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Microbially Mediated Nitrogen Conservation and Loss in Low and High Nitrogen Input Rice Paddies

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Abstract

High rates of fertilizer nitrogen (N) application in rice paddies is a common practice in the majority of the rice-producing countries. However, more than 60% of the applied fertilizer-N is lost to the atmosphere and other terrestrial ecosystems. This causes serious environmental pollution and economic loss. On the other hand, un-fertilized rice paddies have shown maintained soil N status and stable N supply to the rice plant for hundreds of years. Microbial N_2 fixation is known to contribute N to un-fertilized rice paddies. However, N_2 fixation alone cannot fully explain the maintained N nutrition in rice paddies, where favourable conditions for N loss by denitrification exist. In largely anaerobic rice paddies, caused by submerged conditions, nitrification takes place in the oxic rhizosphere and soil-water interface which continuously supply nitrate (NO_3^-) to the anaerobic soil. Fertilizer-N input increases the nitrification rate, NO_3^- concentration and the denitrification rate in rice paddies. Rice paddies under long-term no or low N input have also shown continuous nitrification activity and NO_3^- production. Despite the nitrification activity and NO_3^- production, there is minimal N loss by denitrification in long-term low N input rice paddies. This implies that microbes in low N input rice paddies have evolved to efficiently recycle mineral N within the system, allowing minimal N loss. However, the mechanisms are still unknown.

Dissimilatory nitrate reduction to ammonium (DNRA), a largely overlooked N-cycling process, has been found to compete with denitrification for loss-prone NO_3^- and retain it as ammonium (NH_4^+) and could help to explain the sustained N nutrition in low input paddies. The *nrfA* gene in soil microbes encodes cytochrome *c* nitrite reductase which catalyses the DNRA process. DNRA has been found to be a major NO_3^- consumption pathway (up to 98% of the available NO_3^-) in several forest soils and sediment ecosystems and the NO_3^- partitioning between DNRA and denitrification is thought to be affected by the soil organic

carbon (OC) and NO_3^- availability. This means DNRA can limit NO_3^- loss from soils where a favourable environment for the process exists. However, scant research has been conducted to explore the significance of DNRA and factors affecting the process in arable soils. There are no studies which have explored the effect of N fertilization regimes on the balance between microbial N conservation and loss in rice paddies.

This study used ^{15}N tracing technique, an acetylene reduction assay and quantitative PCR assays to quantify DNRA, denitrification, anammox and N_2 fixation rates and the relevant microbial gene abundances, in laboratory incubation experiments using paddy soils from Australia and Myanmar. The first study used soils from three high N input (>150 kg N ha^{-1}) rice paddies from Southeast Australia for a glasshouse pot experiment and subsequent laboratory experiments. The pot experiment was conducted with two levels of N input, zero and 150 kg urea-N ha^{-1}. The rhizosphere and bulk soils (separated by nylon bag) in the pots were collected separately for the laboratory experiments before the panicle initiation stage of rice. Effects of the history of N fertilization on the N-cycling processes were investigated in the second study using three paddy soils from long-term high N input Australian sites (>150 kg N ha^{-1}) and three paddy soils from low N input (<25Kg N ha^{-1}) Myanmar sites. Long-term no or low N (<25 kg N ha^{-1}) and high N fertilized (~100 kg N ha^{-1}) rice paddies from different geographical regions within Myanmar were used for the final experiment of this thesis.

The glasshouse pot experiment showed that omitting N enhances the DNRA rate in rice paddies. DNRA consumed 1.09-1.40 kg NO_3^--N ha^{-1} day^{-1} in N omitted paddy soils which was >16% compared to N added paddy soils. DNRA rates were similar or higher than denitrification rates in paddy soils without N addition. But DNRA consumed less than 50% of the NO_3^- than by denitrification in paddy soils with N addition. Nitrogen omission did not have a clear effect on the *nrfA* gene abundance and the N_2 fixing activity in long-term high N input paddy soils. DNRA and N_2 fixation did not vary between the rhizosphere and bulk soils but denitrification was lower in the former than the latter. This study confirmed that DNRA plays a significant role in retaining N in rice paddies which do not receive fertilizer-N.

Comparison of the effect of long-term N fertilization regimes on microbial N loss and retention in Myanmar and Australian rice paddies in the second and third study revealed more serious negative consequences of the long-term high N input on microbial N retention. Microbial N_2 fixation was found to add more than 4 kg N ha^{-1} day^{-1} in long-term no or low N input paddies, whereas N_2 fixation was less than 1.8 kg N ha^{-1} day^{-1} in long-term high N input paddies. Furthermore, DNRA was able to retain around 60% of the soil available NO_3^- as NH_4^+, allowing less than 15% to be reduced to N_2 by denitrification, whereas denitrification reduced around 30% of the NO_3^- to N_2 in long-term high N input rice paddies, where DNRA only retained approximately 10% of the NO_3^- as NH_4^+. These results confirmed that microbes can efficiently retain and recycle N in no or low N input paddies, allowing maintained soil N status and stable N supply to rice plants. However, in high N input rice paddies, microbes have less incentives to invest energy in retaining and recycling N. These results suggest that, under high N input systems, microbes (N_2 fixing and DNRA) in rice paddies lose the ability to retain N which exists in low N fertilised systems, in the order of ~ 3 kg N ha^{-1} day^{-1} in our study, which needs to be recompensed with fertilizer-N input.

Soil OC and NO_3^- concentrations were the most important environmental determinants of the fate of NO_3^- in rice paddies. Rates of DNRA consistently showed a positive correlation with the soil OC:NO_3^- ratio and a negative correlation with the soil NO_3^- concentration. In accordance with the previous findings from chemostat experiments using pure bacterial culture, higher soil OC:NO_3^- ratios in this study promoted NO_3^- partitioning to DNRA over denitrification. Artificially manipulating the soil OC:NO_3^- ratio by adding NO_3^- and different

levels of labile OC confirmed that maintaining higher soil OC:NO_3^- ratio can improve N retention when there is high NO_3^- concentration in rice paddies.

This study, for the first time, provides a comprehensive dataset of the effect of fertilizer-N input on the balance between microbial N conservation and loss in rice paddies. These findings suggest that long-term no or low N input paddy systems have unique microbial N regulation mechanisms to maximize N retention and minimize N loss, thus supporting primary productivity in the systems, whereas these mechanisms were not apparent in high input systems. This new knowledge is important for fertilizer-N management in low input paddies such as in Myanmar, where fertilizer-N use is starting to increase. In such a context, fertilizer-N dose should be established based on the examination of the effect of different fertilizer-N rates on the balance between N conservation and loss.

Keywords:

Rice paddies, Nitrogen, Microbial nitrogen transformation, N_2 fixation, Denitrification, Dissimilatory nitrate reduction to ammonium, Organic carbon, Nitrate, Ammonium, *nrfA* gene, Nitrogen fertilizer, low nitrogen input rice paddies, high nitrogen input rice paddies, ^{15}N isotope, Acetylene reduction assay, gene abundances, $^{29}N_2$, $^{30}N_2$, $^{15}NH_4^+$, and quantitative PCR.

Declaration

This is to certify that:

I. This thesis comprises only my original work.
II. All other materials used has been acknowledged.
III. This thesis contains less than 80,000 words, exclusive of tables, illustrations, bibliography and appendices.

Arjun Pandey
Date: 30-10-2018

Preface

This PhD thesis is my original work. This thesis comprises six chapters, an Introduction chapter, a Literature review chapter, followed by three research chapters and a Discussion and conclusions chapter. Among the three research chapters, one has been published in a peer reviewed journal and is in a format as required by the journal and the two other research chapters have been submitted to journals for publication and are also in the format required by the respective journals. Arjun Pandey is the first author of all the research chapters. Arjun Pandey identified the research gaps, designed the experiment, collected and analysed the data and wrote this thesis, including the research chapters. The co-authors supervised and contributed at various stages of this PhD study. The following three research chapters have been included in this thesis and are either published or submitted for publication to a journal:

Chapter 3:

Pandey, A., Suter, H., He, J.Z., Hu, H.W. and Chen, D., 2018. Nitrogen addition decreases dissimilatory nitrate reduction to ammonium in rice paddies. *Applied and Environmental Microbiology*, AEM-00870.

Chapter 4:

Pandey, A., Suter, H., He, J.-Z., Hu, H.-W., Chen, D., 2019. Dissimilatory nitrate reduction to ammonium dominates nitrate reduction in long-term low nitrogen fertilized rice paddies. *Soil Biology and Biochemistry* 131, 149-156.

Chapter 5:

Pandey, A., Suter, H., He, J.Z., Hu, H.W. and Chen, D., 2018. Dissimilatory nitrate ammonification helps maintain nitrogen nutrition in resource limited rice paddies in Myanmar. *Ecology* (submitted).

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I would like to dedicate my PhD thesis to my late father Lok Prasad Pandey; I would not have been where I am without his belief and dream for me. My PhD study would not have been possible without the immense support from my mother, Madhavi Pandey and my loving wife Prashansa Aryal Pandey. Thank you Prashansa for allowing me to pursue my PhD dream and supporting me during the entire journey. I wish to thank all my family members who directly and indirectly supported me throughout my PhD study.

Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5:

List of Figures in Supplementary Materials

Chapter 3

Chapter 4

Chapter 5

List of Figures in the Appendix

List of Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Tables in Supplementary Materials

Chapter 4

Chapter 5

CHAPTER 1: Introduction

1.1 Background

Rice (*Oryza sativa* L.) is the most important staple food for the majority of the world's population (Maclean et al., 2002). Although the crop is cultivated under different water regimes, almost 75% of the world's rice supply comes from continuously flooded rice paddies (Maclean et al., 2002). Unlike other arable crops, the rice plant has arenchyma, longitudinal interconnection of gas spaces extending from leaves, stems and to the roots of the plant, which supply oxygen (O_2) to the roots for respiration and thus enables the survival of the rice plant in submerged conditions (Kirk, 2003; Steffens et al., 2011). The O_2 supplied to the root also leaks into the adjacent soil and creates oxic conditions around the root surface (Revsbech et al., 1999). In addition, the rice plant supplies a considerable amount of organic carbon (OC) through root exudates and decaying plant parts (Ge et al., 2012) which accelerate the development of anaerobic conditions in the soil (Wassmann and Aulakh, 2000). Therefore, paddy soils are comprised of a complex set of oxic and partially oxic environments in the rhizosphere and strictly anoxic environment in the non-rhizosphere (bulk) soil (Kögel-Knabner et al., 2010). These complex set of rhizospheric environment (rhizosphere and bulk soils) in rice paddies favour simultaneous occurrence of several microbial nitrogen (N) transformation processes which have direct effects on the N use efficiency of rice production.

Nitrogen is one of the most important yield-limiting nutrients for arable crops in general and it is the most important yield-limiting nutrient in rice production (Cassman et al., 1998). Rice paddies are one of the biggest fertilizer-N sinks, in the world, which is similar to wheat and maize, although the area under rice is less than that under wheat and maize (Heffer, 2013; FAO, 2018). However, the efficiency of use of fertilizer-N is generally below 40% in rice production (Choudhury and Kennedy, 2005), which is mainly attributed to ammonia (NH_3) volatilization (10-25% of applied N) and denitrification loss (~36% of applied N) (Cassman et al., 1998; Aulakh et al., 2001; Li et al., 2008; Ju et al., 2009).

On the other hand, rice paddies which do not receive fertilizer-N have shown sustained yields (at moderate level) for hundreds, if not thousands, of years (Cassman et al., 1995). Long-term (10- 40 years) experimental trials conducted at several locations in different countries have shown maintained soil organic carbon (OC) and N status, and sustained rice yields without fertilizer-N input (Saleque et al., 2004; Pampolino et al., 2008; Bi et al., 2009; Tong et al., 2009). These and other long-term experiments have documented net N accretion in paddy soils which do not receive fertilizer-N (Cassman et al., 1998; Bi et al., 2009; Tong et al., 2009).

Diazotrophic N_2 fixation contributes to N nutrition of the rice plant in paddies which receive no or low fertilizer-N (Kyaw et al., 2005; Bei et al., 2013). N_2 fixation has been found to supply more than 45 kg N ha^{-1} in un-fertilized rice paddies (Bei et al., 2013). However, N_2 fixation can be negligible when rice paddies receive high doses of fertilizer-N (Kyaw et al., 2005; Bei et al., 2013). The *nifH* gene in diazotrophs encodes the nitrogenase enzyme which catalyses N_2 fixation and the gene is expressed when the diazotrophic cells are N deficient (Jonsson and Nordlund, 2007). When fertilizer-N is applied to rice paddies, diazotrophs utilize the N in fertilizer and become N sufficient and in such conditions the *nifH* gene remains unexpressed, resulting in minimal N_2 fixation (Jonsson and Nordlund, 2007; Nordlund and Hogbom, 2013). Indeed, studies have reported lower *nifH* gene and *Cyanobacterial* (also involved in N_2 fixation) abundances in long-term N fertilized rice paddies compared to un-fertilized rice paddies (Wang et al., 2016; Wang et al., 2016).

Despite the N contribution, diazotrophic N_2 fixation alone cannot fully explain sustained yields and maintained soil N status in un-fertilized rice paddies, where a favourable environment for N loss by denitrification exists. Paddy soils are low in O_2 availability due to the extended period of flooding (Wassmann and Aulakh, 2000), which is a prerequisite condition for the denitrification process (Kraft et al., 2011). Another prerequisite for denitrification is nitrate (NO_3^-) availability. Even though rice paddies are largely anaerobic, nitrification produces NO_3^- in the oxic rhizosphere and the soil-water interface (Arth and Frenzel, 2000). The produced NO_3^- quickly diffuses into the anoxic soil layers and gets denitrified (Arth and Frenzel, 2000; Li et al., 2008). A significant amount of NO_3^- is produced in rice paddies through nitrification of ammonia (NH_3) after fertilizer-N input (Arth and Frenzel, 2000). Rice paddies have shown continuous NO_3^- production also in un-fertilized rice paddies (Ghosh and Kashyap, 2003; Wang et al., 2014). Ammonium concentration in long-term un-fertilized rice paddies is at a level comparable to N fertilized rice paddies (Wu et al., 2011; Wang et al., 2014; Wu et al., 2017), which is contributed by mineralization of soil indigenous N and fixed N_2 (Bacon et al., 1986; Kundu and Ladha, 1995; Ghosh and Kashyap, 2003). This NH_4^+ is then utilized by the nitrifiers to produce NO_3^-. Indeed, research have shown a tight coupling between N_2 fixation and N loss through denitrification in anaerobic systems (Deutsch et al., 2007; Korth et al., 2014).

Denitrifiers utilize the labile OC as an electron donor to reduce NO_3^- stepwise to N_2, promoting N loss (Kraft et al., 2014). Paddy soils receive a significant amount of OC (between 30-50% of net photosynthesized carbon) through root exudates and decaying plant parts (Lynch and Whipps, 1990; Kögel-Knabner et al., 2010) and this fuels the denitrification process. Unlike significant N loss through denitrification from N fertilized rice paddies, there is a minimal N loss via denitrification from un-fertilized rice paddies (Arth et al., 1998; Aulakh et al., 2001). This implies that N-cycling microbes in un-fertilized rice paddies have stringent N regulation strategies to efficiently recycle and retain N in the system.

A series of enzymes encoded by different genes in denitrifiers reduce soil available NO_3^- to produce N_2 (Dong et al., 2009). Nitrate reduction to nitrite (NO_2^-) is encoded by the *nar* gene and the subsequent reduction of NO_2^- to NO and NO to N_2O is encoded by the *nir* and *nor* genes, respectively, and the final step of denitrification (N_2O reduction to N_2) is encoded by the *nos* gene (Zumft, 1997). N_2 is the main denitrification product in paddy soils, where N_2O production is almost two orders of magnitude lower than N_2 production (Arth et al., 1998; Aulakh et al., 2001). A recently identified process of anaerobic ammonium oxidation (anammox) has also been found to produce N_2 mainly in O_2 minimum zones in the oceans (Lam et al., 2009; Trimmer et al., 2013). Hydrazine synthase, encoded by the *hzs* gene in anammox bacteria, catalyses the production of hydrazine (N_2H_4) through the oxidation of NH_4^+ with NO_3^-/NO_2^- which is then oxidised to N_2 (Kartal et al., 2011; Harhangi et al., 2012). Limited research conducted on anammox in rice paddies has found that the process can contribute negligible to 40% of the total N_2 production (Bai et al., 2015; Nie et al., 2015; Yang et al., 2015). These findings have challenged our previous understanding of denitrification as the sole N_2 production pathway. Studies on the anammox process in rice paddies so far were focused on whether the process exists in rice paddies. Little is known about effects of fertilizer-N input on anammox in rice paddies.

Dissimilatory nitrate reduction to ammonification (DNRA), a less known microbial N-cycling process in agricultural ecosystems, competes with denitrification and anammox for loss-prone NO_3^- and transforms it to NH_4^+ which is then retained by clay minerals (Silver et al., 2001). The cytochrome *c* nitrite reductase enzyme encoded by the *nrf* gene in bacteria catalyses the ammonification of NO_3^- (Kraft et al., 2011). The requirement for strong anaerobic conditions, which was believed to be a prerequisite for the DNRA process, has been ruled out by recent research where DNRA was detected under aerobic soil conditions (Silver et al., 2005; Yang et al., 2017). Therefore, DNRA may occur in both the oxic rhizosphere and anoxic bulk paddy soils. However, the factors affecting the DNRA process in soils are largely unknown. Chemostat experiments with pure bacterial culture have suggested that the ratio of OC:NO_3^- is the most important factor determining NO_3^- partitioning between denitrification and DNRA (Kraft et al., 2014; Van Den Berg et al., 2015; Yoon et al., 2015). Recently, the DNRA process has received a great deal of attention during the study of N-cycling in forest and sediment ecosystems (Silver et al., 2005; Dong et al., 2011; McTigue et al., 2016), but contradictory results of the effect of OC and NO_3^- on DNRA has been reported (Silver et al., 2005; Schmidt et al., 2011; Yang et al., 2017). Nevertheless, DNRA has been found to play a significant role in NO_3^- reduction in several forest soils, where more than 75% (in some cases up to 99%) of the available NO_3^- was reduced to NH_4^+ by the process (Silver et al., 2001; Huygens et al., 2007; Yang et al., 2017). However, little is known about the significance of DNRA in rice paddies and the factors affecting the process.

Rice paddies in the majority of the rice-producing countries receive high amounts of fertilizer N (up to 300 kg N ha^{-1}) (Cassman, 1999; Peng et al., 2006; Ju et al., 2009). Long-term N fertilization has shown increased soil NO_3^- production (Wang et al., 2014; Wang et al., 2016), and a reduction in the labile OC fraction (Neff et al., 2002; Cusack et al., 2011; Dou et al., 2016). This have an effect on the OC:NO_3^- ratio in soil and may affect the NO_3^- partitioning between the DNRA and denitrification processes. Long-term high N fertilization has been found to lead to an increase in denitrification rates and the abundance of *narG*, *nirK* and *nosZ* genes in rice paddies (Chen et al., 2012). This implies that the long-term high N fertilization may promote NO_3^- partitioning to denitrification, leading to a negative effect on DNRA. However, little information is available on this. In particular, there are no studies looking into the effects of long-term N fertilization on DNRA activity and the related *nrfA* gene abundance, while the results from short-term N fertilization experiments on DNRA activity in various soil ecosystems are contradictory (Schmidt et al., 2011; Baldos et al., 2015; Minick et al., 2016).

Whilst, the effect of N fertilization on N_2 fixation and denitrification is well studied, the following aspects remain largely unexplored in rice paddies:

1. The effect of long-term N fertilization on the balance between N conservation (N_2 fixation and DNRA) and N loss (denitrification and anammox).
2. The effect of the rhizospheric environments on DNRA and the *nrfA* gene abundance.
3. The effect of long-term N fertilization on NO_3^- partitioning between the DNRA and denitrification (including anammox) processes.
4. The effect of soil OC:NO_3^- ratios on the DNRA and denitrification processes.

1.2 Research aims, research questions and hypotheses

The aims of this study were: (i) to gain insights into the effect of different N fertilization regimes on microbial N conservation and loss pathways; (ii) to develop an understanding on the role of the DNRA process in N cycling in paddy soils; (iii) to examine the soil environmental determinants of the NO_3^- partitioning between DNRA and denitrification in rice paddies. The following research questions were answered to achieve the aims of this study:

1. How do the rhizospheric environments and N addition affect N retention (N_2 fixation and DNRA) and N loss (denitrification and anammox) and the abundance of relevant N-cycling genes in rice paddies?

   The following hypotheses were set:

   I. Oxic environment in rhizosphere affect N loss but does not affect N retention rates.
   II. N addition reduces N retention and increases N loss rates.
   III. N addition reduces the abundances of the microbial genes involved in N retention.
2. How does the history of N fertilization affect microbial N retention and loss and the relevant N-cycling microbial gene abundances in rice paddies?

The following hypotheses were set to answer the research question:

I. N_2 fixation is higher in the long-term low N fertilized than in high N fertilized rice paddies.

II. DNRA transforms the majority of the NO_3^- to NH_4^+ in long-term low N fertilized rice paddies and allows minimal denitrification loss.

III. The microbial gene abundances related to N retention are higher in long-term low N than high N fertilized rice paddies.

3. How does the soil OC and NO_3^- concentration affect the partitioning of NO_3^- between the DNRA and denitrification processes in long-term low and high N fertilized rice paddies?

I. Higher soil OC and lower soil NO_3^- concentration promotes DNRA over denitrification.

II. Retention of NO_3^- as NH_4^+ can be increased by increasing the soil OC:NO_3^- ratio in long-term high N fertilized rice paddies.

1.3 Thesis outline

This thesis is with publications and contains 6 chapters.

CHAPTER 1-Introduction: This chapter includes the thesis outline, background, research aims, research questions and hypotheses. This chapter also presents the relevance of the study and a brief overview of the methods used to answer the research questions.

CHAPTER 2-Literature review: This chapter expands on the background presented in Chapter 1 and presents the synthesized outcomes of the relevant published research and knowledge gaps identified.

CHAPTER 3-Nitrogen addition decreases dissimilatory nitrate reduction to ammonium in rice paddies: This is an experimental chapter and addresses the first research question of this thesis. This study demonstrated that the fertilizer-N addition influences the NO_3^- partitioning between DNRA and denitrification and thereby reduces N retention and increases N loss in paddies. This study also showed that N retention does not differ between the rhizosphere and bulk soils, but N loss does. Fertilizer-N omission treatment in this study did not have a clear effect on the abundance of the microbial genes involved in N retention.

This chapter has been published:

Pandey, A., Suter, H., He, J.-Z., Hu, H.-W., Chen, D., 2018. Nitrogen addition decreases dissimilatory nitrate reduction to ammonium in rice paddies. *Applied and Environmental Microbiology*, AEM. 00870-00818.

CHAPTER 4-Dissimilatory nitrate reduction to ammonium dominates nitrate reduction in long-term low nitrogen fertilized rice paddies: This chapter addresses the second research question. This study demonstrated that the microbes in long-term low N fertilized paddies efficiently conserve N, resulting in minimal N loss, but this was not apparent in high N input paddies. This chapter also demonstrated that the microbial gene abundances associated with N retention are higher in long-term low N fertilized paddies than in high N fertilized paddies.

This chapter has been published.

Pandey, A., Suter, H., He, J.-Z., Hu, H.-W., Chen, D., 2018. Dissimilatory nitrate reduction to ammonium dominates nitrate reduction in long-term low nitrogen fertilized rice paddies. *Soil Biology and Biochemistry*, 131, 149-156.

CHAPTER 5-Dissimilatory nitrate ammonification helps maintain nitrogen nutrition in resource limited rice paddies in Myanmar: This chapter draws upon chapter 4, further corroborating the findings, and addresses the third research question. We observed that the long-term low N fertilized rice paddies are more efficient in N conservation than the high N fertilized rice paddies. We also observed that the SOC:NO_3^- ratio is the most important environmental determinant for the NO_3^- partitioning between the DNRA and denitrification processes and N retention can be improved in long-term high N fertilized rice paddies by manipulating the SOC:NO_3^- ratio.

This chapter has been submitted to a journal for publication.

Pandey, A., Suter, H., He, J.-Z., Hu, H.-W., Chen, D., 2018. Dissimilatory nitrate ammonification helps maintain nitrogen nutrition in resource limited rice paddies in Myanmar. *Ecology* (submitted).

CHAPTER 6-Discussion and conclusions: This chapter presents the synthesis of the research outcomes from Chapter 3, 4 and 5, and discusses the implications of the research outcomes.

1.4 Relevance of the study

Rice paddies comprise a complex set of microsites (rhizosphere and bulk soils) that support different N transformation processes simultaneously. This study focuses on the simultaneous determination of the different microbial N retention (N_2 and DNRA) and N loss (denitrification and anammox) processes and their relevant gene abundances in different rice paddies. Simultaneous determination of the processes addressed the methodological error in the determination of the process rates. Investigation of the processes in the rhizosphere and bulk soils in rice paddies with different levels of soil OC and managed under different N fertilization regimes improved our understanding of the processes. A better understanding of these processes backed by empirical evidence will help us determine efficient N management strategies in low and high N fertilized rice paddies to enhance nitrogen use efficiency of rice production.

1.5 Methods overview

1.5.1 Site selection

Site 1

Rice paddies in the Riverina region of New South Wales (NSW) in Australia were categorised as Site 1 and paddy soils were collected for a glasshouse pot experiment, laboratory incubation experiments and molecular studies. More than one million tons of rice was produced from around 113,600 hectares (ha) of land in Australia in 2013 (FAO, 2018). Most of the rice growing area is concentrated in the Riverina region in Australia (Kinoshita et al., 2015). The recommended average N input for a rice crop in the region is ~150 kg N ha^{-1} but farmers apply up to 200 kg N ha^{-1} (Dunn et al., 2014; DPI, 2015). As with most of the rice-producing countries, urea is the most commonly used fertilizer-N source for rice production in Australia (Dunn et al., 2014). The rice cultivation in the region is largely dependent on irrigation and fields are flooded for much of the growing season (Dunn et al., 2014).

Site 2

Rice paddies in central and lower Myanmar were categorised as Site 2 for this study and paddy soils were collected for laboratory incubation experiments and molecular studies. Myanmar ranks sixth in the world in rice production (Ricepedia, 2015). Approximately 7 million hectares of land was under rice cultivation in the country in 2013 (FAO, 2018). Rice paddies in the country are characterised by no or low N (~25 kg N ha^{-1}) fertilization (Denning et al., 2013; Haggblade and Boughton, 2013). Even though the level of N input is low, the rice yield is around 3.5 tonnes ha^{-1} (Denning et al., 2013; Haggblade and Boughton, 2013). Double rice cropping is the most common practice and rice paddies are continuously flooded during a cropping season but may vary with water availability in some reasons (http://www.fao.org/docrep/005/Y4347E/y4347e18.htm). Very little research has been conducted on N cycling in rice paddies of Myanmar.

1.5.2 Determination of N transformation rates and microbial gene abundances

Nitrogen transformation rates were determined through laboratory incubation of paddy soils collected in Myanmar and Australia. An acetylene reduction assay (ARA) was used to determine the N_2 fixation rate (Unkovich and Baldock, 2008). N_2 fixation results obtained from the ARA technique were calibrated using a $^{15}N_2$ uptake experiment (only for the Chapter 5) (Keuter et al., 2014). Denitrification, anammox and DNRA rates were determined using different ^{15}N isotope combinations (Trimmer and Nicholls, 2009; Song et al., 2013). A combination of $^{15}NH_4^+$ + unlabelled NO_3^- was used to detect the anammox process (Trimmer et al., 2013). Denitrification, anammox and DNRA rates were determined by incubating paddy soils anaerobically with $^{15}NO_3^-$ (99 ^{15}N atom%) and measuring $^{29}N_2$, $^{30}N_2$ and $^{15}NH_4^+$ production with time (Trimmer and Nicholls, 2009; Song et al., 2013). Quantitative PCR (qPCR) analysis was used to determine the abundance of microbial genes involved in each of the N transformation processes.

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CHAPTER 2: Literature Review

2.1 Rice production

Rice (*Oryza sativa* L.) is the most important staple food for the majority of the world’s population (Maclean et al., 2002). In 2013, around 165 million hectares (ha) of land was under rice cultivation globally, which produced 742 million tonnes of rice (FAO, 2018). During the same time, ~733 million tonnes of wheat was produced from 218 million ha of land (FAO, 2018). Use of high yielding verities, fertilizers and improved management practices have increased rice production by threefold since 1970 (Ussiri and Lal, 2013). China is the world’s top rice producing country; more than 202 million tons of rice was produced in 2015 and the yield of rice was just over 6 tonnes ha^{-1} (IRRI, 2015). India is the top rice exporting country, followed by Thailand, Vietnam and Myanmar (IRRI, 2015).

2.2 Rice production systems and the environmental conditions in soils

Approximately 50% of the world’s total rice area is covered by continuously flooded lowland rice which contributes 75% of the total rice production (Bouman et al., 2007; IRRI, 2015). Flooded conditions in rice paddies suppress weeds and support better growth (Bouman et al., 2007). Rice paddies are bunded, flooded and puddled before the transplanting of rice. Puddling of the paddy soil creates a plough pan in the soil at around 20 cm depth which impedes the percolation of water and nutrients into the deeper soil layers where it would be unavailable to the rice plant. Rice is the only cereal crop that can grow in submerged soils. Rice plants have unique longitudinal interconnections of gas spaces known as aerenchyma (Figure 1), which extend from the shoots to the roots (Nishiuchi et al., 2012). The aerenchyma transfers atmospheric O_2 to the submerged roots for root respiration and enables rice to survive in submerged conditions (Kirk, 2003).

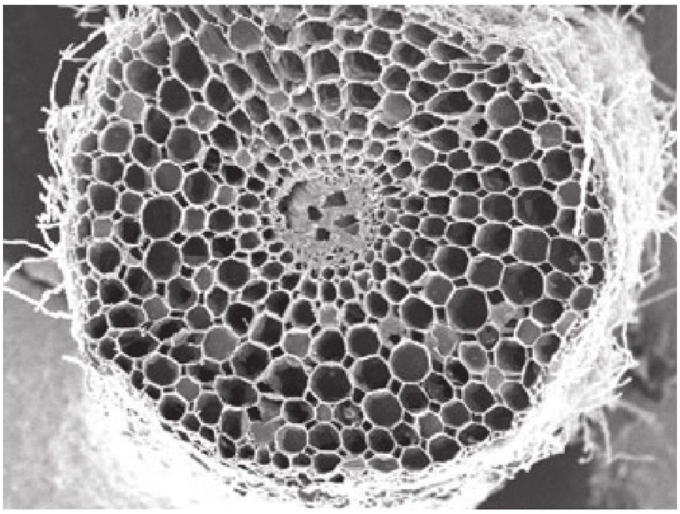

Figure 1. Scanning electron microscope image of the transverse sections of newly formed aerenchymatous tissues in a 51-day-old root (Li et al., 2008).

Flooded paddy soils are comprised of different microsites (Figure 2). Soon after flooding, the trapped O_2 in paddy soils is quickly respired by the soil microorganisms, creating anoxic conditions (Wassmann and Aulakh, 2000). Due to the diffusion of the atmospheric O_2, the top 2 mm of the standing water on the paddy soil surface remains oxic (Revsbech et al., 1999; Kögel-Knabner et al., 2010). Below the oxic surface layer, there exists a strongly anoxic condition in the non-rhizosphere (bulk soil) (Kirk, 2003; Kögel-Knabner et al., 2010), where the O_2 concentration reaches below the detection limit (Revsbech et al., 1999). The O_2 supplied to the roots through aerenchyma also leaks from the roots to the adjacent soil/rhizosphere (Kirk, 2003) and as a result, a very thin (< 1 mm) oxic layer, with an O_2 concentration around 43 – 115 µM L^{-1}, is created around the roots (Revsbech et al., 1999; Schmidt et al., 2011).

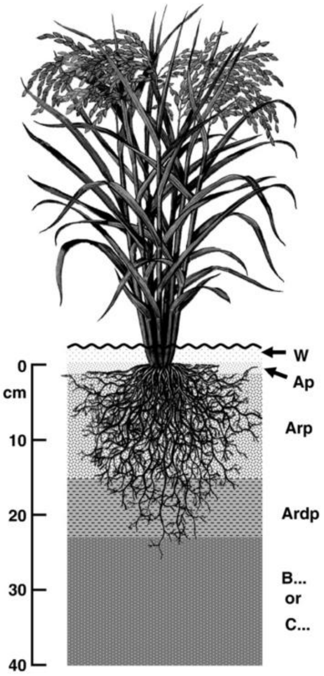

Figure 2. Horizon sequence of paddy soils. **W** is the oxic layer of standing water, **Ap** is the oxic and partially oxic puddled layer, **Arp** is the anoxic puddled layer and **Ardp** is the reduced layer in plough pan, B and **C** are the sub-soil horizons (Kögel-Knabner et al., 2010).

Rice plants supply a significant amount of organic carbon (OC) to the soil and a large portion is in the form of root exudates, secretions, lysates, mucilages, and sloughed-off root cells, and is readily decomposable (Kimura et al., 2004). Roots are allocated with approximately 30-60% of the photosynthesised carbon and up to 90% of that is translocated to the soils (Lynch and Whipps, 1990; Kögel-Knabner et al., 2010). However, only around 5% of the rhizodeposition is stored in the soil and the rest is rapidly used by soil microbes (Hütsch et al., 2002; Ge et al., 2012). The availability of the labile OC varies between the rhizosphere and bulk soils because of the release of the root exudates in the rhizosphere soil (Nie et al., 2014; Zhang et al., 2017). Nie et al. (2014) reported 129 and 91.96 mg dissolved OC kg^{-1} rhizosphere and bulk soils, respectively, when the rice plants were 37 days old. The labile OC is a source of electrons for the reduction processes. Soil microbes utilize OC for the reduction of NO_3^-, Mn^{4+}, Fe^{3+} and SO_4^{2-} for their energy-conserving metabolic processes (Wassmann and Aulakh, 2000; Kögel-Knabner et al., 2010). Like OC, the reduced Fe^{2+} and sulfide, concentrated mainly in bulk soils, also act as electron donors (Begg et al., 1994; Liesack et al., 2000; Lin et al., 2010). The oxidation and reduction products in rice paddies are illustrated in Figure 3. Thus paddy soils are comprised of oxic, anoxic and partially anoxic microsites with different oxidised and reduced substrates that favour survival and growth of diverse soil microorganisms, i.e. aerobic, anaerobic, and facultative (Schmidt and Eickhorst, 2013). These soil microorganisms play an important role in nutrient cycling in paddy soils.

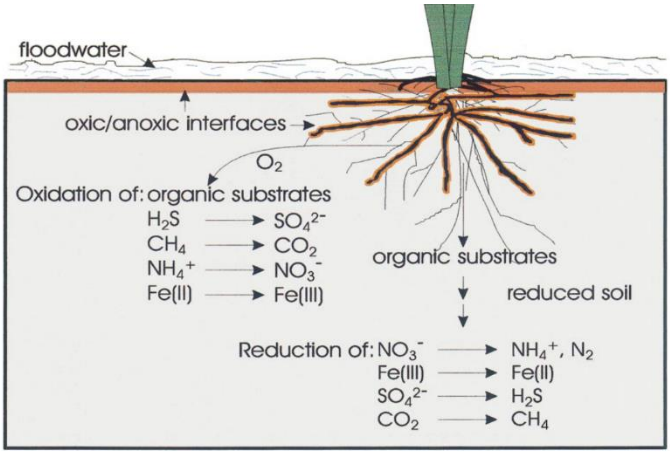

Figure 3. Illustration of redox reactions in the rhizosphere, soil-water interface and the bulk soils in rice paddies (Liesack et al., 2000).

Nitrogen is the most important nutrient for rice production (Cassman et al., 1998). The aerobic, anaerobic and facultative microbes in paddy soils carry out simultaneous oxidation and reduction of different forms of N (Liesack et al., 2000). For example, nitrification occurs in the oxic rhizosphere and soil-water interface which provides oxidised N substrate for downstream reduction processes, such as denitrification and dissimilatory nitrate reduction to ammonium. In addition, several aerobic, anaerobic and facultative N_2 fixing microbes, known as diazotrophs, continuously supply bioavailable N to other N-cycling microbes in paddy soils. This study focuses on N-cycling and the relevant microbial gene abundances in rice paddies which are directly involved in N conservation and loss in rice paddies.

2.3 Nitrogen in rice paddies

2.3.1 Nitrogen supply and efficiency

Among the arable soils, paddy soils are one of the biggest fertilizer-N sinks in the world (Kögel-Knabner et al., 2010; Heffer, 2013). Fertilizer-N use in rice production was more than 16 million tonnes globally in 2010/11, which is around 15% of the total N used in agricultural crops (Heffer, 2013). Rice paddies commonly receive 100-300 kg N ha^{-1} crop^{-1} in the major rice producing countries (Hossain et al., 2005; Ju et al., 2009; Peng et al., 2010; Dunn et al., 2014; Pandey et al., 2014). However, the nitrogen use efficiency (NUE), i.e. the proportion of applied N recovered in aboveground biomass, of rice paddies is generally below 40% (Dobermann and Fairhurst, 2000; Choudhury and Kennedy, 2005). Fertilizer-N input in rice paddies in China was more than 5 million tonnes in 2010/11 (Heffer, 2013) and the NUE of rice production was below 40% (Wang et al., 2001; Peng et al., 2006). Myanmar ranks sixth in the world among the top rice-producing and exporting countries (IRRI, 2015) but N input in rice paddies in Myanmar is very low (~25 kg N ha^{-1}) (Denning et al., 2013; Haggblade and Boughton, 2013; IRRI, 2015), compared to China (~200 kg N ha^{-1}) (Heffer, 2013; IRRI, 2015). Despite the low N application in rice paddies in Myanmar, the yield is moderate (3.5 tonnes ha^{-1}) (Denning et al., 2013; IRRI, 2015).

Several long-term (10-30 years) experimental trials in different countries have shown a stable trend or net accretion of soil OC and total N in un-fertilized rice paddies (Table 1). Experimental trails at 155 sites across Asia have shown that there is little change in indigenous soil N supply to the rice plant over time in un-fertilized rice paddies (Dobermann et al., 2003). Grain yield trend in un-fertilized rice paddies is also stable over time (Dobermann et al., 2003; Shen et al., 2004). This implies that soil microbes in rice paddies which do not receive fertilizer-N have efficient N recycling and retention strategies to maintain soil N status and consistent N supply to rice plants, allowing for sustained rice yields. However, little information is available about these N retention strategies.

Table 1. Change in OC (g kg^{-1} soil) and total N (g kg^{-1} soil) content of paddy soils without fertilizer-N input over time. Results presented here includes only experimental plots which have at least two rice crops per year without additional organic matter.

Experiment duration (years)	$^{a}OC_{start}$	$^{a}OC_{end}$	$^{b}TN_{start}$	$^{b}TN_{end}$	Countries	References
	g kg^{-1} soil					
10	7.6	10.5	0.817	0.95	Nepal	Regmi et al. (2002)
25	16.2	19.9	1.58	2.04	China	Bi et al. (2009)
22	14.8	15.6	1.36	1.54	China	Bi et al. (2009)
30	18.3	20.7	1.94	2.09	Philippines	Cassman et al. (1998)
17	26.4	35.7	2.72	3.22	China	Tong et al. (2009)
17	17.1	16.6	1.76	1.64	China	Tong et al. (2009)
18	16.4	18.2	1.70	1.65	China	Pan et al. (2009)
25	14.0	17.9	1.43	1.55	China	Yan et al. (2007)
15	18.3	20.0	1.49	1.67	Philippines	Pampolino et al. (2008)

a: OC_{start} and OC_{end} are the OC content of the soil at the start and at the end of the long-term experiments, respectively. b: TN_{start} and TN_{end} are the total N content of the soil at the start and at the end of the long-term experiments, respectively.

2.3.2 Mineral nitrogen in rice paddies

Unlike other arable soils where NO_3^- is the predominant form of mineral N, NH_4^+ constitutes the majority of the mineral N in paddies (Li et al., 2008). Ammonium-based fertilizer, mainly urea, is the most commonly applied fertilizer-N in paddies (Dan et al., 2001). Urea hydrolyses with water to form NH_4^+ in soils (Adachi-Pagano et al., 2003; Shang et al., 2015). Ammonium is adsorbed by the clay minerals and is retained in soils for a longer period than other mineral N forms (Huygens et al., 2008). It is also the form of mineral N preferred by primary producers (Silver et al., 2001; Yin et al., 2002; Kraft et al., 2014).

Nitrate-based fertilizers are seldom applied in rice paddies. However, in the presence of O_2, NH_4^+ gets oxidized to NO_3^- via nitrite (NO_2^-) during nitrification (Simek, 2000). In largely anaerobic rice paddies, the nitrification process continuously produces NO_3^- in the oxic rhizosphere and the soil-water interface (Revsbech et al., 1999; Li et al., 2008). The concentration of NH_4^+ is higher in the anoxic bulk soil compared to the oxic root surface layer and partially oxic soil in the rhizosphere where nitrification takes place (Li et al., 2008; Nie et al., 2015). Mass flow of NH_4^+ towards the rhizosphere and soil-water interface takes place due to an evapotranspiration induced percolation of water thus providing substrates for nitrification in the oxic rhizosphere and soil-water interface (Arth and Frenzel, 2000). Nitrate is the most mobile form of mineral N in the soil (Huygens et al., 2007) and is not adsorbed by clay minerals (Huygens et al., 2008; Dimitrov et al., 2010). Although the rhizosphere and soil-water interface have a higher nitrification rate compared to the bulk soils (Li et al., 2008), due to the highly mobile nature of NO_3^- it diffuses to the adjacent anoxic bulk soils resulting in an even distribution of the NO_3^- in the rhizosphere and bulk soils (Li et al., 2007; Li et al., 2008). Nitrate leaching is generally minimal in rice paddies due to the plough pan (Cassman et al., 1998), but NO_3^- is subjected to rapid denitrification loss in flooded conditions (Aulakh et al., 2001; Cai et al., 2007; Xie et al., 2010).

Soil NO_3^- concentration significantly rises after N fertilization in rice paddies and remains high for up to two weeks (Arth et al., 1998; Arth and Frenzel, 2000; Aulakh et al., 2001). Fertilizer-N is applied in split doses in rice paddies, resulting in a high NO_3^- concentration for a longer period (up to 2 months), which declines and remains low (~2 mg NO_3-N kg^{-1} soil) after the last dose of N application (Aulakh et al., 2001; Yang et al., 2013). More than 5-9 mg NO_3^--N kg^{-1} can be detected in paddy soils immediately after fertilizer-N input (~100 kg N ha^{-1}), which can last for up to two weeks (Aulakh et al., 2001; Sik Yoon et al., 2006; Yang et al., 2013). However, the NO_3^- level in un-fertilized paddies is low, around

0.5-2 mg NO_3^--N kg^{-1} soil throughout the rice growing period (Aulakh et al., 2001; Yang et al., 2013). Long-term fertilization increases NO_3^- concentration in rice paddies (Chen et al., 2010; Wang et al., 2014). Due to the highly mobile and unstable nature of NO_3^- and its rapid utilization by soil microbes for redox reaction, quantification of the soil NO_3^- concentrations cannot represent actual NO_3^- production rates. Paddy soil NO_3^- concentration may vary depending on soil microsites (rhizosphere and bulk soil), the measurement timing after fertilizer-N application and plant growth stages (Li et al., 2008). Measurement of nitrification potential of rice paddies can provide better information on the effect of long-term N fertilization on NO_3^- production in paddy soils. Studies have demonstrated an increase in nitrification potential of rice paddies after long-term N fertilization (Table 2).

Table 2. Change in nitrification potential of rice paddies after long-term N fertilization.

References	Nitrogen fertilization (kg N ha^{-1} crop^{-1})	Potential nitrification rate (mg NO_3^--N kg^{-1} soil day^{-1})	Experiment duration (years)
Wu et al. (2011)	0	59.04	22
	180	132	
Zhong et al. (2007)	0	122	23
	167	770	
Jin et al. (2014)	0	8	21
	213	16	
Wu et al. (2017)	0	4.96	20
	157	7.36	
Wang et al. (2014)	0	12.72	6
	180	15.84	

2.3.3 Effects of nitrogen fertilization on the SOC and SOC:NO_3^- ratio

The effects of N fertilization on SOC is not clear. Long-term N fertilization experiments have demonstrated both no effect (Neff et al., 2002; Cusack et al., 2011; Dou et al., 2016) and an increase in SOC stocks in soil (Bi et al., 2009; Tian et al., 2015; Wang et al., 2015). But an accelerated decomposition and reduction in labile OC (light fractions) after long-term N fertilization has been consistently documented (Neff et al., 2002; Ghani et al., 2003; Corre et al., 2010; Cusack et al., 2011; Dou et al., 2016). Regardless of the effects of N fertilization on the SOC pool, in the short-term there is a reduction in the SOC:NO_3^- ratio immediately after fertilization due to a significant rise in NO_3^- concentration. Long-term N fertilization can also reduce the SOC:NO_3^- ratio due to the increased soil NO_3^- concentration as a consequence of the increased nitrification rate (Corre et al., 2010; Putz et al., 2018). This effect of short-term and long-term N fertilization on the labile SOC and SOC:NO_3^- ratio can influence several N transformation processes in rice paddies.

2.4 Co-existing microbial nitrogen loss and conservation processes

The unique characteristic of paddy soils, with large amounts of dissolved OC, simultaneous availability of a range of N substrates and coexistence of oxic and anoxic microsites, favours different microbial N transformation pathways (Figure 4) which play a direct role in N loss and conservation in rice paddies. Anaerobic conditions with readily available OC in paddy soils favour denitrification which mediates the loss of bioavailable NO_3^- as inert N_2 gas (Arth and Frenzel, 2000). The recently identified process of anaerobic ammonium oxidation (anammox) in O_2 minimum zones of oceans has also been found to contribute to N_2 production in rice paddies (Nie et al., 2015). However, little is known about its significance. Dissimilatory nitrate reduction to ammonium (DNRA), a largely overlooked N transformation process in agricultural soil ecosystems, has been found to compete for NO_3^- with denitrification and conserve it as NH_4^+ in several forest soils and sediments (Silver et al., 2001; Huygens et al., 2007; Hardison et al., 2015; Yang et al., 2017). The DNRA process may play a significant role in N conservation in paddy soils, but little information is available on this. Paddy soils are also well known to harbour diverse diazotrophic communities capable of reducing inert N_2 to bioavailable NH_4^+ and this is thought to be the most important N conservation mechanism prevailing in the low input paddy soils (Hoque et al., 2001; Bei et al., 2013). All these processes are directly involved in either N loss or conservation in soil ecosystems. Whilst denitrification and N_2 fixation have been well studied in rice paddies, less is known about DNRA and anammox.

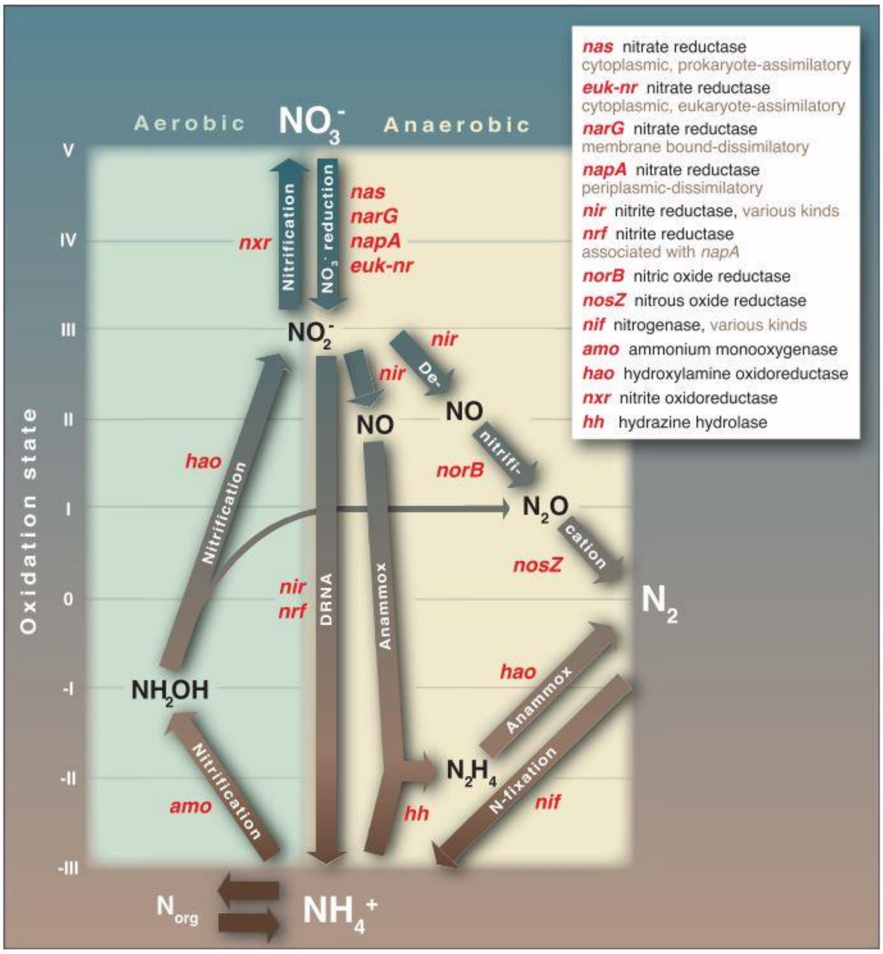

Figure 4. Co-existing microbial N transformation pathway and the relevant microbial genes (Canfield et al., 2010).

2.4.1 Denitrification

Denitrification is the reduction of NO_3^- to N_2 through a sequential process as shown in equation 1 (Zumft, 1997; Lam and Kuypers, 2011). During the process, reduction of NO_3^- to NO_2^- is mediated by *narG* and *napA* genes encoding nitrate reductases. The NO_2^- is then reduced to nitric oxide (NO) by nitrite reductases encoded by *nirS* or *nirK* genes (Zumft, 1997; Dong et al., 2009). Thereafter, the nitric oxide reductase catalyses the reduction of NO to N_2O by combining two molecules of NO as shown in equation 2, which ultimately gets reduced to form diatomic N_2 or escapes to the atmosphere (Zumft, 1997). The *nosZ* gene encodes the nitrous oxide reductase which catalyses the reduction of N_2O to N_2 (Kraft et al., 2011). The bacteria and archaea involved in denitrification are almost exclusively facultative and include over 40 bacterial and archaeal genera (Zumft, 1997; Lam and Kuypers, 2011). *Pseudomonas spp., Paracoccus spp, Escherichia coli,* and *Rhodobacter spp.* are among the important bacteria involved in denitrification (Zumft, 1997).

$$\mathrm{NO_3^-} \rightarrow \mathrm{NO_2^-} \rightarrow \mathrm{NO} \rightarrow \mathrm{N_2O} \rightarrow \mathrm{N_2} \tag{1}$$

$$2\,\mathrm{NO} + 2\,\mathrm{H^+} + 2\,\mathrm{e^-} \rightarrow \mathrm{N_2O} + \mathrm{H_2O} \tag{2}$$

N_2 is the major end product of the denitrification process in rice paddies, whereas N_2O production constitutes only a minor (almost two orders of magnitude lower than the N_2 production) proportion (Arth et al., 1998; Aulakh et al., 2001). N_2O is mainly produced during the drainage of rice paddies (Pandey et al., 2014). Although N_2O production from rice paddies is significant from an environmental viewpoint, it only has a minor contribution towards the N loss (Shang et al., 2011). Emission of N_2O from continuously flooded rice paddies is mostly under the detection limit (Zou et al., 2005; Pandey et al., 2014; Xiong et al., 2015; Adviento-Borbe and Linquist, 2016), but N_2 production is high (Lindau et al., 1990). More than 700 g N ha^{-1} day^{-1} was denitrified to N_2, whereas only 5 g N_2O-N ha^{-1} day^{-1} was produced from rice paddies when applied with 80 kg urea-N ha^{-1} (Mosier et al., 1989; Lindau et al., 1990). Pandey et al. (2014) observed an emission of only 0.31 kg N_2O ha^{-1} during a whole rice growing season from rice paddies fertilised with 100 kg urea-N ha^{-1}. Therefore, N_2 production from paddy soils represents much of the denitrification N loss. However, in situ N_2 measurement poses a significant challenge due to the high background N_2 concentration.

The earth's atmosphere is comprised of ~78% of N_2, and therefore, it is difficult to detect small changes in the N_2 fluxes from soils. N_2 in the atmosphere is composed of $^{14}N^{14}N$, $^{14}N^{15}N$ and $^{15}N^{15}N$, where $^{14}N^{15}N$ and $^{15}N^{15}N$ are 0.7299 and 0.001 % of the total N_2, respectively (Nielsen, 1992). Due to the very low background concentration of ^{15}N-N_2, measurement of any excess ^{15}N-N_2 production after the application of a highly enriched $^{15}NO_3$ in soil under laboratory incubation can be used to determine the denitrification rate (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen et al., 2003). The ratio of different isotopic N_2 molecules can be determined using Gas Chromatograph coupled with Isotope Ratio Mass Spectrometer (GC-IRMS) (Robertson et al., 2016).

Denitrification is the major microbial N transformation pathway mediating N loss from paddy soils (Zhou et al., 2009; Yan et al., 2011). Denitrification accounts for the loss of around 36% of the applied fertilizer-N in rice paddies (Aulakh et al., 2001; Ju et al., 2009). Both short-term and long-term N fertilization in rice paddies increases denitrification N loss (Chen et al., 2012; Nie et al., 2015). Nitrogen fertilization enhances nitrification and the coupled nitrification-denitrification process contributes to a significant N loss from flooded agroecosystems (Arth and Frenzel, 2000; Penton et al., 2013). Anaerobic conditions in the soil and the availability of OC and NO_3^- are the prerequisites for denitrification (Weier et al., 1993; Swerts et al., 1996). Denitrification is inhibited by the presence of O_2 (Aulakh et al., 2001). Readily available OC in the paddy soils can accelerate the development of the anaerobic conditions as O_2 is rapidly consumed during the respiration of OC (Swerts et al., 1996). Furthermore, the OC also works as an electron donor which is required by denitrifies for NO_3^- reduction (Weier et al., 1993). Substantial research has focused on denitrification (Arth et al., 1998; Arth and Frenzel, 2000; Nicolaisen et al., 2004) and related molecular mechanism in rice paddies (Yoshida et al., 2009; Ishii et al., 2011; Yoshida et al., 2012). The rate of denitrification varies within the microsites in rice paddies (Nie et al., 2015). There is a lower denitrification activity in the rhizosphere compared to bulk soil most probably due to the presence of O_2 in the rhizosphere that inhibits denitrification (Nie et al., 2015). Until recently, the denitrification process was thought to be the sole N_2 production process from soils. However, the detection of significant N_2 production by the anammox process from anaerobic sea sediments has challenged this (Thamdrup and Dalsgaard, 2002).

2.4.2 Anaerobic ammonium oxidation

Anammox is the term used for the process of anaerobic ammonium oxidation with NO_3^- /NO_2^- (Thamdrup and Dalsgaard, 2002). The process prevails in anoxic conditions where strictly anaerobic bacteria oxidize NH_4^+, with NO_3^- / NO_2^- as the electron acceptor and N_2 as the final product (Schmid et al., 2005). The process is found to proceed through hydrazine (N_2H_2) and can be illustrated with the following stoichiometry (equation 3-5, equation 6 shows the overall stoichiometry) (Dalsgaard et al., 2003; Risgaard-Petersen et al., 2003; Kartal et al., 2011)

$$\mathrm{NO_2^-} + 2\,\mathrm{H^+} + \mathrm{e^-} \rightarrow \mathrm{NO} + \mathrm{H_2O} \tag{3}$$

$$\mathrm{NO} + \mathrm{NH_4}^+ + 2\,\mathrm{H^+} + 3\mathrm{e^-} \rightarrow \mathrm{N_2H_4} + \mathrm{H_2O} \tag{4}$$

$$\mathrm{N_2H_4} \rightarrow \mathrm{N_2} + 4\,\mathrm{H^+} + 4\mathrm{e^-} \tag{5}$$

$$\mathrm{NH_4}^+ + \mathrm{NO_2^-} \rightarrow \mathrm{N_2} + 2\,\mathrm{H_2O} \tag{6}$$

Anammox yields nearly equal free energy as denitrification (Lam and Kuypers, 2011), and therefore, anammox can be as important as denitrification in anaerobic ecosystems. Unlike denitrification where two NO molecules are reduced to produced N_2, anammox uses one molecule each of NH_4^+ and NO_3^- to produce N_2. This differences in stoichiometry of N_2 production between the pathways can be used to distinguish the two processes. Anammox can be detected by applying $^{15}NH_4^+$ in soils under anoxic conditions where the labelled $^{15}NH_4^+$ reacts with the unlabelled NO_3^- to produce $^{29}N_2$ (Schmid et al., 2005). But for simultaneous measurement of denitrification and anammox, first the soil should be anaerobically pre-incubated to deplete soil NO_3^-/NO_2^- and then $^{15}NO_3^-$ (>98 ^{15}N atom%) tracer can be added to the incubated soil to trace the $^{29}N_2$ and $^{30}N_2$ production (Long et al., 2013). This anaerobic pre-incubation process does not resemble the in-situ conditions in most agricultural ecosystems. However, this method is suitable for paddy soils due to the in-situ anaerobic conditions. After $^{15}NO_3^-$ addition, it combines with NH_4^+ in the soil during the anammox process to produce $^{29}N_2$ (Thamdrup and Dalsgaard, 2002). Denitrification in the same soil will result in the production of $^{30}N_2$ (the $^{14}NO_3^-$ is depleted prior to $^{15}NO_3^-$ addition, so denitrification has to rely only on $^{15}NO_3^-$ as a substrate) (Thamdrup and Dalsgaard, 2002). Anammox is currently believed to be mediated only by the members of order *Brocadiales* related to *Planctomycetes* (Francis et al., 2007; Harhangi et al., 2012). Studies have identified five *Candidatus* genera so far involved in anammox activity in different ecosystems (Harhangi et al., 2012). The *hzs* gene, in anammox bacteria, encodes the subunit of hydrazine synthase, which catalyses the hydrazine (N_2H_4) synthesis from NH_4^+ and NO_2^- and the N_2H_4 is ultimately oxidized to N_2 (Kartal et al., 2011; Wang et al., 2012). Detection of the *hzs* gene, therefore, can be used as a phylogenetic marker for the presence of anammox bacteria (Kartal et al., 2011; Harhangi et al., 2012).

Direct evidence of anammox was first reported by Mulder et al. (1995) in a wastewater treatment plant, and the study of the process came to light after the detection of the process in sea sediments by Thamdrup and Dalsgaard (2002). The importance of the process for agricultural ecosystems was only reported in 2011 (Zhu et al., 2011). Because, both NH_4^+ and NO_3^- (Li et al., 2007), and strong anoxic conditions with very low O_2 concentration (Revsbech et al., 1999) exist in rice paddies, they can be a hotspot for the anammox process. There has been scant research on the anammox process in paddy soils and those studies that have been done have indicated 4-41% of the total N_2 production occurs through this process

(Zhu et al., 2011; Nie et al., 2015; Yang et al., 2015). Rice paddies comprise of complex sets of oxic and anoxic microsites with varying levels of OC (Ge et al., 2012), NH_4^+ concentrations and rates of NO_3^- production (Li et al., 2008), which might lead to differences in anammox activity between the microsites (Nie et al., 2015). However, there is a lack of knowledge on how paddy soils with varying levels of OC and long-term N fertilization regimes influence anammox rate and the related microbial abundance in rice paddies. The investigation of anammox in rice paddies so far has concentrated on whether anammox is a significant process in rice paddies (Zhu et al., 2011; Yang et al., 2015), but no study has investigated the effect of soil OC and long-term N fertilization regimes on the process rate and related microbial abundance.

2.4.3 Dissimilatory nitrate reduction to ammonium

Dissimilatory nitrate reduction to ammonium (DNRA), also known as respiratory nitrite-ammonification, is the process through which highly mobile NO_3^-/NO_2^- is reduced to NH_4^+ (Lam and Kuypers, 2011). DNRA competes with denitrification and anammox for loss-prone NO_3^- and reduces it to NH_4^+, utilizing OC as an electron source (Kraft et al., 2014). Because there is an electrostatic interaction between NH_4^+ and the soil matrix, DNRA can be an important process for retaining soil N (Huygens et al., 2008; Welsh et al., 2014). DNRA can be explained by the following stoichiometry (equation 7) (Lam and Kuypers, 2011; Fernandes et al., 2012):

$$\frac{1}{2}\,\mathrm{NO_3}^- + \frac{1}{2}\,\mathrm{C_2H_3O_2}^- + \frac{1}{2}\,\mathrm{H}^+ + \frac{1}{2}\,\mathrm{H_2O} \rightarrow \frac{1}{2}\,\mathrm{NH_4}^+ + \mathrm{HCO_3}^- \qquad (7)$$

Many common soil bacteria, including *Anaeromyxobacter dehalogenans* and *Desulfitobacterium spp.* have been found to be capable of reducing NO_3^- to NH_4^+ (Welsh et al., 2014). Reduction of NO_3^- to NO_2^- is catalysed by NAR and NAP enzymes encoded by *narG* and *napA* genes (Dong et al., 2009). These genes are related to both DNRA and denitrifying bacteria. But some bacteria unrelated to DNRA or denitrification can also possess these genes and therefore the reduction of NO_3^- to NO_2^- may also be an independent process and not related to any of the downstream processes (DNRA or denitrification) (Welsh et al., 2014). The NO_2^- reduction is catalysed by the cytochrome *c* nitrite reductase encoded by the *nrfA* genes in DNRA bacteria to produce NH_4^+ (Simon, 2002; Dong et al., 2009; Welsh et al., 2014). Therefore, cytochrome *c* nitrite reductase is the analogous target to study the microorganisms associated with DNRA (Welsh et al., 2014). The *nrfA* gene abundance has been extensively studied in aquatic systems (estuaries and ocean sediments) (Dong et al., 2009; Song et al., 2014; Welsh et al., 2014) but there is a lack of study looking at the *nrfA* gene abundance in agricultural systems, including rice paddies.

Denitrification is less efficient mechanism to conserve the energy produced during enzymatic reactions compared to DNRA (Table 3) and the bacterial growth yield per molecule of NO_3^- reduced in ammonifiers is higher than that in denitrifiers (Strohm et al., 2007; Lam and Kuypers, 2011). Therefore, DNRA can have more significance than denitrification in NO_3^- limiting conditions (Lam and Kuypers, 2011).

Table 3. Standard Gibbs free energy (ΔG°) calculated for denitrification and DNRA pathways using acetate as the electron donor (Lam and Kuypers, 2011).

Pathway	Stoichiometry	ΔG° (kJ per reaction)
Denitrification	$\frac{4}{5}\,\mathrm{NO_3}^- + \frac{1}{2}\,\mathrm{C_2H_3O_2}^- + \frac{3}{10}\,\mathrm{H}^+ \rightarrow \frac{2}{5}\,\mathrm{N_2} + \mathrm{HCO_3}^- + \frac{2}{5}\,\mathrm{H_2O}$	-398
DNRA	$\frac{1}{2}\,\mathrm{NO_3}^- + \frac{1}{2}\,\mathrm{C_2H_3O_2}^- + \frac{1}{2}\,\mathrm{H}^+ + \frac{1}{2}\,\mathrm{H_2O} \rightarrow \frac{1}{2}\,\mathrm{NH_4}^+ + \mathrm{HCO_3}^-$	-257

A significant amount of NO_3^- conversion to NH_4^+ through DNRA has been reported in a wide range of ecosystems, including estuary sediments (Dong et al., 2011), forest soils (Silver et al., 2001; Huygens et al., 2008) and agricultural soils (Zhang et al., 2015; Putz et al., 2018). Unlike denitrification, DNRA has shown less sensitivity to O_2 concentration

(Roberts et al., 2014; Yang et al., 2017). The soil OC and NO_3^- concentration is thought to be the important factor determining the NO_3^- partitioning between DNRA and denitrification (Tiedje, 1988). The OC concentration and NO_3^- production varies between the rhizosphere and bulk soil in rice paddies (Wu et al., 2017; Zhang et al., 2017), and therefore, the rate of DNRA can vary between these environments. But Fe (II) and sulfide, which are mainly concentrated in bulk soil (Begg et al., 1994; Lin et al., 2010), can also act as an electron donor for the DNRA process and this can affect the DNRA rate in the rhizosphere and bulk soil. However, there is a lack of studies looking at DNRA rates in the rhizosphere and bulk soils in rice paddies.

Contradictory results have been reported on the effects of OC and NO_3^- on the NO_3^- partitioning between DNRA and denitrification (Yang et al., 2017; Friedl et al., 2018; Putz et al., 2018). Studies have suggested that it is the OC:NO_3^- ratio that affects the NO_3^- partitioning rather than the OC or NO_3^- concentration alone (Kraft et al., 2014; Putz et al., 2018). Denitrification requires six electrons as opposed to eight electrons for DNRA for a complete reduction of a NO_3^- molecule (Kraft et al., 2014). Therefore, denitrification should have a competitive advantage over DNRA in OC limited condition (Van den Berg et al., 2016). On the other hand, bacteria achieve higher growth yields during DNRA compared to denitrification, which implies that DNRA dominates over denitrification when NO_3^- is limiting (Van Den Berg et al., 2015). So in theory, a higher OC:NO_3^- ratio should support more NO_3^- partitioning to DNRA than to denitrification. Recent chemostat experiments using pure bacterial culture have supported this theory (Kraft et al., 2014; Van Den Berg et al., 2015; Yoon et al., 2015). Because un-fertilized rice paddies are low in NO_3^- concentrations, DNRA can have a competitive advantage over denitrification. However, little is known about this in rice paddies and contradictory reports of the relationship between the OC:NO_3^- ratio and DNRA have been documented in other soil ecosystems (Table 4).

Table 4. Summary of the relationships between the soil C:NO_3^- ratio and DNRA and denitrification from literature

Soil sampling sites	[a]C:NO_3^- vs DNRA (R^2)	C:NO_3^- vs Denitrification (R^2)	Reference
Subtropical pasture	[b]none	none	Friedl et al. (2018)
Temperate arable soils	0.95	(-)0.85	Putz et al. (2018)
Forest and grassland	none	[c]NA	Yang et al. (2017)
Pine plantation	(-)0.57	NA	Minick et al. (2016)
Temperate arable soils	0.42	NA	Schmidt et al. (2011)
Tidal sediment	0.84	(-)0.88	Porubsky et al. (2009)
Lowland forest	none	NA	Sotta et al. (2008)
Plantation and forest	0.44	NA	Silver et al. (2005)

[a]C:NO_3^-: C in the C:NO_3^- ratio was either total C or SOC or labile C or dissolved organic C depending on the cited literature.
[b]none: no significant relationship.
[c]NA: Denitrification rate was not determined in the study
(-): Refers to a negative relationship

Long-term N fertilization has shown increased soil NO_3^- concentration in paddy soil (Wang et al., 2016), a reduction in the labile OC fraction (Cusack et al., 2011; Dou et al., 2016), and ultimately a reduction in the soil OC:NO_3^- ratio (Chen et al., 2010; Sun et al., 2015). Such soil conditions may favor denitrification over DNRA. Long-term high N fertilization has been found to increase denitrification rates and the abundance of *narG, nirK* and *nosZ* genes in rice paddies (Chen et al., 2012). This implies that the long-term high N fertilization may promote NO_3^- partitioning to denitrification. However, little information is available on this. Particularly, there are no studies looking into the effects of long-term N fertilization on DNRA activity and the related *nrfA* gene abundance, while the results from short-term N fertilization experiments on DNRA activity in various soil ecosystems are contradictory (Schmidt et al., 2011; Baldos et al., 2015; Minick et al., 2016).

Despite the importance of the DNRA in soil N retention, limited research has been conducted on the process in paddy soils (Yin et al., 2002; Zhang et al., 2015). Using a N transformation model, Zhang et al. (2015) estimated that the DNRA rate can be up to 1.09 mg N kg^{-1} soil day^{-1} in some paddy soils, which indicates DNRA can be an important mechanism for soil N retention in rice paddies. There is a lack of knowledge on the effect of different levels of soil OC, N input and SOC:NO_3^- ratio and the rhizosphere and bulk soils, on the activity and abundance of bacteria involved in the DNRA process in rice paddies. Therefore, there is a need to further investigate this to understand the soil environmental factors affecting the NO_3^- partitioning between DNRA and denitrification. The rate of DNRA can be measured by applying $^{15}NO_3^-$ to the anaerobically incubated paddy soil and quantifying $^{15}NH_4^+$ formation (Silver et al., 2001; Dong et al., 2009). Unlabelled NH_4^+ can be applied together with $^{15}NO_3^-$ to inhibit $^{15}NO_3^-$ immobilization by soil microbes (Bengtson and Bengtsson, 2005) so that the $^{15}NH_4^+$ formation can be attributed to DNRA.

2.4.4 Diazotrophic N_2 fixation

Diazotrophic N_2 fixation is known to contribute a significant amount of N to rice paddies (Roger and Ladha, 1992; Choudhury and Kennedy, 2004; Bei et al., 2013). Diazotrophs constitute a wide range of organisms capable of fixing atmospheric N_2 (Unkovich et al., 2008). Diazotrophic diversity has been widely studied in rice paddies and the molecular mechanism has been well established (Ueda et al., 1995; Xie et al., 2006; Mårtensson et al., 2009). The *nifH* gene in the N_2 fixing bacteria encodes the protein subunit of nitrogenase, which catalyse the N_2 fixation. So, the *nifH* gene is used as a biomarker to study the presence and diversity of N_2 fixers (Ueda et al., 1995). The simultaneous presence of both oxic and anoxic microsites, the different light status on the surface layer of flooded soil compared to soil underneath, and the difference in substrate availability in different microsites in paddy soils favours diverse free-living aerobic, facultative and anaerobic N_2 fixing bacteria (Roger and Ladha, 1992; Ladha and Reddy, 2003).

There is a wide variation in the estimates of N_2 fixation in rice paddies which depends on the soil conditions and fertilization practice (Roger and Ladha, 1992; Choudhury and Kennedy, 2004). The N_2 gas is formed with a strong triple bond between two N atoms and it requires considerable energy to be reduced to NH_4^+ (Santruckova et al., 2010). Labile OC provides energy to heterotrophic diazotrophs and enhances N_2 fixation (Choudhury and Kennedy 2004, Kanungo et al 1997). Presence of rice plants can have a stimulus effect on N_2 fixation due to the secretion of labile OC through root exudates (Bürgmann et al., 2005; Knauth et al., 2005). Application of low level of fertilizer-N (20 kg N ha^{-1}) in paddy soils was found to enhance N_2 fixation but a higher dose of fertilizer-N (60 kg N ha^{-1}) significantly reduced it (Roger and Ladha, 1992; Van Nieuwenhove et al., 2001). High level of mineral N in soil can inhibit the nitrogenase enzyme (Mills et al., 2004). Using $^{15}N_2$ labelling technique, Bei et al. (2013) reported around 45 kg N ha^{-1} of N_2 fixation in rice paddies without N application.

Cyanobacteria, also known as blue-green algae, are aquatic photosynthetic bacteria growing in colonies and are capable of fixing N_2 in rice paddies (Roger and Ladha, 1992; Valiente et al., 1997). There is a wide variation in the estimates of N contribution by *Cyanobacteria* to paddy soils, ranging from 8 to 50 kg N ha^{-1}. A reduction of more than 50% in N_2 fixation by *Cyanobacteria* has been reported when the paddy soils were broadcast with urea (Roger and Ladha, 1992). N_2 fixing heterotrophic bacteria, *Azotobacter and Azospirillum,* confined to oxic zones in rice paddies, especially in the rhizosphere of rice (Choudhury and Kennedy, 2004; Kennedy et al., 2004), are estimated to fix more than 15 kg N ha^{-1}(Ladha and Reddy, 2000; Choudhury and Kennedy, 2004). These bacteria are sometimes associated with rice roots but are not in a symbiotic relationship (Choudhury and

Kennedy, 2004). *Clostridium spp* is an anaerobic N_2 fixing heterotrophic bacterium and is active in anoxic microsites in rice paddies and is considered to be a major N_2 fixing free-living bacterium in paddy soils (Roger and Ladha, 1992; Kawasaki et al., 1998; Sabra et al., 2000; Ladha and Reddy, 2003; Choudhury and Kennedy, 2004).

Heterotrophic microorganisms need labile OC as a source of energy to fix N_2 (Bürgmann et al., 2005). Aerobic heterotrophs, such as *Azotobacter*, can fix a higher amount of N_2 in the rhizosphere where there is higher labile OC than in the bulk soil (Choudhury and Kennedy, 2004). Anaerobic heterotrophs such as *Clostridium* are confined to anaerobic sites and their capacity to fix N_2 also depends on the supply of labile OC (Choudhury and Kennedy, 2004). *Clostridium* is estimated to fix 5-10 mg N g^{-1} C consumed, so the supply of an external source of C can enhance N_2 fixation in paddy soils (Choudhury and Kennedy, 2004; Kennedy et al., 2004). There are some other associative and non-associative N_2 fixers in rice paddies, such as *Herbaspirillum and Burkholderia*, but it is almost impossible to estimate N_2 fixation by individual bacteria in paddy soils (Roger and Ladha, 1992; Choudhury and Kennedy, 2004; Unkovich et al., 2008). Therefore, estimation of total N_2 fixation by aerobic, anaerobic and facultative diazotrophs is the common research approach. Among several techniques used to estimate N_2 fixation in soil ecosystems, $^{15}N_2$ uptake and the acetylene (C_2H_2) reduction assay (ARA) techniques (discussed below) are the most widely used techniques to estimate N_2 fixation in paddy soils (Unkovich and Baldock, 2008).

2.5 Calculation of DNRA, denitrification, anammox and N_2 fixation rates

Application of the ^{15}N isotope pairing technique to estimate the rates of DNRA, denitrification and anammox has been widely used in recent research (Thamdrup and Dalsgaard, 2002; Dong et al., 2009; Zhu et al., 2011). In this technique, soil is anaerobically incubated in airtight vials to deplete indigenous NO_3^- in the soil. Thereafter, $^{15}NO_3$ (>98 ^{15}N

atom%) is applied, with or without unlabelled NH_4^+ and ^{15}N-N_2 ($^{14}N^{15}N$ and $^{15}N^{15}N$) production with time is determined using GC-IRMS. The rates are then calculated using the following equations 8-17 (Thamdrup and Dalsgaard, 2002; Dong et al., 2009):

$$A_{28} = A_{total} \times (1 - F_N) \qquad (8)$$

$$A_{29} = A_{total} \times F_N \qquad (9)$$

where, A_{28} and A_{29} are $^{28}N_2$ and $^{29}N_2$ produced by anammox, $A_{total} = A_{28} + A_{29}$, F_N is the proportion of ^{15}N in the NO_3^- pool (typically > 98%) after the addition of $^{15}NO_3^-$ in anaerobically incubated vials. Similarly, the denitrification process in the same vials can produce $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ as follow:

$$D_{28} = D_{total} \times (1 - F_N)^2 \qquad (10)$$

$$D_{29} = D_{total} \times 2 \times (1 - F_N) \times F_N \qquad (11)$$

$$D_{30} = D_{total} \times F_N^2 \qquad (12)$$

$$D_{total} = \frac{P_{30}}{F_N^2} \qquad (13)$$

where, D represents N_2 production by denitrification, P_{30} represents the total measured production of $^{30}N_2$. As all the $^{30}N_2$ produced is assumed to be due to denitrification, $D_{30} = P_{30}$. Thus, combining the previous equations gives:

$$D_{29} = P_{30} \times 2 \times (1 - F_N) \times F_N^{-1} \qquad (14)$$

$$A_{29} = P_{29} - D_{29} = P_{29} - P_{30} \times 2 \times (1 - F_N) \times F_N^{-1} \qquad (15)$$

$$A_{28} = A_{29} \times F_N^{-1} \times (1 - F_N) \qquad (16)$$

$$A_{total} = F_N^{-1} \times [P_{29} + 2 \times (1 - F_N^{-1}) \times P_{30}] \qquad (17)$$

Possible end-products after the transformation of applied $^{15}NO_3^-$ in anaerobic soils are presented in Figure 5.

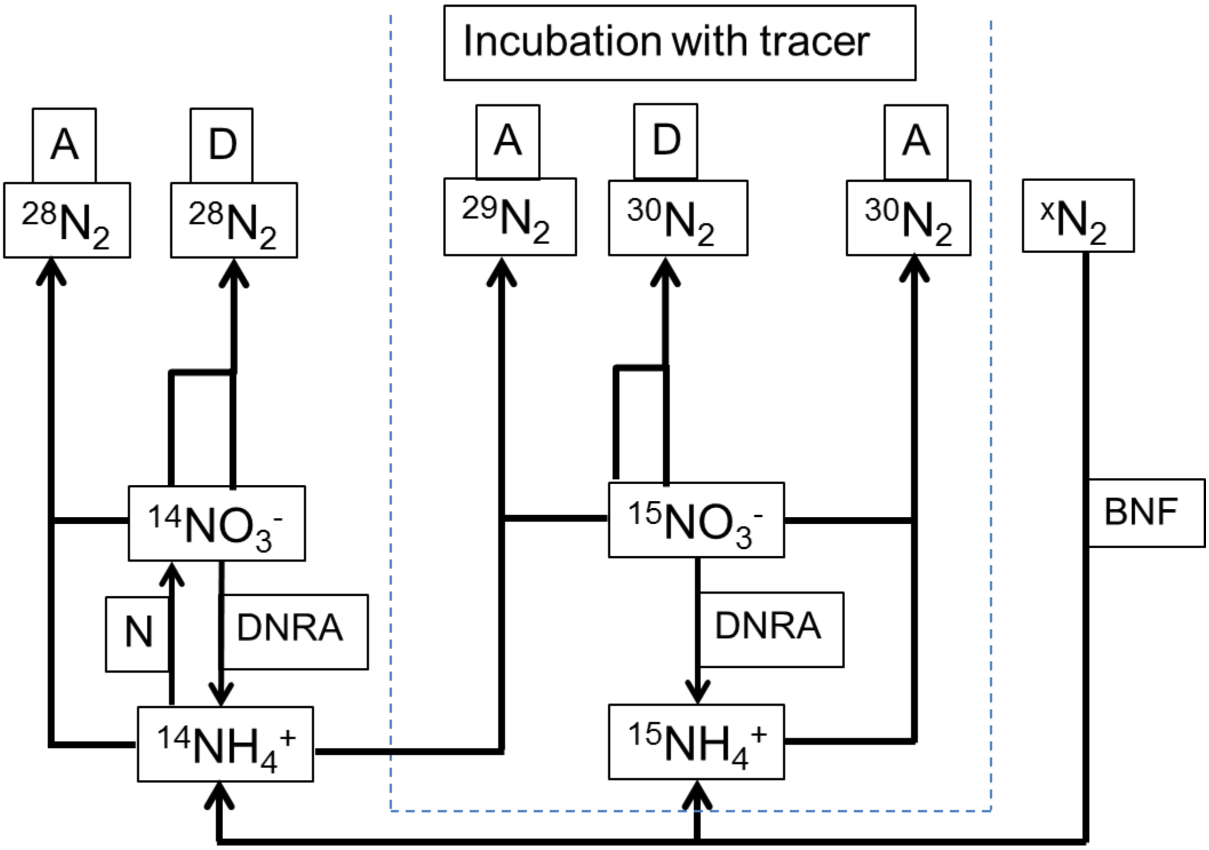

Figure 5. Illustration of biological N_2 fixation (BNF), dissimilatory NO_3^- reduction to NH_4^+ (DNRA), denitrification (D), anammox (A) and nitrification (N) processes. The section within the dotted line represents the anaerobic transformation of an added $^{15}NO_3^-$ tracer in soils after depleting indigenous soil $^{14}NO_3^-$.

Previous studies conducted to determine denitrification and anammox in rice paddies (Zhu et al., 2011; Nie et al., 2015; Yang et al., 2015) used the calculation procedure described above and did not take into account of the possible influence of DNRA in the estimation of the denitrification or anammox (Figure 5). The calculation procedure of denitrification and anammox (equation 8-17) using the conventional isotope pairing technique considers all the $^{30}N_2$ produced to be due to denitrification. But the existence of DNRA can produce $^{15}NH_4^+$ in the same incubation vial which can then combine with the added $^{15}NO_3^-$ to produce $^{30}N_2$ during the anammox process (Figure 5). This $^{30}N_2$ can be disguised as being produced by denitrification and the rates of anammox can be underestimated, while denitrification rates can be overestimated. Further, the $^{15}NH_4^+$ (produced by DNRA) utilized by the anammox process can result in underestimation of the DNRA rate. Therefore, this methodological error should be addressed to achieve a precise estimation of each of the processes. The effect of DNRA (including N_2 fixation) can be addressed using the equations 18-21 (Song et al., 2013).

$$A_{30} = A_{total} \times F_N \times F_A \tag{18}$$

where, A_{30} is the production of $^{30}N_2$ and F_A is the fraction of $^{15}NH_4^+$ in the NH_4^+ pool and can be measured by the determination of $^{15}NH_4^+$ concentration and total NH_4^+ at each time point. Combining the previous equations A_{30} and denitrification can be calculated as:

$$A_{30} = \frac{F_A \times [P_{29} \times F_N - 2 \times (1 - F_N) \times P_{30}]}{F_N \times (1 - F_A) - F_A \times (1 - F_N)} \tag{19}$$

$$D_{total} = \frac{[P_{30} - A_{30}]}{{F_N}^2} \tag{20}$$

Following this DNRA will be further calculated as:

$$DNRA = \frac{P_{15_{NH_4^+}} + A_{30}}{F_N} \tag{21}$$

where, $P_{15_{NH_4^+}}$ is the $^{15}NH_4^+$ production with time.

If the soil has a significant potential for N_2 fixation then the N_2 produced by either denitrification or anammox can be fixed back to NH_4^+. This will again affect the F_A. But this issue is already addressed when we determine the F_A based on the concentration of $^{15}NH_4^+$ in the NH_4^+ pool. Therefore, simultaneous determination of the processes will address the methodological error during the calculation of the process rates.

Among the various techniques used to estimate N_2 fixation, $^{15}N_2$ uptake and the acetylene (C_2H_2) reduction assay (ARA), have been most commonly used in rice paddies, but wide discrepancies exist between the techniques (Roger and Ladha, 1992). The $^{15}N_2$ uptake technique can directly quantify N_2 fixation in soils but there are several problems associated with this technique (Unkovich and Baldock, 2008). Firstly, $^{15}N_2$ is expensive and secondly, it requires careful enclosure in the experimental setup to avoid dilution with the external high background atmospheric N_2 (Chalk et al., 2017). On the other hand, the ARA technique is widely used to estimate potential N_2 fixation in rice paddies (Roger and Ladha, 1992; Unkovich et al., 2008). However, the ARA technique does not directly quantify the N_2 fixation as done by the $^{15}N_2$ uptake technique. But the ARA technique, where C_2H_4 production after the reduction of C_2H_2 is taken as a proxy to N_2 fixation, is sensitive to nitrogenase activity and easy and cheap to use (Mulholland et al., 2006; Unkovich et al., 2008).

Apart from being cheap and easy to handle, there is a major issue associated with the ARA which is the variation in the conversion factor of C_2H_4 produced to N_2 fixed (Seitzinger and Garber, 1987). The nitrogenase enzyme in N_2 fixers catalyses the conversion of N_2 to NH_3 as in the following reaction (equation 22) (Unkovich et al., 2008):

$$\mathrm{N \equiv N + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2} \tag{22}$$

The nitrogenase enzyme can also convert C_2H_2 gas to C_2H_4 gas (equation 23) (Unkovich et al., 2008) which can be used to estimate potential N_2 fixation in soils.

$$\mathrm{HC \equiv CH + 2H^+ + 2e^- \rightarrow H_2C = CH_2} \tag{23}$$

While comparing the two reactions above (equation 22 and 23), each mole of C_2H_2 reduced consumes two electrons but eight electrons are used for N_2 reduction (six for N_2 reduction and two additional electrons for the reduction of the protons) (Unkovich et al., 2008). Thus, one mole of N_2 reduced by nitrogenase, theoretically, would be equal to four moles of C_2H_2 reduced and therefore a conversion ratio of 1:4 can be used while using C_2H_2 reduction to estimate N_2 fixation (Unkovich et al., 2008). But during the reduction of a mole of N_2, two electrons are used in the reduction of two protons (equation 22). Therefore, a conversion ratio of 1:3 is also widely accepted by theoretical and empirical evidence and commonly used as a conversion factor (Unkovich and Baldock, 2008; Keuter et al., 2014). But the actual conversion factor has been found to vary from 0.6 to 7.9 depending on the soil ecosystems (An et al., 2001; Unkovich et al., 2008; Barron et al., 2009; Keuter et al., 2014).

This issue can be dealt with by calibrating the conversion factor against $^{15}N_2$ fixation for the ecosystem where the ARA technique is to be used (Anderson et al., 2004; Keuter et al., 2014). For calibration, only two timepoints (0 h and X h) sampling for $^{15}N_2$ uptake can be used so that only a small volume of the ^{15}N enriched N_2 gas is required (Anderson et al., 2004; Keuter et al., 2014). Thus, the $^{15}N_2$ uptake by soils can be compared with parallel incubation of the soils with C_2H_2 and thereafter a conversion factor can be estimated (Anderson et al., 2004; Barron et al., 2009; Keuter et al., 2014). The C_2H_2 reduction experiment can be conducted with as many timepoints as practical and calibrated with the conversion factor obtained from the parallel incubation experiment (Barron et al., 2009), thus allowing soil specific C_2H_2 reduction to N_2 fixation conversion factors.

2.6 Key knowledge gaps

The N transformation pathways discussed above co-exist in rice paddies and contribute to the loss or retention of N in paddy soils. These processes are interlinked by their substrates use and the enzymes involved (Canfield et al., 2010). Therefore, estimating these pathways in isolation can over or underestimate the N turnover which can lead to a misinterpretation of the actual rates. However, there are no studies investigating all these processes together in agricultural ecosystems, including rice paddies. Apart from this, the following research problems were identified:

1) There are no studies that have investigated how short-term and long-term N fertilization regimes affect the balance between microbial N conservation (N_2 fixation and DNRA) and loss (denitrification and anammox) in rice paddies.

2) The effect of the rhizosphere and bulk paddy soils on DNRA and the associated (*nrf)* gene abundances has never been studied.
3) The effect of soil OC and NO_3^- concentration on the NO_3^- partitioning between the DNRA and denitrification in rice paddies is unknown.

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CHAPTER 3: Nitrogen Addition Decreases Dissimilatory Nitrate Reduction to Ammonium in Rice Paddies

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Nitrogen Addition Decreases Dissimilatory Nitrate Reduction to Ammonium in Rice Paddies

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ABSTRACT Dissimilatory nitrate reduction to ammonium (DNRA), denitrification, anaerobic ammonium oxidation (anammox), and biological N_2 fixation (BNF) can influence the nitrogen (N) use efficiency of rice production. While the effect of N application on BNF is known, little is known about its effect on NO_3^- partitioning between DNRA, denitrification, and anammox. Here, we investigated the effect of N application on DNRA, denitrification, anammox, and BNF and on the abundance of relevant genes in three paddy soils in Australia. Rice was grown in a glasshouse with N fertilizer (150 kg N ha^{-1}) and without N fertilizer for 75 days, and the rhizosphere and bulk soils were collected separately for laboratory incubation and quantitative PCR analysis. Nitrogen application reduced DNRA rates by >16% in all the soils regardless of the rhizospheric zone, but it did not affect the *nrfA* gene abundance. Without N, the amount and proportion of NO_3^- reduced by DNRA (0.42 to 0.52 $\mu g\ g^{-1}$ soil day^{-1} and 45 to 55%, respectively) were similar to or higher than the amount and proportion reduced by denitrification. However, with N the amount of NO_3^- reduced by DNRA (0.32 to 0.40 $\mu g\ g^{-1}$ soil day^{-1}) was 40 to 50% lower than the amount of NO_3^- reduced by denitrification. Denitrification loss increased by >20% with N addition and was affected by the rhizospheric zones. Nitrogen loss was minimal through anammox, while BNF added 0.02 to 0.25 μg N g^{-1} soil day^{-1}. We found that DNRA plays a significant positive role in paddy soil N retention, as it accounts for up to 55% of the total NO_3^- reduction, but this is reduced by N application.

IMPORTANCE This study provides evidence that nitrogen addition reduces nitrogen retention through DNRA and increases nitrogen loss via denitrification in a paddy soil ecosystem. DNRA is one of the major NO_3^- reduction processes, and it can outcompete denitrification in NO_3^- consumption when rice paddies are low in nitrogen. A significant level of DNRA activity in paddy soils indicates that DNRA plays an important role in retaining nitrogen by reducing NO_3^- availability for denitrification and leaching. Our study shows that by reducing N addition to rice paddies, there is a positive effect from reduced nitrogen loss but, more importantly, from the conversion of NO_3^- to NH_4^+, which is the favored form of mineral nitrogen for plant uptake.

KEYWORDS nitrogen, dissimilatory nitrate reduction to ammonium, denitrification, rice paddies, *nrfA*

Rice paddies are one of the biggest nitrogen (N) fertilizer sinks (1); however, the fertilizer N use efficiency of rice production is generally below 40% (2). Extended periods of flooding in rice paddies create an anoxic environment, which induces microbial reduction of soil available nitrate (NO_3^-) (3). Therefore, NO_3^- availability is generally low in rice paddies (4). However, localized oxic conditions in the rice rhizosphere, due to the leakage of O_2 from the roots, enables nitrification and, thus, a

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continuous supply of NO_3^- (5, 6). The NO_3^- reduction processes, such as denitrification and anaerobic ammonium oxidation (anammox), can cause a significant amount of N loss from rice paddies (7, 8). On the other hand, dissimilatory NO_3^- reduction to ammonium (DNRA), which transforms loss-prone NO_3^- to soil-retainable NH_4^+, may contribute to N retention in paddy soils (9).

Denitrification has received much attention during the study of NO_3^- reduction and N loss in rice paddy ecosystems, but little research has been conducted on the DNRA process, which has been found to be an important NO_3^- reduction and N-conserving mechanism in forest and grassland ecosystems (10, 11). During the DNRA process, the nitrate reductase Nrf, encoded by the *nrfA* gene, transforms NO_3^- to NH_4^+ (12), thus reducing the risk of N loss from leaching and denitrification (10). Several recent studies have reported that DNRA can reduce up to 92% of the available NO_3^- in different ecosystems, including from anaerobic sediments to aerobic forest and grassland ecosystems, which suggested that DNRA is not confined to anaerobic ecosystems but is ubiquitous (11, 13, 14). This indicates that DNRA is an important NO_3^--consuming process in various soil ecosystems and can compete with denitrification for NO_3^- and, hence, reduce N loss (13, 15), but little is known about its contribution to NO_3^- reduction in paddy soils.

Compared to denitrification, DNRA yields higher energy per molecule of NO_3^- reduced, and therefore, the theoretical assumption is that the DNRA process can have a competitive advantage over denitrification in soils with low NO_3^- (16, 17). Rice paddies are low in NO_3^- because the anaerobic conditions in the soils due to flooding greatly limit nitrification activity (4). The NO_3^- availability is further restrained by the utilization of NO_3^- by the rice plant (5). Under such conditions, DNRA may have a competitive advantage over denitrification, with more NO_3^- being reduced to NH_4^+ than to N_2. However, the reported effects of soil NO_3^- on DNRA are contradictory. Some studies have reported a positive relationship between NO_3^- and DNRA in soils (11, 15), whereas others have reported a negative relationship between these parameters (13, 18).

Rice paddies commonly receive more than 100 kg N ha^{-1} per crop in major rice-producing countries (19–21). Nitrogen application has been found to reduce biological N_2 fixation (BNF) and increase N loss through denitrification (22). Nitrogen addition can change the dynamics of soil NO_3^- availability because of the increase in nitrification activity (6). This increase in nitrification activity and NO_3^- availability can have an effect on the partitioning of the NO_3^- between the DNRA and denitrification processes (23, 24). Addition of N also influences the structural fraction of soil organic carbon (OC), which can have a direct influence on DNRA activity. Soil labile OC has been considered an important influencing factor for DNRA activity, and the addition of N in soil can reduce soil labile OC (25, 26), which ultimately can reduce DNRA (26). The limited literature available on the effect of N on DNRA in forest soils and sediments is contradictory. In forest soils, Silver et al. (16) and Gao et al. (27) reported no effect of N addition on DNRA activity, whereas Minick et al. (15) reported a positive effect. However, Baldos et al. (26) and Schmidt et al. (18) showed a negative effect of N addition on DNRA. Even though soil pH has been found to influence DNRA (18, 26), the contradictory results of NO_3^- concentration and N input reported by the above-mentioned studies are not due to the soil pH differences (11, 15, 18, 26). There are no studies looking into how N addition can influence DNRA rates and *nrfA* gene abundance and the partitioning of NO_3^- between DNRA and denitrification in paddy soils. A better understanding of how N addition can affect the partitioning of NO_3^- between DNRA and denitrification in rice paddies is of particular significance, as this affects the N use efficiency in rice production.

Therefore, we conducted a glasshouse pot experiment followed by laboratory incubation experiments with the objectives to (i) investigate the significance of DNRA in rice paddies compared to other N transformation processes and (ii) examine the effect of N addition on DNRA rates and *nrfA* gene abundance in rice paddies. The following hypotheses were tested: (i) N addition to rice paddies reduces DNRA rates, (ii)

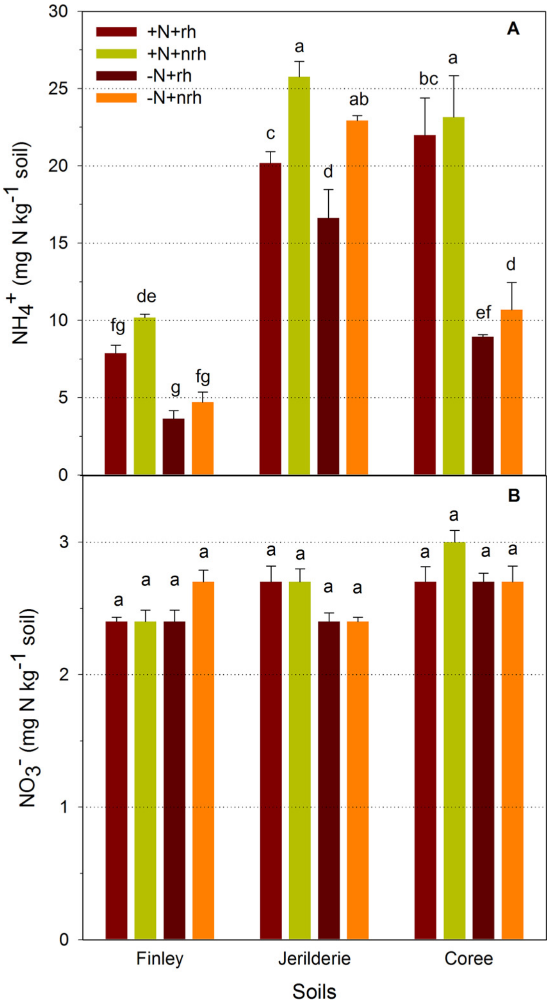

FIG 1 NH_4^+ (A) and NO_3^- (B) concentrations in rhizosphere soil (rh) and bulk soil (nrh) from the three sites (Finley, Jerilderie, and Coree) with (+N) and without (−N) N input. The results for bars with different letters on top are significantly different ($P < 0.05$). Error bars represent ±1 standard error.

N addition influences the partitioning of NO_3^- between DNRA and denitrification, and (iii) the *nrfA* gene abundance is influenced by N addition.

RESULTS

Mineral nitrogen in soils collected from pots. Soil samples collected from the glasshouse pot experiment 75 days after sowing (DAS) of the rice were analyzed for the concentrations of NH_4^+ and NO_3^- (Fig. 1). As expected, the treatments with N input (+N treatment) had a higher NH_4^+-N concentration than the treatments without N input (−N treatments) (Fig. 1A). There was no significant difference in the NO_3^- concentration ($P > 0.05$) between the N treatments (Fig. 1B). The concentration of NO_3^- was 2 to 5 times lower than the NH_4^+ concentration. The concentration of NH_4^+-N in the bulk soil was significantly higher ($P < 0.05$) than that in the rhizosphere soil in all the treatments in all three soils except the −N treatment in the soil from the rice paddy in Finley, Australia (Fig. 1A). However, there was no concentration gradient of NO_3^- between the rhizospheric zones (Fig. 1B).

DNRA rates and partitioning of NO_3^- between DNRA and denitrification. Nitrogen addition had a significant negative effect ($P < 0.05$) on the DNRA rate (Table 1 and Fig. 2A). The DNRA rate was between 0.42 and 0.52 μg N g^{-1} soil day^{-1} in the −N treatments, whereas it was between 0.32 and 0.40 μg N g^{-1} soil day^{-1} in the +N treatments. There was no significant effect of the rhizospheric zone ($P > 0.05$) on the

TABLE 1 Results from the generalized linear model ANOVA

	P value[a]							
Variable	**DNRA**	**Denitrification**	**BNF**	**Anammox**	***nrfA***	***nosZ***	***nifH***	***hzsB***
Site	0.003	0	0	NA	0.036	0.022	0	0.001
Nitrogen	0	0	0.012	0.026	0.286	0	0.343	0.484
Root	0.064	0	0.143	0.007	0.061	0	0.034	0.112
Site × nitrogen	0.515	0.039	0	NA	0.054	0.052	0.1	0.047
Site × root	0.416	0.225	0.082	NA	0.034	0.012	0.667	0
Nitrogen × root	0.609	0.576	0.884	0.069	0.997	0.69	0.86	0.181
Site × nitrogen × root	0.453	0.412	0.293	NA	0.22	0.267	0.696	0.064

[a] The confidence level was set at 95%. NA, not applicable.

DNRA rate (Table 1). The DNRA rates did not vary significantly ($P > 0.05$) between the paddy soils from Jerilderie and Coree regardless of the N treatment, but it was mostly significantly lower ($P < 0.05$) in the Finley soil than in the other two soils (Fig. 2A). Reduction of NO_3^- by DNRA in the rice paddies without N addition was similar to or

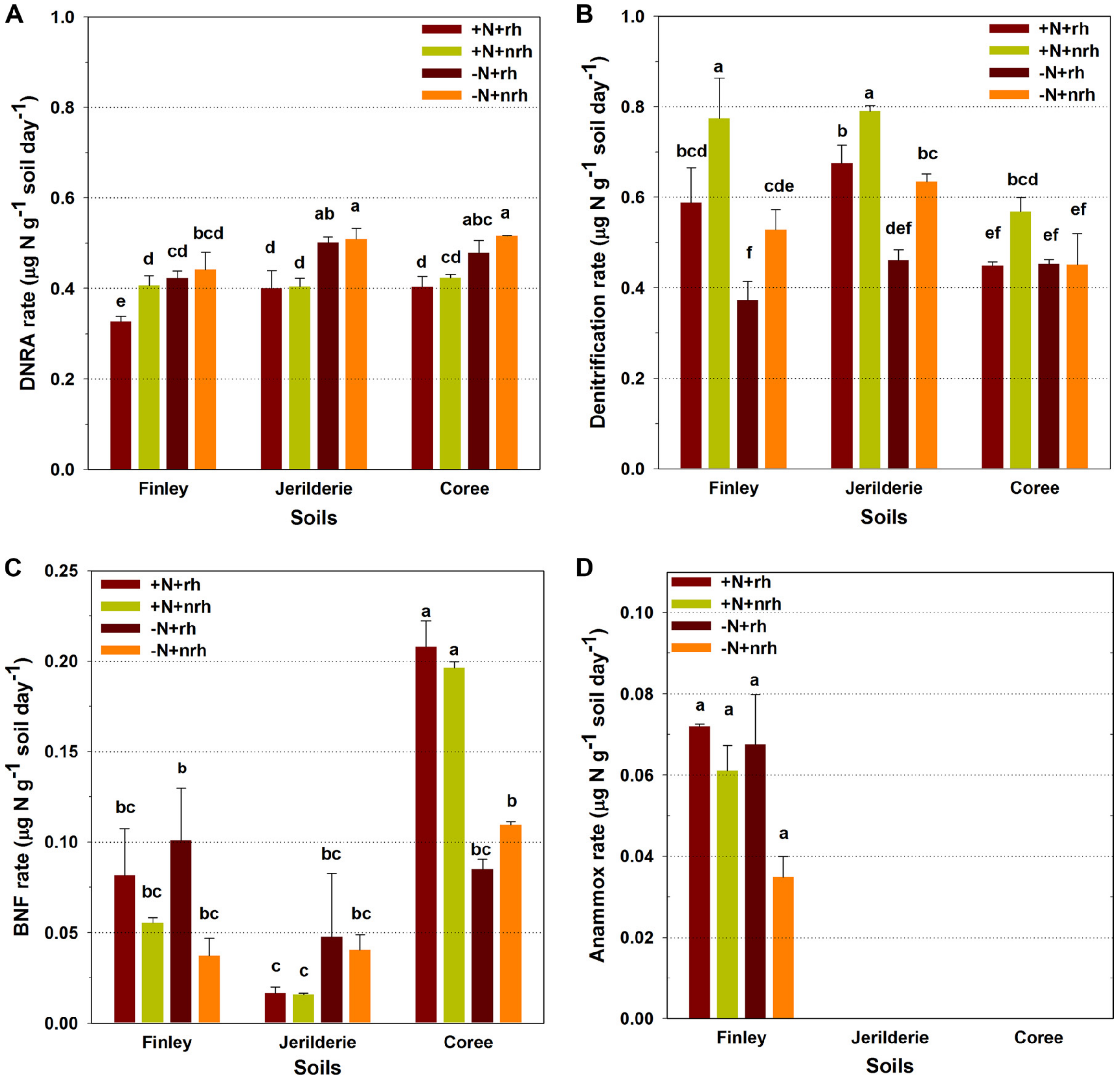

FIG 2 Rate of N transformation by DNRA (A), denitrification (B), BNF (C), and anammox (D) in the paddy soils from the three sites (Finley, Jerilderie, Coree). +N and −N, with and without N input, respectively; rh and nrh, rhizosphere and bulk soils, respectively. The result for bars with different letters on top within a chart are significantly different ($P < 0.05$). Error bars represent ±1 standard error. The *y* axis scales are different for the different panels.

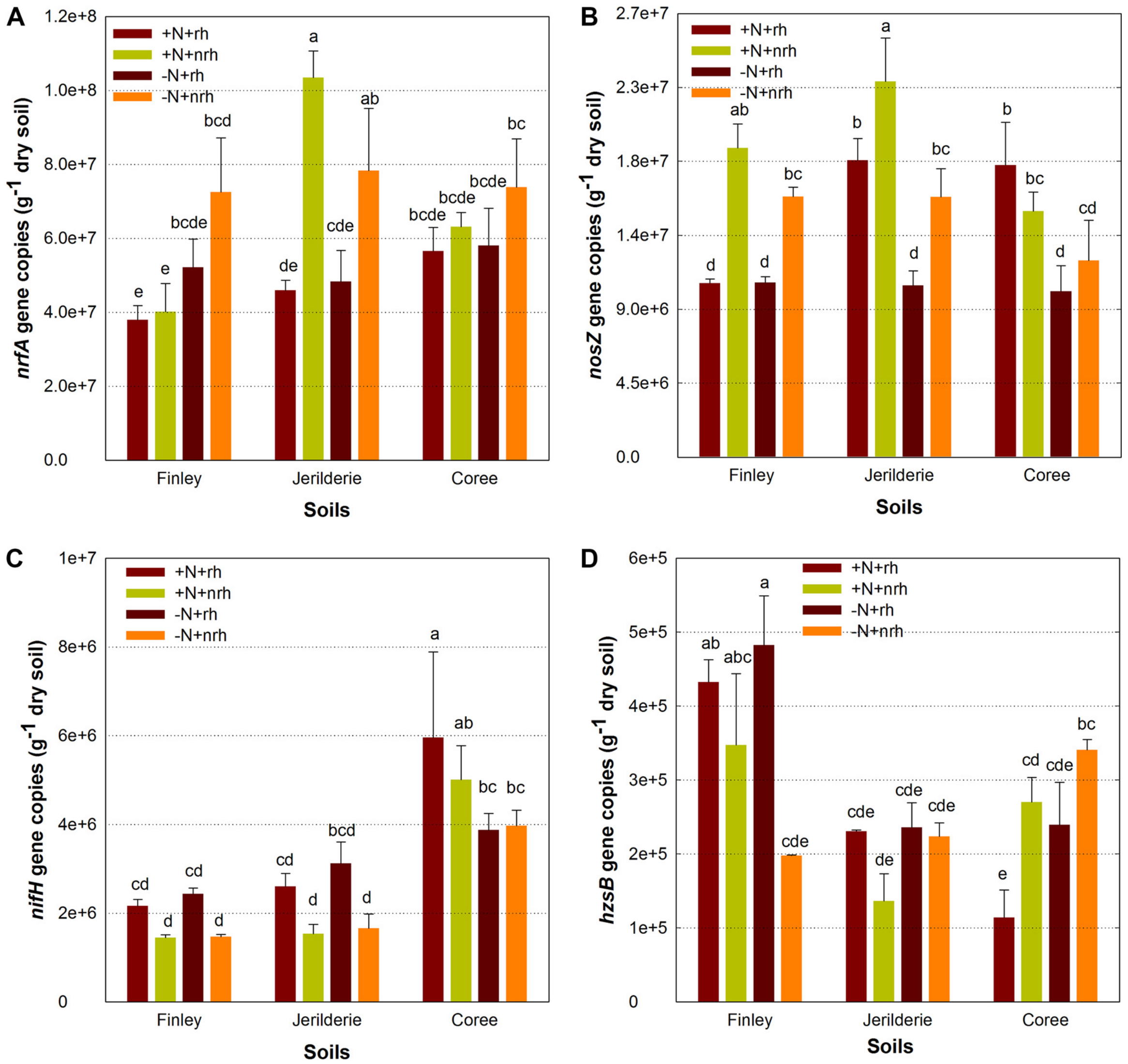

FIG 3 Abundance of the *nrfA* (A), *nosZ* (B), *nifH* (C), and *hzsB* (D) genes in the three sites (Finley, Jerilderie, and Coree). +N and −N, with and without N input, respectively; rh and nrh, rhizosphere and bulk soils, respectively. The results for bars with different letters on top within each panel are significantly different ($P < 0.05$). Error bars represent ±1 standard error.

higher than that by denitrification (Fig. 2A and B). However, when N was added, denitrification reduced up to twice the amount of NO_3^- reduced by DNRA. Both N addition and the rhizospheric zone had a significant effect ($P < 0.05$) on the denitrification rates (Table 1). Nitrogen addition significantly increased the denitrification rates (Fig. 2B). In the rhizosphere, the denitrification rate ranged from 0.45 to 0.67 μg N g^{-1} soil day^{-1} with N application, whereas it was 0.37 to 0.46 μg N g^{-1} soil day^{-1} without N. In the bulk soil, the denitrification rate ranged from 0.58 to 0.79 μg N g^{-1} soil day^{-1} with N application, whereas it was 0.45 to 0.63 μg N g^{-1} soil day^{-1} without N. Denitrification rates were significantly higher (by >10%) in the bulk soil than in the rhizosphere soil (Fig. 2B).

Abundance of genes related to DNRA and denitrification. Neither the addition of N nor the rhizospheric zone had any significant effect ($P > 0.05$) on *nrfA* gene abundance (Table 1). The rhizosphere and bulk soil in the Finley and Coree soils had similar *nrfA* gene abundances, whereas the gene abundance in the bulk soil was higher than that in the rhizosphere soil in the Jerilderie soil (Fig. 3A). A significant positive correlation between the DNRA rates and the *nrfA* gene abundance was observed

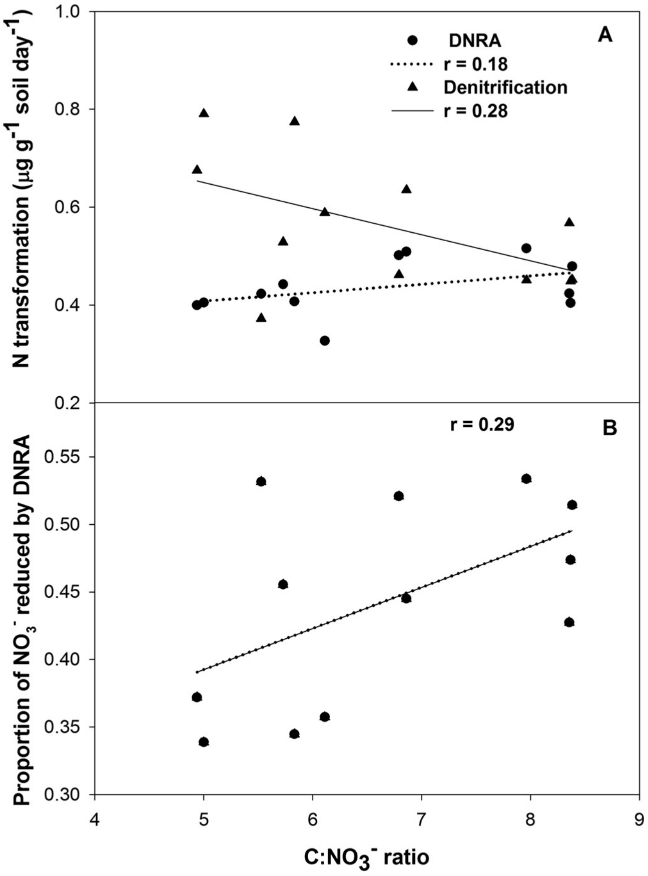

FIG 4 Relationship between the C/NO_3^- ratio and N transformation rates (A) and the proportion of NO_3^- reduced by DNRA (B).

($P = 0.042$, $r = 0.25$). The *nosZ* gene abundance and denitrification rates were significantly correlated ($P < 0.05$, $r = 0.77$) across all soils. There was a significant effect ($P < 0.05$) of the addition of N and the rhizospheric zone on the *nosZ* gene abundance (Table 1). The *nosZ* gene abundance was significantly higher in the +N than in the −N treatment in the Jerilderie and Coree soils, but no such difference was observed in the Finley soils (Fig. 3B). There was a significant difference in the *nosZ* gene abundance between the rhizosphere and bulk soils in the Finley and Jerilderie soils, but no such difference was observed in the Coree soils (Fig. 3B). There was mostly no significant difference in the *narG* and *nirK* gene abundance between the N treatments and the rhizospheric zones (see Fig. S1 in the supplemental material).

Carbon/NO_3^- ratio and DNRA. Pearson's correlation analysis showed that there was a weak but significant positive relationship between DNRA rates and the soil C/NO_3^- ratio ($r = 0.18$, $P < 0.05$; Fig. 4A), but the proportion of NO_3^- reduced by DNRA showed a stronger (but still weak) relationship with the soil C/NO_3^- ($r = 0.29$, $P < 0.05$; Fig. 4B). Denitrification rates had a significant negative relationship with the C/NO_3^- ratio ($r = 0.28$, $P < 0.05$; Fig. 4A).

Biological N_2 fixation and anammox processes in the paddy soils. The biological N_2 fixation rate ranged from 0.02 to 0.21 μg N g^{-1} soil day^{-1}, and the rates were significantly and positively correlated with the *nifH* gene abundance ($P < 0.05$, $r = 0.76$; Fig. 2C). Nitrogen application showed a significant effect on biological N_2 fixation (Table 1). The *hzsB* gene, related to anammox activity, was detected in all the soils (Fig. 3D); however, the contribution of anammox toward the N loss was minimal (0.03 to 0.07 μg N g^{-1} soil day^{-1}) and was detected only in the Finley soils (Fig. 2D).

DISCUSSION

Microbial nitrogen retention in rice paddies. DNRA accounted for a significant proportion of the total NO_3^- reduced (33 to 55%) and a significant amount of NH_4^+ production (0.32 to 0.52 μg g^{-1} soil day^{-1}) in the rice paddies. This represents an important N retention process which can reduce the risk of N loss through NO_3^- leaching and denitrification (10). The amount of NO_3^- transformation by DNRA observed in the rice paddies was similar to that observed in grassland and forest ecosystems (10, 11, 28). Considering arable soils, Schmidt et al. (18) could not detect DNRA without adding glucose in their incubation study, whereas Davis et al. (29) and Inselsbacher et al. (30) observed very low levels (0.03 to 0.2 μg N g^{-1} soil day^{-1}) of DNRA activity. This indicates that rice paddies may have a more favorable environment for DNRA than other arable soils. Compared to other arable soils, rice paddies are generally NO_3^- limiting, as nitrification is limited by oxygen availability (4) and such conditions are thought to be favorable for DNRA (17). Studies have reported that the NO_3^- limiting forest soils and sea sediments is dominated by DNRA rather than by denitrification (10, 23, 31). As well as being NO_3^- limiting, paddy soils receive significant amounts of labile OC from the root exudates (3), and these conditions have been found to enhance DNRA activity in soils (further discussed below) (18).

When N was added to the rice paddies, the DNRA rate was reduced by up to 22%. Our findings of a reduced DNRA rate with N addition are in agreement with those of previous studies in sediments and forest soils (23, 26). Nitrogen addition into the rice paddies increases the nitrification rate and, subsequently, the NO_3^- availability to soil microbes (4, 32), and this can negatively affect DNRA activity (24). Studies conducted in diverse ecosystems have suggested that the most important factor affecting the DNRA activity may be the C/NO_3^- ratio rather than the amount of C or NO_3^- alone (16, 26, 33). A study based on bacterial culture demonstrated that denitrification is the sole NO_3^- reduction process at a low C/NO_3^- ratio, a moderate C/NO_3^- ratio supports both DNRA and denitrification, and at a high C/NO_3^- ratio, DNRA is the sole NO_3^- reduction process (34). The study also found a higher *nrfA* transcript abundance and a lower *nosZ* transcript abundance at higher C/NO_3^- ratios. Similar effects of the C/NO_3^- ratio on DNRA and denitrification were also observed by van den Berg et al. (35). Addition of N can affect the OC fraction in soil and reduce the amount of labile OC (25, 26). A decrease in OC/NO_3^- due to the change in the OC or NO_3^- concentration after N addition can inhibit cytochrome *c* nitrite reductase synthesis, which can result in the accumulation of NO_2^- and favor denitrification instead of DNRA (18, 36, 37). Our results of a positive relationship between the DNRA rate and the C/NO_3^- ratio and a negative relationship between the denitrification rate and C/NO_3^- further support this theory.

Nitrogen addition alone explained 44% of the variability in the partitioning of the NO_3^- between DNRA and denitrification. Our study, however, contradicts the findings of Minick et al. (15) and Yang et al. (11), who documented a negative relationship between DNRA and C/NO_3^- and a positive relationship between DNRA and soil available NO_3^- in forest and grassland ecosystems. The DNRA rate and the *nrfA* gene abundance were not affected by the rhizospheric zone, which suggests that DNRA is not regulated by differences in chemical characteristics between the rhizosphere and bulk soils (3). There are no studies investigating the effect of rhizospheric zones on DNRA and the *nrfA* gene abundance in rice paddies, and the results from the few studies which have looked into the *nrfA* gene abundance in the roots of other arable crops are contradictory (38, 39). The rhizosphere soil in rice paddies receives considerably higher OC from root exudates than from the bulk soil, which should support higher DNRA rates in the rhizosphere soil than the bulk soil, but we did not observe such an effect in our study. There are a number of possible reasons which could have reduced the difference in the DNRA rate between the rhizosphere and bulk soil caused by the difference in the C/NO_3^- ratio. One of them could be the higher sulfide concentration in the bulk soil (40), because sulfide acts as an electron donor and promotes DNRA activity (35, 41). Similarly, Fe(II)-dependent NO_3^- reduction can also favor NH_4^+ production in the bulk soil (42).

A positive correlation between the DNRA rates and the *nrfA* gene abundance was observed, but the relationship was weak ($r = 0.25$), most likely due to the fact that the *nrfA* gene is present in diverse genera of bacteria which are involved in multiple metabolic pathways and the relationship of gene abundance-to-DNRA rate expression is not straightforward (43–45). Several other studies have observed similar results of weak or no significant correlations between the DNRA rate and the *nrfA* gene abundance (44, 46, 47). Such a poor relationship between the process rate and the functional gene abundance is not limited to the DNRA process. A meta-analysis study showed that only one-seventh of studies looking at the relationships between N transformation processes and the relevant gene expression exhibited some sort of significant correlation (48). The N transformation processes that are conducted by a narrow range of microbial taxa can have a significant positive correlation, but the functional genes, such as *nrfA*, which are present in a broad range of microbes may not show a strong correlation (48). Recent studies have found that the organisms capable of DNRA are also capable of reducing N_2O and may switch functions depending on the environmental conditions (49, 50). Studies have suggested that the mRNA-based technique could closely represent protein expression (51, 52), but other studies have demonstrated that there are several confounding factors in this approach (45, 48). Therefore, simultaneous quantification of N transformation rates and related genes abundances, as was done in this study, will aid in the understanding of the N transformation potential of paddy soils. In addition, quantification of a broad range of genes involved in a process can provide meaningful results of ecosystem potential for different N transformation pathways (49, 50).

Nitrogen addition did not have any effect on N_2 fixation or the *nifH* gene abundance in the Finley and Jerilderie soils, but surprisingly, the N input had a beneficial effect on those components in the Coree soil. Nitrogen addition has been found to reduce N_2 fixation in rice paddies as the diazotrophs start using N from the fertilizer instead of fixing N_2 for their N requirement (22, 53), but we did not observe such an effect in this study. The estimated BNF of 0.02 to 0.25 μg N g^{-1} soil day^{-1} in this study may be an underestimate of the total N_2-fixing potential of the rice paddies, as we studied only anaerobic and heterotrophic N_2 fixation. Under field conditions, cyanobacteria and other aerobic microbes, such as *Azobacter*, can fix a significant amount of N_2 into the soil (>50 kg N ha^{-1} rice $crop^{-1}$) (54). The nitrogenase activity and, hence, N_2 fixation were controlled by diazotrophs possessing the *nifH* gene, as shown by the strong positive correlation ($r = 0.76$, $P < 0.05$) between nitrogenase activity and the *nifH* gene abundance, which indicates a coupling between the diazotrophs and enzyme activity.

Microbial nitrogen loss from rice paddies. Denitrification rates were higher in the +N treatments than in the −N treatments. Nitrogen addition increases nitrification rates and NO_3^- availability in rice paddies (4), which ultimately enhance denitrification (55). Extractable NO_3^- in the paddy soil did not provide a good estimate of the nitrification activity or denitrification potential (5, 55). The *nosZ* gene abundance followed a trend similar to the denitrification rate, and these were positively correlated, which indicates that *nosZ* can provide a good estimate of the denitrification potential in rice paddies. Previous work, including the meta-analysis by Rocca et al. (48), found that the *nosZ* gene abundance has the ability to strongly and directly explain the denitrification potential in other ecosystems (56). Recent studies demonstrated that bacteria harboring *nosZ* clade II have a higher affinity for N_2O than those harboring *nosZ* clade I (57–59). The primers used for the *nosZ* gene in this study are able to amplify only *nosZ* clade I and therefore may underestimate the N_2O reduction potential of the soil. Inclusion of *nosZ* clade II could have provided further strength in explaining the denitrification potential of the investigated rice paddies. As discussed above, nondenitrifiers have also demonstrated N_2O reduction capabilities which should also be accounted for while studying the denitrification potential of any ecosystem (50). Quantification of specific genes related only to denitrifiers or DNRA may not provide a meaningful understanding of the NO_3^- reduction potential of the ecosystem, as recent studies demonstrated that the organisms lacking the *nirS*, *nirK*, and typical *nosZ* genes are capable of reducing NO_3^- and N_2O (49, 50). However, simultaneous quantification of the interlinked N transformation processes and their related genes in this study enabled us to understand the significance of the mostly overlooked DNRA process in paddy soil ecosystems.

Nitrogen addition and bulk soil conditions enhanced the *nosZ* gene abundance in the Jerilderie and Coree soils, supporting the findings of previous studies (60, 61). Denitrification was the dominant N_2 loss pathway, accounting for more than 90% of the total N_2 production, with only a minor amount coming from anammox ($<$10%). Denitrification consumed 14 to 30% of the total NO_3^- in the paddy soils. We observed a clear rhizospheric effect on denitrification, with the denitrification rates in the rhizosphere soils being lower than those in the bulk soils, regardless of fertilizer treatment and soil type, as a consequence of the higher redox conditions in the bulk soils, which aligns with the findings of previous studies (55, 62). The abundance of the efficient N_2O-reducing *nosZ* clade II was demonstrated to be higher in the bulk soil than in the rhizosphere soil (38), which may further explain the higher N_2 production in the bulk soil. However, our findings contradict those of Zhao et al. (38), who observed that the abundance of *nosZ* clade I associated with the roots of legume crops was higher than that in the bulk soil. Recent studies have demonstrated that anammox can account for up to 41% of the total N_2 production from rice paddies (7, 55). In this study, anammox was detected only in the Finley soil, consuming about 1.5% of the available NO_3^-. The *nosZ* gene abundance was 10- to 20-fold higher than the *hzsB* gene abundance, reflecting the 10-fold higher rate of denitrification compared to that of anammox.

Conclusion. Fertilizer N addition influences the partitioning of NO_3^- between DNRA and denitrification and ultimately can impact the balance between the microbial N loss and retention in rice paddies by uncoupling of soil N cycling. DNRA transforms a significant proportion, 12 to 20%, of the total available NO_3^- to NH_4^+ in these paddy soils and is reduced by N addition. However, N addition does not affect the *nrfA* gene abundance. Denitrification is the main source of N loss in the studied paddy soils, with anammox providing a minimal contribution. Rhizospheric environments influence microbial N loss, particularly the denitrification rates and the *nosZ* gene abundance, but have a limited effect on microbial N retention processes. Our study shows that by reducing N inputs to these paddy soils, there is a positive effect from reduced N_2 loss but also, more importantly, from the conversion of NO_3^- to NH_4^+ for plant uptake.

MATERIALS AND METHODS

Field sampling and soil physicochemical properties. Three different rice paddies (Finley, 35°29′54.82″S, 145°38′29.46″E; Jerilderie, 35°18′59.6″S, 145°38′37.6″E; Coree, 35°18′55.6″S, 145°38′21.9″E) in the Riverina region of New South Wales, Australia, were selected for soil sampling. The Riverina region is the major rice-growing area in Australia, where more than 800,000 ha of land is under rice cultivation (63). Soil samples (0 to 20 cm) were collected from the three rice paddies just before sowing in 2015. The Finley site had been cultivated with rice continuously for 4 years before the soil sampling, while the Jerilderie and Coree sites were under rice every fourth year. Soil samples were collected randomly within each site and composited and homogenized. The three rice paddies were selected to comprise a range of soil organic C and total N contents.

The collected paddy soils were sieved and processed for physicochemical analysis. Soil chemical properties were determined using the methods described by Rayment and Lyons (64). Soil pH and electrical conductivity (EC) were determined in a soil-water (1:5) suspension. Total soil C and N levels were determined by Dumas high-temperature combustion using isotope ratio mass spectrometry (Sercon Hydra, Crewe, UK). Soil mineral N (NH_4^+ and NO_3^-) (2 M KCl, 1:5 ratio) and Olsen P (after extraction with 0.5 M $NaHCO_3$) were analyzed colorimetrically using a segmented flow autoanalyzer (Skalar, Breda, The Netherlands). Total soil P, Fe, Mn, S, and Zn were analyzed using the inductively coupled plasma (ICP) after digestion of the soils with nitric acid. Soil texture was determined using the hydrometer method. The physicochemical properties of the collected paddy soils are shown in Table 2.

Glasshouse pot experiment and laboratory incubation. The paddy soils were used for a glasshouse pot experiment followed by a laboratory incubation experiment. A pot experiment with a 3-by-2 factorial design with four replicates was established. The first factor was soil types, and the second factor

TABLE 2 Physicochemical properties of the three soils

	Value for soil from the following sites:		
Property	**Finley**	**Jerilderie**	**Coree**
Total C (g kg^{-1})	13.93	19.80	22.10
Total N (g kg^{-1})	1.23	1.77	1.97
NH_4^+ (mg N kg^{-1})	10.34	13.10	16.03
NO_3^- (mg N kg^{-1})	39.78	24.20	12.25
Olsen P (mg kg^{-1})	18.33	42.33	73.29
pH	5.72	5.48	5.75
Total P (mg kg^{-1})	98.20	249	395
Total Fe (g kg^{-1})	25.97	28.11	25.21
Total Mn (g kg^{-1})	0.22	0.39	0.26
Total S (g kg^{-1})	0.14	0.39	0.25
Total Zn (mg kg^{-1})	48.80	46.60	52.90
Clay (%)	20.00	22.00	28.00
Silt (%)	17.00	17.00	14.00
Sand (%)	63.00	61.00	58.00

was urea N input, i.e., with N input (+N; 150 kg N ha^{-1}) and without N input (−N). To examine the effect of the localized conditions in the rhizosphere and bulk soils, nylon rhizo-bags (diameter, 8 cm; height, 15 cm; pore size, 50 μm) were filled with 1 kg of soil and placed at the center of the polyvinyl chloride pots (diameter, 15 cm; height, 25 cm). Thereafter, about 3.7 kg of homogenized soil was filled up to a 20-cm height in the pots. The rhizo-bag allows water and nutrients to flow through its walls but does not allow the rice roots to penetrate through, thus separating the rhizosphere and bulk soils. The pots were kept in the glasshouse for 45 days, during which the soils were irrigated to maintain the moisture at the same level (17 to 20% gravimetric moisture content) as that found in the soils when they were collected from the fields. After 45 days, pregerminated rice seeds were sown in the center of the rhizo-bags. Nitrogen fertilizer was applied in split doses in the +N treatment, as practiced by the rice growers in the Riverina region. The fertilizer N was placed at an ~7-cm soil depth during the application. The first dose of N was applied at a rate of 20 kg N ha^{-1} during the sowing of rice (24 h before the flooding of the pots). The second dose of N was applied at a rate of 76 kg N ha^{-1} 35 days after sowing (DAS), and the third N dose was applied at a rate of 54 kg N ha^{-1} 55 DAS. A basal dose of phosphorus (P; 14.4 kg ha^{-1}), sulfur (S; 12.7 kg ha^{-1}), and zinc (Zn; 7.2 kg ha^{-1}) was also applied in all the treatments at sowing.

Just before panicle initiation (75 DAS), the entire rhizosphere soils and bulk soils were sampled separately from each of the pots. This sampling time was chosen because the rice paddies had received all the N doses and the rice plants had completed their vegetative growth. In addition, the full root growth at this stage enables comparison of the maximum rhizospheric effect on N transformation. The irrigation water was also collected and retained for a subsequent laboratory incubation experiment. The collected soil samples were homogenized and separated into two subsamples. The first subsample was used for laboratory incubation within 24 h of collection to determine the biological N_2 fixation (BNF), DNRA, anammox, and denitrification rates, and the second subsample was used for the analysis of the soil mineral N content and DNA extraction. The subsample for DNA extraction was immediately stored at −80°C for subsequent molecular analysis.

Measurement of DNRA rates. The rate of DNRA was determined through the incubation of the paddy soils with a $^{15}NO_3^-$ tracer following the procedure described by Song et al. (65) with some modification. Approximately 3 g of fresh soil was transferred into a 12-ml screw-top Exetainer vial. The vial was degassed and completely filled with He-purged irrigation water. The vial was sealed and preincubated for 48 h to deplete any oxides of nitrogen (NO_x) and O_2. All the incubations were performed inside an N_2 glove bag. Then, the vial was injected with He-purged $^{15}NO_3^-$ (>99 ^{15}N atom%) and unlabeled NH_4^+. The amount of tracers added was 5.5 μg mineral N g^{-1} soil, which was less than 1% of the total soil N and was also less than the soil mineral N available before the preincubation. The NH_4^+ was added to inhibit NO_3^- immobilization by microbes (28, 66, 67). The soils used for the experiment were relatively high in NH_4^+ content, and in such soils, the level of NO_3^- assimilation by microbes is negligible (10, 16). Therefore, the excess $^{15}NH_4^+$ that formed after the addition of $^{15}NO_3^-$ can be attributed to DNRA.

To trace the reduction of $^{15}NO_3^-$ to $^{15}NH_4^+$, the microbial activity in triplicate vials was stopped with the injection of 200 μl 7 M $ZnCl_2$ at 0, 3, 6, 12, and 24 h after the ^{15}N tracer injection. Any $^{15}NH_4^+$ formation was converted to ^{15}N-N_2 following the hypobromite method, as described by Trimmer and Nicholls (68). Briefly, the NH_4^+ in the soil slurry was extracted using 2 M KCl (1:1), and 7 ml of the extract was transferred to a 12-ml screw-top Exetainer vial (Labco) containing 200 μl 7 M $ZnCl_2$. The headspace of the vial was flushed with He before adding hypobromite (68). The headspace gas was analyzed by continuous-flow isotope ratio mass spectrometry coupled with gas chromatography (GC-IRMS), and $^{15}NH_4^+$ production was calculated using the following equation (69):

$$\text{Excess } ^{15}\text{NH}_4^+ = \text{excess } ^{29}\text{N}_2 + 2 \times \text{excess } ^{30}\text{N}_2 \quad (1)$$

Following the calculation, the DNRA rate corresponding to $^{15}NO_3^-$ enrichment was calculated from the linear slope of the excess amount of $^{15}NH_4^+$ formed in the vials incubated for different time periods ($P_{15}NH_{4^+}$) and the mole fraction of $^{15}NO_3^-$ in NO_3^- (F_N) using the following equation:

$$\text{DNRA rate} = P_{^{15}\text{NH}_4^+} \times F_{\text{N}}^{-1} \tag{2}$$

Measurement of denitrification and anammox rates. Denitrification and anammox rates were determined using the vials prepared in the same way as for the DNRA rate determination. After the preincubation, the first set of vials was injected with $^{15}NH_4^+$ (>99 ^{15}N atom%) as a control, the second was injected with $^{15}NH_4^+$ (>99 ^{15}N atom%) plus unlabeled NO_3^- to determine the presence of anammox, and the third was injected with $^{15}NO_3$ (>99 ^{15}N atom%) to determine the rates of denitrification and anammox (70, 71). The microbial activity in triplicate vials was stopped at 0, 3, 6, 12, and 24 h after the tracer was injected using 200 μl 7 M $ZnCl_2$ solutions. Thereafter, exactly 4 ml of the water in each vial was replaced with high-purity He using a high-precision glass syringe. The formation of $^{29}N_2$ and $^{30}N_2$ in the vials was then determined by GC-IRMS. Calculation of anammox and denitrification rates was based on any excess production of $^{29}N_2$ and $^{30}N_2$ using the methods described by Thamdrup and Dalsgaard (72):

$$D_{\text{total}} = \frac{P_{30}}{F_{\text{N}}^{\,2}} \tag{3}$$

$$A_{\text{total}} = F_{\text{N}}^{-1} \times \left[P_{29} + 2 \times \left(1 - F_{\text{N}}^{-1}\right) \times P_{30}\right] \tag{4}$$

where D_{total} and A_{total} represent the amounts of N_2 produced by denitrification and anammox, respectively. P_{30} represents the rate of production of $^{30}N_2$, P_{29} represents the rate of production of $^{29}N_2$, and F_N represents the fraction of ^{15}N in NO_3^-, which is 0.99 (71).

Measurement of BNF rates. The rates of BNF were determined by the acetylene (C_2H_2) reduction assay (ARA) (73), whereby ethylene is produced from the reduction of C_2H_2 by the nitrogenase enzyme and the rate of ethylene production is taken as a proxy for BNF. Briefly, approximately 3 g of fresh soil sample and 2 ml of irrigation water was transferred into a 12-ml screw-top Exetainer vial. Exactly 10% of the headspace inside the vials was replaced with C_2H_2, and the vials were shaken manually to homogenize the C_2H_2. The vials were then incubated in the dark at room temperature for 0, 6, 12, and 24 h (triplicate vials for each time point) after the C_2H_2 injection. Control vials without C_2H_2 were also prepared to check any endogenous ethylene production. At each time point, 500 μl of the headspace gas was sampled and injected directly into a gas chromatograph-mass spectrometer (GC-MS) with a flame ionization detector (GCMS-QP2010; Shimadzu). The ethylene production rate was calculated as the change in the rate of ethylene production with time (73). A conversion ratio of 1:3 for the amount of ethylene produced to the amount of N_2 fixed was used to convert C_2H_2 reduction to BNF in this study.

DNA extraction, PCR amplification, and quantitative PCR (qPCR) analysis. DNA extraction was performed with 0.25 g of homogenized and freeze-dried soil samples using a MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The DNA concentration and quality were measured using a NanoDrop ND-2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The extracted DNA was stored at −20°C for subsequent molecular work.

The *nrfA* gene, related to DNRA, was amplified using the nrfAF2aw and nrfAR1 primers (12). The nitrogen-fixing gene, *nifH*, was amplified using the primer pair nifHF and nifHRc (74). The *narG* and *nirK* genes, which are associated with the initial stage of NO_3^- reduction, were amplified using the primer pairs narG-F and narG-R (75) and FlaCu and R3Cu (76), respectively. The *nosZ* gene, associated with N_2 production in denitrifiers, was amplified using the primer pair nosZ2F and nosZ2R (77). The *hzsB* gene in anammox bacteria was amplified using the primer pair hzsB_396F and hzsB_742R (78). All the genes were amplified using 5 μl of 5× MyTaq reaction buffer (15 mM $MgCl_2$, 5 mM deoxynucleoside triphosphates; Bioline), 0.5 μl of each primer (10 μM), 0.25 μl of MyTaq DNA polymerase (5 U μl^{-1}; Bioline), and 2 μl of DNA template (~10 ng μl^{-1}). The thermal cycling conditions were as described in the references cited above, except for those for the *nrfA* gene, for which the thermal cycling conditions were as follows: 94°C for 10 min and 50 cycles of 94°C for 15 s, 52°C for 45 s, and 72°C for 20 s, followed by 72°C for 5 min. The amplified PCR product was purified using a Wizard SV gel and PCR cleanup system (Promega, Madison, WI, USA) and cloned using a pGEM-T Easy cloning kit (Promega) according to the manufacturer's instructions. The positive clones were selected and sequenced to confirm that the plasmids were carrying the correct inserts before they were used as standards for the qPCR assay. We could not amplify the *nirS* gene using the universal primers (79).

The abundances of the *narG*, *nirK*, *nosZ*, *hzsB*, *nrfA*, and *nifH* genes were quantified using a Bio-Rad CFX96 optical real-time PCR detection system (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The 20-μl qPCR mixtures contained 10 μl of 2× SensiMix SYBR without carboxy-X-rhodamine (Bioline), 0.5 μl of each primer (10 μM), and 2 μl (10-fold diluted) of DNA template. The standards for qPCR were generated through 10-fold serial dilution of the plasmids carrying the targeted genes. The thermal cycling conditions used for the qPCR were as described in the references cited above, except for those for the *nrfA* gene, the thermal cycling conditions for which were described by Song et al. (65). Gene copy numbers were calculated per gram of soil (dry weight) on the basis of the amount of soil used for DNA extraction.

Statistical analysis. Statistical analysis of the N transformation data was performed in R (version 3.3.2; www.r-project.org). Data were log transformed whenever required to meet the assumptions of statistical inferences before the parametric tests. The distribution of the data was assessed using the Shapiro-Wilk normality test. The rates of production of $^{29}N_2$, $^{30}N_2$, $^{15}NH_4^+$, or ethylene gas were compared using a linear model (lm function in R). Analysis of variance (ANOVA) and Tukey pairwise comparison tests were used to test the statistical significance of the difference in functional gene abundance between the treatments. Pearson's correlation test was performed to examine the correlation between the N transformation rates, soil properties, and the related functional gene abundance using Minitab (version 18) statistical software.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00870-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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Supporting information

Nitrogen addition decreases dissimilatory nitrate reduction to ammonium in rice paddies

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Running Head: Nitrogen addition decreases DNRA

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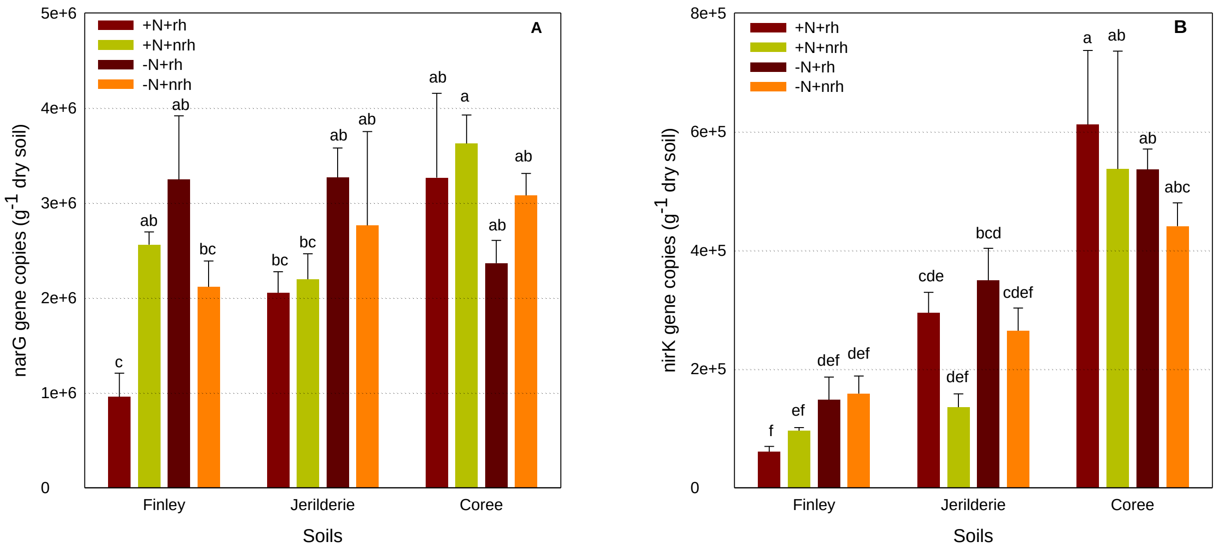

FIGURE S1. Abundance of *narG* (A) and *nirK* (B) gene in the three sites (Finley, Jerilderie and Coree). +N and –N represents with and without N input and rh and nrh represents the rhizosphere and non-rhizosphere soil, respectively. Bars with different letters on top within a chart are significantly different ($p < 0.05$). Error bars represent ±1 standard error.

CHAPTER 4: Dissimilatory Nitrate Reduction to Ammonium Dominates Nitrate Reduction in Long-term Low Nitrogen Fertilized Rice Paddies

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Dissimilatory nitrate reduction to ammonium dominates nitrate reduction in long-term low nitrogen fertilized rice paddies

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ABSTRACT

Dissimilatory nitrate reduction to ammonium (DNRA) and diazotrophic N_2 fixation contribute to nitrogen (N) supply in rice paddies, whereas denitrification contributes to N loss. Continuous N fertilization in rice paddies is known to increase denitrification and reduce N_2 fixation, however little is known about its effect on DNRA and the NO_3^- partitioning between DNRA and denitrification. Here, we investigated the rates of DNRA, denitrification and N_2 fixation, and their relevant microbial gene abundances, in long-term high and low N fertilized rice paddies using a $^{15}NO_3^-$ tracer, an acetylene reduction assay and quantitative PCR analysis, in laboratory incubation studies. We observed that DNRA exceeded denitrification by a factor of eight in low N fertilized rice paddies, while DNRA was almost half of the denitrification rate in high N fertilized rice paddies. The *nrfA* gene abundance, related to DNRA, was significantly higher in the low N fertilized rice paddies and was positively correlated with DNRA rates. However, no clear difference in denitrifying gene (*narG, nirK* and *nosZ*) abundances was observed between the N fertilization regimes. The proportion of total NO_3^- reduced by DNRA had a significantly positive correlation with the soil organic carbon-to-NO_3^- ratio and negative correlation with the soil NO_3^- concentration. N_2 fixation added ten times more N in the low N input than in the high N input paddies. Our findings highlight the self-regulated microbial N cycling in low N input paddy systems which maintain long-term paddy soil N nutrition.

1. Introduction

Mineralization of soil organic nitrogen (N) and application of ammoniacal fertilizers are sources of ammonium (NH_4^+) in paddy soils. In addition, dinitrogen (N_2) fixation catalyzed by the nitrogenase enzyme encoded by the *nifH* gene in diazotrophs also supplies a significant amount of NH_4^+ to paddy soils (Kyaw et al., 2005; Bei et al., 2013). Due to low rates of nitrification, caused by the oxygen (O_2) limited conditions, NH_4^+ is the most abundant form of mineral N in rice paddies (Arth and Frenzel, 2000). However, nitrification in the aerobic rhizosphere and surface water ensures a continuous supply of nitrate (NO_3^-) to paddy soils (Arth and Frenzel, 2000; Li et al., 2008). Nitrate is highly mobile and is prone to leaching and denitrification loss (Silver et al., 2001). Denitrification reduces NO_3^- to N_2 and can contribute to a loss of up to 36% of fertilizer-N applied in rice paddies (Aulakh et al., 2001; Ju et al., 2009). Dissimilatory nitrate reduction to ammonium (DNRA) competes with denitrification for NO_3^- and converts it to NH_4^+ which can be retained by clay minerals (Silver et al., 2001). Therefore an improved understanding of how NO_3^- is partitioned between DNRA and denitrification could enable the manipulation of environmental conditions to enhance N retention in paddy systems.

The ratio of organic carbon (OC):NO_3^- has been suggested as the key environmental determinant for the partitioning of NO_3^- between DNRA and denitrification (Kraft et al., 2014; Yoon et al., 2015). Recent studies using pure bacterial culture have demonstrated that denitrification dominates in growth media with low OC:NO_3^- ratio, whereas DNRA dominates in growth media with high OC:NO_3^- ratio (Kraft et al., 2014; Yoon et al., 2015) and such a relationship applies when either OC or NO_3^- is limiting (van den Berg et al., 2016). However, this relationship has not been as straight-forward in soil-based studies. Recent research investigating the relationship between the soil organic carbon (SOC):NO_3^- ratio and DNRA have reported no correlation in pasture soils (Friedl et al., 2018), a weak (Schmidt et al., 2011) and strong positive correlation (Putz et al., 2018) in temperate arable soils, and a negative correlation in forest ecosystems (Minick et al., 2016). These results suggest that, like in pure bacterial culture study, the relationship between the SOC:NO_3^- and the NO_3^- partitioning between DNRA and denitrification in soil ecosystems may

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depend on whether SOC or NO_3^- is limiting in the system. Paddy soils are characterised by low NO_3^- availability and high labile OC supplied by root exudates (Kögel-Knabner et al., 2010) and this might provide conditions that favor DNRA over denitrification. However, N fertilization can alter the SOC and NO_3^- dynamics in rice paddies and ultimately affect the end product of the microbial NO_3^- reduction. Continuous high N fertilization has shown increased soil NO_3^- concentration (Sun et al., 2015), a reduction in the labile SOC fraction (Neff et al., 2002; Cusack et al., 2011; Dou et al., 2016), and ultimately a reduction in the SOC:NO_3^- ratio (Sun et al., 2015). Such soil conditions may favor NO_3^- partitioning to denitrification over DNRA, although this is poorly documented.

Rice paddies in the majority of the rice-producing countries receive high amounts of fertilizer N (up to 250 kg N ha^{-1}) (Cassman, 1999; Peng et al., 2006), but N use efficiency of the rice production is generally below 40% (Peng et al., 2006). Long-term high N fertilization has been observed to lead to an increase in denitrification rates and the abundance of *narG*, *nirK* and *nosZ* genes, which are related to denitrification, in rice paddies (Chen et al., 2012). This implies that the long-term high N fertilization may promote NO_3^- partitioning to denitrification. Production of N_2 represents the major proportion of N loss by denitrification but ~0.3 kg N_2O, a greenhouse gas with global warming potential of 265 times higher than CO_2 in a 100-year time horizon, is produced when 100 kg fertilizer-N is applied to rice paddies (Pachauri et al., 2014; Pandey et al., 2014). Thus, denitrification poses economic and environmental risks for the farmers and the environment (Ju et al., 2009). Therefore, understanding the effect of N fertilization on the partitioning of NO_3^- between DNRA and denitrification is important to develop efficient N management strategies. However, there are no studies investigating the effects of long-term N fertilization on DNRA activity, NO_3^- partitioning and the *nrfA* gene abundance (encoding the cytochrome *c* nitrite reductase that catalyses DNRA).

Long-term experimental trials have shown that rice paddies which do not receive N fertilizer can maintain soil N status and sustain rice production (Cassman et al., 1998; Pampolino et al., 2008; Bi et al., 2009; Tong et al., 2009). Diazotrophic N_2 fixation has been widely considered as the contributing factor for the maintained soil N status and sustained yields in rice paddies (Roger and Ladha, 1992; Cassman et al., 1998; Ladha and Reddy, 2003). However, the role of DNRA in conserving N, in low N input rice paddies, has been ignored so far. Recently, the DNRA process was found to consume more than 75% of the available NO_3^- in forest (Silver et al., 2001), temperate arable soils (Putz et al., 2018) and sediment ecosystems (Salk et al., 2017), but limited research has been conducted in paddy soil ecosystems. The few studies that have looked at DNRA within rice paddies have indicated that the process may play important role in NO_3^- retention (Yin et al., 2002; Zhang et al., 2015; Pandey et al., 2018). However, these studies applied relatively high rates of $^{15}NO_3^-$ and/or used labile organic OC to fuel DNRA activity, which does not reflect in situ conditions within rice paddies. Also, there are no studies which have investigated the effect of long-term N fertilization on DNRA activity and the NO_3^- partitioning between DNRA and denitrification.

Therefore, there is a need for a comprehensive examination of DNRA, denitrification and N_2 fixation in rice paddies under different N fertilization regimes to better understand the balance between these microbial N loss and retention processes. This study aimed to: (i) examine the effect of N fertilization history on DNRA, denitrification and N_2 fixation in rice paddies; (ii) examine the effect of SOC and NO_3^- on the partitioning of NO_3^- between DNRA and denitrification and the relevant N-cycling microbial gene abundances in rice paddies. The following hypotheses were tested: (i) paddy soils with a history of low N fertilization have higher microbial N input (N_2 fixation) and retention (DNRA) than microbial N loss (denitrification) compared to a high N fertilization history; (ii) the SOC:NO_3^- ratio and the NO_3^- concentration are the most important environmental determinants of the NO_3^- partitioning between denitrification and DNRA; and (iii) the abundance of the microbial genes associated with N retention is higher in long-term low N fertilized than high N fertilized rice paddies.

2. Materials and methods

2.1. Study sites, soil sampling and soil physicochemical properties

Three sites each in Myanmar and Australia were selected for soil sampling in 2016. Myanmar was chosen for soil sampling because of the typically low N fertilization farming systems. Generally, rice paddies in Myanmar receive none or very limited fertiliser-N (25–40 kg fertilizer-N ha^{-1}, as urea or compound fertilizer) (Denning et al., 2013). Soil samples were collected from rice paddies in the townships of Kyauktaga (N 18° 56.9'; E 96° 19.8′), Taungoo (N 18° 12.3′ 47''; E 96° 26.7'') and Pyinmana (N 19° 49'; E 96° 15′) in Myanmar. These sites are characterized by long-term continuous rice-rice cultivation. The selected rice paddy in Kyauktaga did not receive N fertilizer and the rice paddies in Taungoo and Pyinmana received 25 kg urea-N ha^{-1} in the year of soil sampling. Local rice varieties were grown in all the sites which were 130–145 days varieties. Mean annual rainfall in Kyauktaga is 2378 mm, in Taungoo is 1959 mm, and in Pyinmana is 1300 mm. Rice paddies are flooded throughout the rice growing period. Paddy soil samples in Australia were collected from Finley (S 35° 29′, E 145° 38′), Jerilderie (S 35° 18′, E 145° 38′), and Coree (S 35° 18′55.6", E 145° 38′) in the Riverina region of New South Wales. Rice is grown in rotation with other cereals in these sites and are characterized by long-term high N fertilization (150–200 kg N ha^{-1} $crop^{-1}$) (Humphreys et al., 1987; Dunn and Gaydon, 2011). All the selected paddy soils in Australia received 150–157 kg urea-N ha^{-1} in the year of soil sampling. The rice variety grown in all the sites was a 150 days variety. Mean annual rainfall in the region is 407 mm. Rice paddies in the region are flooded for first 120 days starting from 5 cm and reaching to 20 cm during the tillering stage of rice.

Soil samples from all the sites were collected when rice plants were just before panicle initiation stage in 2016, when all the N fertilizer applications were completed. A wet soil sampler was used to collect soil (0–20 cm) from each site. Samples were stored on ice, transported to the laboratory, and homogenized before taking four subsamples. The first subsample was used for laboratory incubation experiments soon after sampling. The second subsample was used to determine mineral N (NH_4^+ + NO_3^-) on fresh soil, and the third subsample was oven dried (40 °C) for soil physicochemical characterization. The fourth subsample was stored in −80 °C for subsequent microbial DNA extraction. Two grams of oven dried soil was used to determine SOC. Prior to analysis, inorganic-C was removed from the sample by washing the soil with 0.1 N HCl twice and then with deionized water (Yang et al., 2017). The soil was then air-dried and analysed for SOC using an elemental analyser (LECO TruMac CN Elemental Analyzer). Total N (TN) was quantified by the Dumas combustion method (LECO TruMac CN Elemental Analyzer). Soil NH_4^+ and NO_3^- (2M KCl, 1:5 soil:solution) were determined colourimetrically using a Segmented Flow Autoanalyzer (Skalar SAN++, Breda, Holland). Soil particle size distribution was determined using the hydrometer method (Gee and Bauder, 1986). The soil physicochemical properties are presented in Table 1.

2.2. Measurement of DNRA rates

The rates of DNRA were determined using a slightly modified method from Trimmer and Nicholls (2009). Briefly, 3 g of fresh paddy soil (~2 g dry weight) was transferred to a 12 mL screw topped Exetainer vial and filled with He gas purged water. The vial was then pre-incubated inside an N_2 glove bag for up to 48 h to deplete soil NO_3^- and any trapped O_2. The duration of pre-incubation for the soils was determined from preliminary NO_3^- depletion trials. Triplicate vials were prepared for each time point, i.e. 0 h, 3 h, 6 h, 9 h and 12 h. The vials (except for controls) were injected with a He purged solution of

Table 1

Physicochemical properties of soils collected from low N input sites in Myanmar and high N input sites in Australia.

Sampling Sites	Soil texture	SOC	TN	pH	SOC:NO_3^- ($\times 10^3$)	*NO_3^--N	*NH_4^+-N
		g kg^{-1}				mg kg^{-1}	
Low N input sites							
Kyauktaga	SiC[a]	15.0 ± 0.38b	1.50 ± 0.04c	5.55 ± 0.03bc	12.56 ± 1.1b	1.20 ± 0.08c	23.85 ± 1.5a
Taungoo	SiC	9.7 ± 0.66d	0.90 ± 0.01c	5.75 ± 0.03b	13.74 ± 0.7b	0.71 ± 0.04d	27.74 ± 3.7a
Pyinmana	SL[b]	11.1 ± 0.51d	1.00 ± 0.01c	6.25 ± 0.04a	19.75 ± 1.0a	0.56 ± 0.03d	22.20 ± 1.1a
High N input sites							
Finley	SL	14.0 ± 0.21c	1.23 ± 0.04b	5.72 ± 0.13b	5.83 ± 0.2c	2.40 ± 0.08b	11.26 ± 0.2b
Jerilderie	SCL[c]	16.5 ± 0.17b	1.77 ± 0.03b	5.48 ± 0.01c	6.34 ± 0.2c	2.70 ± 0.07 ab	27.10 ± 1.2a
Coree	SCL	25.1 ± 0.24a	1.97 ± 0.22a	5.75 ± 0.01b	8.36 ± 0.3c	3.00 ± 0.08a	25.58 ± 5.1a

*: NO_3^--N and NH_4^+ concentration were determined in the freshly collected paddy soils.

Numbers with different letters within a column are significantly different ($P < 0.05$).

[a] SiC: Silty clay.

[b] SL: Sandy loam.

[c] SCL: Sandy clay loam.

$^{15}NO_3^-$ (99 ^{15}N atom%) plus unlabelled NH_4^+. The concentration of $^{15}NO_3^-$ addition was similar to that in the soils before the pre-incubation, i.e. 0.6–1.3 μg $^{15}NO_3^-$-N g^{-1} soil in Myanmar rice paddies and 2.4–3 μg $^{15}NO_3^-$-N g^{-1} soil in Australian rice paddies (Table 1), which was determined immediately after soils were collected from the paddies. The $^{15}NO_3^-$ concentration was maintained at similar level throughout the incubation period by adding $^{15}NO_3^-$ every 1 h from stock solution which was determined from the preliminary NO_3^- depletion trials (Fig. S1). The level of NO_3^- concentration in each paddy soil was maintained at the same level that was measured in the field to ensure N transformation rates reflected those that would occur *in-situ*. Detail on NO_3^- depletion trial and the level of NO_3^- added in the pre-incubated vials have been included in supplementary materials (Table S1 and Fig. S1). Together with $^{15}NO_3^-$, unlabelled NH_4^+ was added at the rate of 0.5 μg NH_4^+-N g^{-1} soil to prevent the microbial immobilization of $^{15}NO_3^-$ injected into the vials. Ammonium can inhibit assimilatory enzymatic activity and repress the synthesis of NO_3^- consuming enzymes but it does not affect dissimilatory NO_3^- reduction (Rice and Tiedje, 1989). Thus, any $^{15}NH_4^+$ formation in the vials can be attributed to the $^{15}NO_3^-$ transformed through the DNRA process. Microbial activity in the triplicate vials at each time point was stopped by injecting 200 μl of 7M $ZnCl_2$. Thereafter, $^{15}NH_4^+$ production in the soil slurry and DNRA rates were determined using the hypobromite method (Risgaard-Petersen et al., 1995) where NH_4^+ is removed from soil with 2 M KCl and then converted to N_2 which is measured by IRMS (Supplementary methods). Rates of DNRA were calculated by taking into account the mass of soil in each vial.

2.3. Measurement of denitrification rates

To determine the denitrification rate, parallel incubation trials were performed over 12 h in vials prepared in the same way as for the DNRA rate measurement. However, only $^{15}NO_3^-$ (99 ^{15}N atom%) was injected into the vials (i.e. no NH_4^+). Microbial activity in triplicate vials at different timepoints was stopped by injecting 200 μl of 7 M $ZnCl_2$. Thereafter, the vials were analyzed for mass-to-charge ratio (m/z) of 28, 29 and 30, which represent $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$, using gas chromatograph coupled with isotope ratio mass spectrometer (GC-IRMS). Only N_2 production was measured to determine the denitrification rate because N_2O production is mostly below detection limit in unfertilized rice paddies and only constitutes less than 1% of fertilizer N application in fertilized rice paddies (Ju et al., 2009; Shang et al., 2011; Pandey et al., 2014). Anaerobic ammonium oxidation (anammox) which also produces N_2 was tested using $^{15}NH_4^+ + NO_3^-$ in another set of vials (Trimmer and Nicholls, 2009). However, only minimal anammox activity was detected in only one of the high N fertilized rice paddies. Therefore any N_2 production was considered to be due to denitrification during the rate calculation (Trimmer and Nicholls, 2009).

2.4. Measurement of N_2 fixation rates

Diazotrophic N_2 fixation was determined using the acetylene reduction assay (ARA) (Keuter et al., 2014). Around 50 g fresh soil (~30 g dry weight) was accurately weighed and transferred to a 500 mL screw topped glass vial (triplicate vials per soil type) equipped with gas sampling ports. The headspace in the vial was made anaerobic by flushing with He. Approximately 10% of the headspace in the vials was replaced with acetylene and the vials were incubated at 25 °C in the dark after shaking briefly. Then an 8 mL gas sample was taken from the headspace at 0 h, 3 h, 6 h, 9 h and 12 h and transferred to new 6 mL vials and analysed for ethylene production using a gas chromatograph mass spectrometer (GC-MS) with flame ionization detector (GC-MS-QP2010, Shimadzu). After each gas sampling, 7.2 mL He and 0.8 mL acetylene gas were injected to the incubation vials to maintain atmospheric pressure inside. Ethylene production was calculated based on a linear increment with time. A theoretical ratio of 3:1 was used to convert the acetylene reduction rate to N_2 reduction rate (Kyaw et al., 2005; Unkovich and Baldock, 2008).

2.5. DNA extraction, PCR amplification and quantitative PCR analysis

The subsample of soil stored at −80 °C was freeze-dried just prior to genomic DNA extraction using a 0.25 g freeze-dried soil sample and the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instruction. The DNA quality was determined using a NanoDrop ND-2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and thereafter stored at −20 °C for subsequent molecular analysis.

Standards for qPCR were prepared from serial dilution of the plasmids carrying positive clones. To generate plasmids, all the genes were amplified in 25 μl PCR reactions using 5 μl of 5x MyTaq Reaction Buffer (15 mM $MgCl_2$, 5 mM dNTPs; Bioline), 0.5 μl of each primer (10 μM), 0.25 μl of MyTaq DNA polymerase (5 U μl^{-1}; Bioline) and 2 μl of (10 ng μl^{-1}) DNA template. The *nrfA* gene was amplified using the primers nrfAF2aw/nrfAR1 (Welsh et al., 2014). The *nifH* gene was amplified using the primer pair nifHF/nifHRc (Rösch and Bothe, 2005). The *narG* and *nirK* genes were amplified using the primers narG-F/narG-R (Bru et al., 2007) and FlaCu/R3Cu (Hallin and Lindgren, 1999), respectively. The *nosZ* gene (Clade I) was amplified using the primer pair nosZ2F/nosZ2R (Henry et al., 2006). Details of all primers are provided in Table S2. The thermal cycling conditions for each gene were as described in the cited references, except for the *nrfA* gene which was as follows: 94 °C for 10 min; 50 cycles of 94 °C for 15 s, 52 °C for 45 s, 72 °C for 20 s followed by 72 °C for 5 min. The PCR product was verified by running 1.5% agarose gels. The amplified PCR products were cloned and sequenced following the protocol described in Song et al. (2014) to confirm the plasmids were carrying positive clones, before using for qPCR.

The abundances of the N-cycling genes were quantified using the Bio-Rad CFX96 optical real-time PCR detection system (Bio-Rad, Laboratories Inc., Hercules, CA, USA). The 20 µl qPCR reaction contained 10 µl of 2× SensiMix SYBR No-ROX (Bioline), 0.5 µl of each primer (10 µM) and 2 µl (10 ng µl^{-1}) of DNA template. The qPCR thermal cycling conditions used for each of the genes were as described in the cited references in Table S2. The amplification efficiency for qPCR runs for all the genes was between 90 and 105%, except for the *nrfA* gene which was 78%.

2.6. Statistical analysis

Differences in soil physicochemical properties were analysed using one-way ANOVA. Rates of production of $^{29}N_2$ and $^{30}N_2$, $^{15}NH_4^+$ or ethylene gas were compared using a linear model ("lm" function in R). Analysis of variance (ANOVA) and Tukey pairwise comparison tests were used to analyse the statistical difference in gene abundances with the significance level set at 95% using Minitab 18. Pearson's correlation matrix was created to compare the correlation between the N transformation rates, related gene abundance and the soil parameters. Linear regression was also used to test the relationship between the soil parameters and the N transformation rates. The data were log transformed whenever necessary before conducting the parametric statistical test.

3. Results

3.1. Soil physicochemical properties

All six paddy soils selected for the experiments were slightly acidic to neutral and the physicochemical properties of soils prior to the incubation assay are presented in Table 1. The long-term high N fertilized paddy soils had 2-6 times higher soil available NO_3^- and also higher SOC (except the Finley soil) than the low N fertilized paddy soils ($P < 0.05$). However, the low N fertilized rice paddies had a considerably higher SOC:NO_3^- ratio (between 13 and 20) compared to the high N fertilized rice paddies (between 6 and 9).

3.2. DNRA, N_2 fixation and denitrification rate and the related microbial gene abundances

The NO_3^- reduced by DNRA (2.21–3.20 µg N g^{-1} soil day^{-1}) exceeded that denitrified (0.17–0.42 µg N g^{-1} soil day^{-1}) by up to eight times in low N input rice paddies, whereas it was about half (0.40–0.42 µg N g^{-1} soil day^{-1}) of the denitrification rate in high N input rice paddies (Fig. 1). The *nrfA* gene abundances were also significantly higher in the low N input than in the high N input rice paddies (Table 2) and were positively correlated with DNRA rates ($R = 0.94$, $P < 0.001$, n = 18). The denitrification rates and the *nosZ* gene abundances were not correlated ($P = 0.189$, n = 18). The *narG* and *nirK* gene abundances were each mostly similar across all the rice paddies (Table 2) and were not significantly correlated with the denitrification rate. Diazotrophic N_2 fixation was significantly higher (2.29–2.98 µg N g^{-1} soil day^{-1}) in the low N input paddies, than in the high N input paddies, where it was less than 0.20 µg N g^{-1} soil day^{-1} (Fig. 1). Similarly, the *nifH* gene abundance was higher in the low N input rice paddies compared to the high N input rice paddies, except between the Taungoo and Coree soils (Table 2). There was a positive correlation between the *nifH* gene abundance and N_2 fixation rates ($R = 0.58$, $P = 0.012$, n = 18).

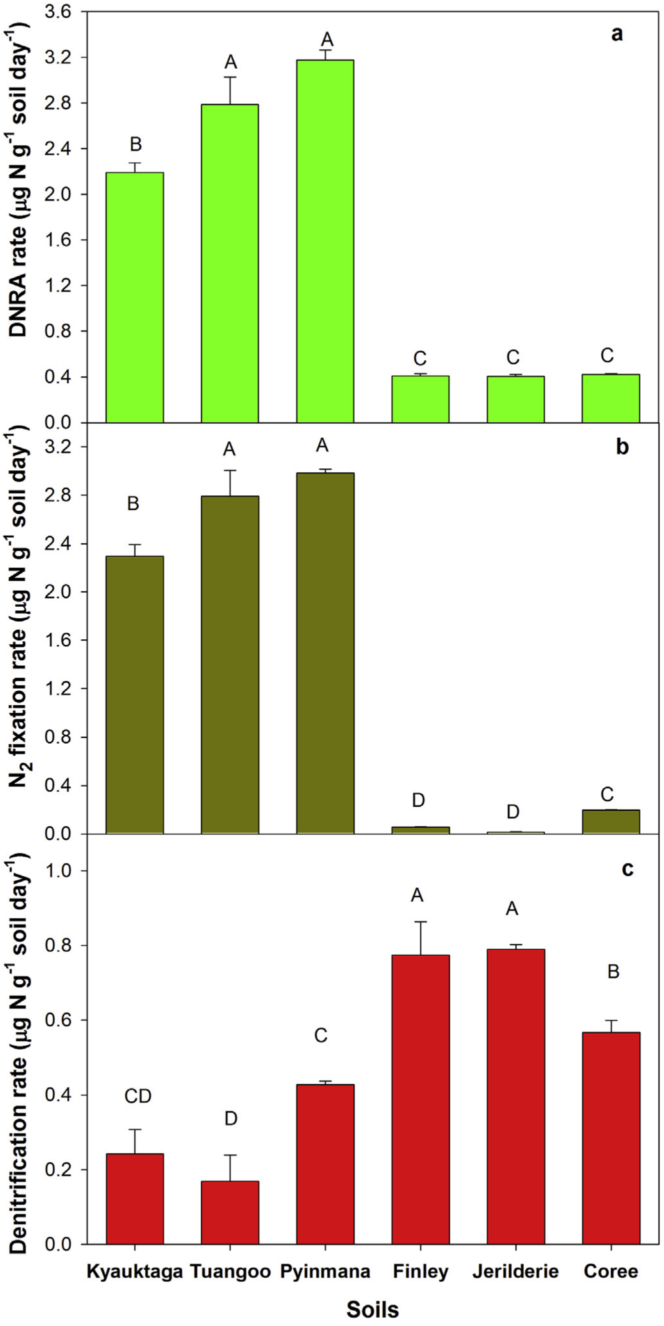

Fig. 1. Rates of N transformations in the six soils (µg N g^{-1} soil day^{-1}): (a) DNRA rates, (b) N_2 fixation rates and (c) denitrification rates. Bars represent means of the N transformation rate with ± 1 standard error of the slope of the linear regression. Bars with different letters on top are significantly different ($P < 0.05$). Note: the Y-axis scales are different for different graphs.

3.3. Environmental determinants of partitioning of NO_3^- between DNRA and denitrification

Rates of DNRA showed a strong positive correlation with the SOC:NO_3^- ratio ($R = 0.92$, $P < 0.001$, n = 18) and a strong negative correlation with NO_3^- ($R = -0.97$, $P < 0.001$ n = 18, Table 3). DNRA rates showed a negative correlation with SOC content ($R = -0.69$, $P = 0.001$, n = 18). The SOC:NO_3^- ratio and soil NO_3^- concentrations together best predicted DNRA rates ($R^2 = 0.96$, $P < 0.001$, n = 18, Table 4). In contrast, denitrification rates exhibited a negative correlation with the SOC:NO_3^- ratio ($R = -0.69$, $P = 0.001$, n = 18) and positive correlation with soil NO_3^- ($R = 0.75$,

Table 2

Gene copy numbers g^{-1} dry soil collected from low N input sites in Myanmar and high N input sites in Australia.

Sampling Sites	*nrfA* ($\times 10^8$)	*nifH* ($\times 10^7$)	*nosZ* ($\times 10^7$)	*narG* ($\times 10^6$)	*nirK* ($\times 10^5$)
Low N input sites					
Kyauktaga	1.82 (± 0.16) b	2.83 (± 0.88) a	1.71 (± 0.58) bc	4.34 (± 0.96) a	1.45 (± 0.19) b
Tuangoo	3.70 (± 0.43) a	0.90 (± 0.05) c	1.21 (± 0.08) c	3.41 (± 0.31) ab	1.26 (± 0.41) b
Pyinmana	3.59 (± 0.36) a	1.44 (± 0.01) b	3.01 (± 0.35) a	3.80 (± 0.71) ab	1.46 (± 0.43) b
High N input sites					
Finley	0.40 (± 0.07) d	0.14 (± 0.07) d	1.88 (± 0.15) bc	2.56 (± 0.13) b	0.96 (± 0.05) b
Jerilderie	1.03 (± 0.07) c	0.15 (± 0.21) d	2.29 (± 0.26) ab	2.20 (± 0.26) b	1.36 (± 0.22) b
Coree	0.63 (± 0.04) d	0.50 (± 0.77) cd	1.50 (± 0.11) bc	3.62 (± 0.30) ab	5.37 (± 0.34) a

Numbers with different letters within a column are significantly different ($P < 0.05$).
Numbers within parenthesis are ± 1 standard error of mean.

Table 3

Results from Pearson's correlation test.

N transformations and relevant genes	Soil Parameters						n
	SOC		NO_3^-		$SOC:NO_3^-$		
	R	P	R	P	R	P	
DNRA	−0.69	0.001	−0.97	< 0.001	0.92	< 0.001	18
nrfA	−0.70	0.001	−0.89	< 0.001	0.87	< 0.001	18
Denitrification	0.36	0.137	0.75	< 0.001	−0.69	0.001	18
nosZ	−0.20	0.419	0.12	0.637	0.24	0.344	18
N_2 fixation	−0.66	0.003	−0.96	< 0.001	0.90	< 0.001	18
nifH	−0.19	0.454	−0.51	0.031	0.48	0.043	18

Significance level was set at $P < 0.05$.

$P < 0.001$, n = 18, Table 3). Soil NO_3^- and SOC together best predicted the rate of denitrification ($R^2 = 0.68$, $P < 0.001$, n = 18, Table 4). The proportion of the total NO_3^- reduced by DNRA had a strong linear relationship with the $SOC:NO_3^-$ ratio ($R^2 = 0.74$, $P < 0.001$, n = 18, Fig. 2a). The *nrfA* gene abundance also displayed a strong positive relationship with the $SOC:NO_3^-$ ratio ($R^2 = 0.75$, $P < 0.001$, n = 18, Fig. 2b). Denitrification rates had a negative relationship with the $SOC:NO_3^-$ ratio ($R^2 = 0.61$, $P < 0.001$, n = 18, Fig. 2c). There was no significant correlation between the *nosZ* gene abundance and SOC and soil NO_3^- concentration and their ratio (Table 3).

4. Discussion

4.1. Dinitrogen fixation and the fate of NO_3^- reduction

The reason the long-term low N fertilized rice paddies were more efficient at fixing atmospheric N_2 was because nitrogenase activity was not suppressed. Long-term N fertilization has been found to suppress nitrogenase activity (Valiente et al., 1997), and reduce the *nifH* gene and *Cyanobacteria* abundances in rice paddies (Wang et al., 2016a,b). The bi-functional enzyme GlnD encoded by the *glnD* gene monitors the N status in the diazotrophic cell and the *nifH* gene is expressed when the cell is N limited. When fertilizer-N is applied, diazotrophs can utilize fertilizer-N and become N sufficient and the *nifH* gene may remain unexpressed and nitrogenase activity is suppressed (Jonsson and Nordlund, 2007), leading to a reduction in N_2 fixation (Mills et al., 2004). In a laboratory experiment, Kyaw et al. (2005) observed that paddy soils without N application received 43 kg N ha^{-1} $season^{-1}$ through N_2 fixation but the amount fell to almost zero when N was applied at 160 kg N ha^{-1}. Our results are consistent with the findings from previous studies and provide further evidence of the inhibitory effect of long-term N fertilization on the nitrogenase activity (Mills et al., 2004).

The partitioning of NO_3^- reduction expressed as a DNRA:denitrification ratio was lower in the high N input than in the low N input rice paddies (0.5:1 and 8:1, respectively) possibly because the higher NO_3^- levels, due to the high N fertilization, inhibit cytochrome c nitrite reductase synthesis, which is encoded by the *nrfA* gene (Tyson et al., 1994; Schmidt et al., 2011). Such inhibition results in NO_2^- accumulation Tyson et al. (1994); Schmidt et al. (2011). The cytochrome *cd_1* nitrite reductase in denitrifiers has higher NO_2^- affinity than the cytochrome *c* nitrite reductase in ammonifiers, resulting in enhanced denitrification (Kraft et al., 2014). In support of this explanation was the up to 8-fold higher *nrfA* gene abundance in the low N input paddy soils (Table 2) and the strong correlation observed between the *nrfA* gene abundance and DNRA activity. Our findings confirm that the increase in soil NO_3^- concentrations, as a consequence of continuous high N fertilization, restrains DNRA activity and the related *nrfA* gene abundance and provides a competitive advantage for denitrifiers. Our findings also suggest that microbial N cycling strategies in low N input rice paddies have evolved to efficiently add and retain N within the system, allowing minimal N loss.

4.2. Environmental controls on NO_3^- partitioning between DNRA and denitrification

Soil NO_3^- concentration and the $SOC:NO_3^-$ ratio played an important role in controlling the fate of NO_3^- reduction in the

Table 4

Results from linear regression test conducted between the N transformation rates and soil parameters.

Response variable	Explanatory variable	Regression coefficient	*P*-value	R^2	*P*-value	*n*
DNRA	Constant	2.23 ± 0.63	0.002	0.96	< 0.001	18
	$SOC:NO_3^-$	0.08 ± 0.03	0.011			
	NO_3^-	−0.95 ± 0.15	< 0.001			
Denitrification	Constant	0.45 ± 0.13	0.011	0.68	< 0.001	18
	NO_3^-	0.43 ± 0.10	0.001			
	SOC	−0.04 ± 0.01	0.037			
BNF	Constant	3.08 ± 0.28	< 0.001	0.95	< 0.001	18
	NO_3^-	−1.99 ± 0.21	< 0.001			
	SOC	−0.10 ± 0.03	0.003			

Significance level was set at $P < 0.05$.

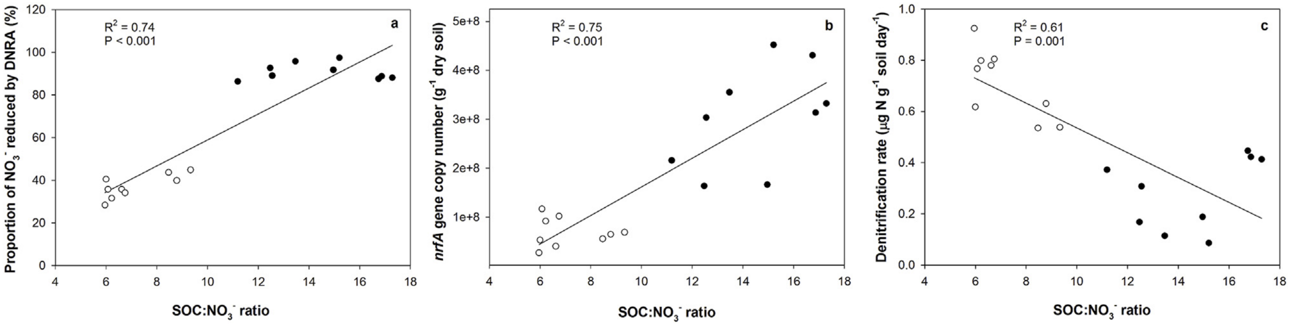

Fig. 2. Relationships of (a) the proportion of NO_3^- reduced by DNRA, (b) the *nrfA* gene abundance and (c) denitrification rates with the SOC:NO_3^- ratio. Empty circles represent high N fertilized soils and filled circles represent low N fertilized soils. R^2 is the regression coefficient and *P* is the statistical significance of the relationships at 95% confidence level.

investigated rice paddies (Table 4 and Fig. 2). Rates of DNRA were negatively influenced by NO_3^- concentrations and positively influenced by SOC:NO_3^- ratios, whereas the opposite effect of these soil parameters on denitrification was observed (Tables 3 and 4). Our findings are similar to the results observed by Putz et al. (2018) in temperate arable soils, who observed a strong positive relationship (R^2 = 0.95) between the SOC:NO_3^- ratio and DNRA rates at soil NO_3^- concentrations of 5–6 μg NO_3^--N g^{-1} soil. Porubsky et al. (2009) also observed similar relationship between DNRA and the SOC:NO_3^- ratio (R^2 = 0.84) in sediments when manipulating the SOC:NO_3^- ratio by adding different levels of labile OC. However, our results contradict with Friedl et al. (2018) where no significant relationship was observed between the SOC:NO_3^- ratio and DNRA or denitrification in pasture soils. The pasture soil used by Friedl et al. (2018) had SOC contents between 4 and 5% and NO_3^- available for DNRA and denitrification was more than 30 μg NO_3^--N g^{-1} soil in the incubation vial. Thus, neither SOC nor NO_3^- was limiting in the soil, which probably led to a lack of an observed significant correlation of the SOC:NO_3^- ratio with DNRA or denitrification in their study. Qualitative and quantitative evidence of DNRA being favored by the higher ratio of electron donor (OC) to electron acceptor (NO_3^-) has been reported in bacterial culture studies (Strohm et al., 2007; Kraft et al., 2014). A study by van den Berg et al. (2016) in pure bacterial culture reported that both DNRA and denitrification can co-exist in a wide range of OC:NO_3^- ratios where neither OC nor NO_3^- is limiting. But DNRA dominates in NO_3^- limiting conditions and denitrification dominates in OC limiting conditions (Yoon et al., 2015; van den Berg et al., 2016). It is highly unlikely that the SOC and NO_3^- concentrations in flooded paddy soils are at such a high level as in pasture soils used by Friedl et al. (2018), regardless of fertilization level (Aulakh et al., 2001; Pampolino et al., 2008; Yang et al., 2013). Yang et al. (2013) observed no more than 15 μg NO_3^--N g^{-1} paddy soil during the rice season when applied with 300 kg urea-N ha^{-1}. Nitrate is mostly limiting in unfertilized rice paddies due to low nitrification activities (Table S3).

Fertilizer-N not only increases NO_3^- concentration in soil but also reduces the labile fraction of SOC due to accelerated decomposition (Neff et al., 2002; Ghani et al., 2003; Corre et al., 2010; Cusack et al., 2011; Dou et al., 2016). This effect of long-term N fertilization on the labile SOC and SOC:NO_3^- ratio can influence several N transformation pathways. Our findings confirm that the NO_3^- partitioning between DNRA and denitrification in rice paddies, where NO_3^- is limiting, are affected by the SOC:NO_3^-. This implies that the N retention in rice paddies could be improved by adding organic carbon to the soil. Improving N retention can help in reducing the amount of fertilizer-N required for rice production. Reduction in fertilizer-N inputs can have an additional advantage by reducing N_2O emission from rice paddies (Shang et al., 2011). However, addition of organic carbon can enhance methane emission, a potent greenhouse gas (Pandey et al., 2014). Therefore, further study is needed to evaluate the effect of changing SOC:NO_3^- ratios on NO_3^- retention and methane emissions from rice paddies.

4.3. Methodological considerations

This study compared N transformation rates between long-term low N input rice paddies of Myanmar and high N input rice paddies of Australia. It is noted that the DNRA, denitrification and N_2 fixation rates were measured separately in the laboratory incubation conditions. The rates represent the N transformation potential for each relevant process in the paddy soils. Apart from N fertilization practice, the N transformation rates in field conditions are also affected by climatic conditions and cultivation practices (e.g. continuous rice in Myanmar, rice-cereal rotations in Australia). Even so, the rates reported in this study are from laboratory incubation experiments conducted under the same conditions which enabled us to perform relative comparison of N transformation rates and correlate the rates with microbial genes and soil properties. Unlike in other arable soils, there is a little effect of soil homogenisation on the N transformation rates in paddy soils because the paddy soil structure is already destroyed during field preparation. Therefore, the findings in this study provide a comprehensive understanding of the N transformation rates in rice paddies with little effect of soil sampling. The ARA method used in this study to determine N_2 fixation does not directly quantify N_2 fixation but the method is cost effective, quick and ethylene is easy to measure due to its low detection limit (Keuter et al., 2014). However, the 3:1 conversion factor, which is most commonly used, to convert C_2H_2 reduced to N_2 fixation, can vary widely depending on ecosystems (Barron et al., 2009). Therefore, the actual conversion factor for the selected rice paddies in this study may vary from the 3:1 conversion factor, but the 3:1 conversion is suitable for the purpose of relative comparison of N_2 fixation in different rice paddies. The rate of N_2 fixation observed in Australian rice paddies in this study is within the range estimated by Gupta et al. (2006) for that agroecological zone.

5. Conclusions

Our study demonstrated that soil microbes in the continuous low N fertilized rice paddies not only potentially fix higher amounts of N_2 but also retain the majority of the loss-prone NO_3^- as soil-retainable NH_4^+ through DNRA, allowing minimal denitrification loss. However, such strategies were not apparent in long-term high N fertilized rice paddies. Difference in soil NO_3^- and SOC:NO_3^- ratio in rice paddies, brought about by the N fertilization history, together play an important role in the partitioning of NO_3^- between DNRA and denitrification where NO_3^- is generally limited due to the anaerobic conditions in paddy soils. This study, for the first time, provides important information on how NO_3^- in paddy soils with different SOC:NO_3^- ratios is partitioned between DNRA and denitrification. Our study highlights the presence of self-regulated N cycling in the natural ecosystems which maintains and sustains the primary productivity without external N input. Nitrogen fertilization is probably important in achieving higher rice yields but it comes with the consequences of unbalancing the microbial N cycling and increasing N loss from paddy soils. Manipulating SOC:NO_3^- ratio by external OC input in high N fertilized rice paddies may improve the N retention. This improved N retention may help in reducing fertilizer-N requirement and also help in reduction of N_2O emissions from rice paddies, generally observed after N fertilization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.01.007.

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Supplementary information:

Dissimilatory nitrate reduction to ammonium dominates nitrate reduction in long-term low nitrogen fertilized rice paddies

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Determination of DNRA rates:

Amount of $^{15}NH_4^+$ production after the reduction of $^{15}NO_3^-$ in the incubation vials were determined following the procedure as described in (Trimmer and Nicholls, 2009). After stopping the microbial activity using $ZnCl_2$ in incubation vials, NH_4^+ ($^{14}NH_4^+$ + $^{15}NH_4^+$) in the soil was extracted using 2 M KCl solution (1:1 soil:solution). Exactly 7 ml of extracted solution was transferred to new 12 ml screwed topped Exetainer vials and the headspace was flushed with high purity He gas. Thereafter, alkaline hypopromite was injected into the vials and mass charge ratios (m/z) 28, m/z 29, and m/z 30 of nitrogen was measured using GC-IRMS. The following equation was used to determine the rate of DNRA based on the linear increment of $^{15}NH_4^+$ production with time:

$$DNRA = r_{14} \times P\ ^{15}NH_4^+$$

where, r_{14} is ^{15}N atom% of $^{15}NO_3^-$ added into the vials, which was is 99% and $P^{15}NH_4^+$ is the rate of production of excess $^{15}NH_4^+$ with time.

Nitrate depletion trial:

Within 24 hours after the collection of paddy soil samples, mineral N (NH_4^+ + NO_3^-) in wet paddy soils was extracted using 2 M KCl (1:5 soil:solution). Concentration of NO_3^- in the extract was determined colourimetrically using a Segmented Flow Autoanalyzer (Skalar SAN++, Breda, Holland) which is presented in Table S3. Around five grams of fresh paddy soil was accurately weighed and transferred to 12 ml screwed topped Exetainer vial and filled with He gas purged water. Triplicate vials were prepared for each time point, i.e. 0 h, 1 h, 2 h, 3 h and 4 h. The vials were then pre-incubated inside an N_2 glove bag for up to 48 h to deplete soil NO_3^- and any trapped O_2. Thereafter exactly the same amount of NO_3^-, i.e. the amount shown in Table S3, was added to the vials to mimic the *in-situ* concentration. Thereafter, triplicate vials were extracted with 2 M KCl (1:1 soil:solution) at each time point and NO_3^- concentration was determined colourimetrically. Example of the rate of depletion of NO_3^- in Coree and Pyinmana soils is presented in Fig. S1. Later during the N transformation rate measurement, the rate of NO_3^- depletion obtained from this trial was used to top up $^{15}NO_3^-$ in the incubation vials to maintain the soil NO_3^- concentration at similar level throughout the incubation.

Table S1. NO_3^- concentration in paddy soils determined immediately after sampling.

Sampling Sites	NO_3^--N	NH_4^+-N
	mg kg^{-1}	
Kyauktaga	1.20±0.08c	23.85±1.5a
Taungoo	0.71±0.04d	27.74±3.7a
Pyinmana	0.56±0.03d	22.20±1.1a
Finley	2.40±0.08b	11.26±0.2b
Jerilderie	2.70±0.07ab	27.10±1.2a
Coree	3.00±0.08a	25.58±5.1a

Table S2. Primers used for PCR and quantitative PCR. Thermocycling conditions used for the qPCR were same as in the cited references.

Targeted Genes	Primers	Sequence (5’ - 3”)	Reference
nrfA	nrfAF2aw	CARTGYCAYGTBGARTA	Song et al. (2014)
	nrfAR1	TWN GGC ATR TGR CAR TC	
nosZ	nosZ2F	CGCRACGGCAASAAGGTSMSSGT	Henry et al. (2006)
(Clade I)	nosZ2R	CAKRTGCAKSGCRTGGCAGAA	
narG	narGG-F	CGCCSATYCCGGC SATGTC	Bru et al. (2007)
	narGG-R	GAGTTGTACCAGTCRGCSGAY TCSG	
nifH	nifHF	AAAGGYGGWATCGGYAARTCCACCAC	Rösch and Bothe (2005)
	nifHRc	TGGGCYTTGTTYTCRCGGATYGGC AT	

Table S3. Literature summary of change in nitrification potential of rice paddies after long-term nitrogen fertilization.

References	Nitrogen fertilization (kg N ha^{-1} $crop^{-1}$)	Potential nitrification rate (mg NO_3^--N kg^{-1} soil day^{-1})	Experiment duration (years)
Wu et al. (2011)	0	59.04	22
	180	132	
Zhong et al. (2007)	0	122	23
	167	770	
Jin et al. (2014)	0	8	21
	213	16	
Wu et al. (2017)	0	4.96	20
	157	7.36	
Wang et al. (2014)	0	12.72	6
	180	15.84	

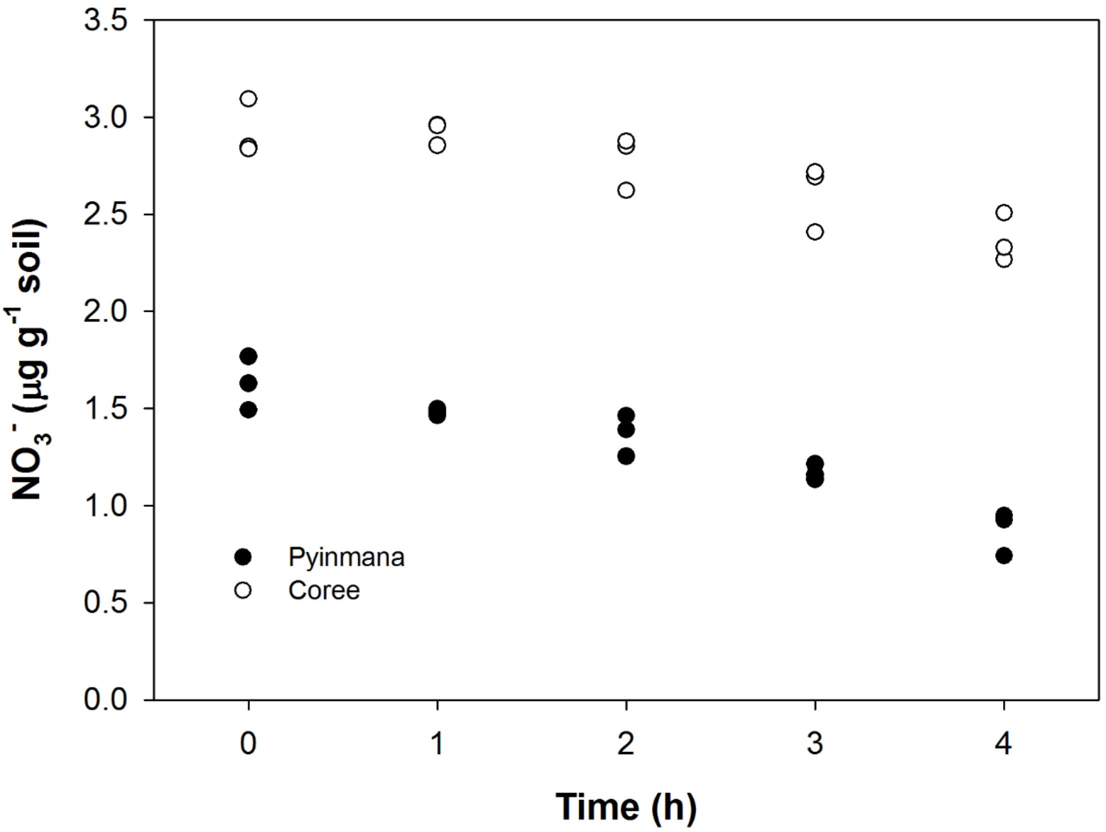

Fig. S1. NO_3^- depletion trial conducted in paddy soil from Pyinmana and Coree.

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CHAPTER 5: Dissimilatory Nitrate Ammonification Helps Maintain Nitrogen Nutrition in Resource Limited Rice Paddies in Myanmar

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Chapter 5

Abstract

Rice paddies have shown maintained soil nitrogen (N) status and sustained yields for decades without fertilizer-N. Rice paddies in Myanmar which receive non or low fertilizer-N have been found to consistently produce more than 3.5 tonnes ha^{-1} of rice grain. Microbial N_2 fixation, known to contribute N to low input rice paddies, cannot fully explain the sustained rice production and maintained soil N status that has existed for hundreds of years in rice production systems without fertilizer-N input, where anaerobic environment provides favourable conditions for denitrification N loss. Dissimilatory nitrate reduction to ammonium (DNRA) may play a key role in limiting denitrification loss by retaining loss-prone NO_3^- as soil-retainable NH_4^+ in low N input rice paddies. However, empirical evidence is still lacking. Here we show that in low N input rice paddies compared to high input paddies, N_2 fixation adds a significant amount of N and the majority of NO_3^- is reduced to NH_4^+, allowing minimal denitrification loss. In laboratory incubation studies, we found that N_2 fixation adds >1.5 µg N g^{-1} soil day^{-1} to the long-term low N input paddies as opposed to <0.7 µg N g^{-1} soil day^{-1} in the high N input paddies. The majority of NO_3^- (30-60%) was reduced to NH_4^+ by DNRA, and minimal NO_3^- (15%) was lost as N_2 from the low N input paddies. In high N input paddies, only 10% of NO_3^- was reduced to NH_4^+, which was less than half of that reduced to N_2 by denitrification. We also found that maintaining a higher SOC:NO_3^- ratio in high N input rice paddies can improve N retention. Our findings highlight the unique microbial N-cycling strategies in low N input paddies which allow minimal N loss and maintained soil N status and sustained rice production.

Keywords: Nitrogen input, Rice paddies, Dissimilatory nitrate reduction to ammonium, Denitrification, N_2 fixation, *nrfA* gene

Chapter 5

Introduction

Rice paddies are one of the biggest fertilizer-N sinks (16 million tonnes per year) in the world (Heffer, 2013) but the N use efficiency of rice production is generally below 40% and the rest of the N is lost to the atmosphere or to other terrestrial ecosystems (Peng et al., 2006). Lost nitrogen represents a serious economic loss to framers, but the impact on the environment and human health is equally, if not more, important (Chen et al., 2008). Nitrogen is the most important yield-limiting nutrient in cereal production (Cassman, 1999), but rice paddies have shown sustained rice production for hundreds, if not thousands, of years without N fertilization (Stewart, 1995). Several long-term (30-40 years) experimental trials at different locations have demonstrated that grain yields (>3 tonnes ha^{-1} $crop^{-1}$) and the soil organic carbon (SOC) levels were stable in the paddies without N fertilization (Dobermann et al., 2003; Saleque et al., 2004; Bi et al., 2009). These and other studies have also demonstrated net N accretion possibly due to the large N input from diazotrophic N_2 fixation and minimal N loss in paddies without N fertilization (Stewart, 1995; Cassman et al., 1998). This implies that low input paddy systems have enclosed N-cycling and efficient conservation strategies which support maintained soil N status and sustained rice yields. Indeed, low N input paddy systems in Myanmar, which is a common practice in the country, have shown >3.5 tonnes ha^{-1} rice yields with non or ~25 kg N ha^{-1} (Denning et al., 2013; Eldridge et al., 2017). This indicates that N-cycling microbes in low N input paddies, such as Myanmar rice paddies, may have evolved to fix and conserve N within the system, allowing minimal N loss.

Rice plants supply a large amount of OC to paddy soils through root exudates and decaying plant parts (Kimura et al., 2004). The OC acts as an electron donor for diazotrophs which is important to meet the high energy requirement for conversion of N_2 to NH_4^+ (Dixon and Kahn, 2004). Diazotrophs can supply more than 45 kg NH_4^+-N ha^{-1} in rice paddies, which receive no fertilizer-N (Bei et al., 2013). N_2 fixation has been demonstrated in marine ecosystems to provide NH_4^+ for nitrification and sustain NO_3^- production which can then be reduced to N_2 by denitrification causing N loss (Deutsch et al., 2007; Korth et al., 2014).

In largely anaerobic paddy soils, nitrification in the aerobic rice rhizosphere and surface water ensures continuous NH_4^+ oxidation and supply of NO_3^- (Arth and Frenzel, 2000; Li et al., 2008). Continuous supply of NO_3^- and OC means that the anaerobic rice paddies provide favourable conditions for denitrification (Mulholland et al., 2008). Significantly higher denitrification N loss compared to the N_2 fixation has been observed in paddies which receive fertilizer-N (Nugroho et al., 1992; Kyaw et al., 2005). Studies have also demonstrated a reduction in the N_2 fixing *nifH* gene abundance and an increase in the *narG* and *nosZ* gene abundance associated with denitrification after long-term high N fertilization (Chen et al., 2012; Wang et al., 2016a; Wang et al., 2016b). Whilst the contributions of N_2 fixation to low input paddy soils have been well documented, this alone could not explain the sustained rice yields and maintained soil N status without N retention mechanisms to limit denitrification loss. Dissimilatory nitrate reduction to ammonium (DNRA), catalysed by the cytochrome *c* nitrite reductase encoded by the *nrfA* gene, has been found to compete with denitrification for NO_3^-, and reduce N loss in forest soils (Silver et al., 2001). The DNRA process has been found to support primary productivity in oligotrophic marine and non-agricultural soil ecosystems by limiting N loss (Ortiz et al., 2014; Michiels et al., 2017). However, less is known about how rice paddies with non or low fertilizer-N input efficiently fix and conserve N. Also, less is known about how long-term high fertilizer-N input affect the NO_3^- partitioning between DNRA and denitrification, and the *nrfA* gene abundance.

The objective of this study was to investigate the N conservation mechanism in low N input paddies. We hypothesize that N_2 fixation contributes significant amounts of N, and the majority of soil NO_3^- is conserved as NH_4^+ through DNRA, thus limiting NO_3^- availability for denitrification loss in paddies which receive low N input. To test our hypothesis, we investigated DNRA and denitrification using ^{15}N-NO_3^- (Trimmer and Nicholls, 2009) and N_2 fixation using an acetylene reduction assay calibrated against $^{15}N_2$ uptake (Barron et al., 2009) in low N (< 25 kg N ha^{-1} crop^{-1}) and high N (>100 kg N ha^{-1} crop^{-1}) input paddy soils in Myanmar, in laboratory incubation experiments. The effect of N input on related microbial N-cycling gene abundances in the paddy soils was investigated.

Materials and methods

Site selection and soil sampling

Rice paddies in Myanmar, where low N input (< 25 kg N ha^{-1}) is a common practice (Denning et al., 2013), were selected for this study. Soils were collected in 2017 from three sites (Pyinmana, Taungoo and Kungyangon) each having both long-term high and low N input rice paddies. Paddy soils in Pyinmana were collected from experimental plots with zero N and high N (100 kg N ha^{-1} crop^{-1}) application for 17 years (N 19º 49’, E 96º 16’; Department of Agricultural Research, Yezin). Similarly, soil samples were collected from another two sites, Taungoo Township (N 18º 95’, E 96º 34’) in Nay Pyi Taw region and Kungyangon Township (N 16º 42’, E 95º 95’) in Yangon region from long-term low (<25 kg N ha^{-1} crop^{-1}) and high (100 kg N ha^{-1} crop^{-1}) N input rice paddies. The high N input rice paddies in the Taungoo and Kungyangon townships did not receive fertilizer-N until 2012 but received a high fertilizer-N dose (100 kg N ha^{-1} crop^{-1}) thereafter. Low and high N input fields were in close proximity to each other and received similar management practices except for the N fertilization.

Soil samples were collected when rice plants were at just before panicle initiation stage, when all the N fertilizer applications were completed. A wet soil sampler was used to collect soil (0-20 cm) from each site and soil was transported on ice immediately after collection and homogenized in the laboratory before taking four subsamples. The first subsample was used for multiple laboratory incubation experiments soon after sampling. The second subsample was used to determine mineral N (NH_4^+ + NO_3^-) in fresh soil. The third subsample was oven dried (40 ºC) for soil physicochemical characterization, and the fourth subsample was stored in -80 ºC for microbial DNA extraction. Chemical characterization of the soil samples was based on the methods for Australasian soils (Rayment and Lyons, 2011). Soil organic carbon (SOC) was determined after removing inorganic carbon (Yang et al., 2017). Soil particle size analysis was done using the hydrometer method (Gee and Bauder, 1986). Physicochemical characteristics of the selected paddy soils are presented in Table 1.

Table 1. Physicochemical properties of soils collected from long-term low and high N input rice paddies.

Sampling Sites	Soil texture	SOC	TN	pH	SOC:NO_3^- ($\times 10^3$)	NO_3^-	NH_4^+	Total P	S	Mn
		g kg^{-1}				mg kg^{-1}				
Low N input sites										
Kungyangon	Clay	18.41	1.44	5.63	18.67	0.99	20.84	115	326	137
Taungoo	Clay loam	9.86	0.75	5.81	18.63	0.53	6.40	182	111	178
Pyinmana	Sandy clay loam	6.68	0.55	7.32	10.67	0.63	6.16	99	58	148
High N input sites										
Kungyangon	Clay	17.25	1.39	5.71	13.25	1.31	24.22	94	305	164
Taungoo	Clay loam	9.73	0.82	5.62	8.38	1.21	17.17	151	133	185
Pyinmana	Sandy clay loam	8.77	0.67	7.13	7.18	1.22	8.71	144	86	178

Measurement of DNRA, denitrification and N_2 fixation rates

The rates of DNRA and denitrification were determined using the method as described in Trimmer and Nicholls (2009). Briefly, triplicate vials were prepared for each time point, i.e. 0, 1.5, 3, 6, 9 and 12 h, by transferring approximately three grams of fresh paddy soil (~2 g dry weight) to each of 12 ml screwed topped Exetainer vials (Labco) and then filling the vials with helium (He) gas purged water. The vials were then pre-incubated inside an anoxic glove bag for 24 h to deplete soil NO_3^- and any trapped O_2. The duration of pre-incubation was determined from preliminary NO_3^- depletion trials. The vials (except for controls) were injected with a He purged solution of $^{15}NO_3^-$ (99 ^{15}N atom%) plus unlabelled NH_4^+ and incubated in the dark inside an anoxic glove bag. The amount of $^{15}NO_3^-$ addition was similar to that in the soils before the pre-incubation (Table 1) and the $^{15}NO_3^-$ concentration was maintained at a similar level throughout the incubation period by adding $^{15}NO_3^-$ from a stock solution which was determined from the preliminary NO_3^- depletion trials. Unlabelled NH_4^+ was added at the rate of 0.5 µg NH_4^+-N g^{-1} soil to prevent the assimilation of $^{15}NO_3^-$ into microbial biomass (Rice and Tiedje, 1989; Silver et al., 2001). Thus, any $^{15}NH_4^+$ formation in the vials can be attributed to the $^{15}NO_3^-$ transformed through the DNRA process. Microbial activity in the triplicate vials at each time point was stopped by injecting 200 µl of 7 M $ZnCl_2$.

Production of $^{29}N_2$ and $^{30}N_2$ at each timepoint was determined by isotope ratio mass spectrometer coupled with gas chromatography (GC-IRMS) with He as a carrier gas. Thereafter, NH_4^+ in the soil slurry was extracted with 2M KCl (1:1 soil:solution). The extracted solution (7 mL) was transferred to a new 12 mL screwed topped Exetainer vial and the headspace was flushed with He gas. The Hypobromite method (Trimmer and Nicholls, 2009) was used to convert $^{15}NH_4^+$ in the extracted solution inside the vials to $^{29}N_2$ and $^{30}N_2$. DNRA and denitrification rates were calculated from excess production of $^{29}N_2$ and $^{30}N_2$ with time (Fig. S1) (Trimmer and Nicholls, 2009). Anaerobic ammonium oxidation (anammox), which also produces N_2, was tested using $^{15}NH_4^+$ + unlabelled NO_3^- in another set of vials (Trimmer and Nicholls, 2009). However, the anammox activity was under the detection limit. Therefore any N_2 production was considered as a denitrification product during the rate calculation.

Diazotrophic N_2 fixation was determined using the acetylene reduction assay (ARA) and calibrated with $^{15}N_2$ uptake (Barron et al., 2009). Triplicate samples of approximately 50 g fresh soil were transferred to triplicate 500 mL screwed topped glass vials equipped with gas sampling ports. Approximately 10% of the headspace in the vials (except in the control vials) was replaced with acetylene gas and incubated at 25 ºC after shaking briefly. Exactly 8 mL of headspace gas from triplicate vials was sampled at 0 h, 3 h, 6 h, 9 h and 12 h and transferred to new 6 mL Exetainer vials (Labco) and analysed for ethylene (C_2H_4) production using gas chromatograph mass spectrometer with a flame ionization detector (GC-MS-QP2010, Shimadzu). The C_2H_4 production rates were calculated based on linear production with time. To determine the conversion ratio for C_2H_4:N_2, we incubated each of the paddy soils with $^{15}N_2$ gas (~10 $^{15}N_2$ atom%) in parallel with acetylene gas. The conversion ratio was then calculated from the C_2H_4 produced to $^{15}N_2$ uptake rates (Barron et al., 2009; Keuter et al., 2014). The conversion ratios for different paddy soils were between 3.5 and 5.5.

Denitrification and DNRA rates against varying OC:NO_3^- ratios

To test the effect of higher soil NO_3^- concentration and different OC:NO_3^- ratios on the NO_3^- partitioning between DNRA and denitrification, we incubated the high N input Taungoo soil anaerobically and amended with 5 µg $^{15}N\text{-}NO_3^-$ g^{-1} soil and different levels of acetate (CH_3COO^-) to achieve OC:NO_3^- ratios of 5, 10, 20, 40 and 80. The high amount of NO_3^- was added to mimic the situation that is generally observed for a few days immediately after fertilization in rice paddies (Aulakh et al., 2001). Acetate was used because it is the most important anaerobic metabolite in submerged paddy soils (Hori et al., 2010). Incubation vials were prepared in the same way as explained above for the determination of DNRA rate measurement, except for the amount of unlabelled NH_4^+ (1 µg N g^{-1} soil) used to inhibit $^{15}NO_3^-$ immobilisation. Triplicate vials were prepared for each time point (0, 3, 6 and 12 h) for each OC:NO_3^- ratio. $^{29}N_2$, $^{30}N_2$ and $^{15}NH_4^+$ concentrations were determined at each time point after stopping the microbial activity by adding 200 µL of 7 M $ZnCl_2$ into the vials (Fig. S2). Denitrification and DNRA rates were calculated as described above.

Quantitative PCR analysis

All the N-cycling genes (Table S1) were amplified using 5 µl of 5x MyTaq Reaction Buffer (15 mM $MgCl_2$, 5 mM dNTPs; Bioline), 0.5 µl each of reverse and forward primers (10 µM), 0.25 µl of MyTaq DNA polymerase (5 U $µl^{-1}$; Bioline) and 2 µl of (10 ng $µl^{-1}$) DNA template. The thermal cycling conditions for each gene were as described in the cited references in Table S1, except for the *nrfA* gene which was as described in Song et al. (2014). Standards for qPCR were prepared from serial dilution of the plasmids carrying positive clones. The abundances of these N-cycling genes were quantified using the Bio-Rad CFX96 optical real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The 20 µl qPCR reaction contained 10 µl of 2× SensiMix SYBR No-ROX (Bioline), 0.5 µl of each primer (10 µM) and 2 µl (10 ng $µl^{-1}$) of DNA template.

Statistical analysis

Rates of production of $^{29}N_2$ and $^{30}N_2$, $^{15}NH_4^+$ or ethylene gas were compared using a linear model (“lm” function in R). Analysis of variance (ANOVA) and Tukey pairwise comparison tests were used to analyze the statistical difference in gene abundances with the significance level set at 95% using Minitab 18. Pearson’s correlation matrix was created to compare the relationships between the N transformation rates, the relevant gene abundance and the soil parameters. The data were log transformed whenever necessary before use for the parametric statistical test.

Results

Rates of N transformations and the related gene abundances

N_2 fixation (calibrated against $^{15}N_2$) was higher ($P < 0.05$) in the long-term low N (1.49-2.08 µg N g^{-1} soil day^{-1}) than in the high N (0.47-0.72 µg N g^{-1} soil day^{-1}) input rice paddies (Fig. 1a). Similarly, DNRA rates were significantly higher ($P < 0.05$) in the long-term low N input rice paddies (1.51-2.67 µg N g^{-1} soil day^{-1}) than in the high N input rice paddies (0.40-0.65 µg N g^{-1} soil day^{-1}; Fig. 1b). Denitrification rates were significantly lower ($P < 0.05$) in the long-term low N input rice paddies (0.18-0.91 µg N g^{-1} soil day^{-1}) than in the high N input rice paddies (0.44-1.57 µg N g^{-1} soil day^{-1}, Fig. 1c). Generally, the *nrfA* and *nifH* gene abundances were higher and the *nosZ* and *narG* gene abundances were lower in the long-term low N input compared to high N input rice paddies but the differences were not always statistically significant (Table 2). DNRA, denitrification and N_2 fixation rates showed significantly positive correlation with the *nrfA* ($R = 0.81$, $P < 0.001$, n = 18), *nosZ* ($R = 0.60$, $P < 0.01$, n = 18) and *nifH* ($R = 0.54$, $P = 0.02$) gene abundances, respectively.

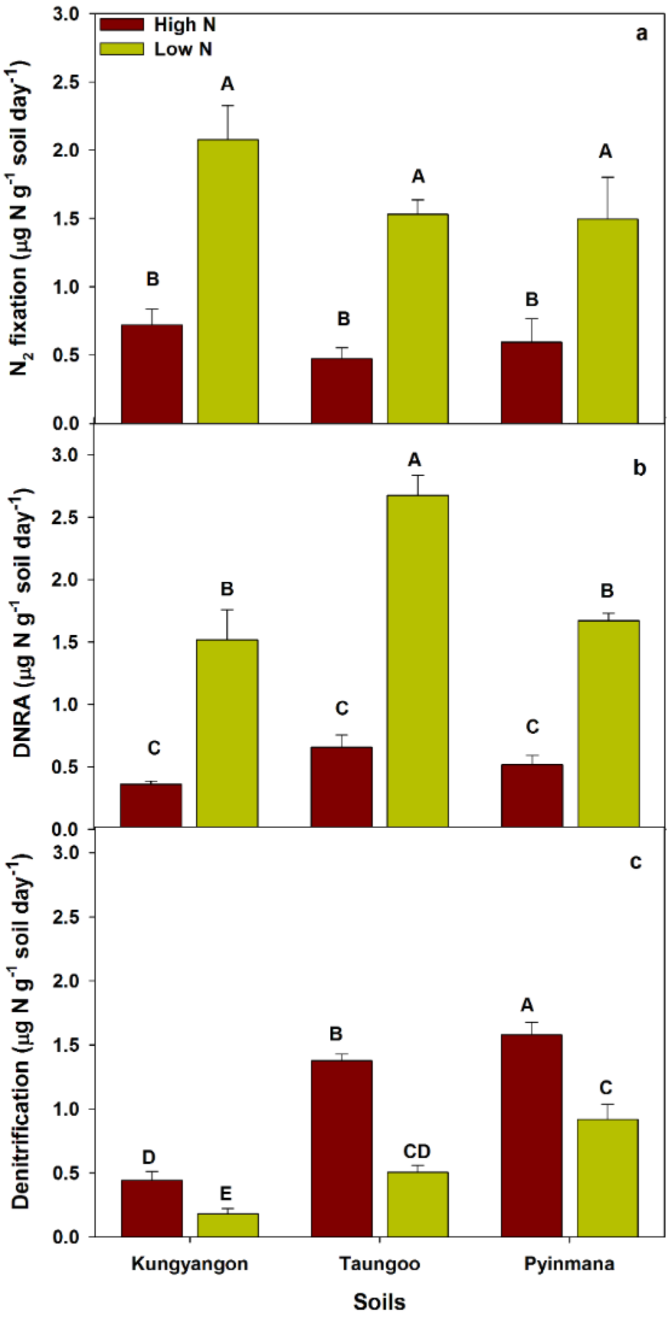

Fig. 1. Rates of N transformations in long-term low and high N input rice paddies (μg g^{-1} soil day^{-1}): (a) N_2 fixation rates, (b) DNRA rates and (c) denitrification rates. Bars represent the means of the N transformation rate with ± 1 standard error of the slope of the linear regression. Bars with different letters on top are significantly different ($P < 0.05$).

Table 2. Gene copy numbers g^{-1} dry soil from long-term low and high N input rice paddies.

Sampling Sites	*nrfA* (×10^8)	*nifH* (×10^7)	*nosZ* (×10^7)	*narG* (×10^6)
Low N input sites				
Kungyangon	4.61 (±0.68) b	6.47 (±1.09) b	0.64 (±0.09) c	6.88 (±0.62) b
Tuangoo	9.00 (±1.19) a	13.4 (±2.88) a	1.12 (±0.07) bc	1.90 (±0.16) c
Pyinmana	5.08 (±0.43) ab	5.96 (±0.35) bc	1.18 (±0.07) bc	1.18 (±0.23) c
High N input sites				
Kungyangon	3.87 (±0.65) b	2.33 (±0.41) cd	0.79 (±0.19) bc	9.64 (±0.79) a
Tuangoo	4.27 (±0.28) b	2.92 (±0.72) bcd	2.29 (±0.52) a	7.67 (±0.17) ab
Pyinmana	0.73 (±0.35) c	1.88 (±0.10) c	1.43 (±0.16) b	2.70 (±0.19) c

Numbers with different letters within a column are significantly different ($P < 0.05$).
Numbers within parenthesis are ± 1 standard error of the mean.

Soil properties and the N transformation processes

Results from the Pearson's correlation test conducted between soil properties and the N transformation processes are presented in Table 3. There was no significant relationship between DNRA, N_2 fixation and the related gene abundance, and SOC content ($P > 0.05$). However, denitrification rates and the *nosZ* gene abundances showed a significant negative correlation with the SOC ($P < 0.05$). DNRA was significantly negatively correlated with soil NO_3^- ($R = 0.88$, $P < 0.001$; n =18) and positively correlated with the SOC:NO_3^- ratio ($R = 0.62$, $P < 0.01$; n =18). Denitrification had no significant correlation with soil NO_3^- and had a significant negative correlation with the SOC:NO_3^- ratio ($R = -0.77$, $P < 0.001$; n =18). The *nrfA* and *nosZ* gene abundances also showed similar relationships with soil properties as the DNRA and denitrification rates, respectively. N_2 fixation and the *nifH* gene abundance showed significant negative correlations with soil NO_3^- and positive correlations with the SOC:NO_3^- ratio.

Table 3. Results from the Pearson's correlation test conducted between the N transformation rates and the related gene abundances, and soil parameters (n = 18).

N transformations	Soil Parameters					
	SOC		NO_3^-		SOC:NO_3^-	
	R	P	R	P	R	P
DNRA	ns	ns	-0.88	<0.001	0.62	0.006
nrfA	ns	ns	-0.71	0.001	0.59	0.01
Denitrification	-0.64	0.004	ns	ns	-0.77	<0.001
nosZ	-0.49	0.038	ns	ns	-0.45	0.064
N_2 fixation	ns	ns	-0.48	0.044	0.59	0.009
nifH	ns	ns	-0.79	<0.001	0.63	0.005

Significance level was set at $P < 0.05$. ns refers to non-significant results.

Effects of varying OC:NO_3^- ratios on the NO_3^- partitioning

During the investigation of the effect of higher NO_3^- and varying OC:NO_3^- ratios on the NO_3^- partitioning, we found that DNRA rates increased significantly ($P < 0.05$) when the OC:NO_3^- ratio changed from 5 to 20, and declined when the ratio changed from 20 to 40 and 80 (Fig. 2). The denitrification rate declined gradually with increasing OC:NO_3^- ratio. The NO_3^- consumption by DNRA was lower than that by denitrification when the OC:NO_3^- ratio was 5 but it surpassed denitrification thereafter (Fig. 2).

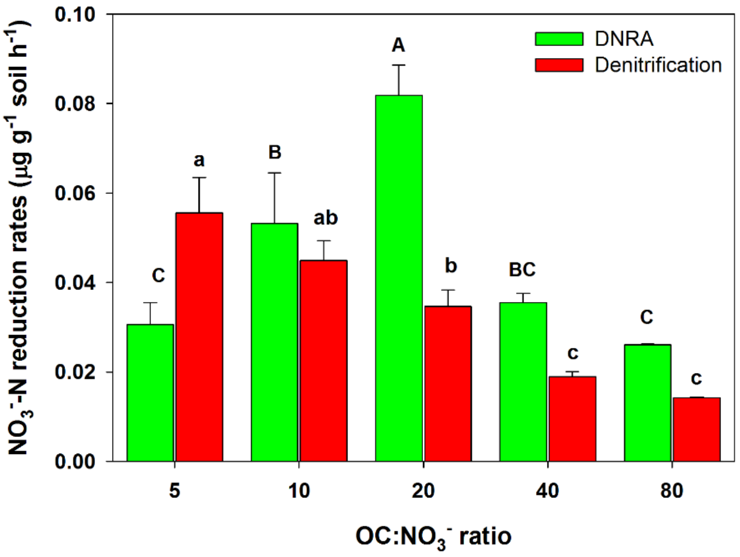

Fig. 2. Rate of reduction of NO_3^- by DNRA and denitrification with addition of 5 µg ^{15}N-NO_3^- g^{-1} soil and 25, 50, 100, 200 and 400 µg acetate g^{-1} soil. Bars represent the means of the NO_3^- reduction rate ± 1 standard error of the slope of the linear regression. Bars with different uppercase and lowercase letters represent significantly different DNRA and denitrification rates, respectively ($P < 0.05$).

Discussion

Balance between N retention and loss in low and high N input rice paddies

Microbial N_2 fixation in long-term high N input rice paddies was less than 40% of that in low N input rice paddies in this study. The bi-functional enzyme GlnD encoded by the *glnD* gene in diazotrophs plays a crucial role in sensing the N level within the cell and influences the N_2 fixing *nifH* gene activity. When the diazotrophic cell is N limited the Glnd enzyme is uridylated, triggering the *nifH* gene for N_2 fixation (Jonsson and Nordlund, 2007). When N is applied to soils, diazotrophs can utilize fertilizer-N and become N sufficient and the *nifH* gene may remain inactive (Jonsson and Nordlund, 2007). Therefore, both short-term and long-term N input in rice paddies reduces N_2 fixation (Valiente et al., 1997; Kyaw et al., 2005). We observed higher NO_3^- and NH_4^+ in high N input than in low N input systems in this study. These inorganic N are known to supress nitrogenase activity through physiological feedback inhibition (Mills et al., 2004). Our finding shows that by converting low input system to high input system, the top 20 cm paddy soil can lose ~2 kg N ha^{-1} day^{-1} from N_2 fixation which needs to be recompensed through fertilizer-N input.

In agreement with our hypothesis, the consumption of NO_3^- was dominated by DNRA in low N input rice paddies, where DNRA rates were up to 4-8 times higher than those that have been observed in other arable and grassland systems (Yang et al., 2017; Putz et al., 2018), and were similar to those in humid forest soils (Silver et al., 2001; Huygens et al., 2008). However, denitrification dominated NO_3^- consumption in the high N input rice paddies, being more than double the DNRA rates. Microbes have higher growth yields during NO_3^- reduction to NH_4^+ compared to reduction to N_2, so they perform DNRA rather than denitrification in NO_3^- limited conditions (Kraft et al., 2014; Van Den Berg et al., 2015). Soil NO_3^- concentration and denitrification potential of rice paddies rises after long-term N fertilization (Chen et al., 2010; Chen et al., 2012). In such conditions, there is little incentive for microbes to perform DNRA which requires eight electrons as opposed to the six required for denitrification (Kraft et al., 2014). In addition, more NO_3^- is partitioned to denitrification when the NO_3^- concentration rises as the cytochrome *c* nitrite reductase in DNRA bacteria cannot keep up kinetically with the nitrate reductase (Schmidt et al., 2011; Kraft et al., 2014). Previous studies have shown that rice paddies that are under long-term N fertilization have significantly higher NO_3^- concentration compared to rice paddies without N application (Zhong et al., 2007; Chen et al., 2010). Nitrogen fertilization not only increases the soil NO_3^- concentration but also reduces the labile OC pool by increasing the decomposition and mineralization activity (Neff et al., 2002; Cusack et al., 2011; Dou et al., 2016). This can change the balance between the electron donor and acceptor and ultimately affect the NO_3^- partitioning.

Environmental determinants of the NO_3^- partitioning between DNRA and denitrification

Among the tested soil properties, the SOC:NO_3^- ratio played the most crucial role in the NO_3^- partitioning between DNRA and denitrification. This is in line with findings from chemostat experiments, which indicated that NO_3^- or OC alone cannot explain the partitioning of NO_3^- between DNRA and denitrification (Kraft et al., 2014; Yoon et al., 2015). Studies using pure bacterial culture have demonstrated that a low OC:NO_3^- ratio promotes denitrification, while DNRA dominates at higher ratios (Kraft et al., 2014; Van Den Berg et al., 2015; Yoon et al., 2015). We observed higher SOC:NO_3^- ratios in the low N input than in the high N input rice paddies (Table 1) and DNRA dominated in the former and denitrification in the latter. Denitrifiers have higher affinity for labile OC, i.e. electron donor, compared to ammonifiers, so denitrification dominates over DNRA in labile OC limited conditions (Kraft et al., 2014; Van den Berg et al., 2016), whereas abundant electron donors at higher OC:NO_3^- ratios but limited electron acceptors provide a competitive advantage to ammonifiers over denitrifiers (Yoon et al., 2015; Van den Berg et al., 2016).

Our OC:NO_3^- ratio manipulation experiment (Fig. 2), the first of a kind in soil ecosystem, confirmed that the OC:NO_3^- ratio plays a crucial role in the partitioning of NO_3^- between DNRA and denitrification. Our findings imply that N retention through DNRA can be improved when there are higher soil NO_3^- concentrations, as a consequence of N fertilization, by manipulating the soil organic carbon pool. Our result, however, contradicts with a few observation that has been observed in forest and pasture soils (Sotta et al., 2008; Friedl et al., 2018) (also see literature summary in Table S2). This contradiction is mainly driven by the high SOC content (>4%) in those studies where slight increase in NO_3^- concentration would still mean relatively high electron donor to electron acceptor ratio. Such a high SOC level are highly unlikely in paddies under continuous rice, so the SOC:NO_3^- ratio management in continuous rice systems can be an important strategy to retain N. Our finding suggests that synchronizing N fertilization with high SOC:NO_3^- ratios through organic matter input can thus maintain soil internal N retention pathways and reduce N loss.

In summary, microbial N cycling strategies in low N input rice paddies in Myanmar have evolved to efficiently add and retain N within the system, allowing minimal N loss (Fig. 3). Our findings highlight the presence of unique microbial N regulation strategies in natural ecosystems which maintain and sustain the primary productivity without external N input (Ortiz et al., 2014; Michiels et al., 2017). Low N input in rice paddies in Myanmar is mainly due to unaffordability and partly due to unavailability of chemical fertilizer in the country (Denning et al., 2013). High N input in rice paddies is a common practice to achieve higher rice yields and increase profits for farmers, and the practice is inevitable in Myanmar. But it has a negative effect on the natural microbial N retention strategies and ultimately on the environment. The reactive N (N_r, all N species except N_2) index, which is N_r lost per kg food produced, of the rice production system in Myanmar was found to be one third of the global rice production N_r index (Chen et al., unpublished). Therefore, creating a market premium for ~3.5 million tonnes of rice that is exported annually from Myanmar which has a lower N_r footprint can provide incentives for farmers to continue existing low input practice.

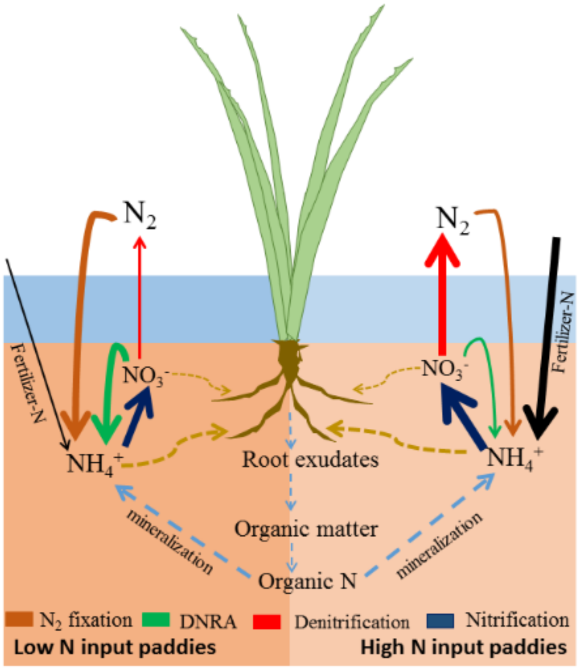

Fig. 3. Illustration of the N pathways in low N input (left) and high N input (right) paddy systems. Solid arrows with different colours represent different N pathways. The thickness of the solid arrows represents the relative magnitude of the N pathways. DNRA and N_2 fixation dominate N transformations in low N input paddies, allowing for minimal N loss, whereas denitrification dominates in high N input paddies.

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Supporting information

This article contains supporting information.

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Chapter 5

Dissimilatory nitrate ammonification helps maintain nitrogen nutrition in resource limited rice paddies in Myanmar

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Supplementary information

Table S1. Primers used for quantitative PCR assay.

Targeted Genes	Primers	Sequence (5’ - 3”)	Reference
nrfA	nrfAF2aw	CARTGYCAYGTBGARTA	(Welsh et al., 2014)
	nrfAR1	TWN GGC ATR TGR CAR TC	
nosZ	nosZ2F	CGCRACGGCAASAAGGTSMSSGT	(Henry et al., 2006)
	nosZ2R	CAKRTGCAKSGCRTGGCAGAA	
narG	narG-F	CGCCSATYCCGGC SATGTC	(Bru et al., 2007)
	narG-R	GAGTTGTACCAGTCRGCSGAY TCSG	
nifH	nifHF	AAAGGYGGWATCGGYAARTCCACCAC	(Rösch and Bothe, 2005)
	nifHRc	TGGGCYTTGTTYTCRCGGATYGGC AT	

Thermal cycling conditions used for qPCR was as in the cited references except for the *nrfA* gene which was as in Song et al. (2014).

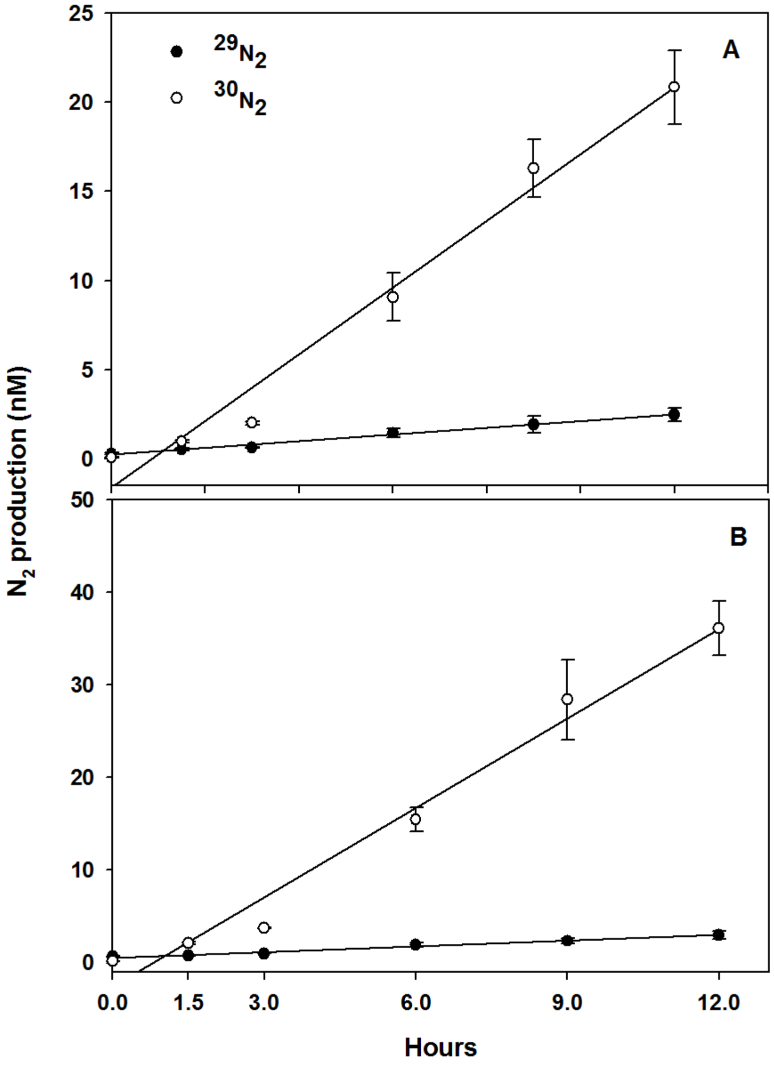

Fig. S1. Production of $^{29}N_2$ and $^{30}N_2$ with time after adding $^{15}NO_3^-$ in paddy soils inside the anaerobically incubated vials. Low N fertilized paddy soil (A) and high N fertilized paddy soil (B) collected from Pyinmana. Error bars represent ± 1 standard error of mean.

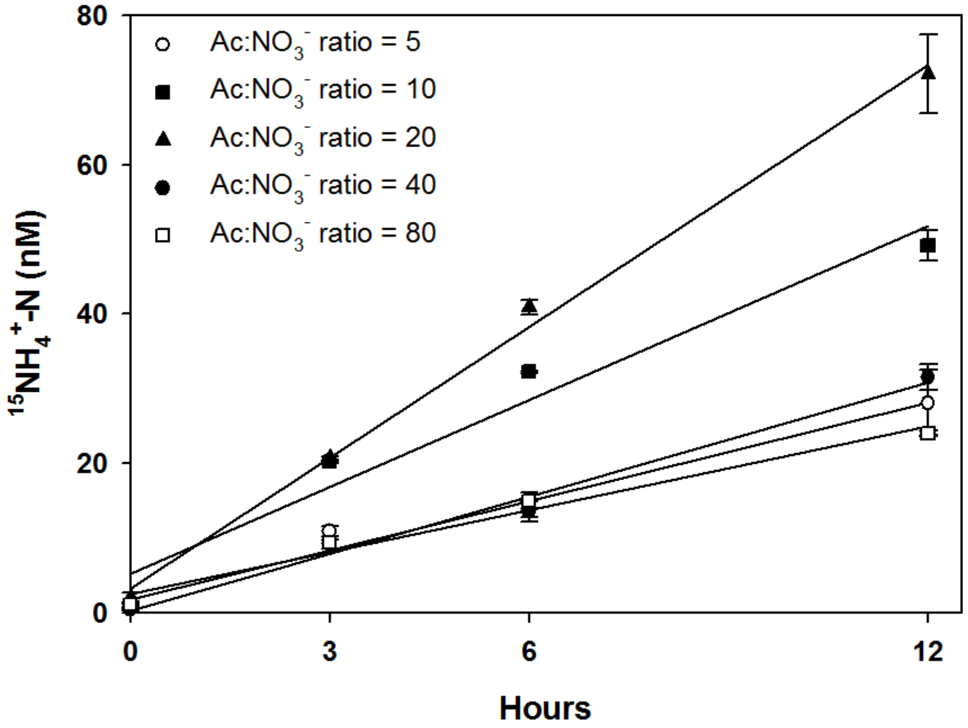

Fig. S2. Rate of production of $^{15}NH_4^+$ in anaerobically incubated paddy soil from Taungoo with 5 µg ^{15}N-NO_3^- g^{-1} soil and 25, 50, 100, 200 and 400 µg CH_3COO^- g^{-1} soil. Error bars represent ± 1 standard error of mean.

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CHAPTER 6: Discussion and Conclusions

Rice paddies have shown maintained N status, stable N supply to rice plants and sustained yields without fertilizer-N (Dobermann et al., 2003; Pampolino et al., 2008; Bi et al., 2009). This implies that N-cycling microbes in un-fertilized rice paddies have evolved and adapted to add and retain N efficiently within the system. On the other hand, rice paddies which receive high fertilizer-N doses have high N loss and low N use efficiency (Aulakh et al., 2001). Such a high N input practice not only increases the economic loss and poses environmental risk but also unbalances natural microbial N-cycling processes in soils (Mills et al., 2004; Chen et al., 2008). A recent study on the effect of fertilizer-N on microbial communities has shown that continuous high N addition promotes the abundance of microbes which are adapted to nutrient rich soils and has the opposite effect on the oligotrophic microbes (Fierer et al., 2012). This effect of N addition can have a negative effect on N retention in paddy soils.

6.1 Nitrogen addition decreases nitrate retention

The background study in Chapter 3 showed that DNRA activity was detected in rice paddies more than a decade ago by Yin et al. (2002), but there were no studies examining the effect of N fertilization on NO_3^- retention through DNRA and the *nrfA* gene abundance.

This study found that DNRA plays a significant role in retaining loss-prone NO_3^- as NH_4^+ in a fertilizer-N omission treatment. DNRA transformed 1.09-1.40 kg NO_3^--N ha^{-1} day^{-1} to NH_4^+ in the top 20 cm when fertilizer-N was omitted, whereas only 0.83-1.04 kg NO_3^--N ha^{-1} day^{-1} was transformed when 150 kg urea-N ha^{-1} was added. When N was added, the NO_3^- partitioning to denitrification and N loss as N_2 increased from 1.17-1.63 kg N ha^{-1} day^{-1} to 1.46-2.05 kg N ha^{-1} day^{-1}. There was no significant effect of the N omission treatment on the *nrfA* gene abundance. Similar effects of N input on DNRA rates have also been observed in temperate arable soils (not paddy soils), where N input reduced the DNRA rate (Schmidt et al., 2011). However, Schmidt et al. (2011) added labile OC and reducing compound to fuel the DNRA activity and did not quantify denitrification rates, and therefore, lack the information on the actual potential of the soil to undertake DNRA and the NO_3^- partitioning between DNRA and denitrification. Findings from Chapter 3 confirmed that the omission of fertilizer-N in rice paddies reduces N loss by denitrification due to the higher competition with DNRA for NO_3^- compared to N fertilized rice paddies. This indicated that DNRA may play an important role in maintaining N nutrition in long-term no or low N input rice paddies. To test this hypothesis, experiments were conducted to examine microbial N retention and loss in the long-term no or low and high N input rice paddies in Chapters 4 and 5.

6.2 Microbes have efficient N conservation strategies in long-term low N input rice paddies

N_2 fixation has been found to be coupled with N loss by denitrification and create N deficient zones in marine and sediment ecosystems (Deutsch et al., 2007; Korth et al., 2014). Therefore, N_2 fixation alone cannot fully explain maintained N nutrition of rice paddies, where there are favourable conditions for N loss by denitrification. DNRA may be playing a key role in low N input rice paddies by retaining loss-prone NO_3^- as NH_4^+ and thus limiting N loss by denitrification and supporting maintained N nutrition of the system. However, little is known about this. In addition, little is known about the effect of long-term N fertilization regimes on the DNRA process and the partitioning of NO_3^- between DNRA and denitrification.

This study examined microbial N conservation and loss processes in several rice paddies under long-term no or low and high N input systems in Myanmar and Australia (Chapters 4 and 5). Considering an average soil bulk density of 1.3 g cm^{-3} on (20 cm soil depth) of the selected paddy soils, N_2 fixation added a minimum of 4 kg N ha^{-1} day^{-1} in long-term low N input systems compared to less than 1.8 kg N ha^{-1} day^{-1} in high N input systems during tillering to panicle initiation stage of rice. Around 60% of the available NO_3^- was transformed to NH_4^+ by DNRA, while minimal NO_3^- (<15%) was lost as N_2 from low N input paddies. In high N input paddies, only 10% of the available NO_3^- was reduced to NH_4^+, whereas around 30% was lost as N_2. This study did not examine the relative contribution of N_2 fixation to the NO_3^- pool in paddy soils. However, assuming that the fixed N_2 contributes around 49-65% to the soil NO_3^- pool, as in the marine and sediment ecosystems (Halm et al., 2009; Korth et al., 2014), denitrification N loss would be much higher without the competition for NO_3^- from DNRA in low N input systems. It is known that mineral N (NH_4^+ and NO_3^+) have an inhibitory effect on nitrogenase activity (Mills et al., 2004) and both NO_3^- and NH_4^+ have similar inhibitory effect on N_2 fixation (Knapp, 2012, review). Therefore, NH_4^+ produced through reduction of NO_3^- by the DNRA process should have limited effect on the amount of N_2 fixed.

Nutrient inefficient microbes have higher fitness in nutrient rich homogenous environment compared to nutrient efficient microbes (Roller and Schmidt, 2015). Therefore, copiotrophic microbes are favoured over oligotrophic microbes, as a consequence of continuous high fertilizer-N input (Fierer et al., 2012) These findings imply that the N-cycling microbes in low N input systems have evolved to efficiently recycle N, resulting in maintained soil N status and sustained rice production. However, there is a little incentive for microbes in long-term high N input rice paddies to conserve N (discussed in section 6.3). These findings are similar to those in non-agricultural oligotrophic systems, where microbial N conservation strategies support primary productivity (Ortiz et al., 2014).

6.3 Environmental determinants of the competition between DNRA and denitrification

Soil NO_3^- and the soil OC:NO_3^- ratio in rice paddies were the most important determinants of the NO_3^- partitioning between DNRA and denitrification. A significantly positive correlation between the soil OC:NO_3^- ratio and DNRA, and a significantly negative correlation between the soil OC:NO_3^- ratio and denitrification was observed across all the rice paddies examined in this study. Increased NO_3^- production in rice paddies, as a consequence of high N input, reduced the soil OC:NO_3^- ratio of the paddy soils. This condition promoted NO_3^- partitioning to denitrification and N loss. This confirmed that the change in the abundance of electron donors and electron acceptors, as a consequence of fertilizer-N input, has an effect on the amount of NO_3^- being lost and retained in the soils. The OC:NO_3^- manipulation experiment in Chapter 5 confirmed that increasing the OC:NO_3^- ratio, by adding a labile OC source in the soil with high NO_3^- concentration, improves NO_3^- retention. This finding provides an important perspective for improving NO_3^- retention in high N input systems. A recent chemostat experiment demonstrated similar findings of higher OC:NO_3^- ratio favouring NO_3^- partitioning to DNRA (Kraft et al., 2014; Van Den Berg et al., 2015; Yoon et al., 2015). However, Friedl et al. (2018) did not find a significant relationship between the soil OC:NO_3^- ratio and DNRA or denitrification in pasture soils with >4% SOC content. But the DNRA rates were still mostly higher than denitrification rates in that study. This finding implies that DNRA rate in soils with very high OC content may get less affected by a slight increase in NO_3^- concentration, which was confirmed by the OC:NO_3^- ratio manipulation experiment in this study (Chapter 5). However, very high OC content is highly unlikely under long-term paddy systems. Therefore, maintaining higher OC in paddy systems during fertilizer-N input can have a positive effect by reducing NO_3^- loss and improving N retention.

6.4 Future directions and recommendations

This study relied on laboratory incubation experiments to examine the rates of N conservation and loss processes. Therefore, future studies should focus on developing techniques for in situ measurement, which could further strengthen our understanding of the processes. This study, however, provides highly reliable measurement of the process rates because all the laboratory incubation experiments were conducted immediately after soil sampling and no external OC sources were added, which has been commonly done in many other studies, to fuel N transformation processes. But this study only represents the rate of N transformation processes in paddy soils that occur between tillering and panicle initiation stages of the rice plant, as the paddy soils were collected during this period. The environmental conditions in rice paddies changes with change in the growth stages of the rice plant. For example, paddy soils receive higher amount of OC from root exudates during the later growth stages of the rice plant compared to initial stages (Zhang et al., 2017). In, addition, the rice plant influences environmental conditions in rhizosphere by supplying O_2. This influences nitrification activity and the NO_3^- availability in paddy soil. Therefore, examining the process rates in situ and throughout the rice growing season can further our understanding of the dynamics of the processes in rice paddies.

Use of fertilizer-N and improved rice varieties helped to drastically improve rice yields globally during the 1960s (Cassman, 1999). However, fertilizer-N use efficiency (NUE) of rice production has rarely exceeded 50% (Ju et al., 2009; Ussiri and Lal, 2013). In this context, managing the SOC:NO_3^- ratio to retain fertilizer-N can improve the NUE of rice paddies. There is no research examining the effect of varying SOC:NO_3^- ratio on the NUE of rice production. Results from this study indicates that increasing the SOC:NO_3^- ratio in fertilized rice paddies can maintain the DNRA rate. Therefore there is a need for more research on the relationship between the SOC:NO_3^- ratio and the NUE of rice production.

Long-term high fertilizer-N input has demonstrated an increase in relative abundance of copiotrophic microbial taxa and the opposite effect on oligotrophic microbial taxa (Fierer et al., 2012). Microbes adapt and evolve according to resource availability in an ecosystem (Roller and Schmidt, 2015). The free energy yield per unit of resource consumed is one of the most important determinants of fitness a microbe to an ecosystem (Linton and Stephenson, 1978; Roller and Schmidt, 2015). Microbes grow rapidly but inefficiently in resource rich ecosystems, whereas slow and efficient growth is favoured in resource poor ecosystems (Pfeiffer et al., 2001). Therefore, microbes with efficient growth are favoured in ecosystems with no external inputs or with limited resources (Roller and Schmidt, 2015). Microbes in un-fertilized agriculture systems probably have evolved towards efficiency rather than rapid growth. Denitrification is energy inefficient compared to DNRA (Strohm et al., 2007; Van Den Berg et al., 2015), and the resource limitation in un-fertilized agricultural systems could favour prevalence of ammonifiers over denitrifiers. In the context of Myanmar rice paddies, high fertilizer-N input is inevitable and probably important to achieve higher rice yields to meet the ever growing global demand (Ray et al., 2013). High fertilizer-N input creates resource rich homogenous environments in paddy soils, and in such environments growth of the energy inefficient microbes, such as denitrifiers, could be favoured (Roller and Schmidt, 2015). Also increasing N deposition in agricultural ecosystems (can be ~50 kg N ha^{-1} in some Chinese regions, on top of fertilizer-N input, He et al., 2007; Ju et al., 2009), can also add to a shift in microbial community structure. Therefore, a detailed study on the effect of different rates of fertilizer-N input on microbial community in rice paddies should be done.

6.5 Conclusions

This study, for the first time, comprehensively and simultaneously examined N transformation pathways that are directly involved in N conservation and loss in rice paddies and how these processes are affected by different N fertilization regimes and soil OC and NO_3^-. Results from this study clearly show that fertilizer-N input in rice paddies negatively affects microbial N conservation processes, i.e. N_2 fixation and DNRA, and enhances N loss by denitrification. This study demonstrated that it is not only N_2 fixation that plays a key role in N nutrition of un-fertilized rice paddies, but DNRA plays an equally important role by retaining the majority of the loss-prone NO_3^- as NH_4^+ and limiting N loss by denitrification. Nitrogen fertilization regimes did not always show a clear effect on the *nrfA* gene abundances but there was a tendency of a higher *nrfA* gene abundance in long-term low N input rice paddies. Anammox was not a significant N_2 producer in the investigated rice paddies. More work is needed to quantify the contribution of DNRA to the NH_4^+ taken up by the rice plant, to understand the contribution of DNRA to the NUE of rice production. Less is known about the contribution of DNRA to N retention in arable soils were NO_3^- is the predominant form of mineral N. So, further studies of DNRA in a range of agricultural soils and cropping systems is needed, as well as strategies to enhance DNRA in these situations.

The SOC:NO_3^- ratio played the most crucial role in determining the pathway of NO_3^- reduction. Higher SOC:NO_3^- ratio always had a positive correlation with DNRA and a negative correlation with denitrification. Lower SOC:NO_3^- ratios, as a consequence of high N input, favoured denitrification and enhanced N loss from rice paddies. The OC:NO_3^- ratio manipulation experiment in this study confirmed that DNRA is the dominant NO_3^- reducer at higher OC:NO_3^- ratios. This finding suggested that N retention by DNRA can be improved in high N input rice paddies by synchronising the N fertilization with higher OC:NO_3^- ratio through labile OC addition to soils.

In summary, this study shows that microbes function efficiently to maintain N nutrition in resource-limited environments, whereas in N cycling is dominated by less efficient microbes in resource-rich environments. DNRA, together with N_2 fixation, plays an important role in N retention in low N input rice paddies and is negatively affected by fertilizer-N input. Maintaining a higher SOC:NO_3^- ratio can improve N retention by DNRA in high N input rice paddies but this may still have a negative effect on N_2 fixation. This study highlights the unique microbial strategies which enable maintenance of N nutrition in resource-limited paddy systems.

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Dissimilatory nitrate reduction to ammonium, denitrification and anaerobic ammonium oxidation in paddy soil

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Abstract

Nitrogen (N) is the most important yield-limiting nutrient for rice production. Flooding of rice paddies for an extended period of time creates anoxic conditions in soil which can favour a simultaneous occurrence of several microbial N transformation processes, such as dissimilatory nitrate (NO_3^-) reduction to ammonium (NH_4^+) (DNRA), denitrification and anaerobic NH_4^+ oxidation (anammox). Little is known about the role of DNRA and anammox in N cycling in paddy soils, and of the simultaneous occurrence of these N transformations. This study utilized a ^{15}N isotopic approach to determine the rates of DNRA, denitrification and anammox processes simultaneously in a paddy soil. The paddy soil was collected from the Riverina region in New South Wales, Australia and studied under laboratory conditions. The rates of the processes were investigated after a week of flooding of paddy soil after a basal dose of N application at the rate of 1.6 g N m^{-2} (farmers practice in the region). Results showed that DNRA contributed to the formation of 0.34 µmole NH_4^+-N hr^{-1} kg^{-1} soil. Denitrification and anammox produced 3.35 µmole N_2 and 0.65 µmole N_2 hr^{-1} kg^{-1} soil, respectively. Denitrification was the major pathway contributing to N_2 production which accounted for 83% of total N_2 produced. Anammox contributed to 17% of total N_2 production. Considering the bulk density of soil (1.3 g cm^{-3}), it can be estimated that DNRA can retain 0.03 g N m^{-2} day^{-1}, whereas denitrification and anammox can contribute to a loss of 0.58 and 0.11 g N m^{-2} day^{-1}, respectively, after the first week of flooding of paddy soil.

Key Words

Paddy soil, DNRA, denitrification, anammox

Introduction

Paddy soils are fundamentally different from other arable soils due to the extended period of flooding. Oxygen in the flooded soil is rapidly consumed and the anaerobic condition (Eh < 0 mV) is developed within a few days of flooding (Das et al. 2016). However, a few millimetres of the water surface are oxic due to the diffusion of atmospheric oxygen. The anaerobic condition with oxic interfaces ensures the continuous availability of different forms of mineral N such as ammonium (NH_4^+) and nitrate (NO_3^-) (Aulakh et al. 2001).

Nitrogen (N) is often the most important yield-limiting nutrient for rice. Rice culture is highly N intensive in the majority of the world's top rice producing countries but the N use efficiency (NUE) of rice production is below 40% due to the rapid loss of N through several pathways (volatilization, denitrification, leaching, runoff) (Fageria et al. 2010). Urea is commonly used as a N source in rice paddies (Palanivell et al. 2016). Hydrolysis of urea as well as mineralization of organic N in soil produce NH_4^+, and the nitrification of produced NH_4^+ (in the presence of O_2) produces NO_2^- and NO_3^- (Simek 2000). Nitrate is highly mobile and is subject to loss through different pathways (denitrification and leaching) while the NH_4^+ is less mobile and is retained in soil (Huygens et al. 2008).

Dissimilatory NO_3^- reduction to NH_4^+ (DNRA) may be an important N retention mechanism prevailing in paddy soils, however there is currently limited information available. As the name suggests, DNRA transforms the highly mobile NO_3^- to less mobile NH_4^+ thus reducing the loss of NO_3^- from denitrification and leaching. Anaerobic microsites with readily available C are thought to be hotspots for the DNRA process in forest soils (Silver et al. 2001). A recent study suggested that DNRA could also be an important N retention mechanism in paddy soils (Zhang et al. 2015). Denitrification is the major microbial N transformation pathway in paddy soils which alone can contribute to the loss of around 33% of the N applied in rice paddies (Aulakh et al. 2001). Denitrification is the process of microbial reduction of NO_3^- to N_2O and/or N_2 (Aulakh et al. 2001). The anaerobic conditions along with readily available C favour rapid reduction of NO_3^- to N_2, thus, loss of N from paddy soils. Use of N fertilizer significantly increases denitrification loss of N in rice paddies (Aulakh et al. 2001). However, recent studies have indicated that denitrification is not the only process producing N_2 in anaerobic systems (Thamdrup & Dalsgaard 2002; Dalsgaard et al. 2003). The process of anaerobic NH_4^+ oxidation (anammox) carried out by strictly anaerobic bacteria has been found to be responsible for around 60% of the total N_2 produced in anaerobic sea sediments (Thamdrup & Dalsgaard 2002). During the anammox process, NH_4^+ is oxidised with NO_3^-/NO_2^- (nitrite) to produce N_2 (Dalsgaard et al. 2005). The first known study of anammox in rice paddies indicated that more than 41% of the total N_2 produced can be due to anammox (Zhu et al. 2011). The finding has challenged our previous view on strategies to reduce N loss from the rice paddies. However, there is still a lack of knowledge on how N input in rice paddies affects anammox rates.

Dissimilatory NO_3^- reduction to NH_4^+, denitrification and annamox have a complex relationship in terms of the use of the N substrates. Therefore, a better understanding of the contribution of each of these processes in paddy soil N cycling can only be achieved by a simultaneous study of these processes. However, there are no reported studies that have looked into all these processes simultaneously in terrestrial ecosystems including paddy soils. Therefore, this study utilized a ^{15}N isotopic approach to determine the rates of DNRA, denitrification and anammox simultaneously during flooding of a paddy soil. The objective of this study was to develop an understanding on the significance of the DNRA, denitrification and anammox processes in N cycling in paddy soils during the first week of flooding.

Methods

Soil sampling

Soil samples (0-20 cm) were collected from a rice paddy in the Riverina region of New South Wales (NSW), Australia; the biggest rice growing area in Australia (Kinoshita et al. 2015). Soil samples were collected from five random positions in the rice paddy just before the sowing of rice, and were gently crushed and homogenised after collection. Soil pH was 5.18 and EC was 131.56 µS cm^{-1}. Total soil organic C was 19.8 g kg^{-1} soil and total soil N was 1.77 g kg^{-1}. Soil NO_3^- + NO_2^- was 17.83 mg kg^{-1} dry soil and NH_4^+ was 72.96 mg kg^{-1} dry soil. Approximately 4.5 kilograms of soil was then placed into four replicate PVC pots (15 cm diameter and 25 cm height) and incubated for one month in the glasshouse. The soil was irrigated occasionally to prevent soil from drying out. After one month of incubation, pre-germinated rice seeds were sown and 64.64 mg urea (equal to 16 kg N ha^{-1} basal application which is equal to farmers' practice) was applied to each pot. The PVC pots were flooded with 3-5 cm water 24 hour after urea application. After one week of flooding, the soils from the replicate pots were sampled, composited and homogenised.

Chemical analysis

Chemical analysis was performed in four replicates. Soil pH and electrical conductivity (EC) were determined in soil-water (1:5) suspension. Total C and total N was determined using Dumas method. Soil NO_3^- + NO_2^- and NH_4^+ were determined after extraction with 2M KCl (1:1, soil:KCl solution).

Measurement of DNRA, denitrification and anammox

The DNRA rates were measured using the method described by Trimmer and Nicholls (2009) and denitrification and anammox was measured using the methods described by Thamdrup and Dalsgaard (2002). Briefly, ~3.5 g of the homogenised soil was transferred to 12.5 ml vials (Exetainer, Labco, UK). The vials were then degassed and purged with Helium (He) gas. The vials were filled with He purged water and then pre-incubated for 48 h to deplete soil NO_3^- and O_2. After the pre-incubation 12 sets of vials were added with 100 µL of He purged either $^{15}NH_4Cl$ (> 99 % ^{15}N, 12 mM N), $^{15}NH_4Cl$ + $K^{14}NO_3$ (> 99 % ^{15}N in $^{15}NH_4Cl$, 12 mM N), or $K^{15}NO_3$ (>99% ^{15}N, 12 mM N). Thereafter, microbial activities in the triplicate vials with each of the tracers were stopped by injecting 200 µL of saturated $ZnCl_2$ solution at 0, 6, 12 and 24 h to measure denitrification and anammox. Exactly 4 ml of water in the vials was replaced with He. The vials were vigorously shaken and the headspace gas was analysed for $^{29}N_2$ and $^{30}N_2$ content using GC-IRMS. In exactly the same way, 12 sets of vials were prepared to measure DNRA, but only with $K^{15}NO_3$ (>99% ^{15}N, 12 mM N) tracer. The vials prepared for DNRA were frozen immediately after $ZnCl_2$ injection at each time point. Thereafter, content of the vials was extracted with 2M KCl (1:1 soil:KCl solution) and the $^{15}NH_4^+$ in the samples was determined using alkaline-hypobromite method (Trimmer & Nicholls 2009). Reference vials without ^{15}N tracer amendment were also prepared for both the methods to calculate natural $^{29}N_2$ and $^{30}N_2$ and $^{15}NH_4^+$ production. The DNRA, denitrification and anammox were calculated using the procedure described by Thamdrup and Dalsgaard (2002).

Results and discussion

Measurement of DNRA, denitrification and anammox

As shown in Figure 1, the NO_3^- level in soil was under the detection limit already after 12 h of pre-incubation. There was no excess $^{29}N_2$, $^{30}N_2$ and $^{15}NH_4^+$ production in the reference vials. There was also no

$^{15}N_2$ ($^{29}N_2$ and $^{30}N_2$) production in the vials with only $^{15}NH_4Cl$ tracer, whereas $^{29}N_2$ was produced in the vials added with $^{15}NH_4Cl + K^{14}NO_3$ tracer, which indicates that there is anammox activity in the soil. After the confirmation of anammox activity, DNRA, denitrification and anammox were calculated from the rate of production of $^{15}NH_4^+$ and accumulation of $^{29}N_2$ and $^{30}N_2$ in the vials with added $^{15}NO_3^-$ (Figure 2). Results showed that the DNRA has the potential for production of 0.34 µmole NH_4^+-N hr^{-1} from the reduction of NO_3^- in per kilogram of paddy soils flooded for a week. Denitrification, however, was a dominant N transformation pathway in the paddy soil which contributed to 3.35 µmole N_2 hr^{-1} kg^{-1} soil, whereas anammox rate was 0.65 µmole N_2 hr^{-1} kg^{-1} paddy soil. Considering the soil bulk density (1.3 g cm^{-3}) of the top 20 cm of the collected soil, DNRA can retain 0.03 g N m^{-2} day^{-1} after the first week of flooding, whereas denitrification and anammox can contribute to a loss of 0.58 and 0.11 g N m^{-2} day^{-1} after the time period. Our study showed that favourable conditions for DNRA, denitrification and anammox are created within a week of flooding of rice paddies. It should be noted that, farmers in the region apply more than 70 kg and 50 kg N ha^{-1} during the second and the third topdressing, respectively. As the application of higher dose of N (in the form of urea) changes the level of NH_4^+ and NO_3^- availability of in the soil it can also change the rate of the processes. As the growth of rice plant is almost negligible, it has little effect on N transformations during the first week of flooding. But during the later stages, rice plants supply significant amount of dissolve organic carbon through the root exudates and decaying plant parts, which further strengthen the soil anaerobic conditions (Aulakh et al. 2001). The availability of labile organic carbon can enhance DNRA and denitrification processes (Aulakh et al. 2001; Silver et al. 2001) but its direct effect on anammox is little known. In addition, the rice plants supply O_2 to its roots through aerenchyma. The supplied O_2 also gets leaked to the adjacent soil which creates aerobic environment around the rice roots. The aerobic environment can influence the nitrification and enhance NO_3^- availability (Li et al. 2008). Therefore the rice plants can have a significant effect on the process rates. Nie et al. (2015) observed a significant effect of rice rhizosphere on the rates of denitrification and anammox where denitrification and anammox were higher in nonrhizosphere than in rhizosphere. However, there are no studies conducted so far that looked at rhizospheric effect on DNRA rates.

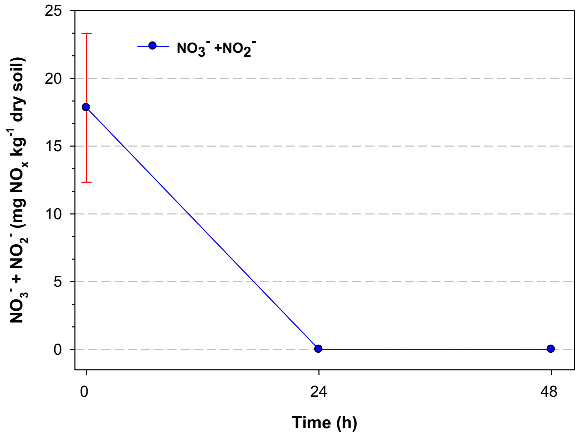


Figure 1. Concentration of NO_3^- + NO_2^- during the pre-incubation. The detection limit for NO_3^- + NO_2^- was 0.5 ppm.

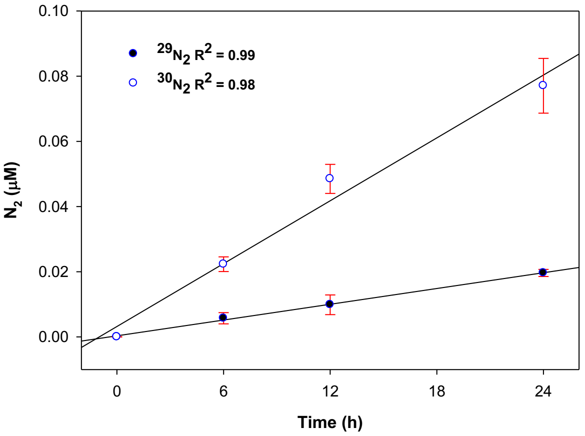
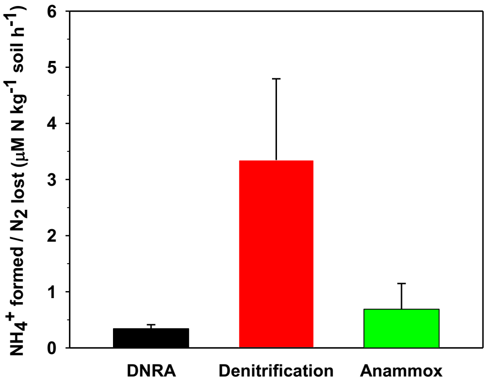


Figure 2. Accumulation of $^{29}N_2$ and $^{30}N_2$ in $^{15}NO_3^-$ added vials at different time period.



Appendix

Figure 3. Rates of DNRA, denitrification and anammox. NH_4^+ produced refers to DNRA rate and N_2 produced refers to denitrification and anammox rates.

Conclusion

Results showed that DNRA, denitrification and anammox are significant N transformation pathways in rice paddies. These processes can start within a week of flooding of rice paddies. This study only investigated the processes rates during the first week of flooding which does not represent the whole rice growing period. The process rates can be affected by the rice roots growth and the higher dose of N that is top-dressed during the later stage of rice growth. Therefore there is a need of study looking at these processes throughout the rice growing period.

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