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Organic carbon determines nitrous oxide consumption activity of clade I and II *nosZ* bacteria: Genomic and biokinetic insights

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ABSTRACT

Harnessing nitrous oxide (N_2O)-reducing bacteria is a promising strategy to reduce the N_2O footprint of engineered systems. Applying a preferred organic carbon source as an electron donor accelerates N_2O consumption by these bacteria. However, their N_2O consumption potential and activity when fed different organic carbon species remain unclear. Here, we systematically compared the effects of various organic carbon sources on the activity of N_2O-reducing bacteria via investigation of their biokinetic properties and genomic potentials. Five organic carbon sources—acetate, succinate, glycerol, ethanol, and methanol—were fed to four N_2O-reducing bacteria harboring either clade I or clade II *nosZ* gene. Respirometric analyses were performed with four N_2O-reducing bacterial strains, identifying distinct shifts in DO- and N_2O-consumption biokinetics in response to the different feeding schemes. Regardless of the N_2O-reducing bacteria, higher N_2O consumption rates, accompanied by higher biomass yields, were obtained with acetate and succinate. The biomass yield (15.45 ± 1.07 mg-biomass mmol-N_2O^{-1}) of *Azospira* sp. strain I13 (clade II *nosZ*) observed under acetate-fed condition was significantly higher than those of *Paracoccus denitrificans* and *Pseudomonas stutzeri*, exhibiting greater metabolic efficiency. However, the spectrum of the organic carbon species utilizable to *Azospira* sp. strain I13 was limited, as demonstrated by the highly variable N_2O consumption rates observed with different substrates. The potential to metabolize the supplemented carbon sources was investigated by genomic analysis, the results of which corroborated the N_2O consumption biokinetics results. Moreover, electron donor selection had a substantial impact on how N_2O consumption activities were recovered after oxygen exposure. Collectively, our findings highlight the importance of choosing appropriate electron donor additives for increasing the N_2O sink capability of biological nitrogen removal systems.

1. Introduction

Nitrous oxide (N_2O) is an important ozone-depleting substance and powerful greenhouse gas that is approximately 265 times more potent than CO_2 (Ravishankara et al., 2009; IPCC, 2014). The atmospheric N_2O concentration has gradually increased by 0.73 ppb per year over the last three decades (Tian et al., 2020). Although N_2O emissions are mainly derived from agricultural activities (Reay et al., 2012), the contribution from wastewater treatment plants (WWTPs) should be closely monitored. The necessity for this is indicated by recent reports claiming that the amount of N_2O emitted during biological nitrogen removal in WWTPs accounts for 0–14.6% of a total nitrogen load and is more substantial than initially expected (Law et al., 2012; Yoon et al., 2017). N_2O is produced during biological nitrogen removal in WWTPs, e.g., from ammonia oxidation, nitrifier denitrification, and incomplete heterotrophic denitrification (Kampschreur et al., 2009). Therefore, recent

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studies have investigated strategies for N_2O emission mitigation, e.g., upgrading the process design to actively remove N_2O in the waste gas stream (Yoon et al., 2017), implementing a refined N_2O monitoring campaign (Gruber et al., 2019), and adjusting operating conditions of WWTPs to reduce N_2O production by activated sludge (Chen et al., 2020; Duan et al., 2021).

Biological mitigation of N_2O emissions is attributed to heterotrophic bacteria harboring *nosZ* gene, which encodes N_2O reductase (N_2OR); this enzyme catalyzes N_2O reduction to N_2 (Sanford et al., 2012; Thomson et al., 2012; Yoon et al., 2016). The *nosZ* gene phylogeny has been distinctively divided into two clades owing to differences in the amino acid sequences. In addition to the canonical clade I *nosZ*, a new lineage of *nosZ* gene (the atypical clade II *nosZ* gene) has been proposed and is found in diverse microbes with unique N_2O metabolism (Sanford et al., 2012; Jones et al., 2013). A recent survey indicates that the clade II *nosZ* gene is more abundant and widespread in engineered systems (Kim et al., 2020a), and clade II type bacteria likely contribute to N_2O consumption due to their higher affinities for N_2O (Zhao et al., 2019). According to enrichment and isolation works (Conthe et al., 2018; Suenaga et al., 2019), clade II type N_2O-reducing bacteria, e.g., *Dechloromonas* and *Azospira*, are promising as a N_2O sink in engineered systems. These clade II N_2O-reducing bacteria likely have a higher growth yield (Yoon et al., 2016), higher affinities for N_2O (Suenaga et al., 2019; Yoon et al., 2016), and N_2OR that is more resilient to oxygen exposure (Suenaga et al., 2018b). Thus, identifying factors that enhance the activities of these effective N_2O-reducing bacteria could lead to effective strategies to curb N_2O emissions.

Carbon sources externally added as electron donors, e.g., ethanol, methanol, and acetate, govern denitrification performances (Adouani et al., 2010; Song et al., 2015; Carlson et al., 2020) and the amount of N_2O emissions in a denitrifying bioreactor (Baytshtok et al., 2009; Song et al., 2015). Although methanol has been widely used for denitrification because it is a cost-effective electron donor (Hallin et al., 2006; Pan et al., 2013), alternatives can be potentially applied based on availability and accessibility. Glycerol, a byproduct of biodiesel production, exhibits promise to replace methanol and ethanol as an electron donor for mixed-culture biomass (Bodík et al., 2009; Schroeder et al., 2020). Despite the practical implementation of an external carbon supply for heterotrophic denitrifying bacteria, their N_2O consumption kinetics and performances fed with different carbon sources have not been systematically investigated. Therefore, further exploration of various carbon sources on the biokinetics of N_2O consumption as the final step of denitrification is necessary.

The long-term continuous supply of an external carbon source as an electron donor shapes denitrifying community compositions (Baytshtok et al., 2009; Carlson et al., 2020). For instance, *Paracoccus, Methylophilus*, and *Methyloversatilis* spp. thrive in methanol-fed conditions (Hallin et al., 2006; Kim et al., 2020b), while *Acidovorax, Comamonas*, and *Thauera* spp. thrive in acetate-fed conditions (Osaka et al., 2006; Lu et al., 2014). Such distinct community compositions suggest that the organic carbon source to improve denitrification (including N_2O consumption) should be carefully selected. Because attaining energy from a supplemented carbon source primarily depends on the genotypes of the denitrifying bacteria (Ribera-Guardia et al., 2014), the presence or absence of genes necessary for metabolizing particular carbon sources will determine the predominant and active denitrifying bacteria and may determine the amount of N_2O emissions. Genome-centric investigations have been performed on a pure culture of denitrifier (Silva et al., 2020) and denitrifying consortia (Orschler et al., 2021), providing profound insights into the organic carbon metabolic potentials in denitrifiers. Nevertheless, no experimental validation has been reported regarding organic carbon effects on N_2O consumption activity, and the knowledge gap between genomic information and the physiological data pertaining to coupling of N_2O reduction and oxidation of organic electron donors remains to be bridged. Filling this gap is of significant importance because WWTPs receive highly mixed organic carbon sources with varying flow rates and concentrations (Kim et al., 2020b; Song et al., 2014). Therefore, as the first step forward, determining the biokinetics of N_2O-reducing bacteria when fed with various organic carbon species can improve our understanding of N_2O emissions from WWTPs supplemented with organic feed.

This study investigated the effects of different organic carbon sources on N_2O consumption biokinetics of canonical denitrifying clade I and clade II type N_2O-reducing bacteria. Given that availability of organic carbon sources is one of the factors that primarily determine N_2O consumption potentials, this study deciphered, revisited, and compared the genomes of the *nosZ*-possessing denitrifying bacteria to acquire insights into their metabolic potentials. Organic carbon sources that are frequently used as an external electron donor for heterotrophic denitrification (acetate, succinate, glycerol, ethanol, and methanol) were added to bacterial cell suspensions. We hypothesized that the organic electron donor may be one of the factors that govern (1) the N_2O-associated biokinetics (e.g., maximum specific growth rate and half-saturation coefficient) and (2) the resilience of N_2O consumption activity to oxygen exposure, in a differential manner in different N_2O-reducers, depending on the energy metabolism-associated gene set they harbor. To verify these hypotheses, we performed online monitoring of N_2O and dissolved oxygen (DO) concentrations in microbial cultures incubated under anoxia with and without oxic intervals, using a micro-respiration system. Furthermore, we queried the genomic libraries of the examined bacteria for genes with possible association with the observed biokinetic data. The findings of this study can contribute to the development of effective mitigation strategies for N_2O produced and emitted from heterotrophic denitrification in WWTPs.

2. Materials and methods

2.1. Bacterial strains and cultivation conditions

The two canonical denitrifiers (*Paracoccus denitrificans* NBRC102528 and *Pseudomonas stutzeri* JCM5965, harboring the clade I *nosZ* gene, were acquired from Biological Resource Center at National Institute of Technology and Evaluation in Japan and Japan Collection of Microorganisms, respectively. Two *Azospira* isolates (*Azospira* sp. strain I13 and *Azospira* sp. strain I09 [NZ_AP021844]) carrying the clade II *nosZ* gene had been enriched and isolated from N_2O-fed bioreactors (Suenaga et al., 2019). The rationale of the selection lies in that (i) both clade I and clade II N_2O reducers were represented, each with two strains, and that (ii) these bacteria are confirmed canonical denitrifying bacteria capable of converting NO_3^- to N_2 via nitrite (NO_2^-), nitric oxide (NO), and N_2O (Yoon et al., 2016; Suenaga et al., 2018b).

The complex medium used for preparation of precultures (hereafter termed preincubation) contained per L: 5.0 g of NaCl, 5.0 g of Bacto™ Peptone (BD-Difco, NJ), and 3.0 g of Oxoid™ Lab-Lemco beef extract (Thermo Scientific, MA). The 50-mL precultures were aerobically incubated at 30 °C for 20–24 h in 100-mL Erlenmeyer flasks shaken at 150 rpm. The bacterial cultures were harvested at the early stationary phase and centrifuged at 4550 g for 5 min. The physiological experiments were performed with a previously described minimal salts medium containing per L: 100.0 mg L^{-1} KH_2PO_4, 188.0 mg L^{-1} $NaHCO_3$, 6.6 mg L^{-1} NaCl, 115.0 mg L^{-1} NH_4Cl, 8.2 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 13.4 mg L^{-1} KCl, and 1 mL L^{-1} trace element solution (Suenaga et al., 2018b). The cell pellets from preincubation were washed with 0.05 × PBS (phosphate buffered saline) and resuspended in 25-mL of this medium for physiological examinations.

2.2. Acquisition and analyses of genome sequences

The genome sequences of *P. denitrificans* NBRC102528 and *P. stutzeri* JCM5965 were downloaded from the NCBI database (accession numbers: NZ_BJUR00000000, RHHC00000000). The genome of *Azospira* sp. strain I13 was previously sequenced in house (Suenaga et al.,

2018a; accession number: NZ_BFBP00000000), and that of the strain I09 was sequenced *de novo*. After aerobic incubation in the preculture medium, *Azospira* sp. strain I09 was harvested by centrifuging at 9100 g for 10 min. The cell pellet was lysed in 10% sodium dodecyl sulfate solution, and DNA was extracted with the phenol-chloroform method (Butler, 2012). RNA and polysaccharide-like contaminants were degraded by cetyltrimethylammonium bromide (10% w/v in 0.7 M sodium chloride solution) and RNaseA (Takara Bio, Shiga, Japan). The remaining DNA solution was further purified with the phenol-chloroform method and precipitated with ethanol. The genomic DNA was dissolved in Tris-EDTA buffer and stored at −30 °C.

Genome sequencing was performed as a hybrid of short-read and long-read sequencing, to ensure high completeness while maintaining high level of accuracy. Short-read Illumina sequencing was performed by Novogene (Beijing, China) on a NovaSeq platform (Illumina, San Diego, CA), generating 150-bp paired-end reads with a targeted throughput of 1 Gb. The adapter sequences and low-quality reads ($Q < 30$) were removed using Trim Galore ver. 0.6.5 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The library for long-read sequencing was prepared with a 1D ligation sequencing kit (SQK-LSK-109; Oxford Nanopore Technologies, Oxford, UK) and sequenced on MinION Mk1B using an R.9.4.1 flow cell. Basecalling was performed using Guppy v3.3.2 using the high-accuracy model (Wick et al., 2019). The adaptor sequences, low-quality reads ($Q < 7$), header (75 bp), and short reads (<1000 bp) were removed using Porechop ver. 0.2.4 (https://github.com/rrwick/Porechop). The short paired-end sequences and the consensus sequences were assembled Unicycler ver. 0.4.7 (Wick et al., 2017).

The gene coding sequences were assigned Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology numbers using GhostKOALA v 2.1 (Kanehisa et al., 2000; 2016), which were subsequently used for prediction of metabolic potentials of the four organisms to utilize the organic species provided as electron donors.

2.3. Micro-respiration system setup and operation

A micro-respirometry system for simultaneous monitoring of DO and N_2O was set up to examine the effect of different organic carbon sources on the biokinetics of N_2O-reducing bacteria (Fig. 1). The cell cultures were incubated in a H-shaped vial (with an effective volume of 10 mL) that can accommodate two microelectrodes and a stir bar (Unisense, Aarhuis, Denmark). Five batch tests were performed per bacterium, each with a different carbon source, i.e., acetate, succinate, glycerol, methanol, or ethanol. At the beginning of each batch test, 10 mL cell suspension was pipetted into the H-shaped vial to which DO and N_2O microelectrodes (Unisense, Aarhus, Denmark) were inserted, leaving no headspace in the vial. Subsequently, 20 μL of concentrated N_2O solution (24 mM at 25 °C) was injected into the vial to achieve an initial N_2O concentration of approximately 50 μmol L^{-1}. The carbon and electron source was added as follows: acetate (320 μmol L^{-1}), methanol (420 μmol L^{-1}), ethanol (210 μmol L^{-1}), glycerol (180 μmol L^{-1}), or succinate (180 μmol L^{-1}). The organic compounds were added in excess to ensure that N_2O reduction activity was not limited by electron donor shortage (Table S1). The micro-respiration system was operated in a water bath maintained at 30 ± 0.2 °C, to allow for comparison with the previous results performed at the same temperature, and stirred at 500 rpm (Yoon et al., 2016; Zhou et al., 2021). N_2O and DO measurements were processed using the Sensor Trace Suite software (Unisense). DO concentration was monitored alongside N_2O concentration, as it is a crucial factor affecting N_2O consumption activity (Massara et al., 2017). After each batch experiment, the cell suspension was fixed by adding 2% glutaraldehyde solution, and the cell count was determined using a microscope (BZ-8100, Keyence, Japan). The details of this procedure are described elsewhere (Lunau et al., 2005). All batch tests were conducted in triplicate.

2.4. Net growth yield measurements

The net biomass yields of the bacterial strains on N_2O were determined for each carbon substrate, as previously described (Sanford et al., 2012; Yoon et al., 2016). A 120-mL glass serum vial was filled with 50 mL of the minimal salts medium (pH 7.5), and the medium was then flushed with 99.99% helium gas for 10 min at 0.1 L min^{-1} to ensure anaerobic incubation condition. Each vial was immediately capped with a rubber plug and sealed with an aluminum crimp. Subsequently, 70 mL of mixed gas (5%/95% of N_2O/N_2) replaced the helium gas in the vial. After sterilization, 2 mL of the preculture suspension and 1 mL of carbon source stock solution were injected into the vial. The carbon source was added in 25-fold excess of N_2O in terms of electron equivalents. The cultures were incubated at 30 °C with shaking at 120 rpm. After the N_2O was completely consumed, 40 mL of the cell suspension was filtered through a 0.22 μm pore size polyvinylidene fluoride membrane (Merk Millipore, Burlington, MA). The membrane was oven-dried at 105 °C for 24 h and then dried in a desiccator until no further decrease in the filter weight was observed. The weight of the salts on the membranes was measured after treating the membrane at 500 °C for 8 h as previously described by Sanford et al. (2012). The net growth yields were calculated with the following equation (Sanford et al., 2012):

$$Y_{X/S} = \frac{\{(m_2 - m_1) - (m_3 - m_1)\} - \{(m'_2 - m'_1) - (m'_3 - m'_1)\}}{n_{N2O}} \quad (1)$$

where $Y_{X/S}$ is the net growth yield [mg biomass (mmol N_2O)$^{-1}$], m and

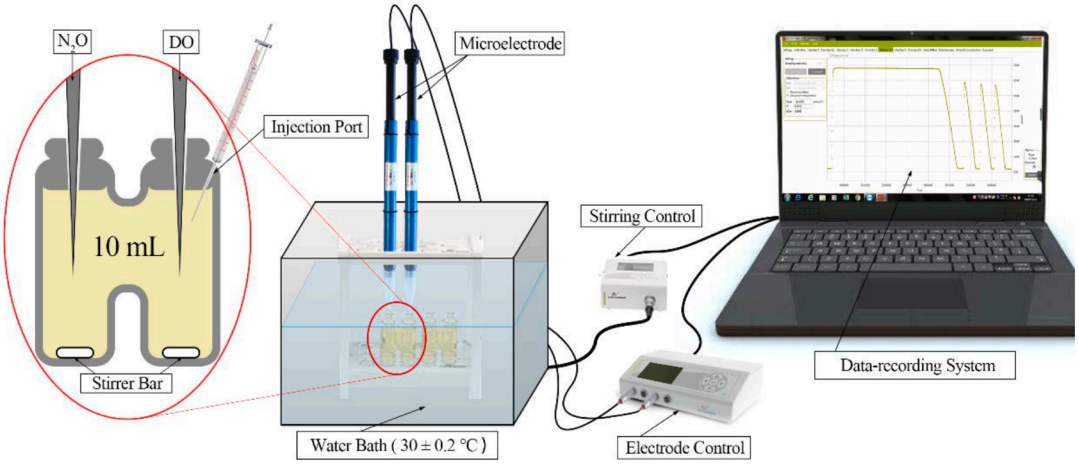

Fig. 1. Diagram of a micro-respiration system.

m' are the weights of the membrane filters measured with the fully grown bacterial culture and the initial bacterial culture, respectively [mg]. The subscripts 1, 2, and 3 denote the pristine membrane [mg], the membrane with retentate after oven-drying (105 °C), and heat-treated (500 °C) membrane with retentate, respectively. n_{N2O} is the amount of N_2O consumed during the incubation [mmol]. The measurement was performed in triplicate.

2.5. Biokinetic parameter calculations

Whole-cell kinetic parameters were estimated by fitting DO and N_2O concentration profiles to the whole-cell Michaelis-Menten equation (Martens-Habbena et al., 2009). As our previous work confirmed that the presence of N_2O had no significant effects on biokinetic parameters associated with DO (Zhou et al., 2021), the possibility of N_2O interference on DO biokinetics was not considered.

$$V = \frac{V_{max} \times [S]}{K_m + [S]} \quad (2)$$

where V is either the consumption rate (measurement interval δ being 15 s) normalized by the number of cells [fmol (cell h)$^{-1}$], V_{max} is the maximum consumption rate [fmol (cell h)$^{-1}$], [S] is the concentration (μmol L^{-1}), and K_m is the whole-cell half-saturation constant (μmol L^{-1}). Nonlinear regression was performed in Origin 9.0 using the least-square method. The specific affinity, as an indicator of effectiveness of N_2O consumption at a low-concentration range, was obtained from the N_2O kinetic parameters using Eq. (3):

$$a_{N2O} = \frac{V_{max,N_2O}}{K_{m,N_2O}} \times 10^{-9} \quad (3)$$

where a_{N2O} is the specific affinity [L (cell h)$^{-1}$], $V_{max,\ N2O}$ is the maximum N_2O consumption rate [fmol (cell h)$^{-1}$], and $K_{m,\ N2O}$ is the whole-cell half-saturation constant for N_2O (μmol L^{-1}).

2.6. Recovery of N_2O consumption activity after DO exposure

The initial presence of DO in the microrespiration experiments enabled observation of N_2O consumption activation (or recovery) following an oxic-to-anoxic transition. As activated sludge in a WWTP, e.g., a Modified Ludzak-Ettinger (MLE) process, is constantly subjected to oxic-to-anoxic transitions and vice versa, such transitional behavior has significant implications to N_2O dynamics in WWTPs (Pomowski et al., 2011; Zhou et al., 2021). After depletion of initially present DO and N_2O, a cycle of adding 20 μL of 25 mmol L^{-1} N_2O solution followed by monitoring N_2O consumption to depletion was repeated until the time-series N_2O concentration profiles were stabilized. The relative N_2O consumption activity during the recovery was estimated by Eq. (4):

$$E = \frac{V_{N_2O}}{V_{max,N_2O}} \quad (4)$$

where E is the relative N_2O consumption activity (dimensionless), and V_{N2O} is the maximum N_2O consumption rate observed during a cycle of N_2O consumption in the course of recovery (fmol (cell h)$^{-1}$). The slope from linear regression of the t-vs-E plot was defined as the reactivation rate of N_2OR, V_{nos} (h^{-1}), serving as a measure of how rapidly N_2O consumption is recovered following oxic-to-anoxic transition under the given condition.

2.7. Analytical and statistical methods

The gaseous N_2O concentration measurements for the determination of net growth yields were taken using a gas chromatography system equipped with an electron-capture detector as described previously (GC-14B, Shimadzu, Japan) (Terada et al., 2013). In accordance with a previous study (Suenaga et al., 2018b), DO and N_2O concentrations were measured using DO and N_2O microelectrodes (Unisense), with detection limits below 0.5 μM and a response time shorter than 0.3 s. The data analysis was performed with Origin 9.0. Statistical analysis was performed using IBM SPSS Statistics version 19.0. The p values lower than 0.05 or 0.01 were considered statistically significant in ANOVA tests.

3. Results

3.1. Genotypes of the tested bacteria regarding organic carbon metabolism

The genome of *Azospira* sp. strain I09 consists of two contigs with 3.77 and 0.397 Mbp lengths, and the completeness is 100% by BUSCO ver. 1 (Simão et al., 2015). The GC content is 64.4%, and 3910 CDSs were detected. Interrogation of the genomes of the four examined N_2O-reducers predicted genomic potentials of these bacteria to utilize various organic electron donors (Fig. 2). All four bacteria possess the genes necessary for metabolizing acetate and succinate to carbon dioxide. *Azospira* sp. strain I13 lacks the gene encoding NAD-dependent alcohol dehydrogenase, which transforms ethanol to acetaldehyde and thus is essential for utilization of ethanol via the tricarboxylic acid (TCA) cycle. The other three strains possess a full suite of functional genes for metabolizing ethanol to CO_2. The two *Azospira* sp. strains lack the genes encoding enzymes that metabolize glycerol, while the genomes of *Pa. denitrificans* and *Ps. stutzeri* harbor the gene necessary for glycerol degradation into dihydroxyacetone phosphate via glycerol-3-phosphate (but not via dihydroxyacetone). The genes encoding glycerol-3-phosphate acyltransferase (EC 2.3.1.15) and acyl-phosphate glycerol-3-phosphate acyltransferase (EC 2.3.1.275) are found in the *Ps. stutzeri* genome, but only the latter is found in the *Pa. denitrificans* genome. Of the four bacteria, only *Pa. denitrificans* harbors a functional gene for methanol conversion into formaldehyde (EC 1.1.1.244).

3.2. Growth characteristics of the N_2O-reducing bacteria

The net biomass yields of the bacteria fed different organic substrates as organic electron donors and N_2O as the sole electron acceptor evaluated the metabolic efficiency of coupling N_2O reduction with oxidation of varying electron donors. Consistent with the findings from the genome analyses (Fig. 2), *Azospira* sp. strains I09, I13, and *Ps. stutzeri* did not consume a significant amount of either methanol or N_2O (Fig. S1). The biomass yield of *Azospira* sp. strain I13 was the highest when fed acetate (5.37 ± 0.48 mg-biomass·mmol-N_2O^{-1}), and this value was 2.87 times higher than the yield observed with ethanol as the electron donor (Fig. 3, Table 1). Acetate generated the largest variance in biomass yields across the organisms. The clade II type *Azospira* strains favored acetate. Their net biomass yields on acetate (15.45 ± 1.07 mg-biomass·mmol-N_2O^{-1} for *Azospira* sp. strain I13 and 11.56 ± 0.62 mg-biomass·mmol-N_2O^{-1} for *Azospira* sp. strain I09) were significantly higher than those of *Pa. denitrificans* (7.88 ± 0.95 mg-biomass·mmol-N_2O^{-1}) and *Ps. stutzeri* (9.13 ± 0.53 mg-biomass·mmol-N_2O^{-1}) ($p < 0.05$). The same trend was obtained with succinate. Among the glycerol-fed cultures, *Ps. stutzeri* exhibited a significantly higher net growth yield than the other three organisms ($p < 0.05$). Net yields were statistically indistinct across the four organisms when ethanol was supplied as the electron donor ($p > 0.05$).

3.3. DO and N_2O respiration dynamics

The choice of organic electron donor significantly affected DO and N_2O respiration dynamics in the four bacterial strains examined. No significant decrease in DO and N_2O concentrations was observed without external organic carbon addition (*Pa. denitrificans* data shown in Fig. S2), indicating that DO and N_2O consumption was coupled with organic carbon oxidation and that the endogenous decay with N_2O was marginal. Regardless of the supplemented organic matter, all four

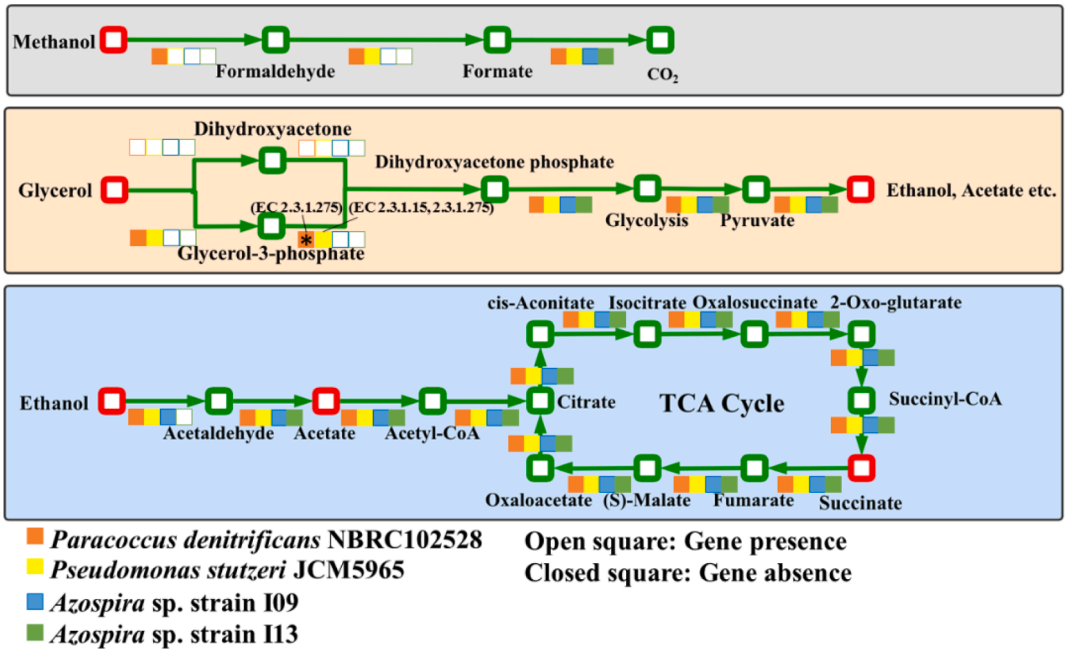

Fig. 2. Carbon source metabolic pathways of *Paracoccus denitrificans* NBRC102528 (orange), *Pseudomonas stutzeri* JCM5965 (yellow), *Azospira* sp. strain I09 (blue), and *Azospira* sp. strain I13 (green). The closed and open squares for each pathway represent the presence and absence of the corresponding functional genes, respectively. * *Ps. stuzeri* harbors the genes for glycerol-3-phosphate acyltransferase (EC 2.3.1.15) and acyl-phosphate glycerol-3-phosphate acyltransferase (EC 2.3.1.275), whereas *Pa. denitrificans* harbors only EC 2.3.1.275.

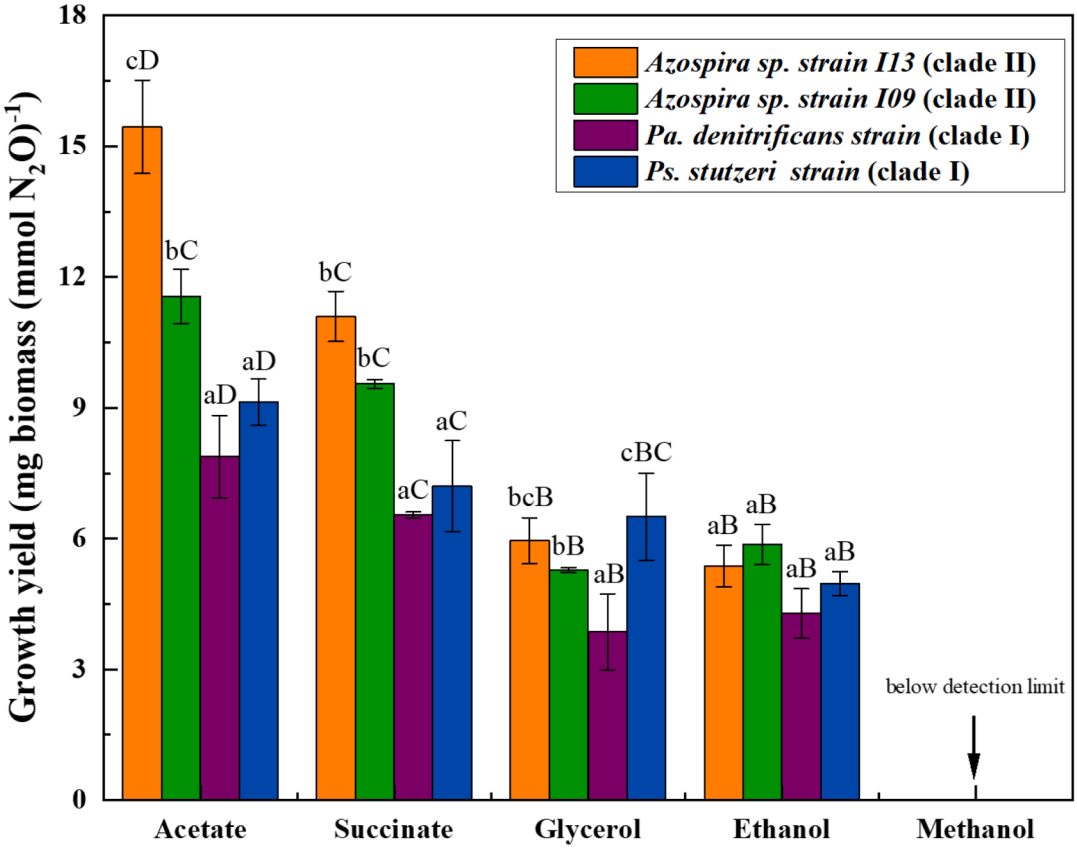

Fig. 3. Net growth yield of the tested bacteria harboring the clade I or clade II *nosZ* gene with different carbon sources in batch culture experiments. The standard errors obtained from the triplicate experiments. Different lowercase and uppercase letters represent significant differences among the bacteria with each carbon source and among the carbon sources within each bacterium at the 0.05 probability level.

Table 1
Summary of N_2O-reducing bacteria biokinetic parameters obtained with a micro-respiration system.

Strains	*nosZ* clade	Carbon source	$K_{m,\ DO}$[μmol L^{-1}]	$V_{max,\ DO}$[fmol (cell h)$^{-1}$]	a_{DO}[$\times 10^{-9}$ L (cell h)$^{-1}$]	$K_{m,N2O}$[μmol L^{-1}]	$V_{max,N2O}$[fmol (cell h)$^{-1}$]	a_{N2O}[$\times 10^{-9}$ L (cell h)$^{-1}$]	$V_{max,N2O}/V_{max,DO}$[-]
Paracoccus denitrificans NBRC102528	I	Acetate	0.97 ± 0.07	9.58 ± 0.64	9.85	1.10 ± 0.12	0.51 ± 0.04	0.46	0.05
		Succinate	0.82 ± 0.25	8.11 ± 1.36	9.88	3.06 ± 0.37	0.58 ± 0.04	0.24	0.07
		Glycerol	0.73 ± 0.19	3.93 ± 0.17	5.35	0.61 ± 0.11	0.14 ± 0.02	0.19	0.04
		Ethanol	0.96 ± 0.33	4.62 ± 0.10	4.79	0.86 ± 0.18	0.14 ± 0.01	0.16	0.03
		Methanol	0.50 ± 0.08	3.79 ± 0.22	7.60	0.67 ± 0.02	0.21 ± 0.06	0.31	0.06
Pseudomonas stutzeri JCM5965	I	Acetate	9.55 ± 1.81	16.83 ± 1.21	1.76	1.01 ± 0.37	2.66 ± 0.28	2.63	0.16
		Succinate	0.88 ± 0.01	7.79 ± 1.02	8.84	0.95 ± 0.10	2.02 ± 0.20	2.13	0.26
		Glycerol	10.91 ± 2.81	13.86 ± 0.69	1.27	1.52 ± 0.05	2.03 ± 0.11	1.33	0.15
		Ethanol	2.07 ± 0.16	5.80 ± 0.14	2.81	1.12 ± 0.36	0.92 ± 0.13	0.83	0.16
		Methanol	0.84 ± 0.07	5.73 ± 0.20	6.79	0.87 ± 0.10	0.60 ± 0.26	0.69	0.10
Azospira sp. strain I09	II	Acetate	0.64 ± 0.05	3.06 ± 0.13	4.81	0.54 ± 0.05	1.24 ± 0.05	2.28	0.41
		Succinate	0.76 ± 0.01	2.98 ± 0.19	3.90	0.28 ± 0.04	0.97 ± 0.03	3.52	0.33
		Glycerol	0.33 ± 0.13	0.37 ± 0.01	1.14	0.19 ± 0.04	0.47 ± 0.04	2.45	1.27
		Ethanol	0.42 ± 0.01	0.42 ± 0.01	0.99	0.59 ± 0.16	0.42 ± 0.01	0.70	1.00
		Methanol	0.17 ± 0.01	0.18 ± 0.05	1.06	0.84 ± 0.33	0.32 ± 0.01	0.37	1.78
Azospira sp. strain I13	II	Acetate	5.57 ± 0.50	13.35 ± 1.00	2.40	2.12 ± 0.02	18.84 ± 0.70	8.88	1.41
		Succinate	2.19 ± 0.35	9.01 ± 0.09	4.11	5.82 ± 0.18	13.14 ± 1.08	2.26	1.46
		Glycerol	0.27 ± 0.01	0.34 ± 0.09	1.30	0.11 ± 0.01	0.12 ± 0.01	1.16	0.35
		Ethanol	0.25 ± 0.06	0.34 ± 0.07	1.14	0.62 ± 0.14	0.31 ± 0.02	1.64	0.91
		Methanol	0.28 ± 0.02	0.47 ± 0.03	1.65	0.59 ± 0.19	0.18 ± 0.08	0.30	0.38

bacterial strains depleted DO prior to N_2O (Fig. 4; the replicates are shown in Figs. S3 and S4). The repeated injections of N_2O (vertical black arrows in Fig. 4) accelerated N_2O consumption. The highest N_2O consumption rate after each N_2O injection differed and depended on the bacteria type and the supplemented organic carbon (summarized in Section 3.4). For *Azospira* sp. strain I09 fed with acetate (Panel a in Fig. 4), the maximum N_2O consumption rates after the second and third N_2O injections, obtained from the linear approximation of the experiment data, were 143 ± 7% (n = 3) and 201 ± 18% (n = 3) of the rate after the first injection, respectively.

3.4. Biokinetic parameters of N_2O-reducing bacteria

Biokinetic parameters (V_{max} and K_m) of the tested bacteria were estimated by fitting the DO and N_2O concentration profiles (Fig. 4) to the whole-cell Michaelis-Menten equation (Eq. (2)). The V_{max} and K_m values of each bacterium were largely dependent on the added carbon source (Table 1). Higher $V_{max,\ DO}$ and $V_{max,\ N2O}$ were obtained when acetate and succinate were added compared with the values obtained with ethanol or methanol as the electron donor (Table 1, Figs. 5 and 6). *Azospira* sp. strain I13 has the largest standard deviation [6.11 fmol (cell h)$^{-1}$] among the $V_{max,\ DO}$ values determined with five different organic electron donors (Table 1). For comparison, the standard deviations of *Pa. denitrificans* and *Ps. stutzeri* were 2.66 and 5.06 fmol (cell h)$^{-1}$, respectively.

The highest N_2O consumption rates ($V_{max,\ N2O}$) were markedly different across the tested bacteria when different carbon sources were injected. *Pa. denitrificans* exhibited substantially lower $V_{max,\ N2O}$ than the other bacterial strains. The $V_{max,\ N2O}/V_{max,\ DO}$ values for *Pa. denitrificans* were 0.05 (acetate), 0.07 (succinate), 0.04 (glycerol), 0.03 (ethanol), and 0.06 (methanol). Even considering that the number of electrons required to reduce a mole of N_2O is half of the number of electrons required for reducing a mole of oxygen, these values indicate a preference for oxygen as the electron acceptor (Table 1). The corresponding $V_{max,\ N2O}/V_{max,\ DO}$ values for *Azospira* sp. strain I13 were 1.41 (acetate), 1.46 (succinate), 0.35 (glycerol), 0.91 (ethanol), and 0.38 (methanol), showcasing a preference for N_2O as an electron acceptor (Table 1). The variance of $V_{max,\ N2O}$ values measured with *Azospira* sp. strain I13 using different organic carbon species was at least 157-fold higher than that of any other bacterial strain examined. Despite being phylogenetically closely affiliated with *Azospira* sp. strain I13, distinct $V_{max,\ N2O}$ values were obtained from microrespirometry experiments with *Azospira* sp. strain I09 with different organic electron donors (Table 1). Interestingly, only *Ps. stutzeri* exhibited a preference for glycerol as the electron donor. This bacterium exhibited 4–41fold higher $V_{max,\ DO}$ [13.9 ± 0.70 fmol (cell h)$^{-1}$] and 4–17 fold higher $V_{max,\ N2O}$ [2.03 ± 0.11 fmol (cell h)$^{-1}$] than the other three bacteria when fed glycerol.

The whole-cell half-saturation constants (K_m) and specific affinities (a_{DO} and a_{N2O}) of the tested bacteria also varied substantially depending on the organic carbon source (Table 1). The minimum and maximum $K_{m,\ DO}$ values ranged from 0.84 ± 0.07 μmol L^{-1} (methanol) to 10.91 ± 2.81 μmol L^{-1} (glycerol) for *Ps. stutzeri* (clade I *nosZ*), and 0.25 ± 0.06 μmol L^{-1} (ethanol) to 5.57 ± 0.50 μmol L^{-1} (acetate) for *Azospira* sp. strain I13 (clade II *nosZ*). Even with the same carbon source, the K_m was significantly different across the tested bacteria. For acetate addition, the $K_{m,\ DO}$ ranged from 0.64 ± 0.05 μmol L^{-1} for *Azospira* sp. strain I09 (the lowest) to 9.55 ± 1.81 μmol L^{-1} for *Ps. stutzeri* (the highest).

The specific affinities for DO (a_{DO}) of *Pa. denitrificans* were higher than those of the other tested bacteria, while those for N_2O (a_{N2O}) were much lower, suggesting once again that *Pa. denitrificans* favors DO over N_2O (Table 1). Acetate-fed *Azospira* sp. strain I13 exhibited the highest a_{N2O} value (8.88×10^{-9} L (cell h)$^{-1}$), which was 19 times higher than the a_{N2O} value of acetate-fed *Pa. denitrificans*.

3.5. Recovery of N_2O consumption after DO exposure

Comparison of the relative N_2O consumption activities, E (Eq. (3)), and the N_2OR reactivation rates, V_{nos} (h^{-1}), evaluated the effect of different electron donors on recovery of N_2O consumption activity following the oxic-to-anoxic transition in the four bacterial strains. Regardless of the bacterium examined or the electron donor added, N_2OR activity increased linearly with time (Fig. 7). The four bacterial strains exhibited noticeably varied E values (Table S2). Intriguingly, the two *Azospira* strains had substantially differing E and V_{nos} values (Fig. 7). With acetate as the electron donor, the time required for complete recovery of N_2O consumption activity (i.e., E = 1.0) was 5.46 h, 4.34 h, 2.73 h, and 1.37 h for *Pa. denitrificans, Ps. stutzeri, Azospira* sp. strain I09, and strain I13, respectively (Table S2). The recovery time differed depending on the supplemented organic carbon source. Also notable were the consistently high reactivation rates observed under the acetate-fed condition in all four bacteria. The V_{nos} values measured with acetate as the electron donor were 0.11 ± 0.01, 0.23 ± 0.02, 0.24 ± 0.01, and 0.35 ± 0.03 h^{-1} for *Pa. denitrificans, Ps. stutzeri, Azospira* sp. strain I09,

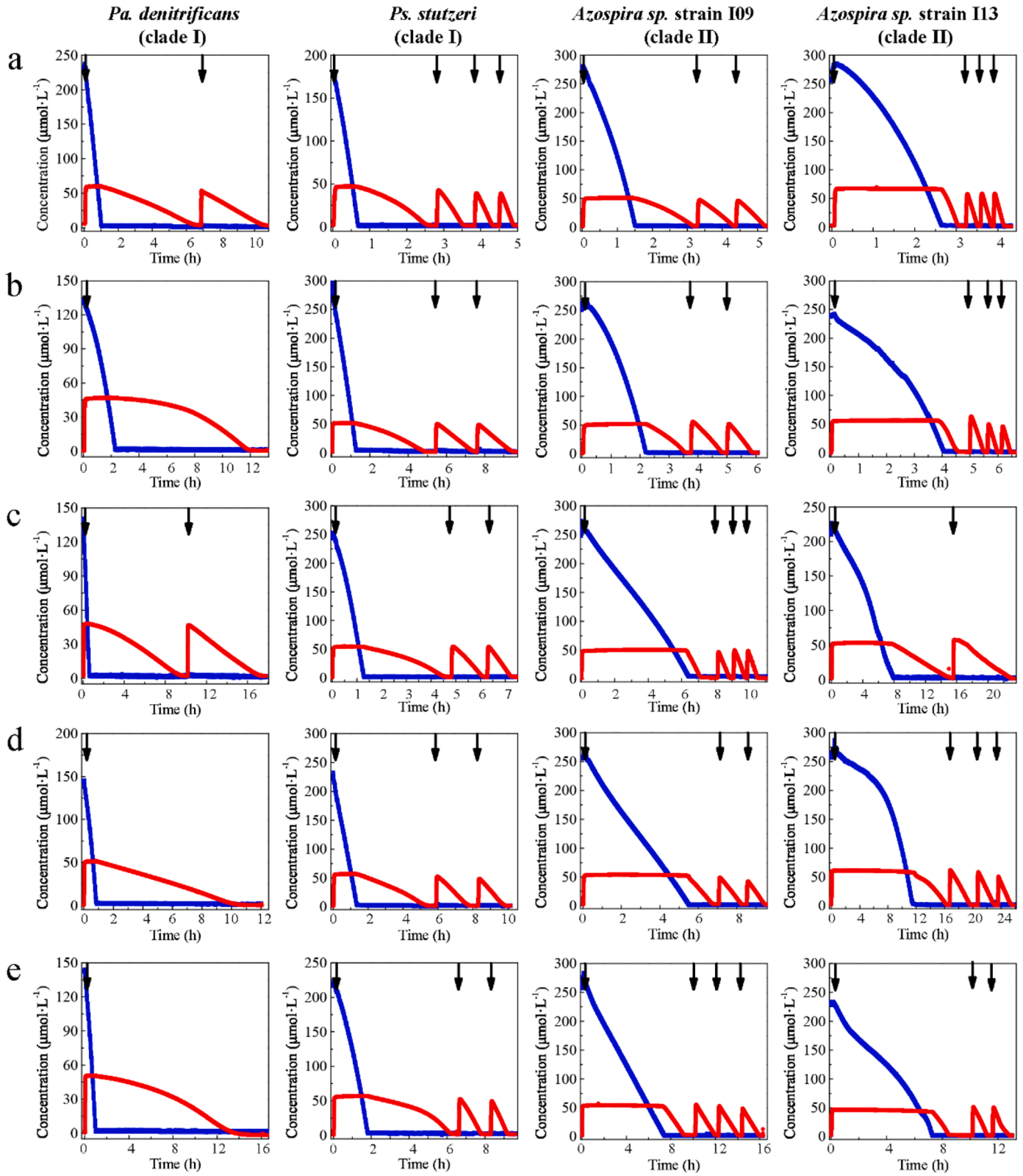

Fig. 4. Dynamics of N_2O (red) and DO (blue) concentration profiles in cultures of *Pa. denitrificans* (clade I), *Ps. stutzeri* (clade I), *Azospira* sp. strain I09 (clade II), and *Azospira* sp. strain I13 (clade II) using acetate (a), succinate (b), glycerol (c), ethanol (d), and methanol (e) as carbon sources. N_2O injections are performed by black vertical arrows. Representative N_2O and DO concentration profiles are shown, and the other replicates are presented in the SI (Figs. S1 and S2).

and strain I13, respectively, all significantly higher ($p < 0.05$) than the V_{nos} values measured with any other electron donors for the respective bacteria. These results also demonstrated that *Azospira* sp. strain I13 was the most resilient bacterium against DO exposure among the four bacterial strains examined under the acetate-feeding scheme (Fig. 7). It should be noted that the N_2O-reducers invariably exhibited the highest $V_{max,\ N2O}$ and V_{nos} values when fed acetate.

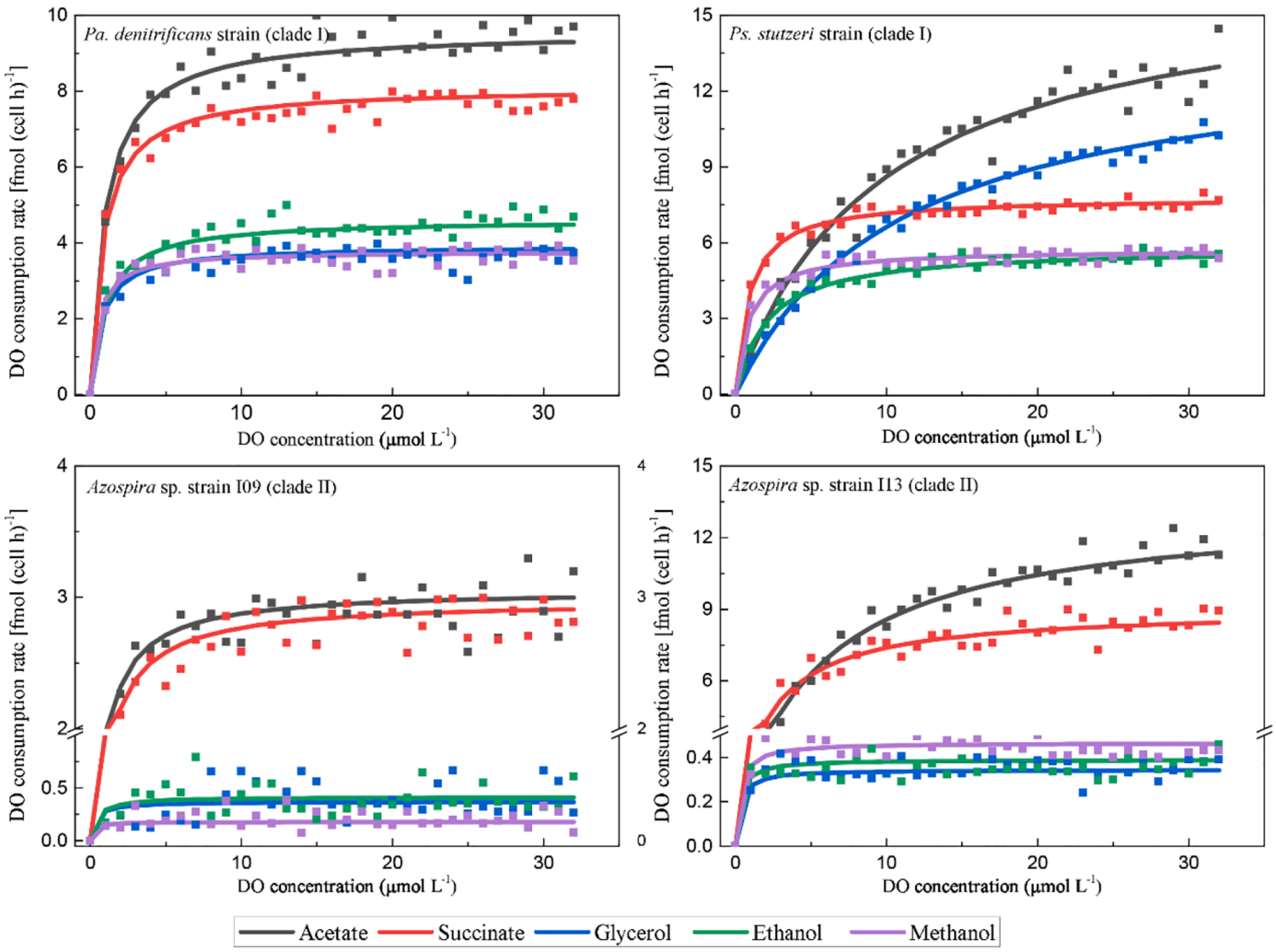

Fig. 5. The whole-cell Michaelis-Menten curves of the DO consumption rate of *Pa. denitrificans* (clade I), *Ps. stutzeri* (clade I), *Azospira* sp. strain I09 (clade II), and *Azospira* sp. strain I13 (clade II) using acetate, succinate, glycerol, ethanol, and methanol as carbon sources. The data were obtained from triplicate experiments.

4. Discussion

4.1. Electron donor preference regulates physiological traits of N_2O consumption

Despite the significance of denitrification reducing reactive nitrogen oxides in wastewaters to nitrogen gas, one of the challenges lies in the accumulation and emission of a highly potent greenhouse gas, N_2O. To limit N_2O emission, harnessing N_2O-reducing bacteria can contribute to N_2O mitigation during denitrification in engineered systems (Suenaga et al., 2019). However, more exploratory research on novel N_2O-reducing bacteria and ecophysiological characterizations is required (Hallin et al., 2018). Although the operating conditions, e.g., pH, DO level, co-existing NO_2^-, and temperature, that facilitate N_2O consumption by these bacteria have been thoroughly investigated (Pan et al., 2012; Suenaga et al., 2018b; Wang et al., 2018; Zhou et al., 2021, 2022), their N_2O consumption associated with various organic carbon species is poorly understood. Given the context, our hypotheses regarded an organic carbon species as a vital factor in (1) N_2O consumption activity in an anoxia environment and (2) N_2OR recovery after DO exposure. The physiological assays carried out using a micro-respiration system, as well as genomic insights, indicated that the organic carbon preference of N_2O-reducing bacteria determines the N_2O consumption rates, N_2O affinity, and activity recovery after DO exposure, verifying the raised hypotheses. As shown in Figs. 5, 6, and Table 1, the application of a micro-respiration system (Fig. 1) illuminated the noticeable differences in their DO and N_2O respiration biokinetics of clade I and clade II *nosZ* canonical denitrifying bacteria and the dependencies upon the type of supplemented organic matter (Table 1). The different DO and N_2O biokinetics with the different electron donors likely provide significant implications for N_2O emissions from denitrification in anoxic tanks of WWTPs, when external organic carbon source is switched, for example, from ethanol to methanol (Baytshtok et al., 2009). Given that the external organic carbon source determines the microbial community composition and its resultant denitrification performances (Baytshtok et al., 2009; Lu et al., 2011, 2014; Carlson et al., 2020), our results underscore that the differences in N_2O consumption activity of the indigenous microbial consortium harboring N_2O-reducing bacteria are potentially caused by external organic carbon source, which plausibly determines a N_2O footprint of a WWTP.

4.2. Metabolic genotype affects the N_2O-related biokinetics of *Azospira* sp. strain I13

The genotype and phenotype investigation of *Azospira* sp. strain I13 elaborated its distinctive and unique N_2O consumption performance. The biokinetic analysis conducted with a micro-respiration system identified the advantage of this strain as a N_2O sink when acetate and succinate were supplied (Fig. 6 and Table 1). The far lower N_2O consumption activities with glycerol, ethanol, and methanol (Fig. 5) were

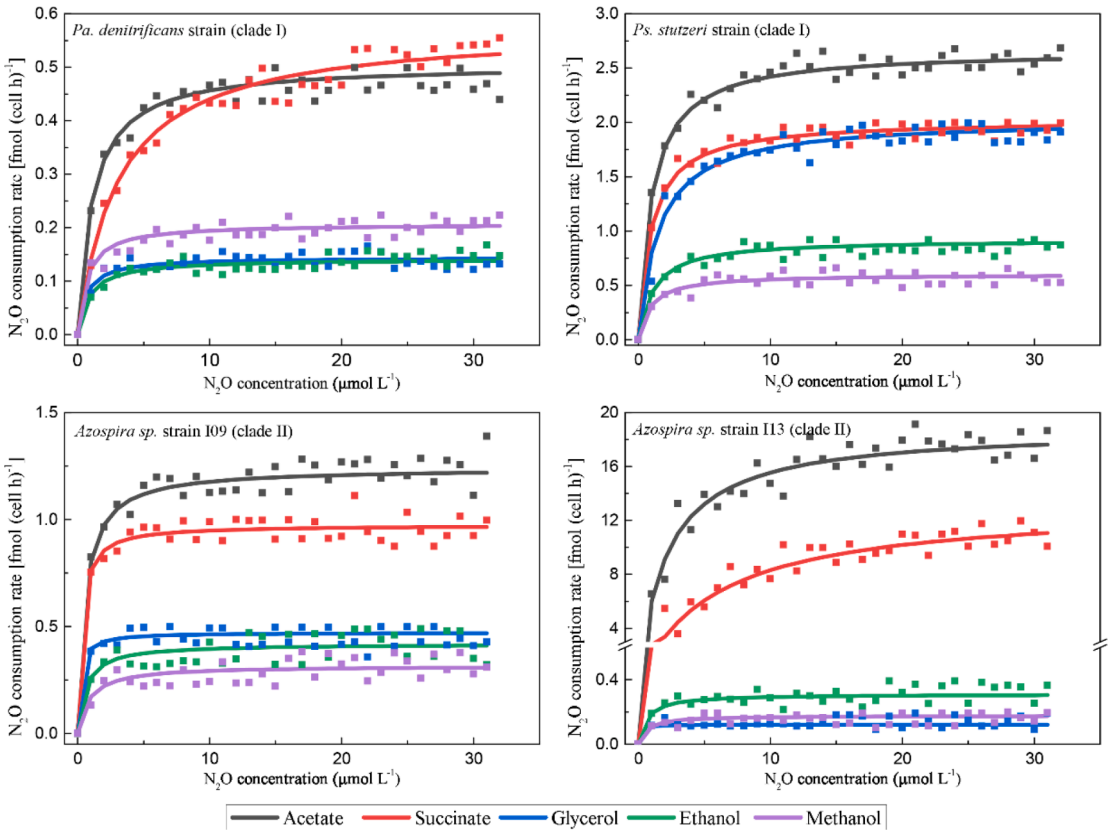

Fig. 6. The whole-cell Michaelis-Menten curves of N_2O consumption rate of *Pa. denitrificans* (clade I), *Ps. stutzeri* (clade I), *Azospira* sp. strain I09 (clade II), and *Azospira* sp. strain I13 (clade II) using acetate, succinate, glycerol, ethanol, and methanol as carbon sources. The data obtained from triplicate experiments.

contextualized by the absence of the key genes enabling their metabolisms (Fig. 2). These trends show why *Azospira* sp. strain I13 had the largest variance of $V_{max,\ N2O}$ among the four tested bacteria when fed the five organic carbon sources (Table 1). The absence of certain genes may force N_2O-reducing bacteria to utilize alternative pathways to utilize electrons for N_2O consumption, likely lowering the consumption rates (Carlson et al., 2020). Our follow-up experiment found that methanol consumption with N_2O respiration was not observed for *Azospira* sp. strain I13 or *Ps. stutzeri*; this is because they lack the methanol dehydrogenase gene (Figs. S1 and 2). Either endogenous respiration or residual carbon from preculture media led to N_2O consumption by bacteria that lack the genes necessary for metabolizing the supplemented organic carbon. Our result shed light on the significance of electron donor selection and expanded our understanding of N_2O-reducing bacterial physiology. This knowledge can support N_2O mitigation in natural and engineered ecosystems.

The limited substrate range may be the general character of the genus *Azospira*. Mining of the *Azospira* genomes in the database revealed *Azospira* genotypes that are analogous to that of *A. oryzae* PS, which lacks genes essential for glycerol metabolism. Hitherto, no *Azospira* species has been found that harbors a complete set of genes needed for methanol metabolism. Although such genotypes may not be shared among all *Azospira* species (genomic information is indeed limited), it is plausible that *Azospira* may not effectively function as an effective N_2O sink when fed with alcohol and polyol, which are cost-effective electron donors (Ribera-Guardia et al., 2014, Table S1). Therefore, effective strategies to control N_2O emissions require further investigation on the physiologies pertinent to specific electron donors.

4.3. Genomic insights into the biokinetics of N_2O-reducing bacteria for suitable external carbon selection

Our experimental study demonstrated that the biokinetics of N_2O-reducing bacteria are largely dependent on the carbon source (Figs. 5 and 6). The acetate and succinate addition resulted in higher $V_{max,\ DO}$ and $V_{max,\ N2O}$ values compared with ethanol and methanol addition (Figs. 5 and 6). These results are consistent with the electron donor preferences of the N_2O-reducing bacteria (Ribera-Guardia et al., 2014) that possess the complete set of genes necessary for the corresponding metabolic pathways (Fig. 2). Acetate and succinate are immediately metabolized in the TCA cycle, while ethanol degradation requires NAD-dependent alcohol dehydrogenase and aldehyde dehydrogenase to consecutively convert ethanol to acetate before entering the TCA cycle via acetyl-CoA (Adouani et al., 2010). Methanol degradation requires methanol dehydrogenase, utilizing a distinct pathway to fully oxidize it to CO_2 (Ribera-Guardia et al., 2014). Therefore, among the tested bacteria, the acetate and succinate additions led to higher DO and N_2O consumption rates compared with the methanol and ethanol additions (Table 1). Interestingly, when glycerol was fed, the $V_{max,\ DO}$ and $V_{max,\ N2O}$ for *Ps. stutzeri* were one to two orders of magnitude higher than those of the other N_2O-reducing bacteria (Figs. 5 and 6). This observation was especially interesting, as it is, to our knowledge, the first report of N_2O reduction biokinetics data with glycerol as the electron donor. This remarkable capability of *Ps. stutzeri* to utilize glycerol as the electron acceptor for N_2O reduction is likely because glycerol-based denitrification involves acyltransferase converting glycerol-3-phosphate into dihydroxyacetone phosphate (Fig. 2). Although *Ps. stuzeri* and *Pa. denitrificans* possess genes for glycerol metabolism, only *Ps. stutzeri* harbors the glycerol-3-phosphate acyltransferase genes for both EC 2.3.1.15 and EC 2.3.1.275 (Fig. 2), which require acyl-CoA and acyl-phosphate as substrates, respectively. *Pa. denitrificans* possesses

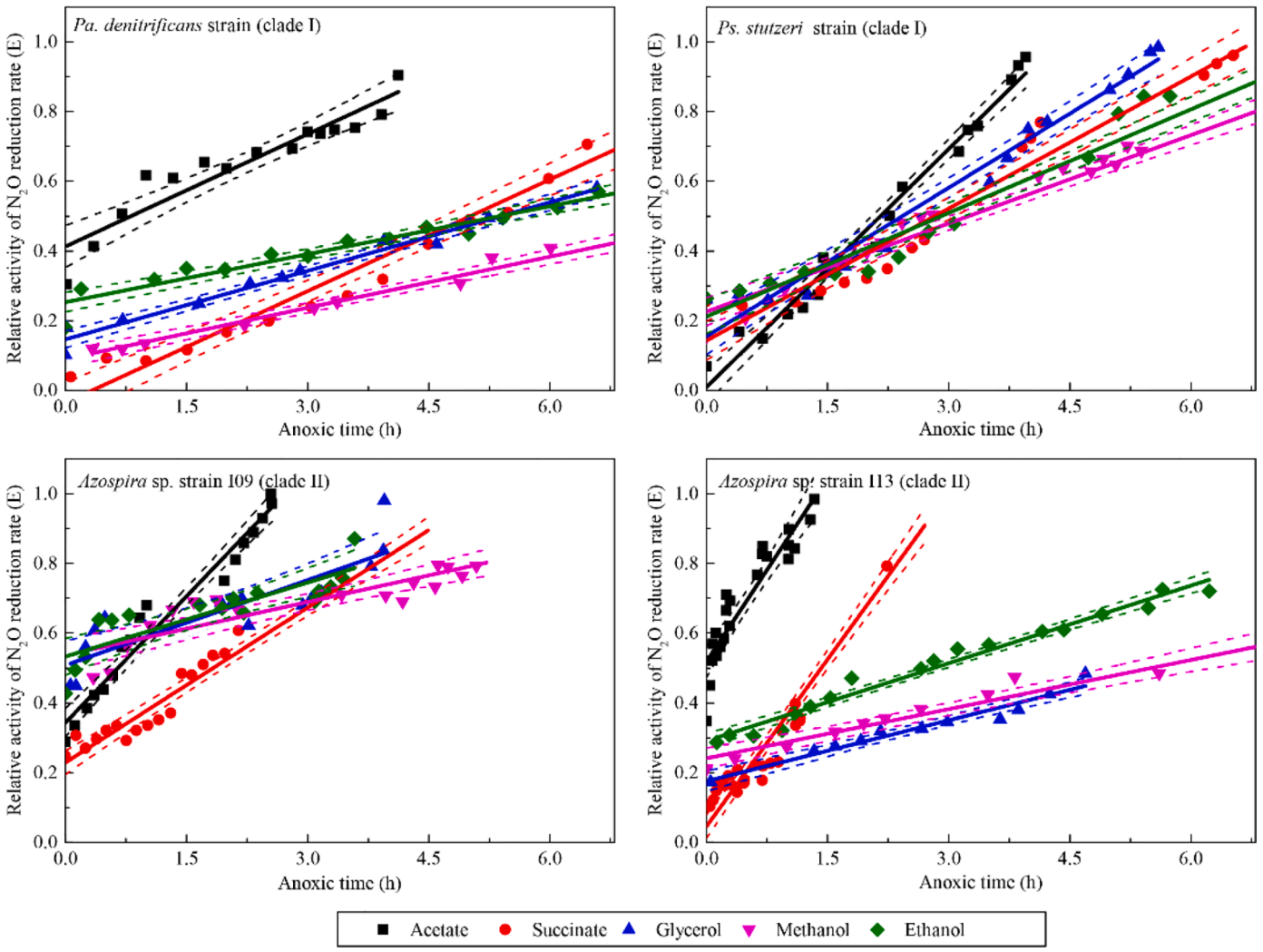

Fig. 7. Recovery of N_2O reduction activity under anoxic conditions. The horizontal axis indicates the time after DO depletion. The data points were derived from N_2O respiration tests performed in triplicate. The solid lines represent the linear model of N_2O reduction recovery in each carbon source, and the dashed lines represent the 95% confidence intervals.

only the latter enzyme, but it may not be able to acquire sufficient acyl-phosphate substrate. Further investigation is necessary to elucidate why *Ps. stutzeri* can achieve high DO and N_2O consumption rates when fed glycerol.

4.4. *Effect of organic carbon source on N_2OR recovery after DO exposure*

The presence or absence of DO drastically alters the N_2O respiration capacity of bacteria (Lu and Chandran, 2010; Suenaga et al., 2018b) because DO exposure can alter gene and protein expressions. N_2OR is reportedly vulnerable to DO exposure (Pomowski et al., 2011). A better understanding of N_2OR activity recovery with different carbon sources is essential for mitigating N_2O emissions in WWTPs where dynamic changes in redox conditions occur. Our investigation of N_2O activity resilience in N_2O-reducing bacteria found that the different organic carbon species caused different lag times in the recovery of N_2O consumption (Fig. 7). At the same organic carbon source, the N_2OR resilience differed among the tested bacteria. When acetate and succinate were applied, *Azospira* sp. strain I13 showed higher N_2O consumption and faster N_2OR recovery than *Pa. denitrificans* and *Ps. stutzeri*, whereas, with glycerol, ethanol, and methanol, the N_2OR recoveries were markedly impaired (Table S2). The recovery times are congruent with the presence or absence of the genes for the different carbon metabolisms (Fig. 2). A delayed N_2OR recovery may boost N_2O emissions in the transiently changing redox conditions often found in WWTPs. In stark contrast, *P. stutzeri* displayed relatively consistent N_2OR resilience regardless of the supplemented carbon source, which may alleviate abrupt N_2O emissions. The contrasting result implies that possessing diverse energy metabolisms genes could be an advantage for mitigating N_2O emissions in engineered systems and agricultural croplands (Jonnasen et al., 2021), as the organic carbon constituents are varied. More N_2O mitigation research is needed to identify and augment highly efficient bacteria amenable to various carbon sources as electron donors. In a MLE process, one of the mostly-widely implemented biological nitrogen removal (BNR) processes, activated sludge is recycled from an aeration tank to an anoxic tank. Our experimental setup mirrors a realistic condition where the redox condition is shifted from aerobic to anoxic conditions as the aerobic activated sludge is recycled to the anoxic tank, to which an organic electron donor is added. Therefore, our biokinetic results regarding the retardation of N_2OR activity recovery after oxygen exposure would prove crucial for enhancing the understanding of N_2O dynamics in BNR systems.

4.5. Carbon sources likely shape different microbial community structures: implications

According to the whole-cell Michaelis-Menten profiles with N_2O as an electron acceptor (Fig. S5), *Azospira* sp. strain I13 could dominate in a denitrifying reactor fed with acetate and succinate, whereas the others tested bacteria with glycerol, ethanol, and methanol. The distinct N_2O biokinetic data suggest that a carbon source likely shapes different predominant species as a N_2O reducer. Microbial community compositions receiving different carbon sources, e.g., methanol or acetate, have been reported (Lu et al., 2014; Song et al., 2015; Carlson et al., 2020) and have shown the predominance of different genera as denitrifiers (Baytshtok et al., 2009; Osaka et al., 2006; (Ma et al., 2015). Acetate-fed biomass reportedly emits less N_2O than methanol-fed biomass (Song et al., 2015). As shown in the current study, given the unique metabolic genotypes and phenotypes of each N_2O-reducing bacterium, a microbial community will have a distinct N_2O consumption activity. This notion is supported by previous work in which an acetate-fed biomass consisting of *Rubrivivax* and *Dechloromonas* (N_2O reducers) exhibited greater N_2O consumption than a methanol-fed biomass that mainly consisted of *Methylotenera* (Song et al., 2015). This is likely because, from the biokinetic point of view, highly efficient N_2O-reducing bacteria, e.g., *Dechloromonas* and *Azospira*, can be dominant in a biomass (Song et al., 2014; Suenaga et al., 2019; Kim et al., 2020). Further studies should investigate the link between the organic carbon source and the N_2O emission in wastewater bioreactors.

Given the efficiency and cost-effectiveness of nitrogen removal in WWTPs, it is imperative to select an appropriate carbon source for N_2O reduction (Ribera-Guardia et al., 2014; Song et al., 2015). It is perceived that a growth yield is a measure of the effectiveness of energy transfer from catabolism to anabolism (Russel and Cook, 1995). The high net growth yield found for the *Azospira* spp. is in keeping with the high metabolic efficiency of *Azospira* when fed acetate and succinate; by contrast, this trend was not observed with glycerol and ethanol (Fig. 3). From a practical point of view, acetate may not be a suitable external organic electron donor for denitrification because it costs seven times more than methanol (Mokhayeri et al., 2009). Glycerol is a good alternate due to its relatively higher N_2O reduction efficiency and lower cost (when crude glycerol is used) than the other carbon sources (Song et al., 2015, Table S1). When glycerol was applied, *Ps. stutzeri* exhibited the highest $V_{max,\ DO}$ and $V_{max,\ N2O}$, which were nearly one order of magnitude higher than those of the other tested bacteria (Figs. 5 and 6). The V_{nos} of *Ps. stutzeri* with glycerol addition was also high, indicating its fast recovery of N_2O consumption activity when transitioning from aerobic to anoxic conditions (Table S2). Based on this short recovery time, biomass harboring *Ps. stutzeri* in a biological nitrogen removal system would rapidly adapt to glycerol when applied as an alternative carbon source. However, not all strains of *Ps. stutzeri* necessarily possess the complete gene set for glycerol metabolism, and more research is needed to clarify this. Additionally, further studies are required to verify a suitable external organic carbon source that is inexpensive and will result in a high N_2O consumption rate in WWTPs.

5. Conclusion

This study systematically compared the N_2O consumption and N_2OR recovery of four N_2O-reducing bacteria harboring either clade I or clade II *nosZ* when fed five different carbon sources. The conclusions are as follows:

- Organic carbon source remarkably affected the performance, biokinetics, and biomass yield of heterotrophic N_2O-reducing bacteria when N_2O served as the sole electron acceptor.
- Of the tested carbon sources, acetate and succinate resulted in the highest DO and N_2O consumption rates, and clade II type *Azospira* sp. strain I13 had a higher consumption rate than clade I type *Ps. stuzeri* and *Pa. denitrificans*.
- The N_2OR resilience after transitioning from aerobic to anoxic conditions heavily depended on the type of N_2O-reducing bacteria and the supplemented carbon source.
- When acetate and succinate were added, the clade II *nosZ* bacteria had higher biomass yields than the clade I *nosZ* bacteria.

Collectively, the performances and biokinetics of N_2O-reducing bacteria are closely associated with their physiological traits and the metabolic potential of the electron donor. Identifying highly efficient N_2O reducers and supplementing them with their preferable organic carbon source would improve N_2O mitigation in WWTPs.

CRediT authorship contribution statement

Chuang Qi: Investigation, Data curation, Writing – review & editing, Software. **Yiwen Zhou:** Investigation, Data curation. **Toshikazu Suenaga:** Formal analysis, Writing – review & editing, Software. **Kohei Oba:** Investigation, Data curation. **Jilai Lu:** Writing – review & editing, Software. **Guoxiang Wang:** Writing – review & editing, Software. **Limin Zhang:** Writing – review & editing, Software. **Sukhwan Yoon:** Writing – review & editing, Software. **Akihiko Terada:** Visualization, Writing – review & editing, Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117910.

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