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Research article

Genome-centric metagenomics reveals the effect of organic carbon source on one-stage partial denitrification-anammox in biofilm reactors

Zejia Zheng [a,b], David J.I. Gustavsson [c,d], Dan Zheng [a], Felix Holmin [c], Per Falås [e], Britt-Marie Wilén [a], Oskar Modin [a], Frank Persson [a,*]

[a] Division of Water Environment Technology, Department of Architecture and Civil Engineering, Chalmers University of Technology, SE-41296, Gothenburg, Sweden
[b] Greenhouse Horticulture, Wageningen University & Research, 6700 AP, Wageningen, the Netherlands
[c] Sweden Water Research AB, c/o Genetor, Raffinaderiet, Fabriksgatan 2, SE-222 35, Lund, Sweden
[d] VA SYD, P.O. Box 191, SE-20121, Malmö, Sweden
[e] Department of Process and Life Science Engineering, Division of Chemical Engineering, Lund University, Lund, SE-22100, Sweden

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ABSTRACT

Nitrogen removal from wastewater with anammox saves energy and resources. Partial denitrification-anammox (PDA) is a promising process alternative for municipal wastewater treatment, given that the understanding about how to control the microbiome and its activity reach sufficient level. Here, two moving bed biofilm reactors were fed with either acetate or propionate to study the role of organic carbon type for microbiome composition and nitrogen turnover during development of PDA. With acetate, 87 % of the removed nitrogen was converted via anammox during stable operation at a rate of 0.52 g N/(m^2·d). With propionate, the anammox contribution was considerably lower (41 %), as was the rate of nitrogen removal (0.27 g N/(m^2·d)). The microbiome composition in the acetate- and propionate-fed reactors was however similar, with an enrichment of metagenome assembled genomes (MAGs) having genes for nitrate reduction (*narG*, *napA*). A large fraction of these MAGs had the potential to accumulate nitrite since they lacked genes for nitrite reduction (*nirS*, *nirK*, *nrfA*). Genes for acetate utilization were common among these MAGs, but the necessary genes for propionate conversion were rare, suggesting that the genetic make-up of the individual denitrifiers had major influence on the nitrogen turnover. One anammox MAG (*Ca.* Brocadia sapporoensis), harboring genes for organic carbon utilization, prevailed in the PDA reactors. Another three anammox MAGs (*Ca.* B. fulgida, *Ca.* B. pituitae and a potentially new species within *Ca.* Brocadia), lacking genes for organic carbon utilization, decreased in abundance in the reactors, indicating the importance of metabolic versatility for anammox bacteria in PDA.

1. Introduction

Anaerobic ammonium oxidation (anammox) plays a crucial role in the biological nitrogen cycle and is increasingly used for nitrogen removal in wastewater treatment (Zhao et al., 2019). It is acknowledged as an energy-efficient alternative to conventional biological nitrogen removal (*i.e.* nitrification-denitrification) in terms of lower biomass production, aeration costs and organic carbon needs (Kartal et al., 2010). The process is carried out by bacteria within *Planctomycetes*, in the candidate families *Ca.* Scalinduaceae, *Ca.* Brocadiaceae, *Ca.* Bathyanammoxibiaceae and *Ca.* Anammoxibacteraceae (Suarez et al., 2022; Zhao et al., 2022). Anammox bacteria are autotrophic and obtain energy from the oxidation of ammonium to nitrogen gas while using nitrite as electron acceptor (van de Graaf et al., 1995). Therefore, ensuring a consistent supply of nitrite is essential for successful implementation of anammox. In practice, partial nitrification (PN) and partial denitrification (PD) represent the two primary methods for generating nitrite. So far, most studies have focused on PN coupled with anammox (PNA), which theoretically can reduce oxygen consumption by 60 % and organic carbon consumption by 100 % compared to the nitrification-denitrification (Cao et al., 2017). However, the performance of PNA systems deteriorate when nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB). NOB repression is particularly hard in mainstream wastewater where the temperature and substrate concentrations are low, and the concentration of free ammonia and free nitrous acid is insufficient for NOB inhibition (Cao et al., 2017; Singh et al.,

* Corresponding author.
E-mail address: frank.persson@chalmers.se (F. Persson).

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2022). PD has recently been suggested as a more stable alternative for provision of nitrite in mainstream treatment (Ma et al., 2020). As opposed to the PN process, PD needs organic matter to support nitrate reduction. The organic content in municipal wastewater is generally sufficient for PD, even after pretreatment (Ma et al., 2020), and decreasing the concentration of organic carbon in the wastewater would benefit the anammox activity in partial denitrification-anammox (PDA) reactors (Izadi et al., 2023). Additionally, nitrate generated by the anammox bacteria (Strous et al., 1998) can be converted by PDA to ensure high effluent quality. Successful operation of PDA has been reported, even at low temperatures corresponding to cold municipal wastewater in temperate climate regions (13–15 °C) (Du et al., 2017). Substantial nitrogen removal via PDA at full-scale wastewater treatment plants (WWTPs) has also been reported (Fofana et al., 2022; Li et al., 2019).

To prevent the denitrifying bacteria to further reduce the formed nitrite, careful process control is needed (You et al., 2020). Recent studies have shown that the denitrification pathway can be truncated, where full denitrification is achieved through the cooperation of different bacteria, each harboring one or more denitrification genes to carry out multiple nitrogen oxide reduction steps (Zhang et al., 2022). Base on the genetic types, denitrifiers can be categorized in three groups (Table S1): group A that solely reduce nitrate to nitrite due to absence of nitrite reductase, group B that possess genes downstream nitrate reductase and do not accumulate nitrite due to simultaneous onset of the denitrification genes, and group C that also have the downstream genes but can accumulate nitrite due to progressive onset of the genes for nitrate and nitrite reductases (Ma et al., 2020). The type of carbon source is one factor that can affect the possibilities for nitrite accumulation (van Rijn et al., 1996; Izadi et al., 2023). Some carbon sources seem to support the growth of denitrifying bacteria in group A, and thus microbial communities selected by different carbon sources can result in varying degrees of nitrite accumulation (Izadi et al., 2023; Shi et al., 2019). The type of carbon source may also affect nitrite accumulation by group C denitrifiers. Batch tests with pure cultures of *Paracoccus denitrificans* and *Pseudomonas stutzeri* showed that some carbon sources promoted nitrite accumulation, while full denitrification was observed with other carbon sources (Blaszczyk, 1993; van Rijn et al., 1996).

In PDA reactors with mixed microbial communities, nitrite provision by denitrifying bacteria to anammox bacteria has been achieved using acetate, methanol, ethanol, glycerol and glucose, although at varying efficiency (Du et al., 2017; Le et al., 2019). Acetate has been successfully used in several studies (Le et al., 2019; Li et al., 2015a; Tao et al., 2023; Xu et al., 2020), but little is known about the effect of increasing the volatile fatty acid (VFA) chain length on the PDA process. This is of importance since hydrolysis and fermentation of wastewater organic matter typically produce a mix of acetate and propionate, with smaller fractions of butyric and valeric acids (Ali et al., 2020; Ossiansson et al., 2023). Recent results suggest that fermentation liquid can indeed govern PDA (Ali et al., 2020), which is promising and warrant further in-depth examination.

The availability of organic carbon also affects the anammox process. Some anammox bacteria are capable of mixotrophic growth where they utilize organic carbon in addition to inorganic substrates (Güven et al., 2005; Kartal et al., 2007a; Lawson et al., 2021). Cross-feeding of secondary metabolites with other bacteria might also be involved (Kallistova et al., 2022). No anammox bacterium have been isolated so far. They usually coexist with other bacteria in the phyla of *Chloroflexi*, *Chlorobi*, *Proteobacteria*, *Acidobacteria*, and *Bacteroidetes* (Zhao et al., 2018). It is possible that anammox bacteria rely on other microbial species to break down complex organic carbon sources into simpler molecules, which are more accessible (Zhao et al., 2018). Overall, the exact mechanism of oxidation of organic carbon sources by anammox bacteria is still not well understood and requires further exploration.

To understand how critical factors, like organic carbon source type, affect the nitrogen and carbon flows in PDA, thorough understanding of the microbial community structure, composition and metabolic potential is necessary. With the advancement of sequencing technology, metagenomics has become a powerful tool that allow for assessing microbial community structure, abundance of key functional genes, as well as the metabolic potential of the key players in the community by genome-centered metagenomics. With regard to anammox bacteria, these approaches have recently revealed new taxa, indicated altered pathways for nitrogen and organic carbon at varying reactor conditions, and suggested cross-feeding of metabolites with other community members (Bovio-Winkler et al., 2023; Yang et al., 2021; Zhao et al., 2018). Metagenomic approaches have also helped in analyzing main factors for nitrite accumulation by denitrifying communities and the denitrification-anammox coupling in PDA reactors (Qian et al., 2024; Zhang et al., 2022). However, main questions about microbial interactions and nitrogen removal pathways in PDA remain unanswered, which prevents understanding of process feasibility and optimization (Izadi et al., 2023).

This study aims to compare two organic carbon types, acetate and propionate, for establishment and maintenance of the PDA process at conditions and nitrogen concentrations relevant for municipal wastewater treatment by evaluating reactor performance, kinetic analysis of functional groups, and genome-centered metagenomics. The study was undertaken in one-stage PDA moving bed biofilm reactors (MBBRs).

2. Materials and methods

2.1. Continuous reactor operation

One MBBR received acetate as carbon source (R_A) and the other propionate (R_P). The two MBBRs had a working volume of 2 L and were operated in a water bath at 17.1 ± 0.4 °C. Stirring (45 rpm) by paddles and purging nitrogen gas ensured homogenous anoxic conditions. Plastic wrapping was used to limit the backflow of air into the overhead volume. Each reactor was inoculated with 225 AnoxKaldnes K5 biofilm carriers from a PNA reactor for sludge liquor treatment at Sjölunda WWTP in Malmö, Sweden (Christensson et al., 2013). The reactors were fed with 20 mg N/L of NO_3^--N and 20 mg N/L of NH_4^+-N. The synthetic wastewater was composed of tap water with additions per liter of 0.0764 g NH_4Cl, 0.144 g KNO_3, 0.68 g KH_2PO_4, 0.014 g $CaCl_2{\cdot}2H_2O$, 0.09 g $MgSO_4{\cdot}7H_2O$, and 1.25 mL trace element solutions A and B (Xu et al., 2020). The trace element solutions were prepared according to van Loosdrecht et al. (2016). Carbon sources, either 0.0586 g/L of CH_3COONa or 0.0265 g/L of CH_3CH_2COOH, were directly added to the synthetic wastewater during the first 5 weeks and added separately during the subsequent weeks to avoid microbial growth in the influent.

Dissolved oxygen (DO) concentrations, pH and temperature were measured by sensors using a HACH digital multimeter (HQ40D) and a pH meter (WTW pH320). Influent and reactor water samples taken 2–3 times a week were filtered through 0.45 μm membranes for measurements of COD, NH_4^+, NO_2^- and NO_3^-. COD was measured using HACH cuvettes (LCK314 and LCK1414), NH_4^+, NO_2^- and NO_3^- were measured using ion chromatography (Metrohm Eco IC).

2.2. Ex-situ batch tests

To measure potential denitrification and anammox activities, *ex-situ* batch tests were conducted on days 3, 23, 43, 67 and 130. Six conical flasks (300 mL) were placed in a water bath at 20 °C, and 30 biofilm carriers were added to each flask. The carriers were separately taken from the reactors (R_A and R_P) and returned to the corresponding reactor after finishing the test. Nitrogen gas was continuously purged to maintain anoxic condition. The nutrient medium in the flasks contained various combinations of nitrogen compounds (NO_3^-, NO_2^-, and NH_4^+ + NO_2^-) at concentrations of 75 mg N/L together with either acetate or propionate (300 mg COD/L) as carbon source, as shown in Table 1. In addition, the nutrient medium contained 22 mM KH_2PO_4 buffer, 0.014 g

Table 1
Details of initial substrates for the batch tests.

Test	NO_3^-	NO_2^-	NH_4^+	Acetate	Propionate	$NaHCO_3$	Source of carriers
	mg N/L	mg N/L	mg N/L	mg COD/L	mg COD/L	mg/L	
Dn-Nitrate_A	75			300			R_A
Dn-Nitrate_P	75				300		R_P
Dn-Nitrite_A		75		300			R_A
Dn-Nitrite_P		75			300		R_P
Amx_A		75	75			315	R_A
Amx_P		75	75			315	R_P

$CaCl_2{\cdot}2H_2O$, 0.09 g $MgSO_4{\cdot}7H_2O$, and 1.25 mL trace element solution A and B (Xu et al., 2020). Concentrations of NO_3^-, NO_2^- and NH_4^+ were measured as previously described (section 2.1) after 1, 60, 120, 180 and 240 min.

2.3. Calculations

2.3.1. Ex-situ batch tests

The *ex-situ* activities of carriers from R_A and R_P were calculated according to the following equation:

$$r_x = -\frac{slope_x}{A} \times \frac{0.24\ L}{30\ carriers} \times \frac{1440\ min/d}{1000\ mg/g}\ (g/(m^2 \cdot d)) \quad 2.1$$

where *slopex* is the slope of the trendline of the corresponding concentration plot (mg N/(L·min)), A is the surface area of each K5 carrier (2.417×10^{-3} m^2/carrier), 0.24 L is the batch reactor working volume, 30 is the number of carriers in each reactor.

2.3.2. Contribution of anammox to total nitrogen removal

The contribution of the anammox reaction to total nitrogen removal was calculated by:

$$\text{Anammox contribution} = \frac{1.02R_3}{1.02R_3 + 0.5R_2} \times 100\ (\%) \quad 2.2$$

where R_2 is the reaction rate of the denitrification of nitrite to nitrogen gas and R_3 is the anammox reaction rate. Detailed calculations of the reaction rates in two reactors are shown in the supplementary material.

2.4. Sequencing and bioinformatic analysis

The biomass samples were collected from the inoculation (seed) and the two reactors (R_A and R_P) at the end of the experiment. Each sample consisted of 5 individual biofilm carriers. The biofilm was first removed from the carriers and DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals) following the manufacturer's protocol. DNA fragmentation was performed using a Covaris E220 system targeting an insert size of 350–400 bp. Libraries were prepared using the SMARTer ThruPLEX DNA-seq Kit (Takara). Paired-end sequencing (150 bp) was carried out using the NovaSeq 6000 system, S4 flowcell and v1.5 sequencing chemistry. The raw sequence reads are deposited at NCBI as BioProject number PRJNA982692. The raw reads were filtered by fastp v0.20.0 and then normalized by BBNorm v38.61 with default settings. Qualified reads from all samples were then co-assembled into contigs using MEGAHIT v1.2.9 (Li et al., 2015b), and those longer than 2000 bp were collected for further analysis. Contig-based annotation was carried out by Prokka (Seemann, 2014). Coverage and detection of genes were calculated by Anvi'o v7.1. MetaBat2 v2.12.1 and Binsanity v0.5.4 were used for binning and the metagenome-assembled genomes (MAGs) from the two software packages were consolidated into one single bin set using Das_tool v1.1.3. Anvi'o v7.1 was used to check for the completeness and contamination of all MAGs. The relative abundance and taxonomy of each MAG were determined separately by CoverM v0.6.1 and GTDB-Tk v1.3.0. Prokka v1.12 was applied for the annotation of all recovered MAGs and phylogenic analysis was performed by Phylophlan v3.0.3 (Asnicar et al., 2020). The average nucleotide identity (ANI) was calculated between anammox MAGs and reference genomes by Pyani v0.2.12 (Pritchard et al., 2016). Co-occurrence network analysis was visualized by Gephi v0.10.1 (Bastian et al., 2009) based on Pearson's correlation coefficient calculated using FastSpar v1.0.0 (Friedman and Alm, 2012; Watts et al., 2019). The connection between two nodes indicates a strong ($|r| > 0.5$) and significant ($p < 0.05$) correlation. The top 30 most abundant MAGs were selected for construction of the network.

3. Results and discussion

3.1. Performance of the reactors

The two MBBRs were operated in parallel for 130 days with either acetate (R_A) or propionate (R_P) as carbon source. After a start-up period (the first 30 days), anoxic conditions and stable influent were maintained with DO concentrations of around 0.15 mg/L and influent concentrations of around 18–19 mg/L of NH_4^+-N and NO_3^--N, and 35–37 mg/L of COD, corresponding to a COD/nitrate ratio of 2 (Fig. 1d–h).

From the effluent concentrations (Fig. 1), it was clear that R_A removed considerably more ammonium and nitrate than R_P. The latter reactor quickly reached stable performance (Fig. S1), with NH_4^+-N and NO_3^--N removal rates of 0.07 ± 0.02 g N/(m^2·d) and 0.21 ± 0.01 g N/(m^2·d), respectively (Table 2). In contrast, removal rates in R_A increased until day 65 (Fig. S1) and subsequently remained stable at that level with NH_4^+-N and NO_3^--N removal rates of 0.24 ± 0.03 g N/(m^2·d) and 0.36 ± 0.06 g N/(m^2·d), respectively (Table 2). It was also clear that the reactors differed in nitrite accumulation, with increasing effluent concentrations of nitrite in R_A after day 70, while no nitrite was detected in R_P. A similar phenomenon of acetate causing higher nitrate to nitrite conversion than propionate was also shown in denitrification batch studies, presumably caused by lower abundance of nitrite-than nitrate reductases in the denitrifying microbiome (Li et al., 2015a).

Increasing ammonium and nitrate removal rates in R_A demonstrated that the interaction between partial denitrification and anammox (PDA) was improving over time in the biofilm, as also seen by the increasing anammox contribution stabilizing at 87 % in R_A (Fig. S2), while in R_P the mean anammox contribution was lower, at 41 % from day 70–130. Recent studies have observed similar anammox contributions to total nitrogen removal (70–94 %) in activated sludge and granular sludge PDA reactors fed with acetate as carbon source (Du et al., 2016; Xu et al., 2020), indicating that efficient nitrite transfer between the denitrifying and anammox populations can be obtained with different biomass configurations. Considering total nitrogen removal, R_A had an average removal rate of 0.52 g N/(m^2·d) during steady state, which was about double that of R_P (Table 2). The removal rate of R_A was in a range relevant for full-scale nitrogen removal at WWTPs and was comparable to that of a PNA MBBR pilot system (Gustavsson et al., 2020).

3.2. Dynamic changes in the denitrification and anammox capacity

Ex-situ batch tests were conducted to investigate changes over time

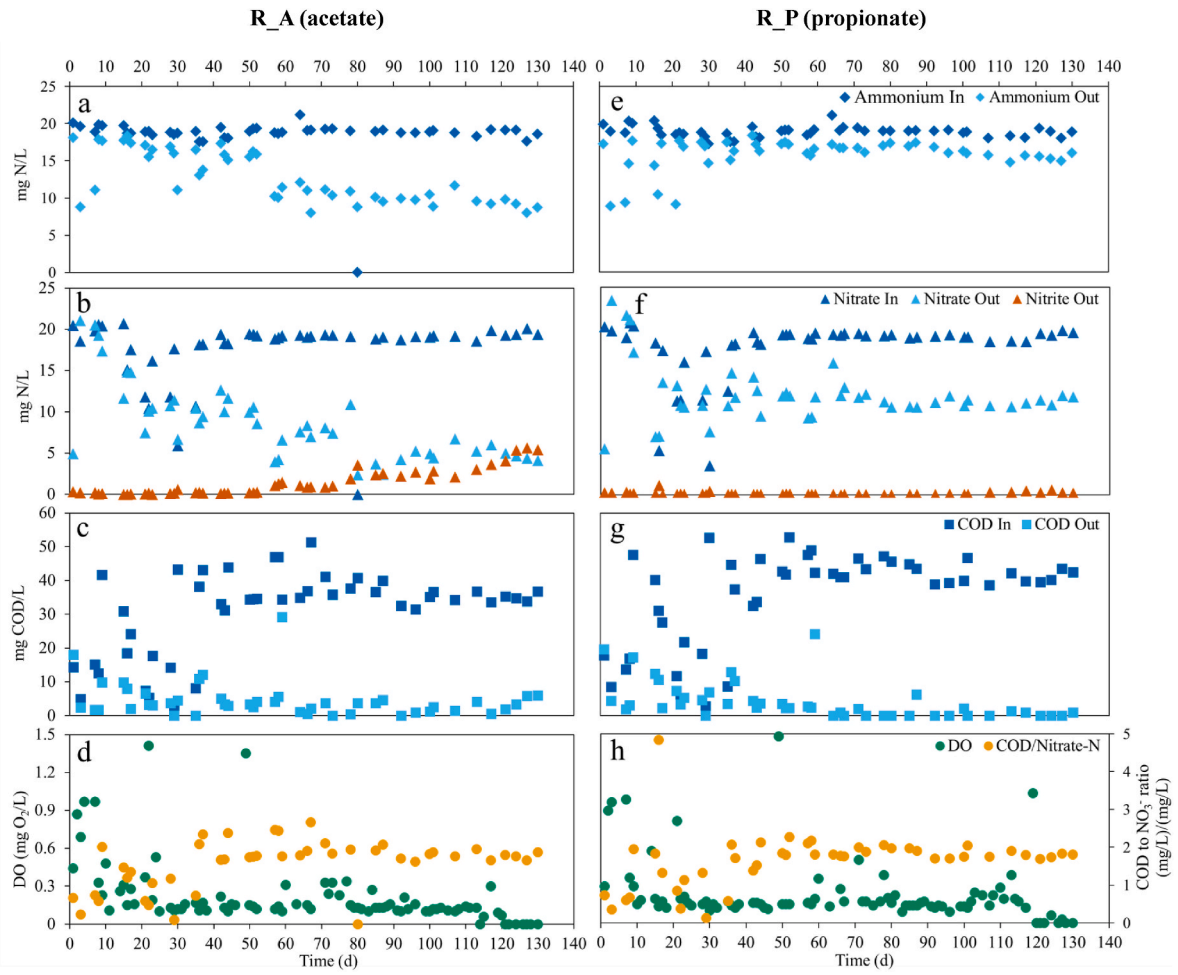

Fig. 1. Operating conditions and effluent concentrations of the PDA reactors R_A fed with acetate (a-d) and R_P fed with propionate (e-h).

Table 2
Comparison of kinetic parameters during steady state (from day 70–130) in R_A and R_P.

	TN loading	TN removal	NO_3^- removal	NH_4^+ removal	NO_2^- accumulation	AMX contribution	COD removal
	(g N/(m^2·d))					%	(g COD/(m^2·d))
R_A, mean	1.00	0.52	0.36	0.24	0.08	87	0.87
R_A, st. dev.	0.04	0.05	0.06	0.03	0.04	11	0.05
R_P, mean	0.95	0.27	0.21	0.07	0.00	41	0.91
R_P, st. dev.	0.04	0.02	0.01	0.02	0.00	13	0.07

of the denitrifying and anammox capacity of the microbiomes in the two biofilm reactors (Table 3).

The biofilm carriers from R_A (acetate) exhibited a gradual increase in nitrate removal capacity from 0.41 to 1.16 g N/(m^2·d), consistent with the progressive enhancement of nitrate removal observed in the reactor (Fig. 1 and S1), while the nitrite reduction capacity decreased over time (Table 3). A progressive nitrite accumulation during the nitrate removal tests with acetate (Fig. S3) highlighted this trend. The biofilm carriers from R_P (propionate) did not show a similar increase in nitrate removal rate, but even for this reactor, the nitrite reduction capacity decreased substantially (Table 3), although nitrite did not accumulate during the nitrate removal rate tests (Fig. S3). This confirms the difference between the two substrates for turnover of nitrogen by the denitrifying communities. The results would suggest a change towards more typical group A and C phenotypes of denitrifiers (Ma et al., 2020) in the acetate-fed biofilm. However, even physiological responses can help in explaining the results. Studies on pure cultures of denitrifying bacteria have shown that VFAs of small size (acetate) can result in higher nitrate- but lower nitrite reduction rates than VFAs of larger sizes (propionate, butyrate etc.) (van Rijn et al., 1996), which is in line with the observed differences between R_A and R_P.

The biofilm communities in the seed were collected from a sludge

Table 3
Potential denitrifying- and anammox activity by *ex-situ* batch tests of biofilm carriers from R_A (acetate) and R_P (propionate). The units are g N/(m^2·d) for all measurements.

Days	Denitrification				Anammox			
	NO_3^- Removal		NO_2^- Removal		NH_4^+ Removal		NO_2^- Removal	
	R_A	R_P	R_A	R_P	R_A	R_P	R_A	R_P
3	0.41	0.21	0.71	0.30	1.10	1.10	1.67	1.67
23	0.42	0.28	0.25	0.17	0.79	0.82	1.08	1.12
42	0.59	0.39	0.30	0.21	0.77	0.58	1.06	0.79
67	1.04	0.77	0.18	0.07	0.27	0.09	0.33	0.14
130	1.16	0.29	0.20	0.07	0.22	0.05	0.30	0.13

liquor treatment PNA reactor and were thereby adapted to conversion of high concentrations of nitrogen, 855 mg N/L in the influent (Christensson et al., 2013). The biofilm niches in the PNA reactor would have included high concentrations of nitrite to be converted via anammox and denitrifying bacteria. This explains the drop in nitrite-reducing capacity over time by both denitrifying and anammox bacteria (Table 3) as the influent nitrogen concentration decreased to municipal wastewater conditions (40 mg N/L). In particular, this resulted in a gradual decrease of anammox activity. The anammox ammonium removal capacities on days 67 and 130 (Table 3) were, however, similar to the actual ammonium removal rates in both reactors (Fig. S1). The anammox bacteria apparently worked at close to full capacity in the reactors during the later phases of the reactor run, suggesting that the anammox activity in the PDA reactor eventually had reached a stable state at the new, lower load.

3.3. Community-wide functional potential showed development of key genes for PDA

A total of 1,930,550 genes were annotated from the contigs in this study. The general distributions of cluster of orthologous groups (COG) categories in R_A and R_P were similar (Fig. S4), indicating a similar general functional potential in the two reactors. To gain insights into the reactor specific potential of the microbiome, the occurrence of key genes for transformation of nitrogen and organic carbon was investigated (Fig. 2). The distribution of key genes in the two reactors was however also similar, indicating that at the gene level, organic carbon sources type (acetate *vs.* propionate) was not a major factor shaping the microbiome. The different nitrogen removal performance of two reactors (Fig. 1, Fig. S1) was apparently not caused by differences in functional gene abundance.

There was, though, an obvious change in abundance of the key genes in R_A and R_P compared with the seed. The *hdh* gene, encoding the last step in the anammox reaction, was the most prevalent nitrogen

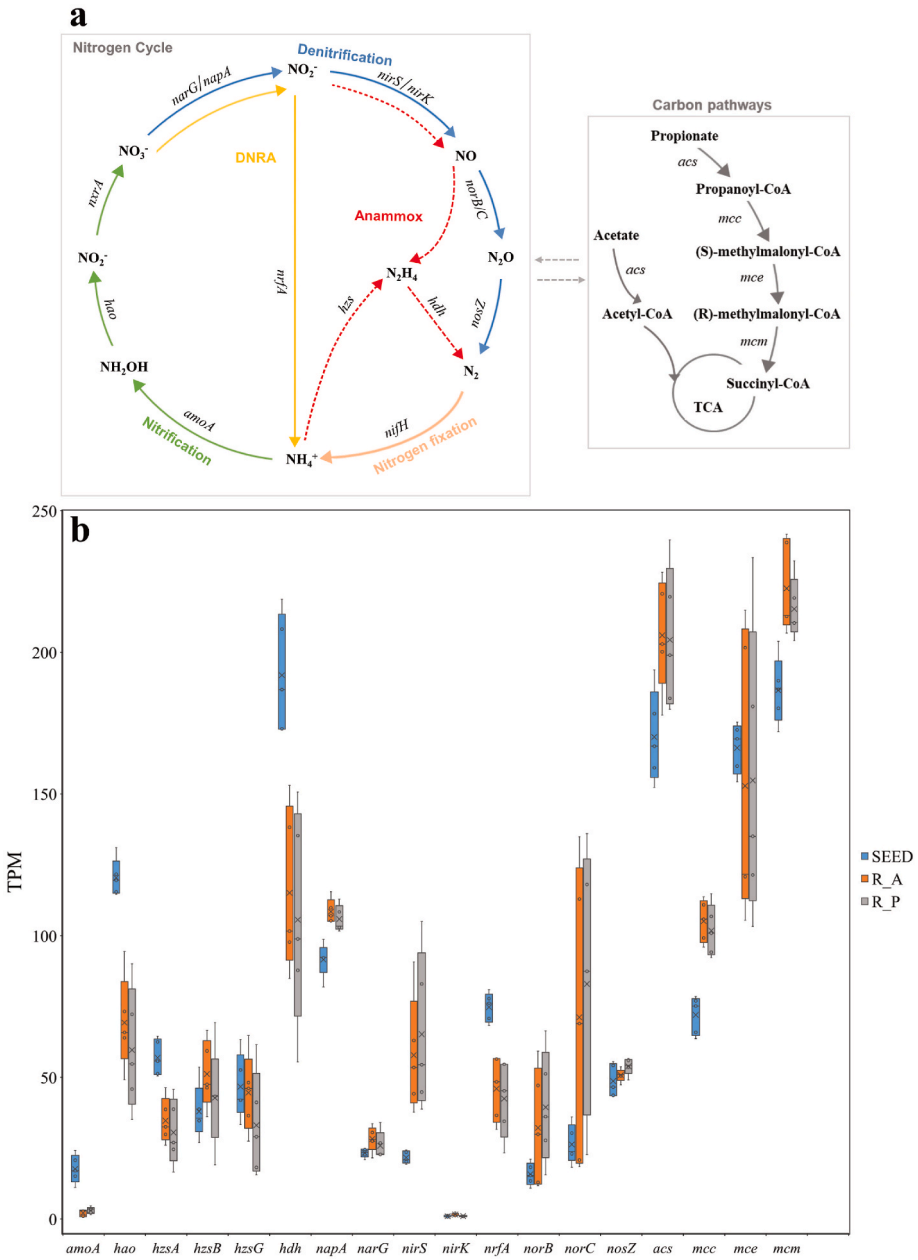

Fig. 2. a) Metabolic pathways related to key genes involved in nitrogen and carbon conversion. b) Normalized occurrence (TPM) of reads of the key genes.

conversion gene in the seed and decreased sharply in both reactors. This explains the loss of anammox capacity over time (Table 3). A marked decrease of *amoA* was also observed (Fig. 2), indicating an expected loss of aerobic ammonium oxidizing capacity in the PDA reactors. In contrast, the relative abundances of the denitrifying genes *napA*, *nirS* and *norB/C* increased in both reactors compared with the seed. *napA* and *nirS* were the main genes for nitrate and nitrite reduction, respectively. The abundance of *nirS* was, however, much lower than that of *napA*, suggesting a higher nitrate-than nitrite reduction capacity, as was shown by the batch tests for R_A or R_P in the later phases of the study period. This finding is crucial for the application of PDA, as the unbalance in nitrate and nitrite reduction capacity of the denitrifiers is essential to generate sufficient nitrite for the growth and activity of the anammox bacteria (You et al., 2020). The *napA* and *narG* genes encodes nitrate reductases of different types, periplasmic and cytosolic, with different operon regulation (Asamoto et al., 2021). The observation that *napA* was the main gene for nitrate reduction in R_A and R_P differs from previous observations of a PDA system where *narG* was the main nitrate reductase gene (Li et al., 2019). This suggests that successful PDA can be reached with nitrate reductase of both types, despite differences in the location and regulation of the enzymes.

The relative abundance of the acetate oxidation related gene *acs* (encoding acetyl-CoA synthetase (ACS), which catalyzes the ligation of acetate with CoA for acetyl-CoA production) increased considerably in both reactors (Fig. 2). Even propionate utilization is catalyzed by ACS through propionate-CoA production (Russ et al., 2012) (Fig. 2a). The increase of *acs* in both reactors compared with the seed indicated the growth of heterotrophic microorganisms necessary for the establishment of PDA. Besides *acs*, propionate oxidation involves *mcc* (Methylmalonyl-CoA carboxyltransferase), *mce* (Methylmalonyl-CoA epimerase) and *mcm* (Methylmalonyl-CoA mutase), which encode for transformation of propionate-CoA to succinyl-CoA by α-carboxylation reactions and further to acetyl-CoA through the TCA cycle (Yang et al., 2022). Although *mcc* and *mcm* increased in abundance in both reactors compared with the seed, the distribution of the mc-genes was similar in R_A and R_P, suggesting that the carbon source type (acetate *vs.* propionate) did not result in a different gene pool.

In short, contig-based metagenomics helped in explaining the development of the microbiome towards a PDA community but did not fully address the interaction between the two carbon sources and the microbiomes. For this, insights in the response of individual microbiome members are necessary.

3.4. Microbial community structure based on genome-centric metagenomics

A total of 293 MAGs were recovered from co-assembly of the 15 biofilm samples (5 individual carriers each from seed, R_A and R_P), with 136 high quality MAGs (completeness >90 %, contamination <5 %) and 157 medium quality MAGs (completeness ≥50 %, contamination <10 %) (Bowers et al., 2017). Over 60 % of the total reads were mapped to these MAGs, indicating a fair representation of the microbial community.

Members of *Proteobacteria* increased considerably in the two reactors compared with the seed (Fig. S5), with enrichment of several families such as *Burkholderiaceae* and *Rhodocyclaceae* which are common in municipal wastewater treatment (Fig. 3). *Burkholderiaceae* have members capable of simultaneous phosphate and nitrate removal (Wang et al., 2020). Its mean relative abundance increased from 2.4 % (seed) to 8.5 % (R_A) and 8.8 % (R_P). *Rhodocyclaceae* increased in mean relative abundance from 0.4 % to 4.7 % (R_A) and 5.3 % (R_P), consisting of well-known denitrifying genera like *Thauera*, *Dechloromonas*, *Dechlorobacter* and *Denitratisoma*. Aerobic ammonium oxidizing bacteria (AOB) within *Nitrosomonadaceae* decreased in both reactors, from 2.8 % in the seed to 0.4 % (R_A) and 0.6 % (R_P), showing the washout of AOB under the anoxic condition. Anammox bacteria, within *Ca.* Brocadiaceae, decreased in mean relative abundance from 27.3 % (seed) to 17.4 % (R_A) and 15.5 % (R_P), matching the decrease of the *hdh* gene (Fig. 2). Interestingly, the microbiome composition on the replicate biofilm carriers from the same sample type (seed, R_A and R_P) varied

		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
Acidobacteriota	*Pyrinomonadaceae-*	0.58	0.57	1.05	0.89	0.48	1.09	0.48	0.42	0.65	1.35	0.57	0.55	0.53	1.30	1.31
	Bryobacteraceae-	0.85	0.81	0.65	0.77	0.84	0.47	0.56	0.75	0.84	0.68	0.60	0.65	0.79	0.61	0.83
Bacteroidota	*Ignavibacteriaceae-*	4.34	4.29	2.84	4.51	5.28	2.45	6.95	5.55	3.36	1.80	2.95	5.75	6.83	1.62	2.23
	Chitinophagaceae-	0.69	0.77	1.28	1.12	0.67	1.70	0.69	0.79	1.32	1.48	1.36	0.96	0.55	1.70	1.57
	Saprospiraceae-	0.63	0.60	1.37	0.76	0.46	0.67	0.20	0.16	0.80	0.63	0.81	0.26	0.07	0.58	0.63
	PHOS-HE28-	0.75	0.78	1.20	0.97	0.51	0.76	0.17	0.12	0.70	0.44	0.71	0.27	0.13	0.45	0.48
	OLB10-	0.92	0.96	0.99	1.04	0.81	0.19	0.49	0.19	0.47	0.26	0.30	0.63	0.25	0.20	0.28
Chloroflexota	*EnvOPS12-*	4.85	5.07	5.27	4.42	5.17	3.16	2.23	1.65	3.88	3.78	4.17	4.54	2.36	3.98	4.56
	SHND01-	0.73	0.64	1.21	0.87	0.57	0.27	0.60	0.31	0.42	0.23	0.31	0.59	0.48	0.18	0.28
	UTPRO1-	1.21	1.05	1.08	1.57	1.22	0.46	1.46	1.02	0.53	0.29	0.38	1.01	1.54	0.23	0.34
	RBG-16-71-46-	0.71	0.58	0.23	0.56	0.68	0.44	1.06	0.90	0.42	0.20	0.42	0.71	1.17	0.16	0.20
	Gemmatimonadaceae-	0.62	0.55	0.52	0.56	0.63	0.33	0.66	0.49	0.62	0.27	0.43	0.64	0.68	0.24	0.33
	OLB16-	0.44	0.44	0.68	0.50	0.37	0.67	0.28	0.18	0.75	0.66	0.67	0.43	0.23	0.62	0.71
	UBA2023-	0.00	0.00	0.00	0.00	0.00	1.43	0.08	0.06	0.79	1.51	1.03	0.50	0.13	1.63	1.41
Planctomycetota	*Brocadiaceae-*	27.45	31.32	23.78	24.05	29.95	14.95	19.68	15.95	23.57	13.02	23.19	19.08	11.92	8.83	14.33
	UTPLA1-	2.88	2.16	0.83	2.71	3.71	0.83	10.35	11.20	0.93	0.59	1.03	6.42	12.78	0.47	0.72
	UBA1924-	0.61	0.56	0.40	0.49	0.61	0.79	0.64	1.47	0.80	0.58	0.71	0.81	0.87	0.61	0.66
Proteobacteria	*Burkholderiaceae-*	2.42	2.15	2.78	2.60	2.08	10.86	4.82	5.21	8.71	12.67	9.37	6.04	4.19	13.16	11.05
	Rhodocyclaceae-	0.46	0.43	0.48	0.39	0.39	6.49	1.56	1.73	5.04	8.42	5.84	3.58	1.45	8.63	7.13
	Beijerinckiaceae-	0.02	0.02	0.02	0.02	0.01	1.47	0.31	0.28	0.81	4.93	0.84	1.02	0.71	4.74	3.94
	Nitrosomonadaceae-	2.52	2.42	3.81	3.30	1.92	0.65	0.22	0.14	0.59	0.61	0.67	0.49	0.26	0.56	0.84
	Ahniellaceae-	0.00	0.00	0.01	0.00	0.00	2.18	0.13	0.21	1.74	1.57	1.69	0.83	0.16	2.71	1.79
	GCA-2729495-	0.77	0.65	0.66	0.71	0.66	0.37	0.59	0.51	0.57	0.29	0.45	0.59	0.70	0.25	0.33
	Abundance<0.5-	15.74	13.43	15.78	16.18	14.19	8.63	17.37	20.14	9.61	9.77	8.10	13.20	21.47	10.28	10.44
	unmapped-	29.87	29.82	33.14	31.03	28.83	38.68	28.45	30.58	32.08	33.99	33.42	30.47	29.76	36.23	33.63
		SEED					R_A					R_P				

Fig. 3. Relative abundance of the MAGs at family level in the microbiomes on individual carriers from the seed, R_A and R_P.

considerably for the reactor samples, with the *Ca.* Brocadiaceae members having a relative abundance between 13-24 % and 9–23 % on the R_A and R_P carriers, respectively (Fig. 3). This suggests variation in the interactions between the microbiome members among the biofilm carriers, presumably resulting in niche differences and varying functions.

3.5. The potential for denitrification and organic carbon conversion among microbiome members

Most MAGs possessed one or more denitrifying genes, while only 9 MAGs contained all four genes for full denitrification (Table S2). This is in line with recent research showing that bacteria capable of full denitrification are rare and that denitrification hence is a result of interspecific cooperation by a consortium of denitrifiers (Gao et al., 2019; Valk et al., 2022).

The *napA* nitrate reducers were dominant in this study, distributed in a wide range of phyla like *Planctomycetota*, *Proteobacteria*, *Acidobacteriota* (Fig. 4). Around 30 % of all reads were mapped to MAGs with genes for nitrate reductase (*napA* or *narG*) (Fig. S6) underlining that the potential for nitrate reduction is common in wastewater microbiomes. The typical group A denitrifiers would have nitrate reductase (*napA* or *narG*) but lack nitrite reductase (*nirS*, *nirK* or *nrfA*). About 16–18 % of the reads were mapped to such MAGs (Fig. S6) with no major change between the seed and the reactor samples, indicating that group A denitrifiers were common but not enriched in the reactors. Furthermore, a large share of the MAGs that were grouped as group A denitrifiers had genes for acetate utilization. Of the 30 most abundant MAGs (Fig. 4), 12 had the genetic make-up for nitrite production using acetate (possess *acs*, *napA/narG*, but lack *nirS/nirK/nrfA*). However, only 3 MAGs (bins 14, 65, and 66) had the corresponding make-up for nitrite production using propionate (possess *acs*, *mcc*, *mce*, *mcm*, *napA/narG*, but lack *nirS/nirK/nrfA*). This indicates higher potential for nitrite accumulation with acetate than propionate and assist in explaining the observed nitrite accumulation in R_A and the higher nitrogen conversion via anammox using acetate compared to propionate (Fig. 1).

There are two main pathways for nitrite reduction (Fig. 2a), the one-electron reduction of nitrite to nitric oxide encoded by *nirS* or *nirK* and the six-electron reduction of nitrite to ammonium (part of dissimilatory nitrate reduction to ammonium, DNRA) encoded by *nrfA*. In this study, *nirS* was the main gene for the one-electron nitrite reduction with a TPM more than 20-fold higher than *nirK* (Fig. 2b). The *nirS*-containing MAGs mainly belonged to *Proteobacteria* and were highly enriched in R_A and R_P compared with the seed where they were virtually absent (Fig. 4). These Proteobacterial MAGs were responsible for the increase in total abundance of MAGs having genes for both nitrate and nitrite oxidation (Fig. S6). The MAGs may be classified as either group B or C denitrifiers, distinguished by the onset mechanisms for the nitrite reductase (Ma et al., 2020). MAGs with *nrfA* were mainly affiliated to *Planctomycetota*, *Chloroflexota* and *Bacteroidota* (Fig. 4). The decrease in abundance of *nrfA* in the reactor samples compared to the seed (Fig. 2) was due to the decrease of anammox bacteria (bin129) in the microbiome. Other DNRA bacteria, like bin204, bin36 and bin191, were relatively stable in abundance (Fig. 4).

The genes *norB* and *norC* encoding for reduction of nitric oxide were mainly detected in the enriched MAGs within *Proteobacteria* that also harbored genes for nitrite reduction (*nirS*), such as MAGs within *Burkholderiaceae* (bin 269), *Rhabdaerophilum* (bins 137 and 282) and *Azonexus* (bins 110 and 274). Unlike the nitrite and nitric oxide reducers centralized in *Proteobacteria*, MAGs with *nosZ* for N_2O reduction were detected in many phyla, even in MAGs having *nrfA* for DNRA (Fig. 4). This supports previous observations suggesting that a variety of members in wastewater microbiomes have potential for N_2O reduction and can function as a sink preventing emissions (Conthe et al., 2019; Valk et al., 2022).

Fermentation of acetate and propionate to methane, and subsequent use of methane for denitrification (DAMO) may also have contributed to the conversion of organic matter and nitrogen in the reactors (Ettwig et al., 2010; Haroon et al., 2013). However, only one potential methanogen (bin37) and one potential DAMO bacterium (bin4) were detected at low abundances among the MAGs (Table S2), indicating that methane turnover was of minor importance.

3.6. Anammox bacteria with metabolic versatility were favored in PDA systems

Two anammox MAGs were highly abundant in the microbiomes of both the seed and the reactor samples, bin129 that was similar to *Ca.* Brocadia fulgida (ANI = 99.8 %) and bin53 similar to *Ca.* Brocadia

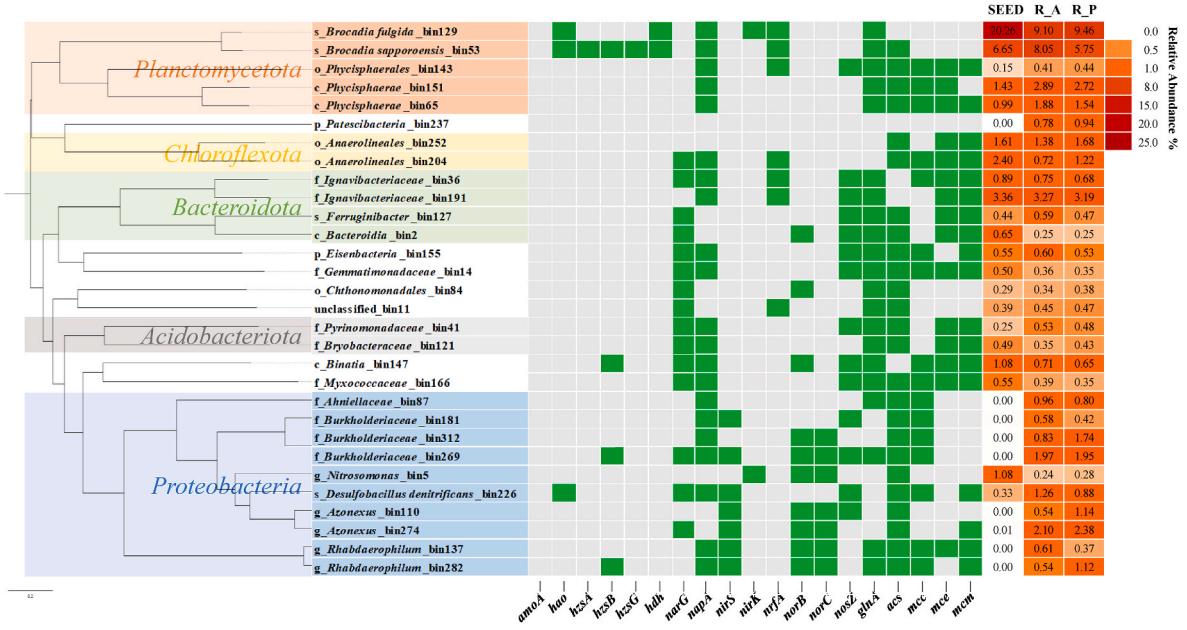

Fig. 4. The most abundant MAGs, mapping the key genes (green: present, grey: absent) and the mean relative abundance in the seed, R_A and R_P microbiomes.(For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sapporoensis (ANI = 99.3 %) (Table S3). Bin129 accounted for 20.3 % in the seed and decreased to 9.1 % in R_A and 9.5 % in R_P (Fig. 4). The second most abundant MAG, bin53, had however similar abundance in the seed of 6.7 % as in R_A and R_P of 8.1 % and 5.8 %, respectively (Fig. 4). Another two MAGs (bins 173 and 190) were identified, although they were not among the most abundant ones in the microbiomes. Bin173 was highly similar to *Ca.* Brocadia pituitae, with ANI of 99.1 % (Table S3). Bin190 belonged to *Ca.* Brocadia, but the highest ANI of 85.3 % with any other known *Ca.* Brocadia genome (Fig. 5) was considerably lower than the proposed species cutoff (ANI >95 %) (Konstantinidis et al., 2017). This suggests that bin190 represents a new species within the genus *Ca.* Brocadia. Both bin173 and bin190 had lower relative abundance in the reactor microbiomes than in the seed (Fig. S7).

The four anammox bins showed differences in the anammox catabolism consisting of nitrite reduction to NO, hydrazine synthesis, and hydrazine oxidation. Bin190 and bin129 had *nirS* and *nirK*, respectively for nitrite reduction, while these genes were absent in bin173 and bin53, as well as in their related genomes (Fig. S7). However, hydroxylamine oxidoreductase (HAO) has been shown to function as an alternative enzyme for nitrite reduction to NO in some anammox bacteria (Ferousi et al., 2021). *hao* was present in bin173 and bin53, as well as in most *Ca.* Brocadia MAGs (Fig. S7), suggesting the use of this mechanism for NO production. The *hzs* genes for hydrazine synthesis were present in all anammox MAGs except bin129 and were also absent in many of the related *Ca.* Brocadia fulgida genomes. Furthermore, *hdh* for hydrazine oxidation was present in all four MAGs and all other *Ca.* Brocadia genomes. It is likely that the absence of *hzs* genes in bin129, which is 86 % complete, and the other *Ca.* Brocadia genomes was caused by incomplete genome binning, rather than actual absence of genes for hydrazine synthesis in these anammox bacteria since they possess *hdh* and originate from reactors with nitrogen removal via anammox.

Apart from the anammox catabolism, bin129 and bin53 also harbored *nrfA* for DNRA. In addition, bin53 had the *acs* gene for oxidation of acetate and propionate (Lawson et al., 2021; Russ et al., 2012). Mixotrophic growth of anammox bacteria has been observed in laboratory studies where small organic acids provided with ammonium and nitrite, resulted in increased cell yield and anammox activity (Yang et al., 2021). DNRA coupled with organic oxidation has furthermore been shown to supply anammox bacteria with extra ammonium (Kartal et al., 2007b). This suggests a metabolic flexibility, where the bin53 potentially capable of organic acid utilization as well as DNRA, could sustain at unaltered relative abundance in the PDA reactors, as opposed to the other anammox strains, lacking this versatility. The organic matter concentration and type can indeed alter the abundance of anammox bacteria (Ji et al., 2022) and metabolic versatility of anammox bacteria may indeed be crucial for long time survival in municipal wastewater treatment (Yang et al., 2021).

3.7. Network analyses showed niche differentiation among the denitrifying and anammox microbiome members

Network analysis showing positive and negative significant correlations among the major MAGs was enabled by having replicate microbiomes for each sample type (Fig. 6). It was clear that many of the denitrifying community members had positive correlations, including potentially nitrate-reducing MAGs with and without genes for nitrite reductase. The high degree of interconnectivity matched well the highly truncated denitrifying gene distribution among these MAGs, indicating a necessary shuttling of intermediates for reducing nitrate to nitrogen gas (Zhang et al., 2023).

In the network, the two major anammox MAGs (bin129 and bin53) were in edge regions with relatively few connections. They had positive within-group correlations with two distinct groups of denitrifying bacteria (Fig. 6), highlighting their different distribution patterns in the microbiomes. The *Ca.* B. sapporoensis bin53 was positively correlated with potentially nitrate-reducing MAGs lacking nitrite reductase genes (group A) bin127, as well as ones having nitrate reductase (group B or C), such as bin143 and bin226. The *Ca.* B. fulgida bin129 correlated positively with other potentially nitrate-reducing MAGs without nitrite reductase genes (e.g. bin121 and bin2). This points to the different niches of the two main anammox bacteria, potentially interacting with different nitrate-reducing bacteria, as also indicated by their differences

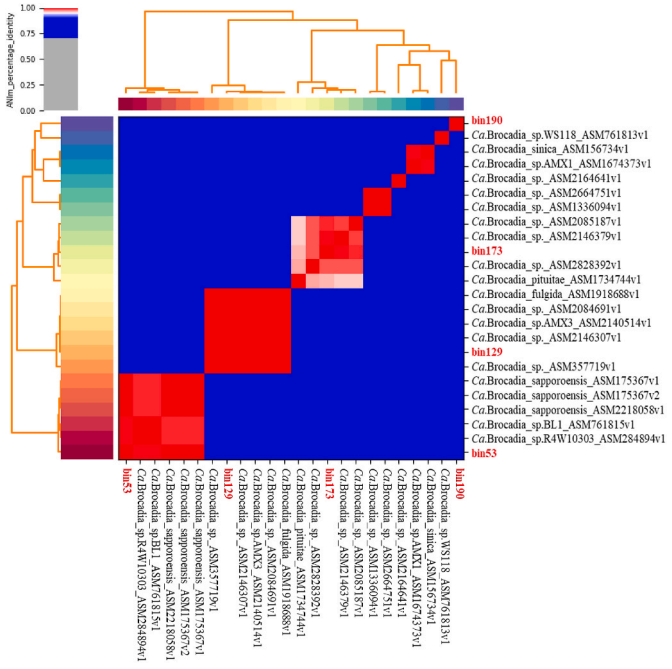

Fig. 5. Heatmap with hierarchical clustering of pairwise average nucleotide identities (ANI) between the anammox MAGs in this study (red) and closely related reference genomes (black).(For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

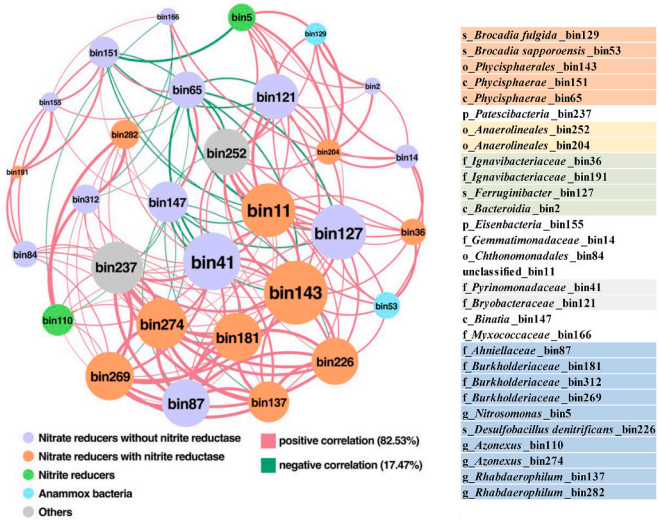

Fig. 6. Network positive and negative correlations of the 30 most abundant MAGs in the microbiomes from the seed, R_A and R_B. The size of each node represents the number of connections, and the thickness of each connection between two nodes (i.e. edge) indicates the correlation coefficient (r), with |r| ranging from 0.5 to 1. The classification and order of the bins (right) follows Fig. 4.

in metabolic potential (Fig. 4, S7). In addition, the *Ca.* B. fulgida bin129 correlated with an AOB MAG (*Nitrosomonas* bin5), showing that these two MAGs were key members in the PNA seed but were less competitive at the PDA conditions. Hence, the diversity among the anammox bacteria likely contributed to functional redundancy during the shift to PDA operation (Louca et al., 2018).

4. Conclusions

The type of organic carbon source had a large impact on the turnover of nitrogen for establishment of one-stage PDA, with more nitrogen removal via anammox with acetate than propionate. Despite this discrepancy, metagenomic analysis did not show significant differences between the two microbial enrichments, suggesting that the organic carbon source did not exert strong selective pressure. The performance of the two PDA reactors was instead attributed to variations in the microbiome metabolism:

1) The enriched denitrifying bacteria exhibited higher nitrate-than nitrite-reducing capacity as observed by higher relative abundance of genes for nitrate-than nitrite reduction in contig-based annotation and by more MAGs containing nitrate reductase genes. This would entail nitrite accumulating potential for use by the anammox bacteria in the PDA reactors.
2) Among the top 30 MAGs, only 3 had the ideal genetic make-up for nitrite accumulation using propionate, while 12 MAGs had the genetic make-up for nitrite accumulation using acetate. Thus, the microbiomes possessed higher nitrite accumulation potential with acetate than propionate, which can explain the higher anammox activity in the acetate-fed reactor.
3) The genetic profile of the anammox bacteria suggested that the potential for organic carbon utilization in *Ca.* Brocadia sapporoensis bin53 contributed to its persistence during the shift from PNA to PDA, while the other three anammox MAGs lacked genes for organic carbon utilization making them more vulnerable.

The large share of nitrogen removal via anammox with acetate at realistic rates for municipal wastewater treatment is promising for future applications. However, the type of carbon source can have a profound impact on process performance by metabolic limitations in the wastewater microbiomes.

CRediT authorship contribution statement

Zejia Zheng: Writing – original draft, Visualization, Investigation, Formal analysis. **David J.I. Gustavsson:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Dan Zheng:** Writing – review & editing, Visualization, Funding acquisition. **Felix Holmin:** Investigation, Formal analysis. **Per Falås:** Writing – review & editing, Investigation. **Britt-Marie Wilén:** Writing – review & editing. **Oskar Modin:** Writing – review & editing, Formal analysis. **Frank Persson:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2025.125972.

Data availability

Data will be made available on request.

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