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a cautionary perspective

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Current Opinion in Microbiology

Review

A tale of two nitrous oxide reductases: a cautionary perspective

Sukhwan Yoon[1], Min Joon Song[1] and Michele Laureni[2]

Nitrous oxide reductases (N2OR) are the sole sink of the potent greenhouse gas nitrous oxide (N_2O) in the environment. Having been studied for decades, N2OR have attracted renewed attention following the discovery of a previously unrecognized clade, now termed clade II. This clade exhibits unexpectedly widespread taxonomic distribution and prevalence across diverse environments, prompting research efforts to define and assign distinct clade-specific traits. In this perspective, we aim to critically review and evaluate dichotomous clade-based classifications, addressing oversimplifications and unresolved ambiguities in linking clade identity to physiological traits like substrate affinity, acid tolerance, and aerotolerance. Growing experimental evidence from N_2O-reducing isolates and enrichments suggests a general difference in substrate affinity between the clades. Recent discoveries of N_2O reduction at pH < 5.0 attribute the long-sought acidophilic N_2O reduction exclusively to organisms possessing clade II *nosZ*, and attempts have also been made to relate clade separation to aerotolerant N_2O reduction. However, it is important to note that such binary characterizations are based on limited observations and lack a solid understanding of the underlying mechanisms, exposing them to bias and oversimplification risks. We emphasize the need for a balanced research effort to establish a robust link between ecophysiology and biochemistry, enabling a more accurate evaluation of clade-based characterizations and, ultimately, a deeper understanding and effective harnessing of N_2O-reducing organisms.

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Nitrous oxide (N_2O) is a potent greenhouse gas responsible for 7.7% of the total radiative forcing from long-lived greenhouse gases, despite constituting merely ca. 330 ppbv of the Earth's atmosphere [1]. Due to the high global warming potential of N_2O, the biogeochemical processes involved with its production and consumption in the environment have been of utmost interest to environmental microbiologists for decades [2]. While a number of distinct pathways lead to N_2O emission from diverse environments, only a single pathway, N_2O reduction to N_2 catalyzed by Nos-type nitrous oxide reductases (heretofore referred to as N2OR), serves as biogeochemical sink of N_2O [2]. A diverse array of microorganisms harbor the *nosZ* gene encoding the catalytic subunit of N2OR and are capable of reducing N_2O to N_2, with N_2O as the terminal electron acceptor for energy conservation. Microbial N_2O reduction has always attracted a fair amount of attention; nevertheless, it was the discovery of the environmental prevalence of a distinct clade of the *nosZ* gene, now referred to as clade II *nosZ*, that sparked a broader interest in this pathway from both scientific and engineering perspectives [3,4].

Historically, N2OR was first discovered through physiological observations of denitrifiers, where N_2O produced as a free intermediate during reduction of NO_3^- and NO_2^- was found to be subsequently reduced to N_2 by an enzymatic reaction [5]. Although reports of N_2O-reducing phenotype in denitrifiers harboring *nosZ* identified as belonging to clade II can be found in the literature, early physiological and biochemical studies focused almost exclusively on denitrifiers with *nosZ* now classified as clade I, such as *Pseudomonas* spp. (renamed as *Stutzerimonas* spp. in databases) and *Paracoccus* spp. [6,7]. Later, the discovery of NosZ-mediated N_2O reduction in *Wolinella succinogens* and the ensuing discoveries from physiological and genomic characterization posed a major challenge to the prevailing paradigm that N_2O reduction is exclusive to denitrifiers [8,9]. Thanks to the expanding genome database and the advent of high-throughput sequencing, far-reaching implications of these earlier findings on nondenitrifier N_2O reduction and the presence of the 'unprecedented' *nos* genes were eventually recognized [3,4]. Clade II *nosZ* not only outnumbers clade I *nosZ* in many environmental microbiomes where nitrogen redox processes are of a great

concern (Table S1) but is also found in taxonomically diverse organisms that include nondenitrifiers [4,10,11]. As such, accumulating evidence increasingly underscores the environmental importance of clade II *nosZ*. In this review, we synthesize key findings on clade I and clade II N_2OR and the microorganisms that harbor and utilize these enzymes. We highlight the latest findings on clade-specific genomic, biochemical, and physiological traits but also caution against potentially oversimplified dichotomies, emphasizing the need to resolve remaining ambiguities.

Phylogenetic and functional split of microorganisms harboring clade I and II *nosZ*

The *nosZ* gene is relatively widespread among prokaryotes, as reported in a recent genomic survey that identified *nosZ* in 12% of all sequenced bacterial and archaeal genomes [12]. Clade I *nosZ* are found mostly in the genomes affiliated to the phylum *Proteobacteria*, while clade II *nosZ* are found across a broader stretch of the bacterial domain that includes the phyla *Campylobacterota*, *Firmicutes*, *Chloroflexi*, *Bacteroidota*, *Verrucomicrobiota*, *Planctomycetota*, *Acidobacteriota*, as well as *Proteobacteria* [12,13]. This broad distribution may be due to higher tendency of clade II *nosZ* for horizontal gene transfer, as suggested by the multiphyletic branches in the *nosZ* phylogeny (Figure 1a). A unique group of haloarchaeal *nosZ* genes exhibits features aligning them with clade I, also challenging the potentially oversimplified notion that clade II is more diverse than clade I [14]. Both clade I and clade II *nosZ* genes are typically encoded within the genomic DNA; however, a plasmid-encoded clade I *nosZ* was reported in *Methylocystis* sp. SC2 [15]. The only consistent clade-specific features of *nos* clusters are the presence of *nosR* in clade I and that of *nosB* in clade II (Figure 1b), despite the literature references to the concerted presence of genes encoding cytochrome *c* and Fe-S proteins as a defining feature of clade II *nos* clusters [3,16,17].

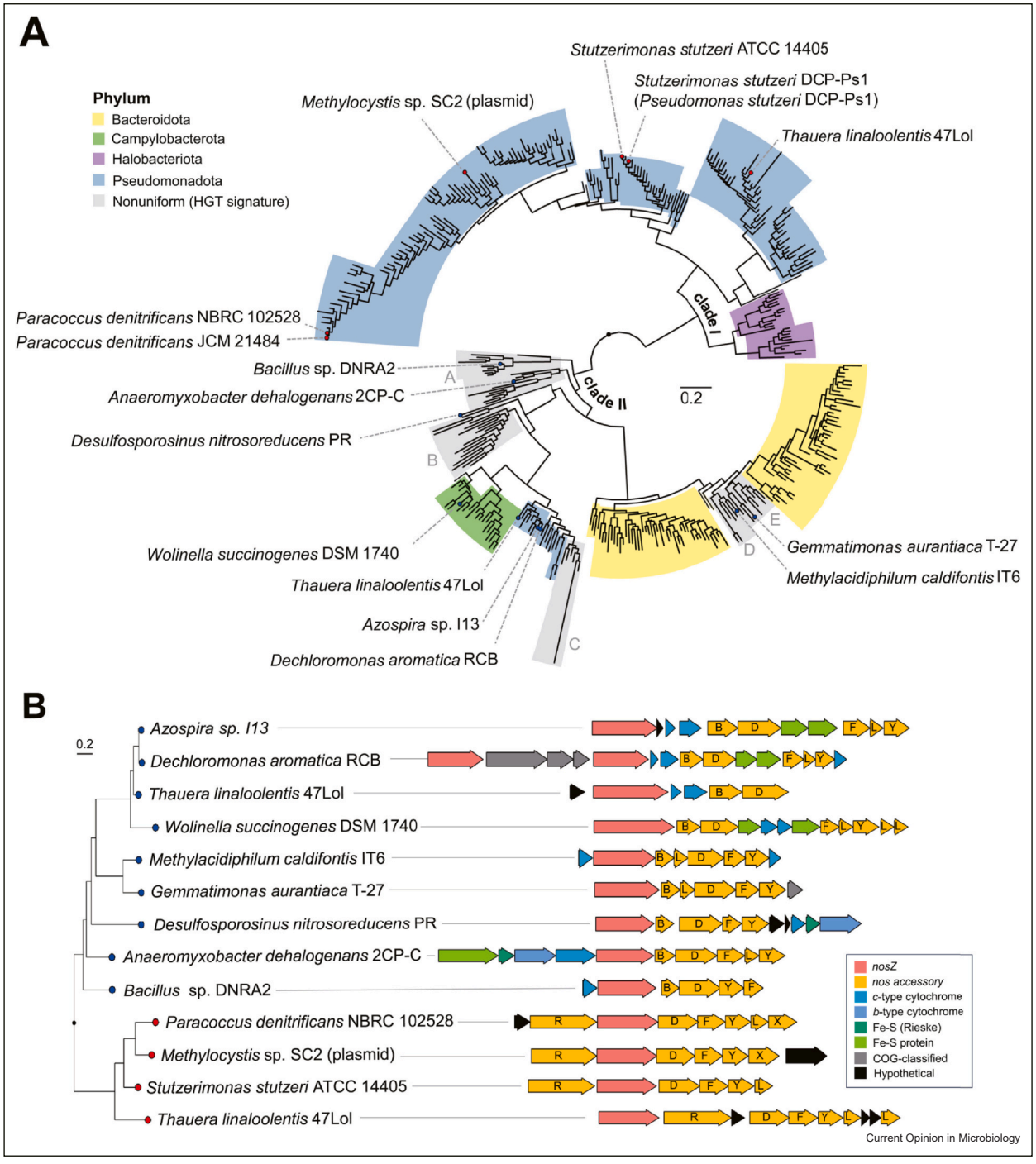
As *nosZ* is regarded to have been inherited largely through vertical evolution, the taxonomic groups containing clade I and clade II *nosZ* are relatively distinct [12,18,19]. For instance, *Pseudomonas* spp. exclusively possess clade I *nosZ*, whereas *Bacillus* spp. exclusively possess clade II *nosZ*. However, several *Betaproteobacteria* genera are shared by organisms harboring clade I *nosZ*, clade II *nosZ*, or both (Figure 1a). *Bradyrhizobium* spp. are typically associated with clade I *nosZ* and *Zooglea* spp. with clade II *nosZ*; however, genomes assigned to these genera with the alternate clade have also been reported [20]. The fact that the only organisms identified with both clades of *nosZ*, *Dechlorobacter hydrogenophilus* LT-1, *Thauera butanivorans* NBRC103042, and *Thauera linaloolentis* 47Lol all belong to *Betaproteobacteria* is unlikely to be a mere coincidence [21]. Inferring *nosZ* type from 16S rRNA-gene-based taxonomic affiliation can thus result in misleading interpretations of N_2O-reducing microbial populations in microbiomes, particularly those with a high abundance of Betaproteobacteria.

Unlike clade I *nosZ*, a substantial fraction of clade II *nosZ* belongs to the microorganisms lacking NO-forming nitrite reductase genes *nirK* and *nirS* [3,4,12,13]. According to the seminal Hallin et al. perspective article, 156 of 187 published genomes containing clade I *nosZ* possess *nirK* or *nirS*, whereas only 54 of 113 genomes with clade II *nosZ* possess either gene [12]. Mounting evidence from metagenomic analyses supports this postulate, ruling out potential culture biases [11,13,20]. Given the prevalence of nondenitrifiers with clade II *nosZ*, its initial discovery in the nondenitrifiers *Wolinella succinogens* and *Anaeromyxobacter dehalogenans* was also unlikely coincidental. A majority of genomes of such nondenitrifier clade II N_2O reducers harbor *nrfA* encoding the cytochrome c_{552} nitrite reductases [3,12,13]. Functional association linking N_2O reduction and dissimilatory nitrate reduction to ammonium (DNRA) remains largely unelucidated; however, a recent study involving a *Bacillus* isolate (strain DNRA2) demonstrated that clade II N2OR facilitates removal of N_2O, a byproduct of DNRA often accounting for several percentage of NO_3^- reduced, alleviating N_2O-induced inhibition of *nrfA* transcription following oxic-anoxic transitions [22].

Differential substrate affinities — a clade-wide trait?

The environmental relevance of the clade I versus clade II *nosZ* dichotomy primarily stems from the role of N_2O reducers in mitigating N_2O emissions [2,23,24]. N_2O reductases inherently mitigate N_2O emissions; without them, denitrification, one of the most prevalent redox reactions constantly occurring in various environments, would produce and emit N_2O in amounts stoichiometric to the NO_3^- reduced [2,13,23]. Particularly consequential in this regard is the role of N2OR-possessing organisms in reducing fugitive N_2O, often present at micromolar or nanomolar concentrations [10,20,25,26]. Biokinetic studies have consistently shown that isolates with clade II *nosZ* genes grouped with those of *Dechloromonas* spp. and *Azospira* spp. exhibit whole-cell Michaelis constants ($K_{m,app}$) indicative of high-affinity N_2O reduction [26–28]. The $K_{m,app}$ value for *Dechloromonas aromatica* RCB was 0.324 μM, equivalent to 15 ppmv in the gaseous phase at 25°C and 1 atm [26]. Consistent with this observation, an independent study reported a $K_{m,app}$ value of 0.866 μM for *Azospira* sp. I13, whose NosZ shares > 85% amino acid identity with those of *D. aromatica* RCB [28]. Furthermore, reactor enrichments with N_2O as the limiting substrate exhibited pronounced expression of *nosZ* affiliated with the *Dechloromonas*-like group, corroborating the hypothesis that

Figure 1



Phylogeny and gene cluster organization of diverse clade I and clade II nitrous oxide reductases. **(a)** Maximum likelihood phylogenetic tree (RAxML-NG) constructed with 375 nonredundant *nosZ* sequences aligned using MAFFT v7.525 and trimmed using trimAl v1.5.0. 663 *nosZ* sequences were extracted from 5776 prokaryotic genomes downloaded from GTDB release 220, and 10 additional sequences were manually retrieved from multiple databases. The *in silico* translated sequences were clustered at 87% identity using CD-HIT v4.8.1 to reduce redundancy. *Wolinella succinogenes* DSM 1740 *nosZ* was manually replaced with the GenBank sequence (CAG26676.1) to reflect biochemical characterization data (also applied to panel b). The tree was visualized using ggtree v3.14.0 on RStudio 2024.12.1. **(b)** *nos* gene clusters of physiologically verified N_2O-reducing organisms described in the text. Only closed genomes were considered. Putative *nos*-cluster genes were predicted using KofamScan v1.3.0, InterProScan 5.69-101.0 (reference: Pfam release 37.0 and NCBIfam release 15.0), and DIAMOND BLASTP (reference: NCBI RefSeq nr database accessed February 2024). *nosB* genes were predicted using OrthoFinder v3.0.1b1 against *nosB* genes in genomes of *D. nitrosoreducens* PR and *A. dehalogenans* 2CP-C. Clusters were visualized using gggenes and arranged according to their positions in the NosZ phylogenetic tree. Accession numbers and taxonomic annotations of *nosZ* sequences used are provided in Table S3.

this particular group of NosZ possesses a distinguishably high affinity that enables their hosts to scavenge N_2O [20,29].

Conversely, several clade I *nosZ*-harboring denitrifiers closely related with the most well-studied denitrifier taxa, *Pseudomonas* spp. and *Paracoccus* spp., exhibited $K_{m,app}$ values orders of magnitude higher than those of *Dechloromonas* spp. and *Azospira* spp. The $K_{m,app}$ values measured for *Pseudomonas stutzeri* DCP-Ps1 and *Paracoccus denitrificans* NBRC102528, sharing 58% identity in their NosZ amino acid sequences, were as high as 35 μM [26,28]. Although not as pronounced as these extremes with a two-order-of-magnitude difference, the whole-cell kinetics data from literature consistently distinguish the two NosZ clades based on their affinity to N_2O [26,28,30,31]. Caution should be taken, however, to avoid making a hasty generalization, assuming that clade II N_2O reducers universally exhibit higher affinity than clade I N_2O reducers. Both clade I and clade II *nosZ* have within-clade diversity that extends well beyond the organisms whose N_2O reduction kinetics data are currently available. Despite the abundance and ecological significance of *Bacillus* spp. (clade II) and *Bradyrhizobium* spp. (mostly clade I) in N_2O-relevant environmental microbiomes, their N_2O reduction kinetics have not yet been reported, nor has the underlying biochemical basis been identified [32,33]. N2OR have been purified and biochemically characterized for decades; however, among clade II Nos, only the one from *W. succinogens* has been purified and examined *in vitro* [34]. The Michaelis constant (K_m) of *W. succinogens* N2OR was comparable to the K_m value of purified clade I *P. denitrificans* Nos, which, confoundingly, was fivefold lower than the $K_{m,app}$ value measured with whole-cell *P. denitrificans* [34–36]. Adding complexity, whole-cell N_2O reduction kinetics vary substantially depending on the methodological approach and/or the incubation condition, for example, temperature and electron donor type, even within a single organism (Table S2) [31]. In summary, while it may be reasonable to highlight the association of clade II Nos with high-affinity N_2O reduction, it would be premature to conclude that clade I and clade II Nos target distinct N_2O concentration ranges.

Another essential yet underexplored aspect of understanding the N_2O sink capabilities of different N_2O reducers concerns the threshold N_2O concentrations required to induce NosZ expression and initiate N_2O reduction. The chemostat observations that *Dechloromonas*/*Azospira*-like clade II N_2O reducers dominate under N_2O-limiting steady-state condition suggest that these organisms, characterized by low $K_{m,app}$ values, may also have lower thresholds; however, systematic physiological study supporting the hypothesis is lacking [20,29]. Exploring these thresholds across different levels of microbial complexity and identifying potential clade-specific trends and correlations with $K_{m,app}$ values would represent an invaluable avenue for future research.

Are acido- and oxygen-tolerances in nitrous oxide reduction clade-specific features?

Two recent breakthrough studies identified microorganisms, both harboring clade II *nosZ*, expanding the previously known pH range for N_2O reduction. Acidity has long been identified as one of the environmental factors typically inhibiting N_2O reduction [37]. Studies with both pure cultures and complex consortia, as well as field experiments, have consistently demonstrated that N_2O emissions from denitrification increase at acidic pH [37–39]. One of the breakthroughs, challenging the perception, found an extreme acidophilic methanotroph affiliated with the phylum *Verrucomicrobia* (*Methylacidiphilum caldifontis* IT6) capable of reducing N_2O at pH 2.0 using methanol as the electron donor [40]. Another study reported N_2O reduction at pH 4.5 by a co-culture of a *nosZ*-lacking *Serratia* strain and an unisolatable *Desulfosporosinus* strain possessing a clade II *nosZ* sharing 48.5% amino acid identity with *A. dehlogenans nosZ* [16]. A follow-up study identified the same group dominating the *nosZ* pool in an N_2O enrichment incubated at pH 4.5, corroborating that *Desulfosporosinus* N2OR were expressed, properly synthesized, and utilized for energy conservation at acidic pH [41]. The *nosZ* genes of *M. caldifontis* IT6 and *Desulfosporosinus* spp. are both located within the subgroups associated with putative horizontal gene transfers (Figure 1a). This, along with the absence of distinguishing feature in their NosZ amino acid sequences compared to those of neutrophilic N_2O reducers (Figure S1), suggests that these acidophilic microorganisms likely acquired the *nosZ* genes through a recent horizontal transfer. The historical elusiveness of acidophilic N_2O reducers may have been due to the rarity of such events in nitrogen-deficient acidic environments, where these organisms were found.

Perhaps, it is not coincidental that *M. caldifontis* IT6 and *Desulfosporosinus* spp., currently the only microorganisms with verified N_2O reduction activity at pH below 5.0, harbor clade II *nosZ* genes [16,40,41]. A recent field study conducted on nitrate-contaminated groundwater at Oak Ridge also linked acidophilic N_2O reduction activity (pH ~4.0) to the abundance of clade II *nosZ* [17]. The profiles of these metagenomic *nosZ* genes were not disclosed; however, as the diversity and uniqueness of these genes were mentioned, it is likely that these *nosZ* genes share only limited similarity with the *Desulfosporosinus*-like *nosZ* group or the *nosZ* gene of *M. caldifontis* IT6 [16,40]. That acidophilic N_2O reduction has been witnessed in such diverse clade II nosZ-harboring N_2O reducers may suggest acid tolerance as a general trait of clade II N2OR. To this end, a closer scrutiny of the

potential roles of the clade II–specific secretion and maturation mechanisms in acid tolerance may also prove to be a highly worthwhile avenue for future research [16]. Another plausible hypothesis from an evolutionary perspective is that the propensity of clade II *nosZ* for horizontal transfers may have facilitated the dissemination of N_2O-reducing capability to diverse acidophilic microorganisms.

Whether clade-specificity is relevant to O_2 resilience of N_2O reduction has also been hypothesized but remains unresolved. N_2O reduction has been regarded as the most oxygen-sensitive step in the denitrification pathway, highly susceptible to O_2 inhibition [42]. The clade II N2OR isolated from *W. succinogens*, an obligately anaerobic organism, was found insensitive to O_2 exposure, in a clear contrast to the response of isolated clade I N2OR [34,43]. These *in vitro* biochemical observations prompted the hypothesis that clade II N2OR is oxygen insensitive at the enzyme level, despite limited supporting biochemical evidence. At whole cell level, however, the physiological results obtained thus far are largely inconsistent. Several lines of evidence support that clade II N2OR do not irreversibly lose its *in vivo* activity under O_2 presence. *Gemmatimonas aurantiaca* T-27 N2OR was expressed only in O_2 presence and became activated as the O_2 level decreased [44]. Likewise, a clade II–dominated reactor culture subjected to alternating oxic-and-anoxic phases retained 90% of its N_2O-reducing capability during the oxic phases ($[O_2] > 6.5$ mg/l) [45]. Conversely, several whole-cell studies clearly demonstrated the absence of N_2O reduction activity in cultures of clade II N_2O reducers, for example, *D. aromatica* RCB, in the presence of O_2 [30]. Further complicating the picture, aerobic denitrification phenotypes have been observed in cultures of denitrifiers possessing clade I *nosZ*, for example, *Stutzerimonas stutzeri* ZoBell. and *Paracoccus denitrificans* JCM21484. [30].

Aerobic N_2O reduction, as a part of denitrification or an independent redox reaction, remains a highly controversial topic [38,46]. In dense microbial cultures, anoxic or microoxic niches can form, allowing even the most O_2-sensitive N_2O reducers to perform N_2O reduction within microenvironments shielded from oxygen [47]. Even organisms with aerotolerant N2OR may have evolved to restrict its expression in the presence of O_2, to channel electrons to O_2 for a higher bioenergetic efficiency [46]. Thus, it is important to acknowledge that aerotolerance at the whole-cell level may depend on factors beyond the O_2-sensitivity of N2OR. Additionally, it is important to note that *in vitro* biochemical assays have been conducted with N2OR from only a limited number of microorganisms, which include that of *W. succinogens* as the only clade II N2OR.

Concluding remarks

Just a little over a decade has passed since two groundbreaking articles, published nearly simultaneously, brought the true *nosZ* diversity into spotlight [3,4]. Early physiological observations have suggested that binary categorization of micro-organisms possessing these structurally distinct Nos enzymes may be possible. The hypothesized dichotomy potentially holds broad biogeochemical and biotechnological implications. Multiple lines of physiological and ecological evidence exist supporting the superior ability of clade II N_2O reducers in metabolizing low concentrations of N_2O. Likewise, clade II N_2O reducers have been repeatedly associated with acidophilic N_2O reduction. Furthermore, biochemical and ecophysiological observations hint to the fact that clade II NosZ may feature higher O_2 tolerance. However, inconsistencies and ambiguities persist, barring definitive conclusions as to whether these supposed dichotomies reflect true distinctions or are artifacts of the still limited number of physiological studies. To date, research on N_2O reduction has been disproportionately focused on readily cultivable organisms or multi-omics-based ecological analyses building up a legacy of correlational and circumstantial evidence regarding NosZ dichotomies. Research should prioritize the elucidation of the biochemical basis, or lack thereof, underlying the observed or hypothesized clade-specific traits. Beyond current approaches, structural analysis, facilitated by recent advances in cryo-electron microscopy (cryo-EM) technology, offers an immediate opportunity. Additionally, expanding the focus to habitats traditionally less studied for reductive nitrogen metabolisms, such as terrestrial and marine hydrothermal systems and oxic groundwater, may enable the enrichment and isolation of previously unrecognized acidophilic or aerotolerant N_2O reducers. These future research efforts will prove essential for reshaping and clarification of the clade I versus clade II framework and for effective utilization of N_2O-reducing organisms in emission mitigation strategies.

Data Availability

We used sequence data available from open databases.

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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Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mib.2025.102631.

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