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Abstract:

In mainstream partial nitritation/anammox (PN/A), suppression of nitrite oxidizing bacteria (NOB) and mitigation of N_2O emissions are two essential operational goals. The N_2O emissions linked to three typical NOB suppression strategies were tested in a covered rotating biological contactor biofilm system at 21°C: (i) low dissolved oxygen (DO) concentrations, and treatments with (ii) free ammonia (FA), and (iii) free nitrous acids (FNA). Low emerged DO levels effectively minimized NOB activity and decreased N_2O emissions, but NOB adaptation appeared after 200 days of operation. Further NOB suppression was successfully achieved by periodic (3 hours per week) treatments with FA (29.3 ± 2.6 mg NH_3-N L^{-1}) or FNA (3.1 ± 0.3 mg HNO_2-N L^{-1}). FA treatment, however, promoted N_2O emissions, while FNA did not affect these. Hence, biofilm PN/A should be operated at relatively low DO levels with periodic FNA treatment to maximize nitrogen removal efficiency while avoiding high greenhouse gas emissions.

Keywords: Deammonification; Nitrous oxide; Carbon footprint; Sewage; Biological

nutrient removal; Nitrification

1. Introduction

Partial nitritation/anammox (PN/A), a cost-effective and energy-efficient nitrogen removal process based on anoxic ammonium oxidation (anammox), has attracted increasing attention (Agrawal et al., 2018). Several successful PN/A applications have been reported in the side stream (sludge line) (Lackner et al., 2014). For mainstream PN/A, i.e. application to the sewage water line, it is more challenging to achieve long-term stability than for the side stream process, which is mainly attributed to the lower temperature in temperate climates (10-15℃ versus >30℃) and lower nitrogen concentrations making nitrite oxidizing bacteria (NOB) suppression difficult (Laureni et al., 2016; Peng et al., 2020; Zhu et al., 2022). The key goal is that the activities of the 'functional' anoxic- ammonium oxidizing bacteria (AnAOB) and aerobic ammonium oxidizing bacteria (AerAOB) can be maintained while unwanted NOB are effectively inhibited. Till now, strategies that relied on the physiological characteristics of these three groups of bacteria have been tested. Specifically for NOB, the observed growth rate (dX_{NOB}/dt) was shown in Eq. 1 (Laureni et al., 2019; Wang et al., 2021), indicating that NOB suppression can be obtained using three main strategies, e.g., reducing bacterial growth rate ($\mu_{NOB,max}$), promoting biomass decay rate (b_{NOB}), and increasing NOB's washout (sludge retention time (SRT) decrease).

$$\frac{\mathrm{dX}_{NOB}}{\mathrm{X}_{NOB} \cdot \mathrm{dt}} = \left(\mu_{NOB,max} - \mathrm{b}_{NOB} - \frac{1}{\mathrm{SRT}}\right) \qquad (\mathrm{Eq.}\,1)$$

To reduce $\mu_{NOB,max}$, low dissolved oxygen (DO) concentrations (Hausherr et al., 2022; Ma et al., 2011) or intermittent aeration (Gu et al., 2022; Peng et al, 2020) are

commonly used in the reaction system because NOB has a higher half-saturation constant of O_2 compared to AerAOB, which means that NOB lose more activity as the DO level decreases (Blackburne et al., 2008). To promote b_{NOB}, free ammonia (FA, the un-ionized form of ammonium) treatment (Wang et al., 2017), free nitrous acid (FNA, the protonated form of nitrite) treatment (Peng et al., 2020), or alternating FA/FNA shocks (Duan et al., 2019) have been successfully applied. The low FA and FNA levels in the mainstream (due to the low nitrogen concentrations and temperature) limit the possibility to directly suppress NOB, but this could be tackled by return-sludge treatment (high FA and FNA conditions could be achieved in the side stream) (Peng et al., 2020). To increase the value of 1/SRT, 'SRT control' was applied to remove NOB (e.g., flocs) while maintaining AnAOB (e.g., biofilm) (Agrawal et al., 2018). An integrated fixed-film activated sludge reactor (IFAS), where AnAOB grow on carriers (long-biofilm) and NOB commonly on flocs (short-floc), or a two-stage PN/A process, where the 'partial nitritation' and 'anammox' processes were separated into two reactors, has been used (Peng et al., 2020; Seuntjens et al., 2018).

The strategies described above are usually accompanied by high ammonium (potentially during FA treatment) and high nitrite concentrations (potentially during FNA treatment), and the strategies can be potentially powerful inducers of nitrous oxide (N_2O) production and emission (Laureni et al, 2016; Peng et al., 2014). That led to variations in N_2O emissions (3.0 – 6.4% of the influent nitrogen loading) from mainstream PN/A systems (Ali et al., 2016; Connan et al., 2018; De Clippeleir et al., 2013). N_2O is a potent greenhouse gas (~265-fold stronger in global warming potential than carbon dioxide (CO_2)) and a strong ozone-depleting substance (Peng et al., 2020; Ravishankara et al., 2009). There are two main routes for AerAOB to produce N_2O, i.e.,

the nitrifier denitrification route (NO_2^- → NO → N_2O) (Kampschreur et al., 2008) and the NH_2OH oxidation route (a side product of the incomplete oxidation process: NH_4^+ → NH_2OH → NO_2^-) (Peng et al., 2014). Nitrite and DO levels are thus critical factors affecting N_2O production. In addition, there is a third N_2O production route when organic carbon is present (Ma et al., 2017), i.e., heterotrophic denitrification (anoxic reduction of NO_2^- or NO_3^- → N_2O).

Only by studying the influences of different NOB suppression strategies on N_2O emissions, the optimal strategy that suppresses NOB activity while limiting N_2O emissions and carbon footprint, can be identified. Although multiple NOB suppression strategies have been employed in various studies, as well as the linked N_2O emissions, there is a lack of research that compares these distinct methods within a single system. This is crucial to ensure a fair comparison, as the results can be influenced by factors such as microbial community composition and types of sludge. Furthermore, there is also a dearth of knowledge regarding the dynamic characteristics of N_2O emissions during the treatment process and between different treatment approaches. Thus, there is a need to further investigate and understand these aspects.

The present research's overall objective was to test the effectiveness of typical NOB suppression strategies and quantify the accompanying N_2O emissions. To achieve that, the N_2O emissions linked to three common NOB strategies, i.e., low DO levels control, periodic FA treatment, and periodic FNA treatment, were characterized.

2. Materials and methods

2.1. Rotating biological contactor (RBC)

An RBC with mature PN/A biofilm was operated at 21 ± 0.6°C for 550 days (Figure 1).

Detailed information on this reactor was presented by Van Tendeloo et al. (2021). The discs' submersion level was fixed at 50%, corresponding to an effective volume of 51 L. With a disc rotation speed of 1.8 rpm, a consecutive exposure of 17 s emerged and submerged condition was achieved. The RBC was covered by an airtight overhead cover, creating a controlled headspace. The O_2 concentration in this headspace (i.e., the emerged DO level) was controlled by the inlet flow rates of N_2 and compressed air to the headspace. Except for days 401 to 450 when the effect of different emerged DO levels was assessed, the conditions were fixed at 0.60 mg O_2 L^{-1} (the values were ranging between 0.19 – 1.84 mg O_2 L^{-1} during days 401 to 450).

The emerged DO levels meant the O_2 concentration in the liquid around the biofilm, which was estimated from the gas phase measurement in the present research (the corresponding O_2 level, which could be dissolved in the liquid film, linked to O_2 in the gas phase). Microorganisms always take up the O_2 from the liquid film. According to previous research, 85–89% of the O_2 input was directly absorbed during the air exposure of the discs (air phase) (Courtens et al., 2014). The off-gas was actively pumped out by a gas analyzer at 330 L h^{-1}, which equalled to the inlet gas flow rate (the total inlet flow rate of N_2 and compressed air).

The synthetic mainstream wastewater was made from tap water supplemented with $(NH_4)_2SO_4$ (47.6 ± 2.3 mg N L^{-1}), $NaHCO_3$ (5 mg HCO_3^- mg^{-1} N), KH_2PO_4 (7.5 mg P L^{-1}), and trace elements solutions A/B (0.01 ml L^{-1}) (de Graaf et al., 1995). The influent flow rate was kept at ~120 L d^{-1} in a continuous mode (Watson-Marlow 323, United Kingdom), resulting in a nitrogen loading rate of 105.2 ± 5.2 mg N L^{-1} d^{-1}.

115 **2.2 Overall Experimental Plan**

116 The experiment was divided into four phases.

117 **2.2.1 Phase – I: Long-term operation at low emerged DO level**

118 In Phase – I (from days 0 to 400), the long-term stability of mainstream PN/A under
119 low emerged DO level was studied. Because of the high initial NOB activity, the
120 emerged DO level was reduced from 0.80 O_2 L^{-1} to 0.60 mg O_2 L^{-1} on day 26 to suppress
121 NOB activity. This value (0.60 mg O_2 L^{-1}) was selected since the reactivation of NOB
122 was observed in the biofilm after the DO increased to values higher than 0.65 mg O_2
123 L^{-1} (Wang et al., 2018). Afterward, a period of up to 374 days was used to verify the
124 stability of NOB inhibition by a low emerged DO level.

125 **2.2.2 Phase – II: Effect of different emerged DO levels on the N_2O emission**

126 In Phase – II (from days 401 to 450), the effect of emerged DO levels on the
127 performance of the PN/A process and N_2O emission was investigated. The DO set
128 point was manipulated within the range of 0.2-1.8 mg O_2 L^{-1}, with increments of 0.4
129 mg O_2 L^{-1} (average experimental value: 0.19, 0.60, 0.91, 1.43, and 1.84 mg O_2 L^{-1}). This
130 adjustment was made by modifying the influent flow rates of N_2 (ranging from 8.8 L h^{-1}
131 to 11.4 L h^{-1}) and compressed air (ranging from 0.2 L h^{-1} to 2.8 L h^{-1}) into the
132 headspace. Each DO level was tested for only 2 days to avoid a strong (irreversible)
133 increase in NOB activity at higher emerged DO conditions. Subsequently, the emerged
134 DO level was returned to ~0.60 mg O_2 L^{-1}. When the NH_4^+ and NO_3^- conversion rates
135 were recovered to the benchmark, the next emerged DO level was tested.

136 **2.2.3 Phase – III: Effect of FA treatment on the N_2O emission**

137 In Phase – III (from days 451 to 497), FA was used as a stressor for NOB suppression.

In total, three FA treatments (3 hours per treatment) were performed with a treatment interval of 7 days (days 453, 460, and 467). The FA treatment condition (29.3 ± 2.6 mg NH_3-N L^{-1} achieved at a pH of ~8.0, temperature of ~21℃, and ammonium concentration of ~735 mg N L^{-1}) was chosen based on the previous research (Van Tendeloo et al., 2021). After the RBC recovered from the FA treatment, the isolated effect of high pH (8.0, 3h) and high ammonium concentration (735 mg N L^{-1}, 3h) shock was tested on days 487 – 490 and days 480 – 484, respectively, to verify the effect of these two factors on the N_2O emissions. The emerged DO level was stable at 0.60 mg O_2 L^{-1} throughout the phase.

2.2.4 Phase – IV: Effect of FNA treatment on the N_2O emission

The NOB activity was positively correlated to the FNA treatment frequency (Duan et al., 2018; Peng et al., 2020). However, the higher treatment frequency and FNA levels would lead to a significant reduction in AerAOB and AnAOB activity. The FA and FNA levels selected in this research were based on the previous research based on realistic values in STP. In Phase – IV (from days 498 to 550), three FNA biofilm treatments were applied. The FNA treatments lasted for 3 hours and were performed with a treatment interval of 7 days (days 498, 505, and 512). The FNA treatment condition (3.1 ± 0.3 mg HNO_2-N L^{-1} achieved at a pH of ~6.0, temperature of ~21℃, and nitrite concentration of ~1205 mg N L^{-1}) was chosen according to Peng et al. (2020). After the RBC recovered from the FNA treatment, the isolated effect of low pH (6.0, 3h) and high nitrite concentration (1205 mg N L^{-1}, 3h) shock was tested on days 531 – 536 and 540 – 547, respectively, to verify the effect of these two factors on the N_2O emissions. During the whole period, the emerged DO level was again stable at 0.60 mg O_2 L^{-1}.

2.3 Online N_2O emission monitoring

The off-gas was dehumidified with a gas cooler (Bühler Technologies, Germany) and analyzed with an online N_2O gas analyzer (Emerson Rosemount CT5800 Quantum Cascade Laser Gas Analyzer, United States, United States) with a range of 0 – 500 ppm (the lowest detection (LOD) was 2 ppm). The data was logged every 30 seconds. Zero and span calibration were accomplished by calibrating the N_2O analyzer measurements against N_2 of instrument gas purity and N_2O reference gas. The sampling flow rate was set at 330 L h^{-1} by a vacuum pump (KNF Laboport, the Netherlands) which equalled the inlet flow rate (N_2 and compressed air) to maintain the gas balance.

The N_2O emission factor (%) was calculated according to the following equations:

$$\mathrm{N_2O\ emission\ factor} = \mathrm{N_2O\ emitted} \div \mathrm{NH_4^+\ converted} \quad (\mathrm{Eq.}\,2)$$

$$\mathrm{N_2O\ emitted} = \sum\left(\mathrm{C}_{N_2O\ gas} \times \mathrm{Q}_{sampling\ pump} \times \Delta\mathrm{t}\right) \quad (\mathrm{Eq.}\,3)$$

$$\mathrm{NH_4^+\ converted} = \mathrm{NH_4^+\ conversion\ rate} \times \mathrm{Q}_{influent} \times \Delta\mathrm{t} \quad (\mathrm{Eq.}\,4)$$

where N_2O emitted represents the released N_2O over a certain amount of time [mg N_2O-N]; NH_4^+ converted represents the converted NH_4^+ over a certain amount of time [mg NH_4^+-N]; C_{N2Ogas} represents the point N_2O concentration in the off-gas [mg N_2O-N L^{-1}]; $Q_{\text{sampling pump}}$ represents the flow rate of the gas sampling pump [330 L h^{-1}]; Δt represents the time interval by which the off-gas N_2O concentration was recorded [h]; NH_4^+ conversion rate is measured by daily sampling [mg N L^{-1} d^{-1}]; $Q_{influent}$ represents the flow rate of the influent (L h^{-1}). The N_2O concentration in the off-gas in ppm was converted to mg N_2O-N L^{-1} based on the volume occupied by 1 mole of an ideal gas at standard temperature and pressure (0°C and 101.3 kPa), which is 22.4 L and corrected for temperature of the gas sample (24°C).

185 The N_2O emission factor is calculated based on the average N_2O emitted within one
186 day (continuous N_2O measurement (per 30 seconds) and daily water samples (for NH_4^+
187 converted measurement)). The data were averaged over the entire period (from the
188 moment the DO value changed to the moment it returned to 0.6 mg O_2 L^{-1}) in the tests
189 at different DO levels. The background values for the indoor N_2O were also measured,
190 which was almost negligible (the average N_2O emission factor was <0.2%).

191 **2.4. Analytical procedures**

192 Liquid samples were taken periodically from the influent and effluent to determine
193 the PN/A performance. After filtering through a 0.2 μm syringe filter (CHROMAFIL Xtra
194 PVDF, Germany) and storing at 4°C, ammonium, nitrite, and nitrate concentrations
195 were measured with a San^{++} Automated Wet Chemistry Analyzer (SKALAR, the
196 Netherlands) and Ion Chromatograph with a Metrosep A Supp 5- 150/4.0 column
197 (Metrohm – Eco IC, Switzerland). The pH and DO values were measured using
198 handheld meters (Hach HQ30d, United States). The microbial community analysis
199 followed the procedure described in Zhu et al. (2022): 16S rRNA gene amplicon
200 sequence variants (ASVs) were determined using the V4 region. The data have been
201 deposited with links to BioProject accession number PRJNA797575 in the NCBI
202 BioProject database.

203 The p-value obtained from the analysis of variance (ANOVA) was compared with a
204 significance level of 0.05 to assess the level of variation. A p-value below 0.05 was
205 considered acceptable evidence of a significant difference. The FA and FNA
206 concentrations were calculated according to Anthonisen et al. (1976).

207 **2.5 Calculations: The relative NO_2^- consumption by AnAOB and NOB**

It was assumed that the decrease in total nitrogen was converted to N_2 by the anammox (the feed did not contain COD in the present study). During the anammox process, the stoichiometric ratio of removed NO_2^-/removed NH_4^+ and produced NO_3^-/removed NH_4^+ are 1.23 and 0.21, respectively (the average of the value reported by Strous et al. (1998) and Lotti et al. (2014)). The percentage of NO_2^- consumed by AnAOB (P(AnAOB)), NOB (P(NOB)), and excess (P(excess)) are shown in Eq. 5 to Eq. 10 (TN presents the total nitrogen).

$$\mathrm{P\ (AnAOB)} = \frac{\mathrm{NO_2^-\ consumed\ by\ AnAOB}}{\mathrm{Influent\ NO_2^- + NO_2^-\ produced\ by\ AerAOB}} \quad (\mathrm{Eq.}\,5)$$

$$\mathrm{P(excess)} = \frac{\mathrm{Remained\ NO_2^-}}{\mathrm{Influent\ NO_2^- + NO_2^-\ produced\ by\ AerAOB}} \quad (\mathrm{Eq.}\,6)$$

$$\mathrm{P(NOB)} = 1 - \mathrm{P(AnAOB)} - \mathrm{P(excess)} \quad (\mathrm{Eq.}\,7)$$

$$\mathrm{NO_2^-\ produced\ by\ AerAOB} = \mathrm{NH_4^+\ removed} - \mathrm{NH_4^+\ consumed\ by\ AnAOB} \quad (\mathrm{Eq.}\,8)$$

$$\mathrm{NO_2^-\ consumed\ by\ AnAOB} = \frac{1.23}{1 + 1.23 - 0.21} \times \mathrm{TN\ removed} \quad (\mathrm{Eq.}\,9)$$

$$\mathrm{NH_4^+\ consumed\ by\ AnAOB} = \frac{1}{1 + 1.23 - 0.21} \times \mathrm{TN\ removed} \quad (\mathrm{Eq.}\,10)$$

3. Results and discussion

3.1 Long-term operation at low emerged DO levels

3.1.1 Decrease the emerged DO level suppressed NOB

Long-term stability is essential for successfully implementing mainstream PN/A at full scale. To assess the potential for this, the performance of the RBC was evaluated for 400 days at a low emerged DO level. Initially, the emerged DO level was set at 0.80 mg O_2 L^{-1}. This, however, boosted NOB activity, resulting in a gradual increase in produced NO_3^- and a NO_2^- consumption by NOB of more than 50% (Figure 2). To suppress NOB,

229 the emerged DO level was decreased to 0.60 ± 0.02 mg O_2 L^{-1} by changing the ratio
230 between the inlet flow rates of N_2 (from 8.8 L h^{-1} to 11.4 L h^{-1}) and compressed air
231 (from 0.2 L h^{-1} to 2.8 L h^{-1}) on day 26. Between days 26 and 200, the NO_3^- production
232 rate (from 33.8 to 5.8 mg N L^{-1} d^{-1}) and the percentage of NO_2^--N consumed by NOB
233 (from about 52% to about 0%) gradually decreased (Figure 2), indicating that NOB
234 activity was effectively reduced by the decrease of emerged DO levels. This is in line
235 with Zhu et al. (2023) and Van Tendeloo et al. (2021) who demonstrated that strict O_2
236 control could achieve complete NOB suppression.
237 The NH_4^+ removal rate remained stable between days 26 and 200 (71.7 ± 5.7 mg
238 NH_4^+-N L^{-1} d^{-1}) (Figure 2A), indicating that decreasing the DO level from 0.8 mg O_2 L^{-1}
239 to 0.60 mg O_2 L^{-1} could effectively suppress NOB activity as well as maintain AerAOB
240 and AnAOB activities. In previous studies, DO levels in a biofilm PN/A system were
241 controlled below 0.20 mg O_2 L^{-1} (to maintain enough AnAOB) to suppress NOB. Still,
242 this strategy lowered AerAOB activity, limiting the overall nitrogen removal rate
243 (Wang et al., 2021). The biofilm in the RBC faced an intermittent aeration mode,
244 switching between anoxic (i.e., submerged DO level of 0 mg O_2 L^{-1}) and low DO (i.e.,
245 emerged DO level of 0.60 mg O_2 L^{-1}) every 17 seconds (i.e., transient anoxia). This
246 might balance the regular supply of O_2 for AerAOB to produce NO_2^-, followed by the
247 removal of the produced NO_2^- during the anoxic period by AnAOB while suppressing
248 NOB activity.
249 After 200 days of operation at an emerged DO level of 0.60 mg O_2 L^{-1}, the NO_2^-
250 consumption by NOB started to increase (e.g., 0% at day 204 versus 30% at day 392)
251 (Figure 2). This might be due to the adaptation of the suppressed NOB to the low DO
252 conditions (i.e., NOB became more efficient in competing with AerAOB for O_2 and with

AnAOB for NO_2^-) (Cao et al., 2018). Microbial community analysis revealed that the enrichment in K-strategists *Nitrospira* (from 0.8% before the DO change on day 26 to 4.7% on day 402), a NOB genus with a higher affinity to O_2 than others (e.g., *Nitrobacter* and *Nitrotoga*) (Wang et al., 2021), in the PN/A biofilm (see supplementary material). The enrichment of *Nitrospira* would eventually result in the failure of the nitrite shunt, which led to the deterioration of the PN/A system (Duan et al., 2019). Cao et al. (2018) also revealed the enrichment of *Nitrospira* during the process of NOB adaptation to low DO level. At the same time, NOB are also likely to maintain structural and genetic integrity by repairing and minimizing damage to the cellular infrastructure at low DO levels (Duan et al., 2019). The adaptation of NOB to low DO levels after the long-term operation (> 200 days) has also been reported by Cao et al. (2018). Even though reducing the emerged DO level from 0.8 mg O_2 L^{-1} to 0.6 mg O_2 L^{-1} could inhibit NOB activity, the adaptation of NOB to the low DO conditions occurred in the long-term run. Thus, to achieve a successful and stable PN/A system, the combined treatment built upon the low DO control to suppress NOB is worthy of further investigation.

3.1.2 Decreasing the emerged DO levels reduced the N_2O emission

To investigate the effect of DO control on N_2O emissions from the RBC, the emerged DO levels varied from 0.19 to 1.84 mg O_2 L^{-1} between days 401 and 450. At an emerged DO level of 0.60 mg O_2 L^{-1}, the average N_2O emission factor was 1.92 ± 0.47% which is within the typical range for a PN/A system (0.1 – 2.4% reviewed by Ali et al. (2016)). As shown in Figure 3, the N_2O emission factor increased with the increase of the emerged DO levels. At a DO of 0.19 mg O_2 L^{-1}, the N_2O emission factor was 1.59 ±

0.05%, while at a DO of 1.84 mg O_2 L^{-1}, it was 2.48 ± 0.20%. One-way ANOVA analysis showed a significant positive correlation (R^2 = 0.9994, $p < 0.05$) between the emerged DO level and N_2O emission factor at the DO level lower than 0.91 mg O_2 L^{-1}. At higher emerged DO levels, the correlation was no longer significant (R^2 = 0.9224, $p > 0.05$).

Since the nitrifier denitrification N_2O production route is linked to the nitrite concentration (Ma et al., 2017), it possibly had only limited contribution to the N_2O emission in this study because of the low nitrite concentration (0.6 ± 0.8 mg N L^{-1}) in the system. Furthermore, heterotrophic denitrification was likely negligible since it could only dominate when heterotrophy was stimulated in the presence of COD (whereas the feed in the present study did not contain COD) (Ma et al., 2017). Therefore, N_2O emission might be mainly attributed to the NH_2OH oxidation pathway. The positive correlation between the DO level and the N_2O emission via the NH_2OH oxidation route was also demonstrated in the nitrifier community of previous research (Domingo-Félez et al., 2014; Wunderlin et al., 2013). Hence, a low DO level is recommended to decrease N_2O emissions from PN/A systems.

The emission of N_2O was dynamic (the average values were used when calculating the N_2O emission factor) and was, amongst others, influenced by changes in operating conditions in the reactor. A shift in emerged DO level caused an immediate change in the emitted N_2O rate. Even when the DO level was not changed, the N_2O concentration in the headspace varied. This might be caused by accumulation of NO_2^-, light, small differences in temperature (± 1℃) and microbial respiration, variable gas pressure, or carbon fixation rate (Lotti et al., 2014). These findings are in line with Qiu et al. (2021), who also found that the observed rate of N_2O production was dynamic even at a constant nitritation activity.

300 **3.2 FA treatment on PN/A biofilm**

301 **3.2.1 NOB suppression was further improved by FA treatment**

302 Long-term operation can trigger adaptation of NOB to low emerged DO conditions
303 (Section 3.1). In Phase – III (from days 451 to 497), FA treatment (29.3 ± 2.6 mg NH_3-
304 N L^{-1}) was therefore implemented in combination with the low emerged DO level
305 (~0.60 mg O_2 L^{-1}) to suppress NOB activity (Figure 4). Before the first FA treatment, an
306 average of 17.5 ± 9.3% of the NO_2^- was consumed by NOB. This value decreased to 0%
307 on day 455 (two days after the first FA treatment), indicating that the activity of NOB
308 was completely suppressed by FA treatment. The following two FA treatments kept
309 the NOB activity low between days 453 and 473 (e.g., 1.9% on day 470). The
310 effectiveness of FA treatment is consistent with Van Tendeloo et al. (2021). After the
311 last FA treatment, the inhibited NOB activity was gradually restored (e.g., 22.7% of the
312 NO_2^- was consumed by the NOB on day 480, 12 days after the last FA treatment),
313 which is also in line with Duan et al. (2019) who reported that stopping FA treatment
314 disrupted the established NO_2^- shunt. In conclusion, FA treatment successfully
315 suppressed low DO-adapted NOB activity, but periodic treatments (3 hours per week)
316 were required to maintain the suppression.

317 There is no unified conclusion regarding the mechanism of FA on NOB suppression.
318 One possibility is that FA directly exhibits inhibitory effects on enzymes involved in
319 nitrite oxidation reactions in NOB (Vadivelu et al., 2006). This hindrance makes
320 electron transfer during the respiration process more challenging for NOB, ultimately
321 reducing their activity. Another possibility suggests that FA may have an inhibitory
322 effect on the synthesis of adenosine triphosphate (ATP) in NOB, which significantly

hampers their anabolism (Yuan, 2007). The precise mechanisms and interactions between FA and NOB are still subjects of ongoing research, and further studies are necessary to comprehensively understand the inhibition mechanisms of FA on various NOB species.

During FA treatment, the NH_4^+ removal rate decreased (68.2 ± 6.1 versus 76.9 ± 4.6 mg N L^{-1} d^{-1}), indicating that AerAOB activity was also influenced by the FA treatment (30 mg NH_3-N L^{-1}). The residual nitrite concentration was increased (9.1 ± 6.7% versus <5% before FA treatment), suggesting that the inhibitory effect of FA treatment on NOB might be stronger than that on AerAOB. A more selective inhibition on NOB guaranteed a stable operation of the PN/A system. Seuntjens et al. (2018) also used a similar FA level (30 mg NH_3-N L^{-1}, 3 hours) to suppress NOB and reported that FA treatment as the sole strategy could not inhibit NOB (increase in relative activity ratio between AerAOB and NOB). This suggests that the combination of FA treatment with a low DO level control is critical to obtain NOB suppression.

3.2.2 FA treatment increased the N_2O emission

N_2O concentrations in the air-phase of the RBC system were measured to assess the effect of FA treatment on the N_2O emissions. Before the FA treatment (benchmark, days 350 to 400), the N_2O emission factor was 1.65 ± 0.10%. It rapidly increased to 2.33 ± 0.36%, 2.40 ± 0.45%, and 2.15 ± 0.30% after each FA treatment (days 453, 460, and 467), respectively (Figure 5), revealing that FA treatment promoted N_2O emission from the RBC system. After the final FA treatment, the N_2O emission factor gradually decreased back to the benchmark within a week. To distinguish the effect of the FA treatment from the influence of the higher ammonium concentration and pH level,

346 separated high pH (~8.0, 3h) and high ammonium concentration (~735 mg N L^{-1}, 3h)
347 shocks were tested on days 488 – 490 and days 480 – 484. Results showed that the
348 high pH level did not affect the N_2O emission factor (1.68 ± 0.08%), whereas the NH_4^+
349 shock promoted the emission of N_2O (2.27 ± 0.13% versus 1.65 ± 0.10% from the
350 benchmark), suggesting that the high ammonium concentration might be the main
351 contributor to the high N_2O emission of FA treatment. Kampschreur et al. (2009) also
352 reported that the N_2O emission increased with increasing ammonium concentration
353 in a full-scale STP.

354 DO and NO_2^- levels are two other essential factors affecting N_2O production by
355 AerAOB (Peng et al., 2014; Wunderlin et al., 2013). The DO levels were always constant
356 during the tests that the residual NO_2^- levels were likely the main influencing factor
357 since it was positively correlated with the N_2O (Peng et al., 2015). After the FA
358 treatment, due to the residual NO_2^- increase (10.7 ± 6.7% versus 4.2 ± 1.7%), the
359 nitrifier denitrification N_2O production pathway might also play a role. The nitrifier
360 denitrification route is nitrite-sensitive because the expression of copper-containing
361 NO_2^- reductase (NirK) is regulated by a nitrite-sensitive transcription repressor protein
362 (Beaumont et al., 2004). The lower residual NO_2^- after the third FA treatment
363 corresponded to the lower N_2O emission factor, which confirmed the role of residual
364 NO_2^-. Due to the operation being kept constant after the FA treatment (e.g., influent
365 ammonium concentrations and the emerged DO levels), the N_2O produced through
366 the NH_2OH oxidation route was likely the same as before. Thus, the N_2O emission
367 increased after the FA treatment might be attributed to the rate increase of nitrifier
368 denitrification route in the present research.

369 The N_2O emission was also dynamic during the FA treatments. At the beginning of

the FA treatment, the N_2O concentration in the air phase rapidly increased (e.g., from 1.03 to 4.23 ppm within 30 minutes during the first FA treatment). Subsequently, the N_2O production gradually decreased (e.g., from 4.23 to 2.31 ppm). A peak was observed after 3 hours of FA treatment, followed by a decrease in N_2O production. Even after 8 hours, the N_2O emission was still higher than the N_2O emission before the FA treatment. The high residual ammonium concentration might have increased the NH_4^+ oxidation rate, which probably yielded more intermediates (e.g., HNO, a byproduct during the oxidation process: $NH_2OH \rightarrow N_2O$) (Law et al., 2012). The NH_4^+ shock caused a rapid increase in N_2O concentration (e.g., from 4.02 to 24.06 ppm in the first shock). Although the value gradually decreased to 16.15 ppm after 3 hours, probably due to the decrease in ammonium concentration, it was still more than 10 times higher than the baseline. At high NH_4^+ levels, not only the NH_2OH oxidation route in AerAOB was promoted, but also N_2O emissions from AnAOB were reported to be enhanced (Jin et al., 2016).

The high pH (8.0) shock had a limited influence on N_2O emission (the drop during the shock can be attributed to the cessation of influent). The effect of pH on N_2O emissions was likely directly related to the influence on the bacterial enzymes and nitrogen compounds rather than corresponding to a shift in bacterial communities (not long enough for a change). A pH range of 6.0 – 8.0 is not expected to affect the ammonia oxidation rate, and was therefore not expected to affect N_2O emissions. It was agreed by Ribera-Guardia and Pijuan (2017) that the N_2O emission was not influenced by a pH decrease (8.0 → 6.5).

3.3 FNA treatment on PN/A biofilm

3.3.1 NOB suppression was further improved by FNA treatment

After stopping the FA treatment, the percentage of NO_2^- consumed by NOB increased to 13.5 ± 5.6 %, showing the recovery of NOB activity. In Phase – IV (from days 498 – 550), FNA treatment (3.1 ± 0.3 mg HNO_2-N L^{-1}) was applied in combination with low emerged DO levels (~0.60 mg O_2 L^{-1}) (Figure 4) to suppress NOB activity. The FNA treatment rapidly inhibited the NOB activity, decreasing the percentage of nitrite consumed by NOB (0%). Wang et al. (2014) also found that FNA treatment (0.24 – 1.35 mg HNO_2^--N L^{-1}) had a significantly higher biocidal effect on NOB than AerAOB. After the last FNA treatment on day 512, the inhibited NOB activity was suppressed for the following 15 days (e.g., only 4.1% of NO_2^- was consumed by the NOB on day 527). That was different from FA treatment in which the activity of NOB was rapidly recovered after the treatment (~6 days versus ~15 days). To conclude, low-DO adapted NOB were successfully suppressed by FNA treatment, but periodic treatment is also required to sustain the suppression. Wang et al. (2016a) also reported that low DO conditions (0.30 − 0.80 mg O_2 L^{-1}) combined with the FNA treatment successfully suppressed NOB in the mainstream PN/A system. This finding is contradicting results of Van Tendeloo et al. (2021) who showed that FNA treatments with similar concentrations failed to suppress NOB activity, which might be attributed to the difference in reactor setup (RBC without overhead cover), operating conditions (higher emerged and submerged DO levels), and the community composition (e.g., dominant NOB genus was *Nitrotoga* versus *Nitrospira* in the present research).

FNA has the ability to enhance the permeability of the cell membrane, facilitating the entry of nitrite into the cells. This change in the intracellular environment can disrupt the functioning of enzymes involved in electron transfer and proton

translocation processes (Philips et al., 2002). Additionally, FNA can inhibit the enzymes responsible for electron transfer and proton translocation in NOB, subsequently impeding ATP synthesis and affecting reactions catalyzed by ATPases. As a result, the activity of NOB is diminished (Frison et al., 2013).

After the FNA treatment, the NH_4^+ removal rate did not differ from the value before treatment (77.9 ± 5.5 versus 76.9 ± 4.6 mg N L^{-1} d^{-1}). In addition, almost no residual NO_2^- (0.8 ± 0.6 mg N L^{-1}) was present in the RBC system. Both results indicated that AnAOB and AerAOB activities were not affected by FNA treatment. In contrast, Peng et al. (2020) reported that FNA treatment exerted an inhibitory impact on both AerAOB and NOB, while the inhibition on NOB was stronger than AerAOB. This difference might be attributed to the higher stability of the biofilm structure in RBC.

3.3.2 FNA treatment did not affect N_2O emission

The N_2O emission factor after FNA treatment (based on the low-DO conditions) was also evaluated. The values were 1.73 ± 0.27%, 1.64 ± 0.24%, and 1.64 ± 0.34% after each FNA treatment, respectively (Figure 6). There was no difference compared to the benchmark (1.72 ± 0.16%) before the first FNA treatment. That differed from the FA treatment, which promoted the N_2O emissions in the RBC system. To distinguish the effect of FNA treatment on the N_2O emission from the effect of a nitrite concentration and pH level, low pH value (~6.0, 3h) and high nitrite concentration (~1205 mg N L^{-1}, 3h) shocks were tested on days 531 – 536 and days 540 – 547. Both the low pH (1.68 ± 0.23%) and high nitrite concentration (1.70 ± 0.16%) did not affect the N_2O emission factor (1.65 ± 0.10% in the benchmark), corresponding to the fact that FNA treatment does not affect N_2O emissions.

Even if the high NO_2^- during the FNA treatment (3h) promoted the N_2O emission, the nitrite concentration was reduced to < 1.0 mg N L^{-1} within two hours. After the FNA treatment, there is no change in the residual NO_2^- (2.6 ± 2.2% versus 4.2 ± 1.7%), suggesting the nitrifier denitrification N_2O production pathway likely still did not play a role, which is again in contrast to the results after the FA treatment (increased residual NO_2^- promoted N_2O emission). In addition, the operation was also constant with that before the treatment, indicating similar N_2O emitted from the PN/A system.

As mentioned above, the N_2O emissions were always dynamic, including during the FNA treatment process. During the FNA treatment, the N_2O production gradually decreased (e.g., from 4.15 to 3.27 ppm within 3 hours in the first FNA treatment). Due to the presence of high NO_2^-, the nitrifier denitrification was likely the primary N_2O production pathway during FNA treatment (Peng et al., 2015). According to previous research, the nitrifier denitrification process is stimulated at moderate NO_2^- range (0 − 50 mg N L^{-1}) (Peng et al., 2015), whereas inhibition appeared at high concentrations (500 − 1000 mg N L^{-1}) (Law et al., 2013). The NO_2^- level used in the present research (1205 mg N L^{-1}) was already beyond the reported suppression range, which might change the detoxification mechanism of NirK (copper-containing nitrite reductase), altering the stimulation threshold of NO_2^- to N_2O emissions (Wang et al., 2016b). That could explain the limited influence of FNA treatment on the N_2O emission. After 3 hours of FNA treatment, the production gradually returned to the benchmark. Due to the sensitivity of the N_2O emission to high nitrite concentration being relatively large (Tallec et al., 2006), the N_2O production rapidly increased at the beginning of the NO_2^- shock (e.g., from 3.91 to 6.33 ppm within 10 minutes in the first NO_2^- shock). Subsequently, the N_2O production gradually increased (e.g., from 6.33 to 10.91 ppm).

That might be explained by the previous findings (Ma et al., 2017) that the nitrite concentration increase could promote the AerAOB denitrification rate (effectively reducing NO_2^- to N_2O). Regarding the low pH (6.0) shock, its impact on N_2O emissions was also limited, similar to the high pH (8.0) shock.

3.4 Implication and outlook

Regarding the suppression of NOB activity in mainstream PN/A systems, scientists have conducted different types of research, but studies of N_2O emissions from this process are still lacking. Strategies to suppress NOB are always accompanied by changes in operating parameters (e.g., pH, ammonium or nitrite concentration, temperature, etc.), leading to dynamic conditions that probably promote N_2O emissions (Kampschreur et al., 2009). Decreasing emerged DO level could suppress NOB activity in the short run while mitigating N_2O emissions. However, NOB adaptation occurred at low DO levels during long-term operation (>200 days). Therefore, in addition to controlling low DO levels, FNA treatment should be performed periodically, since it stabilizes N_2O emissions while suppressing NOB activity. The findings in the present study are a beneficial supplement to the application of PN/A in the mainstream. While some breakthroughs have been made, there are still several issues that still merit further research. First, the correlation between periodical treatment and the levels of FNA is still unclear. Understanding the relationship between the two can provide a basis for selecting the most cost-effective frequency and level of FNA treatment. Second, the stability of the successful strategy proved in the present research, periodical FNA treatment combined with low DO control, should also be tested in the long-term run (> 200 days).

RBC is a type of biofilm-only system, which has higher biomass density and operational stability than the other reactors, such as suspended cell reactors (Soares et al., 2019). Yet, RBC is probably not a preferred system to apply in mainstream anammox. But if use them, several units that are coupled in series or parallel can be applied to treat the large flow wastewater. It would be at least technologically feasible or durable to cover them, mainly to protect the biofilm from weather events and lower the O_2 levels in the headspace (even gas control is economically not feasible). Even though the biomass growth configuration was different from the floccular and granular system (attached growth versus suspended growth), the findings of the present research are generic, and it can be extrapolated to several types of biofilm-based systems (e.g., integrated fixed-film activated sludge (IFAS), moving bed biofilm reactor (MBBR), etc.) (Van Tendeloo et al., 2021). At the level of N_2O emissions, the type of system or the type of growth may not matter too much since it is mainly determined by the nitrite accumulation, O_2 level, and operational parameters.

4. Conclusion

A balance between NOB suppression and N_2O mitigation will be key to achieving a sustainable mainstream PN/A. Decrease emerged DO level suppressed NOB activity while mitigating N_2O emissions. Yet, an adaptation of NOB occurred after 200 days operation. Periodic FA or FNA treatment further suppressed the low DO-adapted NOB. Characterizing the N_2O emissions linked, FNA treatment did not affect (~1.67% versus 1.72% of removed NH_4^+-N) and was, hence, more advantageous than FA treatment (~2.29% versus ~1.65% of removed NH_4^+-N). Thus, low DO conditions with periodic FNA treatment could effectively suppress NOB activity while emitting less N_2O than

510 FA treatment.

511 E-supplementary data for this work can be found in e-version of this paper online.

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Table and Figure Captions

Figure 1

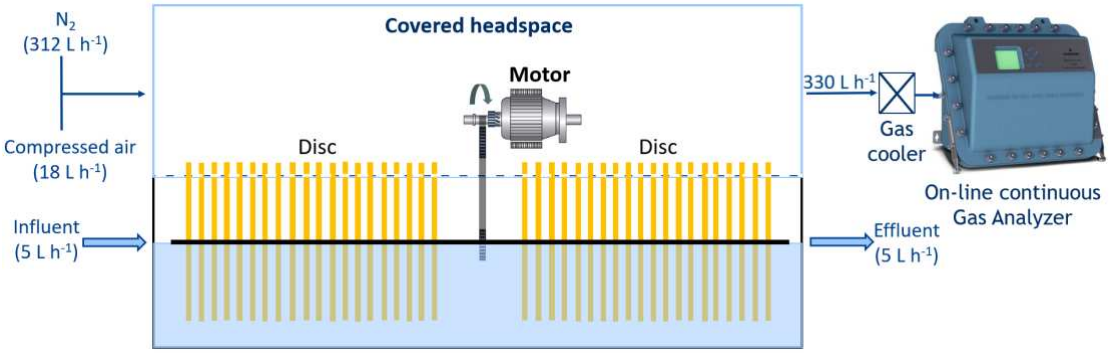


Figure 1. Schematic of the rotating biological contactor (with overhead cover).

Figure 2

Phase	Phase – I	Phase – II
Emerged DO	~ 0.60 mg O_2 L^{-1} (~ 0.8 mg O_2 L^{-1} before days 26)	0.19 – 1.84
Eff. NH_4^+	13.9 ± 3.5 mg N L^{-1}	12.9 ± 5.3

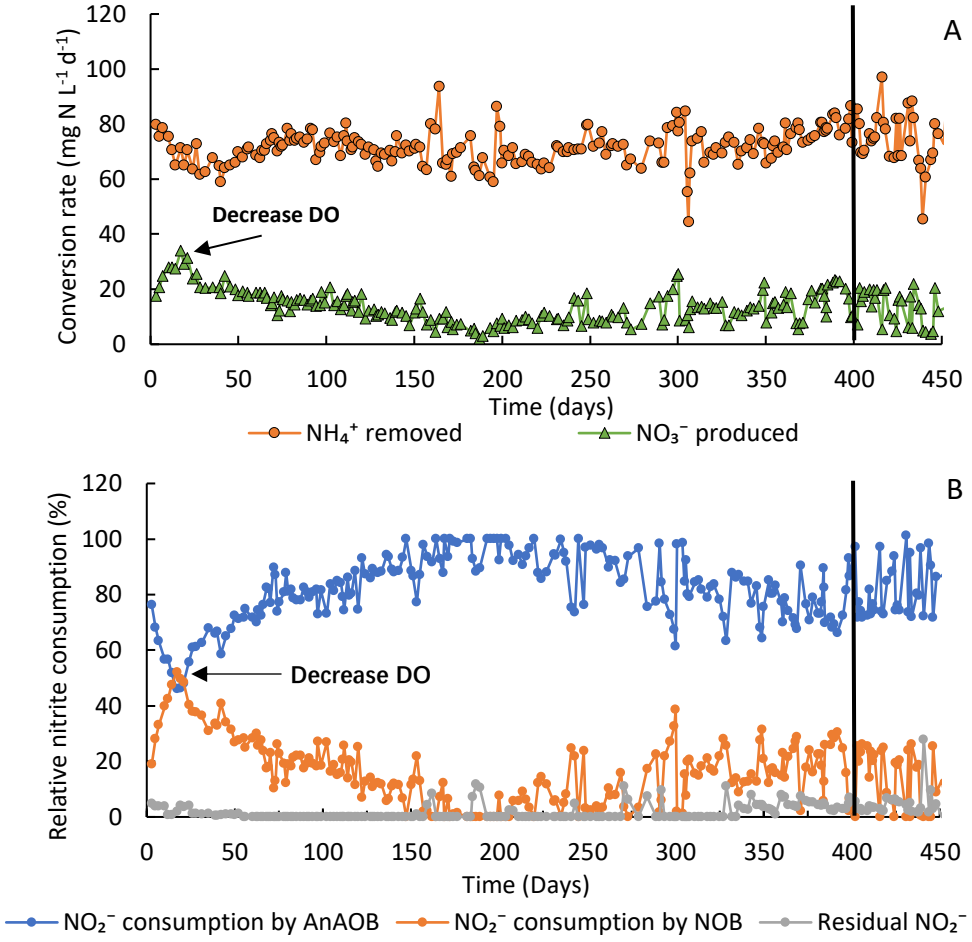
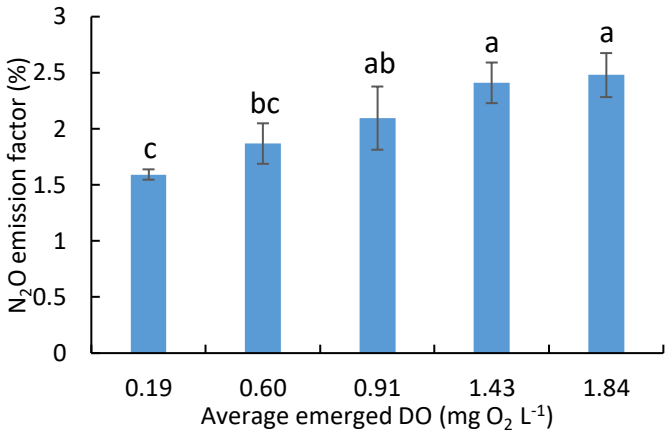


Figure 2. Reactor performance in Phase – I (days 0 to 400) and Phase – II (days 401 to 450). A, volumetric NH_4^+ removal and NO_3^- production rate; B, relative NO_2^- consumption by AnAOB and NOB, and residual NO_2^- (in the effluent).

686 **Figure 3**



687

688 **Figure 3. The average N_2O emission factor under different emerged DO levels.** The

689 error bars depict the standard deviations (n = 3). Significant differences are marked

690 with a letter ($p < 0.05$).

Figure 4

Phase	Phase – III	Phase – IV
DO level	~ 0.60 mg O_2 L^{-1}	
Eff. NH_4^+	13.3 ± 3.8 mg N L^{-1}	
FA	30 mg NH_3-N L^{-1}	/
FNA	/	3.0 mg HNO_2-N L^{-1}

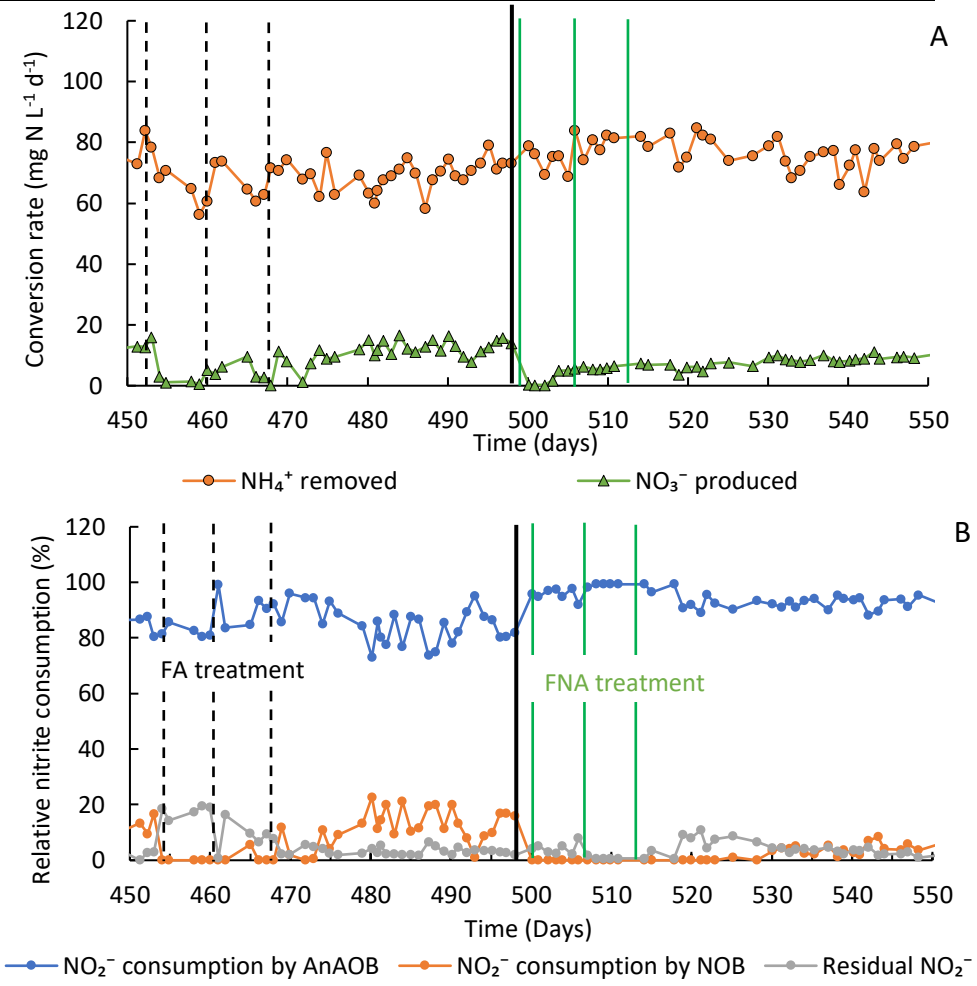


Figure 4. Reactor performance in Phase – III (days 451 to 497) and Phase – IV (days 498 to 551). A, volumetric NH_4^+ removal and NO_3^- production rate; B, relative NO_2^- consumption by AnAOB and NOB, and residual NO_2^- (in the effluent). The black dashed lines indicate FA treatment (30 mg NH_3-N L^{-1}, 3h), whereas FNA treatments (3.0 mg HNO_2-N L^{-1}, 3h) are indicated with a green solid line.

Figure 5

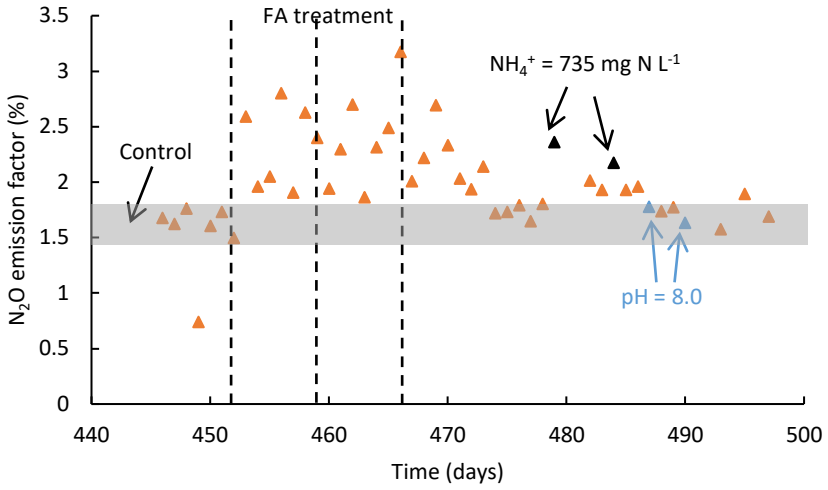


Figure 5. Changes in N_2O emission factors under FA treatment (30 mg NH_3-N L^{-1}, 3h, black dashed lines) and associated NH_4^+ (~735 mg N L^{-1}, 3h, black triangles) or pH shock (~8.0, 3h, blue triangles).

Figure 6

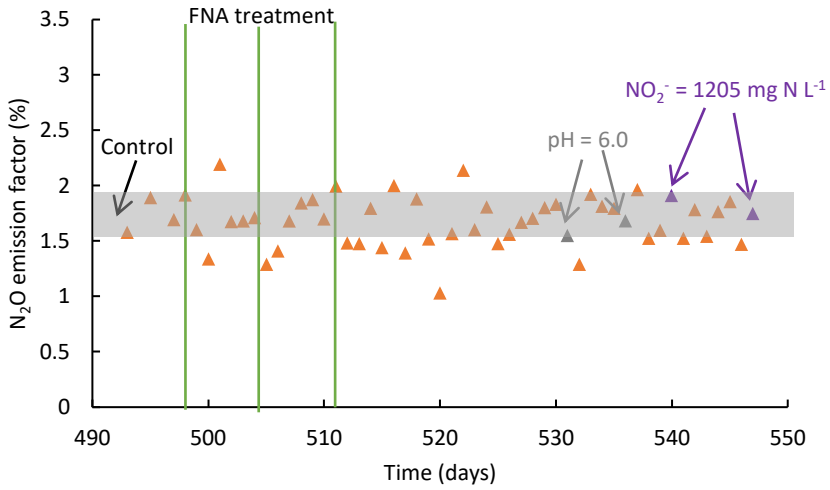


Figure 6. Changes in N_2O emission factors under FNA treatment (3 mg HNO_2-N L^{-1}, 3h, green solid lines) and associated NO_2^- (~1205 mg N L^{-1}, 3h, purple triangles) or pH shock (~6.0, 3h, grey triangles).