1   DIVERSITY AND GENE EXPRESSION PATTERNS OF FUNCTIONAL GROUPS IN

2   SIDESTREAM AND MAINSTREAM WASTEWATER PARTIAL-NITRITATION ANAMMOX

3   BIOFILMS

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16

17   **Abstract**

18   Partial nitritation-anammox (PNA) is today used for nitrogen removal from highly concentrated

19   wastewater after anaerobic sludge digestion (sidestream). However, implementation of PNA for

20   treatment of municipal wastewater (mainstream), with its lower ammonium concentration and lower

21   temperature is challenging, which might be due to differences in microbial community composition

22   and/or activity. To investigate this, we compared side-by-side sidestream and mainstream PNA

23   biofilms using amplicon sequencing of 16S rDNA and rRNA, *hzsB* DNA and mRNA, and the genes

24   *nxrB*, and *amoA*. The two communities were different to each other with relatively more heterotrophic

25   denitrifying bacteria and less anammox bacteria in the mainstream. With *hzsB* and *nxrB* we found

26   microdiversity among *Brocadia* and *Nitrospira*, and turnover (taxa replacement) between sidestream

27   and mainstream. However, in both environments *Brocadia sapporoensis* represented most of the *hzsB*

28   DNA and mRNA reads, despite the different environmental conditions and nitrogen removal rates. All

29   of those populations present in both sidestream and mainstream had no differences in their 16S

30   rRNA:rDNA ratios, supporting recent findings that rRNA:rDNA ratios are poor indicators of bacterial

31   activity. The observed diversity within functional groups and composition differences between

32   sidestream and mainstream add complexity to our view of PNA communities with possible

33   implication for reactor function.

34

35   **Introduction**

36   Excess of reactive nitrogen in the environment contributes to eutrophication (Erisman, *et al*. 2015). To

37   reduce reactive nitrogen discharges into water bodies, removal of nitrogen in wastewater treatment

38   plants (WWTPs) is essential. Biological nitrogen removal from the sidestream of wastewater, i.e.

39   reject water, from dewatering of anaerobic digested sludge, with high ammonium concentration and

40   high temperature, can be achieved by the partial nitritation-anammox (PNA) process (Lackner, *et al*.

41   2014). PNA combines oxidation of part of the wastewater ammonium to nitrite by ammonia oxidising

42   bacteria (AOB) and a subsequent conversion of the nitrite and remaining ammonium to nitrogen gas

43   by anammox bacteria (AMX). PNA communities are often grown in biofilms in granule reactors or in

44   moving bed biofilm reactors (MBBRs) to maintain the slow growing AMX at high concentrations in

45   the reactors (Agrawal, *et al*. 2017).

46

47   Implementation of PNA for the colder, more diluted mainstream of wastewater, which contains the

48   majority of the nitrogen at WWTPs, has been challenging. Low nitrogen removal rates and high NO_3^-

49   production are commonly reported (Gonzalez-Martinez, *et al*. 2016, Gustavsson, *et al*. 2020, Lotti, *et*

50   *al*. 2014, Wu, *et al*. 2016). From a population ecology perspective, multiple scenarios exist to explain

51   the differences in removal rates and nitrate production between sidestream and mainstream PNA.

52   First, the lower substrate concentration and the lower temperature in the mainstream will inevitably

53   result in lower removal rates. Second, some taxa could differ in their metabolic activity in the two

54   environments. For example, high nitrate production by nitrite oxidising bacteria (NOB) is often

55   reported in mainstream PNA (Cao, *et al*. 2017). Third, functional groups might differ in abundance,

56   which in turn could influence ecosystem function. When gradually replacing sidestream with

57   mainstream wastewater a decrease in AMX and AOB abundances was observed by Yang, *et al*.

58   (2018). Fourth, diversity in the accessory genome within bacterial species exist (McInerney, *et al*.

59   2017). A mechanism explaining microdiversity is that sub-populations have different ecological

60   niches, i.e. are ecotypes (Moore, *et al*. 1998). It is possible that differences within the main functional

61   groups in sidestream and mainstream PNA could occur. For instance, cold tolerant strains with an

62   oligotrophic lifestyle might be observed in mainstream, while sidestream conditions might favour

63   eutrophic lifestyles.

64

65   Amplicon sequencing of the 16S gene (rDNA) has recently been employed to investigate the effect of

66   various operational conditions in PNA systems, and to describe community composition (Agrawal, *et*

67   *al*. 2017, Laureni, *et al*. 2016, Persson, *et al*. 2017, Yang, *et al*. 2018). Sequencing of rDNA offers

68   limited resolution to infer closely related populations, but the use of amplicon sequences variants

69   (ASVs) (Callahan, *et al*. 2017) instead of operational taxonomic units (OTUs) would allow potential

70   ecotypes to be elucidated (García-García, *et al*. 2019). Even higher resolution within taxonomic

71   groups could be achieved by sequencing of functional genes, like *hzsB* for anammox bacteria (Wang,

72   *et al*. 2012), *amoA* for AOB (Rotthauwe, *et al*. 1997), and *nxrB* for NOB (Pester, *et al*. 2013), but this

73   approach is rarely used for describing the PNA communities.

74

75   Community composition can be described with amplicon sequencing, but bacteria can be active,

76   growing, dormant or deceased (Blazewicz, *et al*. 2013), and their metabolic status cannot be

77   determined from gene sequencing alone. An alternative is sequencing of 16S rRNA (rRNA), as

78   bacterial growth has been associated with an increase in ribosome production, at least for

79   *Proteobacteria* (Kerkhof and Kemp 1999, Schaechter, *et al.* 1958), and ribosomal degradation is seen

80   for some bacteria during starvation (Deutscher 2003). Thus, estimations of rRNA:rDNA ratios have

81   been considered a measure of activity in the total community (Campbell, *et al.* 2011, Jones and

82   Lennon 2010). However, this metric is not universal, as there are exceptions to the link between

83   rRNA content and activity in bacteria (Blazewicz, *et al.* 2013).

84

85   In this study, we operated pilot-scale MBBRs for PNA, fed with either pre-treated municipal

86   wastewater (mainstream) or sludge liquor from anaerobic sludge digesters (sidestream) from the

87   Sjölunda WWTP, Malmö, Sweden. The aim of this study was to determine, by high throughput

88   amplicon sequencing of rDNA, if different microbial communities and/or taxa abundance were

89   established in the mainstream and sidestream. We also asked if multiple AMX, NOB and AOB

90   populations coexisted in the two environments. To address this question, we sequenced the functional

91   genes *hzsB*, *nxrB* and *amoA*, as well as *hzsB* mRNA from the biofilm on individual mainstream and

92   sidestream carriers. Furthermore, we measured rRNA:rDNA ratios to tests our hypothesis that the

93   rRNA:rDNA ratios of bacterial groups present in both environments would vary due to the different

94   conditions, which would provide insights about the response in activity of specific taxa.

95

96   **Materials and Methods**

97   The sidestream and mainstream pilot MBBRs were located at the Sjölunda WWTP, Malmö, Sweden

98   (Hanner, *et al.* 2003), see Table 1 for operational data. They were filled to 40% with K1® carriers

99   (Veolia Water Technologies AB – AnoxKaldnes, Lund, Sweden). The MBBRs are described in detail

100   elsewhere (Gustavsson, *et al.* 2020). To promote anammox growth in the mainstream MBBR, biofilm

101   carriers were frequently exchanged between the sidestream and mainstream MBBRs. However,

102   biofilm carriers sampled in this study were not exchanged, but kept isolated in each reactor in

103   cylindrical cages (immersed volume 2.5 L) for 128 days until sampling. A steel mesh bottom in the

104   cages allowed water circulation. The cages were filled using carries taken from their respective

105   MBBRs.

106   **Table 1:** Operational data of the pilot reactors from 15 September 2015 to 14 October 2015. Mean

107   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 m^{-2} d^{-1})	1.8 ± 0.25	0.78 ± 0.14
Nitrogen removal rate (g N m^{-2} d^{-1})	1.5 ± 0.19	0.13 ± 0.12
DO (mg L^{-1})	1.0 ± 0.24	1.0 ± 0.15
T ($\circ$C)	28.3 ± 0.80	19.1 ± 0.65

108

Sampling

110   Biofilm carriers from each cage were snap-frozen in an ethanol-dry ice mixture immediately at

111   sampling, kept frozen in dry ice during transportation and then stored at -80°C. Sidestream biofilms

112   had a red colour, while a brown colour was observed in the mainstream biofilms (Figure S1,

113   Supporting information). In addition, a wet weight of 431 mg $\pm$ 36 (average $\pm$ 95% confidence

114   interval) was observed for sidestream biofilms, and 296 ± 13 for mainstream biofilms.

115

Co-extraction of DNA and RNA

117   Carriers with biofilms were thawed in RNA later-ICE (Thermo Fisher Scientific, Waltham, MA

118   USA). The biofilm was removed from the carrier compartments and added to a lysis matrix tube E

119   (MP biomedicals, Santa Ana, CA, USA) with 800 µl of lysis solution of a ZR-duet MiniPrep kit

120   (Zymo Research). Mechanical disruption of the biofilm was done with a FastPrep-24 5G (MP

121   biomedicals) at speed 6 for 40 seconds. Subsequent steps of the DNA-RNA co-extraction were carried

122   out with the ZR-duet kit according to manufacturer instructions. Ribolock RNase inhibitor (40 U/µl;

123   Thermo Fisher Scientific) was added to the extracted RNA. DNA and RNA concentration were

124   measured after extraction using a Qubit 3.0 fluorometer (Thermo Fisher Scientific). Potential traces of

125   genomic DNA were removed from the RNA extraction with a DNA-free DNA Removal kit (Thermo

126   Fisher Scientific). cDNA was synthesised with SuperScript VILO MasterMix (Thermo Fisher

127   Scientific) according to the manufacturer's instructions.

128

129   **Sequencing**

130   PCR amplification of the 16S V4 region was done with primers 515′F (Hugerth, *et al*. 2014) and 806R

131   (Caporaso, *et al*. 2011), using dual indexing of the primers (Kozich, *et al*. 2013). Template DNA (40

132   ng) or undiluted cDNA (2 µl) was amplified in a total volume of 50 µl using a Phusion Hot Start II

133   DNA Polymerase (Thermo Fisher Scientific). The following PCR program was used: activation

134   (98°C, 30 s); 30 cycles of denaturation (98°C, 10 s), annealing (56°C, 30 s) and elongation (72°C, 15

135   s); followed by final elongation (72°C, 10 min). PCR products were purified with Ampure XP

136   (Beckman Coulter, Brea, CA, USA). Purified PCR products were pooled in equimolar amounts.

137   Sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit v3 (Illumina, San

138   Diego, CA, USA).

139

140   PCR amplification of *nxrB*, *hzsB*, and *amoA* was carried out in two steps using Nextera index adapters

141   (Illumina). Primers nxrB169F and nxrB638R (Pester, *et al*. 2013) were used for *nxrB* amplification,

142   amplification of *hzsB* was done with the hzsB_396F and hzsB_742R primers (Wang, *et al*. 2012), and

143   primers AmoA1F mod (Stephen, *et al*. 1999) and AmoA2R (Rotthauwe, *et al*. 1997) were used for

144   *amoA*. The following PCR program was used for all amplicons: activation (98°C, 30 s); 25 cycles of

145   denaturation (98°C, 10 s), annealing (56°C, 30 s) and elongation (72°C, 45 s); followed by final

146   elongation (72°C, 5 min). PCR amplicons were then purified, and a second PCR with 8 cycles of

147 amplification was used to attach the index adapters followed by a second purification. Amplicons

148 were then pooled together in equimolar amounts and sequenced on a MiSeq as described above.

149

150 **Statistics and data analysis**.

151 Samples with less than 30,000 reads were excluded prior to analysis. ASVs were generated, using

152 DADA2 version 1.12 (Callahan, *et al*. 2016). The SILVA 132 database (Quast, *et al*. 2013) was used

153 for taxonomic classification of the 16S amplicons with IDTAXA (Murali, *et al*. 2018). Data was

154 analysed in R (R Core Team 2019) using the packages Phyloseq (McMurdie and Holmes 2013) and

155 Vegan (Oksanen, *et al*. 2019). Raw sequence reads were deposited at the NCBI (Bioproject:

156 PRJNA552732). See table S1 for individual accession numbers of each sample.

157

158 Reads were normalized by proportion prior to estimation of beta diversity between mainstream and

159 sidestream in the rDNA, rRNA, *nxrB* and *hzsB* libraries and for comparisons between the rDNA and

160 rRNA libraries. Beta diversity was estimated with the abundance-based Bray-Curtis index and the

161 presence-absence-based Simpson index, which is not sensitive to richness differences, and is a

162 measure of turnover (Baselga 2010). To estimate differential abundance of ASVs between sidestream

163 and mainstream, DESeq2 was used (Love, *et al*. 2014) without subsampling before the analysis

164 (McMurdie and Holmes 2014). A $p_{(adj)}$ <0.01 value (DESeq2) was used as criterion for statistical

165 significance. Only samples with both rDNA and rRNA libraries available (mainstream, n=6;

166 sidestream, n=7) were used for plots of rDNA vs rRNA. Ratios of rRNA:rDNA were estimated after

167 excluding values of zero in the libraries.

168

169 **Fluorescence in situ hybridization (FISH)**

170 FISH was carried out on suspended biomass as previously described (Suarez, *et al*. 2015). The probe

171 AMX820 (Schmid, *et al*. 2001), labelled with FAM, was used to targ*et al*l *Brocadia* populations. The

172   probes BAN162 (Schmid, *et al*. 2001), labelled with Cy3, and BFU613 (van de Vossenberg, *et al*.

173   2008), labelled with Cy5, were used to target *Brocadia* subpopulations, as they only partially cover

174   the *Brocadia* genus, as determined with ARB 6.0.6 (Ludwig, *et al*. 2004) using the SILVA NR 132

175   SSU *Brocadia* sequences. These probes were applied together with unlabelled competitors (for

176   Ban162, CGG TAG CCC CAA TTG CTT; for Bfu613, GGA TGC CGT TCT TCC GTT GAG CGG)

177   to increase probe specificity, as previously reported (Persson, *et al*. 2014, Suarez, *et al*. 2015).

178

179   **Results and disscusion**

180   **Sidestream and mainstream communities**

181   We compared PNA microbial communities exposed to sidestream and mainstream conditions by

182   sequencing rDNA and rRNA from individual biofilm carriers. Mainstream and sidestream

183   communities, estimated from both rDNA and rRNA, were significantly different, as shown with the

184   abundance-based Bray-Curtis index (Adonis β_{bray}; $p < 0.01$, $r^2 = 0.16$; Figure 1A). Because beta-

185   diversity can also exist among rare taxa (Gobet, *et al*. 2012), we also used the presence-absence

186   Simpson index, which measures species turnover (Baselga 2010). The observed results (Adonis β_{Sim};

187     $p < 0.01$, $r^2 = 0.32$; Figure 1B), suggest that the sidestream and mainstream communities not only

188   differed significantly in their relative abundance of taxa, but also in their identity. The sidestream and

189   mainstream communities also diverged from the initial seed communities, indicating temporal

190   dynamics (Figure S2, Supporting information). Although elucidating the assembly mechanism of

191   PNA biofilms is beyond the scope of this study, sidestream and mainstream were exposed to different

192   environmental conditions and subjected to potential immigration from two different water sources,

193   which would both influence the community composition.

194
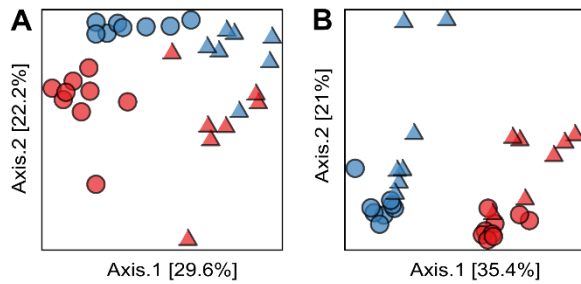
Figure 1: PCoA of rDNA and rRNA libraries, based on the abundance-based Bray-Curtis index (**A**)

and the presence-absence-based Simpson index (**B**). Red: mainstream, Blue: sidestream. Circles:

rDNA, triangles: rRNA.

198

Comparing rDNA and rRNA libraries

200   The rDNA and rRNA libraries of the mainstream and sidestream communities were different in

201   relative read abundance (Adonis β_{bray}; $p < 0.001$, $r^2 = 0.24$; Figure 1A), which could be interpreted as

202   rRNA:rDNA ratios different to one. However, turnover between the rDNA and rRNA libraries was

203   also observed (Adonis β_{sim}; $p < 0.001$, $r^2 = 0.13$; Figure 1B), which would imply that different ASVs

204   were detected by sequencing of rDNA and rRNA. ASVs present in the rRNA, but not the rDNA

205   libraries are known as phantom taxa. They could be the result of PCR errors during reverse

206   transcription, but could also arise due to rDNA under-sampling of rare but highly active taxa (Klein,

207   *et al*. 2016). Supporting the latter suggestion, we commonly observed phantom taxa for genera with

208   high rRNA:rDNA values, like *Competibacter*, *Agitococcus, Romboutsia*, the AMX *Brocadia* and the

209   AOB *Nitrosomonas* (Figure S3, Supporting information). In addition, among genera with low

210   rRNA:rDNA, like *Denitratisoma*, *Dokdonella* and *UTBCD1* (Figure S3, Supporting information),

211   some ASVs did not have any corresponding rRNA reads. This could signal that a high proportion of

212   these taxa in the biofilms were dormant or dead. Extracellular DNA is commonly observed in

213   biofilms (Dominiak, *et al*. 2011) and could inflate rDNA reads for some taxa (Albertsen, *et al*. 2015).

214

215   Despite the differences, relative read abundances of the rDNA and rRNA libraries over the entire

216   dataset were positively correlated at the phylum level (Kendall´s τ = 0.77, p < 0.001, Figure 2) as well

217   as the ASV level (Kendall's τ = 0.59, p < 0.001), and also seen by modelling rRNA abundance of

218   ASVs with beta regression (z =18.0, p<0.001, pseudo-R^2 = 0.44; Figure S4, Supporting information).

219   Thus, ribosomal relative abundance was generally linked to taxa relative abundance. Nonetheless, it

220   appears that rRNA:rDNA ratios varied between phyla; for example, low ratios were observed for

221   *Acidobacteria*, and high ratios were noticed for *Firmicutes* and *Planctomycetes* (Figure 2, S4, table

222   S2). Such different rRNA:rDNA ratios have been observed before across phyla (Denef, *et al*. 2016,

223   Steven, *et al*. 2017).
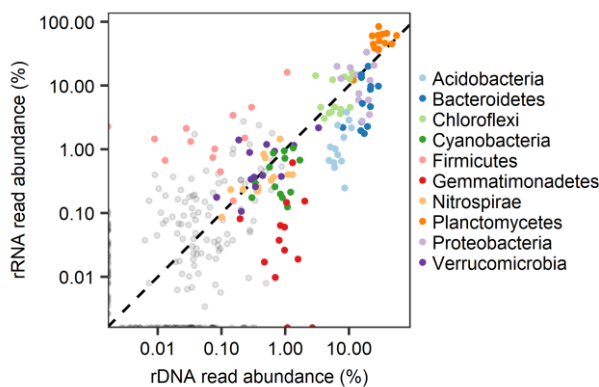
224

225   **Figure 2.** Comparison of rDNA and rRNA of the entire dataset at the phylum-level. Each point

226   indicates the abundance of an ASV in one MBBR carrier; the black dashed diagonal line indicates

227   equal rDNA and rRNA abundance. The colours denote the 10 most abundant phyla.

228

229   *Acidobacteria* are considered slow growing bacteria (Ward, *et al*. 2009), and hence it is possible that

230   their low rRNA:rDNA ratios in fact represent slow growth rates compared to other taxa in the

231   biofilms. This might be supported by the fact that *Acidobacteria* have between one and two copies of

232   the 16S RNA gene, since low copy numbers are associated with oligotrophic lifestyles (Klappenbach,

233   *et al*. 2000, Stevenson and Schmidt 2004). On the other hand, although rRNA:rDNA values larger

234   than one are frequently used as criteria for activity (Blazewicz, *et al*. 2013), different taxa might differ

235   in their rRNA content during growth or dormancy to the extent that rRNA content and growth rate are

236   not linked (Blazewicz, *et al*. 2013). Thus, potentially active taxa might be misclassified as dormant

237   just because their rRNA:rDNA ratio is below one (Steven, *et al*. 2017).

238

239   The disproportionally high read abundances of rRNA at low rDNA abundances for most phyla

240   (Figure S4) confirm previous similar observations, suggesting higher activity among rare taxa

241   (Campbell, *et al*. 2011, Jia, *et al*. 2019, Jones and Lennon 2010, Klein, *et al*. 2016, Wilhelm, *et al*.

242   2014). This phenomenon could perhaps be due to under-sampling (Steven, *et al*. 2017), but higher

243   growth rate among rare taxa could arise due to intraspecific competition or predation of abundant taxa

244   (Jousset, *et al*. 2017). In fact, by using metagenomic data, Jia, *et al*. (2019) observed higher

245   replication rates for taxa at low relative abundances supporting that this is a real phenomenon.

246

247   **Comparing rRNA:rDNA ratios between sidestream and mainstream**

248   We originally expected that the same ASVs present in the relatively different sidestream and

249   mainstream conditions would have different rRNA:rDNA ratios. For example, in the case of *Brocadia*

250   we anticipated a higher rRNA:rDNA in the sidestream because the activity in terms of nitrogen

251   conversion was much higher than in mainstream (Table 1). In addition, the growth rate of anammox

252   bacteria is affected by temperature (Laureni, *et al*. 2015). But differences in rRNA:rDNA ratios

253   between the two environments were not observed for any of the 12 ASVs present across all rRNA and

254   rDNA libraries (Wilcoxon rank sum test, p>0.05; Figure 3).
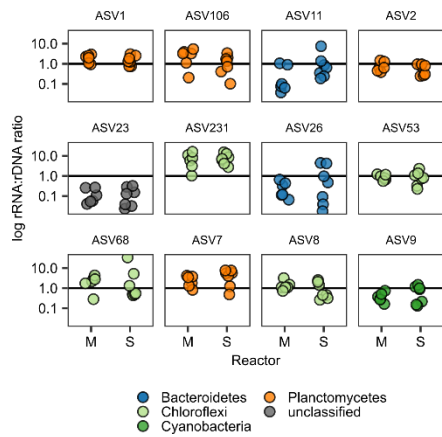
Figure 3: Ratios of rRNA:rDNA for 12 ASVs present in all rDNA and rRNA libraries. Colours denote phylum classification, M = mainstream and S = sidestream. ASV1 and ASV7 are classified as the AMX *Brocadia*.

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; $p_{(adj)} < 0.01$; Figure 4B). Furthermore, potential heterotrophic denitrifying bacteria

275   (HDB) like *Zoogloea* and *Sulfuritalea*, among others, were more abundant in the Mainstream

276   (DESeq2; $p_{(adj)} < 0.01$, Figure 4B) and were in fact more or less absent in the sidestream biofilms.

277   Similarly, Yang, *et al*. (2018) observed a decrease in AMX abundance when a sidestream community

278   was exposed to mainstream conditions, also indicating that sidestream conditions favour AMX growth

279   relative to other bacteria. The larger fraction of HDB in the mainstream suggests more extensive

280   competition for NO_2^- between AMX and HDB; in addition, potential nitrogen loops of nitrification,

281   anammox, denitrification and DNRA may occur (Speth, *et al*. 2016).
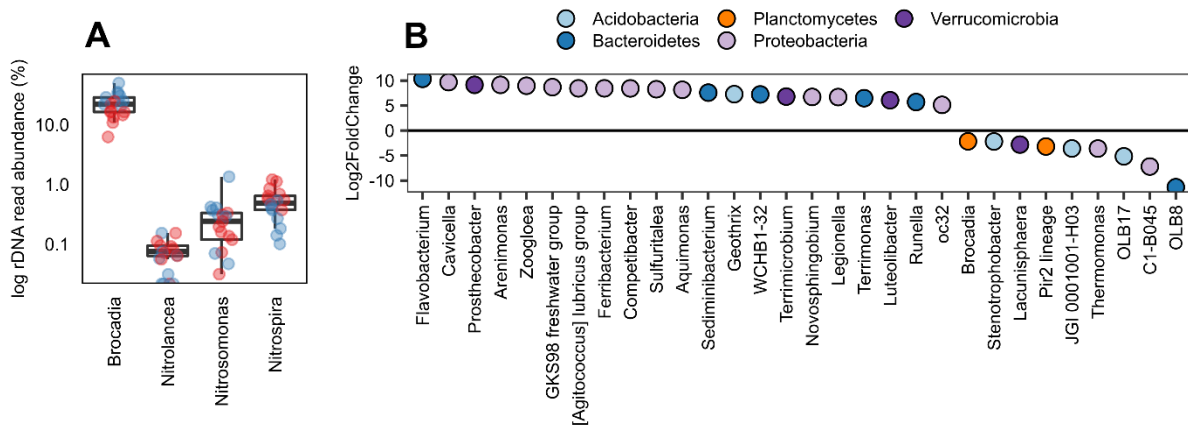

282

283   **Figure 4.** Abundance of taxa at the genus level. **A:** rDNA read abundance of AMX (*Brocadia*), AOB

284   (*Nitrosomonas*) and NOB (*Nitrospira and Nitrolancea*), red: mainstream, blue: sidestream. **B:**

285   Log2FoldChange (LFC) for rDNA genera with differential abundance between mainstream and

286   sidestream biofilms (DESeq2; $p_{(adj)} < 0.01$). Positive LFC corresponds to higher abundance in

287   mainstream, and negative LFC corresponds to higher abundance in sidestream; only the top 30 genera

288   with the lowest significant $p_{(adj)}$ are shown.

289

Microdiversity of nitrogen transforming bacteria

291   Several anammox populations coexisted in the biofilms as assessed by rDNA sequencing (Figure S5-

292   S7, Supporting information). An example of the microdiversity within *Brocadia* can be observed in

293   Figure 5A, where a combination of different FISH probes was used to visualize three different

294   *Brocadia* subpopulations. By sequencing the *hzsB* gene, 119 ASVs within *Brocadia* were detected

295   (Figure 5B, S8, Supporting information), and turnover between sidestream and mainstream was

296   demonstrated (Adonis, β_{sim}, r^2=0.57, p=0.01). Nonetheless, a single ASV was dominant in both

297   sidestream and mainstream (Figure 5B, S8, Supporting information), and represented $77 \pm 3\%$ of the

298   total *hzsB* reads (Figure 5C). As hydrazine synthase (HZS) is a key enzyme for the anammox process,

299   this suggests that this strain was responsible for the bulk anaerobic oxidation of ammonium in both

300   the sidestream and the mainstream, in spite of the different environmental conditions in the reactors.
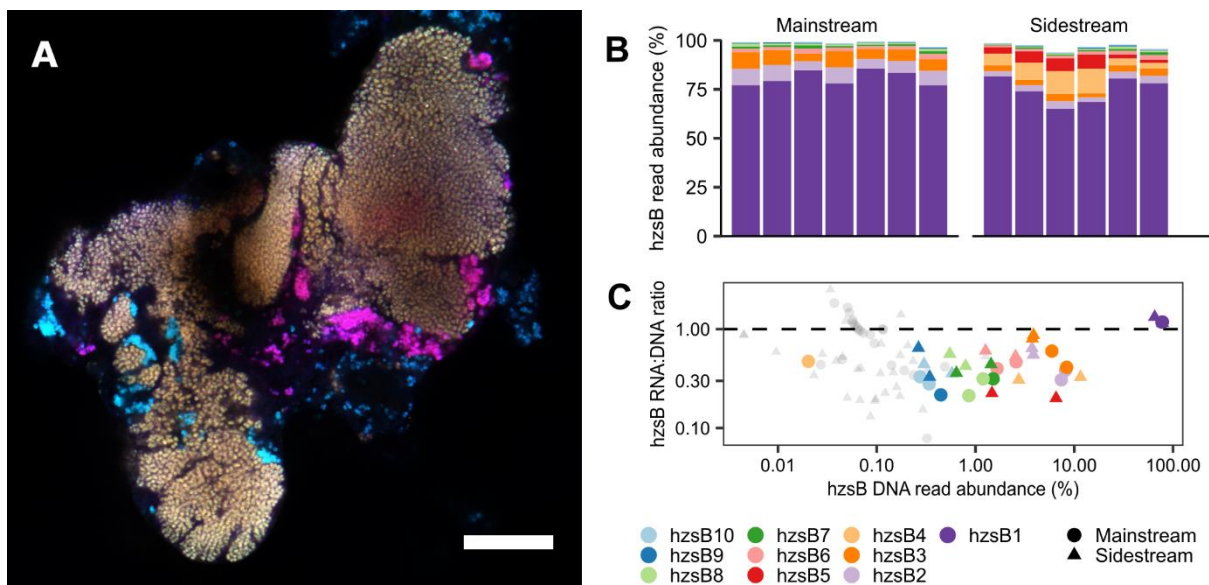
301

302   **Figure 5A:** Multiple *Brocadia* populations (sidestream) targeted with the FISH probes AMX820

303   (Blue), BFU613 (Red) and Ban162 (Green); overlap among probes results in additional colours: Cyan

304   (AMX820 and Ban162), Magenta: (AMX820, BFU613), White: (AMX820, Ban162 and BFU613). **B**:

305   DNA read abundance of the top 10 *hzsB* ASVs; each colour represents a unique ASV; each bar

306   represents a biofilm sample. **C:** RNA:DNA ratios for the top 10 *hzsB* ASVs (n=4). Same colour

307   coding as in B.

308

309   Using rDNA, several *Nitrospira* and *Nitrosomonas* ASVs were observed (Figure S6, S7, Supporting

310   information). Likewise, using the key enzymes *nxrB* and *amoA* we also observed multiple *Nitrospira*

311   ASVs (Figure 6, S9, Supporting information) and *Nitrosomonas* ASVs (Figure S10, Supporting

312   information), respectively. Turnover of *Nitrospira* communities in sidestream and mainstream was

313   observed as assessed with *nxrB* (Adonis, β_{sim}, p=0.004, r^2=0.39), while for *amoA*, low PCR yield in

314   sidestream samples prevented comparison of sidestream and mainstream. In microbial communities

315   the coexistence of closely related taxa is often reported (Goldford, *et al*. 2018). For example, for

316   *Nitrospira*, which is commonly present in wastewater (Daims, *et al*. 2001), multiple populations can

317   coexist in activated sludge (Gruber-Dorninger, *et al*. 2014). Coexistence of multiple AMX

318   populations has also been reported in PNA systems (Bhattacharjee, *et al*. 2017, Laureni, *et al*. 2019,

319   Persson, *et al*. 2014). *Nitrospira* is a heterogeneous group, which not only represents a nitrite

320   oxidising potential, but also contains populations capable of using a myriad of electron donors and -

321   acceptors (Daims, *et al*. 2015, Koch, *et al*. 2015, van Kessel, *et al*. 2015) and the same has also been

322   shown for AMX (Hu, *et al*. 2019, Kartal, *et al*. 2007, Strous, *et al*. 2006, van de Vossenberg, *et al*.

323   2008) and AOB (Bock, *et al*. 1995, Schmidt, *et al*. 2004). Thus, the coexistence of different *Brocadia*,

324   *Nitrosomonas* and *Nitrospira* ASVs, observed in this study, could be a general phenomenon,

325   explained by ecotypes within the AMX, AOB and the NOB utilizing different metabolic pathways

326   and playing various ecological roles. The roles of the less abundant AMX in the PNA biofilms are yet

327   unclear, but read abundance of *hzsB* mRNA (Figure 5C) suggests that their contribution to the

328   anammox process might have been minor.
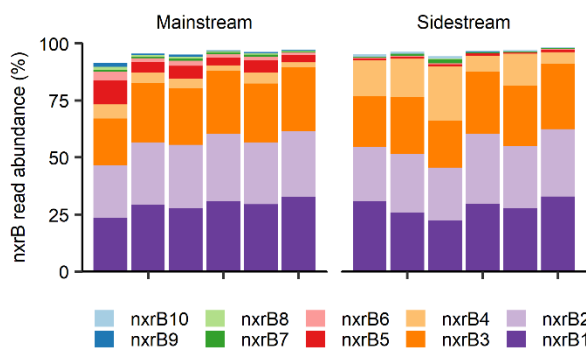
329

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

331

332   The dominant *hzsB* ASV had an identical sequence to *B. sapporoensis*, which is frequently observed

333   in PNA bioreactors (Lotti, *et al*. 2015b, Persson, *et al*. 2014) and is considered relatively fast growing

334   (Lotti, *et al*. 2015a). It has a maximum specific anammox activity at 37°C (Narita, *et al*. 2017), which,

335   together with other factors like nitrogen load and partial nitritation rate, would explain the lower

336   nitrogen removal rates observed in the mainstream compared with the sidestream MBBR. A

337   considerable decrease of anammox rates with decreasing temperature has often been described in

338   PNA reactors (Gilbert, *et al*. 2014, Laureni, *et al*. 2016, Lotti, *et al*. 2015b). Interestingly, no

339   particular cold-tolerated AMX have so far been detected, instead AMX within *Brocadia* are

340   commonly reported in mainstream reactors. This study suggests that *B. sapporoensis*, can in fact be

341   competitive relative to other AMX taxa at both sidestream and mainstream conditions. One possible

342   explanation for such a phenomenon can be mechanisms of adaptation, where temperature changes can

343   result in changes in membrane composition (Rattray, *et al*. 2010) and protein expression (Lin, *et al*.

344   2018). Alternatively, there could be multiple closely related populations with identical *hzsB* and

345   rDNA sequences, but with different accessory genes, and thus metagenomic studies would be needed

346   to resolve such populations.

347

348   Comammox can occur among lineage II in *Nitrospira* (Daims, *et al*. 2015). The majority of *Nitrospira*

349   in this study were from lineage I (Figure S9, Supporting information) and thus the comammox

350   process was likely not important for the nitrogen transformations in the biofilm. Inhibition of NOB is

351   critical for the function of PNA, and several strategies have been proposed for NOB suppression

352   including, but not limited to, DO limitation, intermittent aeration and exposure to free nitrous acid

353   (Malovanyy, *et al*. 2015, Pérez, *et al*. 2014, Wang, *et al*. 2016). However, the observed NOB diversity

354   within *Nitrospira* could impact reactor performance, because microbial diversity might lead to

355   functional redundancy (Allison and Martiny 2008). As NOB in any PNA reactor likely consist of

356   multiple coexisting populations, operational strategies to reduce nitrite oxidation based on knowledge

357   gained from pure cultures or enrichments may not be adequate. In fact, the inhibition of a dominant

358   NOB population, may well lead to the succession of another dominant population. Future studies,

359   combining well-defined reactor experiments with high resolution genomics would gain further

360   insights in how microdiversity of key guilds, as observed here, affects turnover of substrates and

361   thereby reactor performance

362

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