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Distinctive NO and N_2O emission patterns in ammonia oxidizing bacteria: effect of ammonia oxidation rate, DO and pH

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Abstract

This study aims at investigating the relationship between the nitric oxide (NO) and nitrous oxide (N_2O) production rates with the ammonia oxidation rate (AORsp) in an enriched AOB culture. Different concentrations of ammonia were applied in a sequential batch reactor (SBR) performing partial nitritation in order to determine the effect of AORsp on N_2O and NO production rates. Results showed that NO linearly correlates with the AORsp whereas N_2O presents an exponential relationship. The effect of changes on the dissolved oxygen (DO) concentration on the overall NO and N_2O emissions was assessed by increasing and decreasing the DO maintaining a constant pH at 7. When DO decreased the AORsp was maintained at the level achieved with the starting DO and led to lower NO and N_2O emissions than when DO was increased. Finally, the effect of pH on N_2O and NO was also tested by maintaining the DO at 1.5-2 mg O_2/L while pH was gradually decreased from 8 to 6.5. Results show that NO was chemically produced due to the addition of HCl when decreasing the pH whereas N_2O was only produced biologically and was not affected by the addition of HCl.

Keywords: ammonia oxidizing bacteria; ammonium oxidation rate; nitric oxide; nitrous oxide.

1. Introduction

Ammonia oxidation in wastewater treatment plants (WWTP) is generally carried out by ammonia oxidizing bacteria (AOB). They perform the first step of nitrification where ammonia is oxidized to nitrite. During this process nitrous oxide (N_2O) and nitric oxide (NO) can be produced and emitted to the atmosphere [1]. N_2O is a potent green house gas with a global warming potential over 100 years, 265 times higher than carbon dioxide [2]. On the other hand, NO is an important compound that can cause depletion of the ozone layer [3] and it is toxic for living organisms [4]. In order to minimize the N_2O and NO emissions it is very important to understand the characteristics of their production. N_2O and NO are produced through two different routes: (i) the hydroxylamine pathway: N_2O and NO are intermediates of the hydroxylamine (NH_2OH) biological oxidation or produced by chemical decomposition of hydroxylamine and (ii) the nitrifier denitrification pathway: reduction of nitrite by AOBs under oxygen-limiting conditions or elevated nitrite concentrations [5].

There have been many studies reporting the factors affecting N_2O production in AOB. Law et al. [6] studied the effect of pH on N_2O production and revealed that the N_2O production rate of an enriched AOB culture was dependent on the pH which in turn, affected the ammonia oxidation rate. They studied this effect on the range of 6-8.5 and found that the relationship between N_2O production rate and AOR in this range of pH was linear. In another study, the same authors revealed that the relationship between N_2O production specific rate (N_2Osp) and ammonia oxidation specific rate (AORsp) was exponential in an enriched AOB culture [7]. Peng et al. [8] studied the effect of dissolved oxygen (DO) on N_2O production and their results showed that as DO increased the N_2O production rate also increased. Later on Peng et al. [9] reported the combined effect of DO and NO_2^- concentrations on the N_2O production of a nitrifying culture. Results showed that at each DO level, as NO_2^- concentration increased so did the N_2O production rate. On the other hand at each NO_2^- level, N_2O production rate decreased as DO concentrations increased. Moreover other factors such as nitrite [10] and salinity [11] apart from pH and DO also affect N_2O emissions from nitrifying systems.

On the other hand, reports on NO production have been very scarce. Rodriguez-Caballero and Pijuan [12] studied the N_2O and NO emissions in a partial nitrification reactor using different cycle configurations to minimize these emissions and concluded that NO should be also taken into account when implementing mitigation strategies to reduce N_2O, since some of these strategies might result in increased NO emissions. Yu et al. [13] also studied the production of NO and N_2O under transient anoxic conditions in a pure culture of AOB and reported N_2O emissions during transient conditions (from anoxic to aerobic) when ammonia had been accumulated. However, NO was mainly produced during anoxic conditions. The relationship between the ammonia oxidation rate and the NO production rate was found to be linear for a pure culture of *Nitrosomonas europaea* using synthetic wastewater [14]. Kampschreur et al. [15] studied the NO and N_2O emissions in a full-scale wastewater treatment plant treating reject wastewater in a two-reactor nitritation-anammox process. The NO emissions from the nitritation reactor were 0.2% of the N-load and denitrification by AOBs was considered to be the most probable cause of NO and N_2O emission from the nitritation reactor.

Little is known about the factors affecting NO production and its relationship with N_2O. Kozlowski and co-workers [16] conducted a comparison of phenotypes of N. europaea lacking expression of NirK, Nor B and both enzymes. They found a clear implication NorB in N2O production, being this one significantly lower in those mutant strains without the expression NorB. More recently, an study with pure cultures of *N. viennensis* (in the pylum *Thaumarchaeota* from the Ammonia-oxidizing archea, AOA) and *N. multiformis* (from ammonia-oxidizing bacteria) indicated a different role of NO in the metabolism of both groups. While NO seems to play an essential role in the process of ammonia oxidation in AOA, stopping its oxidation if NO is absent, in the case of AOB NO would not be affecting their main metabolic pathway [17].

This study aims at investigating the effect of ammonia oxidation rate on NO production and assesses its relationship with N_2O. Also, the effect of pH and DO on the production of NO and N_2O was explored in an enriched AOB culture.

2. Materials and methods

2.1 Bioreactor set-up and operation

A cylindrical 8L SBR was inoculated with activated sludge from a local domestic WWTP located in Girona (Spain). The mixed liquor temperature was controlled at 30ºC using a water jacket, to mimic the common temperature conditions of reactors treating reject wastewater. The SBR was operated in cycles of 6h, consisting of feed-1 (2 min), aeration-1 (105 min), feed-2 (2 min), aeration-2 (103 min), settling (132 min) and decanting (15 min). 1L of synthetic wastewater (prepared in the laboratory to maintain the same composition during the experimental period) was added in each feeding period, providing a hydraulic retention time (HRT) of 24h. DO was controlled with a programmable logic controller (PLC) between 1.5-2.0 mg O_2/L by adding air or nitrogen gas at 5 L/min. The feed was prepared as to mimic the concentration of ammonia present in anaerobic digester liquor and is detailed below. The feed had a pH of 8 and a molar ratio of ammonium to bicarbonate of 1:1. After feeding, the pH of the reactor increased to 7.5 and decreased afterwards due to the nitrification reaction. When pH reached 7, it was automatically controlled by adding 1M $NaHCO_3$ solution. Cycle studies were carried out on a weekly basis to monitor the nitrification activity of the reactor. Samples for the analysis of ammonia, nitrate and nitrite were taken along the cycle and filtered with 0.22 µm Millipore filters. At the end of the second aerobic phase mixed liquor suspended solids (MLSS) and volatile MLSS (MLVSS) were also analysed.

The synthetic wastewater had the characteristics of a typical anaerobic digester liquor. The wastewater composition was modified from Kuai and Verstraete [18]: 5.63 g/L of NH_4HCO_3 (1 g N-NH_4^+/L), 0.064 g/L of each KH_2PO_4 and K_2HPO_4 and 2 mL of trace element stock solution. The trace element solution included (g/L): 1.25 EDTA, 0.55 $ZnSO_4*7H_2O$, 0.4 $CoCl_2*6H_2O$, 1.27 $MnCl_2*4H_2O$, 0.40 $CuSO_4*5H_2O$, 0.05 $Na_2Mo_4*2H_2O$, 1.37 $CaCl_2*2H_2O$, 1.25 $FeCl_3*6H_2O$ and 44.40 $MgSO_4*7H_2O$.

2.2 Batch tests

Batch tests were conducted in the same parent reactor. Three sets of experiments were carried out. The first set consisted on adding a continuous feed (6.57 mg N-NH_4^+/min) followed with different ammonia concentration pulses to see the effect of the AOR on the N_2O and NO production. The DO and pH were controlled at the same values as in the parent reactor. Samples were taken every 30 minutes to analyse ammonia and nitrite.

The second set of experiments was conducted to explore the effect of DO on N_2O and NO emissions. Three different batch tests were conducted in this set of experiments. In the first batch (2.1) pH was maintained constant at 7 while DO was increased every 15 minutes from 0.5 to 3 mg O_2/L in a stepwise manner. The DO increased from 0.5-1mg O_2/L to 1-2.5 mg O_2/L and 2.5-3 mg O_2/L. The second batch (2.2) mimicked the first but with DO decreasing every 15 min from 3 to 0.5 mg O_2/L in a stepwise mode. In this case the DO decreased in the ranges of 3-2.5, 2-1.5 and 1-0.5mg O_2/L. A pulse of NH_4Cl (50 N-NH_4^+/L) followed by a continuous feed (6.57 mg N-NH_4^+/min) was added in the reactor. In the third batch test (2.3), DO was set at 0 mg O_2/L and pH was maintained at 7 to see the effect of anoxic conditions on the N_2O and NO emissions. No NH_4^+ was added in this test.

The third set of experiments consisted on exploring the effect of pH on N_2O and NO emissions. Five different batch tests were conducted (3.1-3.5). In the first batch (3.1), DO was maintained constant at 1.5-2 mg O_2/L while pH was gradually decreased 0.5 units every 15 minutes from 8 to 6.5. The other batch tests were conducted under the same conditions as batch 3.1. Batch test 3.2 was conducted without addition of ammonia. Batch test 3.3 was carried out without biomass and without the addition of ammonia. In the fourth batch test (3.4) RO water was used without biomass but with the addition of ammonia in the reactor. In batch test 3.5 NaOH was added. All the experiments lasted between 60 and 120 minutes.

Samples for NH_4^+ and NO_2^- were taken every 15 minutes and filtered through 0.22 µm Millipore filters. At the end of each test samples for mixed liquor suspended solids (MLSS) and volatile MLSS (MLVSS) were taken in order to calculate the N_2O and NO production specific rates and the ammonia oxidation specific rate.

2.3 Chemical and Microbial analyses

Samples for ammonia, nitrate, nitrite and phosphate were taken and analyzed via ion chromatography (ICS5000, DIONEX). MLSS and MLVSS were analyzed according to standard methods [19].

Fluorescence in situ hybridization (FISH) was performed as described in [20] using Cy~~t~~5-labelled EUBmix (for all bacteria) and Cy~~t~~3-labelled AOBmix (for AOBs) comprising equal amounts of oligonucleotide probes Nso1225, NEU and NmV. FISH preparations were visualized with a Nikon CS1 confocal laser-scanning microscope (CLSM) using Plan-Apochromat 63 x oil (NA1.4) objective. Thirty images were taken from each sample for quantification. The area containing Cy3-labelled specific probe (AOBMIX) cells was quantified as a percentage of the area of Cy5-labelled bacteria probe (EUBMIX) within each image using pixel counting program.

4-amino-5-methylamino-2′,7-difluorofluorescein diacetate (DAF-FM DA) [21] was used for a visual qualitative assessment of the cellular NO production [22]. In the same procedure DAPI was used for the qualitative assessment of all bacteria. Cell suspension was diluted with 20µM DAF-FM DA solution and incubated for 60 minutes at room temperature and dark conditions. After a 50µg/mL DAPI solution was added to the cell suspension and DAF-FM DA solution and it was kept 15 minutes at 4ºC protected from the light. Then it was centrifuged and washed with a 0.5M TrisHCl solution and incubated for 30 minutes at room temperature in dark conditions before being visualized with an epifluorescence microscope.

2.4 N_2O and NO gas measurements

The N_2O and NO emissions were continuously analysed by commercial gas analysers. NO was analysed via a chemiluminescence gas analyser CLD64 (Eco Physics, Dürten, Switzerland). N_2O was analysed with an infra-red gas analyser V-A 3000 (Horiba, Japan) equipped with a sample conditioning system (series CSS, M&C Tech group). Off gas was collected continuously (at 5 L/min) from the reactor headspace and concentration data was logged every 15 s for the N_2O and every 5 s for the NO concentration.

2.5 Calculations

In order to calculate N_2O emissions equation 1 was used.

$$N_2O\ emitted = \sum\left(C_{N-N_2O} \cdot Q_{gas} \cdot \Delta t\right) \qquad \text{(Eq. 1)}$$

Where

$$C_{N-N_2O} = C_{N-N_2O}\ (\text{ppmv}) \cdot \text{N}_2\text{O molar volume } (0.0402 \text{ at 1atm and } 25^{\circ}\text{C}) \cdot 10^{-6} \cdot 28\left({}^{\text{g N}-\text{N}_2\text{O}}/_{\text{L}}\right)$$

Q_{gas} is the gas flow rate (L/min)

Δt is the time interval by which the off-gas N_2O and NO concentration was recorded

A homologous calculation was done for the NO emission but the concentration of NO (g NO/L) was multiplied by 14 g/mol instead of 28.

In order to calculate the N_2O and NO production rates equations 2 and 3 were used:

$$N_2O\ production\ rate\ (g\ N - N_2O/g\ VSS \cdot min) = \frac{\sum N_2O\ emitted\ (g)}{\Delta t(min) \cdot \frac{gVSS}{L} \cdot V(L)} \qquad \text{(Eq. 2)}$$

$$NO\ production\ rate\ (g\ N - NO/g\ VSS \cdot min) = \frac{\sum NO\ emitted\ (g)}{\Delta t(min) \cdot \frac{gVSS}{L} \cdot V(L)} \qquad \text{(Eq. 3)}$$

Where

V is the volume of the reactor at the moment that the MLVSS were taken

Δt is the interval of time during which the N_2O or the NO production rates were calculated.

Ammonia oxidation specific rate was calculated as follows:

$$AOR_{sp} = \frac{\text{N−NH4+ consumed}}{\text{g VSS·min}} \qquad \text{(Eq. 4)}$$

3. Results

3.1 Reactor performance

After 1 year of operation, stable nitrogen removal was achieved in the AOB-SBR. The reactor was operating with a 91% of ammonia converted to nitrite and nitrate was not accumulated in the effluent, achieving a complete nitritation process. Quantification of the AOB abundance in the biomass through the FISH technique showed that 79.3 ± 3.6 % of the bacterial community was targeted with the AOBmix probe.

Ammonia was consumed and nitrite was produced in both aerobic phases. There was a peak of N_2O and NO at the beginning of the cycle (Fig 1). These emissions were produced during the first 5 minutes of the cycle and then decreased very quickly. The peak of N_2O was much greater than the one of NO (500ppmv and 6ppmv, respectively). The peak of N_2O decreased sharply after the addition of ammonia but the production level of NO showed a gradual increase possibly corresponding to the increase on the nitrite concentration. Also the NO concentrations decreased when DO increased. When ammonium was almost depleted NO decreased to nearly zero. After the second feed there was another peak of NO which was lower than the one observed during the first 5 minutes of the cycle which can be related to the concentration of the ammonia. The pattern of NO in the second aerobic phase was similar to the one in the first aerobic phase showing a gradual increase likely due to an increase on the nitrite concentration and a decrease when DO was decreased. However, N_2O did not show the same pattern on the second aerobic phase since after the second feeding phase, there was a much lower peak of N_2O than in the first feeding phase. This is due to the fact that the production of N_2O also occurred during the settling phase and was emitted during the first 5 minutes of the cycle due to stripping when aeration started [12].

3.2 Correlation of NO and N_2O with AOR

In order to identify the correlation between NO and N_2O production and the ammonia oxidation rate, different concentrations of ammonia were added to the reactor to achieve different ammonia oxidation rates. Figure 2 shows an example of the profiles of NO, N_2O and NH_4^+ obtained in the first set of experiments.

Before the addition of ammonia there was no NO or N_2O emissions, indicating that the oxidation of ammonia by AOB had to be occurring to detect emissions. At minute 20, 50 mg N-NH_4^+/L were added as a pulse followed by a continuous addition of ammonia throughout all the experiment. At minute 90 and 155, two more pulses of 50 mg N-NH_4^+/L were added. After these pulses, a peak of N_2O was observed which decreased as ammonia was decreasing. On the other hand, NO presented a peak after each addition of ammonia. However, differing from the N_2O pattern, NO increased its baseline every time that ammonia was added suggesting an effect of the ammonia concentration on the NO production. The ammonia oxidation rate was 0.70, 0.86 and 1.08 mg N-NH_4^+/g VSS·min, respectively after the addition of each pulse.

Figure 3 shows the results obtained in the first set of experiments that were conducted at DO=1.5-2 mg O_2/L and pH=7-7.3 which are the same parameters used in the parent SBR. The different concentrations of ammonia were added in pulses to study the effect of AORsp in NO (Fig. 3a) and N_2O production rates (Fig. 3b).

Slightly higher NO than N_2O emissions were observed at the lower AORsp range (from 0 to 1 mg N/g VSS·min). At higher AORsp, N_2O emissions overcame the emissions from NO. The relationship between NO production rate and AORsp was lineal (r^2=0.81) whereas the relationship of the N_2O production and the ammonia oxidation rate was exponential (r^2=0.75. An r^2=0.6 was obtained when a linear relationship was fitted into the N2O vs AOR data).

Linear correlations were found with the ammonium concentration (Fig A.1). This is due to the fact that an increase on ammonia resulted in an increased AOR (Fig A.2) which has been previously reported to be the true factor affecting N_2O emissions [7].

During these tests, some sludge samples were taken to conduct a chemical staining for NO. Figure 4 shows the presence of NO inside the biomass extracted from the test conducted at AORsp of 1.08 mg N/g VSS·min (Figure 3). The majority of the biomass was targeted by the NO stain, indicating the biological origin of NO during these tests.

3.3 Effect of DO on NO and N_2O emissions

The second set of experiments was conducted to assess the effect of DO and anoxic conditions on the overall NO and N_2O emissions. Figure 5 shows the profiles of NO, N_2O, NH_4^+, NO_2^-, pH and DO when DO was decreased (a) and increased (b) in a step-wise mode.

In the test were DO was decreased (Figure 5a), N_2O increased in a linear manner and only a small jump on the N_2O signal was observed when the DO was reduced to the lowest set point tested. On the other hand, the NO signal suffered a small decrease every time the DO set point was decreased but within the same DO range, the NO profile was relatively constant.

On the other hand, in the test where DO was increased from 0.5 to 3 mg O_2/L (Figure 5b), N_2O increased within the first two DO set-points and also a jump on the N_2O concentration was detected when moving from the lowest DO to the intermediate set-point tested. Interestingly, the N_2O concentration started to decrease as soon as the DO set-point was increased to 2.5-3 mg O_2/L. NO had a similar pattern as in the other test. Its concentration remained stable under each DO set-point only increasing when the set-point was increased. Table 1 shows a comparison between the rates and ratios obtained during the different DO set-points in both experiments.

Table 1: N_2O and NO emission rates and ratios and AORsp at different DO levels and activity of the AOBs when DO was decreasing and increasing.

DO decreasing					
Range of DO (mg O_2/L)	N_2O production rate (mg N/g VSS ·h)	N_2O produced/NH_4^+ consumed	NO production rate (mg N/g VSS ·h)	NO produced/NH_4^+ consumed	AORsp (mg N-NH_4^+/g VSS ·h)
2.50-3.00	0.06	0.08%	0.06	0.08%	73.65
1.50-2.00	0.08	0.09%	0.05	0.06%	88.59
0.50-1.00	0.14	0.18%	0.05	0.06%	77.18
DO increasing					

DO (mg O_2/L)	N_2O production rate (mg N/g VSS ·h)	N_2O produced/NH_4^+ consumed	NO production rate (mg N/g VSS ·h)	NO produced/NH_4^+ consumed	AOR (mg N-NH_4^+/g VSS ·h)
0.50-1.00	0.06	0.23%	0.08	0.29%	25.47
1.50-2.00	0.16	0.27%	0.14	0.23%	60.78
2.50-3.00	0.18	0.24%	0.20	0.27%	73.64

When comparing both experiments it was observed that both N_2O and NO production were higher in the experiment where the DO was increased from 0.5 to 3 mg O_2/L as compared with the test where the DO was decreased. This might be related to the different behavior in terms of the AORsp detected between both tests (Table 1). In the test started with the lowest DO concentration range, the AORsp increased progressively when the DO was increased, indicating that the AOR was limited by the DO at the beginning of the test. Interestingly, in the batch started with the highest DO range, the AOR remained relatively constant at high values and seemed not to be affected by the DO.

Another experiment was conducted under anoxic conditions to determine the possible effect of oxygen depletion on NO and N_2O emissions in AOB. Figure 6 shows the profiles of NO, N_2O, NO_2^-, pH and DO when DO was 0 mg O_2/L. Results show that as soon as DO was depleted from the mixed liquor, there was a peak of NO and a very low peak of N_2O suggesting that nitric oxide production was more affected by anoxic conditions than N_2O production. The production of NO was significant and after the peak it was slowly decreasing until reaching a stable value at around 15 ppmv. On the other hand, N_2O showed a low peak and afterwards it remained constant at around 5 ppmv, also indicating a continuous production of N_2O during anoxic conditions.

3.4 The effect of pH on N_2O and NO emissions

Figure 7 shows the effect of a step-wise pH decrease from 8 to 6.5 on N_2O and NO emissions. DO was kept constant at 1.5-2 mg O_2/L which are the same conditions as in the parent SBR.

Before ammonia addition, no emissions of NO or N_2O were detected. Around minute 20, ammonia was added which produced a peak on N_2O. This peak is associated to the activation of the ammonia oxidation by AOB and lasted for 10 min approximately, reaching a stable N_2O baseline after the decrease of the peak. Every time that pH was decreased, N_2O also decreased, reaching a new baseline. On the other hand, the NO emissions detected follow a complete different trend. NO increased to a baseline when ammonia was added. But each time the set point of pH was decreased 0.5 points by adding 0.6M HCl, NO increased in the form of a peak. The fact that NO showed a peak when HCl was added suggests a chemical formation of NO. In order to clarify this hypothesis batch tests 3.2-3.5 were conducted.

Figure 8 shows the results of batches 3.2 and 3.3 using biomass diluted with effluent water with high concentrations of nitrite and without ammonia (a) and without biomass neither ammonia but using the effluent water with high nitrite concentrations (b).

When ammonia was not added in the AOB culture (Figure 8a) the production of N_2O was negligible even when pH was changed. However, NO was produced each time HCl was added in a similar fashion as observed in figure 7. In the case when AOB biomass was removed from the reactor (Figure 8b) N_2O was neither produced but the same pattern for NO was observed. This clearly indicates that NO was chemically produced due to the addition of HCl. Further experiments were conducted with RO water that did not contain nitrite (Figure A.3, batch test 3.4). In this case NO emissions were not detected indicating that nitrite was the precursor of the chemical production of NO. Also, a test was conducted with RO water to assess the effect of increasing the pH with NaOH (Fig A.4) but no emissions were detected in that case.

4. Discussion

4.1 Correlation of NO and N_2O vs AORsp

Results showed that the correlation between N_2O and AORsp was exponential whereas the relationship between NO and AORsp was lineal. The exponential correlation between N_2O and AORsp was also found by Law et al. [7] using an enriched AOB culture similar to the one used in this study. In their case the range of AORsp tested was wider (0-5.8 mg $N\text{-}NH_4^+$/g VSS·min) than the one used in this study (0-2 mg $N\text{-}NH_4^+$/g VSS·min). These authors also postulated that at high ammonia and nitrite concentrations (500 mg N/L) and low DO concentrations (0.5-0.8 mg O_2/L), the chemical breakdown of the nitrosyl radical (NOH), an intermediate in NH_2OH oxidation to nitrite could become dominant for the production of N_2O. To avoid this increase on N_2O production, they suggested that AOR should be lower than its maximum level to minimize the N_2O production rate. Also, Schneider et al. [21] reported that the N_2O specific production rate was positively correlated with the AORsp during stable nitritation reporting a linear correlation in their study.

Fewer studies have been focused on NO. Stüven and Bock [14] reported that for a pure culture of *Nitrosomonas europaea* in synthetic wastewater, NO production rate linearly correlated to its ammonia oxidation rate. They postulated that release of NO was due to an imbalanced ammonium oxidation in the oxidation of hydroxylamine. They also postulated that NO production is a side effect of a detoxification mechanism used by AOBs to eliminate the nitrite. This would explain the fact that ammonia oxidizers continuously produce relatively high amounts of NO and, occasionally, nitrogen dioxide (NO_2).

The linear relationship between NO production and the AORsp in this study suggests that the production of NO is higher than its reduction leading to the accumulation of this gas. This is in agreement with Kozlowski et al [17] who found that a pure culture of *N. multiformis* (AOB) had a linear rate of oxygen consumption during ammonia oxidation and this oxygen consumption led to a production of NO till a maximum and then when half of the available oxygen was consumed, NO started being consumed. A possible mitigation strategy would be reducing the AOR and trying to reach the point where AOR is equal or lower than the nitric oxide reduction rate. At the same time, this would also reduce the N_2O emissions. This is in agreement with Kozlowski and co-workers [16] who suggested that the absence of NorB expression alone in *N. europaea* had no effect on growth or substrate oxidation rates or on NH_2OH accumulation but did result in diminished N_2O production in comparison to that of the wild type.

These results highlight the importance of also monitoring NO emissions on those systems where AOB are dominant.

4.2 The effect of changing DO

Higher N_2O and NO emissions were detected in the test with increasing DO. This could be due to the difference on the activity of AOBs. From the results reported in this paper, AOB activity and its emissions seem to be influenced not only by the DO applied but also by the conditions that AOB have been previously exposed to since interestingly, in the batch started with the highest DO range, the AOR remained relatively constant at high values and seemed not to be affected by the DO.

The fact that N_2O emissions decreased when DO increased could be due to a change on the contribution pathway for N_2O production. This was reported by Peng et al. [8] who studied the effect of DO on a nitrifying culture and determined that as DO increased the contribution of the nitrifier denitrification pathway decreased while the contribution of the hydroxylamine oxidation pathway increased. However, later on Peng et al. [9] suggested that nitrifier denitrification was the dominant contribution pathway of N_2O production in an enriched nitrifying sludge with AOBs and NOBs in a wide range of DO and nitrite concentrations. They reported that the hydroxylamine oxidation pathway was only active when DO was high and nitrite was low which is not the case here.

When anoxic conditions were applied in the reactor, an immediately production of NO and N_2O was observed. The production of NO was 7 times higher than that of N_2O. Anoxic conditions in AOB have been suggested to cause an over expression of the nitrite reductase gene and an under-expression of the genes encoding for ammonia oxidation, hydroxylamine oxidation and nitric oxide reduction leading to NO accumulation [24,25]. Yu et al. [13] reported that under anoxic or anaerobic conditions, AOBs can utilize alternate electron acceptors such as nitrite, dimeric nitrogen oxide (N_2O_4) and produce N_2O and NO. They showed a production of NO under strict anoxic conditions which correlates with our results but no N_2O production was reported. Also, Kampschreur et al. [25] reported that oxygen depletion during ammonia oxidation clearly increased NO emissions in an enriched nitrifying culture. However, Law et al. [6] showed that NO was produced under anoxic conditions but N_2O was produced in the transient from anoxic to aerobic. In our study, N_2O was produced under anoxic conditions (Fig. 6). Schmidt [26] reported that the oxidation of hydroxylamine does not depend on oxygen and it is catalyzed by HAO under both oxic and anoxic conditions which could explain the production of N_2O when DO is zero. This would suggest that N_2O emitted under anoxic conditions would be produced through the hydroxylamine pathway.

4.3 The effect of pH

The results of the third set of experiments conducted decreasing the pH revealed that N_2O was produced biologically when ammonia was present and that each time the set point of pH was decreased, N_2O decreased to a new baseline. These results agree with the ones obtained by Law et al. [6] who reported an immediate change on the N_2O production when pH was changed from 7 to 8 till reaching a new baseline in a partial nitritation reactor. They also showed a negligible production of N_2O when ammonia was not present but there was nitrite and pH was changed which corroborates with our results (Figure 8a). On the other hand, NO was produced chemically in the tests. Each time HCl was added, there was a peak of NO that decreased sharply after the addition. This production could be due to the deprotonation of HNO_2 (Eq. 3), since the pka value of the NO_2^-/HNO_2 couple is 3.29 and therefore under acidic conditions NO will be formed [28,29]. The fact that there is a NO peak every time that HCl is added might indicate that there is a sudden local pH drop to values lower than the pH setpoint, originating the NO peaks detected. After the water volume is homogenized the NO returns to its baseline level, that is attributed to that particular pH.

$$2HNO_2 \leftrightarrow NO + NO_2 + H_2O \quad \text{(Eq. 3)}$$

The results from this study highlight the importance of monitoring NO in addition to N_2O. In order to assess operational strategies to mitigate N_2O emissions, NO emissions being controlled could help to diminish N_2O emissions.

5. Conclusions

The main conclusions of this study are:

- NO linearly correlates with the ammonia oxidation rate whereas N_2O has an exponential correlation with the AOR.
- NO and N_2O can be produced under anoxic conditions in a partial nitritation system, being the production of NO much higher than that of N_2O.
- NO is chemically produced when pH is decreased with HCl. N_2O is not affected by this addition.
- NO emissions cannot be neglected in those reactors where AOB are predominant.

Acknowledgments

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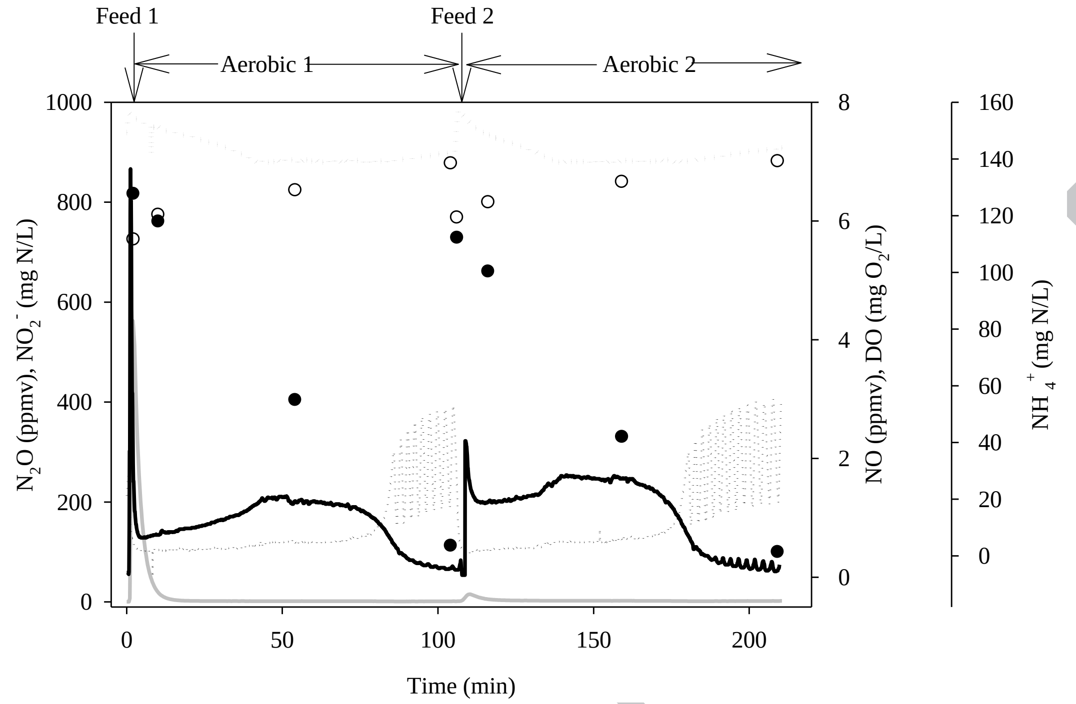


Figure 1: Experimental profiles of N_2O (—), NH_4^+ (●), NO_2^- (○), NO (—), DO (···) and pH (···) during a typical cycle study of the AOB reactor. Nitrate was not detected in any of the samples taken.

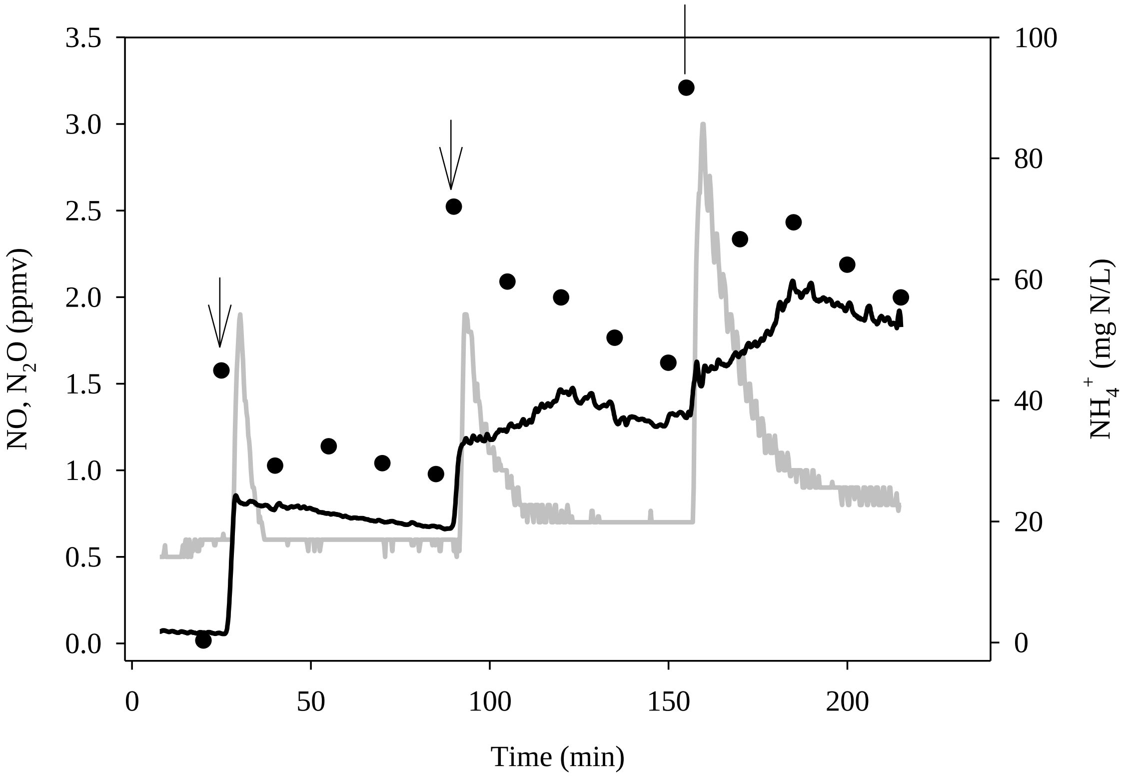
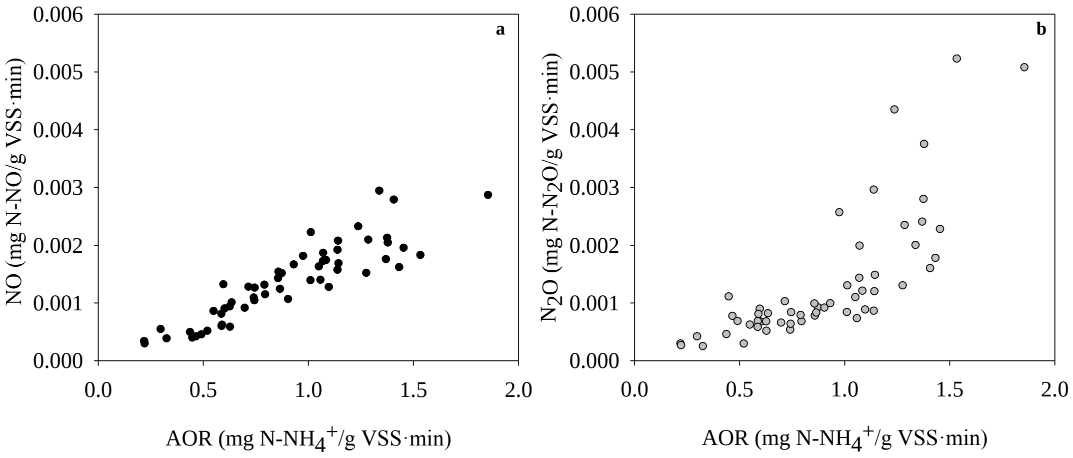


Figure 2: Experimental profiles of NO(—), N_2O (—) and NH_4^+ (●) at pH 7 and DO=1.5-2 mg O_2/L. The arrows represent the time when a pulse of ammonia was added. Nitrate was not detected in any of the samples taken.



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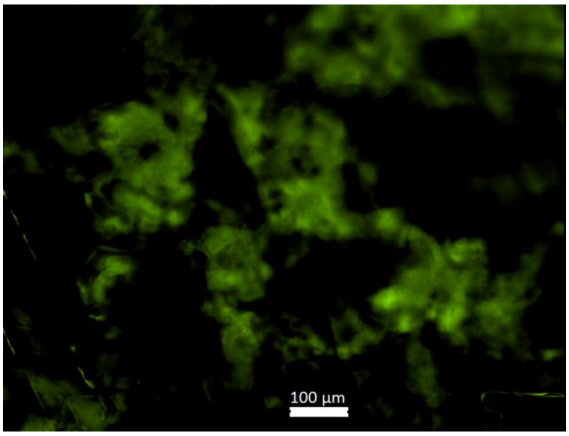

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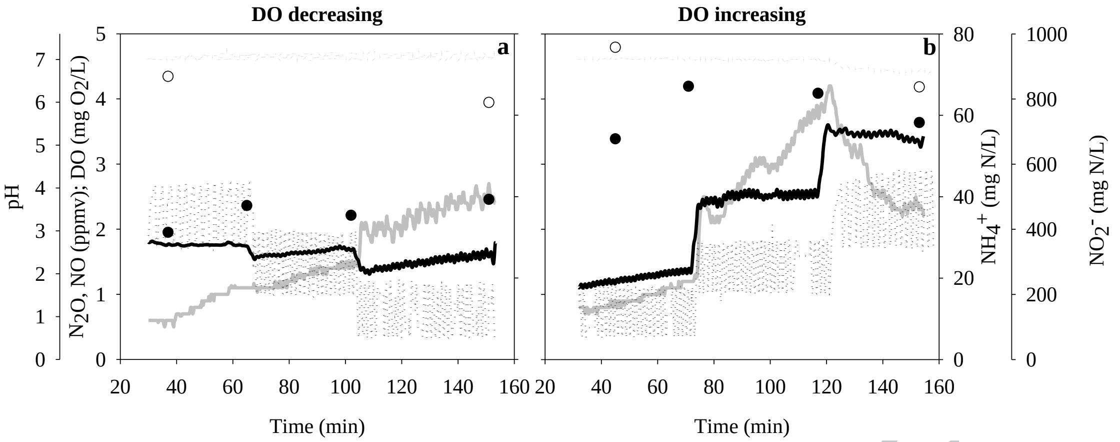

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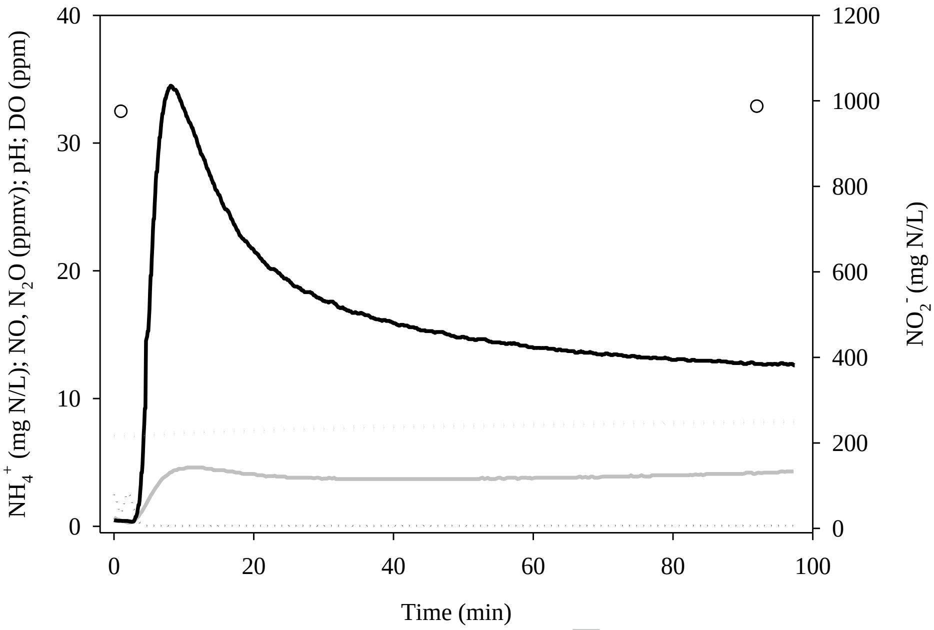

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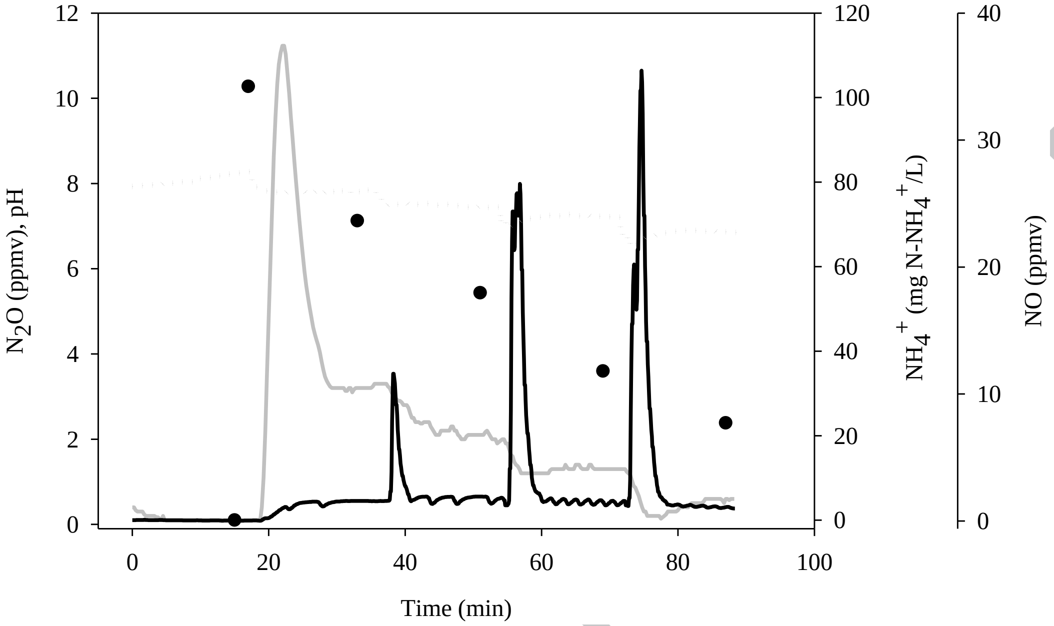

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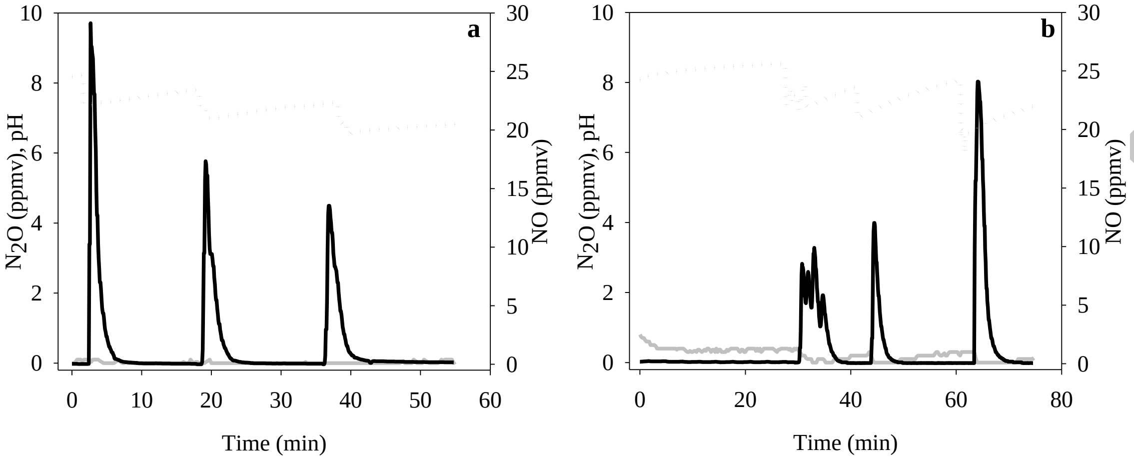

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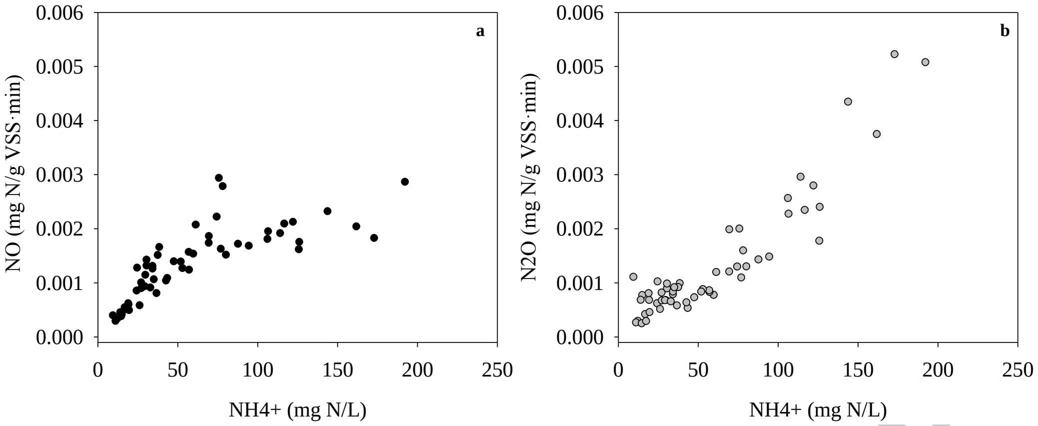


Figure A.1: Correlation between the specific NO production rate and ammonia concentration (a) and the specific N_2O production rate and the ammonia concentration (b).

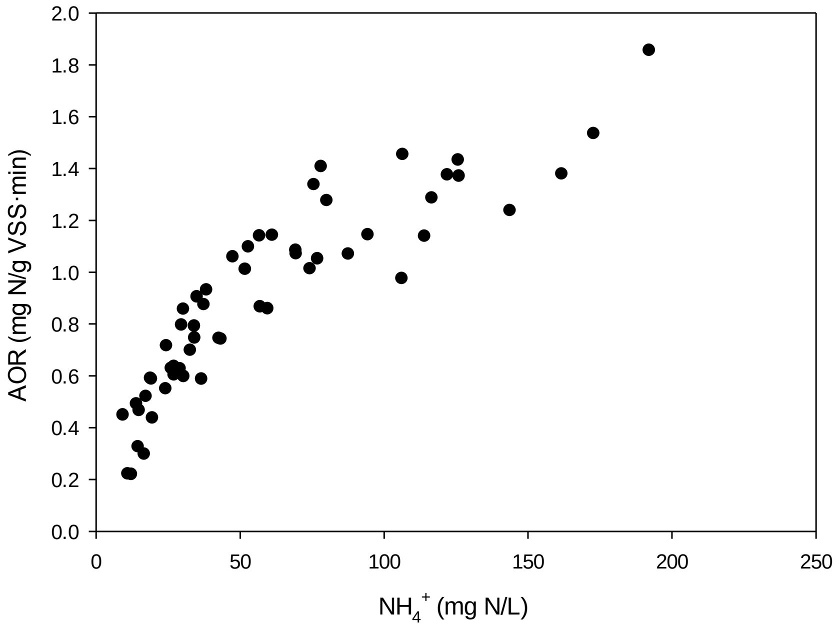
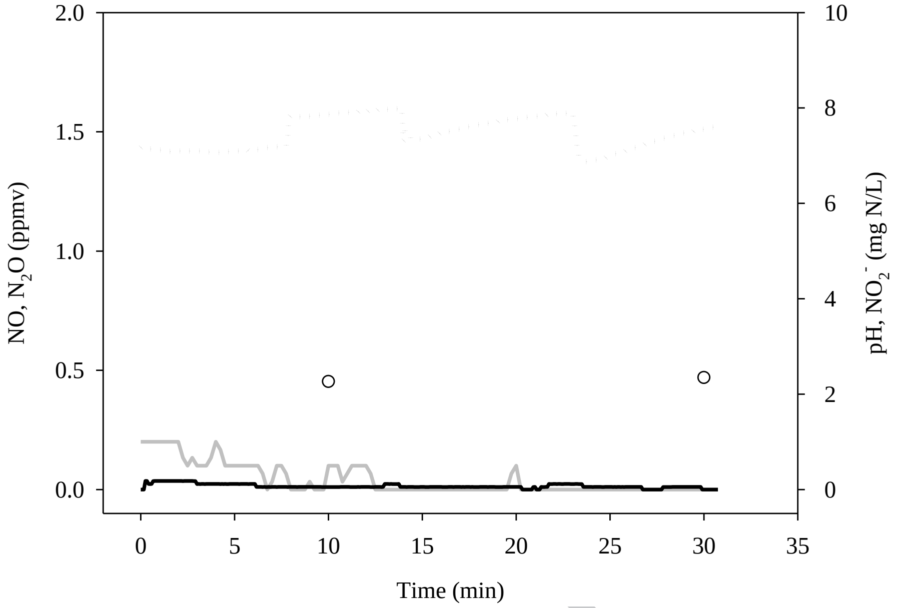


Figure A.2: Correlation between ammonia oxidation rate and the different ammonia concentrations.



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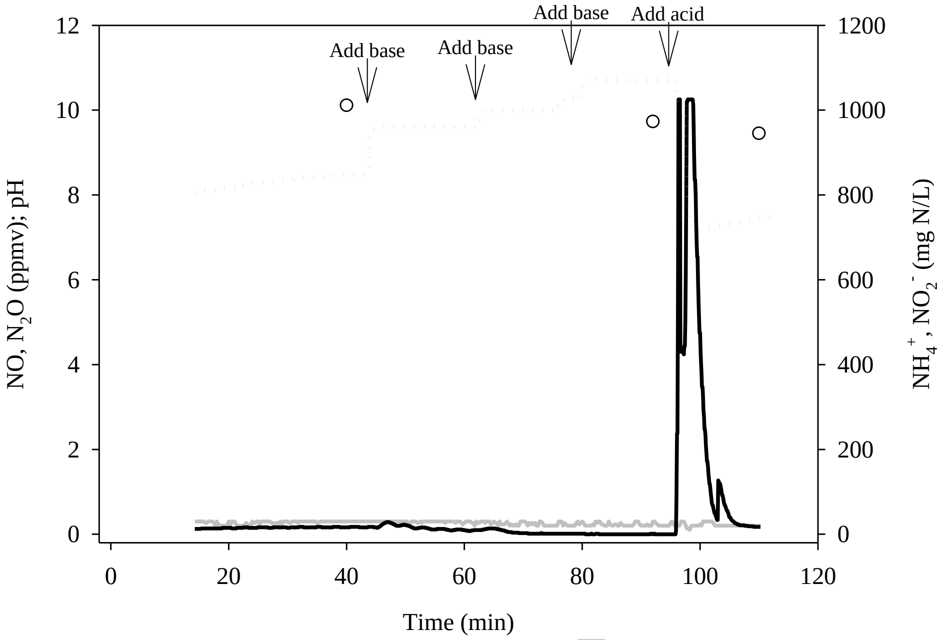

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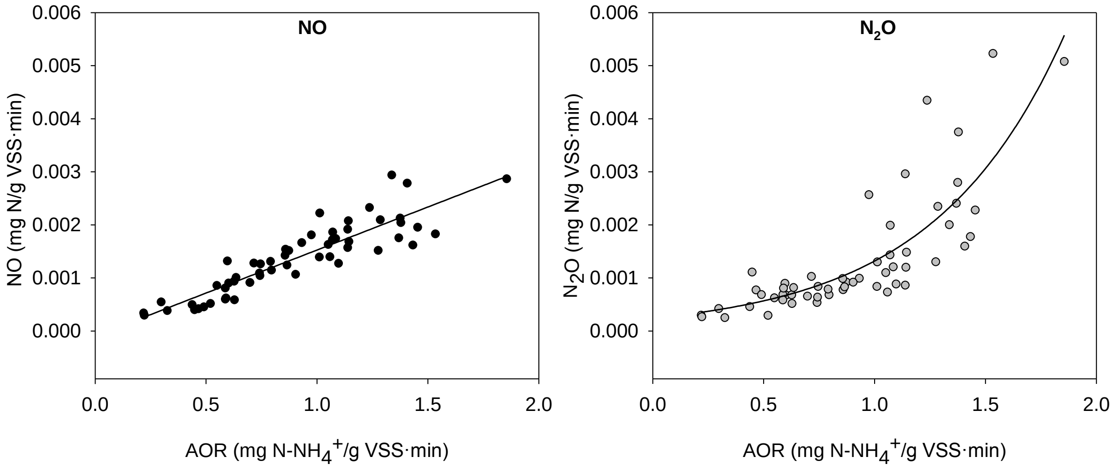
NO
NO (mg N/g VSS·min)
0.006
0.005
0.004
0.003
0.002
0.001
0.000
0.0
0.5
1.0
1.5
2.0
AOR (mg N-NH4+/g VSS·min)
N2O
N2O (mg N/g VSS·min)
0.006
0.005
0.004
0.003
0.002
0.001
0.000
0.0
0.5
1.0
1.5
2.0
AOR (mg N-NH4+/g VSS·min)

Highlights

- Relationship between NO and N_2O production rate and AORsp in an enriched AOB culture is studied.
- Effect of pH and DO on N_2O and NO production are assessed
- NO linearly correlates with the AORsp while N_2O correlates exponentially
- N_2O and NO can be produced under anoxic conditions
- NO is chemically produced when HCl is added