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Evaluation of N_2O sources after fertilizers application in vegetable soil by dual isotopocule plots approach

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ABSTRACT

Nitrogen (N) fertilizer is the major deriver of nitrous oxide (N_2O) emissions in agricultural soil. In the vegetable fields in China both inorganic and organic fertilizers are largely applied as basic sources of nitrogen. Identifying the effects of fertilizer type on soil microbial activities involved in N_2O emissions would be of great help for future development of N_2O reduction strategies. N_2O isotopocule deltas, including $\delta^{15}N^{bulk}$, $\delta^{18}O$ and SP (the ^{15}N site preference in N_2O), have been used to analyze microbial pathways of N_2O production under different treatments, including bio–organic fertilizer treatment, half bio–organic fertilizer and half urea (mixed fertilizer) treatment, urea treatment and no fertilizer treatment. We measured environmental factors, N_2O fluxes and N_2O isotopocule deltas to evaluate the dynamics of N_2O emissions and constructed the dual isotopocule plots ($\delta^{15}N^{bulk}$ vs. SP and $\delta^{18}O$ vs. SP) of the main N_2O emission phases to assess contribution of the involved microbial processes (bacterial nitrification, bacterial denitrification, nitrifier denitrification and fungal denitrification). According to the results of the main N_2O emission phases, we found that bio–organic fertilizer and mix fertilizer treatments had significantly lower N_2O emissions compared to urea treatment, with average N_2O fluxes of 1477 ± 204, 1243 ± 187 and 1941 ± 164 μg m^{-3} h^{-1}, respectively, but there were no significant effects on mineral N and cabbage yield. In addition, the urea treatment and the mixed fertilizer treatment had close and higher nitrogen use efficiency. Furthermore, the $\delta^{18}O$ vs. SP plot was useful for providing insight into microbial processes, showing that fungal denitrification/bacterial nitrification was the dominant microbial pathway and bio–organic fertilizer and mix fertilizer treatments had higher denitrification and N_2O reduction compared to urea treatment. Those findings demonstrated that the partial replacement of urea with bio–organic fertilizer was a better choice, by means of enhancing denitrification to reduce N_2O emissions and also guaranteeing the nitrogen use efficiency and the cabbage yield.

1. Introduction

Nitrous oxide (N_2O) is an important greenhouse gas and a main destroyer of the ozone layer. Microbial activity in agricultural soil are the major anthropogenic source of N_2O production (Smith et al., 2007). The microbial pathways discussed in this respect are bacterial nitrification (BN), bacterial denitrification (BD), nitrifier denitrification (ND) and fungal denitrification (FD) (Crenshaw et al., 2008; Rohe et al., 2017). However, it is still challenging to trace and quantify the source of N_2O from soil because of its temporal and spatial variability and unknown share of abiotic N_2O production (Hayatsu et al., 2008; Wei et al., 2019).

Many studies based on C_2H_2 inhibition, ^{15}N tracing or isotope labeling have been applied to distinguish N_2O produced by nitrification or denitrification (Baggs, 2008; Mueller et al., 2014). However, these methods have some limitations, such as interference with soil systems, uneven diffusion and long-term monitoring. The ^{15}N site preference (SP), the distinct $^{14}N/^{15}N$ isotope deltas difference between the central (α) and terminal (β) N of N_2O, was presented to serve as an indicator for N_2O microbial process (Toyoda and Yoshida, 1999). The SP of N_2O produced by BN or FD (32.8 ± 4.0‰) was found to be distinct from SP produced by BD or ND (−1.6 ± 3.8‰) (Decock and Six, 2013). In

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addition, the enrichment factor ($\varepsilon^{15}N$ or $\varepsilon^{18}O$), the difference of $\delta^{15}N$ or $\delta^{18}O$ in substrate and product, have been reported to be indicative for the formation processes of N_2O (Pérez, 2005). Characteristic isotopic signatures for certain microbial pathways determined in a number of pure culture experiments have been summarized in Toyoda et al. (2017), Denk et al. (2017) and Buchen et al. (2018). Correspondingly, the dual isotopocule plots of $\delta^{15}N^{bulk}$ vs. SP (Yamagishi et al., 2007; Zou et al., 2014) and $\delta^{18}O$ vs. SP (Maeda et al., 2017; Buchen et al., 2018) have been used to understand multiple microbial pathways of N_2O production. However, the $\delta^{15}N^{bulk}$ and $\delta^{18}O$ of N_2O in isotopocule plots are linked to the isotopic composition of the substrate (NH_4^+, NO_3^-, NO_2^- and H_2O). In contrast, the SP is independent of the N_2O precursor isotopic composition (Ostrom and Ostrom, 2011). Additionally, N_2O reduction under denitrifying conditions increases N_2O isotopocule deltas to complicate the attribution of microbial processes (Ostrom et al., 2007; Decock and Six, 2013; Lewicka–Szczebak et al., 2014; Toyoda et al., 2017). Recently, Yamamoto et al. (2017) estimated multiple processes of N_2O production using $\delta^{15}N^{bulk}$ vs. SP in N_2O, which was questioned because the authors did not consider $\delta^{15}N$ of precursors and N_2O reduction (Well et al., 2018). In addition, $\delta^{18}O$–N_2O, which was not assessed in this study, was discussed as a vital index in interpretation of N_2O source processes. Lewicka-Szczebak et al., (2016) reported nearly complete O exchange between N_2O and H_2O occur, suggesting the feasibility using $\delta^{18}O$–N_2O to assess microbial processes under determining $\delta^{18}O$–H_2O. Therefore, if precursors and reduction of N_2O are considered in isotopocule plots, $\delta^{15}N^{bulk}$ vs. SP plot and $\delta^{18}O$ vs. SP plot are conducive to evaluate the source of N_2O. However, it needs further be discussed that the application effect of them on evaluation of N_2O sources in field.

The processes of N_2O emission are affected by the soil moisture and temperature, mineral N, and organic carbon (C) (Beauchamp, 1997). In general, fertilization is one of the most commonly used agricultural managements in field production, which is considered to play a vital role in regulating agricultural N_2O emissions. In China, inorganic and organic fertilizers are largely applied to ensure crop yield and quality in vegetable fields. Most studies show that inorganic fertilizer can promote the nitrification process, while organic fertilizer appears to enhance denitrification and its contribution to N_2O production (Toma et al., 2007; Toyoda et al., 2011). Bio–organic fertilizer as a kind of organic fertilizer has been widely used in China to replace inorganic fertilizer because of its beneficial effect resistance against crop disease (Wu et al. 2009, 2015; Ma et al., 2018). In addition, bio–organic fertilizer might promote alternative N transformation and N_2O production pathways, because of the addition of exogenous microorganisms, which needs to be further explored. Besides, N_2O emissions and source processes, for the soil with added bio–organic fertilizer or part added bio–organic fertilizer, are expected to display a large spatial and temporal heterogeneity, which has not been explored until now.

In this study, we used stable isotope technology to ascertain source partitioning of N_2O in vegetable production systems under different fertilizer treatments and agricultural irrigation conditions. Applied fertilizer qualities include bio–organic fertilizer (O), half urea and half bio–organic fertilizer (mixed fertilizer, OU), urea (U) and no fertilizer (NF). The goal of our study was to (i) evaluate the effects of the replacement of and the partial replacement of urea with bio–organic fertilizer; (ii) quantify the contribution of major N_2O production processes by using dual isotopocule plots ($\delta^{15}N^{bulk}$ vs. SP and $\delta^{18}O$ vs. SP); (iii) to compare the applicability of $\delta^{15}N^{bulk}$ vs. SP and $\delta^{18}O$ vs. SP in the analysis of microbial processes.

2. Materials and methods

2.1. Study site

The study was conducted at the environmental research station of the Chinese Academy of Agricultural Sciences, situated in the Shunyi District, Beijing, China (40°15′N, 116°55′E). The local climate is temperate continental monsoon climate with an annual mean temperature of 12.5 °C and annual mean precipitation of 623.5 mm. The soil of the experiment field is classified as calcareous Fluvo–aquic (Food and Agriculture Organization FAO), with the following properties: silt 64.2%, sand 28.7%, clay7.1%, bulk density 1.4 g cm^{-3}, organic matter 15.5 g kg^{-1}, organic carbon 9.2 g kg^{-1}, total nitrogen 1.1 g kg^{-1}, total phosphorus 0.6 g kg^{-1}, total potassium 18.4 g kg^{-1}, and pH 8.1 (0.01M $CaCl_2$). In addition, the experiment field has been planted with vegetable (Chinese cabbage) for 2 years.

2.2. Field treatments

The Chinese cabbage (*Brassica pekinensis*, New Beijing No.3) were sown on 14 August 2014, with a hill spacing of 50 cm and a row spacing of 50 cm. Bio–organic fertilizer comprised decomposed organic solid waste such as manure and straw, and a novel composite strain of Japanese silicate bacteria combined with actinomycetes from Nocardia (OM, 45%; Humic Acid (HA), 35%; N, 2%; P, 1.5%; K, 1.5%; microbial count $> 4 \times 10^7$ g^{-1}). Urea (N, 46%) were applied with calcium superphosphate ($Ca(H_2PO_4)_2{\cdot}2H_2O$; P_2O_5, 18%) and potassium sulphate (K_2SO_4; K_2O, 52%). The experiment comprised four treatments with different combinations of bio–organic fertilizer and urea: bio–organic fertilizer (O), half bio–organic fertilizer and half urea (mixed fertilizer, OU), urea (U), no fertilizer (NF). Each treatment had four replicates. Those fertilizers were incorporated into soil on 13 august with uniform content at 300 kg N ha^{-1}. In addition, the mount of P_2O_5 and K_2O both was the same, of 225 kg ha^{-1}. The sprinkler irrigations were executed to maintain the requirement of cabbage growth on August 20, September 15 and October 26, respectively.

2.3. Sample collection

Gas samples were collected intensively in the early stage of fertilization and then were collected at one–week or two–week interval until the cabbage was harvested. Meanwhile, the soil samples were collected at 0–20 cm depth on the same day. Gas samples were collected from cylindrical PVC chambers (25 cm diameter and 50 cm height). The base of PVC chambers with water trough were inserted 5 cm into the soil, placed between two rows of cabbages. The PVC chambers were tightly placed on the base and sealed by water. The aluminum–sealed vent was installed on the top of the PVC chamber for sampling. There is a small fan and a temperature probe in the PVC chamber to assure a uniform distribution of gas and measuring temperature variation. At every sampling day at 10 a.m. the chambers were closed for 30 min, before 120 mL of sample gas were extracted using a syringe and injected into a pre-evacuated 120 mL serum bottle. The fresh soil samples were taken back to the lab and divided into two parts, one for measuring WFPS (water filled pore space) and extracting soil water, and the other was sieved though a 2 mm mesh and then stored at −20 °C for inorganic nitrogen analysis.

2.4. Soil analysis

The fresh soil samples were dried at 105 °C for 24 h to measure soil moisture that was used to calculate WFPS. The 20 g of sieved soil samples were thawed at room temperature and then were extracted with 100 mL 2 mol L^{-1} potassium chloride (KCl) for the NO_3^-–N, NH_4^+–N concentrations. The concentrations of NO_3^-–N, NH_4^+–N were measured using a continuous flow analyzer (QuikChem8000, LACHAT, Colorado, USA). In addition, the soil water was extracted from the fresh soil samples by automatic water extraction system (LI–2100 EP, LICA United Technology Limited, Beijing, China).

2.5. N_2O emission fluxes and isotopocule deltas measurement

N_2O concentrations and isotope deltas ($\delta^{15}N^{\alpha}$, $\delta^{15}N^{bulk}$ and $\delta^{18}O$) were measured using an isotope ratio mass spectrometer with a pre-concentrator system (IRMS; Delta V Plus–Precon, Thermo Fisher Scientific, Bremen, Germany). The complete procedure for analysis of N_2O isotope deltas was described by Toyoda and Yoshida (1999). The N_2O concentration was calculated by comparing peak area (N_2O, m/z 44) with those of the primary standard gas (319 ppb, Specialty Gases LLC, Air Liquide America, Houston, USA) (Toyoda et al., 2011; Zou et al., 2014; Li et al., 2017). In addition, the calculation of N_2O fluxes refers to Ding et al. (2019).

N_2O isotopocule deltas (alpha, beta and bulk nitrogen isotope delta, $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$ and $\delta^{15}N^{bulk}$; ^{15}N site preference, SP; oxygen isotope delta, $\delta^{18}O$) as calculated according to:

$$\delta X = R_{sample}/R_{standard} - 1 \cdot (X = {}^{15}N^{\alpha}, {}^{15}N^{\beta}, {}^{15}N^{bulk} \text{and} {}^{18}O) \quad (1)$$

$$SP = 2\delta^{15}N^{\alpha} - 2\delta^{15}N^{bulk} \quad (2)$$

Therein, R represents $^{15}N/^{14}N$ and $^{18}O/^{16}O$ deltas, in the sample and reference gas. The reference gas provides the link to the international isotope ratio scales (Air-N_2 and Vienna Standard Mean Ocean Water). $^{15}N^{\alpha}$, $^{15}N^{\beta}$ and $^{15}N^{bulk}$ are central, terminal and bulk ^{15}N atoms in N_2O molecule, respectively. The primary standard gas of N_2O ($\delta^{15}N^{\alpha}$, −0.4‰; $\delta^{15}N^{\beta}$, −0.15‰; $\delta^{15}N^{bulk}$, −0.28‰; $\delta^{18}O$, 41.95‰; Specialty Gases LLC, Air Liquide America, Houston, USA) was applied for daily calibration of the IRMS. The analytical precision of $\delta^{15}N^{bulk}$, $\delta^{18}O$ and SP were below 0.1, 0.1, and 0.5‰, respectively.

The isotopocule deltas and concentration of N_2O were measured from sampling gas ($\delta_{chamber}$, $C_{chamber}$) and ambient air (δ_{air}, C_{air}). Therefore, isotopocule deltas of soil–drived (δ_{SD}) need be calculated following the mass conservation (Well et al., 2006; Ostrom et al., 2007).

$$\delta_{chamber} \cdot C_{chamber} = \delta_{SD} \cdot C_{SD} + \delta_{air} \cdot C_{air} \quad (3)$$

where the equation is applied when C_{SD}/C_{air} is more than 1.3 in our study, and concentration of soil–drived (C_{SD}) = $C_{chamber} - C_{air}$. The mean value of air concentration of N_2O near vegetable farmland was 321 ± 6 ppb.

In addition, we measured $\delta^{15}N$–NH_4^+ (NH_4^+ diffusion method) and fertilizer using an isotope ratio mass spectrometer with elemental analyzer (EA–IRMS, isoprime100–vario PYRO cube, Elementar, Berlin, Germany), and the NO_3^- after conversion to N_2O using the denitrifier method (*Pseudomonas aureofaciens*, ACTT 13985). The $\delta^{18}O$ of soil water were measured by isotope water vapour analyzer (L115–I, Picarro Inc., Sunnyvale, CA, USA). The analytical precision of $\delta^{15}N$–NH_4^+, $\delta^{15}N$–NO_3^- and $\delta^{18}O$–H_2O were below 2, 0.2 and 0.1‰, respectively.

2.6. Analysis of N_2O production pathways using N_2O isotopocule deltas

As described in the introduction, the values of SP, $\varepsilon^{18}O$ and $\varepsilon^{15}N$ for the major microbial processes had been summarized in previous literature (Buchen et al., 2018; Denk et al., 2017; Toyoda et al., 2017; Lewicka-Szczebak et al., 2017), including bacterial nitrification (BN), fungal denitrification (FD), nitrifier denitrification (ND) and bacterial denitrification (BD) (Table S1, supplementary materials). The enrichment factor of isotope, ε, is difference between the isotope deltas in the product (δ_P) and substrate (δ_S) as follow:

$$\varepsilon \approx \delta_P - \delta_S \quad (4)$$

The O source substrate can be considered as soil H_2O, but the N source substrates are different, as shown in the above four processes. For estimating the contribution of different microbial pathways in N_2O emissions, we constructed $\delta^{15}N^{bulk}$ vs. SP plot and $\delta^{18}O$ vs. SP plot against the isotopic signatures of the major microbial N_2O source signatures. Where, the $\delta^{15}N^{bulk}$ and $\delta^{18}O$ of product (N_2O) for four microbial processes of N_2O production were calculated following the method described by Zou et al. (2014) and Lewicka-Szczebak et al. (2017), respectively (Tables S2 and S3, supplementary materials). The $\delta^{15}N$ of substrates (NH_4^+, NO_3^-) and the $\delta^{18}O$ of substrate (H_2O) were directly measured in our study.

2.7. Calculation the contribution of microbial processes on N_2O emissions

According to the difference of SP, four microbial processes can be divided into low SP value group and high SP value group. Low SP value group includes BD and ND, and high SP value group includes BN and FD. N_2O emissions weighted average value of N_2O isotopocule deltas were used to calculate the contribution of microbial processes. However, the increase in N_2O isotopocule deltas caused by N_2O reduction will affect the evaluation of microbial source. Therefore, two equations were used: (1) calculating two microbial sources assuming the absence of N_2O reduction, and (2) assessing the effect of N_2O reduction by using Rayleigh equation assuming the existence of N_2O reduction in closed–system.

$$\delta_{SD} = x \cdot \delta_{high} + (1 - x)\delta_{low} \quad (5)$$

$$\delta_R = \delta_0 + \varepsilon_R \cdot \ln(1 - f_R) \quad (6)$$

Therein, δ_{SD}, δ_{low} and δ_{high} are N_2O isotopocule deltas from soil-driven, low SP value group and high SP value group, respectively. x is the contribution of microbial process with N_2O isotopocule deltas of high SP value group (Calculated based on SP and $\delta^{15}N^{bulk}$ or $\delta^{18}O$). δ_0 and δ_R are N_2O isotopocule deltas before N_2O reduction and after N_2O reduction, respectively. There are two cases of i and ii for N_2O reduction. Case i: N_2O produced by microbial processes and mixed in soils is later reduced by denitrifying bacteria; $\delta_R = x \cdot \delta_{high} + (1-x) \cdot \delta_{low} + \varepsilon_R \cdot \ln(1-f_R)$. Case ii: both production and reduction of N_2O are carried out by denitrifiers and then N_2O is emitted out soil; $\delta_R = x \cdot \delta_{high} + (1-x) \cdot (\delta_{low} + \varepsilon_R \cdot \ln(1-f_R))$. ε_R represents the enrichment factor of N_2O reduction ($\varepsilon^{15}N_R$: 9.1 to −2.5‰, mean −6.6‰, Lewicka-Szczebak et al., 2015; $\varepsilon^{18}O_R$: 25 to −5‰, mean −15‰, Lewicka-Szczebak et al., 2017; SP_R: 7.7 to −2.3‰, mean −5‰, Lewicka-Szczebak et al., 2017). f_R is the extent of N_2O reduction.

2.8. Statistical analysis

The experiment data were processed using Microsoft Excel 2010, and then plotted these data by Sigmaplot (version 12.5). Processed data was statistically analyzed using IBM SPSS (version 20). Using Shapiro–Wilk and Bartlett test, normality of the residuals and homogeneity of the variances were tested. One–way analysis of variance (ANOVA) with Fisher's Least Significant Difference (LSD, $p < 0.05$) was performed to test effect of different treatments on environmental factors, N_2O fluxes and N_2O isotopocule deltas. Pearson's correlation coefficient (r) was used to test the correlations between N_2O fluxes and soil mineral N content and WFPS as well as between SP and N_2O isotope deltas and WFPS ($p < 0.05$). Data was ln–transformed when data didn't satisfy the assumption of normality.

3. Results

3.1. Environmental factors of soil and air

During the sampling time, the mean soil temperature and maximum and minimum air temperatures were 16.0 °C, 23.6 °C and 13.6 °C, respectively (Fig. 1a). Total rainfall and irrigation were 289.5 mm and are reflected in a WFPS increase (Fig. 1b). The soil mineral N (NH_4^+ and NO_3^-) contents of all treatments peaked at 2–3 days after fertilizer application (Fig. 1c and d), and they were significantly ($p < 0.05$) higher in the O, OU and U treatments than in the NF treatment during the main N_2O emissions phases (Table 1).

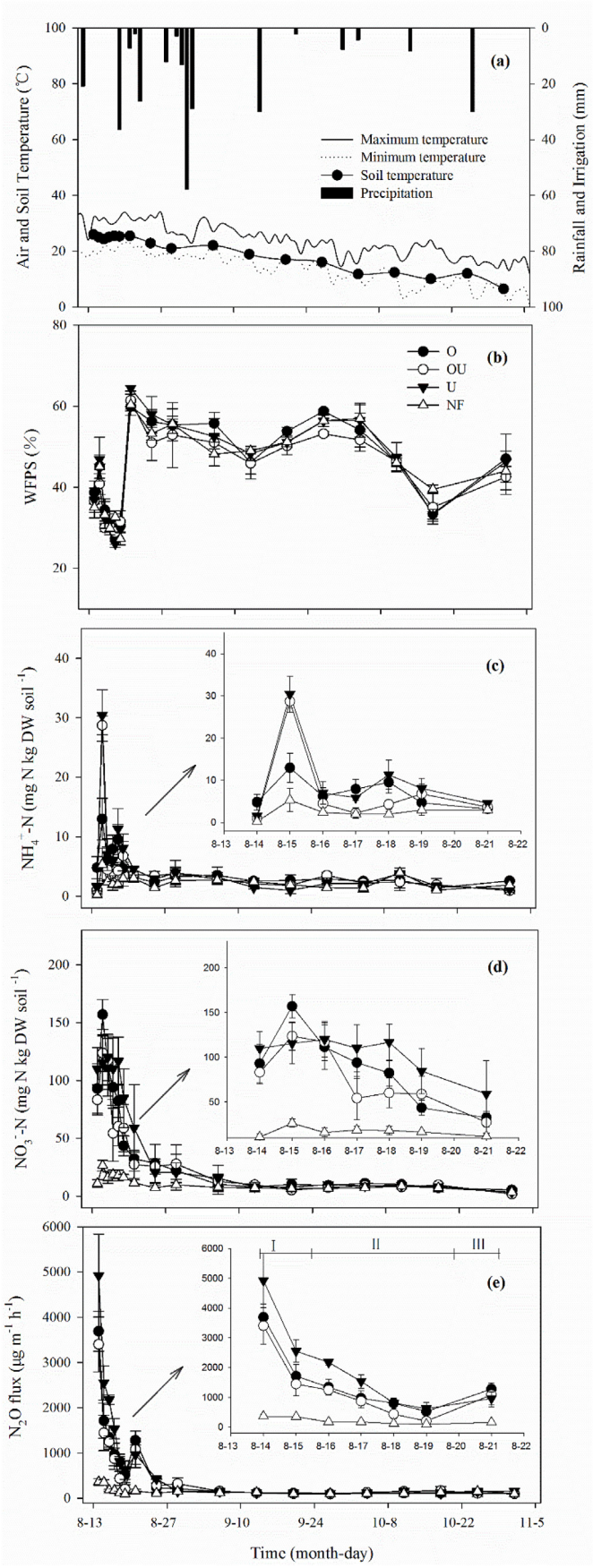

Fig. 1. Time series of (a) soil mean temperature, maximum air temperature, minimum air temperature, and rainfall and irrigation, (b) WFPS, (c) NH_4^+ concentration, (d) NO_3^- concentration, and (e) N_2O flux. Error bars are standard error of mean (n = 4). O, OU, U and NF indicate organic fertilizer, mixed fertilizer, urea and no fertilizer treatments, respectively. The roman numbers denote three N_2O emissions phases. The same below.

3.2. The temporal trend of N_2O emissions

N_2O emissions of the O, OU and U treatments had two major peaks that appeared in the 1st day and 8th day during the experiment period (Fig. 1e). The initial experimental period, with main N_2O emissions, was subdivided in three phases before August 21: phase I, the first N_2O peak from 1 to 2 days; phase II, N_2O with a decreasing trend from 3 to 6 days; phase III, the second N_2O peak in 9 day (Fig. 1e). In all three phases, N_2O emissions of the U treatment were significantly ($p < 0.05$) higher than that of other treatments. There was no significant difference in the O and OU treatments for N_2O emissions, although the OU treatment has lower N_2O emissions. N_2O emissions were significantly ($p < 0.05$) lower for NF treatment than other treatments (Table 1). N_2O emissions were positively correlated with NO_3^-–N for all treatments and negatively correlated with WFPS for the O, OU and U treatments, and positively correlated with NO_3^- and NH_4^+ for the NF treatment (Table 2).

3.3. The variation of N_2O isotopocule deltas

Soil–driven isotopocule deltas of N_2O ($\delta^{15}N^{bulk}$, $\delta^{18}O$ and SP) were only analyzed before August 21, for the three fertilized treatments (O, OU, U), as the N_2O concentration in the chamber headspace was afterwards below the set lower limit ($C_{N2O} < 417$ ppb) (Fig. 2a, b and c). For the NF treatment the N_2O flux was always below the threshold and no N_2O isotope analysis was performed. Large fluctuations in the isotopic composition of soil derived N_2O were observed in all three treatments, with values of $\delta^{15}N^{bulk}$, $\delta^{18}O$ and SP were −39.7‰ to −2.1‰, 17.1‰–66.6‰ and 12.8‰–39.5‰, respectively. The variance analysis indicated that $\delta^{15}N^{bulk}$ values of the OU treatment were significantly ($p < 0.05$) lower than that of the O treatment and significantly ($p < 0.05$) higher than that of U treatment; $\delta^{18}O$ values were not significantly different between the three treatments; SP values were significantly ($p < 0.05$) lower for O treatment than U treatment (Table 1). SP values were positively correlated with $\delta^{15}N^{bulk}$ and $\delta^{18}O$ values, and negatively correlation with WFPS in the three fertilizer treatments (Table 2).

In addition, soil extracted $\delta^{15}N$–NO_3^- and $\delta^{15}N$–NH_4^+ after fertilizer application was similar for three fertilizer treatments, in the range from 0 to 10‰ (Table 1). Furthermore, the $\delta^{15}N$ of the applied fertilizers was in a similar range, with urea (n = 5) and bio–organic fertilizer (n = 5) being −2.2 ± 0.4‰ and 4.2 ± 0.6‰, respectively. The $\delta^{18}O$ value of soil water (n = 6) was −7.3 ± 0.2‰.

3.4. The dual isotopocule plots and microbial N_2O production processes

In Fig. 3, two dual isotopocule plots, $^{15}N^{bulk}$ vs. SP and ^{18}O vs. SP, are presented to disentangle the share of different microbial N_2O formation processes (Bacterial Nitrification, BN; Fungal Denitrification, FD; Nitrifier Denitrification, ND; Bacterial Denitrification, BD) in our study (Fig. 3). Most of samples are located in the mixing area of the four microbial processes, while individual samples are placed outside. Notably, all samples were located away from ND in $\delta^{15}N^{bulk}$ vs. SP plot. Higher SP and $\delta^{15}N^{bulk}$, $\delta^{18}O$ values were observed for phase II, as compared to phase I and III. Regarding fertilizer treatment, U treatment showed higher SP values compared to OU and O treatment. The samples in phase II and U treatment were more closed to high SP microbial group (FD/BN).

Table 1
Average of time series mean of NH_4^+ and NO_3^- concentration and N_2O emissions, N_2O emissions weighted average of isotopocule deltas, mean of initial isotope ratios of NH_4^+ and NO_3^-, and total yield (Y) of cabbage.

Treatment	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})	N_2O (μg m^{-1} h^{-1})	$\delta^{15}N^{bulk}$ (‰)	$\delta^{18}O$ (‰)	SP (‰)	$\delta^{15}N$–NH_4^+ (‰)	$\delta^{15}N$–NO_3^-(‰)	Y (t hm^{-2})
O	87.7 ± 15.1a	7.1 ± 2.2a	1477 ± 204b	−12.8 ± 2.2a	30.6 ± 4.2a	22.7 ± 5.0b	5.3 ± 6.7a	7.2 ± 4.1a	99.5 ± 8.6a
OU	74.9 ± 17.6a	7.3 ± 1.4a	1243 ± 187b	−17.2 ± 2.7b	34.0 ± 4.1a	25.8 ± 5.8 ab	4.4 ± 7.8a	5.3 ± 3.9a	109.4 ± 11.4a
U	102 ± 24.5a	9.8 ± 2.1a	1941 ± 164a	−24.0 ± 3.9c	31.7 ± 2.8a	26.8 ± 6.7a	2.9 ± 8.3a	3.6 ± 5.2a	110.6 ± 12.0a
NF	16.8 ± 3.8b	2.6 ± 0.9b	207 ± 29c	–	–	–	–	–	80.2 ± 5.0b

Results are shown as means ± standard deviation (n = 4). O, OU, U, NF are bio–organic fertilizer, half bio–organic fertilizer and half urea, urea, and no fertilizer treatments, respectively. One-way ANOVA was executed for all treatments. Different letters (a to c) within a column indicated significantly difference ($p < 0.05$). The same below.

Table 2
Relationships of Pearson's correlation coefficients between N_2O fluxes and NO_3^- and NH_4^+ concentration and WFPS throughout growing period of cabbage and between SP and $\delta^{15}N^{bulk}$, $\delta^{18}O$ and WFPS of three fertilizer treatments during three N_2O emissions phases.

Treatment	N_2O			SP		
	NH_4^+	NO_3^-	WFPS	$\delta^{15}N^{bulk}$	$\delta^{18}O$	WFPS
O	0.267	0.559**	−0.283[a]	0.559**	0.692**	−0.691**
OU	0.102	0.601**	−0.34[a]	0.627**	0.454[a]	−0.426[a]
U	0.206	0.701**	−0.44**	0.307	0.651**	−0.651**
NF	0.492**	0.499**	−0.274			

[a] Significant at $p < 0.05$, ** significant at $p < 0.01$.

3.5. The cabbage yields and the nitrogen use efficiency

Total yield of cabbage of the three fertilizer treatments were significantly ($p < 0.05$) higher than those of the NF treatment. There were no significant differences in total yield of cabbage among the different fertilizer treatments, although the total yield of cabbage in the U and OU treatments tended to be higher than that in the O treatment (Table 1).

Generally, the nitrogen absorption capacity of the 1000 kg Chinese cabbage is generally 2.2 kg. Therefore, the nitrogen use efficiency (NUE) can be estimated by a simple equation as $NUE = (Un\text{-}U_0)/FN$. Where, Un and U_0 are the amount of nitrogen absorption capacity for fertilizer treatment and no fertilizer treatment, respectively. FN is the amount of nitrogen applied to the fertilizer treatment. According to the calculation, the NUE of U, OU and O treatments were about 22.3, 21.4 and 14.2%, respectively.

4. Discussion

4.1. Effect of fertilizer on N_2O emissions

During time course, the direct N2O emission had reached the highest levels in one day after fertilization application (Fig. 1e), which was the similar as most studies (Park et al., 2011; Zou et al., 2014; Lin et al., 2019), because the fertilizers supplied large amount of inorganic N that is thought to be the main precursor of N_2O production (Fig. 1c and d). For the three fertilizer treatments, the content of NO_3^-–N was higher than that of NH_4^+–N and was positively correlated with N_2O emissions (Table 1), indicating the major role of NO_3^-–N in promoting N_2O emissions through its effect on denitrification (Barnard et al., 2005). Furthermore, WFPS varied widely in the initial period from 35.2 to 51.6% in phase I, 27.3–34.5% in phase II (were maximum N_2O emissions occurred), to 58.9–64.4% in phase III. Previous research indicated that N_2O emissions mainly came from nitrification (Ruser et al., 2006; Bags and Philippot, 2010) when WFPS is below 60%, whereas fungal denitrification was also found under these moisture conditions (Hayatsu et al., 2008; Seo and DeLaune, 2010). Therefore, we suspected most of N_2O emissions may be derived from fungal denitrification,

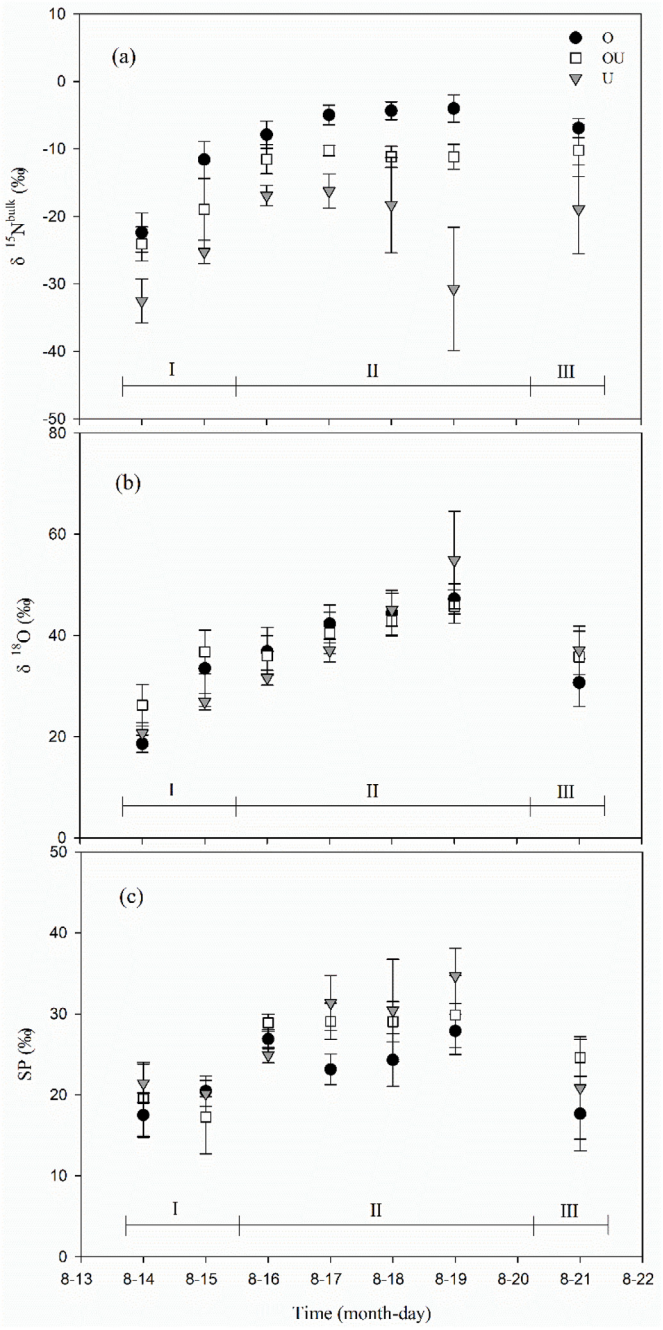

Fig. 2. Time series in (a) $\delta^{15}N^{bulk}$, (b) $\delta^{18}O$ and (c) SP of N_2O in the three fertilizer treatments. The N_2O isotopocule delta has been corrected by equation (3). The results were shown only when N_2O mixing isotopocule delta can be effectively delected ($C_{N2O} > 417$ ppb).

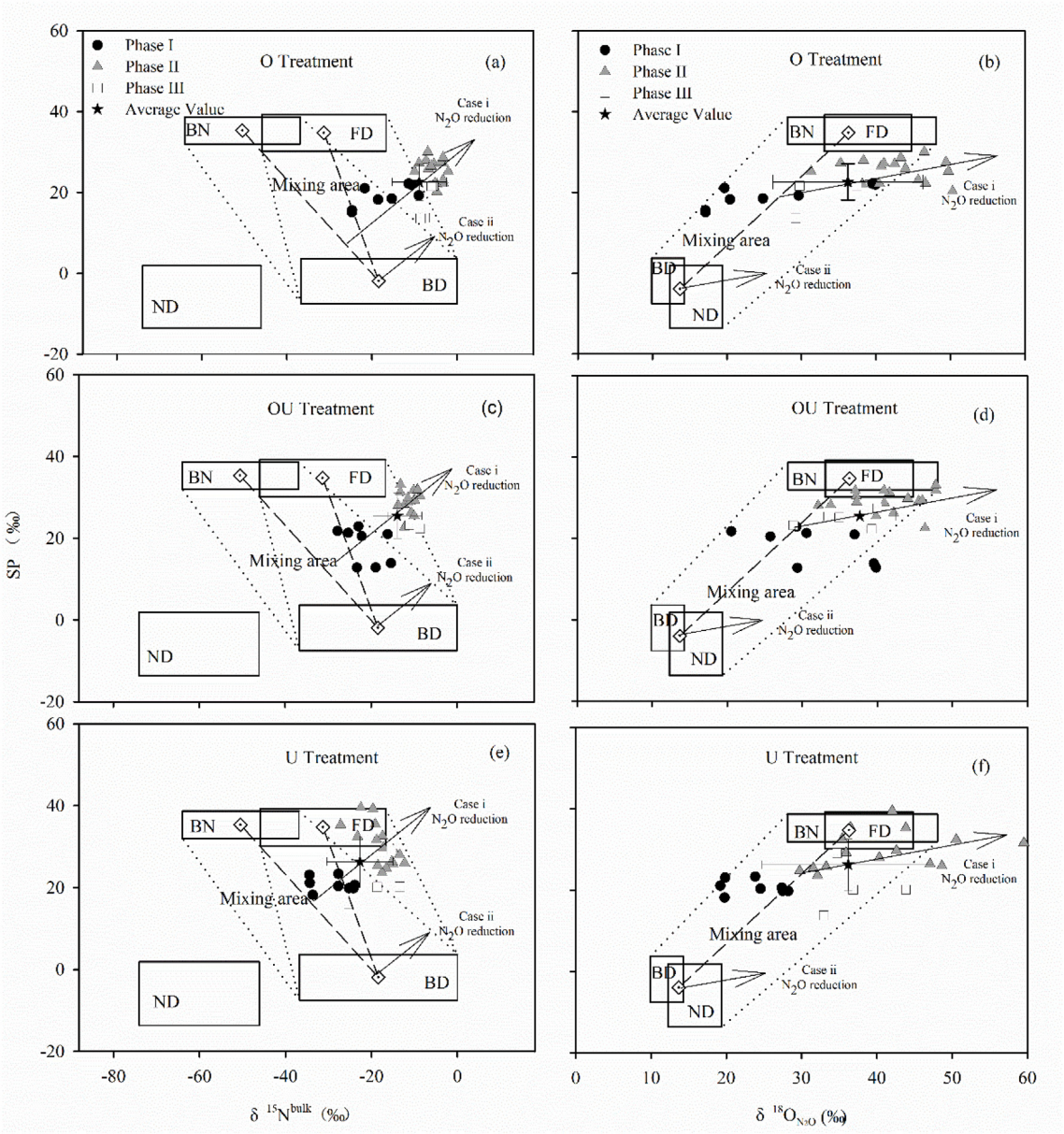

Fig. 3. A graphical representation of two dual isotopocule plots method ($\delta^{15}N^{bulk}$ vs. SP and $\delta^{18}O$ vs. SP) presented to allow an analysis of probable microbial processes occurring. a, c and e denote graphical representation of $\delta^{15}N^{bulk}$ vs. SP of O, OU and U treatments, respectively. b, d and f denote graphical representation of $\delta^{18}O$ vs. SP of O, OU and U treatments, respectively. The box of BN, BD, FD and ND represent ranges of $\delta^{15}N^{bulk}$ vs. SP or $\delta^{18}O$ vs. SP of bacterial nitrification, bacterial denitrification, fungal denitrification and nitrifier denitrification, respectively (Tables S1 and S2, supplementary materials).

especially in the phase II. In addition, although urea is preferentially converted to ammonium nitrogen, it is easily and rapidly evaporated under aerobic conditions and is converted to nitrate nitrogen in large quantities, which might occur too quickly to be detected in our study (Fig. 1e). There were no significant differences in mineral N and cabbage yields between the three fertilizer treatments, but N_2O emissions of the urea treatment were significantly ($p < 0.05$) higher than that of the mixed fertilizer and bio–organic fertilizer treatments (Table 1). This might be related to that most of organic N in bio–organic fertilizer has been mineralized by microorganisms and then was absorbed by cabbages (Parkin, 1987). It is precisely because of the mineralization process that bio-inorganic fertilizer treatment had the lowest the nitrogen use efficiency. However, urea treatment and mixed fertilizer treatment had higher and closer the nitrogen use efficiency, indicating the better effect of the partial replacement of urea with bio–organic fertilizer than that of complete replacement.

4.2. Comparison of the results from two dual isotopocule plots

Multiple microbial pathways can simultaneously exist in soil for N_2O production (Granli and Bøckman, 1994; Hu et al., 2015). It is difficult to ascertain complex mechanisms of N_2O source. The dual isotopocule plots of N_2O are proved to be feasible for distinguishing four main microbial processes (Bacterial Nitrification, BN; Fungal Denitrification, FD; Nitrifier Denitrification, ND; Bacterial Denitrification, BD) of N_2O production (Maeda et al., 2017; Toyoda et al., 2017; Buchen et al., 2018). In our study, the SP values were positively correlated with $\delta^{15}N^{bulk}$ and $\delta^{18}O$ values (Table 2), and the most of samples fell within mixing area of four microbial for $\delta^{18}O$ vs. SP plot either $\delta^{15}N^{bulk}$ vs. SP plot in the three phases.

The $\delta^{18}O$ vs. SP plot approach was simplified to disentangle the contribution of process groups BD/ND and BN/FD and the share of N_2O to N_2 reduction by BD in our study. From the results of calculation by $\delta^{18}O$ vs. SP plot, BN/FD was major process contributing more than 60%

Table 3
Using the dual isotopocule plots to estimate the proportion of microbial pathways under the two cases of N_2O reduction (%)[a].

Phase	Treatment	$\delta^{18}O$ vs. SP			$\delta^{15}N^{bulk}$ vs. SP					
		BN + FD[b]	Fr[c]/casei	Fr/caseii	BN	Fr/casei[d]	Fr/caseii[e]	FD	Fr/casei	Fr/caseii
Total	O	61	18	41	30	88	97	40	80	94
	OU	65	31	66	39	88	97	52	52	92
	U	71	12	36	49	79	95	66	35	72
Phase I	O	61	0	0	34	79	90	45	54	76
	OU	55	21	42	38	72	87	51	33	56
	U	69	0	0	50	56	80	63	0	0
Phase II	O	65	59	92	29	96	99.1	39	93	99.1
	OU	78	42	92	41	96	99.5	55	89	99.2
	U	80	42	93	52	91	99.4	70	71	98
Phase III	O	51	31	52	18	93	96	24	89	94
	OU	68	36	75	33	94	99	44	88	73
	U	55	58	80	38	83	94	50	59	83

[a] N_2O emissions weighted average of isotopocule deltas in three phases were provided in Table S4.
[b] BN and FD represent bacterial nitrification and fungal denitrification, respectively.
[c] F_r is extent of N_2O reduction.
[d] N_2O produced by microbial processes and mixed in soils is later reduced by denitrifying bacteria.
[e] Both production and reduction of N_2O are carried out by denitrifiers and then N_2O is emitted out soil.

for three fertilizer treatments, and urea treatment was the highest and bio-organic fertilizer treatment was the lowest from the perspective of fertilizer treatments (Table 3). The reason might be that the input of bio-organic fertilizer promoted the microbial activity to consume O_2 creating an anaerobic condition (Hill and Cardaci, 2004) and provided abundant organic C as energy for denitrifier growth (Mei et al., 2018), so denitrification might increase after application of bio-organic fertilizer in our study (Bollmann and Conrad, 1998). Additionally, compare to the phase II, the more BD/ND occurred in the phase I and III (Table 3), with relatively high N_2O emissions and water content (Fig. 1b, e). Moreover, WFPS was negatively corrected with SP (Table 2). It indicated water content influences microbial activity. Previous research indicated that the contribution of denitrification increases as WFPS increases (Bateman and Baggs, 2005). The increased WFPS provides the anaerobic conditions and dissolves more inorganic N for N_2O emissions. Those results were highly consistent with 4.1 section of discussion. However, it is very difficult to use $\delta^{18}O$ vs. SP plot to distinguish between FD and BN or between BD and ND, because they have similar range values on SP and $\delta^{18}O$.

From the observation of samples falling into the area of $\delta^{15}N^{bulk}$ vs. SP plot, we found that all samples were located away from ND area. Related research shows that ND mainly occurs in soils with WFPS greater than 70% (Bateman and Baggs, 2005). The WFPS of all treatments were within 27.3%–64.4% for the three phases in our study. Thus, N_2O emissions contributed by ND might be very little. In the $\delta^{15}N^{bulk}$ vs. SP plot, BN vs. BD and FD vs. BD were adopt to simplify the plot and distinguish ND from FD. The results showed that the trends of three fertilizer treatments and three N_2O emission phases were consistent with the $\delta^{18}O$ vs. SP plot. However, the contribution of either BN or FD (most contribution with below 50%) was obviously lower than the contribution BN/FD in the $\delta^{18}O$ vs. SP plot (Table 3), which was more controversial because BN or FD should be the main process in our moisture condition. In addition, the contribution of FD was closer to the contribution of BN/FD for the $\delta^{18}O$ vs. SP plot, indicating that FD might have a greater proportion than BN. In any case, the contradiction between both plot approaches requires further analyses.

4.3. Influencing factors of isotopic source apportionment

There were obvious differences in $\delta^{15}N^{bulk}$ between the three fertilizer treatments (Table 1). On one hand, isotope fractionations of microbial action cause the shift in $\delta^{15}N$ during N_2O emissions (Vitoria et al., 2004; Xue et al., 2009). On the other hand, $\delta^{15}N$ of precursors (NO_3^-, NO_2^- and NH_4^+) might be different depending on their origin (Bergstermann et al., 2011; Lewicka–Szczebak et al., 2015). The $\varepsilon^{15}N$, the difference in $\delta^{15}N$ of product and $\delta^{15}N$ of substrate, is considered as the effective tool to ascertain the main microbial processes (Baggs, 2008). Although we have measured the $\delta^{15}N$ of the N_2O precursors within 0‰–10‰ for three fertilizer treatments and put them into plot to assess possible microbial contributions, the different substrate isotopes and isotopic fractionation of three treatments might influence our judgment of outcome. In addition, this analysis still has some shortcomings, such as isotopic fractionation during denitrification leads to spatial enrichment of NO_3^- in active sites (Bergstermann et al., 2011; Lewicka-Szczebak et al., 2014) and the $\varepsilon^{15}N$ from the same microbial process was also quite different in different researches (Toyoda et al., 2011; Denk et al., 2017), and the endmembers of the microbial process have a large range (Lewicka-Szczebak et al., 2017). Since the uncertainty was relatively high, accurate estimations remain diffidence (Wu et al., 2019). Therefore, the results of $\delta^{15}N^{bulk}$ vs. SP plot were controversial and not recommended in our study.

The $\delta^{18}O$ of N_2O was not significantly different among the three fertilizer treatments (Table 1), which might be mainly due to complex precursor (O_2, H_2O, NO_3^-, NO_2^-) and potential isotope fractionation (Kool et al., 2007). Furthermore, there might be partial O exchange between H_2O and N oxides to change the original isotope fractionation effect (Snider et al., 2012, 2013). However, Lewicka–Szczebak et al. (2016) reported nearly complete O exchange with H_2O occur in static anoxic incubation experiments, which make relatively stable O exchange fractionation (Lewicka–Szczebak et al., 2014). The $\delta^{18}O$ vs. SP plot in our study was based on this theory that H_2O was considered to be sole source of O in N_2O. This method has been used to estimate N_2O production and consumption processes in more and more researches because of the small variable factors (Kool et al., 2011; Lewicka–Szczebak et al., 2017; Ibraim et al., 2019), and it was also well proved in our study.

N_2O reduction to N_2 is considered an important process controlling N_2O emissions (Jinuntuya-Nortman et al., 2008; Ostrom et al., 2007; Well et al., 2013). Many researches indicated N_2O reduction increases $\delta^{15}N^{bulk}$, $\delta^{18}O$ and SP values of N_2O (Ostrom et al., 2007; Decock and Six, 2013; Lewicka–Szczebak et al., 2014; Toyoda et al., 2017). As most researches showed (e.g., Koba et al., 2009; Toyoda et al., 2011; Zou et al., 2014; Lewicka–Szczebak et al., 2017) N_2O reduction was evaluated only when two–part (high SP value group and low SP value group) microbial processes are considered. The results indicated that the extent of N_2O reduction was higher in the treatment with high bio–organic fertilizer content and low moisture content. Meanwhile, there are two cases of N_2O reduction (case i, reduction then mixing;

case ii, mixing then reduction). According to calculation results, the two cases had same proportion of N_2O production processes, but different extent of N_2O reduction (case i was obviously lower than case ii, Table 3). The extent of N_2O reduction in the phase II with low water content was higher than that in other stages. However, when WFPS exceeds 70%, N_2O reduction can become important process for N_2O emissions (Davidson, 1991), whereas the WFPS in our study was 27.3–64.4% in the three phases, indicating that N_2O reduction accounted for a small proportion of N_2O production. So, denitrification was not the primary microbial process. It also further negated the result of $\delta^{15}N^{bulk}$ vs. SP mapping. Furthermore, the total extent of N_2O reduction of three treatments in the plot of $\delta^{18}O$ vs. SP was as high as 12–66%. The reason might be that high pH (8.1) promotes N_2O reduction (Qu et al., 2014).

These results showed that the $\delta^{18}O$ vs. SP plot is useful indicator to reveal the source of the N_2O from the soil. However, this method has its inherent limitation, which is the inability to distinguish BN and FD because they have large overlapping. Furthermore, it is known that N_2O reduction to N_2 is last step of denitrification, whereas most fungi lack N_2O reductase (N_2OR) and can't perform N_2O reduction (Shoun et al., 1992). Therefore, it is difficult to quantify the contribution of them completely. Although there were some indications in our study that FD might contribute more, substantial evidence for this is lacking. Further work needs to explore more effective methods to work out this problem.

5. Conclusions

The $\delta^{18}O$ vs. SP plot was a potentially useful approach to estimate major microbial processes of N_2O production in vegetable fields. Our results showed that fungal denitrification/bacterial nitrification was dominant microbial process on N_2O emissions. Increasing moisture content and bio–organic fertilizer content enhanced denitrification. The total extent of N_2O reduction for the three treatments were between 12% and 66%. In addition, applying mix bio–organic fertilizer significantly reduced N_2O emissions but not the nitrogen use efficiency and cabbage yield, so using the partial replacement of urea with bio–organic fertilizer was a better choice in our study.

Author contributions

Concept and design: Yuzhong Li and Wei Lin. Analysis and interpretation of data: Wei Lin, Junjun Ding, Chunying Xu and Yuzhong Li. Drafting of the manuscript: Wei Lin. Critical revision of the manuscript for important intellectual content: Yuzhong Li, Junjun Ding. Statistical analysis: Lili Mao. Prepared experiments: Qian Zheng and Shang Zhuang. Obtained funding: Yuzhong Li, Junjun Ding and Xiaoying Liu. Study supervision: Qiaozhen Li.

Data accessibility

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Appendix A. Supplementary data

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