Journal of Agricultural Science; Vol. 8, No. 7; 2016
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

A Newly Practice to Mitigate N_2O Emission from Winter Wheat Soil by Intercropping *Isatis indigotica*

Hong-Sheng Wu[1], Ji Li[1], Su-Yun Chen[1], Dong-Yang Liu[2], Joshua Kendall[3], Ya Xu[1], Lu-Ji Yu[4], Dong-Yang Wei[5], Shun-Yi Li[6], Ke-Ke Wang[6], Xiao-Xia Shang[1], Xiao-Hang Fang[5] & Ya-Dong Liu[7]

[1] Department of Agricultural Resources and Environment, Nanjing University of Information Science and Technology, Nanjing, China

[2] College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, China

[3] Department of Population and Community Ecology, Evangel University, Springfield, Missouri, USA

[4] College of Water and Irrigation and Environmental Engineering, Zhengzhou University, Zhengzhou, China

[5] South China Institute of Environmental Sciences, Ministry of Environmental Protection of China, Guangzhou, China

[6] College of Energy Resource and Chemical Engineering, Zhengzhou University, Zhengzhou, China

[7] Public courses, Yangzhou Polytechnic Institute, Yangzhou, China

Correspondence: Hong-sheng Wu, Department of Agricultural Resources and Environment, Nanjing University of Information Science and Technology, Nanjing 210044, China. Tel: 86-25-5869-9873. E-mail: wuhsglobe@sina.com, yzwhs@126.com

Lu-ji Yu, College of Water and Irrigation and Environmental Engineering, Zhengzhou University, Zhengzhou 450001, China. E-mail: yuluji@126.com

Received: April 23, 2016 Accepted: May 24, 2016 Online Published: June 15, 2016

doi:10.5539/jas.v8n7p7 URL: http://dx.doi.org/10.5539/jas.v8n7p7

Abstract

Greenhouse gas (GHG) emitted from agricultural field was received considerable attention worldwide, depending on differed land use and cropping system. An innovative strategy to mitigate agricultural N_2O by intercropping traditional Chinese medicinal herb *Isatis indigotica* in winter wheat field was assessed. By exogenously applying root exudates of *I. indigotica* in a lab incubation study, we testify and quantify whether N_2O emission was inhibited.

Results demonstrated great reduction of N_2O emission from winter field soil intercropping *I. indigotica* (NPKWR-N+P+K+wheat+*I. indigotica*) compared to CK (NPKW-N+P+K+wheat but no *I. Indigotica*) was found. N_2O emission in treatment of NPKWR was decreased by 32% than that in CK during the whole winter wheat growth season, among which the best decreasing N_2O emission was obtained in the stage of grain filling of winter wheat, N_2O emitting from NPKWR was reduced by 60% than that in CK. The N_2O emission intensity per kg of harvested wheat grain treated with *I. indigotica* was declined to 0.15 g N_2O/kg grain from 0.24 g N_2O/kg grain in CK.

qPCR (quantitative fluorescent polymerase chain reaction) analysis indicated nitrifying microbial population in wheat soil was severely suppressed by *I. indigotica*. The number of qPCR gene copy in both soil intercropping *I. indigotica* and exogenously applying root exudates of *I. indigotica* was lower than in CK. Such trend of decreased microbial population number was in agreement with that of N_2O emission from winter wheat field. This suggested that intercropping *I. indigotica* was a practical and simple technique to reduce N_2O emission from winter wheat field which was an effective strategy for mitigating and adapting global change worldwide in agriculture.

Keywords: winter wheat, N_2O emission, *Isatis indigotica*, ammonia oxidizing microbes, nitrifying bacteria, mitigating global change

1. Introduction

Nitrous oxide (N_2O) is a powerful greenhouse gas which also contributes to ozone depletion in the stratosphere (IPCC, 2007), and one of the most important non-CO_2 greenhouse gases (GHGs) with a global warming potential 298 times higher than that of carbon dioxide (CO_2) (IPCC 2001; Reay et al., 2012). Emissions of nitrous oxide (N_2O) from agricultural soils contribute to global warming and stratospheric ozone depletion. Nitrogen fertilization is considered a primary source of N_2O emissions from agricultural soils (Bouwman et al., 2002; IPCC, 2013; Qin et al., 2014; Kimr et al., 2015), where agriculture soils are the strongest single source contributing approximately 58% to total N_2O emissions (IPCC, 2007). Asia is the largest consumer of N fertilizer (FAO, 2012) as it accounts for 60% of the global N fertilizer production.

Most soil-evolved N_2O is produced by nitrification and denitrification processes (Bremner, 1997), where the soil microbes like nitrifying and denitrifying bacteria oxidize organic nitrogen compounds to release amino base and transforms into nitrous oxide (Massimiliano et al., 2014). In well aerated conditions, N_2O emissions from nitrification of ammonium based fertilizers can be substantial and is considered as a by-product of nitrification and may also occur by denitrification of nitrite by denitrifying organisms under oxygen stress (Mosier, 1994). Hence, N_2O emission flux could be seriously associated to the activity of nitrifying and denitrifying bacteria in soil.

Some agricultural practices could be used to mitigate N_2O emission. Management options to limit direct N_2O emissions from N-fertilized soils should emphasize improving N-use efficiency (Angela et al., 2015). Such management options include managing irrigation frequency, timing and quantity; applying N only to meet crop demand through multiple applications during the growing season or by using controlled release fertilizers; applying sufficient N only to meet crop needs; or using nitrification inhibitors. Compared to conventional tillage, reduced tillage or no-tillage plus winter cover crop decreases N_2O emission from winter wheat field (Petersen et al., 2011). By substituting winter grain/forage legumes for wheat, a technically feasible, low-input solution to the N pollution problems particularly N_2O emission could be used in the intensive rice-based cropping systems in the Taihu Lake Plain China (Zhao et al., 2015).

Some chemicals were developed to mitigate GHG. Dicyandiamide (Liu et al., 2015), another nitrification inhibitor, 3,4-dimethylepyrazole phosphate (DMPP) (Pfab et al., 2012), wheat herbicides such as acetochlor, tribenuron-methyl, fenoxaprop-p-ethyl and rice herbicides such as butachlor, bensulfuron-methyl (Jiang et al., 2015; Ju et al., 2011; Yan et al., 2013), returning wheat straw into field soil (Ma et al., 2009), biochar amendment to soil (Woolf et al., 2010; Sean et al., 2015; Xiang et al., 2015) could effectively reduce soil surface N_2O fluxes in the soil.

Are there any other cheaper, easier to practice and more effective approaches or land use pattern to control soil N_2O? Here, we investigated whether it would be possible for traditional Chinese medicinal herbs to mitigate N_2O. *I. indigotica*, as one of traditional Chinese medicines, contains a lot of active physiological and biochemical substances inhibiting many human and animal pathogens (Wang et al., 2006; Shin et al., 2010; Tian & Wang, 2012). Can these active chemicals be secreted from root of *I. indigotica* to suppress the nitrifying bacteria?

In the current investigation, *I. indigotica* was intercropped with winter wheat to testify whether there is any inhibitory effect of N_2O emission from winter wheat soil to explore a newly land use pattern based on our hypothesis of *I. indigotica* decreasing N_2O formation. Hence, our aims are (1) to determine if *I. indigotica* inhibits nitrifying bacteria, (2) to detect if *I. indigotica* reduces N_2O emission form wheat soil.

2. Materials and Methods

2.1 Experimental Site

Field experiment was conducted at Agro-meteorological Experimental Station, Nanjing University of Information Science and Technology, Nanjing China, 18.7055°E, 32.2068°N, where the climate type is belong to subtropical monsoon climate, 1200 mm in precipitation, 15.6 °C for annual mean air temperature, annual extreme high air temperature 39.7 °C， annual extreme low air temperature -10.7 °C, average day sunshine hour 1900 h, free frost period 237 day. Field soil is northern subtropical yellowish brown loam, clay-like soil with 26.1% clay particle (Table 1). The former season crop was maize.

Table 1. Soil physico-chemical properties

Organic matter ($g \cdot kg^{-1}$)	Fast available P ($mg \cdot kg^{-1}$)	Available K ($mg \cdot kg^{-1}$)	Fast available N ($mg \cdot kg^{-1}$)	Total N ($g \cdot kg^{-1}$)	pH
12.01	15.08	87.65	80.28	0.75	7.2

Lab experiment (static-state incubation by exogenously supplying *I. Indigotica* root exudates in lab) was carried out in the agricultural environment lab, Nanjing University of Information Science and Technology, Nanjing China. Soil was taken from the same place, 20 cm plowing layer before growing winter wheat, wind-dried and passed 60 mesh sieves.

2.2 Field Experiment

2.2.1 Plant Materials

Winter wheat (*Triticum aestivum* L.), variety-Ningmai 13 was brought from Jiangsu zhongjiang seed industry Co. Ltd., and the seed of traditional Chinese medicinal herb plant, *Isatis indigotica*, was obtained from Bozhou Chinese medicinal herbal plants market, Anhui, China.

2.2.2 Static Closed Chamber

N_2O gas was sampled with static closed chamber which was made of PVC, with size of 50 cm × 50 cm × 110 cm, comprised of basal seat, main box body and up cover. The thickness of the PVC chamber was 5 mm.

2.2.3 Experimental Design

Two treatments were established, where (1) NPKW (control, N-nitrogen, P-phosphorous, K-potasium, W-wheat): 900 $kg \cdot hm^{-2}$ of 45% compound fertilizer + winter wheat, and (2) NPKWR (R-*Isatis indigotica*): 900 $kg \cdot hm^{-2}$ of 45% compound fertilizer + winter wheat + traditional Chinese medicinal herbal plant *I. indigotica*. The plot area was 16 m^2 (4 m × 4 m), with width of 50 cm and height of 20 cm footpath (ridge) surrounding each independent plot with three replicates for each treatment. All plots were distributed randomly. The used compound fertilizer was obtained from Zhongdong chemical fertilizer company, with total nutrients content of 45% (15% N, 15% P_2O_5 and 15% K_2O) applied as basal fertilizer into soil before growing, while additional 75 $kg \cdot hm^{-2}$ urea was used on April 26, 2014.

2.2.4 Cultivating Plants

Seeds of winter wheat (*Triticum aestivum* L.), and *I. indigotica* were sowed on October 30, 2013. Winter wheat was seeded at the rate of 200 $kg \cdot hm^{-2}$, with column space of 10 cm. *I. indigotica* was seeded at the rate of 18.75 $kg \cdot hm^{-2}$, with column space of 20cm apart from wheat line. The wheat seedlings germinated on November 10, 2013 and the *I. indigotica* germinated 4 days earlier than wheat. All field management practices were the same for each plot. Wheat and *I. indigotica* were harvested on May 30, 2014.

2.2.5 Greenhouse Gases Sampling and Determination

A static closed chamber was used to collect greenhouse gases and gas chromatography was used to analyze N_2O concentration and flux. The static closed chamber was fixed into the field soil in plots while wheat and *I. indigotica* germinated and grew 5 cm high. Inside each chamber (50 cm × 50 cm) was 30 wheat seedlings. Wheat and *I. indigotica* seedlings were in the chamber for the treatment of intercropping traditional Chinese medicinal herbs. Sampling of N_2O began while the mean wheat plant height reached 10cm. Samples were taken every 4-5 days. Samples were taken from 9:00 to 11:00 on a sampling day. Gas sampling frequency was increased after application of fertilizer or rain event. During the winter (mid-December–mid-February), samples were taken every two weeks. Water was added into the chamber linkage to seal the chamber body prior to sampling. Gas sampling was performed at five chambers simultaneously. The chamber air temperature was recorded before and after sampling. Sampling began after sealing of the chamber and a 10 minute wait time for chamber gas mixing. Gases were collected with a 50 ml-volume plastic syringe equipped a three-passage valve at 0, 5, 10, 15 and 20 min.

Greenhouse gas samples were analyzed in Jiangsu Provincial Key Lab for Agricultural Greenhouse Gas Mitigation. Gas chromatographer Agilent 7890A was used to determine N_2O concentration. Standard N_2O was 520 × 10^{-9} $mg \cdot L^{-1}$, obtained from Nanjing shangyuan industrial gas station. Chromatography was performed under the conditions of N_2O detector, FID, detection temperature, 300 °C, chromatograph column, SS-2 m × 2 mm × Porapak Q (60/80), column temperature, 50 °C, converter, nickel catalyst, temperature 375 °C, carrier gas, pure dinitrogen (flow rate 25 $cm^3 \cdot min^{-1}$), inflammable gas, hydrogen (flow rate 45 $cm^3 \cdot min^{-1}$), air (flow rate 400 $cm^3 \cdot min^{-1}$), retention time 1.40 min.

N_2O emission flux from wheat field was analyzed by the chromatograph values based on the slope of the relationship between gas sample concentration vs time and inner air temperature inside chamber body at sampling. Actual N2Oemission flux was calculated by the following formula (Zou et al., 2005):

$$F = 60 \times H \times \frac{dc}{dt} \times \frac{44 \times 1.103}{8.314 \times (273 + T)} \quad (1)$$

Where,

F: determined gas flux ($mg \cdot m^{-2} \cdot h^{-1}$); H: the overall height of chamber; dc/dt: the concentration change of detected gas at unit time; T: mean air temperature inside the chamber during sampling (°C); 44: gas mole mass for N_2O; 8.314: universal gas constant. N_2O emission flux was expressed as mean ± standard deviation of three replicates.

2.2.6 Determination of Biomass and Yield of Winter Wheat and *I. indigotica*

Wheat and *I. indigotica* were harvested on May 30, 2014. A representative sample 1 m × 1 m was harvested, threshed, and impurities were discarded. Yield of wheat and *I. indigotica* were determined dry weight per hectare.

2.3 Lab Incubation Experiment

2.3.1 Experimental Design

This experiment had five treatments: (1) CK (control): 1500 g soil + 800 mL distilled water; (2) RI1 (*Isatis indigotica*): 1500 g soil + 800 mL distilled water + 0.2 mL condensed root exudates of *I. indigotica*; (3) RI2: 1500 g soil + 800 mL distilled water + 0.4 mL condensed root exudates of *I. indigotica*; (4) RI3: 1500 g soil + 800 mL distilled water + 0.8 mL condensed root exudates of *I. indigotica*; (5) RI4: 1500 g soil + 800 mL distilled water + 1.6 mL condensed root exudates of *I. indigotica*. Here, the abbreviated word RI means *I. Indigotica.* The experiment was conducted at temperature 15 °C. Location within the incubators were distributed randomly and maintained for 16 days. The incubator was a simple bottle equipped with gas sampling chamber and thermometer (Figure 1). Before initiation of the experiment, air-tightness of every apparatus was determined by submersion of the chamber to detect the water-tightness of the bottle plug and stopper.

Figure 1. Diagram of bottle incubator

2.3.2 Collecting and Condensing Root Exudates of *Isatis indigotica*

Seeds of *I. indigotica* were disinfected with 10% H_2O_2 for 10 min. The seeds were rinsed and put onto 4 layers of filter paper. Seeds were covered with one filter paper and water was sprayed to keep the paper wet. Damp seeds were incubated at 25 °C. 1/5 MS nutrient solution was added when germinating. Seedlings were moved into conical flask with 1/2 MS nutrient solution when two leaves had emerged. From 9:00 through 13:00

everyday root exudates were collected by moving seedlings to 5000 mL beaker with distilled water. When seedlings had grown to 8-10 leaves, root exudates were collected for two weeks continuously. The collecting root exudates were condensed with a water bath at 50 °C and to concentrate 1000 times. The condensed liquid was filtered to discard the impurities. Filtration was conducted by syringe with inner diameter of filter pore of 0.45 μm. The condensed lroot exudates were stored at 4 °C until use.

2.3.3 N_2O Sampling and Measurement

N_2O was sampled from 9:00 through 11:00 morning. Every 40 minute a gas sample was collected. Three intervals were collected. When collecting gas from bottle incubator, the syringe was pumped 5 times so as to homogenize the gases. The temperature was recorded to correct the calculation of N_2O flux.

N_2O concentration was measured as the mentioned method. However, the calculation formula was modified as below (Zou et al., 2005):

$$F = 60 \times V \times \frac{dc}{dt} \times \frac{44 \times 1.013}{8.314 \times (273 + T) \times m} \quad (2)$$

Where,

F: flux of detected gas ($mg \cdot kg^{-1} \cdot h^{-1}$); V: the gases volume inside the bottle incubator (ml); dc/dt: detected gas concentration inside the bottle in a unit time; T: mean gas temperature inside the bottle during sampling (°C), 44: mole gas mass of N_2O; m: soil weight inside bottle (g); 8.314: universal gas constant.

2.4 Soil Total DNA Extraction

Soil DNA was extracted with MoBio Soil DNA extract Kit based on the manufacturer manual. The final soil microbial DNA was dissolved in 200 μL of TE solution for future use.

2.5 Quantitative Fluorescent Real-Time Polymerase Chain Reaction (qPCR or qRT-PCR) Detectation

2.5.1 Target DNA Primers and Fluorescent DNA Probes Design

Field soil N_2O emission originated from the oxidization and conversion of residual plant body and N fertilizer by soil ammonia oxidizing bacteria and nitrifying bacteria populations. In the present investigation, we select several key soil nitrifying bacteria to simultaneously detect the possible effect of traditional Chinese medicinal herb on these microbes and subsequently effect N_2O emission based on qPCR. Soil nitrifying bacteria included ammonia oxidizing bacteria, ammonia oxidizing archae, *Nitrospira* spp., and *Nitrobacter* spp.. Primer pairs for qPCR were selected as: amoA1F/amoA2R: GGGGTTTCTACTGGTGGT/CCCCTCKGSAAAGCCTTCTTC for ammonia oxidizing bacteria (Rotthauwe, et al., 1997), Arch-amoAF/Arch-amoAR: STAATGGTCTGGCTTAG ACG/GCGGCCATCCATCTGTATGT for ammonia oxidizing archar (Hermansson & Lindgren 2001; Francis et al., 2005), NSR 1113F/NSR 1264R: CCTGCTTTCAGTTGCTACCG/GTTTGCAGCGCTTTGTACCG for *Nitrospira* spp. (Graham et al., 2007), Nitro 1198F/Nitro 1423R: ACCCCTAGCAAATCTCAAAAAACCG/ CTTCACCCCAGTCGCTGACC for *Nitrobacter* spp. (Dionisi et al., 2002).

2.5.2 Standard Working Curve Preparation for qPCR

By using the template of target DNA fragment, amplify the template with responding PCR specific primers and reaction condition. Cut the electrophoresis agrose gel of PCR products to recover the PCR fragments. Link the pMD19-T vector to target DNA fragments with ligase and transform the complex by heat shock. Select the successful transformants to culture and extract the plasmid to verify the positive clone by PCR. Measure the plasmid concentration by Ultraviolet spectroscopy (Nanodrop, USA). Sequence the known concentration of plasmid. Prepare 6 different known concentrations of PCR fragments to conduct qPCR to draw standard working curve of fluorescent intensity vs DNA concentration (microbe's number).

2.5.3 DNA Amplification, Enzymatic Ligation, Transformation

PCR system was consisted Target DNA template coloned plasmid 1 μl, Premix Taq polymerase (containing amble dNTPs) 25 μl, forward primer 1 μl，reverse primer 1 μl, ddH_2O-22 μl. DNA amplifier was used for PCR by Applied Biosystem (Tokyo, Japan) under protocol: initial denaturation 5 min at 94 °C, denaturation 30 s at 94 °C, annealing 30 s at 55 °C, initial elongation 1.5 min at 72 °C, elongation for maturation 10 min, at 72 °C, storing 10 °C forever, with 30 cycles of such proliferation process. The PCR products were identified by electrophoresis and cut the agarose gel to recover the pure PCR products with AxyPrep PCR Cleanup kit (Kangning Life Science Co., Ltd., Suzhou, China). Link the recovered PCR products plasmid to vector with pMD19-T. The enzymatic ligation reaction system was comprised of pMD19-T Vector 1 μl, recovered plasmid of cut gel of PCR products 4 μl，Solution I 5 μl overnight at 16 °C based on the suggestions of manufacturer manual. Take a tube (about 200 μl) of accessible DH5α E. coli cell, add the ligated products. Mix the tube to

blend the system. Put the tube into ice water for an ice bath for 30 min. Then take the iced tube into the hot water at 42 °C for a water bath for 90 s, subsequently ice-bath for 2 min. Add 800 μl LB culture to incubate at 37 °C for 1 h in a shaking incubator with 100 rpm. Centrifuge the culture at 3000 rpm for 2-5 mins. Spread the culture bacteria on the plates to identify transformants. Pick white colony (successful transformants) from plates to 800 μl LB culture to incubate at 37 °C for 4 h in a shaking incubator at 100 rpm again.

2.5.4 Quantitative Fluorescent Real-Time PCR (qPCR) Performance

Measure the weight and concentration of transformants and quantify the DNA concentrations. Prepare a series of concentration gradients of transformaants. qPCR was performed to quantify the transformants with 7500 Real Time PCR System (Applied Biosystem, Tokyo, Japan). The PCR system comprised of transformants 1 μl, forward primer 0.4 μl, reverse primer 0.4 μl, dNTPs+ligase, 2 × All-in-one qPCR mix 10 μl (GeneCopoeia, USA), fluorescent probe dye 50 × ROX Reference Dye 0.4 μl, ddH_2O 7.8 μl, under reaction of initial denaturing at 95 °C for 10min, denaturing at 95 °C for 10 s, initial annealing at 55 °C for 20 s, elongation at 72 °C for 34 s with 30 cycles of such reaction. A standard working curve of amplification plot was obtained wile the qPCR was finished.

2.5.6 qPCR Determination and Quantification of Different DNA from Different Soil Samples

Prepare different standard working curve of amplification plot for different soil DNA respectively. Each soil DNA template was amplified by qPCR under the same condition as standard working curve to obtain soil DNA amplification plot to get Ct value. Replace the responding variable in the equation of standard working curve by the soil DNA Ct value to calculate the actual weight of soil DNA. Different DNA content represents different microbial number.

2.6 Data process and Statistical Analysis

Microsoft Excel 2003 was used to process data and draw figures and tables. Results were presented as mean ± SD of three replicates of treatments or sample for each treatment. One way analysis of variance (ANOVA) was used to statistically test the results. Data process software SPSS 19 was used to perform the statistical analysis at $p < 0.05$. Also SPSS 19 was used to perform the correlation analysis. LSD was used to compare the significance of differences between treatments.

3. Results

3.1 Effect of Intercropping Isatis indigotica on N_2O Emission Flux from Winter Wheat Field Soil

N_2O emission wassignificantly decreased with winter wheat field soil intercropping *I. indigotica*. During different growth stage of wheat, N_2O emission flux from soil was different. Less N_2O flux was emitted from soil during germination and overwinter, while gradually increased when greenish tillering. The largest N_2O emission was obtained during jointing, flowering and heading, full filling stage (Figure 2). Less N_2O was released from winter wheat soil amended with *I. indigotica* (NPKWR) than that from control (NPKW) during the all of the wheat growth stages (Figures 2 and 3). N_2O emission flux declined by 17% compared to control during overwinter, while by 24%, 40%, 18%, 60% compared with CK during greenish tillering, jointing, flowering and heading, filling stage, respectively (Figure 3). An average decrease of 32% of N_2O emission flux from wheat soil intercropping *I. indigotica* was found relative to CK during the entire wheat growth season (Figure 3). N_2O emission intensity per kg of harvested wheat grain was decreased from 0.24 g N_2O/kg harvested wheat grain in the wheat field (CK) to 0.15 g N_2O/kg harvested grain in the field treated with *I. indigotica*, which N_2O emission intensity was reduced by 37.36% in comparison with CK (Figure 4).

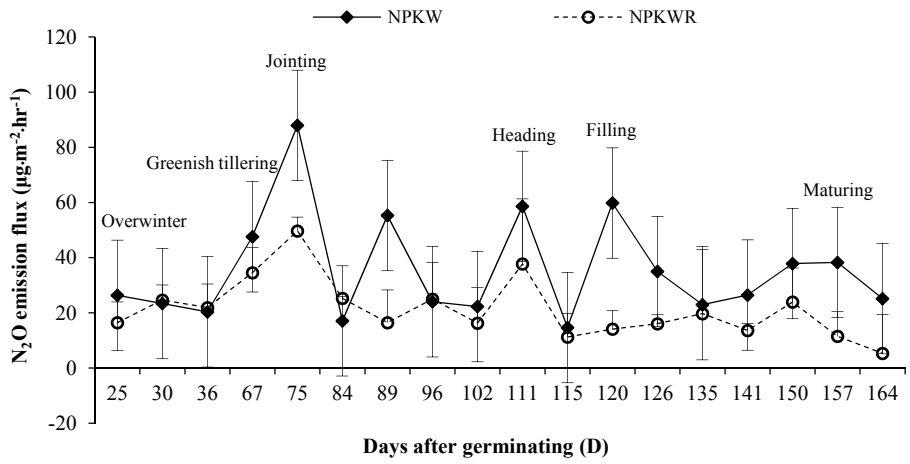


Figure 2. Effect of intercropping *Isatis indigotica* on N_2O emission flux of winter wheat grown in field

Note. Two treatments NPKW (CK) indicated winter wheat cultivated in field soil applied with 900 kg·hm^{-2} of 45% compound fertilizer (15% N, 15% P_2O_5 and 15% K_2O), and NPKWR shown winter wheat grown in field soil applied with 900 kg·hm^{-2} of 45% compound fertilizer (15% N, 15% P_2O_5 and 15% K_2O) and intercropping traditional Chinese medicinal herb plant *I. indigotica*. Each treatment had three replicates and results represented as mean + SD in the column. The bars indicated standard deviation.

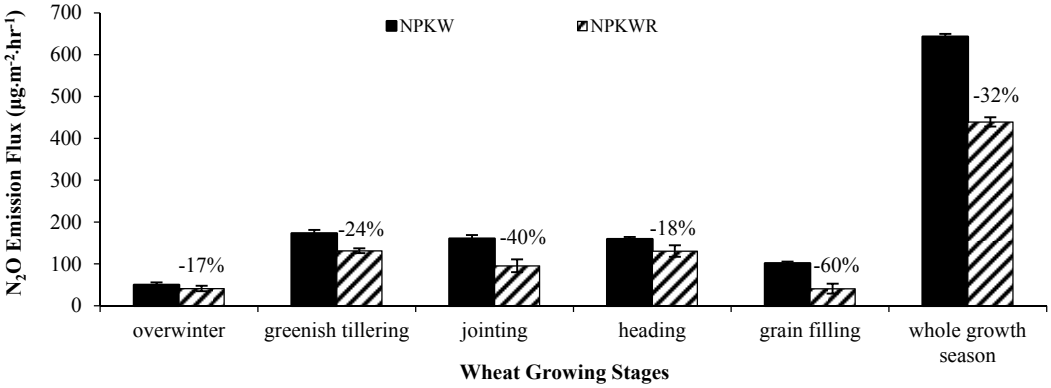


Figure 3. Effect of intercropping *Isatis indigotica* on N_2O emission flux of winter wheat grown in field during different growth stages

Note. Treatments NPKW (CK) indicated winter wheat cultivated in field soil applied with 900 kg·hm^{-2} of 45% compound fertilizer (15% N, 15% P_2O_5 and 15% K_2O), and NPKWR shown winter wheat grown in field soil applied with 900 kg·hm^{-2} of 45% compound fertilizer (15% N, 15% P_2O_5 and 15% K_2O) and intercropping traditional Chinese medicinal herb plant *I. indigotica*. Each treatment included three replicates and results represented as mean + SD in the column. The bars indicated standard deviation. The number on the grey streak suggested the N_2O decrease percentage of treatment NPKWR compared to CK (NPKW) at differed winter wheat growth stages.

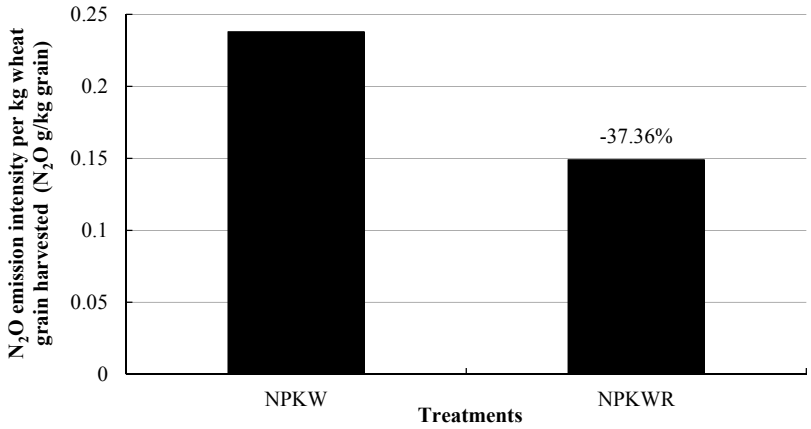
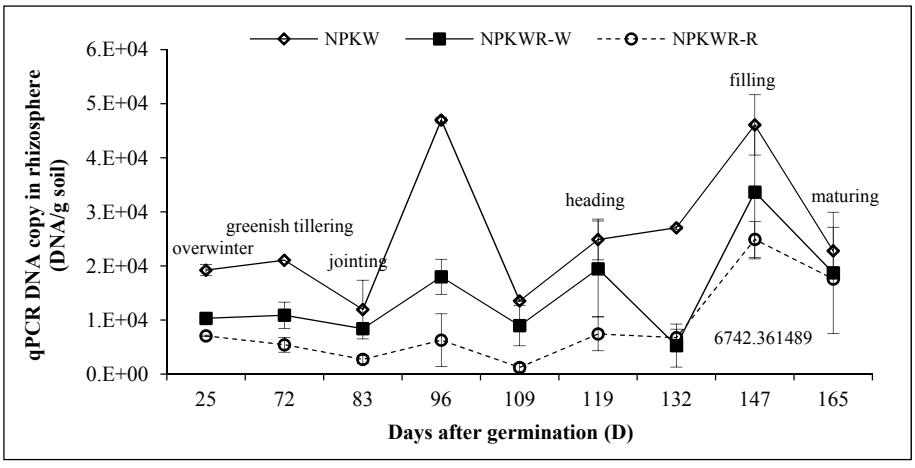


Figure 4. Effect of intercropping *Isatis indigotica* on N_2O emission intensity per kg of wheat grain harvested during the whole winter wheat growing season

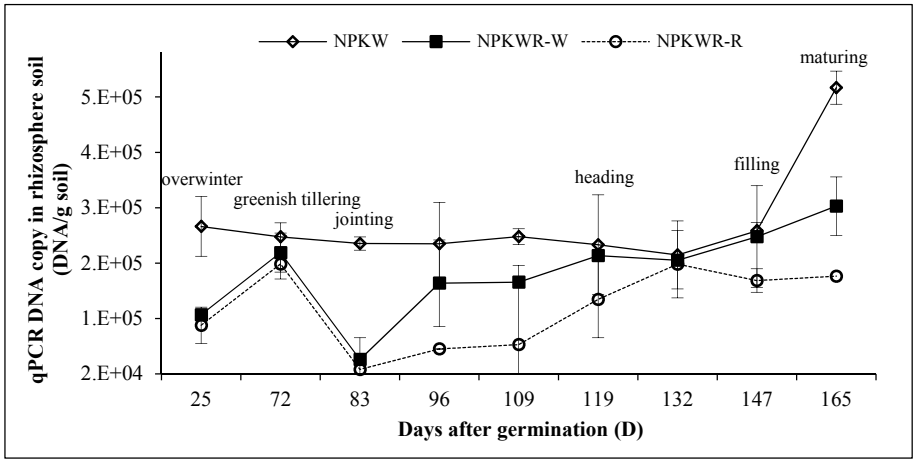
Note. Treatments NPKW (CK) indicated winter wheat cultivated in field soil applied with 900 kg·hm^{-2} of 45% compound fertilizer (15% N, 15% P_2O_5 and 15% K_2O), and NPKWR shown winter wheat grown in field soil applied with 900 kg·hm^{-2} of 45% compound fertilizer (15% N, 15% P_2O_5 and 15% K_2O) and intercropping traditional Chinese medicinal herb plant *I. indigotica*. Each treatment included three replicates and results represented as mean + SD in the column.

3.2 Effect of Intercropping Isatis indigotica on Rhizosphere Soil Microorganism Population (Ammonia Bacteria, Ammonia Archae, Nitrospiro spp. and Nitrobacter spp.) Related to N_2O Formation from Winter Wheat Soil Based on qPCR Analysis

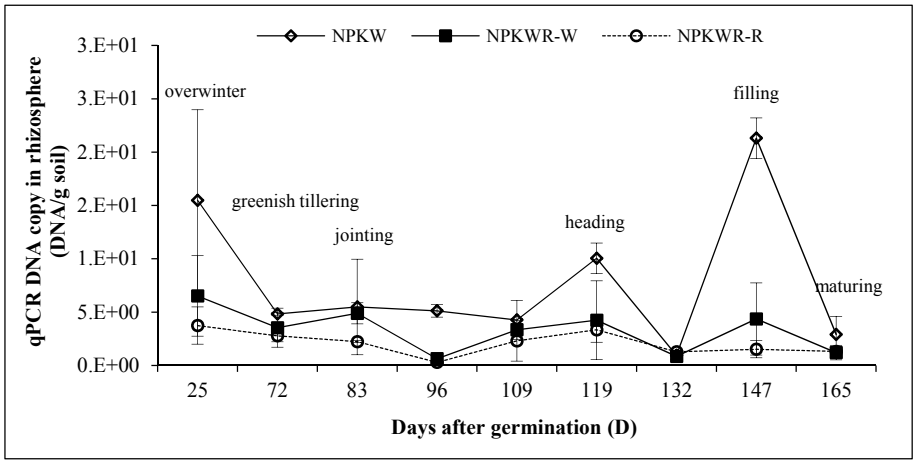
Agricultural soil microorganisms such as ammonia oxidizating bacteria, ammonia oxidizing archae, *Nitrospiro* spp. and *Nitrobacter* spp. was associated with the formation of N_2O directly from soil. In the present study, the number of qPCR DNA copy for such four sorts of microbes in the rhizosphere of *I. indigotica* (NPKWR-R) and in the wheat rhizosphere soil treated with *I. indigotica* (NPKWR-W) was less than that in wheat rhizosphre soil (CK, NPKW), while the number of such four sorts of microbes in the wheat rhizosphere (NPKWR-W) was also less than that in the wheat rhizosphere soil in CK (NPKW) (Figure 5). The change trends of qPCR DNA copy number of four sorts of microbes paralleled to those of N_2O emission from wheat soil. Several peaks of the populations number of four soil microbes responded to the N_2O emission peaks subsequently during the whole wheat growth season, where the dynamic fluctuation of ammonia oxidizing bacteria (Figure 5a), *Nitrospiro* spp. (Figure 5c) and *Nitrobacteria* spp. (Figure 5d) populations was in a higher agreement with N_2O emission from wheat soil.



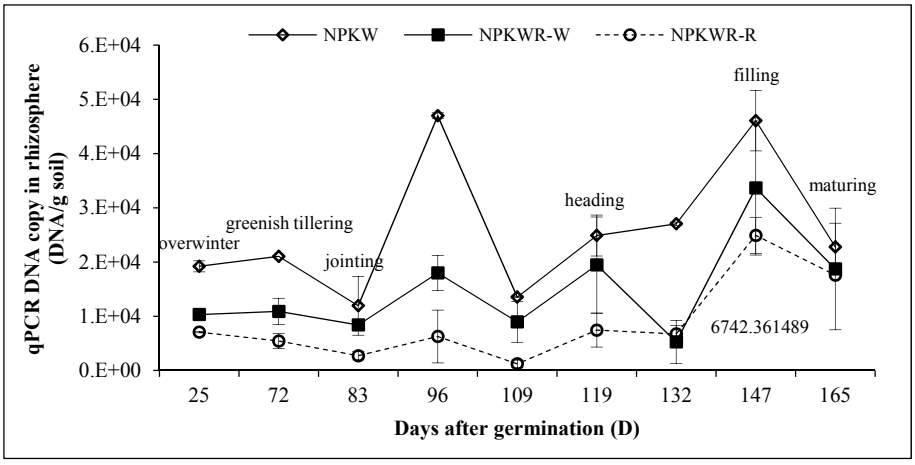
a. qPCR DNA copy number for primers amoA1F/amoA1R (ammonia oxidizing bacteria) in rhizosphere soil



b. qPCR DNA copy number for primers Arch-amoAF/Arch-amoA1R (ammonia oxidizing archae) in rhizosphere soil



c. qPCR DNA copy number for primers NSR1113F/NSR1264R (*Nitrospira* spp.) in rhizosphere soil



d. qPCR DNA copy number for primers Nitro1198F/Nitro1423R (*Nitrobacter* spp.) in rhizosphere soil

Figure 5. Effect of intercropping *Isatis indigotica* on qPCR DNA copy number of nitrifying and denitrifying microbes (ammonia oxidizing bacteria and archae, *Nitrospira* spp., *Nitrobacter* spp.) related to N_2O emission flux in wheat or *R. isatidis* rhizosphere soil in different stages during wheat growth season in field.

Note. NPKW (CK) indicated qPCR DNA copy number in winter wheat rhizosphere cultivated in field soil applied with 900 kg·hm^{-2} of 45% compound fertilizer (15% N, 15% P_2O_5 and 15% K_2O), NPKWR-W shown qPCR DNA copy number in winter wheat rhizosphere grown in field soil applied with 900 kg·hm^{-2} of 45% compound fertilizer (15% N, 15% P_2O_5 and 15% K_2O) in treatment of NPKWR and intercropping traditional Chinese medicinal herb plant *R. isatidi*s, and NPKWR-R shown qPCR DNA copy number in herb *R. isatidis* rhizosphere grown in field soil applied with 900 kg·hm^{-2} of 45% compound fertilizer (15% N, 15% P_2O_5 and 15% K_2O) in treatment of NPKWR. Each treatment included three replicates and results represented as mean + SD in the column. The bars indicated standard deviation. The words on the column suggested the growing stage of winter wheat.

3.3 Effect of Exogenously Adding Root Exudates of Isatis indigotica on N_2O Emission from Soil in a Lab Incubator

N_2O emission from soil amended with root exudates of *I. indigotica* in a lab incubator was significantly inhibited. N_2O emission flux in the treatments of all kinds of concentrations of root exudates of *I. indigotica* (RI1-RI4) was obviously lower than CK and much less decreased N_2O emission flux was obtained with increasing of root exudates of *I. indigotica*, which suggested a suppression of N_2O emission from soil by root exudates of *I. indigotica* (Figure 6).

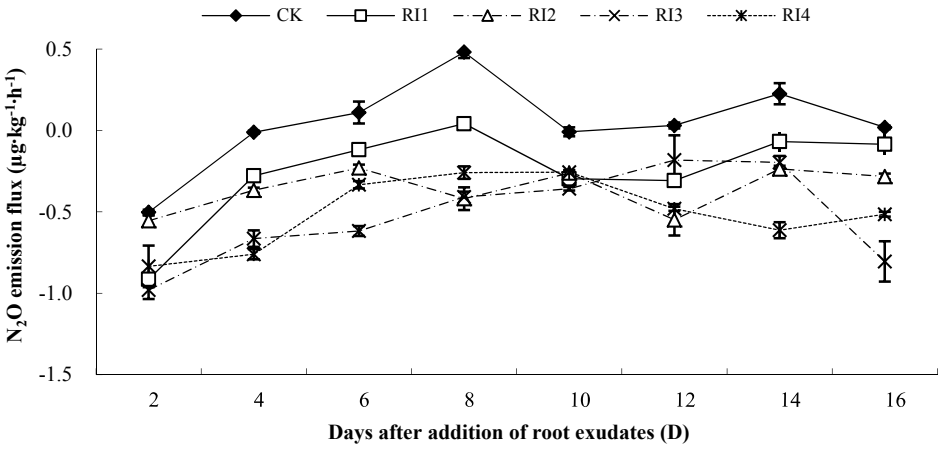
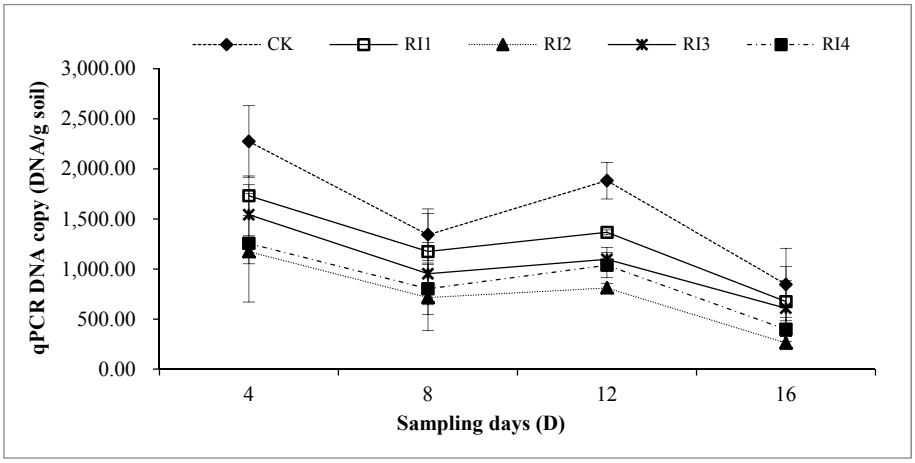


Figure 6. Effect of different concentrations of root exudates of *Isatis indigotica* on N_2O emission from soil in lab bottle incubator

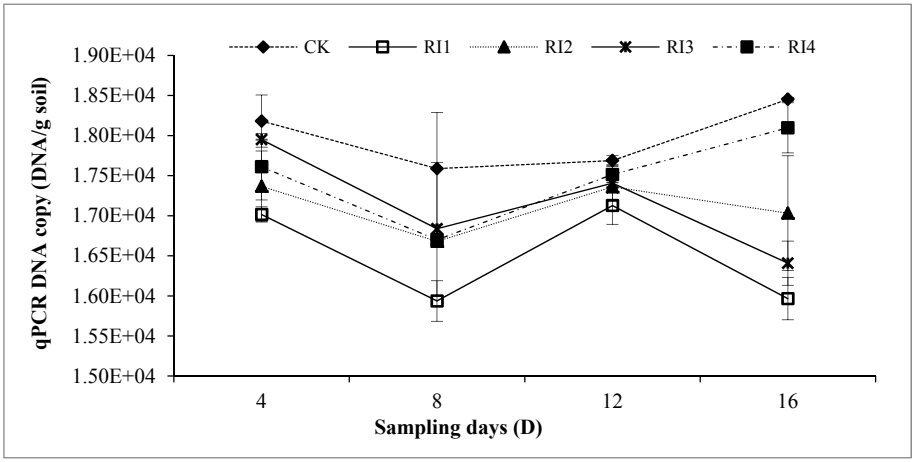
Note. (1) CK indicated no root exudates added into soil, 1500 g soil + 800 mL distilled water, (2) RI1: 1500 g soil + 800 mL distilled water + 0.2 mL condensed root exudates of *I. indigotica*, (3) RI2: 1500 g soil + 800 mL distilled water + 0.4 mL condensed root exudates of *I. indigotica*, (4) RI3: 1500 g soil + 800 mL distilled water + 0.8 mL condensed root exudates of *I. indigotica*, (5) RI5: 1500 g soil + 800 mL distilled water + 1.6 mL condensed root exudates of *I. indigotica* Here, the abbreviated word RI means *I. Indigotica* The experiment was conducted at a lab bottle incubator, room temperature 15 ^{o}C, ambient sunshine for 16 days. Each treatment included three replicates and results represented as mean + SD in the column. The bars indicated standard deviation.

3.4 Effect of Artificially Applying Root Exudates of I. indigotica on Nitrifying Microbes (Ammoinia Oxidizing Bacteria, Ammonia Oxidizing Archae, Nitrospiro spp. and Nitrobacter spp.) in a Lab Bottle Incubator

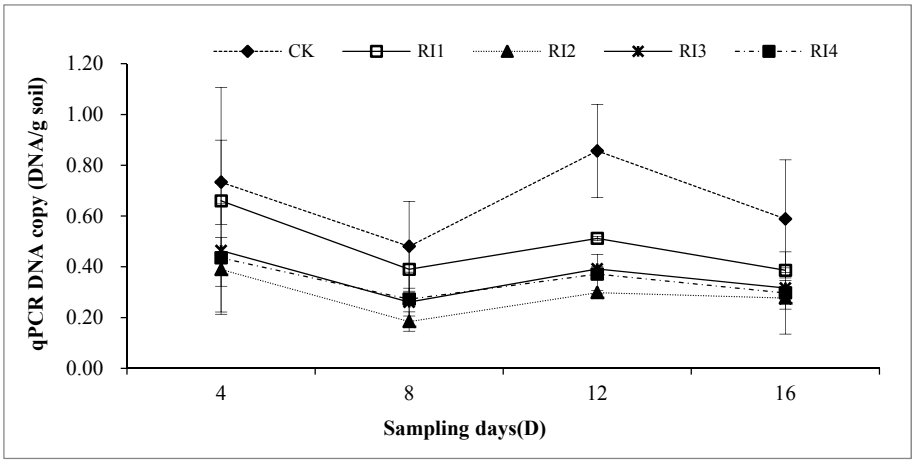
Nitrifying microbes, ammoinia oxidizing bacteria, ammonia oxidizing archae, *Nitrospira* spp. and *Nitrobacter* spp., directly associated to N_2O emission from soil, were affected significantly by root exudates of *I. indigotica.* The qPCR DNA copy number of four tested nitrifying microbes from soil treated with root exudates of *I. indigotica* was lower than CK and stronger depression of qPCR gene copy with increasing root exudates of *I. indigotica* (Figure 7).



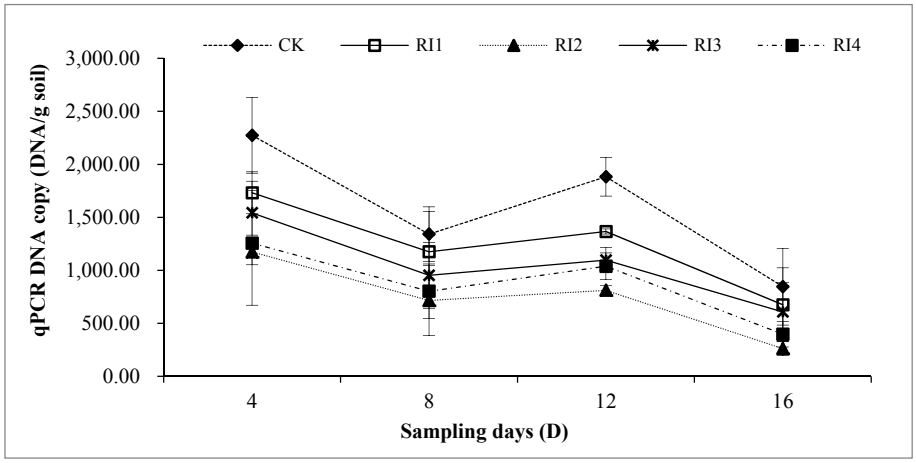
a. qPCR DNA copy number for primers amoA1F/amoA1R (ammonia oxidizing bacteria) in incubator soil



b. qPCR DNA copy number for primers Arch-amoAF/Arch-amoA1R (ammonia oxidizing archae) in incubator soil



c. qPCR DNA copy number for primers NSR1113F/NSR1264R (*Nitrospira spp.*) in incubator soil



d. qPCR DNA copy number for primers Nitro1198F/Nitro1423R (*Nitrobacter* spp.) in incubator soil

Figure 7. qPCR DNA copy number of nitrifying microbes (a. ammonia oxidizing bacteria, b. ammonia oxidizing archae, c. *Nitrospira* spp., and d. *Nitrobacter* spp.) related to N_2O emission flux soil amended with different concentrations of root exudates of *Isatis Indigotica* in lab bottle incubator

Note. (1) CK indicated no root exudates added into soil, 1500 g soil + 800 mL distilled water, (2) RI1: 1500 g soil + 800 mL distilled water + 0.2 mL condensed root exudates of *I. indigotica*, (3) RI2: 1500 g soil + 800 mL distilled water + 0.4 mL condensed root exudates of *I. indigotica*, (4) RI3: 1500 g soil + 800 mL distilled water + 0.8 mL condensed root exudates of *I. indigotica*, (5) RI5: 1500 g soil + 800 mL distilled water + 1.6 mL condensed root exudates of *I. indigotica*. Here, the abbreviated word RI means *I. Indigotica*. The experiment was conducted at lab bottle incubator, room temperature 15 ^{o}C, ambient sunshine for 16 days. Each treatment included three replicates and results represented as mean + SD in the column. The bars indicated standard deviation.

4. Discussions

It is well known that cultivating winter wheat emitted N_2O from soil, which contributes to agricultural GHG and global climate change. Such deleterious gas was partly and significantly controlled and mitigated by intercropping a traditional Chinese medicinal herb, *Isatis indigotica*, in the winter wheat field in the present work. About 1/3 as much as N_2O emission was reduced from winter field soil intercropping *I. indigotica* compared to check (Figures 1 and 2). This reduction of N_2O obviously was associated to *I. indigotica* which released some active compounds from root during growth and was supported by the results of decreased N_2O from incubator by exogenously supplying purified and concentrated root exudates of *I. indigotica* (Figure 6). This was the first report of new land use pattern by co-cropping herbal plant to mitigate N_2O emitted from agricultural soil. Compared to some other agricultural practices such as N fertilizer management (KAISER et al., 1998; Ulrike et al., 2003; Chu et al., 2007; Fub et al., 2011; Aguilera et al., 2013; Fisk et al., 2015; Frederico et al., 2015), reduced tillage (Fub et al., 2011; Fisk et al., 2015; Frederico et al., 2015), drip irrigation and fertigation (Aguilera et al., 2013; Kennedy et al., 2013), organic amendments (Qiu et al., 2013; Aguilera et al., 2013; Fisk et al., 2015), crops straw incorporation (Ma et al., 2009; Yang et al., 2015), biochar (Xiang et al., 2015), nitrification inhibitor (Ranucci et al., 2011; Barneze et al., 2015), this newly developed approach could be more effective and economical as it was simple and easily to conduct in a low-cost manner for farmer. Of course, it needs to be further improved and optimized.

Soil N_2O emission was originated from soil microbial activity which secrets some hydrolases and invertases to catalyze ammonia N nitrifying into all sorts of nitrogen oxides including N_2O. Nitrifying bacteria (ammonia oxidizing bacteria and archae) are the key and dominant populations related to N_2O formation and release from soil (Mei et al., 2004; Ju et al., 2011; Saggar et al., 2013). It is believed that N_2O emission is positively correlated to soil nitrifying bacteria activity. Hence, by inhibiting the activity of soil nitrifying bacteria to mitigate N_2O emission certainly was reasonable and practical. Linking to the current investigation, it could be implied that some active substances suppressed the activity of ammonia oxidizing bacteria and archae and nitrifying bacteria in winter wheat field soil because *I. indigotica* contains a lot of active physiological and biochemical substances

inhibiting many human and animal pathogens. This practical land use pattern could be beneficially developed to decrease greenhouse gas emission so order to avoid global change partly.

5. Conclusions

N_2O emission from winter wheat soil was significantly decreased by *I. indigotica* ascribed to the suppression of nitrifying bacteria and ammonia oxidizing bacteria, potentially considered as a newly strategy for mitigating and adapting global change in agriculture.

Acknowledgements

Part of this work was supported financially from Environmental Protection Ministry of China project Major Science and Technology Program for Water Pollution Control and Treatment (2015ZX07204-002). We would like to thank graduates from Nanjing Agricultural University, Cong Wang, Lei Ma and Yao-jun Zhang, for their assistance in determination of N_2O and performance of qPCR. Special thanks given to Dr. Professor Saman Bowatte and Jahufei Zulfi from AgResearch Limited, New Zealand, for their kind help in improving and language-editing before submission.

References

Aguilera, E., Luis, L., Alberto, S.-C., Josette, G., & Antonio, V. (2013). The potential of organic fertilizers and water management to reduce N_2O emissions in Mediterranean climate cropping systems: A review. *Agri. Ecosyst. Environ., 164*, 32-52. http://dx.doi.org/10.1016/j.agee.2012.09.006

Angela, T.-R., Sonia, G.-M., Mariela, N., Emilia, L.-S., Jose, L. T., & Antonio, V. (2015). N_2O and CH_4 emissions from a fallow–Wheat rotation with low N input in conservation and conventional tillage under a Mediterranean agroecosystem. *Sci. Total Environ., 508*, 85-94. http://dx.doi.org/10.1016/j.scitotenv.2014.11.041

Barneze, A. S., Minet, E. P., Cerri, C. C., & Misselbrook, T. (2015). The effect of nitrification inhibitors on nitrous oxide emissions from cattle urine depositions to grassland under summer conditions in the UK. *Chemosphere, 119*, 122-129. http://dx.doi.org/10.1016/j.chemosphere.2014.06.002

Bouwman, A. F., Boumans, L. J. M., & Batjes, N. H. (2002). Modeling global annual N_2O and NO emissions from fertilized fields. *Glob. Biogeochem. Cycles, 16*, 1080. http://dx.doi.org/10.1029/2001GB001812

Bremner, J. (1997). Sources of nitrous oxide in soils. *Nutr. Cycl. Agroecosys., 49*, 7-16. http://dx.doi.org/10.1023/A:1009798022569

Chu, H. Y., Yasukazu, H., & Kazuyuki, Y. (2007). NO, N_2O, CH_4 and CO_2 fluxes in winter barley field of Japanese andisol as affected by N fertilizer management. *Soil Biol. & Biochem., 39*, 330-339. http://dx.doi.org/10.1016/j.soilbio.2006.08.003

David, U., & Lal, R. (2013). Global Sources of Nitrous Oxide (Chapter 5). *Soil Emission of Nitrous Oxide and Its Mitigation* (p. 133). Springer Science+Business Media Dordrecht.

Dionisi, H. M., Layton, A. C., Harms, G., Gregory, I. R., Robinson, K. G., & Sayler, G. S. (2002). Quantification of Nitrosomonas oligotropha-like ammonia-oxidizing bacteria and *Nitrospira* spp. from full-scale wastewater treatment plants by competitive PCR. *Appl. Environ. Microbiol., 68*, 245-253. http://dx.doi.org/10.1128/AEM.68.1.245-253.2002

Fisk, L. M., Barton, L., Jones, D. L., Glanville, H. C., & Murphy, D. V. (2015). Root exudate car bon mitigates nitrogen loss in a semi-arid soil. *Soil Bio. & Biochem., 88*, 380-389. http://dx.doi.org/10.1016/j.soilbio.2015.06.011

Francis, C. A., Roberts, K. J., & Beman, J. M. (2005). Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc. National Acad. Sci. USA, 102*(41), 14683-14688. http://dx.doi.org/10.1073/pnas.0506625102

Frederico, C. B. V., Marisa, de C. P., Jeferson, D., & Johan, S. (2015). Soil nitrous oxide emissions as affected by long-term tillage, cropping systems and nitrogen fertilization in Southern Brazil Cimélio Bayer, Juliana Gomes, Josiléia Accordi Zanatta. *Soil & Till. Research., 146*, 213-222. http://dx.doi.org/10.1016/j.still.2014.10.011

Graham, D. W., Knapp, C. W., Van Vleck, E. S., Bloor, K., Lane, T., & Graham, C. E. (2007). Experimental demonstration of chaotic instability in biological nitrification. *ISME J., 1*, 385-394. http://dx.doi.org/10.1038/ismej.2007.45

Hermansson, A., & Lindgren, P. E. (2001). Quantification of ammonia oxidizing bacteria in arable soil by real time PCR. *Appl.Environ. Microbiol., 67*, 972-976. http://dx.doi.org/10.1128/AEM.67.2.972-976.2001

Hofstra, N., & Bouwman, A. F. (2005). Denitrification in agricultural soils: Summarizing published data and estimating global annual rates. *Nutri. Cycl. Agroecosys., 72*, 267-278. http://dx.doi.org/10.1007/s10705-005-3109-y

Hosomi. (2015). Mitigation of CH_4 and N_2O emissions from a forage rice field fertilized with aerated liquid fraction of cattle slurry by optimizing water management and topdressing. *Ecol. Engi., 75*, 24-32. http://dx.doi.org/10.1016/j.ecoleng.2014.11.034

IPCC. (2001). *Climate Change 2001: The Scientific Basis*. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change, Cambridge, United Kingdom and New York, USA.

IPCC. (2001). *Climate Change 2001: The Scientific Basis*. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change Cambridge. United Kingdom and New York, USA.

IPCC. (2007). *Climate Change 2007: The Physical Science Basis*. In S. Solomon, et al. (Eds.). Cambridge University Press, Cambridge.

IPCC. (2013). *Climate Change 2013: The Physical Science Basis*. Cambridge University Press, Cambridge, UK.

Jian, X., Liu, D. Y., Ding, W. X., Yuan, J. J., & Lin, Y. X. (2015). Effects of biochar on nitrous oxide and nitric oxide emissions from paddy fi eld during the wheat growth season. *J. Cleaner Prod., 104*, 52-58. http://dx.doi.org/10.1016/j.jclepro.2014.12.038

Jiang, J. Y., Chen, L. M., Sun, Q., Sang, M. M., & Huang, Y. (2015). Application of herbicides is likely to reduce greenhouse gas (N_2O and CH_4) emissions from riceewheat cropping systems. *Atmos. Environ., 107*, 62-69. http://dx.doi.org/10.1016/j.atmosenv.2015.02.029

Ju, X. T., Lu, X., Gao, Z. L., Chen, X. P., Su, F., Kogge, M., … Zhang, F. S. (2011). Processes and factors controllin g N_2O production in an intensively managed low carbon calcareous soil under sub-humid monsoon conditions. *Environ. Pollu., 159*, 1007-1016. http://dx.doi.org/10.1016/j.envpol.2010.10.040

Kaiser, E.-A., Kohrs, K., Kue, C. M., Schung, E., Heinemeyer, O., & Munch, J. C. (1998). Nitrous oxide Release from Arable Soil: Importance of N-Fertilization, Crops and Temporal Variation. *Soil Biol. Biochem., 30*(12), 1553-1563. http://dx.doi.org/10.1016/S0038-0717(98)00036-4

Kennedy, L., Taryn, E., Suddick, C., & Johan, S. (2013). Reduced nitrous oxide emissions and increased yields in California tomato cropping systems under drip irrigation and fertigation. *Agri. Ecosys. Environ., 170*, 16-27. http://dx.doi.org/10.1016/j.agee.2013.02.002

Kim, Y., Youngho, S., David, K., Steffen, K., Edwin, H., John, T., & Ralf, K. (2015). Estimation and mitigation of N_2O emission and nitrate leaching from intensive crop cultivation in the Haean catchment, South Korea. *Sci. Total Environ., 529*, 40-53. http://dx.doi.org/10.1016/j.scitotenv.2015.04.098

Lin, T., & Wang, Z. (2012). Study on Antibacterial Activity of *Isatis Indigotica* Extracts and Preliminary Investigation of Their Antibacterial Mechanism. *Proceedings of the 2012 International Conference on Applied Biotechnology (ICAB 2012)* (pp. 1681-1688), Lecture Notes in Electrical Engineering 251. Springer-Verlag Berlin Heidelberg.

Liu, Y.-N., Li, Y.-C., Peng, Z.-P., Wang, Y.-Q., Ma, S.-Y., Guo, L.-P., … Han, X. (2015). Effects of different nitrogen fertilizer management practices on wheat yields and N_2O emissions from wheat fields in North China. *J. Integra. Agri., 14*(6), 1184-1191. http://dx.doi.org/10.1016/S2095-3119(14)60867-4

Ma, J., Ma, E. D., Xu, H., Yagi, K., & Cai, Z. C. (2009). Wheat straw management affects CH_4 and N_2O emissions from rice fields. *Soil Biol. Biochem., 41*, 1022-1028. http://dx.doi.org/10.1016/j.soilbio.2009.01.024

Massimiliano, De A. M., Clemens, S., Peter, R., Grace, D. W., Rowlings, M. B., & James, M. (2014). Influence of different nitrogen rates and DMPP nitrificationinhibitor on annual N_2O emissions from a subtropical wheat-maize cropping system. *Agri. Ecosys. Environ., 186*, 33-43. http://dx.doi.org/10.1016/j.agee.2014.01.016

McNeill, A., & Murray, U. (2007). The Nitrogen Cycle in Terrestrial Ecosystems in Soil Biology. In P. Marschner & Z. Rengel (Eds.), *Nutrient Cycling in Terrestrial Ecosystems* (Volume 10, p. 40). Springer-Verlag, Berlin, Heidelberg. http://dx.doi.org/10.1007/978-3-540-68027-7_2

Mei, L. J., Yang, L. Z., Wang, D. J., Yin, B., Hua, J., & Yin, S. X. (2004). Nitrous oxide production and consumption in serially diluted soil suspensions as related to in situ N_2O emission in submerged soils. *Soil Biol. Biochem., 36*, 1057-1066. http://dx.doi.org/10.1016/j.soilbio.2004.03.001

Petersen, O. S., James, K. M., Elly, M. H., & Lars, J. M. (2011). Tillage effects on N_2O emissions as influenced by a winter cover crop. *Soil Biol. Biochem., 43*, 1509-1517. http://dx.doi.org/10.1016/j.soilbio.2011.03.028

Pfab, H., Iris, P., Franz, B., Sabine, F., Torsten, M., & Reiner, R. (2012). Influence of a nitrification inhibitor and of placed N-fertilization on N_2O fluxes from a vegetable cropped loamy soil. *Agri. Ecosys. Environ., 150*, 91-101. http://dx.doi.org/10.1016/j.agee.2012.01.001

Qin, S. P., Wang, Y. Y., Hu, C. S., Oenem, O., Li, X. X., Zhang, Y. M., & Dong, W. X. (2012). Yield-scaled N_2O emissions in a winter wheatesummer corn double-cropping system. *Atmos. Environ., 55*, 240-244. http://dx.doi.org/10.1016/j.atmosenv.2012.02.077

Qiu, S.-J., Ju, X.-T., Ingwersen, J., Guo, Z.-D., Stange, C. F., Bisharat, R., … Zhang, F.-S. (2013). Role of Carbon Substrates Added in the Transformation of Surplus Nitrate to Organic Nitrogen in a Calcaeous Soil. *Pedosphere, 23*(2), 205-212. http://dx.doi.org/10.1016/S1002-0160(13)60008-9

Ranucci, S., Teresa, B., Luca, V., Paul, D. T., Lucia, O., Marco, O., … Vincenzo, M. (2011). The influence of management and environmental variables on soil N_2O emissions in a crop system in Southern Italy. *Plant Soil, 343*, 83-96. http://dx.doi.org/10.1007/s11104-010-0674-x

Reay, D. S., Davidson, E. A., Smith, K. A., Smith, P., Melillo, J. M., Dentener, F., & Crutzen, P. J. (2012). Global agriculture and nitrous oxide emissions. *Nat. Clim Chang., 2*, 410-416. http://dx.doi.org/10.1038/nclimate1458

Rotthauwe, J. H., Witzel, K. P., & Liesack, W. (1997). The ammonia monooxygenase structural gene amoa as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol., 63*, 4704-4712.

Saggar, J. S., Singh, D. L., Giltrap, M., Zaman, J., Luo, M., Rollo, D.-G., … van der Weerden, T. J. (2013). Quantifi cation of reductions in ammonia emissions from fertiliser urea and animal urine in grazed pastures with urease inhibitors for agriculture inventory: New Zealand as a case study. *Sci. Total Environ., 465*, 136-146. http://dx.doi.org/10.1016/j.scitotenv.2012.07.088

Sean, D. C. C., Niall, P. M., David, S., Reay, A., Stott, W., Helen, K. G., & Jeanette, W. (2015). Biochar suppresses N_2O emissions while maintaining N availability in a sandy loam soil. *Soil Biol. Biochem., 81*, 178-185. http://dx.doi.org/10.1016/j.soilbio.2014.11.012

Singh, S. K., Shanthy, S., & Kaushal, K. (2013). *Photosynthetic Microorganism-Based CO_2 Mitigation System: Integrated Approaches for Global Sustainability* (Chapter 5, p. 83). Photosynthetic Microorganisms, Springer Briefs in Materials.

Ulrike, S., Reiner, R., & Jean, C. M. (2003). Nitrous oxide fluxes from maize fields: relationship to yield, site-specific fertilization, and soil conditions. *Agri. Ecosyst. Environ., 99*, 97-111. http://dx.doi.org/10.1016/S0167-8809(03)00139-7

Wang, W. Q., Fang, J. G., Liu, Y. H., & Li, Q. (2006). Screening of Anti-endotoxin Components from *Isatis Indigotica*. *J. Huazhong Uni. Sci. Tech., 26*(2), 261-264. http://dx.doi.org/10.1007/BF02895833

Wolf, U., Roland, F., Frank, H., & Heinz, F. (2014). Contribution of N_2O and NH_3 to total greenhouse gas emission from fertilization: Results from a sandy soil fertilized with nitrate and biogas digestate with and without nitrification inhibitor. *Nutr. Cycl. Agroecosyst., 100*, 121-134. http://dx.doi.org/10.1007/s10705-014-9631-z

Xu, Z., Wang, S. Q., & Xing, G. X. (2015). Maintaining rice yield and reducing N pollution by substituting winter legume for wheat in a heavily-fertilized rice-based cropping system of southeast China. *Agri., Ecosyst. Environ., 202*, 79-89. http://dx.doi.org/10.1016/j.agee.2015.01.002

Yan, G. X., Zheng, X. H., Cui, F., Yao, Z. S., Zhou, Z. X., Deng, J., & Xu, Y. (2013). Two-year simultaneous records of N_2O and NO fluxes from a farmedcropland in the northern China plain with a reduced nitrogen additionrate by one-third. *Agri., Ecosyst. Environ., 178*, 39-50. http://dx.doi.org/10.1016/j.agee.2013.06.016

Yang, H. S., Yan, B., Dai, Y. J., Xu, M. M., Roger, T., Wang, X. H., … Bian, X. M. (2015). Soil nitrogen retention is increased by ditch-buried straw return in a rice-wheat rotation system. *Europ. J. Agron., 69*, 52-58. http://dx.doi.org/10.1016/j.eja.2015.05.005

Zhu, T. B., Zhang, J. B., & Cai, Z. C. (2011). The contribution of nitrogen transformation processes to total N_2O emissions from soils used for intensive vegetable cultivation. *Plant and Soil, 343*(1), 313-327. http://dx.doi.org/10.1007/s11104-011-0720-3

Zou, J. W., Huang, Y., & Jiang, J. Y. (2005). A 3-year field measurement of methane and nitrous oxide emissions from rice paddies in China: Effects of water regime, crop residue, and fertilizer application. *Global Biochem. Cycles, 19*(2), 65-73. http://dx.doi.org/10.1029/2004GB002401