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.

GRAPHICAL ABSTRACT
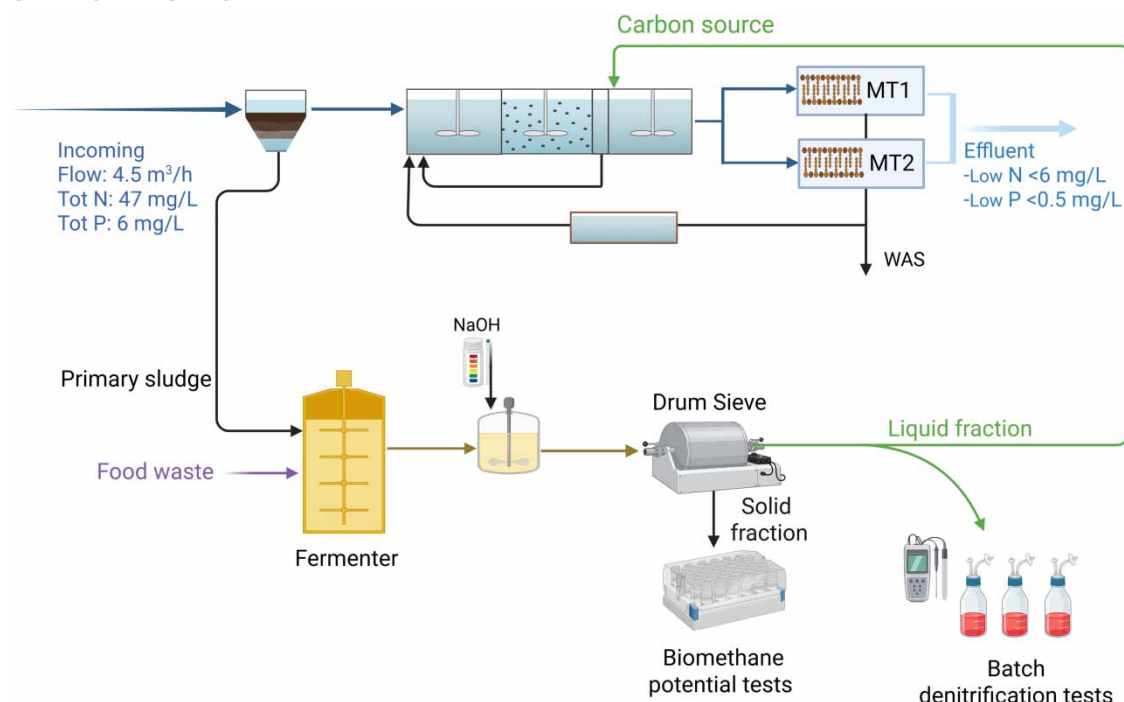
1. INTRODUCTION

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_2O, compared to other carbon sources, like
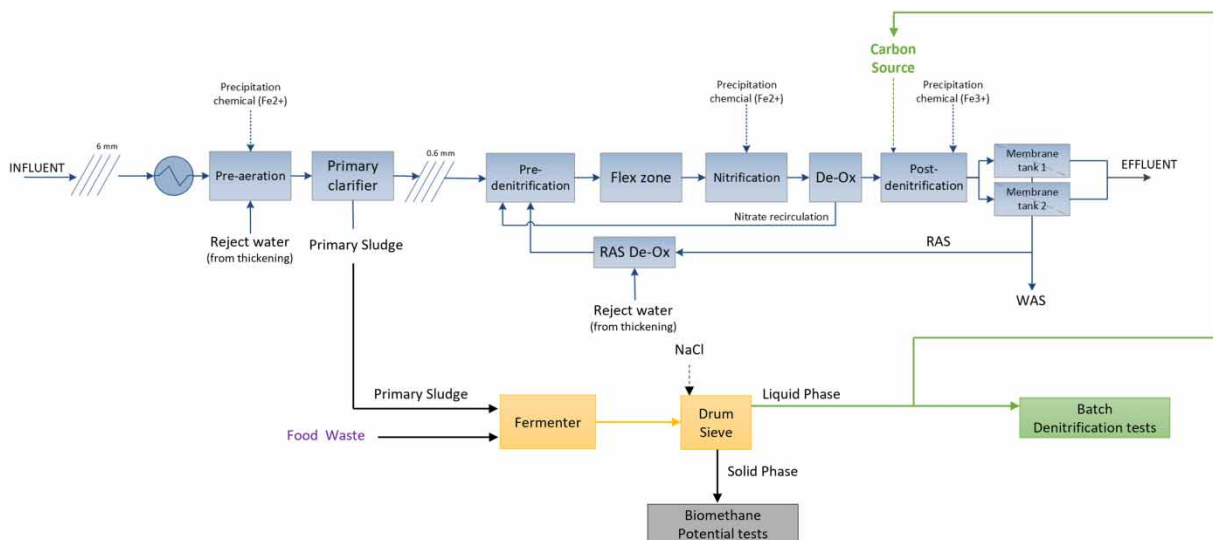
Figure 1 | Experimental scheme of the MBR pilot line (blue), including all the chemical and carbon source additions. The carbon source line production (yellow) includes the fermenter, the separation with a drum sieve and pH control. The liquid phase (green) was used in denitrification batch tests and 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 $\pm$ 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

	Days of operation (d)	Carbon source	Inflow (m^3/h) Av.	Inflow (m^3/h) Sd.	RAS (xQ) Av.	SS bioreactor (g SS/m^3) Av.	SS bioreactor (g SS/m^3) Sd.	Aerated sludge age (d) Av.	Total sludge age (d) 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 (%) Av.	TS (%) Sd.	VS (% of TS) Av.	VS (% of TS) Sd.	sCOD (g/L) Av.	sCOD (g/L) Sd.	NH$_4$-N (mg/L) Av.	NH$_4$-N (mg/L) Sd.	PO4-P (mg/L) Av.	PO4-P (mg/L) Sd.	tVFA (incl. lactic acid) (g/L) Av.	tVFA (incl. lactic acid) (g/L) 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 $n = 15$.
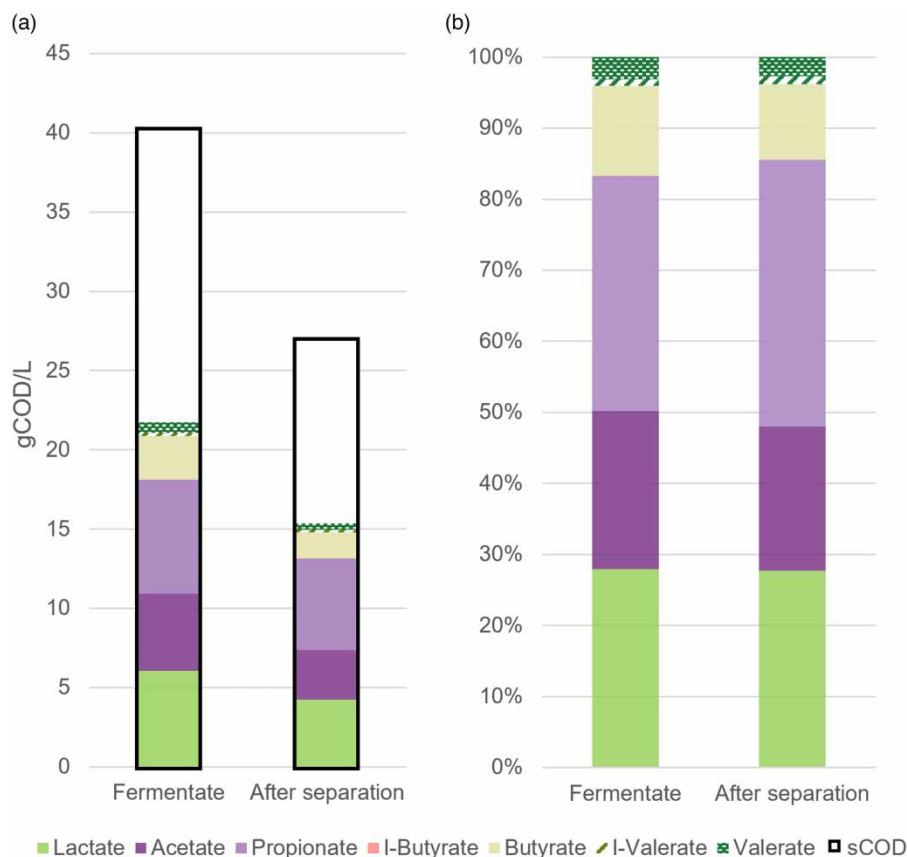
Figure 2 | (a) sCOD and VFA concentration (g sCOD/L) and (b) VFA composition in the fermentate before and after separation.

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:

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

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

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

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

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.
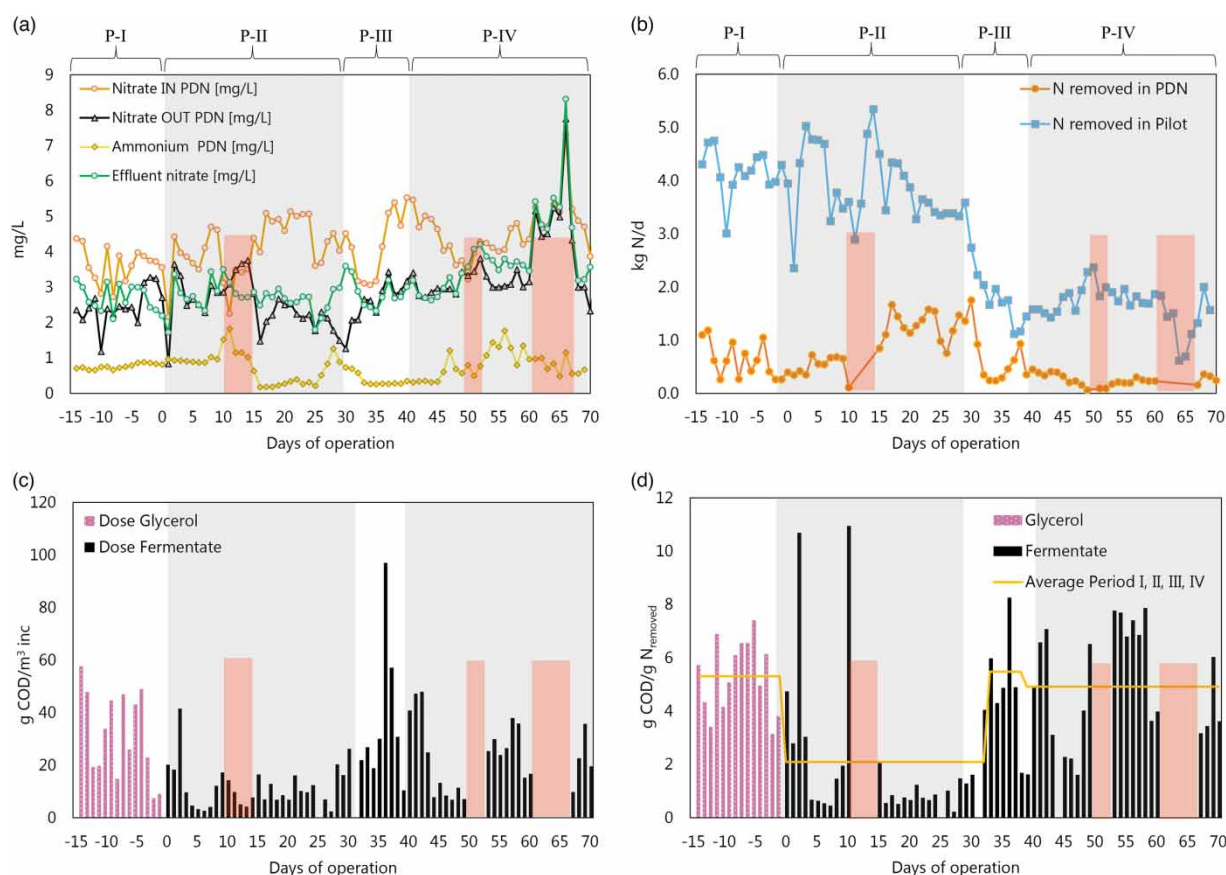

Figure 3 | Denitrification results pilot trials. (a) Nitrate in and out of the post-denitrification zone (PDN), effluent nitrate and ammonium in PDN [mg/L]; (b) nitrogen removed in PDN and nitrogen removed in the overall plant [kg/d]; (c) dose of carbon source [g COD/m³ of incoming water to the pilot]; (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 $NO_{3removed}$ (av. glycerol dose: 31.6 g COD/m^3 $_{incoming\ water}$), 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%), *Sapospiraceae* (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
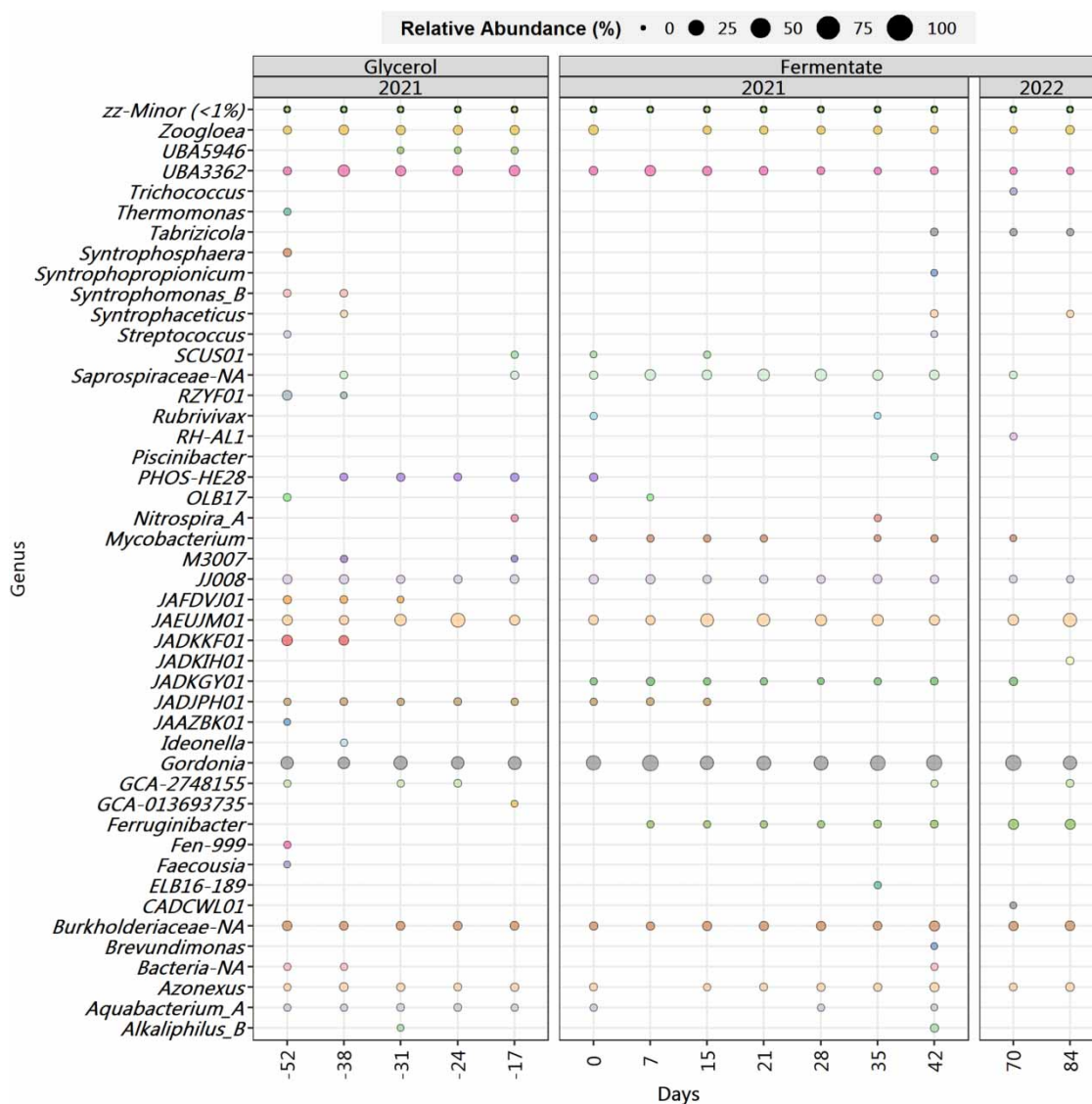
Figure 4 | Microbial community profile (in%) at the genus level in the activated sludge samples. The two carbon sources are shown, glycerol (left) and fermentate (right), during the experimental period. Samples correspond left to right with glycerol: 6, 4, 3, and 2 weeks before the trial and on day −15. 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.

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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