

ARTICLE

Targeted metagenomics reveals extensive diversity of the denitrifying community in partial nitrification anammox and activated sludge systems

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Abstract

The substantial presence of denitrifiers has already been reported in partial nitrification anammox (PNA) systems using the 16S ribosomal RNA (rRNA) gene, but little is known about the phylogenetic diversity based on denitrification pathway functional genes. Therefore, we performed a metagenomic analysis to determine the distribution of denitrification genes and the associated phylogeny in PNA systems and whether a niche separation between PNA and conventional activated sludge (AS) systems exists. The results revealed a distinct abundance pattern of denitrification pathway genes and their association to the microbial species between PNA and AS systems. In contrast, the taxonomic analysis, based on the 16S rRNA gene, did not detect notable variability in denitrifying community composition across samples. In general, *narG* and *nosZa2* genes were dominant in all samples. While the potential for different stages of denitrification was redundant, variation in species composition and lack of the complete denitrification gene pool in each species appears to confer niche separation between PNA and AS systems. This study suggests that targeted metagenomics can help to determine the denitrifying microbial composition at a fine-scale resolution while overcoming current biases in quantitative polymerase chain reaction approaches due to a lack of appropriate primers.

KEYWORDS

activated sludge, functional diversity, metagenomics/community genomics, microbial communities, partial nitrification anammox

1 | INTRODUCTION

The tremendous anthropogenic influence of increasing nitrogen loads to feed the world's population has a significant effect on the global nitrogen cycle (Gruber & Galloway, 2008). As only a small proportion of the nitrogen is captured in our bodies, the main fraction is excreted with urine. If not treated properly, these nitrogen compounds are discharged into open water bodies, resulting in eutrophication.

The removal of nitrogen from wastewater is, therefore, of extreme environmental importance especially in densely populated areas (Kowalchuk & Stephen, 2001). Typical biological treatment technologies like the conventional activated sludge (AS) process are based on two-step nitrification/denitrification. Since the discovery of autotrophic anaerobic ammonium oxidizing bacteria (AnAOB; Strous et al., 1999), there has been a shift from conventional nitrification/denitrification to anaerobic ammonium oxidation combined with

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partial nitrification anammox (PNA) for certain applications over the last two decades. PNA is the completely autotrophic oxidation of ammonium with nitrite as an electron acceptor, converting nitrite and the remaining ammonium from aerobic AOB to nitrogen gas. Driven by the much higher energy-efficiency due to less aeration demands, today, more than 150 PNA installations are already successfully operated worldwide (Lackner et al., 2014).

Initially, only a very limited number of microorganisms, that is, AOB, AnAOB, and nitrite-oxidizing bacteria (NOB) were considered as key players in PNA systems. However, with time many studies reported that denitrifiers are common and usually dominant in PNA systems. Thus, these bacteria may contribute to the overall nitrogen removal potential of the PNA microbiome (Agrawal et al., 2017; Chen et al., 2019; Chu et al., 2015; Pereira et al., 2017; Persson et al., 2017; Y. Wang et al., 2019). Denitrification is a facultative respiratory pathway reducing nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) to nitrogen gas (N_2) catalyzed by four types of nitrogen reductases in sequence: nitrate reductase (*Nar*), nitrite reductase (*Nir*), nitric oxide reductase (*Nor*), and nitrous oxide reductase (*Nos*; Zumft, 1997). Denitrification is not typically linked to phylogeny because denitrifiers are taxonomically diverse, therefore most studies about denitrification focused on functional genes rather than the 16S ribosomal RNA (rRNA) gene (Philippot, 2006). But, in case of PNA systems, most of the previous studies reported the occurrence of denitrifiers in PNA systems based on 16S rRNA gene amplicon sequencing (Agrawal et al., 2018; Cao et al., 2017; Pereira et al., 2017).

There are few studies (Pellicer-Nàcher et al., 2014; Shu et al., 2018; D. Wang et al., 2019), which used quantitative polymerase chain reaction (qPCR) for quantitative analysis of denitrifying functional genes in PNA systems. However, for all denitrifying genes, several primer pairs have been published and continuously reassessed, especially for nitrite reductase (*nirK* and *nirS*; Braker & Tiedje, 2003; Casciotti & Ward, 2001; Throback et al., 2004). Ma et al. (2019) evaluated the coverage of existing primer pairs in silico based on a metagenomic study. The results pointed out that the existing *nirK*, *nirS*, and *norB* primers have low coverage over the entire gene pool, and in consequences are hardly suitable for molecular methods to investigate denitrifiers. Also for *narG* and *nosZ* primers, there are serious issues for both, coverage and specificity (Ma et al., 2019). This implies that the use of currently available primers for assessing denitrifiers can introduce bias in the results. A similar situation appears with primer pairs for the 16S rRNA gene and other functional genes (Orschler et al., 2019).

The use of metagenomics can circumvent the problems associated with PCR-based approaches in understanding the role of denitrifiers, which carry one or more denitrifying genes (Agrawal et al., 2018). To the best of our knowledge, very few metagenomic studies are available on PNA systems, and that limits the focus primarily to AnAOB and few selected denitrifiers (Bhattacharjee et al., 2017; Guo et al., 2016; Lawson et al., 2017; Speth et al., 2016). Nevertheless, these studies are only based on the analysis of a single sample with no observation of the diversity of denitrifying community between

different PNA and AS systems. Moreover, these studies were based on the lab-scale PNA system (Bhattacharjee et al., 2017; Guo et al., 2016; Lawson et al., 2017), or a full-scale PNA reactor at the end of the mainline of a wastewater treatment plant treating potato-processing wastewater at 35°C (Speth et al., 2016). Thus, there is still a lack of knowledge about the extent of diversity of denitrifying community based on profiling of functional genes among full-scale PNA systems, as well as to what extent the denitrifying community of full-scale PNA differs from AS systems.

In this study, we investigated differences and similarities of the denitrification genes and microorganisms associated with them, between two sidestream PNA systems and also compared with the conventional AS processes of the respective wastewater treatment plants (WWTPs). The de novo metagenomic assembly tends to assemble dominant microorganisms (Namiki et al., 2012; Q. Wang et al., 2015), because it is reliant on reference sequence databases containing an unknown amount of extant microbial diversity. Thus, it could result in the limited recovery of denitrification genes. Therefore, we analyzed samples from two different WWTP employing a targeted metagenomic assembly approach. We also show in this study, the impact of determining the composition of denitrifying members based on 16S amplicon sequencing in comparison to the targeted metagenomic assembly approach for genes associated with the denitrification pathway.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Biomass samples were collected from two different wastewater treatment plants in Germany: two samples from conventional AS nitrification–denitrification processes (AS1 and AS2) and two samples from sidestream single-stage sludge-based PNA processes (PNA1 and PNA2; Table S1). Total genomic DNA was extracted from triplicates using the Fast DNA Spin Kit for soil (MP Biomedicals) according to a modified manufacturer's protocol (Orschler et al., 2019) and pooled afterward. The quality of the DNA was checked using gel electrophoresis, and the concentration was measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific).

2.2 | Library preparation and sequencing

Sample preparation and library construction were performed with the Ion Xpress™ Plus Fragment Library Kit (Thermo Fisher Scientific). Enzymatic shearing was used to prepare fragment libraries from genomic DNA for downstream template preparation and was handled according to the manufacturer's protocol. Purified DNA was tagged using the Ion Xpress Barcodes Adapters™ (Thermo Fisher Scientific) and the size selection of the library for 600 base-pair (bp) reads was performed with E-Gel™ Size Select™ II Agarose Gel. Each sample was adjusted to a 60-pM concentration. Template

preparation was performed on the ION chef system with Ion 520™ & Ion 530™ ExT Kit. Sequencing was performed on the Ion Torrent (ION Torrent Ion S5) using the 530 chip. Base-calling, demultiplexing, and initial quality control were conducted by Torrent Suite version 4.4.2 (Thermo Fisher Scientific) with default parameters. Trimmed and quality-filtered reads were used for downstream analyses.

2.3 | Targeted metagenomics for functional analysis

To evaluate the abundance and phylogenetic affiliation of genes associated with nitrogen metabolism, specific genes were assembled using the Xander assembler (Q. Wang et al., 2015). To assemble the sequences, Xander requires the protein profile Hidden Markov Model (HMM) built from a reference set of target genes. For *narG*, *napA*, *nirK*, *nirS*, *norB*, *nosZ*, *nosZa1*, and *nosZa2* genes, nucleotide, and amino acid sequences were downloaded from the Functional Gene Repository (<http://fungene.cme.msu.edu>). The minimal cutoff was set to 100 amino acids. For each gene, a table of the operational taxonomic unit (OTU) counts was made based on k-mer (set value 45) coverage of the representative sequences. The OTU tables were further analyzed in R.

2.4 | Taxonomic affiliation based on the 16S rRNA gene

From the whole metagenome dataset, 16S rRNA gene sequences were extracted with Metaxa2 (version 2.0) using default settings (Bengtsson-Palme et al., 2015). Genus assignment was performed at more than 95% identity with the reference 16S rRNA gene sequence and reported as relative abundance.

2.5 | Statistical analysis

All statistical tests were performed in R. Nonmetric multidimensional scaling (NMDS) analysis was performed using the metaMDS function of the “vegan” package to create an ordination based on the sample dissimilarity. Heatmaps and bar plots diagrams were all generated in R (<http://www.R-project.org/>). Venn diagrams were created using the “VennDiagram” package in R. Proportionality correlation was performed using the “propr” package in R and *p*-values for the correlation analysis were also calculated.

3 | RESULTS

The four samples (two samples from each WWTP: sidestream PNA and AS samples; for more information see Table S1) produced an average of 12 million quality filtered, merged reads, at an average length of 460 bp. The four samples were grouped into two categories by process design for analysis and discussion. The first category

summarizes the AS processes (referred to as AS1 and AS2), whereas the second group includes the sidestream PNA systems (PNA1 and PNA2) with no external inoculum for process start-up. To focus on the denitrification pathway, reads were extracted from the full metagenome dataset and analyzed. From here on, the read abundance is presented as mean read per million (rpm) reads.

The results revealed that the aggregate abundance of genes associated with nitrate (*narG* and *napA*) and nitrous oxide reduction (*nosZ*, *nosZa1*, and *nosZa2*) were higher than the genes associated with the nitrite and nitric oxide reduction (Figures 1 and S1). The total abundance of the nitrate reductase encoding genes in PNA2 (217 rpm) was highest, followed by PNA1 (164 rpm) > AS2 (87 rpm) > AS1 (73 rpm). However, looking at individual genes (i.e., *napA* and *narG*), the respiratory nitrate reductase gene (*narG*) was higher in abundance than the periplasmic nitrate reductase gene (*napA*), especially in PNA systems. The *narG:napA* ratio ranged from 12.0 for PNA1; 8.0 for PNA2; 2.0 for AS2 to 1.0 for AS1. In the AS samples, the *napA* gene was more abundant than in the PNA samples. In AS1, AS2, and PNA1 the cytochrome *cd* containing nitrite reductase gene (*nirS*), as well as the copper-containing nitrite reductase gene (*nirK*) were found. However, the *nirS* gene was dominant in all the samples. In PNA2 only *nirS* was found. The aggregate abundance of the nitrite reductase genes (i.e., *nirS* and *nirK*) was very similar for AS1 (77 rpm) and PNA2 (71 rpm), followed by PNA1 (61 rpm) and AS2 (32 rpm). The abundance of nitric oxide gene (*norB*) was higher in the AS than in the PNA samples (Figure S1). The aggregate read the abundance of the N₂O reductase genes (i.e., *nosZ*, *nosZa1*, and *nosZa2*), exhibited a similar trend to the *nirS*, because *nosZ* gene abundances were higher in the AS and lower in the sidestream PNA in WWTP1 and vice versa in WWTP2. Of the total reads in all samples assigned to *nosZ*, the majority of the reads assigned to the atypical clade II *nosZ* gene, that is, between 62% and 80% of the total reads.

3.1 | Correlation analysis of denitrification genes

To investigate the association between the target genes, we assessed the proportionality between the read abundance of the genes across samples. Results are presented in a heatmap of the proportionality metric, ρ , between read counts from each gene in more detail (Figure 2). *NarG* and *napA* read counts were inversely proportional ($\rho = -1$), while the abundance of the *narG* was strongly proportional with *nirK* ($\rho = 0.99$) and *nosZ* ($\rho = 0.96$). *NapA* and *norB* abundances were also strongly proportional ($\rho = 0.95$). Proportionality clustering revealed that the abundances of the *nirS* and *nirK* had no significant association. However, *nirS*, *nosZa1*, and *nosZa2* have a significant association, while *nirK* and *nosZ* abundances are significantly associated.

3.2 | Taxonomic composition

Additionally, we performed taxonomic annotations of the denitrification target genes to determine the pattern in the abundance of

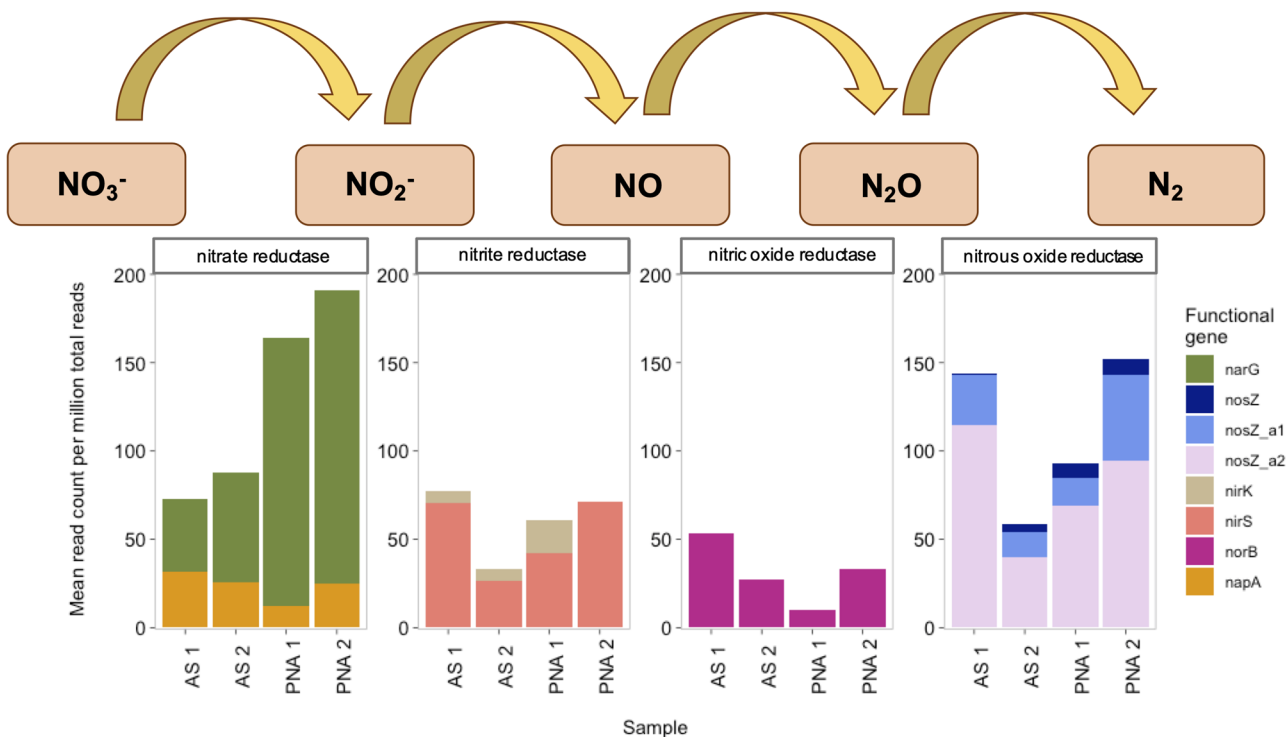


FIGURE 1 Abundance of reads associated with each denitrifying gene across the samples (i.e., *narG*: respiratory nitrate reductase; *napA*: cytoplasmic nitrate reductase; *nirK*: copper-containing nitrite reductase; *nirS*: cytochrome cd containing nitrite reductase; *norB*: nitric oxide reductase; *nosZ*: typical nitrous oxide reductase; *nosZa1*: atypical nitrous oxide reductase clade1; *nosZa2*: atypical nitrous oxide reductase clade 2). AS1 and AS2: conventional activated sludge samples; PNA1 and PNA2: sidestream PNA samples [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

the microorganisms associated with denitrification pathway genes. Also, to determine whether the microorganisms associated with denitrification genes are ubiquitous across the samples, especially between sidestream PNA and AS samples. We used Xander software

that uses a novel data structure combining de Bruijn graphs and HMM to target assembly of specific protein-coding genes from metagenomic data (Q. Wang et al., 2015). We used this data structure to apply powerful graph search techniques to assemble individual genes. Xander analysis disclosed all MatchNames related to the analyzed gene set.

Among the annotated reads for all denitrification genes, *Proteobacteria* was the most abundant phylum, while the abundance varied between samples (Figure S2). The most abundant phyla in the sidestream PNA samples were *Proteobacteria* (32% in PNA1 and 38% in PNA2), *Chloroflexi* (18% in PNA1 and 4% in PNA2), *Chlorobi* (10% in PNA1 and 13% in PNA2), *Bacteroidetes* (8% in PNA1 and 12% in PNA2), and *Ignavibacteriae* (7% in PNA1 and 5% in PNA2). In comparison, in conventional AS samples, *Proteobacteria* (50% in AS1 and AS2) and *Bacteroidetes* (33% in AS1 and 27% in AS2) were the dominant phyla. Besides, in AS2, *Actinobacteria* (10%) was also dominant (Figure S2).

At the class level, *Betaproteobacteria*, associated with at least one gene for each intermediate denitrification pathway, were dominant and ubiquitous across the samples (Figure 3). For other dominant classes, we observed differences between the PNA and AS samples (Figure 3). For example, in PNA samples, *Gammaproteobacteria* associated with *narG*, *napA*, and *nirS* (6% in PNA1 and PNA2); unclassified *Chlorobi* associated with *narG* and *nosZa2* (10% in PNA1 and 15% in PNA2) and *Ignavibacteriae* associated with *nosZa2* (7% in PNA1 and 5% in PNA2) were dominant. Whereas, *Alphaproteobacteria* associated with *narG*, *napA*, and *norB* (6% in AS1 and 7% in AS2);

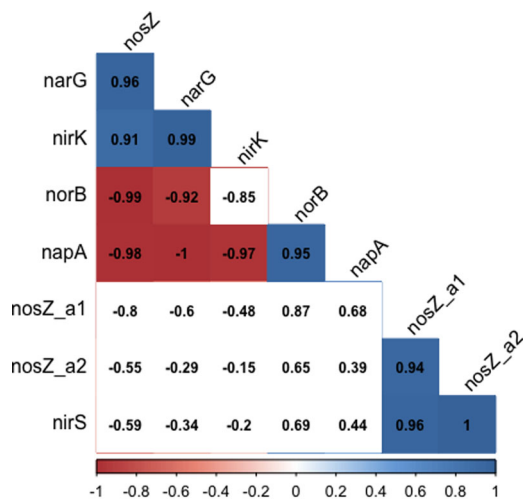


FIGURE 2 Heatmap showing the proportionality for the abundances of each denitrification gene (i.e., *narG*: respiratory nitrate reductase; *napA*: cytoplasmic nitrate reductase; *nirK*: copper-containing nitrite reductase; *nirS*: cytochrome cd containing nitrite reductase; *norB*: nitric oxide reductase; *nosZ*: typical nitrous oxide reductase; *nosZa1*: atypical nitrous oxide reductase clade1; *nosZa2*: atypical nitrous oxide reductase clade 2) [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

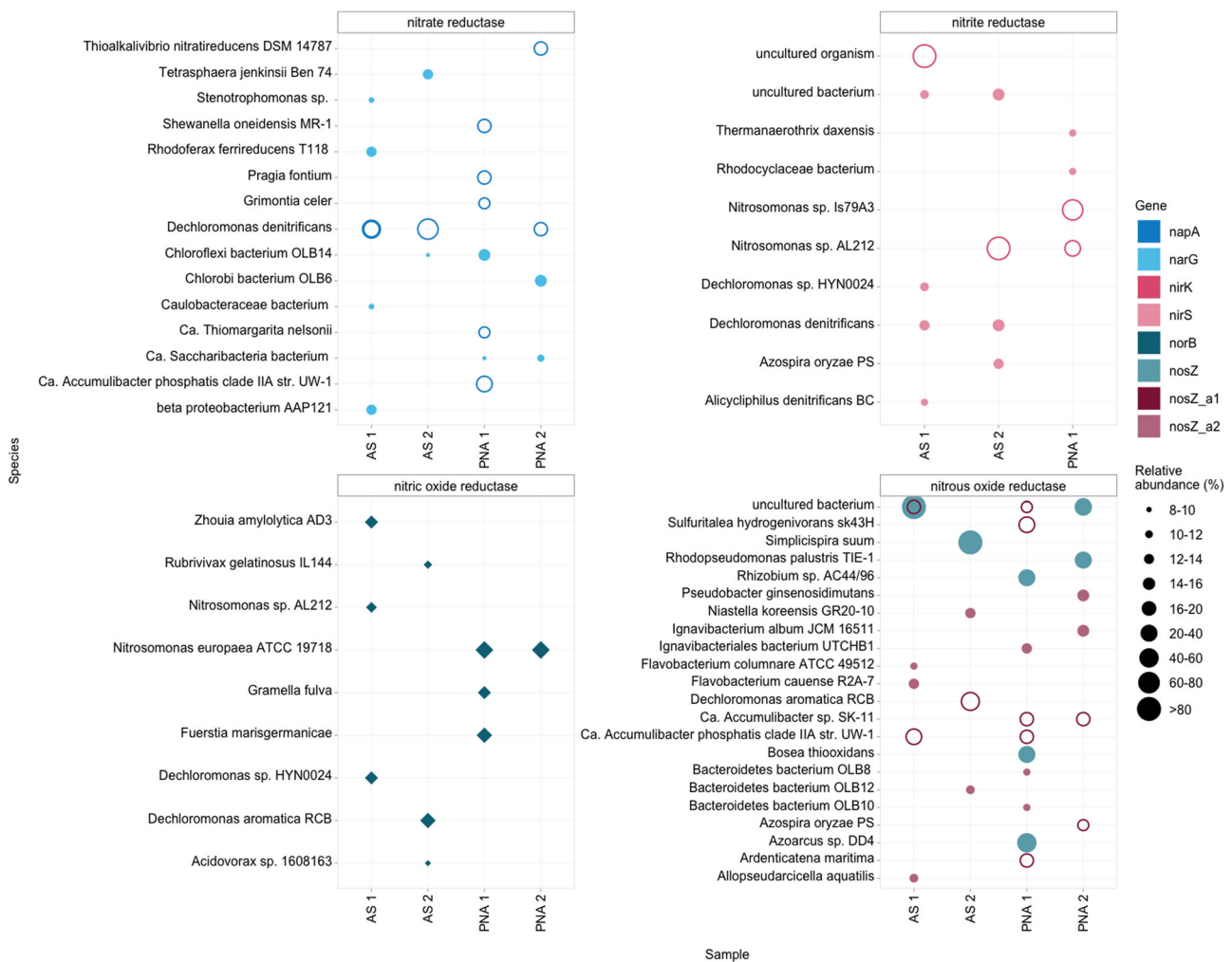


FIGURE 4 Relative abundance of top 50 abundant species found across the samples for each gene, respectively. Functional gene analysis was performed with Xander and adjustment of MatchNames with NCBI database [Color figure can be viewed at wileyonlinelibrary.com]

systems compared to AS systems (Figure S2), though both of them have been reported as common members in the AS microbiome based on 16S rRNA gene analysis (Kragelund et al., 2007; Nielsen et al., 2009). The reason could be a lack of denitrifying genes in *Chloroflexi* and *Chlorobi* present in the AS samples. Kragelund et al. (2007) reported a lack of denitrifying capability of some *Chloroflexi* isolates from AS samples (*napA/narG*, *nirK/nirS*, *norB*, and *nosZ*). In recent years, several studies focused on the heterotrophic activity in anammox systems and the continuous presence of *Chlorobi* and *Chloroflexi* gained in interest (Agrawal et al., 2017; Bhattacharjee et al., 2017; Pereira et al., 2017; Speth et al., 2016). They possess *narG* and *nosZ* and presumably encode a nitrite loop with anammox and NOB, and therefore support anammox growth, as previously reported (Lawson et al., 2017).

The *napA* gene has a higher affinity for nitrate and is usually associated with nitrate-limited environments (Papasprou et al., 2014; Potter et al., 1999). However, PNA and AS systems have typically sufficient nitrate, which could explain the dominance of *narG* in all the samples (Figure 1). Additionally, we observed a clear

separation of dominant *narG* and *napA* communities between PNA and AS samples (Figure 4), except *D. denitrificans*, as it was detected in all four samples. A significant abundance of *narG* in PNA samples supports that partial denitrifiers could support the removal of nitrate through the nitrate–nitrite loop (Agrawal et al., 2017; Bhattacharjee et al., 2017; Speth et al., 2016).

The *nirS* gene was found dominant in all our samples (Figure 1), which is in line with prior studies reporting *nirS* being dominant in PNA and AS systems. Although *nirS* and *nirK* are functionally and physiologically equivalent, it has been reported that the dominance of *nirS* over *nirK* in environments with sufficient nitrite might be due to its higher efficiency for nitrite respiration (Graf et al., 2014; Nadeau et al., 2019). The *nirK* gene found in the samples associated with *Nitrosomonas* species (*N. europaea* ATC 19718 (PNA1/PNA2), *Nitrosomonas* sp. Is79A3 (PNA1), and *Nitrosomonas* sp. AL212 (AS1/AS2/PNA1)) underlines as previously reported that *Nitrosomonas* uses the nitrite pathway only against nitrite toxicity and not for the respiration. There is presumably a key role for *nirK* and *norB* genes in nitrifier denitrification (Schmidt et al., 2004).

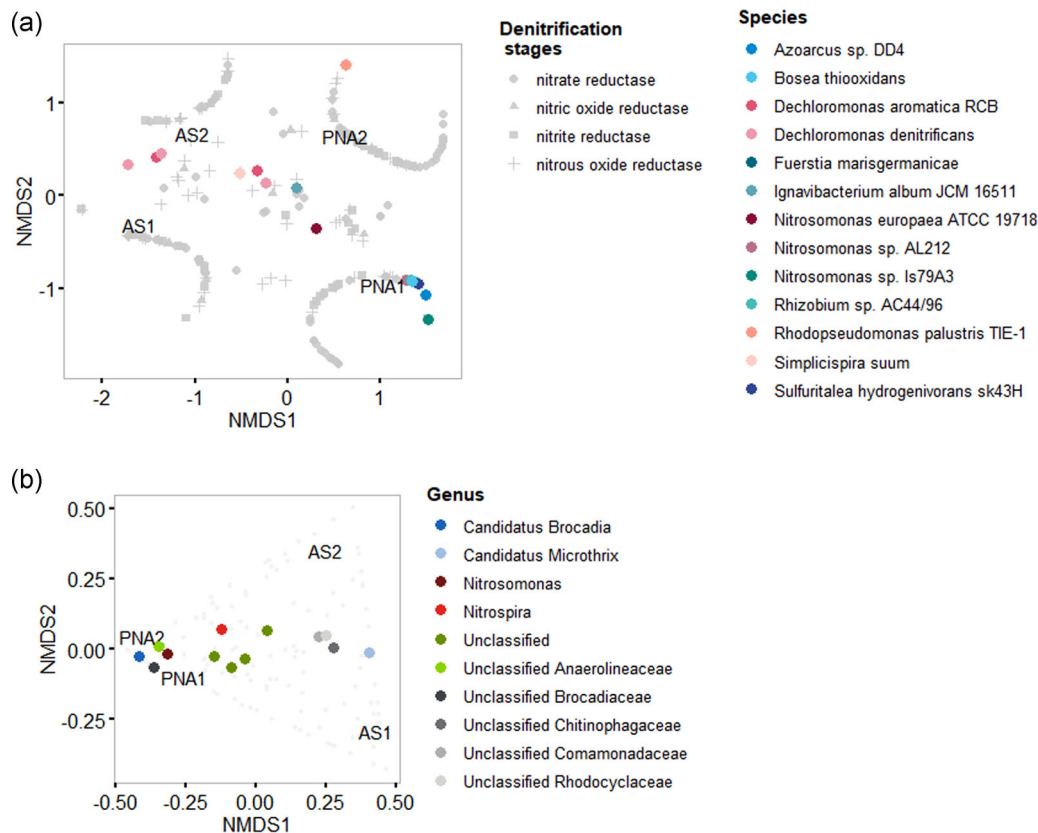


FIGURE 5 Nonmetric multidimensional scaling (NMDS) ordination plots comparing the activated sludge (AS) and sidestream partial nitrification anammox (PNA) samples. NMDS plots were derived from Bray Curtis distance based on; (a) abundance of denitrifiers, which associate with different denitrifying genes, found in the samples, also showing the top 13 abundant species across the samples. (b) The abundance of all microbial members detected in the samples based on the 16S ribosomal RNA gene sequence analysis performed using Metaxa, also showing the top 10 abundant genus across the samples [Color figure can be viewed at wileyonlinelibrary.com]

The presence of *norB* across all the samples with similar relative abundance might suggest its redundant nature, as nitric oxide reductase encoded by *norB* can reduce both nitric oxide and oxygen (Chen & Strous, 2013). Moreover, many denitrifiers and non-denitrifiers contain *norB*, which is advantageous against nitrosative stress and microaerobic conditions (Heylen et al., 2007). Nitric oxide reductase showed a clear separation between PNA and AS systems, presumably due to differences in the environmental conditions (Table S1), as *norB* was associated with *Nitrosomonas*, *Fuerstia*, *Gramella*, *Bacteroidetes*, and *Planctomycetes* in the PNA system, and associated with *Dechloromonas*, *Nitrosomonas*, *Zhoueia*, *Rubrivivax*, and *Acidovorax* in the AS system. For the *norB* gene, nitric oxide reducers, we also found a clear separation between PNA and AS systems.

We found that in all our samples, a significant fraction of *nosZa2* reads could be assigned to members of *Bacteroidetes* and *Ignavibacteriae* (Figures 1 and 4), similar to a previous study (Juhanson et al., 2017). Nevertheless, *Ignavibacteriae* was only predominant in PNA systems and exclusively assigned to the *nosZa2* gene, which agrees with previous studies, often reporting members of *Ignavibacteriae* in PNA systems (Juhanson et al., 2017; Pereira et al., 2017).

The presence and composition of denitrifiers in the PNA systems based on 16S rRNA gene amplicon sequencing has been extensively

reported (Agrawal et al., 2017; Du et al., 2019; Lauren et al., 2015; Persson et al., 2017; D. Wang et al., 2019). Based on the 16S rRNA gene taxonomic composition, it is difficult to accurately determine the denitrifying potential, because it may not denote whether all functional genes are involved in the denitrification process. For example, metagenomic studies on PNA systems revealed that none of the heterotrophic members carried all the genes of the denitrification pathway (Bhattacharjee et al., 2017; Guo et al., 2016; Speth et al., 2016). Moreover, it is known that taxonomic diversity based on denitrifying genes is not congruent with 16S rRNA gene phylogeny (Heylen et al., 2006; Kragelund et al., 2007). Similarly, our results demonstrate the compositional diversity of denitrifiers between samples, which is not visible based on the 16S rRNA gene phylogeny (Figure 5b).

The results from this study show that denitrification pathway genes do show a distinct pattern of abundance and association to the microbial species between sidestream PNA and AS systems. To understand the complex trophic network and relationships between different heterotrophs carrying a particular set of denitrification genes (also counterpart gene for the same function) is not possible from this study alone. Thus, we suggest that more studies (using targeted metagenomic for better resolution or whole genomics) should focus on denitrifying microbial communities and the

functional genes plus more, mainstream as well as sidestream PNA systems need to be examined to decipher the role of denitrifiers in the PNA systems.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

All authors designed the study. Laura Orschler and Shelesh Agrawal performed the experiments and wrote the manuscript. Susanne Lackner supervised the project. All authors contributed comments on the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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