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Article

Fluctuations in Ammonia-Oxidizing Bacteria Biomass Density as a Potential Driver of Seasonal Variations in N_2O Emissions from Activated Sludge Nitrification

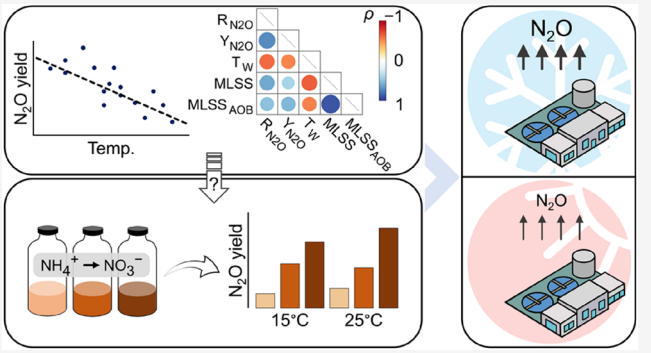
Minho Lee, Fabrizio Sabba, Michele Laureni, and Sukhwan Yoon*

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ABSTRACT: Wastewater treatment plants (WWTPs) exhibit marked seasonality in N_2O emissions. This study aimed at investigating whether the temperature response of the wastewater nitrifying community contributes to this seasonality. NH_4^+ oxidation and N_2O production rates were determined with indigenous activated sludge in the laboratory at the water temperatures measured *in situ* at the time of sampling (14.5−27.5 °C) under nonlimiting O_2 availability (>5 mg L^{-1} throughout all incubations). The N_2O yield, which ranged between 0.004 and 0.028 mol N_2O−N/mol NH_4^+, exhibited a significant negative correlation ($\rho = -0.53$, $p = 0.0015$) with temperature. Interestingly, N_2O−N yield was also positively correlated with mixed-liquor suspended solid (MLSS) concentration ($\rho = 0.41$, $p = 0.017$), a parameter upheld in winter to sustain nitrification rates. This biomass effect was substantiated by subsequent experiments in which N_2O yields of activated sludge (MLSS: 2343 ± 39 and 3760 ± 93 mg L^{-1}) were significantly higher (1.6- to 1.9-fold) than their 2-fold dilutions, regardless of temperature. Higher NH_2OH levels detected in denser activated sludge during nitrification (peak concentration of 0.25 ± 0.13 μM versus 0.09 ± 0.01 μM of the 2-fold dilution) suggested NH_2OH accumulation as a possible mechanistic explanation. These observations suggest that the higher design MLSS for winter performance may contribute to an increase in N_2O emissions from nitrogen removal WWTPs.



KEYWORDS: *activated sludge, nitrification, ammonia oxidation, nitrous oxide, seasonality, biomass density, hydroxylamine*

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INTRODUCTION

Nitrous oxide (N_2O) emissions from wastewater treatment plants (WWTP), though contributing a small fraction to global totals, have received substantial attention due to their concentrated nature and amenability to mitigation.[1,2] Wastewater treatment plants have also served as valuable model systems for investigating the ecophysiology of microbial nitrogen-cycling processes and their complex interactions that collectively underpin N_2O dynamics.[3−5]

Decades of research have led to a scientific consensus that >90% of N_2O emitted during conventional biological nitrogen removal (BNR) processes (e.g., A2O process) is produced from nitrification and denitrification.[6,7] Nitrification, more specifically ammonia oxidation, is largely accountable for N_2O emissions from the aerated segment of activated sludge BNR, where NH_4^+−N is oxidized to NO_3^-_N by the concerted activities of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) and/or the activity of comammox *Nitrospira*.[7,8] N_2O production from ammonia oxidation can occur via different pathways, including microbial and/or abiotic oxidation of NH_2OH, i.e., the immediate product of NH_4^+ oxidation, and NO_2^- reduction to N_2O via NO through nitrifier denitrification.[9−11] Recent studies have indicated that even pathways previously regarded as minor, such as the abiotic oxidation of extracellularly leaked NH_2OH, may have substantial contribution to N_2O emissions, depending on the organisms and environmental conditions.[11,12] For example, N_2O production via this pathway, estimated from NH_2OH accumulation profiles and abiotic decay kinetics, accounted for 0.05−0.12% of NH_4^+ in the ammonia-oxidizing bacterium *Nitrosospira multiformis* and 0.05−0.07% in *Nitrosomonas europaea*, values that are consistent with previous reported N_2O yields for these organisms.[10] N_2O emitted from the anoxic segment, where activated sludge is kept unaerated to accommodate denitrification, i.e., reduction of NO_3^- and NO_2^- to gaseous forms (NO, N_2O, N_2), is attributed to the

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Table 1. Physicochemical and Operational Parameters from the Oxic Tank of Daejeon WWTP

date[a]	T_w (°C)	pH	DO	MLSS (mg L^{-1})	NH_4^+–N (mg L^{-1})	NO_2^-–N (mg L^{-1})	NO_2^-–N (mg L^{-1})	SRT (d)[b]	HRT (d)[b]
09/19/22	26.2	6.70 (0.00)[c]	2.4	4860[d]	0.46 (0.04)	0.38 (0.27)	1.05 (0.06)	14.8	3.7
09/28/22	25.0	6.73 (0.07)	3.3	4860	0.85 (0.11)	0.00 (0.00)	2.20 (0.04)	14.8	3.7
10/25/22	21.5	6.61 (0.02)	2.9	4330	0.69 (0.00)	0.34 (0.24)	3.70 (0.37)	10.4	3.7
11/14/22	21.0	6.46 (0.00)	2.0	4523 (46)	N/A[e]	N/A	N/A	15.3	3.9
01/09/23	14.5	6.48 (0.02)	4.4	6377 (5)	1.83 (0.01)	0.10 (0.00)	3.65 (0.06)	12.6	3.8
02/22/23	14.2	6.46 (0.01)	2.5	6480 (8)	0.35 (0.04)	0.11 (0.00)	2.18 (0.06)	12.8	3.7
03/13/23	16.3	6.50 (0.02)	2.2	4760 (36)	3.75 (0.22)	0.15 (0.04)	3.54 (0.07)	8.4	3.5
03/29/23	17.8	6.54 (0.01)	2.7	5883 (67)	1.83 (0.17)	0.34 (0.03)	2.52 (0.07)	8.4	3.5
05/01/23	20.2	6.50 (0.00)	3.1	4353 (12)	4.84 (0.07)	0.52 (0.11)	3.39 (0.08)	9.2	3.5
06/25/23	26.1	6.47 (0.02)	3.1	3230 (64)	3.79 (0.14)	0.28 (0.08)	10.8 (0.06)	8.6	3.5
07/21/23	25.1	6.60 (0.02)	3.1	2836 (91)	1.71 (0.06)	0.43 (0.14)	3.77 (0.03)	8.6	2.9
08/04/23	27.5	6.51 (0.01)	4.7	5257 (357)	2.37 (0.08)	0.22 (0.03)	4.44 (0.10)	5.8	3.0
08/22/23	27.5	6.44 (0.01)	2.1	2597 (48)	3.35 (0.03)	0.18 (0.01)	3.96 (0.28)	5.8	3.0
09/12/23	27.0	6.45 (0.01)	4	2867 (211)	3.95 (0.10)	0.20 (0.00)	3.46 (0.06)	6.8	2.8
10/08/23	23.5	6.55 (0.02)	3.6	3133 (189)	4.12 (0.18)	0.22 (0.08)	3.33 (0.04)	6.3	3.1
11/14/23	20.3	6.16 (0.01)	2.4	4073 (87)	0.64 (0.13)	0.32 (0.03)	5.38 (0.03)	7.8	3.3

[a]Dates of sampling. [b]Monthly SRT and HRT (hydraulic retention time) of the oxic tank, provided by the operator. [c]The values in the parentheses represent the standard deviations of three independently collected samples on the same date. [d]The MLSS values without standard deviation were provided by the plant operators from the monthly monitoring data. [e]N/A - not provided/measured.

kinetic imbalance between the N_2O-generating and consuming steps of denitrification due to, for example, differential O_2 inhibition and uneven electron distribution from organic e^- donors.[13] Previous studies have assigned widely varying levels of importance to these different N_2O-producing pathways, as technical difficulties in disentangling pathway-specific contributions within complex activated sludge microbiomes, together with inconsistencies across experimental and monitoring data have hindered the emergence of a coherent conclusion.[7,8,14] Due to this complexity, linking field monitoring data to the underlying physiology involved via theoretical extrapolations or modeling has proven challenging, if not impossible.

Temporal variability in N_2O emissions is one such phenomenon that lacks a clear physiological basis despite being consistently observed across multiple long-term monitoring campaigns. Up to a 100-fold difference in N_2O fluxes between peak and trough periods has been commonly observed in the literature reporting data from year(s)-long monitoring campaigns.[8,15−18] Most of these monitoring campaigns were conducted in the global north with distinct summer and winter seasons, and seasonal patterns have been observed in quite a few of these studies, with N_2O emissions usually peaking during winter or early spring.[16,17,19] These observations suggested that the water temperature or operational parameters associated with the seasonal temperature variation may play a consequential role in causing these temporal dynamics. The growing availability of multiomics data has enabled mechanistic investigations into the temporal N_2O emission dynamics.[16,17] One such recent study, integrating long-term on-site monitoring with metagenomic and metaproteomic analyses, attributed pronounced seasonal variability, characterized by wintertime peaks (January−March) exceeding baseline values by more than 20-fold, to temperature-triggered shifts in the activity balance among key nitrifier guilds in the oxic tank, leading to NO_2^- accumulations (up to 1.2 mg L^{-1}) and enhanced nitrifier denitrification activity.[17] However, many uncertainties remain unresolved, as the correlation between NO_2^- accumulation and N_2O emission has not been ubiquitously observed in other monitoring campaigns.[8,20] Moreover, this mechanistic explanation is applicable only to fluctuations in N_2O produced via nitrifier denitrification.[8,17,20]

To address the knowledge gap, the current study systematically investigated temperature effects on N_2O emissions from the activated sludge nitrifying community under highly aerated condition, under which N_2O emissions contribution from nitrifier denitrification is known to be minimal.[10,21] N_2O emissions from incubation of activated sludge samples collected from an A2O-type WWTP between September 2022 and November 2023 with water temperatures ranging from 14.5 to 27.5 °C were examined both with and without biomass dilution and chloramphenicol (inhibitor of *de novo* protein synthesis) amendment under high oxygen tension (>5 mg L^{-1} throughout incubation). Controlled physiological experiments corroborated the key finding of the campaign that the AOB biomass density is a key controlling factor of nitrification-related N_2O emissions. This finding provides practical insight for BNR design and operation, suggesting that seasonal biomass management may influence N_2O mitigation strategies.

MATERIALS AND METHODS

Site Description and Sample Collection

Activated sludge samples were obtained from either an oxic or an anoxic section of an activated sludge train located at the Daejeon municipal WWTP (36°23′5″N 127°24′2″E), depending on the purpose of the experiment. Each train has a total volume of 3,084 m^3, comprising anaerobic, anoxic, and oxic segments of 500 m^3, 1044 m^3, and 1540 m^3, respectively. The average solid retention time (SRT) and aerobic SRT (aSRT) were 10.4 ± 3.6 and 5.1 ± 1.8 days, respectively, during the study period from September 2022 to November 2023 (Table 1). The average solid retention time (SRT) during the winter months (December to February, 14.8 ± 2.9 d) was approximately 2-fold longer than during the summer months (June to August, 7.7 ± 1.3 d). Key operating parameters were provided by the operators (Table S1).

Each sample was collected by filling a presterilized polyethylene bottle to the brim with grab-sampled activated sludge. All samples were collected at 12:00 p.m. on the dates listed in Table 1. Additionally, three 1 mL activated sludge samples were filtered on site using a 0.2 μm syringe filter (Hyundai Micro, Inc., Seoul, South

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Korea), and the filtrate was collected. These samples were transported to the laboratory ~5 km apart from the site in a cooler filled with ice; however, minor nitrogen turnover and partial depletion of dissolved O_2 during transport cannot be ruled out. Upon arrival, a 50 mL aliquot of the raw activated sludge sample was separated from the bottle and stored at −80 °C for microbial composition analysis. The triplicate 1 mL filtrates were stored at −20 °C and later used for chemical characterization. Bacteriostatic N_2O production rates were started within 1 h of sampling, and the growth-uninhibited incubation experiments were prepared with the remaining sample within 3 h of sampling.

Measurement of Bacteriostatic N_2O Production Rates at *In Situ* Temperatures

The N_2O production rates from nitrifying (oxic) and denitrifying (anoxic) activated sludge were measured at multiple time points during the study period, using the *in situ* water temperature at the time of sampling as the incubation temperature. For nitrifying activated sludge, nitrogen turnover and N_2O production were measured with 50 mL of raw oxic activated sludge (collected from the aerated segment) in presterilized 160 mL serum bottles with air headspace. The activated sludge samples, with the background NH_4^+ concentrations ranging from 0.05 mM to 0.35 mM, were amended with additional 1 mM NH_4Cl, and chloramphenicol (Sigma-Aldrich, St. Louis, MO) was added to a concentration of 45 μg mL^{-1} to arrest *de novo* protein synthesis.[22] The bottles were sealed with butyl rubber stoppers and aluminum crimps. A set of control experiments were performed to examine the possible interference of heterotrophic and nitrifier aerobic denitrification and abiotic N_2O production on these aerobic N_2O production rate and yield measurements. Ammonia oxidation was selectively inhibited by 100 μM allylthiourea, and N_2O reduction was inhibited by adding 10% v/v C_2H_2 to the headspace.[23,24] C_2H_2 was produced by reacting calcium carbide with distilled water as described previously.[25] To a subset of incubations, 1 mM KNO_3 was added as the substrate for denitrification.

Denitrification experiments were performed using activated sludge samples from the anoxic segment of the WWTP, prepared identically to the nitrification experiments but without O_2 in the headspace. To remove dissolved O_2, the vials were flushed with >99.999% N_2 gas (DIG Airgas Co., Seoul, South Korea) before sealing. N_2O production and consumption rates of denitrifying activated sludge were determined in two separate sets. N_2O production was observed in the set amended with 1 mM KNO_3 and with 10% of the N_2 headspace replaced with C_2H_2. N_2O consumption was observed in the set amended with 49 μmoles N_2O−N. Reduction of NO_3^- was also observed without C_2H_2 to monitor transient N_2O accumulation.

Headspace N_2O concentrations and dissolved NH_4^+, NO_2^-, and NO_3^- concentrations were measured at four to six time points for each rate determination experiment. At each sampling time point, a 100 μL headspace sample was extracted for measuring N_2O concentration in the headspace, and 0.5 mL cell suspension was extracted for dissolved nitrogen species concentration measurements. The suspension was immediately centrifuged, and the supernatant was stored at −20 °C. The production or consumption rates of the nitrogen species were calculated by performing linear regression on the time series data of their total amounts in the incubation bottle. For each set, an additional replicate was examined in parallel, to ensure that a pH change remained minimal (<0.5) and, for oxic measurements, that O_2 supply was not limited (>5.0 mg L^{-1} dissolved O_2) throughout incubation. Unless otherwise stated, all experiments were performed in triplicate with shaking set to 150 rpm. Conditions for all laboratory incubation experiments are summarized in Table S3 to underscore their subtle differences.

N_2O Production from Oxic Nitrifying Diluted Activated Sludge at 15 and 25 °C without Protein Synthesis Inhibition

Batch incubation experiments without chloramphenicol addition were performed at two different temperatures, 15 and 25 °C, using activated sludge as the inoculum to examine whether temperature selection or adaptation of ammonia-oxidizing population may affect N_2O yield. Each 160 mL serum bottle culture was prepared with ambient air headspace and 50 mL synthetic wastewater and sealed after autoclaving.[26] Filter-sterilized 1 M NaH_2PO_4/Na_2HPO_4 stock solution at pH 7.2 was added to achieve a final phosphate concentration of 10 mM. The medium was amended with $NaHCO_3$ and NH_4Cl to concentrations of 1 and 2 mM, respectively. Inocula were prepared from the oxic activated sludge samples by concentrating 50 mL of raw activated sludge into a 3× concentrate in 1 mL of NH_4^+-free synthetic wastewater medium. After addition of the inoculum, the resulting suspension contained activated sludge biomass at 6% of the original concentration. The culture vessels were incubated in two thermostat incubators with the temperatures set to 15 and 25 °C until no further change to the nitrogen species concentrations was observed. The instantaneous N_2O yield at time t_n ($n > 0$) was estimated using linear regression over a time window spanning from t_{n-1} to t_{n+1}.

Impact of Biomass Density on Nitrification N_2O Emissions and NH_2OH Accumulation

The effect of activated sludge biomass on the N_2O−N yield was examined with an oxic activated sludge sample collected on 11/05/24, when the *in situ* water temperature was 22.4 °C. The activated sludge sample was diluted using two different media: the cell-free filtrate of the activated sludge and the synthetic wastewater described above. The cell-free filtrate was prepared by passing raw activated sludge through a 0.22-μm MCE membrane filter (Merck Millipore Ltd., Carrigtwohill, Ireland). Three different dilutions were prepared in these media, adjusting biomass concentration to 0.06×, 0.5×, and 1× of that of the original activated sludge. The 0.06× incubation employed the same dilution factor used in the 15 °C-versus-25 °C experiments described above, whereas the 1× and 0.5× incubations were designed to represent two realistic MLSS concentrations in oxic activated sludge. Experiments were performed with 50 mL suspensions in 160 mL serum bottle amended with 1 mM NH_4Cl and 45 μg mL^{-1} chloramphenicol, with air in the headspace. Sampling of the aqueous and gaseous phases for monitoring nitrogen species concentrations and N_2O production was performed as described above. To isolate the effect of temperature from that of biomass, the experiment with wastewater filtrate was repeated with an oxic activated sludge sample collected on 05/31/25, incubated at 15 and 25 °C. Additionally, transient NH_2OH accumulation was monitored with the two (1× and 0.5×) concentrations of activated sludge (collected on 11/23/24; 20.0 °C) prepared in cell-free filtrate.

Analytical Methods

Headspace N_2O concentration was measured using an 8890-series gas chromatograph equipped with an HP-PLOT/Q column and a microelectron capture detector (Agilent, Palo Alto, CA). The injector, oven, and detector temperatures were set to 200, 50, and 250 °C, respectively. Gas samples were manually injected using a Hamilton 1700 series gastight syringe (Reno, NV). Dissolved N_2O concentrations were calculated from headspace concentrations under the assumption of quasi-equilibrium, using the dimensionless Henry's constant calculated for the incubation temperature.[27] NH_2OH concentration was measured by chemically oxidizing NH_2OH to N_2O with acidic 2.5 mM $FeCl_3$ solution after removing NO_2^- in the sample by reacting with 1.8 mM sulfanilamide prepared in 0.8 M HCl.[28] The dissolved O_2 (DO) concentration was monitored by using a FireStingO_2 oxygen meter and fiber-optic oxygen sensor spots (Pyroscience GmbH, Aachen, Germany). Dissolved concentrations of NO_3^-, NO_2^-, and NH_4^+ were determined colorimetrically as previously described.[29,30] The MLSS concentrations were measured following Standard Methods 2540D.[31]

Genomic DNA Extraction and 16S rRNA Amplicon Sequencing

Nitrifier communities within microbial samples were analyzed by using 16S rRNA gene amplicon sequencing. Genomic DNA was extracted using a DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). Amplification sequencing was performed targeting the

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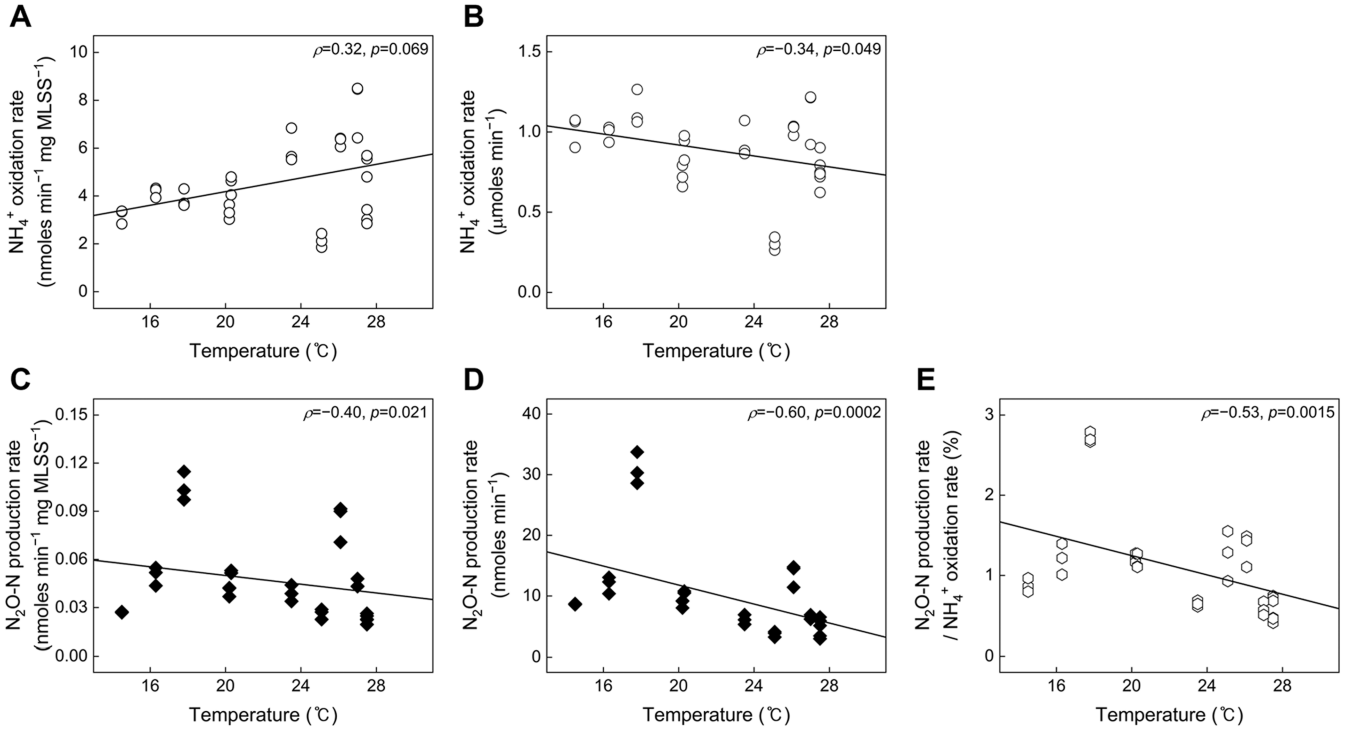


Figure 1. Spearman's rank correlation analyses relating *in situ* activated sludge temperature with NH_4^+−N oxidation rate (A: MLSS-normalized, B: net) and N_2O production rate (C: MLSS-normalized, D: net) and yield (E). Each data point represents an individual replicate. The solid black line represents the least-squares regression line.

V3−V4 region of 16S rRNA with 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) primer sets on a MiSeq platform (Illumina, San Diego, CA) at Macrogen Inc. (Seoul, South Korea), and the data were processed using the QIIME2 v2023.9 pipeline.[32] The ASVs were assigned taxonomy using the SILVA classifier (silva-138−99-nb-classifier.qza) with the confidence threshold set to 0.8.[33,34] Nitrifier ASVs were screened based on the taxonomic annotations. All ASVs annotated as known nitrifier taxa, including *Nitrosomonas* (AOB) and *Nitrospira* (NOB and comammox), were selected for α- and β-diversity analyses.[35,36] Nonmetric multidimensional scaling (NMDS) analysis for the nitrifier ASVs was performed using VEGAN software embedded in R software package v4.3.0.[37] The sequence data were deposited in the NCBI SRA database under accession number PRJNA1332362.

Statistical Analyses

Statistical analyses were performed using R statistical software package v4.3.0. The statistical significance of a pairwise comparison across two conditions was evaluated using two-sample *t*-tests. The strength and statistical significance of a pairwise correlation between two sets of parameters were evaluated using Spearman's rank correlation, as none of the parameters was normally distributed, according to Shapiro-Wilk normality test results. Linear regression analyses were performed using OriginPro 2020 SR1 software (v.9.7.0.188).

RESULTS

Temporal Changes in Activated Sludge Characteristics

The temporal changes in the properties of activated sludge in the oxic segment of Daejeon WWTP were monitored from September 2022 to November 2023 (Table 1; the corresponding data for the anoxic segment are presented in Table S4). The *in situ* water temperature ranged between 14.5 °C (01/09/23) and 27.5 °C (08/04/23). Mixed-liquor suspended solid, which ranged between 2597 mg L^{-1} (08/22/23) and 6480 mg L^{-1} (02/22/23), was the only measured parameter that exhibited a statistically significant correlation with water temperature ($\rho = -0.65$, $p < 0.001$). The concentrations of the inorganic nitrogen species exhibited no discernible trend. NO_3^-−N concentration was comparable to or higher than NH_4^+ concentrations throughout the year. This, together with the low concentration (<1 mg L^{-1}) of NO_3^- in the preceding anoxic tank, indicates that active nitrification occurred regardless of water temperature. A parameter that may potentially affect nitrification, the pH, did not exhibit any temporal trend.

N_2O Production Kinetics in Protein-Synthesis-Arrested Activated Sludge under *In Situ* WWTP Temperature Conditions

Nitrification kinetics measured with chloramphenicol-treated (bacteriostatic) oxic activated sludge identified significant correlations between the water temperature and both the rate and yield of nitrifier N_2O production (Figures 1 and S1). The correlation between the net NH_4^+ oxidation rate and water temperature was only marginally significant ($p = 0.049$) and not statistically significant when normalized to MLSS ($p = 0.069$). The rate of N_2O production varied across the samples taken at different times of the year, with the maximum (617.6 ± 42.7 nmol L^{-1} min^{-1}; 03/29/23; 17.8 °C) approximately 10-fold higher than the minimum (61.9 ± 4.5 nmol L^{-1} min^{-1}; 08/22/23; 27.5 °C). Despite the large variation, both the net N_2O production rate and the MLSS-normalized rate exhibited significant negative correlations with water temperature ($\rho = -0.60$, $p < 0.001$; and $\rho = -0.40$, $p < 0.05$, respectively). The N_2O yield, i.e., μmole N_2O per μmole NH_4^+ oxidized, also exhibited a significant negative correlation with temperature ($\rho = -0.53$, $p < 0.01$).

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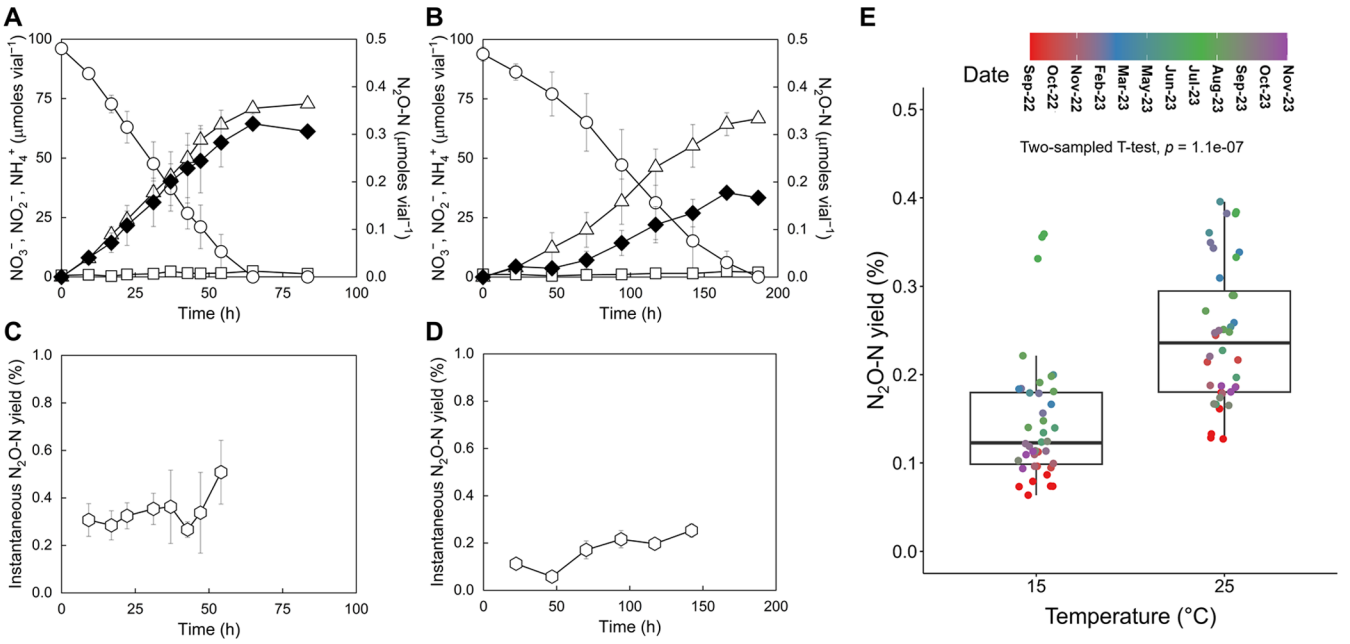


Figure 2. Nitrogen turnover and N_2O production observed during incubation of diluted activated sludge aerobically incubated with 1 mM NH_4^+ at (A) 25 °C and (B) 15 °C. Amounts of NH_4^+ (○), NO_2^- (□), NO_3^- (Δ), and N_2O–N (♦) in the culture vials were monitored. (C, D) Instantaneous N_2O yields corresponding to (A) and (B), respectively. The data points represent the averages of the triplicate cultures. The error bars are the standard deviations of the triplicate measurements. The representative data sets shown here were acquired from the experiments performed with the activated sludge samples collected on 05/01/23 (20.2 °C). (E) Compilation of N_2O yields data from 14 sets of experiments (each set with data from triplicate cultures, each presented as an individual data point) performed with samples collected between Sep 2022 and Nov 2023. The complete data set is presented in Figure S4.

The control experiments showed that NO_3^- reduction activity or associated N_2O production were unlikely to have occurred during aerobic incubation (Figure S2). No N_2O–N was produced in the samples amended with allylthiourea regardless of NO_3^- amendment. Although N_2O–N was measured in substantial amounts at the initial time point (t = 0) in the C_2H_2-amended preparations (0.049 ± 0.009 μmoles vial^{-1}), neither significant NO_3^- reduction nor N_2O production occurred during incubation.

Denitrification kinetics examined with the anoxic activated sludge samples collected on three different dates, 02/11/23 (14.0 °C), 03/29/23 (17.5 °C), 04/25/23 (20.2 °C), and 06/25/23 (26.1 °C; see Table S4 for other parameters) at their respective *in situ* temperatures, suggested that N_2O emission from denitrification is unlikely to be substantially affected by water temperature (Figure S3). All three sets of experiments recorded N_2O consumption rates significantly exceeding the rates of N_2O production from NO_3^-, suggesting against the possibility that temperature may have disproportionate effects on the upstream N_2O-producing and downstream N_2O-consuming steps of denitrification. Additionally, transient N_2O accumulation did not exceed 0.02 μmoles N_2O–N/bottle (0.063 μM dissolved N_2O; at 18 min) during anoxic incubation of 100 mL activated sludge (in 160 mL serum bottles) on 100 μmoles NO_3^- with chloramphenicol amendment (but without C_2H_2; performed only with the 02/11/23 sample). As these results suggested that seasonal temperature variation is unlikely to influence denitrifier N_2O production, anaerobic N_2O production was excluded from further analyses.

Physiological Temperature Effect on Nitrification N_2O Yield Examined with Diluted Activated Sludge

Incubation experiments with diluted activated sludge consortia in minimal medium without chloramphenicol showed that the imposed lab temperature (irrespective of the *in situ* water temperature at the time of sampling) significantly affected the rate and amount of N_2O produced during nitrification, but with higher N_2O yields at 25 °C than at 15 °C ($p < 0.001$; Figures 2 and S4). The experiments performed on 14 different dates all showed similar trends; therefore only the experiment performed on 05/01/23 (Figure 2). NH_4^+ oxidation eventually reached completion at 15 °C but demanded substantially longer time (187 h) than at 25 °C (83 h). No significant accumulation of NO_2^- above the detection limit (1 μM) was observed at either temperature condition, implying that NO_2^- oxidation occurred simultaneously with NH_4^+ oxidation at a comparable rate regardless of the temperature. The total amount of N_2O produced after ~100 μmol NH_4^+ was completely consumed was 0.31 ± 0.06 μmol N_2O–N (an N_2O yield of 0.32 ± 0.07%) at 25 °C, which was significantly larger than 0.17 ± 0.01 μmoles N_2O–N vial^{-1} (0.18 ± 0.01%) at 15 °C ($p < 0.001$). Additionally, instantaneous N_2O yields were consistently larger at 25 °C than at 15 °C throughout the incubation. These results contrasted with the observations from the bacteriostatic kinetics experiments in two aspects: first, a significantly higher, not lower, N_2O production was observed at the higher temperature, and second, the N_2O yields (0.32 ± 0.07% and 0.18 ± 0.01% at 25 and 15 °C, respectively) were an order of magnitude lower.

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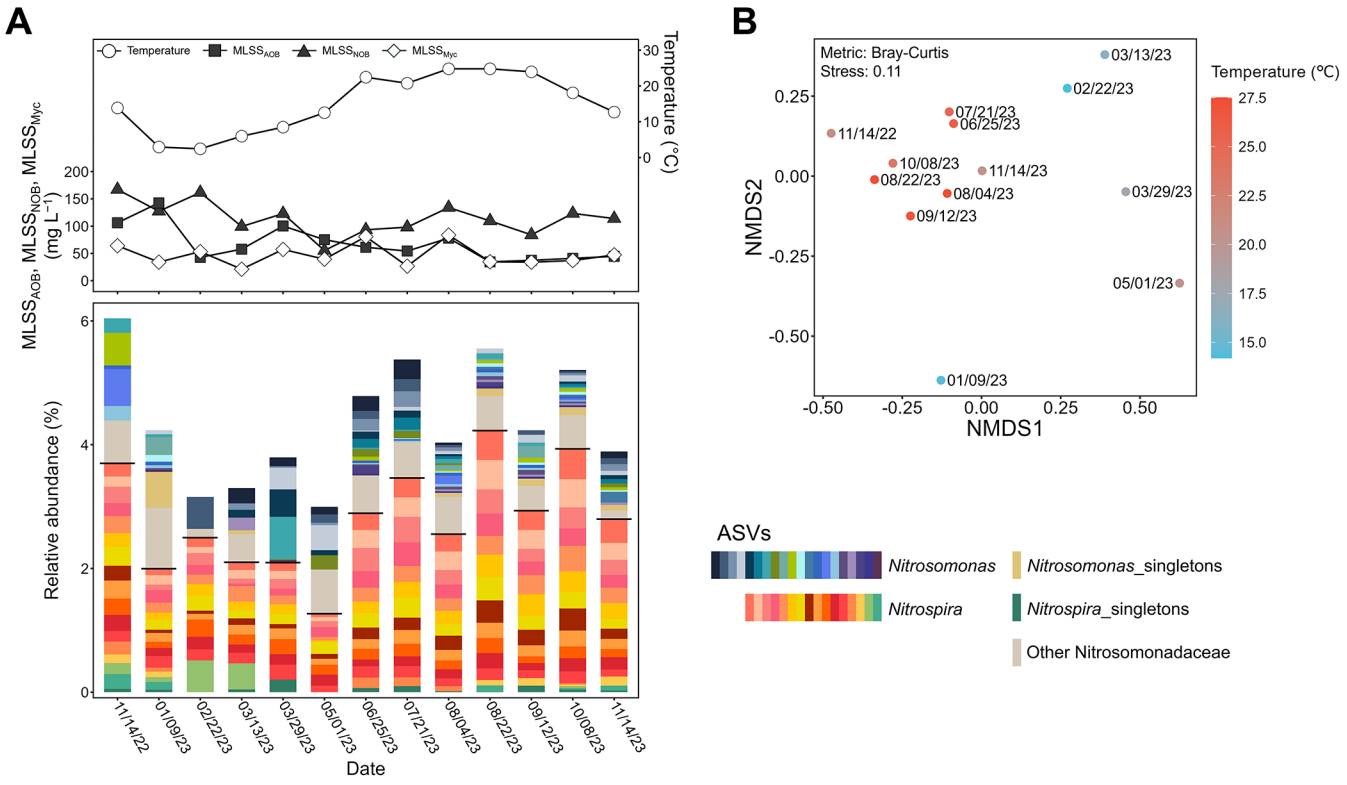


Figure 3. Analyses of the nitrifier community in the activated sludge samples collected at different time points between Nov 2022 and Nov 2023, with water temperature ranging between 14.2 and 27.5 °C. (A) Relative abundances of ASVs affiliated to known ammonia- and nitrite-oxidizing taxa with proxy AOB and NOB biomass concentrations ($MLSS_{AOB}$ and $MLSS_{NOB}$, respectively) estimated as the products of MLSS and their respective relative abundances. The proxy *Mycobacterium* biomass concentration ($MLSS_{Myc}$) was included for comparison with a non-nitrifying taxon. *Nitrosomonas* singletons, *Nitrospira* singletons (each detected in only one sample), and non-*Nitrosomonas* Nitrosomonadaceae ASVs were each pooled and shown as a single color. (B) NMDS plot constructed with the nitrifier ASVs illustrating the β diversity of the nitrifier communities in the activated sludge samples.

Nitrifier Community Composition in the Activated Sludge Microbiomes

Among 302,617 partial 16S rRNA gene reads obtained from sequencing of 13 raw AS samples (see Table S5), a total of 13,902 reads, grouped into 167 ASVs, were affiliated with known nitrifiers (AOB, AOA, NOB, and comammox). *Nitrosomonas* (42 ASVs) and *Nitrospira* (70 ASVs) were the predominant nitrifier taxa, with the two taxa combining for >76.1% of all nitrifier reads in all analyzed samples. The relative abundances of *Nitrosomonas* ranged from 0.51 to 1.70%, and those of *Nitrospira* ranged from 1.27 to 4.22% (Figure 3A). Other putative Nitrosomonadaceae AOB constituted 0−0.98% of the microbial community. The absolute abundance of the Nitrosomonadaceae AOB, estimated using the proxy parameter MLSS × AOB relative abundance ($MLSS_{AOB}$), was generally higher during the low-temperature periods (<18 °C) than during the high-temperature periods, although the difference was not statistically significant (>25 °C; 90 ± 36 mg L^{-1} versus 53 ± 16 mg L^{-1}, respectively; p = 0.11). In contrast, the absolute abundance of NOB ($MLSS_{NOB}$) showed no consistent temperature-dependent pattern. Another genus-level taxon detected in all samples but not involved in nitrification, *Mycobacterium*, also showed no temperature dependence, suggesting that the observed pattern was specific to AOB. Further, only $MLSS_{AOB}$, but neither $MLSS_{NOB}$ nor $MLSS_{Myc}$, significantly correlated with temperature, SRT, or N_2O yield, indicating that the observed relationship between AOB biomass density and N_2O yield was unlikely to be artifacts of SRT-related variations in parameters such as the MLVSS/MLSS and F/M ratios (Figure 4A and Table S1). The nitrifier communities of all four high-temperature-activated sludge microbiomes were closely clustered in the NMDS plot, contrasting with the dispersed distribution of those from the low-temperature-activated sludge microbiomes. Neither ASV with less than two mismatches to previously confirmed comammox *Nitrospira* ASVs nor AOA ASV was identified in any of the samples.

Biomass Density as a Determinant of N_2O Emissions and NH_2OH Accumulation during Activated Sludge Nitrification

Two independent lines of evidence suggested that the AOB biomass density may influence N_2O emission: (1) MLSS and $MLSS_{AOB}$ both exhibited a significant correlation with both temperature and N_2O yield from nitrification (Figure 4A) and (2) N_2O yields measured using undiluted activated sludge (Figure 1) were approximately an order of magnitude higher than those obtained from the experiments using the activated sludge consortia diluted to 0.06X biomass concentration (Figure 2).

This hypothetical biomass effect was verified with N_2O yield measurements performed with a raw activated sludge and its dilutions prepared in a cell-free activated sludge filtrate (Figure 4B). The N_2O yield of the 0.5× activated sludge was 0.54 ± 0.05%, which was approximately half of the N_2O yield measured with the undiluted activated sludge, 1.03 ± 0.02% (p < 0.01). The N_2O yield was further reduced in the 0.06×

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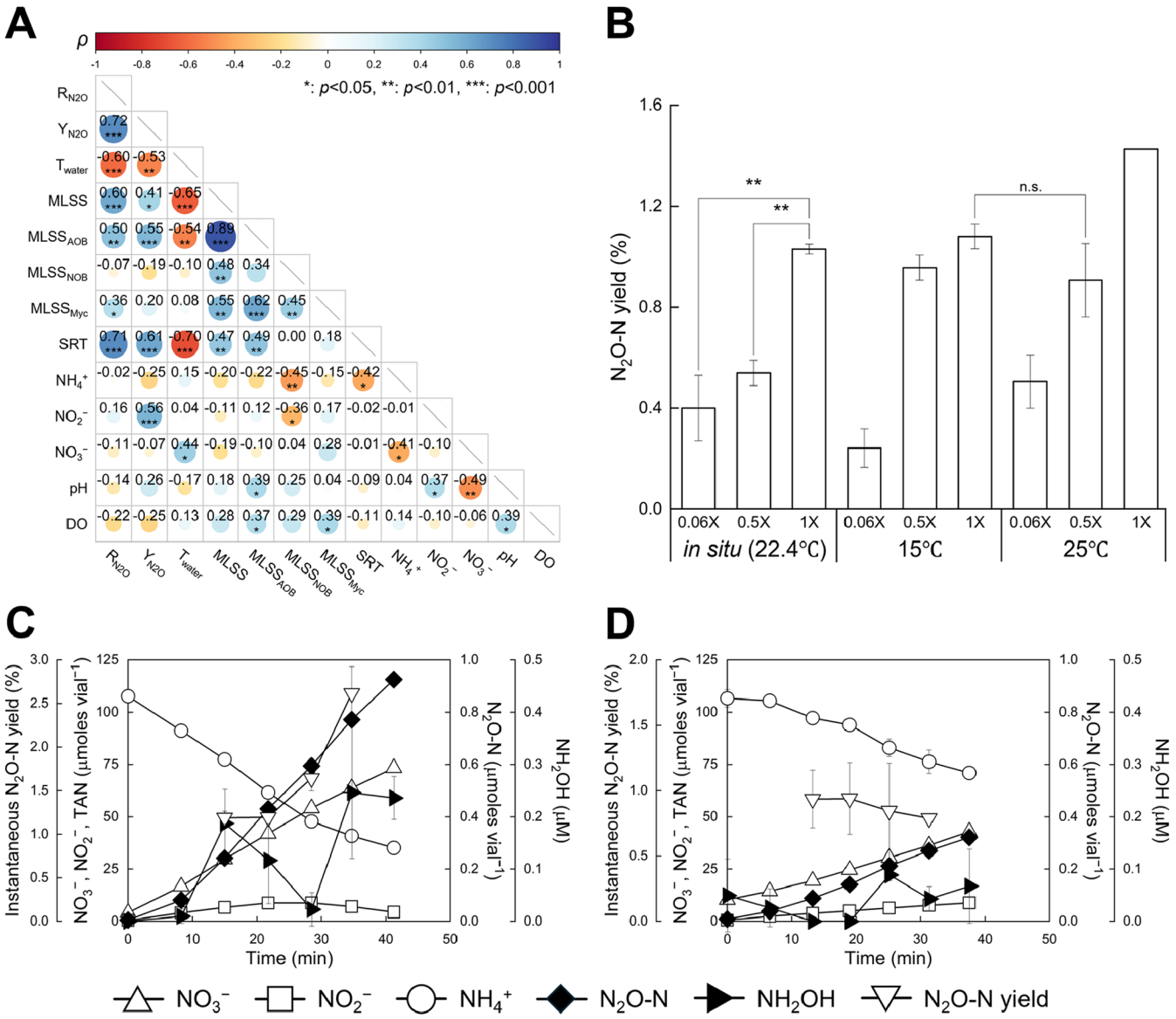


Figure 4. Correlational and experimental evidence suggesting the impact of biomass density on N_2O production from nitrification. (A) Spearman's rank correlation matrix illustrating the pairwise relationships among physicochemical properties of activated sludge samples and experimentally determined nitrification kinetic parameters. R_{N2O}: unnormalized N_2O–N production rate, Y_{N2O}: N_2O yield (g N_2O–N produced per g NH_4^+ consumed), T_W: *in situ* wastewater temperature, NH_4^+: total ammonia nitrogen concentration, NO_2^-: NO_2^- concentration, NO_3^-: NO_3^- concentration. (B) Comparison of N_2O–N yields between three different (0.06×, 0.5×, and 1×) biomass concentrations of activated sludge samples measured at the *in situ* wastewater temperature (22.4 °C; sampled on 11/05/24; MLSS of raw activated sludge: 2343 ± 46 mg L^{-1}) and two different laboratory-imposed temperatures, 15 and 25 °C (sampled on 05/31/25; MLSS of raw activated sludge: 3760 ± 93 mg L^{-1}). Dissolved concentrations of NH_2OH monitored during aerobic batch incubation of (C) undiluted activated sludge (MLSS: 3227 ± 54 mg L^{-1}) and (D) 0.5X activated sludge at 20.0 °C (the *in situ* temperature on 11/23/24) are also presented, with the data points representing averages of triplicate cultures and error bars their standard deviations.

activated sludge to 0.40 ± 0.13%; however, the difference from the 0.5X activated sludge was statistically insignificant. The ensuing experiment, performed at two different temperatures, showed that the biomass effect can counteract the physiological temperature effect. The Q_{10} values for NH_4^+ oxidation rates were 1.83, 1.70, and 1.89 for the 1×, 0.5×, and 0.06× incubations, respectively, indicating consistent temperature sensitivity regardless of biomass density. Contrastingly, Q_{10} values for N_2O production rates varied substantially (2.40, 1.58, and 4.30), suggesting increased mechanistic complexity. Whereas 1× incubation yielded significantly more N_2O per mol NH_4^+ oxidized at 25 °C than at 15 °C, the N_2O yield from 0.5× incubation at 25 °C was statistically indistinguishable from that of 1× incubation at 15 °C ($p > 0.05$). At both incubation temperatures, 17-fold dilutions produced N_2O with a yield less than one-third of that observed with undiluted activated sludge at the respective temperatures.

The biomass level affected transient accumulation of extracellular NH_2OH, which may represent a key intermediate underpinning the observed biomass effect on the N_2O yield (Figure 4C,D). The maximum NH_2OH concentration measured in cell-free aqueous samples during nitrification was 0.25 ± 0.13 μM, even in the undiluted activated sludge; however, at five of six time points (excluding $t = 0$) during the

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incubation, NH_2OH concentrations measured in the undiluted activated sludge was significantly, and at least 2-fold higher than those measured in the 2-fold dilution monitored in parallel (0.14 ± 0.09 μM versus 0.04 ± 0.03 μM on average between 6.6 and 37.5 min; Figure 4C,D).

DISCUSSION

Today, N_2O emissions from BNR systems are known to exhibit seasonal fluctuations, as corroborated by recent and ongoing *in situ* monitoring studies.[8,16,20] However, we found a surprising paucity of studies directly addressing the effect of temperature and/or other seasonally recurring changes in activated sludge properties on N_2O production from key microbial nitrogen turnover processes, namely nitrification (more specifically, ammonia oxidation) and denitrification (i.e., NO_3^- and NO_2^- reduction to gaseous products NO, N_2O, and N_2) at physiological level.[38,39] Through a series of laboratory incubation experiments performed with indigenous activated sludge collected at varying time of the year, we demonstrated here that (1) N_2O production from aerobic nitrification, but not denitrification, exhibits a significant seasonal dependence, and (2) this seasonal variation arises from the combined, yet opposing, influences of temperature and activated sludge biomass (parametrized as MLSS) on nitrification-derived N_2O emissions. Importantly, these findings are based on controlled physiological experiments that preserved the microbiological and physicochemical complexity of activated sludge rather than on correlation analysis alone. Also, the MLSS, NH_4^+ oxidation rates, and N_2O yields were within the range reported in previous *in situ* WWTP monitoring studies, supporting the representativeness of our results.[40,41]

As early as 1970, it was considered self-evident that elevated temperatures would lead to higher N_2O yields from ammonia oxidation.[42,43] Perhaps, the obviousness and consistency of such a tendency may have been the reason that explains the paucity of subsequent reports on the effect of temperature on nitrifier N_2O production. Consistent with this, all experimental sets performed at two laboratory-imposed temperatures in this study showed significantly higher N_2O production upon aerobic incubation at 25 °C than at 15 °C (Figures 2, 4B, and S4), with the sole exception of the 0.5X biomass condition. The results were consistent regardless of the activated sludge used and at all time points during incubation, ruling out temperature selection for nitrifiers with an inherently higher or lower N_2O yield as the underlying mechanism.

A key challenge in this study was reconciling this conventional knowledge with the observed negative correlation between the *in situ* water temperature and nitrification-associated N_2O yield of activated sludge, as the two appeared contradictory. Correlational analyses identified MLSS, or activated sludge biomass, concentration, as the missing link, a hypothesis subsequently confirmed through laboratory experiments in which biomass density was the isolated variable. Simply diluting the same activated sludge sample (MLSS of 3,760 ± 93 mg L^{-1}) 2-fold was sufficient to significantly reduce the N_2O yield at both ends of the *in situ* temperature spectrum, and this impact more than offset the impact of a 10 °C difference in incubation temperature (Figure 4B). The ammonia-oxidizing community of the activated sludge was composed exclusively of AOB of the Nitrosomonadaceae family throughout the year of study, with a complete absence of ASV attributed to comammox *Nitrospira* or AOA, the nitrifier groups generally associated with lower intrinsic N_2O yields.[35,44,45] This observation further supported that intrinsic physiological properties of different groups of microorganisms constituting the nitrifier population were unlikely to have played a significant role in determining the N_2O yield.

The biomass density effect resolved the apparent contradiction between the opposing temperature dependencies of nitrification N_2O yields observed in the two experimental approaches of this study; however, its mechanistic basis remains elusive. The most conventional explanation would be an expansion of O_2-limited microenvironments within biomass flocs in high-density activated sludge, known to promote nitrifier denitrification and heterotrophic denitrification.[10,21,46] An early physiological study with *N. europaea* reported an elevated N_2O yield (0.039 mol N_2O−N/mol NO_2^- produced) in a highly densified culture (1.7×10^7 cells mL^{-1}), which was attributed to increased nitrifier denitrification activity.[46] However, this explanation is quantitatively inconsistent with the current knowledge of nitrifier denitrification based on more recent experimental observations. Although nitrifier denitrification contributes to N_2O production even under the O_2-replete condition, its contribution is minor under such condition.[10] That is, nitrifier denitrification accounted for only 3.1−13.5% of total N_2O production across five *Nitrosomonas* isolates and three *Nitrosospira* isolates under ambient air in the headspace.[10] Here, the absence of consistent N_2O production in ATU- and C_2H_2-amended controls fed with NO_3^- and NO_2^- negated the possibility of a significant contribution from heterotrophic denitrification to N_2O production. Moreover, the absence of heterotrophic denitrification in these control experiments indicates that the anoxic or microoxic pockets were minimal during laboratory incubations under ambient air headspace, further diminishing the possibility of a significant contribution from nitrifier denitrification, also known to proliferate in AOB subjected to limited O_2 availability.[10,47,48]

With nitrifier denitrification excluded as a plausible mechanism, the observed increase in N_2O yield with increasing AOB density may be hypothetically explained with N_2O-forming enzymatic and abiotic reactions involving NH_2OH. Evidence from the literature supports that a substantial fraction, up to several percents, of NH_4^+ oxidized by different groups of ammonia-oxidizing organisms is released extracellularly as NH_2OH, with the extent of release varying by orders of magnitude across organisms.[11,49,50] Consistent with these previous studies, extracellular NH_2OH was detected in our incubations, and its average concentration was approximately 3-fold higher during incubation of 1× activated sludge than with 0.5× activated sludge, suggesting that AOB biomass density affects NH_2OH accumulation in the medium (Figure 4C). The enzymatic reaction that is most consistent with the observations is the one catalyzed by cytochrome P460, in which the iron active center undergoes sequential reactions with two NH_2OH molecules to form N_2O.[9,51] The *in vitro* NH_2OH turnover rate of cytochrome P460 was linearly dependent on the NH_2OH concentration, at concentrations up to 20 mM, supporting a first-order reaction mechanism.[51] Increased biomass density (and thus cytochrome P460 abundance), coupled with elevated NH_2OH concentrations that enhance the per-cell NH_2OH-to-N_2O turnover rate, would cause the N_2O production rate to increase superlinearly with biomass, resulting in higher N_2O yields. This hypothesized mechanism is consistent with the observations in this study.

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Abiotic decay of NH_2OH may also explain the observed increase in N_2O yield with AOB biomass density during nitrification.[11] Abiotic NH_2OH decay to N_2O may occur via multiple pathways, and the contribution of each pathway is not possible to determine in chemically complex activated sludge; however, all known pathways follow non-zero-order kinetics with respect to NH_2OH concentration.[52−54] Thus, we hypothesize that the increased abiotic N_2O yield in more concentrated activated sludge may be attributed to the increased NH_2OH concentration, which in turn promotes its accelerated decay to N_2O. Verification of these hypothetical mechanisms and determination of the relative importance of the aforementioned biotic and abiotic reactions would require targeted experiments using pure minimal-medium cultures of *Nitrosomonas europaea* or other *Nitrosomonas* isolates, thereby sacrificing system complexity. Such experiments therefore fall outside the scope of the present study. Nonetheless, they represent an important direction for future research crucial for improving our understanding of N_2O emissions from nitrification-based BNR systems.

Seasonal adjustment of SRT is a strategy widely practiced at BNR WWTPs in temperate climates to regulate activated sludge biomass density and sustain nitrogen removal efficiency under varying temperature conditions.[55,56] The SRT and MLSS data indicated that the study site also implements this control scheme, which likely contributed to sustained compliance with stringent nitrogen removal limits year-round. Based on the data from our monitoring and controlled laboratory experiments, higher winter MLSS, and consequently higher AOB population (as approximated by $MLSS_{AOB}$), was associated with higher nitrification-linked N_2O emissions and yields in activated sludge samples collected during colder months. Full-scale plant operations are influenced by a wide range of fluctuating factors, such as nitrogen contents and organic carbon concentrations of the influent wastewater, as well as dissolved O_2 concentration of the aerated segment, which vary on hourly time scales.[57,58] Nevertheless, our results suggest that seasonal increases in biomass density can act as potential amplifying factors for nitrification-associated N_2O formation. For example, when combined with episodic surges in influent nitrogen load, the dense winter- or early spring nitrifier communities may result in N_2O effluxes an order of magnitude higher than the summertime average. Although activated sludge systems differ in microbial composition and physicochemical conditions, the operating schemes and parameters of this particular WWTP are representative of A2O-type BNR WWTPs across the globe.[41,59] Moreover, the NH_4^+ oxidation rates (1.9−8.5 nmoles min^{-1} mg $MLSS^{-1}$) and the N_2O yields (0.4−2.8%) measured in our laboratory experiments (Figure 1A,E) are consistent with the range of respective values measured *in situ* at geographically distant full-scale WWTPs.[40,60,61] Therefore, the findings from this study may extend beyond this facility and region, although confirmation across additional plants is warranted.

The extent to which the findings of this study apply to biofilm- and granule-based systems, where the biomass density is, in general, substantially higher than that in suspended activated sludge systems, remains an open question. Several lines of evidence from the literature suggest that the proposed mechanism linking AOB biomass density and NH_2OH accumulation to N_2O formation during ammonia oxidation may be relevant in such systems. Local dissolved N_2O concentrations ranging from approximately 2 μM to 80 μM have been reported at the membrane surface in membrane-aerated bioreactors (MABRs), which are substantially higher than those typically measured in aerated tanks of A2O-type BNR WWTPs.[62−64] Similarly, relatively high N_2O emission factors (0.8−2% of nitrogen loading) have been reported for biofilm- and granule-based partial nitritation systems, which have traditionally been attributed to low DO conditions that stimulate nitrifier denitrification.[6,65,66] These long-observed phenomena may be consistent with the proposed mechanism; however, as consolidated biomass contains extensive oxygen-deprived microenvironments, the contribution of nitrifier denitrification would be difficult to disentangle.[65,67] Moreover, the measurement of NH_2OH in biofilms remains technically challenging, precluding direct validation of elevated local NH_2OH concentrations in biofilms. Accordingly, extension of the present findings linking AOB biomass density, NH_2OH accumulation, and N_2O yields to biofilm- and granule-based processes should be regarded as a hypothesis requiring further verification. If confirmed, these mechanistic insights could have important implications for the design and operation of climate-friendly BNR processes across diverse reactor configurations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.5c13522.

Raw experimental data for determining nitrification and N_2O−N production rates; controls with allylthiourea and/or C_2H_2; temperature effect on anaerobic denitrification; full raw data for experiments determining physiological temperature effect on activated sludge nitrifiers; operating parameters of Daejeon WWTP; summary of experimental conditions; physicochemical properties of wastewater samples; and sequencing statistics (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Sukhwan Yoon − *Department of Civil and Environmental Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 34141, Republic of Korea;* orcid.org/0000-0002-9933-7054; Phone: +82 10-3461-2024; Email: syoon80@kaist.ac.kr

Authors

Minho Lee − *Department of Civil and Environmental Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 34141, Republic of Korea;* orcid.org/0009-0001-4208-5490

Fabrizio Sabba − *Department of Civil and Environmental Engineering, Syracuse University, Syracuse, New York 13244, United States;* orcid.org/0000-0002-6369-9432

Michele Laureni − *Department of Water Management, Delft University of Technology, Delft 2628 CN, The Netherlands*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.5c13522

Author Contributions

S.Y. conceived and supervised the study. M.Le. and S.Y. designed experiments. M.Le. performed experiments. M.Le., F.S., M.La., and S.Y. analyzed experimental and sequencing

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data. M.Le. and S.Y. wrote the manuscript with contributions from F.S. and M.La. All authors critically reviewed the manuscript.

Notes

The authors declare no competing financial interest.

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