




Effect of operating conditions on N₂O production in an anammox sequencing batch reactor containing granular sludge

Tiago Duarte Santos Pereira, Carla Eloísa Diniz dos Santos, Xi Lu, Hussein E. Al-Hazmi, Joanna Majtacz, Eduardo Cleto Pires , Márcia Helena Rissato Zamariolli Damianovic and Jacek Makinia 

ABSTRACT

Nitrous oxide (N₂O) is one of the gases with the greatest impact in the atmosphere due to its persistence and significant contribution to the greenhouse effect. This study provides an insight into the dynamics of N₂O production in wastewater nitrogen removal systems. A 10 L sequencing batch reactor containing enriched anammox biomass was subjected to different operational conditions, i.e., temperature, feed time, NO₂⁻/NH₄⁺ ratio and the initial concentrations of NH₄⁺ and NO₂⁻. Tests showed no significant differences in maximum N₂O production when the system was operated with a shorter feed time and no increase in the operating temperature. A higher N₂O production was observed when the initial NO₂⁻/NH₄⁺ ratio increased from 1.3 to 1.7 and 1.9. The highest initial concentration of NO₂⁻ was linked to an increase in residual N₂O at the end of the batch cycle, probably due to heterotrophic denitrifying metabolism.

Key words | ammonia-oxidizing bacteria, anammox, heterotrophic denitrification, nitrite accumulation, nitrous oxide (N₂O)

Tiago Duarte Santos Pereira (corresponding author)
Carla Eloísa Diniz dos Santos
Eduardo Cleto Pires 
Márcia Helena Rissato Zamariolli Damianovic
 Department of Hydraulics and Sanitation,
 Laboratory of Biological Processes, São Carlos
 School of Engineering,
 University of São Paulo,
 Av. João Dagnone 1100, CEP 13563-120, São
 Carlos, SP, Brazil
 E-mail: tiagodsantosp@gmail.com

Xi Lu
 Institute of Environmental Science and
 Engineering,
 Tongji University,
 1239 Siping Road, Shanghai 200092, China

Xi Lu
Hussein E. Al-Hazmi
Joanna Majtacz
Jacek Makinia 
 Faculty of Civil and Environmental Engineering,
 Gdansk University of Technology,
 ul. Narutowicza 11/12, 80-233 Gdansk, Poland

INTRODUCTION

Nitrous oxide (N₂O), a powerful greenhouse gas, persists in the atmosphere for more than 120 years and has a global warming potential 298 times greater than carbon dioxide (CO₂) (IPCC 2013). Due to its polluting potential, N₂O has attracted attention from the scientific community, which has called for efforts to identify and reduce the sources of this gas. Among the sources are wastewater treatment plants (WWTPs): N₂O is an intermediate product involved in the metabolic pathways of nitrogen removal bioprocesses. Thus, there is a growing concern about the dynamics of the production and emission of N₂O in WWTPs, which are attempting to establish more sustainable units and minimize energy consumption, greenhouse gas emissions and final sludge production (Okabe *et al.* 2011).

doi: 10.2166/wst.2019.237

The anaerobic ammonium oxidation process (anammox) is a low-cost metabolic pathway based on the oxidation of ammonia (NH₄⁺) to nitrogen gas (N₂) using nitrite (NO₂⁻) as the final electron acceptor. In WWTPs, this process is usually preceded by a partial nitrification reactor, to achieve partial oxidation of influent ammonia to nitrite (Law *et al.* 2011; Castro-Barros *et al.* 2015). The combined process can also occur in a single reactor using the deammonification process, known as completely autotrophic nitrogen removal over nitrite (the Canon process). It is only established in the presence of a mixotrophic community dominated by anammox and ammonia-oxidizing bacteria (AOB), by adopting specific operational strategies that allow the inhibition of nitrite-oxidizing bacteria (NOB) (Third *et al.* 2001; Zhang *et al.* 2012).

Because it is completely autotrophic, the Cannon process does not depend on the influent organic matter, which is essential for heterotrophic denitrification in conventional treatment units. In addition, as the complete conversion of nitrogenous compounds to N₂ can be achieved in a single reactor operated under low aeration conditions, the demands for space and energy are reduced (Third *et al.* 2001). However, the deammonification process can produce N₂O (Kampschreur *et al.* 2009; Schreiber *et al.* 2012), which has not been reported as an intermediate or end product of the anammox reaction, although another gaseous intermediate (nitric oxide; NO) has been found in experiments using a pure culture of anammox bacteria (Speth *et al.* 2016). However, nitrifying microorganisms (specifically AOB) and heterotrophic denitrifiers (HET), which coexist with anammox bacteria in deammonification systems, have been identified as contributors to N₂O production in biological nitrogen removal systems (Schreiber *et al.* 2012; Speth *et al.* 2016).

The heterotrophic community may be retained in deammonification systems by using soluble microbial products (SMPs) for their growth, even if the wastewater fed does not contain organic carbon (Liu *et al.* 2016). These heterotrophs play an important role in deammonification systems because they act to reduce the nitrate (NO₃⁻) produced by the anammox metabolism and protect the anammox bacteria from the inhibitory effect of the dissolved oxygen (DO) (Lotti *et al.* 2014). Anammox bacteria are generally reported as slow growing organisms, therefore one of the major challenges related to practical applications of deammonification is to ensure an adequate solid retention time for establishing this community.

In this context, the present study verified the dynamics of N₂O production in a sequencing batch reactor (SBR) containing anammox enriched granular sludge under different operating conditions, i.e., temperature, feed time, influent NO₂⁻/NH₄⁺ ratio and initial concentrations of NH₄⁺ and NO₂⁻.

MATERIALS AND METHODS

Bioreactor set-up and experimental procedure

The experiments were conducted in a 10 L Plexiglass SBR equipped with a bath jacket (Julabo GmbH, Germany) for temperature control. Stirring, maintained at 200 rpm, was ensured by a mechanical stirrer (Heidolph, Germany, model RZR 2021) with an impeller with inclined blades (diameter 5 cm). When required, the DO input, initially set

to vary between 0.08 and 0.6 mgO₂·L⁻¹, was performed by an aeration system which included an air pump (Mistral, model 200), a magnetic valve and a DO probe (Endress + Hauser, Switzerland, model COS22D). The whole system was operated by a programmable logic controller (PLC). pH was measured using a pH meter (Endress + Hauser, Switzerland, model CPS471D), connected to the PLC. pH and DO were measured every 30 seconds. The reactor feed, effluent discharge and the HCl and NaOH dosages were performed using peristaltic pumps (Cole-Parmer, Portugal, model 77200-60).

During the tests, the SBR was operated in eight daily cycles of 180 min. Each cycle was divided into four phases: filling (30 min), mixing or reaction (120 min), sedimentation (20 min) and discarding (10 min). The volume exchange rate, which is defined as the ratio between the volume of medium withdrawn at the end of the cycle and the total working volume, was 75%, once 2.5 L of medium had been added and withdrawn during one cycle. The initial values of the operating cycle phases were based on previous studies (Sobotka *et al.* 2016; Yin, Z. *et al.* 2016; Lu *et al.* 2018). Ten batch tests were carried out to elucidate the dynamics of N₂O production by anammox enriched granular sludge under different operating conditions. The parameters evaluated were: feed time (6 min and 30 min), operating temperature (30 °C and 36 °C), the NO₂⁻-N/NH₄⁺-N ratio (1.3, 1.7 and 1.9), variation of influent NO₂⁻-N and NH₄⁺-N concentrations (with the NO₂⁻-N/NH₄⁺-N ratio at 1.3) and the system's response under two extreme conditions: absence of NH₄⁺-N and absence of NO₂⁻-N. Each condition tested was maintained for at least eight cycles, in which similar performances were observed. One of the monitored cycles was used to evaluate the results.

During the whole experiment, the reactor was fed with synthetic medium, as described by Dapena-Mora *et al.* (2004). NH₄⁺-N and NO₂⁻-N concentrations in the medium were varied using ammonium sulfate ((NH₄)₂SO₄) and sodium nitrate (NaNO₂) were used as the sources of ammonium and nitrite, respectively. The operational conditions tested are shown in Table 1.

Each test was planned to have operational conditions that favored certain biochemical pathways to the detriment of others. In this context, it was possible to compare the results of the tests by relating them to the biochemical pathways and possible microbial groups involved. The average of biomass concentration (in terms of volatile suspended solids; VSS) used in each test was 3.217 gVSS·L⁻¹.

Between each test the SBR was adjusted back to experimental condition T1 (Table 1) for at least 12 hours or until

Table 1 | Initial characteristics of the experiments performed in terms of substrates, temperature, period time, pH, DO and biomass

Test number	Synthetic medium			Temp (°C)	F.T. (min)	pH	DO (mg·L ⁻¹)	VSS (mg·L ⁻¹)
	NO ₂ ⁻ -N /NH ₄ ⁺ -N ratio	NH ₄ ⁺ -N (mgN·L ⁻¹)	NO ₂ ⁻ -N (mgN·L ⁻¹)					
T1	1.3	100	130	33.6	6	7.4–7.8	<0.17	3,217
T2	1.3	100	130	33.2	30	7.4–7.8	<0.16	3,217
T3	1.3	140	182	29.7	30	7.5–7.8	<0.16	2,339
T4	1.3	140	182	36.1	30	7.5–7.8	<0.15	2,339
T5	1.3	200	260	30.1	30	7.5–7.8	<0.16	3,217
T6	1.7	200	340	30.0	30	7.5–7.8	<0.12	3,217
T7	1.9	180	340	30.0	30	7.5–7.8	<0.14	3,217
T8	1.3	200	260	30.1	30	7.5–7.8	<0.16	3,217
T9	–	0	80	30.1	30	7.6–7.8	<0.34	3,217
T10	–	80	0	30.3	30	7.6–7.7	0.3–0.8	3,217

F.T., feed time; VSS, volatile suspended solids.

all NO₂⁻, NH₄⁺ and N₂O had been consumed or the reactor presented a stable N₂O concentration profile.

Origin and characteristics of the anammox-enriched granular sludge

Prior to the start of the tests, granular anammox biomass was collected from a full-scale sidestream treatment system in Zurich (Switzerland), and acclimatized in an SBR reactor for 18 months. Phylogenetic analysis performed by Sobotka et al. (2016) revealed the coexistence of all groups involved in the nitrogen removal cycle, including anammox bacteria (genus *Candidatus* Brocadia 42.9%), heterotrophic bacteria (26.8%), AOB (0.10%) and NOB (0.12%). Approximately 30% belonged to unclassified bacteria groups or were not responsible for the biochemical reactions considered in this study.

Analytical methods

During the tests, a liquid sample was taken at intervals of 10–15 min during the filling and mixing phases. The samples were filtered through an MFV-3 glass fiber filter (porosity 1.2 μm and diameter 47.0 mm). The concentrations of the nitrogenous species (NH₄⁺, NO₃⁻ and NO₂⁻) were then analyzed in the samples filtered using analytical cuvette tests (LCK 303, LCK340, LCK342, Hach Lange GmbH, Germany). The absorbance readings were carried out using the spectrophotometer Benchtop DR3900 (Hach Lange GmbH, Germany).

N₂O concentrations in the liquid phase were monitored online every 15 seconds using a Clark-type microsensor model N₂O-R and a model 6276 multimeter (Unisense A/S, Denmark). The measurement range was 0–2.0 mg·L⁻¹, the detection limit was 0.0004 mg·L⁻¹ and the agitation sensitivity was less than 2%. The VSS analyses followed the gravimetric method 2540 E in *Standard Methods for the Examination of Water and Wastewater* (APHA 2012). Temperature, pH and DO were continuously monitored by both Endress + Hauser pH (model CPS471D) and DO (COS22D) probes.

RESULTS AND DISCUSSION

Effect of feed time on N₂O production

Tests T1 and T2 were conducted to verify the effect of varying the feed time (6 min and 30 min) on N₂O production in the SBR. As shown in Figure 1(b), the strategy of increasing feed time from 6 (Test T1) to 30 min (Test T2) favored the presence of lower concentrations of NO₂⁻ and NH₄⁺ for the first 15 min. In the literature, higher values of ammonia and nitrite half saturation coefficients (K_{NH4} and K_{NO2}) were found for heterotrophs or AOB (in oxygen limited conditions), compared with anammox (Law et al. 2012; Niu et al. 2016; Lu et al. 2018). Thus, at low substrate concentrations, anammox metabolism may be favored due to their higher affinity for the substrate and, consequently, since anammox does not have N₂O as a metabolic intermediary, low N₂O production was expected (Figure 1). However, the increase

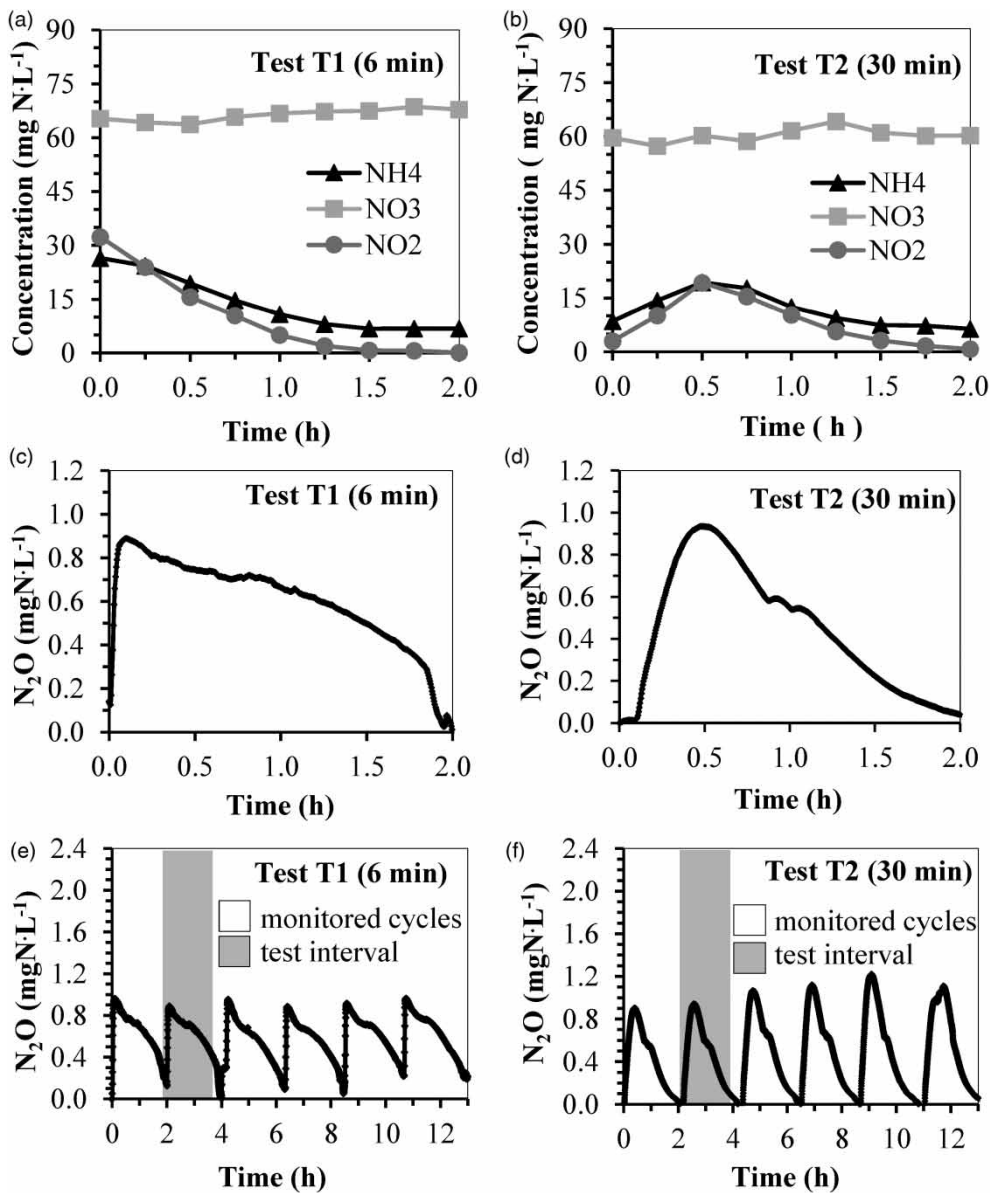


Figure 1 | Effect of the variation of the feed time of the biological reactor (Tests T1 and T2) on nitrogen removal (a and b) and N₂O production (c and d) in the liquid medium during the second cycle of the period monitored (e and f).

in feed time appeared not to favor the decrease in peak N₂O production. Instead of what was predicted, the maximum N₂O production reached values close to 0.900 mgN·L⁻¹ in both tests and no significant difference was observed between Tests T1 and T2 in terms of the peak value of N₂O production (Figure 1).

However, the results of the N₂O concentration profile during the 2 hours of the tests (Figure 2), showed that adopting a longer feed time lead to lower N₂O concentrations in the bulk liquid at most of the intervals analyzed. Only in the range between 21 min and 45 min

were N₂O concentrations higher in Test T2. Nitrogen removal efficiencies were similar (88%) in both cases. The VSS within the SBR did not vary significantly over the course of the experiment and the effect of their concentration on the effluent were negligible. This result was the same in all the conditions tested.

Temperature influence

As shown in Figure 3, in the case of N₂O production, the increase in the operating temperature from 30°C (Test T3)

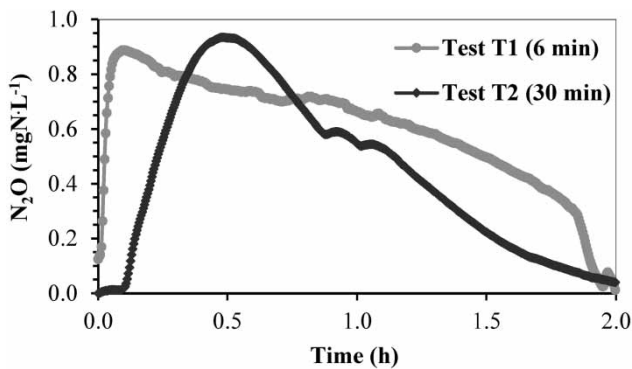


Figure 2 | Comparison between production of and decrease in N₂O during Test T1 (6 min of feeding) and Test T2 (30 min of feeding).

to 36 °C (Test T4) produced no significant effects. The maximum production of N₂O when the reactor was operated at 30 °C was 0.312 mgN·L⁻¹ and it occurred 36 min after the test began. Under the operating temperature of 36 °C, the SBR had the peak N₂O production – 0.300 mgN·L⁻¹ – 34 min after the start of the test (Figure 3).

The ratio of N₂O production to the nitrogen load rate (NLR) was 1.04% and the ratio of N₂O production to the nitrogen removal rate (NRR) for both tests was 1.05%. These values are within the range reported by Ali et al. (2016): 0.1% to 0.6% for anammox reactors, 0.8% to 6.1% for partial nitrification reactors and 0.1% to 3.0% for single-stage nitrification-anammox reactors.

The only significant difference between Tests T3 and T4 was in terms of NO₂⁻-N and NH₄⁺-N consumption rates. In this case, the adjustment of these compounds showed that operation at 36 °C led to a higher nitrogen consumption rate (1.47 gN·gVSS⁻¹·d⁻¹) versus 1.07 gN·gVSS⁻¹·d⁻¹ for the reactor operated under 30 °C. The specific NRR values were 0.49 and 0.72 gN·gSSV⁻¹·d⁻¹ for the reactor at 30 °C and 36 °C, respectively. Sobotka et al. (2016) also observed the increase in the specific removal rate and the anammox activity with increasing temperature, up to a limit of 40 °C, when using an enriched anammox granular biomass.

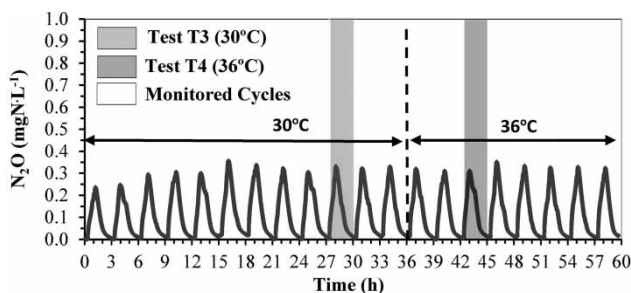


Figure 3 | Effect of the temperature on the N₂O profile in Tests T3 and T4 during a monitoring period of 20 cycles.

Effects of the NLR increase by the change in the NO₂⁻-N and NH₄⁺-N affluent concentrations

In Tests T3 and T5 (Figure 4), the peak of N₂O production changed in response to NO₂⁻-N and NH₄⁺-N concentrations. The maximum N₂O concentration in the liquid phase increased from 0.312 to 0.441 mgN·L⁻¹ when the NO₂⁻-N concentration increased from 182 to 260 mgN·L⁻¹ and the NH₄⁺-N concentration increased from 140 to 200 mgN·L⁻¹. These results indicate a positive correlation between N₂O production and NO₂⁻-N and NH₄⁺-N concentrations. Similarly, the results showed an increase in the maximum production factor (i.e., the N₂O produced per N consumed) from 1.03% to 1.45%. These results agree with previous observations of the deammonification process (Ali et al. 2016).

The increased N₂O production was expected, as high substrate concentrations tend to favor the species with the highest specific growth (Lu et al. 2018). Although heterotrophic bacteria most likely had the autotrophic SMPs as a source of organic matter (Liu et al. 2016), this NLR increase probably favored the heterotrophic denitrifying bacteria in comparison with anammox. A similar result was also observed by Domingo-Félez et al. (2017).

Effect of the NO₂⁻-N/NH₄⁺-N ratio on N₂O production

For tests T5, T6 and T7, a progressive increase in the NO₂⁻-N/NH₄⁺-N ratio was provided. The first test was conducted with a ratio of 1.3 (Test T5) and for subsequent tests the NO₂⁻-N/NH₄⁺-N ratio was increased to 1.7 (Test T6) and 1.9 (Test T7).

An overall assessment of the data presented in Figure 5 highlights a clear correlation between the NO₂⁻-N excess and N₂O accumulation at the end of the reaction phase. By maintaining the NO₂⁻-N/NH₄⁺-N ratio of 1.3, N₂O production was observed, until its maximum, followed by decreases (Figure 5(d)–5(f)). On the other hand, when higher NO₂⁻-N/NH₄⁺-N ratios were applied (Tests T6 and T7), the decrease in N₂O production ceased once the anammox reaction appeared to be finished due to the lack of NH₄⁺, approximately 1.5 hours after the beginning of the test. From this point onwards, it is likely that heterotrophic denitrification by nitrite reduction played the dominant role in N₂O production.

Test T5 gave the lowest value of N₂O (0.441 mg·L⁻¹). When the NO₂⁻-N/NH₄⁺-N ratio was increased to 1.7 in Test T6, and subsequently to 1.9 in Test T7, the N₂O reached its maximum concentration (0.600 mg·L⁻¹) (Figure 5(e) and 5(f)). Although the T6 and T7 tests produced the same peak value of N₂O, the N₂O concentration remaining

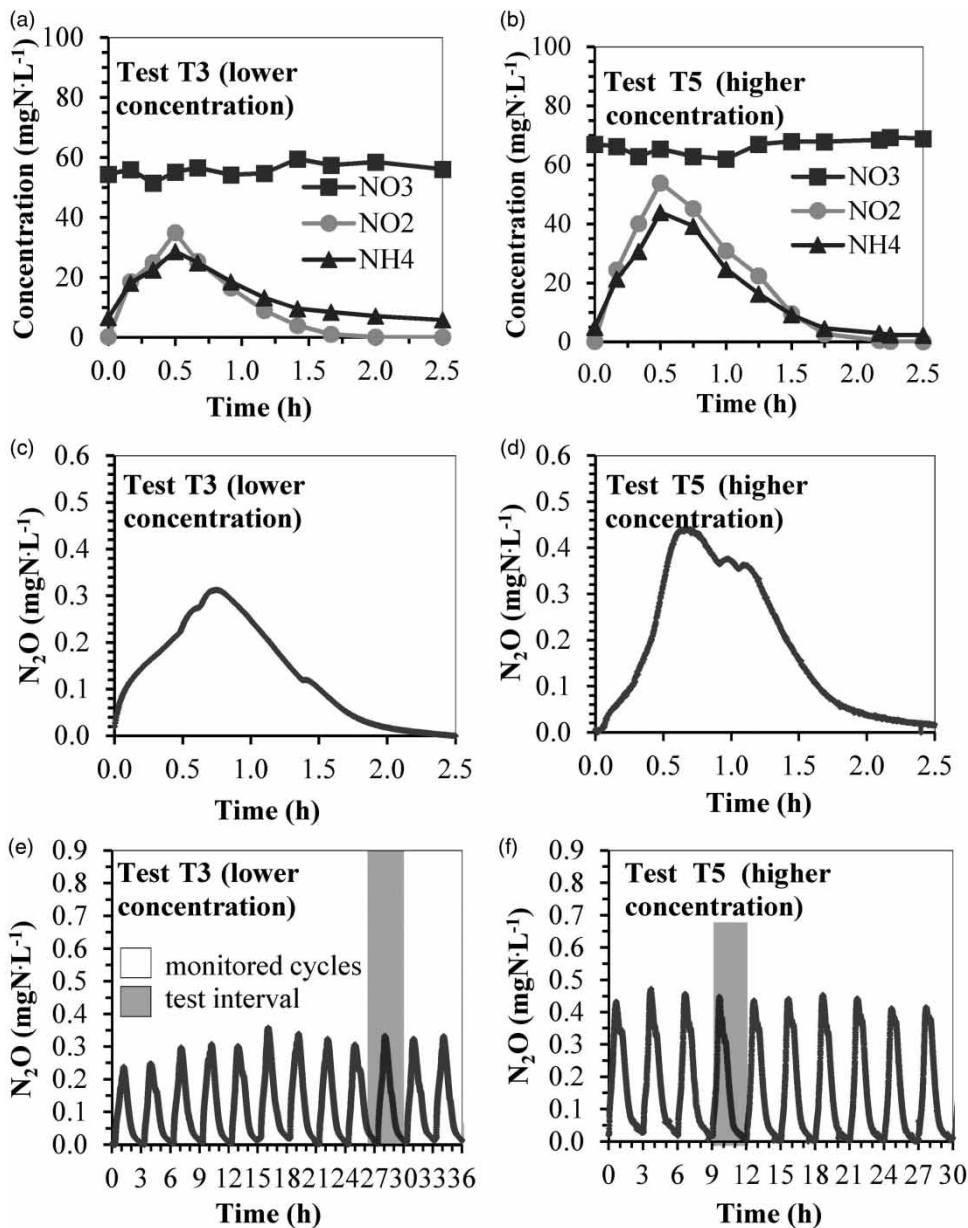


Figure 4 | Effects of the NLR increase by the variation in NO_2^- -N and NH_4^+ -N concentrations from $182 \text{ mgNO}_2^- \cdot \text{N}\cdot\text{L}^{-1}$ and $140 \text{ mgNH}_4^+ \cdot \text{N}\cdot\text{L}^{-1}$ (Test T3) to $260 \text{ mgNO}_2^- \cdot \text{N}\cdot\text{L}^{-1}$ and $200 \text{ mgNH}_4^+ \cdot \text{N}\cdot\text{L}^{-1}$ (Test T5), under the same NO_2^- -N/ NH_4^+ -N ratio (1.3) in: nitrogen removal (a and b) and N_2O concentration profile (c and d) in the liquid medium during the monitored period (e and f).

at the end of the reaction phase was different. A higher NO_2^- -N/ NH_4^+ -N ratio led to a higher final N_2O concentration. The percentage of N_2O consumed from the peak value until the end of the cycle decreased from 100% (Test T5) to 54% (Test T6) and 19% in Test T7. So the N_2O concentration remaining in the bulking liquid was higher when the NO_2^- concentration was increased. The final N_2O concentration values observed in Tests T5, T6 and T7 were 0, 0.270 and $0.484 \text{ mgN}\cdot\text{L}^{-1}$, respectively.

The higher rate of N_2O reduction compared with nitrite and nitrate reduction leads to the conclusion that in anaerobic/anoxic environments, the N_2O produced can be completely reduced to N_2 under favorable conditions (Kampschreur *et al.* 2009). In the present study, however, there was no input of organic matter. With this in mind, heterotrophic denitrification probably occurred by using SMPs as electron donor sources (LIU *et al.* 2016), which may have been insufficient to reduce all available N_2O .

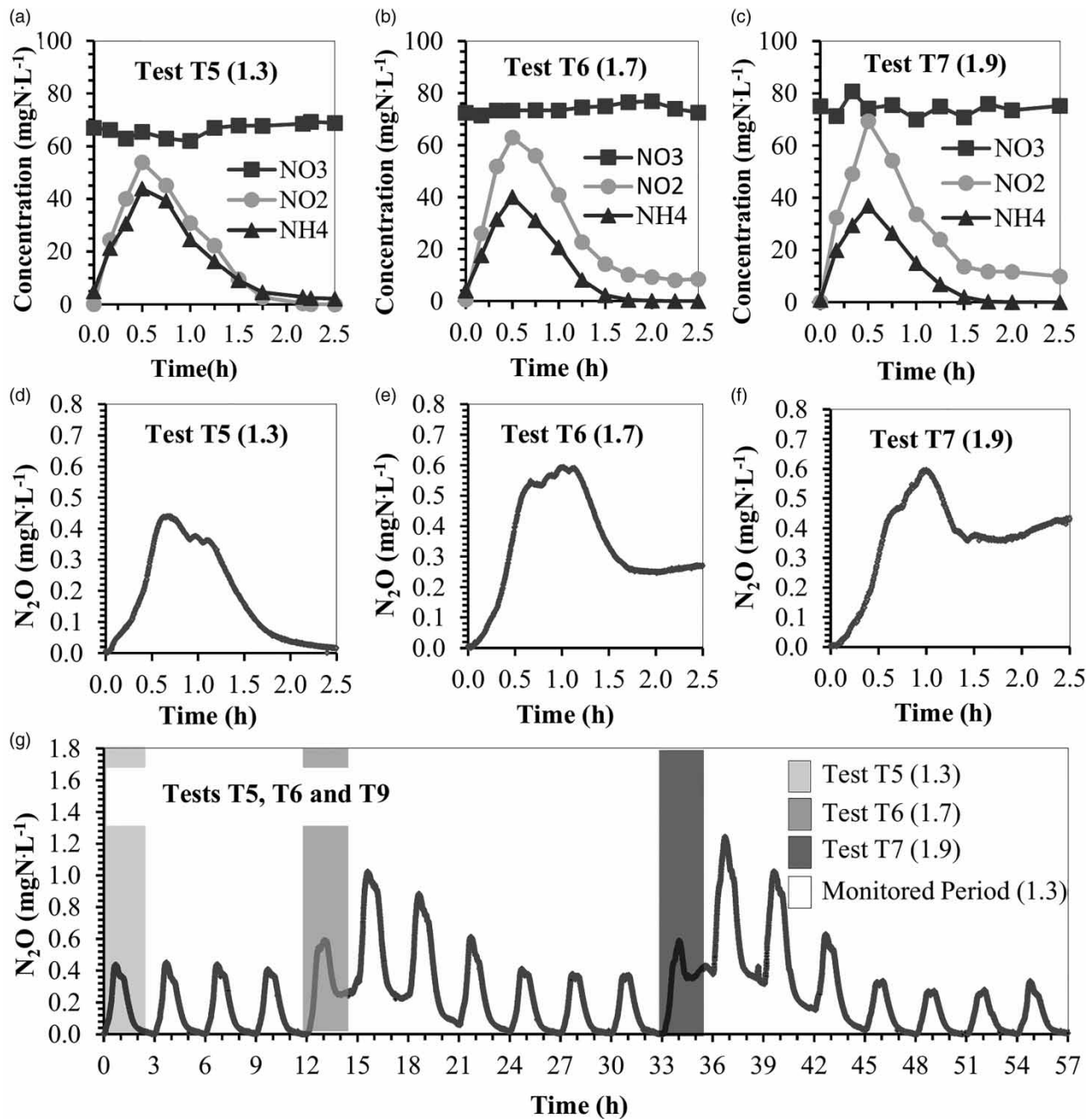


Figure 5 | Effects of the NO_2^-/NH_4^+-N ratio variation from 1.3 (Test T5) to 1.7 (Test T6) and 1.9 (Test T7) on nitrogen removal (a–c) and N_2O profile (d–f) during the period monitored (g).

A positive correlation between the concentration of nitrite and N_2O production has been reported in other studies and NO_2^- is commonly considered to be one of the key parameters in high N_2O emissions (Tallec *et al.* 2006; Kampschreur *et al.* 2009, 2008; Kim *et al.* 2010; Ali *et al.* 2016). Most of these studies, however, were performed with biomass from an activated sludge treatment system under nitrification or denitrification conditions with variable DO

concentrations. Consequently, the authors generally attribute N_2O production to AOB activity by autotrophic denitrification or by ammonia oxidation via hydroxylamine (NH_2OH). In this study, tests were performed with low DO concentrations (less than $0.16 \text{ mgO}_2\text{L}^{-1}$). Thus, the predominant mechanisms tended to be autotrophic denitrification or heterotrophic denitrification. However, for Test T6 and Test T7, there was probably a predominance of heterotrophic

denitrification, since after 1.5 hours of the tests, there was no more NH₄⁺ in the liquid medium (Figure 5(e) and 5(f)).

After Test T7, in which the NO₂⁻-N/NH₄⁺-N ratio applied was 1.9, the SBR was again operated under an initial NO₂⁻-N/NH₄⁺-N ratio of 1.3. The subsequent cycles were monitored in order to verify a possible decrease in accumulated N₂O levels and the process recovery until total consumption of the remaining N₂O (Figure 6). Test T8 gave the highest N₂O value of all the proposed tests (1.24 mgN·L⁻¹). The cycle began with a concentration of 0.385 mgN·L⁻¹ and the net N₂O production was 0.855 mgN·L⁻¹ (Figure 6(a)). This value is higher compared to other tests, which is probably due to the nitrite remaining from the previous cycle. As shown in Figure 6(c), a NO₂⁻-N accumulation of 9.93 mgN·L⁻¹ was observed at the beginning of Test T8. At the end of the reaction phase, 100% of the produced N₂O had been consumed and the final N₂O concentration was similar (0.370 mgN·L⁻¹), at the beginning and end of the test (Figure 6(e)).

This result endorses all previous observed results where all N₂O produced was consumed by the end of the reaction phase when the SBR was operated under the NO₂⁻-N/NH₄⁺-N ratio of 1.3. After the reaction phase in Test T8, however, the N₂O declined until the beginning of the feeding of the next cycle. So over the whole cycle N₂O consumption was higher than production. This result was repeated in the subsequent cycles of Test T8 until no N₂O was observed at the end of a cycle and the concentration of N₂O in the bulk liquid was stable (Figure 6(a)).

Identification of the dominant N₂O production processes (heterotrophic vs autotrophic)

During Test T9 (Figure 7) operational conditions favoring the growth of heterotrophic denitrifiers were adopted: 80 mgN·L⁻¹ of NO₂⁻, no addition of NH₄⁺ and DO maintained below 0.1 mgO₂·L⁻¹. Consequently, there

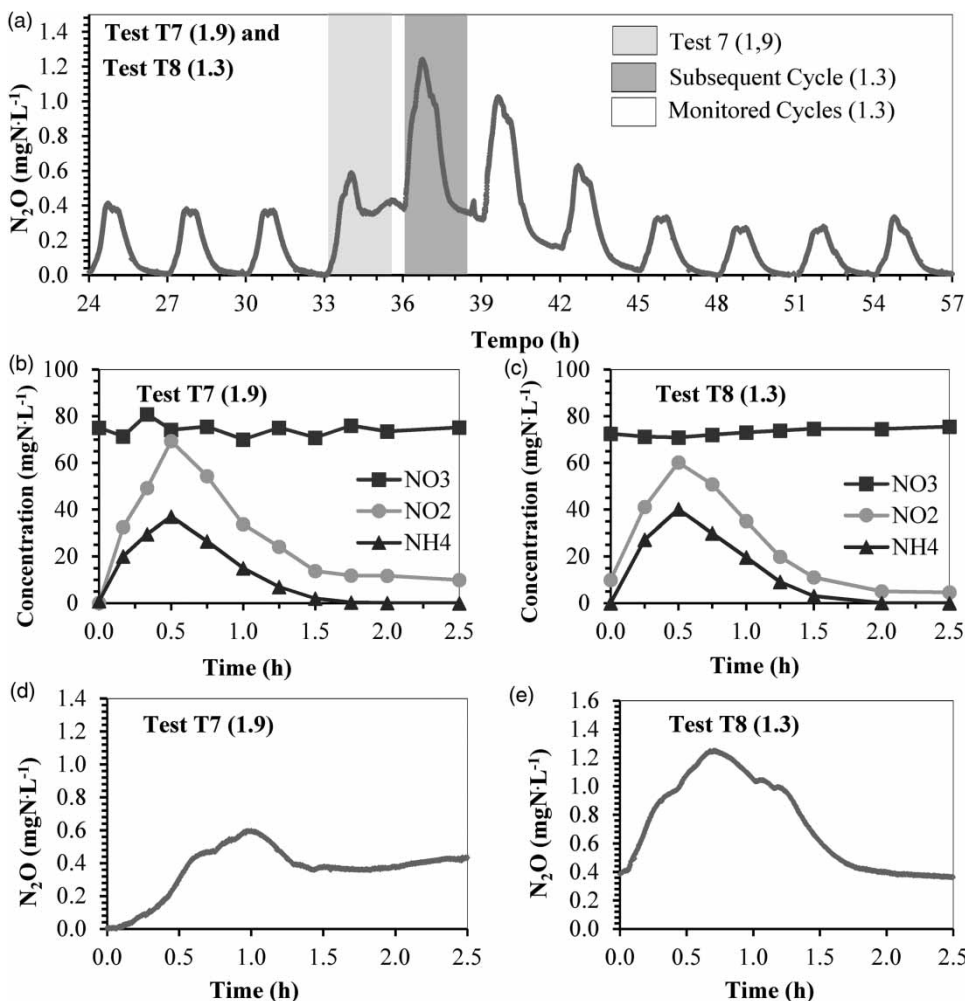


Figure 6 | N₂O concentration profiles (d and e) in the subsequent cycle of Test T7 (a) whose NO₂⁻-N/NH₄⁺-N ratio was reduced from (b) 1.9 (Test T7) to (c) 1.3 (Test T8).

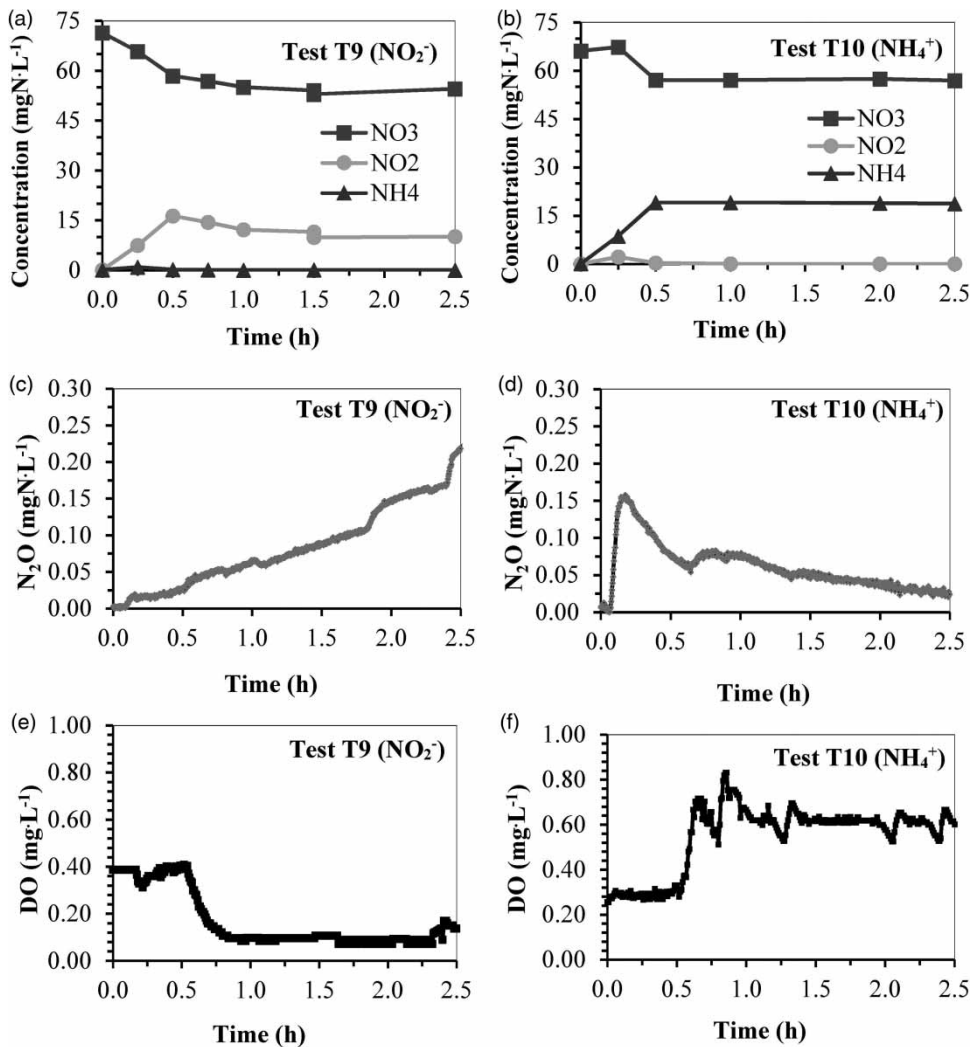


Figure 7 | Effect of the absence of NH₄⁺ (a and c) or NO₂⁻ (b and d) and DO concentration (e and f) on N₂O production and nitrogen removal.

was no substrate for the anammox or AOB and not enough DO for nitrification by NOB. Thus, the N₂O production could be attributed exclusively to heterotrophic denitrification. Higher levels of DO, approximately 0.4 mgN·L⁻¹, were present in the feed phase, probably due to agitation.

The nitrogen removal efficiency observed in Test 8 was 49.38% and 2.24% of the NRR was converted to N₂O at a production rate of 0.66 mgN·gSSV⁻¹·h⁻¹. In a study on the emission of N₂O in low DO conditions Jia *et al.* (2013) used 1 L SBR in the process of simultaneous nitrification and denitrification for nitrogen removal. By adding NO₂⁻ and using nitrification inhibitors, the authors favored heterotrophic denitrification and observed an N₂O production rate of 0.15 mgN·gSSV⁻¹·h⁻¹, 4.5 times lower than the value we found in the present study.

Figure 7(c) shows the observed increase in N₂O production throughout Test T9. Confirming the results obtained in previous tests (T6 and T7), the presence of NO₂⁻ resulted in N₂O accumulation at the end of the cycle (0.221 mgN·L⁻¹). This result highlights that NO₂⁻ and heterotrophic denitrification may be closely related to the accumulation and emission of N₂O. Okabe *et al.* (2011) also related N₂O production in anammox granular biomass to heterotrophic denitrification and to nitrite accumulation. In an anoxic medium, the authors did not observe nitrous oxide emissions when the granular biomass was given a synthetic medium containing NO₂⁻, NH₄⁺ and an inhibitor for AOB and HET (Okabe *et al.* 2011). On the other hand, production was considerable when biomass from the same source was given a medium with only NO₂⁻.

In Test T10 (Figure 7(b) and 7(d)), aeration was provided to maintain the DO close to 0.6 mgO₂·L⁻¹ (Figure 7(f)) in the presence of NH₄⁺ only. Those conditions could favor AOB activity to the detriment of HET and anammox bacteria. These metabolisms were inhibited due to the absence of NO₂⁻. Accordingly, N₂O production could be attributed to oxidation of NH₄⁺ via NH₂OH.

The results showed maximum production (0.157 mgN·L⁻¹) at 15 min, even during the feed phase, which corresponds to 1.38% of the oxidized ammonia in that time. Wunderlin *et al.* (2012) observed this production factor in the range of 1.3% to 3.8% using an inoculum from a pilot plant of activated sludge in an SBR with a DO concentration of 0.6 mg O₂·L⁻¹. During the reaction phase, 89% of N₂O produced was consumed, probably used as a substrate by HET, or emitted from the liquid phase into the atmosphere. The nitrogen removal efficiency observed during the test was only 6.5%, 7.5 times lower compared to the previous test (T9). This result can be attributed to inhibition by DO concentration and the absence of NO₂⁻. Yin, X. *et al.* (2016) and Yin, Z. *et al.* (2016) also observed a reduction in the nitrogen removal efficiency (approximately 50%) when an SBR with enriched anammox biomass operated under anoxic conditions, initially operated with DO concentrations between 0.6 to 1.0 mgO₂·L⁻¹.

The net N₂O production in Test T10 represented only 0.9% of the value obtained at the end of T9. These results indicated that heterotrophic denitrification had a higher impact on net N₂O production than NH₄⁺ oxidation via NH₂OH. The inhibition of HET, and the inhibition of N₂O production via nitrite autotrophic denitrification by AOB, was responsible for the reduction of more than 99% of the N₂O production. This result was as expected, since, as an intermediary of ammonia oxidation and nitrate reduction, significant NH₂OH accumulation is energetically unfavorable (Casciotti *et al.* 2003).

CONCLUSIONS

N₂O production was influenced by the feed time, NLR and NO₂⁻N/NH₄⁺-N ratio. The operating temperature did not influence the production of N₂O. The results highlighted nitrite as a key parameter for N₂O production and accumulation. When nitrite was not added to the medium, there was a reduction of more than 99% of N₂O production. However, the addition of higher concentrations of nitrite in the feed medium, by increasing the NO₂⁻N/NH₄⁺-N ratio, resulted in NO₂⁻ accumulation and consequent N₂O accumulation at the end of the cycle.

As long as nitrite is not added in excess, the anammox reactor studied presents no N₂O residue at the end of the cycle period studied. SBR operation maintaining the NO₂⁻N/NH₄⁺-N ratio lower than 1.3 was effective for minimizing N₂O production and no N₂O accumulation was observed in this study. This result can be used to control N₂O production in deammonification systems with granular anammox sludge in WWTPs, while still maintaining high NRR and efficiency.

AUTHOR DISCLOSURE STATEMENT

The authors declare that they do not have any conflicts of interest and no competing financial interest.

ACKNOWLEDGEMENTS

This study has been financially supported by the Polish National Center for Research and Development under the project 'Reduction of N₂O emissions from wastewater treatment plants – measurements, modeling and process optimization (RENEMO)' (WPN/7/2013, Polish-German sustainability call) and by the Coordination for the Improvement of Higher Education Personnel (CAPES). During this study, Tiago Duarte Santos Pereira and Xi Lu were visiting researchers at Gdansk University of Technology under the project 'CARbon BALAncing for nutrient control in wastewater treatment (CARBALA)', People Maria Curie Actions (FP7-PEOPLE-2011-IRSES). Eduardo C. Pires has a grant from the National Council for Scientific and Technological Development (CNPq, Brazil, Grant no. 307042/2014-6).

REFERENCES

- Ali, M., Rathnayake, R. M. L. D., Zhang, L., Ishii, S., Kindaichi, T., Satoh, H., Toyoda, S., Yoshida, N. & Okabe, S. 2016 [Source identification of nitrous oxide emission pathways from a single-stage nitrification-anammox granular reactor](#). *Water Res.* **102**, 147–157. <https://doi.org/10.1016/j.watres.2016.06.034>.
- APHA 2012 *Standard Methods for the Examination of Water and Wastewater*, 22nd edn. American Public Health Association/American Water Works Association/Water Environmental Federation, Washington, DC.
- Casciotti, K. L., Sigman, D. M. & Ward, B. B. 2003 [Linking diversity and stable isotope fractionation in ammonia-oxidizing bacteria](#). *Geomicrobiol. J.* **20**, 335–353. <https://doi.org/10.1080/01490450303895>.
- Castro-Barros, C. M., Daelman, M. R. J., Mampaey, K. E., van Loosdrecht, M. C. M. & Volcke, E. I. P. 2015 [Effect of aeration](#)

- regime on N₂O emission from partial nitrification-anammox in a full-scale granular sludge reactor. *Water Res.* **68**, 793–803. <https://doi.org/10.1016/j.watres.2014.10.056>.
- Dapena-Mora, A., Van Hulle, S. W. H., Campos, J. L., Méndez, R., Vanrolleghem, P. A. & Jetten, M. 2004 Enrichment of Anammox biomass from municipal activated sludge: experimental and modelling results. *J. Chem. Technol. Biotechnol.* **79**, 1421–1428. <https://doi.org/10.1002/jctb.1148>.
- Domingo-Félez, C., Pellicer-Nàcher, C., Petersen, M. S., Jensen, M. M., Plósz, B. G. & Smets, B. F. 2017 Heterotrophs are key contributors to nitrous oxide production in activated sludge under low C-to-N ratios during nitrification—Batch experiments and modeling. *Biotechnol. Bioeng.* **114**, 132–140. <https://doi.org/10.1002/bit.26062>.
- Kampschreur, M. J., van der Star, W. R. L., Wielders, H. A., Mulder, J. W., Jetten, M. S. M. & van Loosdrecht, M. C. M. 2008 Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment. *Water Res.* **42**, 812–826. <https://doi.org/10.1016/j.watres.2007.08.022>.
- IPCC 2013 Summary for Policymakers. In: *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex & P. M. Midgley, eds). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
- Jia, W., Liang, S., Zhang, J., Ngo, H. H., Guo, W., Yan, Y. & Zou, Y. 2013 Nitrous oxide emission in low-oxygen simultaneous nitrification and denitrification process: Sources and mechanisms. *Bioresour. Technol.* **136**, 444–451. <https://doi.org/10.1016/j.biortech.2013.02.117>.
- Kampschreur, M. J., Temmink, H., Kleerebezem, R., Jetten, M. S. M. & van Loosdrecht, M. C. M. 2009 Nitrous oxide emission during wastewater treatment. *Water Res.* **43**, 4093–4103. <https://doi.org/10.1016/j.watres.2009.03.001>.
- Kim, H. S., Gellner, J. W., Boltz, J. P., Freudenberg, R. G., Gunsch, C. K. & Schuler, A. J. 2010 Effects of integrated fixed film activated sludge media on activated sludge settling in biological nutrient removal systems. *Water Res.* **44**, 1553–1561. <https://doi.org/10.1016/j.watres.2009.11.001>.
- Law, Y., Lant, P. & Yuan, Z. 2011 The effect of pH on N₂O production under aerobic conditions in a partial nitrification system. *Water Res.* **45**, 5934–5944. <https://doi.org/10.1016/j.watres.2011.08.055>.
- Law, Y., Ni, B. J., Lant, P. & Yuan, Z. 2012 N₂O production rate of an enriched ammonia-oxidising bacteria culture exponentially correlates to its ammonia oxidation rate. *Water Res.* **46**, 3409–3419. <https://doi.org/10.1016/j.watres.2012.03.043>.
- Liu, Y., Sun, J., Lai, P., Wang, D., Dai, X. & Ni, B. 2016 Assessment of heterotrophic growth supported by soluble microbial products in Anammox biofilm using multidimensional modeling. *Sci. Rep.* **6**, 275765. <https://doi.org/10.1038/srep27576>.
- Lotti, T., Kleerebezem, R., Lubello, C. & van Loosdrecht, M. C. M. 2014 Physiological and kinetic characterization of a suspended cell anammox culture. *Water Res.* **60**, 1–14. <https://doi.org/10.1016/j.watres.2014.04.017>.
- Lu, X., Pereira, T. D. S., Al-Hazmi, H. E., Majtacz, J., Zhou, Q., Xie, L. & Makinia, J. 2018 Model-based evaluation of N₂O production pathways in the Anammox-nitrified granular sludge cultivated in a sequencing batch reactor. *Environ. Sci. Technol.* **52**, 2800–2809. <https://doi.org/10.1021/acs.est.7b05611>.
- Niu, Q., He, S., Zhang, Y., Ma, H., Liu, Y. & Li, Y. Y. 2016 Process stability and the recovery control associated with inhibition factors in a UASB-anammox reactor with a long-term operation. *Bioresour. Technol.* **203**, 132–141. <https://doi.org/10.1016/j.biortech.2015.12.003>.
- Okabe, S., Oshiki, M., Takahashi, Y. & Satoh, H. 2011 N₂O emission from a partial nitrification-anammox process and identification of a key biological process of N₂O emission from anammox granules. *Water Res.* **45**, 6461–6470. <https://doi.org/10.1016/j.watres.2011.09.040>.
- Schreiber, F., Wunderlin, P., Udert, K. M. & Wells, G. F. 2012 Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological pathways, chemical reactions, and novel technologies. *Front. Microbiol.* **3**, 1–24. <https://doi.org/10.3389/fmicb.2012.00372>.
- Sobotka, D., Czerwionka, K. & Makinia, J. 2016 Influence of temperature on the activity of anammox granular biomass. *Water Sci. Technol.* **73**, 2518–2525. <https://doi.org/10.2166/wst.2016.103>.
- Speth, D. R., Guerrero-cruz, S., Dutilh, B. E. & Jetten, M. S. M. 2016 Genome-based microbial ecology of anammox granules in a full-scale wastewater treatment system. *Nat. Commun.* **7**, 11172. <https://doi.org/10.1038/ncomms11172>.
- Tallec, G., Garnier, J., Billen, G. & Gossailles, M. 2006 Nitrous oxide emissions from secondary activated sludge in nitrifying conditions of urban wastewater treatment plants: effect of oxygenation level. *Water Res.* **40**, 2972–2980. <https://doi.org/10.1016/j.watres.2006.05.037>.
- Third, K. A., Sliekers, O. A., Kuenen, J. G. & Jetten, M. S. M. 2001 The CANON system (Completely autotrophic nitrogen-removal over nitrite) under ammonium limitation. *Syst. Appl. Microbiol.* **596**, 588–596.
- Wunderlin, P., Mohn, J., Joss, A., Emmenegger, L. & Siegrist, H. 2012 Mechanisms of N₂O production in biological wastewater treatment under nitrifying and denitrifying conditions. *Water Res.* **46**, 1027–1037. <https://doi.org/10.1016/j.watres.2011.11.080>.
- Yin, X., Qiao, S., Zhou, J. & Tang, X. 2016 Fast start-up of the anammox process with addition of reduced graphene oxides. *Chem. Eng. J.* **283**, 160–166. <https://doi.org/10.1016/j.cej.2015.07.059>.
- Yin, Z., dos Santos, C. E. D., Vilaplana, J. G., Sobotka, D., Czerwionka, K., Damianovic, M. H. R. Z., Xie, L., Morales, F. J. F. & Makinia, J. 2016 Importance of the combined effects of dissolved oxygen and pH on optimization of nitrogen removal in anammox-enriched granular sludge. *Process Biochem.* **51**, 1274–1282. <https://doi.org/10.1016/j.procbio.2016.05.025>.
- Zhang, L., Jiang, J., Yang, J., Hira, D. & Kenji, F. 2012 High rate nitrogen removal by the CANON process at ambient temperature. *Water Sci. Technol.* **65** (10), 1826–1833. <https://doi.org/10.2166/wst.2012.870>.