Received: 15 October 2020 | Accepted: 11 January 2021

DOI: 10.1111/gcb.15545

Global Change Biology WILEY

INVITED REPORT

Beyond denitrification: The role of microbial diversity in controlling nitrous oxide reduction and soil nitrous oxide emissions

Jun Shan[1] | Robert A. Sanford[2] | Joanne Chee-Sanford[3] | Sean K. Ooi[4] | Frank E. Löffler[5,6] | Konstantinos T. Konstantinidis[7] | Wendy H. Yang[8]

[1]State Key Laboratory of Soil and Sustainable Agriculture, Changshu National Agro-Ecosystem Observation and Research Station, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, China

[2]Department of Geology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

[3]Global Change and Photosynthesis Research Unit, United States Department of Agriculture – Agricultural Research Station,, Urbana, IL, USA

[4]Program in Ecology, Evolution, and Conservation Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

[5]Center for Environmental Biotechnology, Department of Microbiology, Department of Civil and Environmental Engineering, Department of Biosystems Engineering and Soil Science, University of Tennessee, Knoxville, TN, USA

[6]Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA

[7]School of Civil and Environmental Engineering and School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, USA

[8]Departments of Plant Biology and Geology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Correspondence
Wendy H. Yang, Mailing Address: 286 Morrill Hall, 505 South Goodwin Ave, Urbana, IL 61801, USA.
Email: yangw@illinois.edu

Funding information
US National Science Foundation Dimensions of Biodiversity, Grant/Award Number: 1831842, 1831582 and 1831599; National Key Research and Development Program of China, Grant/Award Number: 2017YFD0200100; China Scholarship Council, Grant/Award Number: 201804910070; Chinese Academy of Sciences, Grant/Award Number: 2017362

Abstract

Many biotic and abiotic processes contribute to nitrous oxide (N_2O) production in the biosphere, but N_2O consumption in the environment has heretofore been attributed primarily to canonical denitrifying microorganisms. The *nosZ* genes encoding the N_2O reductase enzyme, NosZ, responsible for N_2O reduction to dinitrogen are now known to include two distinct groups: the well-studied Clade I which denitrifiers typically possess, and the novel Clade II possessed by diverse groups of microorganisms, most of which are non-denitrifiers. Clade II N_2O reducers could play an important, previously unrecognized role in controlling N_2O emissions for several reasons, including: (1) the consumption of N_2O produced by processes other than denitrification, (2) hypothesized non-respiratory functions of NosZ as an electron sink or for N_2O detoxification, (3) possible differing enzyme kinetics of Clade II NosZ compared to Clade I NosZ, and (4) greater *nosZ* gene abundance for Clade II compared to Clade I in soils of many ecosystems. Despite the potential ecological significance of Clade II NosZ, a census of 800 peer-reviewed original research articles discussing *nosZ* and published from 2013 to 2019 showed that the percentage of articles evaluating or mentioning Clade II *nosZ* increased from 5% in 2013 to only 22% in 2019. The census revealed that the slowly spreading awareness of Clade II *nosZ* may result in part from disciplinary silos, with the percentage of *nosZ* articles mentioning Clade II *nosZ* ranging from 0% in Agriculture and Agronomy journals to 32% in Multidisciplinary Sciences journals. In addition, inconsistent nomenclature for Clade I *nosZ* and Clade II *nosZ*, with 17 different terminologies used in the literature, may have created confusion about the

two distinct groups of N_2O reducers. We provide recommendations to accelerate advances in understanding the role of the diversity of N_2O reducers in regulating soil N_2O emissions.

KEYWORDS
atypical *nosZ*, Clade II *nosZ*, denitrification, N_2O reduction, nitrous oxide, non-denitrifier, *nosZ*, *nosZ* Clade II, *nosZ*-II, soil N_2O emissions

1 | INTRODUCTION

Soil nitrous oxide (N_2O) emissions account for approximately 60% of global emissions of N_2O, which contributes to climate change and stratospheric ozone depletion (Ciais et al., 2013). While a variety of biotic and abiotic processes are known to produce N_2O in soils, consumption of N_2O in soils is strictly biological and, until recently, thought to be primarily associated with bacteria that can respire N_2O by reducing it to dinitrogen (N_2) as the last step in the modular denitrification pathway (Baggs, 2011; Butterbach-Bahl et al., 2013; Zhu-Barker et al., 2015). Rates of N_2O consumption (i.e., reduction to N_2) relative to N_2O production determine net soil–atmosphere N_2O fluxes, with the fraction of N_2O produced in soil that is emitted to the atmosphere, known as the N_2O yield, ranging from 0 to 1 (Schlesinger, 2009). In some cases, N_2O consumption can outpace N_2O production such that soils can even act as net sinks for atmospheric N_2O (Chapuis-Lardy et al., 2007). The *nosZ* gene that encodes N_2O reductase (NosZ), the key enzyme responsible for the reduction of N_2O to N_2, therefore, has been the subject of many studies attempting to elucidate controls on N_2O emissions from soils and other environmental systems. In 2012, a genomics-based study identified an "atypical" *nosZ* gene (hereafter, Clade II *nosZ*; Sanford et al., 2012) that is often more abundant in soils than the well-studied "typical" *nosZ* gene (hereafter, Clade I *nosZ*; Orellana et al., 2014), suggesting that our understanding of the role of NosZ in controlling soil N_2O emissions has been incomplete.

2 | POTENTIAL ECOLOGICAL SIGNIFICANCE OF CLADE II N_2O REDUCERS

Nitrous oxide reduction has most commonly been studied in the historical context of canonical denitrification, the microbially mediated process by which nitrate (NO_3^-) or nitrite (NO_2^-) is reduced to the gaseous products N_2O or N_2 (Tiedje, 1988). Complete denitrification involves the sequential reduction of NO_3^-, NO_2^-, nitric oxide (NO), and N_2O catalyzed by the enzymes nitrate reductase (NarG or NapA), nitrite reductase (NirK or NirS), nitric oxide reductase (e.g., QnorB), and N_2O reductase (NosZ), respectively. Fungal denitrifiers (Higgins et al., 2016, 2018) and some bacterial denitrifiers (Haslun et al., 2018; Lycus et al., 2017) have truncated enzymatic pathways for denitrification, with the absence of the *nosZ* gene resulting in N_2O as the terminal product. For denitrifiers with the complete enzymatic pathway, the amounts of N_2O and N_2 released as denitrification end products depend on environmental conditions such as NO_3^- concentrations, pH, and soil aeration (e.g., Firestone et al., 1979; Lycus et al., 2018; Weier et al., 1993). Soil N_2O emissions from denitrification are therefore regulated by both overall denitrification rates and the proportion of N_2O reduced to N_2 (Figure 1a; Firestone & Davidson, 1989).

In contrast to Clade I *nosZ*-possessing microorganisms, which predominantly have the genetic potential to be complete denitrifiers, only some Clade II *nosZ*-possessing microorganisms carry the genes for the entire denitrification pathway. The majority of known microorganisms possessing Clade II *nosZ* lack one or both of the key denitrification genes *nir* and *nor*, and thus are considered non-denitrifiers (Figure 2; Hallin et al., 2018; Jones et al., 2013; Sanford et al., 2012). Because most microorganisms with Clade II *nosZ* do not produce N_2O via denitrification, their abundance and diversity have been linked to lower soil N_2O emissions (Domeignoz-Horta et al., 2015, 2016; Samad et al., 2016) and net soil N_2O consumption (Jones et al., 2014). This suggests that considering N_2O reduction beyond the context of canonical denitrification could improve our ability to accurately predict net soil-atmosphere N_2O fluxes and N_2O yields (Figure 1b).

We present a conceptual framework for explicitly considering how microbial diversity may control N_2O emissions in the environment (Figure 3). This framework integrates the possible roles of (a) diversity of N_2O source processes, such as nitrification, bacterial denitrification, fungal denitrification, chemodenitrification, and dissimilatory nitrate reduction to ammonium (DNRA), that contribute to N_2O production potential in the ecosystem, (b) coupling of N_2O production with N_2O reduction that helps determine N_2O reduction potential, (c) phylogenetic diversity in *nosZ*-possessing microorganisms and the diversity in NosZ function, including respiration, electron sink, and detoxification, that contribute to N_2O reduction potential in the ecosystem, (d) environmental factors that control the expression of Clade I *nosZ* and Clade II *nosZ* and the production of Clade I NosZ and Clade II NosZ, and (e) variation in Clade I NosZ versus Clade II NosZ enzyme kinetics. The complexity of this framework highlights how microbial diversity can affect N_2O reduction rates in many ways, which we discuss here.

2.1 | Coupling of N_2O sources to N_2O sinks

While a variety of biotic and abiotic N_2O-producing processes are now known, the fate of N_2O produced by these diverse sources

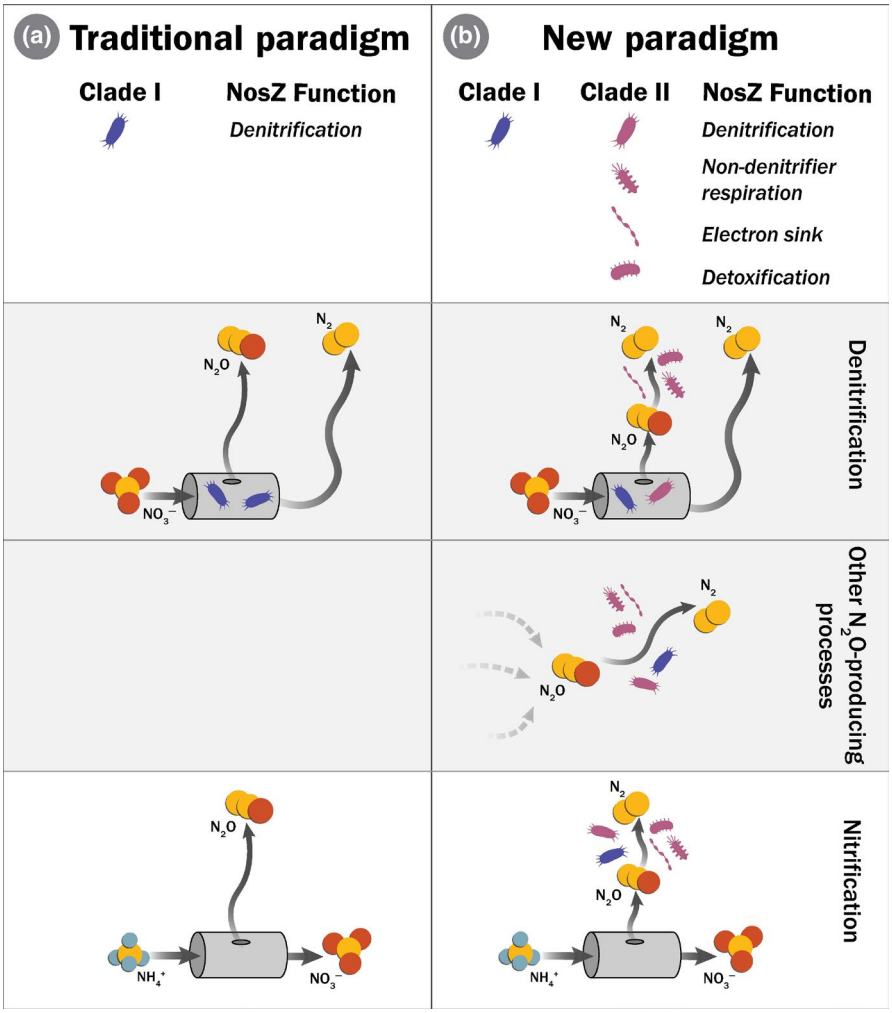

FIGURE 1 (a) The denitrification-focused traditional paradigm of how nitrous oxide (N_2O) reduction regulates soil N_2O emissions and N_2O yield versus (b) the new paradigm that includes greater functional diversity in Clade II N_2O reducers compared to Clade I N_2O reducers. The traditional hole-in-the pipe paradigm proposed by Firestone and Davidson (1989) focuses on denitrification and nitrification as sources of N_2O, which is an intermediate or a by-product of the processes, respectively, and considers only denitrifiers as N_2O reducers. The new paradigm includes non-denitrifiers that can reduce N_2O from many sources, including denitrification, nitrification, chemodenitrification, and dissimilatory nitrate reduction to ammonium [Colour figure can be viewed at wileyonlinelibrary.com]

remains uncertain. The traditional paradigm of controls on soil N_2O emissions explicitly considers only N_2O reduction occurring after N_2O production in the enzymatic sequence of canonical denitrification (Figure 1a). The identification of Clade II N_2O reducers, which are predominantly non-denitrifiers, expands the paradigm to account for the consumption of N_2O produced from a variety of sources (Figure 1b).

The potential coupling of N_2O production and consumption can be predicted from the co-occurrence of *nosZ* and functional genes associated with N_2O production pathways at the ecosystem scale (Graf et al., 2014). This predictive approach has been proposed for denitrification. Known cultured microorganisms with sequenced genomes possessing the S-type nitrite reductase gene (*nirS*) often also possess *nosZ*, whereas microorganisms possessing the K-type nitrite reductase gene (*nirK*) often lack *nosZ*, suggesting that *nirS* microorganisms have greater potential to perform denitrification to the end product N_2 than *nirK* microorganisms (Graf et al., 2014). *nirK* is often more abundant than *nirS* in agricultural ecosystems (Clark et al., 2012, Coyotzi et al., 2017, Philippot et al., 2007) and also in lower pH environments (Bowen et al., 2020; Cuhel et al., 2010). The relative abundance of *nirS* and *nirK* genes in an ecosystem has been suggested to provide constraints on predictions of N_2O reduction versus N_2O release by denitrifiers (Bowen et al., 2020; Cuhel et al., 2010; Jones et al., 2014). However, using *nir* gene abundances to predict soil N_2O emissions inherently considers only denitrifiers as N_2O reducers, does not account for the potential for N_2O production by denitrifiers to be coupled to N_2O reduction by non-denitrifiers, and is limited by the genomic data available from known *nirS*- and *nirK*-expressing microorganisms. With these limitations taken into account, this example illustrates how gene co-occurrence patterns could inform understanding of controls on N_2O emissions across different ecosystems.

Gene co-occurrence of Clade II *nosZ* with various other functional genes associated with N_2O generation within the genomes of individual microorganisms can predict many potential couplings of N_2O production and consumption. For example, the Clade II N_2O reducer *Anaeromyxobacter dehalogenans* lacks *nir* genes to convert nitrite to NO and therefore cannot biochemically generate N_2O via enzymatic denitrification (Figure 2). However, *A. dehalogenans* can reduce both ferric iron (Fe^{3+}) and NO_3^-, with the resulting ferrous iron (Fe^{2+}) and NO_2^- reacting chemically to produce N_2O, which the microorganism then reduces to N_2 to gain additional energy to support its growth (Onley et al., 2018). In this way, *A. dehalogenans* does not directly produce the N_2O that it reduces but rather facilitates chemodenitrification to externally produce N_2O that it can utilize as a respiratory electron acceptor. This microorganism also possesses *nrfA*, the key functional gene for dissimilatory NO_3^- reduction to ammonium

nosZ Type	Representative taxon	(Sub-) Phylum	N-oxide reduction capability
Clade I	*Paracoccus denitrificans* [1]	(Alpha-) Proteobacteria	NO_3^- → NO_2^- → NO → N_2O → N_2
	Bradyrhizobium japonicum [2]	(Alpha-) Proteobacteria	NO_3^- → NO_2^- → NO → N_2O → N_2
	Thauera aromatica K172 [3]	(Beta-) Proteobacteria	NO_3^- → NO_2^- → NO → N_2O → N_2
	Pseudomonas stutzeri DCP-Ps1 [4]	(Gamma-) Proteobacteria	NO_3^- → NO_2^- → NO → N_2O → N_2
	Shewanella loihica PV-4 [5]	(Gamma-) Proteobacteria	NO_3^- → NO_2^- → NO → N_2O → N_2
Clade II	*Dechloromonas aromatica* RCB [6]	(Beta-) Proteobacteria	NO_3^- → NO_2^- → NO → N_2O → N_2
	**Anaeromyxobacter dehalogenans* [7]	(Delta-) Proteobacteria	NO_3^- → NO_2^- NO → N_2O → N_2
	**Wolinella succinogenes* [8]	(Epsilon-) Proteobacteria	NO_3^- → NO_2^- N_2O → N_2
	**Gemmatirosa kalamazoonesis* [9]	Gemmatimonadetes	NO_2^- → NO N_2O → N_2
	**Gemmatimonas aurantiaca* [10]	Gemmatimonadetes	NO_2^- → NO N_2O → N_2
	**Dyadobacter fermentans* [11]	Bacteroidetes	NO_3^- → NO_2^- N_2O → N_2
	Solitalea canadensis [12]	Bacteroidetes	NO_3^- → NO_2^- → NO → N_2O → N_2
	Flavobacterium columnare [13]	Bacteroidetes	NO_3^- → NO_2^- → NO → N_2O → N_2
	Bacillus azotoformans [14]	Firmicutes	NO_3^- → NO_2^- → NO → N_2O → N_2
	**Desulfitobacterium hafniense* [15]	Firmicutes	NO_3^- → NO_2^- NO → N_2O → N_2
	Geobacillus denitrificans NG80-2 [16]	Firmicutes	NO_3^- → NO_2^- → NO → N_2O → N_2
	**Caldilinea aerophila* STL-6-01	Chloroflexi	NO_3^- → NO_2^- NO → N_2O → N_2
	**Opitutus terrae* PB90-1	Verrucomicrobia	NO_2^- → NO N_2O → N_2
	Hydrogenobacter thermophilus [17]	Aquificae	NO_3^- → NO_2^- → NO → N_2O → N_2
	**Pyrobaculum calidifontis*	Crenarchaeota	NO_3^- → NO_2^- NO → N_2O → N_2
	Lepiospira biflexa	Spirochaetes	NO_2^- → NO → N_2O → N_2
	Denitrovibrio acetiphilus [18]	Deferribacteres	NO_3^- → NO_2^- N_2O → N_2
None	*Pseudomonas chlororaphis* [19]	(Gamma-) Proteobacteria	NO_3^- → NO_2^- → NO → N_2O
	Streptomyces thioluteus [19]	Actinobacteria	NO_3^- → NO_2^- → NO → N_2O

* Non-denitrifiers

→ Genetic potential and demonstrated activity

→ Genetic potential only

[1] Baumann *et al.*, 1996; [2] Bedmar *et al.*, 2005; [3] Tschech & Fuchs, 1987; [4] Chee-Sanford & Sanford, unpublished data; [5] Yoon *et al.*, 2015; [6] Coates *et al.*, 2001; [7] Onley *et al.*, 2018; [8] Payne *et al.*, 1982; [9] Chee-Sanford & Sanford, unpublished data; [10] Park *et al.*, 2017; [11] Chee-Sanford & Sanford, unpublished data; [12] Jones *et al.*, 1990; [13] Tekedar *et al.*, 2017; [14] Heylen & Keltjens, 2012; Manachini *et al.*, 2000; [15] Villemur *et al.*, 2006; [16] Liu *et al.*, 2008; [17] Suzuki *et al.*, 2001; [18] Myhr & Torsvik, 2000; [19] Graf *et al.*, 2014

FIGURE 2 Representative taxa of Clade I and Clade II *nosZ*-possessing microbes and their nitrogen oxide reduction capabilities inferred from the functional genes present in their genomes [Colour figure can be viewed at wileyonlinelibrary.com]

(DNRA, also called respiratory ammonification), another process known to generate N_2O (Baggs, 2011; Butterbach-Bahl et al., 2013). Although not yet demonstrated experimentally, *A. dehalogenans* can also potentially reduce the N_2O formed when performing DNRA, thereby limiting DNRA-derived N_2O emissions. Physiological characterization of Clade II N_2O reducers will provide insight into the potential ecological significance of the many possible gene co-occurrence patterns for predicting N_2O emissions in the environment.

2.2 | NosZ enzyme function

The few physiological studies of microorganisms expressing Clade II NosZ suggest that N_2O reduction could serve functions other than yielding energy as a respiratory electron acceptor for growth (Figure 1b; Chee-Sanford et al., 2019; Park et al., 2017; Sullivan et al., 2013; Yin et al., 2019). Based on the respiratory function of NosZ, a common misconception is that the relative thermodynamic favorability of reducing alternative terminal electron acceptors (i.e., O_2, NO_3^-, N_2O) controls N_2O reduction rates. In fact, N_2O reduction yields more free energy than O_2 per electron; however, due to the higher biochemical efficiency of capturing energy aerobically (Chen & Strous, 2013) and the kinetic stability of N_2O (Tolman, 2010), O_2 is favored as an electron acceptor when it is in greater abundance than N_2O. The N_2O yield of denitrification indeed increases as O_2 availability increases in soils (e.g., Burgin & Groffman, 2012; Weier et al., 1993). The N_2O yield also generally increases as soil NO_3^- availability increases (e.g., Chapuis-Lardy et al., 2007; Firestone et al., 1979; Weier et al., 1993), although N_2O reduction has been observed under field conditions with abundant O_2 and NO_3^- in the bulk soil (Yang & Silver, 2016). While N_2O reduction can occur in anoxic microsites present in oxic soils (Sexstone et al., 1985), the identification of non-denitrifying Clade II N_2O reducers opens the possibility that NosZ could serve non-respiratory functions that would lead to N_2O reduction outside of anoxic microsites in unsaturated environments, as described below.

Strict aerobes possessing functional Clade II *nosZ* fundamentally suggest a NosZ function alternative to anaerobic respiration. One such microorganism, *Gemmatimonas aurantiaca*, reduces N_2O only after transitioning from oxic to anoxic conditions (Chee-Sanford et al., 2019; Park et al., 2017). This reduction of N_2O is apparently not associated with growth but rather may function as an electron sink that allows the aerobe to survive transient anoxic periods when O_2 is no longer available (Park et al., 2017). *G. aurantiaca* belongs to one of the most abundant *nosZ*-possessing phylogenetic groups in soil environments, the Gemmatimonadetes phylum (Jones et al., 2013, 2014; Orellana et al., 2014). However, the ubiquity of this alternative NosZ function within the Gemmatimonadetes phylum or across other phyla is not known because it has not yet been tested on a broader diversity of isolates. If it is common, an electron sink function could play an important role in N_2O reduction in the environment during oxic to anoxic transitions, such as following rain events (Krichels et al., 2019; Liptzin et al., 2011).

Nitrous oxide reduction could also serve as a detoxification mechanism for both Clade I and Clade II N_2O reducers. Nitrous oxide

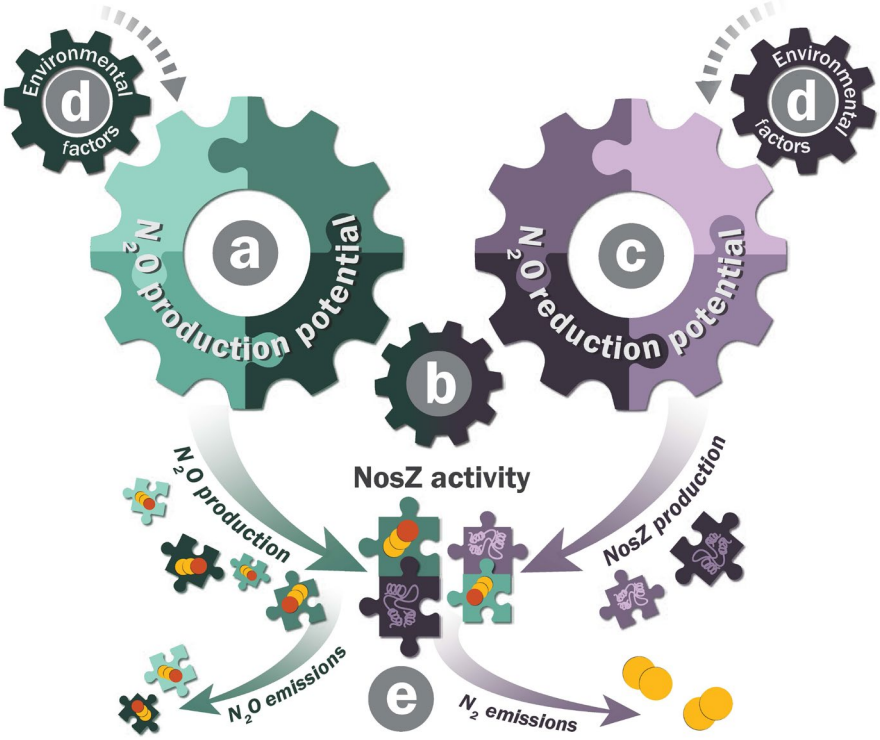

FIGURE 3 Conceptual framework for considering how microbial diversity may mediate controls on emissions of nitrous oxide (N_2O) and dinitrogen (N_2), represented by the yellow and red molecules, in the environment. This framework integrates the possible roles of (a) diversity of N_2O source processes, such as nitrification, bacterial denitrification, fungal denitrification, chemodenitrification, and dissimilatory nitrate reduction to ammonium, represented by the different grades of green, that contribute to N_2O production potential in the ecosystem, (b) the coupling of N_2O production with N_2O reduction that helps determine the N_2O reduction potential, (c) phylogenetic and functional diversity in *nosZ*-possessing microorganisms, represented by the different shades of purple, that contribute to N_2O reduction potential in the ecosystem, (d) environmental factors that control the production of N_2O, the expression of Clade I *nosZ* and Clade II *nosZ*, and the production of Clade I NosZ and Clade II NosZ, represented by the differently shaded purple puzzle pieces, and (e) variation in Clade I NosZ and Clade II NosZ enzyme kinetics. The N_2O produced by different source processes, represented by the differently shaded green puzzle pieces, can be reduced by Clade I NosZ or Clade II NosZ [Colour figure can be viewed at wileyonlinelibrary.com]

can inhibit microbial processes, such as methanogenesis (Balderston & Payne, 1976; Kluber & Conrad, 1998; Tugtas & Pavlostathis, 2007) and reductive dechlorination (Nelson et al., 2002; Yin et al., 2019) by suppressing microbial growth. This toxicity may be caused by the binding of N_2O to cobamides (vitamin B_{12}-type cofactors), thereby preventing cobamide-dependent enzyme reactions essential for many microbial metabolisms (Sullivan et al., 2013; Yin et al., 2019). Although N_2O toxicity has been documented, a detoxification function for NosZ in environmental systems, such as soil, has yet to be demonstrated. This alternative detoxification function of NosZ could potentially predict increasing N_2O reduction rates with increasing N_2O concentrations above a threshold that would cause metabolic inhibition, but currently too little is known about when microorganisms utilize the detoxification function under *in situ* conditions in the environment.

2.3 | N_2O reducer community structure

Although Clade II *nosZ* genes appear to be more abundant than Clade I *nosZ* genes in many soils, wide variation in the relative abundances of the two groups across ecosystems suggests that their relative contribution to N_2O reduction may vary predictably across ecosystems (Hallin et al., 2018; Jones et al., 2013; Orellana et al., 2014; Sanford et al., 2012). We do not yet know which factors determine the relative abundance of Clade I versus Clade II *nosZ*. Higher growth yields with N_2O for Clade II organisms could contribute to their greater abundance in many soils (Sanford et al., 2012; Yoon et al., 2016). As discussed in the next "NosZ enzyme kinetics" section, variation in NosZ enzyme kinetics has also been suggested to contribute to niche differentiation between Clade I and Clade II N_2O reducers (Hallin et al., 2018; Yoon et al., 2016), although there is not yet field-based evidence in support of this hypothesis. In addition, understanding the relationship between phylogenetic and functional diversity in Clade II NosZ could aid the prediction of Clade II N_2O reducer abundance based on environmental conditions more conducive for their potential metabolisms. However, the high Clade II *nosZ* diversity (Graf et al., 2014; Orellana et al., 2014) makes it more challenging to discern factors that regulate the relative abundance of Clade I and Clade II *nosZ* because Clade II microorganisms cannot be treated as a monolithic group.

2.4 | NosZ enzyme kinetics

Differences in NosZ enzyme kinetics could contribute to niche differentiation of N_2O reducers and possibly also contrasting temporal patterns in N_2O reduction by Clade I versus Clade II N_2O reducers. In the few bacterial pure cultures that have been studied thus far, Clade I N_2O reducers have exhibited lower substrate affinity (i.e., high K_s values) and higher maximum N_2O utilization rates (i.e., V_{max}) than observed for Clade II N_2O reducers (Suenaga et al., 2018; Yoon et al., 2016). The lower substrate affinity would restrict N_2O reduction by Clade I N_2O reducers to environments with higher N_2O concentrations. As such, Clade I N_2O reducers could episodically contribute to N_2O reduction when high soil N_2O concentrations occur, such as following stimulation of N_2O production by fertilizer application or large rain events (Krichels et al., 2019; Shcherbak et al., 2014). In addition, the higher maximum utilization rate of Clade I N_2O reducers would favor their dominance where and when high N_2O conditions occur more frequently. In contrast, the higher substrate affinity (i.e., low K_s) of Clade II N_2O reducers (Suenaga et al., 2018; Yoon et al., 2016) would allow them to occupy a low N_2O concentration niche and contribute a steady background rate of N_2O reduction in the environment regardless of N_2O concentration. The high substrate affinity of Clade II NosZ may also enable reduction of N_2O at the sub-atmospheric concentrations observed in soils acting as net N_2O sinks (Yang & Silver, 2016).

Despite these tantalizing ecological implications, NosZ enzyme kinetics can vary as much within clades as between clades (Suenaga et al., 2018; Yoon et al., 2016). Physiological characterization of a greater number of representative taxa across the diversity of Clade I and Clade II N_2O reducers will clarify the potential for enzyme kinetics to cause niche differentiation of N_2O reducers and differing temporal patterns in N_2O reduction by Clade I versus Clade II N_2O reducers. Furthermore, characterization of community-level N_2O reduction kinetics can elucidate if and how diversity in NosZ enzyme kinetics across ecosystems influences patterns in N_2O emissions.

3 | TAKING STOCK OF *NOSZ* STUDIES

Since the identification of Clade II *nosZ* genes as distinctive from Clade I *nosZ* genes, some studies have begun to shed light on the potential ecological significance of the organisms possessing Clade II *nosZ* compared to those with Clade I *nosZ*. Yet, anecdotally, it seems that many *nosZ* studies continue to be conducted in ignorance of the two distinct clades, and in some cases cause confusion about Clade II *nosZ*. A census of peer-reviewed *nosZ* articles published since 2013, 1 year after Clade II *nosZ* was first reported in the literature (Jones et al. 2013; Sanford et al. 2012), provides insight into potential factors hindering the spread of awareness and advances in knowledge about the two distinct *nosZ* clades.

We identified peer-reviewed original research articles discussing *nosZ* and published from 2013 to 2019 by searching the Web of Science using the following topic search terms individually: "nosZ," "atypical nosZ," "nosZ II," "nosZ-II," "nosZII," "clade II nosZ," "nosZ clade II," "nosZ2," "cluster II nosZ," "clade II N_2O," or "clade II nitrous oxide." This search yielded 800 articles that were included in the census. From the collected articles, we extracted information about: (1) the approaches used to evaluate *nosZ*; (2) the primary research disciplines of the journals they were published in; (3) the terminologies used to describe the *nosZ* genes; and (4) efforts to correlate *nosZ* gene or transcript abundances with measures of microbial activity used as proxies for N_2O reduction activity. We report the number of publications in each category for a given variable (e.g., primary research disciplines listed by Web of Science for the journals the *nosZ* articles were published in, the approach used to evaluate *nosZ*, and whether a statistically significant correlation between *nosZ* gene abundance and N_2O reduction-related microbial activity was reported in the article).

This census revealed that most of the 800 peer-reviewed *nosZ* articles published in the 7 years following the first reports of Clade II *nosZ* exhibited no awareness of Clade II (Figure 4). In only 12% of studies did researchers evaluate Clade II *nosZ*. In an additional 8% of *nosZ* articles, the researchers merely mentioned Clade II *nosZ*, sometimes simply to dismiss its potential relevance in the ecosystem being studied (Figure 4). The majority of studies evaluating Clade II *nosZ* used quantitative PCR (qPCR) to enumerate gene or transcript abundances (59%), and few studies used other approaches (e.g., metagenome sequencing, peptide analysis, or microarray analysis) to characterize the communities of Clade II N_2O reducers (Figure S1). The annual number of *nosZ* publications increased over the 7-year time period, nearly tripling during that time. At the same time, the percentage of *nosZ* articles published each year that included evaluation or mention of Clade II *nosZ* increased from 5% in 2013 to 22% in 2019. These trends reflect recognition that N_2O reduction plays an important role in mitigating N_2O emissions and increasing awareness of Clade II *nosZ*. However, the data suggest that most researchers continue to be unaware that studying only Clade I *nosZ* will, for most environmental systems studied, lead to an incomplete understanding of N_2O reduction.

We found that the tendency for researchers to interact primarily within their disciplines (i.e., disciplinary silos) may contribute to the slow spread of awareness about Clade II *nosZ*. The distinction between Clade I and Clade II *nosZ* was initially reported in a multidisciplinary journal that bridges research fields (Sanford et al. 2012). In 2013–2019, of *nosZ* articles published in Multidisciplinary Science journals, 32% mentioned Clade II *nosZ*, which is higher than the percentage of all *nosZ* articles that mentioned Clade II *nosZ* (20%; Table 1). Similarly, 29% of *nosZ* articles published in Microbiology and Biochemistry journals exhibited awareness of Clade II *nosZ* (Table 1). In contrast, two thirds of the 800 *nosZ* articles from 2013 to 2019 were published in journals within the two broadly defined disciplines of (1) Biotechnology, Chemistry and Engineering, and (2) Ecology and Environmental Science; however, only 12% and 18% of the *nosZ* articles in these two broad disciplines, respectively, mentioned Clade II *nosZ* (Table 1). None of the 17 *nosZ* articles published in Agriculture and Agronomy journals mentioned Clade II *nosZ* (Table 1). The disparity in awareness of Clade II *nosZ* among research

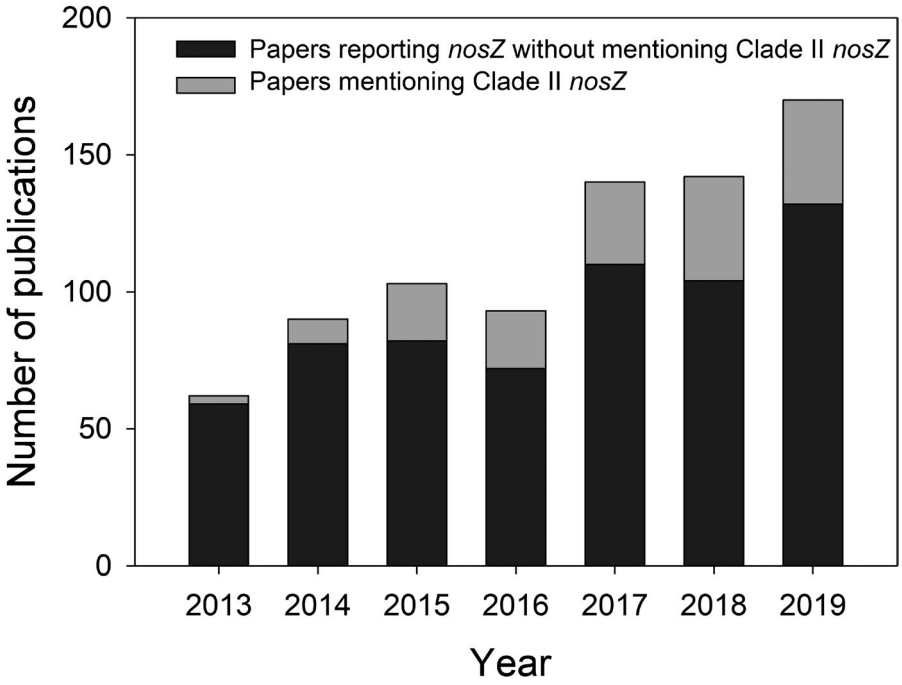

FIGURE 4 Frequency distribution of peer-reviewed *nosZ* articles published annually from 2013 to 2019, the time period following the first reports of Clade II *nosZ* by Sanford et al. 2012 and Jones et al. 2013. The black portion of each bar represents *nosZ* articles that did not evaluate or mention Clade II *nosZ*, and the gray portion of each bar represents *nosZ* articles that evaluated or simply referred to Clade II *nosZ*

disciplines suggests that advances in knowledge about Clade II NosZ and the relevance of diversity in *nosZ* genes to understanding the controls on N_2O emissions could be better conveyed across disciplines.

A lack of consensus on terminology used to refer to Clade I and Clade II *nosZ* may contribute to confusion about the distinction between the two clades. Since the initial studies reporting on "typical *nosZ*" versus "atypical *nosZ*" (Sanford et al. 2012) and "clade I *nosZ*" versus "clade II *nosZ*" (Jones et al. 2013), the peer-reviewed literature has included 17 different terminologies derived from these initial designations to refer to these two groups of *nosZ* genes (Figure 5). In addition, some articles acknowledged the distinction between Clade I and Clade II *nosZ* without using specific terminology to refer to the two clades. Of the 17 specific terminologies used, most appear in three or fewer articles, suggesting that the authors of these articles devised their own terminology. Ultimately, there is no consistent nomenclature used in the published literature, with four terminologies most frequently used: *nosZ*I and *nosZ*II (29%), typical *nosZ* and atypical *nosZ* (19%), *nosZ* Clade I and *nosZ* Clade II (20%), and Clade I *nosZ* and Clade II *nosZ* (13%) (Figure 5). The diverse terminology used to refer to Clade I and Clade II *nosZ* could make it more difficult for researchers regardless of discipline to use keywords to search the literature for recent advances about Clade II N_2O reducers.

Some Clade I *nosZ* articles documented efforts to correlate Clade I *nosZ* gene abundance with microbial activity even though gene abundance typically does not reflect activity (Rocca et al., 2015). Furthermore, most measures of microbial activity used in the published studies did not target N_2O reduction to N_2 by NosZ, but rather, represented total denitrification (N_2O + N_2) and N_2 production without differentiating source processes (e.g., denitrification, chemodenitrification, anammox, Feammox, etc.), or net soil-atmosphere N_2O fluxes. It therefore seems even more unlikely that *nosZ* gene abundance would correlate with the measured process rates. Nonetheless, across the different measures of microbial activity, 11%–50% of articles that included correlation of Clade I *nosZ* gene abundance and activity reported the correlation to be statistically significant (Figure S2). Considering study systems represented by more than 15 publications, Clade I *nosZ* linkage to microbial activity also varied little across study systems; statistically significant correlations were reported for 10%–30% of articles documenting correlations with total denitrification rates and 20%–25% of articles documenting correlations with net soil-atmosphere N_2O fluxes (Figure S3ab). Assessing statistical significance at $p < .05$ leads to false positive (Type I) errors approximately 28% of the time (Hubbard et al., 2003). We therefore infer that the well-constrained and relatively low frequency of significant correlations reported across different measures of N_2O reduction-related activity and study systems provides little support for true correlations between Clade I *nosZ* gene abundances and the measured microbial activities.

4 | CHALLENGES IN STUDYING DIVERSITY OF N_2O REDUCERS

The low number of studies that included evaluation of Clade II *nosZ* may reflect not only a slowly spreading awareness of Clade II N_2O reducers but also the inherent challenges in studying the diversity of N_2O reducers. To test hypotheses about the ecological importance of phylogenetic and functional diversity of N_2O reducers (Figure 1), we must characterize the physiology of N_2O reducers across high phylogenetic diversity (Figure 2) and quantify the relative abundance and activity of Clade I and Clade II N_2O reducers under varying environmental conditions (Figure 3). Here we discuss how accounting for and characterizing phylogenetic

TABLE 1 Research disciplines of journals for original research articles published from 2013 to 2019 that discussed only Clade I *nosZ* or discussed both Clade I *nosZ* and Clade II *nosZ*.

Discipline	Primary Web of Science subject category for journal	Number of articles with Clade I *nosZ* only	Number of articles with Clades I and II *nosZ*	Total number of *nosZ* articles	Percent of *nosZ* articles with Clade II *nosZ*
Agriculture and agronomy	Agriculture	11	0	11	0
	Agronomy	6	0	6	0
	Total	17	0	17	0
Biotechnology, chemistry, and engineering	Biotechnology and applied microbiology	86	3	89	3
	Chemistry	8	1	9	11
	Environmental engineering	30	7	37	19
	Green and Sustainable science & technology	1	0	1	0
	Materials science	1	0	1	0
	Water Resources	25	10	35	29
	Total	151	21	172	12
Ecology and environmental sciences	Ecology	19	5	24	21
	Environmental sciences	144	21	165	13
	Fisheries	1	0	1	0
	Forestry	1	2	3	67
	Geosciences	5	0	5	0
	Limnology	2	1	3	33
	Marine and freshwater biology	7	3	10	30
	Oceanography	3	0	3	0
	Plant Sciences	1	0	1	0
	Soil Science	121	35	156	22
	Total	304	67	371	18
Microbiology and biochemistry	Biochemical research methods	3	1	4	25
	Biochemistry and molecular biology	10	4	14	29
	Microbiology	115	48	163	29
	Total	128	53	181	29
Multidisciplinary sciences	Multidisciplinary sciences	40	19	59	32
	Total	31	40	19	59
	Overall Total	640	160	800	20

and functional diversity are made difficult by methodological challenges.

Physiological differences between Clade I and Clade II N_2O reducers underlie the potential ecological importance of diversity in N_2O reducers, yet many of the Clade II *nosZ* diversity is represented by few representative cultures available for physiological characterization. Advances in molecular-based tools, such as amplicon-based sequencing and shotgun metagenome sequencing, have led to a shift away from cultivation, yet gene sequence information alone reveals little about a microorganism's activity in the environment. For example, Clade II NosZ activity has yet to be demonstrated in the soil isolate *Opitutus terrae*, an abundant soil taxon that possesses Clade II *nosZ* (Sanford et al., 2012). Sequencing surveys in soils have documented the widespread presence and relatively high abundances of members within the Gemmatimonadetes (Jones et al., 2013, 2014; Orellana et al., 2014), yet this phylum is represented by only seven reported isolates, with three of them from soil (DeBruyn et al., 2013; Joseph et al., 2003; Pascual et al., 2016, 2018). Thus far only two of these isolates have been shown to express active Clade II NosZ, *Gemmatimonas aurantiaca* and *Gemmatirosa kalamazoonesis* (DeBruyn et al., 2013; Joseph et al., 2003). Members of the phylum Verrucomicrobia are also abundant in soils (Carbonetto et al., 2014; Fierer et al., 2012; Orellana et al., 2014), but even fewer cultivated strains of Verrucomicrobia have been obtained (Davis et al., 2005; Joseph et al., 2003). Many of these isolates were previously deemed "unculturable" when relying on traditional enrichment and isolation strategies that employ commercial growth media (e.g., nutrient agar) that fail to select for

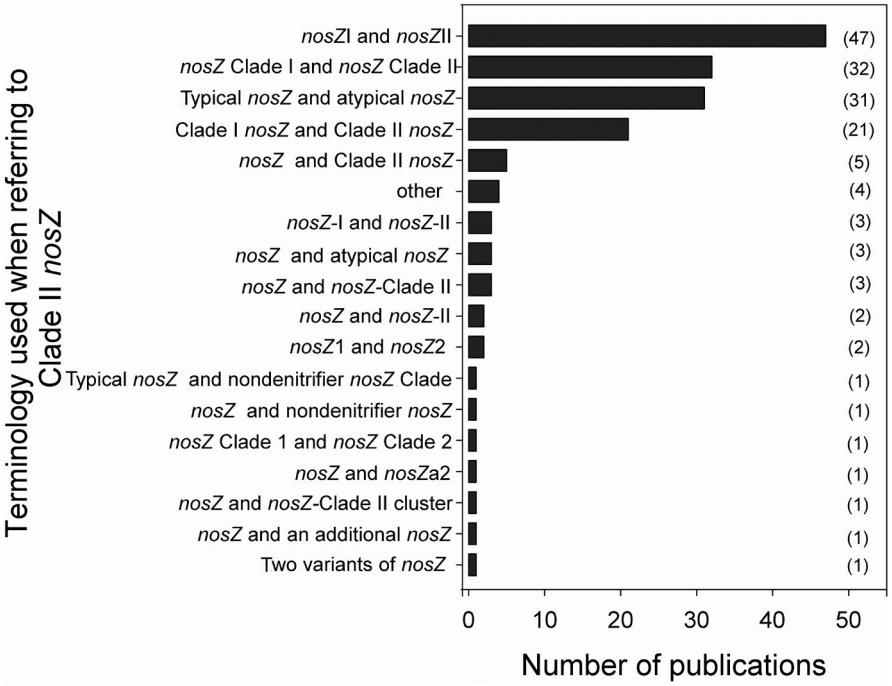

FIGURE 5 Frequency distribution of the usage of specific terminologies for Clade I *nosZ* and Clade II *nosZ* in peer-reviewed articles from 2013 to 2019. The "other" category refers to publications including acknowledgment of the distinction between Clade I and Clade II *nosZ* without using specific terminology to refer to the two clades. The number represented by each bar is shown in parentheses

more fastidious or slow-growing organisms (Davis et al., 2005). The use of nonconventional cultivation approaches and selective growth substrates has yielded novel isolates (Joseph et al., 2003; Vartoukian et al., 2010). However, in general, cultivation efforts are becoming rarer despite their benefit for our understanding of microbial physiology and function. Due to the metabolic diversity of organisms with Clade II *nosZ*, and in order to properly test the hypothesized ecological role of N_2O reducers with this type of *nosZ*, we must invest effort in cultivating a greater number of taxa with different Clade II *nosZ* subclades so that physiological studies can be conducted (Gutleben et al., 2018).

The high phylogenetic diversity in Clade II *nosZ* relative to Clade I *nosZ* presents a challenge in our ability to fully characterize the active populations as microbial communities respond to dynamic environmental conditions. A metagenomic approach can more comprehensively capture the genetic diversity of functional genes, such as Clade I and Clade II *nosZ*, and avoid biases in the representation of genetic diversity associated with the use of primers in amplicon-based sequencing or targeted qPCR approaches. However, the current cost of metagenome sequencing is prohibitive for the large sample numbers typically evaluated using amplicon-based approaches. Moreover, current bioinformatics tools to aid metagenomics data analysis are not yet standardized, easy to use, and publicly available. Therefore, amplicon sequencing- and qPCR-based approaches are more commonly used (Figure S1). These techniques rely on primers designed to effectively target conserved regions of the gene, generally taken from public databases such as National Center for Biotechnology Information (NCBI) or FunGene (Fish et al., 2013). A PCR primer set was previously designed to target conserved regions of Clade II *nosZ* (Jones et al. 2013), and it is commonly used in Clade II *nosZ* studies (Table 2). However, the amplicon size generated by this primer set (~680–700 bp) is suboptimal for next-generation sequencing platforms because the sequencing read lengths are too short to fully cover the amplicon length (i.e., the forward read and reverse read of sequencing do not overlap, such that only the forward or reverse read of each amplicon sequence is available for analysis). In addition, the product generated with this primer pair is outside the optimal size range for qPCR. More importantly, while this primer set provides broad coverage (86.1%) of Clade II *nosZ* identified on the genomes of isolates (Ma et al., 2019), it does not capture the full diversity of Clade II *nosZ* genes, missing potentially important groups that have been observed in natural environments (Chee-Sanford et al., 2020). As a result, this *nosZ*-targeted primer set will not yield accurate results for Clade II *nosZ* gene abundance using qPCR or for relative Clade II *nosZ* gene amplicon abundances using sequencing. Recently, a suite of primers was designed to target specific Clade II subclades from reference genomes and sequences retrieved from environmental samples for effective use in both next-generation sequencing platforms and qPCR (Chee-Sanford et al., 2020). These new primers can be used in independent qPCR assays that would allow quantitative comparisons of the targeted subclades of Clade II *nosZ* in any given sample. These recent technical developments allow better assessments of both Clade I and Clade II *nosZ* and should be applied in future studies in which PCR-based methods are used for DNA and/or transcript analyses. As available *nosZ* sequences increase, refinements to existing primer sets or new primer design can continue to advance amplicon-based molecular approaches (Chee-Sanford et al., 2020).

Characterizing the functional importance of diversity in N_2O reducers in the environment requires distinguishing N_2O reduction by Clade II N_2O reducers from that of Clade I N_2O reducers. However, accurately quantifying rates of N_2O reduction to N_2 in soils is already difficult in general due to the high atmospheric N_2

TABLE 2 Primers for Clade I *nosZ* and Clade II *nosZ*, and the frequency of their use reported in peer-reviewed *nosZ* original research articles published from 2013 to 2019[a]

Target clade	Reference	Number of articles[b]	Primer name	Sequence (5′–3′)	Amplicon size (bp)
Clade I	Henry et al. (2006)	84	nosZ2F	CGGRACGGCAASAAGGTSMSSGT	267
			nosZ2R	CAKRTGCAKSGCRTGGCAGAA	
			nosZ1F	WCSYTGTTCMTCGACAGCCAG	259
			nosZ1R	ATGTCGATCARCTGVKCRTTYTC	
Clade I	Throback et al. (2004)	14	nosZ-F	CGYTGTTCMTCGACAGCCAG	~450
			nosZ1622R	CGCRASGGCAASAAGGTSCG	
Clade I	Jones et al. (2013)	11	nosZ-F	CGCTSTTYMTIGAYAGYCAG	~450
			nosZ-R	SKSACCTTITTRCCITYICG	
Clade I	Kloos et al. (2001)	9	nosZ-F	CGY TGT TCM TCG ACA GCC AG	~700
			nosZ-R	CAT GTG CAG NGC RTG GCA GAA	
Clade I	Rich et al. (2003)	6	nosZ-F–1181	CGCTGTTCITCGACAGYCAG	700
			nosZ-R–1880	ATGTGCAKIGCRTGGCAGAA	
Clade I	Scala and Kerkhof (1998)	5	Nos661F	CGGCTGGGGGCTGACCAA	1100
			Nos1773R	ATRTCGATCARCTGBTCGTT	
			Nos661F	CGGCTGGGGGCTGACCAA	900
			Nos1527R	CTGRCTGTCGADGAACAG	
			Nos1527F	CGCTGTTCHTCGACAGYCA	250
			Nos1773R	ATRTCGATCARCTGBTCGTT	
Clade I	Wyman et al. (2013)	1	nosZF1	GAYGTNCANTAYCARCCNGGNCA	612
			nosZR	CATYTCCAKRTGNADNGCRTGRCA	
Clade II	Jones et al. (2013)	74	nosZ-II-F	CTI GGI CCI YTK CAY AC	689–716
			nosZ-II-R	GCI GAR CAR AAI TCB GTR C	
Clade II	Sanford et al. (2012)	4	NosZ912F	CGTCCCCGGCCTCGTGTA	~912
			NosZ1853R	GAGCAGAAGTTCGTGCAGTAGTAGGG	

[a]See Ma et al. (2019) and Chee-Sanford et al. (2020) for updated information about *nosZ* primers and optimal PCR conditions.
[b]Articles that reported using more than one primer were accordingly counted under each primer used.

background (Groffman et al., 2006). Moreover, the methods for measuring soil N_2 production cannot target specific N_2 production processes because they do not differentiate the sources of N_2 (Almaraz et al., 2020). We therefore can only link N_2O reduction rates to specific taxa in pure culture studies, which may not be indicative of their contribution to N_2O reduction activity in the environment. Alternatively, transcriptomic or proteomic analysis approaches can be used to probe *nosZ* gene transcription or NosZ enzyme synthesis, respectively, as proxies for N_2O reduction activity by specific taxa (Helbling et al., 2012; Hettich et al., 2012; Hultman et al., 2015; Jansson & Hofmockel, 2018). These omics methods necessitate experimentally altering environmental conditions to induce changes in gene transcription or protein synthesis in order to deduce which taxa are responsible for observed N_2O reduction activity. However, gene transcription may not always correlate with enzyme activity (Vogel & Marcotte, 2012), and enzyme quantification is notoriously difficult, especially in soils (Orellana et al., 2019; Wang et al., 2016). Nevertheless, without methods that can target measurement of N_2O reduction rates by different taxa, these omics-based proxies for microbial activity are currently the most appropriate tools for elucidating the functional importance of Clade II N_2O reducers in the environment.

5 | RECOMMENDATIONS FOR ADVANCING UNDERSTANDING OF N_2O REDUCER DIVERSITY

Based on the synthesis of current knowledge about Clade II N_2O reducers and the census of peer-reviewed original research articles discussing *nosZ*, we make the following recommendations to accelerate progress in understanding the role of diversity in N_2O reducers in controlling N_2O reduction rates and N_2O emissions:

1. *Break down disciplinary silos* to facilitate the exchange of knowledge about N_2O reducers and N_2O dynamics in the environment. Thus far, advances in knowledge about Clade II N_2O reducers have been driven by microbiologists, microbial ecologists, and biochemists characterizing the physiological and phylogenetic diversity of bacteria with Clade II *nosZ* as well

as the relative abundances of Clade I *nosZ* and Clade II *nosZ* in the environment. This "bottom-up" approach complemented by a "top-down" characterization of spatiotemporal patterns in soil N_2O concentrations, N_2O reduction rates, and N_2O fluxes by ecosystem ecologists, biogeochemists, and others will allow researchers to more quickly home in on environments, in which hypotheses about the ecological roles of Clade I and Clade II N_2O reducers can be tested and refined.

2. *Create a more cohesive body of literature on Clade I nosZ and Clade II nosZ* using consistent and unambiguous nomenclature. We urge the research community to henceforth adopt the nomenclature of Clade I *nosZ* and Clade II *nosZ* first introduced by Jones et al. (2013) and subsequently used in equal frequency (when considering it together with its minor variant *nosZ* Clade I and *nosZ* Clade II) as the other two most common terminologies (*nosZ*I and *nosZ*II, and typical *nosZ* and atypical *nosZ*). Researchers can use other terminologies as additional keywords to build bridges to prior publications that included those variant terminologies.
3. *Enable physiological characterization of more reference organisms possessing Clade II nosZ* by increasing efforts to isolate novel N_2O-reducing organisms from diverse environments. Given that microbial physiology underlies the hypothesized ecological significance of diversity in NosZ, these efforts will be critical for improving our understanding of the roles of Clade I and Clade II N_2O reducers in controlling soil N_2O emissions.
4. *Expand research efforts on elucidating the drivers of functional diversity in N_2O reducers and variation in N_2O reducer community structure across diverse environments*. This includes better characterizing intra- and inter-clade variation in NosZ enzyme kinetics and N_2O reducer growth yields, exploring NosZ function under different environmental conditions, and investigating niche differentiation between Clade I and Clade II N_2O reducers in diverse environments.
5. *Better characterize the diversity of Clade II nosZ in the environment* through the use of omics and improved amplicon-based sequencing approaches. Metagenomics, transcriptomics, and proteomics will become increasingly accessible as these approaches become more cost-effective for higher sample numbers and standardized bioinformatics approaches become more routine. Meanwhile, amplicon-based sequencing and qPCR remain indispensable tools to detect and quantify *nosZ* genes in environmental matrices. Primers should be continually modified, optimized, and redesigned based on updated sequence information to improve specificity and coverage in detecting and quantifying both Clade I *nosZ* and Clade II *nosZ*. Due to the high diversity in Clade II *nosZ*, we recommend using a suite of primers targeting distinct Clade II *nosZ* subclades (Chee-Sanford et al., 2020) as opposed to using only broad specificity Clade II *nosZ* primers (Jones et al., 2013). To best leverage these molecular data for future meta-analyses that can reveal overarching principles that underlie microbial N_2O reduction in soil, we also urge researchers to use standardized nucleic acid extraction protocols (Plassart et al., 2012) and transparently report the workflow used to process and interpret the data.
6. *Consider the limitations of process rate measurements* in efforts to determine the functional importance of Clade I and Clade II N_2O reducers. Few, if any, methodologies target the activity of NosZ—that is, they do not enable measurement of N_2O reduction rates specifically (Almaraz et al., 2020). As such, we caution that researchers should take into account the limitations and assumptions of the methodologies employed when interpreting their process rate data. Even if N_2O reduction rates by NosZ could be measured specifically, existing methodologies cannot partition the contribution of different N_2O reducers to this activity in the environment. To complement these measurements and to identify dominant taxa with NosZ, transcriptomic, proteomic, or amplicon-based targeted *nosZ* gene expression (i.e., from mRNA pools) approaches should also be used.

6 | CONCLUSION

There is much yet to be learned about Clade II N_2O reducers, but the diversity in N_2O reducers holds promise for improving our understanding of controls on N_2O emissions from soil and other environments. The challenges in studying the phylogenetic and functional diversity of N_2O reducers that we have reviewed here currently make it difficult for researchers to fully address the role of microbial diversity in their studies. However, moving forward, Clade II N_2O reducers should be considered along with Clade I N_2O reducers in interpreting the role of NosZ in controlling N_2O emissions in the environment. The known features of Clade II *nosZ* that bear consideration include (1) its high phylogenetic diversity and representation in a wide range of microbial taxa and (2) its presence largely (though not exclusively) in the genomes of non-denitrifiers. The hypothesized features of Clade II NosZ that warrant further investigation include (1) enzyme function aside from respiration, and (2) differing enzyme kinetics compared to Clade I NosZ. These known and hypothesized features of Clade II N_2O reducers expand the potential ecological role of N_2O reducers beyond the constraints of conditions conducive to denitrification.

ACKNOWLEDGMENTS

The authors appreciate graphic design by Sada Egenriether who created the conceptual diagrams. This study was funded by the US National Science Foundation Dimensions of Biodiversity grants DEB 1831842, 1831582 and 1831599. JS was supported by the National Key Research and Development Program of China (No. 2017YFD0200100) and the China Scholarship Council (No. 201804910070). JS also received support as a member of Youth Innovation Promotion Association, Chinese Academy of Sciences (2017362).

AUTHOR CONTRIBUTIONS

WY conceived the manuscript idea based on discussions with RS, JCS, FEL, and KK; WY, RS, JCS, and JS designed the census; JS

conducted the census and wrote the first draft of the manuscript; JS, WY, RS, JCS, SO, FL, and KK revised the manuscript.

DATA AVAILABILITY STATEMENT

Data from this study are available at the Illinois Data Bank: https://doi.org/10.13012/B2IDB-5788371_V1.

ORCID

Jun Shan https://orcid.org/0000-0002-0276-4456
Wendy H. Yang https://orcid.org/0000-0002-2104-4796

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Shan J, Sanford RA, Chee-Sanford J, et al. Beyond denitrification: The role of microbial diversity in controlling nitrous oxide reduction and soil nitrous oxide emissions. *Glob Change Biol*. 2021;27:2669–2683. https://doi.org/10.1111/gcb.15545