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Hybrid Nitrous Oxide Production from a Partial Nitrifying Bioreactor: Hydroxylamine Interactions with Nitrite

Akihiko Terada,[*,†] Sho Sugawara,[†] Keisuke Hojo,[†] Yuki Takeuchi,[†] Shohei Riya,[†] Willie F. Harper, Jr.,[†,⊥] Tomoko Yamamoto,[†] Megumi Kuroiwa,[‡,§,∥] Kazuo Isobe,[§] Chie Katsuyama,[∥,#] Yuichi Suwa,[∥] Keisuke Koba,[‡,▽] and Masaaki Hosomi[†]

[†]Department of Chemical Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Naka, Koganei, Tokyo 184-8588 Japan

[‡]Department of Environmental and Natural Resource Sciences, Tokyo University of Agriculture and Technology, Saiwai-cho Fuchu, Tokyo, Japan

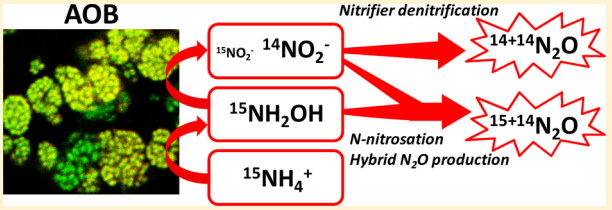
[§]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi, Bunkyo, Tokyo, Japan

[∥]Department of Biological Sciences, Faculty of Science and Engineering, Chuo University, Bunkyo, Tokyo, Japan

[⊥]Department of Systems Engineering and Management, Air Force Institute of Technology, Dayton, Ohio, United States

Supporting Information

ABSTRACT: The goal of this study was to elucidate the mechanisms of nitrous oxide (N_2O) production from a bioreactor for partial nitrification (PN). Ammonia-oxidizing bacteria (AOB) enriched from a sequencing batch reactor (SBR) were subjected to N_2O production pathway tests. The N_2O pathway test was initiated by supplying an inorganic medium to ensure an initial NH_4^+-N concentration of 160 mg-N/L, followed by $^{15}NO_2^-$ (20 mg-N/L) and dual $^{15}NH_2OH$ (each 17 mg-N/L) spikings to quantify isotopologs of gaseous N_2O ($^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$). N_2O production was boosted by $^{15}NH_2OH$ spiking, causing exponential increases in mRNA transcription levels of AOB functional genes encoding hydroxylamine oxidoreductase (*haoA*), nitrite reductase (*nirK*), and nitric oxide reductase (*norB*) genes. Predominant production of $^{45}N_2O$ among N_2O isotopologs (46% of total produced N_2O) indicated that coupling of $^{15}NH_2OH$ with $^{14}NO_2^-$ produced N_2O via *N*-nitrosation hybrid reaction as a predominant pathway. Abiotic hybrid N_2O production was also observed in the absence of the AOB-enriched biomass, indicating multiple pathways for N_2O production in a PN bioreactor. The additional N_2O pathway test, where $^{15}NH_4^+$ was spiked into 400 mg-N/L of NO_2^- concentration, confirmed that the hybrid N_2O production was a dominant pathway, accounting for approximately 51% of the total N_2O production.



INTRODUCTION

Nitrous oxide (N_2O) is emitted from wastewater treatment plants (WWTPs) designed for biological nitrogen removal.[1] This has raised serious concerns related to climate change, because N_2O is a powerful greenhouse gas, approximately 300 times as powerful as CO_2 in terms of radiative heating effects. It also participates in reactions with stratospheric ozone, depleting the protective layer that absorbs ultraviolet radiation from the Sun.[2] It is therefore important to learn more about N_2O production from WWTPs to prevent these dangerous environmental effects.

N_2O is produced during either nitrification or denitrification in WWTPs, and the amount produced was potentially underestimated in the 1990s.[3] High NO_2^- concentrations and aeration intensities, and low dissolved oxygen (DO) concentrations boost N_2O production in WWTPs;[1,4,5] these findings have particular relevance for low-cost and energy-saving nitrogen-removal processes (e.g., nitrification/denitrification via NO_2^- and nitritation coupled to anaerobic ammonia oxidation (anammox)) designed to operate at high NO_2^- (e.g., 15−1680 mg-N/L)[6] and low DO concentrations (e.g., 0.05−1.5 mg/L in full scale anammox systems).[7] N_2O production must be carefully studied under these conditions to develop more sustainable nitrogen-removal processes. The N_2O production factor (i.e., the mass of produced N_2O divided by that of converted nitrogen compounds) is lower in conventional nitrification/denitrification full-scale systems (i.e., between 0.01% and 3.3%)[8] than in full-scale nitritation−anammox systems (between 1.2% and 12%).[9−11] Okabe et al. (2011) showed that N_2O emissions from partial nitrification (PN) offset the N_2O-related benefits of anammox, which does not have a metabolic pathway for N_2O production.[12] There is considerable work to be done on improving N_2O mitigation in

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PN processes, and a better fundamental understanding of N_2O production mechanisms will pave the way for novel strategies and new designs.

Previous research has investigated N_2O production mechanisms in activated sludge systems,[13−15] nitrifying bioreactors,[16,17] and granular sludges.[18] In PN bioreactors, there are putative abiotic and biotic N_2O production pathways (Supporting Information (SI) Figure S1) including (i) heterotrophic denitrification,[19] (ii) denitrification by ammonia-oxidizing bacteria (AOB) also called nitrifier denitrification,[19] (iii) hydroxylamine (NH_2OH) oxidation,[19,20] and (iv) *N*-nitrosation hybrid N_2O production.[21,22] The NH_2OH oxidation pathway can be further broken down into three reactions, (1) interactions of NH_2OH with oxygen ($NH_2OH + 0.5O_2 \rightarrow 0.5N_2O + 1.5H_2O$) as an electron acceptor,[23] (2) chemical decomposition of nitrosyl radicals (HNO), produced via NH_2OH oxidation by hydroxylamine oxidoreductase (HAO) in AOB,[17,24] and (3) NH_2OH oxidation to N_2O by *Nitrosomonas europaea* cytochrome P460, interacted with ferric nitric oxide (NO) complex.[20] The *N*-nitrosation hybrid N_2O production is driven by the interaction of NH_2OH with NO_2^- ($NH_2OH + NO_2^- + H^+ \rightarrow N_2O + 2H_2O$), mediated via abiotic and biotic pathways.[22,25,26] Abiotic pathways reviewed elsewhere[27] have been reported under acidic conditions,[23] whereas biotic pathways, also called codenitrification, have been found for denitrifying bacteria,[25,28,29] fungi,[30] oligotrophic AOB[22] and ammonia-oxidizing archaea (AOA).[21] Reports have proposed alternative N_2O production pathways such as abiotic N_2O production via Fe(II) reduction coupled with NO_3^-/NO_2^- reduction,[31] and photolysis of ammonium nitrate.[32]

Isotopomer analysis is an excellent method for visualizing complex N_2O production mechanisms;[13,15,18] however, the natural abundance of ^{15}N in N_2O presents technical difficulties, including unavailability of international standards and the requirement of preconcentration of N_2O for precise analysis.[33] Furthermore, N_2O isotope/isotopomer techniques cannot distinguish between NH_2OH oxidation and *N*-nitrosation hybrid N_2O production. This challenge can be solved using ^{15}N tracer techniques, which involve labeling nitrogen atoms with ^{15}N.[34] This method has been used to elucidate microbial processes, for example, distribution of anammox and heterotrophic denitrification.[35] Analysis of ^{15}N contents for N_2O production by the denitrifier method, using a quadrupole GC-MS system, can provide useful information on nitrogen dynamics, including potential N_2O production pathways.[36,37] Messenger RNA (mRNA) tracking is another way to investigate metabolic pathways; for example, quantification of mRNA functional genes has revealed how AOB respond to changes in inorganic carbon concentrations[38] and starvation,[39] cope with low DO and high NO_2^- conditions,[40] and produce N_2O.[41]

The aim of this study was to elucidate N_2O production pathways and their relative contributions in a PN activated sludge system, in which AOB were highly enriched. Although only a few works have proven *N*-nitrosation hybrid N_2O production by oligotrophic AOB and AOA biomasses,[22] and AOA pure culture,[21] it is not clear if this hybrid N_2O production is important in an AOB-enriched bioreactor treating high concentration of NH_4^+. A combination of ^{15}N stable isotope labeling, metabolite detection, and mRNA functional gene characterization was used to quantify this hybrid N_2O production.

MATERIALS AND METHODS

Experimental Overview. Biomass with a dense AOB population was subjected to experiments. In an effort to collect direct evidence on the interaction of NH_2OH with NO_2^-, ^{15}N labeled NH_2OH ($^{15}NH_2OH$) and NO_2^- were spiked in a batch test for analysis of the N_2O isotopologs in exhaust gas. Then, the effect of NH_2OH concentration on N_2O production was investigated to consolidate the implication of *N*-nitrosation hybrid N_2O production. One cycle profiles of nitrogen constituents and N_2O isotopologs in a sequencing batch reactor (SBR) were investigated to confirm the occurrence of hybrid N_2O production, concomitant with other N_2O production pathways.

Bioreactors. AOB were enriched in an SBR fed with synthetic inorganic wastewater containing NH_4^+ at 600 mg-N/L, as described elsewhere.[42] Hydraulic and solid retention times were 1 and 20 day, ensuring a volumetric NH_4^+ loading rate of 0.60 g-N/L/d. The DO concentration and pH were set at 1.5 mg/L and 7.8 using DO and pH controllers (Yamagata Toa DKK, Yamagata, Japan), achieving an average NH_4^+ oxidation rate of 0.59 ± 0.02 g-N/L/d with a high NO_2^- accumulation ratio (i.e., NO_2^- concentration divided by total concentration of NO_2^- and NO_3^-) of 0.89 ± 0.06.

Scheme for Batch Experiment with $^{15}NH_2OH$. Biomass was transferred from the SBR to a 1 L reactor vessel to ensure a final mixed liquor volatile suspended solid (MLVSS) concentration of 570 ± 10 mg/L (n = 3). A batch experiment began by addition of a synthetic inorganic ammonia-containing medium (SI Table S1),[43] at an initial NH_4^+ concentration of 160 mg-N/L. NH_4^+, NO_2^-, NO_3^-, gaseous/dissolved N_2O, and gaseous N_2O isotopologs were monitored. The water temperature was 28 °C and pH was controlled at 7.8 by addition of saturated bicarbonate solution. Air was supplied at a flow rate of 1.0 L/min. ^{15}N-labeled compound was not supplied at the start of the experiment (Phase I). After NH_4^+ depletion, ^{15}N-labeled NO_2^- [$^{15}NO_2^-$ (^{15}N 98%), SI Science, Saitama, Japan] was added at 20 mg-N/L (Phase II). This period helped to evaluate the relative magnitude of heterotrophic denitrification. $^{15}NH_2OH$ (^{15}N 98%, SI Science, Saitama, Japan) was then added at 17 mg-N/L (Phase III). The NH_2OH concentrations applied were comparable with those previously applied.[14,44] $^{15}NH_2OH$ addition was repeated twice to confirm N_2O production from $^{15}NH_2OH$. $^{15}NH_2OH$ addition was performed to identify N_2O production from NH_2OH oxidation, *N*-nitrosation hybrid N_2O production, and nitrifier denitrification. The concentrations of N_2O isotopologs ($^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$) were determined using a quadrupole GC-MS system (GCMS-QP2010 Plus, Shimadzu, Kyoto, Japan). Also, ^{15}N contents of N_2O and NO_2^- were carefully monitored. Some of the data points collected in these batch experiments were used to evaluate mathematical models in a study published previously.[45]

The Effect of Several Nitrogen Species on N_2O Production. Because *N*-nitrosation hybrid N_2O production was proposed as the predominant N_2O production pathway in a PN bioreactor, the effect of NH_2OH mixed with several nitrogen species on N_2O production was investigated. Eight 500 mL beakers were prepared by addition of different nitrogen species to water (300 mL). Nitrifying activated sludge was sampled from the SBR and transferred to four beakers, each of which contained NH_2OH (10 mg-N/L) with NO_2^-, NO_3^-, or NH_4^+ each at 400 mg-N/L, or without any nitrogen species.

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The other four beakers did not contain activated sludge, but otherwise the contents were the same as those with the sludge. Dissolved N_2O was measured using an N_2O microsensor (Unisense, Aarhus, Denmark).

Biotic and Abiotic N_2O Production by Coupling NH_2OH with NO_2^-. In order to distinguish between abiotic and biological N_2O production, ^{15}N tracer experiments were conducted by adding $^{15}NH_2OH$. Different amounts of $^{15}NH_2OH$ were added to 500 mL beakers containing water (300 mL) with NO_2^- concentrations of 400 mg-N/L in the presence or absence of nitrifying biomass. After spiking with $^{15}NH_2OH$ at final concentrations of 1, 5, 10, and 20 mg-N/L, the dissolved N_2O concentrations and gaseous N_2O isotopologs were monitored using an N_2O microsensor and quadrupole GC-MS system, respectively.

Batch Experiments in an SBR. One cycle profiles of nitrogen species and N_2O isotopologs were monitored. To this end, an 8-h, one-cycle SBR experiment was carried out. The AOB-enriched biomass was harvested and transferred to a 1 L reactor vessel. ^{15}N-labeled NH_4^+ [$^{15}NH_4^+$ (^{15}N 98%), SI Science, Saitama, Japan] and nonlabeled NO_2^- -containing medium was filled to 1 L to ensure the initial NH_4^+ and NO_2^- concentrations of 200 and 400 mg-N/L, respectively. The MLVSS concentration in the vessel was 1720 ± 70 mg/L. The operation conditions and monitoring contents were identical with the batch experiment spiking $^{15}NH_2OH$ except that gaseous and dissolved N_2O concentrations were measured by a quadrupole GC-MS system and an N_2O microsensor (Unisense, Aarhus, Denmark), respectively.

Chemical Analysis. The samples for nitrogen species analysis were filtered through a membrane filter (DISMIC-13HP045AN, Advantec, Tokyo, Japan). NH_4^+, NO_2^-, and NO_3^- were determined using ion chromatography (ICS-1000; Dionex, Osaka, Japan). MLVSS was assayed according to the Standard Methods for the Examination of Water and Wastewater.[46] The gaseous N_2O concentration was determined using quadrupole GC-MS or GC-ECD (GC-14B, Shimadzu, Kyoto, Japan), as previously reported.[42] The dissolved N_2O concentration was measured using an N_2O microsensor or GC-ECD. For N_2O isotopologues, quadrupole GC-MS with a modified injection port was used to distinguish $^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$ in the produced N_2O, based on previous work.[37] The details are described in the Supporting Information. The contributions to the N_2O production pathways by AOB, i.e., nitrifier denitrification, *N*-nitrosation hybrid reactions, and NH_2OH oxidation, were estimated as described in the SI. The ^{15}N ratio in NO_2^- was obtained by converting NO_2^- into N_2O by the Azide method[47] and the produced N_2O isotopologs were measured by the method described above.

DNA Extraction and Cloning. The nitrifying biomass was retrieved and immediately frozen. DNA was extracted using a FastDNA SPIN Kit (MP Biomedicals, Carlsbad, CA, USA). The DNA concentrations and purities were measured spectrophotometrically (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA). The PCR was performed to amplify the functional gene for bacterial ammonia monooxygenase (*amoA*) (SI Table S2). The extracted DNA was amplified using the PCR[48] for construction of a phylogenetic tree using a neighbor-joining algorithm.[49] Details of the PCR and cloning procedures are given in the SI.

RNA Extraction and Reverse Transcription. RNA was immediately extracted using a FastRNA Pro Soil Direct Kit (MP Biomedicals, Carlsbad, CA, USA). The concentration and purity of the extracted RNA were determined by NanoDrop. Reverse transcription of RNA was performed using a QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, CA). Contamination of genome DNA was excluded during reverse transcription.

Real-Time Quantitative PCR. Complementary DNA from extracted RNA was quantified using the reverse transcription quantitative PCR (CFX96 real-time PCR detection system, BioRad, Hercules, CA, USA). The targeted functional genes of AOB as an expression of mRNA (mRNA) were *amoA* for NH_4^+ oxidation, *haoA* for NH_2OH oxidation, *nirK* for NO_2^- reduction, and *norB* for NO reduction; the primer sequences have been reported elsewhere (SI Table S2).[50,51] These functional genes were amplified and quantified as described in SI.

Fluorescence in Situ Hybridization. Fluorescence in situ hybridization (FISH) was conducted to quantify AOB with respect to total bacteria. NSO1225[52] and EUB338mix[53,54] probes were applied to each nitrifying biomass. The percentage of specific probe-positive cells compared with EUB338mix-positive cells was quantified using Daime imaging software.[55]

Nucleotide Accession Numbers. The bacterial *amoA* gene sequences from this study have been deposited with GenBank, accession numbers KJ930172−KJ930174.

RESULTS

Community Structures in Nitrifying Bioreactors. A phylogenetic tree based on *amoA* is shown in SI Figure S2. All the clones obtained from the SBR-enriched biomass were identified to a single operational taxonomic unit (>98% similarity). The predominant AOB were phylogenetically affiliated with halophilic and halotolerant *Nitrosomonas* spp. and close to *N. europaea* 19178, with 97% similarity. Archaeal *amoA*, quantified using real-time quantitative PCR with the primer set Arch-amoAF-Arch-amoAR,[56] was below the detection limit ($<10^2$ copies/ng-DNA). Quantitative FISH indicated that the β-proteobacterial AOB populations detected by the NSO1225 and EUB338mix probes occupied 83% (±6.7%) over total bacterial populations (SI Figure S3).

$^{15}NH_2OH$ Experiments: Profiles of the Nitrogenous Compounds. Batch profiles of dissolved nitrogen compounds and N_2O in the enriched biomass from the SBR are shown in Figure 1. Approximately 90% of NH_4^+-N was converted to NO_2^--N during Phase I (Figure 1a). The specific NH_4^+ oxidation rate in the enriched SBR biomass, obtained from the linearly approximated NH_4^+ decrease (Figure 1a) was 1.16 g-N/g-VSS/d. The N_2O conversion percentage, obtained from the N_2O production rate (based on the sum of gas and dissolved N_2O concentration divided by the NH_4^+-removal amount), was 0.42% during Phase I. After addition of $^{15}NO_2^-$ at the beginning of Phase II, a negligible amount of N_2O was produced in the reactor (Figure 1b). The addition of $^{15}NH_2OH$ at the beginning of Phase III led to abrupt increases in N_2O production in the liquid, from 0.0054 to 0.31 mg-N/L (Figure 1b). Subsequently, the produced N_2O was transferred from the liquid to gas phases (Figure 1b). The pH decreased slightly from 8.15 to 7.99 because of NH_2OH oxidation to NO_2^- (Figure 1c). The DO concentration changed dynamically; it was relatively low at the beginning of one cycle when NH_4^+ was present (range from 0.88 to 1.30 mg/L) and increased to 3.95 mg/L after NH_4^+ depletion. Spiking with NH_2OH during Phase

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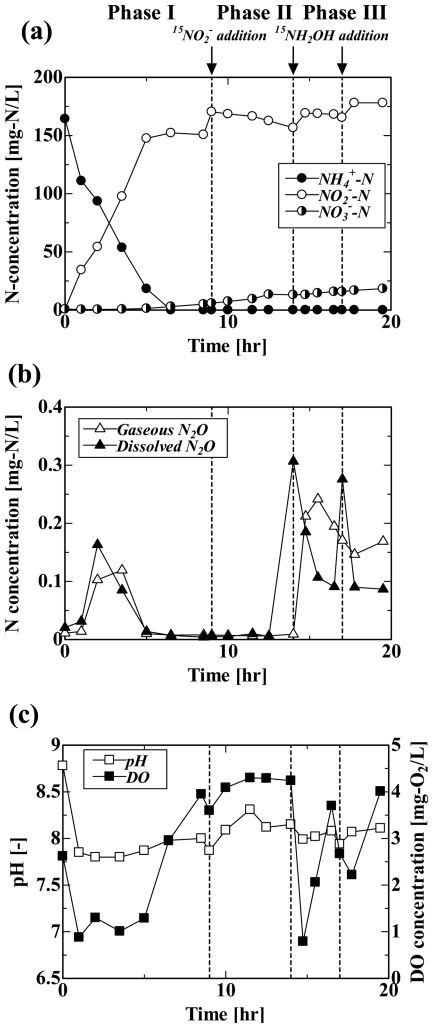

Figure 1. One-cycle profiles of ammonia oxidation and N_2O production tests for nitrifying biomass acclimated in SBR: (a) nitrogen compounds; (b) gaseous and dissolved N_2O; (c) pH and DO. The samples at 7, 14, and 17 h were taken right after the NO_2^- and NH_2OH spikes.

III greatly decreased the DO concentrations in the reactor, from 4.24 to 0.79 mg/L (Figure 1c).

When the solution was spiked with $^{15}NO_2^-$ at the beginning of Phase II, the only change observed was the expected increase in the $^{15}NO_2^-$ pool from a 0.40% background level to 11.5%, as the added ^{15}N was diluted in the bulk medium (Phase I to II; Figure 2a). The increase in ^{15}N in the NO_2^- pool (11.5%) is understandable, because the labeled NO_2^- was diluted in the bulk liquid at NO_2^- concentrations of 150.7 mg-N/L. After spiking with $^{15}NH_2OH$ at the end of Phase II, the ^{15}N percentage in N_2O at the beginning of Phase III increased to 60% (Figure 2a). The second spiking, in Phase III, further increased the ^{15}N percentages in N_2O, to 70% (Figure 2a). The ^{15}N percentages in NO_2^- increased stepwise from 11% to 19% to 26%, resulting from the dual $^{15}NH_2OH$ spiking. The N_2O isotopolog compositions are shown in Figure 2b. Integration of each composition in Phase III shows $^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$ production of 13%, 46%, and 41%. The compositions of N_2O produced via the three putative pathways, that is, nitrifier denitrification, *N*-nitrosation hybrid N_2O production and NH_2OH oxidation were 21%, 49%, and 30% in the SBR-enriched biomass (For details, please see SI).

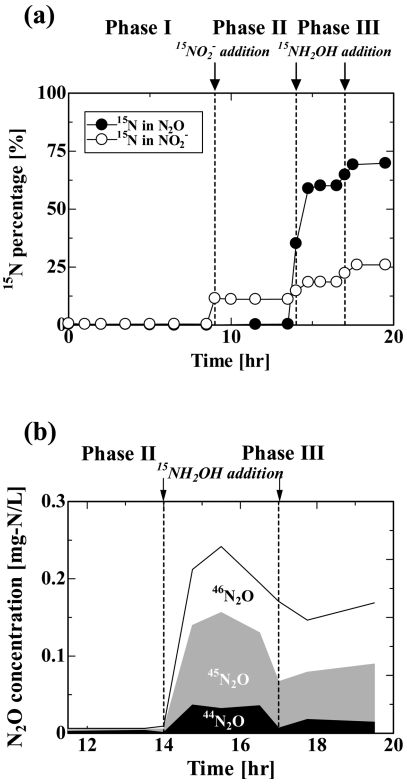

Figure 2. Time courses of ^{15}N percentages in produced N_2O and NO_2^- (a), and compositions of isotopologues of gaseous N_2O (b). The samples at 7, 14, and 17 h were taken right after the NO_2^- and NH_2OH spikes.

$^{15}NH_2OH$ Experiments: mRNA Transcription. The mRNA transcription levels for AOB functional genes are shown in Figure 3. The mRNA transcription level for *amoA* was

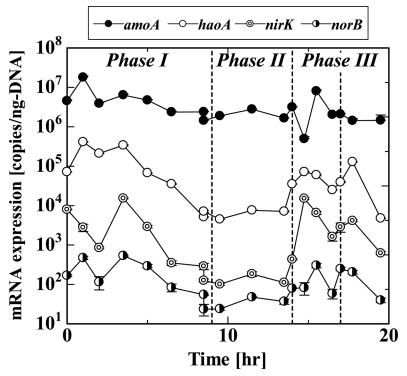

Figure 3. One-cycle profiles of gene transcription level of AOB *amoA*, *haoA*, *nirK*, and *norB* mRNA.

the highest, but remained relatively constant during Phase I compared with those of the other AOB functional genes. The mRNA levels of *haoA*, *nirK*, and *norB* gradually decreased during Phase I, in accordance with the NH_4^+ concentration. The addition of $^{15}NO_2^-$ did not significantly change the mRNA levels (Phase II, Figure 3). Noticeable increases in *haoA*, *nirK*, and *norB* mRNA genes were observed immediately after additions of NH_2OH (Phase III, Figure 3). These functional gene expression levels were nearly 1 order of magnitude higher at the beginning of Phase III than those at the end of Phase II. The most dynamic change in the mRNA transcription level was

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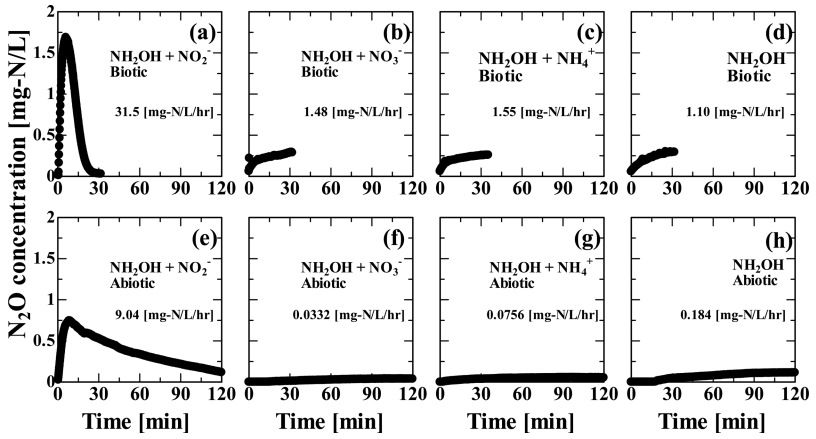

Figure 4. Effects of interactions between NH_2OH with different nitrogenous compounds, in the presence (a–d) or absence (e–h) of activated sludge, on N_2O production: (a) and (e) NH_2OH with NO_2^-; (b) and (f) NH_2OH with NO_3^-; (c) and (g) NH_2OH with NH4+; and (d) and (h) only NH_2OH. The number embedded in each figure represents the initial N_2O production rate estimated by linear approximation.

Table 1. ^{15}N Percentages in N_2O under Biotic and Abiotic Conditions for Different Additions of $^{15}NH_2OH$[a]

	initial N_2O production rate (mg-N/L/h)		^{15}N percentage in N_2O (%)[b]		maximum dissolved N_2O (mg-N/L)		composition of N_2O at 5 min after NH_2OH spiking (%)					
							abiotic			biotic		
initial $^{15}NH_2OH$ concentration (mg-N/L)	abiotic	biotic	abiotic	biotic	abiotic	biotic	$^{44}N_2O$	$^{45}N_2O$	$^{46}N_2O$	$^{44}N_2O$	$^{45}N_2O$	$^{46}N_2O$
1	1.29	5.65	51.2	43.3	0.0925	0.197	0.6	96.4	3.0	9.4	90.1	0.5
5	6.96	16.1	51.1	39.7	0.547	0.735	0.4	97.0	2.6	25.7	74.0	0.3
10	14.5	33.2	50.7	41.5	0.934	1.591	0.3	98.0	1.7	17.4	81.4	1.2
20	24.0	93.3	50.2	40.5	1.480	4.913	0.5	98.5	1.0	19.4	79.3	1.3

[a]Dynamics of dissolved N_2O concentrations and ^{15}N ratios in gaseous N_2O were acquired from Figure S6. [b]^{15}N percentage in N_2O was quantified at 5 min after spiking with $^{15}NH_2OH$, when the highest dissolved N_2O concentration was obtained.

for *nirK*: NH_2OH addition increased its mRNA expression level by a factor of 136 (Phase III, Figure 3 and SI Figure S4). In contrast, the *amoA* gene expression level was relatively constant, despite perturbations imposed on the biomass (Figure 3 and SI Figure S4).

Interactions of NH_2OH with Different Nitrogen Species. The effect of NH_2OH on different nitrogen constituents in N_2O production is shown in Figure 4. Regardless of the presence or absence of nitrifying biomass, the interaction of NH_2OH with NO_2^- produced the most N_2O. The initial N_2O production rate was highest (31.5 mg-N/L/h) in the run with NH_2OH and NO_2^- under biotic conditions (Figure 4a), followed by 9.042 mg-N/L/h under abiotic coupling of NH_2OH with NO_2^- (Figure 4e). In other nitrogen species combinations, the presence of nitrifying biomass produced N_2O at rates 50–250 times higher than in the absence of nitrifying biomass. The N_2O concentration peak was higher in the presence of nitrifying biomass (1.69 mg–N-N_2O/L) than in the absence of nitrifying biomass (0.75 mg–N-N_2O/L) when NH_2OH was combined with NO_2^-. However, the N_2O production profiles were temporally broader without biomass (Figure 4a, e). The biotic N_2O productions were not greatly different (1.098–1.548 mg-N/L/h) when NH_2OH was coupled to either NO_3^- or NH_4^+, or with NH_2OH only.

Effect of $^{15}NH_2OH$ Amount on N_2O Production. The initial $^{15}NH_2OH$ concentration in the reactor, where the NO_2^- concentration was 400 mg-N/L, positively correlated with the initial N_2O production rate, irrespective of the presence (r^2 = 0.961) or absence (r^2 = 0.981) of nitrifying biomass (SI Figure S5). However, biotic conditions produced N_2O approximately 3.6 times faster than abiotic conditions (The initial N_2O production rate with added NH_2OH was 4.34 vs 1.26 h^{-1}; SI Figure S5). In all experiments, the DO concentrations and pH were sufficiently high, ranging from 7.11 to 8.25 mg/L and from 6.40 to 7.40, respectively (data not shown). The effects of abiotic and biotic conditions on the N_2O production rate and ^{15}N ratio are shown in SI Figure S6 and Table 1. Irrespective of the initial $^{15}NH_2OH$ concentration, biotic conditions gave a higher N_2O production rate and maximum N_2O concentration compared with abiotic conditions (Table 1). The ^{15}N percentages in N_2O produced under abiotic conditions were nearly 50%, and the predominant N_2O isotopolog was $^{45}N_2O$. The ^{15}N percentage decreased from 50% to approximately 30% under biotic conditions (SI Figure S6). This trend was consistent, irrespective of the initial NH_2OH concentration (SI Figure S6). Despite a decrease in ^{15}N in N_2O, the most abundant N_2O isotopolog under biotic conditions was $^{45}N_2O$, followed by $^{44}N_2O$ and $^{46}N_2O$ (Table 1). $^{46}N_2O$ production was marginal at 5 min after NH_2OH spiking under biotic conditions: the percentage for $^{46}N_2O$ was 0.3% to 1.3% (Table 1).

$^{15}NH_4$ Experiments. A one-cycle batch test in an SBR system was performed. The initial $^{15}NH_4^+$ and NO_2^- concentrations at 0 h were adjusted at approximately 200 and 400 mg-N/L, respectively. The concomitant NH_4^+ decrease and NO_2^- increase were observed without accumulation of NO_3^- (Figure 5a). The presence of NH_4^+ and NO_2^- at the beginning caused abrupt increases in dissolved and gaseous N_2O concentrations as shown in Figure 5b. On the contrary, the relatively lower NH_4^+ concentration (35.9 mg-N/L) at 3 h

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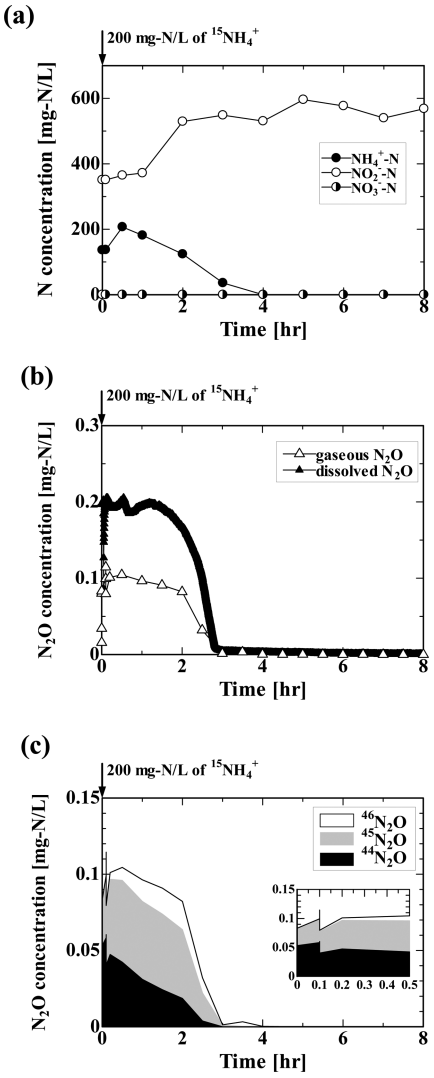

Figure 5. One-cycle profiles of nitrogen compounds (a); gaseous and dissolved N_2O concentrations (b); compositions of gaseous N_2O isotopologues (c); and short-term transitions of gaseous N_2O isotopologues (inset panel (c)).

led to a rapid decrease in the N_2O concentrations close to zero and without subsequent N_2O production. The addition of $^{15}NH_4^+$ at 0 h remarkably increased $^{44}N_2O$ and $^{45}N_2O$ percentages (Figure 5c). From 0.17 to 2.0 h, $^{45}N_2O$ was the most predominant isotopolog, followed by $^{44}N_2O$ and $^{46}N_2O$ in the descending order. Integration of each composition revealed that the compositions of $^{44}N_2O$, $^{45}N_2O$ and $^{46}N_2O$ production were 32%, 52%, and 16%, respectively. The relative contributions of nitrifier denitrification, *N*-nitrosation hybrid N_2O production and NH_2OH oxidation were 43%, 51%, and 6%, respectively (For details, please see SI).

DISCUSSION

N_2O Production via Multiple Pathways. Three different N_2O compounds (i.e., $^{46}N_2O$, $^{45}N_2O$, $^{44}N_2O$) were detected during batch experiments with $^{15}NH_2OH$ and SBR experiments with $^{15}NH_4^+$. This result is strong evidence for the involvement of multiple pathways because each isotopolog can be associated with N_2O production mechanisms using the methodology presented in SI. As a practical matter $^{45}N_2O$ can generally be associated with *N*-nitrosation hybrid N_2O production when $^{15}NH_2OH$ is added to unlabeled NO_2^-, because the mass number (45) is the sum of 15 (either from $^{15}NH_2OH$ or $^{15}NH_4$), 14 (from unlabeled $^{14}NO_2^-$), and 16 (from O). Similarly, $^{44}N_2O$ and $^{46}N_2O$ can generally be associated with nitrifier denitrification and NH_2OH oxidation, respectively. The involvement of multiple pathways was further supported by gene expression profiles that show that genes associated with N oxidation (i.e., *haoA*) and reduction (*nirK* and *nor B*) were simultaneously expressed and further induced by the addition of $^{15}NH_2OH$. The addition of $^{15}NH_2OH$ boosted mRNA levels for *haoA*, *nirK*, and *norB* (Figure 3). The exponential increases in *nirK* mRNA genes after the first NH_2OH spiking (by factors of 136; Figure 3 and SI Figure S4), indicate that electrons produced by NH_2OH oxidation are relayed to NO_2^- and NO reductases in an AOB cell. Yu and Chandran[40] reported increases in expression levels of *norB* and *nirK* genes in AOB at lower DO concentrations. This is consistent with our results showing that expression of these genes was 1 order of magnitude higher after NH_2OH addition (Figure 3 and SI Figure S4), concomitant with a decrease in DO concentration to less than 1.0 mg/L. However, these gene expressions were boosted even at DO concentrations above 2 mg/L when the second $^{15}NH_2OH$ spiking was performed, inconsistent with the previous report.[40] This controversial trend could be explained by the finding that *nirK* expression by *N. europaea* is aerobically induced with an increase in NO_2^- concentration, potentially correlated with expression of gene encoding NO_2^--sensitive *nirK* transcription repressor.[57] So far, an explicit reason for high *nirK* expression is not clear. In addition, recent reports unveiled implications of cytochrome P460[20] and unidentified nitrite reductase of *N. europaea*[58] for N_2O and NO production, respectively, which should be investigated in future. Nevertheless, our results suggest that NH_2OH addition to the bulk liquid accelerates NH_2OH oxidation to NO_2^-, and produces electrons, resulting in noticeable expression of mRNA *nirK* and *norB* genes harbored by AOB, despite a higher DO concentration than previously reported.[40] This ^{15}N tracer study, in combination with mRNA quantification, highlights the role of multiple N_2O production pathways mediated by AOB in PN bioreactors. It is worth noting that Law et al.,[17] postulated a different N_2O production pathway via HNO in a nitrifying bioreactor for NO_2^- accumulation. The discrepancy between our results (multiple pathways) and those of Law et al.[17] (HNO pathway) probably stems from the predominant AOB in nitrifying bioreactors and DO concentrations (the DO range was 1−4 mg/L in our study and mainly <1 mg/L in the previous study[17]).

***N*-Nitrosation Hybrid N_2O Production.** The primary N_2O production mechanism observed in this study was *N*-nitrosation hybrid N_2O production. During the batch experiments with $^{15}NH_2OH$, 49% of the N_2O generated was produced via this mechanism. The SBR tests with $^{15}NH_4$ showed a similar result (i.e., 51% $^{45}N_2O$), which means that the predominance of $^{45}N_2O$ was not an artifact caused by adding NH_2OH. Although hybrid N_2O production was demonstrated by isolated *Nitrososphaera viennensis*[21] and AOB/AOA biomasses,[22] our result is the first evidence, proven by ^{15}N tracer, that N_2O is potentially produced by this pathway in a PN bioreactor. *N*-nitrosation hybrid N_2O formation had not been previously and explicitly proven for AOB-enriched biomass. A biotic hybrid reaction for N_2O production, whereby N_2O is produced with NH_2OH or NH_4^+ as the cometabolized nitrogen species, has previously been reported.[21,22,25,28] A

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hybrid N_2O reaction is also observed under abiotic conditions by coupling NH_2OH with a metal (e.g., Mn or Fe) or NO_2^-.[23,59] Our abiotic tests indicate that interactions between NH_2OH and NO_2^- triggered N_2O production (Figure 4e), in agreement[59] and disagreement[60] with previous reports. This disagreement is probably caused by trace elements in the synthetic wastewater. An additional abiotic N_2O production experiment showed that trace metals in the synthetic medium (SI Table S1) catalyzed abiotic N_2O production (SI and Figure S7). The supplementary abiotic N_2O production test also indicated that involvement of Fe(III)[61] in abiotic N_2O production was insignificant (SI Figure S8). More than 96.4% of the N_2O produced under abiotic conditions was $^{45}N_2O$ (Table 1). The ^{15}N ratios in the produced N_2O were approximately 50%, indicating that N_2O production was derived from $^{15}NH_2OH$ and unlabeled NO_2^-. Abiotic hybrid N_2O production from NH_2OH and NO_2^- is observed at acidic pHs (2−3);[23] this does not apply in the current work. However, we used a synthetic medium as the liquid for N_2O production, so trace elements in the medium may have reduced the activation energy and promoted abiotic hybrid N_2O production at neutral pH. Recent publications reported abiotic hybrid N_2O production in soils at pH 5.5[44] and likely in a wastewater treatment system by coupling NH_2OH and HNO_2 at circumneutral pH.[45,59] The effect of trace metals on hybrid N_2O production warrants future study, and must be considered for nitrifying bioreactors subject to NO_2^- accumulation.

N_2O Production via Biological Mechanisms. The current results showed secondary levels of both $^{46}N_2O$ and $^{44}N_2O$. $^{46}N_2O$ production suggests NH_2OH oxidation, likely explained by biological reactions converting NH_2OH to HNO by HAO in an AOB cell,[62] followed by instantaneous chemical decomposition to N_2O ($2HNO \rightarrow H_2N_2O_2 \rightarrow N_2O + H_2O$),[63] or NH_2OH oxidation to N_2O by *N. europaea* cytochrome P460,[20] and/or *N*-nitrosation hybrid reaction coupling of $^{15}NH_2OH$ with $^{15}NO_2^-$. Quantification of *haoA*, whose expression levels increased after NH_2OH spiking by factors of 10.1, supports the involvement of NH_2OH with HAO in an AOB cell (Figure 3 and SI Figure S4). $^{44}N_2O$ was mainly derived from nitrifier denitrification. In principle, $^{44}N_2O$ may also be produced by heterotrophic denitrification, but in the current study this was unlikely because there were no external carbon sources to drive denitrification and because endogenous decay is unlikely to be driven by NO_2^- in the presence of DO. Law et al.[60] also reported the absence of heterotrophic denitrification in a highly enriched AOB bioreactor. The contribution of nitrifier denitrification to N_2O production may vary in different studies because of (i) different predominant distinct AOB species, (ii) different DO concentrations, and (iii) the amount of added NH_2OH. Phylogenetic differences, even at the species level, affect the amount of N_2O produced by AOB[42,64,65] and its production pathways.[64,65] Furthermore, nitrifier denitrification is more dominant at DO levels lower than those observed during our batch tests (i.e., 0.79−4.30 mg-O_2/L; Figure 1c).[66−68] Higher DO concentrations retard nitrifier denitrification, because oxygen competes with NO_2^- as an electron acceptor and induces NH_2OH oxidation.[67] This may explain the low contribution of nitrifier denitrification to N_2O production.

Implications and Future Research. To the best of our knowledge, this is the first study to prove that N_2O is produced via biotic and abiotic *N*-nitrosation hybrid N_2O production during PN. The implications for these findings are significant for those interested in understanding and controlling N_2O emissions during PN of wastewater. If abiotic N_2O production is neglected, there is great potential to underestimate N_2O emission from PN systems or to incorrectly attribute measured N_2O emissions to only the known biological mechanisms. The current work also showed the merit associated with using labeled N substrates, which permitted N_2O production mechanisms to be distinguished and their relative contributions to be quantified. Future work should also employ this approach for a range of reactor configurations and dynamic operating conditions in an effort to further elucidate the intricacies of the biotic and abiotic pathways responsible for N_2O production.

■ ASSOCIATED CONTENT

Supporting Information

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Additional informtion as noted in the text (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +81-42-388-7069; fax: +81-42-388-7731; e-mail: akte@cc.tuat.ac.jp.

ORCID

Akihiko Terada: 0000-0002-9258-6912

Present Addresses

[#](C.K.) Graduate School of Integrated Arts and Sciences, Hiroshima University.

[▽](K.K.) Center for Ecological Research, Kyoto University.

Notes

The authors declare no competing financial interest.

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