Importance of hydroxylamine in abiotic N₂O production during transient anoxia in planktonic axenic *Nitrosomonas* cultures

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**Graphical Abstract**

**Abstract**

When investigating the N₂O emissions by ammonia oxidizing bacteria, research has mainly focused on identifying and quantifying the biological pathways. This work evaluated previous studies with *Nitrosomonas europaea* (ATCC 19718) and assessed the role of the abiotic reaction of hydroxylamine with free nitrous acid during transient anoxia. In cultivations when transient anoxia is cyclically imposed, nitrous oxide and hydroxylamine peaked every time upon recovery to aerobic conditions. When using the same culture conditions abiotically (i.e., without biomass, but adding hydroxylamine and nitrite), the volumetric N₂O emission rates were very comparable to those from the biological experiments, ranging from 0.04 to 0.08 mg-N/L/h in both abiotic and biotic conditions. These results demonstrate that at the culture conditions tested, abiotically produced N₂O is likely the major source of emission. Therefore, for the correct investigation of the biological pathways, abiotic tests must always be performed and hydroxylamine should be added. To our knowledge there is no means to distinguish abiotic from biological N₂O production in a biological system, even using N¹⁵ labelling. We suggest that the contribution of abiotic N₂O emissions can be minimized by, for example, maintaining lower nitrite concentration and higher pH.

**Keywords:**

Nitrous oxide  
Ammonium oxidizing bacteria  
Abiotic emissions  
Chemical reaction  
Hydroxylamine  
Nitrous acid

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1. Introduction

Nitrous oxide (N\textsubscript{2}O) and nitric oxide (NO) can be emitted during nitrogen removal in lab-scale reactors and wastewater treatment plants (WWTPs). N\textsubscript{2}O and NO are ozone depleting gases. Moreover, due to a 300-fold larger greenhouse effect compared to that of CO\textsubscript{2}, the study of N\textsubscript{2}O emissions has increased over the past years. Main contributors to N\textsubscript{2}O and NO emissions are both nitrification and denitrification processes [1]. Factors identified to contribute to increased emissions are: (i) low dissolved oxygen and high nitrite concentrations in both nitrification and denitrification [1], (ii) low COD/N ratio during denitrification [1], (iii) limited inorganic carbon in nitrification-anaerobic processes [2].

In nitrification systems ammonium oxidizing bacteria (AOB) or archaea (AOA) are reported to be the main contributors to NO/N\textsubscript{2}O emissions [1]. AOB convert ammonium to nitrite in two steps: ammonium oxidation to hydroxylamine (NH\textsubscript{2}OH) by ammonium monoxygenase (AMO) and further hydroxylamine oxidation to nitrite by hydroxylamine oxidoreductase (HAO). It has been recently proposed that the ammonium oxidation could also occur via a three-step reaction [3]. Experiments in vitro showed that NO is an obligate intermediate in hydroxylamine oxidation by HAO, implying that hydroxylamine is biochemically oxidized to NO which is subsequently converted in a chemical oxidation to NO\textsubscript{2} [3]. In vivo studies are still needed to confirm this potential reaction mechanism. Regarding NO/N\textsubscript{2}O production traditionally, two pathways have been described for the production of N\textsubscript{2}O in the AOB metabolism: by hydroxylamine oxidation and by nitrifier denitrification [1,4,5]. However, recent studies have highlighted the complexity and the existence of multiple pathways branching from the traditional ones, with contributions by both abiotic and biological reactions [6–9] (see Fig. 1).

Possible enzymes responsible of hydroxylamine oxidation into N\textsubscript{2}O have been under discussion [10]. Recent studies showed a possible involvement of the cytochrome P460 from the HAO enzyme. This cytochrome can convert two NH\textsubscript{2}OH molecules to N\textsubscript{2}O via an iron mediated catalytic reaction [7]. Incomplete oxidation of hydroxylamine by HAO might also lead to intermediates, such as NO or HNO, that can interact with the cytochrome can convert two NH\textsubscript{2}OH molecules to N\textsubscript{2}O via an iron

\text{HN\textsubscript{2}OH + HNO} \rightarrow N\textsubscript{2}O + 2H\text{O}

(1)

The chemical reaction of hydroxylamine with free nitrous acid has not been considered as a possible contributor to the overall N\textsubscript{2}O emissions until recently [6,15], due to several reasons. Firstly, abiotic controls are usually not executed/reported [4,16–19] and even if these controls are performed, hydroxylamine is not included in the medium [20–23]. Secondly, the complicated chemistry of related N-compounds [24–26] together with the highly reactive hydroxylamine, as the intermediate that may accumulate in the bulk liquid, is not well characterized. Finally, complex engineered ecosystems are usually used to study N\textsubscript{2}O emissions. Thus, (i) diverse AOB consortia may coexist with other potential N\textsubscript{2}O microbial producers (e.g., heterotrophic denitrifiers) and (ii) micro-gradients of substrates and oxygen in colonies, flocs and biofilms are usually present, all resulting in a system difficult to interpret leading to underestimation of some pathways. A better strategy to understand the basic pathways producing N\textsubscript{2}O in AOB and the conditions affecting the extent of N\textsubscript{2}O emissions has been the use of axenic cultures of a single representative species (e.g., Nitrosomonas europaea) cultivated as planktonic cells. This avoids micro-gradients of substrates and oxygen in colonies, flocs and biofilms, allowing for a more direct examination of potential N\textsubscript{2}O pathways [4,8,11,17,18], among others).

Consequently, more research is needed to reassess the biological pathways, while acknowledging the extent of abiotic contributions. Abiotic reaction of hydroxylamine with nitrite is likely to occur in biological systems with nitrite and hydroxylamine accumulation [5,10,12,23]. Thus, part of this biologically produced hydroxylamine and free nitrous acid can abiotically react forming N\textsubscript{2}O.

Hydroxylamine accumulation in the liquid can be the direct or indirect key factor promoting N\textsubscript{2}O emissions. During nitrification, hydroxylamine has been reported to accumulate in the bulk liquid in the range of 0.01–1 mg·N-NH\textsubscript{2}OH/L [2,17,19,27,28]. This is supposed to be happening when conditions in the reactor favors specific growth rates close to the maximum [18]. At these high conversion rates, the rate of ammonium conversion to hydroxylamine and its further oxidation to nitrite can be imbalanced, resulting in hydroxylamine accumulation. For instance, imposing transient anoxia disturbances in a continuous aerobic axenic culture of Nitrosomonas europaea triggered hydroxylamine build up in the bulk liquid [4,19]. Studies of NO/N\textsubscript{2}O production during transient anoxia are important, as most of the engineered systems are subject to sudden dissolved oxygen (DO) changes. In addition, switching conditions between aerobic and anaerobic in a single tank are imposed in conventional biological nitrogen removal processes. Both the effect of DO changes and transient anoxia on NO/N\textsubscript{2}O emissions have been extensively studied [4,17,18,22,23], among others). During transient anoxia NO was only produced in anaerobic conditions, and no N\textsubscript{2}O emissions were detected [4,18,19]. However, immediately after recovery to aerobic conditions N\textsubscript{2}O was emitted simultaneously with the hydroxylamine accumulation [4,18,19]. Yu et al. [18], could not find a correlation between N\textsubscript{2}O emissions and gene expression, instead, they linked emissions to a metabolic shift from low to maximum specific activity. However, no abiotic controls containing both hydroxylamine and nitrite were performed in any of these studies [18,19].

In the present study, the dynamics of N\textsubscript{2}O emission from a Nitrosomonas europaea axenic culture during transient anoxia are reported, discussed and compared to abiotic tests. Culture conditions were abiotically replicated, by adding nitrite and hydroxylamine in the
concentrations measured just after the switch to aerobic conditions in the biological system. The aim was to assess the consequences of transient anoxia in _N. europaea_ and the contribution of abiotic emissions on N\(_2\)O formation. Furthermore, some recommendations are provided for future research in order to reduce the contribution of abiotic N\(_2\)O emissions when investigating biological production pathways in either axenic cultures or natural and engineered ecosystems.

2. Materials and methods

2.1. _Nitrosomonas europaea_ cultivation

_Nitrosomonas europaea_ (ATCC 19718) planktonic cultures were cultivated in dark in triplicate chemostats (6 L total volume, 4 L operating volume, 21 °C, pH 7.5 ± 0.1) at a dilution rate of 0.45 d\(^{-1}\) [19]. The growth medium contained 20 mM NH\(_4\)\(^+\) and (per liter): 0.2 g of MgSO\(_4\)·7H\(_2\)O, 0.02 g of CaCl\(_2\)·2H\(_2\)O, 0.087 g of K\(_2\)HPO\(_4\), 2.52 g EPPS (3-(4-(2-hydroxyethyl)-1-piperazine) propanesulfonic acid), 1 mL of 13% EDTA-Fe\(^{3+}\), 1 mL of trace elements solution (10 mg of Na\(_2\)MoO\(_4\)·2H\(_2\)O, 172 mg of MnCl\(_2\)·4H\(_2\)O, 10 mg of ZnSO\(_4\)·7H\(_2\)O, 0.4 mg of CoCl\(_2\)·6H\(_2\)O, and 100 mL of distilled water), 0.5 mL of 0.5% phenol red, and 0.5 mL of 2 mM CuSO\(_4\)·5H\(_2\)O [19]. In order to mimic conditions that can be found in engineered systems during biological nitrogen removal, transient anoxia was imposed and the culture adaptation was followed in time. Although not applicable across the board, the 'rule of thumb' for preliminary design of many pre-anoxic systems is 25% anoxic, 75% aerobic, so the transient aerobic-anoxic cycling was imposed accordingly. To impose transient anoxia, air was substituted by filtered N\(_2\) once per day during 6 h for 13 consecutive days (both at a flow rate of 2.7 L/min). Gaseous N\(_2\)O (gas-filter correlation, Teledyne API 320E, San Diego, CA), and NO (chemiluminescence, CLD-64, Ecophysics, Ann Arbor, MI) were measured online once every 2 min. Hydroxylamine concentration was measured spectrophotometrically [29]. More details on further reactor dynamics, physiochemical analysis and genomics/proteomics can be found in Ref. [19].

2.2. Abiotic batch tests

The conditions used for the cultivation of _N. europaea_ [18,19] were also used in abiotic tests to assess the N\(_2\)O emission rates through the reaction of hydroxylamine and free nitrous acid. In order to achieve a comparable gas composition (in ppmv), air flow to working volume ratio was maintained as in the _N. europaea_ cultures [19] (0.68 min\(^{-1}\)) and as in Yu et al., [17] (0.29 min\(^{-1}\)), while the ratio headspace to total volume was also kept to 1:3. Aerobic conditions were used in all abiotic tests, as in biological systems hydroxylamine accumulation is only related to continued ammonium oxidation, which only occurs under aerobic conditions. Reaction mixture contained 20 mM NH\(_4\)\(^+\) and (per liter): 0.2 g of MgSO\(_4\)·7H\(_2\)O, 0.02 g of CaCl\(_2\)·2H\(_2\)O, 0.087 g of K\(_2\)HPO\(_4\), 2.52 g EPPS (3-(4-(2-hydroxyethyl)-1-piperazine) propanesulfonic acid), 10 mL of 1.3% EDTA-Fe\(^{3+}\), 1 mL of trace elements solution (10 mg of Na\(_2\)MoO\(_4\)·2H\(_2\)O, 147 mg of MnSO\(_4\)·H\(_2\)O, 10 mg of ZnSO\(_4\)·7H\(_2\)O, 0.482 mg of CoSO\(_4\)·7H\(_2\)O, and 100 mL of distilled water), and 0.5 mL of 2 mM CuSO\(_4\)·5H\(_2\)O as described previously [18,19].

Medium containing all the metal ions was sparged with air and pH control was applied at the start to reach the target set point of 7.5 ± 0.1. Afterwards, solid NaN\(_2\)O was added to a final concentration of 230 mg-N-N\(_2\)O\(^2-\)/L (5 · 10\(^{-3}\) mg-N-FNA/L), so similar concentrations to those reported during biological cultivations were used. No N\(_2\)O was detected during the period when only nitrite was present with metals. Finally, after approximately half an hour, the necessary volume of a 14 mg-N/L hydroxylamine hydrochloride solution was added to achieve the desired concentrations for each test according to Table 1. Hydroxylamine reaction with metals producing nitrous oxide was ruled out in a previous work using comparable metals composition [6].

2.3. Calculations

The N\(_2\)O measurement in parts per million gas volume (ppmv) was converted to concentration of N\(_2\)O in mg-N/L gas. The N\(_2\)O emission rate (mg-N/h) was obtained from the N\(_2\)O concentration multiplied by the air flow used, followed by a normalization for the working volume to obtain the volumetric emission rate (mg-N/L/h). Details about these calculations and free nitrous acid calculations are provided in the Supplementary Material.

3. Results

3.1. N\(_2\)O production during transient anoxia in _Nitrosomonas europaea_ cultivation

An axenic steady-state chemostat culture of _Nitrosomonas europaea_ was exposed to transient anoxia. Full details of the experiments and discussion of transcription analysis, proteomics and metabolic network modelling can be found in Ref. [19]. To illustrate the changes during transient anoxia, the data is used here focusing on gas emissions and hydroxylamine dynamics (Fig. 2). The culture had minimal N\(_2\)O or NO formation under steady state conditions [19]. Transient anoxia was imposed for 6 h a day during 13 days. The nitrite concentration remained high (ca. 230 mg-N-N\(_2\)O\(^2-\)/L, 5 · 10\(^{-3}\) mg-N-FNA/L) during the whole operation [19]. The switch to aerobic conditions resulted in immediate ammonia consumption [19] and maximum hydroxylamine accumulation varied from 0.2 ± 0.05 to 0.07 ± 0.01 mg-N/L from day 1 to day 13 (Fig. 2A).

NO/N\(_2\)O gas emissions were measured throughout the reactor operation. Nitric oxide (NO) production immediately increased after switching to anoxic period and then decreased gradually during the rest of the operation (Fig. 2B). In contrast to NO, N\(_2\)O was only emitted immediately after the switch to aerobic conditions (Fig. 2C). The highest N\(_2\)O and NO emissions were observed in the first day of transient anoxia and the concentrations in the gas reached 1.8 ppmv N\(_2\)O and 9 ppmv NO (Fig. 2C). For hydroxylamine, accumulation in the liquid was detected at the same time with N\(_2\)O emission (Fig. 2A). Similar with the N\(_2\)O emission pattern, maximum NH\(_2\)OH values were reached in the first day (0.2 ± 0.05 mg-N/L) and decreased to 0.07 ± 0.01 mg-N/L on day 13 (Fig. 2A). Hydroxylamine was also detected during anoxic conditions at a certain constant concentration of ca. 0.07 ± 0.01 mg-N/L (Fig. 2A). However, that low and steady hydroxylamine concentration could be linked to the background absorbance, due to the presence of iron ions that produce a slight yellowish tinge. This was noticed also in a previous study, where background solutions were prepared with fresh cultivation medium [6].

3.2. Abiotic N\(_2\)O production in aerobic conditions

When replicating the _N. europaea_ culture conditions without any biomass, N\(_2\)O emissions were detected immediately after the addition of hydroxylamine. N\(_2\)O concentration peaked from 0.9 to 3 ppmv depending on experimental conditions (Fig. 3 and Table 1).

For the same nitrite concentration (230 mg-N-N\(_2\)O\(^2-\)/L, 5 · 10\(^{-3}\) mg-N-FNA/L) and medium composition, but different hydroxylamine concentrations (0.05–0.2 mg-N/L), the total N\(_2\)O emissions increased from 0.040 ± 0.002 to 0.14 ± 0.01 mg-N (Fig. 3A, Table 1). The ratio of air flow to working volume was 0.68 min\(^{-1}\), like in the biological experiments performed in this study.

For a lower air flow and working volume ratio, similar with conditions used in Yu et al., [18] the total N\(_2\)O emissions were the same for 0.1 mg-N/L of hydroxylamine concentration but 1.6-fold lower for 0.2 mg-N/L hydroxylamine (Fig. 3B, Table 1).

Calculated yields assuming hydroxylamine reaction with free nitrous acid producing N\(_2\)O emissions as in reaction (1) ranged from 20 ± 1% to 40 ± 2% (Table 1), clearly indicating the presence of side products.
reactions because the NH$_2$OH was completely converted at the end of experiments.

4. Discussion

4.1. Biological N$_2$O sources

In the studies of Yu et al., [18,19] (Fig. 2 and Table 2), N$_2$O emissions from _N. europaea_ cultures imposed to transient anoxia were only observed under recovery to aerobic conditions. That was also the case in other studies with pure cultures where different dissolved oxygen concentrations were tested [23]. Higher N$_2$O emissions correlated with lower oxygen tensions [22,23]. The increased N$_2$O production during recovery from anoxia was initially explained by an imbalanced metabolism during the transition from low to high cell specific activity [18]. However, a more recent study related the N$_2$O emissions to the proteomic level of cytochrome P460 [19]. On the other hand, the model proposed in Ref. [19] could not accurately predict the N$_2$O emissions during the aerobic period compared to the good representation obtained for NO emissions.

One of the processes involved in nitrous oxide emissions is the nitrifier denitrification pathway. NIR and NOR have been identified as the enzymes responsible of performing successive transformation of NO$_2^-$ into NO and then N$_2$O in _Nitrosomonas europaea_ [5,11,30] (Fig. 1). This pathway is active during anoxic and microaerobic conditions, where nitrite is used instead of oxygen as terminal electron acceptor, thus the eventually remaining oxygen can be used for ammonia oxidation. Moreover, Kozoluzh et al., [11] demonstrated that a double mutant lacking NOR and NIR was unable to produce N$_2$O during anaerobic or hypoxic conditions. However, the same double deficient mutant did produce N$_2$O during aerobic conditions, thus the authors suggested the presence of other enzymes not yet characterized as a possible explanation for aerobic emissions [11]. All this indicates that the main NO emissions measured during the anoxic phase (Fig. 2B) are likely due to nitrifier denitrification [18,19]. However, it is surprising that in spite of NO toxicity, this was not further transformed to N$_2$O. However, it is surprising that in spite of NO reactivity, this was not further transformed to N$_2$O. Genomics and proteomics analyses suggested that long term response to cyclic transient anoxia lead to downregulation of detoxification proteins (0.6, 0.5 and 0.8-fold for CypP460, c-S54 and NOR, respectively), whereas energy conversion was favored (2-fold change of AMO) [19]. Conversely, the N$_2$O emissions were just observed during the aerobic period (Fig. 2C) showing that other pathway than the denitrification was contributing to the N$_2$O production.

N$_2$O emissions during aerobic conditions are mainly related to hydroxylamine oxidation pathways, and the involved enzymes are still being investigated. So far, it has been reported that enzymatic extracts of HAO were able to catalyze the production of NO and N$_2$O in vitro, but its potential effect in vivo remains to be assessed [31]. Recently, cytochrome P460 has been shown to catalyze N$_2$O production from hydroxylamine, however the experiments were performed also in vitro [7]. Finally, Terada et al. highlighted the importance of the hydroxylamine reaction with nitrite either biocatalyzed or by abiotic transformations at high ammonia oxidation rates and nitrite concentrations in a partial nitritation reactor [9]. The operation conditions were similar to those used in our study (i.e., high nitrite concentrations). The proteomic and genomic analysis performed by Yu et al. revealed an adaptation of the biomass to repeated transient anoxia exposure [19]. However, these experiments were performed in conditions where abiotic emissions can occur. In view of all these possible biological conversions, the biological contribution to N$_2$O emissions during the aerobic period of the _N. europaea_ cultivations [18,19] cannot be excluded.

4.2. Importance of abiotic N$_2$O production in biological experiments with _N. europaea_

Most of the studies focused on the biologically emitted N$_2$O, even if different chemical reactions are known to produce N$_2$O [6,13-15,32]. In the present study, abiotic experiments confirmed that chemical N$_2$O production is possible under the conditions used for the cultivation of _N. europaea_ (Fig. 2), as well as in the conditions from a previous study [18]. The high nitrite concentration (ca. 230 mg-N/L) at pH 7.5 leads to a free nitrous acid concentration in the range of ca. 0.005 mg-N/L, which can react with hydroxylamine to produce N$_2$O. Hydroxylamine was added in the abiotic experiments in the concentrations measured during the anoxic cultivations performed previously (Table 1). Similarly, other studies have also reported hydroxylamine accumulation in the liquid in the range 0.01–1 mg-N-NH$_2$OH/L [2,17,27,28], and these concentrations are in agreement with those reported during the recovery from transient anoxia.

Most remarkably, the measured abiotic N$_2$O emissions rates (0.044–0.082 mg-N/L/h), (Table 1) were directly comparable with those recorded during recovery to aerobic conditions in biological experiments (0.043–0.085 mg-N/L/h, Table 2). Thus, it becomes clear that at the conditions at which _N. europaea_ was cultivated, abiotic N$_2$O emissions were fast enough to contribute significantly to the total emissions or be the only pathway of N$_2$O formation. In our abiotic experiments replicating conditions used by Yu et al. [18] the N$_2$O emissions were up to 3 ppmv, which represented about 1/3 of the peak emissions reported in Ref. [18]. However, because no hydroxylamine data were reported in Ref. [18], an accurate comparison with this study is not possible as the rate and ppmv of abiotic N$_2$O produced depends on the amount of hydroxylamine that is reacting.

It should be noted that the hydroxylamine measured in the continuous cultivation is the result of a balance between its continuous production by ammonia oxidation and consumption by either transformation to nitrite or N$_2$O. This indicates that the net accumulation of hydroxylamine might be very sensitive to a small change in one of the main conversions. Thus, the amount of hydroxylamine transformed chemically to N$_2$O in the continuous biological experiments can be different from the residual hydroxylamine measured and therefore different from the amount used in the abiotic tests. That would explain why the total N$_2$O produced during the continuous biological cultivations is higher than the total N$_2$O produced in the abiotic batch test. A biological contribution to N$_2$O emissions in the continuous cultivation

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**Table 1**

<table>
<thead>
<tr>
<th>Abiotic batch test</th>
<th>Initial hydroxylamine concentration (mg N/L)</th>
<th>Air flow/working volume ratio (min$^{-1}$)</th>
<th>Total N$_2$O emitted (mg N)</th>
<th>Yield relative to NH$_2$OH (%)</th>
<th>Maximum N$_2$O emission rate (mg N/h)</th>
<th>Volumetric maximum N$_2$O emission rate (mg N/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.68</td>
<td>0.040 ± 0.002</td>
<td>40 ± 2</td>
<td>0.06 ± 0.01</td>
<td>0.048 ± 0.008</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.68</td>
<td>0.056 ± 0.02</td>
<td>28 ± 8</td>
<td>0.08 ± 0.02</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>0.68</td>
<td>0.14 ± 0.01</td>
<td>35 ± 3</td>
<td>0.106 ± 0.002</td>
<td>0.0815 ± 0.001</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>0.29</td>
<td>0.056 ± 0.005</td>
<td>28 ± 2</td>
<td>0.0570 ± 0.0005</td>
<td>0.0440 ± 0.0005</td>
</tr>
<tr>
<td>5</td>
<td>0.2</td>
<td>0.29</td>
<td>0.083 ± 0.004</td>
<td>20 ± 1</td>
<td>0.080 ± 0.002</td>
<td>0.065 ± 0.002</td>
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</table>
cannot be ruled out, because it was not possible to assess the amount of hydroxylamine chemically transformed to N₂O. A study with mixed populations of nitrifiers in aggregates performed in a partial nitrification reactor [9] found that half of the N₂O emitted was produced through the reaction of hydroxylamine and nitrite (without elucidating the proportion of abiotic and biotic contributions). However, in the present work and in literature [18,19], axenic cultures of planktonic cells were used, thus gradient effects due to aggregated biomass and N₂O emissions by other pathways than that of *N. europaea* can be ruled out.

In the study of Yu et al. [18], N₂O emissions in the *N. europaea* culture during transient anoxia were associated to differences in specific cell activity upon the anoxic/aerobic switch and the N₂O production was assumed to be biological. In the later work, protein analysis revealed a 0.6-fold change on cytochrome P460 protein content adaptation after 13 days imposing transient anoxia cycling [19]. However, the source of reducing equivalents for NO and N₂O formation from nitrite remained unknown, and it is generally assumed to be provided by an endogenous reductant pool [4]. Remarkably, with the abiotic and the biotic results reported in the present study, most of the emissions found during the biological experiments [18,19] could be explained by the chemical transformation (i.e., volumetric rates were comparable). The unknown source of reducing equivalents (i.e., electron donor) for the nitrite reduction, claimed in Refs. [4,18], would be automatically hydroxylamine.

We believe that the main reason why the chemical reaction of hydroxylamine with free nitrous acid has been given little attention in the past, is that hydroxylamine has not been regularly included in control tests when trying to identify possible abiotic N₂O production. For instance, Poth et al. performed controls with heat-killed biomass incubated with ammonia and nitrite [20], Goreau et al. used HgCl₂-killed biomass in ammonia containing flasks [22], Shaw et al. performed non-

**Fig. 3.** Averaged data from two abiotic duplicates replicating biological culture conditions to assess the amount of N₂O produced chemically. Experiments were abiotically performed at 21 °C, pH 7.41, 230 mg-N-NO₂⁻/L (5 · 10⁻³ mg-N-FNA/L). Air flow to liquid volume ratio: (A) 0.69 min⁻¹, (B) 0.28 min⁻¹.
Table 2

N₂O emissions during 3 different days in biological experiments with repeated transient anoxia conditions.

<table>
<thead>
<tr>
<th>Day</th>
<th>Total N₂O emitted during recovering from transient anoxia (mg-N)</th>
<th>Maximum N₂O emission rate (mg-N/h)</th>
<th>Volumetric maximum N₂O emission rate (mg-N/L/h)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1.05</td>
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<td>7</td>
<td>0.29</td>
<td>0.21</td>
<td>0.053</td>
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<tr>
<td>13</td>
<td>0.25</td>
<td>0.17</td>
<td>0.043</td>
</tr>
</tbody>
</table>

5. Conclusions

In the present work we showed that N₂O abiotic emissions from the reaction of FNA and hydroxylamine occurred at the same conditions and in comparable rates with those measured during recovery from anoxia in cultures of Nitrosomonas europaea. Thus, we propose that abiogenic N₂O production from hydroxylamine reaction with FNA should be considered when describing the N₂O emission pathways. Additionally, to reduce the impact of the abiogenic pathway on the assessment of the biologically produced N₂O, low nitrite concentration and high pH should be maintained during cultivation of AOB to reduce the amount of FNA available in the culture.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2017.10.141.

References


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