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Published in:
Environmental Science and Technology

Link to article, DOI:
[10.1021/acs.est.9b02237](https://doi.org/10.1021/acs.est.9b02237)

Publication date:
2019

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Suenaga, T., Hori, T., Riya, S., Hosomi, M., Smets, B. F., & Terada, A. (2019). Enrichment, isolation, and characterization of high-affinity N₂O-reducing bacteria in a gas-permeable membrane reactor. *Environmental Science and Technology*, 53, 12101-12112. <https://doi.org/10.1021/acs.est.9b02237>

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**Enrichment, isolation, and characterization of high-affinity
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Journal:	<i>Environmental Science & Technology</i>
Manuscript ID	es-2019-022378.R1
Manuscript Type:	Article
Date Submitted by the Author:	23-Aug-2019
Complete List of Authors:	Suenaga, Toshikazu; Tokyo University of Agriculture and Technology, Chemical Engineering Hori, Tomoyuki; National Institute of Advanced Industrial Science and Technology (AIST), Riya, Shohei; Tokyo Noko Daigaku, Chemical Engineering Hosomi, Masaaki; Tokyo University of Agriculture and Technology, Chemical Engineering Smets, Barth F.; Technical University of Denmark, Environmental Engineering Terada, Akihiko; Tokyo University of Agriculture and Technology, Chemical Engineering

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Hollow fiber membrane

Enrichment reactor

N₂OFR1 v.s. N₂OFR2 v.s. NO₃FR

$N_2O \rightarrow N_2$
N₂O reducing bacteria
clade I, clade II

Enrichment

Isolation

Characterization of
N₂O reducing activity

Manuscript submitted to *Environmental Science and Technology* (Research article)

Enrichment, isolation, and characterization of high-affinity N₂O-reducing bacteria in a gas-permeable membrane reactor

Running title: Enrichment and isolation of high-affinity N₂O reducers

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1 **Abstract**

2 The recent discovery of nitrous oxide (N₂O)-reducing bacteria suggests a potential biological
3 sink for the potent greenhouse gas N₂O. For an application towards N₂O mitigation,
4 characterization of more isolates will be required. Here, we describe the successful enrichment
5 and isolation of high-affinity N₂O-reducing bacteria using an N₂O-fed reactor (N₂OFR). Two
6 N₂OFRs, where N₂O was continuously and directly supplied as the sole electron acceptor to a
7 biofilm grown on a gas-permeable membrane, were operated with acetate or a mixture of
8 peptone-based organic substrates as an electron donor. In parallel, a NO₃⁻-fed reactor (NO₃FR),
9 filled with a non-woven sheet substratum, was operated using the same inoculum. We
10 hypothesized that supplying N₂O vs. NO₃⁻ would enhance the dominance of distinct N₂O-
11 reducing bacteria. Clade II type *nosZ* bacteria became rapidly enriched over clade I type *nosZ*
12 bacteria in the N₂OFRs whereas the opposite held in the NO₃FR. High-throughput sequencing
13 of 16S rRNA gene amplicons revealed the dominance of Rhodocyclaceae in the N₂OFRs.
14 Strains of the *Azospira* and *Dechloromonas* genera, canonical denitrifiers harboring clade II
15 type *nosZ*, were isolated with high frequency from the N₂OFRs (132 out of 152 isolates). The
16 isolates from the N₂OFR demonstrated higher N₂O uptake rates ($V_{\max}$: 4.23×10^{-3} - 1.80×10^{-2}
17 pmol/h/cell) and lower N₂O half-saturation coefficients (K_{m, N_2O} : 1.55-2.10 μ M) than a clade I
18 type *nosZ* isolate from the NO₃FR. Furthermore, the clade II type *nosZ* isolates had higher
19 specific growth rates on N₂O than nitrite as an electron acceptor. Hence, continuously and
20 exclusively supplying N₂O in an N₂OFR allows the enrichment and isolation of high-affinity
21 N₂O-reducing strains, which may be used as N₂O sinks in bioaugmentation efforts.

Introduction

Nitrous oxide (N₂O) emissions are a global concern as N₂O is a potent greenhouse gas as well as a substance with ozone destruction potential^{1,2}. Atmospheric N₂O concentrations have increased at a rate of 0.75 ppb/year during the last century³ and are expected to increase further. Biological nitrogen removal in wastewater treatment plants (WWTPs) has drawn attention as N₂O emissions are more substantial than initially expected⁴. N₂O is produced as a byproduct of ammonia oxidation mediated by ammonia-oxidizing autotrophic microorganisms⁵ and is an obligatory intermediate in denitrification mediated by heterotrophic microorganisms. Studies on the mechanisms of N₂O production^{6–9} and the quantification of N₂O emissions from various treatment bioreactors^{10–12} abound. Whereas these studies have substantially advanced our knowledge, it should be noted that N₂O emissions are the net effect of production and consumption, and a comprehensive understanding of N₂O consumption mechanisms awaits us. Community analyses based on 16S rRNA¹³ and functional gene encoding N₂O reductase (*nosZ*)¹⁴ have been performed to identify microorganisms responsible for N₂O consumption in wastewater treatment bioreactors. Nevertheless, studies on N₂O reducers in WWTPs have so far been limited¹⁵ and further research is necessary to broaden our knowledge of N₂O reducers.

Previously, bacteria harboring the *nosZ* gene, encoding NosZ, were considered canonical denitrifiers, which also catalyze nitrite (and often nitrate) reduction¹⁶. Genomic analyses of N₂O-reducing bacteria have revealed two distinct classes of *nosZ* sequences, allowing for the classification of canonical denitrifiers into two distinct groups^{17,18}. Most well-investigated canonical denitrifiers, *e.g.*, the genera *Bradyrhizobium*, *Pseudomonas*, and *Paracoccus*, possess clade I type *nosZ*. The clade II type *nosZ* is phylogenetically, being present in 14 bacterial phyla¹⁹, and is ubiquitous in natural environments^{17,20}. In addition, non-denitrifying N₂O-reducing

bacteria have recently been identified from soils and sediments^{17,18}. They lack most denitrification enzymes except nitric oxide reductase (Nor) and NosZ, and most of them possess a clade II type *nosZ* gene¹⁹. Such non-denitrifying N₂O reducers, as well as canonical denitrifiers with clade II type *nosZ*, potentially function as N₂O sinks^{15,18,19,21,22}.

N₂O-reducing bacteria that possess clade II type *nosZ* may be better N₂O consumers than those possessing clade I type *nosZ*, because former has a higher affinity for N₂O. Isolates carrying clade II type *nosZ* of the genera *Dechloromonas* and *Anaeromyxobacter* have lower N₂O affinity constants (K_{m,N_2O} =0.324 and 1.34 μ M) than clade I type *nosZ* isolates *Pseudomonas stutzeri* and *Shewanella loihica* (K_{m,N_2O} =35.5 and 7.07 μ M)^{23,24}. However, the kinetics of N₂O reduction have so far been examined for a limited set of isolates: the conclusion that clade II type *nosZ* bacteria always display high affinity for N₂O may be premature. Surprisingly, in a recent chemostat study, clade I type *nosZ* bacteria remained dominant over clade II type *nosZ* bacteria, irrespective of the electron donor or electron acceptor (N₂O) limitation across the examined dilution rates²⁵. In addition, in biofilters operated specifically for N₂O mitigation, the predominant N₂O-reducing bacteria carried clade I type *nosZ*, and their abundances changed with different operating conditions^{26,27}. Clearly, it is not yet fully established which N₂O-reducing bacteria are favored in which environments. Further exploration of N₂O reducers is, therefore, needed.

For enriching N₂O-reducing bacteria, we used gas-permeable membrane biofilm reactors²⁸. The reactor is fed with N₂O as a sole electron acceptor (N₂O-fed reactor or N₂OFR) to enrich unexplored and/or high-affinity N₂O-reducing bacteria. In an N₂OFR (**Fig. 1**), gaseous N₂O is supplied via the membrane lumen directly to the biofilm grown on the outer membrane surface, which results in the countercurrent supply of N₂O and the carbon substrates, provided via the

aqueous phase^{29,30}. We assume that the direct N₂O supply to a biofilm onto a gas-permeable membrane without any bubbles would curb biofilm sloughing and the resultant washout of enriched bacteria^{31,32}, ensuring the retention of diverse N₂O-reducing bacteria possessing various physiological traits in the biofilm without creating any liquid-gas interfaces. This property is distinct from chemostat-based enrichments where bacterial community composition is determined by a dilution rate^{25,33}. In addition, a biofilm-based enrichment system would provide a range of niches along the biofilm depth. Whereas the biofilm base adjacent to the membrane receives high N₂O concentration, the N₂O concentration decreases towards the biofilm surface, providing a local environment where high-affinity N₂O reducers thrive (**Fig. 1**)³⁴. Such stratified environments may select for a range of N₂O-reducing bacteria, possessing various affinities for N₂O.

Hence, we investigated the feasibility to enrich N₂O-reducing bacteria in a gas-permeable membrane reactor by continuously supplying N₂O (N₂OFR) and examined the enrichment yielded diverse or mainly high-affinity N₂O reducers. In parallel with the N₂OFR, a biofilm reactor fed with nitrate (NO₃FR) was operated to examine whether electron donor and acceptor types affect the selection of *nosZ* clade types. The enrichment process was monitored by *nosZ* targeted real-time qPCR and high-throughput sequencing of 16S rRNA gene amplicons, followed by the isolation of N₂O-reducing bacteria and examination of their N₂O reduction activity.

Materials and Methods

Enrichment reactors

Two types of N₂O-fed reactors, one with acetate (N₂OFR1) and one with a peptone-based mixed

91 substrate (N_2OFR_2) as electron donor, and one NO_3^- -fed reactor, with the mixed substrate as
92 electron donor (NO_3FR), were continuously operated for 191 days. N_2OFR s received N_2O as a
93 sole electron acceptor to enrich N_2O -reducing bacteria. NO_3FR received NO_3^- as a sole electron
94 acceptor to enrich canonical denitrifying bacteria. The volume of each enrichment reactor was
95 consistently 94.2 mL. During the operation, limiting and excess amounts of electron acceptors
96 were supplied in Phases 1 (day 0 to 71 in N_2OFR s, day 0 to 38 in NO_3FR) and 2 (day 71 to 191
97 in N_2OFR s, day 0 to 38 in NO_3FR), respectively (**Table 1**). N_2OFR s contained a bundle of gas-
98 permeable hollow fiber membranes (Model MHF 200TL, Mitsubishi Chemical Co. Inc., Tokyo,
99 Japan) mounted in a cylindrical reactor (**Fig. 1, SI**), where the lumen was continuously flushed
100 at 3 mL/min with an $\text{N}_2\text{O} : \text{N}_2$ mixture. N_2O loading rates were set by controlling the N_2O partial
101 pressure; 2.67 ± 0.25 kPa- N_2O in Phase 1 and 38.5 ± 2.3 kPa- N_2O in Phase 2. The N_2O partial
102 pressure was increased to enhance N_2O transfer to the biofilms, developed on the gas-permeable
103 membrane. The N_2O supply rate was calculated based on the specific N_2O transfer coefficient
104 of the used hollow fiber membranes (5.91 mmol- N_2O /day/ m^2 /kPa), attained from an
105 experiment following the previous study³⁰ (**SI**). The expected N_2O concentration in each phase
106 in the absence of microorganism was estimated from the N_2O partial pressure as detailed in
107 **Table 1**. Electron balances in the enrichment reactors were calculated based on the supply rates
108 of electron donors and acceptors as detailed in **SI**. This calculation assumes the respiration (but
109 not fermentation) that electron donor is oxidized to CO_2 whereas NO_3^- and N_2O are reduced to
110 N_2 . In the NO_3FR a non-woven sheet of polyethylene (Nippon Vilene, Tokyo, Japan)³⁵ served
111 as a substratum for biofilm growth and nitrate was supplied via the liquid feed (**Fig. S1**).
112 Hydraulic retention time (HRT) was 7 hours in all reactors. Each reactor was inoculated with
113 10 mL of biomass from a full-scale conventional activated sludge system treating municipal

wastewater treatment (Tokyo Japan), containing, on average, 130 mg/L of BOD, 34 mg-N/L of total nitrogen and 3.4 mg-P/L of total phosphorus. In the inoculum, clade II type *nosZ* was one order of magnitude higher than clade I type (day 0 of **Fig. S3**).

Media composition

The synthetic medium contained (per 1 L of distilled water): 100 mg of KH_2PO_4 , 6.6 mg of NaCl, 8.20 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 13.4 mg of KCl, and 188 mg of NaHCO_3 , supplemented with 1 mL of trace element solution. The trace element solution contained (per 1 L of distilled water): 10 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g of $\text{FeCl}_3 \cdot 7\text{H}_2\text{O}$, 2 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 4 g of $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g of $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.1 g of H_3BO_4 , 0.3 g of Na_2SeO_3 , and 10 g of citric acid³⁶. In addition, the medium was supplemented with organic carbon and nitrogen sources: a mixture of CH_3COONa (170 mg/L) and NH_4Cl (22.8 mg) for $\text{N}_2\text{OFR1}$; a mixture of bacto-peptone (48.8 mg/L), beef extract (64.4 mg/L), dextrin (21.3 mg/L) and yeast extract (62.0 mg/L) for $\text{N}_2\text{OFR2}$; and the same mixture as for $\text{N}_2\text{OFR2}$ plus KNO_3 (253 mg/L) for NO_3FR . All media were sterilized by autoclaving and purged for 20 min with N_2 gas before use. CO_2 stripping during N_2 gas purge was minimal (< 2%).

Chemical analyses

Samples for water quality analyses were centrifuged at $9,900 \times g$ for 10 min, and afterward, the supernatant was filtered using a $0.45 \mu\text{m}$ membrane filter (A045A025A, Advantec, Tokyo, Japan). NO_3^- , NO_2^- and NH_4^+ were analyzed using ion chromatography systems ICS1000 and ICS90 (Dionex, Boston, MA). Dissolved organic carbon (DOC) and dissolved total nitrogen (DTN) were analyzed by a TOC analyzer, installing a total nitrogen measurement unit (TOC-V, Shimadzu, Kyoto, Japan). pH was measured using a portable meter (D-53, HORIBA, Kyoto, Japan) equipped with a pH sensor (9625-10D, HORIBA, Kyoto, Japan). Average pH values in

effluent of N₂OFR1, N₂OFR2, and NO₃FR were 8.01 ($\pm$ 0.40), 7.45 ($\pm$ 0.42), and 8.46 ($\pm$ 0.41), respectively. N₂O concentration in supply gas was determined by a GC-ECD (GC-14B, Shimadzu, Kyoto, Japan).

Biofilm sampling and DNA extraction

Biofilm samples, developed on the hollow fiber membranes of N₂OFR1 and N₂OFR2 (**Fig. S2**), were sampled through a dedicated port (P3 port in **Fig. 1**) by inserting a needle. A large portion of biofilm suspension (2 mL) was removed by suction. The samples were stored at -20°C until DNA extraction. In case of NO₃FR, a 10 $\times$ 10 mm piece of non-woven sheet was cut and taken, followed by dislodging the biomass with a sterile brush for the collection. DNA was extracted using a FastDNA Spin kit (MP Bio., CA) according to the manufacturer's instructions, followed by measurement using Nanodrop2000c (Thermo Scientific, MA). Extracted DNA was stored at -20°C until analysis.

Real-time qPCR analysis

Gene abundance for 16S rRNA, N₂O reduction (i.e., clades I and II *nosZ*) and canonical heterotrophic NO₂⁻ reduction (i.e., *nirS* and *nirK*) were quantified by real-time qPCR with reported primer sets (**Table S1**) using a CFX96 Real-Time PCR Detection System (BioRad Laboratories, CA). PCR conditions for the amplification of genes of 16S rRNA, *nirS*, *nirK*, clade I *nosZ*, and clade II *nosZ* are modified from earlier protocols and described in **SI** ^{16,17,37–39}.

Microbial community analysis

Amplicons of 16S rRNA gene were generated using the universal primers 515f-806r ⁴⁰ and sequenced on a MiSeq DNA sequencer (Illumina, CA) in paired-end sequencing mode. The PhiX, low-quality (Q<30), and chimeric sequences were removed (Mothur ver. 1.31.2) ⁴¹ and

the paired-end sequences were assembled as reported previously⁴². Operational taxonomic units (OTUs) were defined based on a 97% sequence similarity cut-off, and the alpha and beta diversities of libraries were calculated with QIIME (version 1.7.0)⁴³. A PCoA plot was generated from weighted UniFrac analysis of libraries after equalizing the sequence number ($n = 22,580$). Sequences have been deposited in DDBJ under BioProject accession number PRJDB4563 (**Table S2**).

Isolation from the reactor enrichments

Isolation medium was identical to the enrichment medium, solidified by addition of 2 w/v% agar powder (Wako, Saitama, Japan). Besides, diluted-nutrient broth medium and bromothymol blue medium, described elsewhere^{44,45}, were used for isolation from the inoculated activated sludge. The enrichment culture from each reactor was sampled (**Fig. 1**) and serially diluted in phosphate buffered saline after dispersion (PBS, pH 7.5). The diluted suspensions were spread on respective isolation media, followed by 10-day incubation in a 5% N₂O, 20% CO₂, and 75% N₂ atmosphere in an anaerobic chamber (AJ-1, Sanshin, Kanagawa, Japan). Visible colonies were transferred in individual wells of a 96-well plate, followed by re-incubation for 2 weeks in the same condition and liquid media devoid of oxygen. Growth in individual wells was monitored by absorbance of 600 nm (Infinite F50, Tecan, Switzerland), and wells with positive growth were retained for DNA extraction and sequencing^{46–47} with detailed protocols found in **SI**. The 16S rRNA gene sequences of representative isolates were deposited in the DDBJ/Genbank/EBI nucleotide sequence database under accession numbers LC097197 to LC097214.

Biokinetic parameter estimation based on N₂O respirometry

N₂O reduction activities of the isolates were characterized via a respirometric system using N₂O and O₂ amperometric microsensors (Unisense, Aarhus, Denmark). The set-up of the system, which monitors N₂O respiration based on depletion of dissolved N₂O, was reported earlier ⁴⁸. *Azospira* sp. strains I09, I13 and *Dechloromonas* sp. strain I20, acquired from the N₂OFRs, and *Alicyclophilus denitrificans* strain I51 isolated from the NO₃FR, were examined for N₂O respiration kinetics. The preparation of pre-growth and harvesting is described in SI. Because N₂O-reducing activities of *Azospira* sp. strains I09 and I13 decreased by 70-86% by washing with 0.05 × PBS and centrifugation as previously observed ⁴⁸, cell suspensions after pre-growth on enrichment media were neither washed nor centrifuged. Instead, the suspensions were diluted with the media excluding the carbon source and transferred to a double port chamber (volume: 3.06 mL) equipped with O₂ and N₂O microsensors (OX-MR and N2O-MR, response time < 45sec). To initiate the respiration test, 10 µL of a highly concentrated N₂O solution (27.05 mM at 20°C) was injected into the chamber yielding initial dissolved N₂O concentrations between 100-140 µM. After 5 min for intensive mixing of N₂O, organic carbon was spiked. In case of *Azospira* sp. strains I09, I13, and *Dechloromonas* sp. strain I20, 20 µL of 100 mM sodium acetate was added at an initial concentration of 650 µM (in stoichiometric excess compared to N₂O). In case of *Alicyclophilus denitrificans* strain I51, 25 µL of autoclaved broth medium (2,050 mg-C/L), consisting of 2.38 g/L of bacto-peptone and 3.04 g/L of yeast extract, was added. O₂ and N₂O concentrations were continuously recorded at 1 Hz. N₂O concentration profiles were subject to the Smooth 2D-data function of Sigma Plot 13.0 (Systat Software, San Jose, CA) to remove high-frequency noise ⁴⁹. The N₂O profiles were used to estimate instantaneous N₂O reduction rates, v_t , and corresponding N₂O concentrations, C_t . These (v_t , C_t) profiles were fit, using least squared error minimization, with the Michaelis-Menten equation

to estimate maximum N₂O reduction rate $v_{\max}$ and N₂O half-saturation coefficient K_{m,N_2O} . In parallel, the rate of abiotic N₂O loss as the background (v_b : 4.48 μ M/h) was measured without bacterial cells and subtracted from $v_{\max}$. This subtraction allowed to estimate the maximum N₂O reduction rate $v_{\max}$ under anoxic conditions. After N₂O respirometry, the cell number of the bacterial suspension was determined by microscopy (The details provided in **SI** and in previous work ⁵⁰). Cell-specific N₂O reduction rates, $V_{\max}$, were calculated by dividing $v_{\max}$ by the estimated cell numbers.

Maximum specific growth rates were estimated from growth curves, based on OD at 600 nm (OD600) using an online monitoring system (OD Monitor C&T, Taitec, Aichi, Japan), and fitting to equations [1] and [2]:

$$\frac{dX}{dt} = \mu X \quad [1]$$

$$\ln X = \mu t + \ln X_0 \quad [2]$$

, where t is time [h], and X_0 is OD600 at $t=0$. The preparation of isolate cell suspensions and the media composition are described in **SI**.

Results

Changes in chemical parameters during the operation of enrichment reactors

During Phase 1, average dissolved organic carbon removal efficiencies in N₂OFR1, N₂OFR2 and NO₃FR were 76%, 66% and 78%, respectively, and during Phase 2, they improved to 92%, 88% and 84%, respectively (Details, see **Table S3**). Neither NO₂⁻ nor NO₃⁻ was detected in either N₂OFRs during the experimental period (**Table S3**). The supplied N₂O and intruding O₂ contributed 98% and 2%, respectively, of the terminal electron acceptors in Phase 1 of the N₂OFR1 and N₂OFR2 (**Fig. S3** and **SI**).

The transition of microbial communities as revealed by qPCR and high-throughput sequencing

The *nosZ* to 16S rRNA relative gene abundance in the three reactors was monitored by real-time quantitative PCR (**Fig. 2**). The clade II type *nosZ* was at higher abundance in the N₂OFRs than in the NO₃FR, whereas the clade I type *nosZ* was at higher abundance in the NO₃FR than in the N₂OFRs. In both N₂OFR1 and N₂OFR2, clade II type *nosZ* significantly increased in abundance from ca. 10⁴ to ca. 10⁵ copies/ng-DNA around day 70-110 and subsequently decreased (**Fig. S4A, B**). On the other hand, in the NO₃FR, the relative abundance of clade I type *nosZ* increased and afterward remained stable (**Fig. 2C and S4C**). The quantification of the *nosZ* clade II type should be considered semi-quantitative in view of the well-known low PCR amplification efficiency associated with these primers (amplification efficiency: 75.8% here vs. 75%¹⁷ and 70%²⁵).

The microbial community compositions were compared among the three enrichment cultures on the basis of 16S rRNA gene amplicon libraries (**Fig. 3A-C**). The rapid changes in microbial community compositions were (**Fig. 3A-C**) likely caused by the high diversity and evenness of the inoculum (**Table S2**). Proteobacteria and Bacteroidetes dominated all enrichment cultures (**Fig. S5A**). With acetate (in N₂OFR1), the N₂O-reducing enrichments were predominantly comprised of Betaproteobacteria from 13.2% on day 0 to 63.5% on day 79 and steady afterward (**Fig. 3A**). With the peptone-based mixed substrate (in N₂OFR2), the enrichments were dominated by Betaproteobacteria, Gemmatimonadetes, Sphingobacteriia, Bacteroidia, and Clostridia (**Fig. 3B**). The relative abundances of Firmicutes abruptly increased to 36.4% on day 12 whereas that of Bacteroidetes reached a maximum of 40.9% on day 190. The Betaproteobacteria abundance in the N₂OFR2 sharply increased on day 79, with the change

in N₂O-supplying rates (*i.e.*, from day 71) (**Fig. 3B**). In the NO₃FR, Gammaproteobacteria became dominant with relative abundance as high as 55.5% on day 79 (**Fig. 3C**). At the order level, Rhodocyclales dominated the enrichment in N₂OFR1; and were also dominant though less abundant in N₂OFR2 (**Fig. S5B**). Throughout the operation, an OTU phylogenetically close to *Pseudomonas stutzeri*, known as a fast-growing clade I type N₂O-reducing bacterium, was detected at a highest relative abundance of 2.9% on day 12 in N₂OFR1 but thereafter decreased below 1% abundance.

OTUs with relative abundances above 10% at any time of observation are summarized in **Fig. 3D-F**. The analysis identified 7 dominant OTUs for each enrichment. In N₂OFR1, OTUs of the genera *Azospira*, *Dechloromonas* and *Azonexus* (family Rhodocyclaceae, class Betaproteobacteria) together comprised more than 50% of the entire microbial community. The Rhodocyclaceae family was also predominant in N₂OFR2. One specific OTU 8208 (*Dechloromonas* sp.) was enriched in both N₂OFRs, whereas most of the other OTUs were unique to individual N₂OFR. The predominant OTUs in the NO₃FR were phylogenetically distinct from those in N₂OFRs: OTU 12912 (*Pseudoxanthomonas* sp.) was the most dominant member with relative abundance above 38.3%, whereas the abundances of OTU12912 in the N₂OFRs were below 0.6%. The taxonomic information of predominant OTUs (relative abundance > 1%) was summarized as a heatmap image (**Fig. S6**).

PCoA plots indicate that the microbial communities in the three reactors dramatically changed during the first 12 days (**Fig. 4**). Until the end of the operation, a distinctive microbial community was established in each reactor, as the convergence of the microbial communities was observed (**Fig. 4** and **Table S2**). The alpha diversity was lower in N₂OFR1 than N₂OFR2 potentially due to the proliferation of non-denitrifying fermentative bacteria with the peptone-

274 based medium.

275 **Isolation of N₂O-reducing bacteria from the three reactors and activated sludge**

276 In total, 219 isolates were obtained from the inoculum and the enrichments from the N₂OFRs
277 and NO₃FR on days 35 and 191 based on colony growth in a 5% N₂O, 20% CO₂, 75% N₂
278 atmosphere. All isolates were classified in 12 OTUs (at 97% sequence similarity of 16S rRNA
279 genes (**Table S4**)). Hereafter, the isolates are termed ISO_OTU with a number to distinguish
280 the OTUs.

281 ISO_OTU 03, closely related to *Azospira suillum* PS (NR074103) (*i.e.* also called
282 *Dechlorosoma suillum* PS), was the dominant isolate from N₂OFR1 (108/108 isolates) and
283 N₂OFR2 (5/44 isolates). ISO_OTU 07, related to *Dechloromonas* sp. strain JM (AF323491),
284 was the most frequent isolate from N₂OFR2 (19/44 isolates). The phylogeny of these isolates
285 (termed ISO_OTU) is compared with the 16S rRNA from the enrichments (termed
286 ENRICH_OTU) and shown in **Fig. 5**. Based on 16S rRNA gene sequence similarity, the
287 sequence of ISO_OTU 03 is identical (100% sequence similarity) to ENRICH_OTU 10027,
288 the dominant OTU in N₂OFR1. Likewise, ISO_OTU 07 and ENRICH_OTU 8208, which
289 became predominant in both N₂OFRs, have 98.4% similarity. These two ISO_OTUs were not
290 isolated from the inoculum nor from the NO₃⁻ enrichment culture (**Fig. 5**) and can, therefore, be
291 considered unique to the N₂O enrichment. Both ISO_OTUs have a clade II type *nosZ* confirmed
292 by end-point PCR and the *nosZ* sequences affiliated with the Rhodocyclaceae (**Fig. S7**). Besides,
293 these ISO_OTUs harbored a *nirS* gene and displayed NO₂⁻ reduction activity, as confirmed by
294 end-point PCR and physiological testing, respectively (details found in **Table S4**). Hence, the
295 two isolates ISO_OTU03 and ISO_OTU07 appear to be canonical denitrifying types.

296 Isolates from N₂OFR2, which were not retrieved from N₂OFR1, included ISO_OTUs 01,

04, 08, 09, and 10 phylogenetically identical to *Aeromonas hydrophila* (NC_008570), *Alicyclophilus denitrificans* (KM210250), *Acidovorax temperans* (NZ_JXYQ000000000), *Achromobacter* sp. (EU727196), and *Pleomorphomonas koreensis* strain NBRC 100803 (NZ_AULH000000000), respectively. Their closely related ENRICH_OTU exhibited relatively low abundances (0.005-7.5% of the total) in N₂OFR2. End-point PCR confirmed that ISO_OTUs 01, 04, and 08 belong to the *nosZ* clade I lineage (**Table S4**). However, ISO_OTUs 09 and 10 could not be classified in either *nosZ* clade. Their physiological traits could not be further investigated because of our inability to maintain them in cultivation.

From the NO₃FR, ISO_OTUs 02, 04, 08, and 11 are phylogenetically identical to *Stenotrophomonas* sp. (KC252893), *Alicyclophilus denitrificans*, *Acidovorax temperans*, and *Acidovorax* sp. (GU184188), respectively. From the inoculum (WWTP activated sludge), five isolates, termed ISO_OTUs 00, 01, 05, 06, and 08, were obtained. ISO_OTUs 00, 05, and 06 were phylogenetically identical to *Ralstonia pickettii* (NR102967), *Pseudomonas nitroreducens* (NZ_BDAI000000000), and *Neisseriales bacterium* (AB769224), respectively. Except ISO_OTU 02 and ISO_OTU10, isolates from the inoculum and NO₃FR (51/66 isolates) carried the clade I type *nosZ*, and none of them carried the clade II type *nosZ*.

N₂O reduction activities of the isolated strains

As representatives of isolates carrying the clade II type *nosZ*, we examined isolates *Azospira* sp. strains I09 and I13 (ISO_OTU 03) and *Dechloromonas* sp. strain I20 (ISO_OTU 07) for N₂O reduction activity. An isolate of *Alicyclophilus denitrificans* strain I51 (ISO_OTU 04), repeatedly isolated from NO₃FR (**Fig. 5**), was selected as a representative carrying the clade I type *nosZ*. The isolate *Stenotrophomonas* sp. I54 (ISO_OTU 02) was likewise selected as another candidate from NO₃FR. End-point PCR using the currently available primer sets for

320 *nosZ* (**Table S4**), however, suggests that *Stenotrophomonas* sp. I54 does not carry a currently
 321 known *nosZ* gene. Spiking NO_2^- to a vial with *Stenotrophomonas* sp. I54 cell suspension
 322 accumulated N_2O but no further reduction proceeded (data not shown). Hence, this isolate was
 323 subsequently excluded from the N_2O reduction activity test.

324 The N_2O reduction activities of the four isolates (*Azospira* sp. strains I09 and I13,
 325 *Dechloromonas* sp. strain I20 and *Alicyclophilus denitrificans* strain I51) were significantly
 326 inhibited in the presence of O_2 , but N_2O reduction commenced immediately upon O_2 depletion
 327 (**Fig. S8** for *Azospira* sp. strain I09). The cell-specific N_2O reduction rates ($V_{\max}$) of *Azospira*
 328 sp. strains I09 and I13, and *Dechloromonas* sp. strain I20 were $4.23 \pm 2.29 \times 10^{-3}$, 1.79 ± 0.24
 329 $\times 10^{-2}$, and $1.80 \pm 0.46 \times 10^{-2}$ pmol/h/cell, respectively (**Fig. S9** and **Table 2**). The respective
 330 $K_{\text{m},\text{N}_2\text{O}}$ values were 1.55 ± 0.61 , 2.10 ± 0.26 , and 2.04 ± 0.13 μM (**Table 2**). The clade I type
 331 *nosZ* isolate, *Alicyclophilus denitrificans* strain I51, had a lower $V_{\max}$ ($3.78 \pm 2.29 \times 10^{-3}$
 332 pmol/h/cell) than *Azospira* sp. strain I13 and *Dechloromonas* sp. strain I20, and it had a higher
 333 $K_{\text{m},\text{N}_2\text{O}}$ (8.98 ± 1.83 μM) than the clade II type *nosZ* isolates (**Table 2**). The a_0 values of the
 334 isolates *Dechloromonas* sp. strain I20 (ISO_OTU 07) and *Azospira* sp. strain I13 (ISO_OTU03)
 335 were 21.7- and 20.4-fold higher, respectively, compared with that of isolate *Alicyclophilus*
 336 *denitrificans* strain I51 affiliated with the clade I type *nosZ* (**Table 2**). The specific growth rates
 337 (μ [h^{-1}]) of the four isolates obtained in the enrichment reactors differed with electron acceptor
 338 and donor (**Table 2**). The clade II type *nosZ* isolates displayed higher growth rates with N_2O
 339 than nitrite whereas the clade I type *nosZ* isolate showed the opposite pattern (**Table 2**).

340

341 Discussion

342 The effect of electron donor and acceptor on clade I/II type *nosZ* gene abundance

We demonstrate, for the first time, that N₂OFRs with gas-permeable membranes allow for the selective enrichment of N₂O-reducing bacteria especially those carrying the clade II type *nosZ* gene (**Fig. 2A-B**). In light of the long-term operation of N₂OFRs and NO₃FR, niche differentiation between the two distinct *nosZ* clade types in the biofilm reactors appears governed by the type of electron acceptor, *i.e.*, N₂O vs. nitrate, rather than that of electron donor, *i.e.*, acetate vs. peptone-based substrate (**Fig. 2A-B**). Previous research has suggested that oxygen level ⁵¹, affinity for N₂O ²³, dilution rate ²⁵ and pH ^{22,52} are determining factors of clade I and II type *nosZ*. Our results indicate the significance of electron acceptor on the predominance of the clade II type *nosZ*. Our successful enrichment of clade II type *nosZ* bacteria through N₂O supply contrasts with the enrichment of *nosZ* clade I type bacteria in a chemostat continuous culture with N₂O as a sole electron acceptor ²⁵. Another study suggests the effect of chemostat dilution rate on selection of clade I versus clade II type *nosZ* N₂O reducers, with lower dilution rates favoring clade II type *nosZ* types ³³. In biofilm-based systems, hydraulic dilution rates do not control selection of predominant bacteria. Instead, extremely long solid retention times and multiple environmental niches are created in biofilms. Such heterogeneity of environmental conditions enriches not only the limited types of bacteria optimally adapted to one situation (as in a chemostat) but various types of bacteria including high-affinity N₂O reducers (**Fig. 2** and **Fig. 3**).

Supply of nitrate (NO₃FR) enriched clade I type *nosZ* bacteria rather than the clade II type bacteria (**Fig. 2C**), indicating the dominant role of electron acceptor type on the enrichment of the *nosZ* clade in biofilm-based systems. The concomitant increases in *nosZ* clade I and *nirS/K* gene abundances from day 0 to 35 (**Fig. S4C**) suggests the enrichment of canonical denitrifying bacteria.

Despite the enrichment of clade II type *nosZ*, the conditions imposed in N₂OFRs did not favor the growth of non-denitrifying N₂O reducers, consistent with observations in chemostat enrichments^{25,33}. Yoon *et al.* (2016)²⁴ report that the non-denitrifying N₂O-reducer, *Anaeromyxobacter dehalogenans* strain 2CP-C, has one order of magnitude lower $\mu_{\max}$ value than canonical denitrifying N₂O reducers, *Pseudomonas stutzeri* strain DCP-Ps1 and *Dechloromonas aromatica* strain RCB. Conthe *et al.* (2018)²⁵ underpin that N₂O-limitation does not provide a selective advantage for non-denitrifying N₂O reducers. In a recent metagenomic study, Lawson *et al.* (2017)⁵³ detected a clade II type non-denitrifying N₂O reducers of the phylum Chlorobi and suggested it thrives off extracellular proteins, excreted from anammox bacteria in a reactor devoid of external organic carbon supply. The selective growth on extracellular compounds indicates the importance of selecting an electron donor to enrich clade II type non-denitrifying N₂O reducers. Although electron donor choice may not be the sole determinant to enrich non-denitrifying N₂O reducers¹⁵, our observations suggest that canonical denitrifying N₂O reducers thrive when carbon substrate are abundant. The condition between clade II type non-denitrifying and canonical denitrifying N₂O reducers as a function of type and supply of carbon source is needed.

The effect of electron donor and acceptor on predominant bacterial taxa

We show that in a biofilm-based reactor, N₂OFR, fed with N₂O as sole electron acceptor, enriched the genus *Dechloromonas*, canonical denitrifying bacteria possessing the clade II type *nosZ*^{17,18}. Despite our hypothesis that N₂OFRs allow enrichment of phylogenetically diverse N₂O reducers, only Betaproteobacteria were enriched in N₂OFRs. The genus *Dechloromonas* has recently been documented as capable of reducing N₂O^{23,33} but is better known for its ability for anaerobic degradation of (per)chlorate^{54–56} and benzene⁵⁷. In stark contrast, the clade I type

nosZ canonical denitrifiers, e.g., *Pseudomonas stutzeri*, were not detected in either N₂OFR enrichment, even though this bacterium, with high maximum specific growth rate and affinity constant for N₂O²³, was detected as a predominant type in a chemostat enrichment fed with N₂O and acetate²⁵. The difference regarding the presence of *P. stutzeri* between this and previous studies corroborates that a membrane bioreactor, fed with N₂O and acetate, provides niches, just like chemostat reactors, for high-affinity (high a_0) N₂O reducers, possessing a lower maximum specific growth rate and affinity constant for N₂O than *P. stutzeri* (**Table 2**). A detailed explanation of why *r*-strategist N₂O-reducers like *P. stutzeri* are not enriched in biofilm awaits further study.

The peptone-based mixed substrate supplied to N₂OFR2 resulted in the enrichment of a more diverse microbial community including potential N₂O-reducing bacteria (**Table S2**). N₂O-reducing bacteria affiliated with phyla Bacteroidetes, Firmicutes and Gemmatimonadetes were only enriched in N₂OFR2 (**Fig. S5A and S6**). Species of Bacteroidetes and Gemmatimonadetes, e.g., *Gemmatimonas aurantiaca*, recently identified as N₂O-reducing bacteria⁵⁸, are known to degrade proteins^{59–61}. The phylum Chlorobi, often found in anammox systems^{53,62}, was probably not enriched due to the lack of an essential ingredient in the medium⁵³. In addition, some OTUs in N₂OFR2 (e.g., Clostridiaceae and Comamonadaceae^{63,64}) that are neither denitrifying nor N₂O-reducing, are potentially fermentative. These fermentative taxa may contribute to the increase on alpha diversity in N₂OFR2 (**Table S2**). For selective enrichment of a broader set of non-denitrifying N₂O reducers, more complex growth media may need to be provided.

Biokinetics of isolates suggests promising N₂O-reducers

Azospira sp. and *Dechloromonas* sp. isolates were only obtained from N₂OFRs and not from

NO₃FR nor the inoculum, underscoring the significance of the enrichment procedure to attain clade II type *nosZ* canonical denitrifiers. Members of the Rhodocyclaceae, including the genera *Dechloromonas* and *Azospira*, have often been detected or isolated from activated sludge as major heterotrophic denitrifiers^{65–68} and also as autotrophic denitrifiers oxidizing H₂ or Fe²⁺⁶⁹. In this family, the genera *Dechloromonas* and *Azospira* have the documented metabolic property of N₂O reduction^{23,48}. This study extends our knowledge on the microbial species that can perform N₂O reduction in WWTPs¹⁵.

Higher substrate affinity for N₂O (a_0) governs the selection of predominant N₂O-reducing bacteria, supported by the higher a_0 values of the isolates *Dechloromonas* sp. strain I20 (ISO_OTU 07) and *Azospira* sp. strain I13 (ISO_OTU03) (**Table 2**). The high a_0 values are caused by the relatively low K_{m,N_2O} values, which are slightly higher but comparable with the values of known clade II type N₂O-reducing bacteria, such as *Flavobacterium* sp. (0.5 μ M)²⁴ and *Anaeromyxobacter dehalogenans* (1.34 μ M)²³ (**Table 2**). In stark contrast, the K_{m,N_2O} of *Alicyclophilus denitrificans* strain I51 (ISO_OTU 02), an isolate of the clade I type *nosZ* lineage, is much higher (8.98 ± 1.83 μ M) (**Table 2**). Moreover, the clade II type *nosZ* isolates favored N₂O over nitrite as an electron acceptor, in contrast to the clade I type *nosZ* isolate. Our biokinetic analysis supports the hypothesis that clade II type *nosZ* bacteria may function as N₂O sinks more effectively than clade I type *nosZ* bacteria²³.

The dominant isolate *Stenotrophomonas* sp. strain I54 (ISO_OTU 02, 14 out of 45 isolates) phylogenetically identical to ENRICH_OTU9398 (7% at 12 day), enriched in NO₃FR and subsequently isolated in the presence of NO₃⁻, lacks a *nosZ* gene (**Table S4**). Additional analysis with the BLAST genome information⁷⁰ revealed the absence of a *nosZ* motif in the genomes of family Xanthomonadaceae (taxid: 32033) (details in **SI**). Continuous NO₃⁻ feeding

does not necessarily lead to success in enriching N₂O-reducing bacteria, but may also enrich partial denitrifiers.

The isolates affiliated with both *nosZ* clade I and clade II span a limited phylogenetic range, suggesting bias due to the cultivation method. Different cultivation methods might be required to access more phylogenetically diverse isolates.

The implication for active N₂O-reducing bacteria

The successful enrichment of high-affinity N₂O-reducing bacteria in this study could pave the way for a novel N₂O mitigation strategy in WWTPs⁴. As a means to alleviate N₂O emissions from WWTPs, bioreactor conditions have been optimized that would enrich N₂O-reducing bacteria and passage of exhaust gas through an N₂O-reducing biofilter has been explored^{26,71}. In addition, N₂O mitigation based on bioaugmentation by N₂O-reducing strains has been proposed^{52,72,73}. The previous augmentation with N₂O-reducing bacteria was not successful in the long-term⁷⁴, likely due to the discrepancy between the actual reactor conditions and optimal conditions for bacterial activity. Given that dissolved N₂O concentration in activated sludge systems ranges from 0.02 to 10 μM (**Fig. S10**), the high K_{m,N_2O} value, *e.g.*, 35.5 ± 9.3 μM for *P. stutzeri* DCP-Ps1²³, would not suffice for *in situ* N₂O reduction despite the high V_{max} . The lower K_{m,N_2O} found in the clade II type *nosZ* bacteria *Dechloromonas* sp. and *Azospira* sp. (**Table 2**) presents a clear advantage over the clade I type *nosZ* bacteria especially in the N₂O concentration range observed in wastewater treatment bioreactors (**Fig. S10**). Non-denitrifying N₂O-reducing bacteria, *e.g.*, *Anaeromyxobacter* sp., isolated from rice paddies⁷⁵, probably have too low specific growth rates and prefer to thrive in oligotrophic environments. Yet, canonical denitrifiers with high N₂O affinities, *i.e.*, *Dechloromonas* sp. and *Azospira* sp., as identified here, may operate as effective N₂O sinks under conditions of high-nitrogen turnover conditions.

Together, the clade II type *nosZ* bacteria possessing canonical denitrifying genes (*nirS* or *nirK*) are plausible candidates for N₂O mitigation in engineered bioreactor systems.

By supplying N₂O via a gas-permeable membrane in continuous-flow reactors, we can selectively enrich N₂O-reducing bacteria possessing the clade II type *nosZ*. In the N₂OFRs, the *Dechloromonas* and *Azospira* genera of the Rhodocyclaceae family became dominant. From the enrichment, we successfully isolated bacterial types of these genera with a high degree of success (132 out of 152 isolates). Representative isolates of both genera, exhibited higher N₂O affinities than the typical clade I *nosZ* canonical denitrifying bacteria. At the dissolved N₂O concentration range observed in WWTPs, the biokinetic traits of these representative strains show excellent potential to serve as an N₂O sink. This study, therefore, underscores the success of enrichment by a membrane-based N₂O-supplied biofilm reactor permitting the isolation of clade II type *nosZ* N₂O-reducing bacteria.

Conflict of Interest

The authors declare that there are no conflicts of interest in this manuscript.

Acknowledgments

We acknowledge Dr. Tomo Aoyagi, Dr. Kang Song, Ms. Kanako Mori and Ms. Fumi Shimura for technical support on reactor operation and 16S rRNA gene amplicon sequencing. This work was supported by the Grant-in-Aid for Challenging Exploratory Research (26630420, 16K12616), the Grand-in-Aid for Scientific Research (17H01893), the Grand-in-Aid for JSPS Fellows (16J08601), the Open Partnership Joint Research Projects (Japan-Denmark) from Japan Society for the Promotion of Science (JSPS), and the Danish Free Research Council

(N2OMAN grant nr. DFF-1335-00100)

Supporting Information

The additional information of materials and methods, Fig.S1-S10 and Table S1-S4 are supplied as Supporting Information.

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Fig. 1. Schematic diagram of an N_2O -fed reactor

Fig. 2. Dynamics of relative abundances of *nosZ* clade I type (triangle) and clade II type (square) versus 16S rRNA genes in (a) N₂OFR1, (b) N₂OFR2 and (c) NO₃FR. The value on day 0 refers to the inoculum.

Fig. 3. Structural changes of microbial communities at the class level (Proteobacteria) in (a) N₂OFR1, (b) N₂OFR2, and (c) NO₃FR and at the OTU level in (d) N₂OFR1, (e) N₂OFR2, and (f) NO₃FR. OTUs with relative abundances more than 10% were extracted from the 16S rRNA gene amplicon sequencing results as dominant species (in bold OTU belonging to family Rhodocyclaceae). The name of each OTU was chosen after the closest species in BLAST analysis. (g) Principal coordinate analysis (PCoA) plot of amplicon-sequencing data. The statistical analysis was performed by UniFrac analysis of equal numbers of the bacterial sequences ($n = 22,580$), and the dot size reflects alpha-diversity (Shannon, **Table S2**).

Fig. 4. Variation of principal coordinate analysis (PCoA) plot of deep-sequencing data. The statistical analysis was performed by UniFrac analysis of equal numbers of the bacterial sequences (n = 22,580), and the plot size reflects alpha-diversity (Shannon, Table S2).

Fig. 5. Phylogenetic tree based on the 16S rRNA gene sequences from community DNA in each reactor (ENRICH_OTU **) and isolated strains (ISO_OTU **). Scale bar represents nucleotide substitutions per site. Bootstrap values are derived from 1000 replicates, indicated as >750 (closed circles) and in the range of 500 to 750 (open circles) at each node. Isolated strains from inoculum activated sludge are in column with label “AS”.

Table 1. Reactor setup and operating conditions

Reactor	Electron donor	Electron donor supply [gC/L/day] (Influent organic carbon concentration [mgC/L])	Electron acceptor	Electron acceptor supply [gN/L/day] (Influent nitrogen concentration [mgN/L])		Electron donor/acceptor supply ratio [gC/gN] (e ⁻ -eq/e ⁻ -eq)	
				Phase 1 *	Phase 2 **	Phase 1	Phase 2
N ₂ OFR1	Acetate	0.184 (53.7 ± 7.0)	N ₂ O	0.147 (0.404) ^{***}	2.12 (5.83) ^{***}	1.24 (5.83)	0.0865 (0.404)
N ₂ OFR2	Mixed substrate	0.248 (72.5 ± 11.1)	N ₂ O	0.147 (0.404) ^{***}	2.12 (5.83) ^{***}	1.68 (9.85)	0.117 (0.683)
NO ₃ FR	Mixed substrate	0.229 (66.9 ± 16.6)	NO ₃ ⁻	0.134 (39.1 ± 7.2)	0.392 (114.5 ± 8.3)	1.71 (2.00)	0.584 (0.682)

*: N₂OFRs: 0 - 71 day, NO₃FR: 0 - 38 day

**: N₂OFRs: 71 - 191 day, NO₃FR: 38 - 191 day

***: Estimated maximum equilibrium concentration from N₂O partial pressure

Table 2 N₂O reduction activity of reference and newly isolated strains. *n.d.* represents no growth.

Isolated strains	<i>nosZ</i> type	$V_{\max}$ [$\times 10^{-3}$ pmol/hr/cell]	K_{m,N_2O} [μ M]	a_0 [$\times 10^{-9}$ L/cell/hr]**	μ [h ⁻¹]			Reference
					N ₂ O-Acetate	N ₂ O-Pep.,Yeast ext.	NO ₂ ⁻ +Pep.,Yeast ext.	
<i>Dechloromonas</i> sp. I20 (ISO_OTU 07)	clade II	18.0 $\pm$ 4.6	2.04 $\pm$ 0.13	8.82	0.198	0.328	0.155	This study
<i>Azospira</i> sp. I09 (ISO_OTU 03)	clade II	4.23 $\pm$ 2.29	1.55 $\pm$ 0.61	2.73	0.0822	0.157	0.0922	This study
<i>Azospira</i> sp. I13 (ISO_OTU 03)	clade II	17.9 $\pm$ 2.4	2.10 $\pm$ 0.26	8.53	0.159	0.259	0.210	This study
<i>Flavobacterium</i> sp.	clade II	-	0.5	-	-	-	-	76
<i>Dechloromonas aromatica</i> RCB	clade II	7.748 $\pm$ 0.57*	0.324 $\pm$ 0.078	23.9	0.182	-	-	23
<i>Anaeromyxobacter dehalogenans</i> 2CP-C	clade II	0.287 $\pm$ 0.040*	1.34 $\pm$ 0.35	2.14	0.00762	-	-	23
<i>Alicyclophilus denitrificans</i> I51 (ISO_OTU 04)	clade I	3.78 $\pm$ 2.29	8.98 $\pm$ 1.83	0.421	<i>n.d.</i> ***	0.0910	0.180	This study
<i>Shewanella loihica</i> PV-4	clade I	7.49 $\pm$ 0.40*	7.07 $\pm$ 1.13	1.06	0.0892	-	-	23
<i>Pseudomonas stutzeri</i> DCP-Ps1	clade I	69.9 $\pm$ 7.4*	35.5 $\pm$ 9.3	1.96	0.326	-	-	23
<i>Paracoccus denitrificans</i>	clade I	-	5	-	-	-	-	77
<i>Pseudomonas denitrificans</i>	clade I	-	30 – 60	-	-	-	-	78
<i>Pseudomonas aeruginosa</i>	clade I	-	100	-	-	-	-	79
<i>Bradyrhizobium japonicum</i>	clade I	0.085	-	-	-	-	-	80
<i>Paracoccus denitrificans</i>	clade I	2.33 - 2.89	-	-	-	-	-	81

* Converted using the 0.28 pg dry-weight per bacterial cell (Madigan and Martinko, 2006)

** Attained dividing $V_{\max} \times 10^{-9}$ by K_{m,N_2O}

*** *n.d.* represents no growth.