DIVERSITY AND GENE EXPRESSION PATTERNS OF FUNCTIONAL GROUPS IN SIDESTREAM AND MAINSTREAM WASTEWATER PARTIAL-NITRITATION ANAMMOX BIOFILMS

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Abstract

Partial nitrification-anammox (PNA) is today used for nitrogen removal from highly concentrated wastewater after anaerobic sludge digestion (sidestream). However, implementation of PNA for treatment of municipal wastewater (mainstream), with its lower ammonium concentration and lower temperature is challenging, which might be due to differences in microbial community composition and/or activity. To investigate this, we compared side-by-side sidestream and mainstream PNA biofilms using amplicon sequencing of 16S rDNA and rRNA, *hzsB* DNA and mRNA, and the genes *nxrB*, and *amoA*. The two communities were different to each other with relatively more heterotrophic

denitrifying bacteria and less anammox bacteria in the mainstream. With *hzsB* and *nxrB* we found microdiversity among *Brocadia* and *Nitrospira*, and turnover (taxa replacement) between sidestream and mainstream. However, in both environments *Brocadia sapporoensis* represented most of the *hzsB* DNA and mRNA reads, despite the different environmental conditions and nitrogen removal rates. All of those populations present in both sidestream and mainstream had no differences in their 16S rRNA:rDNA ratios, supporting recent findings that rRNA:rDNA ratios are poor indicators of bacterial activity. The observed diversity within functional groups and composition differences between sidestream and mainstream add complexity to our view of PNA communities with possible implication for reactor function.

Introduction

Excess of reactive nitrogen in the environment contributes to eutrophication (Erisman, *et al.* 2015). To reduce reactive nitrogen discharges into water bodies, removal of nitrogen in wastewater treatment plants (WWTPs) is essential. Biological nitrogen removal from the sidestream of wastewater, i.e. reject water, from dewatering of anaerobic digested sludge, with high ammonium concentration and high temperature, can be achieved by the partial nitrification-anammox (PNA) process (Lackner, *et al.* 2014). PNA combines oxidation of part of the wastewater ammonium to nitrite by ammonia oxidising bacteria (AOB) and a subsequent conversion of the nitrite and remaining ammonium to nitrogen gas by anammox bacteria (AMX). PNA communities are often grown in biofilms in granule reactors or in moving bed biofilm reactors (MBBRs) to maintain the slow growing AMX at high concentrations in the reactors (Agrawal, *et al.* 2017).

Implementation of PNA for the colder, more diluted mainstream of wastewater, which contains the majority of the nitrogen at WWTPs, has been challenging. Low nitrogen removal rates and high NO_3^- production are commonly reported (Gonzalez-Martinez, *et al.* 2016, Gustavsson, *et al.* 2020, Lotti, *et al.* 2014, Wu, *et al.* 2016). From a population ecology perspective, multiple scenarios exist to explain

the differences in removal rates and nitrate production between sidestream and mainstream PNA. First, the lower substrate concentration and the lower temperature in the mainstream will inevitably result in lower removal rates. Second, some taxa could differ in their metabolic activity in the two environments. For example, high nitrate production by nitrite oxidising bacteria (NOB) is often reported in mainstream PNA (Cao, *et al.* 2017). Third, functional groups might differ in abundance, which in turn could influence ecosystem function. When gradually replacing sidestream with mainstream wastewater a decrease in AMX and AOB abundances was observed by Yang, *et al.* (2018). Fourth, diversity in the accessory genome within bacterial species exist (McInerney, *et al.* 2017). A mechanism explaining microdiversity is that sub-populations have different ecological niches, i.e. are ecotypes (Moore, *et al.* 1998). It is possible that differences within the main functional groups in sidestream and mainstream PNA could occur. For instance, cold tolerant strains with an oligotrophic lifestyle might be observed in mainstream, while sidestream conditions might favour eutrophic lifestyles.

Amplicon sequencing of the 16S gene (rDNA) has recently been employed to investigate the effect of various operational conditions in PNA systems, and to describe community composition (Agrawal, *et al.* 2017, Lauren, *et al.* 2016, Persson, *et al.* 2017, Yang, *et al.* 2018). Sequencing of rDNA offers limited resolution to infer closely related populations, but the use of amplicon sequences variants (ASVs) (Callahan, *et al.* 2017) instead of operational taxonomic units (OTUs) would allow potential ecotypes to be elucidated (García-García, *et al.* 2019). Even higher resolution within taxonomic groups could be achieved by sequencing of functional genes, like *hzsB* for anammox bacteria (Wang, *et al.* 2012), *amoA* for AOB (Rotthauwe, *et al.* 1997), and *nrxB* for NOB (Pester, *et al.* 2013), but this approach is rarely used for describing the PNA communities.

Community composition can be described with amplicon sequencing, but bacteria can be active, growing, dormant or deceased (Blazewicz, *et al.* 2013), and their metabolic status cannot be

determined from gene sequencing alone. An alternative is sequencing of 16S rRNA (rRNA), as bacterial growth has been associated with an increase in ribosome production, at least for *Proteobacteria* (Kerkhof and Kemp 1999, Schaechter, *et al.* 1958), and ribosomal degradation is seen for some bacteria during starvation (Deutscher 2003). Thus, estimations of rRNA:rDNA ratios have been considered a measure of activity in the total community (Campbell, *et al.* 2011, Jones and Lennon 2010). However, this metric is not universal, as there are exceptions to the link between rRNA content and activity in bacteria (Blazewicz, *et al.* 2013).

In this study, we operated pilot-scale MBBRs for PNA, fed with either pre-treated municipal wastewater (mainstream) or sludge liquor from anaerobic sludge digesters (sidestream) from the Sjölanda WWTP, Malmö, Sweden. The aim of this study was to determine, by high throughput amplicon sequencing of rDNA, if different microbial communities and/or taxa abundance were established in the mainstream and sidestream. We also asked if multiple AMX, NOB and AOB populations coexisted in the two environments. To address this question, we sequenced the functional genes *hzsB*, *nxB* and *amoA*, as well as *hzsB* mRNA from the biofilm on individual mainstream and sidestream carriers. Furthermore, we measured rRNA:rDNA ratios to test our hypothesis that the rRNA:rDNA ratios of bacterial groups present in both environments would vary due to the different conditions, which would provide insights about the response in activity of specific taxa.

Materials and Methods

The sidestream and mainstream pilot MBBRs were located at the Sjölanda WWTP, Malmö, Sweden (Hanner, *et al.* 2003), see Table 1 for operational data. They were filled to 40% with K1[®] carriers (Veolia Water Technologies AB – AnoxKaldnes, Lund, Sweden). The MBBRs are described in detail elsewhere (Gustavsson, *et al.* 2020). To promote anammox growth in the mainstream MBBR, biofilm carriers were frequently exchanged between the sidestream and mainstream MBBRs. However, biofilm carriers sampled in this study were not exchanged, but kept isolated in each reactor in

cylindrical cages (immersed volume 2.5 L) for 128 days until sampling. A steel mesh bottom in the cages allowed water circulation. The cages were filled using carries taken from their respective MBBRs.

Table 1: Operational data of the pilot reactors from 15 September 2015 to 14 October 2015. Mean values +/- S.D.

	Sidestream	Mainstream
Influent NH_4^+ (mg N L^{-1})	880 ± 48	26 ± 5.0
Effluent NH_4^+ (mg N L^{-1})	55 ± 12	21 ± 4.6
Effluent NO_2^- (mg N L^{-1})	6.1 ± 2.1	0.25 ± 0.52
Effluent NO_3^- (mg N L^{-1})	120 ± 13	1.7 ± 1.3
Ammonium loading rate (g N $\text{m}^{-2} \text{d}^{-1}$)	1.8 ± 0.25	0.78 ± 0.14
Nitrogen removal rate (g N $\text{m}^{-2} \text{d}^{-1}$)	1.5 ± 0.19	0.13 ± 0.12
DO (mg L^{-1})	1.0 ± 0.24	1.0 ± 0.15
T ($^{\circ}\text{C}$)	28.3 ± 0.80	19.1 ± 0.65

Sampling

Biofilm carriers from each cage were snap-frozen in an ethanol-dry ice mixture immediately at sampling, kept frozen in dry ice during transportation and then stored at -80°C . Sidestream biofilms had a red colour, while a brown colour was observed in the mainstream biofilms (Figure S1, Supporting information). In addition, a wet weight of $431 \text{ mg} \pm 36$ (average $\pm$ 95% confidence interval) was observed for sidestream biofilms, and 296 ± 13 for mainstream biofilms.

Co-extraction of DNA and RNA

Carriers with biofilms were thawed in RNA later-ICE (Thermo Fisher Scientific, Waltham, MA USA). The biofilm was removed from the carrier compartments and added to a lysis matrix tube E (MP biomedical, Santa Ana, CA, USA) with 800 μl of lysis solution of a ZR-duet MiniPrep kit (Zymo Research). Mechanical disruption of the biofilm was done with a FastPrep-24 5G (MP

biomedicals) at speed 6 for 40 seconds. Subsequent steps of the DNA-RNA co-extraction were carried out with the ZR-duet kit according to manufacturer instructions. Ribolock RNase inhibitor (40 U/μl; Thermo Fisher Scientific) was added to the extracted RNA. DNA and RNA concentration were measured after extraction using a Qubit 3.0 fluorometer (Thermo Fisher Scientific). Potential traces of genomic DNA were removed from the RNA extraction with a DNA-free DNA Removal kit (Thermo Fisher Scientific). cDNA was synthesised with SuperScript VILO MasterMix (Thermo Fisher Scientific) according to the manufacturer's instructions.

Sequencing

PCR amplification of the 16S V4 region was done with primers 515'F (Hugerth, *et al.* 2014) and 806R (Caporaso, *et al.* 2011), using dual indexing of the primers (Kozich, *et al.* 2013). Template DNA (40 ng) or undiluted cDNA (2 μl) was amplified in a total volume of 50 μl using a Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific). The following PCR program was used: activation (98°C, 30 s); 30 cycles of denaturation (98°C, 10 s), annealing (56°C, 30 s) and elongation (72°C, 15 s); followed by final elongation (72°C, 10 min). PCR products were purified with Ampure XP (Beckman Coulter, Brea, CA, USA). Purified PCR products were pooled in equimolar amounts. Sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA).

PCR amplification of *nxB*, *hzb*, and *amoA* was carried out in two steps using Nextera index adapters (Illumina). Primers nxB169F and nxB638R (Pester, *et al.* 2013) were used for *nxB* amplification, amplification of *hzb* was done with the hzb_396F and hzb_742R primers (Wang, *et al.* 2012), and primers AmoA1F mod (Stephen, *et al.* 1999) and AmoA2R (Rotthauwe, *et al.* 1997) were used for *amoA*. The following PCR program was used for all amplicons: activation (98°C, 30 s); 25 cycles of denaturation (98°C, 10 s), annealing (56°C, 30 s) and elongation (72°C, 45 s); followed by final elongation (72°C, 5 min). PCR amplicons were then purified, and a second PCR with 8 cycles of

amplification was used to attach the index adapters followed by a second purification. Amplicons were then pooled together in equimolar amounts and sequenced on a MiSeq as described above.

Statistics and data analysis.

Samples with less than 30,000 reads were excluded prior to analysis. ASVs were generated, using DADA2 version 1.12 (Callahan, *et al.* 2016). The SILVA 132 database (Quast, *et al.* 2013) was used for taxonomic classification of the 16S amplicons with IDTAXA (Murali, *et al.* 2018). Data was analysed in R (R Core Team 2019) using the packages Phyloseq (McMurdie and Holmes 2013) and Vegan (Oksanen, *et al.* 2019). Raw sequence reads were deposited at the NCBI (Bioproject: PRJNA552732). See table S1 for individual accession numbers of each sample.

Reads were normalized by proportion prior to estimation of beta diversity between mainstream and sidestream in the rDNA, rRNA, *nxrB* and *hzsB* libraries and for comparisons between the rDNA and rRNA libraries. Beta diversity was estimated with the abundance-based Bray-Curtis index and the presence-absence-based Simpson index, which is not sensitive to richness differences, and is a measure of turnover (Baselga 2010). To estimate differential abundance of ASVs between sidestream and mainstream, DESeq2 was used (Love, *et al.* 2014) without subsampling before the analysis (McMurdie and Holmes 2014). A $p_{(adj)} < 0.01$ value (DESeq2) was used as criterion for statistical significance. Only samples with both rDNA and rRNA libraries available (mainstream, n=6; sidestream, n=7) were used for plots of rDNA vs rRNA. Ratios of rRNA:rDNA were estimated after excluding values of zero in the libraries.

Fluorescence in situ hybridization (FISH)

FISH was carried out on suspended biomass as previously described (Suarez, *et al.* 2015). The probe AMX820 (Schmid, *et al.* 2001), labelled with FAM, was used to target *all Brocadia* populations. The

probes BAN162 (Schmid, *et al.* 2001), labelled with Cy3, and BFU613 (van de Vossenberg, *et al.* 2008), labelled with Cy5, were used to target *Brocadia* subpopulations, as they only partially cover the *Brocadia* genus, as determined with ARB 6.0.6 (Ludwig, *et al.* 2004) using the SILVA NR 132 SSU *Brocadia* sequences. These probes were applied together with unlabelled competitors (for Ban162, CGG TAG CCC CAA TTG CTT; for Bfu613, GGA TGC CGT TCT TCC GTT GAG CGG) to increase probe specificity, as previously reported (Persson, *et al.* 2014, Suarez, *et al.* 2015).

Results and discussion

Sidestream and mainstream communities

We compared PNA microbial communities exposed to sidestream and mainstream conditions by sequencing rDNA and rRNA from individual biofilm carriers. Mainstream and sidestream communities, estimated from both rDNA and rRNA, were significantly different, as shown with the abundance-based Bray-Curtis index (Adonis β_{bray} ; $p < 0.01$, $r^2 = 0.16$; Figure 1A). Because beta-diversity can also exist among rare taxa (Gobet, *et al.* 2012), we also used the presence-absence Simpson index, which measures species turnover (Baselga 2010). The observed results (Adonis β_{Sim} ; $p < 0.01$, $r^2 = 0.32$; Figure 1B), suggest that the sidestream and mainstream communities not only differed significantly in their relative abundance of taxa, but also in their identity. The sidestream and mainstream communities also diverged from the initial seed communities, indicating temporal dynamics (Figure S2, Supporting information). Although elucidating the assembly mechanism of PNA biofilms is beyond the scope of this study, sidestream and mainstream were exposed to different environmental conditions and subjected to potential immigration from two different water sources, which would both influence the community composition.

Figure 1: PCoA of rDNA and rRNA libraries, based on the abundance-based Bray-Curtis index (A) and the presence-absence-based Simpson index (B). Red: mainstream, Blue: sidestream. Circles: rDNA, triangles: rRNA.

Comparing rDNA and rRNA libraries

The rDNA and rRNA libraries of the mainstream and sidestream communities were different in relative read abundance (Adonis β_{bray} ; $p < 0.001$, $r^2 = 0.24$; Figure 1A), which could be interpreted as rRNA:rDNA ratios different to one. However, turnover between the rDNA and rRNA libraries was also observed (Adonis β_{sim} ; $p < 0.001$, $r^2 = 0.13$; Figure 1B), which would imply that different ASVs were detected by sequencing of rDNA and rRNA. ASVs present in the rRNA, but not the rDNA libraries are known as phantom taxa. They could be the result of PCR errors during reverse transcription, but could also arise due to rDNA under-sampling of rare but highly active taxa (Klein, *et al.* 2016). Supporting the latter suggestion, we commonly observed phantom taxa for genera with high rRNA:rDNA values, like *Competibacter*, *Agitococcus*, *Romboutsia*, the AMX *Brocadia* and the AOB *Nitrosomonas* (Figure S3, Supporting information). In addition, among genera with low rRNA:rDNA, like *Denitratisoma*, *Dokdonella* and *UTBCD1* (Figure S3, Supporting information), some ASVs did not have any corresponding rRNA reads. This could signal that a high proportion of these taxa in the biofilms were dormant or dead. Extracellular DNA is commonly observed in biofilms (Dominiak, *et al.* 2011) and could inflate rDNA reads for some taxa (Albertsen, *et al.* 2015).

Despite the differences, relative read abundances of the rDNA and rRNA libraries over the entire dataset were positively correlated at the phylum level (Kendall's $\tau = 0.77$, $p < 0.001$, Figure 2) as well as the ASV level (Kendall's $\tau = 0.59$, $p < 0.001$), and also seen by modelling rRNA abundance of ASVs with beta regression ($z = 18.0$, $p < 0.001$, pseudo- $R^2 = 0.44$; Figure S4, Supporting information). Thus, ribosomal relative abundance was generally linked to taxa relative abundance. Nonetheless, it appears that rRNA:rDNA ratios varied between phyla; for example, low ratios were observed for *Acidobacteria*, and high ratios were noticed for *Firmicutes* and *Planctomycetes* (Figure 2, S4, table S2). Such different rRNA:rDNA ratios have been observed before across phyla (Denef, *et al.* 2016, Steven, *et al.* 2017).

Figure 2. Comparison of rDNA and rRNA of the entire dataset at the phylum-level. Each point indicates the abundance of an ASV in one MBBR carrier; the black dashed diagonal line indicates equal rDNA and rRNA abundance. The colours denote the 10 most abundant phyla.

Acidobacteria are considered slow growing bacteria (Ward, *et al.* 2009), and hence it is possible that their low rRNA:rDNA ratios in fact represent slow growth rates compared to other taxa in the biofilms. This might be supported by the fact that *Acidobacteria* have between one and two copies of the 16S RNA gene, since low copy numbers are associated with oligotrophic lifestyles (Klappenbach, *et al.* 2000, Stevenson and Schmidt 2004). On the other hand, although rRNA:rDNA values larger than one are frequently used as criteria for activity (Blazewicz, *et al.* 2013), different taxa might differ

in their rRNA content during growth or dormancy to the extent that rRNA content and growth rate are not linked (Blazewicz, *et al.* 2013). Thus, potentially active taxa might be misclassified as dormant just because their rRNA:rDNA ratio is below one (Steven, *et al.* 2017).

The disproportionally high read abundances of rRNA at low rDNA abundances for most phyla (Figure S4) confirm previous similar observations, suggesting higher activity among rare taxa (Campbell, *et al.* 2011, Jia, *et al.* 2019, Jones and Lennon 2010, Klein, *et al.* 2016, Wilhelm, *et al.* 2014). This phenomenon could perhaps be due to under-sampling (Steven, *et al.* 2017), but higher growth rate among rare taxa could arise due to intraspecific competition or predation of abundant taxa (Jousset, *et al.* 2017). In fact, by using metagenomic data, Jia, *et al.* (2019) observed higher replication rates for taxa at low relative abundances supporting that this is a real phenomenon.

Comparing rRNA:rDNA ratios between sidestream and mainstream

We originally expected that the same ASVs present in the relatively different sidestream and mainstream conditions would have different rRNA:rDNA ratios. For example, in the case of *Brocadia* we anticipated a higher rRNA:rDNA in the sidestream because the activity in terms of nitrogen conversion was much higher than in mainstream (Table 1). In addition, the growth rate of anammox bacteria is affected by temperature (Laureni, *et al.* 2015). But differences in rRNA:rDNA ratios between the two environments were not observed for any of the 12 ASVs present across all rRNA and rDNA libraries (Wilcoxon rank sum test, $p>0.05$; Figure 3).

Figure 3: Ratios of rRNA:rDNA for 12 ASVs present in all rDNA and rRNA libraries. Colours denote phylum classification, M = mainstream and S = sidestream. ASV1 and ASV7 are classified as the AMX *Brocadia*.

We conclude that, at least for PNA systems, the rRNA:rDNA ratios are not a direct proxy for metabolic activity. This would agree with recent studies in soil using stable isotope probing showing that rRNA:rDNA ratios underestimate active taxa and are a poor predictor of rRNA synthesis (Papp, *et al.* 2018a, Papp, *et al.* 2018b). For some taxa, ribosomes may not be degraded during slow/no growth conditions. For *Nitrosomonas*, stable ribosomal content has been observed during inhibition (Wagner, *et al.* 1995) and preservation of old ribosomes appears to occur in *Thaumarchaeota* (Papp, *et al.* 2019). Furthermore, presence of rRNA is not necessarily an indication of active rRNA production, therefore it might be useful to complement measurements of rRNA:rDNA ratios with other methods such as stable isotope probing (Papp, *et al.* 2018a).

Sidestream and mainstream communities

As also reported for other PNA MBBRs (Agrawal, *et al.* 2017, Persson, *et al.* 2017), the dominant taxa in the biofilms were AMX (Figure 4A), with *Brocadia* being the only AMX genus detected. Relative read abundance of *Brocadia* rDNA was higher in the sidestream than the mainstream biofilms (DESeq2; $p_{\text{adj}} < 0.01$; Figure 4B). Furthermore, potential heterotrophic denitrifying bacteria

(HDB) like *Zoogloea* and *Sulfuritalea*, among others, were more abundant in the Mainstream (DESeq2; $p_{(adj)} < 0.01$, Figure 4B) and were in fact more or less absent in the sidestream biofilms. Similarly, Yang, *et al.* (2018) observed a decrease in AMX abundance when a sidestream community was exposed to mainstream conditions, also indicating that sidestream conditions favour AMX growth relative to other bacteria. The larger fraction of HDB in the mainstream suggests more extensive competition for NO_2^- between AMX and HDB; in addition, potential nitrogen loops of nitrification, anammox, denitrification and DNRA may occur (Speth, *et al.* 2016).

Figure 4. Abundance of taxa at the genus level. **A:** rDNA read abundance of AMX (*Brocadia*), AOB (*Nitrosomonas*) and NOB (*Nitrospira* and *Nitrolancea*), red: mainstream, blue: sidestream. **B:** Log2FoldChange (LFC) for rDNA genera with differential abundance between mainstream and sidestream biofilms (DESeq2; $p_{(adj)} < 0.01$). Positive LFC corresponds to higher abundance in mainstream, and negative LFC corresponds to higher abundance in sidestream; only the top 30 genera with the lowest significant $p_{(adj)}$ are shown.

Microdiversity of nitrogen transforming bacteria

Several anammox populations coexisted in the biofilms as assessed by rDNA sequencing (Figure S5-S7, Supporting information). An example of the microdiversity within *Brocadia* can be observed in Figure 5A, where a combination of different FISH probes was used to visualize three different *Brocadia* subpopulations. By sequencing the *hzsB* gene, 119 ASVs within *Brocadia* were detected

(Figure 5B, S8, Supporting information), and turnover between sidestream and mainstream was demonstrated (Adonis, β_{sim} , $r^2=0.57$, $p=0.01$). Nonetheless, a single ASV was dominant in both sidestream and mainstream (Figure 5B, S8, Supporting information), and represented $77 \pm 3\%$ of the total *hzsB* reads (Figure 5C). As hydrazine synthase (HZS) is a key enzyme for the anammox process, this suggests that this strain was responsible for the bulk anaerobic oxidation of ammonium in both the sidestream and the mainstream, in spite of the different environmental conditions in the reactors.

Figure 5A: Multiple *Brocadia* populations (sidestream) targeted with the FISH probes AMX820 (Blue), BFU613 (Red) and Ban162 (Green); overlap among probes results in additional colours: Cyan (AMX820 and Ban162), Magenta: (AMX820, BFU613), White: (AMX820, Ban162 and BFU613). **B:** DNA read abundance of the top 10 *hzsB* ASVs; each colour represents a unique ASV; each bar represents a biofilm sample. **C:** RNA:DNA ratios for the top 10 *hzsB* ASVs (n=4). Same colour coding as in B.

Using rDNA, several *Nitrospira* and *Nitrosomonas* ASVs were observed (Figure S6, S7, Supporting information). Likewise, using the key enzymes *nxB* and *amoA* we also observed multiple *Nitrospira* ASVs (Figure 6, S9, Supporting information) and *Nitrosomonas* ASVs (Figure S10, Supporting information), respectively. Turnover of *Nitrospira* communities in sidestream and mainstream was

observed as assessed with *nxB* (Adonis, β_{sim} , $p=0.004$, $r^2=0.39$), while for *amoA*, low PCR yield in sidestream samples prevented comparison of sidestream and mainstream. In microbial communities the coexistence of closely related taxa is often reported (Goldford, *et al.* 2018). For example, for *Nitrospira*, which is commonly present in wastewater (Daims, *et al.* 2001), multiple populations can coexist in activated sludge (Gruber-Dorninger, *et al.* 2014). Coexistence of multiple AMX populations has also been reported in PNA systems (Bhattacharjee, *et al.* 2017, Lauren, *et al.* 2019, Persson, *et al.* 2014). *Nitrospira* is a heterogeneous group, which not only represents a nitrite oxidising potential, but also contains populations capable of using a myriad of electron donors and - acceptors (Daims, *et al.* 2015, Koch, *et al.* 2015, van Kessel, *et al.* 2015) and the same has also been shown for AMX (Hu, *et al.* 2019, Kartal, *et al.* 2007, Strous, *et al.* 2006, van de Vossenberg, *et al.* 2008) and AOB (Bock, *et al.* 1995, Schmidt, *et al.* 2004). Thus, the coexistence of different *Brocadia*, *Nitrosomonas* and *Nitrospira* ASVs, observed in this study, could be a general phenomenon, explained by ecotypes within the AMX, AOB and the NOB utilizing different metabolic pathways and playing various ecological roles. The roles of the less abundant AMX in the PNA biofilms are yet unclear, but read abundance of *hxsB* mRNA (Figure 5C) suggests that their contribution to the anammox process might have been minor.

Figure 6: Read abundance of the top 10 *nxB* ASVs.

The dominant *hxsB* ASV had an identical sequence to *B. sapporoensis*, which is frequently observed in PNA bioreactors (Lotti, *et al.* 2015b, Persson, *et al.* 2014) and is considered relatively fast growing

(Lotti, *et al.* 2015a). It has a maximum specific anammox activity at 37°C (Narita, *et al.* 2017), which, together with other factors like nitrogen load and partial nitrification rate, would explain the lower nitrogen removal rates observed in the mainstream compared with the sidestream MBBR. A considerable decrease of anammox rates with decreasing temperature has often been described in PNA reactors (Gilbert, *et al.* 2014, Laureni, *et al.* 2016, Lotti, *et al.* 2015b). Interestingly, no particular cold-tolerated AMX have so far been detected, instead AMX within *Brocadia* are commonly reported in mainstream reactors. This study suggests that *B. sapporoensis*, can in fact be competitive relative to other AMX taxa at both sidestream and mainstream conditions. One possible explanation for such a phenomenon can be mechanisms of adaptation, where temperature changes can result in changes in membrane composition (Rattray, *et al.* 2010) and protein expression (Lin, *et al.* 2018). Alternatively, there could be multiple closely related populations with identical *hzsB* and rDNA sequences, but with different accessory genes, and thus metagenomic studies would be needed to resolve such populations.

Comammox can occur among lineage II in *Nitrospira* (Daims, *et al.* 2015). The majority of *Nitrospira* in this study were from lineage I (Figure S9, Supporting information) and thus the comammox process was likely not important for the nitrogen transformations in the biofilm. Inhibition of NOB is critical for the function of PNA, and several strategies have been proposed for NOB suppression including, but not limited to, DO limitation, intermittent aeration and exposure to free nitrous acid (Malovanyy, *et al.* 2015, Pérez, *et al.* 2014, Wang, *et al.* 2016). However, the observed NOB diversity within *Nitrospira* could impact reactor performance, because microbial diversity might lead to functional redundancy (Allison and Martiny 2008). As NOB in any PNA reactor likely consist of multiple coexisting populations, operational strategies to reduce nitrite oxidation based on knowledge gained from pure cultures or enrichments may not be adequate. In fact, the inhibition of a dominant NOB population, may well lead to the succession of another dominant population. Future studies, combining well-defined reactor experiments with high resolution genomics would gain further

insights in how microdiversity of key guilds, as observed here, affects turnover of substrates and thereby reactor performance

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