Comammox and Unknown Candidate AOBs Contribute to Nitrite Accumulation in An Integrated A-B stage process that Incorporates Side-stream EBPR (S2EBPR)

Yuan Yan[a,#], Jangho Lee[a,#], IL Han[a], Zijian Wang[a], Kester McCullough[a,b], Stephanie Klaus[b], Da Kang[a,c], DongQi Wang[a], Anand Patel[a], Guangyu Li[a], Jim McQuarrie[d], Beverley M. Stinson[e], Christine deBarbadillo[f], Paul Dombrowski[g], Charles Bott[b,*], April Gu[a,*]

[a] School of Civil and Environmental Engineering, Cornell University, Ithaca, United States

[b] Hampton Roads Sanitation District, Virginia Beach, United States

[c] Department of Environmental Engineering, College of Environmental & Resource Sciences, Zhejiang University, China

[d] Denver Metro Wastewater Reclamation District, Denver, Colorado, United States

[e] AECOM, Laurel, Maryland

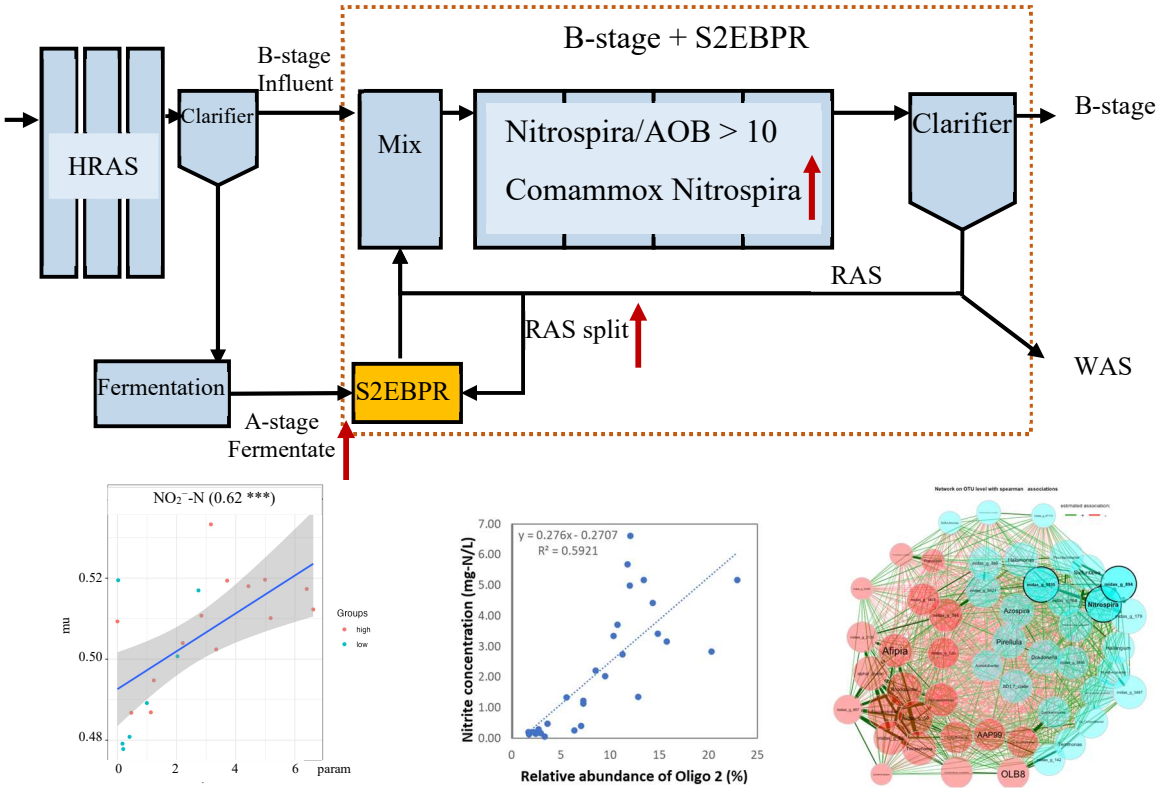
[f] DC Water and Sewer Authority, Washington, District of Columbia

[g] Woodard & Curran, Inc, Enfield, Connecticut, United States

[#] Joint first authors

[*] Corresponding authors

Graphical abstract



Highlights

- Nitrite accumulation was achieved in HRAS-P(d)N-S2EBPR system via partial nitrification with *Nitrospira*/AOB >10.
- The positive correlation between *Nitrospira* oligo type 2 and nitrite accumulation suggested the contribution of comammox *Nitrospira* in partial nitrification.
- The integration of S2EBPR and elevating fermentate VFA encourages the development of heterotrophic nitrifiers from family Comamonadaceae and order Rhodocyclaceae.

Abstract

In a pilot-scale A-B process integrated with side-stream enhanced biological phosphorus removal (EBPR) for treating municipal wastewater, average of 7.62 ± 2.17 mg-N/L nitrite accumulation with nitrite accumulation ratio of 49% was achieved by partial nitrification, which was verified through relative higher ammonia oxidation activity (1.94 ± 0.89 mgN/gSS/h) than nitrite oxidation rate (1.06 ± 0.99 mgN/gSS/h). *Nitrospira* dominated the ammonia oxidizing community in a B-stage nitrification tank throughout the operation periods with one to two orders of magnitude higher than canonical aerobic oxidizing bacteria (AOB). The higher ammonia oxidation activity along with increased *amoB*/*nxr* ratio in nitrite accumulation period suggested the contribution of comammox *Nitrospira* as AOB. Phylogenetic investigation suggested that AMO and HAO were embedded in heterotrophic organisms that belong to the family Comamonadacea and the order Rhodocyclaceae. These organisms may function as heterotrophic nitrification. The integration of S2EBPR provides PAOs suitable environment, such as low ORP and favorable VFA concentrations and composition, but also exerts additional selective pressure on NOB activity than AOB, which may be beneficial for main-stream partial nitrification.

1 Introduction

The adsorption/bio-oxidation (A/B) process is a promising engineering innovation towards carbon capture, energy recovery, and efficient nutrient removal. It diverts organic carbon to anaerobic digestion and thus manipulates the C/N in B-stage reactors through carbon flow redirection (Ren and Pagilla, 2022). The first stage (A-stage) is designed for high-rate carbon capture and removal using high-rate activated sludge (HRAS), and the second stage (B-stage) is for nutrient (mostly nitrogen) removal (Sancho et al., 2019). HRAS is widely operated with a low hydraulic retention time (HRT), a low sludge retention time (SRT) and a low dissolved oxygen (DO) concentration less than 0.5 mg L^{-1} (Boehnke et al., 1997). After HRAS, the wastewater COD/N ratio could be lowered to 0.67 (Lotti et al., 2015), which is suitable for B-stage short-cut nitrogen removal processes to achieve "carbon-free" autotrophic nitrogen removals.

Partial nitrification with anammox (PN/A) process has emerged to be one of the promising nitrogen removal processes, which can save 100% carbon, 60% aeration and reduce 80% sludge production (Jetten et al., 1997). It consists of two step processes where aerobic ammonia-oxidizing bacteria (AOB) oxidize half of the ammonia to nitrite and, then anaerobic ammonia-oxidizing bacteria (anammox) bacteria convert ammonia and nitrite into dinitrogen. Nitrite accumulation through partial nitrification in B-stage via the out-selection of nitrite oxidizing bacteria (NOB) is crucial for PN/A and it has been successfully demonstrated for side-stream high-strength wastewater (Lackner et al., 2014). A number of operational strategies have been suggested to enable side-stream NOB out-selection and consequently nitrite accumulation, including high temperature, low DO, low SRT, high pH, and high free nitrous acid (FNA) and/or free ammonia (FA) concentration (Hellinga et al., 1998; Joss et al., 2009; Van Dongen et al.,

64 2001; Xu et al., 2012). However, these strategies were not readily applicable in mainstream
65 treatment since the inhibitory levels of 0.02 mg/L FNA and 1 mg/L FA is unavailable with ca. 50
66 mg-N/L ammonia at neutral pH in the mainstream (Wang et al., 2017). Also, it has been shown
67 that NOB can adapt to high concentration of FA (An et al., 2021) and low DO (Liu and Wang,
68 2013), which makes it harder to be out-selected in mainstream process. Therefore, maintaining
69 long-term stable NOB out-selection still remains a challenge for main-stream wastewater
70 treatments and the underlined mechanisms need to be explored.

71 Most PN/A studies focus only on nitrogen removal, however, phosphorus co-occur with nitrogen
72 and is also a critical nutrient that needs to be treated in wastewater (Rittmann et al., 2011).
73 Enhanced biological phosphorus removal (EBPR) process has been widely applied worldwide in
74 full-scale facilities. Its performance and stability strongly rely on the activity of phosphorus
75 accumulating organisms (PAOs) with favorable influent rbCOD/TP ratio >15 (Gu et al., 2008).
76 Therefore, EBPR is considered incompatible with B-stage autotrophic nitrogen removal due to
77 the insufficiency of readily biodegradable carbon in A-stage effluent (B-stage influent). The
78 implementation of a side-stream anaerobic biological sludge hydrolysis and fermentation reactor
79 called side-stream EBPR (S2EBPR) can be an alternative option to address the carbon shortage
80 challenge of coupling EBPR with B-stage nitrogen removal process. S2EBPR takes advantage of
81 the volatile fatty acids (VFAs) generated via hydrolysis/fermentation of side-stream return
82 activated sludge (RAS) and mixed liquor, and thus overcome the reliance on the influent
83 carbon (Vollertsen et al., 2006; Barnard et al., 2017; Onnis-Hayden et al., 2020; Gu et al., 2018;
84 Wang et al., 2019). However, the influence of implementing S2EBPR on the PN/A removal
85 system, especially on the nitrite accumulation remains unknown. We hypothesize that the
86 incorporation of S2EBPR would affect the nitrifying populations because of the extended

87 anaerobic conditions in the side-steam reactor with low oxidation reduction potential (ORP,
88 <300 mV) and high VFAs. In addition, PAOs and the surplus of sludge fermentate from the
89 S2EBPR reactor to the down-stream B-stage would compete oxygen with nitrifiers (Carvalheira
90 et al., 2014).

91 Recently, our team has reported a successful incorporation of S2EBPR into a A-B stage process
92 with downstream Annamox polishing to achieve simultaneous short-cut N removal and
93 biological P removal for main-stream treatment (Klaus, 2019; McCullough et al., 2020). This
94 pilot-scale HRAS-P(d)N-S2EBPR-Anammox system provides us good opportunity to explore
95 the interacting impacts of PAOs and nitrogen removal related microorganisms. In this study, we
96 focused on the comprehensive examination of the nitrite accumulation and AOB and NOB
97 population dynamics in B-stage processes that generated nitrite with intermittent aeration AvN
98 (ammonia versus NO_x^--N (nitrite+nitrate) control strategy. Process performance, batch activity tests
99 were performed to investigate the underlying processes involved in the nitrite accumulation. 16S
100 rRNA gene amplification sequencing and quantitative PCR were applied to reveal the microbial
101 community composition and dynamics with emphasis on nitrification-relevant populations. To
102 our knowledge, this is the first study that revealed the influence of implementing S2EBPR on B-
103 stage nitrification population and, provided new insights on the distinctive nitrifying populations
104 that contributed to nitrite accumulation in this HRAS-P(d)N-S2EBPR process that is different
105 from those typically observed in previous WWTPs. The results pointed out a possible new
106 strategy in achieving nitritation to enable annamox for main-stream municipal WW treatment.

2 Material methods

2.1 Pilot reactor setup and Operation conditions

The pilot plant operated by Hampton Roads Sanitation District (HRSD) is located at Chesapeake-Elizabeth WWTP in Virginia Beach, VA, USA. It mainly consisted of an A-stage HRAS process followed by the B-stage process, a parallel S2EBPR reactor and a final stage Annamox polishing tank (Figure S1). The detailed parameters of A-B process have been introduced elsewhere (Klaus et al., 2020; Printz, 2019). The A-stage HRAS consisted of three bioreactors in series (V_{total} = 511 L) followed by an intermediate clarifier with effluent going to the B-stage process. A portion of A-stage wasted activated sludge (WAS) was splited into a combined fermenter and thickener. The B-stage consisted of four CSTRs bioreactors in series (Vtotal = 606 L) each with independent aeration control via Ammonia versus NOx (nitrite + nitrate) (AvN) control (Regmi et al., 2014) and mechanical mixing. The B-stage system was operated in a fully intermittent configuration in which all CSTRs were intermittently aerated for the same interval and duration, and it was maintained at a 5-hour HRT using a flow-paced (2.0 L min^{-1}) influent progressive cavity pump (Seepex BW2, Bottrop, Germany) with a variable frequency drive (VFD) and magnetic flow meter (Rosemount 8705, Houston, TX). In the studied period, B-stage reactors were switched from clarifier wasting to aeration tank wasting (hydraulic wasting) and recycling. A portion of B-stage RAS and the A-stage WAS fermentate were added to the side-stream reactor for simultaneous VFA production via hydrolysis and fermentation, and for PAOs selection. The HRT of side-stream reactor varies depending on the percentage of B-stage RAS sent to the reactor, but it maintained around 4 hours during most of the operation period in this study (Figure S2). The SRT of the S2EBPR reactor was estimated as $SRT_{total} - SRT_{B\text{-}stage}$, resulting in an average SRT of 6 days.

130 **2.2 Chemical analysis: sCOD, VFA, NH4, NO2, NO3, PO4**

131 Total and soluble COD, OP, total ammonia nitrogen (NH_4^+-N + NH_3-N), NO_2^--N, and NO_3^--N
132 were measured with HACH TNTplus kits (Loveland, Colorado) and a HACH DR2800
133 spectrophotometer (Loveland, CO). Nutrient and soluble COD samples were filtered through
134 0.45 μm and 1.5 μm filters respectively. The sCOD measurement includes readily biodegradable
135 and colloidal COD fractions. Daily pH and temperature readings of the reactors were recorded
136 using a handheld pH and temperature meter (Beckman Coulter, Brea, CA). Readings for DO
137 were recorded using a handheld DO sensor (Insite Model 10, LA, USA).

138 Concentrations of acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids
139 were determined by GC-FID as previously described in (Kinyua et al., 2017). VFA
140 concentrations were determined from a 0.5-100 mg/L calibration curve prepared from
141 commercial standards (Absolute Standards).

142 **2.3 AOB and NOB activity analysis**

143 AOB and NOB activities were analysis weekly on site. About 4L mixed liquor suspended solids
144 (MLSS) were withdrawn from the last B-stage aerobic tank and pre-aerated for 30 min to oxidize
145 the residue carbon. Ammonium chloride was spiked to reach 25 mg-N/L final concentration. DO
146 and pH were maintained around 3-5 mg/L and 7, respectively. The samples were taken every 15
147 minutes for a total of 1 hour. After filtered by 0.45 μm filter, the filtrate was used to analyze the
148 NH_4^+-N, NO_2^--N, and NO_3^--N concentrations using HACH TNT kits (HACH Loveland, CO).
149 AOB activity was calculated as the sum of nitrite and nitrate production rates, and NOB activity
150 was calculated as the nitrate production rates by linear regression.

151 **2.4 Quantification of nitrifying candidates**

152 Quantitative polymerase chain reaction (qPCR) was performed on a CFX96 real-time PCR
153 detection system (Bio-Rad, USA) to determine the concentrations of functional genes including
154 *amoA*, *nxrB*, *amoB* and 16S. The amoA-1F/2R primer for the canonical ammonia
155 monooxygenase (AMO) enzymes in AOB (Rotthauwe et al., 1997), and the *nxrB* 169F/638R
156 primer for the beta subunit of nitrite oxidoreductase (NXR) (Pester et al., 2014) were used to
157 target canonical AOB and NOB. The *amoB* 148F/485R primer were used to target the beta
158 subunit of AMO in *Nitrospira* that can do complete ammonia oxidation (comammox) (Cotto et
159 al., 2020). All functional genes were then normalized by 16S rRNA with primer set 341f/534r
160 (He et al., 2007). Calibration curves were obtained using 10-fold serial dilutions of known
161 concentrations of cloned DNA samples that were prepared following the instruction from TOPO
162 TA cloning kit (Thermo Fisher, USA) in our laboratory. The R^2 of standard curves were above
163 0.99 and the amplification efficiency was around 95%. The detailed qPCR reaction conditions
164 were summarized in Table S1.

165 **2.5 Bioinformatics and statistical analysis**

166 2.5.1 Microbial population analysis

167 Sludge samples in effluent from B-stage were monthly collected for DNA extraction. Genomic
168 DNA was extracted using Instagene matrix (Kim et al., 2013) and then used for both 16S
169 ribosomal RNA (16S rRNA) sequencing and functional genes quantification.

170 The V4 region of the 16S rRNA genes were amplified using primers 515F-Y (Parada et al.,
171 2016) and 926R (Quince et al., 2011). Each primer contained a unique six base index sequence
172 for sample multiplexing, as well as Illumina flow cell binding and sequencing sites (Bartram et

173 al., 2011). The 25 µl PCR mix contained 1× ThermoPol buffer, 0.2 µM forward primer, 0.2 µM
174 reverse primer, 200 µM dNTPs, 15 µg bovine serum albumin, 0.625 U *Taq* DNA polymerase
175 (New England Biolabs, USA), and 10 ng of template. The PCR was prepared in triplicate as
176 follows: 95°C for 3 min, 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 68°C for 1 min, and a
177 final extension of 68°C for 7 min. Equal quantities of 16S rRNA gene amplicons were pooled,
178 excised from an agarose gel, and purified using the Wizard SV Gel and PCR Clean-Up System
179 (Promega, WI, USA). A 3 pM library containing 10% PhiX Control v3 (Illumina, CA, USA) was
180 sequenced on a MiSeq (Illumina, CA, USA) using a 2 x 250 cycle MiSeq Reagent Kit v2.
181 Paired-end reads were assembled using the paired-end assembler for Illumina sequences
182 (PANDAseq version 2.8) (Masella et al., 2012). The raw paired-end reads were assembled for
183 each sample and processed in Mothur v. 1.39.4 following the MiSeq standard operating
184 procedure (SOP) (Kozich et al., 2013). High-quality reads were obtained after quality control and
185 chimera screening and then clustered at a 97% similarity to obtain the operational taxonomic
186 units (OTUs).

187 To minimize the inflation of rare species in the community analysis, we removed OTUs that are
188 lower than 0.01%. The filtered-OUT matrix was used to perform statistical analyses in R-4.2.3.
189 The matrix was also used to predict the bacterial metagenomes using Phylogenetic Investigation
190 of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) pipeline (Douglas et al.,
191 2020) with implemented tools EPA-ng (Barbera et al., 2019), gappa (Czech et al., 2020), and
192 castor (Louca and Doebeli, 2018). The co-occurrence patterns of microbial community and the
193 correlation between the environmental variables and the most abundant 50 taxa were done
194 through microbiomeSeq package (Ssekagiri et al., 2017; Torondel et al., 2016). All bioinformatic
195 figures were generated with ggplot2 package (Villanueva and Chen, 2019, p. 2).

196 2.5.2 Oligotyping analysis

197 Oligotyping analysis was performed according to (Eren et al., 2013; Srinivasan et al., 2021) to
198 reveal the sub-clusters (i.e. microdiveristy) within the dominant nitrifying population. A total of
199 65,24316S rRNA gene amplicon sequences classified as *Nitrospira* were extracted and formatted
200 as required by the oligotyping pipeline. After quality filtering, 18 nucleotide positions were
201 selected for entropy decomposition (102, 120, 134, 135, 149, 151, 160, 181, 197, 199, 210, 219,
202 240, 262, 265, 375, 488, 491), which were used to generate a total of 205 raw oligotypes. After
203 denoising and elimination based on the -s (oligotypes that occurred in at least 3 samples) and -M
204 (a minimum count of 30 (~0.1%)) parameters, 3 dominant oligotypes, presumably representing
205 micro-diversity of *Nitrospira* sub-groups, were finally identified. The representative sequence of
206 the 3 dominant oligotypes were retrieved and blasted in the NCBI's nt database
207 (http://www.ncbi.nlm.nih.gov/).

208 **3 Results and Discussion**

209 **3.1 Simultaneous nitrite accumulation and efficient P removal in A-B-stage-S2EBPR**
210 **process**

211 The HRAS-P(d)N-S2EBPR treatment line was designed to perform nitrogen and phosphorus
212 removal simultaneously. To overcome the shortage of influent VFA and enable bio-P removal in
213 main-stream treatment line, a side-stream reactor was implemented to facilitate bio-P removal
214 through receiving supplemental carbon from A-stage sludge fermenter (Figure S1). With the
215 increase in the A-stage WAS fermentate supplement flow and RAS slit ratio to the S2EBPR
216 reactor (Figure S2), the nitrite accumulation was observed over a period of approximately 150
217 days (Figure 1). During this period, the effluent nitrite concentration raised to a maximum level

of 6.55 mg-N/L with an average concentration of 2.65 ± 1.89 mg-N/L (Figure 1). The average nitrite accumulating ratio (NAR) was 49%, suggesting that a significant nitrite accumulation occurred. Figure 2A shows the N mass balance in the system and changes of the various N species along the B-stage reactors in series. As ammonia decreased along the B-stage zones, the nitrite began to accumulate from 3.9 g/d to 20.7 g/d (Figure 2A). Previous research indicated that nitrite can inhibit PAO activity by increasing maintenance energy and disturbing the TCA cycle (Zhou et al., 2011). Nevertheless, our results indicated an efficient P removal (77%-92%) could be achieved concurrently with nitrite accumulation (0.2 ± 0.05 - 2.7 ± 1.9 mg-N/L) during this same period (Supplementary text in SI).

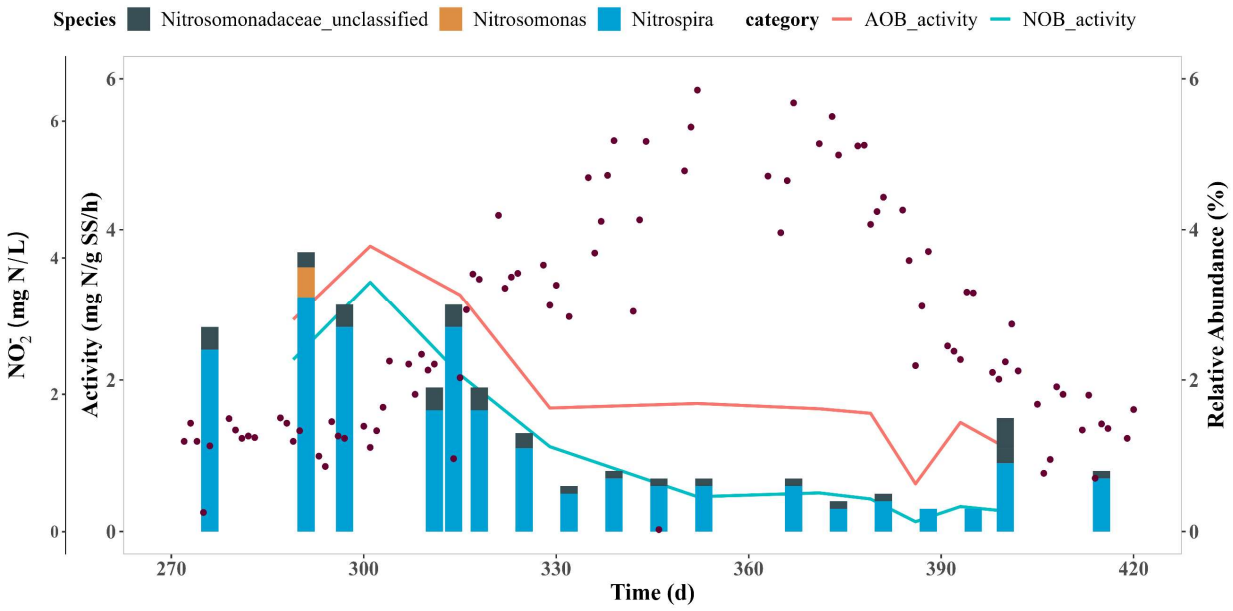


Figure 1 AOB and NOB activities, nitrite accumulation, and the relative abundance of AOB and NOB throughout the operation period for this study.

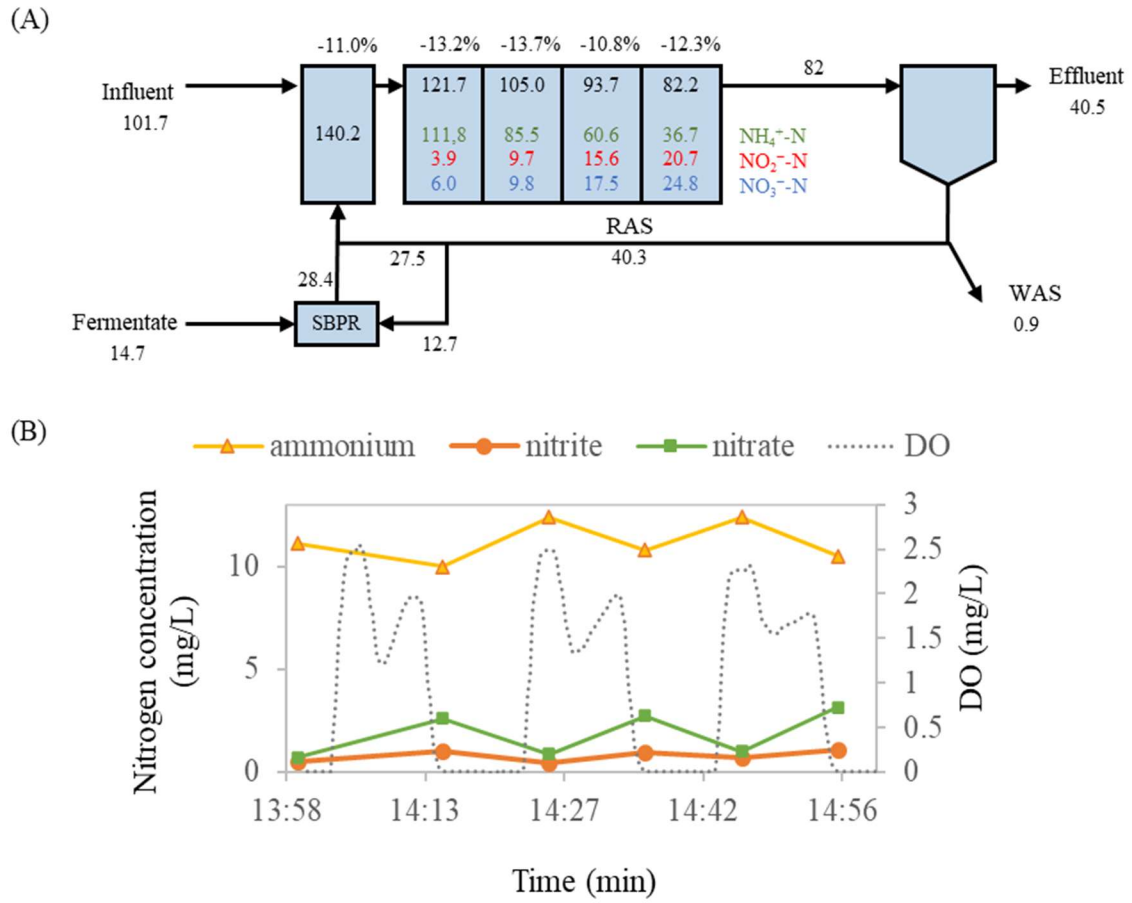


Figure 2 (A) Nitrogen mass balance of the pilot plant in study period (units are in g/d; values with the percentage sigh represent the removal rate, values of TIN, ammonia, nitrite, and nitrate are colored with black, green, red, and blue, respectively The B-stage effluent will go into the following anammox reactor; (B) Intermittent aeration profile taken on Day 522, showing nitrite accumulating during aerobic periods and being denitrified during anoxic periods. Reproduced from McCullough et al., (2020).

3.2 Nitrite accumulation due to partial nitrification

Since nitrite can be accumulated either through partial nitrification or partial denitrification pathways, both AOB/NOB activities were evaluated over long-term on site to identify the dominant mechanisms involved in the nitrite accumulation (Figure 1). Examination of the N

241 species during the aerobic/anoxic cycle showed that nitrite only accumulated in aeration periods
242 while it was consumed after aeration was off (Figure 2B), suggesting that nitrite accumulation
243 was likely due to partial nitrification (PN) rather than partial denitrification. This was further
244 supported by the AOB and NOB activities assessment. During the study period, the average
245 AOB and NOB activities were 1.9 ± 0.9 and 1.1 ± 1.0 mgN/gSS/h, respectively (Figure 1). AOB
246 activities were higher than the NOB activities during the period of study, consistent with the
247 nitrite accumulation via partial nitrification (Figure 1). This evidence led to our conclusion that
248 the higher nitrite accumulation in this study period is likely attributed to, at least mostly, partial
249 nitrification.

250 **3.3 Unknown AOB that contributed to the High AOB activity**

251 3.3.1 Low and negligible known AOB, NOB abundance

252 Temporal Microbial communities via 16S rRNA gene sequencing analysis revealed the
253 microbial community structure dynamics and patterns during the study period (Figure 3). The
254 average relative abundance of known AOB, including *Nitrosomonas* and *Nitrosomonadaceae*
255 were remarkably low at 0.07% and 0.2%, respectively. The only known NOB is *Nitrospira*, has a
256 relative abundance ranging from 1.7-6%. Their abundances were depicted in Figure 1 to show
257 the variation trends. In our system, the relative abundances of both known AOB and NOB are
258 significantly lower than that typically reported in mainstream PN/A systems (0.23-4.4% and 0.2-
259 53%) (summarized in Table S2). Particularly interesting, the relative abundance of *Nitrospira*
260 was much higher than the known AOB, which did not align with the higher ammonia oxidation
261 activity compared to nitrite oxidation activity.

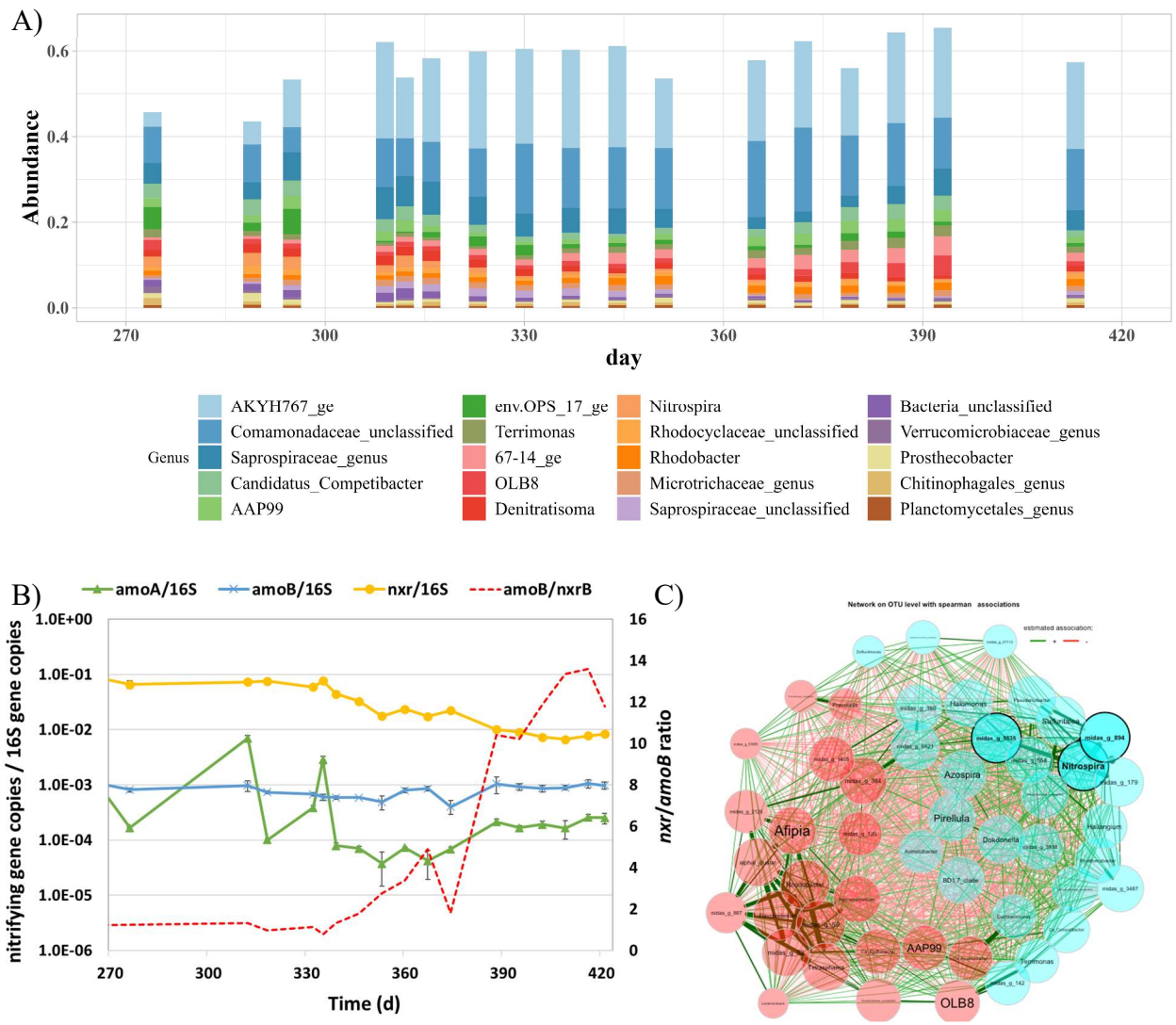


Figure 3 (A) Relative abundance of microbial community during nitrite accumulation period at genus level by 16S rRNA sequencing. (B) Quantitative nitrifying genes, *amoA* indicating ordinary proteobacteria AOB, *amoB* indicating comammox *Nitrospira*, and *nxr* indicating NOB *Nitrospira*, normalized by 16S rRNA genes. (C) Network analysis on OTU level with Spearman associations; The size of the nodes is proportional to its own total degree. The width of the edges is proportional to the correlation between the two nodes to which it corresponds. Positive and negative correlations between taxa (nodes) are indicated by blue and red color of the edges respectively.

271 To further verify the abundances of nitrifiers in the sludge, the *amoA* genes in AOB and the *nxr*
272 genes in NOB were quantified and normalized to 16S rRNA genes (Figure 3B). The average
273 *nxr*/16S ratio was 8.3×10^{-3} in this study period. Generally, *Nitrospira* genome harbor 1-6 copies
274 of *nxr* (Palomo et al., 2018; Pester et al., 2014), while AOB in betaproteobacteria contains 2-3
275 copies of *amoA* (Norton et al., 2002). Considering the copy difference, the abundance of *nxrB*
276 was still one order of magnitude higher than *amoA,* suggesting a higher known *NOB* abundance
277 than known AOB abundance (Figure 3B). Both 16S sequencing and functional gene
278 quantification confirmed that the abundance of known AOB (*Nitrosomonas* and
279 *Nitrosomonadaceae*) was lower than NOB (*Nitrospira*) throughout the treatment periods, yet the
280 system still achieved partial nitrification.

281 Theoretically, the numerical ratio of AOB to NOB in a balanced nitrifying system should be
282 higher than 2:1 (Arciero et al., 1991; Winkler et al., 2012). However, the current investigation
283 shows a disparity between the rates of nitrite and ammonia oxidation, and the abundance of
284 nitrifying biomass. Similar observations have been reported before (Wang et al., 2015; Fitzgerald
285 et al., 2015; Zhang et al., 2018; Gao et al., 2021). Wang et al. (2015) observed NOB:AOB ratios
286 from 9:1 to 5000:1 in five activated sludge reactors through qPCR. Zhang et al. (2018) achieved
287 stable nitrite accumulation with higher abundance of *Nitrospira* comparing to AOB. Several
288 scenarios can potentially explain the higher relative abundance of known NOBs but lower nitrite
289 oxidation activity in our system: 1) there might be a group of *Nitrospira* who can perform not
290 only nitrite oxidation, but also ammonia oxidation, such as complete ammonia oxidizer
291 (comammox); 2) underestimation of AOB in the system due to unknown AOBs; and 3) some
292 substances in wastewater could have more severe inhibitory effects on NOB than AOB.

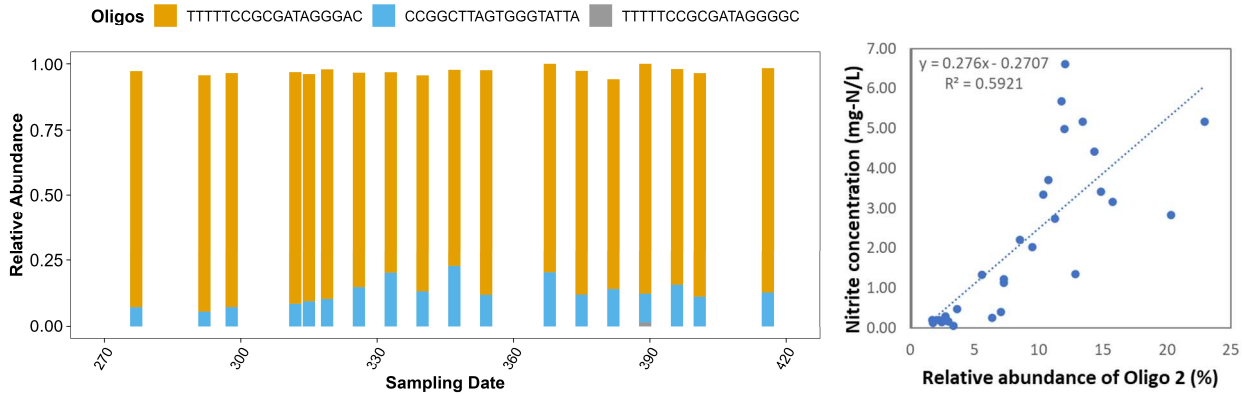
293 3.3.2 Comammox *Nitrospira* may contribute to nitrite accumulation.

294 Roots et al., (2019) reported that the abundance of Comammox *Nitrospira* was much higher than
295 both AOB and ammonia oxidizing archaea (AOA) in their in nitrification reactor (Roots et al.,
296 2019), challenging the commonly held assumption that comammox play a minor role in
297 wastewater treatment bioreactors. Among the versatile *Nitrospira* genus, the comammox
298 *Nitrospira* encode a phylogenetically distinct *amo* gene from the canonical AOB and AOA,
299 allowing them to fully oxidize ammonia to nitrate (Daims et al., 2016; Koch et al., 2015). High
300 presence of comammox was reported in nitrite accumulating condition, indicating a potential
301 correlation between the comammox and nitrite accumulation, given the higher ammonia
302 oxidation rate than the nitrite oxidation rate in comammox *Nitrospira* (Shao and Wu, 2021). A
303 recent isotope labelling test has proven that comammox species (*N. nitrosa* and *N. nitrificans*)
304 provide nitrite for anammox bacteria (*Brocadia*) (van Kessel et al., 2015). The pure cultured *N.*
305 *inopinata* was recorded to accumulate nitrite during complete nitrification (Kits et al., 2017).
306 Given the function versatility of comammox species and the high abundance of *Nitrospira*, it's
307 possible that a portion of the comammox *Nitrospira* in our system functioned as AOB, leading to
308 the accumulation of nitrite.

309 The presence of comammox *Nitrospira* in our system was determined by quantifying the *amoB*
310 gene that was specifically found in comammox (Cotto et al., 2020; Roots et al., 2019) (Figure
311 3B). Results indicated an occurrence of higher abundance of comammox *Nitrospira* and nitrite
312 accumulation. In addition, *amoB* was present and remained much more stable than both *nxrB* and
313 *amoA* (Figure 3B). The average of *amoB* ratio was 7.8×10^{-4} per 16S gene copies. The *amoB*/*nxrB*
314 ratio was deployed to indicate the relative abundance of comammox *Nitrospira* to the total
315 *Nitrospira*. Results clearly indicate that comammox *Nitrospira* abundance increased from 1% to

316 13.6% during nitrite accumulation period. Considering that *Nitrospira* genome contains 1-5
317 times more copies of *nxr* (1-2 copies in most comammox genomes but 4 copies in *N. nitrificans*
318 and 5 copis in *N. mosocoviensis*) than *amo* (single copy) (Palomo et al., 2018; Pester et al.,
319 2014), the relative abundance of comammox *Nitrospira* to the total NOB ranges from 13.6% to
320 68% in our system.

321 The increasing abundance of comammox *Nitrospira* motivated us to employ the oligotyping
322 analysis to further elucidate the micro-diversity of *Nitrospira*. This analysis identified 3
323 dominant oligotypes of *Nitrospira* and revealed their changes in microbial communities over
324 time (Figure 4A).



325

326 Figure 4 A) Oligotypes (3 total) of 16S rRNA gene amplicon sequences classified as genus
327 *Nitrospira* throughout the investigation period; 2) Positive correlation between the relative
328 abundance of Oligotype 2 (CCGGCTTAGTGGGTATTA) and the nitrite concentration.

329 Though the oligotype 1 consistently dominated the *Nitrospira* (> 71%), Oligotype 2 gradually
330 increased in tandem with the increasing nitrite accumulation. The similar increasing trends of
331 *amoB*/*nxrB* and the relative abundance of oligotype II indicates a positive relationship between
332 the comammox and the oligotype II *Nitrospira*. Note that comammox *Nitrospira* do not form

333 a monophyletic group in 16S rRNA gene sequence or *nxrB*-based phylogenetic analyses, but are
334 instead interspersed with strict nitrite-oxidizing *Nitrospira* (Daims et al., 2015; van Kessel et al.,
335 2015). Different comammox *Nitrospira* encode *amo* orthologs that dissimilar to each other, with
336 only ~60% amino acids identity between members of the different clades (Daims et al., 2015),
337 making it difficult to design a uniform comammox qPCR primers (Beach and Noguera, 2019;
338 Cotto et al., 2020). The *amoB* primer set used in this study was specifically optimized for clade
339 A comammox (Cotto et al., 2020). However, it is possible that clade B comammox also played a
340 role in our S2EBPR-PN/A processes, but current primers did not capture the whole comammox
341 community. Nevertheless, oligotyping and qPCR analysis indicated that a group of strict nitrite-
342 oxidizing *Nitrospira* decreased, while another group of *Nitrospira*, which might belong to
343 comammox and can oxidize ammonia increased ($r^2 = 0.60$, Figure 4B), partially contributing to
344 the nitrite accumulation. We also retrieved the representative sequence for the oligotypes
345 TTTTTCCGCGATAGGGAC (1) and CCGGCTTAGTGGGTATTA (2) and aligned them in the
346 nt database. However, the majority of the top hits found in NCBI's nt database are labelled as
347 "uncultured bacteria", indicating that the *Nitrospira* detected in our system is phylogenetic far
348 from the records of known reference sequences in *Nitrospira* genera. The genome information of
349 the *Nitrospira* in S2EBPR system needs further investigation.

350 3.3.3 Atypical ammonia oxidation bacteria

351 Fitzgerald et al. (2015) had proposed that the abundance of ammonia oxidizing organisms may
352 be underestimated, particularly in low DO condition (Fitzgerald et al., 2015). Despite the
353 presence of complete nitrification to nitrate, no known AOB were identified in their low DO
354 nitrification systems. To address this issue, they did pure culture and found 5 isolates that had not
355 previously been recognized as AOB but can oxidize NH_4 under both autotrophic and

356 heterotrophic conditions. They proposed that genus *Pseudomonas* and *Rhodococcus*, and family
357 Xanthomonadaceae and Sphingomonas are involved in heterotrophic nitrification under low-DO
358 conditions.

359 To further explore the potential ammonia oxidizers in our samples, the functional potential of
360 bacterial assemblages was predicted with PICRUSt2 in both enzymes and pathways. The
361 dominated enzyme genes are related to carbohydrate metabolism (e.g., glycolysis/gluconeogesis
362 metabolism), amino acid metabolism, DNA polymerase, and electron transport, which is
363 conceivable that all those functions are vital for microorganisms. Then we specifically aimed at
364 genes that can oxidize ammonia into nitrite. However, only three OTUs have the potential to
365 hold the methane/ammonia monooxygenase subunits (AMO, EC:1.14.18.3 and 1.14.99.39) that
366 oxidize ammonia into hydroxylamine, and they all belong to unclassified genus under order
367 Rhodocyclaceae. Another 11 OTUs belonging to Comamonadaceae_unclassified have the
368 potential to hold hydroxylamine oxidase (HAO, EC: 1.7.2.6) that can further oxidize
369 hydroxylamine to nitrite (Table S3 and Figure S4). Indeed, AMO and HAO were purified from
370 heterotrophic denitrifiers back in 1996 (Moir et al., 1996; Wehrfritz et al., 1996), and
371 heterotrophic nitrification has drawn a lot attention nowadays. 134 isolates were identified at the
372 genus/species level, and members from family Comamonadaceae and order Rhodocyclaceae
373 were reported to be aerobic denitrifiers (Duan et al., 2022).

374 In addition, the co-occurrence microbial patterns during nitrite accumulation periods revealed
375 that genus Nitrospira, midas_g_894, and midas_g_5535 are the main hubs for this specific
376 microbiome structure (Figure 3C). The latter two belong to the family Saprospiraceae and
377 Rhodocyclaceae, respectively, and the species in those families have all been shown to play a
378 role in autotrophic/heterotrophic nitrification (Duan et al., 2022; Mehrani et al., 2022).

379 Though the relative abundance of OTUs that are predicted to hold *hao* only represents a small
380 fraction of the entire Comamonadaceae_unclassified group (Figure S4), the prediction guides us
381 to look into the function played by Comamonadaceae. Though most heterotrophic nitrifiers can
382 oxidize ammonia to N_2O or N_2 by a full nitrification and denitrification pathway, some of them
383 are not capable of aerobic denitrification and ends with nitrite (Duan et al., 2022). A test with
384 enriched Comamonadaceae (~96%) showed ammonium oxidation potential under aerobic
385 conditions, with nitrite as the main product (Bao and Li, 2017), indicating the importance of
386 Comamonadaceae in partial nitrification. Some research also indicates that they contribute to
387 both nitrogen and phosphorus removal and can function as PAO (Ge et al., 2015). Our unique
388 coupling of A-B-stage with S2EBPR created unprecedented selection forces and conditions for
389 both P and N removal relevant organisms in this system, which would conceivably lead to
390 enrichment of organisms with versatile functions different from those in conventional EBPR or
391 PDN systems for main-stream treatment. In the current study, the relative abundance of
392 Comamonadaceae family remained stable at unusually high relative abundance level. (13%) in
393 the study period (Figure 3A) and they might contribute to nitrite accumulation.

394 It's important to note that the predicted functional potential was based on the current database,
395 which only includes the sequences of known AMO. Considering the fact that the *amo* genes in
396 comammox *Nitrospira* are phylogenetically distinct from previously identified AMOs (van
397 Kessel et al., 2015), it is possible that some other groups of bacteria are oxidizing ammonia but
398 cannot be captured by the current database.

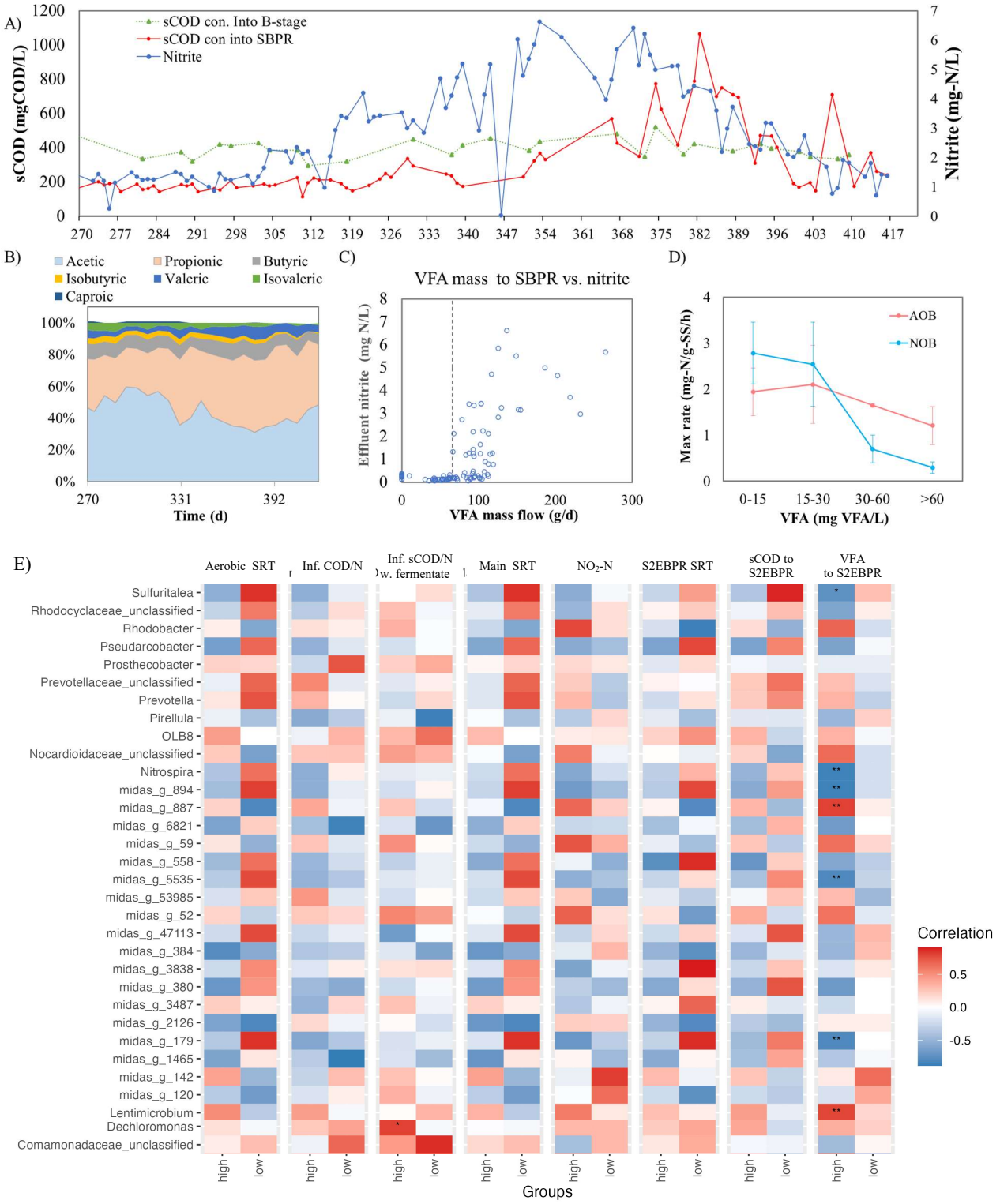
399 **3.4 Possible factors on nitrite accumulation**

400 Another possible scenario to explain nitrite accumulation is that NOB activities were more
401 severely inhibited than that on AOB. Multiple strategies have been postulated to suppress NOB

402 in partial nitrification reactors, including low DO, transient anoxic condition, FNA inhibition, FA
403 inhibition, and VFA composition (Eilersen et al., 1994; Jiang et al., 2019; Park et al., 2010;
404 Wang et al., 2014). The average DO is 1.1 ± 0.9, 1.8 ± 0.7, 1.3 ± 0.5, 0.76 ± 0.51 mg/L in 4
405 CSTRs, respectively, in line with the DO range that is suitable for partial nitrification (Cao et al.,
406 2017; Regmi et al., 2014). However, more and more evidence show that DO regulation is not
407 reliable to suppress NOB alone (Park et al., 2010). Also, the FNA concentrations in both B-stage
408 influent and the mixing zone (B-stage influent + flow from Side-stream reactor) are nearly zero,
409 which did not provide inhibition pressure on NOB.

410 Compared to the conventional B-stage nutrient removal system, the coupling of S2EBPR with
411 PN created unique environmental selection conditions differ from those in conventional EBPR or
412 short-cut N system without EBPR. A split of RAS will flow through S2EBPR, where the unique
413 feature of S2EBPR system exerted different condition for nitrifying populations since they were
414 exposed to extended longer anaerobic time (HRT of anaerobic time is 3.65 hrs.) with lower ORP
415 (-383 ± 17 mv) in comparison to conventional nutrient removal processes where AOB and NOB
416 generally experience shorter anoxic conditions (1-2 hrs) with shallow ORP level (~-150 mv). In
417 addition, high level (126.7 ± 72.1 mg/L as COD) and more complex VFA (Figure 5B and Table
418 S4, containing acetate, propionate, butyric, valeric acids) were added in side-stream reactor.
419 Indeed, the sCOD concentrations into SBRP highly covariates with the nitrite accumulation in B-
420 stage (Figure 5A). The sCOD mass in S2EBPR in this Period is 288 mg/L. When the carbon
421 mass in S2EBRP decreased at the end of the study period, the nitrite accumulation also declined.
422 This trend cannot be observed in B-stage reactor, indicating that side stream EBPR reactor is the
423 place where NOB was inhibited. Spearman correlation analysis indicated that the VFA
424 concentration into S2EBPR abundance is negatively correlated with the abundance of *Nitrospira*

425 and this influence is more profound when the VFA mass flow into S2EBPR is high (> 90 g/d)
426 (Figure 5E). The unique conditions in the S2EBPR, including the low ORP environment and the
427 VFAs concentration and composition, may play roles for the nitrite accumulation and they are
428 further discussed in the following sections.



429

430 Figure 5 (A) Soluble COD concentrations in SBPR, B-stage and nitrite accumulation; (B) VFA

431 speciation in A-stage fermentate; (C) VFA mass flow into SBPR and nitrite accumulation; (D)

VFA concentration in SBPR and AOB/NOB activities; (E) The Spearman correlation between the most abundant 50 taxa and numerical environmental variables. The Spearman correlation test is performed and associated p-values are adjusted for multiple testing.

3.4.1 VFA Inhibit NOB activity and alter NOB metabolic pathway:

Back to 1994, Eilersen et al. has reported that nitrite oxidation is more easily to be inhibited by VFA than ammonia oxidation, especially acetic, propionic, and butyric acids (Table S5). Especially, the butyric acid exerted strong inhibition on NOB activity (IC_{50} = 399 mg/L) comparing to acetic (IC_{50} = 3707 mg/L) and propionic (IC_{50} = 3082 mg/L). Following research confirmed that NOB activity was more susceptible to VFA inhibition comparing to AOB and the inhibition level is proportional to the VFA molecular weight (Delgado et al., 2004; Gomez et al., 2000). All those researchers came to this conclusion since the nitrate production decreased when they added VFAs into NOB consortia. However, a later study showed that VFA (1500 mg/L) addition did not influence the CO_2 fixation that is proportional to nitrite oxidation in NOB consortia (*Nitrospira* spp.), indicating other metabolic pathway was activated by VFAs rather than activity inhibition (Oguz et al., 2006). Though some *Nitrobacter* and *Nitrospira* benefit from the presence of simple organic compounds, e.g., pyruvate, formate, urea (Daims et al., 2016; Palomo et al., 2018), only comammox *Nitrospira* was reported to utilize fatty acids (acetate and propionate) (Xu et al., 2022), and even have potential to synthesize and degrade poly(3-hydroxybutyrate) (Palomo et al., 2018; Yang et al., 2020).

Comparing to the conventional WWTP that rely on mono external carbon source, such as methanol or acetate, our system's A-stage fermentate contained a higher and stable proportion (58.9%) of high molecular size VFAs, including propionic, butyric, iso-butyric and isovaleric acids (Figure 5B). A-stage fermentate addition in S2EBPR may alter comammox *Nitrospira*

towards organic matter utilization pathway and down regulate the nitrite oxidation enzymes. Notably, nitrite accumulation was not observed until the VFA mass flow was increased higher than 66 g/d (Figure 5C). The activity tests also indicated that higher residual VFA concentration (> 30 mg/L) in B-stage resulted lower NOB activity (in terms of nitrate production rates) ($p < 0.01$, Figure 5D), confirming the role of residual VFAs in alternating the NOB activity (Figure 5E). All those results indicated that implementation of S2EBPR with VFAs supplement seem to favor the proliferation of versatile *Nitrospira* over canonical *Nitrospira*. This speculation was supported by quick increase of the relative abundance of comammox *Nitrospira* over the total *Nitrospira* community (indicated as *amoB*/*nxrB*) (Figure 4).

3.4.2 Longer exposure to anaerobic conditions with lower ORP impact NOB:

In our system, S2EBPR induced an extended anaerobic phase (~3.7 hours) where AOB and NOB have to survive/maintain their activities for a longer time than typical duration in conventional WWTPs. Some research reported that the endogenous decay rate of AOB (0.020 d^{-1}) was slower than that of NOB (0.035 d^{-1}) in anoxic condition (Cui et al., 2019), this decay difference was also supported by the FISH staining analysis Morgenroth et al., (2000). However, other results showed identical decay rates of AOB and NOB under both anoxic and anaerobic conditions (Munz et al., 2011; Salem et al., 2006), The differences may have been due to wastewater characteristics, operation parameters, and the composition of the microbial communities and the related survival strategies (Geets et al., 2006; Hao et al., 2010). The ORP in S2EBPR was −383 ± 17 mV, which is significantly below the +100 to +350 mV in normal nitrification tank, and +50 to −50 mV in denitrification tank (Environmental, 2008). Generally, the endogenous decay rates of nitrifiers decreases with the decreasing ORP condition, as $b_{aerobic} >> b_{anoxic} > b_{anaerobic}$ (Geets et al., 2006; Munz et al., 2011; Ruiz-Martínez et al., 2018; Salem et al., 2006). We assume that the

actual decay rates in our system would be even lower than 0.02 d^{-1}. Especially, the abundance of *Nitrospira* was much higher than AOB throughout the experiment periods, indicating that the decay differences between AOB/NOB did not play a significant role in NOB out-selection.

Other than low ORP, NOB is prone to be sensitive to the aerobic/anoxic perturbation. The nitrite oxidation enzyme reactivation time of NOB is longer than AOB when they are exposed to aerobic condition again (Gilbert et al., 2014; Kornaros et al., 2010). This characteristic has been applied as intermittent aeration strategy in partial nitrification tank to suppress the NOB activity (Kornaros et al., 2010). With more RAS split into S2EBPR (Figure S2), more AOB and NOB population would undergo anaerobic and VFA exposure. With less versability, AOB may slow down their metabolic activity while NOB may seek for other alternative pathway to maintain their energy. This explains why they need a longer (enzyme) reactivation time (lag phase) to change back to autotrophic nitrite oxidation in intermittent aeration systems (Gilbert et al., 2014) and thus lead to nitrite accumulation.

4 Conclusions

Nitrite accumulation was observed along with good bio-P removal in a pilot-scale S2EBPR implemented A-B stage process treating municipal wastewater.

(1) Average of 7.62 ± 2.17 mg-N/L nitrite accumulation with nitrite accumulation ratio of 49% was achieved by partial nitrification, which was verified through relative higher ammonia oxidation activity (1.94 ± 0.89 mgN/gSS/h) than nitrite oxidation rate (1.06 ± 0.99 mgN/gSS/h).

(2) The integration of S2EBPR provides PAOs with a suitable environment, such as low ORP and high VFA concentrations, but also inhibits NOB activity and provides additional selective pressure nitrifying community.

(3) *Nitrospira* dominated the autotrophic nitrification community throughout the operation periods with one to two orders of magnitude higher than AOB. The positive correlation between *Nitrospira* oligo type 2 and nitrite accumulation suggested the contribution of comammox *Nitrospira* as AOB.

(4) Phylogenetic investigation suggested that AMO and HAO were embedded in heterotrophic organisms that belong to the family Comamonadaceae and order Rhodocyclaceae. These organisms may function as heterotrophic nitrifiers in response to elevated VFA addition.

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