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Minireviews

MINIREVIEWS

Ecological and physiological implications of nitrogen oxide reduction pathways on greenhouse gas emissions in agroecosystems

Sukhwan Yoon[1,*], Bongkeun Song[2], Rebecca L. Phillips[3], Jin Chang[1] and Min Joon Song[1]

[1]Department of Civil and Environmental Engineering, KAIST, 291 Daehakro, Yuseonggu, Daejeon 34141, South Korea, [2]Department of Biological Sciences, Virginia Institute of Marine Sciences, College of William and Mary, 1375 Greate Rd, Gloucester Point, VA 23062, USA and [3]Ecological Insights Corporation, 130 69th Street SE, Hazelton, ND 58544, USA

*Corresponding author: Sukhwan Yoon, Department of Civil and Environmental Engineering, KAIST, 291 Daehakro, Yuseonggu, Daejeon 34141, South Korea, Tel: +82 42 350 3631; Fax: +82 42 350 3610; E-mail: syoon80@kaist.ac.kr

One sentence summary: This review summarizes the recent discoveries regarding the ecology and physiology of Nitrogen-oxide reduction pathways and their implications to greenhouse gas emissions in agroecosystems.

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ABSTRACT

Microbial reductive pathways of nitrogen (N) oxides are highly relevant to net emissions of greenhouse gases (GHG) from agroecosystems. Several biotic and abiotic N-oxide reductive pathways influence the N budget and net GHG production in soil. This review summarizes the recent findings of N-oxide reduction pathways and their implications to GHG emissions in agroecosystems and proposes several mitigation strategies. Denitrification is the primary N-oxide reductive pathway that results in direct N_2O emissions and fixed N losses, which add to the net carbon footprint. We highlight how dissimilatory nitrate reduction to ammonium (DNRA), an alternative N-oxide reduction pathway, may be used to reduce N_2O production and N losses via denitrification. Implications of *nosZ* abundance and diversity and expressed N_2O reductase activity to soil N_2O emissions are reviewed with focus on the role of the N_2O-reducers as an important N_2O sink. Non-prokaryotic N_2O sources, e.g. fungal denitrification, codenitrification and chemodenitrification, are also summarized to emphasize their potential significance as modulators of soil N_2O emissions. Through the extensive review of these recent scientific advancements, this study posits opportunities for GHG mitigation through manipulation of microbial N-oxide reductive pathways in soil.

Keywords: nitrogen cycling; agricultural carbon footprint; denitrification; dissimilatory nitrate reduction to ammonium; fungal denitrification; chemodenitrification

INTRODUCTION

Nitrate (NO_3^-) is the most oxidized form of nitrogen (N) and often the dominant form in soil and aquatic environments where oxygen (O_2) is readily available to microbial communities (Canfield, Glazer and Falkowski 2010). When transported to anoxic environments or upon sudden transition of the surroundings to anoxia, NO_3^- serves as an alternative electron acceptor for the residing microorganisms (Postma *et al.* 1991; Aulakh, Doran and Mosier 1992; Giles *et al.* 2012). Nitrate is first reduced to nitrite (NO_2^-), an intermediate that is considered

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the 'gateway' to a suite of reductive processes (Jetten 2008). The reductive transformation of NO_2^- is a crucial exit path for N introduced to the terrestrial systems through biological nitrogen fixation or fertilization (Tiedje *et al.* 1983; Galloway *et al.* 2004).

The increasing concern in greenhouse gas (GHG) emissions and climate change has recently rekindled scientific interests in biotic and abiotic pathways that utilize the reactive N-oxides as electron acceptors (Canfield, Glazer and Falkowski 2010; Montzka, Dlugokencky and Butler 2011; Doane 2017). Microbial denitrification is a stepwise reduction pathway of NO_3^- and NO_2^- to gaseous N species including nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2). These gaseous losses following agricultural fertilization contribute to the problem of low crop N use efficiency, which is typically below 40% (Canfield, Glazer and Falkowski 2010). Nitrogen fertilizer production processes (e.g. Haber-Bosch process for ammonium synthesis) are energy-intensive and thus, synthetic fertilizers generally have high carbon dioxide (CO_2) emission factors. One plausible approach for reducing agricultural GHG emissions is to control gaseous N losses from soil denitrification, allowing for more efficient use of fertilizer N (Garnett 2011; Zhang *et al.* 2013a).

Microbial denitrification is a major source of N_2O, a potent greenhouse gas with ~320 times higher global warming potential than CO_2 (Thomson *et al.* 2012). Paradoxically, the last step of denitrification pathway, N_2O reduction to N_2, is the only known biological sink of N_2O. Management of the kinetic balance between production and consumption of N_2O represents a path forward toward N_2O emission mitigation, but the kinetic balance is complicated by the multitude of N_2O-producing pathways. The prerequisite for this approach is greater understanding of the regulatory mechanisms for prototypical (prokaryotic) denitrification and other understudied pathways leading to N_2O emissions, including fungal denitrification, codenitrification, and chemodenitrification (Spott, Russow and Florian Stange 2011; Wei *et al.* 2014; Buchwald *et al.* 2016). In this review, we summarize the ecology and physiology of microbes participating in N-oxide reduction and their linkages to GHG emissions in agroecosystems. We also propose potential strategies for mitigating N_2O emissions, and thereby carbon footprints, for agroecosystems.

FUNDAMENTALS OF BIOTIC AND ABIOTIC NITRATE/NITRITE REDUCTION PATHWAYS

In reduced anoxic environment, dissimilatory reduction of NO_3^- or NO_2^- typically results in production of N_2O, N_2, and NH_4^+ as the end-products (Kraft, Strous and Tegetmeyer 2011; Rütting *et al.* 2011; Matocha, Dhakal and Pyzola 2012; Heil, Vereecken and Brüggemann 2016). Reduction of NO_3^- occurs exclusively via NO_2^- as the intermediate; thus, NO_2^- is a common gateway for biotic and abiotic pathways of all known NO_3^- and NO_2^- reduction pathways (Fig. 1): prokaryotic and fungal denitrification, dissimilatory NO_3^-/NO_2^- reduction to NH_4^+ (DNRA), chemodenitrification, and codenitrification (Kraft, Strous and Tegetmeyer 2011). Canonical denitrification mediated by bacteria completes reduction of NO_2^- to N_2 via a stepwise reaction involving NO and N_2O as intermediates, with each step catalyzed by different enzymes (Kraft, Strous and Tegetmeyer 2011). Nitrous oxide is the end product of denitrification in the organisms lacking N_2O reductase genes (*nosZ*) or when environmental conditions are not conducive to N_2O reductase expression or activity (Hallin *et al.* 2017). Several species of eukaryotic fungi are capable of incomplete denitrification producing N_2O

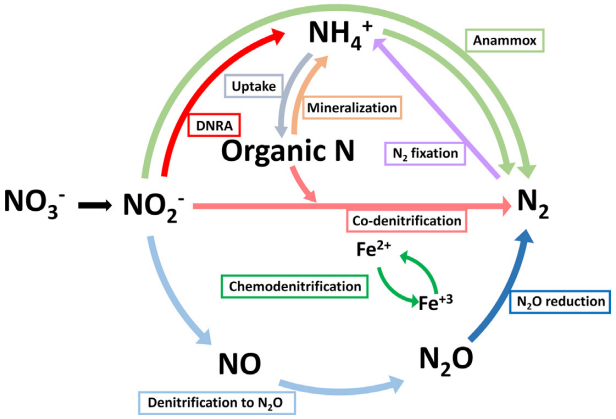

Figure 1. A graphical summary of the reductive nitrogen oxide transformation reactions discussed in this review. Both biotic and abiotic pathways are included and the pathways previously regarded as negligible, e.g. chemodenitrification and codenitrification, are also included in the network.

as the end product, and evidence is lacking that indicates fungal N_2O reductase activity or the presence of a *nosZ* orthologue in any closed fungal genome to date (Shoun and Tanimoto 1991; Maeda *et al.* 2015; Higgins *et al.* 2018). Dissimilatory NO_3^-/NO_2^- reduction to NH_4^+, also termed respiratory ammonification, is a microbially-mediated pathway of NO_3^-/NO_2^- reduction to NH_4^+ catalyzed by cytochrome *c* nitrite reductase (Nrf) (Kraft, Strous and Tegetmeyer 2011; Welsh *et al.* 2014). The electrons for denitrification and DNRA reactions are acquired from oxidation of various organic and inorganic electron donors, including, but not limited to glucose, pyruvate, lactate, acetate, hydrogen, sulfide and ferrous iron (Brunet and Garcia-Gil 1996; Straub *et al.* 1996; Robertson *et al.* 2016).

The fate of NO_3^-/NO_2^- in anoxic environment is further complicated by biotic and abiotic N_2- and N_2O- producing pathways that had been previously considered as minor contributors. These pathways include anaerobic ammonia oxidation (anammox), codenitrification and chemodenitrification. Although anammox is an important exit path for fixed nitrogen from the marine environments (Burgin and Hamilton 2007), the significance of anammox in agroecosystems is questionable and will not be addressed further in this review. Codenitrification is an understudied biotic NO_2^- reducing pathway characterized as an enzymatic reaction between a nitroso group and organic nitrogen or hydoxylamine (NH_2OH) to release N_2 and/or N_2O (Spott, Russow and Florian Stange 2011). Chemodenitrification is reduction of NO_2^- coupled to oxidation of ferrous iron (Fe^{2+}) and has been recognized as a potentially significant source of N_2O in soil environments where microbial NO_2^- accumulation and ferric iron (Fe^{3+}) reduction to ferrous iron (Fe^{2+}) provide consistent feeds of these reactants (Matocha, Dhakal and Pyzola 2012; Zhu-Barker *et al.* 2015; Heil, Vereecken and Brüggemann 2016).

DENITRIFICATION VS. DNRA COMPETITION

Implications to N use efficiency and GHG emissions in agroecosystems

Nitrogen fertilizer use has steadily increased globally over the past 100 years, as rapid population growth has called for greater agricultural yields (Erisman *et al.* 2008). Production of N fertilizers, mostly in forms of reduced ammonia (NH_3)-N, still relies heavily on the century-old Haber–Bosch process. Due to the high

energy cost of the process, N fertilizers have high GHG emission factors. Emission factors for production and application of urea and NH_4NO_3, the most common N fertilizers, were calculated as 3.5 and 7.2 kg CO_2eq kg N^{-1}, respectively, in a recent life cycle analysis study (Williams, Audsley and Sandars 2010). Generally, less than half of N applied as fertilizer is incorporated into plant biomass. A substantial portion of applied N is lost via denitrification subsequently converting the products of nitrification, i.e. NO_2^- and NO_3^-, to N_2 and N_2O (Beeckman, Motte and Beeckman 2018). Besides, both nitrification and denitrification are significant sources of N_2O in agricultural soils (Bateman and Baggs 2005; Philippot *et al.* 2011).

Enhancing DNRA activity in fertilized soils has been suggested as a possible approach for improving N use efficiency in agroecosystems (Goulding, Jarvis and Whitmore 2008; Zhang *et al.* 2013a; Yoon *et al.* 2015b; Putz *et al.* 2018). Along with denitrification, DNRA is one of the two major pathways for NO_3^-/NO_2^- reduction in anoxic soil environments (Tiedje *et al.* 1983; Burgin and Hamilton 2007). Microbes performing denitrification and DNRA compete for common electron acceptors, NO_3^- and/or NO_2^-. Unlike denitrification, where N_2O is produced as an intermediate and often escapes to the atmosphere, DNRA does not lead to direct production of N_2O, although trace N_2O evolution was observed in microbial cultures reducing NO_3^-/NO_2^- via DNRA (Stremińska *et al.* 2012; Sun, de Vos and Heylen 2016). If DNRA were a dominating reduction pathway in agricultural soil, NO_3^- and NO_2^- could be reduced back to NH_4^+. Ammonium, the predominant form of NH_3/NH_4^+ at circumneutral pH, tends to adsorb onto negatively charged soil particles and thus is less prone to leaching than NO_3^- and NO_2^- (Laima *et al.* 1999; Fitzhugh, Lovett and Venterea 2003). Thus, in a hypothetical DNRA-dominant agricultural soil, N loss would be substantially reduced. Promoting DNRA will improve the fertilizer-N use efficiency and reduce the carbon footprint associated with the production and field application of fertilizer-N (Zhang *et al.* 2015; Beeckman, Motte and Beeckman 2018). The potential saving in the life-cycle GHG emissions via DNRA enhancement is illustrated in Fig. 2, wherein the GHG emissions (in kg CO_2eq/kg N to crops) are compared between a denitrification-dominant system and a DNRA-dominant system. The calculations are based on rather bold assumptions and simplifications; nevertheless, the comparison clearly shows the comparative advantage of a DNRA-dominant system. Therefore, DNRA activity enhancement has been regarded as a viable strategy to decrease GHG emissions in agroecosystems (Silver, Herman and Firestone 2001; Silver *et al.* 2005; Rütting *et al.* 2011; Welsh *et al.* 2014; Zhang *et al.* 2015; Yoon *et al.* 2015b).

Environmental factors promoting DNRA activities

What determines the bifurcation of two major dissimilatory NO_3^-/NO_2^- reduction pathways? Can DNRA activity be stimulated to dominate N-oxide reduction over denitrification in agricultural soils? These questions remain largely unresolved. Nevertheless, several clues to the search for the environmental factors leading to enhancement of DNRA activities can be found from the previous studies reporting DNRA activities in aquatic and terrestrial ecosystems. Traditionally, DNRA had been perceived as a minor microbial reaction relative to denitrification; however, more recent findings have discovered that DNRA accounts for significant fraction of NO_3^- reduction in diverse environments and is in fact the dominant dissimilatory NO_3^--reduction pathway in certain microenvironments (Gardner *et al.* 2006; Scott *et al.* 2008; Gardner and McCarthy 2009; Koop-Jakobsen and Giblin 2010; Nizzoli *et al.* 2010; Dong *et al.* 2011; Dunn *et al.* 2013; Nogaro and Burgin 2014; Roberts *et al.* 2014; Jahangir *et al.* 2017). These local environments, where DNRA was observed to have significant contributions to the fate of NO_3^-, have been generally characterized by: (i) temporal or spatial richness of labile organics, (ii) high salinity, (iii) high sulfide concentration, (iv) low dissolved O_2, (v) high pH, and/or (vi) high temperature. The vast majority of these investigations targeted marine or estuarine environments. Whether similar conditions would promote DNRA in agricultural soils has not yet been elucidated, due to the paucity of data on DNRA activities in terrestrial environments. Bacterial *nrfA* genes are recovered in substantial quantities in soils and detectable DNRA activities have been observed in several isolated occasions (Orellana *et al.* 2018; Pandey *et al.* 2018; Putz *et al.* 2018; Pandey *et al.* 2019). Consistent correlation between geochemical parameters and enhancement of DNRA activity has not yet been observed in terrestrial ecosystems (Silver, Herman and Firestone 2001; Yin *et al.* 2002; Welsh *et al.* 2014; Zhang *et al.* 2014). Nevertheless, multiple cases of DNRA dominance in sparsely fertilized rice paddy soils were recently reported with more than five-fold higher DNRA rates than denitrification rates, suggesting the feasibility of DNRA enhancement in agricultural soils (Pandey *et al.* 2019). The C-to-NO_3^- ratios of the three rice paddies with dominating DNRA activity were approximately twice those of other three rice paddies where denitrification reaction was dominant, consistent to the observation of the same authors that urea application to rice paddy soils significantly reduced the DNRA rates (Pandey *et al.* 2018). Another recent study reported that incorporating a key rotation into a cereal production system significantly increased organic C-to-NO_3^- ratio and enhanced DNRA activity (Putz *et al.* 2018). This correlation suggests plants and/or plant microbiomes may locally influence the competition between DNRA and denitrification. These experimental evidences altogether point to the likelihood that C-to-NO_3^- and/or redox potential is indeed an important determinant of the competition between DNRA and denitrification.

Recently, several independent research groups examined the competition between denitrification and DNRA in simplified systems, i.e. pure cultures of *Shewanella loihica* PV-4 and NO_3^--enrichment cultures (Kraft *et al.* 2014; van den Berg *et al.* 2015; Yoon, Sanford and Löffler 2015a; Yoon *et al.* 2015b; van den Berg *et al.* 2016; Kim, Park and Yoon 2017). The genome sequence survey has identified several bacteria harboring the genes involved in both denitrification and DNRA pathways including *Opitutus terrae*, *Shewanella loihica*, *Marivirga tractuosa*, and *Intrasporangium calvum* (Sanford *et al.* 2012; Vuono *et al.* 2019). *Shewanella loihica* PV-4 was used as a model organism for a series of experiments investigating the effects of environmental parameters previously hypothesized as influential to the selection of denitrification and DNRA pathways (Yoon, Sanford and Löffler 2015a; Yoon *et al.* 2015b; Kim, Park and Yoon 2017). As previously hypothesized decades ago, the organic C-to-NO_3^- ratio was a potent regulatory parameter affecting NO_3^- fate in anoxic *S. loihica* PV-4 cultures (Tiedje *et al.* 1983). The continuous culture of strain PV-4 incubated with excess of electron donor (lactate) exhibited stimulated DNRA activity. When the electron donor was limited, denitrification, rather than DNRA, was stimulated (Yoon *et al.* 2015b). Enrichment of activated sludge with similar electron acceptor-limiting chemostat was able to select for organisms affiliated to the genus *Geobacter* that were capable of energy conservation via DNRA (van den Berg *et al.* 2015). In a long-term chemostat experiment emulating NO_3^-/NO_2^- reduction in tidal flat sediments, the generation rate, i.e. the dilution rate of the chemostat reac-

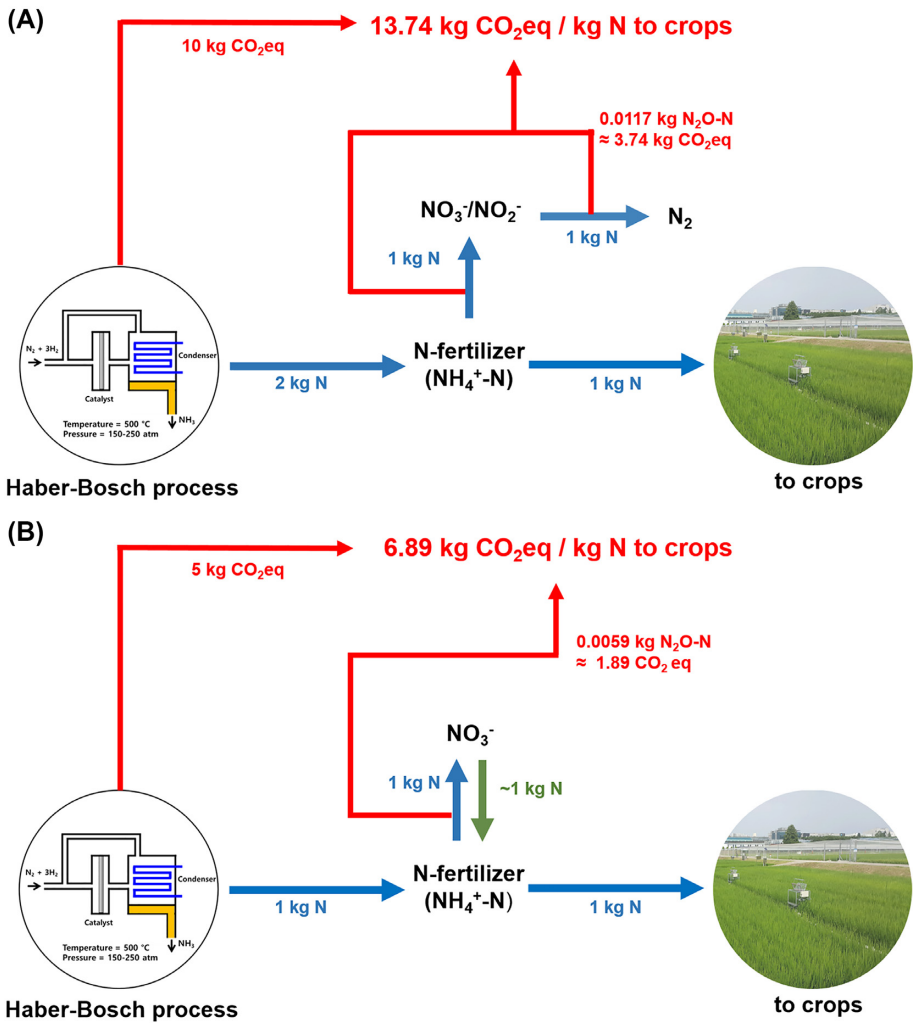

Figure 2. The comparison between net GHG emission in (A) denitrification-dominant agricultural soils and (B) hypothetical DNRA-dominant agricultural soils. The net GHG emission was calculated per kg of N conveyed to the crop biomass. For simplicity of presentation, the following assumptions were made. (i) An emission factor of 5 kg CO_2eq/kg N was assumed for production and application of N fertilizers. (ii) Microbial N assimilation was assumed to be negligible. (iii) An emission coefficient of 0.0117 kg N_2O-N / kg N applied was assumed for N_2O emissions from N fertilizers (US EPA 1995). (iv) Nitrification and denitrification were assumed to contribute equally to N_2O emissions and thus, the emission coefficient of nitrification alone would be 0.0059 kg N_2O-N/kg N.

tor, was the major determinant of NO_3^- fate (Kraft *et al.* 2014). The importance of the C-to-N ratio was also observed, as limitation of organic carbon resulted in greater rates of denitrification and lower rates of DNRA. All of these results from the pure culture and enrichment experiments were in agreements with what was observed in the aquatic and soil environments. High C-to-N ratios led to predominance of DNRA, suggesting C-to-N ratio may be a determining factor in the bifurcation of DNRA and denitrification.

Accumulations of NO_2^- occur in the environment due to the imbalance between the rates of the reactions producing NO_2^- (e.g. NO_3^--to-NO_2^- reduction and NH_4^+ oxidation), and the reactions consuming NO_2^- (e.g. denitrification, DNRA, anammox, abiotic nitrosation, and NO_2^- oxidation) (Smith *et al.* 1997; Philips, Laanbroek and Verstraete 2002; Shen, Ran and Cao 2003). The NO_2^--to-NO_3^- ratio was identified as another factor determining the relative activities of denitrification and DNRA; however, contrasting results were observed between the experiments performed in three independent research groups (Kraft *et al.* 2014; Yoon, Sanford and Löffler 2015a; van den Berg *et al.* 2017). While replacement of NO_3^- with NO_2^- as the electron acceptor reduced the DNRA activity in the tidal flat enrichment, the DNRA-dominant enrichment acquired from activated sludge was not affected by switching the electron acceptor from NO_3^- to NO_2^- (Kraft *et al.* 2014; van den Berg *et al.* 2017). In the pure culture of *S. loihica* strain PV-4, increased NO_2^--to-NO_3^- ratios resulted in enhanced DNRA activity, although the effect was less influential than the effect of the organic C-to-NO_3^- ratio. In this instance, the effect of NO_2^- on rates of denitrification and DNRA were only observed when the culture was operated in either C- or N-limiting conditions. The DNRA activity of *S. loihica* strain PV-4 also positively correlated with the temperature and pH within ranges of 21°C–37°C and 6.0–8.0, respectively (Yoon *et al.* 2015b; Kim, Park and Yoon 2017). These laboratory experiments have certainly improved the understanding of biogeochemical controls on NO_3^-/NO_2^- reduction pathways, but these findings should be interpreted with caution when addressing N dynamics in complex natural systems.

Is DNRA enhancement in agricultural soils feasible?

The idea of enhancing DNRA activities in agricultural soils to reduce N loss and N_2O emissions is certainly enticing. However, the feasibility of the approach remains questionable, with our limited understanding of the DNRA-vs-denitrification competition in soil systems. The results from the previous investigations suggest that enhancement of DNRA activity may be attainable by amending soil properties; however, such biostimulation approaches may not be practical for application in agricultural soils. Maintaining soil at highly reducing condition (i.e. highly-negative redox potential) would require prolonged flooding and organic carbon application leading to increased life-cycle CO_2 emissions. The effects of pH lack sufficient experimental evidences to be developed into a generalized solution, although liming can be relatively easily used to alter soil pH. In spite of the odds, the recent report of dominant DNRA activity in sparsely fertilized rice-paddy soils suggest DNRA activity stimulation may be possible via improved soil management, even though enhanced understanding of DNRA-vs.-denitrification competition would be a prerequisite (Pandey *et al.* 2019).

Establishing an engineered microbiome with increased population of DNRA-catalyzing organisms may be a plausible alternative approach, as a significant correlation between *nrfA*-possessing population and soil's DNRA activity was recently reported, and a recent metagenomic analysis of peatland-transformed farm soils suggested that low *nrfA* population could be one of the reasons for apparent absence of DNRA activity (Espenberg *et al.* 2018; Putz *et al.* 2018). Contrary to the common perception that DNRA only takes place in highly reducing environments, the results of the experiments performed with pure cultures of organisms capable of respiring on NO_3^-/NO_2^- reduction to NH_4^+, e.g. *Shewanella oneidensis*, suggest that DNRA (or respiratory ammonification) can occur regardless of the redox potential as long as NO_3^-/NO_2^- and an electron donor are available under anoxia (Sanford, Cole and Tiedje 2002; Cruz-García *et al.* 2007; Strohm *et al.* 2007; Decleyre *et al.* 2015). By establishing a DNRA-catalyzing population that outnumbers the denitrifying populations through bioaugmentation, DNRA may become, at least temporarily, the dominating NO_3^-/NO_2^- reduction pathway in agricultural soils. Bioaugmentation has been often dismissed as being notoriously unsuccessful; however, several successful cases have been reported, including the case of N_2O reduction enhancement by inoculation of soybean root nodules with *nosZ*-possessing *Bradyrhizobium* spp. (Itakura *et al.* 2013; Akiyama *et al.* 2016). Inoculation of soybean root nodules with a mixture of 63 *Bradirhizobium diazoefficiens* isolated from agricultural fields in Japan significantly reduced the N_2O emissions in the 2-year long field experiment, suggesting that use of diverse inoculum acclimated to the local environment may be key to success of bioaugmentation approach (Itakura *et al.* 2013). In this context, securing a collection of fast-metabolizing, resilient DNRA-catalyzing organisms would be a prerequisite for this bioaugmentation approach. Unfortunately, the pool of soil isolates with verified DNRA capability is very limited and poorly characterized. Currently, there is no isolation method specifically targeting DNRA-catalyzers and the only way to identify whether an isolate is a DNRA-catalyzing organism is to measure NH_4^+ production after anoxic incubation on NO_3^-, which is a time-consuming and labor-intensive process (Lycus *et al.* 2017). Thus, development of a high-throughput screening method is warranted for rapid screening of DNRA-catalyzing microorganisms from environmental samples, which will be crucial in increasing the chance of success in developing DNRA bioaugmentation approach. Besides, the expansion of soil DNRA bacterial library would help enhance the ecophysiological understanding of DNRA processes in terrestrial environments, which, as mentioned above, would be a prerequisite for developing soil management techniques promoting DNRA.

DENITRIFICATION PARADOX

Source or sink of N_2O?

Denitrification can be a source or a sink of N_2O, depending on the relative activities of the enzymes producing N_2O and those reducing N_2O. The net N_2O emission from an anoxic environment is determined by the difference between the rates of N_2O production and its reduction to N_2 (Betlach and Tiedje 1981; Thomson *et al.* 2012). Nitric oxide (NO) reduction is not regarded as a rate-limiting step in denitrification, as suggested by the minimal concentrations of NO observed during denitrification reactions (Betlach and Tiedje 1981; Bergaust *et al.* 2010). Thus, the rate of N_2O production via microbial denitrification is dependent on the rate of NO_2^- reduction to NO mediated by either type of nitrite reductases (Cu-type: NirK or Fe-type: NirS). The rate of N_2O consumption is solely dependent on the rate of N_2O reduction by the microbes with expressed NosZ (Thomson *et al.* 2012). Therefore, a net N_2O production from denitrification is dependent on the balance between NirK/NirS activity and NosZ activity.

Denitrifiers are ubiquitous in diverse natural habitats. The 'canonical', or complete denitrifiers, possess the suite of enzymes encoding for the complete cascade of reductions from NO_3^- to N_2. Denitrifiers with truncated denitrification pathways, i.e. incomplete denitrifiers, lack one or more of these denitrification genes. Such incomplete denitrifiers are not rare, and *nosZ* gene is absent in a substantial portion of denitrifying population in the environment (Jones *et al.* 2008; Graf, Jones and Hallin 2014; Lycus *et al.* 2017). Out of 68 organisms possessing *nirK* or *nirS* genes with their genomes sequenced as of 2008, 25 organisms lacked *nosZ* gene, and four of 13 phenotypically confirmed denitrifying soil isolates did not possess *nosZ* gene. Incomplete denitrifiers without *nosZ* gene are obvious sources of N_2O, as their denitrification end-product is N_2O. A significant correlation was observed between the *nosZ*-to-16S rRNA gene abundance ratios and the rates of potential N_2O production in a field study investigating spatial patterns of denitrification (Philippot *et al.* 2009). In a subsequent study, addition of *Agrobacterium tumefaciens* C58, an incomplete denitrifier without *nosZ* gene, to agricultural soils significantly increased the $N_2O/(N_2O+N_2)$ product ratios (Philippot *et al.* 2011). Soils with comparable *nosZ*-to-(*nirK*+*nirS*) gene abundance ratios responded differently to the addition of *A. tumefaciens* C58, suggesting a possible discrepancy between the functional gene abundances and the activities of the expressed enzymes *in situ*. This discrepancy may be due to the presence of dormant *nosZ*-, *nirK*- and/or *nirS*-possessing organisms or the lack of proper gene quantification tools for comprehensive coverage of these divergent genes, especially *nosZ*. Even the most frequently used primers for quantification of these genes had been designed with limited sequence information and thus, are not capable of accurately capturing their vast diversity (Braker, Fesefeldt and Witzel 1998; Throbäck *et al.* 2004; Henry *et al.* 2006). Identification of active N_2O reducers and development of more reliable tools for molecular quantification of these active players

are warranted in the future studies for improved estimation of N_2O sink potential.

Conversely, organisms that carry the *nosZ* gene but lack the genes encoding enzymes required for upstream denitrification reactions are likely to function as important N_2O sinks in the environments. Recent expansion of microbial genome databases has revealed that sizable subgroups of *nosZ*-possessing organisms lack either *nirK* or *nirS* gene in their genomes, and thus, are incapable of reducing NO_2^- to NO (Sanford *et al.* 2012; Jones *et al.* 2013; Graf, Jones and Hallin 2014; Jones *et al.* 2014; Hallin *et al.* 2017). Two of such organisms, *Anaeromyxobacter dehalogenans* and *Wolinella succinogens*, are able to use N_2O as the sole electron acceptor, suggesting that such organisms with 'standalone' *nosZ* genes may be active N_2O sinks in the environment (Sanford *et al.* 2012; Yoon *et al.* 2016; Hein, Witt and Simon 2017). Phylogenetically, *nosZ* genes are classified to two distinct groups, namely clade I and clade II *nosZ*, the latter of which was only recently identified (Sanford *et al.* 2012; Jones *et al.* 2013). Interestingly, the distribution of organisms with standalone *nosZ* genes is biased towards the group with clade II *nosZ*. As of 2017, 49% of the sequenced microbial genomes with clade II *nosZ* contained neither *nirK* nor *nirS* gene, while only 17% of genomes with clade I *nosZ* lacked either NO-forming nitrite reductase (Hallin *et al.* 2017). Along with the physiological observations that clade II *nosZ*-possessing organisms generally exhibited higher affinity for N_2O (Yoon *et al.* 2016), this uneven phylogenetic distribution of standalone *nosZ* genes suggests that these clade II *nosZ*-possessing organisms may have evolved to take advantage of trace N_2O released from various N-metabolism reactions. In support of this hypothesis, a significant correlation was observed between the abundance and diversity of the clade II *nosZ* and soil N_2O sink capability (Jones *et al.* 2014; Domeignoz-Horta *et al.* 2015).

Environmental controls on denitrifier N_2O production

The sensitivity of N_2O reductase to acidic pH may lead to increased N_2O emissions from denitrification (Bergaust *et al.* 2010; Liu *et al.* 2010; Dörsch, Braker and Bakken 2012; Liu, Frostegård and Bakken 2014; Qu *et al.* 2014; Brenzinger, Dörsch and Braker 2015; Kim, Park and Yoon 2017). Significantly increased N_2O accumulation was observed in pure cultures of neutrophilic denitrifiers, namely *Paracoccus denitrificans* and *Shewanella loihica*, at incubation pH lower than 6.5 (Bergaust *et al.* 2010; Liu, Frostegård and Bakken 2014; Kim, Park and Yoon 2017). This effect of pH could not be explained solely by the modest shift in *nosZ* transcription or inactivation of expressed N_2O reductases. Thus, loss of N_2O reduction capability was also attributed to posttranscriptional regulation or disrupted formation of functional enzyme complexes. Complete or partial loss of N_2O reduction activity at acidic pH has been regarded as a general physiological trait shared among denitrifiers and non-denitrifying N_2O reducers, as similar accumulation of N_2O has been repeatedly observed in NO_3^--reducing acidic soil enrichments and isolates (Liu *et al.* 2010; Dörsch, Braker and Bakken 2012; Qu *et al.* 2014; Brenzinger, Dörsch and Braker 2015). Direct incubation of field soils with pH varying from 4.0 to 8.0 with NO_3^- amendment resulted in increasing N_2O-to-(N_2O+N_2) product ratio with the decrease in pH and N_2O reduction was completely inactive in the acidic soil at pH 4.0 (Liu *et al.* 2010). Chinese agricultural soils acidified due to long-term mismanagement also showed higher N_2O-to-(N_2O+N_2) product ratios than the fertilized soils at circumneutral or alkaline pH (Qu *et al.* 2014). Different responses of complex microbial communities extracted from the soils of distinct origin (pH varying between 5.4 and 7.1) upon exposure to acidic pH suggested that long-term adaptation of denitrifiers and non-denitrifying N_2O-reducers may take place over time, enabling N_2O utilization at moderately acidic pH (Dörsch, Braker and Bakken 2012; Brenzinger, Dörsch and Braker 2015). Whether N_2O-reducing capabilities of the clade I and clade II *nosZ*-possessing organisms differ under low-pH stress has not yet been investigated and would certainly be an interesting topic for future research.

Several studies have investigated the influence of O_2 on N_2O emissions from denitrification, including nitrifier denitrification (Tallec *et al.* 2006; Wonderen *et al.* 2008; Lu and Chandran 2010; Bergaust *et al.* 2012; Rosamond, Thuss and Schiff 2012; Qu *et al.* 2016). The consensus of the reports in the literature indicates that the entire denitrification pathway, from NO_3^- to N_2, is inhibited by the presence of O_2. Presumably, denitrifiers have evolved to maximize energy conservation efficiency by shutting down the respiratory pathways yielding less energy than aerobic respiration (Bonin, Gilewicz and Bertrand 1989; Körner and Zumft 1989; Qu *et al.* 2016). Expressions and syntheses of the enzymes in the denitrification pathway are, in most observed cases, completely inhibited under fully aerobic conditions (i.e. under atmosphere containing 21% O_2). Under suboxic conditions, the sensitivity of denitrification enzymes to O_2 often leads to accumulation and release of intermediates, including N_2O (Körner and Zumft 1989; Qu *et al.* 2016). Nitrous oxide reductase is widely accepted as being the most sensitive to O_2 of the enzymes mediating the different steps of the denitrification pathway. Several researchers have reported enhancement of N_2O emissions from denitrifying isolates and consortia at suboxic conditions, which was attributed to partial inactivation of N_2O reductase (Takaya *et al.* 2003; Wunderlin *et al.* 2012). More recent findings from experiments with axenic and enrichment cultures have suggested that the O_2 sensitivity of N_2O reductase expression and activity may vary among different groups of N_2O reducers (Lu and Chandran 2010; Desloover *et al.* 2014; Park, Kim and Yoon 2017; Suenaga *et al.* 2018). The increased N_2O emissions observed in suboxic soils and activated sludge tanks were also partly attributed to nitrifier denitrification by ammonia oxidizing bacteria (AOB) (Kool *et al.* 2011; Aboobakar *et al.* 2013; Zhu *et al.* 2013). Nitrifier denitrification is one of several mechanisms where N_2O is produced and released from AOB (Kozlowski, Price and Stein 2014; Kozlowski, Kits and Stein, 2016). Many AOB isolates, including a model organism *Nitrosomas europaea*, possess *nirK* and/or *norB* and are capable of denitrification producing N_2O upon introduction of hypoxia (Arp, Chain and Klotz 2007; Kozlowski, Kits and Stein, 2016). Experiments with ^{15}N- and ^{18}O-labeled NO_2^- have verified the significance of nitrifier denitrification pathway for production of N_2O by AOB; however, controversy exists regarding the effect of O_2 on nitrifier denitrification (Shaw *et al.* 2006; Kool *et al.* 2011). As nitrification is out of the scope of this review, the topic will not be discussed here in further detail.

Bioavailability of copper (Cu) may also be an important regulator of N_2O emission from microbial denitrification in agricultural soils. As mentioned above, two of the key enzymes in the denitrification pathway, NirK and NosZ, are cuproenzymes, requiring Cu as the necessary constituent of their reactive centers (Murphy *et al.* 1995; Brown *et al.* 2000). In contrast to NirK, which has a Cu-independent alternative (NirS), NosZ is the only enzyme capable of reducing N_2O in the environment. The active heterodimeric form of NosZ has high requirement for Cu, as each monomeric unit contains a tetranuclear Cu site (Cu_Z) and a dinuclear Cu site (Cu_A). Despite the theoretical dependency of

NosZ on free Cu ion availability and observation of N_2O accumulation in denitrifying cultures deprived of Cu, the data were equivocal (Granger and Ward 2003). Previous investigations of denitrifiers using microbial consortia from a freshwater lake and oxygen minimum zones of the Pacific Ocean and the Arabian Sea concluded N_2O reductase activity is rarely limited by Cu deficiency (Twining, Mylon and Benoit 2007; Ward *et al.* 2008). The threshold for N_2O reductase activation in denitrifiers was regarded to be orders of magnitudes lower than the free Cu concentrations attainable with naturally occurring ligands. Two recent research results, however, suggest ecological scenarios in which N_2O reduction may actually be suppressed due to limited Cu bioavailability (Felgate *et al.* 2012; Chang *et al.* 2018). A set of chemostat experiments with two denitrifying species, *Paracoccus denitrificans* and *Achromobacter xyloxidans* suggested that Cu deficiency-induced N_2O emission enhancement may be relevant in C-deprived, N-rich environments (Felgate *et al.* 2012). Another recent study demonstrated that Cu limitation may result in reduced N_2O reductase activity in ecological niches cohabited by denitrifiers and methanotrophs, as methanobactin, the copper chelator exuded by methanotrophs and utilized for Cu uptake, may limit Cu availability to denitrifiers (Chang *et al.* 2018). These observations indicate that Cu availability should not be overlooked as a regulatory factor of N_2O emissions from soil environments.

Outside the prokaryotic domains—fungal and chemical NO_2^- reduction pathways

Fungal denitrification of NO_3^- or NO_2^- to N_2O was first reported over two decades ago (Shoun *et al.* 1992), yet N_2O emissions from denitrification are still commonly attributed to bacteria. Several studies indicated that fungi contribute a large proportion of the total N_2O efflux from agroecosystems (Laughlin and Stevens 2002; Long *et al.* 2013). The enzymes and genes involved in fungal denitrification were only recently reported (Shoun *et al.* 2012), which clarified fundamental physiological differences between bacterial and fungal denitrification pathways. One main difference is the intracellular loci of the denitrification reactions. Bacterial denitrification occurs in the periplasm while fungal denitrification occurs majorly in an intracellular organelle, mitochondria (Mothapo *et al.* 2015). Many of the enzymes involved in fungal denitrification are somewhat different from those of bacteria. The presence and activity of ubiquinone-dependent dissimilatory NO_3^- reductases analogous to bacterial NO_3^- reductases were confirmed in the mitochondrial fraction of *Fusarium oxysporum* and *Aspergillus nidulans*; however, dissimilatory NO_3^- reductase activity was not detected in another denitrifying fungal species *Cylindrocarpon tonkinese* (Uchimura *et al.* 2002; Watsuji *et al.* 2003). This finding suggests a distinct denitrification pathway in fungi, where assimilatory NO_3^- reductases may be involved. Nitrite reduction during fungal denitrification is carried out exclusively by Cu-dependent NO_2^- reductases (NirK), which are homologs of bacterial NirK (Long *et al.* 2015). Conversion of NO to N_2O is mediated by cytochrome P450 NO reductases encoded by *p450nor* genes, which are found in diverse fungi (Kobayashi *et al.* 1996; Takaya *et al.* 1999; Higgins *et al.* 2018). The bacterial homologs of P450 NO reductases (P450 family CYP105) were found in *Actinobacteria* and *Proteobacteria* and are presumed to have NO detoxification function (Higgins *et al.* 2018). No analogue of the genes encoding bacterial N_2O reductase has been identified in any fungal denitrifier genomes, indicating N_2O is the end-product of fungal denitrification (Shoun *et al.* 2012).

Fungal denitrification was first discovered in *F. oxysporum* (Bollag and Tung 1972; Shoun and Tanimoto 1991), and denitrification activity has been reported for 155 species belonging to 77 different genera (Mothapo *et al.* 2015). These fungal denitrifiers produce N_2O mostly under microaerophilic conditions (Maeda *et al.* 2015). Most of denitrifying fungal isolates belong to the phylum *Ascomycota*; however, denitrification activity was also observed in several isolates belonging to *Basidiomycota* and *Zygomycota* phyla. As NO_2^- reductase (*nirK*) and NO reductase (*p450nor*) genes are common genetic markers for fungal denitrifiers, several attempts have been made to design PCR-based methods to detect denitrification potential in fungal isolates and soil samples (Long *et al.* 2015; Wei *et al.* 2015; Higgins *et al.* 2016; Novinscak *et al.* 2016). Despite the efforts, exhaustive coverage of fungal *nirK* and *p450nor* is not yet possible with these PCR methods, and molecular detection and quantification of denitrifying fungi still remains a challenging task. The failure to detect either gene in several N_2O-producing fungal isolates may also raise a possibility that an alternative denitrification pathway may exist for fungi.

Traditionally, relative contribution of fungal denitrification to soil N_2O emissions was determined *ex situ* using the substrate induced respiration-inhibition (SIRIN), a method utilizing cycloheximide for selective inhibition of fungal denitrification activity (Anderson and Domsch 1973; Laughlin and Stevens 2002; McLain and Martens 2006; Yanai *et al.* 2007; Crenshaw *et al.* 2008; Laughlin *et al.* 2009; Long *et al.* 2013; Marusenko, Huber and Hall 2013; Wei *et al.* 2014; Chen, Mothapo and Shi 2015b; Rex *et al.* 2018). Table 1 summarizes the results of the selected experimental studies reporting the rates of fungal denitrification based on the SIRIN method. Fungal contribution to soil N_2O production ranged from 30%–98% for the examined grassland and cropland soils, with the highest fungal contribution observed in andisol-type agricultural soil planted with radish (Wei *et al.* 2014). Chen, Mothapo and Shi (2014) reported that fungi contributed 40% to 51% of the total N_2O emissions observed for soils under several agricultural management scenarios: conventional farming, integrated crop and livestock systems, organic farming, plantation forestry and abandoned agriculture fields. Fungal denitrification accounted for 80% of N_2O produced in a crop field amended with organic fertilizers (Wei *et al.* 2014). Manure application to arable soils is generally perceived to cause increased N_2O emissions and the role of fungal denitrification in such manure-amended agricultural fields is an active area of investigation (Dambreville *et al.* 2006; Dambreville, Morvan and Germon 2008; Clark *et al.* 2012).

Several microcosm studies have investigated the impacts of soil properties on fungal N_2O production, and results point to predictive variables such as organic carbon, pH and soil water content (Laughlin *et al.* 2009; Chen, Mothapo and Shi 2015a; Chen, Mothapo and Shi 2015b). Amendment of soil with additional acetate selectively stimulated fungal N_2O production in microcosms of fungi-rich grassland soil (Laughlin *et al.* 2009). Higher relative contribution of fungi to total denitrification activity was observed in the soil microcosms amended with cellulose as compared to microcosms amended with glucose (Chen, Mothapo and Shi 2015b). In a similar microcosm study, the contribution of fungal denitrification to net N_2O production was larger in soil samples with acidic pH and lower water-

Table 1. Reports of fungal N_2O production in soil microcosms determined using the SIRIN method.

Soil type	Land use	Incubation conditions	N form added	N amount added	Cycloheximide dose (mg g^{-1})	Fungal N_2O production (μg N g dw^{-1} d^{-1})	Post-cycloheximide inhibition (%)	Reference
Entisol	arable	aerobic	KNO_3	100 mg N g dw^{-1}	2	3.1–6.4	70–80	Chen *et al.* (2015)
Ultisol	arable	aerobic	KNO_3	100 mg N g dw^{-1}	2	1.5–8.5	52–71	Chen *et al.* (2015)
Tropical peatland	arable	aerobic	n/a[a]	n/a[a]	15	0.6	81	Yanai *et al.* (2007)
Andisol	arable	anaerobic	n/a[a]	n/a[a]	5	0.6–1.7	30–84	Wei *et al.* (2014)
Deep inceptisol	grassland	aerobic	$^{15}NH_4^+$, $^{15}NO_3^-$	3.2 μmoles N g dw^{-1} [b]	3	0.3	96	Laughlin *et al.* (2009)
Deep inceptisol	grassland	aerobic	$^{15}NH_4^+$, $^{15}NO_3^-$	6.7 μmoles N g dw^{-1} [b]	15	0.2	89	Laughlin and Stevens (2002)
Ultisol	arable	anaerobic	$Na^{15}NO_3$	1 mM $Na^{15}NO_3$	15	0.2	65	Long *et al.* (2013)
Entisol	grassland	aerobic	NH_4^+, NO_3^-	200 mg g dw^{-1}	1.5	0.002	79	McLain and Martens (2006)
Aridisol	grassland	aerobic	n/a[a]	n/a[a]	1.5	0.002	85	Crenshaw *et al.* (2008)
Alfisol	grassland	anaerobic	$CO(^{15}NH_2)_2$	1000 kg N ha^{-1}	8	0.001	76	Rex *et al.* (2018)
Aridisol	grassland	aerobic	n/a[a]	n/a[a]	1.5	0.0001–0.005	70–98	Marusenko, Huber and Hall (2013)

[a]n/a: information not available.
[b]both NH_4^+ and NO_3^- added.

filled pore space (i.e. more intruding O_2) (Chen, Mothapo and Shi 2015a). Such reports of experimental observations regarding contribution of fungal denitrification to N_2O production are scarce and largely inconsistent; nonetheless, they invariably point towards significance of N_2O production from fungal denitrification. Recently, experimental techniques using isotopic fractionation of $^{14}N/^{15}N$ and $^{16}O/^{18}O$ and ^{15}N site preference of N_2O are under development for *in situ* differentiation of bacterial versus fungal N_2O production (Sutka *et al.* 2008; Rohe, Well and Lewicka-Szczebak 2017; Yamamoto *et al.* 2017). Successful application of these experimental techniques will allow for more reliable assessment of the significance of N_2O production from fungal denitrification in agricultural soils.

Apart from fungal denitrification, fungi reportedly produce N_2 and N_2O via biotic nitrosation, which is a process known as codenitrification. In this instance, NO_2^- and reduced N compounds including azide, NH_4^+, salicylhydroxamic acid, and hydroxylamine react to form N_2 and N_2O (Tanimoto *et al.* 1992; Spott and Florian Stange 2011; Spott, Russow and Florian Stange, 2011). Codenitrification has notable similarity to anammox, as both use N from NO_2^- and reduced N compounds to produce a hybrid form of N_2 (Spott and Florian Stange 2007). Unlike anammox, however, fungal codenitrification can utilize organic N compounds instead of NH_4^+, and the hybrid-N end product may be N_2O, as well as N_2 (Su, Takaya and Shoun 2004). Ammonia fermentation, or fungal DNRA, is another understudied fungal reaction with potential importance in the soil N-cycling (Zhou *et al.* 2002). Observed only in strict absence of O_2 in selected groups of fungal isolates (18 isolates belonging to 14 species), ammonia fermentation couples NO_3^- reduction to oxidation of ethanol or acetate via substrate-level phosphorylation (Zhou *et al.* 2002; Takasaki *et al.* 2004; Stief *et al.* 2014).

N_2O production may also occur via an abiotic nitrosylation process termed chemodenitrification, in which oxidized forms of inorganic N (e.g. NO_2^- and NO) react with a metal center (e.g. Fe(II)) to form N_2O (van Cleemput 1998; Kampschreur, Kleerebezem and de Vet 2011; Buchwald *et al.* 2016). Chemodenitrification of NO_2^- coupled to oxidation of Fe(II) at near-neutral pH results in formation of N_2O. Reports of the experimental results verifying N_2O production from chemodenitrification have so far been limited to laboratory experiments, as chemodenitrification requires consistent supply of Fe(II), which is unlikely in the soil environments due to its high reactivity (Kampschreur, Kleerebezem and de Vet 2011; Jones *et al.* 2015; Buchwald *et al.* 2016). Potential recycling of Fe(II) by metal-reducing organisms, e.g. *Geobacter* spp., *Shewanella* spp., and *Anaeromyxobacter*, may theoretically serve as the source of Fe(II) for chemodenitrification in the soil environments (Kampschreur, Kleerebezem and de Vet 2011; Melton *et al.* 2014); however, such biotic-abiotic interplay has not yet been experimentally verified. Recently, *Anaeromyxobacter dehalogenans*, an iron-reducing bacterium abundant in agricultural soils, was found to be capable of utilizing chemodenitrification to bridge its truncated denitrification pathway (Onley *et al.* 2017). The discovery of the novel hybrid denitrification pathway in an iron-reducing bacterium may not be a coincidence, as energy yield from limited amount of electron acceptors can be maximized by adopting this strategy.

Microbiological insights to agricultural practices

Potential solutions to the agricultural N_2O emissions problem, as can be deduced from these recent discoveries, may appear rather straightforward. For example, a simple approach might

be to enhance the soil N_2O sink capacity. This might be achieved by promoting microbial N_2O-reducer populations and/or accommodating environmental conditions favorable for NosZ expression and activity. Developing practical soil treatment techniques to bring about the desired alterations in the microbial populations and activities is challenging. Biochar amendment is one of such promising techniques, which had been inadvertently found to reduce soil's N_2O emissions (Lehmann, Gaunt and Rondon 2006). Large volume of follow-up studies confirmed consistent reduction in N_2O emissions upon biochar addition to soils in multiple laboratory and field studies (van Zwieten *et al.* 2010; Zhang *et al.* 2010; Harter, Krause and Schuettler 2014; Agegnehu *et al.* 2016; Harter, Weigold and El-Hadidi 2016a). Anoxic soil incubations with biochar-amendment consistently resulted in reduced $N_2O/(N_2+N_2O)$, suggesting complete denitrification to N_2 was favored in biochar-amended soils (Cayuela *et al.* 2013; Obia *et al.* 2015; Harter, Weigold and El-Hadidi 2016a). These observations were supported by significant increases in the *nosZ* gene abundances accompanying the reduced N_2O emissions in the microcosm studies, where concomitant increases in the abundances of the genes encoding N_2O-producing enzymes, *amoA*, *nirK/nirS* and *norB*, were not significant or not as pronounced (van Zwieten *et al.* 2010; Harter, Krause and Schuettler 2014). Increased *nosZ* transcription and significant alterations to the composition of both clade I and clade II *nosZ* genes and transcripts further supported enhancement of N_2O reduction in the biochar-amended soils (Harter, Weigold and El-Hadidi 2016a; Harter *et al.* 2017). Although several mechanistic explanations have been suggested, e.g. increased pH, improved water-holding capacity, reduced availability of carbon and nitrogen and gas entrapment, a definitive scientific rationale is yet lacking in these recent findings (Harter, Krause and Schuettler 2014; Harter *et al.* 2016b). Nevertheless, these experimental observations suggest the feasibility of controlling soil's N_2O dynamics by amending soil properties, subsequently enhancing N_2O-reducing microbial population and their activities.

For soils assessed with high fungal denitrification activities, an intuitive approach for N_2O emission mitigation is to repress fungal N_2O production by inhibiting fungal growth. In a field experiment performed in fertilized grassland, application of frequently used fungicides, mancozeb and chlorothalonil, effectively suppressed N_2O emission, indicating a potential use of fungicide for mitigation of N_2O emission (Kinney *et al.* 2004). A caveat with this approach, however, is that such synthetic organic fungicides are considered as soil contaminants due to their toxicological and non-target effects on soil ecosystems and naturally, the microbial community within (Burrows and Edwards 2002). In fact, abundances of prokaryotes involved in N-cycling were significantly reduced in the soils repetitively treated with chlorothalonil as compared to the controls without fungicide amendment (Zhang *et al.* 2016). Bicarbonate salts have been used to inhibit fungal activity and growth and have the following benefits over the synthetic fungal inhibitors: (i) no harmful effect on human and animals; (ii) lower risk of developing fungal resistance; and (iii) lower cost of application. (Deliopoulos, Kettlewell and Hare 2010 and references therein). Application of bicarbonate fungicides would, in theory, effectively inhibit fungal denitrification and contribute to mitigation of agricultural N_2O emissions; however, this aspect has not yet been experimentally examined in field scale.

Traditional agricultural soil managements, such as liming and tillage, have also been re-examined in the context of the effects on N_2O emissions, as these management schemes are highly relevant to two consequential parameters that affect soil N_2O emissions: pH and O_2 diffusivity. Consistent with the aforementioned scientific findings regarding the pH effect on N_2O reduction activities, liming significantly reduced N_2O emissions from agricultural soils, especially those originating from denitrification (van Kessel *et al.* 2013; Hansen, Clough and Elberling 2014; Yamamoto *et al.* 2014; García-Marco *et al.* 2016; Wang *et al.* 2018). In inherently acidic (pH < 5.0) soils of a Japanese tea field, partial substitution of conventional organic N fertilizers with calcium cyanamide, a N fertilizer doubling as liming agent, resulted in >30% reduction in the annual N_2O emission (Yamamoto *et al.* 2014). More pronounced liming effect was observed with the pulse N_2O emissions from NO_3^--amended agricultural soils upon flooding (Hansen, Clough and Elberling 2014). The integrated N_2O flux from the limed soil with higher pH during the flooding period was <50% of that from the untreated soil. These observations are consistent with the findings from the experiments with pure cultures or enrichments and a recent global meta-analysis by Wang *et al.* (2018), where the authors reported significant negative correlations between soil pH and N_2O emissions. Unlike liming, no-till management of agricultural soils have yielded mixed results in terms of N_2O emissions (van Kessel *et al.* 2013; Zhao *et al.* 2016). The reduced O_2 diffusivity and improved carbon storage in no-till soils would be favorable for complete denitrification and N_2O reduction; however, little consistency could be found in the field experiments assessing the effects of tillage on N_2O emissions (Mutegi *et al.* 2010; Garland *et al.* 2011; García-Marco *et al.* 2016; Badagliacca *et al.* 2018). The meta-analyses of the compiled field data suggested that the different environmental settings (e.g. temperature and precipitation) and physicochemical properties of the examined soil systems (e.g. soil texture and drainage characteristics), as well as the length of no-tillage management (long term vs. short term), could have affected the extents to which N_2O-producing upstream reactions and N_2O-reducing downstream reaction were influenced by the different tillage schemes (van Kessel *et al.* 2013; Zhao *et al.* 2016).

As such, the enhanced understandings of the microbial physiology and ecology gained from the laboratory experimentations are, in fact, directly relevant to N_2O emission management of agricultural fields. These new insights not only are helpful in interpreting the effects of various soil management schemes to N_2O emissions, but are also key to devising and experimentally verifying novel, climate-smart soil management techniques.

CONCLUSIONS

The demand for agricultural application of N fertilizers have been on the rise for decades, and this trend is expected to continue for the foreseeable future. Better management practices aimed toward enhancing N use efficiency and mitigating GHG emissions depend upon improved understanding of the N-oxide reductive pathways in soil. The recent scientific findings reviewed here have substantially advanced knowledge of organisms and ecological conditions that influence GHG emissions and alter the fate of fixed N. Nevertheless, new challenges and questions for microbial ecologists and biogeochemists remain. How can long-term stimulation of DNRA activity be made possible in soil communities, based on physiological understanding of microbial isolates? What are the impacts of interactions with macroorganisms, e.g. crop plants and earthworms, on the biotic and abiotic reactions associated with soil N_2O emissions? What are the environmental parameters affecting the extents of fungal pathways, including denitrification, codenitrification and DNRA, in soils? These are a few examples of yet unanswered

questions that need to be resolved to comprehensively address GHG mitigation strategies that incorporate linkages between the soil N cycle and crop production. The answers to such intricate questions can be sought through trans-disciplinary efforts that integrate microbial ecology, microbial physiology, molecular biology, bioinformatics, geochemistry, and biochemistry. Initiation and expansion of such research would be facilitated by public awareness of the global environmental and economic costs associated with excess agricultural N currently lost through N-reductive pathways and GHG emitted in the process.

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