Eelgrass Sediment Microbiome as a Nitrous Oxide Sink in Brackish Lake Akkeshi, Japan

TATSUNORI NAKAGAWA1*, YUKI TSUCHIYA1, SHINGO UEDA1, MANABU FUKU1, and REIJI TAKAHASHI1

1College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, 252–0880, Japan; and 2Institute of Low Temperature Science, Hokkaido University, Kita-19, Nishi-8, Kita-ku, Sapporo, 060–0819, Japan

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Nitrous oxide (N2O) is a powerful greenhouse gas; however, limited information is currently available on the microbiomes involved in its sink and source in seagrass meadow sediments. Using laboratory incubations, a quantitative PCR (qPCR) analysis of N2O reductase (nosZ) and ammonia monoxygenase subunit A (amoA) genes, and a metagenome analysis based on the nosZ gene, we investigated the abundance of N2O-reducing microorganisms and ammonia-oxidizing prokaryotes as well as the community compositions of N2O-reducing microorganisms in situ and cultivated sediments in the non-eelgrass and eelgrass zones of Lake Akkeshi, Japan. Laboratory incubations showed that N2O was reduced by eelgrass sediments and emitted by non-eelgrass sediments. qPCR analyses revealed that the abundance of nosZ gene clade II in both sediments before and after the incubation as higher in the eelgrass zone than in the non-eelgrass zone. In contrast, the abundance of ammonia-oxidizing archaella amoA genes increased after incubations in the non-eelgrass zone only. Metagenome analyses of nosZ genes revealed that the lineages Dechloromonas-Magnetospirillum-Thiocapsa and Bacteroidetes (Flavobacteria) within nosZ gene clade II were the main populations in the N2O-reducing microbiome in the in situ sediments of eelgrass zones. Sulfur-oxidizing Gammaproteobacteria within nosZ gene clade II dominated in the lineage Dechloromonas-Magnetospirillum-Thiocapsa. Alphaproteobacteria within nosZ gene clade I were predominant in both zones. The proportions of Epsilonproteobacteria within nosZ gene clade II increased after incubations in the eelgrass zone microcosm supplemented with N2O only. Collectively, these results suggest that the N2O-reducing microbiome in eelgrass meadows is largely responsible for coastal N2O mitigation.

Key words: nitrous oxide-reducing microbiome, nosZ, amoA, eelgrass sediments, sulfur-oxidizing Gammaproteobacteria, Flavobacteria

Nitrous oxide (N2O) is a powerful, long-lived greenhouse gas (45) and causes the depletion of the stratospheric ozone layer (54). In natural ecosystems, N2O is predominantly produced through the microbial processes of denitrification, nitrification, and nitrifier-denitrification (27, 72, 83). Among these mechanisms, coastal and estuarial N2O sources are assumed to be mainly due to sediment denitrification (46, 47). N2O emissions from an open ocean have recently been attributed to nitrification by ammonia-oxidizing archaea (AOA) (40, 61). However, limited information is currently available on the relationship between N2O emissions and nitrifiers in seagrass sediments. Moreover, the consumption of N2O was previously reported in sediments of eelgrass (Zostera marina) meadows (47). Considerable sediment N2O uptake has recently been reported in pristine shallow coastal ecosystems (20, 41), and rapid N2O reduction has been discovered in the suboxic ocean (4).

Denitrification is the sequential reaction of nitrate to the gaseous products N2O and/or nitrogen gas (N2) via nitrite (NO2−) and nitric oxide (NO). Nitrous oxide reductase (NosZ) is a key enzyme that catalyzes the reduction of N2O to N2 during denitrification under sufficiently low molecular oxygen conditions (11, 34). Therefore, the step of N2O reduction by the enzyme NosZ is a major process that influences N2O flux to the atmosphere (72, 84). Many prokaryotes, including more than 60 genera of bacteria, have the ability to denitrify heterotrophically (65). Autotrophic denitrifiers are also able to utilize nitrate (NO3−) and/or NO2− as the electron acceptor and reduce N2O to N2 using the enzyme NosZ (64). Some autotrophic denitrifying sulfur oxidizers have the nosZ gene on the whole genome, such as Thiobacillus denitrificans and Sulfuritalea hydrogenivorans within Betaproteobacteria (5, 33), Sedimenticola thiotauroi within Gammaproteobacteria (19), and Sulfurimonas denitrificans within Epsilonproteobacteria (67). Chemolithoautotrophic denitrifiers within Gammaproteobacteria and Epsilonproteobacteria play an important role in the nitrogen cycle in the oxygen minimum zone in the ocean (37). nosZ genes have recently been classified into two phylogenetically distinct NosZ clades: the first encodes typical NosZ proteins, now designated as nosZ gene clade I, while the other encodes atypical NosZ proteins, now designated as nosZ gene clade II, including the denitrifying members of Gammaproteobacteria, Epsilonproteobacteria, and the phylum Bacteroidetes as well as the non-denitrifying microorganisms of genera such as Anaeromyxobacter, Dyadobacter, and Ignavibacterium (29, 60). PCR and metagenome analyses based on the nosZ gene revealed that nosZ gene clade II is more abundant and widespread than nosZ gene clade I in several environments such as soil (29, 30, 43, 51), wastewater treatment plants (29), marine water (70), and marine sediments (3, 56, 79). Furthermore, previous studies demonstrated that non-denitrifying N2O-reducing bacteria within nosZ gene clade II played an important role in the consumption of N2O within soil (14, 52, 81). However, limited information is currently available on the distribution and community structure of microorganisms with the capacity to reduce N2O in eelgrass sediments.

* Corresponding author. E-mail: nakatats@brs.nihon-u.ac.jp; Tel: +81–466–84–3359; Fax: +81–466–84–3359.

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Seagrass meadows provide the stabilization of sediment, a habitat for ecologically diverse and productive ecosystems that reduce the exposure of humans, fishes, and invertebrates to bacterial pathogens in coastal environments (16, 38). Sediments inhabited by seagrasses are generally anoxic due to sulfide produced by sulfate-reducing bacteria that utilize sulfate as an electron acceptor for the mineralization of organic matter accumulated by seagrasses (9). Molecular ecological studies based on 16S rDNA previously revealed the predominance of sulfur-oxidizing bacteria (SOB) within Gammaproteobacteria and/or Epsilonproteobacteria together with sulfate-reducing bacteria in seagrass sediments (10, 12, 17, 73). Some SOB within Gammaproteobacteria and/or Epsilonproteobacteria possess the nosZ gene (22, 28). Since high denitrification rates have been reported within the surface of seagrass sediments (8), we hypothesized that SOB possessing the nosZ gene may be responsible for the N₂O sink in seagrass sediments alongside their role in sulfide detoxification for seagrasses growing in sulfidic sediments (24, 75).

To test this hypothesis, we characterized the microbiomes responsible for N₂O reduction in eelgrass meadow sediments using the high-throughput sequencing of the nosZ gene and quantitative PCR (qPCR) analyses of nosZ and ammonia monoxygenase subunit A (amoA) genes. Since PCR primers for nosZ gene clade II have limitations for investigating the diversities of the genera Anaeromyxobacter and sulfuricolous and the phylum Bacteroidetes due to PCR bias (29), we examined the community structures of the N₂O-reducing microbiome based on nosZ gene clades I and II. The community structures of microbiomes in sediments were compared between non-eelgrass and eelgrass zones. Laboratory incubations of sediment microcosms were conducted for sediments from both zones to elucidate the relationship between the community structures of the N₂O-reducing microbiome and N₂O sink. Since few studies have investigated the influence of nitrogen fertilizers on the coastal areas, such as the sharp increase in (NH₄)₂SO₄ due to increased precipitation, we examined the influence of ammonium on nitrification and N₂O production in non-eelgrass and eelgrass zone sediments.

**Materials and Methods**

**Study area and sampling**

The study area was Lake Akkeshi, a brackish lake located in Hokkaido, Japan. Most of the lake (31.8 km²) is covered with the eelgrass Zostera marina (80). Sediment core samples were obtained at two different positions, a non-eelgrass zone (43°03′54″N, 144°53′24″E) (n=1) (Fig. S1A and C) and an eelgrass zone (43°03′18″N, 144°53′24″E) (n=1) (Fig. S1B and D), by a diver using a plastic corer (8 cm in diameter and 50 cm in length) on 28 July, 2015. A sample of the surface water was also obtained in a 1-L steel corer (7.0 cm in diameter and 4.0 cm in length). While the color of the sediment obtained from the non-eelgrass zone was dark brown, that from the eelgrass zone was black and sulfidic (Fig. S1F and G). The sediments collected were placed into sterilized 50-mL plastic tubes. The concentrations of NO₃⁻ and SO₄²⁻ in the pore water of sediments were 8.1 μM and 20.2 mM in the non-eelgrass zone and 5.2 μM and 20.9 mM in the eelgrass zone, respectively. Samples were transferred to the laboratory in an ice-cooled box within 3 d. Sediments were centrifuged in the sterile 50-mL plastic tubes (5,800×g; 4°C, 10 min) and then mixed well after the supernatant had been discarded. Sediments for the incubation test were kept on ice until incubation experiments. Sediments for DNA extraction were stored at −80°C. One liter of seawater from the two different zones was filtered using Nalgene Rapid-Flow Filters ( pore size, 0.2 μm; Thermo Fisher Scientific, Waltham, MA, USA).

**Cultivation of sediment microcosms and N₂O analysis**

Sediment incubation experiments (three biological replicates) were performed for the non-eelgrass zone and eelgrass zone. Fifty-milliliter serum bottles (GL Sciences, Tokyo, Japan) containing 5 g of sediments and 15 mL of filter-sterilized seawater (Fig. S1F and G) were closed with butyl rubber stoppers (Nichiden-Rika Glass, Kobe, Japan), capped with an aluminum crimp seal (GL Sciences), and then incubated at 20°C for 7 d in the dark. The bottles were shaken by hand for a few seconds once a day during the 7-d incubation (except on day 6 of the incubation). Three treatments were prepared for two different zones: one as a control (not amended) designated with the sample names N1 for the non-eelgrass zone and E1 for the eelgrass zone, one treated with 3.2 mM NH₄Cl seawater (N2 for the non-eelgrass zone and E2 for the eelgrass zone) to enhance the activity of ammonia oxidizers, and one spiked with 0.3 μl of 99.5% N₂O (N3 for the non-eelgrass zone and E3 for the eelgrass zone) in order to confirm differences in net N₂O absorption between the non-eelgrass and eelgrass zones. Each treatment was conducted in triplicate, giving a total of nine bottles in each zone. The gas in the headspace of the bottle after day 7 of the incubation was withdrawn via the closed butyl rubber stopper using a Pressure-Lok precision analytical syringe (VICI Precision Sampling, Baton Rouge, LA, USA) for the N₂O analysis. N₂O concentrations were measured with a gas chromatograph equipped with an electron capture detector (GC-14B; Shimadzu, Kyoto, Japan). Sediment and seawater in serum bottles after day 8 of the incubation were centrifuged in the sterile 50-mL plastic tubes (5,800×g; 4°C, 10 min) and then stored at −80°C after the supernatant had been discarded.

**Nucleic acid extraction**

Each wet sediment (~0.5 g) before (in situ) and after the incubation was added to a plastic tube containing lysis solutions and beads in ISOIL for the Beads Beating Kit (Nippon Gene, Toyama, Japan), mixed vigorously for 45 s, and then incubated at 65°C for 1 h. Nucleic acids were extracted according to the manufacturer’s protocol, and extracted nucleic acids in 20 μL of Tris-EDTA (TE) buffer were then stored at −20°C. Twenty-four DNA samples were extracted from the incubated sediments (N1, N2, N3, E1, E2, and E3) and in situ sediments were designated with the sample names Ni for the non-eelgrass zone and Ei for the eelgrass zone.

**qPCR of nosZ and amoA genes**

Regarding the quantification of nosZ and amoA gene copies, each DNA solution extracted from sediments before (in situ) and after the incubation was quantified by real-time PCR in a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) using the SYBR Premix Ex Taq (Tli RNase H Plus) kit (Takara Bio, Kusatsu, Japan). Twenty-three of the DNA samples were used for qPCR: duplicate DNA samples from N3 due to the loss of samples, and triplicate DNA samples from the remaining sediment samples. The following detection primer sets were used: nosZ2F and nosZ2R for nosZ gene clade I (26), modified nosZ-II-Fn (5′-CTN GGN CCN YTK CAY AC-3′) and nosZ-II-Rn (5′-GCN GAR CAR AAN TCB GTR C-3′) for nosZ gene clade II (29), GenAOAF and GenAOAR for the AOA gene (44), and amoA-1F and amoA-2R for the beta-proteobacterial
ammonia-oxidizing bacteria (AOB) amoA gene (57). Standard curves were generated from a plasmid containing each of the following cloned genes: the nosZ clade I gene fragment of Pseudomonas stutzeri NBRC14165 amplified with the PCR primers nosZ 1F (15) and nosZ2R for nosZ gene clade I (26), the nosZ clade II gene fragment of the environmental clone G3H008 amplified with the PCR primers nosZ-II-Fn and nosZ-II-Rn (Fig. S2), the AOA amoA gene fragment of Nitrosopumilus sp. NM25 amplified with the PCR primers CrenAMO_F and CrenMO_R (23), and the AOB amoA gene fragment of Nitrosomonas stercorea KYUHI-S, amplified with the PCR primers amoA-1F and amoA-2R. Each reaction was performed in a volume of 25 μL containing 2 μL of diluted DNA solution (one fiftieth of template DNA), 0.2 μM of each primer (1.0 μM of each primer only for nosZ gene clade I), and 12.5 μL of SYBR Premix Ex Taq (Tli RNase H Plus). Cycling conditions were as follows: for nosZ clade I (26), an initial denaturation step at 95°C for 3 min, followed by 6 cycles at 95°C for 15 s, 65°C for 30 s, and 72°C for 30 s, and then 40 cycles at 95°C for 15 s, 65°C for 15 s, 72°C for 30 s, and 80°C for 15 s. Fluorescence intensity was measured at 80°C. Regarding nosZ clade II (29), cycling conditions were an initial denaturation step at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, 72°C for 30 s, and 80°C for 30 s. Fluorescence intensity was measured at 80°C. Cycling conditions for AOA amoA (44) were an initial denaturation step at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s, 55°C for 30 s, 72°C for 30 s, and 80°C for 1 s. Fluorescence intensity was measured at 80°C. Regarding AOB amoA (2), cycling conditions were an initial denaturation step at 95°C for 3 min, followed by 40 cycles at 95°C for 10 s, 57°C for 30 s, 72°C for 30 s, and 80°C for 1 s. Fluorescence intensity was measured at 78°C. After each run, the amplicon was visualized on an agarose gel to confirm the specific product bands of the expected size. Efficiencies for nosZ clade I, clade II, AOA amoA, and AOB amoA amplification were estimated at 62, 62, 108, and 82%, with R² of 0.963, 0.999, 0.980, and 0.998, respectively.

Metagenomic library construction and sequencing of nosZ genes

The library for the shotgun metagenomic analysis for nosZ genes was created with the tagmentation-based Nexera DNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer’s protocol, and samples were then stored at -20°C. Twenty-one DNA samples were used for tagmentation: a single DNA sample from N1 due to the loss of samples, duplicate DNA samples from N3 due to the loss of samples, two duplicate DNA samples from the remaining sediment samples. The DNA quality of each library was verified by 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA), and quantified with the Qubit dsDNA HS assay kit and Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). The sequencing of composite DNA samples was performed using the MiSeq V2 reagent kit (2×150 bp) on MiSeq (Illumina). The initial quality filtering of paired-end reads was performed with MiSeq software version 2.5.0.5 (Illumina) to remove some reads with base calls below the threshold (Q30) and the trim sequences of tag and adapter. All paired-end Illumina reads were imported into CLC Genomic Workbench version 8.5.1 (CLCBio; QIAGEN, Aarhus, Denmark), and some reads that were shorter than 90 nucleotides were discarded from the libraries, resulting in 38,029,139 reads (Table S1). Due to the limitation of computer memory, 30% of the N1, N2, and N3 reads were used in subsequent analyses. To detect nosZ reads in the metagenomes derived from each sediment sample, publicly available NosZ amino acid references were downloaded from FunGene (http://fungene.cme.msu.edu) (18) of the Ribosomal Database Project (RDP) and the National Center for Biotechnology Information (NCBI), and then imported into the CLC Genomic Workbench. NosZ-encoding reads were identified by blastx (1) against NosZ amino acid references with an e-value of more than 10–15 from the references, the amino acids of uncultured bacteria with an e-value of more than 10–15 in the NosZ amino acid references were aligned with archetype amino acid sequences using the CLUSTAL W program in MEGA version 7 (35). The phylogenetic tree was constructed by the maximum-likelihood method in MEGA7, resulting in 1,986 of nosZ gene reads (Table S1). Similarly, the numbers of nitric oxide reductase subunit B (norB) and amoA gene reads in the metagenomes were investigated as described in the supplementary information. Operational taxonomic units (OTUs) were defined as sequence groups based on the lineages constructed from the phylogenetic tree in order to compare nosZ-based diversity with a rarefaction analysis using Analytic Rarefaction 1.3 (https://strata.uga.edu/software/index.html).

Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyze the significance of differences in N₂O concentrations and the abundance of nosZ and amoA genes before and after the incubation. Pearson’s product moment correlation (PPMC) analysis was performed to assess the relationship between the increased abundance of amoA genes and elevated concentrations of N₂O in the headspace of bottles after the 7-d incubation. ANOVA and PPCM analyses were performed with SPSS Statistics version 20 (IBM, Armonk, NY, USA).

Nucleotide sequence accession number

All metagenomics reported in the present study were deposited in the DNA Data Bank Japan (DDBJ) Sequence Read Archive (DRA) under accession number DRA006867. The corresponding tables between the sample names and deposit IDs of nosZ gene reads on DRA006867 have been submitted to FigShare (http://dx.doi.org/10.6084/m9.figshare.6720428).

Results

N₂O emissions and sink

N₂O concentrations in the headspace after the incubation were significantly higher in non-eelgrass sediment bottles than in eelgrass sediment bottles (P<0.010) (Fig. 1A), indicating that N₂O emissions were higher from non-eelgrass sediments than from eelgrass sediments. Even though ammonium was supplemented to sediments, the N₂O concentration in eelgrass sediment bottles (0.022 μmol L⁻¹) was similar to that in eelgrass sediment bottles without ammonium (0.009 μmol L⁻¹) (Fig. 1B). In contrast, the N₂O concentration in the headspace of non-eelgrass sediment was 90-fold higher than that in eelgrass sediment, implying low N₂O emissions from eelgrass sediments. In addition, when a high concentration of N₂O was injected into sediment bottles, N₂O concentrations in the headspace after the incubation were significantly lower in eelgrass sediment bottles than in non-eelgrass sediment bottles (P<0.011) (Fig. 1C), suggesting that eelgrass sediments have the capability to absorb more N₂O than non-eelgrass sediments.

qPCR of nosZ and amoA genes

The abundance of nosZ gene clade II in in situ sediments was significantly higher in eelgrass zone sediment E1 than in non-eelgrass zone sediment N1 (P<0.000) (Fig. 2A). Similarly, after the incubation, the abundance of nosZ gene clade II was higher in all types of bottles with eelgrass zone sediments E1, E2, and E3 than in all bottles with non-eelgrass zone sediments N1, N2, and N3. Although the target lengths of PCR products for nosZ gene clade I were detected by qPCR for all samples, the abundance of nosZ gene clade I was not elucidated because the fluorescent intensities of amplicons for the non-targeted region were as strong as those of the target PCR products after qPCR.
The abundance of the AOA amoA genes in incubated bottles with non-eelgrass zone sediments N1, N2, and N3 was approximately 10-fold higher than that in the in situ non-eelgrass zone sediment Ni (Fig. 2B). In contrast, no significant changes were observed in the abundance of AOA amoA genes between before and after the incubation among eelgrass sediment samples. In AOB amoA genes, no significant differences were noted between before and after the incubation for both sediment samples (Fig. 2C). The abundance of AOB amoA genes in in situ sediments from the non-eelgrass zone (Fig. 2C, Ni) was significantly higher than that of AOA amoA genes in in situ sediments from the non-eelgrass zone (Fig. 2B, Ni) (P=0.002). However, there was no significant change in the abundance of amoA genes between AOA (Fig. 2B, Ei) and AOB (Fig. 2C, Ei) in the eelgrass zone (P=0.082).

**nosZ gene metagenome**

Shotgun metagenomic analyses based on the nosZ gene revealed that nosZ gene reads mainly fell into the lineages *Dechloromonas-Magnetospirillum-Thiocapsa*, *Bacteroides*, *Myxococcales*, *Anaeromoxybacter-Oipitatus*, *Rhodothermus-Thermomicrobium*, *Epsilonproteobacteria*, and *Gemmaminadeti-Egnivibacteria* within nosZ gene clade II, and into the lineages *Alphaproteobacteria* and *Gammaproteobacteria* within nosZ gene clade I in the non-eelgrass and/or eelgrass zones (Fig. 3A). nosZ genes within *Bacteroidetes* and *Epsilonproteobacteria* were detected as predominant members using shotgun metagenomic sequencing (Fig. 3A and B) even though they were not detected by the cloning analysis (Fig. S2). The community structural proportions of nosZ gene clade II were approximately four-fold higher than those of nosZ gene clade I in in situ sediments for both zones (Fig. 3B). This result was consistent with previous findings on nosZ gene clade II (51, 56, 70). Furthermore, the numbers of nosZ gene reads were slightly higher than those of norB gene reads (Table S1).

In the eelgrass zone, the reads of nosZ gene clade II were occupied by the dominant members of the lineages *Dechloromonas-Magnetospirillum-Thiocapsa* (approximately 30%) and *Bacteroidetes* (approximately 30%) in in situ and incubated sediments (Fig. 3B, Ei, E1, E2, and E3). In the lineage *Dechloromonas-Magnetospirillum-Thiocapsa*, the reads of the sulfur-oxidizing gammaproteobacterial nosZ gene dominated (more than approximately 50%) in in situ and incubated sediments in the eelgrass zone (Table 1, Ei, E1, E2, and E3). The majority of sulfur-oxidizing Gammaproteobacteria were related to sulfur- and sulfide-oxidizing symbionts, such as *Thiolapillus brandeum* isolated from a hydrothermal vent (48), ‘Candidatus Thiodiazotropha endoloripes’ and ‘Candidatus Thiosymbion oneisti’ in seagrass sediments (53), the marine bivalve mollusk *Solemya veleicina* gill symbiont (58), and...
endosymbionts of the deep-sea tubeworms *Ridgeia piscesae* and *Tevnia jerichonana* (22). Further predominant *nosZ* gene reads were related to the giant sulfur-oxidizing bacterium *Candidatus Thiomargarita nelsonii* (77) and anaerobic phototrophic nitrite oxidizer *Thiocapsa* sp. KS1 (25) in the *in situ* sediment of the eelgrass zone. In addition, the spiking of N$_2$O into serum bottles (Table 1, E3 and N3) induced an increase in unclassified *nosZ* reads related to the cytochrome c N$_2$O reductase (*cNos*) of *Epsilonproteobacteria*, which dominated in the gill chamber epibiosis of the deep-sea shrimp (28).

In both zones, the lineage *Bacteroidetes* was dominated by *nosZ* reads related to marine *Flavobacteria* (*Lutibacter, Seonamhaeicola, Maribacter, Arenibacter, Aquimarina, Cellulophaga, Bizonia, and Muricada*), such as *Lutibacter profundi* (78), *Flavobacteriales bacterium ALC-1* (7), and *Flavobacteriaceae bacterium NORP142* (74) (Table 2). In N$_2$O-supplemented sediment E3 in the eelgrass zone, in which the proportion of *Epsilonproteobacteria* increased (Fig. 3B), epsilonproteobacterial reads were related to *S. gotlandica* GD1 (36), *Sulfurospirillum multivorans* DSM 12446 (62), and *Arcobacter* spp. (13, 71). In the non-eelgrass and eelgrass...
zones, approximately 90% of alphaproteobacterial reads within nosZ gene clade I fell into the lineage Azospirillum-\textit{Thioploca-Magnetospirillum-\textit{Thiocapsa}} (Fig. 3A); however, most alphaproteobacterial reads were related to uncultured bacteria. A rarefaction analysis showed that there was no significant difference in diversity among samples (Fig. S3).

**Discussion**

**qPCR and shotgun metagenomic analyses**

The qPCR analysis based on the nos\textit{Z} gene revealed that the abundance of N\textsubscript{2}O-reducing microbes within nos\textit{Z} gene clade II in \textit{in situ} sediments of the eelgrass zone was 3.7-fold higher than that in the non-eelgrass zone (Fig. 2A). A previous study reported that PCR primers for nos\textit{Z} gene clade II are limited by PCR bias (29). Although \textit{Bacteroidetes} and \textit{Epsilonproteobacteria} were detected in incubated sediment E3 (sample ID: aA8) with N\textsubscript{2}O in the eelgrass zone as predominant members using shotgun metagenomic sequencing (Fig. 3B), nos\textit{Z} genes related to \textit{Bacteroidetes} and \textit{Epsilonproteobacteria} were not detected in the same sediment E3 (sample ID: aA8) by a PCR-dependent analysis based on the nos\textit{Z} gene in the present study (Fig. S2). This result suggests an underestimation of the abundance of nos\textit{Z} gene clade II within \textit{Bacteroidetes} (\textit{Flavobacteria}) and \textit{Epsilonproteobacteria}. However, a shotgun metagenomic analysis based on the nos\textit{Z} gene onlydetected sulfide-oxidizing \textit{Gammaproteobacteria} within the lineage \textit{Dechloromonas-Magnetospirillum-\textit{Thiocapsa}}, but also successfully identified N\textsubscript{2}O-reducing microbes within \textit{Flavobacteria} and \textit{Epsilonproteobacteria} from incubated sediment E3 of the eelgrass zone (Fig. 3). Therefore, a parallel analysis (qPCR and shotgun metagenomic sequencing) demonstrated that sulfide-oxidizing \textit{Gammaproteobacteria} and marine \textit{Flavobacteria} were the dominant N\textsubscript{2}O-reducing microbes in \textit{in situ} sediments of the eelgrass zone. However, a new qPCR primer set needs to be designed to accurately evaluate the enumeration of assemblages. In addition to the contribution of N\textsubscript{2}O-reducing microbes, since the numbers of nos\textit{Z} reads detected were higher than those of nor\textit{B} reads in both \textit{in situ} sediments (Table S1), the net N\textsubscript{2}O emission in \textit{in situ} sediments also appears to have been suppressed by the lower abundance of microbes possessing nitric oxide reductase, which produces N\textsubscript{2}O.

**Sulfide scavengers for N\textsubscript{2}O reduction in sulfidic sediments**

Laboratory incubation tests (Fig. 1) revealed N\textsubscript{2}O absorption by eelgrass sediments, which were black and sulfidic due to the vigorous sulfate-reducing activity of SRB in eelgrass zone (Fig. S1E). The continuous supply of hydrogen sulfide (H\textsubscript{2}S) from the bottom sediment and dissolved dioxygen (O\textsubscript{2}) from surface water appeared to be responsible for the growth of SOB within surface sediments in the eelgrass zone. The abundance of nos\textit{Z} gene clade II in \textit{in situ} sediments in the eelgrass zone was approximately four-fold higher than that in \textit{in situ} non-eelgrass zone sediments (Fig. 2A). However, previous studies reported that sulfide inhibits the activity of N\textsubscript{2}O-reducing microbes (42, 50, 68). Furthermore, the reduction of N\textsubscript{2}O by \textit{Aeranomryxobacter dehalogenans} was inhibited in laboratory culture medium amended with 0.2 mM sulfide (50). Sulfide concentrations may be reduced by SOB activity in seagrass sediments (24, 75). Therefore, SOB may act as sulfide scavengers, reducing sulfide stress for the Nos\textit{Z} enzyme activity of N\textsubscript{2}O-reducing microbes. In addition, the

**Table 1. Average relative taxonomic distribution of nos\textit{Z} gene reads within the \textit{Dechloromonas-Magnetospirillum-\textit{Thiocapsa}} lineage at the genus level.**

| Genus of the lowest E-value (Accession numbers) | Sulfur-oxidizing bacteria | N3 | N2 | N1 | Ni | Ei | E1 | E2 | E3 |
|-----------------------------------------------|--------------------------|----|----|----|----|----|----|----|----|
| \textit{Thiolapillus brandeum} (WP_041065396)  | S                         | 9  | 5  | 15 | 22 | 15 | 10 | 6  | 10 |
| \textit{Candidatus Thiidiazotropha} spp. (WP_069019464, WP_069128191) | S                         | 9  | 16 | 8  | 14 | 10 | 12 | 13 |
| \textit{Solemya viessiana} gill symbiont (OOG42363) | S                         | 9  | 12 | 15 | 2  | 10 | 8  | 6  | 4  |
| \textit{Candidatus Thiisymbion onecisti} (WP_089723768) | S                         | 9  | 5  | 15 | 14 | 8  | 9  | 15 | 4  |
| \textit{Candidatus Thiomargarita} spp. (OAD21140, KHD07088) | S                         | 2  | 8  | 9  | 1  | 7  | 1  | 1  | 0  |
| \textit{Gammaproteobacteria} bacterium LUC14_002_19_P1 (OQQ30387) | S                         | 9  | 8  | 6  | 4  | 5  | 2  | 2  | 0  |
| \textit{Thiocapsa} spp. (EGV19470, CI65417) | S                         | 0  | 2  | 0  | 5  | 4  | 3  | 4  | 4  |
| \textit{Gammaproteobacteria} bacterium LUC003_010 (OQQX42463) | S                         | 9  | 6  | 6  | 1  | 4  | 4  | 6  | 10 |
| endosymbiont of \textit{Ridgea piscesae} (WP_060528325, KRT60036) | S                         | 2  | 3  | 0  | 0  | 3  | 6  | 2  | 0  |
| \textit{Sulfuricella denitrificans} (WP_009206857) | S                         | 0  | 0  | 0  | 0  | 1  | 6  | 1  | 0  |
| \textit{Thioploca ingrica} (BAP57181) | S                         | 0  | 0  | 0  | 0  | 1  | 3  | 0  | 0  |
| endosymbiont of \textit{Tevnia jerichonana} (vent Tica) (EGW55450) | S                         | 0  | 3  | 0  | 1  | 0  | 2  | 0  | 0  |
| sulfur-oxidizing symbiont (WP_005958984) | S                         | 4  | 0  | 3  | 0  | 1  | 0  | 0  | 0  |
| \textit{Sulfuritalea} sp. (AIC84795) | S                         | 2  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| marine sediment metagenome (GAG5455S) | nd                         | 2  | 5  | 0  | 10 | 4  | 10 | 8  | 19 |
| \textit{Sedimenticola} spp. (WP_029133254, WP_048659760) | nd                         | 4  | 5  | 15 | 9  | 5  | 7  | 5  | 7  |
| \textit{Gammaproteobacteria} bacterium RIFOXYD12_FULL_61_37 (OGT89854) | nd                         | 3  | 6  | 0  | 8  | 5  | 9  | 5  | 1  |
| \textit{Magnetospirua} sp. (CCQ73153) | nd                         | 0  | 2  | 0  | 2  | 4  | 0  | 0  | 2  |
| \textit{Magnetopirillum} sp. (CAM47903, CDK98645, BAE51890, KIM60076, OAN53142, OAN47899) | nd                         | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  |
| \textit{Gammaproteobacteria} bacterium HGW-Gammaproteobacteria-1 (PKM46105) | nd                         | 0  | 1  | 3  | 1  | 2  | 4  | 0  | 0  |
| \textit{Thauera phenylacetica} (ENO96869) | nd                         | 0  | 0  | 0  | 0  | 0  | 2  | 1  | 2  |
| \textit{Candidatus Accumulibacter} phosphatis (ACV36679) | nd                         | 0  | 0  | 0  | 0  | 0  | 0  | 2  | 0  |
| \textit{Dechlorosoma} aromatica (AAZ46320) | nd                         | 4  | 2  | 0  | 4  | 0  | 0  | 1  | 1  |
| \textit{Maritimibacter} sp. (WP_085526419) | nd                         | 0  | 0  | 3  | 0  | 0  | 0  | 8  | 2  |
| unclassified bacteria | nd                         | 23 | 11 | 6  | 7  | 6  | 5  | 10 | 19 |

\* Data (%) represent the mean of biologically independent samples (n=3). Only N1 is shown (n=1), and only N3 is shown (n=2).
\* S represents the sulfur-oxidizing bacterium.
\* nd represents not determined.
formation of FeS and FeS\textsubscript{2} appeared to contribute to decreasing sulfide concentrations in eelgrass sediments.

**Production of N\textsubscript{2}O by nitrifiers**

The production of N\textsubscript{2}O is attributed to nitrification and denitrification in marine sediments at low oxygen concentrations (31). In the non-eelgrass zone, laboratory incubation tests indicated net N\textsubscript{2}O emission from incubated sediments (Fig. 1A and B). The abundance of AOB was higher than that of AOA in in situ sediments of the non-eelgrass zone (Fig. 2 and C). This result supports recent findings indicating that AOB outnumbered AOA in estuarine sediments influenced by human activity (39, 76, 82). However, the abundance of AOA markedly increased after the incubation for non-eelgrass sediments only (Fig. 2B). In the non-eelgrass zone, the increased concentration of N\textsubscript{2}O in the headspace of bottles and elevated copy numbers of AOA amo\textsubscript{A} genes after the incubation in both samples without ammonium and supplemented with ammonium (Fig. S4) showed positive correlations ($r=0.922, \ P<0.01$ for sediments incubated without NH\textsubscript{4}Cl; $r=0.774, \ P=0.07$ for sediments incubated with NH\textsubscript{4}Cl). AOA may grow under microaerobic conditions coupled with the production of N\textsubscript{2}O (55). Furthermore, dioxygen was presumed to be quickly depleted in the serum bottles. Therefore, N\textsubscript{2}O production by AOA and AOB appeared to contribute to N\textsubscript{2}O emissions in the non-eelgrass zone in addition to N\textsubscript{2}O production by denitrification. In contrast, net N\textsubscript{2}O emissions from sediments after the incubation were lower in the eelgrass zone than in the non-eelgrass zone (Fig. 1A and B). Furthermore, no significant increase in AOA or AOB amo\textsubscript{A} genes occurred after the incubation in eelgrass sediment bottles (Fig. 2B and C). Therefore, the production of N\textsubscript{2}O by nitrification appeared to be inhibited in incubated bottles from the eelgrass zone.

**Heterotrophic and autotrophic N\textsubscript{2}O reduction**

nos\textsubscript{Z} genes within *Flavobacteriia* were detected from in situ sediments in the non-eelgrass and eelgrass zones (Fig. 3B). *Flavobacteriia* are one of the most abundant populations in aquatic environments (32) including seagrass sediments (12, 17, 73). They are proficient at degrading high-molecular-weight organic matter (32). Recent metagenomic analyses using next-generation sequencers demonstrated that some *Flavobacteriia* possess nos\textsubscript{Z} genes, suggesting their capacity to reduce N\textsubscript{2}O (7, 74, 78). Heterotrophic N\textsubscript{2}O reduction is
preferable to gain energy, such as N\textsubscript{2}O reduction using acetate as the electron donor: 2 N\textsubscript{2}O+1.5 C\textsubscript{2}H\textsubscript{4}O\textsubscript{2}→2 N\textsubscript{2}+HCO\textsubscript{3}→1.5 H\textsuperscript{+} (\Delta G\textsuperscript{°}=–600 kJ per reaction) (37). N\textsubscript{2}O reduction may be one of the preferential reactions to yield energy for Flavobacteria in the absence of dioxygen as an electron acceptor in sulfidic sediments.

Sulfur-oxidizing N\textsubscript{2}O-reducing microbes within nosZ gene clade II were one of the highest populations in sulfidic sediments of the eelgrass zone (Fig. 3B and Table 1). Autotrophic denitrification coupled with sulfide oxidation, 2 NO\textsubscript{3}→5 HS\textsuperscript{−}+7 H\textsuperscript{+}→N\textsubscript{2}+5 S\textsuperscript{2−}+6 H\textsubscript{2}O (\Delta G\textsuperscript{°}=–1,260 kJ per reaction), is a favorable reaction for chemolithotrophs (37). The sulfur-oxidizing gammaproteobacterium T. brandeum (48) and sulfur-oxidizing epsilonproteobacterium S. gordianica (36) have the ability to grow autotrophically with nitrate as an electron acceptor. Since nosZ genes related to T. brandeum and S. gordianica were predominantly detected from sediments in the eelgrass zone in the present study, autotrophic denitrification by SOB also appears to be responsible for the N\textsubscript{2}O sink in sediments in the eelgrass zone. The highest rate of complete denitrification was reported at 40 m within the peak of H\textsubscript{2}S in the marine oxygen minimum zone off Peru, suggesting that autotrophic SOB reduced N\textsubscript{2}O with H\textsubscript{2}S in the oxygen minimum zone (63).

A previous study reported that the N\textsubscript{2}O consumption rates of the heterotropic N\textsubscript{2}O reducers, P. stutzeri, Shewanella loihica, Dechloromonas aromatica, and A. dehalogenans, were 4.16, 0.464, 0.461, and 0.0171 μmol min\textsuperscript{−1} mg biomass\textsuperscript{−1}, respectively (81). Similarly, the N\textsubscript{2}O consumption rate of the autotrophic N\textsubscript{2}O reducer Thiohalorhabdus denitrificans was 180–300 nmol min\textsuperscript{−1} mg protein\textsuperscript{−1} (69). Although no significant differences have been reported in N\textsubscript{2}O reduction rates between heterotrophic and autotrophic N\textsubscript{2}O reducers, the substrate affinity of clade II nosZ N\textsubscript{2}O reducers for N\textsubscript{2}O is generally higher than that of clade I nosZ N\textsubscript{2}O reducers (59, 81). The \textit{K_m} value of soil Flavobacteria sp. for N\textsubscript{2}O was 0.5 μM (6). Thus, the predominance of clade II nosZ N\textsubscript{2}O reducers in eelgrass sediments appears to be due to differences in affinity for N\textsubscript{2}O between clade I and clade II nosZ N\textsubscript{2}O reducers. Since the surface of eelgrass zone sediments was covered with dead leaves (Fig. S1D and E), the heterotrophic denitrifier Flavobacteria may play an important role in the N\textsubscript{2}O sink with organic matter as an electron donor. However, soil Flavobacteria sp. have been shown to produce N\textsubscript{2}O as oxygen tension increases (6). The Flavobacteria nosZ phylotype was detected as the predominant member at the N\textsubscript{2}O peak within marine oxygen-deficient zones in the Eastern Tropical North Pacific (ETNP) (21). Further studies are needed to clarify whether heterotrophic and autotrophic N\textsubscript{2}O reducers contribute to the N\textsubscript{2}O sink in \textit{in situ} eelgrass sediments.

Conclusions

A shotgun metagenomic analysis based on the nosZ gene coupled with quantitative and physiological experiments suggested an N\textsubscript{2}O sink due to the N\textsubscript{2}O-reducing microbiome in sediments of the eelgrass zone. Seagrass meadows are widely distributed along coastal environments worldwide (66). Therefore, N\textsubscript{2}O-reducing microbiomes in seagrass meadows play an important role in the global nitrogen cycle, and have the potential to mitigate N\textsubscript{2}O from coastal environments worldwide. Future studies on the vertical distribution of N\textsubscript{2}O-reducing microbiomes coupled with the vertical dynamics of dissolved N\textsubscript{2}O, sulfide (HS\textsuperscript{−}), NO\textsubscript{3}−, O\textsubscript{2}, ferrous (Fe\textsuperscript{2+}), the oxidation-reduction potential, and stable isotopic composition of NO\textsubscript{3}− (49) in sediments, and also on sulfur-oxidizing symbionts involved in N\textsubscript{2}O reduction are needed in order to provide a comprehensive understanding of the role of seagrass sediment microbiomes as an N\textsubscript{2}O sink. Since rapid precipitation is expected to increase, the effects of NO\textsubscript{3}− outflow by nitrogen fertilizers on the production of N\textsubscript{2}O in eelgrass zone sediments warrant further study.

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