IDENTIFICATION OF nosZ-EXPRESSING MICROORGANISMS CONSUMING TRACE N₂O IN MICROAEROBIC CHEMOSTAT CONSORTIA DOMINATED BY AN UNCULTURED Burkholderiales

Daehyun D. Kim¹, Heejoo Han¹, Taeho Yun¹, Min Joon Song¹, Akihiko Terada², Michele Laureni³,⁴ and Suhkwan Yoon¹✉

© The Author(s) 2022

Microorganisms possessing N₂O reductases (NosZ) are the only known environmental sink of N₂O. While oxygen inhibition of NosZ activity is widely known, environments where N₂O reduction occurs are often not devoid of O₂. However, little is known regarding N₂O reduction in microoxic systems. Here, 1.6-L chemostat cultures inoculated with activated sludge samples were sustained for ca. 100 days with low concentration (<2 ppmv) and feed rate (<1.44 µmoles h⁻¹) of N₂O, and the resulting microbial consortia were analyzed via quantitative PCR (qPCR) and metagenomic/metatranscriptomic analyses. Unintended but quantified intrusion of O₂ sustained dissolved oxygen concentration above 4 µM; however, complete N₂O reduction of influent N₂O persisted throughout incubation. Metagenomic investigations indicated that the microbiomes were dominated by an uncultured taxon affiliated to Burkholderiales, and, along with the qPCR results, suggested coexistence of clade I and II NosZ reducers. Contrastingly, metatranscriptomic nosZ pools were dominated by the Dechloromonas-like nosZ subclade, suggesting the importance of the microorganisms possessing this nosZ subclade in reduction of trace N₂O. Further, co-expression of nosZ and ccoN/O/cydAB genes found in the metagenome-assembled genomes representing these putative N₂O-reducers implies a survival strategy to maximize utilization of scarcely available electron acceptors in microoxic environmental niches.

The ISME Journal; https://doi.org/10.1038/s41396-022-01260-5

INTRODUCTION

Climate change, brought about by a dramatic increase in greenhouse gas (GHG) emissions over the last two centuries, is one of today's most serious environmental concerns [1, 2]. Nitrous oxide (N₂O) is one of the three major GHGs along with carbon dioxide (CO₂) and methane (CH₄) [1, 3]. The concentration of N₂O in the atmosphere has increased steadily over the past century due primarily to the increased anthropogenic nitrogen inputs to the environment and currently stands at 332 ppb, a concentration approximately 20% higher than in the pre-industrial era [3]. As N₂O has 298 times higher per-mole global warming potential than CO₂ over a 100-year scale, the contribution of N₂O to the net global warming potential is substantial (estimated to be 6.2%) despite its low atmospheric concentration [2]. Thus, scientific and engineering endeavors to understand and mitigate N₂O emissions are an indispensable part of global efforts to curb climate change and ensure a sustainable future.

Unlike CO₂, >90% of N₂O emitted to the atmosphere has a biological origin [3]. Net emission from an environmental N₂O source is determined by the difference between the rates of its production and removal. While a multitude of microbial-mediated processes including nitrification and denitrification result in production of N₂O, N₂O can be removed only via biological reduction catalyzed by a family of enzymes referred to as nitrous oxide reductases (NosZ) [4, 5]. This reaction had long been regarded merely as a part of the chain of reactions constituting the denitrification pathway, i.e., the stepwise reduction of NO₃⁻ to N₂ via NO₂⁻, NO, and N₂O; however, the significance of the reaction as a growth-supporting standalone terminal electron acceptor reaction has only recently been recognized, as genomic and physiological investigations have repeatedly suggested that denitrification occurs largely in a modular manner in the environment and also that a substantial portion of nosZ possessing microorganisms lack either nir gene [6-9]. Further, discovery of a novel nosZ clade, later termed clade II NosZ, led to recognition of the true diversity of nosZ and nosZ-harboring microorganisms [6, 10]. These recent findings altogether signified the potential importance of nosZ-harboring N₂O-reducing microorganisms as a N₂O sink.

The nosZ-harboring N₂O reducers are often categorized into two subgroups based on the type of nosZ they possess [7, 11]. The clade I and clade II NosZ have distinguishable features in their amino acid sequences suggesting differences in their structures and secretion mechanisms [6, 12]. The possibility that these differences may have ecophysiological implications for N₂O reduction in diverse environmental settings has recently been a topic of broad interest. Specifically, as N₂O concentration measured at its source, e.g., agricultural soil and activated sludge,
is rarely below low micromolar concentrations, particular attention has been paid to differences in their ability to compete for trace N$_2$O [13, 14]. Several previous research results suggested that clade II nosZ generally have higher affinity for N$_2$O than clade I nosZ [15–17]. Measurements of whole-cell N$_2$O reduction kinetics yielded significantly lower apparent half-saturation constants for the microorganisms harboring clade II nosZ, e.g., *Dechloromonas* spp. and *Azospira* spp., than those harboring clade I nosZ [16, 18]. The microorganisms possessing clade II nosZ were selectively enriched in bioreactors of different configurations where biological low concentration of N$_2$O was observed [19, 20]. In recent experiments that examined the feasibility of removing N$_2$O from activated sludge bioreactor exhaust gas via biotrickling filtration, *Flavobacterium* spp., putatively possessing a clade II nosZ, showed the highest relative abundance among the biofilm-forming microorganisms [19]. In another recent study where the N$_2$O-reducing biofilm was selectively enriched on the membrane surface of a gas-permeable membrane reactor, *Azospira* spp. and *Dechloromonas* spp., putatively possessing clade II nosZ, dominated the microbial population [20]. Contradicting observations have also been reported. A long-term chemostat enrichment of an activated sludge-seeded culture with N$_2$O as the sole and limiting (i.e., undetectable in the reactor) electron acceptor observed clade I nosZ dominating the nosZ pool [21]. The inconsistency among these pioneering works may have stemmed from spatially non-uniform distribution of gaseous N$_2$O within the reactors, and also possibly from molecular quantitative analyses with reliability and/or resolution issues [16, 17, 22]. Another possible explanation is that the clade I-vs.-clade II distinction may not be sufficient for rationalizing different capabilities of N$_2$O-reducing microorganisms to consume trace N$_2$O, as several strains of clade I nosZ-harboring microorganisms have been found to exhibit whole-cell half-saturation constants (K$_{n_{app}}$) as low as those of *Azospira* spp. and *Dechloromonas* spp. and/or higher specific affinity (a$_{N_{2}O} = V_{m}/K_{m,app}$) [23].

An often overlooked environmental factor that may also have caused such conflicting observations is the role of trace O$_2$. Many denitrifying and non-denitrifying N$_2$O reducers are facultative anaerobes and prefer O$_2$ as the electron acceptor over N$_2$O when both electron acceptors are available [24]. In most laboratory settings where microbial N$_2$O reduction is observed, sub- or low-micromolar O$_2$ is often regarded irrelevant, as the amounts or the rates of N$_2$O supplied to the experimental cultures far exceed those of O$_2$ [17, 20, 21]. Under such conditions, the proportion of microorganisms growing on O$_2$ respiration would be negligible relative to those dependent on N$_2$O respiration. In environmental systems where N$_2$O emission is of great concern, however, O$_2$ penetrating into the anoxic niches may be substantially higher in concentration than N$_2$O produced in situ or transported from neighboring sources. The dissolved oxygen concentrations in anoxic activated sludge tanks are, in general, orders of magnitudes higher than the dissolved N$_2$O concentrations [25, 26]. In agricultural soils, N$_2$O reduction would occur most actively where N$_2$O is most readily available, i.e., at the oxic-anoxic interface subjected to O$_2$ intrusion and frequent oxic-to-anoxic and anoxic-to-oxic transitions [27]. Microorganisms expressing cb$eta$-type cytochrome oxidases and/or cytochrome bd respiratory oxygen reductases are capable of thriving under such microoxic conditions [28]. Several recent studies suggested transcriptomic evidence of microaerobic respiration and denitrification simultaneously occurring under microoxic conditions; however, how reduction of trace N$_2$O and the microbial community responsible for the reaction would be affected by co-occurring microaerobic O$_2$ respiration has not yet been explored [29, 30].

In this study, a chemostat reactor was designed for enrichment of microbial consortia capable of reducing N$_2$O supplied at a feed rate several orders of magnitude lower than previously applied for enrichment of N$_2$O-reducing microorganisms. To ensure spatially uniform N$_2$O-limiting condition throughout incubation, the reactor culture was fed N$_2$O dissolved in the inflowing medium, instead of gaseous N$_2$O previously used for enriching N$_2$O-reducing consortia. Unintended O$_2$ penetration into the reactor at a rate exceeding the N$_2$O feed rates provided a serendipitous opportunity to emulate environments where reduction of trace N$_2$O occurs with microaerobic respiration. A gene-centric investigation of these microaerobic reactor cultures persistently reducing <50 nM N$_2$O was performed via quantitative PCR (qPCR) analyses and nosZ-targeting metagenomic analyses. Further, the metatranscriptomes of the reactor microbiomes were analyzed to identify the actively expressed nosZ genes among those found with high relative abundance in the gene-centric analyses. Analyses of the functional gene profiles in the reconstructed metagenome assembled genomes (MAGs) and transcription profiling of the MAGs enabled identification of the nosZ possessing minorities that are likely to have substantially contributed to the observed reduction of trace N$_2$O. The findings in this study not only substantiate the importance of *Dechloromonas*-like clade II nosZ harboring microorganisms as a consequential environmental N$_2$O sink, but also provide novel insights into N$_2$O reduction occurring in microoxic environments where O$_2$ is consistently introduced and consumed.

**MATERIALS AND METHODS**

**Operation of chemostat reactors**

The activated sludge samples used as the inocula for the reactors were collected from the anoxic compartment of an active sludge tank at the Daejeon municipal wastewater treatment plant (WWTP; 36°23’N 127°24’28’E) immediately before each set of experiments (December 2019 and July 2020). A pre-sterilized 1-L polyethylene bottle was completely filled with the activated sludge sample, capped immediately to minimize oxygen ingress, and transported to the laboratory in a cooler filled with ice. Upon arrival at the laboratory, 100 mL of the sample was subsampled and stored at −20 °C, and the rest was stored at 4 °C.

Three bioreactor experiments were performed. For convenience, the N$_2$O-fed chemostat enrichment performed with the December 2019 sample is hereafter referred to as N2OR1 and the repeat of the experiment (with slight modifications to the incubation condition as described below in detail) and the N$_2$O-free control experiment performed with the July 2020 sample as N2OR2 and N2FCR, respectively. The modified MR-1 medium, used for all three reactor experiments, contained per liter of double-distilled water, 0.50 g NaCl, 0.29 g KH$_2$PO$_4$, 0.50 g K$_2$HPO$_4$, 0.27 g NH$_4$Cl, 1 mL of 1000X trace metal solution (Table S1), and 1 mL of 1000X vitamin stock solution (Table S2; added to the autoclaved medium through a 0.22-µm syringe filter) [31]. Sodium acetate was added to a concentration of 5.0 mM as the sole source of electrons and carbon. Acetate has been known to support growth of taxonomically diverse N$_2$O-reducing microorganisms and is a non-fermentable substrate [32]. The amount added was stoichiometrically sufficient to ensure excess supply of electron donor throughout incubation, and acetate concentration was measured at the end of each incubation to experimentally verify this electron donor excess.

The reactor system was constructed with a 2-L pyrex glass bottle with a side-port serving as the main chamber for enrichment of microbial culture (Fig. S1). The bottle was capped with a Duran 4-port GL45 connection system (DWK Life Sciences, Wertheim, Germany) and the side-port with a septum-lined cap. For chemostat operation, fresh medium was supplied into the main chamber from a 5-L pyrex glass bottle prepared with a 4 L initial volume of autoclaved acetate-amended MR-1 medium at a flowrate of 42 mL h$^{-1}$ (i.e., a dilution rate of 0.026 h$^{-1}$). The medium bottle was replaced with a new one when the volume of the remaining medium dropped below 500 mL. Waste culture medium was pumped out from the main chamber into a waste collection bottle at the same flowrate as the fresh medium influx. The medium bottle was equilibrated with a gas stream of N$_2$ containing 200 ppmv (N2OR1) or 2000 ppmv (N2OR2) (Special Gas, Daejeon, South Korea; these concentrations are nominal values). For chemostat operation, fresh medium in the side-port with a 0.22-µm syringe filter was added was stoichiometrically sufficient to ensure excess supply of electron donor throughout incubation, and acetate concentration was measured at the end of each incubation to experimentally verify this electron donor excess.

The reactor culture was mixed with a magnetic stirrer rotated at 200 rpm. A stream of >99.9999% N$_2$ gas, gassed through the waste collection bottle at a constant flowrate of 200 mL min$^{-1}$ to prevent reverse ingress of contaminating O$_2$ into the main chamber. Liquid flow was controlled with a two-channel peristaltic pump (LabSciTech, Corona, CA) and gas flow with gas flowmeters (Dwyer Instrument, Michigan City, IN).
Operation of the two N2O-fed reactors, N2OR1 and N2OR2, differed only in the start-up strategy. The N2OR1 reactor was started as a batch reactor fed with continuous stream of N2O-containing N2 gas. The main chamber was flushed with >99.9999% N2 gas until no further decrease in O2 concentration was observed, and the gas supply was switched to N2 gas containing 200 ppmv N2O (Special Gas, Daejeon, South Korea). The source of O2 contamination was uncertain, and dissolved O2 concentration could not be reduced below 10 μM during the preconditioning procedure. After the headspace N2O concentration reached 200 ppmv, the 1600-mL aqeous phase of the reactor was inoculated with 80 mL of the activated sludge sample. After 57 hours of initial incubation as a batch reactor, N2OR1 was transitioned to continuous operation and examined for 98 days and 61 volume changes.

Improvements to the experimental designs for the N2OR2 experiment were based on the findings from the N2OR1 experiment. To measure the O2, a tracer, the reactor system was abiotically operated prior to inoculation with the liquid and gas compositions and flowrates identical to those used for the chemostat operation that followed. The main chamber and the waste collection bottle were flushed with N2 gas and the medium bottle with N2O gas containing 2000 ppmv N2O, both with the same flowrate (200 mL min⁻¹). After the dissolved oxygen concentration in the main chamber had stabilized, the gas flow into and out of the main chamber was shut down and the liquid flow was switched on. The amounts of O2 and N2O in the main chamber were monitored over next three days to measure the rates at which N2O and O2 (via contamination) were introduced into the reactor. After these rates were calculated, rates assuming ideal reactor operation, 2.05 and 0 μmol h⁻¹, respectively (See supplementary materials). After inoculation with 80 mL activated sludge, the reactor system was operated and monitored for 101 days through 63 volume changes. In parallel with N2OR2, a control reactor system, N2FCR, was set up with an identical configuration, medium compositions and inoculant, but without N2O in the N2 gas stream passed through the medium bottle. The operation of N2FCR was halted on day 59 due to atmospheric oxygen contamination caused by a screw cap that had been accidentally left loosened after sampling on day 45.

**Analytical methods**

The dissolved O2 concentrations in the medium bottle and the main chamber of the reactor systems were measured using a FireSting-O2 optical oxygen meter and fiber-optic oxygen sensor spots (Pyroscience, Aachen, Germany) attached on the inner walls of glass bottles (see supplementary information for detailed description of the method). N2O concentration was measured by manually injecting headspace gas samples into a gas chromatograph with an ECD after filtering the sample with a 0.22-μm pore-size syringe filter (Advantec, Inc., Tokyo, Japan).

**Extraction and sequencing of nucleic acids**

Cell suspensions were collected from N2OR1, N2OR2, and N2FCR at varying time points for monitoring of the reactor cultures via quantitative PCR (qPCR) and 16S rRNA gene amplicon sequencing. At each sampling time point, a 15 mL cell suspension was collected and subsequently distributed to three 5 mL microcentrifuge tubes in 4.5-mL aliquots. Cell pellets were collected from these cell suspensions via centrifugation at 15,000 × g, and DNA was extracted using DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), according to the protocol provided by the manufacturer. The V6-V8 regions of 16S rRNA gene were amplified using the 926F-1392R primer set (Table S3), and sequencing of the amplicons was performed at Macrogen Inc. (Seoul, South Korea) with the MiSeq platform (Illumina, CA) for cell fixation and RNA preservation [35]. Total RNA was extracted from the cells collected on the membrane filter, shredded immediately after vacuum filtration with a razor blade treated with RNaseZAP (Sigma-Aldrich). The filter pieces were placed into a 2 mL RNase-free screw cap tube containing 50 μg of 0.1 mm diameter glass beads (Omni International, Kennesaw, GA). The cells were disrupted with a Bead Ruptor Homogenizer (Omni International, Kennesaw, GA) for 5 minutes at the maximum speed. The tube was flash-frozen in liquid nitrogen three times during the homogenization process to prevent warming of its contents. The total RNA was isolated using TRIzol reagent (Ambion, Austin, TX), and purified using RNeasy MinElute Cleanup Kit (Qiagen) [17, 36]. Ribosomal RNA was removed using MICROExpress Bacterial mRNA Enrichment Kit (Thermo Fisher Scientific, Waltham, MA), and the sequencing library was prepared using TruSeq Stranded mRNA Kit (Illumina) and barcoded using the TruSeq RNA CD Index Kit (Illumina), according to the instruction provided by the manufacturer.

**Quantitative PCR analyses**

Time-series qPCR analyses were performed for real-time quantitative monitoring of the relative abundances of nosZ genes in the N2OR1, N2OR2, and N2FCR reactor cultures. TaqMan qPCR assays were performed using the pre-designed primers-and-probe sets targeting the 16S rRNA genes, the two clade I nosZ groups (NosZG1 and NosZG2) and the two clade II nosZ groups (NosZG3 and NosZG5; summarized in Table S3) [17, 37]. Calibrator curves for the qPCR were constructed with serial dilutions (10⁻¹ to 10⁻⁸ copies per μL) of purified TOPO PCR2.1 vectors (Thermo Fisher Scientific, Waltham, MA) containing the respective PCR amplicons as inserts [17]. The detection limits were below 10⁻⁸ copies per μL for all qPCR reactions, as the standard deviations of Cq values were less than 0.35 (the average Cq value was 33.0) at this plasmid concentration.

**Metagenome data processing**

Low-quality reads were removed from the raw metagenomic reads using Trimmomatic v0.36 software with the parameters set as follows: LEADING: 3, TRAILING: 3, SLIDINGWINDOW: 4:15, and MINLEN: 70 [38]. De novo assembly of the quality-trimmed reads was performed using metaSPAdes v3.14.0 with default parameters [39]. Potential gene-coding sequences in the assembled contigs were predicted using Prodigal v2.6.3, and the translated amino acid sequences were annotated using DIAMOND blastp against the NCBI nr database (accessed on 04/15/2020) [40, 41]. For each gene-coding sequence, only the best hit with the highest bitscore was considered in the downstream analyses. The coding sequences were assigned KEGG Orthology (KO) numbers using the GhostKOALA server (https://www.kegg.jp/ghostkoala). The blastp-based functional annotations were cross-checked with KO number assignments [42]. The predicted nosZ sequences in N2OR1 and N2OR2 metagenomes were translated in silico and aligned along with 97 full-length reference NosZ sequences from the nr database using MUSCLE v3.8.31 with parameters set to default values (Table S4) [43]. The alignment was uploaded to CIPRES Science Gateway v3.3 (https://www.phylo.org/), and a maximum likelihood phylogenetic tree was constructed using RAxML v8.2.12 (1000 bootstrapss) with the protein substitute model and matrix options set to ’PROTGAMMA’ and ’AUTO’, respectively [44]. For quantitative comparison with the qPCR data, each phylogenetic group of the metagenome-derived nosZ sequences was categorized into one of the four target groups via in silico PCR of its representative sequence using Geneious software v9 (Biomatters, Auckland, New Zealand). The relative abundances of the nosZ sequences assigned to each target group were tallied, and the ratios among these tallied relative abundances were compared with the ratios among the NosZG1-5 qPCR counts. For the N2OR1 culture not subjected to a separate 16S rRNA gene amplicon sequencing analysis, microbial community composition analysis was performed with the 16S rRNA sequences extracted from the endpoint
shotgun metagenome data. Putative 16S rRNA reads were extracted from the quality-trimmed reads using Meta-RNA3 with molecule type and e-value set to "ssu" and "1E-10", respectively [45]. Full-length 16S rRNA genes were reconstructed from these reads using EMIRGE v0.6.10 with SILVA SSU database release 138 as reference [46, 47]. The reconstructed 16S rRNA genes were assigned taxa using RDP classifier v2.10.2 based on SILVA SSU database release 138 with minimum threshold set to 0.8 [47, 48].

Quantitative metagenomic and metatranscriptomic data processing

The quality-trimmed raw metagenomic reads were mapped on to the assembled contigs using the Burrows-Wheeler Aligner (BWA)-MEM algorithm with default parameters [49]. The resulting sequence alignment mapping (sam) files were sorted using SAMtools v1.10, and the length-normalized read coverage of each contig was calculated using BEDTools (v2.26.0) genomcov tool [50, 51]. The read coverage values of the contigs were further normalized by the total number of mapped reads in each sample, yielding the reads-per-kilobase-per-million-mapped-reads (RPKM) values. The RPKM value of an entire contig was taken as the relative abundances of the gene-coding sequences of interest found within the contig, as using longer sequences as mapping templates generally yield less-biased coverage data [52].

The transcription levels of the functional genes of interest were estimated by mapping the metatranscriptomic reads onto the gene-coding sequences in the metagenomes. The quality-trimmed metatranscriptomic reads were mapped onto the rRNA database consisting of the short subunit (SSU) and long subunit (LSU) rRNA sequences from SILVA Ref NR 99 database release 138 and 3S rRNA sequences from the Rfam v14.4 database using the BWA-MEM algorithm [49]. The non-rRNA metatranscriptomic reads, i.e., the reads not mapped onto any of these rRNA sequences, were extracted and organized into a separate fastq format file using SAMtools v1.10 [49, 50]. These reads were mapped on to the gene-coding sequences of interest in the metagenomic contigs, and counting of the reads was performed using RSEM v1.3.3 [53]. The reads counts were normalized with the gene lengths and the total number of non-rRNA metatranscriptomic reads for conversion to RPKM values.

Reconstruction and analyses of Metagenome Assembled Genomes (MAGs)

Metagenome assembled genomes (MAGs) were constructed from the shotgun metagenome data. In addition to the contigs generated with metaSPAdes, another set of contigs was constructed via de novo assembly using MEGAHIT v1.2.9 with default parameters [54]. The two sets of contigs were then run in parallel, to maximize the number of extractable MAGs. The contigs were binned into MAGs using MetaBAT v2.15 [55]. Prior to binning, contigs with <2 kb length or <2 mean read coverage over the entire length were excluded using the ‘jgi_summarize_bam_contig_depths’ script in MetaBAT and the coverage data were constructed using BWA-MEM [55]. The MAGs were refined using RefineM as follows [56]. 1) The contigs, with genomic properties, i.e., GC content, tetranucleotide frequency distribution, and/or mean read coverage substantially deviating from the mean values were filtered out. 2) The gene-coding sequences in the contigs in a MAG were assigned taxonomy using DIAMOND v0.9.31.132 with GTDB protein database release 80 as reference, and obvious outliers were removed using ‘taxon_profile’ and ‘taxon_filter’ scripts in RefineM v0.1.1 with parameters set to default values [56]. 3) The 16S rRNA gene sequences in a MAG were assigned taxonomy using DIAMOND v0.9.13.132 with reference to GTDB SSU database release 80, and the contigs carrying 16S rRNA genes identified as incongruent to the consensus classification of the MAG were screened out using the ‘ssu_erroneous’ script with default parameters. The quality of the MAGs were assessed via the lineage-specific workflow of CheckM v1.1.2, and those with <75% completeness or >10% contamination were excluded [57]. The two sets of remaining MAGs, one from metaSPAdes assembly and the other from MEGAHIT assembly, were pooled, and an average nucleotide identity (ANI) matrix showing pairwise comparisons among the MAGs was constructed using Mash and ANImf algorithms implemented in dRep v2.5.0 [58]. The pairs of MAGs with >99% ANI were dereplicated using the ‘dereplicate’ command in dRep v2.5.0 (58). The MAGs from N2OR1 and N2OR2 metagenomes, constructed and processed independently up to this stage, were pooled and dereplicated using the same procedure. The read coverages of the finalized set of MAGs were computed by mapping the quality-trimmed reads onto the contigs in the MAG bins using the BWA-MEM algorithm [49].

The taxonomic affiliation of each unique MAG was determined using the Microbial Genomes Atlas (MiGA) server (http://microbial-genomes.org/; accessed on 01/27/2021) [59]. Phylogenetic marker genes were identified in the MAGs using PhyloSift v1.0.1 with markers database 4 (release 2018-02-12) as reference [60]. The phylogenetic relationship among the MAGs was inferred by aligning the translated amino acid sequences of the concatenated marker gene sequences using MUSCLE v3.8.31 with default parameters [43]. After removing poorly aligned regions with Gblocks v0.91b, the alignment file was uploaded on CIPRES Science Gateway version 3.3 (https://www.phylo.org/), and a maximum-likelihood phylogenetic tree with 1000 bootstraps was constructed using RAxML v8.2.12 [44, 61].

The transcription profiles of the protein-coding genes of interest in the MAGs were reported from the gene count data generated with RSEM. The genes of interest included nosZ and other nitrogen-cycling functional genes and cydAB and ccoN1/ccoO1/ccmNO genes encoding cytochrome bd ubiquinol oxidases and cbb3-type cytochrome c oxidases, respectively, putatively involved in high-affinity O2 reduction. The median transcript abundance of single-copy marker (SCM) genes that have been reported to be constitutively expressed in bacterial cells served as a measure of overall transcription activity in the microorganism represented by a MAG [62–64]. The list of the analyzed genes is summarized in Table S5. The KO-based annotations were used for reconstruction and transcription profiling of the KEGG pathway modules for the MAGs [65].

RESULTS

Operation of the chemostat reactors

Despite the low N2O feed rates, biotic N2O removal was consistently observed throughout the entire study periods in the N2OR1 and N2OR2 reactors (Fig. S2). The actual N2O concentration in the headspace of the N2OR1 reactor after equilibration with N2 gas containing 200 ppmv N2O plateaued at 230.0 ± 0.3 ppmv (5.61 ± 0.01 μM in the aqueous phase). The dissolved O2 concentration was 9.5 ± 0.2 μM. After inoculation, the headspace N2O concentration eventually decreased to 109.1 ± 2.6 ppmv after 57 h of batch incubation. After the reactor operation was transitioned to the continuous mode of operation with N2O supplied as dissolved in the influent medium, the N2O concentration in the headspace of the reactor decreased below the detection limit (<2 ppmv, equivalent to <49 nM in the aqueous phase at 25 °C) within 90 min. Throughout 98 days of continuous operation, N2O concentration in the headspace was sustained below the detection limit, indicating a constant N2O consumption rate of 0.13 μmol L⁻¹ h⁻¹. During the chemostat operation, dissolved O2 concentration hovered between 8.0 μM and 10 μM (9.0 ± 0.4 μM from 33 datapoints collected between day 3 and 98).

The N2OR2 experiment was started with abiotic continuous operation of the reactor, during which the O2 penetration rate and N2O supply rates were estimated (see supplementary information and Fig. S3). After inoculation with the activated sludge sample at 88 h, the dissolved O2 concentration decreased from 71.6 μM to 6.3 μM within 6 h. A rapid decrease in N2O concentration followed the drop in O2 concentration, and the N2O concentration dropped below the detection limit at 100 h. As observed with N2OR1, N2O was undetectable throughout 101 days of reactor operation and O2 concentration remained nearly constant within 4.0-5.0 μM range (4.8 ± 0.2 μM from 24 datapoints collected between day 5 and 101). Assuming that O2 penetration and N2O supply rates were constant throughout the reactor operation, the steady-state rates of O2 and N2O consumption were estimated as 14.3 ± 0.1 μmol L⁻¹ h⁻¹ and 0.90 ± 0.04 μmol L⁻¹ h⁻¹, respectively. Acetate concentrations in the reactor effluent measured at the end of incubation were 4.15 ± 0.01 mM and 4.17 ± 0.07 mM for N2OR1 and N2OR2, respectively, indicating that the reactor cultures were not electron donor-limited.

Microbial community compositions of the reactor cultures

The microbial communities of N2OR1, N2OR2, and N2FCR reactor cultures were invariably dominated by microorganisms belonging to a single taxon affiliated to the Burkholderiales order (Fig. 1). The endpoint analysis of the N2OR1 microbial community showed that
29,587 out of 38,727 reads mapped to 74 unique 16S rRNA genes reconstructed from the metagenome sequencing of the N2OR1 reactors throughout the study period. The genus-level taxa apart from the strain T34 (Fig. 1A). The amplicon sequence variants (ASVs) affiliated to the strain T34 in the microbial community of N2FCR showed the migration of the N2OR2 and N2FCR microbial communities over the course of incubation.

The taxonomic affiliations and relative abundances of 16S rRNA genes reconstructed from the shotgun sequence reads obtained from metagenome sequencing of the N2OR1 reactor culture collected on day 98. The relative abundance of the amplicon sequence variants (ASVs) from the N2OR2 and N2FCR reactor cultures with the uncultured strain T34 as the closest taxon. The nonmetric multidimensional scaling (NMDS) plot showing the migration of the communities the over the course of incubation.

The non-metric multidimensional scaling (NMDS) plot showing migration of the N2OR2 and N2FCR reactor communities clearly illustrates the similarity between progression of enrichment in the two reactors (Fig. 1D). The NMDS plots based on unweighted UniFrac and Blay-Curtis distance metrics, also showed the two reactor communities were heading in the same direction (Fig. S4). This similarity suggests that the selective pressure that shaped the N2OR2 reactor community, and probably the N2OR1 community as well, was not the availability of trace N_2O as the electron acceptor. Rather, it was more likely that O_2 penetrating into the reactor was responsible for the overwhelming enrichment of microorganisms affiliated to the strain T34 and shaping of these unique microbial communities. The substantially lowered relative abundance (2.2% relative abundance) of the microorganisms affiliated to the strain T34 in the microbial community of N2FCR as well as the nonculturable microorganisms confirmed their capability to efficiently utilize low micromolar dissolved O_2.

Quantitative monitoring of nosZ genes in N_2O-amended and N_2O-free chemostats

Monitoring of the four nosZ groups (targeted by the NosZG1, NosZG2, NosZG3, and NosZG5 qPCR) in both N2OR1 and N2OR2 reactor cultures identified the NosZG2 (Acidovorax-like clade I nosZ) and NosZG5 (Dechloromonas-like clade II nosZ) groups as the most abundant groups of nosZ genes (Fig. 2). In N2OR1, the 16S rRNA gene copy number increased rapidly from 4.0 ± 0.3 × 10^3 to 7.1 ± 4.3 × 10^3 copies mL^-1 within 15 days of transition to continuous operation on day 2. In N2OR2 operated without the initial batch incubation, the 16S rRNA gene copy number dropped precipitously after inoculation, down to 5.0 ± 0.3 × 10^3 copies mL^-1. These observations imply that the vast majority of cells not
The time-series nosZ qPCR data from the N2FCR cannot be directly compared with the corresponding data gathered from N2OR1 and N2OR2; however, interesting observations could be gleaned from this data (Fig. 2). While neither NosZG1 nor NosZG3 was detected beyond day 10, the NosZG2 and NosZG5 groups both persisted until day 28, with copy numbers of $8.9 \pm 1.5 \times 10^2$ copies mL$^{-1}$ and $1.4 \pm 0.4 \times 10^3$ copies mL$^{-1}$, respectively. The persistence of NosZG2 and NosZG5 populations suggests that at least substantial portions of these nosZ possessing microorganisms grew on microaerobic respiration alone.

**Genomic and transcriptomic profile of nosZ in the chemostat cultures consuming trace N$_2$O.**

Metagenomic analyses of the N2OR1 and N2OR2 cultures collected at the end of incubation identified 41 (10 clade I and 31 clade II) and 27 (9 clade I and 18 clade II) unique nosZ-coding sequences in the assembled contigs, respectively (Fig. S6). All unique clade I nosZ sequences belonged to the NosZG2 target group and 28 out of 49 clade II nosZ sequences belonged to the NosZG5 target group. Consistent with the qPCR assays, none were affiliated with nosZ of NosZG1, and the NosZG3 group was also underrepresented (7 sequences; Fig. S7).

The quantitative compositions of the nosZ gene pools were substantially different between the N2OR1 and N2OR2 cultures (Fig. 3). In the N2OR1 culture, the relative abundance of the clade I nosZ genes was comparable (2.3 RPKM) to those belonging to the clade II nosZ (2.5 RPKM), mostly due to the high abundance of the nosZ genes affiliated to *Oryzomicrobium terrae* (0.9 RPKM) and *Acidovorax* spp. (0.7 RPKM). Among clade II nosZ, those affiliated to *Dechloromonas* spp. (0.9 RPKM) and *Azospira oryzae* (0.5 RPKM), both grouped with the NosZG5 target group, had the highest relative abundance. Contrastingly, the nosZ pool in the N2OR2 was dominated by the clade II nosZ genes affiliated to *Bradyrhizobium* spp. (0.5 RPKM) and *Dechloromonas* spp. (2.0 RPKM), together constituting 86.8% of the entire nosZ pool in the metagenome. These results were in line with the qPCR-based group-specific quantification of nosZ, in that the NosZG2 and NosZG5 target groups were the most abundant groups and also that NosZG2 was the most abundant group in N2OR1 on day 98 while NosZG5 was the most abundant group in N2OR2 on day 101. None of the nosZ genes without affiliation to the qPCR target groups had RPKM value above 0.08 in either reactor, confirming that the vast majority of nosZ genes were captured with the group-specific qPCR assays.

Metatranscriptomic investigation of the nosZ expression profiles clarified that the microorganisms harboring NosZG5 nosZ are likely the main players in consumption of trace N$_2$O observed in N2OR1 and N2OR2 reactors (Fig. 3). In both N2OR1 and N2OR2, the vast majority of nosZ transcripts (47.4 out of 62.3 RPKM total and 116.2 out of 125.3 RPKM total, respectively) were assigned to the NosZG5 target group. More specifically, the nosZ transcripts affiliated to *Dechloromonas* spp. (22.8 RPKM), *Azospira oryzae* (12.7 RPKM), and *Bradyrhizobium* spp. (11.4 RPKM) were the most abundant in N2OR1, and those affiliated to *Bradyrhizobium* spp. (74.3 RPKM) and *Dechloromonas* spp. (41.9 RPKM) were the most abundant in N2OR2. The nosZ genes affiliated to *Oryzomicrobium terrae*, despite being the most abundant nosZ in the N2OR1 metagenome, was transcribed at a much lower level (6.3 RPKM) than the nosZ belonging to NosZG5. The clade II nosZ gene affiliated to an unidentified *Bacteroidetes* bacterium (7.6 RPKM) was the only transcribed non-NosZG5 nosZ over 1 RPKM in N2OR2.

**Analyses of the metagenome-assembled genomes (MAGs).**

After quality-screening and dereplication, 14 and 16 MAGs were recovered from the N2OR1 and N2OR2 metagenomes, day 98 for N2OR1 and day 101 for N2OR2. As to the comparison between the abundances of NosZG2 and NosZG5, no unequivocal conclusion can be drawn from the qPCR data alone.

![Fig. 2 Time series of nosZ and 16S rRNA gene copy numbers (per mL sample) in N2OR1, N2OR2, and N2FCR reactor cultures quantified using TaqMan qPCR targeting NosZG1, NosZG2, NosZG3, and NosZG5 groups as well as 16S rRNA gene. The closed and open circles indicate the clade type of each nosZ group (closed: clade I, open: clade II).](image-url)
respectively (Table S6). The genome size (the cumulative length of contigs in a MAG bin) and the GC content of the recovered MAGs ranged between 0.51-1.47 Mbp and 32.0-40.7%, respectively (Table S6). All reconstructed MAGs, with a single exception (MS_R2_24), were assigned taxa at the phylum level. The phyla represented by the MAGs included Proteobacteria, Bacteroidetes, Spirochaetes, Actinobacteria, Firmicutes, Acidobacteria, Ignavibacteriae, Chloroflexi, and Chlorobi (Table S6). In general, the MAGs exhibited low level of similarity to reference genomes in the database; therefore, taxon assignment at genus level was possible only for MS_R1_15 (Oryzomicrobium), MH_R1_24 (Acidovorax), MH_R1_8 (Acinetobacter), and MS_R2_31 (Azovibrio). The lowest average amino acid identity (AAI) of the concatenated marker sequences of MAGs to their closest matches in the database was 38.8%, suggesting the phylogenetic novelty of the reconstructed MAGs (Table S6).

Closely related MAGs tended to share similar inventories of the genes involved in nitrogen cycling and oxygen scavenging (Fig. 4). Seven MAGs, three from N2OR1 and four from N2OR2, were found to contain nosZ genes (Fig. 4). Two N2OR1 MAGs, MH_R1_24 and MH_R1_12, harbored clade I nosZ genes affiliated to the Comamonadaceae family (NosZ2G). The other nosZ-containing N2OR1 MAG, MS_R1_15, was phylogenetically affiliated to the genus Oryzomicrobium (NosZ2G). The nosZ found in this MAG was the most abundant nosZ in the N2OR1 metagenome with 95.8% identity at the amino acid level to that of Oryzomicrobium tereae (WP_054620228.1). Three clade II nosZ genes and a clade I nosZ gene were found in the MAGs obtained from N2OR2. The nosZ gene binned in MS_R2_11, a MAG taxonomically affiliated to the Rhizobiales order of the class Alphaproteobacteria, was the highly expressed clade II nosZ gene 83.0% identical at amino acid level to nosZ of Bradyrhizobium sp. Y36 (WP_097659381.1; NosZ2G). MH_R2_13, affiliated to the order Rhodocyclales, contained a nosZ gene with 93.5% AAI to nosZ of Dechloromonas sp. CZR5 (WP150430259; NosZ2G). The concatenated marker sequence of the other MAG with a clade II nosZ, MS_R2_34, showed only 40.3% AAI to that of its closest relative in the database (Algibacter lectus; GCA_009807485). The nosZ in this MAG shared 80.8% AAI with nosZ of an uncharacterized bacterium (TRZ66679.1; NosZ2G). It should be noted that this MAG was the only one with nosZ and nrfA, but neither nirK nor nirS. A MAG affiliated to the order Rhodocyclales, MS_R2_18, contained a clade I nosZ sharing 91.6% AAI with nosZ of Zoogloea sp. (KAB2967761.1; NosZ2G).

Of the 30 MAGs extracted from the N2OR1 and N2OR2 metagenomes, 24 MAGs included genes encoding either cbb3-type cytochrome c oxidase (ccoNO) or cytochrome bd ubiquinol oxidase (cydAB), the two terminal oxidases associated with O2 scavenging capability under microoxic conditions (Fig. 4). Among the seven nosZ-containing MAGs, only MH_R2_13 lacked either terminal oxidase. Most notable of the MAGs possessing ccoNO and/or cydAB genes were MH_R1_2_6_11 and MH_R2_22 identified as close relatives of the uncultured strain T34. The 16S rRNA gene found in MH_R2_22 was 100% identical to one of the ASVs assigned as the uncultured strain T34 (T34_ASV3 in Fig. 1C).
The closest isolated relative of the two MAGs, in terms of the marker sequence identity, was *Hydromonas duriensis* (57.4% and 60.9% AAI with MH_R1_2_6_11 and MH_R2_22, respectively). The two MAGs shared 98.3% average nucleotide identity (ANI), indicating that the two microorganisms represented by these MAGs belong to the same species. Both MAGs had small genome sizes for *Proteobacteria* with 0.6 Mbp and 0.5 Mbp for MH_R1_2_6_11 (from N2OR1) and MH_R2_22 (from N2OR2), respectively, which can hardly be due to incomplete genome assembly, as the genome completeness of MH_R1_2_6_11 and MH_R2_22 were 95.1% and 87.6%, respectively. As anticipated, 87.5% of the N2OR1 reads and 97.7% of the N2OR2 reads covered by the binned contigs mapped onto MH_R1_2_6_11 and MH_R2_22, respectively.

### Transcription of nosZ and cytochrome oxidase genes in the MAGs

The genes encoding the cytochrome bd ubiquinol oxidase (*cydB*, *cydAB*), cbb3-type cytochrome c oxidase (*ccoNO*), and nitrous reductase (*nosZ*) were among the highly expressed genes in many of the MAGs (Fig. 5). The transcription level of *ccoN* and *ccoO* genes were remarkably high (>11-fold higher than the median transcription level of the single copy marker (SCM) genes) in MH_R1_2_6_11 and MH_R2_22, suggesting that these close relatives of the uncultured strain T34 thrived on O2 scavenging. The *nosZ* gene in the *Oryzobacterium*-affiliated MAG, MS_R1_15, was transcribed at a 6-fold higher level than the median of the SCM genes, suggesting that this microorganism may have contributed to removal of trace N2O; however, transcription of *ccoN* and *ccoO* genes were several folds higher, suggesting that the main mode of survival for this microorganism was more likely O2 scavenging than N2O reduction. The two *Acidovorax* nosZ of MH_R1_24 and MH_R1_12 had no mapped transcriptome reads, suggesting that these microorganisms are unlikely to have contributed to the N2O reducing activity of the N2OR1 culture. *Dechloromonas* nosZ of MH_R2_13 and *Bradyrhizobium* nosZ of MS_R2_11, both grouped with the NosZGS target group, showed the highest transcription levels, with 14- and 37-fold higher than the median of the SCM genes in the respective MAGs. As in MAG MS_R1_15, ccoNO was relatively highly expressed, suggesting the microorganisms simultaneously utilized O2 and N2O; however, unlike MS_R1_15, the nosZ transcription level in MS_R2_11 was approximately four-fold higher than that of ccoNO. The two minor nosZ-containing MAGs in N2OR2, MS_R2_18 and MS_R2_34, also showed simultaneous expression of the cytochrome oxidase genes and nosZ. These observations suggest that under the conditions where the electron acceptors are scarce, many nosZ possessing microorganisms may adopt a strategy of simultaneously utilizing O2 and N2O.

### DISCUSSION

Identification of the active sinks of N2O is crucial for understanding of dynamics and emission of N2O in natural and built environments [17, 18, 66, 67]. This study, by combining culture-based experiments with metagenome and metatranscriptome analyses, identified the groups of microorganisms that are likely to function as such crucial N2O sinks. The designed N2O supply rates to the N2OR1 and N2OR2 reactors were sufficiently low to ensure sub-micromolar steady-state N2O concentration and limit N2O-dependent microbial growth in the reactors, and provision of N2O in dissolved form and constant stirring ensured constant uniform N2O limitation in the reactor cultures. Further, operation of the reactors as chemostats ensured that microorganisms with the specific growth rates lower than the dilution rate were eventually washed out [68]. Thus, persistence of N2O reduction activity in the reactors attested the existence of microorganisms that can utilize <50 nM dissolved N2O as an electron acceptor, corroborating the previous hypothesis that specific guilds of N2O reducing microorganisms may have evolved to benefit from trace N2O leaked from cohabiting denitrifiers [66, 67].

Penetration of O2 into the reactors was unavoidable, and due to the low N2O feed rate, dissolved O2 concentration in the reactor (9.03 ± 0.42 µM and 4.80±0.22 µM in N2OR1 and N2OR2, respectively).
respectively) was always orders of magnitude higher than dissolved N₂O concentration (<50 nM), complicating the search for the microorganisms contributing to high-affinity N₂O reduction. Despite the overwhelming dominance of the close relatives of the strain T34 in the N₂O-fed reactors, their participation in N₂O reduction was ruled out due to the following reason: 1) The N₂FCR reactor free of N₂O was also dominated by this same group of bacteria; 2) the sums of nosZ copy numbers, as quantified with the qPCR assays, were orders of magnitudes lower than the 16S rRNA gene copy numbers in the N2OR1 and N2OR2 reactors; and 3) neither of the two MAGs affiliated to the uncultured strain T34 contained any nosZ gene.

The results of qPCR and metagenome analyses both suggest that the vast majority of the nosZ-possessing microorganisms that avoided washout in the reactors were those with NosZG2 and NosZG5. The qPCR results did not yield conclusive results regarding the quantitative comparison between NosZG2 and NosZG5 in either reactor. However, the metagenome analyses clearly showed NosZG5 being substantially more abundant than NosZG2 in N2OR2, where N₂O was supplied and consumed at a 7-fold higher rate than N2OR1, suggesting that the microorganisms harboring NosZG5 benefited from their capability to compete for trace N₂O (Fig. 3). The dominance of NosZG5 in the metatranscriptomic nosZ pools (82.8% and 92.4% in N2OR1 and N2OR2, respectively) further suggested that the NosZG5 target group was largely responsible for N₂O reduction, regardless of their relative abundance. These observations were consistent with the previous measurements of N₂O consumption kinetics, where Dechloromonas spp. and Azospira spp., possessing nosZ belonging to NosZG5 consistently exhibited the lowest half-saturation constants among diverse N₂O-reducing microorganisms [16, 18, 69].

What remains uncertain is whether sustenance, i.e., growth at a rate higher than the dilution rate, of N₂O-consuming microorganisms harboring the NosZG5 group in the chemostats required O₂ as a supplementary electron acceptor. Selection of the NosZG5 group in N2OR2 suggest that the capability to efficiently utilize submicromolar N₂O substantially contributed to growth and selection of these microorganisms over other nosZ-possessing microorganisms. It should be noted that the recovery of MAG (MH_R2_13) with NosZG5 nosZ but neither ccoNO nor cydAB, was not sufficient to infer the presence of N₂O reducers entirely independent of O₂. Rather, the simultaneous elevated transcription of NosZG5 nosZ, ccoNO, and cydAB in the more abundant microorganism represented by MS_R2_11 suggest that reduction of N₂O was mainly carried out by the microorganisms of NosZG5 simultaneously utilizing O₂ and N₂O. The near complete absence of clade II nosZ of NosZG3 in N2OR1 and N2OR2 contrasted with the previous reverse transcription (RT)-qPCR analyses of anoxic tank activated sludge samples [17]. Possibly, the microorganisms belonging to NosZG3 may not be as well suited for utilization of micromolar O₂ as a growth-supporting electron acceptor. Also possible is that the microorganisms possessing NosZG3 and NosZG5 nosZ may have distinguishable capability in utilizing trace N₂O for main or supplementary growth-supporting electron acceptor, even though the two nosZ groups have been referred to as a single clade in most literature.

The microorganisms affiliated to the uncultured strain T34 of the order Burkholderiales dominated both N2OR1 and N2OR2 cultures, presumably thanks to their capability to scavenge oxygen utilizing the cbb₃-type cytochrome c oxidase (encoded by ccoNO) [70–72]. Thus, little information could be gathered regarding N₂O-reducing microorganisms from the 16S rRNA gene amplicon sequencing-based community analyses. Moreover, the abundance of the nosZ genes in the metagenomes could not serve as an indicator for estimating the contributions of different nosZ-possessing microorganisms to removal of trace N₂O, due to the enrichment of nosZ-possessing microorganisms equipped with ccoNO or cydAB, as exemplified by the abundance of NosZG2 nosZ in the N2OR1 metagenome, which were hardly expressed (Fig. 3). These drawbacks with DNA-based analyses suggest the necessity of RNA- or protein-level investigation for identification of true contributors to N₂O reduction in environmental microbiomes, as the environmental niches where denitrification occurs are rarely completely segregated from neighboring oxic environments and, as in N2OR1 and N2OR2, O₂ availability would largely exceed N₂O availability [25, 73]. The comparison of the qPCR data and the metagenome data confirmed the predictive power of the NosZG1-G5 qPCR that the authors had developed earlier [17]. One of the nosZ genes with the highest level of transcription was the gene that showed >83% identity at the amino acid level with the nosZ gene found in the genome of an uncharacterized...
Bradyrhizobium isolate (strain Y36; GCF_002531575.1). All other nosZ genes in the database affiliated to Bradyrhizobium spp. or the order which the genus belongs to, Rhizobiales, are classified as clade I; however, this particular nosZ, belonged to the NosZG5 target group of clade II, as can be shown in the nosZ phylogenetic tree presented in Fig. S6 [74, 75]. Misclassification was unlikely, as the MAG MS_R2_11 containing this gene and the genome of Bradyrhizobium sp. Y36 also share high level of similarity (76% ANI). The high abundance of this nosZ in both N2OR1 and N2OR2 transcriptomes implies involvement of this atypical member of Rhizobiales in high-affinity N2O reduction. Additional investigations into the evolutionary history of this Bradyrhizobium nosZ may be worthwhile, as the presence of this clade II nosZ in Rhizobiales may be a rare evidence of horizontal transfer of a nosZ gene [76]. Several studies have suggested the potential significance of non-denitrifying N2O reducers as environmental N2O sinks [6, 10, 74]. The nosZ genes affiliated to the microorganisms lacking either nirK or nirS often constitute substantial portions of agricultural soils’ nosZ pools; however, previous attempts to enrich N2O-reducing consortia in either suspended-sediment or biofilm bioreactors failed to identify such microorganisms as significant constituents [10, 17, 20, 21, 74]. The phylogenetic placement of the recovered nosZ genes, as well as the near absence of non-denitrifier nosZ in the MAGs, suggest against non-denitrifier N2O reducers playing a vital role in the high-affinity N2O reduction observed in N2OR1 and N2OR2 reactors. Perhaps, more important than the (nirK + nirS)/nosZ gene ratio in determining N2O emissions from anoxic environments where denitrification occurs may be the abundance and activity of the N2O-reducing microorganisms capable of sustained consumption of trace N2O, as previously suggested [77].

The oxygen levels measured in the reactors may strike many as being too high to be categorized as microoxic. The long-perceived boundary demarcating the O2 level that would provide anaerobes selective advantage over aerobes, the ‘Pasteur Point’, is approximately 0.2%, and many biogeochemical models have predicted the critical O2 level as low micromolar concentrations (<10 µM) [78–80]. The O2 levels observed in N2OR1 and N2OR2 after they had attained pseudo-steady state were consistent with this range of concentrations. However, recent investigations, mostly performed with Unisense microsensors (including the new STOX sensors with improved sensitivity) have reported microbial consumption of O2 in the sub-to-low nanomolar concentration range and suggested low-nanomolar concentrations as the critical O2 levels for aerobic growth [30, 81, 82]. Given that the typical range and suggested low-nanomolar concentrations as the critical O2 level as low micromolar concentrations (<10 µM) approximately 0.2%, and many biogeochemical models have predicted the critical O2 level as low micromolar concentrations (<10 µM) may have served as the main growth-supporting electron acceptor for some microorganisms that contributed to N2O reduction, as suggested from the transcription profile of MAGs MS_R1_15 (NosZG2), wherein ccoNO transcripts were found in substantially higher abundance than nosZ transcripts. Further, simultaneous expression of nosZ and ccoNO observed in MS_R2_11 (NosZG5) with the most highly-expressed nosZ in the N2OR2 microbiome suggested that the presence of low-micromolar O2 may even be crucial for sustenance of microorganisms that play the most consequential roles in removing trace N2O. Evidence of simultaneous expression of nosZ and ccoNO has also been found in transcriptomes of marine coastal sediments and a sequencing batch reactor during the microoxic phase in recent studies, although measurements of N2O concentrations or consumption rates were not accompanied [29, 30]. The interplay between oxygen scavenging and N2O reduction may be a crucial underlying mechanism enabling reduction of trace N2O at denitrification hotspots, e.g., anoxic tanks in activated sludge WWTP andoxic-anoxic interfaces in agricultural soils, where complete anoxia is rarely attained, keeping N2O emissions from these hotspots in check.

DATA AVAILABILITY
All raw sequence data and the finalized MAGs were deposited in the NCBI database under the BioProject accession number PRJNA789121.

REFERENCES
1. Montzka SA, Dlugokencky EJ, Butler JH. Non-CO2 greenhouse gases and climate change. Nature 2011;476:43–50.
2. Masson-Delmotte V, Zhai P, Pirani A, Connors SL, Péan C, Berger S, et al. (eds). Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge: Cambridge University Press; 2021. (in press).
3. Wuebbles DJ. Nitrous oxide: no laughing matter. Science. 2009;326:56–7.
4. Kool DM, Dolfing J, Wragge N, van Groenigen JW. Nitrifier denitrification as a distinct and significant source of nitrous oxide from soil. Soil Biol Biochem. 2011;43:174–8.
5. Yoon S, Song B, Phillips RL, Chang J, Song MJ. Ecological and physiological implications of nitrogen oxide reduction pathways on greenhouse gas emissions in agroecosystems. FEMS Microbiol Ecol. 2019;95:fiz066.
6. Sanford RA, Wagner DD, Wu Q, Chee-Sanford JC, Thomas SH, Cruz-Garcia C, et al. Unexpected nondenitrifier nosZ nitrous oxide reduce gene diversity and abundance in soils. Proc Natl Acad Sci USA. 2012;109:19709–14.
7. Hallin S, Philippot L, Löffler FE, Sanford RA, Jones CM. Genomics and ecology of novel N2O-reducing microorganisms. Trends Microbiol. 2018;26:43–55.
8. Graf DR, Jones CM, Hallin S. Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N2O emissions. PLoS One. 2014;9:e114118.
9. Rosco TA, Bergauttu LL, Bakken LR, Yavitt JB, Shapleigh JP. Modulation of nitrogen oxide reducing soil bacteria: linking phenotype to genotype. Environ Microbiol. 2017;19:2507–19.
10. Jones CM, Graf DR, Bru D, Philippot L, Hallin S. The unaccounted yet abundant nitrous oxide-reducing microbial community: A potential nitrous oxide sink. ISME J. 2013;7:417–26.
11. Frostegård Å, Vick SH, Lim NY, Bakken LR, Shapleigh JP. Linking meta-omics to the kinetics of denitrification intermediates reveals pH-dependent causes of N2O emissions and nitrite accumulation in soil. ISME J. 2022;16:26–37.
12. Simon J, Eisin G, Krones PMH, Zumft WG. The unprecedented nosZ gene cluster of Wolinella succinogenes encodes a novel respiratory electron transfer pathway to cytochrome c nitrous oxide reductase. FEBS Lett. 2004;569:7–12.
13. Foley J, De Haas D, Yuan Z, Lant P. Nitrous oxide generation in full-scale biological nutrient removal wastewater treatment plants. Water Res. 2010;44:831–44.
14. Zheng J, Doskey PV. Simulated rainfall on agricultural soil reveals enzymatic regulation of short-term nitrous oxide profiles in soil gas and emissions from the surface. Biogeochemistry. 2016;128:327–38.
15. Kem M, Simon J. Three transcription regulators of the Nos family mediate the adaptive response induced by nitrate, nitric oxide or nitrous oxide in Wolinella succinogenes. Environ Microbiol. 2016;18:899–912.
16. Suenaga T, Rya S, Hosomi M, Terada A. Biotic characterization and activities of N2O-reducing bacteria in response to various oxygen levels. Front Microbiol. 2018;9:697.
12

71. Rauhamäki V, Bloch DA, Wikström M. Mechanistic stoichiometry of proton translocation by cytochrome cbb3. Proc Natl Acad Sci USA. 2012;109:7286–91.
72. Borisov VB, Gennis RB, Hemp J, Verkhovsky MI. The cytochrome bd respiratory oxygen reductases. Biochim Biophys Acta - Bioenerg. 2011;1807:1398–413.
73. Lee A, Winther M, Priemé A, Blunier T, Christensen S. Hot spots of N2O emission move with the seasonally mobile oxic-anoxic interface in drained organic soils. Soil Biol Biochem. 2017;115:178–86.
74. Orellana L, Rodriguez-R L, Higgins S, Chee-Sanford J, Sanford R, Ritalahti K, et al. Detecting nitrous oxide reductase (nosZ) genes in soil metagenomes: method development and implications for the nitrogen cycle. MBio 2014;5:e01193–14.
75. Ormeño-Orrillo E, Martínez-Romero E. A genomotaxonomy view of the Bradyrhizobium genus. Front Microbiol. 2019;10:1334.
76. Tong W, Li X, Wang E, Cao Y, Chen W, Tao S, et al. Genomic insight into the origins and evolution of symbiosis genes in Phaseolus vulgaris microsymbionts. BMC Genom. 2020;21:186.
77. Conthe M, Lycus P, Arntzen MØ, da Silva AR, Frostegård Å, Bakken LR, et al. Denitrification as an N2O sink. Water Res. 2019;151:381–7.
78. Goldblatt C, Lenton TM, Watson AJ. Bistability of atmospheric oxygen and the Great Oxidation. Nature. 2006;443:68–9.
79. Brewer PG, Hofmann AF, Peltzer ET, Ussler W III. Evaluating microbial chemical choices: The ocean chemistry basis for the competition between use of O2 or NO3– as an electron acceptor. Deep Sea Res Part I Oceanogr Res Pap. 2014;87:35–63.
80. Bianchi TD, Dunne JP, Sarmiento JL, Galbraith ED. Data-based estimates of suboxic, denitrification, and N2O production in the ocean and their sensitivities to dissolved O2. Global Biogeochem Cycles 2012;26:GB2009.
81. Stolper DA, Revsbech NP, Canfield DE. Aerobic growth at nanomolar oxygen concentrations. Proc Natl Acad Sci USA. 2010;107:18755–60.
82. Zakem E, Follows M. A theoretical basis for a nanomolar critical oxygen concentration. Limnol Oceanogr. 2017;62:795–805.
83. Liengaard L, Nielsen LP, Revsbech NP, Priemé A, Blunier T, Christensen S. Hot spots of N2O emission move with the seasonally mobile oxic-anoxic interface in drained organic soils. Soil Biol Biochem. 2017;115:178–86.
84. Shcherbak I, Robertson GP. Nitrous oxide (N2O) emissions from subsurface soils of agricultural ecosystems. Ecosystems. 2019;22:1650–63.
85. Qu Z, Bakken LR, Moltstad L, Frostegård Å, Bergaust LL. Transcriptional and metabolic regulation of denitrification in Paracoccus denitrficans allows low but significant activity of nitrous oxide reductase under oxic conditions. Environ Microbiol. 2016;18:2951–63.
86. Desloover J, Roobroeck D, Heylen K, Puig S, Boeckx P, Verstraete W, et al. Pathway of nitrous oxide consumption in isolated Pseudomonas stutzeri strains under anoxic and oxic conditions. Environ Microbiol. 2014;16:3143–52.

ACKNOWLEDGEMENTS
This work was financially supported by the National Research Foundation of Korea (NRF) (Grant No. 2020R1C1C1007970). MS was supported by NRF (Grant No. 2021R1I1A1A01054770). ML was supported by a VENI grant from the Dutch Research Council (NWO) (Project No. VI.Veni.192.252). AT was supported by the Japan Society for the Promotion of Science (JSPS) (Project No. 20H04362).

AUTHOR CONTRIBUTIONS
SY conceived and supervised the study. DDK, HH, MJS, and SY designed experiments. DDK performed experiments. DDK, TY, and SY analyzed experimental and sequencing data, with contributions from HH and MJS. DDK and SY wrote the manuscript, with contributions from HH, TY, MJS, AT, and ML. All authors critically reviewed the manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41396-022-01260-5.

Correspondence and requests for materials should be addressed to Sukhwan Yoon.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.