de DQO (<12 g/L y <20 g/L con pretratamiento de hidrólisis térmica) y mayor liberación de nutrientes, lo que incrementó los requerimientos de dosificación de la fuente de carbono y por ende los costos. A pesar de estas diferencias, la eficiencia en la desnitrificación fue consistente con todos los fermentados (7-9 mg NO_3^--N/gVSS·h), independientemente de su origen. Sustituyendo el uso de metanol con fermentados redujo las emisiones de CO_2, aunque requirió una inversión de capital significativa. El análisis económico confirmó que RA y mezclas LP:RA son las opciones más rentables, reduciendo los costos en hasta 0,039 EUR/$m^3_{\text{agua-tratada}}$, mientras que el uso de digestato aumentó los costos en hasta 0,026 EUR/$m^3_{\text{agua-tratada}}$. Los resultados demuestran una vía viable para sustituir metanol por fuentes de carbono provenientes de residuos, cumpliendo los objetivos climáticos y de calidad del agua.

Keywords: Fuentes de carbono, Desnitrificación, Fermentación, Ácidos grasos volátiles, Recuperación de recursos, Tratamiento de aguas residuales.

Dedication

To my family and to the love of my life. Always.

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List of publications

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I. Carranza-Muñoz, A., Malovanyy, A., Baresel, C., Singh, A., Schnürer, A. (2025). Integrated Resource Recovery from Co-Fermentation of Food Waste and Sludge: Balancing Denitrification Potential and Biogas Recovery (Submitted).

II. Carranza-Muñoz, A., Olsson, J., Malovanyy, A., Baresel, C., Machamada-Devaiah, N., Schnürer, A. (2024). Impact of thermal hydrolysis on VFA-based carbon source production from fermentation of sludge and digestate for denitrification: experimentation and upscaling implications. Water Research. Volume 266, 2024 (122426), ISSN 0043-1354. https://doi.org/10.1016/j.watres.2024.122426.

III. Carranza-Muñoz, A., Malovanyy, A., Singh, A., Baresel, C., Karlsson, J., Stark-Fujii, K., Schnürer, A. (2025). Replacing methanol with internally produced VFA-based carbon source for denitrification at the Henriksdal WWTP. Water Science and Technology,92 (1), 139-152. https://doi.org/10.2166/wst.2025.086.

IV. Carranza-Muñoz, A., Storm, B., Malovanyy, A., Schnürer, A., Baresel, C. (2025). The cost and feasibility of replacing methanol with internally produced VFA as an external carbon source: Is It Worth It? (manuscript)

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The contribution of Andrea Carranza-Muñoz to the papers included in this thesis was as follows:

I. Contributions included participation in experimental planning, experimental work, and data analysis. Main writer of the manuscript, prepared in close collaboration with the co-authors.

II. Contributions included participation in experimental planning, experimental work, and data analysis. Main writer of the manuscript, prepared in close collaboration with the co-authors.

III. Contributions included participation in experimental planning, experimental work, and data analysis. Main writer of the manuscript, prepared in close collaboration with the co-authors.

IV. Contributions included participation in developing the methodology, performing the data handling and conducting the result analysis. Main writer of the manuscript, prepared in close collaboration with the co-authors

In addition to the papers mentioned above, Andrea Carranza-Muñoz contributed to the following papers during the timeframe of the doctoral project, which are not included in this thesis.

Academic Papers:

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II. Andersson, S. L., Baresel, C., Andersson, S., Westling, K., Eriksson, M., Carranza-Muñoz, A., Persson, G., Narongin-Fujikawa, M., Johansson, K., & Rydberg, T. (2024). Chemical-Saving Potential for Membrane Bioreactor (MBR) Processes Based on Long-Term Pilot Trials. Membranes, 14(6), 126. https://doi.org/10.3390/membranes14060126

Technical Reports:

I. Grahn, D., Storm, B., Carranza-Munoz, A., Persson, E., Hedayati, A., Chi Johansson, N., Hernández Leal, M., Held, J. (2025). Enhanced Methane Production Through Carbon Dioxide Utilization at Biogas Plants (978-91-7883-699-4 (ISBN)). (C-rapport, Issue. http://urn.kb.se/resolve?urn=urn:nbn:se:ivl:diva-4583

II. Sehlén, R., Moestedt, J., Carranza-Muñoz, A., Ahlström, M., Wärff, C. (2024). Effektivare användning av organiskt material i avloppsvatten. Rapport 2024–19. SVU Rapport. https://vattenbokhandeln.svensktvatten.se/wp-content/uploads/2024/12/2024-19-SVU-rapport-Effektivare-anvandning-av-organiskt-material-i-avloppsvatten.pdf

III. Baresel, C., Bornold, N., Rahmberg, M., Malovanyy, A., Lindblom, E., Carranza-Munoz, A. (2023). *Resultat från FoU-samarbete Syvab-IVL* (978-91-7883-524-9 (ISBN)). B-rapport; B2469. http://urn.kb.se/resolve?urn=urn:nbn:se:ivl:diva-4234

IV. Andersson, S. L., Westling, K., Andersson, S., Karlsson, J., Narongin, M., Carranza Munoz, A., Bornold, N., & Baresel, C. (2021). *Long term trials with membrane bioreactor for enhanced wastewater treatment coupled with compact sludge treatment - pilot Henriksdal 2040, results from 2020* (978-91-7883-305-4 (ISBN)). B-rapport B2425 http://urn.kb.se/resolve?urn=urn:nbn:se:ivl:diva-3801

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Abbreviations

AD	Anaerobic Digestion
AF	Anaerobic Fermentation
BMP	Biochemical Methane Potential
BOD	Biological Oxygen Demand
CAPEX	Capital Expenditure
COD	Chemical Oxygen Demand
sCOD	Soluble Chemical Oxygen Demand
tCOD	Total Chemical Oxygen Demand
CSTR	Continuous Stirred Tank Reactor
EBPR	Enhanced Biological Phosphorus Removal
EPS	Extracellular Polymeric Substances
FW	Food Waste
GHG	Greenhouse Gas
HRT	Hydraulic Retention Time
LCCA	Levelized Cost of Avoided CO_2 Emissions
LCOT	Levelized Cost of Treated Water
MBBR	Moving Bed Biofilm Reactor
MBR	Membrane Bioreactor
NADH	Nicotinamide Adenine Dinucleotide
MS	Mixed Sludge
NPV	Net Present Value
OPEX	Operational Expenditure
O&M	Operation and Maintenance
Pe	Person Equivalent

PS	Primary Sludge
SRT	Solids Retention Time
TAN	Total Ammonia Nitrogen
THP	Thermal Hydrolysis Pre-treatment
TN	Total Nitrogen
TP	Total Phosphorous
TS	Total Solids
VFA	Volatile Fatty Acids
tVFA	Total Volatile Fatty Acids
VS	Volatile Solids
VSS	Volatile Suspended Solids
WAS	Waste Activated Sludge
WWTP	Wastewater Treatment Plant
Y_{OHO}	Anoxic Growth Yield

1. Introduction

The treatment of wastewater has long been central to the development of societies, environmental protection, and public health. As environmental requirements become stricter and the need for climate mitigation increases, wastewater treatment plants (WWTPs) are expected not only to remove pollutants but also to contribute to resource recovery and reduce emissions [1]. Treatment of wastewater is critical, as one of the most important global problems is the degradation of water bodies resulting from excessive loads of nutrients. Eutrophication continues to cause serious consequences for freshwater and marine ecosystems, leading to oxygen depletion, biodiversity loss, and ecosystem degradation [2]. One of the most affected regions in the world is the Baltic Sea [3], a particularly sensitive marine environment where oxygen depletion caused by eutrophication has been ongoing for decades [4]. The recovery of the Baltic Sea is dependent on reductions of nutrient discharges from all the surrounding countries, and thus, initiatives such as the Baltic Sea Action Plan Fund, supported by Sweden and Finland, are being implemented [3, 5]. In line with this, Sweden has introduced stricter national regulations on WWTPs aimed at reducing nutrient pollution [6], supported by the new European Union directive on wastewater treatment, which, in addition to nutrients, also introduces targets for energy efficiency and emissions reduction [7].

The new directive proposes discharge limits of 8 mg/L of total nitrogen (TN) and 0.7 mg/L of total phosphorus (TP) for large WWTPs. In Sweden, these limits will be implemented, and in sensitive zones such as Stockholm, the TN limit is set at 6 mg/L but can be even lower depending on the total nitrogen load per year. The TP limit is 0.20 mg/L [6, 7]. Some WWTPs can achieve such low TN concentrations by using advanced configurations and optimal conditions [8], but the Nordic region faces unique challenges. The combination of low influent chemical oxygen demand (COD), low C/N ratios, and cold climate significantly limits biological processes, including nitrogen removal [8, 9]. Biological nitrogen removal at WWTP is often achieved through a combination of nitrification/denitrification. Denitrification is a microbial process in which nitrate (NO_3^-) is sequentially reduced to nitrogen gas (N_2) via nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O), using organic carbon as an electron donor [8]. This process requires a sufficient supply of readily biodegradable carbon, which is sometimes limited in municipal wastewater. For example, to meet the strict targets, the three largest WWTPs in Stockholm have redesigned their processes and estimated the future need for thousands of cubic meters of methanol per year as an external carbon source for biological nitrogen removal. Fossil-based methanol is commonly used as a carbon source because of its high carbon content and operational simplicity. However, it contributes significantly to the indirect CO_2 emissions at WWTPs [10]. At the same time, geopolitical instability in Europe, especially the war in Ukraine, has caused severe volatility in the price of methanol. Since the beginning of this thesis project in 2020, the price of methanol has increased from approximately 200 €/ton to around 700 €/ton in early 2025 [11]. Additionally, the use of methanol in the EU is also expected to increase as more WWTPs adopt it to comply with stricter nitrogen removal requirements.

These challenges present both environmental and economic pressures for WWTPs. In parallel, the Nordic water sector, including all the WWTPs, has also committed to achieving climate neutrality, aiming to reduce both direct and indirect emissions, as well as energy and chemical consumption [12]. The rising cost of methanol, its significant CO_2 emissions, and the increasing demand for external carbon sources make methanol an unsustainable long-term solution. A range of alternative carbon sources for denitrification has been previously proposed, including ethanol, glycerol, cellulose, industrial wastewaters, and volatile fatty acids (VFA) [13-15]. VFAs are widely recognised as the most effective carbon sources for denitrification [16, 17, 18, Paper I-III] and are also suitable for enhancing biological phosphorus removal when required [19, 20]. A potential alternative to replace the carbon source is therefore the internal production of VFAs through anaerobic fermentation of waste streams such as primary sludge, waste-activated sludge or food waste [21, 22, Paper I-IV]. However, there is a continued need to explore new substrates and optimise process configurations to improve carbon recovery and process integration. The effects of different carbon sources on the water line are partly understood but require further evaluation, particularly at larger scales, with specific attention to nutrient recirculation, fermentate handling and process stability. Additionally, redirecting organic matter from digestion towards fermentation affects important aspects of WWTPs, including biogas production, sludge handling volumes, and greenhouse gas emissions (GHG). These interconnected impacts must be considered to determine whether internal carbon source production and implementation represent a feasible and sustainable alternative under increasingly strict discharge and climate targets.

In this context, Stockholm's largest WWTP operators, Stockholm Vatten och Avfall, Käppalaförbundet, and Syvab AB, have faced growing concerns over methanol demand during the last decade. In response, the three operators, together with IVL Swedish Environmental Research Institute, initiated this project to develop alternative carbon sources. Finding sustainable alternatives to fossil-based carbon sources is crucial for reducing emissions, lowering costs, and enhancing resource recovery efficiency in wastewater treatment. Evaluating whether internally produced carbon sources can replace methanol for denitrification is therefore critical, along with understanding how such a shift affects plant operation and economics, as well as CO_2 emissions under increasingly strict discharge and climate targets.

1.1 Aim

The aim of this thesis is to assess the potential of using both novel and more explored organic waste streams, fermented and unfermented, as substitutes for fossil-based methanol as external carbon sources for denitrification in large wastewater treatment plants. The work addresses the production of alternative carbon sources and their practical application in biological nitrogen removal, while also evaluating the broader impacts on sludge handling and disposal, biogas production, operational costs, and CO_2 emissions. The goal is to support the transition toward more self-sufficient, cost-effective, and climate-friendly carbon source solutions in wastewater treatment.

The research is structured around four main objectives:

- Identifying suitable waste streams and assessing their potential to be used for volatile fatty acids (VFA) production through fermentation, with considerations of substrate composition, pre-treatment processes, and their impact on the microbial community involved in fermentation. [Paper I-II]

- Evaluating the performance and process implications of using different alternative carbon sources, including fermented and unfermented waste streams, compared to more commonly used carbon sources like methanol, acetate, and glycerol. This includes assessing nitrate removal efficiency and the broader effects on the water treatment line. [Paper I-III]
- Assessing biogas losses and process trade-offs by quantifying the effects of redirecting organic matter toward carbon source production on methane yields, sludge production and costs in full-scale WWTP configurations. [Paper I-IV]
- Investigating microbial community dynamics in denitrification with alternative carbon sources: understanding how different substrates influence microbial communities involved in nitrate reduction. [Paper III]

2. Wastewater Treatment and Nitrogen Removal

Nitrogen removal remains a fundamental and sometimes challenging aspect of wastewater treatment, particularly under stringent environmental regulations. Biological denitrification often relies on external carbon sources, with fossil-based methanol being the most commonly used. However, concerns about climate impact, costs, and sustainability have driven interest in alternative carbon sources produced from organic waste streams. This chapter provides an overview of the regulatory context, the nitrogen problem, the denitrification process, and the different external carbon sources for denitrification.

2.1 Evolution of wastewater treatment and European environmental policy

Wastewater treatment began with ancient drainage systems developed by the Mesopotamians, Greeks, and Romans [23]. Modern systems emerged in the mid-1800s as cities abandoned the failed "solution to pollution is dilution" approach, highlighted by crises like London's Thames: "The great Stink". The twentieth century introduced key advances such as the introduction of the concept of Biological Oxygen Demand (BOD) (1912), the activated sludge process (1913), and nutrient removal (late 1960s). From the 1970s, environmental regulations in the UK, USA, and Europe drove the development of today's advanced treatment systems [23, 24].

EU water policy evolved from weak discharge limits into comprehensive regulation. Milestones include the 1991 Urban Wastewater Treatment Directive and the 1999 Water Framework Directive. Since 2015, court rulings have pushed stricter compliance, especially on diffuse pollution. Regional initiatives such as the Nordic collaboration on climate mitigation in the water sector have also been introduced, aiming to reduce emissions, achieve energy neutrality, and minimise the use of chemicals [12]. In 2024, the European Union issued a revised Urban Wastewater Treatment Directive, introducing stricter discharge limits and performance targets. For large WWTPs (over 150,000 pe) in sensitive areas, the directive mandates tertiary treatment with limits of 8 mg/L total nitrogen (82.5% removal) and 0.7 mg/L total phosphorus (87.5% removal), along with targets for reduced GHG emissions, and energy neutrality by promoting energy recovery and operational efficiency [7]. In Sweden, stricter nutrient discharge limits apply to large WWTPs located on the east coast, with total nitrogen requirements below 6 mg/L to protect the Baltic Sea. These regulatory conditions have directly shaped the research presented in Papers I-IV.

Environmental policy has evolved to transform wastewater from a pollutant into a resource, aligning treatment with climate and public health goals. Yet it presents a core dilemma: reaching very low nutrient concentrations often requires increasing demands of energy and chemicals. Low phosphorus typically depends on chemical precipitation or the addition of external carbon, while nitrogen removal to low levels often requires external carbon for denitrification. The challenge is clear: how to reduce nutrients without increasing emissions or chemical use, especially those produced from fossil-based sources.

2.2 The nitrogen problem and traditional removal in urban wastewater

Nitrogen (N) is a major nutrient essential for the growth of microorganisms, plants, and animals [8]. As a main component of proteins and energy metabolism, it is widely applied as a fertiliser along with other macronutrients [25]. However, it is also released as ammonium (NH_4^+) during protein degradation in the liver (0.16 g N/g protein), and around 80% of it is excreted in the urine [26]. This makes protein catabolism the primary source of urinary nitrogen. As a result, nitrogen accumulates in urban wastewater and agricultural runoff [8, 27], making urbanisation and intensified agriculture the main drivers of the nitrogen problem [28]. The Haber-Bosch process produces most of the ammonia, of which 80% is used in fertilisers, accounting for approximately 2% of global CO_2 emissions [29, 30]. This has created a nitrogen imbalance, as excess nitrogen disrupts natural cycles, leading to eutrophication, an overload of nitrogen and phosphorus that triggers algae blooms, oxygen depletion, and severe damage to aquatic ecosystems, as seen in the Baltic Sea [3].

To address some of these challenges, several wastewater treatment plants continue to focus on removing nitrogen and phosphorus from the wastewater.

Even though nutrients can be recovered [31], recovery from urban wastewater remains limited due to diluted streams, large water volumes, and high chemical demand of the recovery processes [32]. Additionally, pathogens and emerging contaminants, such as microplastics and pharmaceuticals, have decreased the direct reuse of sludge and water in agriculture [33]. For now, most efforts still focus on their removal, while better recovery technologies are being further developed.

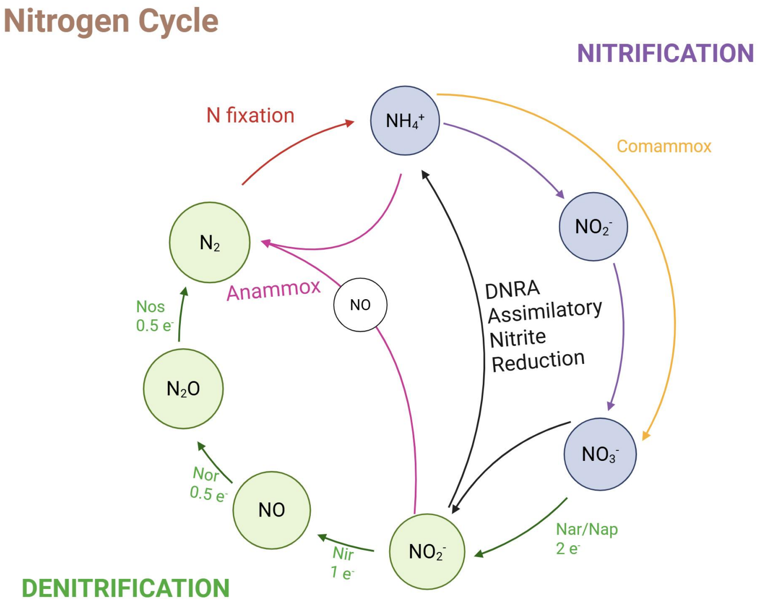

Figure 1 The nitrogen cycle

Nitrogen can exist in several oxidation states, and bacteria can alter these states depending on whether conditions are aerobic or anaerobic. These changes can go in either direction (oxidation or reduction), but nitrogen is primarily present in raw urban wastewater in its reduced form as ammonium (NH_4^+) and organic nitrogen (at a ratio 5:3) [8, 34]. The traditional methods for nitrogen removal in urban wastewater treatment rely on biological processes that transform NH_4^+ into nitrogen gas (N_2), following different pathways from the nitrogen cycle (Figure 1) [35]. Nitrogen is removed from the water phase and transferred to a) the solid phase: around 12-20% of the incoming N is used for cell growth and incorporated in the sludge, and b) the gas phase: as nitrogen gas (N_2) [32]. Efficient nitrogen removal in biological processes relies on a balanced interaction among various microbial groups [36]. Among nitrogen transformations, nitrification, denitrification, anammox, and assimilation are the most relevant for wastewater treatment. Pathway selection impacts nutrient removal efficiency, energy use, and chemical demand [37]. In recent years, alternatives such as SHARON®, ANAMMOX®, CANON®, and BABE® have targeted specific steps in the nitrogen cycle, but they require high initial ammonium levels and are sensitive to fluctuating conditions. As a result, conventional nitrification-denitrification remains the most widely applied approach due to its resilience, operational stability, and flexibility with varying loads and temperatures [8]. Nevertheless, it comes with trade-offs, including a high oxygen demand for nitrification (4.59 g O_2 per g NH_4^+-N) and often a need for external carbon to complete denitrification (4–7 g COD per g NO_3^--N). Addressing these carbon demands is a central focus of this thesis, as explored in Papers I-III.

In conventional nitrification-denitrification, ammonium is first oxidised to nitrate by *ammonia-oxidising bacteria* (AOB) and *nitrite-oxidising bacteria* (NOB), both aerobic chemoautotrophs [8]. Once ammonium is converted to nitrate (Equation a), denitrification takes place (Equation b).

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

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2^{\uparrow} \qquad b)$$

The following section discusses how carbon source selection influences this process.

2.3 The denitrification process and the need for external carbon sources

Denitrification is the biological reduction of NO_3^- or NO_2^- to N_2, serving as an alternative respiratory pathway under anoxic conditions. It occurs through two pathways: the assimilatory pathway, where nitrate is converted to NH_4^+ for cell synthesis, and the dissimilatory pathway, where nitrate is reduced stepwise to NO_2^-, nitric oxide (NO), nitrous oxide (N_2O), and finally to N_2 (Figure 1). This reduction sequence is catalysed by four key enzymes: nitrate reductase (Nar/Nap/Nas), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) (Figure 2) [8, 32, 34, 36].

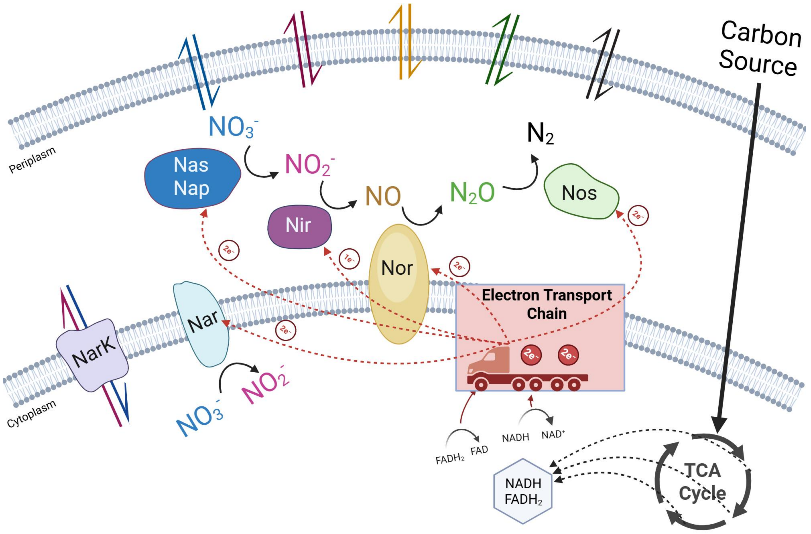

Figure 2 The denitrification process

The efficiency of nitrate reduction is based on the transfer of electrons generated through carbon metabolism by heterotrophic denitrifiers [38]. Regardless of the electron donor used, this enzyme-driven pathway remains the same, producing nicotinamide adenine dinucleotide (NADH), which acts as an electron donor, via an electron transport chain, to specific denitrification reductases (Figure 2). The efficiency of this electron transfer is proposed to be closely tied to the type and availability of the carbon source [38]. Limited or slowly metabolised carbon sources can lead to the accumulation of intermediate compounds, i.e. NO_2^-, NO and N_2O, limiting complete nitrogen reduction to N_2 [39-42]. N_2O is a potent GHG, having a global warming potential approximately 300 times that of CO_2 [43]. As a result, inefficient electron transfer caused by insufficient carbon supply or poorly metabolised substrates can significantly increase N_2O emissions from WWTPs [44]. In contrast, rapidly metabolised carbon sources, such as acetate, ethanol or methanol, provide electrons efficiently, preventing intermediate build-up [38, 42]

The carbon source used by denitrifying bacteria in wastewater treatment depends on the process configuration. In pre-denitrification zones, soluble carbon present in the wastewater is used to reduce recycled nitrate. However, the amount of nitrate reduced in these zones is often limited by the amount of available COD in the incoming water, the internal recirculation rate, the reactor volume, hydraulic and solid retention times in the anoxic tank and temperature [8]. When influent sCOD is insufficient for denitrification, post-denitrification relies on the COD from the hydrolysis of particulate matter, which is strongly influenced by temperature and other factors [8]. As a result, external carbon sources are often required to meet low N effluent levels. Tchobanoglous et al. [8] suggests that external carbon is typically needed to reach levels below 6 mg/L. Although some studies and plants have achieved this without carbon [45], design calculations indicate that heavily loaded WWTPs will likely depend on external carbon in the future.

2.4 External carbon sources for denitrification

Sufficient carbon is essential for denitrification, and the type of carbon source has a significant impact on efficiency. Denitrifying bacteria can utilise a wide range of substrates, each influencing kinetics, microbial community structure, and emissions. Since the 1990s, extensive research has explored different external carbon sources for denitrification [39, 46, 47], with studies also extending to enhanced biological phosphorus removal (EBPR) systems [48]. These include *solid-phase:* biodegradable polymers and cellulose; *gas-phase*: methane (involving different microbial pathways); and the most common, *liquid-phase*: methanol, ethanol, acetic acid, and other Volatile Fatty Acids (VFAs), simple sugars, industrial by-products, and increasingly, fermentates from waste [13, 22, 38, 44, Papers I-III].

Traditional liquid carbon sources like methanol (equation c - [44]), ethanol, and acetic acid (equation d - [8]) are effective, but have drawbacks related to fossil-based production emissions [49], rising costs, and competition from sectors like maritime transport [50]. Oxidation-reduction equation for wastewater (e) [8], for comparison:

$$5CH_3OH + 6NO_3^- \rightarrow 3N_2 + 5CO_2 + 7H_2O + 6OH^- \qquad \text{c)}$$

$$5CH_3COOH + 8NO_3^- \rightarrow 4N_2 + 10CO_2 + 6H_2O + 8OH^- \qquad \text{d)}$$

$$C_{10}H_{19}O_3N + 10NO_3^- \rightarrow 5N_2 + 10CO_2 + 3H_2O + NH_3 + 10OH^- \qquad \text{e)}$$

Among them, acetic acid and other VFAs are particularly effective [16, 38, 51], with acetic acid consistently outperforming methanol, glycerol, glucose, and other VFAs, due to its direct link to the TCA cycle and efficient electron transfer [16, 39]. In mixed carbon sources, after acetic acid is depleted, denitrifiers switch to other VFAs and then to soluble proteins and alcohols [16, 52]. The typical carbon demand is around 4 g BOD_5/g NO_3-$N_{removed}$, but it varies based on system conditions and the carbon source [8, 36]. Methanol, for example, has a lower biomass yield than other carbon sources, allowing more COD to be directed towards nitrate reduction instead of bacterial growth [8]. The total COD demand equals the COD needed for NO_3 and NO_2 reduction plus the COD for biomass growth, expressed as COD/N + Y_{OHO} (anoxic growth yield). This is determined either theoretically or via batch tests, considering nitrate's oxygen equivalent (2.86 g O_2/g NO_3-N) and nitrite's (1.71 g O_2/g NO_2-N) [8, 36].

2.4.1 Methanol

Methanol is the most used carbon source for denitrification in WWTPs, due to its effectiveness and previously low price. It has a high COD content (1,200 -1,500 g COD/L), with typical carbon consumption ratios of 4 to 5 g COD/g NO_3^--$N_{removed}$. Its low biomass growth yield (0.2 to 0.3 gVSS/gCOD) results from the growth of specialised methylotrophic bacteria. While beneficial, this may also reduce the population of denitrifiers that consume other sources (e.g. VFAs), affecting process kinetics and creating 'methanol dependency' [8]. Methanol is associated with lower N_2O emissions compared to acetate or glycerol [53-55], but has toxicity risks at high doses, alongside safety hazards and CO_2 emissions from production.

2.4.2 Ethanol

Another conventional carbon source is ethanol. With a high COD content (~1,600–1,700 g COD/L), it supports a broader range of heterotrophic bacteria compared to methanol, resulting in typically higher denitrification rates, particularly in low-temperature conditions where methanol-based processes may become limiting [14]. Ethanol leads to higher biomass production (0.4–0.5 g VSS/g COD), which can increase sludge handling requirements. Compared to methanol, ethanol is less toxic and safer to handle. It is usually bio-based (e.g., from sugar fermentation), although fossil-based production also exists, affecting its climate impact depending on the supply chain.

2.4.3 Glycerol

Glycerol, a by-product of biodiesel production, is a low-cost and highly available source. It has a COD content of ~800-1,160 g COD/L and supports a wide range of heterotrophic bacteria, resulting in higher biomass yields (0.5–0.6 g VSS/g COD) than methanol. Glycerol shows slower and less predictable kinetics, as it requires hydrolysis into simpler compounds [14, 39, Paper III]. Challenges include accumulation of process intermediates (NO_2, NO, N_2O) and variability in purity depending on its source.

2.4.4 Acetic acid and other volatile fatty acids

VFAs, primarily acetic, propionic, and butyric acids, are highly effective carbon sources for denitrification. They have a COD content ranging from 1,070 to 1,600 g COD/L [38, 56] with moderate biomass yields (0.4–0.8 g VSS/g COD) [14, Paper I-III]. VFAs are typically produced through chemical manufacturing via fossil-based petrochemical routes such as carbonylation, the Reppe, Larson, Fischer-Tropsch, and the oxo process, using methanol and ethylene as substrates [22, 57, 58], influencing CO_2 emissions [57]. However, they can also be produced from the fermentation of organic streams (Figure 3), including waste streams such as sludge, food and agricultural waste and other industrial residuals [13, 22, 38, 44, Papers I-III]. The resulting fermentate from mixed cultures contains a mixture of organic compounds, including VFAs, alcohols, and other soluble proteins, sugars, and lipids [22, 57, Papers I-III]. Their VFA profile depends on fermentation conditions [44, Paper I-III].

2.4.5 Lactic acid

Lactic acid, a simple organic acid, has been studied as an effective carbon source for denitrification due to its high solubility, fast microbial uptake, and role as an electron donor [17, 18]. It can be produced through the fermentation of carbohydrate-rich waste streams, with a COD content of approximately 1,066 g COD/L and a biomass yield of around 0.4–0.5 g VSS/g COD. Lactic acid supports denitrification rates comparable to those of acetic acid [14, 18], and is rapidly converted into acetic and propionic acids, further sustaining the process [59]. However, large-scale application is limited due to availability and production costs.

2.5 Microbiology of denitrification

Denitrification is catalysed by heterotrophic bacteria carrying the functional genes nar, nir, nor, and nos. Typical denitrifiers in WWTPs include genra *Thauera, Propionivibrio, Pseudomonas,* and *Paracoccus* [39, 60, Paper III], while fungi contribute marginally, they are often linked to higher N_2O emissions [39, 60]. The microbial community structure is highly influenced by the type of carbon source and temperature [39, 61]. Methanol promotes a specialised community dominated by methylotrophic bacteria such as *Methylotenera* and *Methyloversatilis*, characterised by unique metabolic pathways and more intricate microbial networks, but with a longer acclimation time and lower metabolic versatility than VFAs or ethanol [39, 62]. Acetate promotes stable and diverse communities dominated by *Thauera* and *Propionivibrio*, associated with high denitrification gene abundance and strong process stability [61]. In contrast, glucose selects for fast-growing taxa like *Pleomorphomonas* and *Ottowia*, resulting in comparably higher biomass yields, more soluble microbial products, and lower stability of the microbial community structure. Temperature further shifts community dynamics, with some bacteria, such as *Actinobacteria,* prevailing at 10 °C, particularly in ethanol-fed systems [60]. Most studies show that substrate type and temperature directly shape microbial community structure, denitrification efficiency, process robustness, and the potential for N_2O emissions. However, other results have shown that changing the carbon source from glycerol to a fermentate did not necessarily alter the core microbial community, even though better denitrification was measured [Paper III].

3. Production of Carbon Sources from Organic Streams

VFAs and other intermediate organic compounds, such as alcohols and reduced sugars, offer a viable alternative to the use of fossil-based methanol in wastewater treatment plants, as they promote denitrification [16, 38, 39, Papers I-III]. However, chemically produced VFAs remain expensive and generate high GHG emissions. As an alternative, microbial production of VFAs through anaerobic fermentation (AF) of organic waste streams by pure [63], and mixed cultures [22, 64, 65, 66, Papers I-II] have gained increasing attention. Such microbial processes not only support circular resource use but also reduce dependence on fossil-based chemicals, which is a central objective of this thesis. VFA production through AF is influenced by both the choice of substrate and operational conditions [22, Papers I-III]. In WWTPs, primary sludge (PS) and waste-activated sludge (WAS) are the most available substrates, but typically yield low concentrations of sCOD and VFAs [67, 68, Papers I-II]. Various pre-treatments, particularly thermal hydrolysis pre-treatment (THP), have shown potential to enhance hydrolysis and increase VFA yields from these streams, although reported outcomes are often contradictory [69-74]. This was further investigated in Paper II. Additionally, Paper II explored the novel use of digestate as a substrate for VFA production, offering a potential pathway to improve resource efficiency and close the carbon loop within the WWTP.

Furthermore, the use of other waste substrates, such as food waste (FW) and various industrial streams, offers strong potential as carbon sources for denitrification, whether used directly [75, 76; in preparation, Paper I] or after fermentation [17, 77-79, 80, Paper I]. Fermented FW can yield significantly higher concentrations of VFA and sCOD compared to sewage sludge [18, 59, 77, 81, Paper I]. However, FW is also a highly demanded resource, increasingly used for energy production, material recovery, and as fertiliser after anaerobic digestion [82]. This competition highlights the importance of using FW wisely. Co-fermentation with sludge and FW is a possible method to enhance VFA yields while reducing dependence on FW alone [83-86]. These co-fermentation proportions were investigated in Paper I.

3.1 VFA production via anaerobic fermentation

AF is an intermediate phase of anaerobic digestion (AD), one of the most widely used bioprocesses for valorising organic-rich residues into bioenergy in the form of biomethane [87]. Anaerobic digestion involves multiple microorganisms [88] operating through four sequential phases (Figure 3): hydrolysis, acidogenesis, acetogenesis, and methanogenesis [87]. However, for VFA production, the methanogenic phase must be inhibited to avoid the conversion of VFAs and intermediates into methane and CO_2. Methanogens are particularly sensitive to pH, temperature, organic loading, and HRT, which can be controlled to suppress their activity while accumulating acidogenesis products [87, 89].

During hydrolysis, complex organic material is broken down by hydrolytic microorganisms into soluble compounds [90]. This process is driven by membrane-bound or extracellular enzymes that degrade biopolymers such as carbohydrates, proteins, and lipids into monomers like sugars, peptides, amino acids, and long-chain fatty acids, increasing soluble COD [91, 92]. Hydrolysis is often the rate-limiting step for VFA production, constrained by an inherently slow solubilisation process and substrate composition, but also by pH, temperature and inhibitors [32, Paper I]. Despite its importance, enzyme activity in mixed substrates like sludge and food waste remains poorly understood, and current metabolic models offer limited results [91, 93].

In acidogenesis, hydrolysis products are taken up by bacteria and fermented into VFAs, alcohols, lactic acid, ethanol, hydrogen, and CO_2 via various metabolic pathways [59]. Acidogenic pathways are typically classified as acetate–ethanol, propionate, butyrate, mixed-acid, or lactate types, based on the dominant products [59, 90]. The amount and composition of the different products depend on the substrate, microbial community, and environmental conditions [74, 94]. As with hydrolysis, fermentation of complex substrates is not yet fully understood, particularly the microbial interactions that influence VFA profiles [93].

During acetogenesis (or anaerobic oxidation), VFAs, alcohols, lactic acid, ethanol, H_2, and CO_2 are further oxidised into acetate, H_2, CO_2 and formate [88, 95]. This step is beneficial when acetate is the target product, given its high efficiency as a carbon source for denitrification [51]. However, in processes aiming to produce a broader VFA mixture, acetogenesis can act as a competing pathway by reducing the proportion of higher VFAs such as propionate and butyrate.

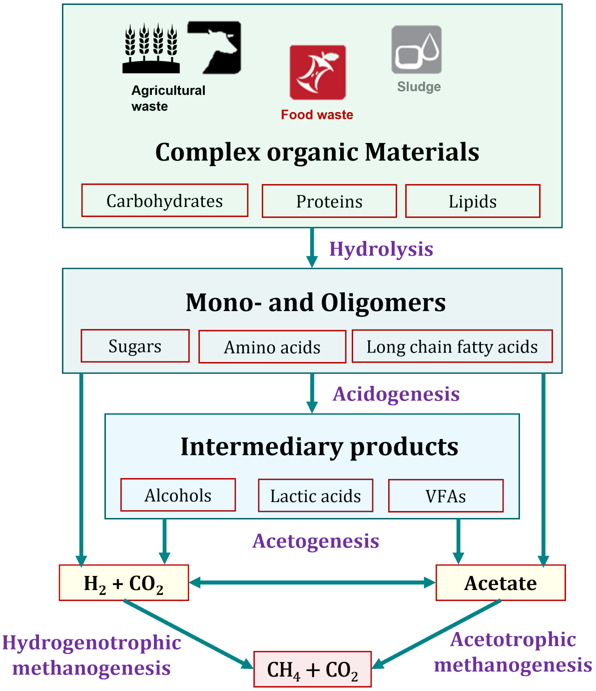

Figure 3 Anaerobic degradation of organic streams to CH_4 and CO_2

3.1.1 Fermentation Pathways of Carbohydrates, Proteins, and Lipids

After hydrolysis of carbohydrates, reduced sugars undergo mainly glycolysis to produce pyruvate and NADH [96]. Pyruvate is then metabolised into a range of non-gaseous end products, including VFAs, alcohols and ketones, depending on the fermentation pathway [97]. Amino acids undergo deamination, producing total ammonia nitrogen (TAN = NH_4^+ + NH_3) and carbon skeletons like pyruvate, acetyl-CoA, or succinate, which are further fermented into VFAs, primarily acetate [98; Paper II]. A common route is the Stickland reaction, where one amino acid is oxidised, generating VFAs, hydrogen, and TAN (increasing pH [32]), while another is reduced to VFAs using the generated hydrogen [99]. The diversity of amino acids leads to varying end products, including acetate, propionate, lactate, ethanol, CO_2, NH_3, and minor amounts of H_2 or sulphur compounds [100]. However, macromolecular interactions also influence these dynamics; for example, protein degradation can be affected by the presence of VFAs, carbohydrates, or lipids [101, 102]. Finally, lipid hydrolyses into glycerol and long-chain fatty acids (LCFAs). Glycerol follows either a reductive pathway, forming 1,3-propanediol, or an oxidative pathway, leading to pyruvate and subsequently VFAs. LCFAs are degraded to acetate and hydrogen through β-oxidation [91].

3.1.2 Parameters influencing VFA yield and composition

Temperature

Temperature strongly influences VFA production by affecting microbial activity, hydrolysis rates, and methanogenic activity [103]. Microbes can function in different temperatures [94, 104], but most fermentation processes operate under mesophilic (25–45 °C) or thermophilic (45–60 °C) conditions, as these ranges maximise hydrolytic and acidogenic activity [105]. Higher temperatures, in general, improve reaction rates and VFA production [106] but come with higher energy demand and greater sensitivity to disturbances. Mesophilic systems typically offer more diverse and stable microbial communities, giving better tolerance to substrate changes, and reduced risks for inhibition [104, 107], in both AD and AF. Additionally, fermentation at lower temperatures (psychrophilic) is also possible, but leads to slower VFA production [20]. Based on these trade-offs, a mesophilic range was selected for all the experiments presented in the papers of this thesis [Papers I–III].

Hydraulic Retention Time (HRT)

HRT is an important parameter in VFA production by balancing process stability, microbial growth, and methanogen suppression [108]. To prevent washout and process failure, HRT must exceed the doubling time of the microorganisms involved [109]. Hydrolytic and acidogenic bacteria typically grow faster than methanogens, allowing them to stay active at shorter HRTs (3 to 5 days [110]), while methanogens generally require more than 10 days to establish, facilitating their washout at shorter HRT [8]. Some studies reported methanogenic activity even at HRTs as low as 4 days, but these systems still favoured VFA accumulation over methane [111, 112]. Shorter HRTs promote the production of short-chain VFAs like acetate, propionate, and butyrate, while longer HRTs (7 days) support production toward valerate and caproate [113, 114]. Furthermore, HRT also influences the balance between lactic acid and other VFAs, with shorter HRTs (1-2 days) favouring lactic acid, while longer HRTs (3-5 days) promote its conversion into acetate, propionate, and butyrate [115]. In the experiments presented in the papers of this thesis, an HRT of 4 days was applied, which was sufficiently low to suppress methanogenic activity in most cases [Papers I-III], except for the digestate trials [Paper II].

pH

pH is one additional important parameter in VFA production, directly affecting both hydrolysis and acidogenesis and defining VFA concentration and acid profile [22, 116, Paper I, Paper II]. Methanogens typically require near-neutral pH (~6.5–8.0) for growth [87, 117, Paper II], but hydrolytic and fermentative bacteria tolerate broader pH ranges and can remain active under acidic or alkaline conditions [116, 118, Paper I]. This differential sensitivity makes pH control an effective strategy to suppress methanogenesis and promote VFA accumulation. Optimal pH for VFA production is influenced by substrate composition and microbial community structure [59, 100]. Enzymatic hydrolysis is generally most efficient at neutral pH [119], but degradation pathways differ: Carbohydrates degrade most effectively at pH 5.5–6.5 [120], proteins around pH 7 [121, 122, Paper I], and lipids are generally near neutrality, depending on the microbes present [123].

At extreme pH levels (<3 or >12), enzyme activity is significantly reduced, resulting in reduced solubilisation and low VFA production [57, 59, 90, 100]. Below the pKa of VFAs (~4.7), undissociated acids can diffuse into microbial cells, disrupt metabolism, and inhibit growth [124, 125]. pH adjustment has been shown to improve hydrolysis and fermentation: raising pH from 3.5 to 6 increased VFA yields from 15 to 28% [126]. In line with this, adjusting pH from 5.3 to 6 in Paper II, sharply increased sCOD and VFA concentrations, improving yields by up to 45%. Nevertheless, some systems show adaptation to acidic conditions. Feng et al. [127] observed efficient solubilisation under acidic conditions, and VFA production even at pH of 3.9 was observed in Paper I, reflecting the complex and substrate-dependent role of pH in acidogenic fermentation.

Pre-treatments Strategies

There has been considerable focus on increasing the hydrolysis rates to enhance the effectiveness of the AF processes. The low hydrolysis rates are related to the complex structure that the sludge could have (i.e., cell walls and extracellular polymeric substances), which becomes hard to break down [67]. The most common pre-treatments for VFA production include chemical pre-treatments, which employ alkaline solutions that improve the degradation by decreasing recalcitration [70, 71, 128, 129]. Biological pre-treatments are also commonly used and include the use of enzymes and biosurfactants [72, 130], and physical pre-treatments including thermal hydrolysis pre-treatment (THP), ultrasonic, microwave, and pressure [131]. THP, being the most widely used technology to enhance AD, employs high temperatures (> 165 °C) and pressure (> 4 bar) to disrupt the floc structure and microbial cell walls [132]. THP has also been studied as a pre-treatment to enhance VFA production from sludge, with mixed results: some report improved yields [69, 106, 133, 134, Paper II], while others noted no enhancement of VFA production due to the presence of refractory Maillard reaction by-products [135, Paper II]. In Paper II, the THP of sludge and digestate was investigated, and results revealed higher total carbon solubilisation compared to untreated materials after fermentation. However, it only resulted in higher VFA yields in the digestate trials, while the yields of sludge remained unchanged.

3.2 Methods and challenges

Experimental and analytical findings are often difficult to compare due to differences in scale, as many studies are limited to batch and semi-batch trials conducted under tightly controlled conditions. These limitations raise questions about the scalability of lab-scale results when applied to large-scale systems, especially with substrates that have significant seasonal variations, such as waste streams. In addition to scale-related factors, differences in experimental design, analytical approaches, and reporting methods across studies further complicate comparison. To address these gaps, this thesis included both bench [Paper I-II] and pilot-scale [Paper I and III] fermentation trials using a range of WWTP-relevant substrates. This section discusses methodological differences with the existing literature, as well as the analytical uncertainties encountered during laboratory work.

3.2.1 Fermentation in Bench Scale vs. Pilot Scale

Fermentation trials are typically conducted in batch, bench, and pilot-scale reactors, depending on the aim of the study. However, implications between scales must be carefully considered, as differences in operational conditions, mixing efficiency, and microbial activity can significantly affect the results. While batch and bench scales provide the control needed to test specific process parameters, pilot trials are essential in biological processes to evaluate system performance, stability, and scalability under realistic operational conditions that are difficult to replicate at the lab scale.

During this thesis, the fermentation trials to evaluate continuous performance of VFA production and denitrification in steady-state were conducted in bench-scale [Paper II] and pilot-scale [Paper I] reactors under mesophilic conditions (34 – 37 °C) with a fixed HRT of 4 days. The bench-scale experiments described in Paper II were carried out in 6.15 L reactors fed semi-continuously six days a week, while the pilot-scale system used in Paper I and III consisted of 0.9 m^3 reactors fed automatically every 30 minutes. Although both systems [Paper I-III] operated under similar HRT and temperature conditions, they differed substantially in terms of setup, control, and operational complexity. The bench-scale trials allowed for tighter control over variables such as stable temperature and sampling, and used consistent, well-characterised substrates collected weekly and stored at 4 °C. This ensured shorter experiment durations, higher reproducibility, and lower standard deviations (Figure 4 and Figure 5). In contrast, the pilot-scale setup better reflected full-scale WWTP conditions but introduced significant variability reflected in large standard deviations, mainly due to fluctuations in substrate (PS and FW) composition, mechanical issues, and difficulties maintaining stable operation. These factors affected process performance and made comparisons between trials more challenging (Figure 4 and Figure 5). These differences in results showed that bench-scale systems were effective for process optimisation but may overestimate stability, while pilot trials better captured the influence of operational variability and real-world conditions but were harder to control.

3.2.2 THP pilot-scale vs. large-scale

The CAMBI THP process consists of three chambers. Dewatered sludge (16–18% dry solids) is first homogenised and preheated in a pulper to approximately 100 °C using recovered steam. The sludge then enters reactors at 160–180 °C and 6 bars for 20 to 30 minutes, followed by rapid depressurisation to promote cell disruption and organic matter solubilisation. The system utilises a recycled steam stream from the reactor and flash tank, returning it to the pulper chamber. In contrast, the THP mini-pilot plant (model 2L/5L; Cambi, Norway) used in Paper II had only two chambers, the reactor and the flash tank. Therefore, no steam was recycled, theoretically losing some of the VFAs that could have been volatilised. The pilot was operated as closely as possible to large-scale conditions in batch mode at 165 °C and 6 bar for 30 minutes (effective contact time). However, it was not possible to feed sludge dewatered to 18% TS, as required in full-scale operation, due to a physical constraint: the inlet pipe to the bench-scale fermenter was too narrow to handle highly dewatered sludge. As a result, mixed sludge with 6% TS from Käppala WWTP was used for both the THP pilot and subsequent fermentation trials.

To address this and quantify the potential differences, sludge samples were obtained from a full-scale CAMBI THP system treating a mix of PS and WAS at HIAS (Norway) and tested in the bench-scale fermenters to compare the performance of pilot- and full-scale THP-treated sludges [Paper II]. Due to the feeding limitations, the THP-HIAS sludge was diluted to 7.5% TS before being added to the fermenters. The THP-HIAS sample resulted in the same solubilisation and lower VFA yield (357±28 g sCOD/kg VS_{in} and 136±1.1 g VFA_{COD}/kg VS_{in}) compared to the pilot-scale THP sample (349±20 g sCOD/kg VS_{in} and 208±3.3 g VFA_{COD}/kg VS_{in}), suggesting that pilot conditions achieved better fermentation performance. The pH during fermentation was slightly higher for full-scale samples (5.7) compared to the THP-pilot samples (5.3), likely due to differences in organic load and sludge alkalinity. These results suggested that despite the absence of steam recycling in the pilot system (which could theoretically lead to VFA losses through volatilisation), the pilot samples yielded more VFAs. Nonetheless, the pilot-scale results provided a valuable baseline for understanding substrate behaviour following THP. These findings underline the importance of validating whether pilot-scale THP systems can deliver comparable solubilisation and VFA yields. While previous studies have focused on sludge from full-scale THP systems [69] or lab-scale THP setups [52, 80, 106, 136], pilot-scale data remained scarce.

3.2.3 Analytical methods

All samples in Papers I–III were centrifuged and filtered (0.45 µm) before spectrophotometric analysis. VFAs (C1–C6) and lactic acid were quantified via HPLC (Agilent 1100 Series) after acidification and further filtration (0.22 µm). Total and volatile solids (TS, VS) were measured following standard methods 2540B and 2540E, respectively [137]. Several challenges were encountered with these methods, particularly regarding comparability with other studies. One issue was the omission of lactic acid in VFA measurements in some studies [Paper I], and the second was whether VS values were corrected for VFA loss during the drying process [Paper I-II]. Additionally, spectrophotometric interference from colour and particulates in THP samples caused uncertainty in data interpretation [Paper II].

Lactic acid vs. no Lactic acid measurements

Lactic acid, although not classified as a VFA, significantly affects both fermentation dynamics and the carbon profile relevant for denitrification. This was particularly evident in substrates like food waste, where lactic acid both contributed to carbon availability and to the VFA profile [Paper I, III]. Lactic acid is not always reported together with VFAs [113, 138, 139], which may contribute to differences in how solubilisation and VFA yields are interpreted, and limit the comparability with other studies.

TS and VS correction for VFA

Organic material is often measured as VS, defined as the fraction of TS that combusts at 550 °C. To calculate VS, TS are first determined by drying a well-mixed sample at 103–105 °C and weighing the remaining residue [137]. VS consists of macromolecules (carbohydrates, proteins, and lipids), VFAs, and small quantities of other organic residues (nucleic acids, organic colloids) [8]. However, VFAs volatilise during TS and VS measurement, causing underestimation if not corrected. In Papers I, II, and III, a correction factor for VFA loss (including lactic acid) was applied following Vahlberg et al. [140]. This correction, rarely reported in other studies, significantly affects yields per kg VS_{in}. Without it, process performance can be overestimated, especially for VFA-rich substrates like food waste.

Spectrometry measurements in THP sludge

Cell tests (Hach, Germany – Paper II) were used to determine sCOD, tCOD, TN, NH_4-N, and PO_4-P in filtered samples. However, THP-treated samples showed inconsistent spectrophotometric results, particularly for NH_4-N and tCOD, likely due to matrix alterations from high-temperature exposure during THP. A distinct yellow colour was observed, suggesting chemical or optical interferences not reported in the literature. In THP-treated mixed sludge, NH_4-N concentrations did not significantly increase compared to untreated samples (82 ± 49 to 109 ± 13 mg/L), despite a large increase in TN (108 ± 15 to 929 ± 281 mg/L) and confirmed protein degradation (external lab). The discrepancy was more pronounced in digestate samples, where NH_4-N unexpectedly decreased after THP (from 802 ± 73 to 395 ± 18 mg/L), contradicting the expected release of ammonium from peptide bond break. In Addition, tCOD consistently increased when measured after THP, particularly in the digestate, where it increased by 6 g COD/L (~11%).

3.3 VFA-based carbon source production from fermented waste streams

Carbon sources can be produced from the fermentation of a wide range of organic streams, including industrial wastewater streams, food waste [59, 81, 141, 142, Paper I] and waste streams generated in wastewater treatment plants, such as primary sludge [20, 85, 110, Paper I-II] and waste-activated sludge [69, 73, 143, Paper II]. Additionally, Paper II explored production from digestate. These streams differ significantly in composition, biodegradability, and microbial accessibility, which directly affect hydrolysis efficiency and fermentation outcomes. While primary sludge and food waste are often rich in readily degradable carbohydrates and lipids, waste activated sludge and digestate are more complex, containing higher fractions of recalcitrant compounds or partially stabilised material. Despite numerous studies demonstrating the potential of PS, WAS and FW [21, 22], results vary widely across the literature due to differences in pre-treatment methods, process configurations, retention times, and pH control strategies. Furthermore, VFAs are not the only important parameter when evaluating a carbon source; soluble COD and nutrient (NH_4, and PO_4) concentrations are also relevant (Section 4). Additionally, in WWPTs where biogas is produced, the impact on biogas production must also be considered (section 5).

3.3.1 Production from sewage sludge [Paper I-III]

Sewage sludge, a by-product of municipal wastewater treatment, is widely studied for its potential to produce VFA [22, 85]. However, sludge characteristics vary significantly between WWTPs, which affects yields. It typically refers to the combination of PS and WAS, which have distinct characteristics. Efficient VFA production from sludge often requires process optimisation, co-fermentation, or pre-treatment to enhance solubilisation and acidogenesis.

Primary Sludge [Paper I]

PS has been used for VFA production since the 1980s due to its high carbon content [144]; however, most of this carbon is in particulate form, with only 1–10% soluble, resulting in slow hydrolysis and the process bottleneck [67]. Traditional setups like the activated primary tank (APT) recycle PS to use the VFAs [145, 146], but these are often inadequate for modern, high-load WWTPs with strict effluent limits. Separate side-stream fermenters are more commonly used now.

PS usually contains ~30-50% carbohydrates, ~20-30% proteins, and ~10-30% lipids [136, 147, 148], although these proportions can vary considerably depending on the origin of the sludge, whether it is chemically enhanced, and on seasonal effects. In Paper I, PS had an unusually high carbohydrate content (63%), which likely contributed to the relatively high solubilisation yield (450 g COD/kg VS_{in}, Figure 4). Reported VFA yields from untreated PS range from 130 to 350 g VFA_{COD}/kg VS_{in} [110, 149, 150]. VFA yield in Paper I (236 g VFA_{COD}/kg VS_{in}) falls mid-range and towards the upper end of all evaluated substrates (Figure 5). The stabilised pH of 5.2 promoted VFA yields, but the low TS limited the final sCOD and VFA concentrations compared to other substrates evaluated (Figure 6). Propionate and acetate were the dominant VFAs (Figure 7), with propionate prevailing, in contrast to most studies where acetate is typically dominant [8, 78, 150, 151]. The comparably higher propionate share in Paper I could be attributed to the microbial community prevailing in the inoculum, as previously described [152, Paper I], as well as to other factors, such as differences in substrate composition. In the experiments presented in Paper I, an inoculum from an acid fermenter treating FW + PS was used, whereas most previous studies have used digestate from a methanogenic process.

PS remains the most used substrate for VFA production in WWTPs due to its availability and potential yields [85, 110, 146, 149, 151, Paper I]. However, the VFA profile from PS in this study differed from most previous studies, highlighting the variability in PS characteristics and the need to continue evaluating it under different conditions. Moreover, PS alone may be insufficient to meet the carbon demand of large WWTPs [150, Paper IV], and co-fermentation with more concentrated substrates or the use of pre-treatment strategies may be needed to reach sufficient amounts. Common co-substrates include WAS and FW.

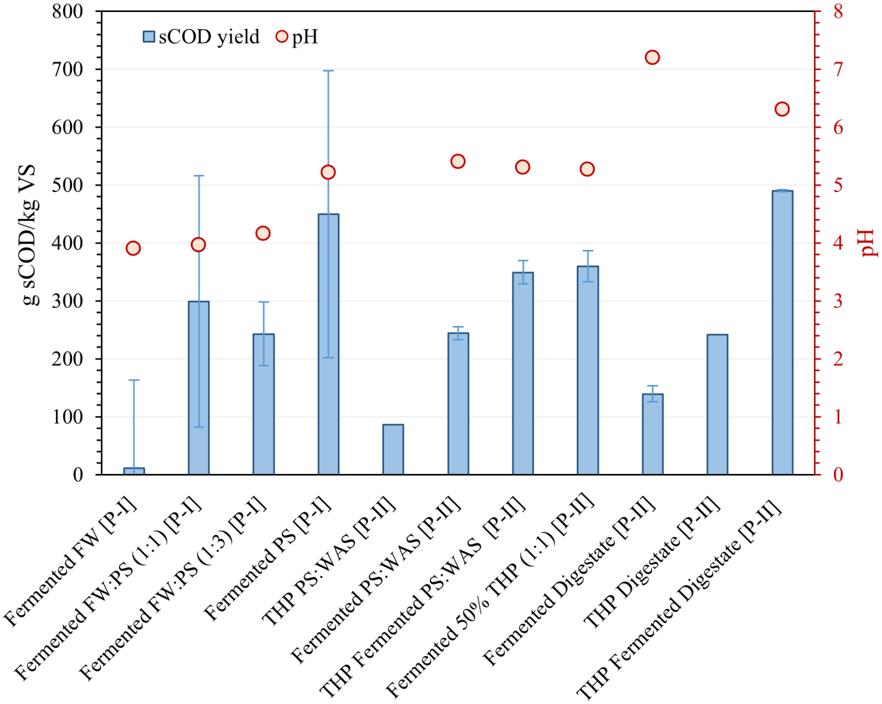

Figure 4 Soluble COD yields (g sCOD/kg VS_{in}) and pH obtained from the fermentation of different substrates. Data are from Papers I and II. Bars represent sCOD yield, and red circles indicate pH at the end of fermentation.

Waste-Activated Sludge

WAS is the main by-product of biological WWTP processes, and typically contains 40 to 60% proteins [153], but has low fermentable organic content and poor degradability, limiting its VFA and biogas yields. Its low degradability is related to the presence of microbial cells and extracellular polymeric substances (EPS), which form dense flocs with other organic and inorganic particles [154, 155]. EPS, mainly composed of proteins and carbohydrates, along with nucleic acids and humic substances, creates structured biofilms that immobilise enzymes, limiting hydrolysis and slowing degradation [156-159].

Fermentation of PS typically results in comparably higher VFA yields than WAS (11.3–25 g COD/kg VSS_{in}) due to a higher readily fermentable organic fraction [150]. VFA and sCOD concentrations from WAS fermentation alone have been reported in the range of 0.1–3 g COD/L [150, 160], which is significantly lower than values obtained from other substrates unless enhanced by alkaline [161] or physical [Paper II] pre-treatments. Fermentation of WAS alone was not investigated.

Co-fermentation of PS and WAS (with and without THP) [Paper II]

Co-fermentation of PS and WAS can enhance VFA production by balancing substrate properties and improving process stability [150, 151]. WAS, due to its high microbial biomass and extracellular polymeric substances (EPS), is less biodegradable and results in lower VFA yields than PS [151]. However, PS alone can also result in limited sCOD and VFA concentrations, which may be insufficient for some plants and can have a great impact in downstream biogas production. Thermal hydrolysis pre-treatment (THP) has been shown to improve solubilisation in mixed sludge, but its effect on VFA yields varies across studies [69, 80, 106, 134, 135, 162, Paper II].

sCOD and VFA yields vary with the PS:WAS ratio and composition of the sludge, but typically peak at 50:50, while mono-fermentation of either PS or WAS gives lower yields [150, 151]. A ratio of 65:35 (5%TS) was used in the trials of Paper II, obtaining concentrations of sCOD of 14.0 g COD/L and VFA of 10.5 g VFA_{COD}/L (Figure 6), corresponding to a sCOD yield of 244 ± 11 g COD/kg VS_{in}, lower than the one obtained by PS alone (Figure 4). Furthermore, the VFA yield obtained was 223 ± 6 g VFA_{COD}/kg VS_{in}, statistically equal to all other fermentation trials with sludge. Protein degradation was the most extensive, followed by carbohydrates, whereas lipid degradation remained limited, which contributed to the moderate fermentation performance. This configuration represented a baseline scenario against which the impacts of THP were compared.

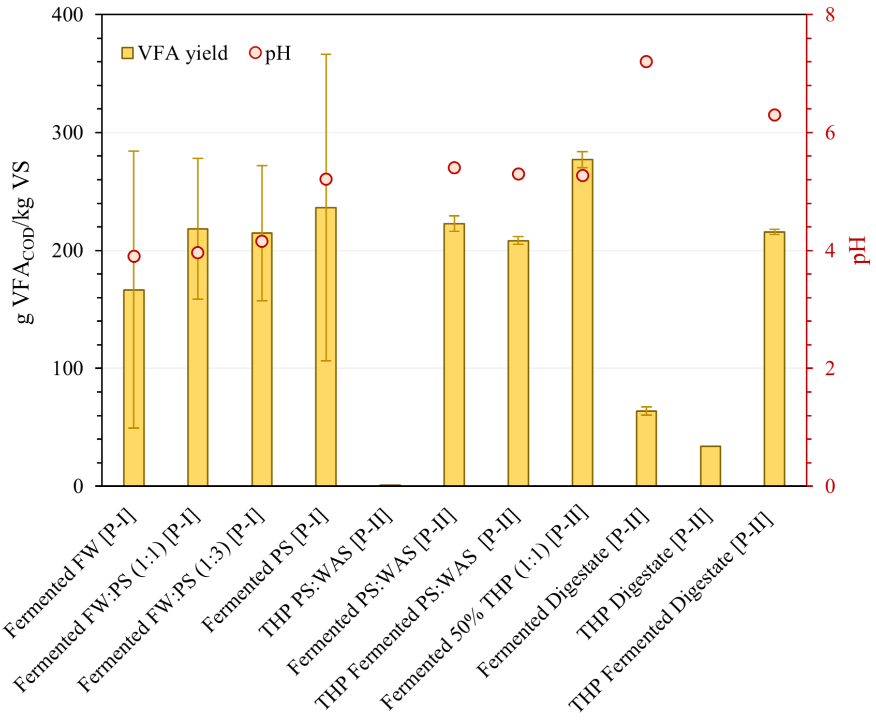

Figure 5 VFA yields (g VFA$_{COD}$/kg VS$_{in}$) and pH achieved during fermentation of different substrates. Data include results from Papers I and III. Bars represent VFA yield, and red circles indicate corresponding pH values.

In Paper II, mixed sludge (65:35) was treated using a THP mini-pilot plant, and the resulting hydrolysate was fermented in bench-scale reactors under the same conditions as the untreated mixed sludge trials. Several studies have reported improved VFA production following thermal hydrolysis of sludge [106, 134], with studies by Morgan-Sagastume et al. [69] and Zhang et al. [80] showing significantly higher VFA yields from THP-treated samples compared to untreated controls. However, the fermentation in those studies operated at higher fermentation pH (6.0-6.5) as compared to experiments in Paper II, where the pH stabilised at lower values (5.1–5.4), likely affecting acidogenic activity and limiting the conversion of solubilised organics into VFAs. Although THP led to higher solubilisation (sCOD: 19 ± 0.5 g COD/L; 31%), the VFA yield (208 ± 3 g VFA$_{COD}$/kg VS$_{in,}$ Figure 5) was slightly lower but not significantly different from the sludge sample with no pre-treatment. This suggests that THP improved solubilisation but not fermentation efficiency, aligning with other studies that proposed that thermal hydrolysis may lead to the formation of Maillard reaction products (MRPs), which can inhibit fermentation processes [135, 162]. To address this, Paper II tested a novel approach using a 50:50 mix of untreated and THP-treated sludge, which achieved higher VFA yields (Figure 5). This is likely due to the dilution of inhibitory compounds while still benefiting from the solubilisation effect of THP. This strategy could reduce the required THP volumes and enhance VFA production.

3.3.2 Production from food waste [Paper I]

FW is a significant component of organic municipal solid waste, presenting environmental and management challenges due to its high organic content and biodegradability [82]. It is widely used for anaerobic digestion (AD) in Sweden, primarily for biogas production [163], with the digestate often certified for agricultural use [164]. However, this certification is not possible when FW is co-digested with sewage sludge, limiting nutrient recovery. FW is also a valuable substrate for producing VFAs [77, Paper I], VFAs, lactic acid [59, 165], and hydrogen [166] through AF. Growing demand for FW in energy and material recovery, including fertiliser production, has increased competition for this resource [82]. To achieve optimal VFA production and quality while minimising food waste input, Paper I evaluated fermentation and co-fermentation using both pure FW [142, 167, Paper I] and FW mixed with PS [78, 141, Paper I] in different proportions.

Food Waste

Fermentation of FW in pilot-scale trials resulted in no significant increase in the soluble carbon concentration (82 ± 8 g sCOD/L) compared to untreated FW (88 ± 20 g sCOD/L) and showed an average net carbon solubilisation of only 11 ± 152 g sCOD/kg VS_{in} [Paper I]. Solubilisation was likely affected by the high initial VFA content (37 ± 9 g VFA_{COD}/L, incl. lactic acid) and by the low pH (3.9), which limited enzymatic activity as seen in other studies [81, 152] (Figure 6). Even though no solubilisation occurred, the sCOD concentration in fermented FW was the highest among all other substrates evaluated, including those with THP pre-treatment (Figure 6), highlighting the strong potential of FW.

In the trials described in Paper I, FW fermentation led to a 34% increase in tVFA, reaching 56 ± 7 compared to 37 ± 9 g VFA_{COD}/L in untreated FW. Initially dominated by lactic acid (51%), the VFA profile shifted after fermentation, with lactic acid dropping to 27% of the total and acetic and propionic acid shares increasing by 40% and 21%, respectively (Figure 7). This suggests a conversion of lactic acid to acetate and propionate, likely via the lactate pathway, as seen in reactors with high FW content [168]. Fermented FW yielded 167 ± 117 g VFA_{COD}/kg VS_{in} (Figure 5), among the lowest of different substrates, but still proposing an active fermentation at pH 3.9. Results were consistent with those reported by Jiang et al. [138], who obtained 32 g VFA_{COD}/kg VS_{in} at pH 3 and 137 g VFA_{COD}/kg VS_{in} at pH 5 under mesophilic conditions.

Co-fermentation of FW and PS

Production of lactic acid and other VFAs from FW could be more economically viable than from sludge [163]. Co-fermentation of PS and FW is a high organic content substrate mix that has been studied at both batch and semi-continuous scales [78, 169], and at pilot scale, showing promise for the production of short [Paper I] and longer-chain [113] VFA, depending on the HRT.

Paper I evaluated FW:PS ratios of 1:1 and 1:3 in a pilot-scale reactor to identify an optimal substrate mix for short-chain VFA and soluble carbon production, including lactic acid. In general, co-fermentation improved the overall solubilisation of organic matter, particularly of proteins, compared to FW alone, but was lower than that of PS alone (Figure 4), likely due to the slightly higher pH conditions at higher PS ratios. Despite these differences, higher FW shares resulted in more available COD for fermentation. For instance, sCOD concentrations reached 82, 70, and 42 g/L with 100%, 50%, and 25% FW, respectively, while PS alone only reached 5.9 g/L (Figure 6). These differences align with the large gap in substrate characteristics, FW before fermentation contained 88 g COD/L sCOD and 37 g VFA_{COD}/L, compared to just 1.4 and 0.9 g/L in PS, respectively [Paper I].

Interestingly, VFA yields (215–218 g VFA_{COD}/kg VS_{in}) were similar regardless of the FW:PS ratio and comparable to mixed sludge (PS+WAS) fermentation (223 g VFA_{COD}/kg VS_{in}) (Figure 5). However, they were slightly below the values reported by Owusu-Agyeman et al. [113] and Yang et al. [103]. This difference could be related to the comparatively lower pH conditions in the reactors in Paper I (4 instead of 5), as well as to differences in analytical approach, since neither of the cited studies included lactic acid or VS correction in their methods. Given that lactic acid may represent up to 50% of the total VFA content in food waste (Figure 7), its exclusion could lead to an overestimation of other VFAs, VS and a different representation of the carbon profile (Section 3.2.3).

In terms of VFA composition, VFA profiles from co-fermentation resembled those of fermented FW more than PS (Figure 7, Paper I), highlighting the strong influence of food waste on microbial activity and the resulting VFA distribution. Furthermore, while some studies identified acetic acid as the dominant product in food waste and primary sludge fermentation [8, 170], Paper I observed a dominance of propionic acid (excluding lactic acid), particularly under low pH conditions. This shift may be linked to the conversion of lactic acid into propionic acid, consistent with microbial fermentation pathways at low pH.

Solubilisation is clearly influenced by substrate shares and pH, while VFA yields appear less sensitive to these factors [Paper I]. Based on these results, a 25% FW share offers a practical compromise between carbon source production, energy recovery (chapter 5), and operational feasibility (chapter 5).

3.3.3 Production from digestate [Paper II]

Digestate is a stabilised sludge by-product from AD and is generally considered to have low biodegradability due to prior degradation of readily available organics. It contains methanogens and has high alkalinity, creating near-neutral pH conditions that favour rapid VFA conversion to methane rather than VFA accumulation [Paper II]. While VFA production from sludge (PS and WAS) has been extensively studied, digestate had not been explored before [Paper II]. However, it presents a unique opportunity to recover carbon without reducing biogas yields from the WWTP's AD. To overcome the limitations of using digestate as a fermentation substrate, THP can be applied to increase the solubilisation of organic matter, making it more bioavailable for acidogenic bacteria. Although some studies have explored the THP of digestate to improve dewaterability and methane production [171-173], its potential for subsequent fermentation and VFA production has, to date, only been investigated in Paper II.

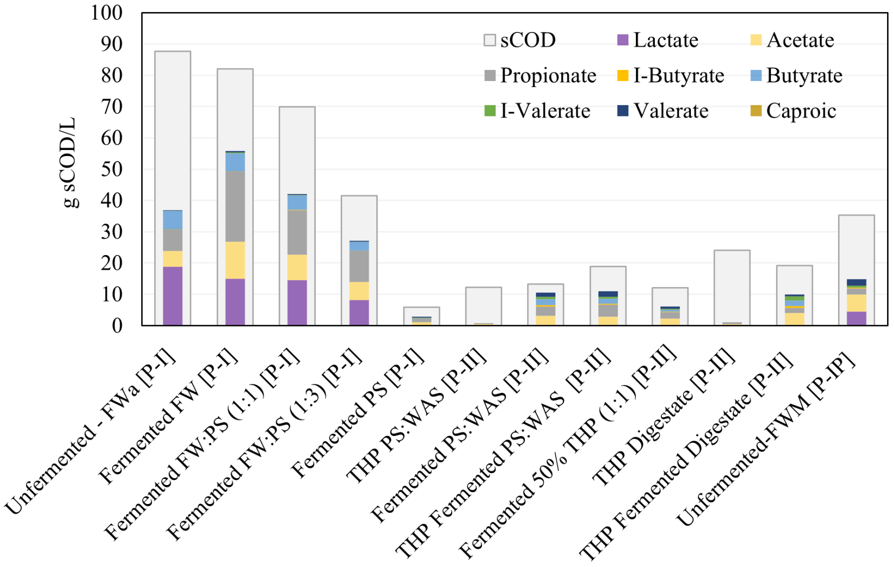

Figure 6 Concentrations of sCOD and VFAs, including lactic acid, in the carbon sources produced from waste streams in Papers I, and II and Carranza-Muñoz et al. [76; in preparation]. IP: in preparation.

In Paper II, digested sludge was evaluated as a substrate for VFA production, both with and without THP. Fermentation of digestate without THP resulted in a neutral pH value (7.2), leading to a significant loss of VFA. In contrast, fermentation of THP-digestate resulted in a higher carbon solubilisation (39.1 ± 0.1%) than THP-sludge and untreated digestate and virtually produced the same VFA yield (235 ± 2 g VFA_{COD}/kg VS_{in}, 9.9 ± 0.9 g/L) as fermentation of THP-sludge (Figure 5 and Figure 6). Protein degradation was the primary mechanism, linked to acetate production ($R^2 > 0.75$), making it the dominant VFA (Figure 7), while carbohydrate degradation was limited, potentially avoiding the formation of inhibitory Maillard products. The THP-digestate fermenter was stabilised at pH 6.3, which supported VFA accumulation but also enabled low methane production (215 mL CH_4/kg VS_{in}). Overall, the results indicated that while THP-digestate is a promising substrate for VFA production, longer trials should be conducted to investigate methane formation and nutrient content further.

3.3.4 Microbial community structure

Microbial communities in AF are highly dynamic and influenced by substrate composition, operational conditions, and environmental factors [174, 175, Paper I]. In paper I, it was evident that substrate played an important role: food waste typically favoured members within phylum Firmicutes, including *Lactobacillaceae* and *Clostridia*, while sewage sludge selected for more Proteobacteria and Bacteroidota, including orders like *Burkholderiales* [176, Paper I]. This indicates a microbial shift in response to substrate variation. The assembly of the microbial community during mixed culture fermentation of FW and PS depends on various parameters, such as the chemical and microbial composition of the substrate [177], as well as the microbial composition of the selected inoculum [152], and the prevailing pH [178]. For example, low pH selected for fast-growing acidogens like *Lactobacillus*, promoting lactic acid and acetate production, while more neutral pH favour more diverse fermentative communities capable of producing a broader VFA spectrum [Paper I].

In Paper I, clear microbial shifts were observed, driven by changes in substrate. However, despite advances in species-level identification using large databases and full-length 16S sequencing [93], functional redundancy and the presence of feed-derived taxa complicate predicting VFA yields based solely on community composition [179-181]. Still, the strong link between substrate, operational conditions, and dominant fermentative guilds remains the main driver of VFA production performance.

3.4 Carbon source production from THP waste streams [Paper II]

THP is widely used on an industrial scale to increase biogas yield and improve sludge dewaterability. At smaller sales, the THP concentrate hydrolysate has been evaluated as a potential carbon source for denitrification [52, 182, Paper II]. As THP is used to enhance the hydrolysis in AF, it increases the solubilisation of organic matter [132, Paper II], but with low VFA to sCOD ratios (Figure 6). The small amounts of VFAs present likely originate from amino acid breakdown under high temperature conditions and sudden changes in pressure [Paper II], rather than from microbial fermentation. Despite this, THP hydrolysates still contain substantial amounts of soluble organic carbon that could be further degraded or directly utilised by denitrifying bacteria. For this reason, unfermented THP-treated samples were evaluated as carbon sources in Paper II.

THP of mixed sludge (PS+WAS) in Paper II resulted in an organic matter solubilisation of 15%, which falls within the previously reported range of 8% to 25%, depending on the substrate composition [133, 135]. The resulting sCOD concentration was 12 g/L. The proportion of WAS plays an important role, as THP applied to WAS alone generally results in higher solubilisation degrees, up to 49%, compared to the mixtures with PS [69, 183]. Although often considered a low-reactivity substrate, digestate achieved an sCOD concentration of 24 g/L, and a solubilisation degree of approximately 40% through THP [Paper II], similarly to Yang et al. [184]. These differences in carbon solubilisation are closely tied to macromolecular composition. THP is especially effective at solubilising proteins and carbohydrates, while lipids are largely unaffected [136, 185]. As such, substrates rich in proteins and carbohydrates, such as WAS and digestate, respond more favourably to THP. On a side note, special attention should be given to the nutrient content, particularly the formation of NH_4-N from the breakdown of amino acids, which can influence their application in denitrification.

3.5 Carbon source from direct use of waste streams [76; in preparation, Paper I]

Using some streams directly without prior treatment simplifies application and improves economic feasibility. In this context, “direct use” refers to streams that are applied after being received and properly handled at the WWTP, including any legally required steps such as hygienisation for food waste, but without additional pre-treatment or fermentation.

The direct use of waste streams from production processes in the agricultural products processing and food industries has proven to be a viable alternative carbon source for denitrification [9, 13, 46, 186, 187]. However, since each waste stream has a unique composition, it must be assessed within the specific treatment context to determine its suitability, potential toxicity, and the presence of heavy metals or other contaminants that may impact the wastewater treatment process or effluent quality. In this thesis, three such streams were used directly as carbon sources for denitrification, without any pre-treatment: Food Waste I (U–FWa), Food Waste II (U–FWb), and Food Waste Mix (U–FWM).

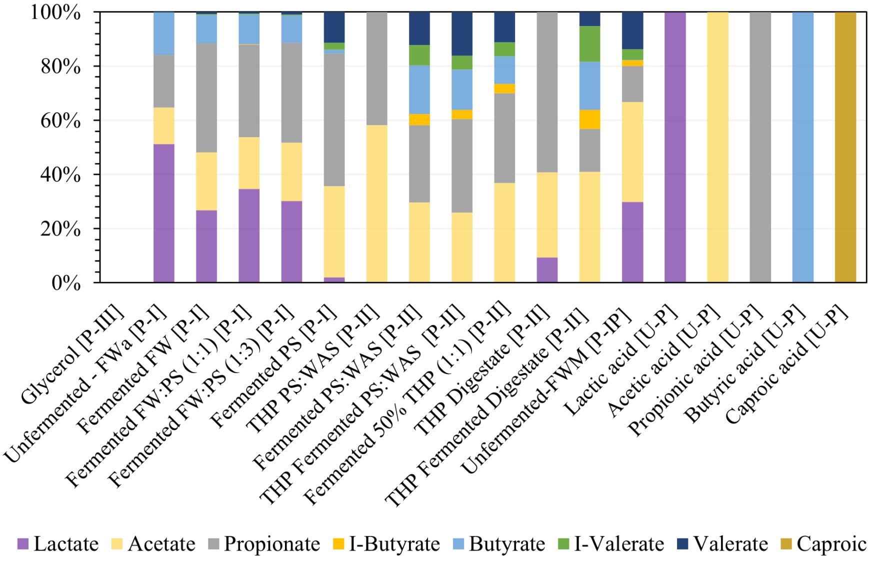

Figure 7 VFA composition and lactic acid shares in all carbon sources used for denitrification. Data are from Papers I–III, and Carranza-Muñoz et al. [76; in preparation]. Bars represent the relative share (%) of each VFA compound. U-P: unpublished data, IP: in preparation.

U–FWa and U–FWb were both hygienised food waste streams obtained from the Södertörn biogas plant operated by Biokraft AB (Huddinge, Sweden). U–FWa consisted of complete food waste samples that were delivered weekly to the research centre and were further sieved to 0.6 mm in our laboratory [Paper I], while U–FWb was the reject water stream from Södertörn plant’s centrifuges (Unpublished). The third stream, U–FWM, was collected at Himmerfjärdsverket WWTP (Södertälje, Sweden) and comprised a mixture of waste streams that the plant receives and uses for biogas production. The main components of U–FWM were grease from fat traps (54%), fruit slurry (18%), food waste (19%), alcohol and soft drinks (6%), and residues from breweries (3%) [76; in preparation].

The main challenge of using waste streams is the variability of the substrate [188, Paper I, Paper III]. This is particularly true for untreated streams, as there is no equalisation zone to buffer the differences. For example, during the pilot trials performed [76; in preparation], U–FWM had an average sCOD concentration of 35 g sCOD/L, with a standard deviation of 27%. In comparison, during the pilot trials in Paper I, U–FWa had an average sCOD concentration of 88 g sCOD/L, with a variability of 22%. The total VFA concentrations, including lactic acid, varied by 15% for U–FWM and 25% for U–FWa. Monitoring with untreated streams should be more rigorous than with conventional or sludge-derived carbon sources to ensure accurate characterisation and appropriate dosing. Further discussion on toxic substances in untreated substrates is presented in Section 4.6.

4. Application of Fermented and Untreated Waste Streams in Denitrification

Denitrifying bacteria can utilise a broad range of carbon sources containing VFAs and other biodegradable soluble organics as electron donors [39, 51]. However, the selection of an external carbon source is a critical operational decision in wastewater treatment plants, as it directly affects nitrogen removal efficiency, sludge production, operating costs, carbon footprint and effluent quality. Because different carbon sources vary in denitrification kinetics, carbon utilisation efficiency, and by-product formation [189], their performance, dosing strategies, and potential downstream impacts must be carefully assessed [13]. Conventional carbon sources, such as methanol, remain widely used due to their reliable performance, stable supply, and simple dosing. In contrast, waste-based alternatives face challenges such as substrate variability, fluctuating VFA yields, operational complexity, and competition with biogas production for organic matter [21, 22, 67, 77]. Even though waste-derived carbon sources offer clear benefits in terms of circularity and resource recovery, their large-scale implementation remains limited [190].

This chapter presents an evaluation of fermented and untreated liquid carbon sources derived from food waste, sewage sludge, and digestate, including samples subjected to THP. These sources were assessed through a combination of laboratory-scale batch tests [76; in preparation, Papers I–III] and pilot-scale applications [76; in preparation, Paper III]. Batch experiments focused on maximum denitrification rates, anoxic yields, and carbon consumption ratios, while pilot-scale trials investigated these parameters under real and dynamic operational conditions.

4.1 Suitability of external carbon sources

Denitrifying bacteria rely on readily biodegradable carbon, especially VFAs, as electron donors to drive the reduction of nitrate to nitrogen gas under anoxic conditions [8, 51]. All tested fermentates and untreated waste-derived carbon sources tested in the experiments presented in this thesis supported denitrification. With few exceptions, the denitrification rates observed were not significantly different from those achieved with acetic acid when tested with the same inoculum, and most sources outperformed methanol (Figure 10). In contrast, glycerol and the hydrolysates from unfermented THP-treated samples exhibited lower rates. Although these THP liquids had high sCOD concentrations (Figure 6), their low VFA/sCOD ratios indicated that the carbon was largely composed of less readily biodegradable compounds, such as soluble proteins, reduced sugars, and humic substances [191, Paper II]. While these findings suggest potential constraints for THP liquids, other studies reported favourable outcomes using THP hydrolysates compared to conventional carbon sources such as methanol or acetate [182]. Therefore, the source-specific composition and degradability of alternative carbon streams must be carefully assessed to determine their applicability.

4.2 Methods and challenges

Comparing experimental results in denitrification is often complicated by differences in scale and microbial community composition. Batch denitrification tests represent the maximum biological denitrification rate, while pilot-scale and full-scale systems are subject to operational constraints such as reactor design, flow and load variability, and resource competition. This highlights the importance of evaluating both approaches [192, 193, Paper III]. Additionally, denitrification performance is strongly influenced by the degree of microbial adaptation to each carbon source, as well as variability in the microbial composition of the activated sludge used as inoculum [44, 194, Paper I-III]. These factors can significantly affect denitrification outcomes, particularly when evaluating alternative carbon sources. In this study, both batch and pilot-scale tests were employed [76; in preparation, Papers I–III], each with inherent strengths and limitations that influence the interpretation of the results.

4.2.1 Batch vs. Pilot Scale Tests

Batch denitrification tests were conducted using all carbon sources, as detailed in Papers I-III. These tests were conducted using the liquid fraction of the fermentate after separation, with activated sludge from different WWTPs or MBBR K5 carriers as inoculum. Batch tests using activated sludge were performed in 5-L reactors, following the methodology described by van Loosdrecht et al. [36]. This setup allowed for controlled addition of nitrate (25–30 mg/L), excess carbon source (150–200 mg/L), and pH adjustments to determine maximum denitrification rates and substrate consumption ratios.

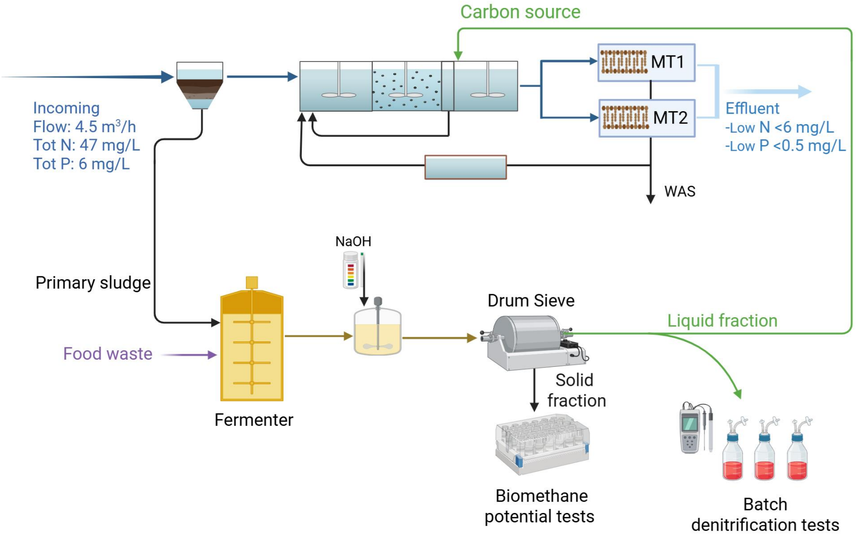

Figure 8 Schematic overview of the pilot-scale trials conducted at Henriksdal WWTP using fermented mixed FW and PS (1:3 %v/v) as a carbon source [Paper III].

However, batch test conditions do not fully replicate real-world treatment plant operations. To address this, pilot-scale trials were also conducted [76; in preparation, Paper III] using more realistic process configurations. For example, Paper III describes a membrane bioreactor (MBR) pilot simulating the future Henriksdal WWTP, which included pre- and post-denitrification zones and membrane tanks, operated at an average dynamic flow of 4.5 m^3/h (Figure 8). Carbon was dosed in the post-denitrification zone. Additionally, Carranza-Muñoz et al. [76; in preparation], includes another MBR pilot representative of the Himmerfjärdsverket WWTP, featuring an activated sludge system with a three-step feed and post-denitrification zone, treating 12 m^3/h. In this case, the carbon source was added in the last pre-denitrification zone of the system (Figure 9). Unlike batch tests, no excess nitrate was added in these pilots. Denitrification performance in pilot trials was influenced by several factors, such as resource competition (as other bacteria consume carbon), reactor design, and lower concentrations. As a result, pilot-scale denitrification rates were considerably lower than those observed in batch tests, which is a common outcome in denitrification process studies [195]. Therefore, combining both approaches provided complementary insight: batch tests reveal maximum biological potential, while pilot-scale systems capture real operational outcomes.

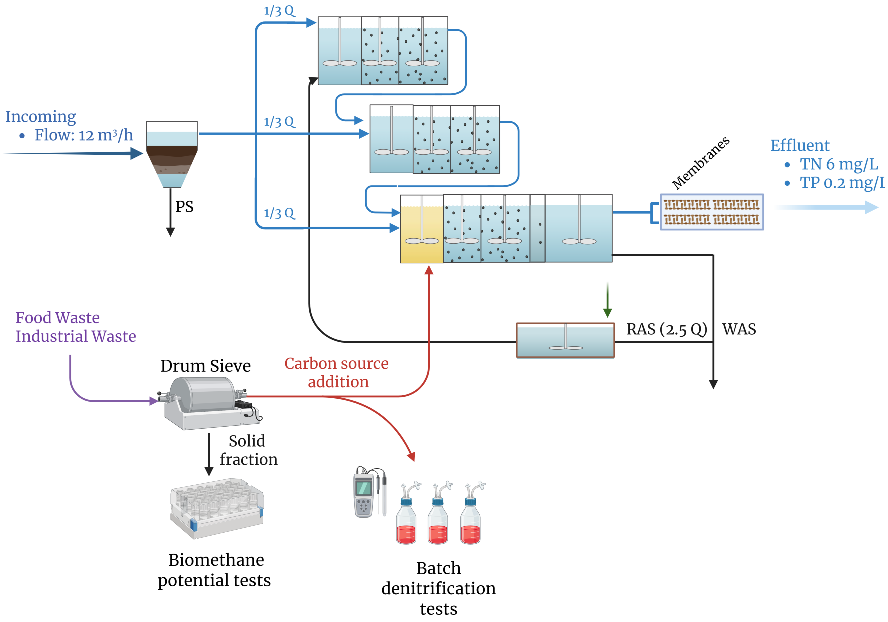

Figure 9 Schematic overview of the pilot-scale trials conducted at Himmerfjärdsverket WWTP using unfermented food waste mix (U-FWM) as a carbon source [76, in preparation].

4.2.2 Variability in activated sludge samples

Factors such as microbial community structure, the availability and type of biodegradable COD, and temperature during the anoxic phase all significantly influence denitrification performance [8]. These factors are, in turn, shaped by the characteristics of the influent and the operational history of the treatment system, which determine the composition and activity of the microbial population in activated sludge [196]. As a result, denitrification potential and carbon source utilisation can vary considerably between systems, even under similar external conditions. This variability poses a challenge when comparing the effectiveness of different carbon sources across studies, particularly in batch experiments using different sludge inocula. In this thesis, such variability was also present, as different activated sludge inocula were used depending on availability and timing. To address this, reference compounds such as methanol, glycerol, or acetic acid are often included to provide a baseline for comparison and help contextualise performance under varying microbial conditions [16, 190, Paper I-III].

In Papers I-III, acetic acid or methanol was used as the reference, and the tested carbon sources were compared against them under different inoculum conditions (Figure 10). It is of importance to consider inoculum variability when interpreting denitrification performance and assessing the feasibility of waste-derived carbon sources, particularly when comparing results from batch denitrification tests.

4.3 Denitrification – Batch tests

Denitrification rates obtained from batch tests using all carbon sources evaluated in Papers I–III and in Carranza-Muñoz et al. [76; in preparation] are presented in Figure 10. Each symbol in the figure corresponds to the activated sludge inoculum used, and comparisons were made within the same inoculum to control for microbial variability. This allowed each carbon source to be evaluated relative to a consistent reference.

In Papers I-III, the alternative carbon sources in all trials, excluding those produced from unfermented THP samples of sludge and digestate, achieved denitrification rates equal to or exceeding those of the conventional reference compounds, consistent with previous studies [17, 80, 167, 197]. Among the reference compounds, acetic acid consistently delivered the highest rates, followed by propionic and lactic acids, while methanol and glycerol showed comparably lower performance. These results align with the literature, where acetic acid is recognised for its efficient electron transfer and energy production in denitrifying pathways due to its simple molecular structure [38, 39, 51]. Interestingly, despite containing complex mixtures of organics, the fermentates performed comparably to or better than pure acetic acid in all cases [17, 80, 167, 197], suggesting that denitrifiers efficiently utilised the available VFAs and other soluble COD components [39] (Figure 10). This is consistent with the known substrate preference hierarchy, where acetic acid is consumed first, followed by other VFAs, soluble proteins, and eventually more complex compounds [16, 52]. Additionally, as shown in Figure 7 and Figure 10, denitrification rates for unfermented mixed FW (U-FWM) and fermented FW often fell within or exceeded the reference range, despite containing lower concentrations of acetic acid. This highlights the ability of the microbial community to effectively utilise other fermented FW derivatives, including lactic acid and other VFAs [17, 80, 167, 195].

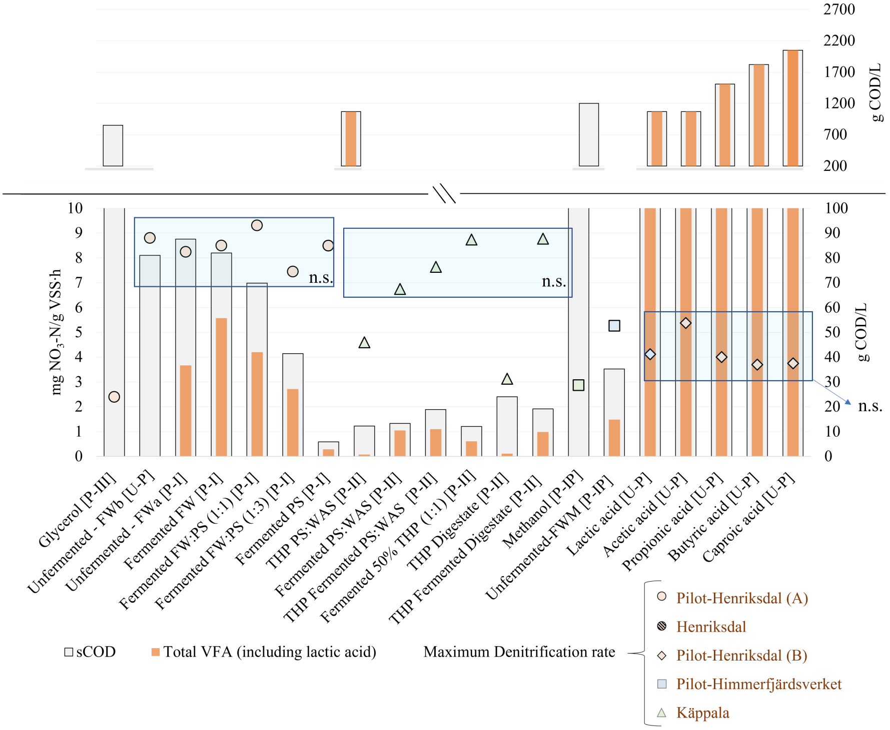

Figure 10 Denitrification rates from batch tests using various carbon sources evaluated throughout the project. The bars correspond to concentrations of sCOD (grey), lactic acid, and individual VFAs (orange). Symbols indicate the activated sludge inoculum used, with different shapes representing samples from different wastewater treatment plants.

Within each inoculum, statistically significant differences were generally absent (noted as "n.s. - not significant" in Figure 10 where applicable), except for unfermented THP samples and glycerol, which consistently underperformed compared to all other carbon sources tried. The THP-derived hydrolysates [Paper II] also showed limited denitrification rates, with values below 5 mg NO_3^--N/g VSS·h (Figure 10). This may be attributed to the lower VFA share of sCOD (Figure 6) and presence of more recalcitrant organics, consistent with previous findings showing that thermal hydrolysis liquids perform less effectively than fermentation liquids as carbon sources for denitrification [52, 198]. However, this contrasts with the results reported by [182], who found higher denitrification rates with sludge THP hydrolysates than with acetic acid.

Additionally, results from Kim et al. [75] and Carranza-Muñoz et al. [76; in preparation] revealed that unfermented substrates, in this case FW, can deliver comparable performance under batch conditions. For example, unfermented food waste mix (U-FWM) achieved a denitrification rate of 5.3 mg NO_3^--N/g VSS·h with Himmerfjärdsverket sludge, nearly double that of methanol (2.8 mg NO_3^--N/g VSS·h). This reinforces the flexibility of waste-derived substrates in supporting denitrification, provided that operational variables are controlled. However, as will be discussed in subsequent sections, nutrient content and carbon-to-nutrient ratios must also be considered when assessing overall suitability for full-scale application.

Despite the lack of statistical significance, results also suggested a trend between the VFA/sCOD ratio and denitrification rate ($R^2 = 0.702$), which supports the idea that carbon sources with simpler molecular structures, such as VFAs, are preferred by heterotrophic denitrifiers [16, 38]. Furthermore, the anoxic biomass yield (Y_{OHO}) results obtained in this study [Papers I-III] aligned with literature values [8, 16, 80] and reflected the observed variability between carbon sources. Conventional compounds, such as methanol (0.2–0.3), sodium acetate (0.5), and glycerol (0.34–0.4), showed relatively low biomass yields, whereas sludge- and food-waste-derived fermentates consistently presented higher yields (Y_{OHO} above 0.6). This is an important finding, as it helps explain the higher sludge handling volumes observed in WWTPs when switching to fermentate-based carbon sources, a point further discussed in the following sections.

These findings demonstrate that diverse, low-emission organic wastes can achieve denitrification rates comparable to pure compounds like acetic acid, supporting their use as sustainable, cost-effective carbon sources in WWTPs. Additionally, all fermentates achieved similar denitrification performance, regardless of substrate type or pre-treatment.

4.4 Denitrification – Pilot trials

To evaluate the real-world performance of waste-derived carbon sources, two pilot-scale MBR systems were operated under conditions representative of full-scale WWTPs. These trials, conducted at Henriksdal and Himmerfjärdsverket, evaluated both fermented and unfermented waste-based carbon sources in comparison to conventional ones. Important metrics such as denitrification rate, carbon consumption, and microbial adaptability were monitored to determine feasibility and performance under dynamic operational conditions. Although pilot-scale denitrification trials have been reported previously [192], studies of this scale incorporating an integrated fermenter with an automated solid–liquid separation system remained very limited. Only a few other complete pilot studies using alternative configurations with real fermentates have been published [199]. Moreover, the specific configuration applied in this work, co-fermentation of PS and FW combined with dosing in a pilot-scale MBR, had not been previously studied at a pilot scale. While unfermented food waste had been tested in full-scale systems [75], this had not been done in MBR configurations, and potential challenges such as fat-related fouling and operational stability were still largely unexplored.

4.4.1 MBR Henriksdal pilot [Paper III]

Glycerol, which was used in this pilot for 2 years prior to the trials, served as the reference carbon source in these trials. Glycerol had an average soluble carbon concentration of 850 g sCOD/L, with no detectable ammonium or phosphate content. Methanol is planned to be used at Henriksdal WWTP upon completion of the plant reconstruction. The alternative tested was a fermented carbon source, produced in a 0.9 m^3 mesophilic pilot fermenter fed with 75% primary sludge and 25% food waste (denoted as Fermented FW: PS 1:3 in Figures 4, 5, 6, 7 and 10). The resulting fermentate was rich in propionate, lactate, and acetate (Figure 7) and was separated via drum sieving after pH adjustment before being dosed (Figure 8).

The specific denitrification rate in the post-denitrification zone was approximately 30% higher when using the fermentate (1.3±0.6 mg NO_3^--N/g VSS·h) compared to glycerol (0.9±0.1 mg NO_3^--N/g VSS·h), after adjusting for temperature. Moreover, the fermentate achieved the same effluent nitrate target (3 mg NO_3^--N/L) while requiring 50% less carbon (COD) per gram of nitrate removed. The lower rates observed in the pilot compared to batch tests (Figure 10) likely reflected the partial and dynamic utilisation of the post-denitrification tank volume, which responds to fluctuations in influent nitrate load. During periods of higher nitrate loading, a larger portion of the reactor volume is engaged, potentially enabling higher denitrification rates closer to batch-scale values.

Notably, the transition from glycerol to fermentate did not require microbial acclimation. This was confirmed by 16S rRNA analysis, which showed no relevant shift in the microbial community and rapid adaptation to the new substrate, achieving higher denitrification efficiency without requiring an extended adaptation phase. This smooth transition may be partly explained by previous glycerol exposure, as glycerol-acclimated sludge is known to express active fatty acid biosynthesis and degradation pathways, supporting efficient uptake of VFAs [62]. These findings support the practical viability of fermentate as a high-performing carbon source and suggest that WWTPs operating on glycerol could transition relatively easily to more efficient VFA-rich alternatives.

4.4.2 MBR Himmerfjärdsverket pilot [76; in preparation]

In the Himmerfjärdsverket pilot, methanol, long established as the standard carbon source at this facility and currently used at full scale, was replaced by unfermented food waste mix (U-FWM) to evaluate its performance in a three step-feed pre-denitrification system (Figure 9). Methanol had been the primary carbon source at the Himmerfjärdsverket pilot for years and served as a benchmark for operational stability and performance.

During the pilot trials, methanol exhibited a 33% higher carbon consumption ratio, requiring 5.02 g COD/g NO_3^--$N_{removed}$, compared to U-FWM, which required only 3.8 g COD/g NO_3^--$N_{removed}$. This is consistent with the trends observed in batch denitrification tests. Unlike glycerol or fermentate, methanol degradation relies on specialised enzymes produced by methylotrophic bacteria, which are adapted exclusively to methanol metabolism and do not utilise other carbon sources during denitrification [62]. As these bacteria become dominant, they may outcompete other functional groups in the system, reducing the capacity to utilise influent sCOD in the pre-denitrification and potentially limiting overall nitrogen removal efficiency. This is particularly important in step-feed systems like the one used in Himmerfjärdsverket (Figure 9), where influent is introduced at multiple pre-denitrification zones to boost the use of influent COD. Pilot results showed a gradual decline in effluent nitrate concentrations following the introduction of U-FWM. However, inconsistent dosing during the first week, due to operational constraints, made it difficult to determine whether the initially elevated effluent nitrate levels reflected a true microbial adaptation lag or simply insufficient substrate availability. As dosing stabilised, a clear reduction in NO_3^--N concentrations was observed, indicating that U-FWM can effectively support denitrification when dosed in the required volumes. Nevertheless, continuous and prolonged operation is required to fully evaluate the long-term performance and reliability of unfermented substrates as a methanol substitute, especially due to changes in the substrate over time.

4.5 Microbial dynamics

To evaluate how carbon source shifts influence microbial communities, 16S rRNA sequencing was performed on samples from the Henriksdal MBR pilot during periods of glycerol and fermentate use [Paper III]. Additional DNA samples were collected during the transition from methanol to unfermented food waste mix (U-FWM) in the Himmerfjärdsverket pilot , although results were not yet available at the time of writing and will be included in a future publication [76; in preparation].

As expected in activated sludge systems, the bacterial community was diverse and shaped by operational conditions, as seen in previous studies [196]. Dominant families in this study included Mycobacteriaceae, Sapospiraceae, Burkholderiaceae, Rhodocyclaceae, and Chitinophagaceae, among others (Figure 11). Known denitrifying genera such as *Zoogloea*, *Hyphomicrobium*, and *Paracoccus* were detected but in low abundance. The shift from glycerol to fermentate resulted in minor changes in community composition. Some families, such as Mycobacteriaceae and Saprospiraceae, increased slightly with fermentate, while genra *Zoogloea* and *Hyphomicrobium* were more associated with glycerol (Figure 6). A notable difference was the higher relative abundance of Candidatus Saccharibacteria UBA5946 during glycerol use, a taxon previously linked to partial denitrification and nitrite accumulation [200]. This finding is consistent with observations from batch tests [Paper III] and may also suggest the accumulation of other denitrification intermediates, such as N_2O.

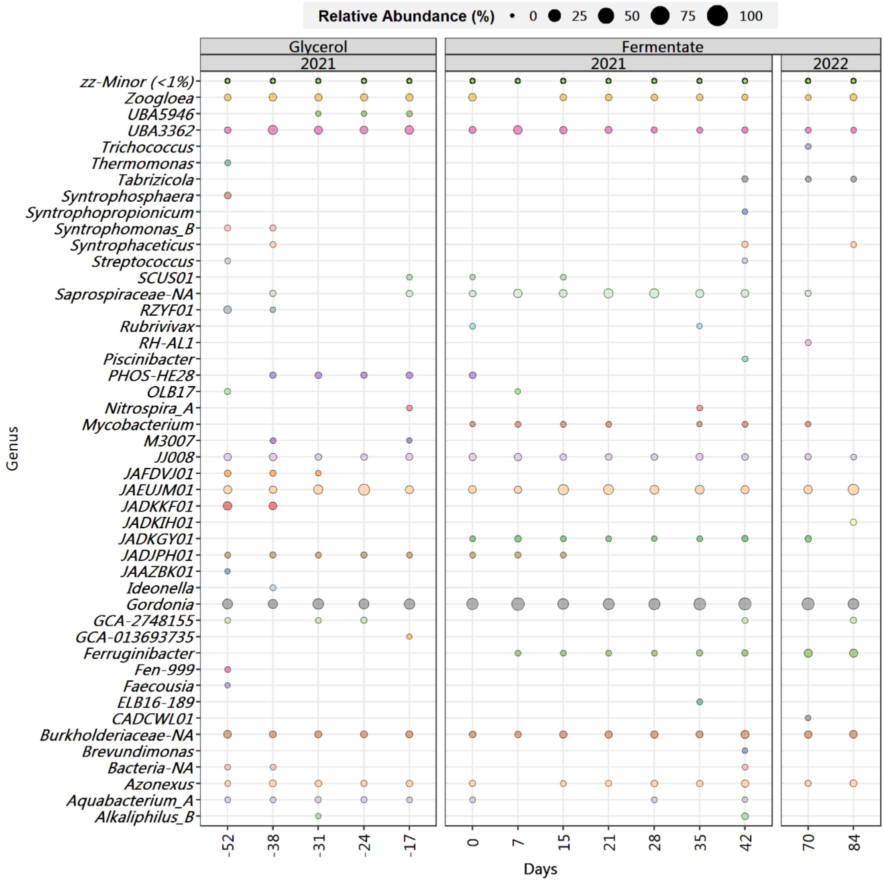

Figure 11 Microbial community profile (in%) at the genus level in the activated sludge samples during dosing of glycerol (left) and fermentate (right), over the experimental period.

Although previous studies have shown that carbon source type can influence microbial community structure [39, 194, 201], no major shifts were observed in this study (Figure 11). This could be explained by two factors: glycerol, despite slower kinetics, supports the utilisation of a broad range of carbon sources [39]; and the fermentates complex composition likely prevented the selective enrichment typically seen with pure substrates. Most studies investigating microbial shifts in response to carbon source changes have been conducted at lab scale or in batch systems [39, 61]. While transitions between methanol and ethanol have been studied before in pilot scale [202], the evaluation presented in Paper III is one of the first pilot-scale studies to examine the microbial response to a shift from a pure substrate (glycerol) to a complex, waste-derived fermentate in an MBR system.

Overall, improved denitrification appeared driven more by carbon utilisation than by changes in microbial composition. The complex organic profile of the fermentate likely supported broader microbial activity without promoting selective enrichment, in contrast to the more targeted effects of pure substrates [39, 194, 201]. Glycerol, despite lower kinetics, offers a broader utilisation range, which may explain the relatively stable community structure observed during its use [Paper III].

4.6 Operational considerations for full-scale use

Batch and pilot-scale tests confirmed the technical feasibility of using waste-derived carbon sources for denitrification, but full-scale implementation requires considering more than just nitrate removal. Factors such as nutrient content, GHG emissions, heavy metals, and downstream process effects also play a critical role. This section summarises the results from Papers I-III on these aspects, with a focus on the practical implications of using fermented and unfermented organic waste at WWTPs.

4.6.1 Nutrient composition of the carbon source – Ammonium, phosphate, and organic compounds

Fermentates from organic streams often contain higher concentrations of nitrogen and phosphorus compared to conventional carbon sources, as these nutrients are both present in the substrates and released during fermentation [203, 204]. Ammonium is released from the degradation of amino acids [205], and phosphate has been shown to be released from fibre-bound complexes and disrupted cell membranes [206, 207].

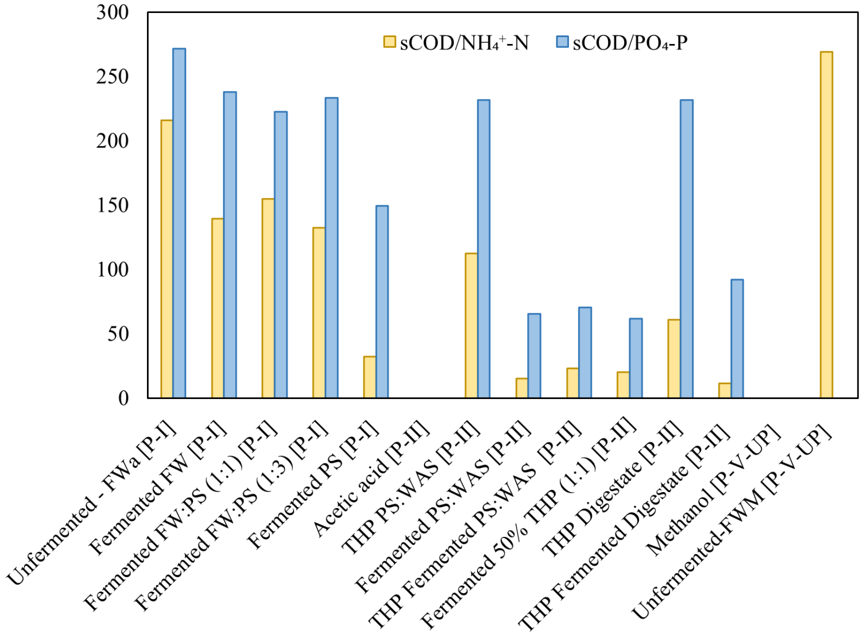

Figure 12 Ratios of soluble COD to ammonium (sCOD/NH_4^+-N, yellow) and soluble COD to phosphate (sCOD/PO_4-P, blue) in carbon sources produced from food waste streams, as well as unfermented substrates and reference chemicals. Bars represent nutrient ratios. Data are based on results from Papers I, II, [76; in preparation].

In Paper I, fermentates from FW and mixed FW:PS (1:1 and 1:3) showed favourable sCOD/NH_4^+-N ratios above 130, while fermented PS alone dropped below 50. All FW-based fermentates and FW had sCOD/PO_4-P ratios above 150, indicating that phosphorus availability played a minor role under the tested conditions, also suggested in pilot trials [Paper III - Figure 12]. By comparison, Soares et al. [203] reported sCOD/NH_4^+-N and sCOD/PO_4-P ratios of 17 and 117, respectively, significantly lower than those observed in this study, but still sufficient to sustain biological nutrient removal (BNR) under their tested conditions. In contrast, Paper II showed that fermentates from protein-rich substrates like WAS and digestate had much lower sCOD/NH_4^+-N ratios (16–11) (Figure 12), posing potential challenges for post-denitrification use. In the Henriksdal pilot trials [Paper III], nutrient contributions from fermentate remained minimal, representing only 0.3–1.1% of the total nitrogen load and ≤0.3% of the phosphorus load, with no impact on effluent P concentrations or chemical dosing. These findings confirm that while nutrient content varies by substrate, low contributions and balanced carbon-to-nutrient ratios, especially when incorporating FW, can support stable operation without affecting overall system performance. When needed, strategies such as nutrient recovery or filtration can help manage excess loads without compromising the carbon fraction [191, 208].

4.6.2 Potential for greenhouse gas emissions

The main GHGs emitted from WWTPs are nitrous oxide (N_2O) and methane (CH_4). N_2O is primarily produced during biological nitrogen removal, while CH_4 is mainly associated with anaerobic processes such as sludge treatment and storage. Both are potent greenhouse gases with a significantly higher climate impact than CO_2. When evaluating waste-derived carbon sources, it is important to consider not only denitrification performance but also potential effects on GHG emissions, particularly N_2O formation during denitrification and CH_4 linked to upstream fermentation or sludge handling.

No direct GHG emissions were analysed during the trials in the present work. However, the accumulation of NO_2^--N, observed in several batch tests, has been shown to be closely associated with increased N_2O emissions in activated sludge systems [53, 209]. Accumulation of intermediates may result from enzymatic competition among denitrification enzymes (NAR, NIR, NOR, NOS), which disrupts the reduction pathway and promotes accumulation [210]; or by the enrichment of bacteria lacking genes for complete NO_2^- reduction [211].

In line with these mechanisms, *Candidatus* Saccharibacteria UBA5946, a taxon associated with partial denitrification and intermediate build-up [200] was found in higher abundance during the use of glycerol compared to fermentate, consistent with batch test results showing a nitrite peak of 4.5 mg NO_2^--N/L [Paper III]. This behaviour mirrors findings from full-scale studies, where N_2O emissions increased proportionally with glycerol dosing [212]. In contrast, fermentates resulted in comparably lower nitrite accumulation: 0.2 mg NO_2^--N/L for PS:WAS fermentates [Paper II], and up to 1.2 mg NO_2^--N/L for FW and PS fermentates [Paper II], with similar levels observed for U-FWM [76; in preparation]. Methanol, tested with sludge from Himmerfjärdsverket, showed the lowest accumulation at just 0.04 mg NO_2^--N/L [76; in preparation]. These results pointed to the fact that the selection of a carbon source can influence the risk of formation of denitrification intermediates (NO_2, NO and N_2O). While acetate and glycerol (major components of many fermentates) have been linked to higher N_2O production [53, 54], alcohol-based sources, such as methanol and ethanol, tend to produce lower emissions [55]. Additionally, environmental factors such as low pH, more commonly associated with acid-based carbon sources, can worsen N_2O generation by disrupting electron flow and enzyme activity [42]. This points to the possibility that alcohol-based carbon sources may emit less N_2O than VFA-based alternatives, a factor that should be considered when selecting carbon sources for full-scale application. However, longer trials with direct N_2O measurements are needed to confirm possible differences and assess their relevance under operational conditions.

4.6.3 Heavy metals

The potential presence of heavy metals is an important consideration when bringing FW as a new substrate into a WWTP. Therefore, monitoring their concentrations in the produced carbon sources was an important part of this evaluation. Heavy metal concentrations were monitored during the pilot trials in the Himmerfjärdsverket pilot [76; in preparation] to evaluate potential accumulation in activated sludge from dosing unfermented food waste mix (U-FWM). Samples collected on the last day of methanol dosing, on day 44 and on day 151 (107 days after U-FWM addition), showed no significant changes in primary metals, such as arsenic, cadmium, lead, nickel, and vanadium, all of which remained within expected ranges for municipal sludge. Some increases were noted in barium (from 76±19 to 140±35 mg/kg TS), copper (from 200±50 to 270±68 mg/kg TS), and zinc (from 250±63 to 340±85 mg/kg TS), while cobalt and chromium decreased slightly; however, none of the changes were statistically significant, and all values remained within commonly reported limits. Total solids content was similar. These findings suggest that the use of untreated FWM as a carbon source did not lead to measurable accumulation of heavy metals in the sludge during the trial period, addressing one of the WWTP's concerns on the use of FW as a carbon source. Similarly, previous studies observed no negative impact of long-term dosing of industrial wastewater as an external carbon source [213]. However, together with the present study, the findings suggest that extended monitoring is important to assess potential long-term accumulation, especially considering the seasonal variability in waste stream composition.

4.6.4 Impact of carbon sources on downstream processes

Downstream impacts are critical to evaluate when implementing alternative carbon sources, as successful denitrification alone does not ensure stable plant performance. In both the Henriksdal [Paper III] and Himmerfjärdsverket [76; in preparation] MBR pilot trials, no significant changes were observed in suspended solids concentrations, sludge output, or solids retention time, despite the higher anoxic growth yields associated with fermentates. This suggests that neither fermented nor unfermented food waste-based carbon sources led to solids accumulation under the tested conditions. A foaming issue occurred at the Himmerfjärdsverket pilot but not at Henriksdal. The exact cause was unclear, but foaming has been a recurring issue at Himmerfjärdsverket and may be linked to local operational conditions, though a contribution from the carbon source cannot be ruled out.

Furthermore, membrane performance remained unaffected using fermentate, with no observed changes in transmembrane pressure, permeability, chemical cleaning frequency, or signs of irreversible fouling. Additional data on membrane behaviour during these trials are provided in the report by Andersson et al. [214]. This is consistent with a previous study using food waste fermentate as a carbon source in a pilot-scale MBR for nitrogen removal and membrane fouling assessment [215]. In contrast, a small-scale study using WAS fermentate reported increased production of proteins and humic substances in bound EPS, contributing to membrane fouling [216]. Nevertheless, the findings in Paper III, and Carranza-Muñoz et al. [76; in preparation] suggest that food waste-based carbon sources do not compromise biological or membrane stability in the short term; however, longer trials are necessary to confirm their long-term effects, especially with other substrates.

4.7 Challenges and opportunities for improvement

The denitrification trials confirmed the potential of waste-derived fermentates as effective carbon sources for denitrification [76; in preparation, Papers I-III]. However, several operational challenges were noted during the pilot trials [76; in preparation, Papers III], which must be addressed for full-scale implementation. An important consideration is the management of fermentation by-products, particularly hydrogen sulphide (H_2S), which was observed at high concentrations during the fermentation of food waste and primary sludge (FW:PS) [Paper III]. The low reactor pH (<5) likely contributed to both enhanced sulphate reduction and a shift in the gas–liquid equilibrium, leading to increased H_2S transfer into the gas phase, with concentrations occasionally exceeding 9000 ppm. Although gas volumes were low, $FeCl_3$ dosing and pH adjustment (to 6.5 using NaOH) before separation in the sieve were necessary to reduce H_2S emissions to safe levels (<5 ppm). H_2S generation and control during fermentation has also been reported in other studies involving WAS [217-219], PS [220], and FW [221]. In full-scale plants, off-gas management can be handled in various ways, and NaOH dosing may not be required to the same extent. Nevertheless, the uncertainty regarding H_2S emissions under different conditions, as highlighted by the results in Paper III (a 900% variation between measurements), highlights the need for further investigation. In the case of the present study, the cost of NaOH could represent up to 10% of the methanol cost savings in the worst-case scenario and down to 3% in others.

Additionally, the drum sieve used for liquid-solid separation included an automatic water flush system that diluted the carbon source by approximately 30%, lowering sCOD and VFA concentrations. However, neither the pH adjustment nor the physical treatment steps (separation, dilution, or short-term storage) altered the VFA composition, crucial for full-scale reliability. To the author's knowledge, no other pilot-scale fermentation using this separation method has been published.

Overall, fermentates and untreated substrates proved technically viable as carbon sources, but attention to gas emissions, separation steps, and long-term monitoring is essential for safe and reliable large-scale operation. These pilot trials revealed challenges relevant for full-scale implementation. Broader impacts, including biogas production, sludge yield, and costs, are discussed in the next chapter.

5. Operational Implications of Fermentation in Full-Scale WWTPs

The use of internally produced carbon sources for denitrification at WWTPs has proven effective at both lab and pilot scale, but its full-scale implementation raises several operational questions. Introducing a fermentation process affects not only nitrogen removal but also energy recovery, sludge disposal, and overall plant economics. These impacts are particularly relevant in Sweden, where most WWTPs produce biogas from sludge [222]. Diverting part of the sludge organics toward VFA production for denitrification reduces the carbon available for biogas production, directly impacting energy output and revenue, as well as influencing sludge production and treatment costs.

Despite its growing relevance due to increasing methanol prices and effluent regulations, the integration of fermentation into sludge lines has received limited attention in full-scale analyses [110]. Most studies focus on VFA production potential or denitrification efficiency, without considering how this choice affects biogas production, operational costs (OPEX), capital investments (CAPEX), and CO_2 emissions. These gaps were addressed in Paper IV, which presents a detailed techno-economic assessment of implementing fermentation in full-scale WWTPs. The assessment included changes in carbon source requirements (5.5), biogas production (5.3 and 5.6), impacts on sludge production (5.6), and the specific implementation context of Stockholm's major WWTPs (5.4). A full techno-economic analysis is presented in Section 5.7, covering CAPEX, OPEX, carbon source demand, and biogas trade-offs, followed by an evaluation of CO_2 emissions and avoided CO_2 emissions in Section 5.8.

5.1 Biogas production and carbon source recovery in WWTPs

Anaerobic digestion and biogas production have long been central to sludge stabilisation and energy recovery in WWTPs [8]. In 2022, total biogas production in Sweden was 2.3 TWh, of which around 130 WWTPs contributed to 31% of the production [223]. Biogas represents a revenue for most WWTPs, and sludge treatment and disposal represent up to 50% of total operating costs [110, 224, 225]. Thus, any measure that impacts sludge volumes or biogas production will have a significant effect on the economy.

Integrating fermentation for VFA recovery into the sludge line of WWTPs introduces a fundamental trade-off: the use of soluble organics for carbon source production reduces the carbon available for methane production. This affects energy recovery, particularly in plants where biogas is used for combined heat and power generation or sold and upgraded for use as vehicle fuel. These trade-offs are still not fully investigated in integrated fermentation/digestion systems, especially regarding their technical performance and economic feasibility [110, 226]. Furthermore, methane yields are highly substrate-dependent, and the macromolecule composition of the substrate also makes a difference; carbohydrates typically yield 0.415 L CH_4/g VS, proteins 0.496 L CH_4/g VS, and lipids 1.014 L CH_4/g VS [227, 228]. As methane has a calorific value of about 9.97 kWh/Nm^3, even small shifts in substrate characteristics and use can translate into considerable energy and revenue changes. Additionally, the introduction of a fermentation step can improve hydrolysis efficiency and increase overall sludge biodegradability, potentially compensating for some of the methane losses even after VFAs have been extracted [Papers I–IV]. Still, the overall benefit, in terms of costs and emissions, depends on the interaction between digestion performance, VFA extraction, and sludge disposal. Given the variability in substrate composition and treatment history, accurately estimating methane potential is essential.

5.2 Biochemical methane potential tests methods and their relevance

Biochemical Methane Potential (BMP) tests are essential for evaluating the methane yield of organic substrates and have direct implications for digester design, energy recovery, and economic performance in WWTPs [229, 230]. BMP also provides understanding into substrate biodegradability and can indicate how much methane is potentially lost when soluble carbon is diverted to fermentation for VFA recovery. This is an important step in evaluating the trade-off between carbon recovery for denitrification and biogas production.

BMP tests are widely used, but results sometimes lack reproducibility because of variations in methods, leading to international efforts to standardise the procedure [231]. Despite these challenges, BMP remains a reliable method for estimating the methane potential. In Papers I–III included in this thesis, BMP tests were used to quantify the maximum methane yield from various substrates, including food waste, primary sludge, mixed sludge, and digestate. These were also compared to their corresponding solid fractions after pre-treatment, fermentation and VFA extraction, with cellulose used as a reference control. All tests were conducted using AMPTS II systems (BPC instruments AB, Sweden) under mesophilic conditions (37 °C) for up to 30 days, or shorter if the methane production plateaued earlier. Each setup included triplicates, blanks, and controls, with an inoculum-to-substrate ratio of 3:1 and an organic loading rate of 3 kg VS/m^3 in all tests.

5.3 Methane potential results

BMP tests were used to quantify the maximum methane yield from various fractions studied in the present thesis, including food waste, primary sludge, mixed sludge, and digestate (Figure 13). The results illustrated that the unfiltered, unfermented food waste mix sample FWM [76; in preparation] exhibited the highest methane yield, with values above 770 NmL CH_4/g VS. This sample was filtered and used directly as a carbon source without fermentation. When the liquid fraction (rich in VFAs) was removed through filtration, the methane potential measured in the remaining solid fraction decreased by about 30%, as expected due to the loss of soluble organics. In contrast, when a food waste sample was fermented (Fermented FW [Paper I]) and the VFAs in liquid fraction were removed, the BMP remained statistically the same as the unfermented, unfiltered FW sample. A similar pattern was observed in the FW:PS mixes (1:1 and 1:3 %v/v). In these cases, the behaviour was more influenced by FW than the PS due to the distribution of VS in the mix. Given that FW had 6.4 times higher TS compared to PS, the actual VS ratios shifted to approximately 10:90 and 50:50 FW to PS, respectively.

For the sludge samples, the methane potential of fermented PS solid fractions was significantly lower than that of the unfermented, unfiltered PS sample. In contrast, samples with mixed sludge (PS:WAS) generally maintained their methane potential, regardless of whether they underwent thermal hydrolysis (THP) or only fermentation (Figure 13). For example, the solid fraction of fermented PS yielded only 163 NmL CH_4/g VS, a 51% reduction compared to unfermented, unfiltered PS [Paper I, Figure 9], directly impacting the economic balance of this carbon source in subsequent cost analyses. However, the impact varied depending on the macromolecular composition of the substrates. FW-rich streams exhibited higher biogas potential in their solid fractions after fermentation compared to the unfermented, unfiltered samples, due to the incomplete hydrolysis of macromolecules, which were less affected by solubilization and acidification due to low pH during fermentation (Figure 4 and Figure 5) and produced more biogas [227, 228, Paper I].

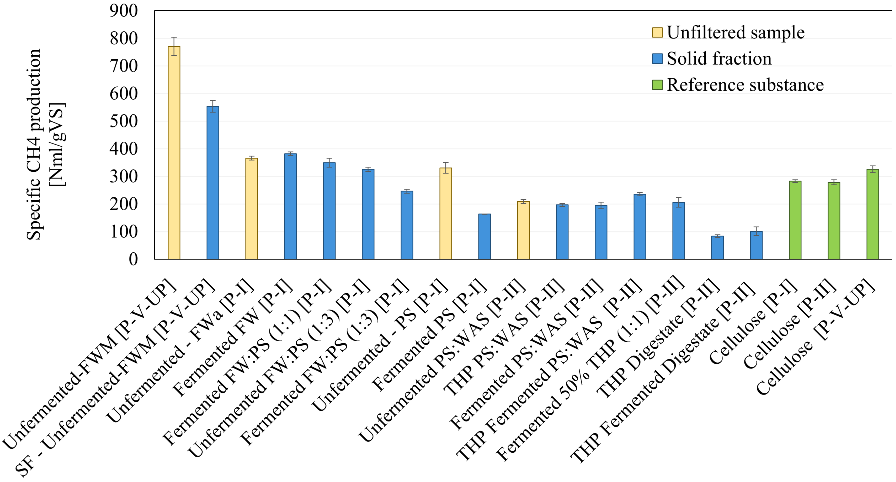

Figure 13 Specific methane production (NmL CH_4/g VS) from different carbon sources, including unfiltered samples (yellow) and their corresponding filtered solid fractions (blue), obtained through BMP tests. Cellulose was used as the reference substance (green). Data are presented as the average of triplicate tests with error bars indicating standard deviation.

In other cases, fermentation even improved methane production in the solid fraction. BMP tests from Paper II showed that fermented THP-treated mixed sludge reached 236 NmL CH_4/g VS, slightly higher than the unfermented control. This was likely due to enhanced hydrolysis and improved bioavailability of the organic matter following THP and fermentation. Interestingly, the fermented THP-treated digestate also showed a residual methane potential post-fermentation (101 NmL CH_4/g VS), suggesting that digestate streams could be repurposed for carbon recovery without compromising biogas production, and even increasing it [Paper II]. A strong correlation was observed between lipid content and BMP, further demonstrating that substrate composition is an important parameter influencing methane production.

5.4 Implementation context: Stockholm's Major WWTPs

As nitrogen limits tighten and effluent targets approach 6 mg TN/L and 0.2 mg TP/L, the demand for external carbon sources is set to increase across many WWTPs in Sweden. This is particularly relevant for Stockholm's three largest plants, Henriksdal, Käppala, and Himmerfjärdsverket, operated by Stockholm Vatten och Avfall, Käppalaförbundet, and Syvab AB, respectively, which together serve around 2.7 million people. Papers I–IV contributed to this effort by assessing the feasibility and performance of these alternatives through batch [Papers I–III] and pilot-scale [76; in preparation, Paper III] trials. The focus was on whether fermentates or raw waste streams could replace methanol, with acceptable trade-offs in energy, emissions, and supply security.

Adding up the projected 2040 carbon dosing needs for all three plants, the combined demand is estimated to exceed 7,000 tons of COD per year, equivalent to approximately 5,000 tonnes of methanol per year. Due to the different treatment configurations of each facility, carbon source requirements and potential for replacement vary. The following sections provide an overview of each plant's configuration and context, organised by size.

5.4.1 Henriksdal WWTP

Henriksdal (Figure 14a) is the largest WWTP in Sweden, serving around 850,000 pe, and will reach 1.6 million when it is fully loaded. It utilises a conventional activated sludge (AS) system, combined with membrane bioreactor (MBR) technology with hollow-fibre membranes (Veolia, France). The biological treatment line includes primary sedimentation, pre-denitrification, nitrification, and post-denitrification steps. Internal nitrate recirculation (NR) of $4Q_{in}$ and return activated sludge (RAS) of $4Q_{in}$ were designed to optimise nitrogen removal [Paper III]. Carbon addition takes place in the post-denitrification zone, and the future operation is expected to achieve full capacity by 2040. The current effluent target is 6 mg TN/L and 0.20 mg TP/L.

5.4.2 Käppala WWTP

Käppala WWTP (Figure 14b) is under renovation and will treat wastewater for approximately 785,000 pe in 2040, employing a moving bed biofilm reactor (MBBR) system, combined with AS lines. The treatment will include pre-denitrification, nitrification, and post-denitrification, followed by secondary clarifiers and a reject water treatment. Käppala uses free-floating plastic K5 biofilm carriers (AnoxKaldnes, Sweden) to facilitate bacterial growth. However, limitations in nitrate recirculation and lack of RAS flow made the requirements for carbon source volume and quality more challenging [Paper II]. Käppala may need to comply with N limits below 6 mg/L, potentially approaching 5 mg/L, due to its specific permit requiring a lower annual nitrogen load.

5.4.3 Himmerfjärdsverket WWTP

Himmerfjärdsverket (Figure 14c) will serve approximately 350,000 pe in 2040. Their new design utilises an MBR system similar to Henriksdal, but it includes a three step-feed biological line. Each step incorporates pre-denitrification and nitrification, and the third step has post-denitrification, followed by hollow-fibre membranes (Veolia, France). Himmerfjärdsverket also receives food waste and industrial waste, making it a particularly interesting case for internal carbon source production or direct utilisation of waste streams [76; in preparation]. The facility features reject water treatment, which significantly reduces nitrogen recirculation into the main line, providing an advantage in reducing external carbon needs.

Although also an MBR, Himmerfjärdsverket has a smaller treatment volume than Henriksdal, which may enable more modular or distributed strategies for fermentation and carbon source separation.

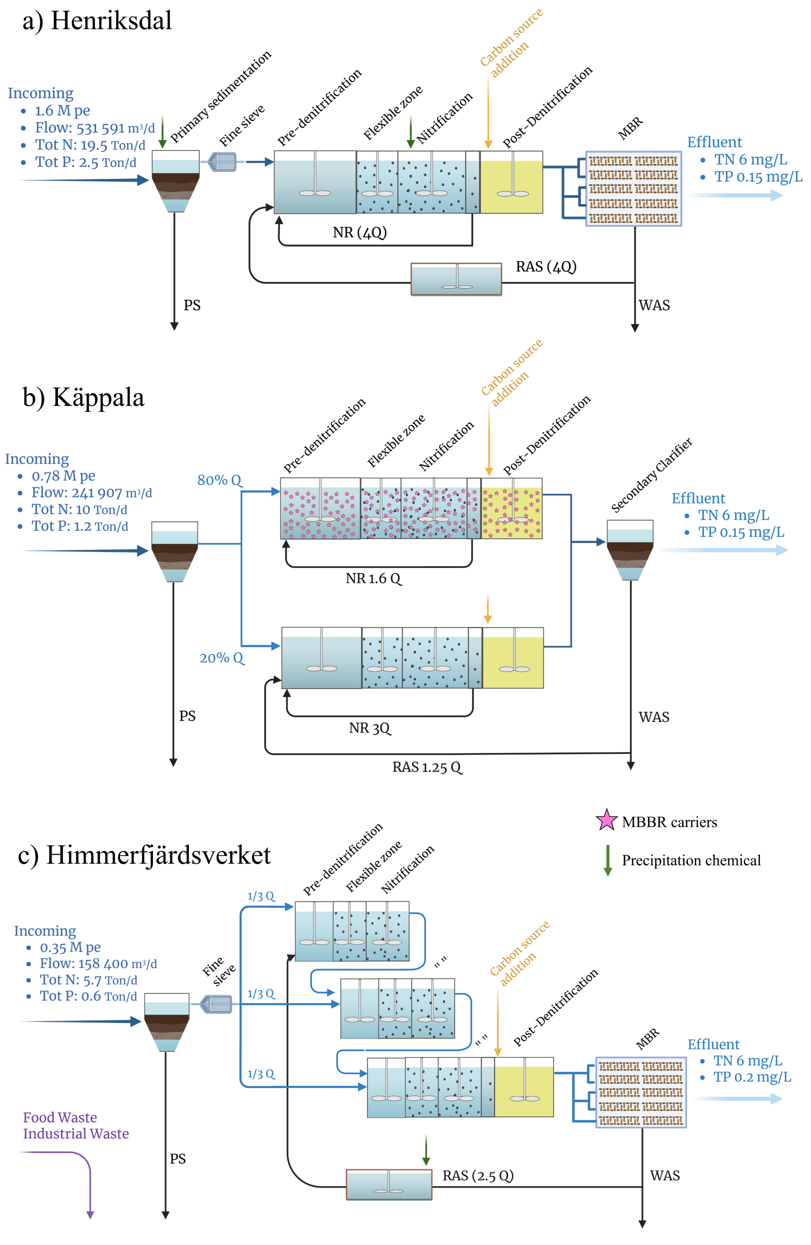

Figure 14 Process configurations of the three wastewater treatment plants included in this study: a) Henriksdal b) Käppala, c) Himmerfjärdsverket.

In summary, MBR and MBBR both achieve nitrogen removal but operate differently. MBRs offer stable conditions, high solids operation, but require more energy, while MBBRs consume less energy and can reduce carbon demand through biofilm oxygen gradients [8]. However, in our results, MBBRs required more external carbon due to limited nitrate recirculation [Paper IV]. Reject water treatment at Himmerfjärdsverket and Käppala reduced carbon demand, with reject streams contributing 10–30% of total nitrogen [32]. In the following sections, two WWTP models sized to the same population equivalent (pe: 785,000), based on Käppala and Henriksdal, were used to assess carbon demand, implementation scenarios, and cost analysis, considering process differences but not aiming to compare water treatment systems.

5.5 Carbon source requirements and fermenter size

Paper IV presented the evaluation of the replacement of methanol (as a base scenario) with 8 internally produced fermentates under four full-scale scenarios (two WWTP configurations: MBBR and MBR, and two effluent targets: 6 and 8 mg TN/L) (Figure 15). These scenarios were modelled using design data from Henriksdal and Käppala. The analysis focused on the amounts of carbon required for denitrification, the infrastructure needed for fermentate production, and the associated operational challenges.

Carbon source demand was estimated for each scenario using mass balances of the WWTPs, along with fermentate data on sCOD and VFA concentrations, carbon consumption ratios ($COD/NO_3\text{-}N_{removed}$), denitrification rates, and nutrient loads (NH_4^+, PO_4^{3-}). Fermentates from mixed sludge and THP-treated streams contain low concentrations of sCOD, increasing the required carbon source volumes by between 20 and 500 times compared to methanol. These sources compromised their carbon efficiency by creating an additional nitrogen load, particularly under the stringent 6 mg TN/L effluent scenarios. In contrast, fermented food waste and FW:PS mixes performed better in terms of carbon efficiency, requiring considerably lower dosing volumes (typically between 7 and 90 m^3/day – 1.6 and 20 times larger than methanol) and manageable additional nutrient impacts, around 0.3 to 1% of the incoming load [Paper III].

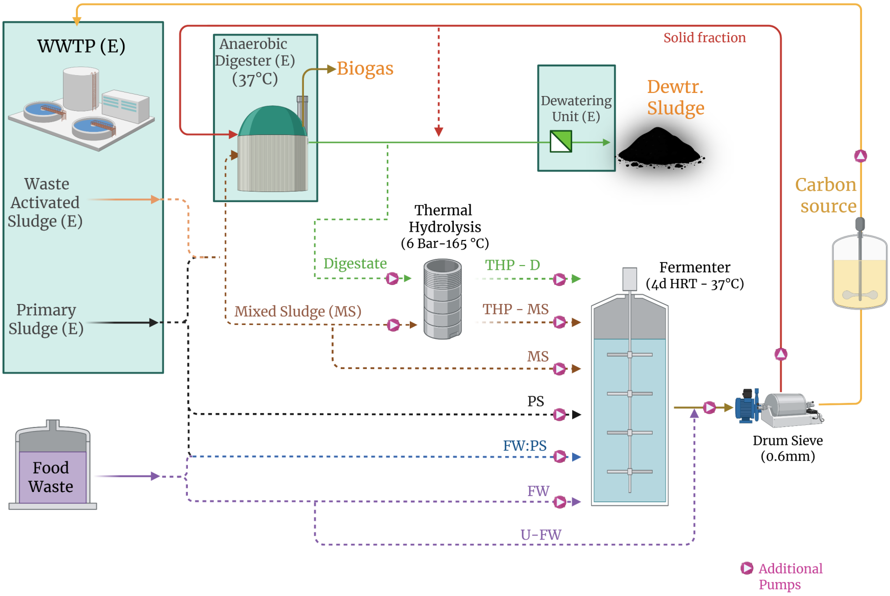

Figure 15 Additional proposed process. E: existing processes in WWTPs. D: digestate, MS: Mixed sludge (PS:WAS), PS: Primary sludge, FW: Food waste, U-FW: Unfermented FW.

The fermenter sizes reflected these trends, as they were calculated based on the carbon source demand and the VFA yields obtained during lab- and pilot-scale trials [76; in preparation, Paper I-III]. Fermented mixed sludge (MS) and PS required the largest fermenters, exceeding 4,000 m³ in some scenarios, while fermented FW and FW:PS mixes needed significantly smaller volumes (300-500 m³) due to higher sCOD concentrations both in the substrates and in the fermentates. Moreover, fermented PS and THP-fermented digestate were unable to fully meet the carbon demand in some scenarios, requiring supplemental methanol addition to remove the required nitrate load, particularly in the most demanding scenarios (6 mg/L). In contrast, fermented FW:PS consistently met the full carbon demand while maintaining compact infrastructure requirements.

Complete replacement of methanol is feasible with certain substrates, but high nutrient loading, especially ammonium, can undermine the benefits unless additional treatment, such as nitrogen recovery or filtration [191], is implemented in cases like PS- and digestate-based systems. Substrate availability, fermenter sizing, and nutrient impacts must be technically and quantitatively assessed, including calculations of required fermenter volume, substrate demand, nutrient loading, and associated energy needs, to determine whether full replacement is practically feasible. These factors were evaluated before proceeding to OPEX, CAPEX, and CO_2 emissions, which are discussed in the following sections.

5.6 Effects of fermentation on biogas production and dewatered sludge

Sludge disposal and biogas production are critical factors in this assessment, both economically and operationally. As previously discussed, sludge disposal could be one of the highest operational costs in WWTPs, up to 100 €/ton wet weight, while biogas represents a significant revenue (~1 €/Nm^3 CH_4). This makes both parameters relevant for evaluating the feasibility of replacing methanol with internal carbon sources. The methane production estimations used in the large-scale calculations were directly based on the BMP values obtained from the experimental tests presented in section 5.2 [76; in preparation, Paper I-III]. The BMP results were used to estimate biogas production from both the recirculated solid fraction and the main digestion process in each scenario.

Methane production varied significantly depending on the carbon source strategy. Scenarios using fermented FW, FW:PS mixes, or THP fermented digestate achieved the highest methane production volumes, either the same or exceeding the methanol base scenario (~16,000 Nm^3 CH_4/d). THP fermented digestate scenarios reached up to 19,550 Nm^3 CH_4/d, while FW-based configurations benefited from the high methane potential of the recirculated solids (21,856 Nm^3 CH_4/d). In contrast, PS- and MS-based fermentates showed consistently lower methane production (as low as 10,200 Nm^3 CH_4/d). Sludge volumes followed a similar trend to methane production, increasing as methane production increased, which balanced costs and revenue. Systems using FW, FW:PS, or digestate produced higher sludge volumes, up to 206 tons/day in the THP-fermented digestate scenario, compared to MS- or PS-based systems, which stayed around 170–180 tons/day depending on the case, and to the methanol base scenario (180 tons/day). This difference is because digestate-based processes use sludge after digestion, whereas PS or MS fermentation uses streams before the digester. These results reinforce the main trade-off: carbon source production impacts both energy recovery and sludge disposal costs. FW and FW:PS mixes offered the most balanced outcome, with high biogas yields and a manageable increase in sludge volumes. In contrast, fermented MS and THP-fermented MS, although technically feasible as methanol replacements, come with higher operational burdens, lower methane production, and increased sludge volumes, factors fully considered in the OPEX and CAPEX assessments presented in the following sections.

5.7 Economic considerations of internal carbon source production systems [Paper IV]

Replacing methanol with internally produced carbon sources requires not only technical viability but also economic justification at full scale. Paper IV presents a comprehensive techno-economic assessment of alternative carbon sources, using the same cases as presented above. The assessment considered both capital and operational costs, infrastructure needs, and CO_2 emissions.

Cost estimates showed that of the evaluated carbon sources, the FW-based fermentates and FW:PS mixes offered the most favourable economics. These carbon sources benefited from higher sCOD and VFA concentrations, comparatively low requirements and smaller infrastructure. Additionally, they benefited from the revenue of receiving and treating FW (23 €/m^3 FW) (Figure 16). In contrast, fermentates from sludge and digestate were more expensive due to comparably lower sCOD and VFA content, larger fermenter volume requirements, higher energy use, and, in some cases, the need for thermal hydrolysis (THP). Raw FW mixes, although operationally simple, faced challenges in certification due to constraints in Sweden when mixed with sludge.

The CAPEX analysis included in Paper IV revealed that THP-based options were by far the most expensive. Investments for THP-fermented digestate ranged between 18 and 19 M€, while THP-fermented MS required 14.5–15.2 M€, driven almost entirely by the cost of the THP unit (88–90% of the total investment). In contrast, non-THP fermentation options such as FW, FW:PS mixes, and PS required significantly lower investments, typically between 1.1 and 2.7 M€, depending on the scenario and TN target. Among them, FW:PS mixes (1:1 and 1:3) consistently fell into the lower range, benefiting from higher VFA concentrations and reduced food waste handling infrastructure compared to FW alone. CAPEX for fermented PS ended up in the higher range due to lower VFA concentration requiring larger fermenters and storage volumes.

OPEX results (Figure 16b) showed that sludge disposal remained as one of the largest operational costs, while biogas remained the most critical revenue source. Food waste handling and methanol replacement (valued at 827 €/ton) also had a substantial positive impact on OPEX in several scenarios.

THP-fermented digestate was the most expensive alternative from an OPEX perspective, with annual costs rising to +2.31 M€/year in MBR configurations compared to methanol. Despite offering better dewaterability (30% TS compared to 24% TS for non-THP fermentates), it was not enough to compensate for the higher energy and operation and maintenance (O&M) costs. In fact, digestate processing after the digester inherently carries larger sludge loads. In contrast, THP-fermented MS performed relatively well, especially in MBBR configurations, achieving OPEX reductions of –1.49 M€/year at 6 mg/L TN compared to methanol. Similarly, fermented MS offered favourable results (–1.08 M€/year in MBBR 6 mg/L). These cases benefited from producing less sludge, despite higher fixed and variable OPEX costs. On the downside, these configurations still contained higher concentrations of nutrients. FW:PS (1:3) consistently delivered one of the most cost-effective solutions, balancing manageable sludge production with good carbon source quality and the benefit of food waste handling revenue. Nevertheless, the economic benefit was sensitive to dosing requirements; at 8 mg/L TN, reduced carbon demand also reduced the volume of food waste required, reducing the associated revenue without decreasing the fixed costs of storage or fermenters. Fermented PS had weak performance in MBBR but remained competitive in MBR systems at 6 mg/L, due to lower overall carbon demands and smaller infrastructure requirements. Breaking down the cost components, Figure 16b shows that sludge disposal dominated in most scenarios, followed by fixed O&M (driven by CAPEX) and food waste revenue. Methanol replacement represented a significant avoided cost in all cases. However, its impact is sometimes outweighed by higher sludge-related costs, particularly when external substrates such as FW increase sludge generation. Nevertheless, all carbon sources, except THP digestate, led to lower OPEX compared to the baseline methanol scenario (Figure 16).

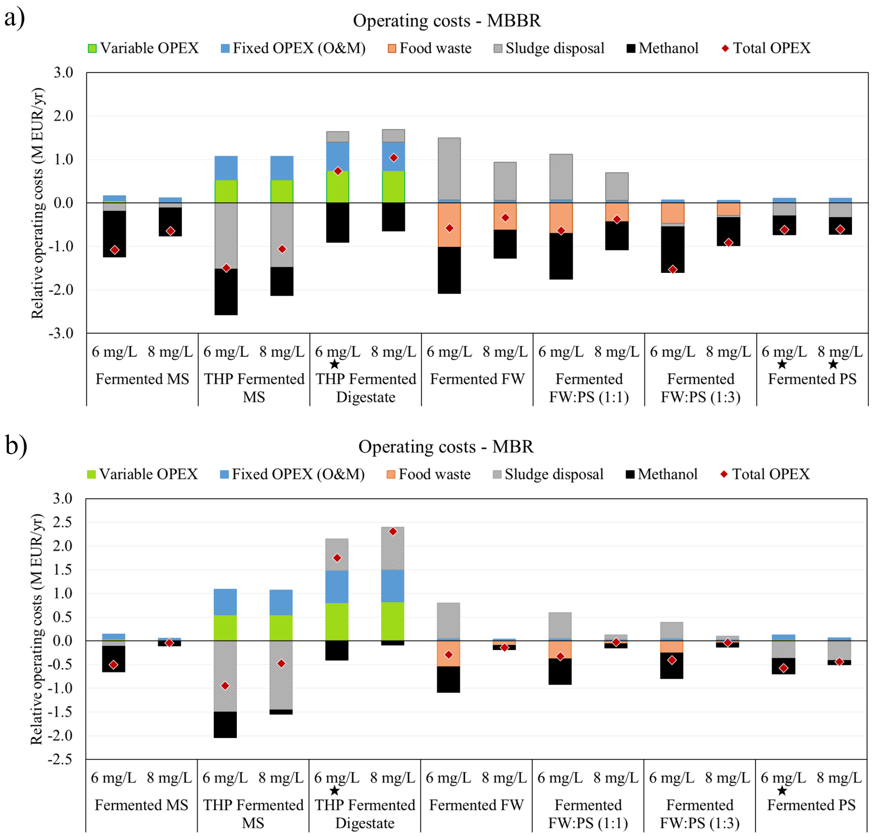

Figure 16 Relative OPEX performance of internal carbon source strategies compared to methanol base scenario under a) MBBR and b) MBR operation. Note*: Full methanol replacement was not achieved in MBBR and MBR 6 mg/L scenarios with THP-fermented mixed sludge, and in MBBR (6 and 8 mg/L) and MBR (6 mg/L) scenarios with fermented primary sludge.

Additionally, to address the uncertainty of future market conditions, a sensitivity analysis was carried out considering key parameters such as CAPEX (50%), electricity price (50%), methanol price (30%), biogas price (50%), sludge disposal costs (50%), and food waste acquisition costs (50%). The sensitivity analysis confirmed that economics were mainly driven by substrate availability, sludge handling costs, and methanol prices. For mixed sludge, sludge disposal fees determined whether thermal hydrolysis was cost-effective, which remained favourable even with a 50% increase in investment. Food waste remained economically beneficial despite higher prices, while methanol price significantly influenced the levelized cost across all scenarios. In contrast, electricity prices had little effect. These dynamics were particularly evident in FW-based scenarios and at increased TN limits, where lower carbon demand reduced both OPEX and food waste intake.

Overall, FW and FW:PS mixes emerged as the most balanced and economically viable alternatives to methanol, offering cost-effective carbon source production with manageable sludge volumes and strong methane yields. Where FW is unavailable, mixed sludge fermentation, with or without thermal hydrolysis, can support methanol replacement, though at higher costs. Sludge- and digestate-based fermentates remain technically feasible but are constrained by higher CAPEX, OPEX, low sCOD, and high nutrient concentrations. Their future viability depends on improvements in nutrient recovery, sludge handling costs, or energy prices.

5.8 CO_2 emissions: Methanol vs. Fermentation-based carbon sources

To assess the climate impact of replacing methanol with internally produced carbon sources, Paper IV evaluated avoided CO_2 emissions compared to methanol (Figure 17) and calculated the Levelized Cost of Avoided CO_2 Emissions (LCCA) for each alternative. LCCA combines economic performance with emissions savings, providing a metric for cost-efficient carbon reduction in WWTPs. The emission factors used in the analysis were based on Swedish and EU sources and included fossil-based methanol production (2,003 kg CO_2e/ton), electricity consumption (15 kg CO_2e/MWh), methane slip from anaerobic digestion (2.3 wt%, 27 kg CO_2e/kg CH_4 and district heating offset benefits (-49.6 kg CO_2e/MWh). Transport-related emissions for methanol and alternative carbon sources were also included based on typical distances (300 km) and fuel type (diesel).

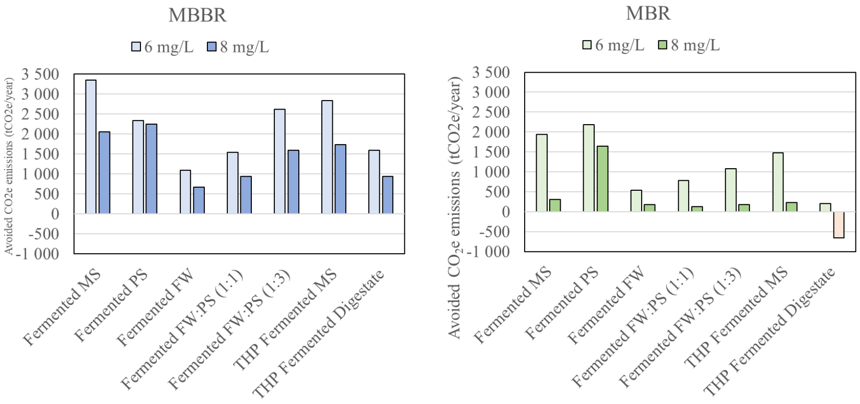

Figure 17 Avoided CO_2 emissions associated with the replacement of methanol, including emissions from the construction and operation of alternative processes. MS: mixed sludge, PS: primary sludge, FW: food waste, THP: thermal hydrolysis.

Total avoided emissions ranged from approximately 172,000 to 3.3 million kg CO_2e/year, depending on plant configuration and carbon source. The highest avoided emissions were achieved with fermented mixed sludge (3.3 million kg CO_2e/year in MBBR 6 mg TN/L), followed by PS-based fermentates and THP-treated mixed sludge (Figure 17). In contrast, food waste-based fermentates consistently delivered the lowest emissions savings, reflecting their lower dosing volumes and already high carbon efficiency. Nevertheless, FW and FW:PS fermentates achieved the lowest LCCA, offering the best combination of carbon efficiency, high methane production, and lower energy and infrastructure demands. In contrast, PS- and THP-based fermentates showed higher LCCA due to lower VFA concentrations, higher nutrient loads, and greater impacts on methane production. These trends align with previous findings by Castro-Fernandez et al. [83], highlighting that food waste achieves better carbon recovery and climate performance compared to PS. Overall, FW-based options remain the most effective in balancing both cost and emissions, while PS-based alternatives are less favourable from a climate perspective but remain feasible where FW availability is limited. Mixed sludge represented a better carbon source option than PS alone.

6. Concluding remarks and future suggestions

6.1 Concluding remarks

This thesis evaluated the production and application of VFA-rich carbon sources from organic waste streams as an alternative to fossil-based methanol for denitrification in large WWTPs. The work combined biological and process engineering perspectives to assess the operational, environmental, and economic trade-offs of implementing internal carbon source production. The general conclusions can be summarised as follows:

- VFAs from waste streams are viable alternatives to fossil methanol for biological nitrogen removal in WWTPs.
- VFAs can be effectively produced from primary sludge, food waste, and digestate after pre-treatment, with digestate demonstrated as a viable but previously underexplored substrate for fermentation.
- Despite low carbon solubilisation yields FW and FW:PS mixes achieved higher sCOD and VFA concentrations among the different evaluated carbon sources, required lower dosing, and had smaller biogas losses.
- Fermenting a mix of untreated and THP-treated sludge (50:50), improved VFA yields, compared to fermented and THP fermented mixed sludge.
- Substrate type defined both the microbial community composition and the VFA profile during fermentation, with FW promoting lactic acid and dominance of *Lactobacillaceae*, while PS was associated with higher propionate production and taxa such as *Prevotellaceae* and *Streptococcaceae*.
- All VFA-based carbon sources, regardless of origin achieved similar denitrification rates as pure acetic acid and outperformed methanol and glycerol in nitrate removal efficiency.
- Changing the carbon source from glycerol to FW:PS fermentate in pilot trials did not significantly change the core microbial community but improved denitrification performance. This is likely because glycerol fosters broad heterotrophic populations capable of utilising diverse

substrates, and the fermentate itself contains a wide mix of compounds, reducing selective pressure for specific taxa

- Variations in anoxic biomass yields between carbon sources had the most significant impact on sludge production and, consequently, operational costs.
- FW and FW:PS mixes were the most cost-effective alternatives to methanol, even when the price of FW increased. Mixed sludge, with or without thermal hydrolysis, is a viable option when FW is not available but comes with higher costs.
- Replacing methanol with alternative carbon sources reduced fossil CO_2 emissions and operational costs in most scenarios but required significant capital investment and increased sludge handling complexity. Only the THP digestate option showed higher costs and lower CO_2 savings compared to methanol.
- The techno-economic assessment confirmed that the feasibility of implementing the use of alternative carbon sources depends strongly on plant configuration, discharge limits, and substrate selection. Plants with higher carbon source requirements had higher CAPEX but lower levelized costs, making full-scale application more feasible.

6.2 Suggestions for future research

Based on the results achieved in this thesis, the following research is proposed to further advance the implementation of fermentation-based carbon sources in WWTPs:

- Further optimisation of VFA production from digestate, including extraction and process conditions.
- Long-term pilot testing to evaluate process stability, microbial dynamics with other carbon sources, and measure N_2O emissions with fermented carbon sources.
- Development of strategies to manage ammonium and phosphate removal from the fermentates without affecting the quality of the carbon source.
- Exploring more effective carbon source dosing strategies at WWTP.

- Extended environmental assessments, including N_2O emissions and life cycle impacts beyond fossil CO_2.
- Development of practical guidelines for implementation, including evaluation and experimentation of separation technologies beyond rotary sieves.
- Investigation of odour management strategies to ensure a safe working environment and minimise impact on surrounding areas.

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Popular science summary

Wastewater treatment plays a crucial role in protecting rivers, lakes, and coastal environments from pollution. One of the challenges for plants is removing nitrogen, which, if discharged into water bodies, can cause algae's uncontrollable growth, oxygen depletion, and damage to ecosystems, such as the Baltic Sea. To remove nitrogen, treatment plants rely on bacteria that convert nitrogen compounds into nitrogen gas to remove it from the water. However, these bacteria require an easily degradable carbon source to complete this process, and sometimes an additional carbon source is needed. Many wastewater treatment plants today rely on methanol as an additional, fossil-based chemical, serving as a carbon source. However, methanol is costly, contributes to greenhouse gas emissions, and depends on the fossil fuel supply. As treatment plants face stricter climate targets and higher chemical costs, there is growing interest in finding sustainable alternatives to these challenges.

This research investigates whether wastewater treatment plants can produce their own carbon source by fermenting waste streams already handled on site, such as, sewage sludge, or digestate, or an additional one as food waste Through a process called anaerobic fermentation, these wastes are broken down to produce volatile fatty acids (VFAs), which bacteria can use in the nitrogen removal process. The study evaluates how different waste materials perform in producing VFAs, how effective these VFAs are for nitrogen removal, and how switching from methanol to VFAs affects the entire treatment plant. Effects include costs, biogas production, sludge handling, and carbon dioxide emissions. The results show that fermentates from food waste and mixtures of food waste with sewage sludge are the most efficient and cost-effective options, offering high carbon quality with manageable operational impacts. Interestingly, all carbon sources, whether produced from food waste, sewage sludge, or digestate, performed equally well in nitrogen removal. This means that the choice of material is mainly driven by costs, emissions, and operational impacts, rather than biological performance. Using only sewage sludge or digestate is technically possible, but comes with higher costs and lower carbon efficiency.

By replacing methanol with carbon sourced from waste, treatment plants can reduce their dependence on fossil chemicals, lower CO_2 emissions, and contribute to a more circular and climate-friendly wastewater management system. This solution supports both environmental goals and the economic sustainability of future wastewater treatment.

Populärvetenskaplig sammanfattning

Avloppsrening fyller en viktig funktion för att skydda sjöar, vattendrag och kustvatten från förorening. En av utmaningarna för reningsverken är kväverening, vilket är nödvändig för att undvika algblomning, syrebrist och förstörda ekosystem, något som drabbat t.ex. Östersjön. För att uppnå kväverening använder reningsverken bakterier som omvandlar kväveföreningar till kvävgas och på så sätt avskiljer kvävet från vattenfasen. För detta behöver bakterierna tillgång till lättnedbrytbar kolkälla och även om detta finns i avloppsvattnet behövs ibland ett tillskott. Många reningsverk använder idag metanol med fossilt ursprung som kolkälla. Metanol är dyrt, genererar stora utsläpp av växthusgas och är beroende av tillgång till fossilt bränsle. I takt med att reningsverken ställs inför strängare klimatmål och ökade kemikaliekostnader växer intresset för att hitta hållbara alternativ till metanol. Denna studie undersöker om avloppsreningsverk kan producera sin egen kolkälla genom att fermentera reströmmar som redan hanteras på anläggningen, såsom avloppsslam eller rötrest, eller genom att tillföra en ytterligare fraktion som matavfall. Genom en process kallad anaerob fermentering bryts dessa material ner och bildar flyktiga fettsyror (VFA), som bakterier kan använda i kvävereningsprocessen. Studien utvärderar hur effektiv produktionen av VFA från olika reströmmar är samt hur bra produkten fungerar för kväverening. Dessutom utvärderas hur ett byte från metanol till VFA påverkar hela reningsverket med avseende på kostnader, biogasproduktion, slamhantering och koldioxidutsläpp. Resultaten visar att fermentat från matavfall och blandningar av matavfall med avloppsslam är de mest effektiva och ekonomiskt fördelaktiga alternativen, då de erbjuder hög kvalitet på kolkällan med hanterbara driftmässiga konsekvenser. Intressant nog visade sig alla kolkällor – oavsett om de producerats från matavfall, avloppsslam eller rötrest – vara lika effektiva i kvävereningen. Det innebär att valet av material främst bör baseras på kostnader, utsläpp och driftspåverkan snarare än biologisk prestanda. Att enbart använda avloppsslam eller rötrest är tekniskt möjligt, men förknippat med högre kostnader och lägre koleffektivitet.

Genom att ersätta metanol med kolkällor baserade på reströmmar kan reningsverk minska sitt beroende av fossila kemikalier, sänka koldioxidutsläppen och bidra till ett mer cirkulärt och klimatvänligt avloppssystem. Denna lösning främjar både miljömål och den ekonomiska hållbarheten i framtidens avloppsrening.

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Vi ses…

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Impact of thermal hydrolysis on VFA-based carbon source production from fermentation of sludge and digestate for denitrification: experimentation and upscaling implications

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ABSTRACT

Stricter nutrient discharge limits at wastewater treatment plants (WWTPs) are increasing the demand for external carbon sources for denitrification, especially at cold temperatures. Production of carbon sources at WWTP by fermentation of sewage sludge often results in low yields of soluble carbon and volatile fatty acids (VFA) and high biogas losses, limiting its feasibility for full-scale application. This study investigated the overall impact of thermal hydrolysis pre-treatment (THP) on the production of VFA for denitrification through the fermentation of municipal sludge and digestate. Fermentation products and yields, denitrification efficiency and potential impacts on methane yield in the downstream process after carbon source separation were evaluated. Fermentation of THP substrates resulted in 37–70 % higher soluble chemical oxygen demand (sCOD) concentrations than fermentation of untreated substrates but did not significantly affect VFA yield after fermentation. Nevertheless, THP had a positive impact on the denitrification rates and on the methane yields of the residual solid fraction in all experiments. Among the different carbon sources tested, the one produced from the fermentation of THP-digestate showed an overall better potential as a carbon source than other substrates (e.g. sludge). It obtained a relatively high carbon solubilisation degree (39 %) and higher concentrations of sCOD (19 g sCOD/L) and VFA (9.8 g VFA_{COD}/L), which resulted in a higher denitrification rate (8.77 mg NOx-N/g VSS•h). After the separation of the carbon source, the solid phase from this sample produced a methane yield of 101 mL CH_4/g VS. Furthermore, fermentation of a 50:50 mixture of THP-substrate and raw sludge produced also resulted in a high VFA yield (283 g VFA_{COD}/kg VS_{in}) and denitrification rate of 8.74 mg NOx-N/g VSS•h, indicating a potential for reduced treatment volumes. Calculations based on a full-scale WWTP (Käppala, Stockholm) demonstrated that the carbon sources produced could replace fossil-based methanol and meet the nitrogen effluent limit (6 mg/L) despite their ammonium content. Fermentation of 50–63 % of the available sludge at Käppala WWTP in 2028 could produce enough carbon source to replace methanol, with only an 8–20 % reduction in methane production, depending on the production process. Additionally, digestate production would be sufficient to generate 81 % of the required carbon source while also increasing methane production by 5 % if a portion of the solid residues were recirculated to the digester.

1. Introduction

Stricter nutrient discharge limits, such as those recently proposed in the new EU legislation for urban wastewater treatment (European Parliamentary Research Service, 2023) or those already approved by the Swedish government, will increase the reliance on external carbon sources at wastewater treatment plants. The Swedish mitigation plan for improving the status of the Baltic Sea, which is affected by eutrophication, already applies stringent effluent requirements on large WWTPs (>100,000 person-equivalent (pe)) (Naturvårdsverket, 2016).

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Consequently, redesign and optimisation of WWTP processes are underway in Sweden. Most of them include dosing external carbon sources in denitrification zones, demand for which is estimated to increase with increasing population. Fossil-based methanol (MeOH) is the preferred carbon source for denitrification because of its high sCOD content and relatively low cost (~EUR 375/ton in Q3 2023) (Methanex Corporation, 2023), but it raises environmental concerns due to the high CO_2 emissions associated with its production and transportation (Methanol Institute, 2022). The methanol consumption for the three WWTPs serving ~2.8 Mpe in the Stockholm area is estimated to reach 6000 tons annually by 2040, significantly influencing operating costs and CO_2 emissions.

The extensive scientific literature on alternative carbon sources for denitrification has been reviewed in several recent publications (Fu et al., 2022; H. Wang et al., 2021). The focus in most previous studies has been on exploring potential carbon sources such as glycerol, methane, ethanol, different pure volatile fatty acids (VFAs), by-products from different industries (Ahmed et al., 2023; Fass et al., 1994; Lee and Welander, 1996) and fermentate liquids produced by sludge fermentation under different conditions (Elefsiniotis et al., 2004; Luo et al., 2019). The results evidence that acetic acid is the most effective carbon source, giving higher denitrification rates than methanol, glycerol or glucose (Pan et al., 2023). This is attributable to its molecular structure, which allows for more efficient production and transfer of electrons and energy, thereby promoting denitrification (Wei et al., 2022). When using a fermentation liquid with a mixture of organic compounds as a carbon source, acetic acid is depleted first, after which the denitrification bacteria use the remaining VFAs (Elefsiniotis et al., 2004), followed by soluble proteins and finally other organic compounds (Guo et al., 2017). Despite this preference of denitrifiers for short-chain carboxylic acids (e.g. acetic acid), denitrification performance remains similar when the carbon source is a mixture of soluble organic compounds. This has led to growing interest in producing carbon sources through fermentation or co-fermentation of organic waste streams at WWTPs to decrease costs and emissions. Studies to date have investigated the fermentation process under different parameters, e.g. temperature (Elefsiniotis and Li, 2006; Ossiansson et al., 2023), hydraulic retention time (HRT) (Khan et al., 2019) and substrates, e.g. food waste and other organic streams as a sole substrate or mixed with sludge (Atasoy et al., 2018; Zhou et al., 2018). Furthermore, pre-treatments can improve carbon solubilisation and VFA yields in sludge fermentation. Thermal hydrolysis pre-treatment (THP), a commonly used method for enhancing anaerobic digestion (AD), uses high temperatures and changes in pressure to destroy floc structures and microbial cell walls (Barber, 2016). THP is currently used on an industrial scale to increase biogas yield and sludge dewaterability, while at a pilot scale, the concentrate produced by THP has been evaluated as a carbon source for denitrification (Barlindhaug and Ødegaard, 1996; Chen et al., 2024; Guo et al., 2017), and as an enhancer for anaerobic fermentation. In terms of fermentation, some studies have observed a positive effect of THP on VFA production from sludge (Hosseini Koupaie et al., 2021; Morgan-Sagastume et al., 2011; Xiang et al., 2023; Zhang et al., 2019), whereas others have found that THP does not enhance biodegradability or VFA production due to the presence of refractory by-products from the Maillard reaction (Castro-Fernandez et al., 2023).

While in-depth investigation has focused on the production of internal carbon sources for denitrification, it has so far only been evaluated using primary (PS) and waste-activated sludge (WAS) and not digested sludge. Using digestate as substrate could enable the production of a carbon source without biogas reduction and with significant reductions in sludge handling volumes, resulting in cost savings. Previous studies have examined the effects of THP (at different temperatures) on digestate for improving dewaterability and enhancing biogas yield (Cai et al., 2021; Nordell et al., 2022; Svensson et al., 2018), but not for further fermentation. The Norwegian company Cambi now offers the option of post-THP (SolidStream) as a pre-treatment for improved dewatering, although the performance of this application has not been fully documented.

Additionally, little attention has been given to the overall implementation of the process in full-scale WWTP or its impact on biogas production and sludge management, which directly impacts WWTP operating costs. Most WWTPs in Sweden produce biogas through AD of sludge, contributing 31 % of the national biomethane production (Klackenberg, 2023). Using sewage sludge for other purposes, e.g. producing a carbon source for denitrification, could theoretically reduce biogas production. This would affect the production of renewable energy in Sweden and would represent a significant revenue loss for WWTPs (of ~EUR 1/Nm^3 CH_4).

In the present study, we compared internal carbon sources for denitrification produced from fermentation of raw and THP-treated sewage sludge and digestate. The aims were to determine the effect of THP on fermentation, denitrification and biogas loss after VFA extraction and to evaluate the potential of digestate as a substrate for VFA production compared with sludge. Additionally, we investigated the potential benefits of feeding a mix of THP sludge and raw sludge to the fermentation. A pilot-scale THP Cambi-pilot plant, two bench-scale fermenters, denitrification test reactors and biomethane potential (BMP) tests were used in the study. The results obtained were used in process calculations and preliminary cost estimates for Sweden's third-largest WWTP, Käppala (Stockholm), in 2028. Käppala WWTP is currently undergoing construction to increase capacity, which will increase the external carbon source requirement, so identifying the most beneficial scenario for producing an internal carbon source for denitrification is of great interest.

2. Materials and methods

2.1. Experimental design

Four different experiments were conducted, as shown in Fig. 1. Each experiment consisted of two fermenters (F1, F2). Fermenter F1 was used as a control and was fed with either raw digestate or a mixture of PS and WAS, while Fermenter F2 was fed with the corresponding thermally hydrolysed substrate [Cambi-Pilot]. After fermentation, the fermentates were centrifuged, resulting in a solid fraction and reject water containing sCOD and VFA (referred to hereafter as carbon source). The carbon source was used in denitrification activity tests, and the solid fraction was used in BMP tests. Thermally hydrolysed sludge (THP-sludge) and thermally hydrolysed digestate (THP-digestate) without fermentation were also centrifuged and evaluated as carbon sources.

In experiments 1 and 2, the fermenters were fed with a mix of PS+WAS (35:65 on a total solids basis; PS_{65}/WAS_{35}) with a final TS of 3.5 %. The same substrate mix was used in experiment 3 but with a higher final TS content (5 %), while digestate was used as the substrate in experiment 4. In experiments 1, 3, and 4, fermenter F2 was fed with 100 % THP-substrate, whereas in experiment 2, F2 was fed with a mixture of 50 % raw sludge and 50 % THP-sludge (volume-based) (Table 1). To validate the results from Cambi-Pilot, an additional experiment was performed with full-scale THP-sludge taken from a thermal hydrolysis Cambi process (HIAS, Norway) (results included in the supplementary material).

2.2. Substrates and inoculum

The PS, WAS and digestate used as substrates were collected weekly from Käppala WWTP. The anaerobic reactors used for sludge treatment and biogas production at Käppala WWTP, from which the digestate was taken, operate under mesophilic conditions (37 °C) and an average retention time of 20 days. This digestate was used as substrate in experiment 4 and as inoculum for all fermentation experiments and BMP tests. Activated sludge from the post-denitrification zone of Käppala WWTP and carriers from the end of the pre-denitrification MBBR zone at

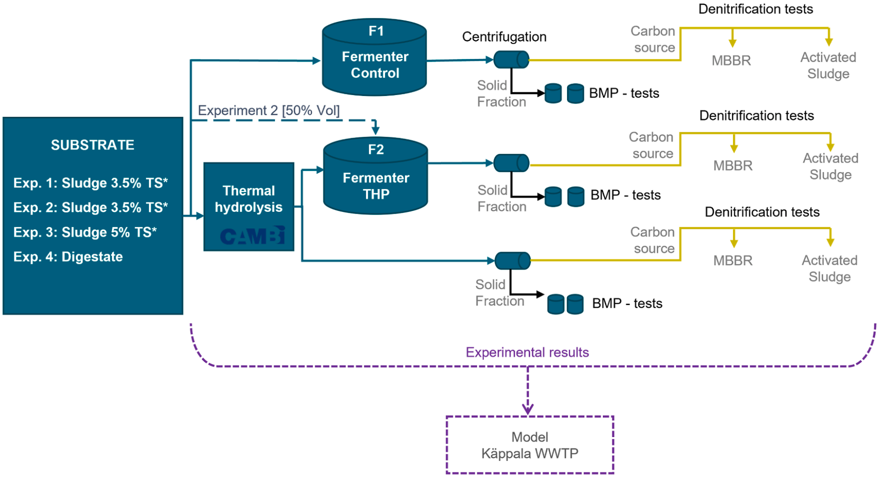


Fig. 1. Experimental scheme: Experiments 1–4. The process included thermal hydrolysis, fermentation, denitrification tests on the fermentation liquid and biomethane potential (BMP) tests on the solid fraction. It also included a control fermenter with no thermal hydrolysis. MBBR: moving bed biofilm reactor. * Mix 65 % PS:35 %WAS (TS based).

Margretelund WWTP (Åkersberga, Sweden) were used as inocula in the denitrification tests (the MBBR lines at Käppala are currently under construction and no samples were available). The characteristics of the substrates and inocula are summarised in Table 1.

2.3. THP pilot plant

THP mini-pilot plant (model 2 L/5 L; Cambi, Norway- Figure S1) was operated in batch mode at 165 °C and 6 bar for 30 mins (effective contact time). The pilot was preheated before each run and then fed with different substrates once the desired temperature was reached. It was operated once a week. The thermally hydrolysed samples were stored in the fridge at 4 °C until fed into the fermenters. In the last run of each experiment, additional samples were stored in a freezer at −18 °C to be used as a carbon source for denitrification and for the BMP tests.

2.4. Fermentation

The fermentation trials were performed in a bench-scale dolly reactor system (Belach Bioteknik AB) (Figure S1). Two twin reactors, each with a total volume of 8 L and an active volume of 6.15 L, were fed semi-continuously at 1.75 L per day, six days a week, reaching an HRT of 4 days. A 5 L gas-tight sample bag (SKC, USA) was used during the feeding of the reactors to prevent oxygen leakage and avoid impacting the gas flow meters. Gas samples were collected and sent for GC analysis to determine gas composition on days 2, 7, 10, 15, 17 and 18 of each experiment. Both were operated in mesophilic conditions at 37 °C. The inoculum was sourced from the AD at Käppala WWTP (Table 1). At the beginning of each trial, the fermenters were inoculated, and methanogen activity was inhibited by adding 2-bromoethanoesulfonic acid sodium salt to a final concentration of 30 mM, following the procedure described by Gong et al. (2021). The fermenters' organic loading rate (OLR) ranged between 7.3 and 11.0 g VS/L•d in all trials (Table 1). Each experiment operated for four HRTs plus an additional two days (18 days) to ensure a steady state. During the last three days of each trial, all chemical analyses were performed, and one additional sample per day was collected and frozen for subsequent denitrification and BMP tests (figure S2-monitoring plan).

In order to investigate the influence of pH, experiments 2 and 3 were each extended by an additional four days, and the pH was increased to 6 in all fermenters, using NaOH in experiment 2 and Na_2CO_3 in experiment 3. Only VFA and sCOD were measured during this additional period.

2.5. Denitrification tests

After the fermentation trials were completed, six samples were selected as the most suitable carbon sources for denitrification. The selected samples were centrifuged in the laboratory in a centrifuge (Megafuge 40, Thermo Fisher Scientific) at 4100 rpm for 5 min, and the reject water obtained was used as a carbon source. Batch denitrification activity tests were performed with activated sludge or MBBR carrier AnoxKaldnes k5 (800 m^2/m^3) as inoculum (Table 1). Tests with activated sludge were performed in 5-L reactors, following the methodology (test DEN.CHE.1) described by van Loosdrecht et al. (2016). Starting reactor concentrations were 25 mg NO_3–N/L and 165 mg sCOD/L, corresponding to a C/N ratio of 6.6 and a C/VSS ratio of 0.05–0.1 g COD/VSS. The sludge was not washed. For the tests performed with MBBR biofilm media, a carrier filling ratio of 45 % (same design in Käppala WWTP) was used in 5-L reactors, and initial concentrations were the same as those for activated sludge. The temperature was controlled at 20°C, and the pH was adjusted to 7 after adding the carbon source in all experiments. The mixing was done with mechanical metal stirrers.

2.6. Biomethane potential tests

After centrifugation of the selected samples and removal of the liquid phase/carbon source, the solid fraction was evaluated in BMP tests (Fig. 1). Three sets of Automatic Methane Potential Test System II (AMPTS II) from BPC Instruments AB (Lund, Sweden) were used, each consisting of 15 glass bottles (500 mL) with 80 % active volume. The inoculum-to-substrate ratio was set to 3:1 (3.6 g $VS_{inoc.}$/1.2 g $VS_{subs.}$), and the organic load was 3 kg VS/m^3 in all tests. The volume was adjusted by the addition of distilled water. The tests were performed under standard mesophilic conditions (37 °C) for 30 days. The inoculum

Table 1

Characteristics of substrates and inocula for fermentation (experiments 1–4) and denitrification, as well as organic loading rates and inflows for fermentation. PS: Primary sludge, WAS: waste-activated sludge, TS: total solids, VS: volatile solids, TCOD: total chemical oxygen demand, sCOD: soluble COD, VFA: volatile fatty acids, TN: total nitrogen, NH4-N: Ammonium nitrogen, PO4-P: phosphate phosphorous, av: average and sd: standard deviation ($n = 9$ for all samples; except protein, lipids and carbohydrates where 2 samples of 1 batch were sent to the external laboratory).

		Raw substrate								Thermally hydrolysed substrate								Inoculum Fermentation [Digester]		Inoculum Denitrification [Activated sludge]	
Experiment		1		2		3		4		1		2		3		4					
		PS + WAS 3.5%TS		PS + WAS 3.5%TS		PS + WAS 5%TS		Digestate		TH 3.5%TS		50% TH Sludge 50% raw sludge 3.5%TS		TH 5%TS		TH Digestate					
Parameter	unit	av.	sd.	av.	sd.	av.	sd.	av.	sd.	av.	sd.	av.	sd.	av.	sd.	av.	sd.	av.	sd	av.	sd
TS	[%]	3.6	0.5	3.7	0.1	5.4	0.4	5.8	0.4	3.6	0.4	3.8	0.3	5.8	0.0	5.8	0.2	3.1	0.0	0.22	0.02
VS	[% of TS]	83.3	1.0	83.2	0.7	82.2	0.8	67.2	0.7	83.1	1.1	82.3	0.9	83.6	0.1	68.2	1.7	64.6	1.2	0.18	0.01
TCOD	[g/L]	42.4	7.0	46.5	0.5	70.2	3.5	57.9	6.1	46.9	7.7	48.8	2.9	74.7	4.2	64.3	3.0	36.0	0.0	–	–
sCOD	[g/L]	1.5	0.2	1.3	0.2	2.5	0.9	0.0	0.1	10.7	0.5	4.6	0.3	12.3	1.3	24.1	0.6	0.9	0.2	0.1	0.0
VFA	[g/L]	0.9	0.1	0.8	0.1	1.4	0.4	0.1	0.0	1.5	0.1	1.1	0.1	1.5	0.5	3.4	0.6	0.6	0.1	–	–
TN (filtered)	[mg/L]	73	24	75	5	107	15	1	57	845	56	414	43	929	281	2 006	105	761	507	6.8	2.2
NH4-N	[mg/L]	52	16	45	2	71	49	802	73	127	26	73	7	109	13	395	18	748	283	0.2	0.5
PO4-P	[mg/L]	23	6	36	8	42	4	37	8	38	28	47	6	53	3	104	11	–	–	0.9	0.5
Protein	[mg g^{-1}VS]	295	–	316	–	365	–	454	–	322	–	308	–	320	–	484	–	–	–	–	–
Lipids	[mg g^{-1}VS]	120	–	135	–	146	–	62	–	138	–	127	–	141	–	60	–	–	–	–	–
Carbohydrates	[mg g^{-1}VS]	585	–	549	–	489	–	484	–	540	–	564	–	540	–	455	–	–	–	–	–

(Table 1) was degasified for seven days prior to the start of the test. Six samples from the fermentation step and one sample of untreated sludge [PS_{65}/WAS_{35}, TS-basis] were analysed. All samples were analysed in triplicate, and each BMP set had triplicate control samples (cellulose) and triplicate blank samples (inoculum).

2.7. *Chemical analyses and analytical methods*

Soluble and total COD, total nitrogen (TN), ammonium-nitrogen (NH_4-N), phosphate (PO_4-P) and VFA were analysed in triplicates using spectrophotometric cuvette tests from Hach (Germany). Before the cuvette tests were used, the samples were prepared by centrifugation and filtration through 0.45-μm acetate filters. In addition, to quantify VFA species (C1-C6) and lactic acid, the samples were further filtered (0.22 μm) and acidified 10 % with 37 % H_2SO_4 and analysed using a high-performance liquid chromatograph (HPLC) Agilent 1100 Series with a refractive index detector and an ion exclusion column (Rezex ROA - Organic Acid $H+$, 300 × 7.80 mm, Phenomenex). The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mL/min). Protein (determined by organic nitrogen using the single factor: 6.25) and lipid content were analysed on sludge samples by an external laboratory (Agrilab AB, Sweden) where the following methods were used: TS determination - Standard Methods: 2540 B, total nitrogen modified method from SS-ISO 13 878, ammonium-nitrogen modified method AN 5226 based on ISO 11,732. Finally, carbohydrates were calculated using the remaining VS.

For statistical analysis, Welch's ANOVA was applied to the data, not assuming homogeneity of variance, to determine the significance of the response variables between scenarios ($p < 0.05$). Tukey's test was used to quantify differences between specific pairs of samples.

2.8. *Käppala WWTP – case study*

Käppala WWTP currently serves a population of 615 000 pe but is under reconstruction to increase the treatment capacity and meet new discharge nutrient requirements (TN <6 mg/L; total $P < 0.2$ mg/L). By 2028, the plant will serve an estimated 641,000 pe. The new design involves eleven separate treatment lines, nine with conventional activated sludge treating 68 % of incoming water and two with MBBR treating 32 %. All process reactors will have pre- and post-denitrification zones to achieve the nitrogen removal goal, requiring an external carbon source according to design calculations. Estimations of incoming water characteristics and seasonal variations were based on eight years of historical data, as well as design documentation provided by the consultant (Ramboll, Sweden). System boundaries for the calculations in this study included the primary sedimentation tanks, biology reactors (including nitrification and pre-post denitrification in both activated sludge and MBBR systems), secondary clarifiers and anaerobic digesters. Calculations and mass balances for assessing different carbon sources, effluent concentrations, sludge production and biogas production were based on design dimensions (Ramboll, Sweden), as well as specific activity rates, microbial performance, and other parameters from Tchobanoglous et al. (2014). The results obtained from the experimental phase of this study (THP, fermentation, denitrification, and BMP) were used to simulate scenarios using internal carbon sources, with methanol (design carbon source) as a benchmark for comparison. The Supplementary Material provides specific values and assumptions used in calculations and economic analysis and a description of Käppala WWTP.

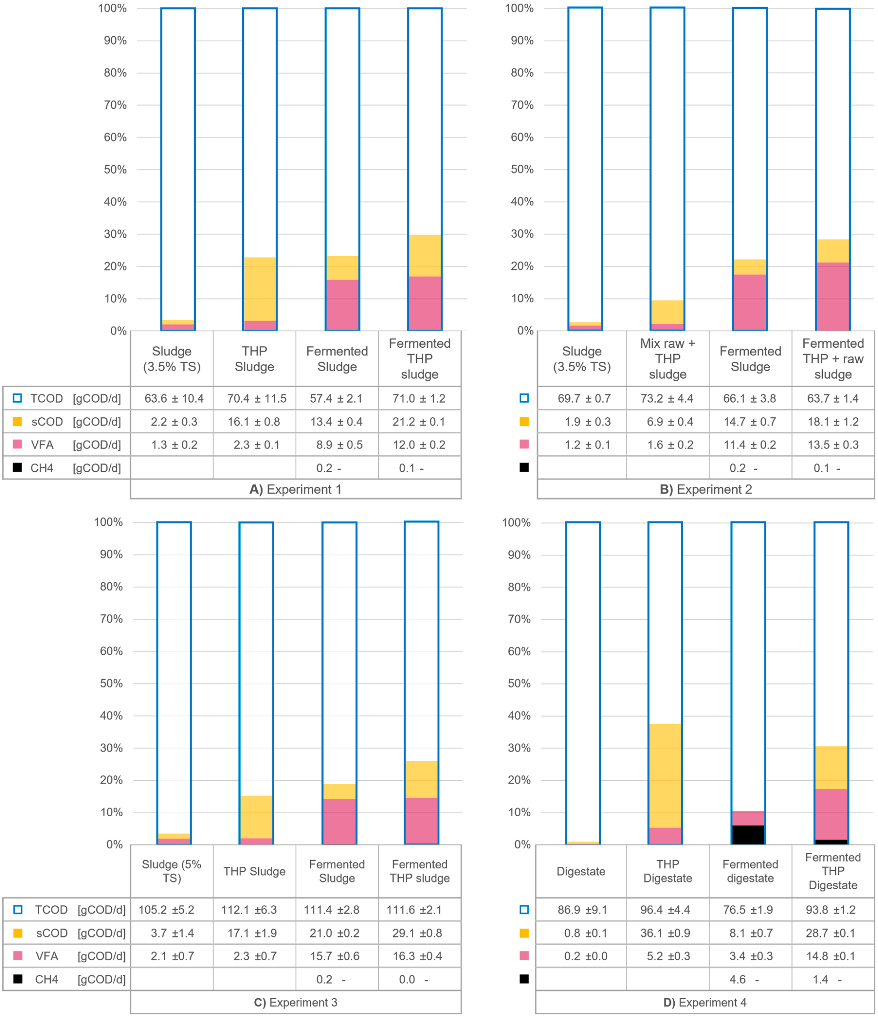
3. Results and discussion

3.1. *Thermal hydrolysis of mixed sludge and digestate*

Three different substrates were subjected to THP in the Cambi-Pilot: one digestate sample and two mixed sludge samples with lower (3.5 % TS) and higher (5 % TS) total solids (Fig. 1). The highest sCOD concentration after THP was obtained with the digestate (24 g sCOD/L), while corresponding values for the lower and higher TS sludge were 10.7 ± 0.5 and 12.2 ± 1.3 g sCOD/L, respectively. These values represented a carbon solubilisation degree of 23, 15 and 41 % for low and high TS sludges and the digestate, respectively (Table 1).

The sCOD increase after THP of sludge was similar to that observed in a previous study using full-scale THP treatment of mixed PS+WAS, where the solubilisation degree was 25 % (Zhang et al., 2019). However, Castro-Fernandez et al. (2023) found lower values (8 % solubilisation degree) also for mixed PS+WAS. The difference between the studies could be linked to different proportions of WAS in the substrate mixture. THP of WAS alone generally results in a higher solubilisation degree than when WAS is mixed with PS, resulting in a range of 19–49 % (Morgan-Sagastume et al., 2011; Zhou et al., 2021). A study by Yang et al. (2019) investigated a THP process with digestate and found 40 % carbon solubilisation and a final sCOD concentration of 15 g sCOD/L, which aligns with our results.

The higher carbon solubilisation post-THP, usually observed for digestate and WAS, may be attributable to differences in macromolecule composition. THP is known to have a more significant effect on proteins and carbohydrates, with lipids remaining unaffected (Li et al., 2022; Wilson and Novak, 2009). WAS is known to have higher protein and lower fat content than PS (Xiao and Zhou, 2020). In our digestate sample, proteins and carbohydrates accounted for 94 % of the VS, while in sludge samples, they accounted for 85–88 % (Table 1). This likely explains the higher solubilisation observed for digestate, as also reported by Svensson et al. (2018). Further, decomposition of amino acids to VFA, which is typically promoted at temperatures above 190 °C (Chen



	Sludge (3.5% TS)	THP Sludge	Fermented Sludge	Fermented THP sludge
□ TCOD [gCOD/d]	63.6 ± 10.4	70.4 ± 11.5	57.4 ± 2.1	71.0 ± 1.2
🟨 sCOD [gCOD/d]	2.2 ± 0.3	16.1 ± 0.8	13.4 ± 0.4	21.2 ± 0.1
🟥 VFA [gCOD/d]	1.3 ± 0.2	2.3 ± 0.1	8.9 ± 0.5	12.0 ± 0.2
■ CH4 [gCOD/d]			0.2 -	0.1 -

A) Experiment 1

	Sludge (3.5% TS)	Mix raw + THP sludge	Fermented Sludge	Fermented THP + raw sludge
□	69.7 ± 0.7	73.2 ± 4.4	66.1 ± 3.8	63.7 ± 1.4
🟨	1.9 ± 0.3	6.9 ± 0.4	14.7 ± 0.7	18.1 ± 1.2
🟥	1.2 ± 0.1	1.6 ± 0.2	11.4 ± 0.2	13.5 ± 0.3
■			0.2 -	0.1 -

B) Experiment 2

	Sludge (5% TS)	THP Sludge	Fermented Sludge	Fermented THP sludge
□ TCOD [gCOD/d]	105.2 ±5.2	112.1 ±6.3	111.4 ±2.8	111.6 ±2.1
🟨 sCOD [gCOD/d]	3.7 ±1.4	17.1 ±1.9	21.0 ±0.2	29.1 ±0.8
🟥 VFA [gCOD/d]	2.1 ±0.7	2.3 ±0.7	15.7 ±0.6	16.3 ±0.4
■ CH4 [gCOD/d]			0.2 -	0.0 -

C) Experiment 3

	Digestate	THP Digestate	Fermented digestate	Fermented THP Digestate
□	86.9 ±9.1	96.4 ±4.4	76.5 ±1.9	93.8 ±1.2
🟨	0.8 ±0.1	36.1 ±0.9	8.1 ±0.7	28.7 ±0.1
🟥	0.2 ±0.0	5.2 ±0.3	3.4 ±0.3	14.8 ±0.1
■			4.6 -	1.4 -

D) Experiment 4

Fig. 2. Carbon characteristics presented as load [gCOD/d], including total (T-) and soluble (s-) chemical oxygen demand (COD), volatile fatty acids (VFA) and methane (CH_4) in a) experiment 1, b) experiment 2, c) experiment 3 and d) experiment 4. $n = 9$ for SD.

et al., 2019; Körner, 2021), seemed to have occurred at 165 °C, as indicated in this study by the increase in VFA concentration in post-THP samples (before fermentation). The VFA concentration in THP-digestate, which had the highest protein content of the substrates tested, was twice as high (3.4 g VFA_{COD}/L after THP) as in the THP-sludge samples (1.1–1.5 g VFA_{COD}/L) (Table 1).

3.2. *Influence of THP on acidogenic fermentation of sludge and digestate*

3.2.1. *Solubilisation – effect on carbon*

The degree of carbon solubilisation during fermentation was generally low relative to that following THP alone (Fig. 2, Table 2). Higher carbon solubilisation was observed after fermentation of the untreated substrates (controls) compared with the THP-substrates, which was expected and supported previous findings (Zhang et al., 2019). However, acidogenic fermentation of THP-sludge and THP-digestate yielded higher total concentrations of soluble carbon and VFA compared with the controls (no THP) in all experiments (Fig. 2). Total carbon solubilisation degree, including THP plus fermentation of sludge, ranged from 24 to 31%, whereas in the controls (fermentation of untreated sludge), it was 16–18 % (Table 2). Fermentation of THP-digestate resulted in the highest total solubilisation degree (39.1 ± 0.1%) despite methane formation (Fig. 2) and a decrease in sCOD concentration during fermentation (from 24.1 ± 0.6 to 19.2 ± 0.1 g sCOD/L). Methane was also formed in the control fermenters in experiments 1–3 but at a lower level than in experiment 4 with digestate (Fig. 2). The formation of methane and pH are discussed later in Section 3.2.3.

During fermentation, significantly greater carbohydrate degradation was observed in the fermenters fed with THP-sludge (experiments 1–3) compared with the controls fed with untreated sludge (Fig. 4a). In contrast, experiment 4 (THP-digestate) showed low to no degradation of carbohydrates, with protein being the only macromolecule degraded (Fig. 4a). This differs from a previous study on the fermentation of THP-WAS, which, in spite of high protein content, showed high carbohydrate degradation (Shana et al., 2013). Lipids (<13 % of VS in all substrates) showed the lowest degree of degradation during fermentation. Lipid degradation is usually slow and requires longer retention times than carbohydrates and proteins (Law et al., 2023), but the fact that lipids were not reduced in the fermentation step is beneficial to the downstream biogas production (Section 3.4).

In experiment 2, fermenter F2 was fed with a 50:50 mixture of raw sludge and THP-sludge, which was expected to benefit the fermentation process. The results indicated that soluble carbon concentration and carbon solubilisation degree were only slightly lower in this fermenter (12.1 ± 0.1 g sCOD/L and 24 %, respectively) than in the fermenter fed with 100 % THP-sludge in experiment 3 (14. 1 ± 0.8 g sCOD/L and 31 %, respectively) (Table 2). These results show that reducing THP reactor volume by 50 % would decrease carbon solubilisation by only 25 % during fermentation. The possible influence of refractory products on fermentation is discussed in Section 3.2.2.

3.2.2. *VFA yield*

Despite the increase in the total degree of carbon solubilisation in all fermenters fed with THP-substrate compared with their controls, the VFA yield of the fermenters fed with THP-sludge in experiments 1 and 3 was not significantly higher than in their control fermenters ($p = 0.60$ and $p = 0.56$, respectively), indicating low conversion efficiency of solubilised organic matter to VFA (Fig. 3). However, in experiment 4, fermentation of THP-digestate resulted in a significantly ($p < 0.001$) higher yield (235 ± 2 g VFA_{COD}/kg VS_{in}) than the corresponding control, leading to a concentration 9.9 ± 0.9 g VFA_{COD}/L. The use of the mixed 50 % raw and 50 % THP sludge in experiment 2 was also beneficial for the fermentation process, yielding a significantly ($p = 0.01$) higher VFA (283 ± 7 g VFA_{COD}/kg VS_{in}) than the control (248 ± 4 g VFA_{COD}/kg VS_{in}).

The low VFA yield in experiments 1 and 3 contradicts the findings by Morgan-Sagastume et al. (2011) and Zhang et al. (2016), who reported that THP of sludge increases VFA yield during fermentation compared with untreated sludge. However, the pH in those studies was between 6.0 - 6.5, compared with 5.1 and 5.4 in our sludge fermenters, which

Table 2

Characteristics during the stable period of fermenters fed with untreated (F ctrl) and thermal hydrolysis pre-treated (THP) substrates in experiments 1–4. PS: Primary sludge, WAS: waste-activated sludge. ($n = 9$ for all samples; except protein, lipids and carbohydrates where $n = 3$ – samples taken in three different days.).

		Fermented Raw substrate (control)								Fermented THP substrate							
Experiment		1		2		3		4		1		2		3		4	
	Substrate	PS+WAS 3.5%TS		PS+WAS 3.5%TS		PS+WAS 5% TS		Digestate		TH 3.5%TS		50% TH Sludge 50% raw sludge 3.5%TS		TH 5%TS		TH Digestate	
Parameter	unit	av.	sd.	av.	sd.	av.	sd.	av.	sd.	av.	sd.	av.	sd.	av.	sd.	av.	sd.
TS	[%]	2.7	0.1	3.1	0.1	5.2	0.2	4.9	0.1	3.3	0.0	3.0	0.0	5.5	0.4	5.4	0.1
VS	[% of TS]	81.9	0.4	83.0	1.7	81.9	0.3	66.5	0.2	82.3	0.5	80.0	0.4	81.0	1.4	68.4	0.3
TCOD	[g/L]	38.2	1.4	44.0	2.5	74.3	1.9	51.0	1.3	47.3	0.8	42.5	0.9	74.4	1.4	62.5	0.8
sCOD	[g/L]	8.9	0.3	9.8	0.5	14.0	0.1	5.4	0.5	14.1	0.1	12.1	0.8	19.4	0.5	19.2	0.1
VFA	[g/L]	5.9	0.3	7.6	0.1	10.5	0.4	2.2	0.2	8.0	0.1	9.0	0.2	11.0	0.3	9.9	0.1
TN (filtered)	[mg/L]	500	43	618	58	1 038	127	1 711	28	915	10	800	69	1 330	106	2 102	113
NH4-N	[mg/L]	430	21	447	20	871	143	1 301	206	582	8	604	11	856	103	1 642	72
PO4-P	[mg/L]	178	4	197	11	203	17	118	5	217	1	187	5	268	21	208	6
Protein	[mg g^{-1}VS]	333	13	311	13	289	2	410	8	313	14	334	8	316	8	373	7
Lipids	[mg g^{-1}VS]	134	16	141	8	158	2	63	0	160	3	143	12	162	4	70	1
Carbohydrates	[mg g^{-1}VS]	533	3	548	21	553	4	527	8	527	17	522	21	522	6	557	6
OLR fermenter	[kgVS/d]	7.3	0.9	7.4	0.2	11.0	0.7	9.4	0.7	7.4	1.0	7.3	0.0	11.8	0.1	9.6	0.5
Inflow fermenter	[L/d]	1.5	0.0	1.5	0.0	1.5	0.0	1.5	0.0	1.5	0.0	1.5	0.0	1.5	0.0	1.5	0.0
Solubilisation degree Thermal hydrolysis	[% - sCOD/ TCOD in]	–	–	–	–	–	–	–	–	22.6	–	22.6	–	14.5	–	41.0	–
Solubilisation degree Fermenter	[% - sCOD/ TCOD in]	18.2	0.7	18.72	1.1	16.5	0.3	10.3	1.0	12.9	0.2	17.4	1.8	12.1	2.7	0 (−15.6)	0.2
Solubilisation degree TH + Fermenter	[% - sCOD/ TCOD in]	18.2	0.7	18.72	1.1	16.5	0.3	10.3	1.0	31.0	0.2	23.8	1.8	24.4	1.1	39.1	0.1

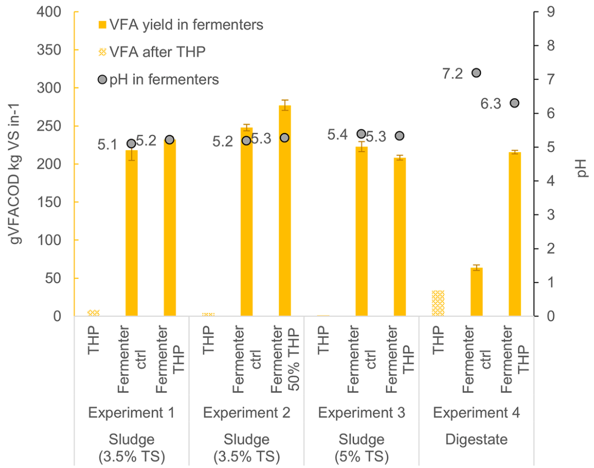


Fig. 3. Volatile fatty acid (VFA) yield and pH in the fermenters run in experiments 1–4, which were fed with different sludges (1–3) and digestate (4), un-treated (ctrl) or pre-treated with thermal hydrolysis (THP).

possibly affected the overall sCOD conversion efficiency (Fig. 3). The importance of pH is discussed in Section 3.2.3.

Another potential explanation for the low VFA yield could be attributed to the Maillard reaction, which has been suggested as a cause for the ineffectiveness of THP for enhanced fermentation and VFA production (Castro-Fernandez et al., 2023; Yan et al., 2022). Maillard reaction products (MRPs), such as melanoidins and other refractory dissolved organic compounds, are formed from the reaction of reducing sugars with amines triggered by increasing temperatures above 140 °C (Ngo et al., 2021; Wilson and Novak, 2009). MRPs can be toxic to biochemical processes, including anaerobic digestion (Q. Wang et al., 2021). In Experiment 2, the highest VFA yield was observed while the fermenter received only half of the substrate from THP, likely diluting the inhibitory compounds. Similary, in Experiment 4, the fermenter fed with THP-digestate resulted in a significantly higher VFA yield than the control, even after accounting for methane production. This outcome suggests that the limited degradation of carbohydrates (as seen in Fig. 4a) may have reduced the likelihood of the Maillard reaction occurring. Additionally, Experiments 1 and 3, which involved sludge with higher carbohydrate content, showed no significant difference in VFA yield compared to their controls, further supporting the hypothesis that inhibition may be linked to the presence of certain compounds generated during the THP.

The dominant carboxylic acids observed were acetic and propionic acids, with lower levels of longer carbon-chain acids in all experiments (Fig. 4a). In contrast, Xiang et al. (2023) observed a pattern of increase in acetate and a simultaneous decrease in propionate during the fermentation of THP-sludge, compared to untreated sludge. Specific

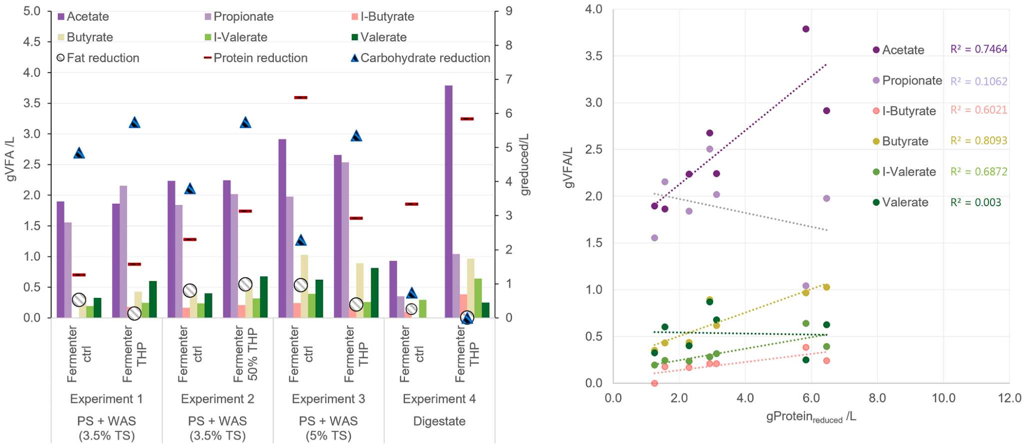


Fig. 4. a) Carboxylic acids (C2-C5), protein reduction (red dashes), carbohydrate reduction (blue triangles) and lipid reduction (grey circles) in fermenters fed with different sludges (experiments 1–3) and digestate (experiment 4), un-treated (ctrl) or pre-treated with thermal hydrolysis (THP). b) Yield (g/L) of volatile fatty acids (VFA) versus reduction in proteins, using results from all fermentation experiments.

VFA production in the fermentation of mixed waste streams is a complex process involving a dynamic community of microorganisms with somehow unpredicted behaviour (Regueira, Lema, et al., 2020). Generally, the VFA species formed during fermentation seem to be determined mainly by the composition of the substrate (Regueira, Bevilacqua, et al., 2020), degradation of macromolecules and operating conditions, and not by the THP per se (Liu et al., 2022; Morgan-Sagastume et al., 2011; Zhang et al., 2019).

Our results suggest that acetate production is mainly linked to protein degradation. This is supported by the observation that acetate concentrations were highest in fermenters with the greatest extent of protein degradation. Additionally, a linear positive correlation was observed between levels of acetic and butyric acid and protein reduction ($R^2 > 0.75$) (Fig. 4b). While protein degradation does not follow fixed stoichiometry, acetate has been identified as the main degradation product. The yield ratio of acetate to protein (g Ac/g Prot) tends to increase with higher pH (Regueira, Lema, et al., 2020). This trend was also evident in our results (Fig. 4b). No other correlations were seen between the reduction in macromolecules and the production of specific acids. However, carbohydrate degradation appeared to follow propionate and valerate formation in experiments 1–3 (Fig. 4a), similar to findings by Rafay et al. (2022).

3.2.3. *Influence of pH*

The fermentation inoculum (digestate from Käppala WWTP) had an initial pH of 7.1 ± 0.2 (Table 1). During the first 5–10 days of fermentation, the pH decreased and stabilised between 5.1 and 5.4 in experiments 1–3 (Fig. 3). Detailed pH, VFA and sCOD profiles throughout the experiments are included in the supplementary material (Figure S3). pH plays a crucial role in the acidogenic fermentation process because carboxylic acids, including VFA, are weak acids (pKa 4.75–4.90). At lower pH, a higher proportion of VFA exists in their undissociated form, negatively affecting microbial growth (Infantes et al., 2012). A pH between 5.5 and 7.0 is beneficial for higher VFA yields during fermentation (Agnihotri et al., 2022).

To determine if the low VFA yields in experiments 2 and 3 resulted from low pH rather than poor substrate biodegradability (i.e. MRPs), the pH was adjusted to 6 after the fermentation trials ended on day 19. The fermentation was continued for an additional 4 days. An immediate increase in sCOD and VFA concentrations was seen in both experiments. Specifically, in experiment 2, the sCOD concentration increased by 35 % [F1-control] and 23 % [F2$_{50\ \%}$raw and $_{50\ \%}$THP]. In experiment 3, the increases were 28 % [F1-control] and 2 % [F2 THP-sludge]. The increase in VFA yield was greater in the fermenters fed with raw sludge (45 % and 19 % in experiments 2 and 3, respectively) than in those fed with THP-substrate in those experiments (31 % and 19 %, respectively). The results suggest that increasing pH had an immediate positive effect on the solubilisation of carbon and VFA production, especially in the fermenters with untreated sludge. However, the process was only operated for one retention time, and longer-term experiments are needed to confirm these indications.

In experiment 4, the pH stabilised at 6.3 in the fermenter fed with THP-digestate (without pH control), which led to increased methanogen activity while still allowing the accumulation of VFAs (Fig. 3). In contrast, the pH in the control fermenter (untreated digestate) was higher (7.2), favouring methanogenesis and converting most VFA to methane (Fig. 2d, Fig. 3). The observed methanogen activity (215 mL CH_4/kg VSin) could also have been influenced by the feed of untreated digestate, which likely contained active methanogens.

3.3. *Performance of carbon sources in denitrification*

Based on the results obtained during THP and fermentation, six samples were chosen for evaluation as carbon sources in denitrification tests (Fig. 1). The selection criteria were the levels of VFA and soluble COD. One sample from experiment 2 (fermented THP$_{50\ \%}$), all three samples from experiment 3 and two samples from experiment 4 (THP-digestate with and without fermentation) were selected. Comparing denitrification rates between studies is difficult due to variations in microbial communities and operating conditions, so reference substances are commonly used to contextualise results (Elefsiniotis et al., 2004; Grana et al., 2024). In this study, acetic acid was used as the reference, which resulted in a denitrification rate of 8.1 mg NOx-N/g VSS•h in activated sludge (Fig. 5) and 0.5 g NOx-N/m^2•d in MBBR. The denitrification profiles can be found in the supplementary material (S4-S10).

The results obtained for the six carbon sources tested (Fig. 6) suggested a correlation between the VFA/sCOD ratio and denitrification rate ($R^2 = 0.702$). This is consistent with the notion that carbon sources with simpler molecular structures, e.g. VFAs, are preferred by heterotrophic denitrifiers due to their ability to produce electrons and energy more efficiently, thereby promoting the denitrification process (Elefsiniotis et al., 2004; Wei et al., 2022).

The lowest denitrification rates (<5 mg NOx-N/g VSS•h) were observed with carbon sources produced by non-fermented THP substrates (both sludge and digestate) (Fig. 5). Despite the high soluble carbon content, these had low VFA concentrations and hence low VFA/sCOD ratio. This suggests that the sCOD was composed mainly of longer chain products, including soluble protein, reduced sugars and humic acid substances, that were not converted to VFA, and cannot be used as ready biodegradable material (Chen et al., 2024). Moreover, despite a decrease in nitrate concentrations, no reduction in sCOD was seen during these denitrification tests (Figure S6 and Figure S9 – Supplementary material), indicating simultaneous production and consumption of organic matter, as reported previously for similar carbon sources (Guo et al., 2017).

The fermentation liquid from the control fermenter in experiment 3 displayed a significantly *($p = 0.004$)* higher rate (6.8 mg NOx-N/g VSS•h) than the hydrolysis liquid from unfermented THP-sludge (4.6 mg NOx-N/g VSS•h). This result aligns with previous research indicating that thermal hydrolysis liquids perform less effectively than fermentation liquids as carbon sources for denitrification (Guo et al., 2017; Sun et al., 2016). The carbon sources from fermented sludge and unfermented THP-substrates resulted in significantly ($p = 0.01$) lower rates compared to the control acetic acid. However, contrary to our results, some studies have reported higher denitrification rates with fermentation liquid (Liu et al., 2016) and THP liquid (Barlindhaug and Ødegaard, 1996) than with methanol, acetic acid and propionic acid.

The carbon sources produced by fermentation of THP-substrates (F2 THP) achieved the highest denitrification rates in all experiments. Specifically, fermented THP-digestate and fermented 50:50 mixed raw-THP sludge resulted in 8.8 and 8.7 ± 0.3 mg NOx-N/g VSS•h, respectively, followed by fermented THP sludge (7.6 ± 0.8 mg NOx-N/g VSS•h) (Fig. 5). No significant difference was found between these rates and the acetic acid reference ($p = 0.51$). These results indicate that the addition of THP prior to fermentation improved the quality of the carbon source produced. Additionally, in experiment 3, the denitrification rate using fermented THP-sludge was 13 % higher than that of the control fermented untreated sludge and 66 % higher than for THP-sludge without fermentation. Despite the lack of impact of THP on VFA yield (Fig. 3), it positively influenced the denitrification rates obtained.

Interestingly, THP-fermented digestate, despite its low VFA/sCOD ratio (<0.5), achieved one of the highest denitrification rates (Fig. 5). To our knowledge, no previous study has investigated fermented THP-treated digestate as a carbon source for denitrification. One contributing factor to the high rate of this substrate could have been the higher acetate and butyrate concentrations in the sample. Previous studies have shown that acetic and butyric acid led to higher denitrification rates than other acids (Elefsiniotis and Wareham, 2007; Grana et al., 2024). However, the preference for single VFAs seems to be dependent on the microbial community and not on electron transfer efficiency alone (Li

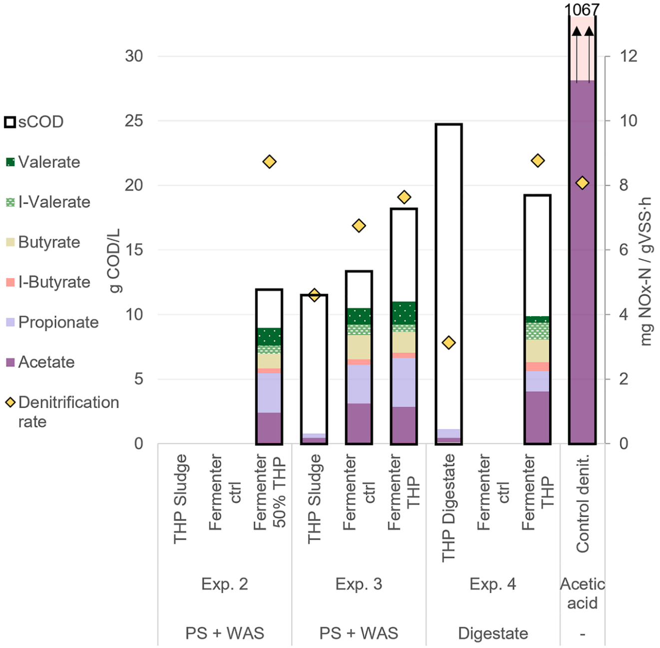


Fig. 5. Carboxylic acids (C2-C6; g COD/L), soluble chemical oxygen demand (sCOD, black rectangles, g/L) and denitrification rates (yellow diamonds, mg NOx-N/g VSS•h) for carbon sources produced during fermentation of sludges and digestate, untreated (Ctrl) and THP. Experiments 2–4. Acetic acid sCOD=1067 g/L (reference).

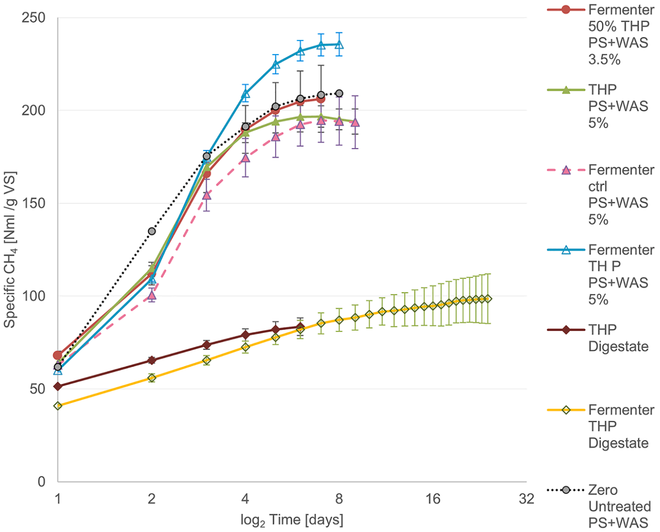


Fig. 6. Biomethane potential (BMP) from sludge solid phase samples recovered after centrifugation of fermentates from the 4 experimental trials performed in the present study (Fig. 1) and THP-treated sludge and digestate.

et al., 2023).

Protein degradation during fermentation was correlated with the denitrification rate (R^2=0.7), likely due to the higher acetate and butyrate production resulting from this degradation. Another contributing factor to the high rate of fermented THP-digestate could be the relatively low biomass growth yield obtained ($Y_{OHO\ gCOD/gCOD}$), which leaves more carbon source available for denitrification (Zhang et al., 2016).

The nitrate removal efficiency exceeded 96 % with fermented THP-substrates, while the unfermented THP-based carbon sources achieved ~70 %. However, the presence of other nitrogen fractions (e.g. NH_4-N and organic nitrogen) in the carbon sources reduced total nitrogen removal efficiency to 75–88 % with fermented THP-substrates and to below 50 % with unfermented THP substrates (Table 2). The release of NH_4-N and PO_4-P during the fermentation of proteins and carbohydrates is a significant concern when using carbon sources from sludge fermentation for denitrification. Nevertheless, process calculations in this study (see Section 3.4) showed that the effluent limits were met with all carbon sources.

Finally, two denitrification tests with MBBR as inoculum were performed with acetic acid and with the carbon source from fermented THP-digestate. They achieved denitrification rates of 0.4 and 0.5 gNOx-N/m^2•d, respectively (p = 0.003), which despite being in the low range (Owusu-Agyeman et al., 2023; Sapmaz et al., 2022), indicated comparable performance to that obtained with AS. The results reveal that digestate-based carbon sources had similar or higher efficacy than acetic acid in both inocula.

The high rate obtained using the fermented THP-digestate is a novel finding and holds promise for the integration of a process to produce carbon sources at WWTP. Additionally, the fermentate from 50:50 mixed raw and THP sludge showed a high denitrification rate (Fig. 6), illustrating the potential for reducing energy consumption and investment costs by requiring 50 % of the THP system volume compared to the other scenarios (Fig. 1).

3.4. Downstream biogas production

In the full-scale installation, the solid phase obtained after centrifugation and removal of the VFA-rich carbon source was assumed to be used for biogas production. To simulate this process and evaluate biogas loss by removal of VFA, the methane potential of the solid phase samples was determined in BMP tests (Figure S9). In total, one sample of mixed raw sludge and the solid phase of the six samples from which the liquid phase was used in the denitrification tests (Fig. 1) were evaluated. The methane produced by the non-centrifuged mixed raw sludge represented the zero scenario when calculating biogas loss.

All samples reached their maximum methane potential within 10 days after initiation of the BMP test, except for the fermented THP-digestate, which continued to produce methane until day 25 (Fig. 6). The slower methane production rate in that sample suggested the presence of more complex organic matter. Digestate is known to contain high residual protein coupled with recalcitrant material, such as collagen, which is known to be difficult to degrade (Ekstrand et al., 2022). The mixed untreated raw sludge (zero) sample achieved a BMP of 209 NmL CH_4/g VS, which is within the normal range for mixed PS and WAS (160–350 NmL CH_4/g VS) (Schnürer and Jarvis, 2010). No significant difference was found between the BMP from the zero scenario and that from the samples originating from fermented $THP_{50\ \%}$, THP-sludge and untreated fermented sludge (p = 0.9) (Table 3). In other words, methane yield (Nm^3/kg VS) was not lower for any of the mixed sludge samples where the VFA-rich liquid phase had been extracted compared with the zero scenario, regardless of the method of VFA production. The fermented THP-sludge sample exhibited a higher BMP value (236±6 NmL CH_4/g VS) than the zero scenario. While the difference was not significant (p = 0.06), it indicated that when mixed sludge was subjected to both THP and fermentation, the methane yield per g VS could increase, even after extracting VFA. One influencing factor for the results was that the lipid share of VS was higher in the fermented sludge and the fermented THP-sludge (with the highest BMP) than in the other samples. This was indicated by a positive correlation (R^2=0.9421) between the lipid content in mg g^{-1} VS in the fermentates (Table 2) and their methane potential (Table 3). Lipids have the highest energy yield of all macromolecules (1.014 L CH_4/g VS), compared with 0.496 L CH_4/g VS for proteins and 0.415 L CH_4/g VS for carbohydrates (Cirne et al., 2007; Magdalena et al., 2018).

The sample from the fermented THP-digestate, which exhibited the highest denitrification rate, had a yield of 101 mL CH_4/g VS, while the sample from unfermented THP-digestate had a yield of 84 mL CH_4/g VS. Similarly, Svensson et al. (2018) obtained BMP of around 50 mL CH_4/g TS (VS 68 %) for dewatered digestate cake exposed to THP (165 °C). These results demonstrate the potential of utilising the digestate for carbon source production without reducing biogas production, as the substrate is taken after AD. Additionally, recirculating part of the solid fraction could even increase biogas production.

3.5. Evaluation of carbon sources

The experimental results were used as a base for scaling-up calculations to determine potentially feasible scenarios at Käppala WWTP. Evaluation criteria included carbon solubilisation, VFA yield, denitrification rate, sCOD/TN ratio, sCOD/TP ratio and BMP (Table 3). Among the scenarios considered, the best overall results were obtained for: fermented 50:50 mixed raw and THP sludge in experiment 2; fermented sludge and fermented THP-sludge in experiment 3; and fermented THP-digestate in experiment 4 (marked * in Table 3). These scenarios were further assessed in process calculations and full-scale implementation

Table 3

Summary of results of the different scenarios, including Carbon solubilisation, VFA yield, Denitrification rate, C/N ratio, TP ratio and BMP. TN: total nitrogen, TP: total phosphorous. The red numbers in the upper right corner represent the ranking of scenarios used to select the most suitable. n = 9.

	Carbon Solubilisation	VFA yield	Denitrification rate	sCOD/ TN	sCOD/ TP	BMP
	[g sCOD/TCOD in]	[g VFACOD/kg VS in]	[mg NOx-N/g VSS.h]	–	–	[mL CH_4 /g VS]
Fermented * 50 % THP PS+WAS Exp. 2	**3** 23.8 ± 1.8	**5** 279 ± 7	**5** 8.7 ± 0.3	**3** 15.1	**4** 61.8	**4** 206 ± 18
THP PS+WAS Exp. 3	**1** 14.7	**1** 1.1	**2** 4.6 ± 0.02	**2** 10.0	**4** 72.8	**4** 197 ± 6
Fermented * ctrl PS+WAS Exp. 3	**2** 15.5 ± 0.3	**3** 223 ± 6	**3** 6.8 ± 0.1	**3** 12.9	**4** 63.6	**4** 195 ± 12
Fermented * THP PS+WAS Exp. 3	**3** 22.3 ± 1.1	**3** 208 ± 3	**3** 7.6 ± 0.8	**3** 13.8	**4** 69.6	**5** 236 ± 6
THP Digestate Exp. 4	**5** 41.0	**2** 34.1	**1** 3.1 ± 0.3	**2** 11.4	**5** 112.8	**5** 84 ± 5
Fermented * THP Digestate Exp. 4	**5** 39.1 ± 0.1	**4** 235 ± 2	**5** 8.8 ± 0.4	**1** 9.1	**4** 87.9	**5** 101 ± 7

for Käppala WWTP in 2028.

3.6. Upscaling results - Käppala WWTP 2028

Käppala WWTP has a set yearly average discharge limit of 6 mg TN/L. In this study, projected data for Käppala 2028 were used for assessment, including an average inflow of 1.97 m^3/s, with 32 % entering the MBBR reactors and 68 % the activated sludge reactors. The inflow characteristics were: BOD_5 60 g/p.d, Tot-N 12.8 g/p.d, Tot-P 1.6 g/p.d, SS 90 g/p.d and alkalinity 88 g/p.d, and monthly variations were calculated using historical data from 2013 to 2020. All input data are presented in Supplementary Material. A summary of the main results is presented in Table 4.

In the base scenario with methanol, the estimated early average nitrate removal requirement in the post-denitrification at Käppala was 629 kg NO_3/d, consuming on average 3 m^3 MeOH/d with a dose of 18 gCOD/m^3 incoming water. This consumption corresponded to a cost of EUR 0.3–1 million per year, depending on methanol prices.

To estimate the amount of internal carbon source needed for nitrate reduction, the concentrations of sCOD and VFA, the NH_4-N load accompanying the carbon source, and the denitrification rates were considered. The nitrate load to be removed increased in all scenarios compared with the base (methanol) scenario due to higher NH_4 (Table 2). The volume of carbon source required to reach 6 mg TN/L ranged between 382 and 708 m^3/d and was ~200 times higher than that of methanol (Table 4). The scenario with fermented THP-digestate had an estimated requirement of 517 m^3/d, which was relatively high due to the high ammonium content in the carbon source. Furthermore, the dose (g COD/m^3 incoming water) was higher in all internal carbon sources scenarios than in the base scenario, meaning that more carbon was used overall. One explanation is the extra nitrogen in the system, but it should also be noted that anoxic growth yield (Y_{OHO}) is lower in methanol than in VFA or other carbon sources (Tchobanoglous et al., 2014).

The amount of substrate required to produce each carbon source was estimated to evaluate the feasibility of internal production at Käppala WWTP. The estimates were based on VFA yield (g VFA/kg VS_{in}) and any VS losses during THP and fermentation, as obtained in the bench-scale fermenter trials. The activated sludge produced with different carbon sources was based on bacterial growth yield (Y_H) and carbon source addition (Table 4). The WAS level increased in all scenarios by between 0.5 and 1.9-tonVS/d (3 % and 11 %), compared with the base scenario. Furthermore, with a fermenter operating with HRT of 4 d, the sludge requirement in scenarios using substrates from experiments 2 and 3 was 46–60 % of total sludge production at Käppala, which can be considered feasible (Table 4). On the other hand, the volume of digestate seemed to be insufficient to replace all the carbon source required, as calculations showed a need for 120 % digestate. However, using digestate would considerably reduce the costs of sludge handling, which for Käppala WWTP accounted for EUR 2.3 M in 2022. Since transportation of centrifuged digestate accounts for a large part of the operating costs (OPEX) of WWTPs, a reduction in the amount could have a great impact on the general costs.

Loss of biogas was calculated using the BMP of the different carbon sources obtained in the tests, and the loss of VS due to THP, fermentation and separation of VFA-based carbon source (Table 3). The estimated total biogas production in Käppala 2028 with methanol (not using any sludge for VFA production) was an average of 13,209 Nm^3 CH_4/d. The use of sludge for the production of internal carbon sources led to a reduction in biogas production: 15 % with fermented 50:50 mixed raw and THP-sludge, 20 % with fermented untreated sludge, and 9 % with fermented THP-sludge. If the digestate were used, the reduction of biogas would be 0 %, and there could even be an increase in biogas production, e.g. by recirculating back 50 % of the residue and producing an extra 1351 Nm^3 CH_4/d (Table 4).

Energy consumption in a thermal hydrolysis system usually varies between 17 and 34 kWh/m^3, but in most cases, it has a positive impact on the overall energy balance of a WWTP and could even be energetically self-sufficient (Ferrentino et al., 2023). Due to the complexity of the current energy flows at Käppala, we did not include THP energy calculations in this study. In terms of costs, without including capital costs, using fermented THP-digestate would be beneficial, even compared with the base scenario. The extra biogas produced (+0.4 M €/year - Table 4), in addition to the reduction in methanol costs (−0.3 M €/year), shifted the balance towards this scenario (Table 4). However, as mentioned, the nitrogen content is a concern, and longer-term trials should be performed to evaluate this scenario fully. Finally, a more extensive economic assessment, including capital costs, will facilitate the decision-making.

Analysis of an approach for producing internal carbon sources to replace methanol in Käppala WWTP by 2028 indicated the benefits of including THP before acidogenic fermentation without exponentially increasing the OPEX. The sludge scenarios analysed could be

Table 4
Results from process calculations and costs associated with scaling-up calculations for the 4 selected scenarios. The red values represent an optional recirculation of digestate. Methanol price=444€/m^3.

	Unit	Methanol	Fermented 50 % THP PS+WAS Exp. 2	Fermented ctrl PS+WAS Exp. 3	Fermented THP PS+WAS Exp. 3	Fermented THP Digestate Exp. 4
Nitrate requirement	Ton NO_3–N /d	0.6	1.1	1.0	1.0	1.5
CS requirement	m^3/d	3	708	491	382	517
Dose	gCOD/m^3 incoming water	18	55	47	46	57
PS production	Ton VS/d	33	33	33	33	33
WAS production	Ton VS/d	17	19	17	17	18
PS+WAS production	Ton VS/d	50	52	51	50	51
Digestate production	Ton VS/d	25	26	25	25	25
Sludge requirement for CS production	Ton VS/d	–	31	32	24	–
Digestate requirement for CS production	Ton VS/d	–	–	–	–	31
	VS% av TS					
	Ton TS/d		39	40	31	46
% of total produced	%	0 %	59 %	63 %	48 %	123 %
Volume fermenter	m^3	240	5 107	2 989	2 185	3 349
Methane production	Nm^3 CH_4/d	13 209	11 262	10 588	12 046	13,209 + 1351
Cost methanol	M euros/ year	−0.3	0	0	0	0
Biogas revenue	M euros/ year	4.3	3.6	3.4	3.9	4.3 + 0.4
Cost of sludge handling	M euros/year	−3.6	−3.7	−3.7	−3.6	−3.7
Total	M euros/year	0.3	−0.1	−0.2	0.3	0.6 + 0.4

implemented in practice to internally produce enough carbon source for denitrification. The digestate could be sufficient to replace 80 % of the carbon source on a yearly average basis, although there is great variation between winter and summer months due to seasonal temperature variation (Figure S4).

4. Conclusions

- The inclusion of THP before acidogenic fermentation had an overall positive effect on the production and quality of the carbon source used for denitrification and further generation of biogas.
- Fermentation of THP-digestate resulted in a better carbon source for denitrification compared with fermented raw and/or THP-mixed sludge. It achieved a higher degree of carbon solubilisation (39 %) and VFA (9.8 g VFA_{COD}/L), which resulted in a higher denitrification rate (8.77 mg NOx-N/g VSS•h).
- Carboxylic acid production during fermentation may be correlated with specific macromolecule degradation. For example, protein degradation led to an increase in acetate and butyrate, while carbohydrate degradation seemed to be related to propionate and valerate production.
- Process calculations based on Käppala WWTP 2028 showed that all carbon sources tested could replace fossil-based methanol and meet the effluent nitrogen limit (6 mg N/L) despite their high nitrogen content. Using THP-digestate could potentially also increase biogas production at Käppala WWTP and decrease the costs of sludge handling.

CRediT authorship contribution statement

Andrea Carranza Muñoz: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jesper Olsson:** Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Andriy Malovanyy:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Christian Baresel:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Nethra Machamada-Devaiah:** Validation, Investigation, Data curation. **Anna Schnürer:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122426.

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III

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Replacing methanol with internally produced VFA-based carbon source for denitrification at the Henriksdal WWTP

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ABSTRACT

To meet future nitrogen removal targets, Henriksdal wastewater treatment plant (WWTP) will require external carbon addition, estimated at 8 tons COD/day by 2040, due to low influent BOD/TN ratios, precipitation chemical use, and low temperatures. Methanol, the projected option, is fossil-based and contributes to indirect greenhouse gas emissions. This study evaluated a volatile fatty acid (VFA)-based fermentate produced from primary sludge and food waste in a 2 m^3 mesophilic fermenter as a carbon source for denitrification. The filtrated fermentate was tested as carbon source in both batch denitrification tests and pilot-scale MBR (4.5 m^3/h), where it was dosed for 70 days, replacing glycerol currently used at Henriksdal and Henriksdals WWTP. In batch tests, the fermentate achieved a 40% higher denitrification rate than glycerol. Pilot trials showed a 30% higher denitrification rate and 50% lower carbon consumption while maintaining effluent concentration below 3 mg NO_3^- -N/L. Microbial analysis revealed no significant community changes with the carbon source transition, indicating effective VFA uptake by existing microorganisms. Full-scale projections suggested that replacing methanol would require 10% of the plant's primary sludge plus food waste. Although this sludge use would reduce biogas production, methane potential tests showed that recycling of the fermentate solid fraction would result in only 2% lower biogas production, representing a minor trade-off.

Key words: carbon source, denitrification, microbiology, resource recovery, VFA, wastewater

HIGHLIGHTS

- VFA-based fermentate achieved 40% higher denitrification rate than glycerol in batch tests.
- Carbon consumption ratio of fermentate was 50% lower than that of glycerol in pilot trials.
- Changing glycerol to fermentate as carbon source did not change the dominant bacterial communities in sludge.
- Replacing methanol with VFA-based fermentate will require 10% of PS and external FW, reducing biogas production by 2% in full-scale.

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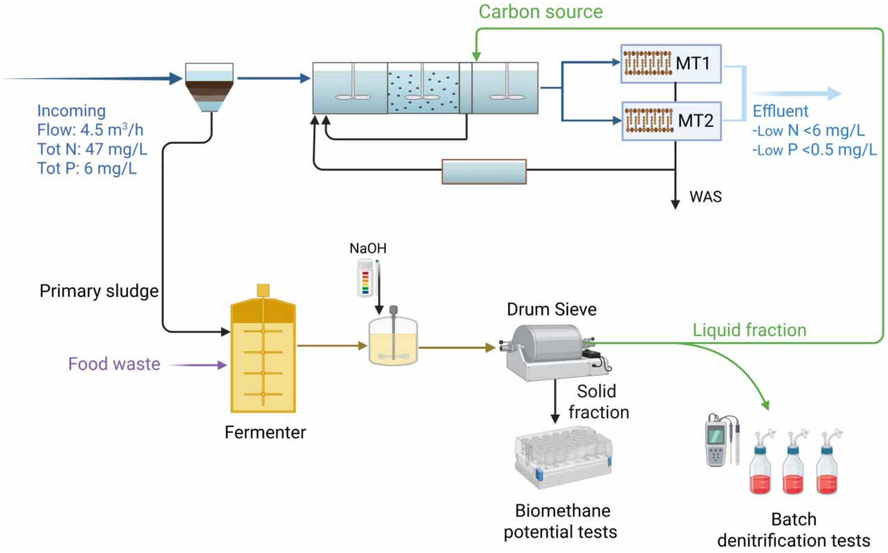
GRAPHICAL ABSTRACT



1. INTRODUCTION

The Swedish government and the European Union (EU) have set new discharge limits, requiring many wastewater treatment plants (WWTPs) to meet 8 mg/L TN and 0.7 mg/L TP, with some plants in Sweden facing stricter targets as low as 6 mg/L TN and 0.2 mg/L TP (Swedish Environmental Protection Agency 2018; European Parliament 2024). As the Baltic Sea is considered a sensitive water body by the Swedish government, Henriksdal WWTP (Stockholm, Sweden) is required to meet stricter discharge targets. Therefore, it is being reconstructed with membrane bioreactor (MBR) technology to increase capacity and comply with the new limits. The process includes biological nitrogen removal via nitrification and denitrification, and chemical phosphorus precipitation using Fe^{2+} (ferrous sulphate) (Figure 1). Heterotrophic denitrification is the most widely used nitrogen removal process, which requires an electron donor (theoretically 2.86 g COD/g NO$_3^-$-N) to reduce nitrate (NO$_3^-$) to nitrogen gas (N$_2$). The carbon present in influent wastewater is usually sufficient to carry out complete denitrification, but additional external sources are sometimes needed to reach low nitrate levels ($<$5 mg/L), particularly when BOD/TKN ratios are low and hydrolysis is reduced at low temperatures, both of which limit denitrification (EPA 2013; Tchobanoglous *et al.* 2014; Fu *et al.* 2022). This is the case at Henriksdal WWTP, where low effluent P limits require high iron doses in the primary sedimentation tanks, reducing the BOD/TN ratio in the influent to the biology reactors to 3:1. The dose of an external carbon source needed in the post-denitrification zone has been projected to be ∼1,500 tons of MeOH/y (3,000 tons COD/y) by 2040, influencing operational costs and indirect CO$_2$ emissions. Fossil-based methanol is a commonly preferred carbon source for denitrification (Fu *et al.* 2022) because of its high carbon content (1,500 g COD/L MeOH) and previously low cost (∼200 €/ton). However, recent geopolitical factors have raised prices to ∼700 (Methanex Corporation 2025), and high CO$_2$ emissions associated with the production also conflict with the EU directive's goals for energy neutrality and emission reduction (European Parliament 2024). Therefore, identifying alternative carbon sources from locally available organic waste is important to enhance circularity, reduce emissions, and improve self-sufficiency.

The application of alternative carbon sources for denitrification has been summarised by several authors (Christensen & Harremoës 1977; Fu *et al.* 2022; Ahmed *et al.* 2023), but its use in large-scale systems remains limited. The most efficient carbon source for denitrification is known to be acetic acid, followed by other volatile fatty acids (VFAs) and lactic acid (Elefsiniotis *et al.* 2004). VFAs are effective carbon sources for denitrification due to their simple structure and high energy yield, leading to higher rates and fewer intermediates, like NO$_2$ and N$_2$O, compared to other carbon sources, like

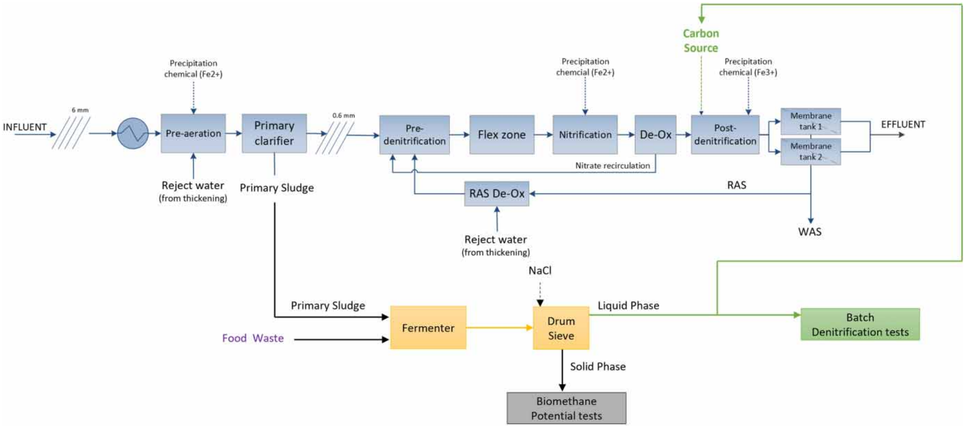


Figure 1 | Experimental scheme of the MBR pilot line (blue), including all the chemical and carbon source additions. The carbon source line production (yellow) includes the fermenter, the separation with a drum sieve and pH control. The liquid phase (green) was used in denitrification batch tests and pilot trials, and the solid phase (black) was used in the biomethane potential test.

methanol, ethanol or glycerol (Wei *et al.* 2022). One way to produce VFAs and other organic compounds in a cheaper way is through the fermentation of waste streams like sewage sludge and food waste (FW) (Atasoy *et al.* 2018; Liu *et al.* 2020; Battista *et al.* 2022), both of which are available at many WWTPs in Sweden. However, using sludge for purposes other than biogas production is a concern since it represents a significant revenue source for the facility included in the present study (~0.5–1 €/N m^3 CH_4). Furthermore, depending on the process and the substrate, a carbon source produced via fermentation of sewage sludge and FW can contribute to additional ammonium and phosphate loads and affect effluent concentrations, which should be considered during process design and operation.

Understanding microbial dynamics when different carbon sources are used is crucial. The roles of individual microbes in ecosystems are complex, resulting from the metabolic activities and interactions of diverse microbial species (Sato *et al.* 2019). Recent investigations have characterised the microbial community in acetate and glucose-fed systems (Wu *et al.* 2023) and methanol-fed systems (Pan *et al.* 2023). However, most studies are conducted in controlled environments, and the impact on the microbial community by changing the carbon source to a fermentate in a continuous system remains unknown. Fermentate contains microbes and organic compounds besides VFAs, which might influence the activated sludge microbial community.

This study aimed to investigate the application of a fermentate as a carbon source for denitrification in a pilot-scale MBR system replicating Henriksdal WWTP. The fermentate was produced through continuous fermentation of FW and PS in a pilot (2m3) at mesophilic conditions. The evaluation included batch denitrification tests and pilot-scale trials, comparing performance with glycerol and previous trials with methanol as carbon source. Additional assessments addressed nutrient loading, changes in microbial community dynamics, potential biogas loss, and full-scale implications for Henriksdal WWTP in Stockholm, Sweden.

2. MATERIALS AND METHODS

2.1. Pilot-scale MBR

An MBR pilot representing a scale of 1:6,500 of the future Henriksdal WWTP was used in this study, with a full description provided by Andersson *et al.* (2024). The process followed Henriksdal WWTP's dynamic inflow and included an aeration tank, a primary sedimentation tank, bioreactors for nitrification and pre- and post-denitrification, two separate membrane tanks, and a sludge line (Figure 1). The trials were conducted during the winter of 2021–2022, with average inflow characteristics of 3.6 m^3/h, 150.7 mg TOC/L, 46.5 mg TN/L, 37.1 mg NH_4^+-N/L, and 5.8 mg TP/L. Nitrogen WTW sensors

(Xylem, USA), were located in the primary clarifier, nitrification zone, post-denitrification zone, RAS-detox tank, and effluent tank, collecting minute-resolution data throughout the experiment. Furthermore, influent and effluent samplers collected flow-proportional composite samples (every 0.43 m^3) for analysis.

The MBR pilot trial was conducted over 85 days and divided into four periods with varying operational conditions, including the use of either glycerol or fermentate as a carbon source (Table 1). During the experiment, the pilot inflow (Q_{in}) ranged from 4.51 to 2.18 m^3/h. The returned activated sludge (RAS) was maintained at 4 Q_{in} (same as Henriksdal WWTP) until day 32, after which it was adjusted due to practical limitations in the pilot setup. Carbon dosing was controlled by the effluent nitrate sensor to maintain an effluent concentration of 3 mg NO_3^--N/L throughout all experimental periods.

2.2. Carbon sources

2.2.1. Glycerol

The carbon source used in the reference period was refined glycerol, a by-product from biodiesel production using vegetable oils as raw material (Perstop, Sweden). The sCOD concentration was, on average, 850 g COD/L, with no detectable levels of ammonium or phosphate in the batches used. The Stockholm Water Company (Stockholm, Sweden) currently uses glycerol at the Henriksdal WWTP, while the methanol tanks are being constructed (2028). The same glycerol was used as a carbon source for denitrification in the pilot between October 2020 and the beginning of this trial in November 2021.

2.2.2. Fermentate

A pilot-scale fermenter with an active volume of 0.9 m^3, operated under mesophilic conditions, with a hydraulic retention time (HRT) of 4 days and an organic loading rate (OLR) of 13.4 ± 4.8 kg VS/m^3·d. The reactor was continuously fed with a mixture of 75% PS and 25% FW (%v/v), producing approximately 215 L/day of fermentate (Table 2). The organic acid profile of the fermentate was dominated by lactate (28%), acetate (22%) and propionate (33%) (Figure 2). The fermented sludge from the reactor was pumped hourly into a separation system consisting of a 50 L equalisation tank with automatic chemical dosing (NaOH) for pH adjustment to a target value of 6.5. It then passed through a 0.6 mm diameter drum sieve, where the liquid fraction was collected in a storage tank for use as a carbon source, and the solid fraction was stored at −18 °C util use for biochemical methane potential (BMP) tests.

Table 1 | Operational conditions during four different periods of the pilot trials

			Inflow (m^3/h)		RAS (xQ)	SS bioreactor (g SS/m^3)		Aerated sludge age (d)	Total sludge age (d)	
	Days of operation (d)	**Carbon source**	**Av.**	**Sd.**	**Av.**	**Av.**	**Sd.**	**Av.**	**Av.**	**Temp. (°C)**
Period I	−15 to 0	Glycerol	4.3	0.4	4.0	7,763	230	4.4	14.5	18.6–17.9
Period II	0–31	Fermentate	4.2	0.6	4.0	7,759	745	4.9	15.8	18.0–16.0
Period III	32–38	Fermentate	2.2	0.2	6.6	6,714	93	4.2	13.6	16.0–14.2
Period IV	39–70	Fermentate	2.2	0.1	2.5	6,411	228	5.7	18.2	14.2–13.4

FW, food waste; PS, primary sludge; RAS, recirculated activated sludge.

Table 2 | Characteristics of substrates and fermentate

	TS (%)		VS (% of TS)		sCOD (g/L)		NH_4-N (mg/L)		PO4-P (mg/L)		tVFA (incl. lactic acid) (g/L)	
	Av.	**Sd.**	**Av.**	**Sd.**	**Av.**	**Sd.**	**Av.**	**Sd.**	**Av.**	**Sd.**	**Av.**	**Sd.**
PS	3.3	1.2	92	1.2	1.8	1.2	50	21	30	12	1.0	0.6
FW	15	2.4	93	0.8	64	42	527	273	338	73	26	17
Fermentate	7.0	2.9	90	2.3	32	19	419	147	181	45	22	8.5

Average and standard deviation of total solids (TS) and volatile solids (VS) were estimated with $n = 14$. Soluble chemical oxygen demand (sCOD), ammonium (NH_4^+-N), phosphate (PO_4^{3-}-P), and total volatile fatty acids including lactic acid (tVFA) were estimated in the substrates with $n = 13$, and in the fermentate with $n = 15$.

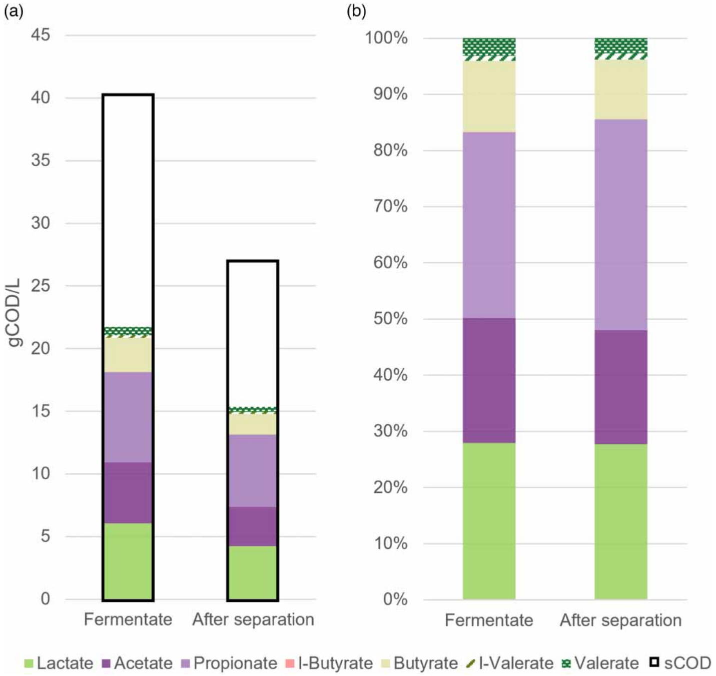


Figure 2 | (a) sCOD and VFA concentration (g sCOD/L) and (b) VFA composition in the fermentate before and after separation.

2.3. Laboratory tests

2.3.1. Batch denitrification tests

The liquid fraction of the fermentate was used as a carbon source. Activated sludge taken from the RAS-detox zone tank of the MBR pilot (Figure 1) was diluted three times and used as inoculum. The tests were performed in three 5 L reactors (duplicates and a reference), following the methodology for the test DEN.CHE.1, described by van Loosdrecht *et al.* (2016). The starting concentrations were 25 mg NO_3^--N/L and 165 mg sCOD/L, corresponding to a C/N ratio of 6.6 and a C/VSS ratio of 0.05–0.1. The inoculum was not washed, and the pH was adjusted to 7 with NaOH after the addition of carbon sources. rNO_x-N endogenous and rNO_x-N exogenous were calculated as the linear regression of the nitrate decreasing slope in mg N/L·min, in the reference reactor and the average of the biological copy reactors, respectively. Denitrification rate, specific anoxic yield, carbon utilisation rate and carbon consumption ratio were calculated using the following formulas:

$$\text{Denitrification rate } q = 60 * \frac{rNO_{x\,\text{exo}} - rNO_{x\,\text{endo}}}{X_{\text{VSS}}} \left[\frac{\text{mg } NO_x - N}{\text{gVSS} \cdot \text{h}}\right]$$

$$\text{Specific Anoxic yield } \; Y_{\text{OHO}} = 1 - 2.86\, \frac{(r_{NO_x\text{-N exo}} - r_{NO_x\text{-N endo}})}{r_{\text{COD}}} \left[\frac{\text{g COD}}{\text{gCOD}}\right]$$

$$\text{Carbon utilisation rate } r_{\text{COD}} = \frac{\text{COD consumed}}{X_{\text{VSS}} \cdot \text{time } (h)} \left[\frac{\text{mg COD}}{\text{gVSS} \cdot \text{h}}\right]$$

$$\text{Carbon consumption ratio} = \frac{\text{COD consumed}}{NO_3^-\text{-N removed}} \left[\frac{\text{g COD}}{\text{g } NO_3^-\text{-N}}\right]$$

2.3.2. BMP tests

One sample of the solid fraction obtained after the drum sieve (on day 70), one sample of untreated sludge [mix of PS_{75}/FW_{25}], and one sample of PS were evaluated in BMP tests. All samples were stored at -18 °C before use. The tests were conducted in triplicate using an Automatic Methane Potential Test System II (AMPTS II) from BPC Instruments AB (Lund, Sweden). Glass bottles (500 mL) were used as reactors, with an 80% active volume. The inoculum used was digestate obtained from the digesters at Henriksdal WWTP, with an inoculum-to-substrate ratio of 3:1, and an organic load of 3 kg VS/m^3 in all tests. The tests were performed at standard mesophilic conditions (37 °C) for 30 days. Cellulose was used as a control substance, and blank controls without substrate were included to determine background gas production from the inoculum.

2.3.3. Chemical analyses and analytical methods

Soluble and total chemical oxygen demand (sCOD and COD), total nitrogen (TN), ammonium nitrogen (NH_4^+-N), and phosphate (PO_4-P) were measured using spectrophotometric cell tests from WTW (Xylem, USA). Samples were centrifuged and filtered through 0.45 μm acetate filters before testing. For VFA species C1–C5 (acetic, propionic, butyric and valeric acids) and lactic acid, samples were further filtered (0.22 μm) and acidified 10% with 37% H_2SO_4 to be analysed using a high-performance liquid chromatography (HPLC) Agilent 1,100 Series with a refractive index detector and an ion exclusion column (Rezex ROA – Organic Acid H+, 300 × 7.80 mm, Phenomenex). The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mL/min.). For statistical analysis, t-tests were conducted to determine the significance of the response variables between different carbon sources ($p < 0.05$).

2.4. 16s RNA gene amplicon sequencing

Samples from the Ras-deox zone were collected for microbial community analysis for both carbon sources. During the glycerol phase, samples were taken 6, 4, 3, and 2 weeks prior to the trial and on day –17. Samples during the fermentate addition period were taken on days 0, 7, 15, 21, 28, 35, 42, 70, and 1 week after the end of the trials (84). DNA extraction from activated sludge samples, amplicon sequencing and data analysis, were performed following the methodology described by Eliasson *et al.* (2023).

2.5. Henriksdal WWTP

The Henriksdal WWTP, located in Stockholm, served 850,000 population equivalents (pe) according to 2023 data and is currently undergoing a capacity upgrade to accommodate an increasing population (1.6 Mpe ~yearly av. 531,000 m^3/d) and to meet new effluent limits of 6 mg TN/L and 0.20 mg TP/L. The upgrade included the transition from conventional activated sludge (CAS) to MBR technology. The inflow characteristics used were biological oxygen demand (BOD_5) – 60 g/p·d, TN – 12 g/p·d, TP – 1.6 g/p·d, suspended solids (SS) – 90 g/p·d and alkalinity – 88 g/p·d. The new process featured increased capacity in the activated sludge reactors, with pre- and post-denitrification, and filtration using hollow fibre membranes. The plant was designed for biological nitrogen removal through nitrification and pre- and post-denitrification, and phosphorus chemical precipitation using Fe^{2+} (ferrous sulphate) dosed at 8–12 g Fe/m^3 in the primary sedimentation tanks, and (3–4 g Fe/m^3) in the biological reactors (Figure 1). Due to the stringent phosphorus discharge limit, significant iron dosing is required, reducing the BOD entering the biological stage. As a result, an external carbon source is needed in post-denitrification, with a projected methanol dose of 15–25 g COD/m^3 corresponding to ~1,800 m^3 MeOH/year. The boundaries of the calculations included the primary sedimentation tanks, biological reactors, membrane tanks and anaerobic digesters. The dimensions, inflow characteristics, population projections, and production rates were based on documents by the design consultant (SWECO, Sweden). Specific activity rates and other design values were taken from Tchobanoglous *et al.* (2014). The results from the pilot trials and BMP tests were used for scale-up calculations.

3. RESULTS AND DISCUSSION

3.1. Fermentate separation

The reactor fed with FW and PS produced a fermentate with average concentrations of 32 ± 19 g sCOD/L and 22 ± 8.5 $gVFA_{COD}$/L (Table 2). The fermenter emitted high concentrations of H_2S during operation, likely due to a combination of sulphate-reducing bacteria activity and low pH (<5), which resulted in higher proportion of H_2S in the gas phase. Sulphate is commonly present in wastewater, and sulphate reduction can proceed even at low pH levels (Koschorreck 2008), such as those observed during fermentation. In line with this, bacteria from the phylum *Desulfobacterota*, containing sulphate-reducing bacteria, were found in low abundance in the fermenter (Supplementary material, Figure S1). The fermenter off-gas contained ~2% CH_4, 80% CO_2, 1.8% O_2, 318 ppm NH_3, 502 ppm and H_2S concentration exceeded the measurement instrument's limit of 9,000 ppm. Although gas volumes were low, control measures were required during fermentate handling, including filtration. A common strategy to control H_2S formation is the addition of Fe^{2+} or Fe^{3+} to precipitate dissolved sulphide as FeS (Zhang *et al.* 2008). In the present study, 8–10 $gFeCl_3/m^3$ of incoming water was dosed in the primary sedimentation tank. However, at pH below 6.5, the formation of iron salts is inefficient (Boon 1995), and it has been recorded that FeS precipitation is 40% lower at acidic than at neutral pH (Nielsen *et al.* 2008). Since the pH in the fermentate ranged from 4.9 and 4.1 throughout the trial, it was adjusted to 6.5 with NaOH (3–30 mL/L fermentate) prior to the separation process. The H_2S levels were then effectively reduced to <5 ppm as it was not detected in the sensor located in drum sieve.

The drum sieve had an automatic pressurised water flush system, which diluted the fermentation liquid by 30%. As a result, the sCOD and VFA concentrations were 30% lower after the separation (Figure 2(a)). However, no changes in the VFA composition were observed during the different treatments, including pH adjustment, separation, dilution and storage of the carbon source (Figure 2(b)), which is important to consider in large-scale systems.

3.2. Denitrification trials

3.2.1. Batch denitrification tests

Both carbon sources (fermentate and glycerol) were evaluated in batch denitrification assays using acclimated activated sludge. The MBR pilot had operated with glycerol as a carbon source for 1.5 years prior to the start of this trial, and heterotrophic denitrifiers were therefore assumed to be acclimated. Tests with fermentate were performed by the end of the trials when the microbial process was considered fully acclimated to this new source. The fermentate resulted in a 57% higher denitrification rate compared to glycerol ($p <= 0.001$), with rates of 8.5 ± 0.1 and 5.4 ± 0.1 mg NO_{x-N}/g VSS·h, respectively. These findings are consistent with previous research. Yuan Pan *et al.* (2023) reported higher rates compared with the present study but overall but found that acetate (50 mg NO_{x-N}/g VSS·h) resulted in a 40% higher rate than glycerol (36 mg NO_{x-N}/g VSS·h), while methanol and glucose were 35 and 160% lower, respectively. Additionally, previous tests using the same inoculum as in this study showed glycerol (2.5 mg NO_{x-N}/g VSS·h) to be the least effective among all tested carbon sources, with acetic acid (4.9 mg NO_{x-N}/g VSS·h), ethanol, and methanol performing 104, 70, and 18% better in batch tests, respectively (Andersson *et al.* 2023).

The superior performance of fermentate can be explained by its composition. As shown in Figure 2, it contained high concentrations of VFAs and other soluble compounds (g sCOD/L), with lactate, acetate, and propionate as dominant acids. VFAs are readily available for microbial uptake and directly enter the TCA cycle, facilitating electron transfer and energy production in the denitrification process (Wei *et al.* 2022). Glycerol, on the other hand, follows a slower metabolic pathway, explaining the lower rate. Glycerol must first be converted to α-glycerol phosphate, then catalysed to glycerone-P, to finally be converted into pyruvate during glycolysis, after which it finally enters the TCA cycle to complete decomposition. Furthermore, while no tests with methanol were conducted in this study its use as a carbon source is well documented (Fu *et al.* 2022). Compared to glycerol and fermentate, degradation of methanol requires specialised enzymes secreted by methylotrophic bacteria that do not grow with other carbon sources, and causes 'carbon dependency' (Zhang *et al.* 2024). As these bacteria become dominant, they do not facilitate the uptake of influent sCOD to be used in pre-denitrification, potentially resulting in lower overall nitrogen removal.

Further, differences were observed in NO_2^--N accumulation during the denitrification batch tests. Glycerol caused a peak accumulation of 4.5 mg NO_2^-N/L compared to 1 mg NO_2^--N/L with the fermentate (Supplementary material, Figures S2 and S3), indicating a more incomplete or partial denitrification with glycerol. This is important, as NO_2^- build-up has been directly linked to increased N_2O emissions in activated sludge systems (Alinsafi *et al.* 2008; Adouani *et al.* 2010). For this, two explanations have been proposed: (1) enzymatic imbalance caused by competition for electrons between nitrate reductase (NAR) and nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS), which can disrupt the reduction pathway and lead to N_2O accumulation (Vasilaki *et al.* 2020); and (2) the enrichment of bacterial populations with a lack or loss of genes for further reducing NO_2 and other intermediates (Roco *et al.* 2017). Full-scale data form Alessio *et al.* (2023) found N_2O emissions to be proportional to the amount of glycerol dosed (as a carbon source) in WWTP. In contrast, fermentate did not exhibit signs of NO_2^-N accumulation during the batch denitrification tests (Supplementary material, Figure S2), indicating a more complete denitrification and the potential for lower N_2O emissions compared to glycerol in full-scale operation. The carbon source type influences N_2O generation, with acetate (a major component of the fermentate) and glycerol shown to produce more N_2O than ethanol (Adouani *et al.* 2010; Hu *et al.* 2018), while methanol produces similar emissions to ethanol but is more sensitive to oxygen and nitrite inhibition (Lu & Chandran 2010). This suggests alcohol-based carbon sources may emit less N_2O than VFAs, a factor worth considering for full-scale application. In addition, environmental conditions, such as low pH, often linked to the use of acids but better managed with fermentates, can also affect N_2O production due to the influence on electron flow and enzyme competition (Pan *et al.* 2012).

Despite the better performance of the fermentate, the carbon consumption ratios in batch tests were similar: 3.9 g COD/g NO_3^--N_{removed} for glycerol and 3.7 g COD/g NO_3^--N_{removed} for fermentate. Typically, lower consumption ratios indicate higher denitrification efficiency, but in the case of glycerol, a similar ratio may reflect slower carbon uptake. However, these ratios differed significantly during pilot-scale trials, as discussed in section 0. An additional observation during the denitrification test with fermentate was the release of phosphate, possibly linked to bio-P activity previously seen in this pilot (Andersson *et al.* 2023).

3.2.2. Pilot trials

The trials were divided into four operational periods (Table 1). During the trials, three technical disruptions occurred: (1) a nitrification issue disrupted the $NO_3^- - N$ in the post-denitrification zone between days 10–15; and (2–3) carbon source pump failures on days 50–52 and 60–65 temporarily stopped dosing, leading to increased nitrate concentrations in the effluent (Figure 3). Despite these events, the effluent nitrate concentration remained relatively stable overall, with an average of 3.1 ± 0.9 mg NO_3^--N/L.

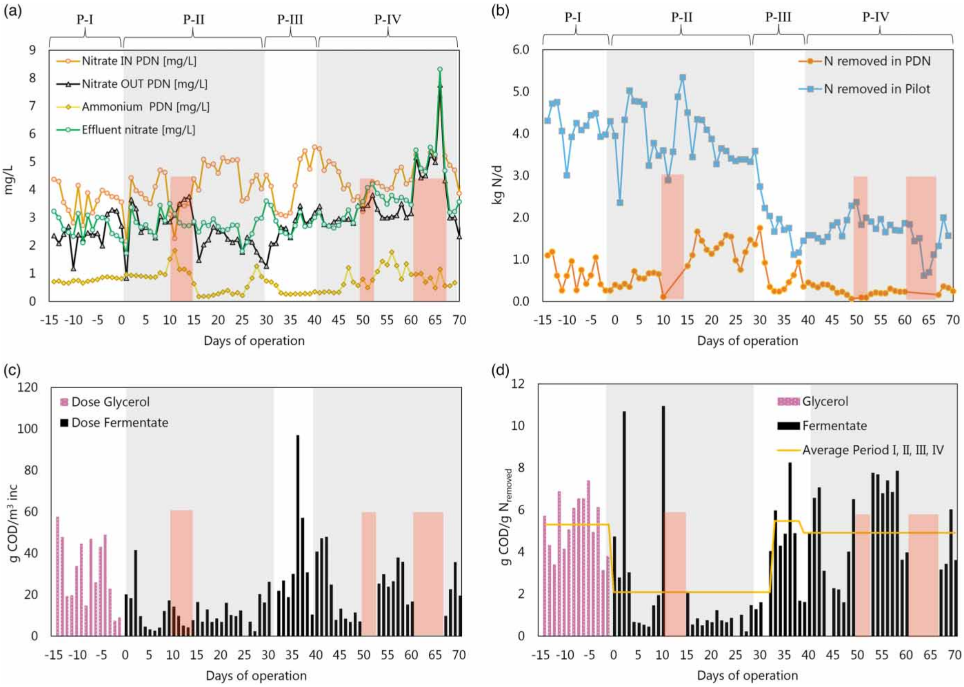


Figure 3 | Denitrification results pilot trials. (a) Nitrate in and out of the post-denitrification zone (PDN), effluent nitrate and ammonium in PDN [mg/L]; (b) nitrogen removed in PDN and nitrogen removed in the overall plant [kg/d]; (c) dose of carbon source [g COD/m^3 of incoming water to the pilot]; (d) carbon source consumption in kg COD added per kg NO_3 removed in the PDN. The red areas represent days with operational failures.

Periods I [days −15 to 0] and II [days 0 to 32] had the same operational conditions, with glycerol and fermentate (liquid phase) as carbon sources, respectively (Table 1). Fermentate exhibited a better denitrification performance than glycerol, consistent with batch results (section 3.2.1) and previous findings using acclimated sludge with acetate and glucose (Pan *et al.* 2023). The carbon consumption ratios during Period I was 5.3 ± 1.3 g COD/g $NO_{3\text{removed}}$ (av. glycerol dose: 31.6 g COD/$m^3{}_{\text{incoming water}}$), 50% higher ($p = 0.0002$) than in Period II with fermentate (2.1 ± 2.7 kg COD/kg NO_3) (Figure 3(c) and 3(d)). The higher consumption observed with glycerol may be attributed to its higher anoxic yield and longer metabolic pathway compared to VFAs.

Nitrate removal in the post-denitrification zone averaged 1.0 ± 0.4 kg $NO_3^-{-}N$/d in Period II, corresponding to 24% of the plant incoming nitrogen (4.1 ± 0.8 kg N/d), a clear improvement over the 0.6 ± 0.3 kg $NO_3^-{-}N$/d (14% reduction) observed with glycerol in Period I. Assuming 8% nitrogen assimilation into biomass, pre-denitrification nitrate removal was higher in Period I (3.4 kg N/d), than in Period II (2.7 kg N/d), likely due to a seasonal drop in influent temperature from 18.2 °C to 16.0 °C (Table 1), which reduced pre-denitrification performance and led to increased nitrate load to the post-denitrification zone (Figure 3(a)). The specific denitrification rate in the post-denitrification zone was 30% higher with fermentate (1.3 ± 0.6 mg NO_3^--N/gVSS · h) compared to glycerol (0.9 ± 0.1 mg $NO_3^-{-}N$/gVSS · h), adjusted with a temperature correction factor (1.026). Lower rates observed in the pilot compared to batch tests are likely because only a portion of the post-denitrification zone was being active, as the system responds dynamically to nitrate load. Under higher nitrate conditions, a larger volume would be utilised, and the observed rates would likely approach those from the batch tests. Furthermore, the results showed that the microbial community adapted easily to the VFA-based carbon source, achieving higher efficiency without the need for longer adaptation periods. According to Zhang *et al.* (2024), sludge acclimated to glycerol exhibits active fatty acid biosynthesis and degradation metabolism, enabling effective utilisation of VFAs in wastewater. This could have helped the pre-denitrification efficiency and facilitated the shift to the VFA-based fermentate.

During Periods III and IV, the pilot inflow was reduced from 4.5 to 2.5 m^3/h, and the RAS flow was adjusted to 4.8 Q_{in} and 2.5 Q_{in}, respectively, due to technical issues with one of the membrane cassettes (unrelated to this trial). These changes altered the overall nitrogen balance in the system. Nitrate removal in the post-denitrification decreased to 0.45 kg NO_3^--N/d in Period III and 0.25 kg NO_3^--N/d in Period IV (Figure 3(b)), corresponding to 25 and 14% of the incoming nitrogen load, respectively. Interestingly, fermentation performance improved during these periods, as indicated by an increase in the VFA/sCOD ratio of the fermentate from 50% in Period II to 65% in Period III (Supplementary material, Figure S5). Despite this improvement, no corresponding increase in denitrification efficiency was observed. Although higher VFA/sCOD ratios are typically associated with better carbon source quality and enhanced denitrification performance, no such effect was seen here. This suggests that under the operational conditions of Periods III and IV, other factors, such as lower flow, altered sludge retention time, or reduced biomass activity due to lower temperatures, may have constrained performance, limiting the benefit of the improved carbon quality, or that simply the denitrifiers are able to uptake most of the other soluble compounds as easily. The denitrification rate remained stable between Periods II and III but declined considerably in Period IV.

The fermentate, produced from a mixture of 25% FW and 75% primary sludge, outperformed glycerol in pilot trials and both glycerol and methanol in batch denitrification tests. Despite operational disruptions, it demonstrated resilience and effectiveness as a carbon source for sustained denitrification. Furthermore, methanol was used in the same pilot between 2017 and 2019 (2 years prior to this study), with yearly average carbon consumption ratios ranging from 0.3 to 0.95 g COD/g TN_{removed} in the overall pilot (Andersson *et al.* 2023), which spans both below and above the values observed in this study with fermentate (0.52 g COD/g TN_{removed} in the overall pilot), further highlighting the strong potential for replacing methanol with fermentate. Finally, although enzyme concentrations were not measured in this study, their role in regulating denitrification kinetics is well established in single-stage acclimated denitrification reactors. Future research should explore enzyme dynamics in continuous pilot-scale systems to better understand metabolic limitations and optimise the use of complex substrates like fermentate at a larger scale.

3.2.3. Influence of carbon source composition: ammonium, phosphate, and organic compounds

No detectable levels of ammonium or phosphate were present in the glycerol batches used. Accordingly, during Period I, the use of glycerol did not introduce any additional ammonium or phosphorus load. In contrast, fermentates from organic streams typically contain higher concentrations of these nutrients, as they are present in the substrates and are released during fermentation (Wei *et al.* 2021). In Period II, the fermentate contributed to approximately 0.01 kg N/day, corresponding to 0.3% of the pilot's nitrogen load. As the dose increased in Periods III and IV, the ammonium contribution rose to 1.1%

and 0.6%, respectively. Phosphate additions were similarly low, accounting for 0.09%, 0.3%, and 0.2% of the total phosphorus load in Periods II, III, and IV, respectively. These increases were negligible, with no observed effect on the effluent phosphorus concentration (<0.05 mg TP/L) or on chemical consumption for phosphorus precipitation.

The additional nitrogen load (0.3–1.1%) also had no adverse impact on process performance, as the RAS flow, maintained at ratios of 4 and 2, effectively recirculated this nitrogen to the pre-denitrification zone for biological removal or was oxidised in the membrane tanks. Although the excess nutrients did not affect our trials, they can vary significantly depending on the substrate used in the fermentation, sometimes affecting its applicability as a carbon source. In particular, low sCOD/N and sCOD/P ratios can introduce excess nutrient loads. Nevertheless, previous studies have shown that such fermentates may still be compatible with biological nutrient removal systems under specific conditions (Soares *et al.* 2010; Carranza Muñoz *et al.* 2024). Moreover, nutrient recovery (Serra-Toro *et al.* 2022) and filtration strategies (Chen *et al.* 2024) that remove nutrients without affecting the soluble carbon fraction offer promising approaches to address these challenges, when necessary.

Furthermore, the results confirmed that using fermentate containing soluble organic compounds as a carbon source did not affect membrane performance. No changes were observed in cleaning chemicals consumption, transmembrane pressure (TMP), permeability, or obvious irreversible membrane fouling, consistent with findings by Tang *et al.* (2017). Additional data on the membrane performance is available in the report by Andersson *et al.* (2023).

3.3. Effect of fermentate on activated sludge microbial community

A large diversity of representatives within *Bacteria* and *Archaea* are typically found in activated sludge processes, and the community composition is influenced by several different factors. Throughout the experimental period, the dominating families identified in the activated sludge samples included *Mycobacteriaceae* (18.2%), *Saprospiraceae* (9.28%), JAEUJM01 (4.2%), *Burkholderiaceae* (4.0%), *Rhodocyclaceae* (4.0%), and *Chitinophagaceae* (3.1%) (Supplementary material, Figure S8). These represent commonly observed microbiota in activated sludge from municipal wastewater, although specific abundances and types of *Bacteria* vary depending on operational conditions and treatment processes (Shchegolkova *et al.* 2016). The microbial community analysis indicated some changes in the dominant bacterial families following the transition from glycerol to fermentate as the carbon source, including increased in relative abundances of *Mycobacteriaceae* and *Saprospiraceae* (Supplementary material, Figure S8).

Typical denitrifying microorganisms in activated sludge processes include bacteria from the genera *Thauera*, *Paracoccus*, *Pseudomonas*, and *Rhodopseudomonas,* among others (Fang *et al.* 2020; Wu *et al.* 2023). In this study, known denitrifying genera detected included *Zoogloea*, *Hyphomicrobium*, *Ferruginibacter* (Figure 4), and *Acidovorax*, *Comamonas*, *Paracoccus*, and *Thauera* in lower abundance. In contrast with findings by Wu *et al.* (2023), who used laboratory-scale reactors fed with glycerol and acetate as carbon sources, the process in this study showed no presence of *Pleomorphomonas* and *Propionivibrio,* which were reported as dominant denitrifiers in their study. Only minor differences were observed in community composition with the change in carbon source. Specifically, *Zoogloea* and *Hyphomicrobium* showed slightly higher relative abundance when glycerol was used (RA 4% and 0.5%) compared to fermentate (RA 2.5 and 0.5%). No significant differences were observed for other known denitrifying genera. Interestingly, *Candidatus* Saccharibacteria UBA5946 was present at significantly higher relative abundance ($p < 0.05$) during the glycerol feeding period (Figure 4 and Supplementary material, Figure S6). This species has previously been reported as dominant (~40%) in reactors operating under partial denitrification conditions (Xiujie *et al.* 2019), which could be a sign of possible accumulation of denitrification intermediates, as also observed in our batch tests. Additionally, members of the *Chitinophagaceae* family were more abundant during the fermentate period ($p < 0.05$). These organisms are known aerobic heterotrophs involved in the degradation of organic matter in activated sludge (Oh *et al.* 2019). Other microorganisms that showed significantly higher RA ($p < 0.05$) with a specific carbon source are presented in Supplementary material, Figures S6 and S7. However, these were not found to be connected to the denitrification efficiency. Changes in the microbial community did not reflect any noticeable differences in denitrification rates.

These findings suggest that observed improvement in denitrification was primarily driven by more efficient utilisation of the carbon source, rather than significant shifts in the microbial community composition. The VFAs in the fermentate were more readily metabolised than glycerol, leading to higher denitrification rates with the same microbial population. Although previous studies have shown that the type of carbon source can influence both microbial community structure and the capacity to utilise a broad range of organics (Wawrik *et al.* 2005; Xu *et al.* 2018; Pan *et al.* 2023), no major microbial changes were observed in this study. The following two factors could explain this outcome: first, glycerol, despite having lower

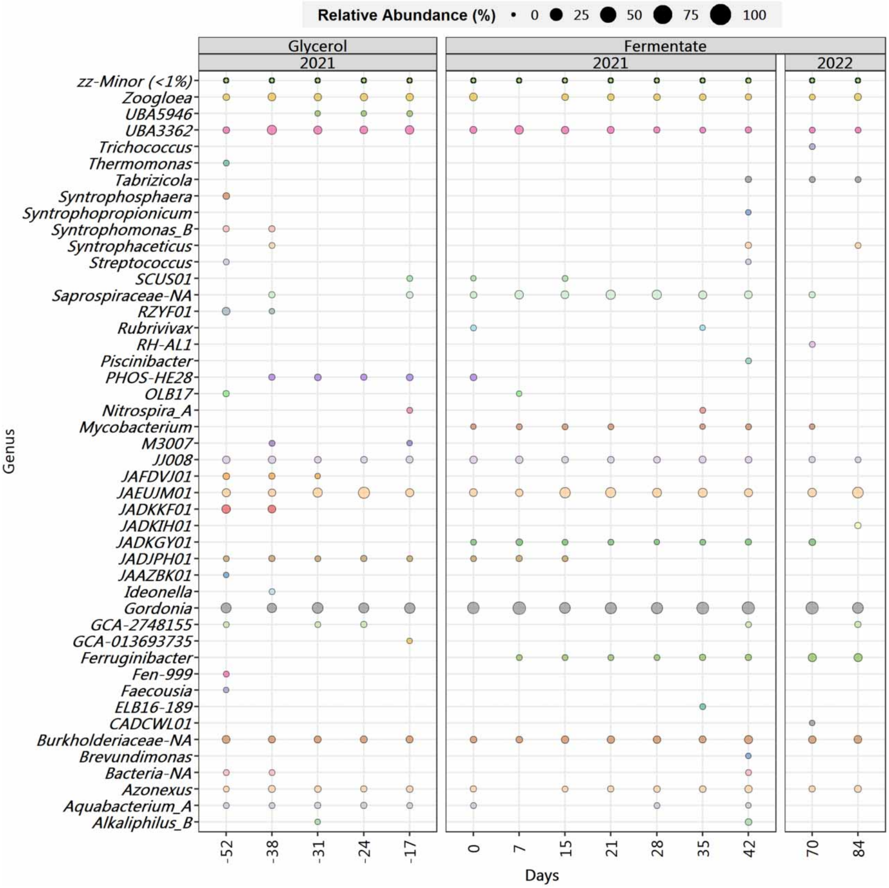


Figure 4 | Microbial community profile (in%) at the genus level in the activated sludge samples. The two carbon sources are shown, glycerol (left) and fermentate (right), during the experimental period. Samples correspond left to right with glycerol: 6, 4, 3, and 2 weeks before the trial and on day −15. Left to right with fermentate: days 0, 7, 15, 21, 28, 35, 42, 70, and 84.

denitrification kinetics, exhibits a great utilisation capacity for a broad range of carbon sources (Pan *et al.* 2023); and second, the fermentate used here consisted of a complex mixture of soluble organics rather than a single carbon compound. This diversity may have prevented the selective enrichment of specific microbial groups, as commonly observed with pure substrates.

3.4. Impact on biogas production

After the liquid phase is separated for use as a carbon source, the remaining solid fraction could be directed into the anaerobic digester to support biogas production. During our trials, the methane potential of the solid fraction from the fermentation process was evaluated through BMP tests and compared with two untreated reference samples, PS alone and a mix of PS and FW at a 75/25 ratio. The BMP of the untreated PS/FW mixture was 325.6 ± 7.7 NmL CH_4/g VS, while the solid fraction from the fermented PS/FW after separation of the VFA-rich liquid, showed a 20% lower methane yield of 257.3 ± 7.1 NmL CH_4/g VS (Supplementary material, Figure S4). This aligns with previous studies showing that VFA extraction during acidogenic fermentation reduces the remaining methane potential in the solid fraction of sludge samples (Carranza Muñoz *et al.* 2024). The untreated PS alone yielded 330.8 ± 19.3 NmL CH_4/g VS. These values were used for full-scale calculations.

3.5. Full-scale implications

To meet the TN effluent limit of 6 mg/L, the requirement for external carbon at Henriksdal WWTP was estimated at 7.7 tons COD/d, corresponding to the removal of approximately 1.5 tons/d of nitrate in the post-denitrification step. The design target assumes final effluent concentrations of 3 mg NO_3^--/L, 1 mg NH_4^+-N/L, and 1 mg N_{inert}/L. Methanol was identified as the preferred carbon source. Based on design calculations (SWECO, Sweden), the projected methanol demand for the full-scale plant in 2040 was estimated at 5.2 m^3/day, corresponding to a dosage of approximately 16 g COD/m^3. Based on the results from this study, replacing methanol with the VFA-based carbon source would require 237 m^3/d of fermentate, assuming an average sCOD – 32 g COD/L (Table 2). Taking into account 30% dilution during separation and 30% of the carbon retained in the solid fraction, this would require a total production of approximately 338 m^3/day of raw fermentate, with a TS content of 6.3% and a VS content of 92.6% of TS. Given the mesophilic conditions and a HRT of 4 days used in the fermentation trials, the required fermenter volume would be approximately 1,352 m^3. The approximate substrate requirement would be 8.7 tons VS of PS and 11.2 tons VS of FW per day based on the fermentation yields. This corresponds to around 10% of the projected PS generated at Henriksdal WWTP in 2040 (85.5 tons VS of PS), while the required FW would be externally obtained and transported to the plant.

Furthermore, based on the BMP results, diverting 10% of the primary sludge to fermentate production would reduce methane generation in the digesters by approximately 2,869 m^3 CH_4/d. However, recirculating the solid residue from the fermentate (BMP 257.3 ± 7.1 NmL CH_4/g VS) would reduce this loss to 600 m^3 CH_4/d, equivalent to only 2% of the total biogas production (~41,000 m^3 CH_4/d). The current price that Stockholm Water Company receives for untreated biogas is around 0.5 €/m^3 CH_4 (assuming a 65% methane concentration), which gives a corresponding decrease in income of 300 €/d. In comparison, the daily cost of methanol dosing (5.2 m^3/day) is estimated at 3,600 €, based on a market price of 700 €/ton (Methanex Corporation 2025). The fact that no FW is currently handled at Henriksdal WWTP means that additional costs would be associated with its transport and pre-processing. Nevertheless, the results from this study demonstrate the technical feasibility of replacing methanol with an internally produced VFA-based carbon source using primary sludge and FW. A more detailed cost–benefit analysis is needed to assess the economic viability of this substitution fully.

4. CONCLUSIONS

This study demonstrated the feasibility of replacing the external carbon source with a VFA-rich fermentate produced from primary sludge and FW at the Henriksdal WWTP. The fermentate outperformed glycerol in both batch and pilot-scale trials, achieving a 40% higher denitrification rate, a 50% lower carbon consumption ratio, and maintaining effluent nitrate concentrations below 3 mg NO_3^--N/L. It has also shown higher performance than methanol and other previously tested carbon sources. Microbial analysis indicated stable community composition, suggesting that improved performance was due to more efficient carbon utilisation rather than shifts in microbial structure. Full-scale projections indicated that producing the required volume of fermentate to replace the external carbon needs would require the co-fermentation of 10% of Henriksdal's primary sludge with external FW. The resulting reduction in methane production was limited to 2%, representing a minor trade-off. Nevertheless, additional long-term trials are suggested to validate the results obtained from the BMP tests, and further investigation of N_2O emissions associated with using fermentate as a carbon source. This transition supports reduced fossil carbon dependency and enhanced resource recovery. Additionally, if Henriksdal implements biological phosphorus removal, VFA would be a suitable and necessary carbon source, unlike methanol. These results highlight the need for supportive policies that encourage circular carbon management and integration of waste streams for sustainable WWTP operation.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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Many wastewater treatment plants rely on fossil methanol for nitrogen removal, increasing CO_2 emissions and hindering sustainability. This thesis explored sustainable alternative carbon sources through lab and pilot trials, assessing fermentation of waste streams, denitrification performance, effect on biogas, and economic and environmental implications. Food waste and mixtures performed best, offering high carbon quality, cost-effectiveness, and low emissions. Sludge and digestate were viable but more costly. All carbon sources achieved effective denitrification, supporting increased nitrogen removal and climate goals.

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