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Jiyun Li,[†] Kangning Xu,[‡] Tingsheng Liu,[†] Ge Bai,[†] Yanchen Liu,[†] Chengwen Wang,[*,†] Min Zheng[*,ϕ]

[†]School of Environment, Tsinghua University, Beijing 100084, P.R. China

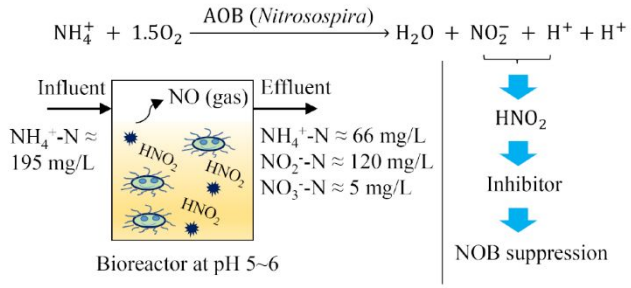
[‡]College of Environmental Science and Engineering, Beijing Forestry University, Beijing 100083, P.R. China

[ϕ]Advanced Water Management Centre, The University of Queensland, St Lucia, QLD 4072, Australia

Corresponding Authors

*E-mail addresses: wangcw@tsinghua.edu.cn (Chengwen Wang); m.zheng@awmc.uq.edu.au (Min Zheng)

ABSTRACT GRAPHIC



ABSTRACT

Partial nitritation providing suitable effluent for subsequent anammox is a critical step in a two-stage autotrophic nitrogen removal system. This study demonstrates an innovative approach for attaining partial nitritation in an acidic bioreactor operating at a slightly low pH (i.e., 5–6). This approach is based on our hypothesis in this study that acid-tolerant ammonia-oxidizing bacteria (AOB) can produce nitrite and protons to self-sustain free nitrous acid (FNA, $NO_2^- + H^+ \leftrightarrow HNO_2$) at a ppm-level, as an inhibitor of nitrite-oxidizing bacteria (NOB). With influent nitrogen of about 200 mg/L and operating conditions of high dissolved oxygen, long sludge retention time and moderate temperature, a lab-scale acidic bioreactor with FNA up to 2 mg HNO_2-N/L successfully established stable nitrite accumulation in the effluent for 200 days with an average ratio ($NO_2^-/(NO_2^- + NO_3^-)$) exceeding 95%. A 16S rRNA amplicon sequencing analysis showed that *Nitrosospira* was the dominant AOB in the biomass of the bioreactor, while *Nitrosomonas* and *Nitrospira*, two typical nitrifying genera in neutral wastewater treatment, both disappeared after the start-up of partial nitritation. Kinetic characterization revealed that *Nitrosospira* had a substrate affinity of 11.4–16.5 mg total ammonia ($NH_4^+ + NH_3$)/L. It also revealed that less than 3.5 mg HNO_2-N/L FNA did not inhibit AOB activity significantly. Acidic operation is economically attractive because it can be achieved via acidophilic ammonia oxidation without adding chemical acid. However, hazardous gas, nitric oxide (NO), should be removed from gas produced by acidic nitrifying bioreactors.

1. INTRODUCTION

Excessive release of nitrogen nutrients from wastewater can lead to water eutrophication.[1-3] Since the discovery of anaerobic ammonium oxidation (anammox) twenty years ago, an innovative autotrophic nitrogen removal process (i.e., partial nitritation/anammox, PN/A) has been increasingly attractive in wastewater treatment. Researchers around the world have widely studied the PN/A process and well-established it in treating concentrated and warm streams.[4] However, the achievement of stable partial nitritation is still a key barrier challenging the process's application in some engineered systems, particularly for the colder and more diluted main-stream process.[5]

Achieving partial nitritation depends on over-selection of nitrite-oxidizing bacteria (NOB) by ammonia-oxidizing bacteria (AOB).[2] To date, researchers have proposed lots of approaches to eliminate NOB. In 1998, Hellinga et al.[6] introduced an approach using combined high temperature of 30−40°C and short sludge retention time (SRT) of 1.5 days for treating anaerobic digester liquor, as called the SHARON process, demonstrating the first successful application of short-cut nitrogen removal process. At present, many other factors have been studied, such as dissolved oxygen (DO),[7-9] aerobic duration control,[10] granular,[11] inorganic carbon,[12] sulfide,[13] weak magnetic field,[14] ultrasonic,[15,16] as well as side-stream sludge treatment[17-19]. Nevertheless, some of them have drawbacks like instability. For example, it has been reported that NOB community *Nitrospira* can shift and adapt to oxygen limitation conditions during long-term reactor operation.[9] Moreover, as demonstrated in the pilot-scale, many approaches

60 are unsuitable for full-scale application.[20-23] Therefore, an alternative approach to
61 achieve stable partial nitritation is required.

62 Free nitrous acid (FNA, i.e., HNO_2) is a weak acid (Equation 1) yet a true inhibitor
63 on NOB.[24-25]

$$NO_2^- + H^+ \leftrightharpoons HNO_2,\ pK_a = 3.25 \quad (1)$$

64 where pK_a is the acid dissociation constant with zero ionic strength at 25°C.

65 FNA inhibitory effect has been considered essential for suppression of NOB activity
66 in partial nitritation bioreactors that fed ammonium-rich wastewaters, including reject
67 water (anaerobic digestion liquor),[26-28] landfill leachate,[29] swine water,[30] black water,[31]
68 and urine wastewater.[32] Attributed to oxidation of high-strength ammonium (Equation
69 2), high-level nitrite produced can ionize relatively high-concentration of HNO_2 (i.e.,
70 ppm level), thereby inhibiting NOB activity. To our knowledge, there has been no
71 attempt to utilize the FNA inhibitory effect in treating diluted wastewaters without
72 plentiful ammonium.

$$NH_4^+ + 1.5O_2 \xrightarrow{AOB} NO_2^- + 2H^+ + H_2O \quad (2)$$

73 Ionization from HNO_2 to nitrite is in a pH-sensitive equilibrium (Equation 3).[24] This
74 indicates that pH decrease can increase HNO_2 concentration greatly. Specifically, 1
75 ppm HNO_2 can be reached with total nitrite nitrogen (TNN = NO_2^-−N + HNO_2−N) of
76 100 mg/L at pH 5.4, whereas the TNN requirement is 1300 mg/L at pH 6.5
77 (Temperature at 22°C). Therefore, we hypothesized that acidic operation of a nitrifying
78 bioreactor (i.e., 5−6) can generate ppm-level FNA, as an inhibitor for the suppression
79 of NOB activity.

$$HNO_2(\text{mg N/L}) = \frac{\text{Total nitrite as N (mg/L)}}{10^{\text{pH}} \times e^{-\frac{2300}{273 + \text{Temp(°C)}}}} \quad (3)$$

Some previous studies have reported that nitrification can work out at low pH,[33-35] even at extreme pH 2.2.[36] The low pH in these nitrifying bioreactors was attained by acidification of AOB-catalyzed reaction[37] on the wastewater where alkalinity is limited relative to total ammonium.[36] Full nitrification ($NH_4^+ \rightarrow NO_3^-$) was reported in these studies, and some acid-tolerant AOB were identified as crucial players under this circumstance. However, to date, an acidic partial nitritation process ($NH_4^+ \rightarrow NO_2^-$) has not been demonstrated yet.

This study aims to assess the feasibility of achieving stable partial nitritation in a lab-scale acidic nitrifying bioreactor. We used source-separated urine as influent because alkalinity is limited relative to total ammonium in this type of wastewater,[32,36] which is a key to drop pH in bioreactor. Other operating conditions of the bioreactor included high DO (> 4 mg/L), long SRT (~150 days) and room-controlled temperature (22-25°C). In general, these parameters favor NOB growth rather than suppression. Within 250-day operation, nitrogen conversions were monitored routinely and microbial communities were analyzed monthly. Once achieving stable partial nitritation, the combined effects of pH, ammonia and nitrite concentrations on AOB activity were explored. Potential emission of nitrogen oxides was also discussed.

2. MATERIALS AND METHODS

2.1 Bioreactor Set-up and Operation

The diagram of a continuous-flow bioreactor is illustrated in Figure 1. The bioreactor

101 has a hollow fiber ultrafiltration membrane (0.02 μm pore size and 0.03 m^2 surface
102 area) module immersed inside. The effluent pump operated in a time-based cycle (8
103 minutes on and 2 minutes off). The programmable controller controlled the inflow
104 according to up and down floating signal response. When water level in the bioreactor
105 was relatively low, the feed pump began to operate and closed at a relatively high water-
106 level. So, an average working volume of the bioreactor was 12 L. Air flushing was
107 created on the membrane surface using a relatively high air flow rate (2 L/min) to
108 mitigate fouling development. Thus, DO concentration was above 4 mg/L.

109 The entire operation of the bioreactor consisted of two phases: initial start-up (0−49
110 days) and maintenance of partial nitritation (50−250 days). In the initial start-up phase,
111 the reactor was operated to achieve a pH drop to a relatively low level (e.g., < 4.5). On
112 day 49, 10 mg/L nitrite nitrogen was spiked in the reactor to form a ppm-level FNA for
113 initial suppression of NOB activity. Afterward, the reactor was operated with nitrite
114 accumulation and stable partial nitritation was maintained in the second phase.

115 Seed sludge was from a nitrification plant (Tsinghua Campus Water Reuse) that
116 treated domestic wastewater on the Tsinghua University campus with stable ammonia
117 removal efficiency over 90%. Feeding urine wastewater containing 100–400 mg/L
118 total nitrogen was collected from a male toilet urinal with tap water rinse. Attributed to
119 dilution, mole ratio of alkalinity (calculated as $CaCO_3$) to total ammonia nitrogen (TAN
120 = NH_4^+−N + NH_3−N) was 1:1.4, which is higher than a theoretical value (1:2) from
121 urea hydrolysis reaction.[38] Average pH was 9.02 ± 0.12, which is consistent with the
122 pH level in literature.[32,36] TNN and nitrate nitrogen concentrations were both at a

123 negligible level (< 3 mg/L).

124 Hydraulic retention time (HRT) of the bioreactor was initially set to 3 days, and
125 shortened to 2 days on day 191 and then to 1.5 days on day 228. Except for sampling,
126 sludge was almost not discharged during the overall operation. So, SRT was calculated
127 as long as about 150 days.

128 Routine operation profiles were monitored by measuring total nitrogen and TAN in
129 influent, and TAN, TNN, and nitrate nitrogen in effluent 3−4 times a week, as well as
130 mixed liquor volatile suspended solids (MLVSS) 1−2 times a month. pH and
131 temperature were recorded daily and microbial community was analyzed monthly.

132

133 **2.2 Batch Activity Assays**

134 Batch assays were conducted in replicate plastic flasks with a working volume of
135 400 mL to assess the effects of pH, ammonia and nitrite concentrations on the ammonia
136 oxidation activity of sludge. All assays were performed in duplicate in volumetric flasks.
137 Sludge samples were taken from the bioreactor under the operating condition of stable
138 partial nitritation. After replacing supernatant of the samples by 1x PBS buffer, small
139 amounts of ammonium (as NH_4Cl) or nitrite (as $NaNO_2$) were added. DO concentration
140 was controlled above 4 mg/L and pH was adjusted using 1 mol/L sodium hydroxide
141 (NaOH) or hydrochloric acid (HCl). Each activity assay lasted for 3−6 hours and liquid
142 samples were taken every 0.5−1.0 hours. TAN, TNN, and nitrate nitrogen
143 concentrations were measured after membrane (0.45 μm) filtration. The AOB activity
144 (mg N/(L·h)) is calculated as the slope of the sum of the TNN and nitrate nitrogen

concentrations versus the time. The activity divided by biomass concentration (g MLVSS/L) represents specific AOB activity (mg N/(g MLVSS·h)).

2.3 Analytical Methods

Chemical analysis and calculations. Total nitrogen, TAN, TNN, nitrate nitrogen, and MLVSS concentrations were measured according to standard methods.[39] DO, pH, and temperature were measured using pH and DO instruments (Lohand Biological DG150; WTW Multi 3420i). Calculation of NH_3 concentration is according to Equation 4,[24] as follows:

$$\mathrm{NH_3(mg\ N/L)} = \frac{\text{Total ammonia as N (mg/L)} \times 10^{\mathrm{pH}}}{10^{\mathrm{pH}} + e^{\frac{6344}{273 + \mathrm{Temp(°C)}}}} \qquad (4)$$

Nitrite accumulation ratio (%) is calculated as the TNN concentration divided by the sum of total TNN and nitrate concentrations in effluent.

DNA extraction, 16S rRNA sequencing, and data analysis. Microbial DNA was extracted using the Fast DNA™ SPIN Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer's protocol. DNA quality was checked by running 3 μL DNA solution on a 1% argose gel. The 16S rRNA gene-targeted amplicons were then analyzed using a high-throughput sequencing method. Primer 515F 5'-barcode-(GTGCCAGCMGCCGCGG)-3' was used to amplify the V4-V5 region of the bacterial 16S ribosomal RNA gene suitable for Illumina MiSeq sequencing. The sequencing data was saved in the NCBI Sequence Read Archive (SRA) database (Accession Number: SUB5916177). The QIIME (version 1.17) was used to demultiplex and filter the alignment sequences. Sequences of 97% similarity of operational units (OTUs) were

166 clustered using UPARSE (version 7.1 http://drive5.com/uparse/) and UCHIME was
167 used to identify and remove chimeras. The silva (SSU115) 16S rRNA database was
168 used to analyze the classification of each 16S rRNA gene sequence using the RDP
169 classifier (http://rdp.cme.msu.edu/) using a 70% confidence threshold.[40]

170 *Apparent substrate affinity estimation.* Monod model (Equation 5) was used to
171 determine apparent affinity constant (K_m) of the ammonia oxidation reaction.

$$V = V_{max}\frac{S}{S + K_m} \quad (5)$$

172 Where S is the TAN or NH_3 concentration, V is the measured specific AOB activity
173 and V_{max} is the maximum activity.

174 The K_m was estimated by using a nonlinear fit by measuring V values at different S
175 concentrations (Origin software 8.0). The inverse of the Fisher information matrix is
176 calculated to obtain a linear approximation of the covariance matrix to calculate 95%
177 confidence intervals.

178

179 **3. RESULTS**

180 **3.1 Bioreactor Performance**

181 Figure 2A shows nitrogen conversion within the overall operation of the nitrifying
182 bioreactor. With the influent TAN increase from 240 to 400 mg/L, the effluent TAN
183 and nitrate both gradually increased in the start-up phase. The bioreactor performed
184 nitrification well (i.e., ammonia converted to nitrate without nitrite accumulation). The
185 TAN removal efficiency was only 60% as alkalinity in the influent (i.e., source-
186 separated urine) was limited relative to the total ammonium.[36] Also attributed to that,

187 the ammonia oxidation process gradually drove a pH decrease down to 4.2 in the
188 nitrifying bioreactor (Figure 2B). On day 49, we spiked 10 mg/L nitrite nitrogen into
189 the mixed liquor of the bioreactor. At the low pH (i.e., 4.2), this small amount of nitrite
190 produced much HNO_2 at 1.33 mg N/L, which inhibited NOB activity straight away. As
191 observed, the nitrite in the effluent increased cumulatively, indicating that AOB indeed
192 worked under the conditions of both low pH and high-level FNA, while suppressing
193 NOB selectively. The TNN concentration in the effluent reached up to 150 mg/L in one
194 week and stabilized at 121 ± 22 mg/L with the average influent TAN nitrogen
195 decreasing to 195 ± 33 mg/L after that. The nitrate nitrogen concentration was less than
196 15 mg/L, showing an average nitrite accumulation ratio exceeding 95% in the effluent
197 (Figure 2C). That is a good indication of attaining stable partial nitritation performance
198 in the acidic nitrifying bioreactor.

199 The pH in the bioreactor stably kept below 6. Thus, during the maintenance of partial
200 nitritation, the nitrite of around 120 mg N/L can ionize HNO_2 at a concentration of up
201 to 2 mg N/L (Figure 2C). HNO_2 at this ppm level is known to have a robust inhibitory
202 effect on NOB activity.[24,25] By contrast, other operating conditions such as high DO,
203 long SRT, and moderate temperature are not expected to affect NOB growth
204 significantly. As NOB had relatively high activity before day 49 even with pH as low
205 as 4.5, the acidic condition itself should not play a critical role in suppressing the NOB
206 activity. Therefore, the NOB suppression likely resulted from *in situ* inhibition by FNA.
207 To further reveal the role of FNA, we adjusted the pH of the nitrifying bioreactor to a
208 neutral level (i.e., 7) by using 1 N NaOH within operating day 74−85 (see arrow in

209 Figure 2B). The operation at neutral pH substantially reduced HNO_2 concentration from
210 a ppm (mg/L) level to ppb (μg/L). High ammonia removal coincided with extra
211 alkalinity supply. However, the effluent nitrite only increased in 5 days and then
212 reduced with considerable nitrate build-up. The results indicated that the acid operation
213 with a ppm-level FNA is of importance for maintaining the stability of the partial
214 nitritation process.

215 The effect of shortening HRT on the partial nitritation performance was also
216 examined. The HRT was reduced from 3 to 2 days on day 191 and then to 1.5 days on
217 day 228. Overall, the nitrogen conversion from ammonia to nitrite was unchanged. With
218 the use of HRT of 1.5 days, the average ammonia removal loading rate reached to 78.4
219 ± 3.6 g N/(m^3·d), which is close to a practically useful rate in wastewater treatment.[41]
220 The pH in the reactor increased after changing the HRT to 2 days or 1.5 days, but
221 decreased again in a few days. This result suggests that acidic process was only
222 temporarily deteriorated after increasing influent ammonia loading rate. Once the AOB
223 received more food, they would grow and further remove the ammonium supplied.
224 Since ammonia oxidation is the driver for dropping pH, it is necessary to explore the
225 AOB dominant community and its activity affected by pH and substrate (NH_4^+ or NH_3)
226 limitations or nitrite/HNO_2 inhibition. These results are presented in the following.
227

228 **3.2 Community Analysis**

229 Nine samples in total were collected from the bioreactor throughout the operation,
230 for each in one month. The microbial communities were analyzed by using 16S rRNA

231 gene-targeted amplicon sequencing. The resulting DNA gene pruning sequence was
232 separated from these samples into 838 OTUs. The Good's Coverage Estimator on the
233 OTUs calculated from each sample showed that the test captured 99% of the sample
234 species. The sequencing results showed that the seed sludge contained two dominant
235 nitrifying genera, *Nitrosomonas* and *Nitrospira*, with relative abundances of 0.89 ± 0.04%
236 and 2.55 ± 0.35%, respectively. Both are the most-representative nitrifying
237 communities in conventional wastewater treatment at neutral pH.[42-44] However, after
238 the start-up of partial nitritation, their abundances substantially decreased down to an
239 undetectable level (Figure 3). The disappearance of *Nitrospira* genus (NOB or
240 Comammox)[45] might be due to FNA suppression, whereas for AOB *Nitrosomonas*
241 likely because the ammonia oxidation rate of this genus very often ceases at a pH
242 slightly below 6.[46] Interestingly, AOB *Nitrosospira* genus became predominant in the
243 nitrifying community, with a read abundance increasing up to 1.87 ± 0.49% on day 114
244 (Figure 3). In addition, no known ammonia-oxidizing archaea (AOA) was detected in
245 this work.

246 The bioreactor operation under the acidic condition shifted the microbial community
247 structure. *Proteobacteria* remained predominated, however during the period of partial
248 nitritation, many phyla such as *Chloroflexi*, *Bacteroidetes*, *Nitrospirae* and
249 *Actinobacteria* disappeared (Figure S1). At a genus level, abundances of
250 *Mizugakiibacter*, *Comamonas* and *Rhodanobacter* were much increased. The reason
251 might be that these bacteria can survive in the acidic environment and resist to the ppm-
252 level HNO_2. As the neutral activated sludge ecosystem contains a core community of

253 abundant organisms,[44] the community shift in the acidic sludge will be of interest in
254 further studies.
255
256 **3.3 Effect of pH, Ammonia and Nitrite Concentrations on AOB Activity**
257 In order to shed light on the ammonia removal capacity in acidic nitrifying bioreactor,
258 batch tests were carried out by using biomass inoculum collected under stable partial
259 nitritation period from day 145 to 182. The combined effects of pH, ammonia and nitrite
260 concentrations on the AOB activity were investigated.
261 Figure 4A depicts the short-term effect of four pH values from 5 to 8 with increment
262 of 1 on the AOB activity. Initially, 10 mg/L TAN was used as a substrate. The specific
263 AOB activity gradually increased with the pH increase, from 0.54 ± 0.08 mg N/(g
264 MLVSS·h) at pH 5 to 1.59 ± 0.08 mg N/(g MLVSS·h) at pH 8. The effects of
265 nitrite/FNA concentrations were examined in six groups of assays with different initial
266 TNN concentrations at pH 5 (TAN concentration was 100 mg/L). As shown in Figure
267 4B, the specific AOB activity was decreased with the nitrite/FNA concentration
268 increase, and substantially reduced to an undetected level when HNO_2 concentration
269 was above 3.5 mg N/L (equal to 150 mg/L TNN at pH 5). The results indicated that the
270 acidic operation partly inhibited AOB activity besides efficient suppression on NOB
271 activity.
272 Substrate (i.e., TAN or NH_3) limitation for AOB was studied in two groups of assays
273 using two pH values. The TAN concentrations at pH 5 and 6 were controlled to be 25,
274 45, 90, 500 mg/L and 2, 5, 15, 40 mg/L, respectively. This set-up had the same range

of 0−2 μM NH_3 in the two assays. The specific AOB activity did increase with the increased TAN or NH_3 concentrations (Figure 4C and D). The data fitting using the Monod model estimated apparent substrate affinity (expressed as K_m value in terms of TAN) as 16.5 ± 1.8 and 11.4 ± 4.8 mg N/L at pH 5 and 6, with R^2 square correlation coefficients were 0.98 and 0.94, in respect. If considering NH_3 as a substrate, the estimated K_m values were 0.07 ± 0.01 μM (pH 5) and 0.36 ± 0.12 μM (pH 6). The results showed that the AOB activities near the apparent K_m value of total ammonia ($NH_3 + NH_4^+$) are likely independent of pH, which is consistent with kinetic estimations on some isolated *Nitrosospira* strains.[47] The TAN concentration in the bioreactor of around 60 mg/L was much higher than the estimated K_m values in terms of TAN, indicating that substrate (i.e., $NH_4^+ + NH_3$) concentration was not limited for the AOB growth in the acidic bioreactor in this work.

4. DISCUSSION

4.1 Acidic Operation as Innovative Approach to Achieve Stable Partial Nitritation

Lots of approaches have been proposed over the last decade to achieve partial nitritation.[4,6-19] This study, for the first time, demonstrates an effective approach using acidic operation to attaining partial nitritation, as indicated by nitrite accumulation ratio exceeding 95% in the effluent over the start-up period (Figure 2). The performance was successfully maintained over 200 days and no NOB community was adapted, indicating the stability of the established process compared with other reported strategies such as low DO.[9] The stable nitrite accumulation was likely obtained through efficient

297 suppression on NOB activity by FNA, according to a well-known inhibitory effect of
298 FNA on NOB.[24,25] Conventionally, the use of FNA inhibitory effect on NOB required
299 high-level nitrite generation from ammonium-rich wastewaters.[26-32] However, the
300 achievement of *in situ* FNA inhibition in this study was due to the acidic operation.
301 With the accumulated nitrite of about 120 mg NO_2^--N/L at pH 5.2, high FNA
302 concentration at a ppm-level (i.e., up to 2 mg HNO_2-N/L) was obtained, even though
303 the diluted urine wastewater was treated. Therefore, it can be concluded that the
304 established approach is suitable and useful in treating more diluted wastewaters, which
305 have been thought to be a critical factor challenging the stability of partial nitritation
306 process in previous studies.[5,23]

307 In this study, FNA was self-sustained in the acidic bioreactor with nitrite and protons
308 production from *in situ* ammonia oxidation reaction. This requires acid-tolerant and
309 FNA-resistant ammonia oxidizers to perform acidophilic ammonia oxidation. 16S
310 rRNA sequencing showed that *Nitrosospira* were the dominant ammonia-oxidizers.
311 The result agrees with the dominant AOB community reported in a full nitrification
312 biofilm reactor operating at pH around 5.[36] The result also indicated that *Nitrosospira*
313 can play a crucial role in maintaining the acidic environment in the partial nitritation
314 bioreactor. Since *Nitrosospira* can work in the acidic soil environment with low
315 ammonia concentration,[48] they may over-select *Nitrosomonas* through substrate
316 competition. Moreover, some recent studies also reported new acid-tolerant AOB genus,
317 such as *Nitrosococcus*-related AOB[36] and *Candidatus Nitrosoglobus terrae*.[49] These
318 microorganisms can grow at the acidic environment (e.g., pH 5) and even survive to

319 extremely low pH 2, suggesting that they may be enriched in future acidic partial
320 nitritation studies and potentially enable to self-sustain FNA in the acidic nitrifying
321 bioreactor.

322 We would further clarify that the acidic operation was attained without adding
323 chemical acid (e.g., HCl). This approach is thus different from many previous studies
324 on the effect of pH on the partial nitritation process. For example, Park et al.[50]
325 recommended that having pH 8 is favorable for nitrite accumulation. However, this pH
326 adjustment requires an enormous amount of chemicals because sewage and activated
327 sludge both have strong buffering capacities. Therefore, the previous approach using
328 chemical-based pH adjustment is not economical in biological wastewater treatment.
329 By contrast, the biological approach proposed in this study, i.e., that is, acidophilic
330 ammonia oxidation for *in situ* protons production, is more economically attractive.

331 Previous studies have reported that stable partial nitritation can be obtained in
332 sequencing batch reactors (SBR) for treating high-strength streams that contain
333 ammonium of about or above 1 g N/L.[26-32] This is achieved by alternating NH_3 and
334 HNO_2 inhibition on NOB activity through dynamic pH variation with AOB-produced
335 protons in each SBR operating cycle. Predictions using a model incorporating the
336 dynamic pH indicated that the SBR partial nitritation requires a minimal ammonium
337 nitrogen concentration of 750 mg/L in influent.[51] In comparison to that, the acid
338 operating approach in this study can significantly reduce the requirement of ammonium
339 in influent (e.g., 200 mg/L, maybe lower with further optimization), thereby increasing
340 the practicality of partial nitritation process.

341 It should also be noted that with the low TAN in the influent, the build-up of acidic
342 operation in our system is due to the relative low alkalinity to total ammonium in the
343 collected urine wastewater. Rare was reported about the acidic operation in other
344 studies because diluted wastewaters may have an adequate ratio (i.e., a mole ratio of
345 $CaCO_3$ alkalinity to total ammonium > 1).[10,11] It may also be related to the
346 denitrification process that re-generates alkalinity following ammonium oxidation in
347 the conventional nitrogen removal processes. For the application of the acidic operation
348 strategy, further studies should concern about the consumption of alkalinity in
349 wastewater treatment systems.
350

351 **4.2 Implementation of Nitrogen Removal from Wastewater via Anammox**

352 The stable partial nitritation in the acidic nitrifying bioreactor can provide suitable
353 effluent for followed anammox in a two-stage autotrophic nitrogen removal system.
354 However, due to low pH (i.e., < 6) in the effluent from the partial nitritation bioreactor,
355 it may be not suitable to use the acidic stream as feeding to anammox directly.[52] Here,
356 this study recommends a by-pass flow for future implementation (detailed in Figure S2).
357 A preliminary test has showed that pH in the combined stream increased to 6.8 if the
358 acidic effluent was mixed with influent urine wastewater at a volumetric ratio of
359 95%/5%. This indicates that pH in the feeding of post-anammox can reach almost a
360 neutral level with optimization of the mixing ratio. Besides, the by-pass flow can adjust
361 NO_2^-/NH_4^+ ratio to a theoretical value (e.g., 1.32) required by anammox bacteria.
362 Therefore, this configuration should be ideal for attaining nitrogen removal via post-

363 anammox.

364 Kinetic characterization revealed that low pH and high-level HNO_2 could both partly
365 restrict AOB activity in the acidic partial nitritation process (Figure 4A and B). The
366 result explains the transient pH increase over 6 after shortening HRT to 1.5 days. As
367 such, more research needs to investigate the effects of pH and HNO_2 on other newly-
368 discovered acid-tolerant AOB (e.g., *Ca. Nitrosoglobus terrae*[49]). It is possible to further
369 increase ammonium removal capacity by introducing these organisms in acidic partial
370 nitritation bioreactor.

371

372 **4.3 Emission of Nitrogen Oxides via Chemical Nitrite Oxidation**

373 Previously reports showed that nitric oxide (NO) can be produced via chemical
374 nitrite oxidation reactions in acid solution, including Equations 6–8, etc. Rates of these
375 reactions are correlated with acidity and increase much at pH less than 4.[53]

$$2HNO_2 \leftrightharpoons NO + NO_2 + H_2O \quad (6)$$

$$NO + 0.5O_2 \leftrightharpoons NO_2 \quad (7)$$

$$2NO_2 + H_2O \leftrightharpoons HNO_2 + NO_3^- + H^+ \quad (8)$$

376 In this work, abiotic production rates of NO and nitrogen dioxide (NO_2) were
377 evaluated by using short term batch test (without biomass). With aeration of the
378 bioreactor effluent with total nitrite nitrogen of 120 mg/L at pH 5.1, NO rather than
379 NO_2 was observed as the main product of chemical nitrite oxidation reactions (Figure
380 S3). The NO chemical production rate, in that case, was estimated to be 3.7 g N/(m^3·d).
381 This result suggests that most of the nitrogen loss (i.e., 1.8–4.1% of influent nitrogen)

might be due to NO emission. The NO emission of the partial nitritation process under acidic operation is substantially higher than that of during normal operation (e.g., 0.005% of influent nitrogen load).[54] Thus, the hazardous gas NO should be removed from gas produced by the acidic nitrifying bioreactors. NO removal is achievable by using a biological filtration method,[55] while needing further tests.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website (PDF).

AUTHOR INFORMATION

Corresponding Authors

*E-mail addresses: wangcw@tsinghua.edu.cn (Chengwen Wang);

m.zheng@awmc.uq.edu.au (Min Zheng)

Notes

The authors declare no competing financial interest.

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577

578 **Table 1**. Average Nitrogen Conversion Efficiencies and Nitrite Accumulation Ratios during Stable Partial Nitritation Phases in the Bioreactor.[a]

Time (days)	Influent TAN measured (mg/L)	Effluent TAN measured (mg/L)	Effluent TNN measured (mg/L)	Effluent Nitrate nitrogen measured (mg/L)	Nitrogen loss calculated (%)[b]	Nitrite accumulation ratio calculated (%)
95−190	205.6 ± 35.1	70.4 ± 20.7	122.2 ± 21.4	4.1 ± 1.5	3.7 ± 16.5	96.7 ± 3.8
191−227	176.1 ± 20.0	58.2 ± 16.5	122.7 ± 25.7	5.8 ± 4.6	2.2 ± 10.8	95.2 ± 4.0
228−250	180.0 ± 6.7	62.4 ± 8.6	110.2 ± 19.0	4.9 ± 8.1	1.6 ± 8.4	95.2 ± 8.7

579 [a]Average value ± standard deviation.

580 [b]Nitrogen loss is calculated by the influent TAN minus the sum of TAN, TNN and nitrate nitrogen in the effluent.

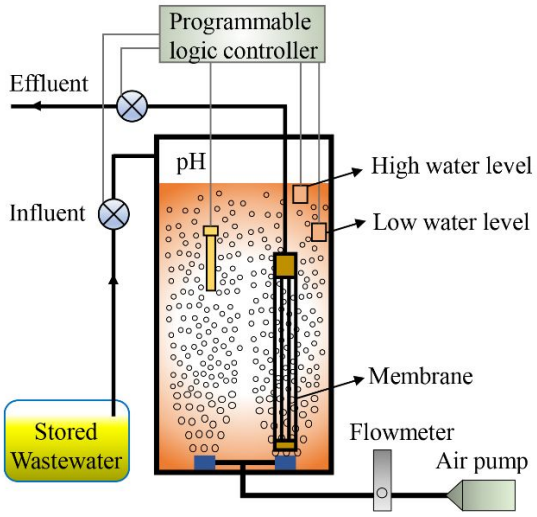
Figure Legends

Figure 1. Diagram of a continuous-flow bioreactor. The effluent is through the ultrafiltration membrane.

Figure 2. (A) Influent TAN (NH_4^+−N + NH_3−N) and effluent TAN, TNN (NO_2^-−N + HNO_2−N), and nitrate nitrogen concentrations; (B) pH values in influent, effluent and reactor as well as HRT operating parameters. Arrows represents a 10-day operating period with pH adjustment to 7 by using 1 mol/L NaOH; (C) Nitrite accumulation ratio in the effluent and HNO_2 concentration in the bioreactor.

Figure 3. Changes in relative abundances of major nitrifying genera, including *Nitrosomonas*, *Nitrosospira*, and *Nitrospira*. Error bars represent standard deviations.

Figure 4. Measured specific AOB activities in different assays. The experimental conditions including: pH increase from 5 to 8 with increments of 1 and initial TAN concentration of 10 mg/L (A); TNN concentrations increase from 20, 35, 50, 100, 150, and 200 mg/L with initial TAN concentration of 100 mg/L at pH 5 (B); TAN concentrations increase from 25, 45, 90 to 500 mg/L at pH 5 (C); and from 2, 5, 15 to 40 mg/L at pH 6 (D). The red lines represent the fit of the results using the Monod model. Error bars represent standard deviations.



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599 **Figure. 1** Diagram of a continuous-flow bioreactor. The effluent is through the

600 ultrafiltration membrane.

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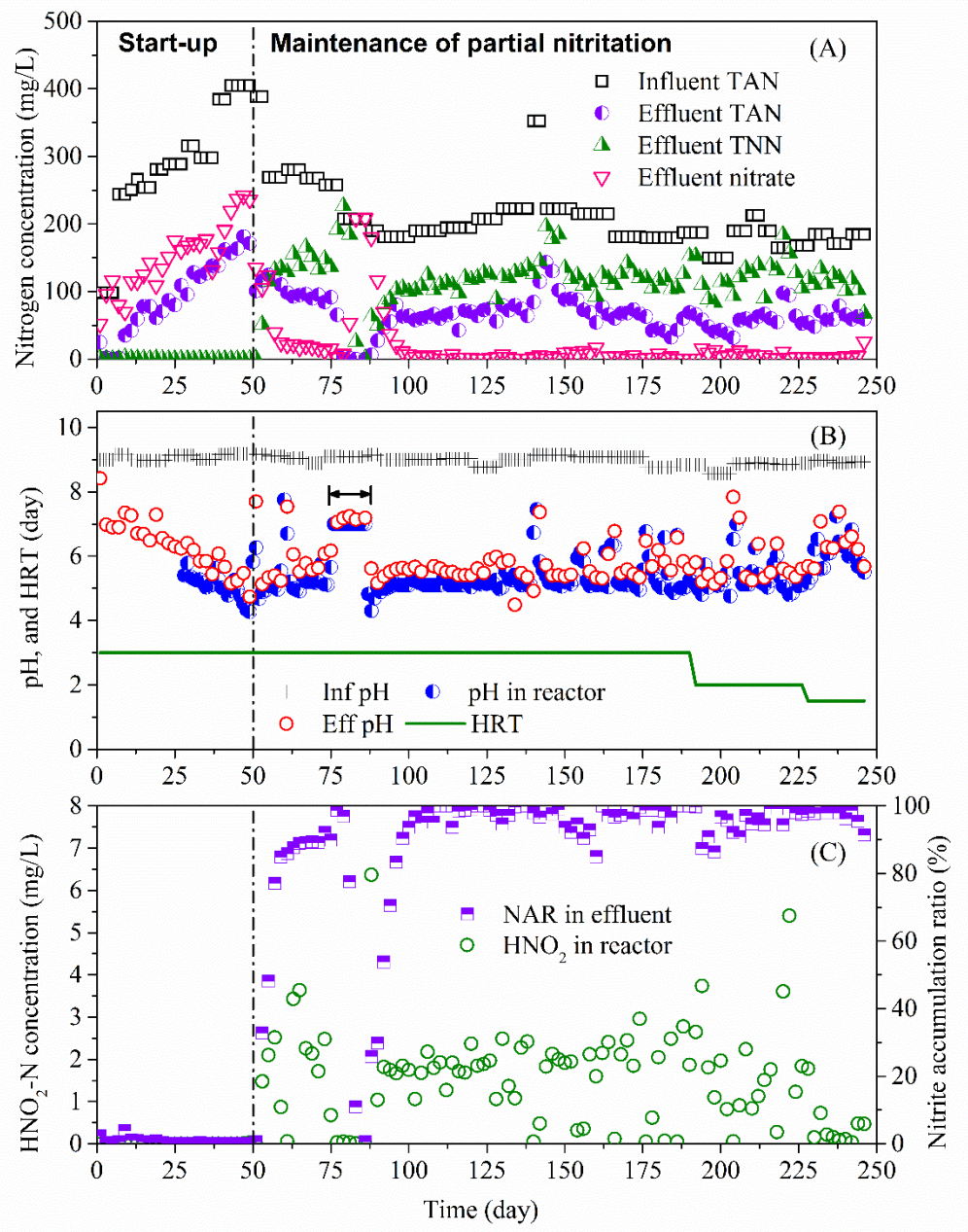

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603 **Figure 2.** (A) Influent TAN (NH_4^+–N + NH_3–N) and effluent TAN, TNN (NO_2^-–N +

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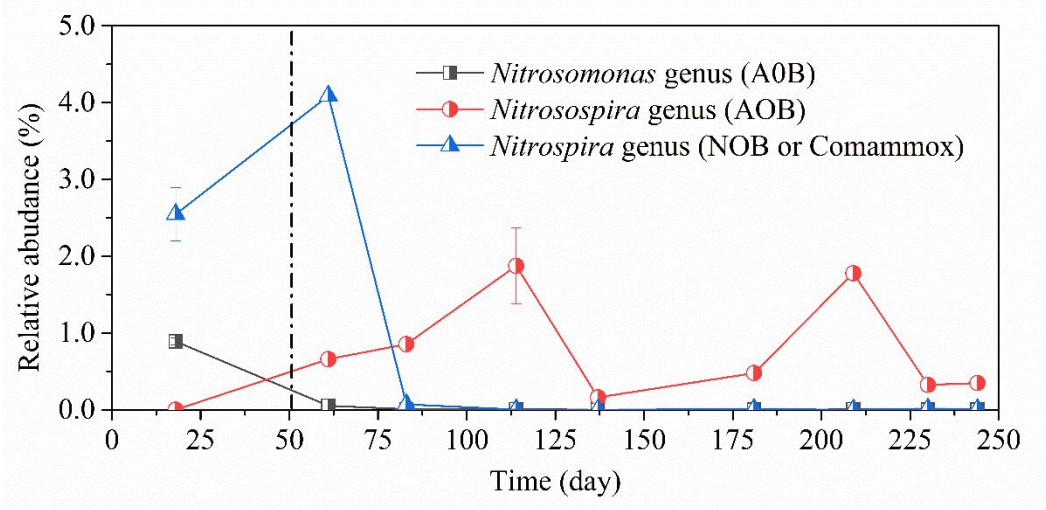

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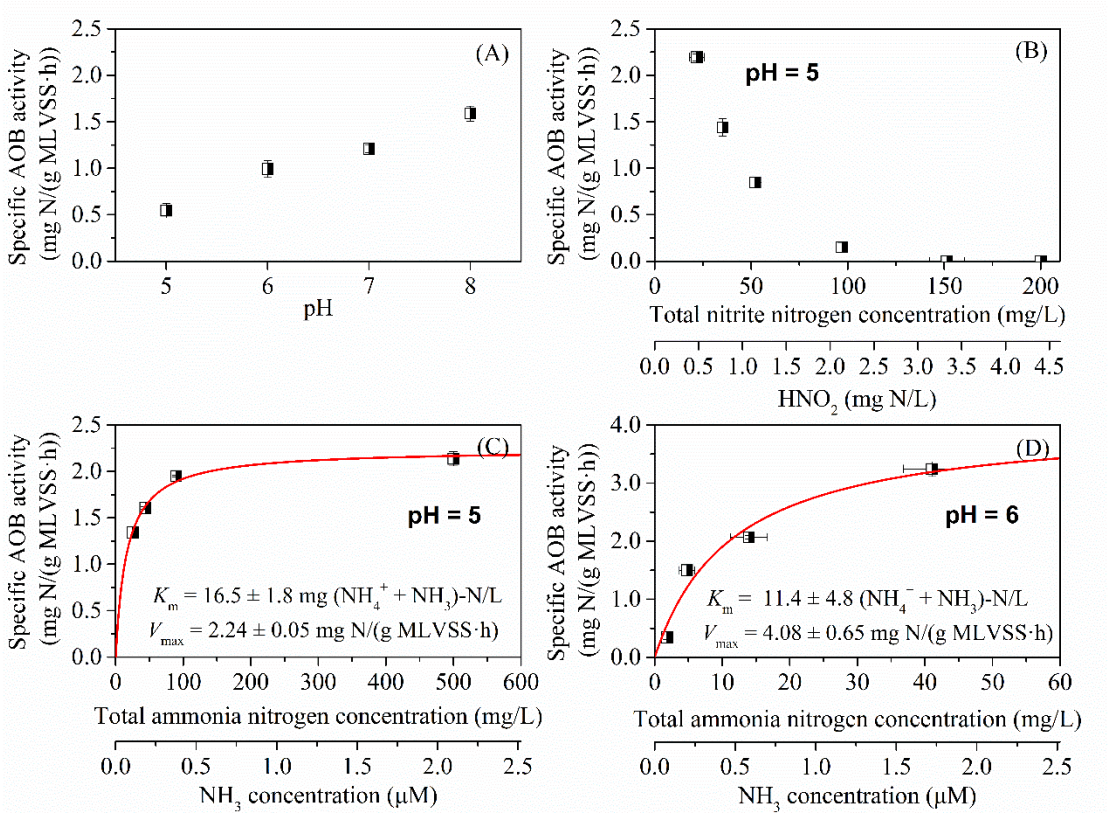

611

612 **Figure 4.** Measured specific AOB activities in different assays. The experimental
613 conditions including: pH increase from 5 to 8 with increments of 1 and initial TAN
614 concentration of 10 mg/L (A); TNN concentrations increase from 20, 35, 50, 100, 150,
615 and 200 mg/L with initial TAN concentration of 100 mg/L at pH 5 (B); TAN
616 concentrations increase from 25, 45, 90 to 500 mg/L at pH 5 (C); and from 2, 5, 15 to
617 40 mg/L at pH 6 (D). The red lines represent the fit of the results using the Monod
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