Return-sludge treatment with endogenous free nitrous acid limits nitrate production and N₂O emission for mainstream partial nitritation/anammox

1   **Return sludge treatment with endogenous free nitrous acid limits nitrate**

2   **production and N₂O emission for mainstream partial nitritation/anammox**

3

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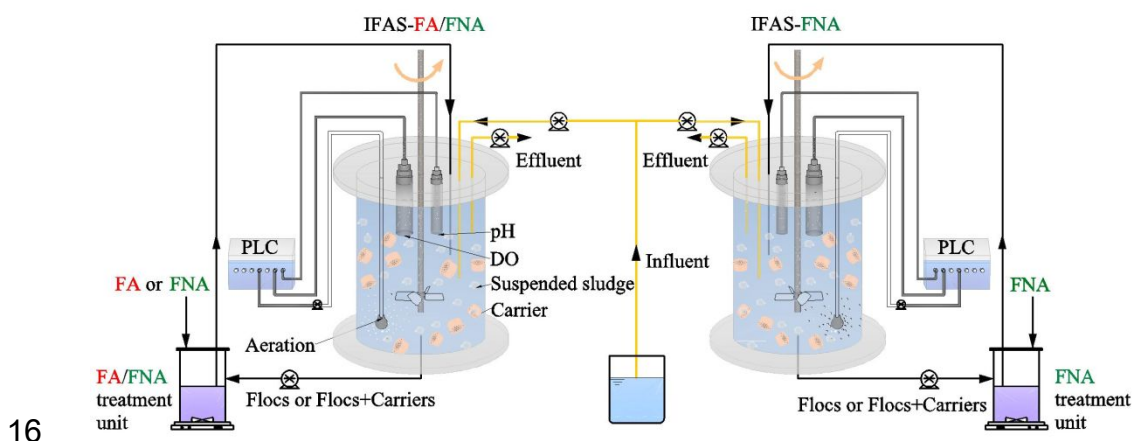
17   **Abstract**

18   Nitrite oxidizing bacteria (NOB) and nitrous oxide (N_2O) hinder the development of

19   mainstream partial nitritation/anammox. To overcome these, endogenous free

20   ammonia (FA) and free nitrous acid (FNA), which can be produced in the side stream,

21   were used for return sludge treatment for two integrated-film activated sludge reactors

22   containing biomass in flocs and on carriers. The repeated exposure of biomass from

23   one reactor to FA shocks had a limited impact on NOB suppression, but inhibited

24   anammox bacteria (AnAOB). In the other reactor, repeated FNA shocks to the

25   separated flocs failed to limit the system's nitrate production since NOB activity was

26   still high on the biofilms attached to the unexposed carriers. In contrast, the repeated

27   FNA treatment of flocs and carriers favoured aerobic ammonium-oxidizing bacteria

28   (AerAOB) over NOB activity with AnAOB negligibly affected. It was further

29   revealed that return sludge treatment with higher FNA levels led to lower N_2O

30   emissions under similar effluent nitrite concentrations. On this basis, weekly 4-hour

31   FNA shocks of 2.0 mg HNO_2-N/L were identified as an optimal and realistic

32   treatment, which not only enabled nitrogen removal efficiencies of ~65% at nitrogen

33   removal rates of ~130 mg N/L/d (20 °C), but also yielded the lowest cost and carbon

34   footprint.

35   **Keywords:** mainstream partial nitritation/anammox; endogenous free nitrous acid;

36   free ammonia; integrated-film activated sludge; nitrous oxide

37

38   **1. Introduction**

39   Partial nitritation/anammox (PN/A) is conceived as a resource- and cost-effective

40   technology for biological nitrogen removal from waters low in biodegradable

41   organics.[1] During this autotrophic nitrogen removal process, part of the ammonium

42   (NH_4^+) is aerobically oxidized to nitrite (NO_2^-) by aerobic ammonium-oxidizing

43   bacteria (AerAOB), and the residual NH_4^+ is further oxidized with the produced NO_2^-

44   to form dinitrogen gas by anoxic ammonium-oxidizing bacteria (AnAOB). Since

45   PN/A does not require organic carbon, it enables to maximally redirect sewage carbon

46   to sludge in the upstream carbon treatment stage, which can be achieved with high-

47   rate activated sludge technologies. So, instead of the traditional Adsorption-

48   Biooxidation or AB process[2] based on nitrification/denitrification, an update has been

49   proposed, still with high-rate activated sludge in the A-stage, but now followed by

50   PN/A in the B-stage (e.g. Verstraete et al.[3]). For resource efficiency, the generated

51   sludge from the A-stage should be sent to anaerobic sludge digestion, enabling energy

52   recovery as biogas, or even recover other commodities.[4] Therefore, mainstream PN/A

53   is a game changer enabling energy-autarkic water resource recovery facilities

54   (WRRF).

55

56   So far, it has been challenging to achieve stable and high nitrogen removal rates and

57   efficiencies for mainstream PN/A, as the microbial competition control is demanding,

58   for instance due to some features inherent to A-stage effluent, such as low nitrogen

59   levels, organics levels necessitating co-occurrence of heterotrophic conversions, low

60   temperatures and varying flow rates.[5] In PN/A process, AerAOB and AnAOB are the

61   desired microbes collaborating to remove nitrogen autotrophically, while NOB are

62   undesired, compete for nitrite with AnAOB, leading to a higher oxygen consumption

63   and lower nitrogen removal efficiency. A multitude of strategies has been proposed to

64   steer the microbial competition for substrates and space in PN/A process. They can be

65   summarized in the conceptual physical/metabolic selection framework of (i) targeting

66   retention of desired/rejection of undesired microorganisms, labeled as IN/OUT

67   control, while also (ii) focusing on promotion of desired/suppression of undesired

68   activities, labeled as ON/OFF control.[5] Lv et al.[6] utilized a novel composite carrier to

69   enhance the proliferation of AerAOB and AnAOB under mainstream conditions.

70   Differential control of sludge retention times (SRT) has been proposed as successful

71   for IN/OUT control, and can be easily achieved in so-called hybrid reactors, i.e. based

72   on the coexistence of suspended and attached growth.[7-10] In such reactors, the biofilm

73   SRT is very high, which is ideal to retain the slowly growing AnAOB, while the floc

74   SRT is controlled at sufficiently low values just retaining enough AerAOB while

75   washing out NOB. The PN/A process in IFAS reactor configuration has been piloted

76   in the study by Kowalski et al.[11] However, the major barrier hindering the application

77   of mainstream PN/A in hybrid reactors is recurrent nitratation. NOB proliferation can

78   be induced by disturbances in operational conditions, such as temperature, flocculent

79   SRT, etc.[8,12,13] In such cases, IN/OUT strategies should be combined with ON/OFF

80   strategies to ensure their rapid suppression and swift restoration of the treatment

81   efficiency.

82

83   Inhibitors have been used as ON/OFF control strategy to favor AerAOB over NOB

84   activity, such as sulfide, perchlorate, formic acid, volatile fatty acid, free ammonia

85   (FA) and free nitrous acid (FNA).[14] Among them, FA and FNA are endogenous

86   inhibitors, as they can be readily available in WRRF with anaerobic digestion, which

87   may economically and environmentally be beneficial.[15] However, under mainstream

88   conditions FA and FNA are low, and have limited inhibitory impacts on AerAOB and

89   NOB. Higher FA and FNA concentrations can be achieved in the side stream, i.e. the

90   sludge line where FA is rich in the digester effluent and FNA can be produced using a

91   nitritation reactor. Sidestream-derived FA and FNA can be applied for NOB

92   suppression without AnAOB in the return sludge line.[16] Further investigations

93   revealed that mainstream partial nitritation (without anammox) was achieved and

94   maintained under the conditions of repetitive exposure to high FNA (1.83 mg N/L,

95   endogenously feasible) and FA (210 mg N/L, endogenously unfeasible) levels with

96   prolonged contact time of 24 hours (h) and treatment cycle (defined as the time

97   required to expose all of biomass to FNA/FA shock) of 4.5 days (d).[15,17] Wang et al.[18]

98   proposed that a promising technology combining IFAS reactor with FNA treatment

99   can potentially achieve stable operation of mainstream single-stage PN/A. However,

100   relevant research remains scarce and insights are needed on how AerAOB, NOB and

101   AnAOB respond to FA and FNA exposure at endogenously available levels and

102   treatment cycle.

103

104   As any biological nitrogen removal process, PN/A process can be a potential source

105   for nitrous oxide (N_2O) emission, which is not only a potent greenhouse gas with

106   approximately 265-fold stronger global warming potential than carbon dioxide, but

107   also a strong ozone depleting substance.[19,20] In PN/A processes, N_2O is produced by

108   AerAOB via two pathways: i) the sequential reductions from NO_2^- to nitric oxide (NO)

109   and to N_2O as the end product, termed as the nitrifier denitrification pathway;[21,22] and

110   (ii) N_2O as a side product during incomplete oxidation of hydroxylamine (NH_2OH) to

111   NO_2^-, known as NH_2OH oxidation pathway.[23,24] N_2O production can be affected by a

112   number of factors, i.e. dissolved oxygen (DO), NO_2^-, NH_4^+, pH, alkalinity, etc.[22,25-27]

113   DO and NO_2^- levels have been recognized as the most important affecting factors

114   which are able to shift N_2O production pathways.[21,28] Given the dynamic DO and

115   NO_2^- levels during PN/A operation, the process can become a strong N_2O emitter

116   under certain conditions. CastroBarros et al.[29] observed that average 2.0% of the total

117   incoming nitrogen was emitted as N_2O in a full-scale sidestream PN/A process, which

118   can be minimized by adequate aeration. At lab scale, N_2O emissions were 4.6% and 5%

119   of the nitrogen load in mainstream PN/A bioreactors, as reported by Connan et al.[30]

120   and DeClippeleir et al.[31], respectively. Wang et al.[32] revealed FNA-based sludge

121   treatment on lab-scale nitrifying culture under mainstream conditions led to a

122   decreased biomass-specific N_2O production rate. As N_2O emission could be a

123   significant or even major contributor (up to 80%) to the carbon footprint of WRRF,[33]

124   systematic and online investigation is needed to provide in-depth insights into the

125   conditions triggering N_2O emission and possible mitigation strategies.

126

127   In this study, realistic FA/FNA doses to return sludge were tested as a way to increase

128   AerAOB/NOB activity ratio in a mainstream one-stage PN/A IFAS system. The

129   immediate inhibitory impact of FA/FNA shocks on the activities of AerAOB, NOB

130   and AnAOB were investigated in batch reactor, while long-term recovery was

131   assessed in two IFAS reactors for a period of 300 d. Shifts in microbial activities and

132   community composition in flocs and carriers were monitored, and the effect of

133   recurrent stressor exposure on the process' N_2O emissions was followed on line. A

134   preliminary assessment was done of the costs and associated carbon footprint of the

135   proposed endogenous stressor concepts. The overall goal was to achieve realistic

136   suppression treatment conditions to maximize N removal, while obtaining low N_2O

137   emissions.

138

139   **2. Materials and methods**

140   *2.1. Inoculation and bio-augmentation*

141   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

167   (Orbisint CPS11D) and controlled in the range of 7.0-7.5 by a programmable logic

168   controller (PLC). DO levels were continuously monitored by a DO sensor (Oxymax

169   COS61) and PLC-controlled in different ranges, as one of the operational strategies

170   for NOB suppression (detailed in Figure 1&2). The targeted nitrogen loading rate and

171   volume exchange ratio were 200 mg N/L/d and 33%, respectively, resulting in a cycle

172   time of 2 h and hydraulic retention time of 6 h. The two reactors were operated in

173   sequencing batch mode, which was achieved by timers controlling overhead stirrers

174   (ES Overhead Stirrer, Velp Scientifica), air pumps (Tetra TEC APS 150), and influent

175   and effluent pumps (DriveSure panel mount pumps, PMD24C, Watson-Marlow).

176   Each 2-h cycle consisted of 40-minute (min) continuous feeding (aerobic), 40-min

177   aerating, 30-min settling, 5-min decanting and 5-min resting. The SRT was not

178   controlled, but monitored by measuring volatile suspended solid (VSS) concentration

179   in the effluent. The IFAS reactors were started up in anaerobic phase for enrichment

180   of AnAOB (50 mg N/L NH_4^+ and 50 mg N/L NO_2^-) and subsequent aerobic phase (50

181   mg N/L NH_4^+ and DO below 1.0 mg O_2/L) for co-culture of AerAOB and AnAOB.

182   The details were presented in S1 of Supporting Information.

183

184   *2.3. FA and FNA inhibition*

185   After start-up, FA and FNA were used as stressors for the return-sludge treatment,

186   schematic diagram of which was presented in Figure S2 of Supporting Information.

187   One of the two IFAS reactors was exposed to FA shock initially and FNA shock

188   subsequently, which was named as IFAS-FA/FNA. The other implementing FNA-

189   based return-sludge treatment was termed as IFAS-FNA. Before applying stressors, a

190   desktop calculation was performed to determine realistic FA and FNA ranges based

191   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

217   each test, 100 mL flocs and 15 random carriers (~1.2-1.3% of the total carriers) were

218   taken from each reactor and brought separately in a nitrogen-free medium. Aerobes

219   and anaerobes were characterized separately in aerobic test and anoxic tests. For

220   aerobic tests, the biomass was added into open glass flasks and constantly mixed by a

221   magnetic stirrer. For the anoxic test, the sludge was flushed with N_2 gas in the

222   penicillin bottles, then sealed completely and constantly mixed by a magnetic stirrer.

223   50 mg NH_4Cl-N/L and 50 mg $NaNO_2$-N /L were spiked at the beginning of all the

224   batch tests. Concentration changes of NO_2^-, NO_3^- and NH_4^+ were monitored by

225   periodical sampling and measurement (details below). At the end of the tests with

226   flocs, the biomass concentration was determined. Aside from N-measurements, DO

227   and pH were also measured. During the whole period, DO concentrations for aerobic

228   tests are all above 7.0 mg O_2/L and pH was close to 7.5. Each test was performed in

229   duplicate. The maximum potential activities of AnAOB, AerAOB and NOB in flocs

230   or biofilm on the carriers in the IFAS reactor were estimated by extrapolating the

231   maximum microbial activities measured in the batch tests by means of the flocculent

232   biomass concentration and the number of carriers in the reactor (IFAS-FA/FNA: 1254;

233   IFAS-FNA: 1119).

234

235   *2.5. Physicochemical water and biomass analyses*

236   To monitor the system performance, samples were taken periodically from influent,

237   reactor and effluent for analysis of NH_4^+, NO_2^-, NO_3^-, COD, total suspended solids

238   (TSS) and VSS concentrations. NH_4^+-N, NO_2^--N and NO_3^--N were measured with

239   San^{++} Automated Wet Chemistry Analyzer. VSS and TSS were measured using

240   standard methods.[36] COD was photometrically determined using Nanocolor test tubes

241   (Macherey-Nagel, Germany).

242

243   *2.6. Online N₂O 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).

292   This observation was consistent with that in preliminary batch test. The NOB growth

293   was probably inhibited by FA treatment, since FA concentration above 6 mg N/L

294   could cease the growth of *Nitrobacter*.[39] The elevated effluent NH_4^+ concentration

295   (Figure 1A) and decreased N removal rate by AerAOB (Figure S6) revealed that

296   ammonia oxidation rate (AOR) was also negatively affected by FA treatment,

297   resulting in a low TN removal efficiency (20% - 40%) (Figure 1B).

298

299   Adaptation to FA stress occurred after two-week exposure. The effluent NO_3^-

300   concentration, NAR and N removal rate by NOB started to increase on around 40th d

301   (Figure 1 & Figure S6), which was not altered by shifting the treated biomass from 50%

302   flocs to 100% flocs (Phase III in Figure 1). By comparing the maximum NOB activity

303   in flocs and carriers in Phase I to those in phase III and VII (Figure 3A), it was found

304   that the FA treatment on flocs pushed more NOB to grow on carriers, which

305   explained why the NOB was still so active after inclusion of FA treatment on flocs of

306   IFAS-FA/FNA. Therefore, in the following operational Phase IV, we exposed 100%

307   flocs + carriers to FA shock. However, on around 120th d N removal rate by AnAOB

308   decreased rapidly (Figure S6). The poor performance of AnAOB leads to a sudden

309   drop of TN removal efficiency from ~60% to ~20% during Phase IV (Figure 1B). The

310   inhibitory impact of FA on AnAOB was reversible and repeatable, which was

311   demonstrated by the subsequently alternate favored and suppressed AnAOB activity

312   with FA treatment on 100% flocs (Phase V) and FA treatment on 100%flocs +

313   carriers (Phase VI), respectively (Figure 1). It was further demonstrated by batch tests

314   that applying FA stress on carriers led to a ~61% reduction of the maximum AnAOB

315   activity on carriers (Phase VI against Phase III in Figure 3A), but releasing the stress

316   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

367   increase AOR, DO level in IFAS-FNA was then increased from 1.0 to 2.0 mg O_2/L.

368   Effluent NH_4^+ immediately dropped. However, the increased AOR leading to the

369   build-up of effluent NO_3^- and NAR (Figure 2). Step-wise decrease of DO

370   concentration in latter phase (V) managed to suppress NOB activity again. This

371   observation revealed that the FNA treatment on return sludge should be combined

372   with limited DO levels. Wang et al.[15] observed that DO limitation (0.3-0.8 mg O_2/L)

373   largely increased NAR in the mainstream partial nitritation reactor with FNA-based

374   return-sludge suppression technology. This is in line with our findings. A second

375   increase of NAR took place when new inoculum MAS sludge was introduced in

376   phase VII due to the increased NOB population (VSS data in Figure S1). By applying

377   a longer contact time (Phase VIII) and a higher FNA level (Phase IX) on flocs and

378   carriers, the NAR and effluent NO_3^- returned to low levels (Figure 2). The

379   investigated suppression treatment conditions in Phase V-IX yielded similar TN

380   removal around 60%. Hence, from the economic point of view (detailed in the

381   following discussion), we gradually released the suppression treatment conditions in

382   latter phases (X-XII).

383

384   The suppression treatment conditions for final Phase XII were FNA 2.0 mg HNO_2-

385   N/L + 4 h. During this phase, IFAS-FNA achieved stable performance with ~65% TN

386   removal and approximately 130 mg N/L/d TN removal rate (Figure 2B). The

387   relatively high removal efficiency and rate (Figure 2B) along with minimum N

388   oxidation by NOB (Figure S7) indicated a successful implementation of FNA-based

389   return-sludge suppression technology in mainstream PN/A process. The proposed

390   technology combining hybrid bioreactor and FNA-based return-sludge treatment has

391   better system performance than mainstream biofilm-based PN/A process, which had a

392   low TN removal efficiency (~40%) due to residual NO_2^- and NO_3^-.[46] The proposed

393   IFAS + FNA technology yielded a comparable TN removal efficiency with reported

394   values for PN/A process based on IFAS reactors (~70%), but much higher removal

395   rate than those from literature (20-55 mg N/L/d).[8,9] In fact, Wang et al.[18] found the

396   application of FNA-based return-sludge technology will potentially increase TN

397   removal efficiency from ~30% to ~60% in IFAS. The proposed IFAS + FNA

398   technology was potentially feasible under lower temperature conditions, since

399   AnAOB activity in biofilm was less affected and even adapted to low temperature (<

400   13 °C)[12] and the level range of FNA treatment became broader under low temperature.

401

402   The results in batch activity test illustrated activity distribution in flocs and carriers of

403   IFAS-FNA (Figure 3B). In general, all microorganisms preferred to grow on carriers

404   when FNA suppression was applied on separate flocs (Phase IV). FNA suppression

405   on flocs and carriers would exert inhibitory impact on the activity of both AerAOB

406   and NOB in both flocs and carriers, but more significant inhibition on NOB. It has

407   been reported that FNA at levels of 0.24-1.35 mg HNO_2^--N/L was significantly more

408   biocidal to NOB than AerAOB.[45] Different from FA treatment (Figure 3A), FNA

409   treatment on flocs and carriers has very limited effect on AnAOB activity (Figure 3B).

410   By comparing phase V to phase VI and IX, it was found that higher FNA level (from

411   2.0 to 3.0 mg N/L) and longer contact time (from 4 to 24) can help to suppress more

412   NOB activity especially on flocs (Figure 3B).

413

414   The role of FNA on NOB suppression can be further clarified in operational phase

415   VIII and XIII in IFAS-FA/FNA (Figure 1). Switching from FA treatment to FNA

416   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

442   production via NH_2OH oxidation pathway positively correlates to DO levels.[28,48] The

443   poor correlation coefficients in our setups (R^2 = 0.0075 for IFAS-FA/FNA, R^2 =0.12

444   for IFAS-FNA, details refer to Figure S8) indicated that DO was not a key factor

445   affecting N_2O emission and thus NH_2OH oxidation pathway was not likely to be the

446   main N_2O source in our study. Since both IFAS reactors were mostly operated above

447   1.0 mg O_2/L, heterotrophic denitrification was expected to play a minor role in N_2O

448   production as well. Nitrifier denitrification pathway is a nitrite-sensitive process due

449   to the fact that the expression of copper-containing NO_2^- reductase (NirK) is regulated

450   by a nitrite-sensitive transcription repressor protein.[49] The dependency of N_2O

451   production via nitrifier denitrification on the nitrite concentration was further

452   demonstrated with the aid of site preference analysis and mathematical modeling.[24,28]

453   In this study, N_2O emission factors were positively correlated with nitrite

454   accumulation (Figure 4), indicating that nitrifier denitrification was the main N_2O

455   production mechanism.

456

457   For IFAS-FA/FNA, at similar NO_2^- levels, the N_2O emission factors in the groups of

458   benchmark and FA treatment (solid symbols) were higher than those in the group of

459   FNA treatment (open symbols, Figure 4A). In terms of IFAS-FNA, we further

460   compared N_2O emission factors under different FNA treatment conditions. It was

461   found that at similar NO_2^- levels, higher FNA (2.0 and 3.0 mg HNO_2-N/L) treatment

462   would lead to lower N_2O emissions compared to benchmark and FNA level of 0.5 mg

463   HNO_2-N/L (Figure 4B). Other studies showed a stimulation of the N_2O production via

464   nitrifier denitrification by moderate nitrite concentrations of 0-50 mg N/L,[24] but an

465   inhibition by very high nitrite levels (500-1000 mg N/L).[50] The FNA levels for sludge

466   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

541   **Acknowledgement**

548

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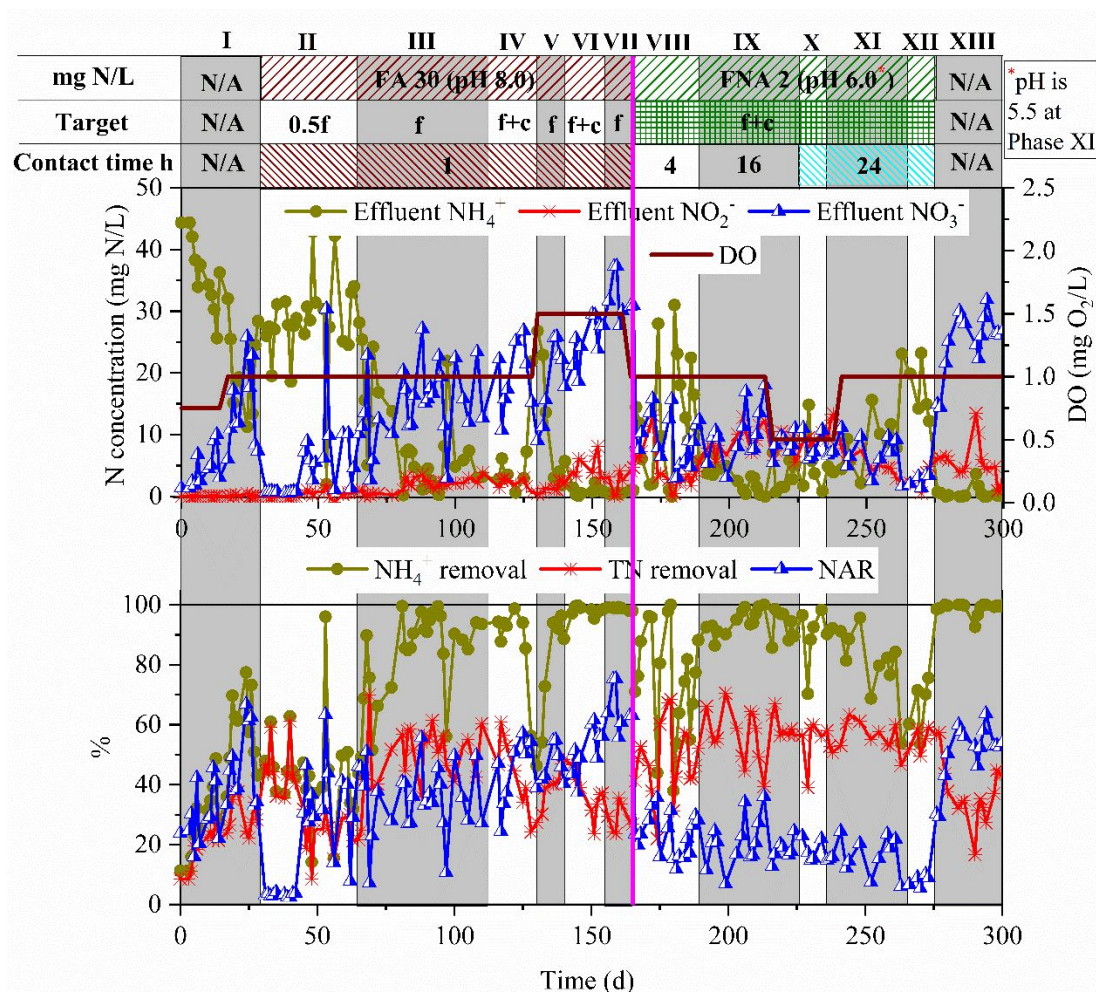732   Stage Partial Nitritation/Anammox Process. *Ecol. Eng.* **2015**, *75*, 413–420.

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.
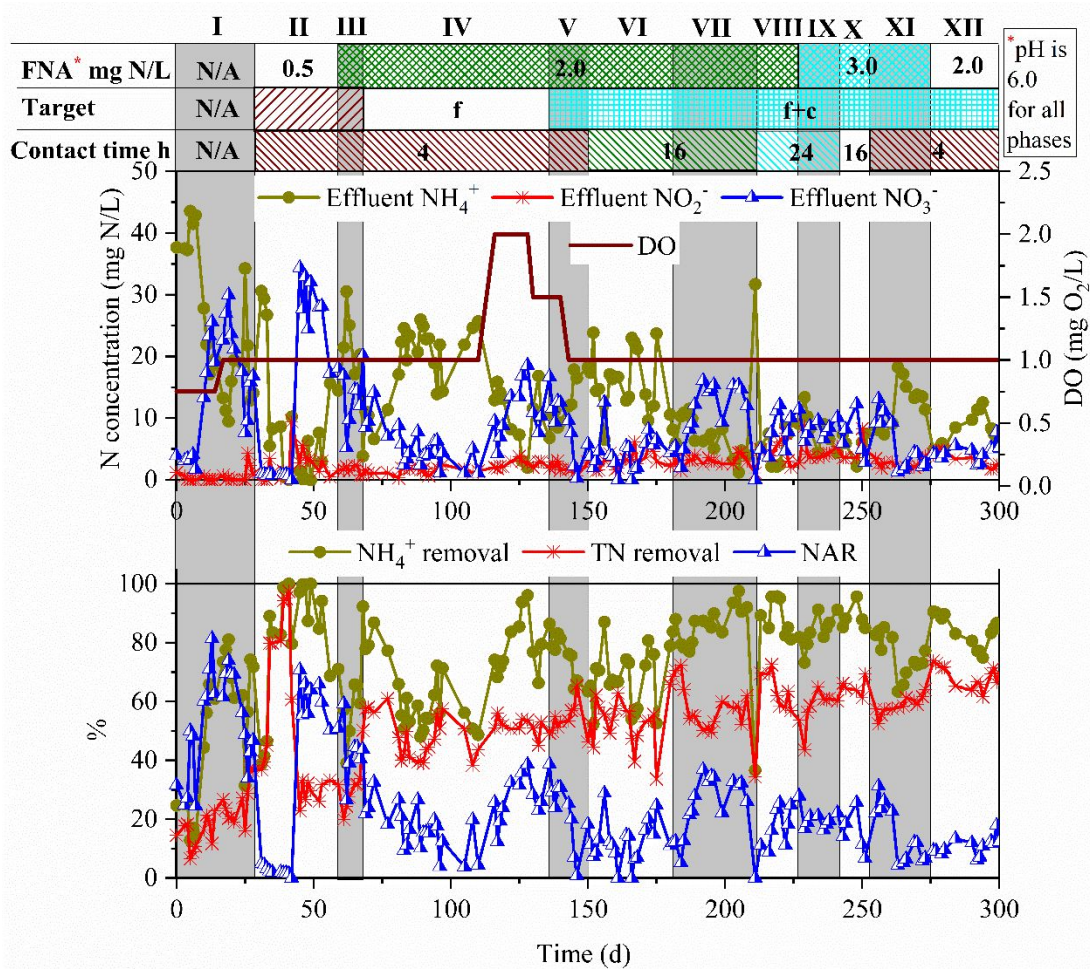
Figure 2. Reactor performance of IFAS-FNA during different operational phases. Main variables among operational phases: average DO concentrations, FNA levels, treated biomass target and contact time. Flocs and carriers are abbreviated as 'f' and 'c', respectively. A) nitrogen concentrations in effluent and average DO concentration inside reactor and B) NH$_4^+$ removal efficiency, total nitrogen (TN) removal efficiency and nitrate accumulation ratio (NAR).
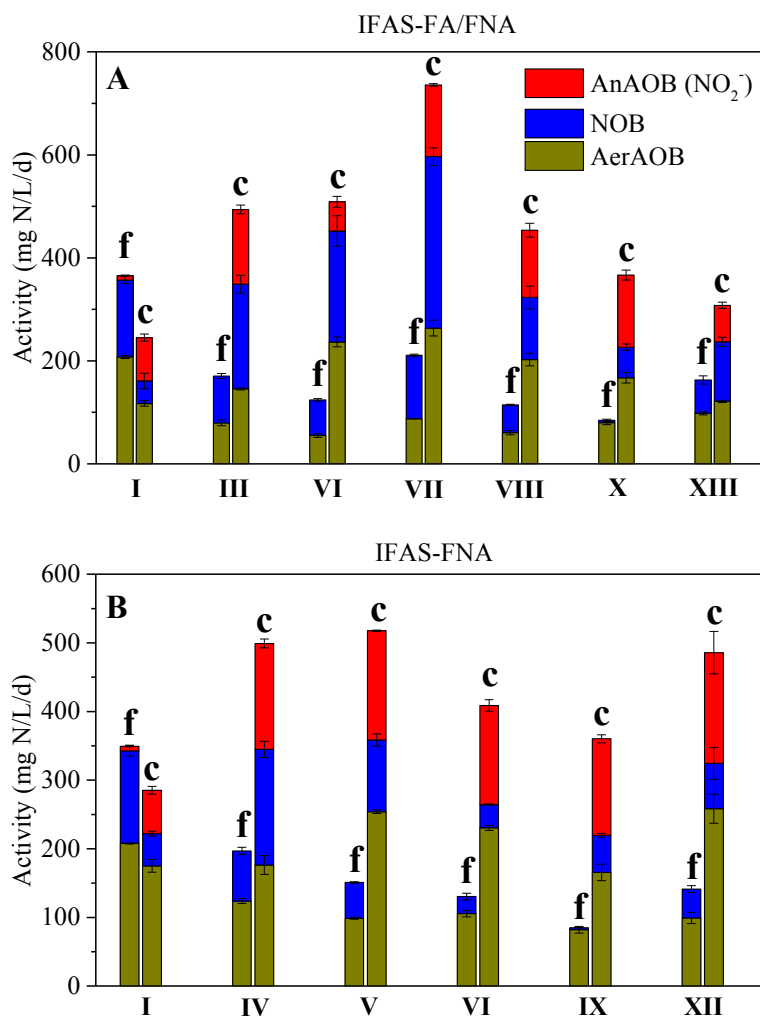
Figure 3. Dynamics of the maximum activities of AerAOB, NOB and AnAOB by flocs and carriers in batch tests fitted to IFAS-FA/FNA (A) and IFAS-FNA (B) reactors over the different operational phases. Flocs and carriers are abbreviated as 'f' and 'c', respectively.
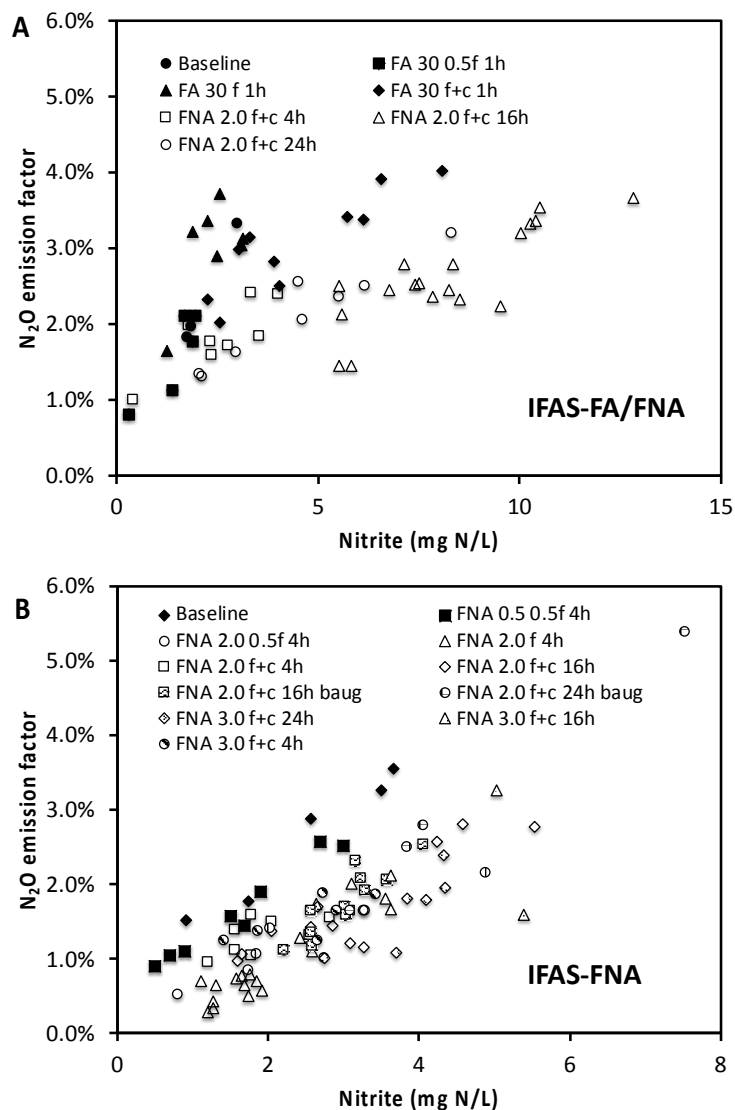
Figure 4. The impact of varying nitrite accumulations and suppression treatment conditions on N$_2$O emission factors from IFAS-FA/FNA (A) and IFAS-FNA (B). Note: each symbol legend consists of the stressor used for return sludge treatment, the stressor level in mg N/L, treated biomass with f representing flocs and c representing carriers, and the contact time for each treatment, successively. In some symbol legend, baug means the inclusion of bio-augmentation.
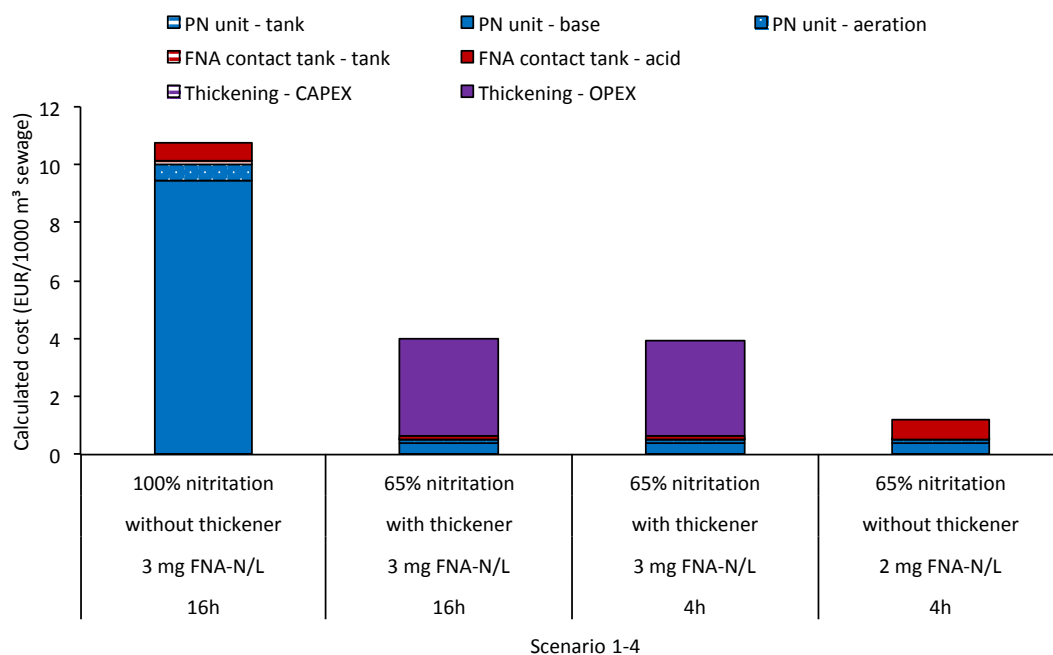
Figure 5. Calculated costs for the four proposed endogenous FNA treatment scenarios, expressed per 1000 m³ raw sewage, as extra cost compared to a baseline treatment scenario without FNA treatment step and with sidestream PN/A instead of sidestream FNA production.