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Effects of 3,4-dimethylpyrazole phosphate and dicyandiamide on nitrous oxide emission in a greenhouse vegetable soil

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ABSTRACT

A laboratory study was conducted to determine the effect of 3,4-dimethylpyrazole phosphate (DMPP) and dicyandiamide (DCD) on nitrous oxide (N_2O) emission, mineral nitrogen (NH_4^+-N, NO_3^--N) contents, as well as ammonia oxidizing and denitrifying microbes in a greenhouse vegetable soil. Five treatments were established at 55% and 75% water filled pore space (WFPS): no fertilizer; urea; urea + manure (UM); urea + manure + DCD (UMDCD) and urea + manure + DMPP (UMDMPP). The application rate of urea and manure was 1800 kg N/ha and 1000 kg N/ha, respectively. DMPP and DCD were applied at the rate of 0.5% and 2% of urea-N application rate, respectively. Compared to UMDCD treatment, UMDMPP treatment significantly reduced N_2O emission and NO_3^--N content and delayed ammonia oxidation, and showed a stronger inhibition effect on ammonium-oxidizing bacteria at both WFPS. Moreover, the copy numbers of *nirS* and *nirK* genes decreased significantly in the presence of DMPP at both WFPS, but were not affected by DCD. These results suggest that the application of DMPP is more effective than DCD on N_2O mitigation in high N level vegetable soil, although the application rate of DMPP was one quarter that of DCD.

Keywords: nitrification inhibitors; denitrification; ammonia oxidizing archaea; *nirK*- and *nirS*-harboring denitrifiers

The excessive application of nitrogen (N) fertilizer causes soil N loss, such as nitrous oxide (N_2O) emission, which can bring a negative effect to environment (Dobbie et al. 1999). Nowadays, large amounts of mineral N fertilizer and manure are used in vegetable fields to maximize the production in China. According to investigations conducted by Chen et al. (2004) and Ju et al. (2004), the average N application rate in greenhouse vegetable soil was 1000 kg N/ha and 2848 kg N/ha in Beijing and Shandong province, respectively. Vegetable cultivation was estimated to contribute 20% of the direct N_2O emission from Chinese croplands (Zheng et al. 2004). Therefore, it is necessary to explore an optimal management practice to reduce N_2O emission in greenhouse vegetable soil.

The use of nitrification inhibitors (NIs), such as 3,4-dimethylpyrazole phosphate (DMPP) and dicyandiamide (DCD), have been proved to be an effective management measure in reducing N_2O emission in grassland and cropland systems (Weiske et al. 2001, Di and Cameron 2012). However, few studies have estimated the effect of DMPP and DCD on N_2O mitigation in greenhouse vegetable soil. DMPP and DCD can slow down the ammonia-oxidizing process by inhibiting the activity of ammonia monooxygenase, thereby reducing N_2O emission (Di and Cameron 2012). Ammonia-oxidizing process, the first and rate-limiting step of nitrification, is traditionally thought to be mainly regulated by autotrophic ammonia-oxidizing bacteria (AOB) (Kowalchuk and Stephen 2001). Recently, ammonia-oxidizing archaea (AOA) is also considered to be responsible for ammonia-oxidizing process to a certain extent (Francis et al. 2005). Denitrification is generally

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responsible for N_2O emission under high soil water filled pore space (WFPS), where oxygen was limited (> 70% WFPS) (Dobbie et al. 1999). The rate-limiting step of denitrification was catalyzed by nitrite reductase, which was encoded by *NirS*-harboring denitrifiers and *NirK*-harboring denitrifiers (Braker et al. 1998). Researches have shown that NIs affected the abundance of AOB and AOA in grassland and cropland soils (Di and Cameron 2011, Gong et al. 2013) and *nirS* gene abundance in a low-fertility agricultural soil (Dong et al. 2013). However, little information is known about the response of the ammonia-oxidizing and denitrifying microbes to NIs it high N level vegetable soil. Therefore, the objective of this study was to investigate the effect of DMPP and DCD on N_2O emission, mineral N content, as well as the copy numbers of AOA *amoA*, AOB *amoA*, *nirS* and *nirK* genes at 55% and 75% WFPS in a greenhouse vegetable soil by a laboratory experiment, so as to provide a certain theoretical foundation for N_2O mitigation in high N level greenhouse vegetable soil.

MATERIAL AND METHODS

Soil preparation. A greenhouse vegetable soil (0–20 cm) that had not been fertilized was collected from a vegetable field in a Beijing suburb (China, 116°23'E, 39°54'N) in June 2012. The soil was sieved (< 2 mm) and stored at 4°C prior to analyses. The soil had following characteristics: organic carbon 3.4%; total nitrogen 0.19%; total phosphorus 0.11%; total potassium 2.31%; soil pH (1:2.5 H_2O) 8.03.

Experimental design. Five treatments were established at 55% and 75% WFPS in a laboratory study: no fertilizer (CK); urea (U); urea + manure (UM); urea + manure + DCD (UMDCD); urea + manure + DMPP (UMDMPP). The applied manure was obtained from a commercial intensive dairy farm nearby, and the chemical compositions of cattle manure were: total carbon 18.5%; total nitrogen 1.57%; total phosphorus 0.88%; total potassium 0.85%; total calcium 1.23%; total magnesium 3.48%; dry matter content 66.5%. The application rate of urea and manure was 1800 kg N/ha and 1000 kg N/ha, respectively. DMPP and DCD were applied at the rate of 0.5% and 2% of urea-N application rate, respectively. Each treatment had four replicates to monitor N_2O emission and three replicates to determine soil chemical and biological indexes. For each replicate, 100 g soil (dry-weight basis) was mixed with appropriate quantities of fertilizer and/or NIs, and weighed into an incubation bottle (300 mL). All bottles were incubated in darkness for 150 days at 25°C. Aeration was performed every 1–2 days. Deionized water was added regularly to maintain certain soil moisture.

Sampling and analysis. Soil organic carbon was measured according to the traditional Walkley-Black method. Total nitrogen and phosphorus were determined by Micro-Kjeldahl method and molybdenum blue colorimetric method, respectively. Total potassium was measured by using the flame photometer (FP640, Shanghai, China). Gas samples (35 mL) were taken from the headspace of bottles with a syringe every day during the first week, and on days 9, 12, 16, 19, 23, 30, 37, 44, 51, 59, 73, 80, 90, 103, 122, 136, 150 during the incubation period. N_2O concentrations were analyzed by gas chromatograph (Agilent GC-7890A, Wilmington, USA) with an electron capture detector (ECD). Cumulative N_2O emission was calculated by the method of Menéndez et al. (2012). Soil samples (10 g) collected on days 1, 3, 5, 9, 16, 23, 30, 37, 44, 59, 73, 90, 103, 122, and 150 were used to analyze NH_4^+-N and NO_3^--N contents using a continuous flow injection analyzer (Alliance, Paris, France).

DNA was extracted using MoBio Powersoil™ DNA isolation kits (San Diego, USA) according to the manufacturer's instruction. Prior to extraction, soil samples were collected on days 3, 30 and 90 and stored at –80°C. Copy numbers of AOB *amoA*, AOA *amoA*, *nirK* and *nirS* genes were determined by quantitative PCR using SYBR Green PCR Master Mix on an ABI StepOne Plus System (Applied Biosystems, Foster City, USA). The above-mentioned genes were amplified using the primers listed in Table 1. A 25 µL reaction mixture contained 12.5 µL 2X SYBR Green qPCR Master Mix, 0.5 µL each primer (Table 1), 2 µL DNA template and 9.5 µL sterilized water. Thermal cycling for the PCR consisted of 95°C for 3 min followed by 35 cycles at 94°C for 30 s, 72°C for 30 s, 72°C for 8 min, and data collection at 95°C for 10 s. Standard curves were generated by 10-fold serial dilution of the copy numbers of plasmids containing a cloned bacterial *amoA*, archaeal *amoA*, *nirS* or *nirK* gene. For these genes, amplification efficiencies were 82–95%, R^2 values were > 0.99, and no signal was observed in negative controls.

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Table 1. RT-PCR primers used for amplification of functional target genes

Target gene	Primer	Primer sequence 5'–3'	Temperature (°C)	Reference
AOB *amoA*	*amo*A–1F	GGGGTTTCTACTGGTGGT	60	Rotthauwe et al. (1997)
	*amo*A–2R	CCCCTCKGSAAAGCCTTCTTC	60	
AOA *amoA*	*amo*AF	STAATGGTCTGGCTTAGACG	60	Francis et al. (2005)
	*amo*AR	GCGGCCATCCATCTGTATGT	60	
nirK	*nir*K 1F	GGMATGGTKCCSTGGCA	55	Braker et al. (1998)
	*nir*K 5R	GCCTCGATCAGRTTRTGGTT	55	
*nir*S	*nir*S cd3aF	GTSAACGTSAAGGARACSGG	55	Throbäck et al. (2004)
	*nir*S R3cd	GASTTCGGRTGSGTCTTGA	55	

AOB – ammonia oxidizing bacteria; AOA – ammonia oxidizing archaea

All qPCR reactions were done by performing melt curve analysis and agarose gel electrophoresis to confirm the amplification of specific products.

Statistical analysis. All results were calculated based on oven-dried (105°C) weight. The Duncan test at the $P = 0.05$ level and one-way analysis of variance (ANOVA) was used to analyze the experimental results. All statistical analyses were conducted with the software SPSS 16.0 (SPSS, Chicago, USA).

RESULTS AND DISCUSSION

The application of urea or urea + manure significantly increased NH_4^+, NO_3^- contents and N_2O emission at both WFPS (Figures 1 and 2), which resulted from the hydrolysis of urea and subsequent nitrification and denitrification. Because manure provided additional mineral N, UM treatment showed significantly higher NH_4^+ content than U

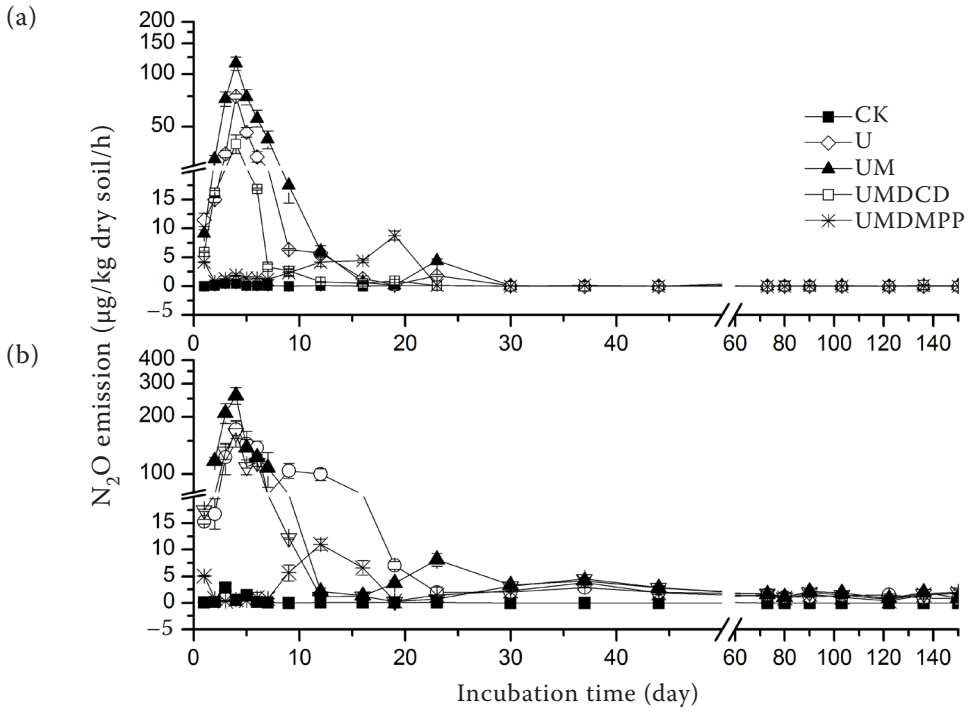

Figure 1. Nitrous oxide (N_2O) emission during incubation period at 55% water filled pore space (WFPS) (a) and 75% WFPS (b). CK – no fertilizer; U – urea; UM – urea + manure; UMDCD – urea + manure + dicyandiamide (DCD); UMDMPP – urea + manure + 3,4-dimethylpyrazole phosphate (DMPP). Error bars indicate the standard deviation of the mean ($n = 4$)

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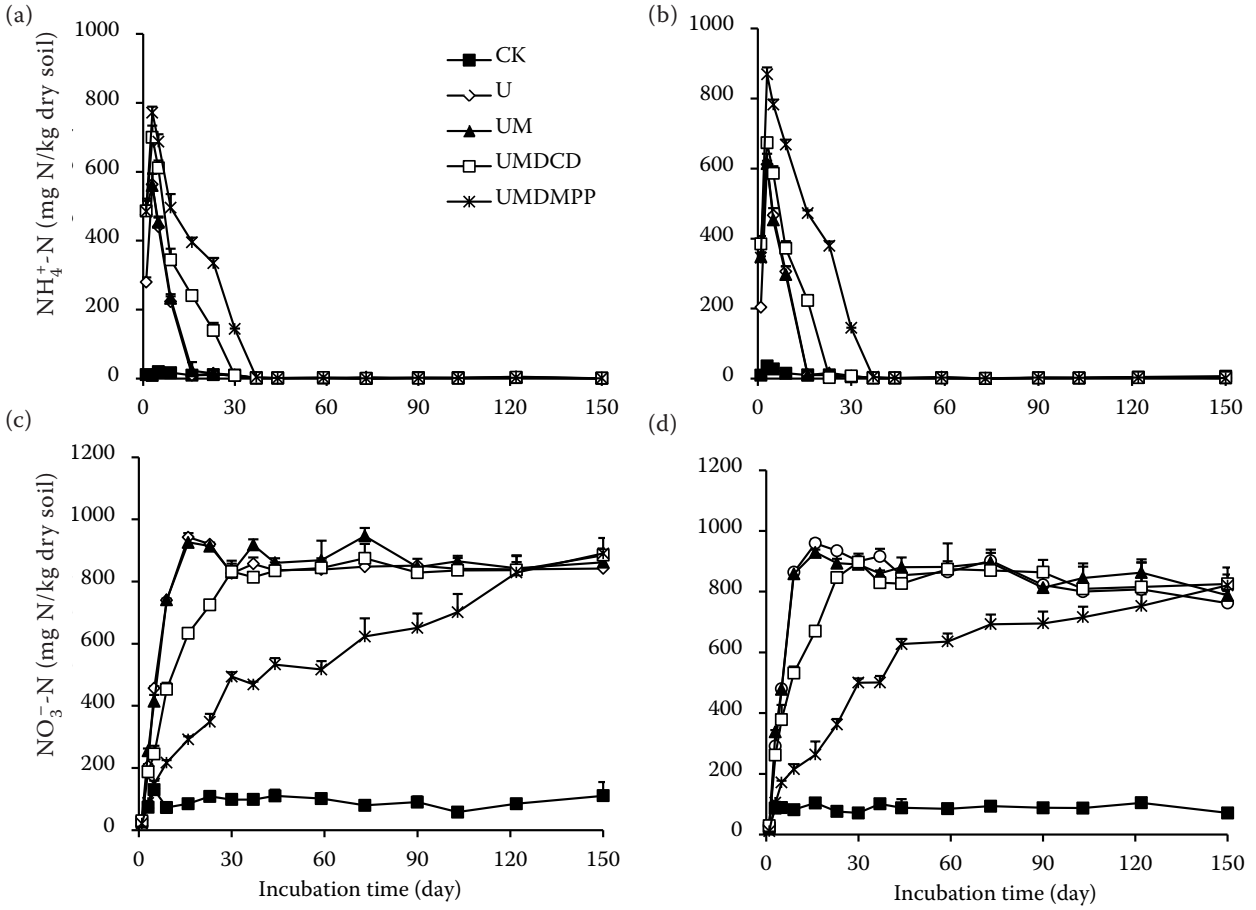

Figure 2. Soil ammonia (a, b) and nitrate (c, d) contents at 55% water filled pore space (WFPS) (a, c) and 75% WFPS (b, d). CK – no fertilizer; U – urea; UM – urea + manure; UMDCD – urea + manure + dicyandiamide (DCD); UMDMPP – urea + manure + 3,4-dimethylpyrazole phosphate (DMPP). Error bars indicate the standard deviation of the mean (n = 3)

treatment on the first day (Figures 2a,b). Higher NH_4^+ content might result in higher N_2O emission under UM treatment than that under U treatment through nitrification during the first week at both WFPS (Figure 1). However, the cumulative N_2O emissions were lower under UM treatment than that under U treatment at 75% WFPS during a 150-day incubation period (Figure 1b), which could be attributed to the change of denitrifier communities induced by application of manure at such an anoxic condition (Miller et al. 2012).

The application of NIs significantly decreased N_2O emission at both WFPS (Figure 1). It is noted that the response of DMPP and DCD to soil WFPS was different. DMPP showed an equal effect in decreasing N_2O emission at both WFPS, which was inconsistent with the results obtained by Chen et al. (2010) and Menéndez et al. (2012). Chen et al. (2010) found that DMPP was more effective to reduce N_2O emission at 40% WFPS than that at 60% WFPS with addition of urea, while Menéndez et al. (2012) reported that the highest inhibition effect on N_2O emission was achieved at 80% WFPS relative to 40% and 60% WFPS with addition of ammonium sulfate nitrate + DMPP. The difference in the effect of DMPP on N_2O emission at different WFPS could be largely due to the difference in soil texture and fertilizer level, etc., which influenced the effect of DMPP (Barth et al. 2001). Unlike the DMPP, DCD had a stronger inhibitory effect on N_2O emission at 55% WFPS than that at 75% WFPS (Figure 1). It could be due to the separation of DCD with NH_4^+ was more readily accessible at high WFPS since DCD is highly water-soluble (Zerulla et al. 2001), suggesting that the effect of DCD on N_2O mitigation was preferable at relatively low WFPS. Figures 1 and 2 demonstrate that in greenhouse vegetable soil

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DMPP was more effective than DCD in reducing N_2O emission and NO_3^--N content and delaying NH_4^+ oxidation. It could be ascribed to (1) slower degradation of DMPP due to the absorption of DMPP by soil colloid and organic matter, which resulted in less readily separation of DMPP with NH_4^+ (Zerulla et al. 2001); (2) rapid mineralization of DCD (Weiske et al. 2001).

High N fertilizer application rate stimulated AOB growth at both WFPS significantly, while the abundance of AOA was restrained (Figures 3a,b), indicating AOB might play a more important role than AOA in nitrification and N_2O emission in vegetable soil. The difference in the response of AOB and AOA to fertilization treatment could be related to soil pH studied here, since AOB populations could increase with increasing soil pH (Nicol et al. 2008), while AOA grew preferentially under low-nutrient and low-pH soil environments (Erguder et al. 2009). DMPP showed a stronger and longer inhibition effect on AOB abundance than DCD at both WFPS (Figure 3a), suggesting that the application of DMPP was more effective in inhibiting nitrification in vegetable soil. Fertilization also increased the copy numbers of *nirK* gene significantly at both WFPS, while the copy numbers of *nirS* gene were increased only at 75% WFPS (Figures 3c,d), which suggested that *nirK*-harboring denitrifiers were more sensitive to environment. The copy numbers of *nirK* gene were 5.9–13.5 times greater than those of *nirS* gene under all treatments (Figures 3c,d), which was similar with the result reported by Bao et al. (2012). The more abundant denitrifiers of *nirK*-harboring than those of *nirS*-harboring indicated that *nirK*-harboring denitrifiers might have a greater contribution to denitrification and N_2O emission in the vegetable soil. As can be observed in Figures 3c,d, DMPP decreased the copy numbers of *nirS* and *nirK* genes significantly on days 3 and 30 at both WFPS ($P < 0.01$), which could be ascribed to restrained denitrification

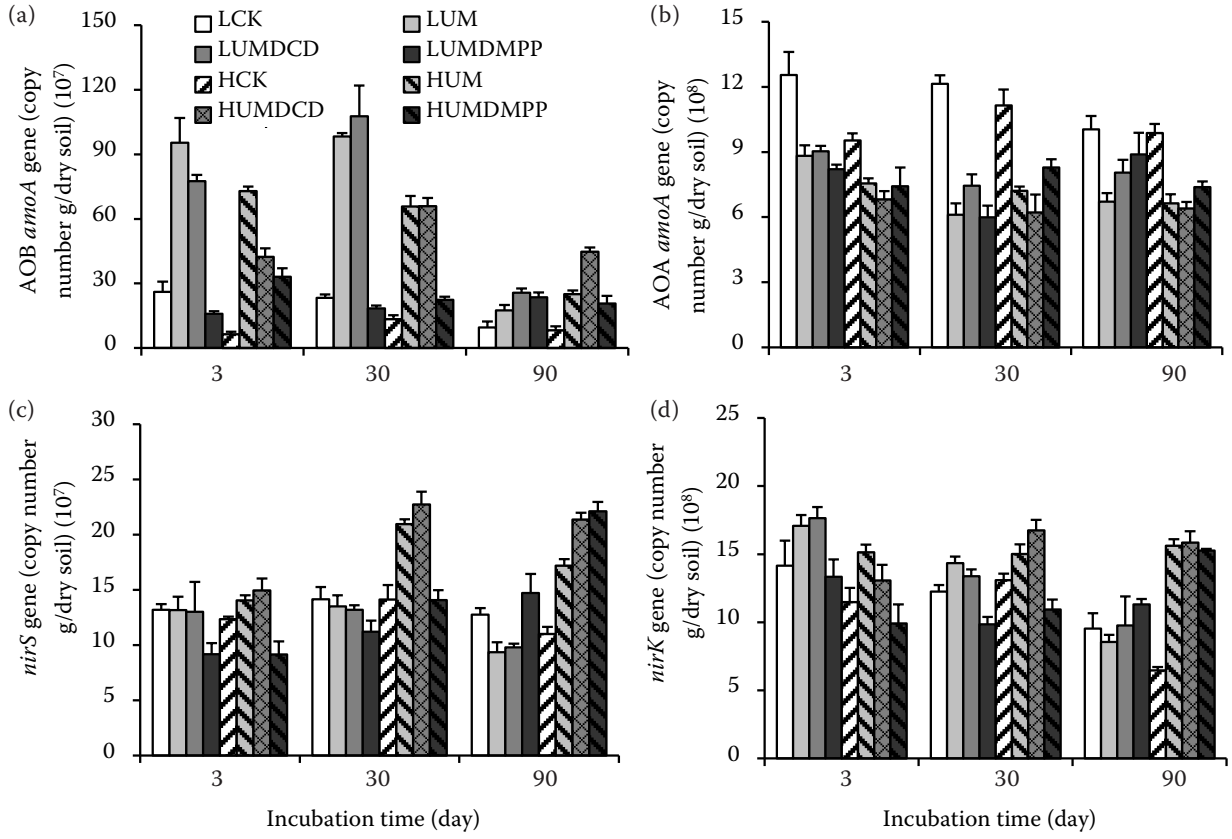

Figure 3. The copy numbers of ammonia oxidizing bacteria (AOB) *amoA* gene (a), ammonia oxidizing archaea (AOA) *amoA* gene (b), *nirS* gene (c) and *nirK* gene (d) at 55% water filled pore space (WFPS) (L) and 75% WFPS (H). CK – no fertilizer; U – urea; UM – urea + manure; UMDCD – urea + manure + dicyandiamide (DCD); UMDMPP – urea + manure + 3,4-dimethylpyrazole phosphate (DMPP). Error bars indicate the standard deviation of the mean ($n = 3$)

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due to reduced NO_3^- content in the presence of DMPP. Similar result was also found by Dong et al. (2013) in a laboratory incubation experiment for *nirS* gene abundance. Previous report had revealed that AOB harbored *nirK* gene (Garbeva et al. 2007), therefore, the strong inhibition effect of DMPP on AOB abundance might decrease the copy numbers of *nirK* gene to some extent. In contrast to DMPP, DCD had no effect on copy numbers of *nirK* and *nirS* genes (Figures 3c,d), which was consistent with the report obtained by Jha et al. (2013). These results suggested that DMPP was more effective than DCD on N_2O mitigation by inhibiting ammonia oxidizing and denitrifying microbes in high N level greenhouse vegetable soil.

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