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

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Running title: Nitrogen oxide reduction and GHG emissions

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**Keywords:**

Nitrogen cycling, agricultural carbon footprint, denitrification, dissimilatory nitrate reduction to ammonium, fungal denitrification, chemodenitrification

## 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.

## INTRODUCTION

Nitrate ($NO_3^-$) is the most oxidized form of nitrogen (N) and often dominant in soil and aquatic environments where oxygen ($O_2$) is readily available to microbial communities (Canfield *et al.*, 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 *et al.*, 1992, Giles *et al.*, 2012). Nitrate is first reduced to nitrite ($NO_2^-$), an intermediate that is considered 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 *et al.*, 2010, Montzka *et al.*, 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 et al. 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 *et al.*, 2011b, 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 $N_2O$, $N_2$, and $NH_4^+$ end-products (Kraft *et al.*, 2011, Rütting *et al.*, 2011, Matocha *et al.*, 2012, Heil *et al.*, 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 (Figure 1): prokaryotic and fungal denitrification, dissimilatory $NO_3^-$ /$NO_2^-$ reduction to $NH_4^+$ (DNRA), chemodenitrification, and codenitrification (Kraft *et al.*, 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 *et al.*, 2011). $N_2O$ 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 $N_2O$ 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 another microbially-mediated pathway of $NO_3^-/NO_2^-$ reduction to $NH_4^+$ catalyzed by cytochrome *c* nitrite reductase (Nrf) (Kraft *et al.*, 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 $N_2O$- production 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 *et al.*, 2011b). 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 *et al.*, 2012, Zhu-Barker *et al.*, 2015, Heil *et al.*, 2016).

**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 $CO_2$eq kg $N^{-1}$, respectively, in a recent life cycle analysis study (Williams *et al.*, 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 *et al.*, 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 *et al.*, 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 (Streminíska *et al.*, 2012, Sun *et al.*, 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 *et al.*, 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 *et al.*, 2018). The potential saving in the life-cycle GHG emissions via DNRA enhancement is illustrated in Figure 2, wherein the GHG emissions (in kg $CO_2$eq/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 *et al.*, 2001, Silver *et al.*, 2005, Rütting *et al.*, 2011, Welsh *et al.*, 2014, Yoon *et al.*, 2015b, Zhang *et al.*, 2015).

**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, 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: (1) temporal or spatial richness of labile organics, (2) high salinity, (3) high sulfide concentration, (4) low dissolved $O_2$, (5) high pH, and/or (6) 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, Pandey *et al.* 2019, Putz *et al*., 2018). Consistent correlation between geochemical parameters and enhancement of DNRA activity has not yet been observed in terrestrial ecosystems (Silver *et al.*, 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 of those from 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 rate (Pandey *et al*., 2018). Another recent study reported that incorporating a ley 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 *et al.*, 2015a, Yoon *et al.*, 2015b, van den Berg *et al.*, 2016, Kim *et al.*, 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 *et al.*, 2015a, Yoon *et al.*, 2015b, Kim *et al.*, 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) stimulated DNRA and not denitrification. 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 reactor, 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 ratio 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 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 *et al.*, 2002, Shen *et al.*, 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 *et al.*, 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 21-37ºC and 6.0-8.0, respectively (Yoon *et al.*, 2015b, Kim *et al.*, 2017). These pure culture experiments have certainly improved the understanding of biogeochemical controls on $NO_3^-$/$NO_2^-$ reduction pathways, but these results 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 anoxic conditions (Sanford *et al.*, 2002, Cruz-Garcia *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 successful enhancement of $N_2O$ reduction activity by inoculation of soybean root nodules with *nosZ*-possessing *Bradyrhizobium* spp. (Itakura *et al.*, 2012, 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 two-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.*, 2012). 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 activity.

## 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 $N_2O$. A 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 intermediates observed in 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 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 full 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 the *nosZ* gene is absent in substantial portion of denitrifying population in the environment (Jones *et al.*, 2008, Graf *et al.*, 2014, Lycus *et al.*, 2017). Out of 68 organisms possessing *nirK* or *nirS* genes with their genome sequenced as of 2008, 25 organisms lacked *nosZ* gene, and four of 13 phenotypically confirmed denitrifying isolates from soil 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 *et al.*, 1998, Throbäck *et al.*, 2004, Henry *et al.*, 2006). Identification of active $N_2O$ reducers and 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 *et al.*, 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 *et al.*, 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 suggest 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 *et al.*, 2012, Liu *et al.*, 2014, Qu *et al.*, 2014, Brenzinger *et al.*, 2015, Kim *et al.*, 2017). Pure cultures of neutrophilic denitrifiers, namely *Paracoccus denitrificans* and *Shewanella loihica*, increased accumulation of $N_2O$ in the headspace at incubation pH lowered than 6.5 (Bergaust *et al.*, 2010, Liu *et al.*, 2014, Kim *et al.*, 2017). This effect of pH could not be explained by the modest shift in *nosZ* transcription or inactivation of expressed $N_2O$ reductases. Thus, loss of $N_2O$ reduction capability was attributed to posttranscriptional regulation or disrupted formation of functional enzyme complex. Complete or partial activity of $N_2O$ reductase 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 *et al.*, 2012, Qu *et al.*, 2014, Brenzinger *et al.*, 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 *et al.*, 2012, Brenzinger *et al.*, 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 *et al.*, 2012, Qu *et al.*, 2016). The consensus of the reports in the literature indicates 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 *et al.*, 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 the N-oxide reductase enzyme 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 (Desloover *et al.*, 2014, Lu and Chandran, 2010, Park *et al.*, 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 *et al.*, 2014,

Kozlowski *et al.*, 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 *et al.*, 2007, Kozlowski *et al.*, 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 *et al.*, 2007, Ward *et al.*, 2008). The threshold for $N_2O$ reductase activation in denitrifiers were regarded to be orders of magnitudes lower than low end of 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 co-habited 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 conclude that fungi contribute a high proportion of $N_2O$ to total $N_2O$ flux in 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 in certain fungal denitrifiers. 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 nitric oxide (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).

ungal 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 *et al.*, 2013, Wei *et al.*, 2014, Chen *et al.*, 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 et al (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 et al., 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 *et al.*, 2015a, Chen *et al.*, 2015b). Amendment of soil with additional acetate selectively stimulated fungal $N_2O$ production in microcosms of fungi-rich grassland soil, suggesting that fungal denitrification may actually be a dominant $N_2O$ producing pathway in soils (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 *et al.*, 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-filled pore space (i.e., more intruding $O_2$) (Chen *et*

*al.*, 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 *et al.*, 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 *et al.*, 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 other than $NH_4^+$, and the hybrid-N end product may be $N_2O$ as well as $N_2$ (Su *et al.*, 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 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 a non-hybrid form of $N_2O$ (van Cleemput, 1998, Kampschreur *et al.*, 2011, Buchwald *et al.*, 2016). Chemodenitrification of $NO_2^-$, through oxidation of Fe(II), at near-

neutral pH results in formation of $N_2O$ under anoxic conditions. 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 *et al.*, 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 *et al.*, 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 *et al.*, 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 *et al.*, 2014, Agegnehu *et al.*, 2016, Harter *et al.*, 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 *et al.*, 2016a). These observations were supported by significant increases in *nosZ* gene abundances accompanied the reduced $N_2O$ emissions in the microcosm studies, while concomitant increase in genes encoding $N_2O$-producing enzymes, *amoA*, *nirK/nirS*, and *norB*, were not significant or not as pronounced (van Zwieten *et al.*, 2010, Harter *et al.*, 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 *et al.*, 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 *et al.*, 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: 1) no harmful effect on human and animals; 2) lower risk of developing fungal resistance; and 3) lower cost of application. (Deliopoulos et al., 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. 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 *et al.*, 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 *et al.*, 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 risen with global demands on food production, 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 understanding the complexity of N-oxide reductive pathways in soil. The recent scientific findings reviewed here have substantially advanced knowledge of organisms and 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 include microbial ecology, microbial physiology, molecular biology, bioinformatics, geochemistry, and biochemistry, among many others. Initiation and application of such research would be advanced by public awareness of the global environmental and economic costs associated with of excess agricultural N currently lost through N-reductive pathways to the atmosphere.

## FUNDING

This work was supported by the "R&D Center for reduction in Non-$CO_2$ Greenhouse gases" (2017002420002) funded by Korea Ministry of Environment (MOE); National Research Foundation (2018H1D3A2063734); and US Department of Agriculture (2014-67019-21614).

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# Figure 1.

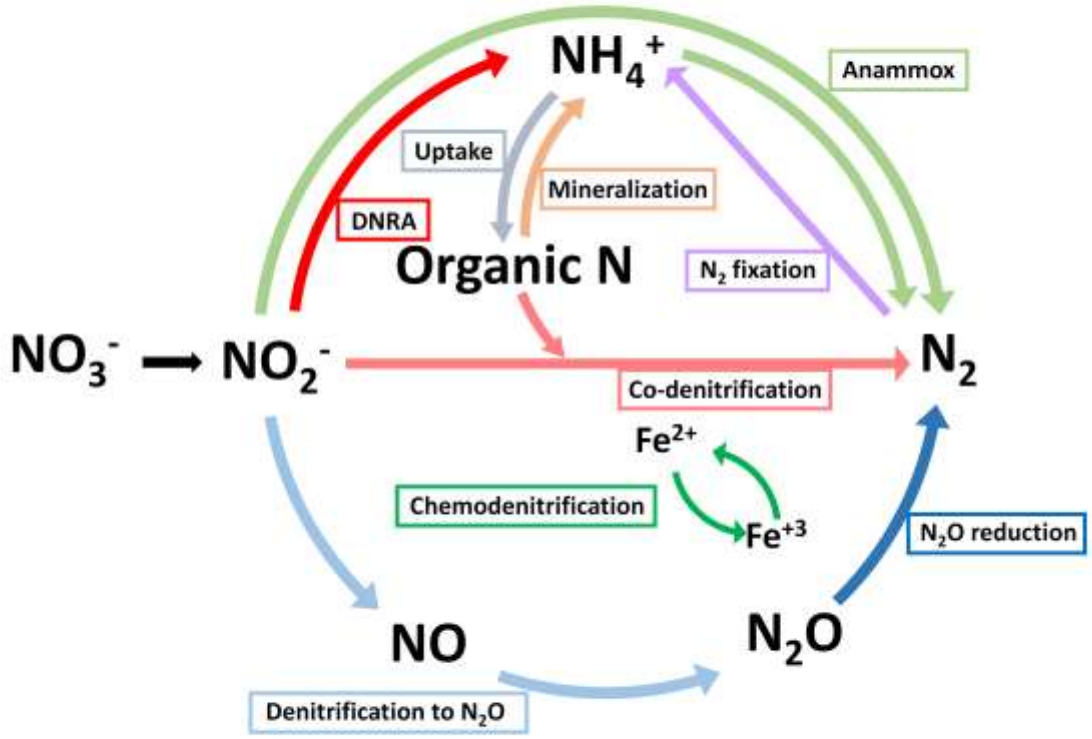


**Figure 1.** A graphical summary of the reductive nitrogen oxide transformation reactions discussed in this review. Both biotic and abiotic pathways are included and the pathways previously regarded as negligible, e.g., chemodenitrification and codenitrification, are also included in the network.

# Figure 2.

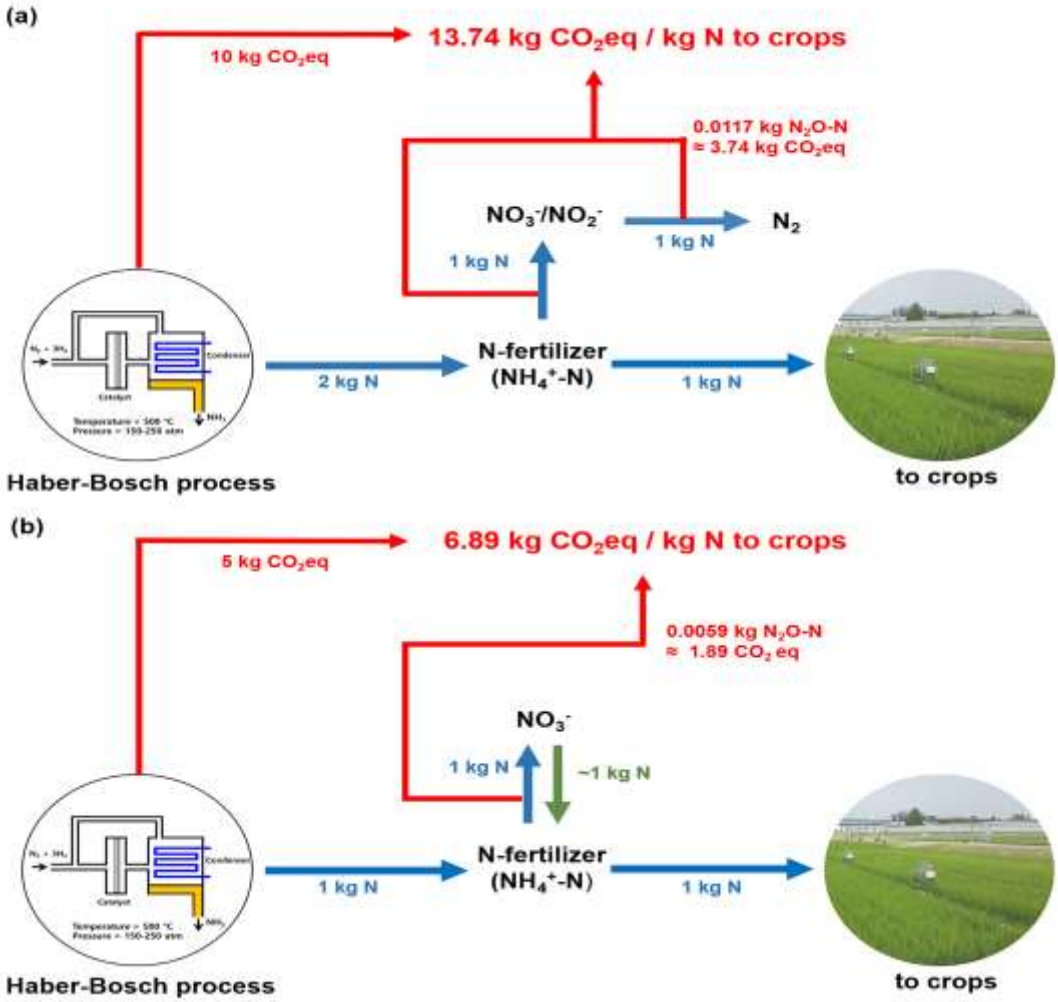


**Figure 2.** The comparison between net GHG emission in a) denitrification-dominant agricultural soils and b) hypothetical DNRA-dominant agricultural soils. The net GHG emission was calculated per kg of N conveyed to the crop biomass. For simplicity of presentation, the following assumptions were made. 1) An emission factor of 5 kg $CO_2$eq/kg N was assumed for production and application of N fertilizers. 2) Microbial N assimilation was assumed to be negligible. 3) 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). 4) 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.

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-cyclohexamide 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 et al. (2013) |

[a] n/a: not available.

[b] both $NH_4^+$ and $NO_3^-$ added.