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Importance of hydroxylamine in abiotic N_2O production during transient anoxia in planktonic axenic *Nitrosomonas* cultures

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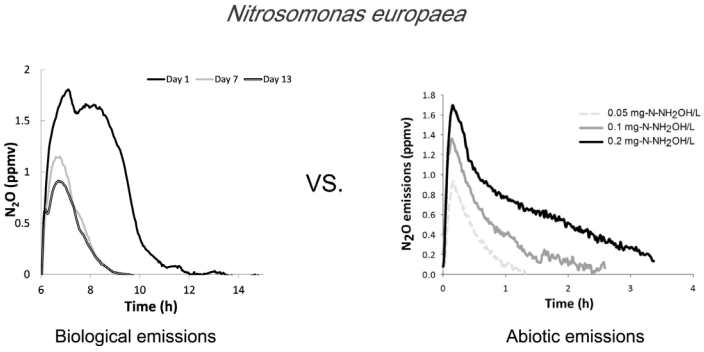
GRAPHICAL ABSTRACT



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ABSTRACT

When investigating the N_2O 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 N_2O 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 N_2O 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 N_2O production in a biological system, even using N^{15} labelling. We suggest that the contribution of abiotic N_2O emissions can be minimized by, for example, maintaining lower nitrite concentration and higher pH.

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1. Introduction

Nitrous oxide (N_2O) and nitric oxide (NO) can be emitted during nitrogen removal in lab-scale reactors and wastewater treatment plants (WWTPs). N_2O and NO are ozone depleting gases. Moreover, due to a 300-fold larger greenhouse effect capacity compared to that of CO_2, the study of N_2O emissions has increased over the past years. Main contributors to N_2O 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 nitritation-anammox processes [2].

In nitrification systems ammonium oxidizing bacteria (AOB) or archaea (AOA) are reported to be the main contributors to NO/N_2O emissions [1]. AOB convert ammonium to nitrite in two steps: ammonium oxidation to hydroxylamine (NH_2OH) 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_2^- [3]. In vivo studies are still needed to confirm this potential reaction mechanism. Regarding NO/N_2O production traditionally, two pathways have been described for the production of N_2O 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_2O 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_2OH molecules to N_2O 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 be further converted to N_2O either by abiotic or biological reactions [8]. Finally, the importance of N_2O production from hydroxylamine and nitrite (Eq. (1)) with biological and abiotic contributions in a partial nitritation system was also highlighted [9]. Regarding nitrifier denitrification, the existence of nitrite reductase (NIR) capable of converting nitrite to NO and nitric oxide reductase (NOR) catalysing the conversion of NO to N_2O have been demonstrated in AOB [10,11]. However, not all AOB have a NOR-related enzyme encoded in the genome [8], which is also the case in some AOA [12]. Thus, abiotic conversion of NO to N_2O has also been postulated when NOR is missing in the genome [8,12]. Surprisingly, abiotic N_2O emissions were not considered in most of the proposed models, although chemical N_2O production is known to happen [1,5,13,14]. Recent studies highlighted the contribution of abiotic N_2O emissions produced by reaction of hydroxylamine with free nitrous acid (FNA) [6,9,15] (Eq. (1)).

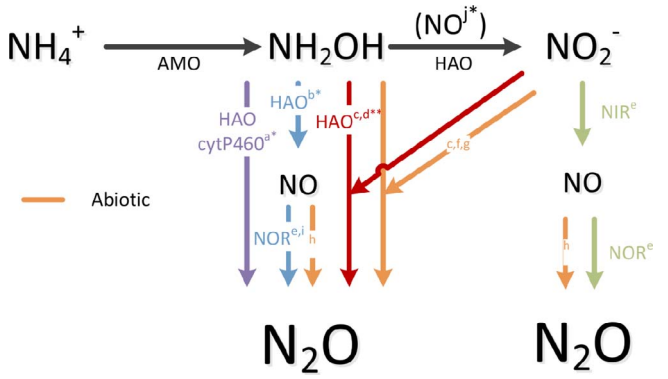

Fig. 1. Summary of N_2O emission pathways in ammonia oxidizing bacteria. Enzymes responsible of each pathway: AMO – Ammonia Monoxygenase, HAO – Hydroxylamine Oxidoreductase, HAO cytP460 – Cytochrome P460 of HAO, NIR – Nitrite reductase, NOR – Nitric oxide reductase. The pathways indicated by orange arrows account for putative abiotic contribution. * – tested in vitro with enzymatic extracts, ** – proposed pathway. References a [7], b [31], c [9], d [33], e [11], f [6], g [15], h [8], i [4], j [3].

$$NH_2OH + HNO_2 \rightarrow N_2O + 2H_2O \quad (1)$$

The chemical reaction of hydroxylamine with free nitrous acid has not been considered as a possible contributor to the overall N_2O 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_2O emissions. Thus, (i) diverse AOB consortia may coexist with other potential N_2O 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_2O in AOB and the conditions affecting the extent of N_2O 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_2O 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_2O.

Hydroxylamine accumulation in the liquid can be the direct or indirect key factor promoting N_2O emissions. During nitrification, hydroxylamine has been reported to accumulate in the bulk liquid in the range of 0.01–1 mg-N-NH_2OH/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_2O 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_2O 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_2O emissions were detected [4,18,19]. However, immediately after recovery to aerobic conditions N_2O was emitted simultaneously with the hydroxylamine accumulation [4,18,19]. Yu et al. [18], could not find a correlation between N_2O 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_2O 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_2O formation. Furthermore, some recommendations are provided for future research in order to reduce the contribution of abiotic N_2O 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 (6L total volume, 4L 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 MgSO4·7H_2O, 0.02 g of $CaCl_2$·2H_2O, 0.087 g of K_2HPO_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_2MoO_4·2H_2O, 172 mg of $MnCl_2$·4H_2O, 10 mg of $ZnSO_4$·7H_2O, 0.4 mg of $CoCl_2$·6H_2O, and 100 mL of distilled water), 0.5 mL of 0.5% phenol red, and 0.5 mL of 2 mM $CuSO_4$·5H_2O [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_2O (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_2O 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_2O, 0.02 g of $CaCl_2$·2H_2O, 0.087 g of K_2HPO_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_2MoO_4·2H_2O, 147 mg of $MnSO_4$·H_2O, 10 mg of $ZnSO_4$·7H_2O, 0.482 mg of $CoSO_4$·7H_2O, and 100 mL of distilled water), and 0.5 mL of 2 mM $CuSO_4$·5H_2O 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 $NaNO_2$ was added to a final concentration of 230 mg-N-NO_2^-/L ($5 \cdot 10^{-3}$ mg-N-FNA/L), so similar concentrations to those reported during biological cultivations were used. No N_2O 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_2O measurement in parts per million gas volume (ppmv) was converted to concentration of N_2O in mg-N/L gas. The N_2O emission rate (mg-N/h) was obtained from the N_2O 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_2O 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_2O 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-NO_2^-/L, $5 \cdot 10^{-3}$ 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_2O 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_2O was only emitted immediately after the switch to aerobic conditions (Fig. 2C). The highest N_2O and NO emissions were observed in the first day of transient anoxia and the concentrations in the gas reached 1.8 ppmv N_2O and 9 ppmv NO (Fig. 2C). For hydroxylamine, accumulation in the liquid was detected at the same time with N_2O emission (Fig. 2A). Similar with the N_2O emission pattern, maximum NH_2OH 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_2O production in aerobic conditions

When replicating the *N. europaea* culture conditions without any biomass, N_2O emissions were detected immediately after the addition of hydroxylamine. N_2O 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-NO_2^-/L, $5 \cdot 10^{-3}$ mg-N-FNA/L) and medium composition, but different hydroxylamine concentrations (0.05–0.2 mg-N/L), the total N_2O 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_2O 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_2O emissions as in reaction (1) ranged from 20 ± 1% to 40 ± 2% (Table 1), clearly indicating the presence of side

Table 1
Abiotic batch experiments conditions to test the impact of hydroxylamine reaction with nitrite in the emission of N_2O in pure cultures conditions. Tests were done at pH 7.41, 21 °C temperature, 230 mg-N/L of nitrite and 1.33 L working volume. Each test was done by duplicate.

Abiotic batch test	Initial hydroxylamine concentration (mg-N/L)	Air flow/working volume ratio (min^{-1})	Total N_2O emitted (mg-N)	Yield relative to NH_2OH (%)	Maximum N_2O emission rate (mg-N/h)	Volumetric maximum N_2O emission rate (mg-N/L/h)
1	0.05	0.68	0.040 ± 0.002	40 ± 2	0.06 ± 0.01	0.048 ± 0.008
2	0.1	0.68	0.056 ± 0.02	28 ± 8	0.08 ± 0.02	0.06 ± 0.01
3	0.2	0.68	0.14 ± 0.01	35 ± 3	0.106 ± 0.002	0.0815 ± 0.001
4	0.1	0.29	0.056 ± 0.005	28 ± 2	0.0570 ± 0.0005	0.0440 ± 0.0005
5	0.2	0.29	0.083 ± 0.004	20 ± 1	0.080 ± 0.002	0.065 ± 0.002

reactions because the NH_2OH was completely converted at the end of experiments.

4. Discussion

4.1. Biological N_2O sources

In the studies of Yu et al., [18,19] (Fig. 2 and Table 2), N_2O 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_2O emissions correlated with lower oxygen tensions [22,23]. The increased N_2O 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 correlated the N_2O 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_2O 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_2O 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, Kozolwski et al., [11] demonstrated that a double mutant lacking NOR and NIR was unable to produce N_2O during anaerobic or hypoxic conditions. However, the same double deficient mutant did produce N_2O 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_2O. However, it is surprising that in spite of NO reactivity, this was not further transformed to N_2O. 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 CytP460, c-554 and NOR, respectively), whereas energy conversion was favored (2-fold change of AMO) [19]. Conversely, the N_2O emissions were just observed in the aerobic period (Fig. 2C) showing that other pathway than the denitrification was contributing to the N_2O production.

N_2O 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_2O in vitro, but its potential effect in vivo remains to be assessed [31]. Recently, cytochrome P460 has been shown to catalyze N_2O 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_2O emissions during the aerobic period of the *N. europaea* cultivations [18,19] cannot be excluded.

4.2. Importance of abiotic N_2O production in biological experiments with *N. europaea*

Most of the studies focused on the biologically emitted N_2O, even if different chemical reactions are known to produce N_2O [6,13–15,32]. In the present study, abiotic experiments confirmed that chemical N_2O 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_2O. Hydroxylamine was added in the abiotic experiments in the concentrations measured during the axenic 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_2OH/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_2O 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_2O emissions were fast enough to contribute significantly to the total emissions or be the only pathway of N_2O formation. In our abiotic experiments replicating conditions used by Yu et al. [18] the N_2O 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_2O 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_2O. 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_2O 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_2O produced during the continuous biological cultivations is higher than the total N_2O produced in the abiotic batch test. A biological contribution to N_2O emissions in the continuous cultivation

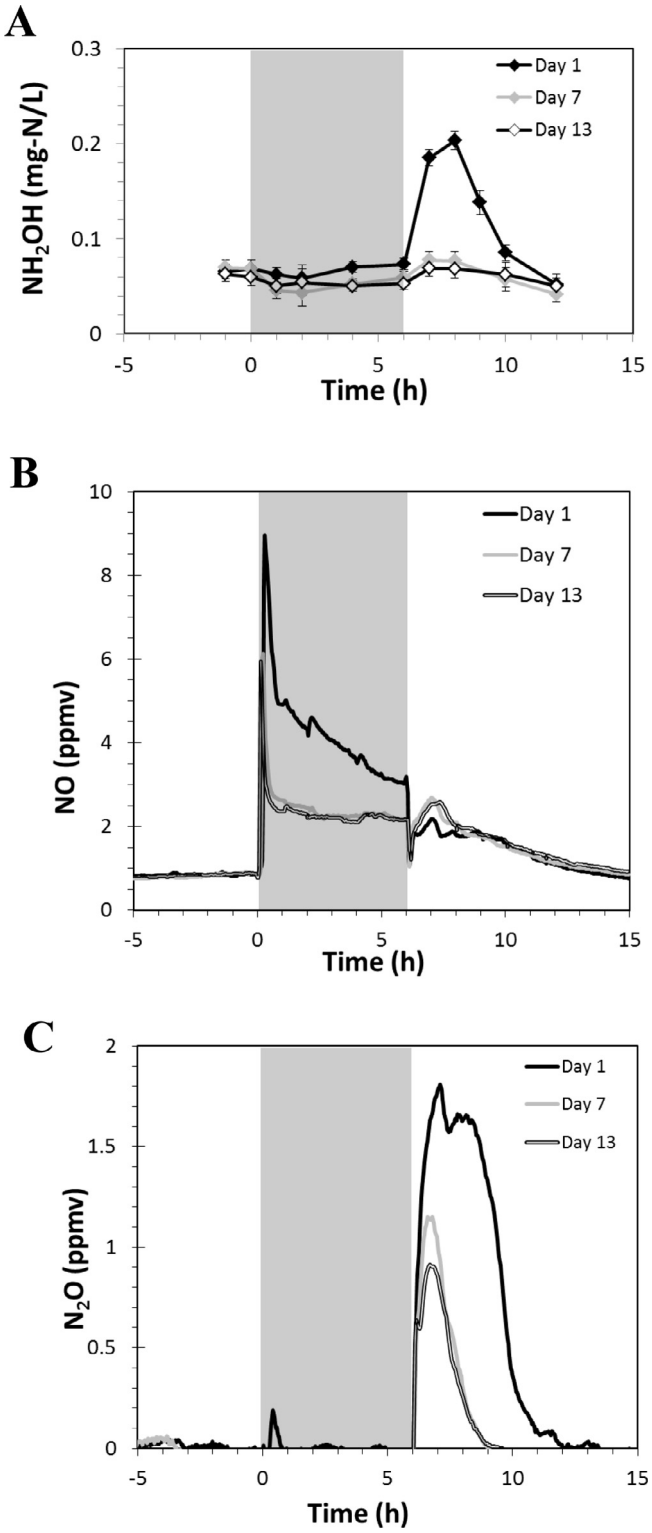

Fig. 2. Hydroxylamine concentration and NO/N_2O emissions during 3 different days of continuous operation where transient anoxia was imposed in a *Nitrosomonas europaea* axenic culture. Grey box indicates anoxic period before the transition to aerobic period. (A) Hydroxylamine bulk concentration, (B) NO emissions, (C) N_2O emissions. Adapted from Ref. [19]

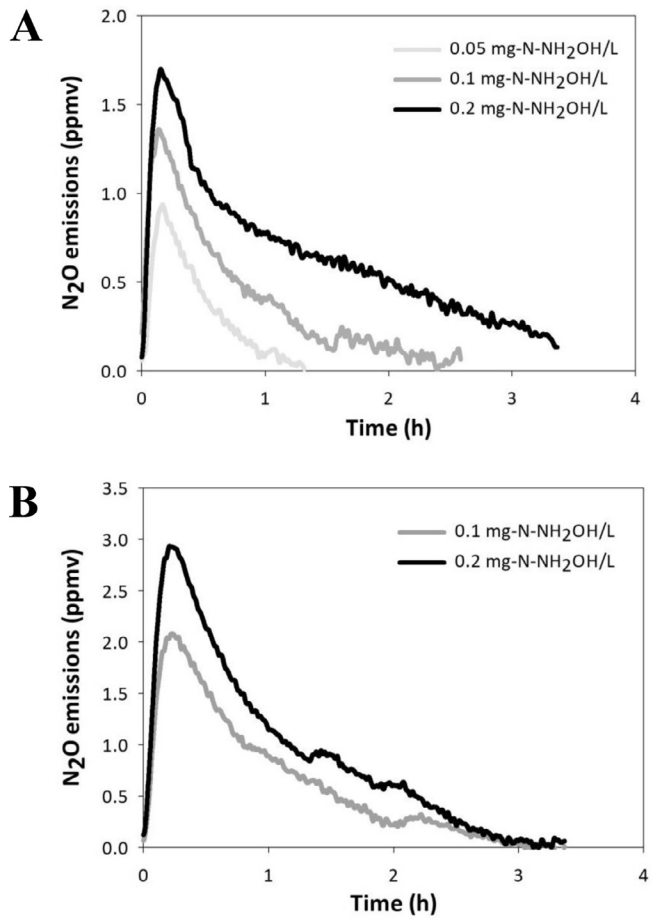

Fig. 3. Averaged data from two abiotic duplicates replicating biological culture conditions to assess the amount of N_2O produced chemically. Experiments were abiotically performed at 21 °C, pH 7.41, 230 mg-N-NO_2^-/L ($5 \cdot 10^{-3}$ mg-N-FNA/L). Air flow to liquid volume ratio: (A) 0.69 min^{-1}, (B) 0.28 min^{-1}.

cannot be ruled out, because it was not possible to assess the amount of hydroxylamine chemically transformed to N_2O. A study with mixed populations of nitrifiers in aggregates performed in a partial nitrification reactor [9] found that half of the N_2O 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 N_2O emissions by other pathways than that of *N. europaea* can be ruled out.

In the study of Yu et al. [18], N_2O emissions in the *N. europaea* culture during transient anoxia were associated to differences in specific cell activity upon the anoxic/aerobic switch and the N_2O 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 N_2O formation from nitrite remained unknown, and it is generally assumed to be provided by an endogenous reductant pool [4]. Remarkably, with the biotic and the abiotic 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 N_2O production. For instance, Poth et al. performed controls with heat-killed biomass incubated with ammonia and nitrite [20], Goreau et al. used $HgCl_2$-killed biomass in ammonia containing flasks [22], Shaw et al. performed non-

Table 2
N_2O emissions during 3 different days in biological experiments with repeated transient anoxia conditions.

Day	Total N_2O emitted during recovering from transient anoxia (mg-N)	Maximum N_2O emission rate (mg-N/h)	Volumetric maximum N_2O emission rate (mg-N/L/h)
1	1.05	0.34	0.085
7	0.29	0.21	0.053
13	0.25	0.17	0.043

inoculated controls in nitrite and ammonia [21], whereas Beaumont et al. did not mention any abiotic control [16]. Summarizing, when trying to assess abiotic N_2O production, either killed biomass or non-inoculated tests were used. The main problem is however that the medium generally used for the control tests did not contain hydroxylamine. Only Anderson et al. showed that control tests containing hydroxylamine and nitrite did produce N_2O [15], but they ruled out the real importance of the chemical reaction based on the (wrong) assumption that hydroxylamine cannot accumulate in the biological cultures. Recently, Kozlowski et al., showed that chemical controls with hydroxylamine and nitrite resulted in lower abiotic N_2O emissions compared to those from the equivalent biological system for different AOB genera [8]. Although this seems to be in disagreement with results obtained in our study, the differences in medium composition (i.e., higher concentration of nitrite or the presence of metals) can still trigger the abiotic reaction [6,9,15]. Nitrite concentrations used in the chemical controls from Ref. [8] were ca. 3.5 mg-N-NO_2^-/L (2.2–6.9 · 10^{-5} mg-N-FNA/L), two orders of magnitude lower than those used in the current and most axenic studies [4,17–19] during transient anoxia (ca. 230 mg-N NO_2^-/L, so ca. 5 · 10^{-3} mg-N-FNA/L). Conditions used by Kozlowski et al. [8] are in agreement with the recommendations given in the present work to study N_2O emissions (i.e., low nitrite concentration, usage of planktonic and axenic cultures). Nevertheless, further research is needed to assess the contribution of abiotic hydroxylamine reaction with free nitrous acid under different cultivation conditions.

4.3. Implications and recommendations

In view of our current results, all results derived from research assuming that transient anoxia produces N_2O exclusively through biological pathways should be reconsidered. Because of the "hybrid" biotic/abiotic pathway, a last reaction step involving the fully abiotic reaction of biologically produced NH_2OH with HNO_2 to emit N_2O is possible. N^{15} labelling can be used to follow metabolic fluxes in axenic cultures and quantify the amount of hydroxylamine transformed to N_2O, obtaining a rationing between different possible pathways, as performed by Terada et al. [9]. These experiments should also be performed in pure cultures of planktonic cells, which could provide insight in the predominant pathways at different conditions and for different AOB species. Nevertheless, with N^{15} labelling it is not possible to differentiate between abiotic and biological production through this "hybrid" pathway, as the reaction is the same.

Other strategies could be conducted to rule out abiotic N_2O emissions, for instance, by applying low ammonia concentrations together with high dilution rates in continuous cultures, so that nitrite (and free nitrous acid) concentration remains at low levels. Similarly, a higher pH in the bulk liquid will lead to lower free nitrous acid concentrations. Besides changing the concentrations of nitrate/nitrous acid, strategies can be directed to decrease the hydroxylamine concentration in order to prevent abiotic and biological emissions. Thus, hydroxylamine measurements are essential to provide information on conditions that trigger its accumulation in the bulk. Finally, including nitrification intermediates, like hydroxylamine, when performing abiotic controls is vital to assess the impact of abiotic N_2O emissions in each culture conditions.

5. Conclusions

In the present work we showed that N_2O 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 abiotic N_2O production from hydroxylamine reaction with FNA should be considered when describing the N_2O emission pathways. Additionally, to reduce the impact of the abiotic pathway on the assessment of the biologically produced N_2O, 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.

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