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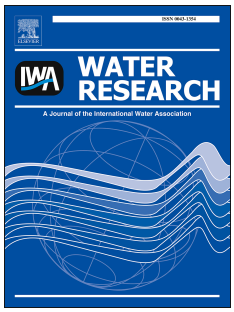

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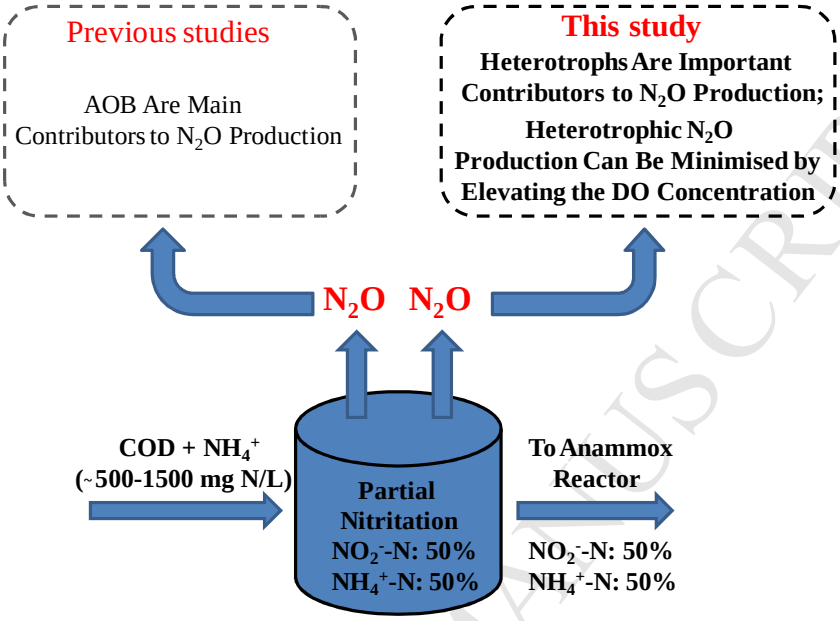
Graphical Abstract



Heterotrophic denitrification plays an important role in N_2O production from nitritation reactors treating anaerobic sludge digestion liquor

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Abstract

Nitrous oxide (N_2O) emissions from nitritation reactors receiving real anaerobic sludge digestion liquor have been reported to be substantially higher than those from reactors receiving synthetic digestion liquor. This study aims to identify the causes for the difference, and to develop strategies to reduce N_2O emissions from reactors treating real digestion liquor. Two sequencing batch reactors (SBRs) performing nitritation, fed with real (SBR-R) and synthetic (SBR-S) digestion liquors, respectively, were employed. The N_2O emission factors for SBR-R and SBR-S were determined to be 3.12% and 0.80% of the NH_4^+-N oxidized, respectively. Heterotrophic denitrification supported by the organic carbon present in the real digestion liquor was found to be the key contributor to the higher N_2O emission from SBR-R. Heterotrophic nitrite reduction likely stopped at N_2O (rather than N_2), with a hypothesised cause being free nitrous acid inhibition. This implies that all nitrite reduced by heterotrophic bacteria was converted to and emitted as N_2O. Increasing dissolved oxygen (DO) concentration from 0.5 to 1.0 mg/L, or above, decreased *aerobic* N_2O production from 2.0% to 0.5% in SBR-R, whereas aerobic N_2O production in SBR-S remained almost unchanged (at approximately 0.5%). We hypothesised that DO at 1 mg/L or above suppressed heterotrophic nitrite reduction thus reduced aerobic heterotrophic N_2O production. We recommend that DO in a nitritation system receiving anaerobic sludge digestion liquor should be maintained at approximately 1 mg/L to minimise N_2O emission.

Keywords: Nitrous oxide; Heterotrophic denitrification; Nitritation; Anaerobic digestion liquor; Free nitrous acid; Dissolved oxygen

1. Introduction

Nitrous oxide (N_2O) is not only a potent greenhouse gas, with a global warming potential of approximately 265 times stronger than carbon dioxide (CO_2) (IPCC, 2013), but also leads to the destruction of the stratospheric ozone layer (Ravishankara et al., 2009). Wastewater treatment systems have been identified as a source of N_2O. N_2O is produced during both nitrification and denitrification processes (Desloover et al., 2012; Law et al., 2012; Ahn et al., 2010; Kampschreur et al., 2009). Nitrification is a two-step process, with ammonium (NH_4^+) being first oxidized to nitrite (NO_2^-) by ammonium-oxidizing bacteria (AOB) and then further to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB). Although N_2O is not an obligatory intermediate of nitrification, it can be produced by AOB through two main pathways: i) N_2O as the final product of AOB denitrification, and ii) N_2O as the by-product of incomplete oxidation of hydroxylamine (NH_2OH, an intermediate of NH_4^+ oxidation to NO_2^-) (Ni et al., 2014; Law et al., 2012; Wunderlin et al., 2012; Yang et al., 2009). In contrast, N_2O is an obligatory intermediate of denitrification. The complete heterotrophic denitrification consists of sequential reductive reactions from NO_3^- to NO_2^-, nitric oxide (NO), N_2O and finally to nitrogen gas (N_2), carried out by heterotrophs. N_2O can accumulate when N_2O reduction is slower than N_2O production (Pan et al., 2013; Desloover et al., 2012; Wunderlin et al., 2012; Law et al., 2012).

Nitrogen removal from the anaerobic sludge digestion liquor in a side-stream process has become a common practice in wastewater treatment plants (WWTPs) (Kampschreur et al., 2008; Mulder et al., 2001). The sludge digestion liquor has a high ammonium concentration (500–1500 mg N/L) and an unfavourable chemical oxygen demand to nitrogen (COD/N) ratio for the conventional nitrification and denitrification process. One treatment option is nitritation ($NH_4^+ \rightarrow NO_2^-$) followed by the anammox process (Kampschreur et al., 2008; van

Dongen et al., 2001). The nitritation process converts around 50% of the ammonium to nitrite, thus producing a mixture of nitrite and ammonium with a molar ratio of around 1:1, which is suitable for the subsequent anammox process.

N_2O emissions from nitritation systems treating anaerobic sludge digestion liquor have been extensively reported with the results showing huge variations. For instance, the N_2O emission factors were determined to be 2.2–19.3% of the NH_4^+-N oxidized in nitritation reactors treating real digestion liquor (Pijuan et al., 2014; Gustavsson et al., 2011; Kampschreur et al., 2008). In contrast, in nitritation reactors receiving synthetic digestion liquor, the N_2O emission factors were in the range of 0.7 to 1.6% of the NH_4^+-N oxidized (Kong et al., 2013; Rodriguez-Caballero and Pijuan, 2013; Rodriguez-Caballero et al., 2013; Rathnayake et al., 2013; Ahn et al., 2011; Law et al., 2011), which are much lower than those in systems receiving real digestion liquor. This implies that it may be possible to run a nitritation reactor with a relatively low N_2O emission factor, if the underlying reasons for the higher N_2O emission factors can be identified.

While simulating the ammonium and bicarbonate concentrations in real digestion liquor, synthetic digestion liquor does not comprehensively mimic other substances such as heavy metals and various types of organic carbon, which have been shown to influence N_2O production (Kampschreur et al., 2011; Zhu and Chen, 2011; Lu and Chandran, 2010). In addition, operational conditions applied in different studies, such as dissolved oxygen (DO) concentration and pH level, were also different. These factors have also been reported to affect N_2O production (Wunderlin et al., 2012; Kampschreur et al., 2009; Tallec et al., 2008; Schulthess et al., 1994).

The aim of this study is to identify the causes for the much higher N_2O emissions from nitritation systems receiving real anaerobic sludge digestion liquor than from those receiving synthetic digestion liquor. Two lab-scale sequencing batch reactors (SBRs) performing nitritation were operated. One SBR was fed with real digestion liquor and the other with synthetic digestion liquor. N_2O emissions from the two SBRs were monitored and compared. Experiments were designed to investigate various potential causes for the higher N_2O emission from the SBR receiving real digestion liquor. A potential strategy to mitigate N_2O emission was proposed based on findings, and experimentally demonstrated.

2. Materials and methods

2.1. Characteristics of digestion liquor

The real digestion liquor was collected from the liquid drainage of the full-scale centrifuge performing dewatering of the digested sludge at a local WWTP. Its main characteristics are shown in Table 1. The synthetic digestion liquor was used to simulate real digestion liquor. Its main characteristics are also shown in Table 1.

(Approximate position for Table 1)

2.2. Reactor set-up and operation

Two lab-scale SBRs performing nitritation were operated. The return activated sludge from a domestic wastewater treatment plant in Brisbane, Australia, was used as the inoculum. One SBR (named as SBR-R) had a working volume of 4 L and was fed with real digestion liquor. The other SBR (named as SBR-S) had a working volume of 8 L and received synthetic digestion liquor. The two SBRs were both operated with a cycle time of 6 h, consisting of 25 min settling, 8 min decanting, 5 min anoxic reaction I, 5 min feeding I (aeration on), 120 min

aerobic reaction I, 35 min anoxic reaction II, 5 min feeding II (aeration on), 120 min aerobic reaction II, 35 min anoxic reaction III, and 2 min sludge wasting (aeration on). In each feeding period, 0.5 L of real digestion liquor and 1 L of synthetic digestion liquor were pumped into SBR-R and SBR-S, respectively, which resulted in a hydraulic retention time (HRT) of 24 h in both SBRs. In each cycle, 91 and 182 mL of mixed liquor were wasted from SBR-R and SBR-S, respectively, giving rise to a sludge retention time (SRT) of 11 days in both SBRs. The reactors were mixed using a magnetic stirrer at 250 rpm in all phases except for the settling and decanting phases. The mixed liquor temperature was controlled at 33 ± 1 °C using a water jacket, mimicking the temperature typical for the reactors treating digestion liquor at full-scale WWTPs. During the feeding, aerobic reaction and wasting phases, aeration was supplied with constant air flow rates, leading to DO concentrations between 0.4 and 0.6 mg/L (0.5 mg/L on average) in both reactors. The real and synthetic digestion liquors had a pH of 7.6 and 8.0, respectively. As such, the pH in the SBR-R and SBR-S increased to around 7.1 and 7.4, respectively, after feeding and then dropped gradually with ammonium oxidation during a typical cycle. A $NaHCO_3$ solution (1 M) was added automatically using a programmable logic controller (PLC) when pH dropped below a pre-determined pH set-point of 6.4. During the settling phase, biomass settling caused a N_2O concentration gradient across the SBR columns. Therefore, anoxic reaction I was introduced after decanting to equilibrate the N_2O concentration across the SBR columns by mixing, in order to determine N_2O production during the settling phase. Anoxic reactions II and III were introduced to mimic the full-scale nitritation reactors, where the aeration is generally discontinuous (Gustavsson et al., 2011; Kampschreur et al., 2008). In full-scale operations, the volumetric ammonium loading rate may vary with time, and thus different aerobic time may be required to achieve a constant ammonium conversion ratio. Consequently, anoxic time is often included to keep the cycle time constant.

The gas and liquid phase N_2O in the two SBRs were measured and compared every 3–4 days using on-line gas analysers and liquid microsensors, further described in section 2.6. Cycle studies in the two SBRs were carried out every week by analysing the ammonium, nitrite and nitrate concentrations with a sampling interval of 30 min throughout the 6 h cycle. The mixed liquor volatile suspended solids (MLVSS) concentrations were monitored once a week. Fluorescence in-situ hybridization (FISH) was performed to examine the microbial composition of the two SBRs while both achieved stable performance. The sampling and measurement procedures are as described in section 2.7.

2.3. Batch tests to investigate factors leading to higher N_2O emission from SBR receiving real digestion liquor

Based on the N_2O results obtained from the two SBRs, we proposed the following potential causes for the higher N_2O emission from SBR-R than from SBR-S: i) lower pH in real digestion liquor resulted in the higher N_2O emission, ii) lower copper concentration in real digestion liquor led to the higher N_2O emission, iii) COD supporting heterotrophic denitrification contributed to the higher N_2O emission from SBR-R, iv) possible inhibitory substances in real digestion liquor, which might affect AOB metabolism leading to increased N_2O emission from SBR-R. The inhibitory substances could be divided into two categories: a) non-adsorbable, soluble substances, therefore would stay in the liquid phase, b) adsorbable substances, which would adsorb into sludge. Five tests (T1–T5) were designed to test these potential causes, as summarised in Table 2. In each test, N_2O was monitored over two consecutive cycles (6 h each) and N_2O emission factors in each cycle were determined. The operating conditions of each cycle in these tests were identical to the normal conditions (see section 2.2), except that the conditions specified in Table 2 were applied. The tests were done in duplicate.

(Approximate position for Table 2)

2.4. Batch tests to investigate anoxic N_2O reduction by heterotrophs

The tests in Table 2 revealed that heterotrophic denitrification was likely a key contributor to the higher N_2O emission in SBR-R. We subsequently designed and carried out two tests to investigate nitrogen conversions during heterotrophic denitrification. Both tests were done directly in SBR-R. One test was performed with the same operating conditions as specified in section 2.2. The other test was conducted under the same conditions with the exception that N_2 stripping was applied at 2 L/min during the anoxic phase. For each test, the anoxic emitted N_2O in the gas phase and the anoxic accumulated N_2O in the liquid phase were monitored over two consecutive cycles. The net anoxic N_2O production (emitted amount + accumulated amount) in both cases was then compared. As the N_2 sparging would actively strip off the dissolved N_2O rendering it unavailable (or at least less available) for further reduction to N_2, the comparison of anoxic N_2O production with and without N_2 sparging would reveal the extent of anoxic N_2O reduction ($N_2O \rightarrow N_2$) during normal anoxic conditions (no stripping).

2.5. DO control as a potential N_2O mitigation strategy

Based on the N_2O results obtained from the above batch tests, experiments were designed and carried out to investigate if aerobic N_2O production from SBR-R could be reduced by increasing DO levels. To this end, the average aerobic DO levels in SBR-R were increased from 0.5 mg/L (i.e. normal operation) to 0.7, 1.0, 1.8 and 3.0 mg/L, respectively. Correspondingly, the average aerobic DO levels in SBR-S were also increased to 0.7, 1.0, 1.8 and 3.0 mg/L, respectively, as control tests. At each DO level, N_2O was monitored in two consecutive cycles and net aerobic N_2O production was determined

2.6. N_2O monitoring and emission calculation

The gas phase N_2O concentration was analysed with an infrared analyser (URAS 14 Advance Optima, ABB) and data was logged every 3 s. A t-shaped tubing joint was fitted onto the gas sampling tube connecting the gas outlet of the reactor and the gas analyser. This allowed the excess gas flow to escape from the system during aerated phases and gas influx into the system during non-aerated phases. During aerated phases, the flow rate of the analyser was always lower than the total flow rate in the reactor. The liquid phase N_2O was measured online using a N_2O microsensor (N_2O-100, Unisense A/S. Aarhus, Denmark). A two-point calibration of the microsensor was done before and after each measurement.

The net N_2O produced (mg N_2O-N) in the SBRs during each phase in a cycle was calculated using Eqs. (1) and (2):

$$\text{Net } N_2O \text{ produced} = M_{N_2O\text{-}N,\ liq,end} - M_{N_2O\text{-}N,\ liq,begin} + N_2O \text{ emitted} \quad (1)$$

$$N_2O \text{ emitted} = \Sigma((C_{N_2O\text{-}N,off\text{-}gas} - C_{N_2O\text{-}N,air}) \times Q_{air} \times \Delta t) \quad (2)$$

where $M_{N_2O\text{-}N,\ liq,end}$=mass of dissolved N_2O at the end of the phase (mg N_2O-N); $M_{N_2O\text{-}N,\ liq,begin}$=mass of dissolved N_2O at the beginning of the phase (mg N_2O-N); $C_{N_2O\text{-}N,off\text{-}gas}$=$N_2O$ concentration in the off-gas of the SBR (mg N_2O-N/L); $C_{N_2O\text{-}N,air}$=N_2O concentration in the air (mg N_2O-N/L); Q_{air}=the flow rate of the aeration during an aerated phase (L/h) or gas flow rate through the analyser during a non-aerated phase (L/h); Δt=time interval over which the off-gas N_2O concentration was recorded. N_2O concentration in the off-gas in mg N_2O-N/L was calculated from ppmv (parts per million volume) recorded by the analyser based on the ideal gas law at standard pressure (101.3 kPa) and a temperature of 25 °C (i.e. the temperature of the gas sample).

The N_2O emission factor (mg N_2O-N/mg NH_4-N oxidized) was determined based on the total amount of N_2O emitted in the entire 6 h cycle relative to the total ammonium conversion in the particular cycle (Law et al., 2011; Ahn et al., 2010). N_2O emission rate (mg N_2O-N/h) was calculated by multiplying the gas phase N_2O concentration by the known gas flow rate. The volumetric N_2O emission rate (mg N_2O-N/L/h) was calculated by dividing the N_2O emission rate by the volume of the mixed liquor in each SBR.

2.7. Chemical and microbial analyses

Mixed liquor samples were taken using a syringe and immediately filtered through disposable Millipore filter units (0.22 µm pore size) for the analyses of ammonium, nitrite, nitrate and SCOD. The ammonium, nitrite and nitrate concentrations were analyzed using a Lachat QuikChem8000 Flow Injection Analyzer (Lachat Instrument, Milwaukee, Wisconsin). The MLVSS, SCOD and TCOD concentrations were determined according to the standard methods (APHA, 1998). The HCO_3^- concentration was calculated from the total inorganic carbon (TIC) as a function of pH and temperature (Metcalf and Eddy, 2003). TIC was determined by the standard method at a total carbon analyser (Tekmar Dohrmann DC-190). The metal concentration was measured using inductively coupled plasma optical emission spectrometry (Perkin Elmer ICP-OES Optima 7300DV, Perkin Elmer, USA).

The method described by Daims et al. (2001) was used to prepare the biomass samples for FISH analysis. The following probes were used: NSO190 (Mobarry et al., 1996), specific for Betaproteobacterial AOB; NEU (Mobarry et al., 1996), specific for *Nitrosomonas* spp.; Nsv443 (Mobarry et al., 1996), specific for *Nitrosospira* spp.; NIT3 (Wagner et al., 1996), specific for *Nitrobacter* spp.; Ntspa662 (Daims et al., 2000), specific for the *Nitrospira* genus; and EUB-mix (EUB338, EUB338-II, and EUB338-III) (Daims et al. 1999), covering most

bacteria. All the probes were either labelled with FITC, or Cy3, or Cy5. FISH-probed samples were visualised using a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Jena, Germany) and images were collected using a Zeiss Neofluar ×40/1.3 oil objective. FISH images were analysed using DAIME version 1.3 to determine the biovolume fraction of the bacteria of interest.

3. Results and discussion

3.1. Reactor performance and N_2O emissions

The two SBRs achieved stable performance two months after their start-up. In both reactors, 50 ± 5% of the NH_4^+-N in the feed was converted to NO_2^--N at the end of each cycle, resulting in both effluent ammonium and nitrite concentrations of 430 ± 40 mg N/L in SBR-R, and 500 ± 50 mg N/L in SBR-S (Figs. 1A and B). Nitrate was below 10 mg N/L at all times in both reactors (Figs. 1A and B). The effluent TCOD and SCOD were determined to be 245 ± 16 and 240 ± 14 mg/L, respectively, for SBR-R, and 25 ± 3 and 16 ± 4 mg/L, respectively, for SBR-S. The other characteristics of the effluent of SBR-R and SBR-S are shown in Table 1. Microbial community analyses with FISH revealed that the dominant population of AOB in both SBR-R and SBR-S was *Nitrosomonas*, at 65 ± 5% and 80 ± 3% of the entire microbial communities, respectively. In contrast, NOB were not detected (< 1%) in either reactor, which supported the negligible nitrate production. The remaining fractions were believed to be heterotrophs, which were at 35 ± 5% and 20 ± 3%, respectively, in SBR-R and SBR-S. The higher fraction of heterotrophs in SBR-R could be attributed to the presence of COD in the real digestion liquor, whereas in comparison no COD existed in the synthetic digestion liquor and the heterotrophs in SBR-S could only grow utilizing the bacterial lysate (Hao et al., 2009). The MLVSS concentrations in SBR-R and SBR-S were 610 ± 30 and 400

275 ± 30 mg/L, respectively. The higher MLVSS concentration in SBR-R relative to SBR-S was
276 probably again due to the COD loading to SBR-R.
277
278 (Approximate position for Fig. 1)
279
280 Figs. 1C and D show that N_2O production occurred during both non-aerated (settling and
281 anoxic phases) and aerated phases. In both SBRs, the liquid phase N_2O started accumulating
282 while entering the anoxic phase due to the absence of active stripping, reaching 0.40 and 0.13
283 mg N_2O-N/L in SBR-R and SBR-S, respectively, towards the end of the anoxic phases. The
284 dissolved N_2O was subsequently stripped into the gas phase in the following aerobic phase,
285 resulting in peaks of volumetric N_2O emission rate at around 3.8 and 1.9 mg N/h/L in SBR-R
286 and SBR-S, respectively, at the start of each aerobic phase. In contrast to the non-aerated
287 phases, N_2O produced in aerobic phases was immediately stripped. Figs. 1C and D clearly
288 show that the volumetric N_2O emission rate and liquid phase N_2O concentration in SBR-R
289 were much higher than those in SBR-S. The N_2O emission factor in SBR-R was determined
290 to be 3.12 ± 0.16%, which was much higher than that (0.80 ± 0.09%) in SBR-S, as also
291 summarised in Table 3. Further analyses indicate that most of the N_2O was produced in the
292 aerobic phase in both SBRs, accounting for around 65% of the net N_2O production in the
293 typical cycles (Figs. 1E and F).
294
295 (Approximate position for Table 3)
296
297 **3.2. Identifying key contributing factors for higher N_2O emission from SBR receiving**
298 **real digestion liquor**

299 In order to investigate the reasons for the higher N_2O emission from SBR-R than from SBR-S,
300 five tests were performed with results presented in Fig. S1 and further summarized in Table 3.
301
302 The N_2O emission factor in T1 (synthetic digestion liquor as feed to SBR-R; $1.11 \pm 0.03\%$)
303 was comparable ($p>0.05$) to that in T2 (SBR-R effluent + NH_4^+ + HCO_3^- as feed to SBR-R;
304 $1.22 \pm 0.08\%$), and was only slightly higher ($p<0.05$) than during normal operation for SBR-
305 S ($0.80 \pm 0.09\%$). This indicates that a slightly lower pH (7.6 vs. 8.0), the potential non-
306 biodegradable inhibitory substances, and the lower copper concentration (0.01 vs. 0.20 mg/L)
307 in real digestion liquor were not the main factors leading to the higher N_2O emission from
308 SBR-R. Since the N_2O production in SBR-S was primarily due to the AOB-related pathways
309 (Law et al., 2011), N_2O production in T1 and T2 were believed to be due to AOB. N_2O
310 emission factor increased substantially from $1.11 \pm 0.03\%$ to $2.48 \pm 0.08\%$ while using
311 synthetic digestion liquor + milk powder (T3) instead of synthetic digestion liquor (T1) as the
312 feed to SBR-R. This suggests that COD supporting heterotrophic denitrification was likely
313 the main contributor to the higher N_2O emission from SBR-R than from SBR-S, and that the
314 potential biodegradable inhibitory substances in real digestion liquor did not play a dominant
315 role in N_2O production from SBR-R. The slightly lower ($p<0.05$) N_2O emission factor in T3
316 ($2.48 \pm 0.08\%$) than under the normal operation of SBR-R ($3.12 \pm 0.16\%$) might be because
317 the milk powder could not be utilized as efficiently as the COD present in real digestion
318 liquor, thus a lower N_2O emission in T3 was observed. Also, the liquid phase N_2O only
319 accumulated to approximately 0.10 mg N_2O-N/L in T1 during the anoxic phase (see Fig. S1-
320 A). In contrast, the liquid phase N_2O accumulated to up to 0.50 mg N_2O-N/L in T3 over the
321 anoxic phase (see Fig. S1-C). Given the fact that the only difference between the feed in T1
322 and in T3 was organic carbon, heterotrophic denitrification was most likely the primary
323 contributor to the anoxic N_2O production.

In SBR-S, the use of the SBR-R effluent (T4) resulted in a similar ($p>0.05$) N_2O emission factor ($0.98 \pm 0.09\%$) to under normal operation ($0.80 \pm 0.09\%$). This confirms that the real digestion liquor did not contain non-adsorbable, non-biodegradable, soluble inhibitory substances that would significantly cause N_2O emission. In contrast, a significant increase in N_2O emission (from $0.98 \pm 0.09\%$ to $1.91 \pm 0.04\%$) was observed when real digestion liquor (T5) rather than SBR-R effluent + NH_4^+ + HCO_3^- (T4) was used as the feed to SBR-S. This supports that COD-related heterotrophic denitrification was likely mainly responsible for the higher N_2O emission from SBR-R. However, the N_2O emission factor in SBR-S ($1.91 \pm 0.04\%$) was lower relative to that in SBR-R ($3.12 \pm 0.16\%$) while the two reactors received the real digestion liquor. This could be due to the fact that the heterotrophs in SBR-S had a lower COD utilization efficiency in comparison to the heterotrophs in SBR-R, thereby leading to a lower N_2O emission.

Previous studies in nitritation systems treating anaerobic sludge digestion liquor indicated that AOB were the main contributors to N_2O production (Wunderlin et al., 2013; Gustavsson et al., 2011; Kampschreur et al., 2008). In contrast, the above batch test results demonstrated that the COD in real digestion liquor contributed significantly to the N_2O emission, strongly suggesting the contribution of heterotrophic bacteria to N_2O production in nitritation systems receiving real digestion liquor.

3.3. Anoxic N_2O reduction in SBR receiving real digestion liquor

Net anoxic N_2O production with and without N_2 sparging in SBR-R was compared in order to qualitatively investigate the extent of N_2O reduction in SBR-R. The net anoxic N_2O production in the presence of N_2 sparging (Fig. S1-F) was determined to be 0.68 ± 0.02 mg

349 N_2O-N/L, which was comparable ($p>0.05$) to the net anoxic N_2O production without N_2
350 sparging (0.73 ± 0.10 mg N_2O-N/L). This indicates that anoxic N_2O reduction probably did
351 not occur in SBR-R. In other words, all nitrite reduced by heterotrophs in this reactor was
352 converted to N_2O rather than N_2. If the sludge in SBR-R did reduce N_2O anoxically, the
353 amount of N_2O reduced should be substantially higher in the absence of N_2 (much higher
354 availability of liquid N_2O) than in the presence of N_2. The enhanced N_2O reduction without
355 N_2 sparging would lead to a low net N_2O production in this case, which contradicts our
356 experimental results. One possible explanation for the cessation of N_2O reduction is the
357 inhibition of N_2O reduction by free nitrous acid (FNA). Zhou et al. (2008) demonstrated that
358 N_2O reduction was completely inhibited by FNA when the FNA concentration was greater
359 than 0.004 mg HNO_2-N/L. Based on the pH, nitrite concentration and temperature in SBR-R,
360 the FNA concentrations in SBR-R were determined according to Anthonisen et al. (1976), to
361 have varied between 0.05 and 0.32 mg HNO_2-N/L during a typical cycle. While the
362 inhibitory threshold reported in Zhou et al. (2008) was for a denitrifying phosphorus removal
363 sludge and hence may not be directly applicable to our sludge, the FNA range in our reactors
364 was 1 – 2 orders of magnitude higher, and is expected to be seriously inhibitory to N_2O
365 reduction by the heterotrophic bacteria in the sludge.
366

367 **3.4. Effect of DO concentrations on aerobic N_2O production**

368 The results reported above suggest that i) the increased N_2O was due to heterotrophic nitrite
369 reduction and ii) N_2O produced was not reduced to N_2 by the sludge likely due to FNA
370 inhibition. With the above, we hypothesised that N_2O emission could be reduced by
371 inhibiting nitrite reduction. A higher DO would help to achieve this goal (Hiatt and Grady,
372 2008). Therefore, a series of tests at different DO levels were conducted to: i) further verify

that heterotrophic reduction was primarily responsible for the higher N_2O emission in SBR-R and ii) develop an N_2O mitigation strategy.

The effect of DO concentration on aerobic N_2O production in both SBR-S and SBR-R is shown in Fig. 2 and Fig. S2. The aerobic N_2O production in SBR-S was not significantly affected ($p>0.05$) by the tested DO concentrations (between 0.5 and 3.0 mg/L) and always remained at $0.52\% \pm 0.02$ of the NH_4^+-N oxidized (see Fig. 2). This indicates that DO did not have a significant effect on the AOB-induced aerobic N_2O production among the tested DO levels (0.5-3.0 mg/L), given the fact that AOB play a dominant role in N_2O production in SBR-S (Law et al., 2011). Fig. S2-(A-D) indicates that the aerobic N_2O production rate increased with increased DO concentration. Fig. S2-(A-D) also indicates that the specific AOB activity increased with increased DO concentration, as reflected by the fact that a shorter aerobic duration was required to achieve 50% ammonium conversion. This suggests that the increased specific AOB activity may be the reason for the increased aerobic N_2O production rate. This is in agreement with that reported by Law et al. (2011). Unfortunately, the specific AOB activity could not be accurately determined due to the varying pH (between 6.4 and 7.4) during a typical cycle, which would result in varying specific AOB activity (Law et al., 2011). In contrast, the aerobic N_2O production in SBR-R decreased substantially (from $2.00 \pm 0.05\%$ to $0.68 \pm 0.03\%$ of the NH_4^+-N oxidized) ($p<0.05$) when DO increased from 0.5 to 1.0 mg/L, and then remained almost unchanged ($p>0.05$) with the further increase in DO level up to 3 mg/L ($0.54 \pm 0.13\%$ of the NH_4^+-N oxidized at a DO level of 3.0 mg/L). The decreased N_2O emission at the higher DO levels was most likely due to the fact that higher DO inhibits heterotrophic nitrite reduction (Hiatt and Grady, 2008), thereby decreasing N_2O production. Although a higher DO is also expected to inhibit N_2O reduction, this does not necessarily add further to the already strong FNA-related inhibition of N_2O reduction.

The decreased N_2O emission at higher DO levels further confirms our finding that COD-supported heterotrophic denitrification played a vital role in the N_2O production in a nitritation system receiving real digestion liquor. The comparable ($p>0.05$) aerobic net N_2O production among SBR-S ($0.52 \pm 0.02\%$ of the NH_4^+-N oxidized), SBR-R at DO=1.0 mg/L ($0.68 \pm 0.03\%$ of the NH_4^+-N oxidized) and SBR-R at DO=3.0 mg/L ($0.54 \pm 0.13\%$ of the NH_4^+-N oxidized) indicates that, heterotrophic nitrite denitrification in SBR-R was largely suppressed when DO concentration was higher than 1.0 mg/L.

(Approximate position for Fig. 2)

3.5. Reducing N_2O emission in nitritation systems receiving nitrogen-rich wastewater

This study showed, for the first time, that COD-supported heterotrophic denitrification plays an important role in the N_2O production in nitritation systems. The study further showed that increasing DO from 0.5 to 1.0 mg/L (or above) significantly decreases aerobic N_2O production (from $2.00 \pm 0.05\%$ to $0.68 \pm 0.03\%$ and $0.54 \pm 0.13\%$) due to the suppression of heterotrophic nitrite reduction. Therefore, operating a nitritation reactor at a DO of 1 mg/L or above is a potential strategy for reducing N_2O emission from nitritation systems receiving nitrogen-rich wastewater.

While increasing DO to mitigate N_2O emission, energy consumption will increase accordingly, thus increasing indirect CO_2 emission. To evaluate the total operational carbon footprint of implementing the N_2O mitigation strategy via increasing DO, we performed a desktop scaling-up study on a full-scale WWTP with a population equivalent (PE) of 350,000. We assumed that an SBR with a working volume of 250 m^3 was used to treat the anaerobic sludge digestion liquor at an average ammonium load of 250 kg NH_4^+-N/d. The study was

performed with DO concentrations of 0.5 and 1.0 mg/L, based on the N_2O emission data obtained in this study. The total operational carbon footprints in the two cases are compared in Table 4. With the increase of DO from 0.5 to 1.0 mg/L, the total operational carbon footprint is estimated to decrease by 60%. The decreased operational carbon footprint can be attributed to the decreased N_2O emission despite the additional CO_2 emission associated with the increased aeration. Therefore, mitigating N_2O emissions via increasing DO could reduce the total operational carbon footprint, indicating it has a potential to be developed into a practical strategy. However, higher DO would also increase energy costs. The exact economic outcome will therefore depend on the price tag for carbon emissions. With the current energy price in Australia at \$0.16 /kWh, the costs would be balanced by a carbon price of \$2.4 /tonne CO_2-eq.

(Approximate position for Table 4)

4. Conclusions

The causes for the much higher N_2O emissions from nitritation systems receiving real anaerobic sludge digestion liquor than from those receiving synthetic digestion liquor were investigated. The main conclusions are:

- Heterotrophic denitrification supported by the organic carbon present in real digestion liquor is the key contributor to the higher N_2O emission from nitritation systems receiving real anaerobic digestion liquor.
- Heterotrophic denitrification plays an important role in N_2O emission from nitritation systems receiving anaerobic sludge digestion liquor.
- Heterotrophic nitrite reduction in nitritation systems receiving anaerobic digestion

liquor likely stopped at N_2O (rather than N_2), with a hypothesised cause being free nitrous acid inhibition.

- DO at 1 mg/L or above suppress heterotrophic nitrite reduction thus reduce aerobic heterotrophic N_2O production. We recommend that DO in a nitritation system receiving anaerobic sludge digestion liquor should be maintained at approximately 1 mg/L to minimise N_2O emission.

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Table 1 - Characteristics of the influent and effluent of both SBR-R and SBR-S (with standard errors where applicable)

Parameter	Influent of SBR-R	Influent of SBR-S	Effluent of SBR-R	Effluent of SBR-S
NH_4^+-N (mg/L)	861 ± 13	1,000	430 ± 40	500 ± 50
HCO_3^- (mg/L)	3,300 ± 36	4,347	Not determined	Not determined
Total COD (TCOD) (mg/L)	345 ± 15	Below detection limit	245 ± 16	25 ± 3
Soluble COD (SCOD) (mg/L)	285 ± 6	Below detection limit	240 ± 14	16 ± 4
Cu (mg/L)	0.01 ± 0.01	0.20	0.01 ± 0.01	0.07
Iron (mg/L)	1.65 ± 0.62	0.52	0.39 ± 0.16	0.24
Zn (mg/L)	0.03 ± 0.01	0.25	0.01 ± 0.01	0.08
Mn (mg/L)	0.03 ± 0.01	0.71	0.01 ± 0.01	0.25
Co (mg/L)	0.02 ± 0.01	0.20	0.02 ± 0.01	0.07
As (mg/L)	0.02 ± 0.01	Below detection limit	0.02 ± 0.01	Below detection limit
Cr (mg/L)	0.02 ± 0.01	Below detection limit	0.02 ± 0.01	Below detection limit
Ni (mg/L)	0.03 ± 0.01	Below detection limit	0.02 ± 0.01	Below detection limit
pH	7.6 ± 0.1	8.0	6.4 ± 0.1	6.4 ± 0.1

Table 2 - Summary of experimental design

Reactor	Test	Key condition	Aim
SBR receiving real digestion liquor (SBR-R)	Normal operation	Feed: Real digestion liquor	Control test
	T1	Sludge was washed using SBR-S effluent; Feed: Synthetic digestion liquor	To evaluate the effect of adsorbable substances in real digestion liquor on N_2O emission
	T2	Feed[a]: SBR-R effluent + NH_4^+ + HCO_3^-	To evaluate the effect of lower pH in the feed, possible non-biodegradable inhibitory substances and lower Cu level in real digestion liquor on N_2O emission
	T3	Feed: Synthetic digestion liquor + milk powder[b]	To evaluate the effect of COD and possible biodegradable inhibitory substances on N_2O emission
SBR receiving synthetic digestion liquor (SBR-S)	Normal operation	Feed: Synthetic digestion liquor	Control test
	T4	Sludge was washed using SBR-R effluent; Feed[a]: SBR-R effluent + NH_4^++HCO_3^-	To evaluate the effect of non-adsorbable, non-biodegradable, soluble substances in real digestion liquor on N_2O emission
	T5	Sludge was washed using SBR-R effluent; Feed: Real digestion liquor	To confirm the findings from the above tests

[a] Biodegradable COD (bCOD) was expected to be quite low in SBR-R effluent. In T2 and T4, concentrated NH_4HCO_3 and $NaHCO_3$ solution was added to the feed to make it contain a similar level of NH_4HCO_3 to that in real and synthetic digestion liquor, respectively.

[b] Milk powder resulted in a bCOD concentration of around 100 mg/L in the feed, which was to roughly mimic the bCOD concentration in real digestion liquor. 1 g milk powder contains around 0.3 g protein, 0.3 g fat and 0.4 g carbohydrate.

Table 3 - N_2O emission factors in different tests (with standard errors)

Reactor	Test[a]	N_2O Emission factor (mg N_2O-N/mg NH_4^+-N oxidized)
SBR receiving real digestion liquor (SBR-R)	Normal operation	3.12 ± 0.16%
	T1	1.11 ± 0.03%
	T2	1.22 ± 0.08%
	T3	2.48 ± 0.08%
SBR receiving synthetic digestion liquor (SBR-S)	Normal operation	0.80 ± 0.09%
	T4	0.98 ± 0.09%
	T5	1.91 ± 0.04%

[a] See Table 2 for the testing conditions

Table 4 - Comparison of operational carbon footprint from nitritation systems operated at DO concentrations of 0.5 and 1.0 mg/L on a desktop scaling-up full-scale WWTP

Parameter	DO=0.5 mg/L	DO=1.0 mg/L
Aerobic N_2O production (mg N_2O-N/mg converted-N (%))	2.00	0.68
Annual N_2O emission (kg/y)	2,870	980
CO_2 equivalent emissions for N_2O emissions (kg CO_2-eq/y)[a]	760,000	260,000
Aeration flow rate (m^3/d)[b]	96,000	104,000
Annual energy requirements for aeration (kwh/y)	93,700	102,000
CO_2 equivalent emissions for aeration (kg CO_2-eq/y)[c]	51,000	55,500
Annual operational carbon footprint (kg CO_2-eq/y)	**811,000**	**315,500**
Annual decrease in operational carbon footprint at DO=1.0 mg/L (kg CO_2-eq/y)	**(811,000-315,500)/811,000=60%**	

[a] 0.544 kg CO_2-eq/kWh (UKWIR, 2008)

[b] Aeration flow rates shown here were scaled up from lab-scale in proportion to reactor volume

[c] 265 kg CO_2-eq/kg N_2O (IPCC, 2013)

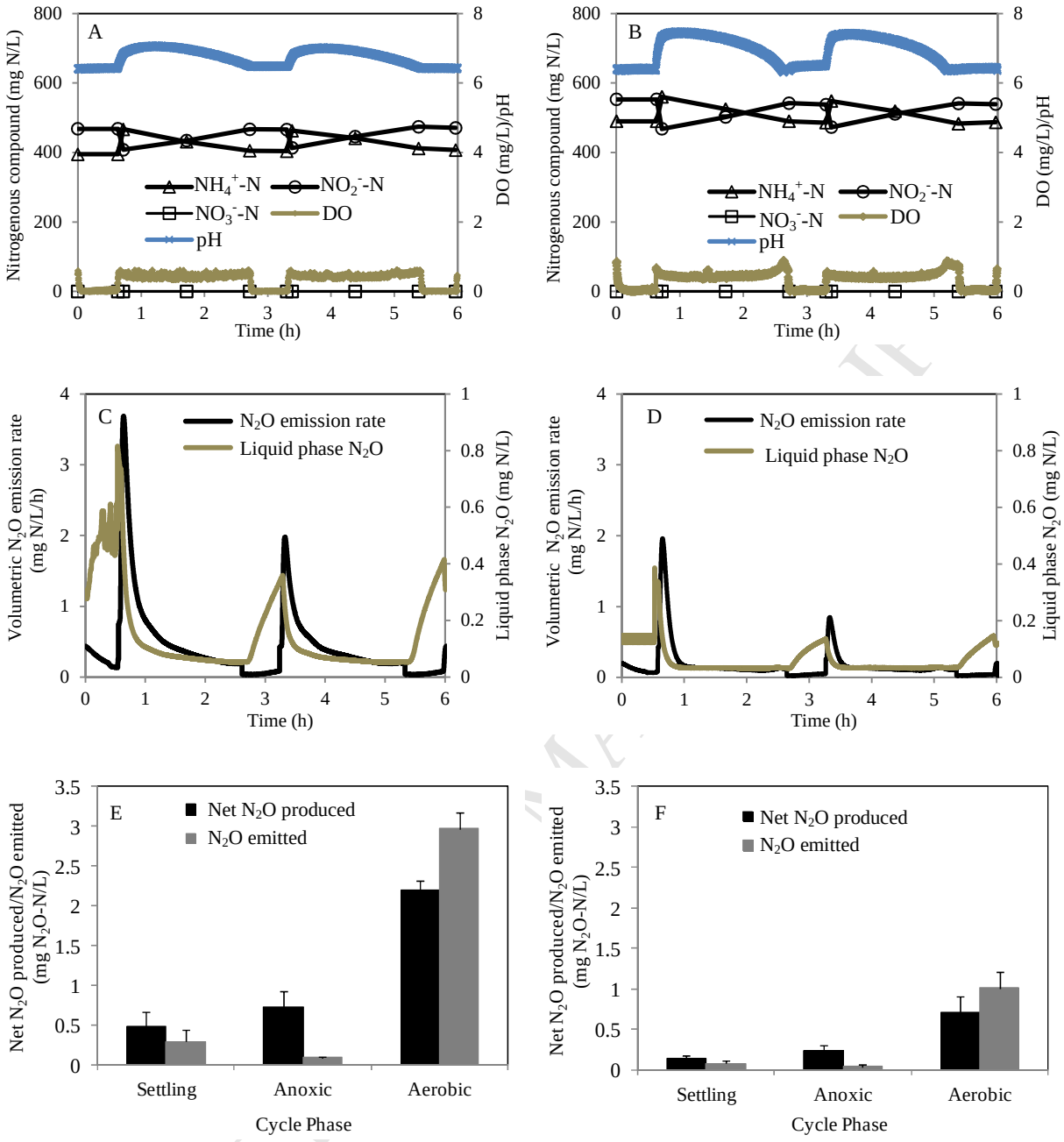

Fig. 1 - (A and B) Experimental profiles of ammonium, nitrite, nitrate, DO and pH; (C and D) Volumetric N_2O emission rate and liquid phase N_2O profiles over a typical 6 h cycle; and (E and F) Net N_2O produced and emitted during settling, anoxic and aerobic phases of a typical cycle. (A, C and E: SBR receiving real digestion liquor; B, D and F: SBR receiving synthetic digestion liquor). Cycle phases in sequence: 25 min settling, 8 min decanting, 5 min anoxic reaction I, 5 min feeding I, 120 min aerobic reaction I, 35 min anoxic reaction II, 5 min feeding II, 120 min aerobic reaction II, 35 min anoxic reaction III, and 2 min sludge wasting

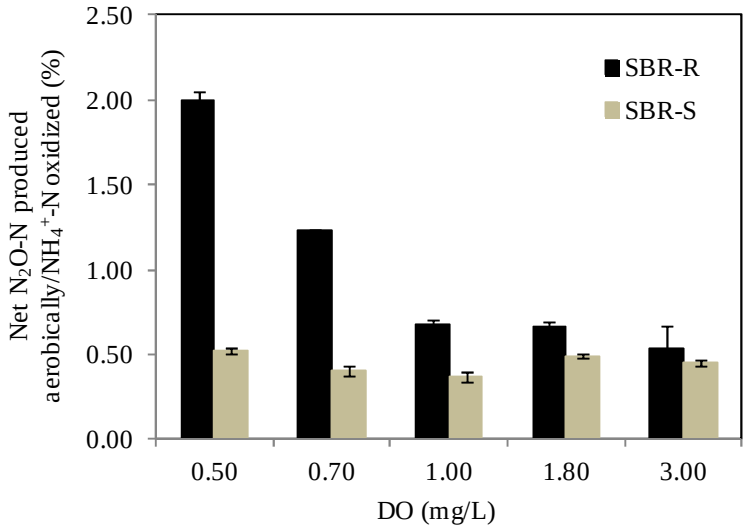

Fig. 2 - Effect of DO concentration on *aerobic* N_2O production

Highlights

- Heterotrophic denitrification plays a crucial role in N_2O emission.
- Heterotrophic nitrite reduction likely stopped at N_2O rather than N_2.
- DO at 1 mg/L or above reduce aerobic heterotrophic N_2O production.
- DO should be about maintained at 1 mg/L to minimise N_2O emission.

Heterotrophic denitrification plays an important role in N_2O production from nitritation reactors treating anaerobic sludge digestion liquor

(Supplementary material)

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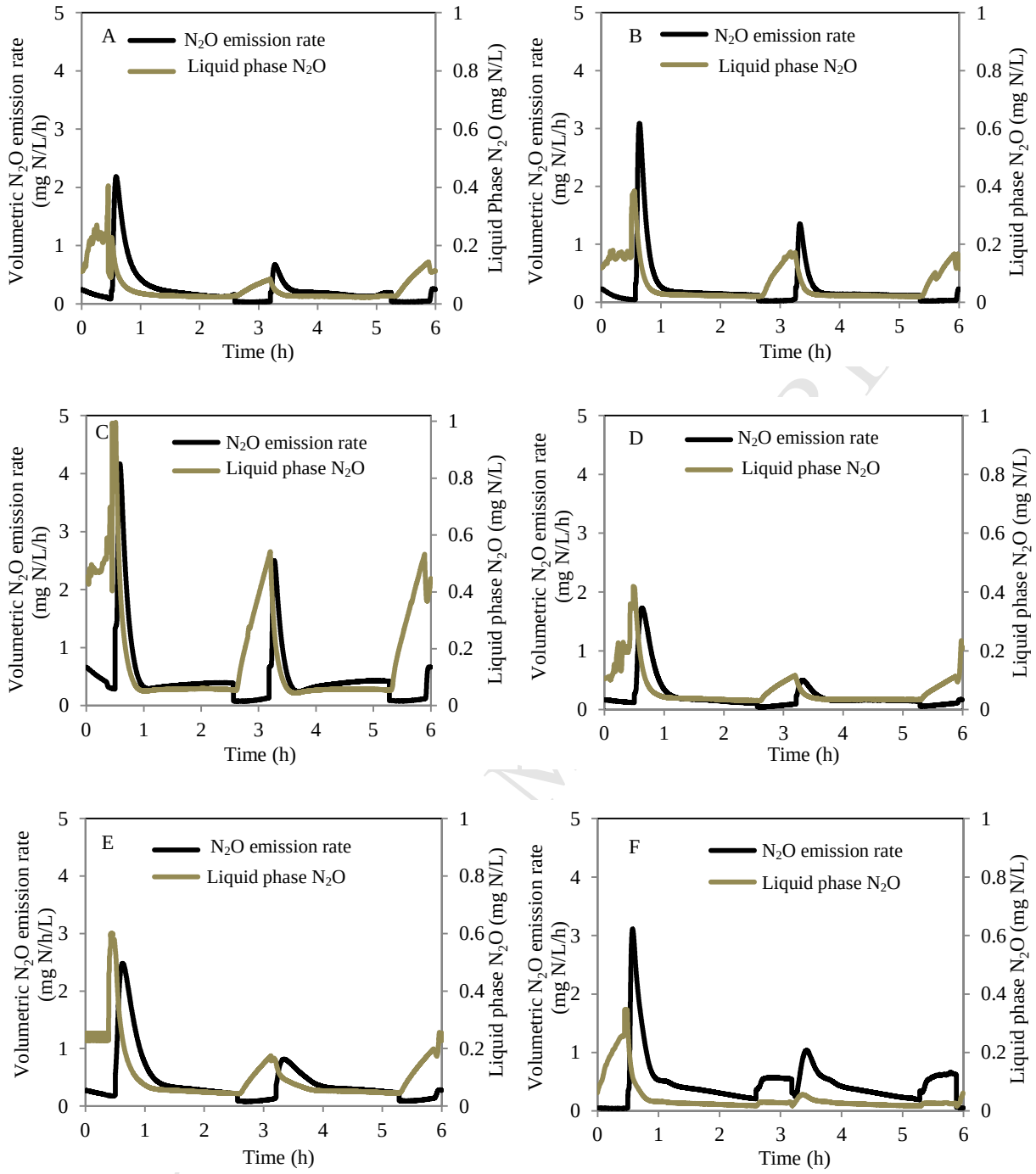
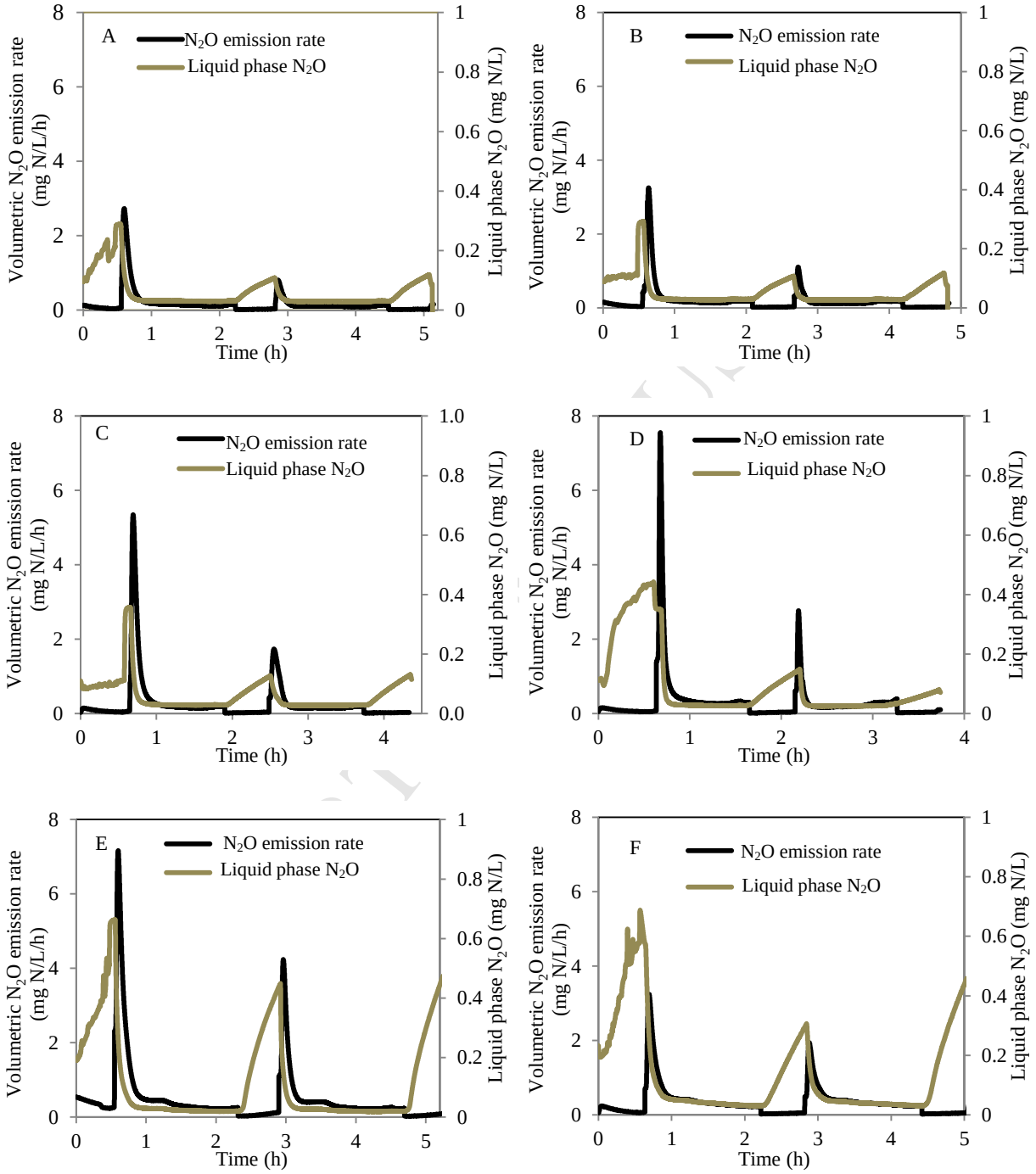


Fig. S1 - Volumetric N_2O emission rate and liquid phase N_2O profiles under different testing conditions. A: T1; B: T2; C: T3; D: T4; E: T5; F: N_2 stripping during the anoxic phase of SBR-R. See Table 2 for the explanations of T1-5. Cycle phases in sequence: 25 min settling, 8 min decanting, 5 min anoxic reaction I, 5 min feeding I, 120 min aerobic reaction I, 35 min anoxic reaction II, 5 min feeding II, 120 min aerobic reaction II, 35 min anoxic reaction III, and 2 min sludge wasting. DO and pH profiles in the cases of T2, T5 and N_2 stripping are

similar to those in Fig. 1C, and DO and pH profiles in the cases of T1, T3 and T4 are similar to those in Fig. 1D.



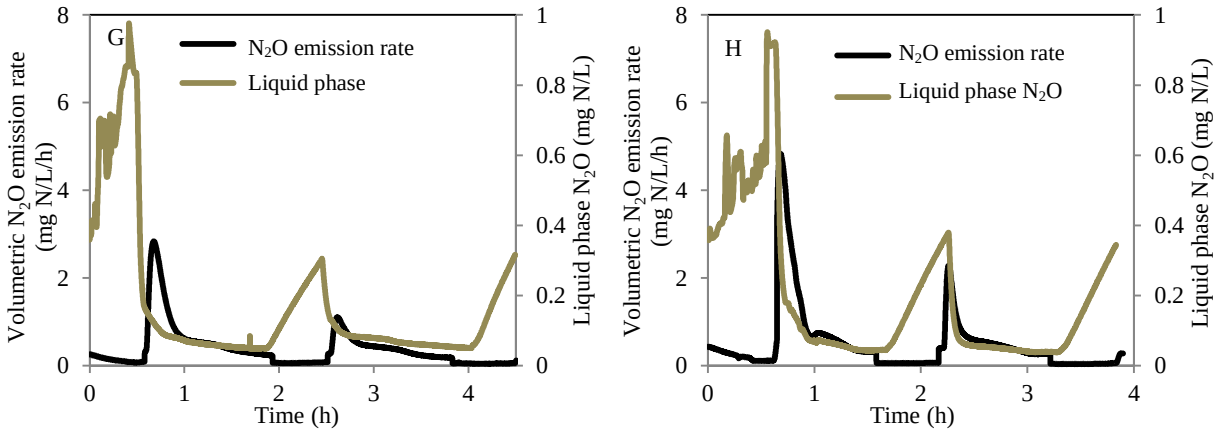


Fig. S2 - Volumetric N_2O emission rate and liquid phase N_2O profiles at different aerobic DO levels. A: DO=0.70 mg/L in SBR-S; B: DO=1.00 mg/L in SBR-S; C: DO=1.80 mg/L in SBR-S; D: DO=3.00 mg/L in SBR-S; E: DO=0.70 mg/L in SBR-R; F: DO=1.00 mg/L in SBR-R; G: DO=1.80 mg/L in SBR-R; H: DO=3.00 mg/L in SBR-R. The aerobic phase began when N_2O emission rate started increasing, and the aerobic phase ended when liquid phase N_2O started accumulation. The duration of the aerobic period decreased with increased DO levels to achieve 50% ammonium conversion and to avoid excessive aeration since the specific AOB activity increased with the increased DO levels.