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Title:
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Date:
2017-12-01

Citation:
Shi, X., Hu, H. W., Zhu-Barker, X., Hayden, H., Wang, J., Suter, H., Chen, D. & He, J. Z. (2017). Nitrifier-induced denitrification is an important source of soil nitrous oxide and can be inhibited by a nitrification inhibitor 3,4-dimethylpyrazole phosphate. Environmental Microbiology, 19 (12), pp.4851-4865. https://doi.org/10.1111/1462-2920.13872.

Persistent Link:
https://hdl.handle.net/11343/293326

Title page

Nitrifier-induced denitrification is an important source of soil nitrous oxide and can be inhibited by a nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP)

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Running title: Nitrifier-induced denitrification contributes to soil N_2O production

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version record. Please cite this article as doi:10.1111/1462-2920.13872.

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Originality-significance statement

Nitrifier-induced denitrification is documented as a pathway of gaseous nitrogen emission, but its relative contribution to nitrous oxide (N_2O) production in agricultural soils remains largely unknown. In this study, by combining a dual-isotope (^{15}N-^{18}O) labelling technique with transcriptional analysis of the key functional genes involved in N_2O formation, we provide novel evidence that nitrifier-induced denitrification can be a significant source of soil agricultural N_2O that can be mediated by ammonia-oxidizing bacteria and inhibited by the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP). Our findings contribute to the differentiation of multiple biological pathways leading to agricultural N_2O production, and have direct implications for explicitly incorporating microbial communities into future N_2O mitigation strategies.

Summary

Soil ecosystem represents the largest contributor to global nitrous oxide (N_2O) production, which is regulated by a wide variety of microbial communities in multiple biological pathways. A mechanistic understanding of these N_2O production biological pathways in complex soil environment is essential for improving model performance and developing innovative mitigation strategies. Here combined approaches of the ^{15}N-^{18}O labelling technique, transcriptome analysis, and Illumina MiSeq sequencing were used to identify the relative contributions of four N_2O pathways including nitrification, nitrifier-induced denitrification (nitrifier denitrification and nitrification-coupled denitrification) and heterotrophic denitrification in six soils (alkaline vs. acid soils). In alkaline soils, nitrification and nitrifier-induced denitrification were the dominant pathways of N_2O production, and application of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) significantly reduced the N_2O production from these pathways; this is probably due to the observed reduction in the expression of the *amoA* gene in ammonia-oxidizing bacteria (AOB) in the DMPP-amended treatments. In acid soils, however, heterotrophic denitrification was the main source for N_2O production, and was not impacted by the application of DMPP. Our results provide robust evidence that the nitrification inhibitor DMPP can inhibit the N_2O production from nitrifier-induced denitrification, a potential significant source of N_2O production in agricultural soils.

Introduction

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Nitrous oxide (N_2O) is the third major anthropogenic greenhouse gas with 298-fold higher global warming potential than CO_2 and is involved in the destruction of stratospheric ozone (Ravishankara et al., 2009; IPCC, 2013). Globally, soil ecosystem is considered as the largest source of N_2O production (estimated at approximately 6.8 Tg N_2O-N per year), due to the extensive use of synthetic nitrogen (N) fertilizers (*ca.* 140 Tg N per year) (IPCC, 2013). An emerging body of studies have suggested that soil microbial communities are key drivers in N_2O production and consumption pathways (Hu et al., 2015). Due to the growing demand for greater food production and therefore agricultural expansion, the global consumption of N fertilizers is projected to increase annually at a rate of 1.4% between 2014 and 2018 (FAO, 2015); this would greatly contribute to the conspicuous elevation in atmospheric N_2O concentrations (Shcherbak et al., 2014). To effectively mitigate N_2O emissions and to promote the reliable estimation of future N_2O production, approaches that can accurately differentiate soil N_2O production from different biological pathways (Baggs et al., 2008; Hu et al., 2015) are in urgent need.

Nitrification and heterotrophic denitrification are believed to be the predominant sources of terrestrial N_2O emissions (Wrage et al., 2001; Philippot, 2007). Nitrous oxide can be emitted as a by-product of ammonia oxidation, the first step of nitrification, or as an intermediate product of heterotrophic denitrification (Hu et al., 2015). Recently, nitrifier denitrification (ND) (Fig. S1) catalysed by the nitrite reductase (NIR) and nitric oxide reductase (NOR), was found to be an important pathway in various soil environments (Wrage et al., 2001; Shaw et al., 2006; Kool et al., 2010; Zhu et al., 2013). Wrage et al. (2005) reported that ND was a major source of N_2O accounting for 44% and 40% of soil N_2O emissions by using the stable isotope labelling and inhibition approach respectively. Nitrifier denitrification was shown to comprise up to 57% of total soil N_2O production by using an improved dual-isotope labelling method (Kool et al., 2011a). Given the evidence that ND contains the reduction step of nitrite to N_2O, low oxygen availability was originally considered as the most effective factor facilitating the occurrence of ND (Lipschultz et al., 1981). Physiological studies, however, have expanded its occurrence to hypoxic conditions, indicating that ND may not be a fully anoxic process (Wrage et al., 2004; Klotz and Stein, 2011). It has been reported that up to 72.7% of total N_2O was ascribed to ND pathway in a laboratory incubation experiment at 21% oxygen concentration (Zhu et al., 2013). Therefore, the ND pathway can be an important contributor to N_2O emissions across diverse

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environmental conditions and requires an improved mechanistic understanding of the involved microbial modulators and their links to N_2O fluxes.

Most of early information on the microbial modulators for ND-derived N_2O has focused on *Nitrosomonas europaea* as a model organism (Kozlowski et al., 2014). Shaw et al. (2006) reported that all of the tested nine ammonia-oxidizing bacteria (AOB) strains within the *Nitrosomonas* and *Nitrosospira* genera were capable of mediating ND, suggesting that ND could be a universal trait of AOB phylogenetic clusters. However, this perception was challenged by recent genetic and physiological analysis which demonstrated that two oligotrophic species of AOB, *Nitrosomonas* sp. Is79A3 and *Nitrosomonas ureae* Nm10 cannot perform ND due to the lack of the nitric oxide reductase genes (*norB* and/or *norY*) encoding NOR (Kozlowski et al., 2016a). As for ammonia-oxidizing archaea (AOA), the nitrite reductase gene (*nirK*) encoding the copper-containing NIR can be found in all published AOA genomes (Bartossek et al., 2010; Kerou et al., 2016). Nevertheless, unlike AOB, recent studies of the soil AOA isolate *Nitrososphaera viennensis* EN76^T have provided direct evidence that the N_2O produced by AOA is attributed to abiotic reaction of nitric oxide (NO) under anoxic conditions, rather than the enzymatic reduction from NO to N_2O through ND pathway (Kozlowski et al., 2016b). Therefore only AOB are able to carry out ND, but the significance of this pathway in soil N_2O emissions remains unclear.

In order to combat the continuous increase in atmospheric N_2O concentration, various mitigation strategies have been proposed to manipulate soil properties and microbial communities, such as the use of inhibitors to inhibit nitrification process (Thomson et al., 2012). The nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) is widely used for improving N use efficiency and reducing N_2O emissions in agricultural ecosystems (Shi et al., 2016b). However, the observed inhibition efficacy in decreasing N_2O emissions is highly variable, ranging from no significant effect to as high as 75% of inhibition of N_2O production in various agricultural systems (Di and Cameron, 2012; Scheer et al., 2014). Previous studies reported that DMPP inhibited nitrification through decreasing the abundance and metabolic activity of AOB (Kleineidam et al., 2011; Shi et al., 2016b). However, other studies found that DMPP also inhibited the activity of AOA (Chen et al., 2015b). Therefore, the molecular mechanism underpinning the reduced soil N_2O emissions by DMPP amendment is still in debate and largely unknown. Given the potential role of ND in soil N_2O production, it is necessary to understand the effect of DMPP inhibition on ND-derived N_2O production in different agricultural soils.

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The objectives of this study are to examine the relative contributions of multiple biological pathways to soil N_2O production and to elucidate the microbial mechanism underlying the reduction of N_2O emission from DMPP treatments in agricultural soils. To achieve this, a dual-isotope (^{18}O-^{15}N) labelling technique (Kool et al., 2011a) was used to differentiate four N_2O production pathways, *i.e.* nitrification (NN), ND, nitrification-coupled denitrification (NCD) and heterotrophic denitrification (HD) (Fig. S1). In addition, transcriptional analysis of the key functional genes involved in NN and heterotrophic denitrification was performed, and high-throughput transcriptome analysis of the 16S rRNA gene was used to link the changes in active soil microbial communities with N_2O emissions. We hypothesized that: (i) ND is an important source of N_2O production in soils where AOB are the dominant ammonia oxidizers; and (ii) the response of biological N_2O production to the application of DMPP is different between the alkaline and acid agricultural soils.

Results

Soil physical and chemical properties

Soil physical and chemical properties were highly variable across the six sampling sites (Table 1). Generally, soil pH (1:5 H_2O) was higher in the cropping soils (vegetable and wheat soils, range from 7.1 to 8.4, alkaline soils) than in the pasture soils (range from 5.4 to 5.8, acid soils). Soil organic matter and total N contents in the acid pasture soils were approximately two to four times higher than in the alkaline cropping soils. The nitrate-N (NO_3^--N) concentrations were significantly higher than the ammonium-N (NH_4^+-N) concentrations in all soils except one pasture soil KP. The wide range of variation in soil properties provided chances to investigate the relative importance of different N_2O production pathways across a broad environmental gradient.

Mineral N concentrations and total N_2O emissions

The concentrations of NH_4^+-N and NO_3^--N and the total N_2O emissions were measured during the 36-hour microcosm incubation. In alkaline soils, the addition of DMPP significantly increased ($P < 0.01$) the NH_4^+-N concentrations from 9.0-22.7 mg N kg^{-1} soil in control to 20.8-53.9 mg N kg^{-1} soil in the DMPP treatments (Fig. 1a). Meanwhile, compared to control, significant lower ($P < 0.05$) NO_3^--N concentrations in the DMPP treatments were observed in alkaline soils (Fig. 1b). In acid pasture soils, however, DMPP had no impact on the dynamics of mineral N concentrations (Figs. 1a and 1b). Consistent with dynamics of mineral N, DMPP significantly decreased the gross nitrification rates in alkaline soils rather

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than in acid soils (Table 2). Similarly, the percentage of N_2O-N emitted per unit NO_3^--N produced was only significantly decreased by DMPP in alkaline soils (Table 2). DMPP significantly reduced the total N_2O production by 27.8-55.8% ($P < 0.05$) in alkaline soils, whereas no influence of DMPP on N_2O production was found in acid soils except one soil sampled from the DP site (Fig. 1c).

Relative contributions of the four biological pathways to N_2O production

By using the ^{15}N-^{18}O labelling technique in the 36-hour microcosm incubation, the relative contributions of the four biological N_2O pathways were compared across the six agricultural soils (Fig. 2 and Table S1). In alkaline soils, nitrification and nitrifier-induced denitrification (including ND and NCD) played a dominant role in N_2O production, with the relative contribution of NN to N_2O ranging from minima of 19-41% to maxima of 51-57% and the contribution of ND ranging from minima of 0-21% to maxima of 44-65%. The maximal contribution of NCD to N_2O varied between 16-33%. The application of DMPP clearly decreased the relative contribution of the ND-derived N_2O rather than the NN- or NCD-derived N_2O in alkaline soils (Figs. 2a, 2b and 2c). Compared with NN and nitrifier-induced denitrification, heterotrophic denitrification constituted a relatively small proportion and its relative contributions to total N_2O production were not largely changed by the application of DMPP. However, heterotrophic denitrification gradually became the dominant N_2O production pathways with decreasing soil pH and comprised 83% of N_2O production in the most acidic soil KP (pH 5.4) (Figs. 2d, 2e and 2f). As for other biological pathways in acid soils, NN constituted minimally 17-38% and maximally 17-56% of N_2O production, while nitrifier-induced denitrification (ND and NCD) accounted for a relatively minor proportion, ranging from maximal 0.6-39% of N_2O production for ND and maximal 0.4-20% for NCD. Similar to the alkaline soils, only the fraction of ND to total N_2O production was decreased by the application of DMPP in acid soils (Fig. 2).

The total N_2O emissions from alkaline soils, varying from 32.0 to 321 μg N_2O kg^{-1} soil, were two to four times higher than those from acid soils, which ranged between 13.9 and 78.5 μg N_2O kg^{-1} soil (Table 2). In alkaline soils, nitrifier denitrification, maximally accounting for 14-210 μg N_2O kg^{-1} soil, played a more or an equally important role in N_2O production compared with NN at the maximal amount of 18-171 μg N_2O kg^{-1} soil and NCD at the maximal amount of 5.24-71.3 μg N_2O kg^{-1} soil in alkaline soils. DMPP showed higher efficacy in decreasing the N_2O production from ND (by 41.4-72.7%) in comparison to NN (by 22.8-35.7%) and NCD (by 34.2-60.9%). Unexpectedly, the inhibition of DMPP on the

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N_2O production from HD in alkaline soils was also significant ($P < 0.05$). In contrast to alkaline soils, the N_2O produced from HD was the main pathway in acid pasture soils, maximally ranging from 9.63 to 18.6 μg N_2O kg^{-1} soil (Table 2). There was no significant influence of DMPP on HD-derived N_2O production in these acid soils.

Transcriptional analysis of key N cycling genes

The expression levels of key N cycling genes related to the biological N_2O pathways were quantified using reverse transcription quantitative PCR (RT-qPCR) to examine their responses to the application of DMPP (Fig. 3). For ammonia oxidizers, the transcriptional levels of AOA *amoA* gene ranged from 1.17×10^5 to 1.76×10^6 copies g^{-1} soil and were not affected by the application of DMPP in all soils (Fig. 3a). In contrast, the transcriptional levels of AOB *amoA* gene in alkaline soils were substantially higher than those in acid soils, and were significantly decreased by 55.9-83.6% by the addition of DMPP ($P < 0.05$). No significant impact of DMPP on the AOB *amoA* gene transcripts was found in acid soils (Fig. 3b). For the heterotrophic denitrifiers, the transcriptional levels of *narG*, *nirK* and *nosZ* genes were determined for the six soils (Figs. 3c, 3d and 3e). The application of DMPP significantly decreased the *nirK* gene transcripts in the alkaline vegetable soil CV and the acid pasture soil TP ($P < 0.05$). By contrast, the transcriptional level of the gene *nosZ* was significantly higher in the DMPP treatment compared to control in the acid soil TP ($P < 0.05$). There were no significant changes in the transcriptional levels of *narG* gene or bacterial 16S rRNA gene (Fig. 3f) in the DMPP treatment compared to control. The transcriptional levels of the genes *nirS*, *qnorB* and *norY* were below the detection limit of RT-qPCR.

Community profiles by terminal-restriction length polymorphism (T-RFLP), cloning and phylogenetic analysis

T-RFLP analysis of the transcripts of the AOB *amoA* gene and denitrifier *nirK* gene was performed to assess the impact of DMPP on the community structures of ammonia oxidizers and denitrifiers. Among all the soil samples, there were four distinct terminal restriction fragments (TRFs) for the AOB *amoA* gene transcripts digested with MspI, of which TRF-59 and TRF-255 were the two most dominant genotypes (Fig. 4a). The AOB community structures in the treatments with and without DMPP amendment were quite similar to each other, while the relative abundances of TRF-59 were decreased by the addition of DMPP in all soils except the acid soil KP. Non-metric multidimensional scaling (NMDS) ordinations based on the Bray-Curtis dissimilarity matrix showed that the active

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AOB assemblages were distinct between the alkaline vegetable soils (WV and CV) and the acid pasture soils, with the exception of the alkaline wheat soil which clustered together with the acid pasture soils (Fig. 4b). This result was further corroborated by the PerMANOVA analysis ($P < 0.05$). For the denitrifier *nirK* gene transcripts, six distinct TRFs were obtained for the MspI digestion, of which TRF-51 and TRF-224 were the dominant genotypes (Fig. 4c). Similar to the AOB community structure, the NMDS analysis suggested that the *nirK*-containing denitrifiers in the alkaline vegetable soils were clearly divergent from those in pasture soils and the wheat soil (Fig. 4d). The PerMANOVA analysis confirmed the significant dissimilarity in microbial community among the six soils ($P < 0.05$), similar to the community structure of AOB.

The community composition and phylogenetic distribution of active AOB were further determined by cloning and sequencing the AOB *amoA* transcripts in control of soils in which DMPP showed inhibition (alkaline soils) and no inhibition (acid soils) (Fig. 5). The results showed that the active AOB communities (based on 39 positive clones, others are negative ones and not shown) in alkaline soils were affiliated within the *Nitrosospira* lineage (Fig. 5a), with 46.2% of active AOB players belonging to Cluster 3a, followed by an unrecognized cluster (33.3%) (Fig. 5b). The active AOB communities in acid pasture soils were analysed based on 33 positive clones, and were mostly classified into the *Nitrosospira* (Fig. 5c), of which Cluster 3a and Cluster 9 accounted for 36.4% and 18.2%, respectively (Fig. 5d). Only one clone was affiliated within the *Nitrosomonas* lineage.

Impacts of DMPP addition on the community compositions of active soil microorganisms

The impact of DMPP on the total soil microbial community structure across different soils was determined by targeting the 16S rRNA gene on the Illumina MiSeq sequencing platform. The high-throughput sequencing yielded a total of 1,879,645 quality-filtered sequences for the 48 soil samples (2 treatments × 4 replicates × 6 soils), corresponding to an average of 39,159 sequences per sample. Across all soil samples, Proteobacteria, Actinobacteria and Acidobacteria were the three most abundant phyla, accounting for 29.8-49.9%, 15.3-28.7% and 7.20-27.6% of the total bacterial sequences, respectively (Fig. S2a). The total microbial community compositions were similar in the treatments with and without DMPP amendment, as revealed by NMDS ordinations based on the Bray-Curtis dissimilarity matrix (Fig. S2b).

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The neighbour-joining phylogenetic trees for AOA, AOB and nitrite-oxidizing bacteria (NOB) were constructed using the representative sequence under each group's operational taxonomic units (OTUs), respectively (Fig. S3). The results suggested that the OTUs affiliated within the *Nitrososphaera*, *Nitrosospira* and *Nitrospira defluvii* were the most abundant sequences, respectively, belonging to AOA, AOB and NOB in both alkaline and acid soils. Based on the result of phylogenetic trees, OTUs under the same phylogenetic classification were merged and used for the calculation of relative percentage of individual phylogenetic lineages for each soil (Table S3). The results showed that DMPP had no impact on the proportion of the AOA *Nitrososphaera* in the six soils, but significantly decreased the relative percentage of AOB *Nitrosomonas* in the alkaline soil WV. Of particular interest, the addition of DMPP clearly decreased the relative percentage of the NOB *Nitrospira moscoviensis* in all alkaline soils, although the difference was not statistically significant (Table S3).

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Discussion

Nitrifier-induced denitrification as a potential significant source of agricultural N_2O in alkaline soils

Previous studies have focused on quantifying N_2O emission rates and understanding the main environmental factors that influence the patterns of soil N_2O fluxes (Kachenchart et al., 2012; Bell et al., 2015), but limited effort was devoted to distinguishing the relative importance of multiple biological pathways for N_2O production. Several approaches such as acetylene inhibition (Offre et al., 2009; Zhong et al., 2014), N_2O isotopomer analysis (Sutka et al., 2006; Ishii et al., 2014), and ^{15}N tracer (Baggs, 2011) have shed some light into the relative contributions of nitrification and heterotrophic denitrification to soil N_2O production. However, the dual-isotope labelling technique is the only available method so far that enables the simultaneous distinction of the four biological pathways, *i.e.* NN, ND, NCD and HD for N_2O production. Despite its advantage in quantifying the contribution of nitrifier denitrification to N_2O production, it should be noted that the dual-isotope method assumes that NO_3^- is the substrate and obligatory intermediate for NCD (Kool et al., 2011b). However, given that the direct use of nitrite by heterotrophic denitrifiers is energetically equal to NO_3^- reduction, and the suppression of NO_3^- on the heterotrophic reduction of nitrite is not a proofed rule (Liu et al., 2013), the dual-isotope labelling method could therefore cause an overestimation of the contribution of ND to total N_2O production. Our current study therefore combined ND and NCD into one pathway called "nitrifier-induced denitrification". Although the dual-isotope labelling method requires further improving, it still offers a valuable tool for improving our understanding of N_2O production from different biological pathways (Kool et al., 2011b).

The ND-derived N_2O accounted for maximally 65% of the total N_2O production in the alkaline cropping soils and 39% in the acid pasture soils (Fig. 2), indicating that ND has great potential to become a significant source of soil N_2O production. In alkaline soils, the contribution of ND-derived N_2O was higher than in acid soils. This might be due to the competitive advantage of AOB under alkaline conditions (Shen et al., 2008) and the capacity of major phylogenetic lineages of AOB to produce N_2O through ND pathway (Shaw et al., 2006). It should be noted that in acid soils, the amount of NN-derived N_2O was greater than the amount of ND-derived N_2O, possibly because AOA, which cannot perform ND, were the dominant ammonia oxidizers responsible for nitrification in these acid soils, although the transcriptional abundance of AOA *amoA* gene was lower than their counterpart AOB in the

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studied acid soils. However, a pervious study suggested that the dynamics in the transcriptional abundance may not always reflect protein production and activity (Nicol et al., 2008). One key factor taken into consideration is the half-life of the *amoA* mRNA, which could vary greatly depending on the growth conditions of the organisms. It should be noted that mRNA can be lost during reverse transcription, thus causing the conversion of only a proportion of mRNA into cDNA. In ammonia-limited acid soils, AOA tend to represent the functionally-dominant ammonia oxidizing group mainly due to their extremely high affinity for ammonia (He et al., 2007, 2012). The functional dominance of AOA over AOB in acid soils has been convincingly confirmed by DNA-stable isotope probing (SIP) experiments and the cultivation of obligate acidophilic AOA strains (Lehtovirta-Morley et al., 2011; Zhang et al., 2012). However, the batch growth experiments and instantaneous microrespirometry measurements showed that AOA are not able to produce N_2O through ND pathway (Kozlowski et al., 2016b). Apart from NN, the relative contribution of NCD to N_2O production was also much higher in alkaline soils (maxima of 16-32%) than acid soils (maxima of 0.4-20%). The potential reason is that higher rates of nitrification might cause oxygen-limiting micro-hot-spots and the accumulated nitrite/nitrate may induce the occurrence of NCD in alkaline soils. Overall, the results indicated that nitrifier-induced denitrification (NN and NCD) played a more crucial role in producing N_2O in alkaline soils than acid soils. The proportion of HD-derived N_2O was higher in acid soils than in alkaline soils, which can be supported by the lower transcriptional level of the *nosZ* gene in these soils (Fig. 3e). Liu et al. (2010) also observed that the decreased expression of the N_2O reductase was associated with the increased product ratio of N_2O to N_2 under acidic conditions (Liu et al., 2010). The additional explanation of high HD-derived N_2O in acid soils is that fungal communities could predominate in these acid soils and were responsible for the increase in N_2O production, due to denitrifying fungi generally lack the *nosZ* gene and therefore contribute to higher heterotrophic denitrification-derived N_2O production (Philippot et al., 2011; Chen et al., 2015a).

Nitrifier-induced denitrification can be inhibited by the nitrification inhibitor DMPP

The impacts of DMPP on the four biological N_2O pathways and the transcriptional activity and community structure of the key N-cycling functional genes were examined in our study. The addition of DMPP showed significant inhibition effect on nitrification in alkaline cropping soils, but not in acid pasture soils (Fig. 1). Similar to the process of nitrification, the amount of N_2O production from NN and nitrifier-induced denitrification (ND and NCD) were

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significantly decreased by DMPP in alkaline soils (Fig. 2 and Table 2). The transcriptional analysis of the functional genes is a useful approach to identify the active microbes (Philippot and Hallin, 2005). The functional genes encoding the enzymes catalyzing ammonia oxidation (AOA/AOB *amoA* genes) and denitrification (the *narG*, *nirS/nirK*, *nosZ* genes) are often used as the proxy for predicting the genetic potential for N_2O production and consumption (Morales et al., 2010; Zhong et al., 2014; Gao et al., 2016). In our study, along with the changes in N_2O production, the transcriptional level of AOB *amoA* gene was significantly decreased by DMPP in all alkaline soils, while no significant influence of DMPP on AOA transcripts was found in any of the studied soils (Fig. 2). These findings suggest that DMPP could reduce the NN and nitrifier-induced denitrification derived N_2O through inhibiting the growth and activity of AOB in alkaline soils. Considering that the AOB-harbouring functional genes (*norB*/*norY*) encoding NOR have the capacity for producing N_2O through ND pathways (Kozlowski et al., 2014, 2016), when the total abundance of AOB (based on the biomarker *amoA* gene) was decreased by DMPP, the AOB capable of mediating ND pathways could be decreased as well, which might explain the reduced N_2O originating from the ND. Additionally, the first step of NN, the oxidation of ammonia to nitrite (via hydroxylamine) can provide the substrate for ND and NCD pathways (Fig. S1), thus the nitrifier-induced denitrification (ND and NCD) derived N_2O could be decreased due to the reduced supply of hydroxylamine/nitrite as a consequence of DMPP inhibition. Given the *norB* orthologues in AOB suffering from a complex and diverse evolutionary history and gene transfer events, the bio-markers standing for the genetic potential of ND is still scarcely available (Cantera and Stein, 2007). The *norY* gene encoding another NOR within AOB has been reported recently (Ishii et al., 2014), but the gene transcript was below the detection limit of RT-qPCR in our study. However, the Sanger sequencing of the AOB cloning library and MiSeq sequencing of the 16S rRNA gene revealed that the *Nitrosospira* lineage is the numerically dominant and metabolically active AOB in studied soils, and might be the main AOB lineage responsible for providing the substrate for nitrifier-induced denitrification and thus contributing to N_2O production (Figs. 5 and S2, Table S3). This study further confirmed our previous observation that the addition of DMPP blocked the assimilation of ^{13}C-CO_2 assimilation into the AOB *amoA* gene using the DNA-SIP technique in the alkaline soil CV (Shi et al., 2016b). Our findings were consistent with early studies that DMPP decreased nitrification and the abundance of AOB *amoA* gene (Di and Cameron, 2011; Kleineidam et al., 2011). However, as far as we know, we provide the first evidence that DMPP has the potential to decrease nitrifier-induced denitrification (ND and NCD)-derived N_2O production

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in alkaline soils. Overall, the high potential of DMPP in inhibiting nitrification and N_2O production in alkaline soils could be supported by the competitive dominance of AOB under alkaline conditions (Shen et al., 2008). The incapability of DMPP in acid soils was supported by our previous microcosm studies and DNA-SIP results that AOA were the dominant ammonia oxidisers in acid soils and could not be effectively inhibited by DMPP (Shi et al., 2016a, 2016b). Furthermore, the high soil organic matter contents in acid soils (Table 1) could also result in high adsorption rate and therefore low availability (and thus inactivation) of DMPP (Roco and Blu, 2006).

In addition to ammonia oxidizers, two *Nitrospira* species belonging to NOB were recently discovered and their capacity of completing ammonia oxidation (comammox) was demonstrated (Daims et al., 2015; van Kessel et al., 2015). In our study, the sequences belonging to the same cluster as comammox species were found within the *Nitrospira moscoviensis* lineage (Fig. S3c), and the relative percentages of the *Nitrospira moscoviensis* were decreased by addition of DMPP in soils where DMPP significantly decreased the total N_2O production (Tables 1 and S3). To our knowledge, however, there is no published evidence that the comammox *Nitrospira* bacteria have the capacity to produce N_2O. The N_2O produced from heterotrophic denitrification was substantially decreased by DMPP only in alkaline soils, although heterotrophic denitrification played a less important role in the total N_2O production compared with nitrification and nitrifier-induced denitrification (Fig. 2). In our study, the transcriptional levels of the *nirK* and *nosZ* genes were significantly decreased and enhanced, respectively, by DMPP addition (Fig. 3). However, controversial reports exist with respect to how DMPP affects the denitrifying functional genes and enzyme activities (Florio et al., 2014; Kou et al., 2015). It is assumed that the application of DMPP can indirectly decrease the N_2O produced from heterotrophic denitrification through reduce the nitrate formation from nitrification. In addition, DMPP showed no impact on the transcriptional level of the soil bacteria and the microbial community structure, which was also supported by recent studies that DMPP is an environmental-friendly nitrification inhibitor without adverse effect on plants, earthworms and non-target soil microbes (Duan et al., 2017; Kong et al., 2017).

Conclusions

By using the dual-isotope (^{15}N-^{18}O) labelling technique, RT-qPCR, and Illumina MiSeq sequencing, we provided evidence that nitrifier-induced denitrification (ND and NCD) can be an important pathway leading to N_2O production in alkaline cropping soils. Compared to NN

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and nitrifier-induced denitrification, heterotrophic denitrification accounted for a larger proportion of total N_2O production in acid soils. Our results showed that the application of DMPP significantly decreased the N_2O produced from nitrifier-induced denitrification in alkaline soils, accompanied by a significant decline in the transcriptional level of AOB belonging to the *Nitrosospira* lineage. Our findings contribute to the quantitative differentiation of the relative contributions of multiple biological pathways to soil N_2O production and have direct implications for incorporating biological N_2O pathways and their microbial modulators into future development of microbial community-based mitigation strategies.

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Experimental procedures

Field sites and sample collection

Soil samples were collected in June 2016 from six sites including vegetable, wheat cropping and pasture fields in Victoria, Australia. Two vegetable growing soils were collected from Werribee (WV; 37°56′ S, 144°40′ E) and Clyde (CV; 38°08′ S, 145°20′ E); one wheat cropping soil was collected from Horsham (HW; 36°45′ S, 142°07′ E); three pasture soils were collected from Dookie (DP; 36°25′ S, 145°42′ E), Terang (TP; 38°15′ S, 142°52′ E) and Kingston (KP; 37°22′ S, 143°57′ E). All the soil samples were taken from 0 to 10 cm by mixing soil cores collected from multiple random locations in the fields, and transported on ice to the laboratory. The samples were thoroughly homogenized and sieved to < 2 mm before the construction of laboratory microcosms.

Soil physicochemical properties

Soil physicochemical properties were measured using methods described in Shi et al. (2016b). Briefly, soil pH was determined using a ratio of 1:5 (fresh soil: H_2O/0.01 M $CaCl_2$) with an Orion Star A211 pH-meter (Thermo Scientific Inc., Melbourne, Australia). Soil organic matter was determined using the $K_2Cr_2O_7$ wet oxidation and colorimetric method. Total N was determined using the classic Dumas method of combustion on an isotope ratio mass spectrometer (Sercon Hydra, Crewe, UK). Soil exchangeable NH_4^+-N and NO_3^--N were extracted using a ratio of 1:5 (fresh soil:1 M KCl, w/v) by shaking at 180 rpm for 1 h, and the filtered solution was measured by a Segmented Flow Analyzer (SAN++, Skalar, Breda, Holland). The soil samples were equilibrated with 1M ammonium acetate at pH 7.0 for 30 min using mechanical shaking at a soil/solution ratio of 1:10 and the suspensions were used for the analysis of cation exchange capacity. Electrical conductivity was determined using a ratio of 1:5 (fresh soil: H_2O) by shaking at 180 rpm for 1 h, and measured with the Orion Star A211 pH-meter (Thermo Scientific). Particle size analysis was measured using the sieve and hydrometer procedures. The detailed information about the soil properties is shown in Table 1.

Dual-isotope (^{15}N-^{18}O) labelling soil microcosm incubation

Soil microcosm incubation was carried out in 160 ml serum glass vials containing 20 g of soils (oven dry-weight equivalent). For each soil, two sets of four treatments were established using a dual isotope labelling technique: (TR_1) ^{18}O-H_2O + NH_4^+ + NO_3^-; (TR_2) H_2O + NH_4^+ + ^{18}O-NO_3^-; (TR_3) H_2O + NH_4^+ + ^{15}N-NO_3^-; and (TR_4) H_2O + ^{15}N-NH_4^+ + NO_3^-,

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with four replicates for each treatment. All microcosms received NH_4NO_3 to provide N at a rate of 50.0 mg NH_4^+-N kg^{-1} soil and 50.0 mg NO_3^--N kg^{-1} soil. The labelled ^{18}O compound was added to bring the H_2O and NO_3^- pools to an enrichment of 1.0 atom% ^{18}O excess, and the labelled ^{15}N compound was added to bring the NH_4^+ and NO_3^- pools to an enrichment of 10.0 atom% ^{15}N excess. The gross nitrification rates were calculated using the data of the total NO_3^- concentrations and the signature of ^{15}N-NO_3^- in the TR_3 treatment as previously described (Kirkham and Bartholomew, 1954). The percentage of N_2O-N emitted per unit NO_3^--N produced is calculated according to Zhu et al. (2013). The N_2O production from autotrophic nitrification and heterotrophic denitrification (Zhu et al., 2013) can be differentiated by comparing the ^{15}N-N_2O production between the isotope labelling treatments of $^{14}NH_4{}^{15}NO_3$ and $^{15}NH_4{}^{14}NO_3$. The N_2O emitted from nitrification and nitrifier denitrification can be distinguished by the addition of ^{18}O-H_2O with the assumption that the origins of oxygen in N_2O are different between nitrifier denitrification and nitrification, *i.e.* theoretically 100% of oxygen in N_2O derived from nitrification originates from O_2, while half of oxygen in N_2O derived from nitrifier denitrification from O_2 and half from H_2O (Andersson et al., 1983; Wrage et al., 2005). This approach was further improved through incorporating the O exchange between H_2O and N oxides during nitrification and denitrification (Kool et al., 2009, 2010). In the improved approach, the comparison of the actual and theoretical O incorporation is used to determine whether the assumptions (1) the maximal contribution of N_2O production derives from NN and NCD, and the minimal contribution derives from ND; and (2) the maximal contribution of N_2O production is from ND, and the minimal contribution derives from NN and NCD) should be maintained or rejected. The detailed data for this study is provided in Table S1.

One set of treatments was amended with the nitrification inhibitor DMPP (1% of applied NH_4^+-N, the recommended dose in agricultural practice), and the other set was treated as control (without DMPP addition). All the microcosms were incubated in the dark of 60% water-filled pore space (WFPS) for 36 hours at 25°C. The N_2O concentrations, the signatures of ^{18}O-N_2O (in the TR_1 and TR_2 treatments) and ^{15}N-N_2O (in the TR_3 and TR_4 treatments) were measured at the Stable Isotope Facility, University of California, Davis. At the end of incubation, soils (20 g) were sampled for the analysis of NH_4^+-N and NO_3^--N concentrations and their ^{15}N signatures by using the micro-diffusion method (Shi et al., 2016b).

Nucleic acids extraction and RT-qPCR

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Soil RNA was isolated from 1.0 g of soils using the MoBio Powersoil® RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Genomic DNA was removed using DNase Max™ Kit (MoBio Laboratories) according to the instructions prior to reverse transcription. Reverse transcription was performed with 500 ng of RNA as template using SuperScript® IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). To elucidate the genetic potential for different processes leading to N_2O production, the specific functional genes including AOA and AOB *amoA* genes for ammonia oxidation; The *narG*, *nirS*/*nirK* and *nosZ* genes (encoding the nitrate reductase, nitrite reductase and nitrous oxide reductase, respectively) were used for heterotrophic denitrification. The abundance of bacterial 16S rRNA gene was also determined. Further information for the primers used is provided in Table S2.

Each RT-qPCR reaction was performed in a 20 µl volume containing 10 µl of SYBR® Premix Ex Taq™ (TaKaRa Biotechnology, Otsu, Shiga, Japan), 0.5 µl of each primer (10 µM) and 1 µl of cDNA template. Two negative controls were always included for each RT-qPCR run. One control contained soil RNA template and all DNase/RT reagents except for the final addition of reverse transcriptase and the other control used water as a template to ensure that all reagents were free of potential contaminant. Amplification was performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Herculers, CA, USA). The thermal cycling conditions for the functional genes are provided in Table S2. Melting curve analysis was conducted at the end of each run to evaluate the specificity of RT-qPCR amplicons. The RT-qPCR data presented in this study were derived from independent extractions of four replicates. The amplification efficiencies ranged between 85-105% and R^2 values were >0.99 for all the genes.

T-RFLP analysis

The community compositions of active AOB and heterotrophic denitrifiers were characterized by T-RFLP analysis of the *amoA* and *nirK* genes. The fluorescent label (FAM) was attached to the 5' end of the forward primer of each primer pair. The PCR reactions in a 25 µl mixture contained 2.5 µl of 10× NH_4 reaction buffer, 0.3 µl of each primer (10 µM), 1 µl of $MgCl_2$ solution (50 mM), 0.5 µl of dNTP mix (20 mM), 1.5 U of BioTaq DNA polymerase (Bioline, Melbourne, VIC, Australia) and 1 µl of cDNA template. Thermal cycling conditions for AOB *amoA* and *nirK* genes were as follows: an initial denaturation of 30 s at 95°C, 36 cycles of 30 s at 95°C, 30 s at 56°C for AOB *amoA* or at 60°C for *nirK*, and 35 s at 72°C, followed by a final elongation of 72°C for 8 min. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA, USA)

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and quantified using the Qubit dsDNA HS Assay Kit (Invitrogen) according to manufacturer's instructions.

Restriction digest was conducted in a 10 µl mixture containing 250 ng purified PCR products, 5 U of the restriction enzymes MspI for *amoA* and *nirK* (BioLabs, Melbourne, Australia), 1 µl of 10 × NEBuffer and 0.1 µl BSA. Digests were incubated at 37°C for 3 h, followed by 95°C for 10 min to denature the restriction enzyme. GeneScan 500-LIZ internal size standard was used for analysing the TRFs using ABI PRISM 3500 Genetic analyser (Applied Biosystems, CA, USA). TRFs profiles were analysed using Genemapper version 4.0 (Applied Biosystems) with a local southern size calling method and a peak amplitude threshold setting of 50. TRFs of peak height less than 2% of the total peak were removed from the downstream analysis, and fragment length that differed by less than 1 bp were combined into the same TRF.

Cloning library and phylogenetic analysis

To explore the phylogenetic affiliation of the active ammonia oxidizers, two clone libraries were constructed of the AOB *amoA* gene transcript using the pooled transcripts from the control treatments of soils in which DMPP showed either inhibition (alkaline soils) or no inhibition (acid soils). PCR amplification for AOB *amoA* gene was performed using the primers amoA-1F/amoA-2R. The pooled PCR products for each library were purified and ligated into the pGEM-T Easy vector (Promega), and the resultant ligation products were transformed into the JM109 competent cells (Promega). For each clone library, a total of 50 clones were randomly selected based on the colour selection and sequenced at the Macrogen Sequencing Department, Korea. The obtained sequences were aligned with reference sequences using MEGA 6.0 (Tamura et al., 2013). Phylogenetic trees were constructed using a neighbour-joining tree with Kimura 2-parameter distance (1000 replicates) to produce Bootstrap values. The sequences retrieved in this study have been deposited in GenBank with accession numbers KY775468-KY775477 for the alkaline cropping soils and KY775478-KY775487 for the acid pasture soils.

Illumina MiSeq sequencing and data processing

Changes in the soil bacterial community structures for the six different soils in response to DMPP addition were determined by targeting the V3 and V4 regions of the bacterial 16S rRNA gene using the primers Bakt_341F/Bakt_805R (Klindworth et al., 2013). The 25 µl PCR reaction mixture contained 12.5 µl of Premix Ex Taq (TaKaRa), 0.5 µl of each primer

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with Illumina overhang adapator (10 μM) and 2 μl of five folds diluted cDNA template. The PCR products were purified with a Wizard SV Gel and PCR Clean-Up System (Promega). The second round of PCR were conducted with 50 μl PCR reactions including 5 μl of purified DNA as template, 5 μl of each Nextera XT Indexe primer (Illumina, Hayward, CA, USA) and 25 μl of SYBR SensiMix No-Rox reagent (Bioline). Amplification conditions were as follows: 95°C for 15 s, 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. The library concentrations were diluted into 4 nM with 10 mM Tris pH 8.5, and quantified using Qubit dsDNA HS Assay Kit (Invitrogen). The pooled library was finally diluted and denatured with NaOH (0.2N), and sequenced on the Illumina MiSeq sequencing platform.

The obtained raw sequences were split by individual samples, quality filtered and de-noised using Quantitative Insights Into Microbial Ecology (QIIME) following the standard operation procedure (Caporaso et al., 2010). The resultant high-quality reads were clustered into operational taxonomic units (OTUs) at a 97% identity level using the chimera filtering approach UPARSE (Edgar, 2013). The taxonomic classification was conducted using the Ribosome Database Project Classifier with a confidence threshold of 0.80 against the SILVA database (Wang et al., 2007; Quast et al., 2013). The representative sequences annotated as N-cycling microorganisms were obtained using the *filter_fasta.py* script in QIIME for phylogenetic analysis. The OTUs belonging to the same phylogenetic classification were merged and used for calculating the relative percentage of different phylogenetic lineages for different soils.

Statistical analysis

The copy numbers of gene transcript were log-transformed prior to statistical analysis. One-way analysis of variance based on the Duncan test was performed for the statistical analysis of mineral N, N_2O production and the transcriptional levels of functional genes between treatments with and without DMPP using the SPSS 17.0 (IBM, USA). Differences at $P < 0.05$ were considered to be statistically significant. Non-metric multidimensional scaling was used to visualize the Bray-Curtis dissimilarity matrices based on the relative abundances of AOB *amoA* and *nirK* gene TRFs or OTUs. A permutational multivariate analysis of variance (PERMANOVA) test with 999 permutations was performed to check the significance of Bray-Curtis dissimilarity between different treatments by using the 'adonis' function in 'vegan' package of R software.

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Acknowledgements

This work was funded by Australian Research Council (DE150100870 and DP160101028). The authors would like to thank Dr. Shukee Lam, Longlong Xia and Dr. Christopher Weston for their assistance with soil collection in the field.

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Table 1 Soil physical and chemical properties.

	Werribee vegetable WV	Clyde vegetable CV	Horsham wheat HW	Dookie pasture DP	Terang pasture TP	Kingston pasture KP
pH (1:5 H_2O)	7.9	7.1	8.4	5.8	5.8	5.4
pH (1:5 $CaCl_2$)	7.6	6.5	7.6	5.2	5.0	4.4
Organic matter (%)	1.7	4.0	1.5	6.0	10.0	4.0
Total nitrogen (%)	0.16	0.29	0.10	0.35	0.39	0.20
NH_4^+ -N (mg kg^{-1})	4.12	3.81	0.83	1.92	0.91	22.85
NO_3^--N (mg kg^{-1})	173.3	52.9	8.83	31.8	6.95	3.49
[1]CEC (cmol(+)kg^{-1})	26.5	13.8	42.2	11.2	10.5	5.61
Elect. conductivity (dS/m)	0.98	0.30	0.17	0.23	0.11	0.05
Texture	sandy clay	loamy sand	clay	silty loam	sandy loam	clay loam
Particle size (%)						
Sand (0.02 – 2 mm)	24.5	77.0	30.7	51.9	60.6	38.1
Silt (0.002 – 0.02 mm)	21.2	8.70	8.70	27.5	23.8	32.5
Clay (< 0.002 mm)	54.3	14.3	60.6	20.6	15.6	29.4

[1]CEC represents cation exchange capacity.

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Table 2 The gross nitrification rates and the amount of N_2O derived from different biological pathways in six soils amended with or without DMPP over the 36-hour microcosm incubation. Different letters indicate a significant difference between treatments ($P < 0.05$). Numbers in brackets represent standard errors (mean ± SE, $n = 4$).

Soil types	Treatments	µg N_2O kg^{-1} soil							[2]Nitrification rates	Percentage of N_2O emitted per unit NO_3^- produced
		[1]NN_{max}	NN_{min}	ND_{max}	ND_{min}	NCD_{max}	HD	Total N_2O		
WV	Control	171 (± 5.1) a	100 (± 2.1) a	210 (± 2.3) a	66.7 (± 7.1) a	73.1 (± 2.0) a	11.0 (± 0.2) a	321 (6.4) a	12.3 (± 0.6) a	0.71 (± 0.0) a
	DMPP	110 (± 0.1) b	81.3 (± 0.6) b	57.3 (± 1.0) b	0.00 b	28.6 (± 0.5) b	3.47 (± 0.4) b	142 (5.5) b	10.2 (± 0.2) b	0.38 (± 0.0) b
CV	Control	53.1 (± 0.6) a	20.0 (± 1.2) a	66.8 (± 1.2) a	0.00 b	33.7 (± 0.6) a	16.7 (± 0.1) a	104 (1.4) a	6.00 (± 0.1) a	0.36 (± 0.0) a
	DMPP	31.1 (± 0.4) b	15.2 (± 0.4) b	35.5 (± 1.3) b	3.16 (± 0.5) b	16.4 (± 0.3) b	11.2 (± 0.0) b	61.9 (1.1) b	4.97 (± 0.3) b	0.29 (± 0.0) b
HV	Control	18.4 (± 0.4) a	13.2 (± 0.1) a	14.0 (± 0.2) a	3.53 (± 0.8) a	5.24 (± 0.5) a	4.88 (±0.1) a	32.0 (0.7) a	1.75 (± 0.3) a	0.81 (± 0.1) a
	DMPP	14.2 (± 0.2) b	10.8 (± 0.1) b	8.17 (± 0.3) b	1.31 (± 0.4) a	3.45 (± 0.1) b	4.10 (± 0.4) a	23.1 (0.6) b	0.66 (± 0.0) b	0.46 (± 0.1) b
DP	Control	44.1 (± 1.3) a	29.7 (± 2.2) a	30.3 (± 2.4) a	0.00 a	15.8 (± 1.4) a	18.6 (± 0.5) a	78.5 (2.3) a	4.34 (± 0.2) a	0.52 (± 0.0) a
	DMPP	40.1 (± 0.6) ab	29.3 (± 1.2) a	22.6 (± 1.9) a	0.00 a	11.7 (± 1.0) a	17.4 (± 0.9) a	69.3 (2.1) b	4.36 (± 0.1) a	0.40 (± 0.0) b
TP	Control	11.8 (± 1.4) a	8.90 (± 1.1) a	7.97 (± 0.3) a	1.10 (± 0.7) a	3.93 (± 0.2) a	9.63 (± 1.0) a	26.5 (2.1) a	0.80 (± 0.1) a	0.59 (± 0.1) a
	DMPP	14.1 (± 0.2) a	12.7 (± 0.5) a	3.26 (± 1.0) b	0.00 b	1.79 (± 0.6) a	10.8 (± 0.6) a	26.7 (1.3) a	0.90 (± 0.1) a	0.50 (± 0.1) a
KP	Control	2.34 (± 0.4) a	2.31 (± 0.4) a	0.08 (± 0.5) a	0.00 a	0.05 (± 0.3) a	11.5 (± 0.5) a	13.9 (0.2) a	0.25 (± 0.1) a	0.30 (± 0.1) a
	DMPP	3.29 (± 0.3) a	3.26 (± 0.3) a	0.08 (± 0.0) a	0.00 a	0.04 (± 0.0) a	11.4 (± 0.3) a	14.8 (1.1) a	0.26 (± 0.1) a	1.27 (± 0.7) a

[1]The abbreviations are: NN, nitrification; ND, nitrifier denitrification; NCD, nitrification coupled denitrification; HD, heterotrophic denitrification.

[2]The unit for the gross nitrification rate is mg N kg^{-1} soil h^{-1}.

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Figure legends

Fig. 1. Measured NH_4^+-N (a) and NO_3^--N (b) concentrations and total N_2O emissions (c) in control and the DMPP treatments at the end of the 36- hour microcosm incubation across the six soils (WC, Werribee vegetable soil; CV, Clyde vegetable soil; HW, Horsham wheat soil; DP, Dookie pasture soil; TP, Terang pasture soil; KP, Kingston pasture soil). The asterisks above the bars indicate a significant difference ($P < 0.05$) between the control and DMPP treatment. The means ± standard deviations of the mean ($n = 4$) are shown.

Fig. 2. The relative contributions of the four biological pathways (NN, nitrification; ND, nitrifier denitrification; NCD, nitrification-coupled denitrification; HD, heterotrophic denitrification) for N_2O production in control and the DMPP treatments across alkaline soils (WV (a), CV (b) and HW (c)) and acid soils (DP (d), TP (e) and KP (f)) over the 36-hour microcosm incubation. The minima and maxima of N_2O derived from different pathways are calculated from the ^{18}O-^{15}N isotope labelling experiments.

Fig. 3. The transcriptional levels of AOA *amoA* (a), AOB *amoA* (b), *narG* (c), denitrifier *nirK* (d), *nosZ* (e) and bacterial16S rRNA (f) genes in control and the DMPP treatment across the six soils (WC, Werribee vegetable soil; CV, Clyde vegetable soil; HW, Horsham wheat soil; DP, Dookie pasture soil; TP, Terang pasture soil; KP, Kingston pasture soil) at the end of the 36-hour microcosm incubation. The means ± standard deviations ($n = 4$) are shown.

Fig. 4. Terminal restriction fragment length polymorphism fingerprints of the AOB *amoA* (a) and denitrifier *nirK* (c) genes transcripts digested by *MspI* across the six soils (WC, Werribee vegetable soil; CV, Clyde vegetable soil; HW, Horsham wheat soil; DP, Dookie pasture soil; TP, Terang pasture soil; KP, Kingston pasture soil). The 'C' refers to the control treatment and the 'D' refers to the treatment with DMPP addition. Error bars represent standard errors ($n = 4$). NMDS ordinations derived from the Bray-Curtis dissimilarity matrices are based on the relative abundance of AOB *amoA* (b) and denitrifier *nirK* (d) genes terminal restriction fragments. The stress values for both NMDS plots were lower than 0.12 indicating that these data were well-represented by the two-dimensional ordinations.

Fig. 5. Phylogenetic trees of the active AOB *amoA* gene transcript retrieved from the alkaline soils (a) (n = 39) and the acid soils (c) (n =33). The relative percentages of different phylogenetic lineages to the total positive clones in the alkaline soils (b) and the acid soils (d) are shown. The sequences identified in this study are highlighted in blue, and the red numbers

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in the parenthesis represent the numbers of sequences with 98% sequence identity. Numbers at the nodes indicate the levels of bootstrap support based on the neighbour-joining analysis of 1,000 resampled data sets. The scale bar represents 5% nucleic acid sequence divergence, and bootstrap values (> 50%) are indicated at branch points.

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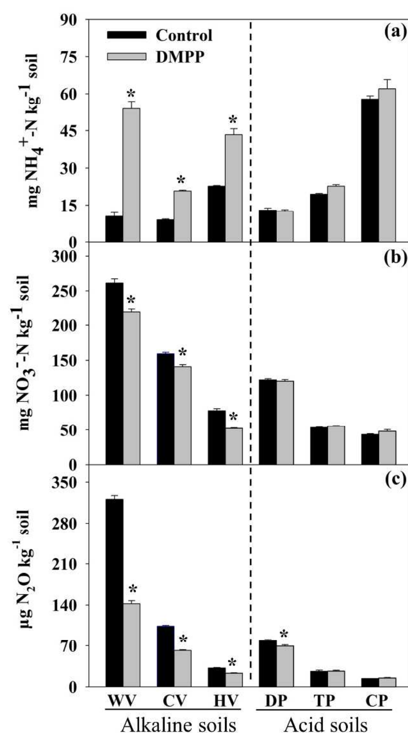

Measured NH4+-N (a) and NO3--N (b) concentrations and total N2O emissions (c) in control and the DMPP treatments at the end of the 36- hour microcosm incubation across the six soils (WC, Werribee vegetable soil; CV, Clyde vegetable soil; HW, Horsham wheat soil; DP, Dookie pasture soil; TP, Terang pasture soil; KP, Kingston pasture soil). The asterisks above the bars indicate a significant difference ($P < 0.05$) between the control and DMPP treatment. The means ± standard deviations of the mean ($n = 4$) are shown.

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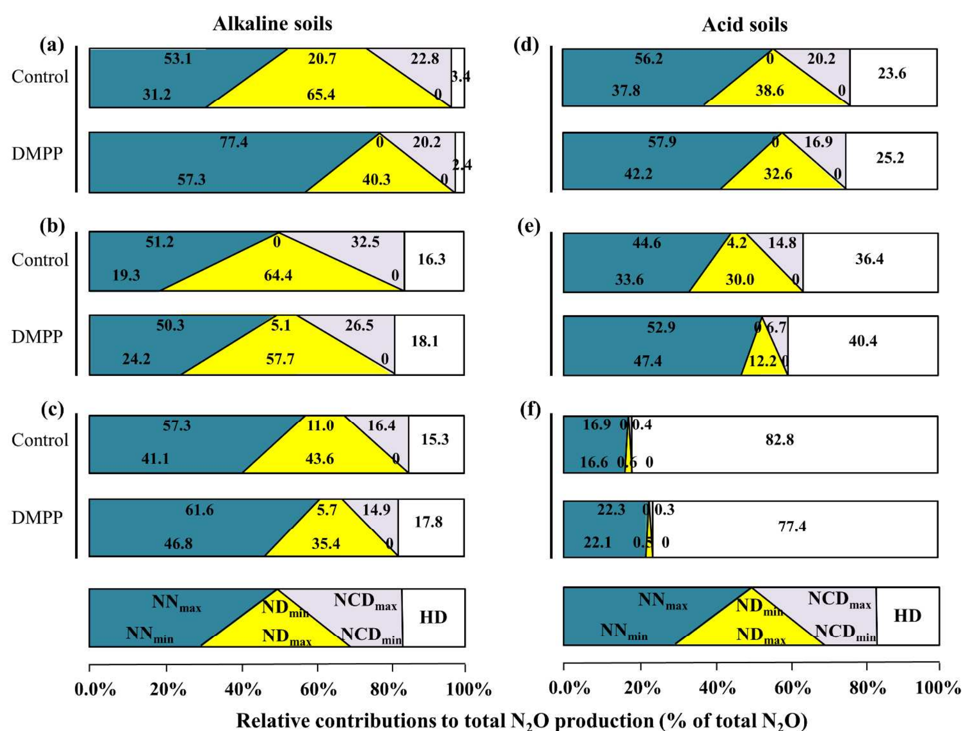

The relative contributions of the four biological pathways (NN, nitrification; ND, nitrifier denitrification; NCD, nitrification-coupled denitrification; HD, heterotrophic denitrification) for N2O production in control and the DMPP treatments across alkaline soils (WV (a), CV (b) and HW (c)) and acid soils (DP (d), TP (e) and KP (f)) over the 36-hour microcosm incubation. The minima and maxima of N2O derived from different pathways are calculated from the 18O-15N isotope labelling experiments.

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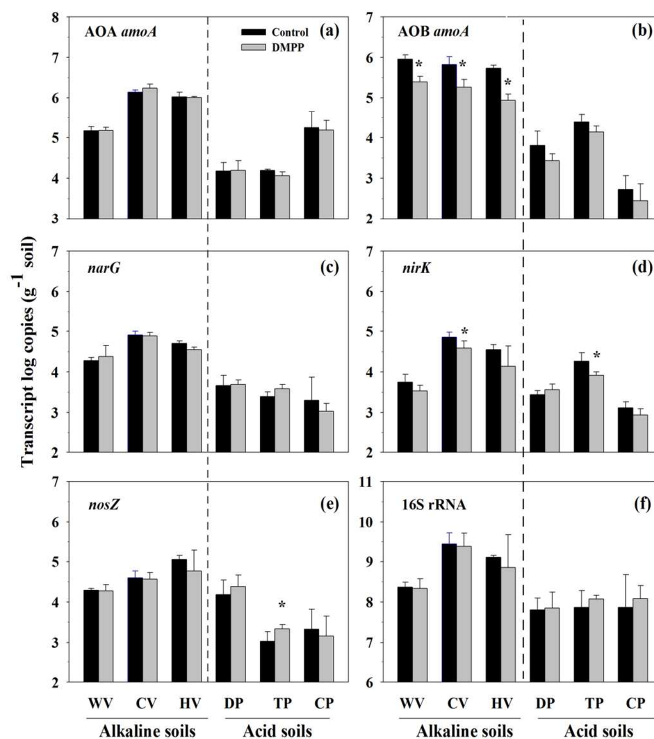

The transcriptional levels of AOA amoA (a), AOB amoA (b), narG (c), denitrifier nirK (d), nosZ (e) and bacterial16S rRNA (f) genes in control and the DMPP treatment across the six soils (WC, Werribee vegetable soil; CV, Clyde vegetable soil; HW, Horsham wheat soil; DP, Dookie pasture soil; TP, Terang pasture soil; KP, Kingston pasture soil) at the end of the 36-hour microcosm incubation. The means ± standard deviations (n = 4) are shown.

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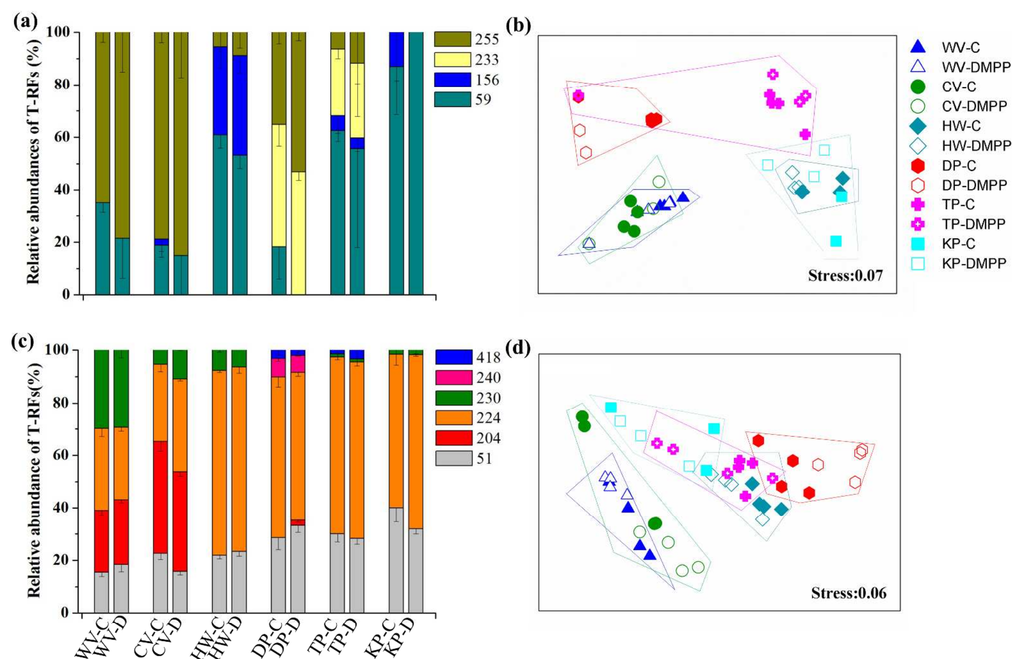

Terminal restriction fragment length polymorphism fingerprints of the AOB amoA (a) and denitrifier nirK (c) genes transcripts digested by MspI across the six soils (WC, Werribee vegetable soil; CV, Clyde vegetable soil; HW, Horsham wheat soil; DP, Dookie pasture soil; TP, Terang pasture soil; KP, Kingston pasture soil). The ‘C’ refers to the control treatment and the ‘D’ refers to the treatment with DMPP addition. Error bars represent standard errors (n = 4). NMDS ordinations derived from the Bray-Curtis dissimilarity matrices are based on the relative abundance of AOB amoA (b) and denitrifier nirK (d) genes terminal restriction fragments. The stress values for both NMDS plots were lower than 0.12 indicating that these data were well-represented by the two-dimensional ordinations.

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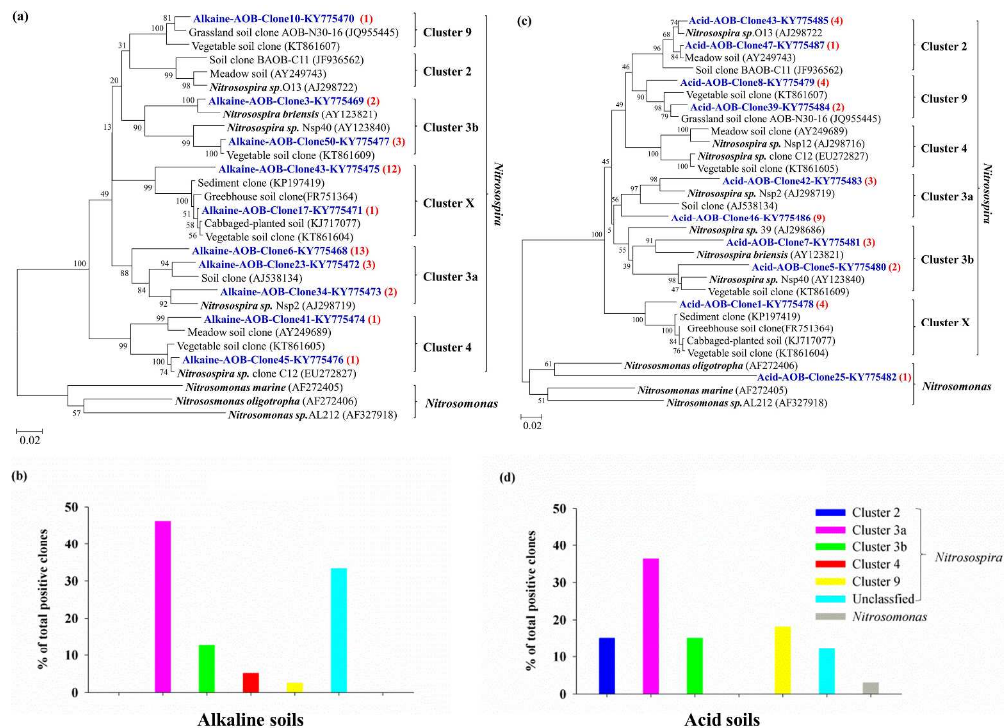

Phylogenetic trees of the active AOB amoA gene transcript retrieved from the alkaline soils (a) (n = 39) and the acid soils (c) (n =33). The relative percentages of different phylogenetic lineages to the total positive clones in the alkaline soils (b) and the acid soils (d) are shown. The sequences identified in this study are highlighted in blue, and the red numbers in the parenthesis represent the numbers of sequences with 98% sequence identity. Numbers at the nodes indicate the levels of bootstrap support based on the neighbour-joining analysis of 1,000 resampled data sets. The scale bar represents 5% nucleic acid sequence divergence, and bootstrap values (> 50%) are indicated at branch points. ‼ ┼

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