Return-sludge treatment with endogenous free nitrous acid limits nitrate production and N_2O emission for mainstream partial nitritation/anammox

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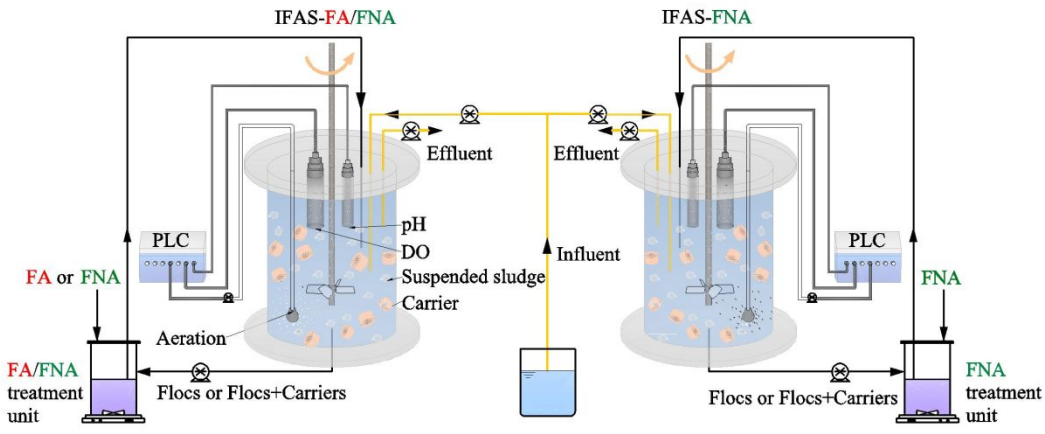

Abstract

Nitrite oxidizing bacteria (NOB) and nitrous oxide (N_2O) hinder the development of mainstream partial nitritation/anammox. To overcome these, endogenous free ammonia (FA) and free nitrous acid (FNA), which can be produced in the side stream, were used for return sludge treatment for two integrated-film activated sludge reactors containing biomass in flocs and on carriers. The repeated exposure of biomass from one reactor to FA shocks had a limited impact on NOB suppression, but inhibited anammox bacteria (AnAOB). In the other reactor, repeated FNA shocks to the separated flocs failed to limit the system's nitrate production since NOB activity was still high on the biofilms attached to the unexposed carriers. In contrast, the repeated FNA treatment of flocs and carriers favoured aerobic ammonium-oxidizing bacteria (AerAOB) over NOB activity with AnAOB negligibly affected. It was further revealed that return sludge treatment with higher FNA levels led to lower N_2O emissions under similar effluent nitrite concentrations. On this basis, weekly 4-hour FNA shocks of 2.0 mg HNO_2-N/L were identified as an optimal and realistic treatment, which not only enabled nitrogen removal efficiencies of ~65% at nitrogen removal rates of ~130 mg N/L/d (20 °C), but also yielded the lowest cost and carbon footprint.

Keywords: mainstream partial nitritation/anammox; endogenous free nitrous acid; free ammonia; integrated-film activated sludge; nitrous oxide

1. Introduction

Partial nitritation/anammox (PN/A) is conceived as a resource- and cost-effective technology for biological nitrogen removal from waters low in biodegradable organics.[1] During this autotrophic nitrogen removal process, part of the ammonium

(NH_4^+) is aerobically oxidized to nitrite (NO_2^-) by aerobic ammonium-oxidizing bacteria (AerAOB), and the residual NH_4^+ is further oxidized with the produced NO_2^- to form dinitrogen gas by anoxic ammonium-oxidizing bacteria (AnAOB). Since PN/A does not require organic carbon, it enables to maximally redirect sewage carbon to sludge in the upstream carbon treatment stage, which can be achieved with high-rate activated sludge technologies. So, instead of the traditional Adsorption-Biooxidation or AB process[2] based on nitrification/denitrification, an update has been proposed, still with high-rate activated sludge in the A-stage, but now followed by PN/A in the B-stage (e.g. Verstraete et al.[3]). For resource efficiency, the generated sludge from the A-stage should be sent to anaerobic sludge digestion, enabling energy recovery as biogas, or even recover other commodities.[4] Therefore, mainstream PN/A is a game changer enabling energy-autarkic water resource recovery facilities (WRRF).

So far, it has been challenging to achieve stable and high nitrogen removal rates and efficiencies for mainstream PN/A, as the microbial competition control is demanding, for instance due to some features inherent to A-stage effluent, such as low nitrogen levels, organics levels necessitating co-occurrence of heterotrophic conversions, low temperatures and varying flow rates.[5] In PN/A process, AerAOB and AnAOB are the desired microbes collaborating to remove nitrogen autotrophically, while NOB are undesired, compete for nitrite with AnAOB, leading to a higher oxygen consumption and lower nitrogen removal efficiency. A multitude of strategies has been proposed to steer the microbial competition for substrates and space in PN/A process. They can be summarized in the conceptual physical/metabolic selection framework of (i) targeting retention of desired/rejection of undesired microorganisms, labeled as IN/OUT

control, while also (ii) focusing on promotion of desired/suppression of undesired activities, labeled as ON/OFF control.[5] Lv et al.[6] utilized a novel composite carrier to enhance the proliferation of AerAOB and AnAOB under mainstream conditions. Differential control of sludge retention times (SRT) has been proposed as successful for IN/OUT control, and can be easily achieved in so-called hybrid reactors, i.e. based on the coexistence of suspended and attached growth.[7-10] In such reactors, the biofilm SRT is very high, which is ideal to retain the slowly growing AnAOB, while the floc SRT is controlled at sufficiently low values just retaining enough AerAOB while washing out NOB. The PN/A process in IFAS reactor configuration has been piloted in the study by Kowalski et al.[11] However, the major barrier hindering the application of mainstream PN/A in hybrid reactors is recurrent nitratation. NOB proliferation can be induced by disturbances in operational conditions, such as temperature, flocculent SRT, etc.[8,12,13] In such cases, IN/OUT strategies should be combined with ON/OFF strategies to ensure their rapid suppression and swift restoration of the treatment efficiency.

Inhibitors have been used as ON/OFF control strategy to favor AerAOB over NOB activity, such as sulfide, perchlorate, formic acid, volatile fatty acid, free ammonia (FA) and free nitrous acid (FNA).[14] Among them, FA and FNA are endogenous inhibitors, as they can be readily available in WRRF with anaerobic digestion, which may economically and environmentally be beneficial.[15] However, under mainstream conditions FA and FNA are low, and have limited inhibitory impacts on AerAOB and NOB. Higher FA and FNA concentrations can be achieved in the side stream, i.e. the sludge line where FA is rich in the digester effluent and FNA can be produced using a nitritation reactor. Sidestream-derived FA and FNA can be applied for NOB

suppression without AnAOB in the return sludge line.[16] Further investigations revealed that mainstream partial nitritation (without anammox) was achieved and maintained under the conditions of repetitive exposure to high FNA (1.83 mg N/L, endogenously feasible) and FA (210 mg N/L, endogenously unfeasible) levels with prolonged contact time of 24 hours (h) and treatment cycle (defined as the time required to expose all of biomass to FNA/FA shock) of 4.5 days (d).[15,17] Wang et al.[18] proposed that a promising technology combining IFAS reactor with FNA treatment can potentially achieve stable operation of mainstream single-stage PN/A. However, relevant research remains scarce and insights are needed on how AerAOB, NOB and AnAOB respond to FA and FNA exposure at endogenously available levels and treatment cycle.

As any biological nitrogen removal process, PN/A process can be a potential source for nitrous oxide (N_2O) emission, which is not only a potent greenhouse gas with approximately 265-fold stronger global warming potential than carbon dioxide, but also a strong ozone depleting substance.[19,20] In PN/A processes, N_2O is produced by AerAOB via two pathways: i) the sequential reductions from NO_2^- to nitric oxide (NO) and to N_2O as the end product, termed as the nitrifier denitrification pathway;[21,22] and (ii) N_2O as a side product during incomplete oxidation of hydroxylamine (NH_2OH) to NO_2^-, known as NH_2OH oxidation pathway.[23,24] N_2O production can be affected by a number of factors, i.e. dissolved oxygen (DO), NO_2^-, NH_4^+, pH, alkalinity, etc.[22,25-27] DO and NO_2^- levels have been recognized as the most important affecting factors which are able to shift N_2O production pathways.[21,28] Given the dynamic DO and NO_2^- levels during PN/A operation, the process can become a strong N_2O emitter under certain conditions. CastroBarros et al.[29] observed that average 2.0% of the total

incoming nitrogen was emitted as N_2O in a full-scale sidestream PN/A process, which can be minimized by adequate aeration. At lab scale, N_2O emissions were 4.6% and 5% of the nitrogen load in mainstream PN/A bioreactors, as reported by Connan et al.[30] and DeClippeleir et al.[31], respectively. Wang et al.[32] revealed FNA-based sludge treatment on lab-scale nitrifying culture under mainstream conditions led to a decreased biomass-specific N_2O production rate. As N_2O emission could be a significant or even major contributor (up to 80%) to the carbon footprint of WRRF,[33] systematic and online investigation is needed to provide in-depth insights into the conditions triggering N_2O emission and possible mitigation strategies.

In this study, realistic FA/FNA doses to return sludge were tested as a way to increase AerAOB/NOB activity ratio in a mainstream one-stage PN/A IFAS system. The immediate inhibitory impact of FA/FNA shocks on the activities of AerAOB, NOB and AnAOB were investigated in batch reactor, while long-term recovery was assessed in two IFAS reactors for a period of 300 d. Shifts in microbial activities and community composition in flocs and carriers were monitored, and the effect of recurrent stressor exposure on the process' N_2O emissions was followed on line. A preliminary assessment was done of the costs and associated carbon footprint of the proposed endogenous stressor concepts. The overall goal was to achieve realistic suppression treatment conditions to maximize N removal, while obtaining low N_2O emissions.

2. Materials and methods

2.1. Inoculation and bio-augmentation

The inoculum for the two IFAS reactors consisted of (i) sidestream PN/A biofilm

142 from an in-house lab-scale rotating biological contactor (RBC), and (ii) mainstream
143 nitrification/denitrification activated sludge flocs from WRRF Antwerpen-Zuid
144 (Antwerpen, Belgium). The PN/A biofilm from the RBC served mainly as source for
145 AnAOB. The RBC (liquid volume of 50 L and biofilm disk area of ~2.8 m^2) was
146 loaded around 4.6 g $N/m^2/d$.[34] WWRF Antwerpen-Zuid treats the sewage of 171000
147 inhabitant equivalents, and screening and sand/grit removals precedes a conventional
148 activated sludge system. Sludge was collected from one of the oxidation ditches
149 performing nitrification and denitrification (loaded 28-42 g $N/m^3/d$).
150
151 To compensate biomass loss and improve flocculation, bio-augmentation was
152 performed on two occasions for each reactor, which were indicated in Figure S1
153 (Supporting Information). The new sludge for bio-augmentation was taken from a
154 pilot-scale reactor, which performed PN/A under mainstream conditions treating the
155 A-stage effluent at WRRF Nieuwveer (Breda, the Netherlands).[35]
156
157 *2.2. IFAS set-up and operation*
158 The PN/A inoculum and K1 carriers were added into two 4.5-L bioreactors operated
159 in a temperature-controlled room at around 20°C. The two reactors were first operated
160 in moving-bed biofilm mode to facilitate biofilm formation on the carriers and
161 enhance AnAOB growth. Then the two bioreactors were changed to the hybrid IFAS
162 mode, through addition of nitrifying sludge flocs. The details of the start-up strategies
163 are described in S1 (Supporting Information). The two IFAS reactors, with co-
164 existing suspended and attached biomass, were operated for over 300 d (schematic
165 diagram of IFAS reactor configuration was shown in Figure S2 of Supporting
166 Information). The pH in the reactors was continuously monitored by pH probe

(Orbisint CPS11D) and controlled in the range of 7.0-7.5 by a programmable logic controller (PLC). DO levels were continuously monitored by a DO sensor (Oxymax COS61) and PLC-controlled in different ranges, as one of the operational strategies for NOB suppression (detailed in Figure 1&2). The targeted nitrogen loading rate and volume exchange ratio were 200 mg N/L/d and 33%, respectively, resulting in a cycle time of 2 h and hydraulic retention time of 6 h. The two reactors were operated in sequencing batch mode, which was achieved by timers controlling overhead stirrers (ES Overhead Stirrer, Velp Scientifica), air pumps (Tetra TEC APS 150), and influent and effluent pumps (DriveSure panel mount pumps, PMD24C, Watson-Marlow). Each 2-h cycle consisted of 40-minute (min) continuous feeding (aerobic), 40-min aerating, 30-min settling, 5-min decanting and 5-min resting. The SRT was not controlled, but monitored by measuring volatile suspended solid (VSS) concentration in the effluent. The IFAS reactors were started up in anaerobic phase for enrichment of AnAOB (50 mg N/L NH_4^+ and 50 mg N/L NO_2^-) and subsequent aerobic phase (50 mg N/L NH_4^+ and DO below 1.0 mg O_2/L) for co-culture of AerAOB and AnAOB. The details were presented in S1 of Supporting Information.

2.3. FA and FNA inhibition

After start-up, FA and FNA were used as stressors for the return-sludge treatment, schematic diagram of which was presented in Figure S2 of Supporting Information. One of the two IFAS reactors was exposed to FA shock initially and FNA shock subsequently, which was named as IFAS-FA/FNA. The other implementing FNA-based return-sludge treatment was termed as IFAS-FNA. Before applying stressors, a desktop calculation was performed to determine realistic FA and FNA ranges based on endogenous nitrogen from the sludge reject water (details in S2 and Figure S3 in

192 supporting information). Three sets of batch experiments were executed to evaluate
193 the impact of varying FA/FNA shock conditions, such as levels, contact time, pH etc.
194 on AerAOB, NOB and AnAOB in inoculum (Table S1 and Figure S4). Based on the
195 results (detailed description in S2 of Supporting Information), initial treatment
196 conditions of FA 30 mg NH_3-N /L (pH 8.0) + 1 h for IFAS-FA/FNA and FNA 0.5 mg
197 HNO_2-N /L (pH 6.0) + 4 h for IFAS-FNA were selected.
198
199 For each FA shock, certain fraction of biomass was withdrawn from IFAS and
200 exposed to FA inhibitor at a level of 30 mg NH_3-N/L, which was achieved by dosing
201 780 mg NH_4^+-N/L and raising pH to 8.0 at temperature of 20 °C. The 1-h FA
202 treatment was terminated by washing the treated biomass with ammonium-free
203 synthetic feed and transferring them back to IFAS reactor. Similar treatment method
204 was applied in FNA-based return-sludge treatment. Different combinations of FNA
205 levels (0.5, 2.0 and 3.0 mg HNO_2-N/L) and contact times (4, 16 and 24 h) were
206 applied to inhibit IFAS biomass. FNA levels of 0.5, 2.0 and 3.0 mg HNO_2-N/L were
207 achieved by dosing 195, 780 and 1170 mg NO_2^--N/L, respectively and dropping pH to
208 6.0 with 1 M HCl, at 20 °C. Preliminary tests were performed to monitor the
209 dynamics of nitrite and ammonium under treatment conditions during a period of 24
210 h. The results showed that their concentrations remained unchanged. Hence, during
211 the subsequent FA/FNA shocks, only pH was measured and controlled constantly to
212 ensure an accurate FA/FNA concentration.
213
214 *2.4. Batch activity rate determination*
215 The maximum potential activities of AerAOB, NOB and AnAOB in flocs and carriers
216 were determined in batch activity tests in a temperature-controlled room at 20 °C. For

each test, 100 mL flocs and 15 random carriers (~1.2-1.3% of the total carriers) were taken from each reactor and brought separately in a nitrogen-free medium. Aerobes and anaerobes were characterized separately in aerobic test and anoxic tests. For aerobic tests, the biomass was added into open glass flasks and constantly mixed by a magnetic stirrer. For the anoxic test, the sludge was flushed with N_2 gas in the penicillin bottles, then sealed completely and constantly mixed by a magnetic stirrer. 50 mg NH_4Cl-N/L and 50 mg $NaNO_2$-N /L were spiked at the beginning of all the batch tests. Concentration changes of NO_2^-, NO_3^- and NH_4^+ were monitored by periodical sampling and measurement (details below). At the end of the tests with flocs, the biomass concentration was determined. Aside from N-measurements, DO and pH were also measured. During the whole period, DO concentrations for aerobic tests are all above 7.0 mg O_2/L and pH was close to 7.5. Each test was performed in duplicate. The maximum potential activities of AnAOB, AerAOB and NOB in flocs or biofilm on the carriers in the IFAS reactor were estimated by extrapolating the maximum microbial activities measured in the batch tests by means of the flocculent biomass concentration and the number of carriers in the reactor (IFAS-FA/FNA: 1254; IFAS-FNA: 1119).

2.5. Physicochemical water and biomass analyses

To monitor the system performance, samples were taken periodically from influent, reactor and effluent for analysis of NH_4^+, NO_2^-, NO_3^-, COD, total suspended solids (TSS) and VSS concentrations. NH_4^+-N, NO_2^--N and NO_3^--N were measured with San^{++} Automated Wet Chemistry Analyzer. VSS and TSS were measured using standard methods.[36] COD was photometrically determined using Nanocolor test tubes (Macherey-Nagel, Germany).

242

243 *2.6. Online N_2O emission monitoring*

244 The off-gas flows from the two IFAS reactors were analyzed on line for N_2O
245 concentration with a gas analyzer (Rosemount™ CT5800 Continuous Gas Analyzer),
246 preceded by a condenser. Data were logged every 5 s. The N_2O analyzer has a range
247 of 0-500 ppmv with a LOD (lowest detection) of 2 ppm. Zero calibration and span
248 calibration were done by calibrating the analyzer measurements against nitrogen gas
249 of instrument gas purity and N_2O reference gas. The detection method is very stable
250 thus requiring less frequent calibration. The flow rate of the sampling pump (Mini
251 Diaphragm vacuum pump, Laboport) was constant at 5.5 L/min, which was higher
252 than the aeration rate of the IFAS reactors (around 2 L/min) avoiding N_2O escape.
253 The N_2O emitted (mg N_2O-N) over a certain amount of time was calculated using the
254 equation: $N_2O\ emitted = \sum(C_{N2Ogas} \times Q_{sampling\ pump} \times \Delta\ t)$, with more details in
255 section S3 of Supporting Information.

256

257 *2.7. Microbiome analysis*

258 To assess the microbial community present in IFAS reactors and population shift
259 responding to stressors, a V4 16S rRNA gene sequencing approach was used. Total
260 DNA content was extracted using the Powerfecal kit (Qiagen) following the
261 manufacturers protocol. V4 region of the 16S rRNA gene was amplified using
262 primers altered for dual-index paired-end sequencing (described by Kozich et al.[37]).
263 Denoising of the raw data was performed using the DADA2 pipeline (v 1.1.6), as
264 described in Callahan et al.[38]. The resulting object was further analyzed in the R
265 environment using an in-house built R package (Tidyamplicons,
266 www.github.com/SWittouck/tidyamplicons). Detailed method description and results

267 can be found in S4 and Figure S5 of Supporting Information, respectively.

268

269 **3. Results and discussion**

270 *3.1. Realistic FA treatment suppresses NOB marginally*

271 The treatment conditions in different operational phases were summarized in Table S2.
272 The ratio between the accumulated nitrate and removed ammonium (NAR) is used as
273 an indicator for NOB suppression level in PN/A. For instance, NAR of 100%
274 indicates a full nitrification with NOB activation. In contrast, when NOB are
275 completely inhibited, nitrate will be solely produced by AnAOB and NAR ranges
276 from 0 to 11% dependent on relative activities of AerAOB and AnAOB.[5] Figure 1
277 compares the effluent quality, N removal, and NAR before (Phase I) and after (Phase
278 II-VII) FA treatment in IFAS-FA/FNA. Before FA treatment in Phase I, effluent
279 NH_4^+ kept decreasing from 45 to 10 mg N/L, NO_2^- was close to zero and NO_3^-
280 gradually built up, peaking at 27 mg N/L (Figure 1A). NH_4^+ removal reached close to
281 80% at maximum, but total nitrogen (TN) removal was poor (mostly below 30%) due
282 to residual ammonia and nitrate accumulation (NAR close to 65% at maximum)
283 (Figure 1B). Figure 3A displays the maximum potential activity of AerAOB, NOB
284 and AnAOB by flocs and carriers in batch tests fitted to IFAS-FA/FNA. Before FA
285 treatment (Phase I), AerAOB and NOB preferred to grow on flocs, while most of
286 AnAOB grew on carriers, which make separate SRT control possible.[8]

287

288 From operational phase II on, the FA-based return-sludge treatment was applied in
289 IFAS-FA/FNA. We firstly treated half of the floccular biomass in the presence of 30
290 mg NH_3-N/L for 1 h twice per week. The minimum effluent NO_3^-, NAR and N
291 removal rate by NOB confirmed successful suppression of NOB by FA (Figure 1).

This observation was consistent with that in preliminary batch test. The NOB growth was probably inhibited by FA treatment, since FA concentration above 6 mg N/L could cease the growth of *Nitrobacter*.[39] The elevated effluent NH_4^+ concentration (Figure 1A) and decreased N removal rate by AerAOB (Figure S6) revealed that ammonia oxidation rate (AOR) was also negatively affected by FA treatment, resulting in a low TN removal efficiency (20% - 40%) (Figure 1B).

Adaptation to FA stress occurred after two-week exposure. The effluent NO_3^- concentration, NAR and N removal rate by NOB started to increase on around 40th d (Figure 1 & Figure S6), which was not altered by shifting the treated biomass from 50% flocs to 100% flocs (Phase III in Figure 1). By comparing the maximum NOB activity in flocs and carriers in Phase I to those in phase III and VII (Figure 3A), it was found that the FA treatment on flocs pushed more NOB to grow on carriers, which explained why the NOB was still so active after inclusion of FA treatment on flocs of IFAS-FA/FNA. Therefore, in the following operational Phase IV, we exposed 100% flocs + carriers to FA shock. However, on around 120th d N removal rate by AnAOB decreased rapidly (Figure S6). The poor performance of AnAOB leads to a sudden drop of TN removal efficiency from ~60% to ~20% during Phase IV (Figure 1B). The inhibitory impact of FA on AnAOB was reversible and repeatable, which was demonstrated by the subsequently alternate favored and suppressed AnAOB activity with FA treatment on 100% flocs (Phase V) and FA treatment on 100%flocs + carriers (Phase VI), respectively (Figure 1). It was further demonstrated by batch tests that applying FA stress on carriers led to a ~61% reduction of the maximum AnAOB activity on carriers (Phase VI against Phase III in Figure 3A), but releasing the stress was able to recover this activity to ~96% of its original value (Phase VII against

317 Phase III in Figure 3A). Similar observations reported that a concentration of 38 mg
318 NH_3-N/L inhibited 50% of AnAOB activity, and this inhibitory impact was
319 reversible.[40]
320
321 Other studies showed that FA inhibited NOB more strongly than AerAOB.[41-43] This
322 more selective inhibition was confirmed by the experimental results from a short
323 batch test in Figure S4, but not apparent from the long-term observations in the parent
324 reactor (IFAS-FA/FNA). In phase VII (Figure 1), effluent NO_3^- and NAR exceeded 30
325 mg N/L and 70%, respectively, indicating a full activation of NOB. The TN removal
326 of phase VII was below 30% (Figure 1B) and the maximum potential activity of NOB
327 even surpassed AerAOB (columns in Figure S6). It was concluded that FA treatment
328 on separate flocs would not be effective due to the selective NOB growth on carriers.
329 Meanwhile, FA treatment on both flocs and carriers would not be an option either
330 since AnAOB activity was largely inhibited. These two findings allowed no
331 possibility of applying greater stress with either higher FA level or longer contact
332 time in IFAS-FA/FNA and demonstrated the failure of using FA inhibitor as return-
333 sludge treatment in single-stage PN/A system. Same FA treatment conditions were
334 tested by Seuntjens et al.[14] for activated sludge from two different WRRF. He
335 revealed that the FA shock (30 mg NH_3-N /L for 1 h) can not be used as a single
336 parameter for increasing the relative activity ratio between AerAOB and NOB in both
337 short-term and long-term tests. Instead, the combination of starvation, sulfide and FA
338 could be more effective. Over 90% of NAR was achieved by applying 210 mg NH_3-
339 N/L FA for 1 d on 22% of biomass within 40 d.[17] Different from inhibition, FA
340 concentrations up to 210 mg NH_3-N/L exerted a biocidal effect on microbes, killing
341 living cells.[17,44] However, according to our calculation based on Nieuwveer WRRF,

342 the FA level in the study by Wang et al.[17] cannot realistically reached with
343 endogenous nitrogen, and has a huge inhibitory impact on AnAOB if extrapolating to
344 single-stage PN/A.
345
346 *3.2 FNA-based return-sludge treatment limits nitrate production*
347 Figure 2 compares the effluent quality, N removal and nitrate accumulation ratio
348 (NAR) before (Phase I) and after (Phase II-XII) FNA treatment in IFAS-FNA. Before
349 FNA treatment, similar trends of effluent nitrogen, efficiencies and removal rates in
350 IFAS-FNA was observed as those in IFAS-FA/FNA in phase I (Figure 2 & Figure S7).
351 Once implementing FNA shock (0.5 mg HNO_2-N/L for 4 h), NOB activity was
352 immediately suppressed (phase II). Similarly, NOB adaptation to FNA suppression
353 took place after approximately two weeks, leading to a build-up of NO_3^- in effluent
354 and consequently high NAR and low TN removal efficiency (Figure 2A&B).
355 Therefore, in the following phases (III – IX), FNA levels, treated biomass (50%flocs,
356 100%flocs and 100%flocs+carriers) and contact times were modified with the aim to
357 create greater stress conditions for NOB. It was observed that the presence of 2.0 mg
358 HNO_2-N/L FNA would prevent NOB adaptation effectively, leading to a gradual
359 decrease of effluent NO_3^- and NAR and increase of TN removal efficiency in phase
360 III and IV (Figure 2 & Figure S7). NAR was successfully maintained at a low level in
361 the following operational phases (III – IX) (Figure 2). Consistent with our observation,
362 Wang et al.[45] found partial nitritation with over 80% of nitrite accumulation ratio can
363 be established within 15 d by exposing 1.35 mg HNO_2-N/L FNA to 22% sludge for
364 24 h per d.
365
366 In phase IV, the NH_4^+ removal efficiency was unsatisfactory (below 60%). To

increase AOR, DO level in IFAS-FNA was then increased from 1.0 to 2.0 mg O_2/L. Effluent NH_4^+ immediately dropped. However, the increased AOR leading to the build-up of effluent NO_3^- and NAR (Figure 2). Step-wise decrease of DO concentration in latter phase (V) managed to suppress NOB activity again. This observation revealed that the FNA treatment on return sludge should be combined with limited DO levels. Wang et al.[15] observed that DO limitation (0.3-0.8 mg O_2/L) largely increased NAR in the mainstream partial nitritation reactor with FNA-based return-sludge suppression technology. This is in line with our findings. A second increase of NAR took place when new inoculum MAS sludge was introduced in phase VII due to the increased NOB population (VSS data in Figure S1). By applying a longer contact time (Phase VIII) and a higher FNA level (Phase IX) on flocs and carriers, the NAR and effluent NO_3^- returned to low levels (Figure 2). The investigated suppression treatment conditions in Phase V-IX yielded similar TN removal around 60%. Hence, from the economic point of view (detailed in the following discussion), we gradually released the suppression treatment conditions in latter phases (X-XII).

The suppression treatment conditions for final Phase XII were FNA 2.0 mg HNO_2-N/L + 4 h. During this phase, IFAS-FNA achieved stable performance with ~65% TN removal and approximately 130 mg N/L/d TN removal rate (Figure 2B). The relatively high removal efficiency and rate (Figure 2B) along with minimum N oxidation by NOB (Figure S7) indicated a successful implementation of FNA-based return-sludge suppression technology in mainstream PN/A process. The proposed technology combining hybrid bioreactor and FNA-based return-sludge treatment has better system performance than mainstream biofilm-based PN/A process, which had a

low TN removal efficiency (~40%) due to residual NO_2^- and NO_3^-.[46] The proposed IFAS + FNA technology yielded a comparable TN removal efficiency with reported values for PN/A process based on IFAS reactors (~70%), but much higher removal rate than those from literature (20-55 mg N/L/d).[8,9] In fact, Wang et al.[18] found the application of FNA-based return-sludge technology will potentially increase TN removal efficiency from ~30% to ~60% in IFAS. The proposed IFAS + FNA technology was potentially feasible under lower temperature conditions, since AnAOB activity in biofilm was less affected and even adapted to low temperature (< 13 °C)[12] and the level range of FNA treatment became broader under low temperature.

The results in batch activity test illustrated activity distribution in flocs and carriers of IFAS-FNA (Figure 3B). In general, all microorganisms preferred to grow on carriers when FNA suppression was applied on separate flocs (Phase IV). FNA suppression on flocs and carriers would exert inhibitory impact on the activity of both AerAOB and NOB in both flocs and carriers, but more significant inhibition on NOB. It has been reported that FNA at levels of 0.24-1.35 mg HNO_2^--N/L was significantly more biocidal to NOB than AerAOB.[45] Different from FA treatment (Figure 3A), FNA treatment on flocs and carriers has very limited effect on AnAOB activity (Figure 3B). By comparing phase V to phase VI and IX, it was found that higher FNA level (from 2.0 to 3.0 mg N/L) and longer contact time (from 4 to 24) can help to suppress more NOB activity especially on flocs (Figure 3B).

The role of FNA on NOB suppression can be further clarified in operational phase VIII and XIII in IFAS-FA/FNA (Figure 1). Switching from FA treatment to FNA treatment resulted in immediate suppression of NOB activity (Phase VIII in Figure 1

417 & 3A), while releasing from FNA suppression immediately triggered NOB activity
418 (Phase XIII in Figure 1 & 3A). The results in Phase X, XI and XII also verified that
419 pH 5.5 has no negative impact on the system performance of IFAS-FA/FNA (Figure
420 1). Hence, the proposed FNA-based return-sludge suppression technology would be
421 applicable even without thickener since FNA level of 2.2 mg N/L can be reached at
422 pH 5.5 based on our calculation.
423
424 The microbiome in flocs and carriers were analyzed using V4 16S rRNA gene
425 amplicon sequencing approach to assess their importance in the growth of targeted
426 bacterial groups (AerAOB, NOB, AnAOB) under varying suppression treatment
427 conditions (Figure S5). The microbiome results were highly consistent with process
428 data (Figure 1&2) and batch activity results (Figure 3). As for AnAOB, a clear
429 difference in their importance was observed between flocs and biofilms samples,
430 demonstrating that AnAOB were more prevalent in the biofilms. In terms of NOB and
431 AerAOB, it was confirmed that FA shocks had a limited influence on NOB
432 abundance, whilst FNA shocks largely lowered NOB levels in both flocs and carriers
433 with AerAOB presence mostly not being affected
434
435 *3.3. FNA-based return-sludge treatment limits N_2O emissions*
436 DO and NO_2^- levels are two important factors affecting N_2O production by
437 AerAOB.[21,28,47] The N_2O emission factors obtained from daily analysis of the two
438 IFAS reactors were correlated to both DO concentrations and NO_2^- accumulation
439 (Figure S8 and Figure 4). Figure 4 presents the impacts of varying NO_2^-
440 accumulations and suppression treatment conditions on N_2O emission factors from
441 both IFAS reactors. Both modeling and isotopic measurements support that N_2O

production via NH_2OH oxidation pathway positively correlates to DO levels.[28,48] The poor correlation coefficients in our setups (R^2 = 0.0075 for IFAS-FA/FNA, R^2 =0.12 for IFAS-FNA, details refer to Figure S8) indicated that DO was not a key factor affecting N_2O emission and thus NH_2OH oxidation pathway was not likely to be the main N_2O source in our study. Since both IFAS reactors were mostly operated above 1.0 mg O_2/L, heterotrophic denitrification was expected to play a minor role in N_2O production as well. Nitrifier denitrification pathway is a nitrite-sensitive process due to the fact that the expression of copper-containing NO_2^- reductase (NirK) is regulated by a nitrite-sensitive transcription repressor protein.[49] The dependency of N_2O production via nitrifier denitrification on the nitrite concentration was further demonstrated with the aid of site preference analysis and mathematical modeling.[24,28] In this study, N_2O emission factors were positively correlated with nitrite accumulation (Figure 4), indicating that nitrifier denitrification was the main N_2O production mechanism.

For IFAS-FA/FNA, at similar NO_2^- levels, the N_2O emission factors in the groups of benchmark and FA treatment (solid symbols) were higher than those in the group of FNA treatment (open symbols, Figure 4A). In terms of IFAS-FNA, we further compared N_2O emission factors under different FNA treatment conditions. It was found that at similar NO_2^- levels, higher FNA (2.0 and 3.0 mg HNO_2-N/L) treatment would lead to lower N_2O emissions compared to benchmark and FNA level of 0.5 mg HNO_2-N/L (Figure 4B). Other studies showed a stimulation of the N_2O production via nitrifier denitrification by moderate nitrite concentrations of 0-50 mg N/L,[24] but an inhibition by very high nitrite levels (500-1000 mg N/L).[50] The FNA levels for sludge treatment in this study resulted in nitrite concentrations of 195, 780 and 1170 mg N/L,

467 the last two of which are within the inhibitory range. High FNA levels might alter the
468 detoxification mechanism of NirK, the enzyme contributing to nitrifier
469 denitrification[51], and thus change the stimulation threshold of nitrite on N_2O
470 emission.[32] Single-stage mainstream PN/A process was expected to emit more N_2O
471 than conventional activated sludge process due to higher prevailing nitrite levels. The
472 proposed endogenous FNA concept, capable of inhibiting N_2O production via the
473 nitrifier pathway, potentially alleviates this concern.
474
475 *3.4. Estimating costs and environmental benefits of the endogenous FNA concept*
476 We performed a preliminary desktop extrapolation for the proposed concept based on
477 endogenous FNA production and recurrent return-sludge exposure. Calculations were
478 done based on data of an existing full-scale AB WRRF with sidestream PN/A
479 (Nieuwveer). The average wastewater characteristics and typical WRRF operational
480 conditions are based on average data for 2017, and can be found in Table S3. This
481 WRRF contains a mainstream A- and B-stage, each with their set of settlers, and the
482 respective resulting sludge sent to belt thickeners and an anaerobic digester producing
483 biogas from the waste sludge. The reject water from dewatering is treated in the side
484 stream by a PN/A installation. Figure S3 is a simplified schematic representation of
485 the proposed endogenous FNA concept, which only requires minimal retrofitting of
486 the existing PN/A system to a nitritation system to produce an FNA-rich liquor next
487 to the construction of a contact tank to mix the FNA-rich liquor from the nitritation
488 reactor and the return sludge from the settler or thickener. The treated biomass along
489 with the FNA-rich liquor are mixed with raw sewage and return sludge flow at the
490 head of the main stream. The FNA effect hence seizes since the diluted nitrite
491 concentrations are low (~4 mg N/L, in scenario 4 below), which will be rapidly

492 removed in the mainstream N removal process.

493

494 Four scenarios have been involved based on assumptions, lab observations and
495 realistic FNA level boundaries described in S5 of Supporting Information and shown
496 in Figure 5: 100% nitritation without thickener & FNA treatment at 3.0 mg HNO_2-
497 N/L for 16 h (scenario 1), 65% nitritation with thickener & FNA treatment at 3.0 mg
498 HNO_2-N/L for 16 h (scenario 2), 65% nitritation with thickener & FNA treatment at
499 3.0 mg HNO_2-N/L for 4 h (scenario 3) and 65% nitritation without thickener & FNA
500 treatment at 2.0 mg HNO_2-N/L for 4 h (scenario 4). Comparison of the different
501 scenarios yielded three important messages. Firstly, 100% nitritation (versus 65%) or
502 inclusion of thickener allows reaching higher FNA levels, such as 3.0 mg HNO_2-N/L.
503 However, the inclusion of thickener (scenario 2) saves up to ~63% of the total cost
504 compared to increasing nitritation efficiency in nitritation unit (scenario 1). Secondly,
505 contact time is a cost factor, but not significant. For example, a weekly sludge
506 exposure period of 4 h (scenario 3) leads to a 0.5% lower cost than a contact time of
507 16 h (scenario 2). Thirdly, since 2.0 mg HNO_2-N/L can be reached in the absence of
508 thickener and reasonable pH levels (~5.5) based on the desktop calculation, ~70% of
509 the costs can be further reduced by leaving out the thickener (scenario 4 vs 3). Hence,
510 the cheapest scenario for the proposed technology is to apply a realistic FNA level
511 that can be reached by 65% nitritation without a thickener, coupled to shorter contact
512 time. As shown in Figure 5, acid dosage to the contact tank is the major operational
513 cost in cheapest scenario 4 (over 50%). We demonstrated experimentally that the
514 cheapest scenario (2.0 mg HNO_2-N/L FNA with 4-h contact time) is able to achieve
515 desirable and stable PN/A process performance. Compared with literature,[18,45] the
516 concluded FNA treatment conditions in this study also resulted in cost savings due to

517 shorter contact time (4 h vs. 24 h) and longer treatment cycle (7 d, 4.5 d).

518

519 According to Zessner et al.[52], total costs for sewage treatment costs are around 30
520 EUR/inhabitant/year for a plant in Austria with nitrogen and phosphorus removal. If
521 per inhabitant 0.25 m^3 sewage is treated daily, the wastewater treatment cost is 0.33
522 EUR/m^3. An additional treatment cost of 0.0012 EUR/m^3 sewage seems acceptable
523 (Figure 5), as this would only increase the cost with 0.36%, while the gain in terms of
524 process stability and robustness can be considerable. This rough cost-benefit analysis
525 is hence very promising and spurs the further development of the proposed concept.

526

527 In terms of environmental assessment, we considered the carbon footprint from
528 electricity consumption (majority from aeration), avoided carbon emissions from
529 electricity generation from the biogas, and the carbon footprint from N_2O emissions
530 from mainstream and sidestream PN/A processes. Based on assumptions listed in
531 Table S4, such as oxygen transfer efficiency, biogas yield, a fixed N_2O emission
532 factor from the side stream, etc, it was concluded that for a neutral carbon emission,
533 the N_2O emission factor for mainstream PN/A should not exceed 0.78%. To this end,
534 NO_2^- accumulation in IFAS-FNA should be controlled below 2 mg N/L. The ratio of
535 NH_4^+/NO_2^- in the partial nitritation process can be regulated by DO concentrations
536 and the volumetric NH_4^+ loading rate.[53] The introduction of non-aerated phases was
537 found to improve the activity of AnAOB and limited that of NOB.[54] Hence, future
538 research may focus on realizing nitrite control in PN/A process by using combined
539 strategies of aeration and feeding based on effluent NH_4^+ and NO_2^- set points.

540

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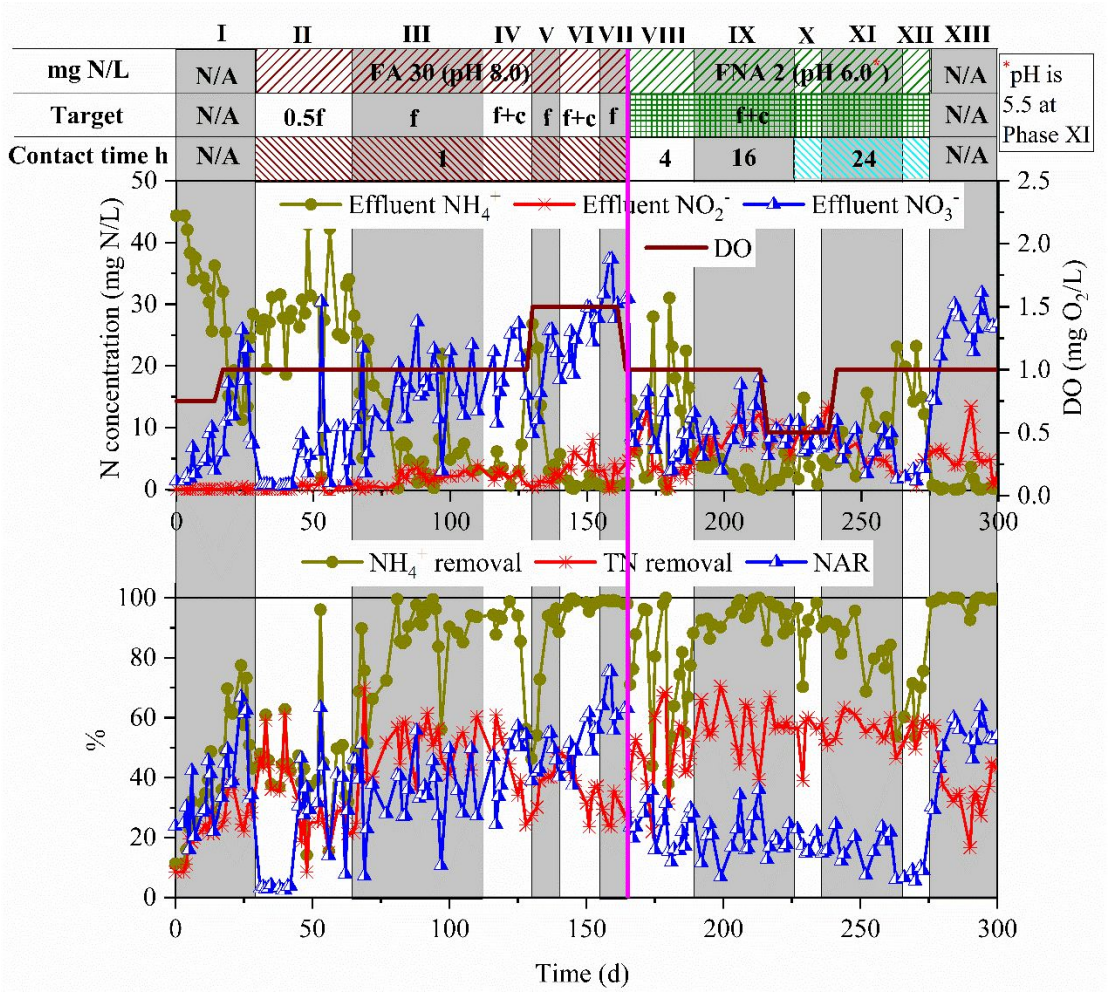

Figure 1. Reactor performance of the IFAS-FA/FNA reactor during different operational phases (FA treatment in I-VII; FNA treatment in VIII-XIII). Main variables among operational phases: average DO concentrations, FA/FNA levels, treated biomass target and contact time. Flocs and carriers are abbreviated as 'f' and 'c', respectively. A) nitrogen concentrations in effluent and average DO concentration inside reactor and B) NH_4^+ removal efficiency, total nitrogen (TN) removal efficiency and nitrate accumulation ratio (NAR). Pink line indicates the shift from FA treatment to FNA treatment.

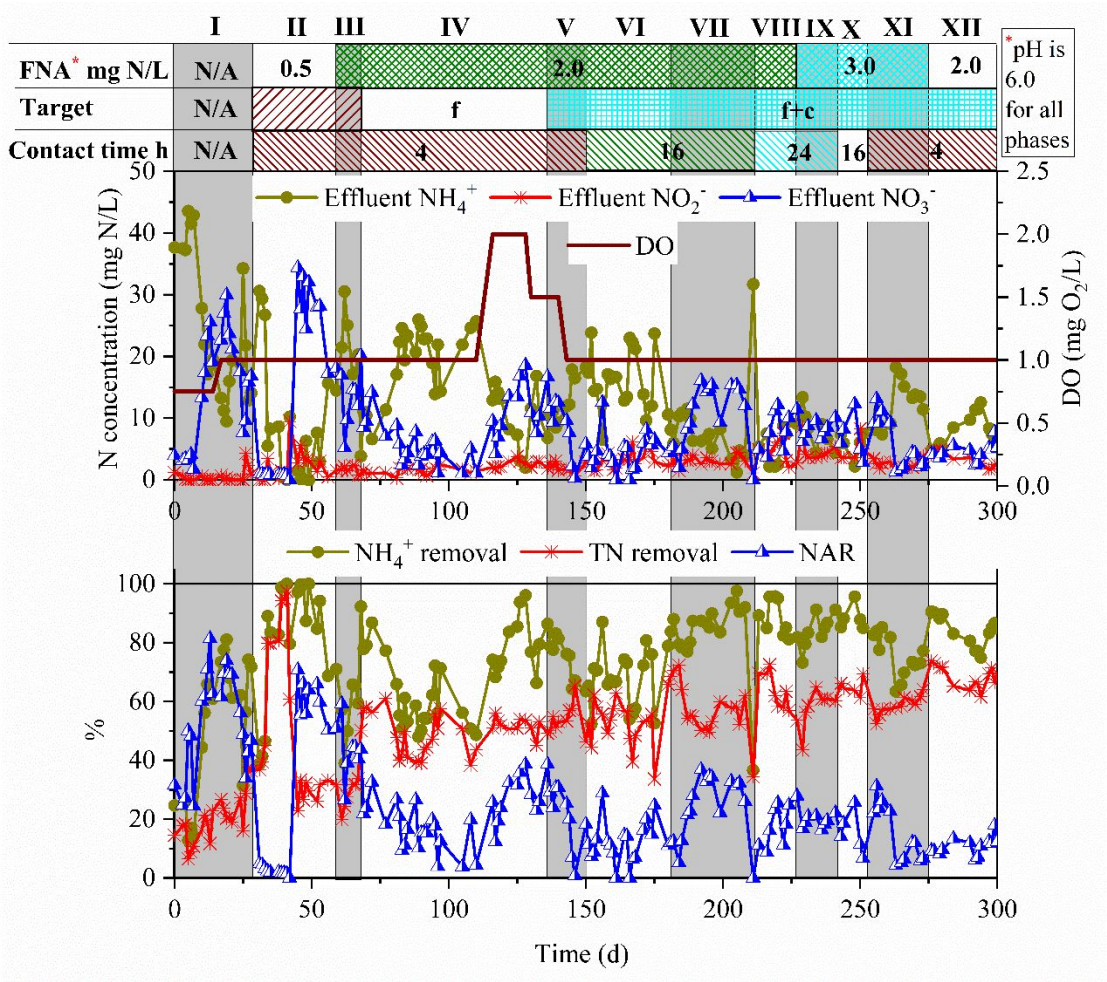

Figure 2. Reactor performance of IFAS-FNA during different operational phases. Main variables among operational phases: average DO concentrations, FNA levels, treated biomass target and contact time. Flocs and carriers are abbreviated as 'f' and 'c', respectively. A) nitrogen concentrations in effluent and average DO concentration inside reactor and B) NH_4^+ removal efficiency, total nitrogen (TN) removal efficiency and nitrate accumulation ratio (NAR).

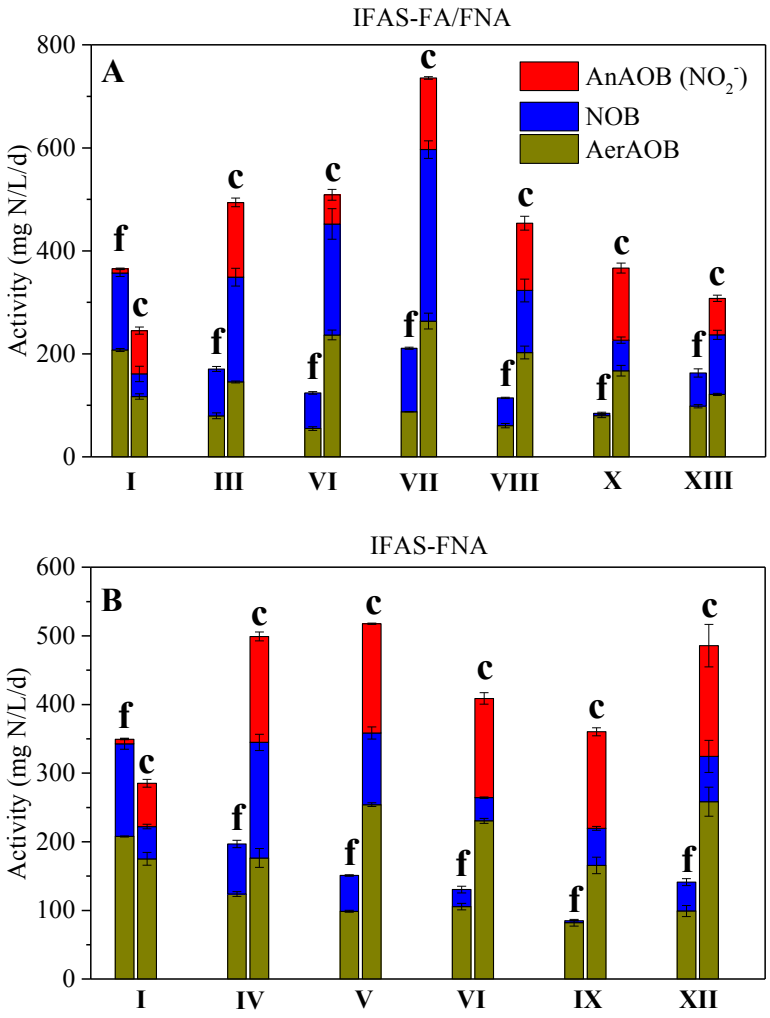

Figure 3. Dynamics of the maximum activities of AerAOB, NOB and AnAOB by flocs and carriers in batch tests fitted to IFAS-FA/FNA (A) and IFAS-FNA (B) reactors over the different operational phases. Flocs and carriers are abbreviated as 'f' and 'c', respectively.

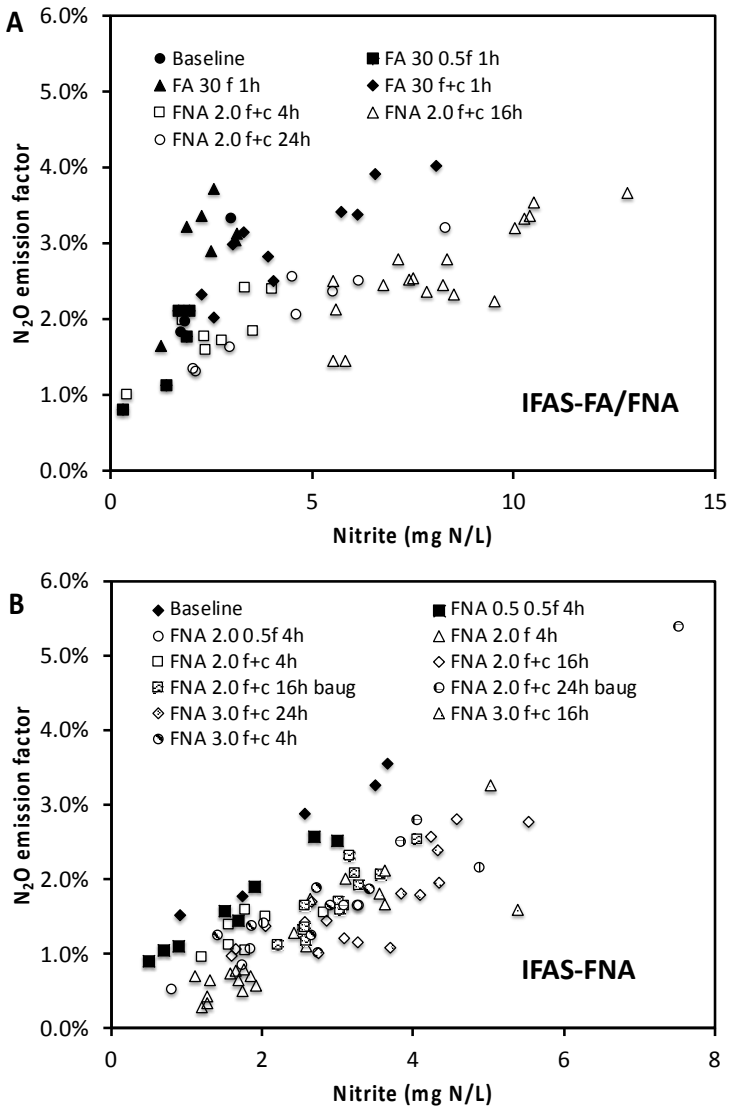

Figure 4. The impact of varying nitrite accumulations and suppression treatment conditions on N_2O emission factors from IFAS-FA/FNA (A) and IFAS-FNA (B). Note: each symbol legend consists of the stressor used for return sludge treatment, the stressor level in mg N/L, treated biomass with f representing flocs and c representing carriers, and the contact time for each treatment, successively. In some symbol legend, baug means the inclusion of bio-augmentation.

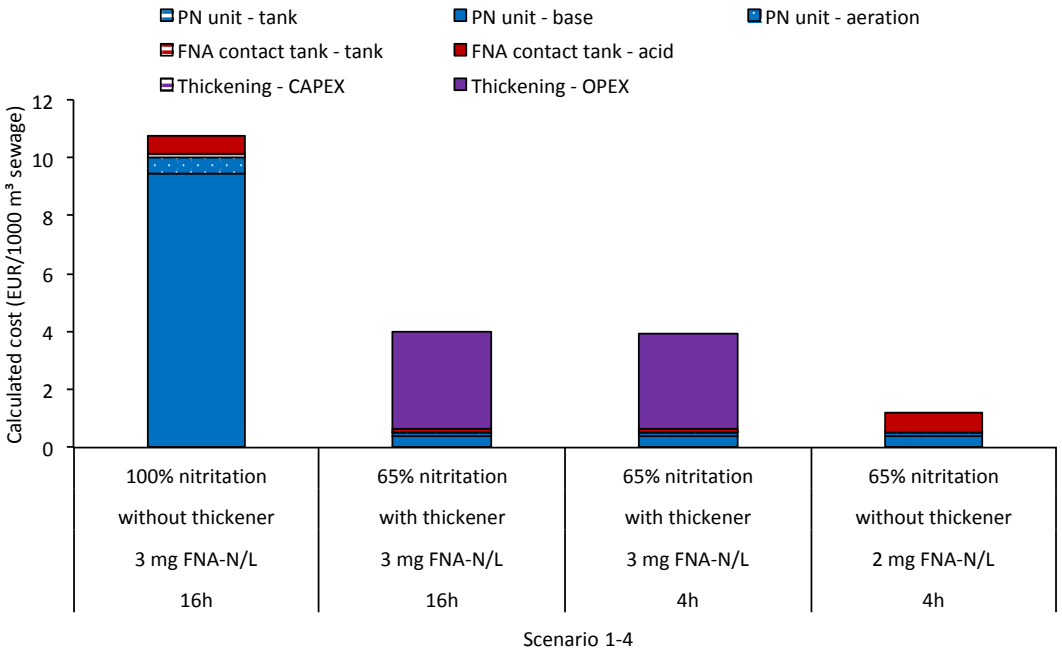

Figure 5. Calculated costs for the four proposed endogenous FNA treatment scenarios, expressed per 1000 m³ raw sewage, as extra cost compared to a baseline treatment scenario without FNA treatment step and with sidestream PN/A instead of sidestream FNA production.