Probing the biological sources of soil N₂O emissions by quantum cascade laser-based ¹⁵N
isotopocule analysis

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Abstract

Isotopocule analysis by quantum cascade laser spectrometry (QCL) is a promising approach for *in situ*, real-time tracking of the biological sources of soil N₂O emissions. However, background atmospheric N₂O is an important source of variability in the measurement of isotopocule ratios (i.e., $^{14}\text{N}^{15}\text{N}^{16}\text{O}/^{14}\text{N}^{14}\text{N}^{16}\text{O}$ and $^{15}\text{N}^{14}\text{N}^{16}\text{O}/^{14}\text{N}^{14}\text{N}^{16}\text{O}$) of gas samples. Here, a method based on Keeling plot for determining the intramolecular ^{15}N distribution in N₂O is introduced. The sensitivity and reliability of this method are examined against N₂O of known ^{15}N site preference (SP), and N₂O produced from fungal and bacterial isolates, soils with different moisture contents and organic amendments, and a soil chamber under field conditions. The isotopocules of N₂O determined by standard gases supported that the Keeling plot method was more reliable than the averaging method. Using this method, SP of N₂O was greater in fungal than bacterial denitrifiers, as well as in soil at 60% water filled pore space (WFPS) than 80-100% WFPS. This method also determined that the SP of N₂O was distinct between soils of different substrate complexity. Further, we observed a N₂O SP between -18.9 ‰ and 2.2‰ in a soil flux chamber deployed in a corn field after two d of rainfalls that is consistent with the SP of N₂O produced from bacterial denitrification and nitrifier denitrification. Our data demonstrate that this Keeling plot method provides accurate discrimination of biological sources when N₂O is analyzed by the QCL system.

Keywords: Nitrous oxide; Isotopocule; Isotopic site-preference; Quantum cascade laser spectrometer

1. Introduction

Nitrous oxide is a potent greenhouse gas and ozone depleting substance (IPCC, 2007). Roughly 70% of anthropogenic N₂O originates from agricultural production systems (US-EPA, 2015). Microbes of diverse physiologies and ecologies, e.g., autotrophic and heterotrophic nitrifiers, and heterotrophic bacterial and fungal denitrifiers, are known to contribute to soil N₂O emissions (Mothapo et al., 2015; Wrage et al., 2001; Zumft, 1997). However, their significance often varies spatially and temporally with management-associated changes in soil conditions. As such, simultaneous *in situ* real-time monitoring and source identification of soil N₂O emissions is critical to the holistic understanding of management impacts on soil N₂O emissions.

Natural abundances of isotopocules, the molecules that have the same chemical constitution and configuration, but differ in either number or position of isotopic substitutions (Coplen, 2011; Sutka et al., 2006; Toyoda et al., 2015). The two N and one O atoms of N₂O are structured linearly, resulting in two ¹⁵N isotopocules: ¹⁴N¹⁵N¹⁶O (¹⁵N^α) and ¹⁵N¹⁴N¹⁶O (¹⁵N^β). ¹⁵N-site preference (SP), i.e., the difference between δ¹⁵N^α and δ¹⁵N^β is closely associated with biological processes, with 32.7 ± 5.0 ‰ (standard deviation) for bacterial NH₄⁺ oxidation (n = 4 observations from individual studies), 33.0 ± 1.6 ‰ for bacterial NH₂OH oxidation (n = 18), -2.2 ± 3.2 ‰ for bacterial denitrification (n = 26), -1.0 ± 4.3 ‰ for nitrifier denitrification (n = 26), and 35.2 ± 4.3 ‰ for fungal denitrification (n = 31) (Decock and Six, 2013).

Quantum cascade laser (QCL) spectroscopy has been developed for simultaneous and real-time quantification of the isotopocule abundances and fluxes of N₂O. However, the variation in the isotopocule composition of the background (i.e., the atmospheric N₂O) may impose uncertainty on the measurement of ¹⁴N¹⁵N¹⁶O and ¹⁵N¹⁴N¹⁶O in samples. Current methods for resolving this issue include (1) using synthetic air as the background (Köster et al., 2013), (2)

concentrating the sample N₂O (Mohn et al., 2012), and (3) averaging a number of readings to improve the measurement reliability (Yamamoto et al., 2014). The first two methods are infeasible for *in-situ* gas measurement in real time under field conditions. For the third method, arbitrary selection of the measurement period is an issue. Here we report a calculation method based on Keeling plot for reliably estimating the SP of N₂O samples in laboratory microcosms and field chambers using the QCL system. This method has been validated by testing SP of N₂O of known $\delta^{15}\text{N}^{\alpha}$ and $\delta^{15}\text{N}^{\beta}$, N₂O produced by fungal and bacterial cultures, N₂O emitted from soils subjected to different water and organic treatments, and N₂O in a soil chamber under field conditions.

2. Materials and methods

2.1. Instrument description

The fluxes and SP of N₂O were analyzed using a dual QCL N₂O measurement system (Model CWQCL-200-D, Aerodyne, Billerica, MA). This system includes two continuous wave quantum cascade (CWRT-QC) lasers for the detection of two or more gas species. The lasers are thermoelectrically cooled in a hermetically sealed housing and operate in continuous mode. The instrument uses a 200 m path length, 2.0 L volume, multiple pass absorption sampling at a pressure of 50 Torr. Two thermoelectrically cooled infrared detectors are provided: one for sample analysis and one for power normalization and to lock the frequency of the lasers to a reference cell. TDL WINTEL software (Aerodyne, Billerica, MA) is used for operating the instrument and for performing data acquisition and analysis. The data acquisition rate is adjustable to 1 Hz. The instrument is supplied with thermoelectrically cooled detectors for long term monitoring.

2.2. Calculation method

The QCL system measures the total N₂O concentration, represented by the ¹⁴N¹⁴N¹⁶O reading, and provides the ¹⁴N¹⁵N¹⁶O and ¹⁵N¹⁴N¹⁶O readings that are used to calculate the ratios of ¹⁵N^α/¹⁴N (R¹⁵N^α) and ¹⁵N^β/¹⁴N (R¹⁵N^β) by dividing ¹⁴N¹⁵N¹⁶O or ¹⁵N¹⁴N¹⁶O by ¹⁴N¹⁴N¹⁶O readings, respectively. These ratios are then used to determine δ¹⁵N^α and δ¹⁵N^β of N₂O. While ambient air as the background shows fairly stable ¹⁴N¹⁴N¹⁶O readings, its ¹⁴N¹⁵N¹⁶O and ¹⁵N¹⁴N¹⁶O readings fluctuate considerably perhaps because these readings are more prone to instrumental random errors (Fig. 1), leading to uncertainty when estimating the R¹⁵N^α and R¹⁵N^β of sample gases. To minimize the background variation, we formulated an equation (Equation 1) based on the principle of mass balance, which is similar to Keeling plot used in the isotopic analysis of CO₂ in the air by spectroscopic systems (Pataki et al., 2003). This equation is derived from the concept that the concentration and isotopocule composition of total N₂O measured at any point is a mixture of the concentration and isotopocule composition of N₂O in sample and air (i.e., Equations 2 and 3).

$$R^{15}N_{\text{total}} = R^{15}N_{\text{sample}} + (R^{15}N_{\text{air}} - R^{15}N_{\text{sample}}) \times \rho_{\text{air}} \quad (\text{Equation 1})$$

$$R^{15}N_{\text{total}} = R^{15}N_{\text{sample}} \times \rho_{\text{sample}} + R^{15}N_{\text{air}} \times \rho_{\text{air}} \quad (\text{Equation 2})$$

$$\rho_{\text{sample}} + \rho_{\text{air}} = 1 \quad (\text{Equation 3})$$

R¹⁵N_{total}, R¹⁵N_{air}, and R¹⁵N_{sample} represent ¹⁵N/¹⁴N of total, air-portion, and sample-portion of N₂O, respectively, and ρ_{air} and ρ_{sample} are the air-portion, and sample-portion of N₂O as a fraction of total N₂O.

R¹⁵N_{total} and ρ_{air} can be directly estimated from the QCL ¹⁴N¹⁴N¹⁶O, ¹⁴N¹⁵N¹⁶O and ¹⁵N¹⁴N¹⁶O readings. Given the QCL frequency resolution of 1 Hz, multiple data points can be collected during the measurement period (~ 2 - 4 minutes) as the sample gas rises and falls back

to baseline. These individual data points are then used to calculate $^{15}\text{N}_{\text{sample}}$ via a linear regression (Equation 1), with $R^{15}\text{N}_{\text{total}}$ and ρ_{air} as the independent variables. An example of $^{14}\text{N}^{14}\text{N}^{16}\text{O}$, $^{14}\text{N}^{15}\text{N}^{16}\text{O}$, and $^{15}\text{N}^{14}\text{N}^{16}\text{O}$ readings of a sample gas during 112 sec and the corresponding linear regression for estimating $R^{15}\text{N}^{\alpha}_{\text{sample}}$ and $^{15}\text{N}^{\beta}_{\text{sample}}$ are given in Fig. 1. This method is also suitable for the calculation of $R^{18}\text{O}_{\text{sample}}$. The isotopocule abundances are then estimated by the standard curves with known $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, and $\delta^{18}\text{O}$ of N_2O gases from Swiss Federal Laboratories for Materials Science and Technology (EMPA) (Mohn et al., 2014) plotted against $R^{15}\text{N}^{\alpha}$, $R^{15}\text{N}^{\beta}$, and $R^{18}\text{O}$ measured by QCL system with $r^2 = 0.999$, respectively. Bulk ^{15}N isotope ratio ($\delta^{15}\text{N}^{\text{bulk}}$) and intra-molecular ^{15}N distribution in N_2O are calculated by the following equations, respectively.

$$\delta^{15}\text{N}^{\text{bulk}} = (\delta^{15}\text{N}^{\alpha} + \delta^{15}\text{N}^{\beta})/2 \text{ (Equation 4)}$$

$$^{15}\text{N SP} = \delta^{15}\text{N}^{\alpha} - \delta^{15}\text{N}^{\beta} \text{ (Equation 5)}$$

2.3. Laboratory validation experiments

This calculation method was validated against the N_2O gases of known $\delta^{15}\text{N}^{\alpha}$ and $\delta^{15}\text{N}^{\beta}$ (Mohn et al., 2014) as well as N_2O gases generated from fungal and bacterial cultures and soils subjected to different water and organic treatments. Briefly, 15-60 mL of a sample N_2O gas as mentioned above was injected by a syringe into a 1 L PVC jar that was sealed with a screw cap having two sampling ports. Thereafter, one port was open to the atmosphere and the other was connected to the QCL system for gas measurements. This setup created a continuous flow from air through the PVC jar to the QCL system and resulting N_2O gas in the PVC jar was analyzed for $R^{15}\text{N}_{\text{total}}$ and ρ_{air} by the QCL system over time. Because the sample gas injected into the PVC jar was continuously diluted by air inflow, the gas measurement continued until $^{14}\text{N}^{14}\text{N}^{16}\text{O}$

reading was back to the background level. The system pressure was well maintained during the measurement period.

The first test was to determine how reliable N_2O isotopocule abundance could be estimated using the Keeling plot method. Five standard gases were used, three having N_2O at 90 ppm and also with known $\delta^{15}\text{N}^\alpha$, $\delta^{15}\text{N}^\beta$, and $\delta^{18}\text{O}$ measured by EMPA, one having N_2O at 50 ppm, and one having N_2O at 10 ppm. These gases were injected by syringes into the 1 L PVC jars at 15, 30, 45, and 60 mL, respectively, and resulting N_2O concentrations were in the range of 0.5-4.7 ppm. Then, the concentration and isotopocule abundance of N_2O in the 1 L PVC jars were analyzed by the QCL system as described above. This Keeling plot calculation method was also compared with the averaging method, which selected a 1-min data period when the N_2O mixing ratios were highest during each measurement.

The second test was to estimate the ^{15}N SP of N_2O generated by six hermetically cultured denitrifying bacterial strains and eight N_2O -producing fungal strains (Mothapo et al., 2013). Bacterial and fungal strains were cultured using 30 mL of liquid LB or Czapek medium, respectively, in 120-mL amber jars. Both media contained 5 mM NaNO_2 and NaNO_3 . Czapek medium also contained 100 $\mu\text{g mL}^{-1}$ streptomycin to prevent bacterial contamination. Fungal and bacterial media were hermetically incubated for 1-wk or 24-h, respectively, and then headspace gas was withdrawn for the QCL analysis as described above. Measurements were made on three replicates and three consecutive incubation-periods.

The third test was to assess the ^{15}N SP of N_2O generated from soils of organic (ORG) and conventional (CON) farming systems subjected to different water and organic treatments as described previously (Chen et al., 2015a; Chen et al., 2015b). Briefly, soils (equivalent to 20 g dry weight) from ORG and CON farming systems were packed into 120 mL amber jars. To test

soil moisture effects, soil water was raised to 40-100% water filled pore space (WFPS) by adding glucose (2 mg C g⁻¹ soil) and NH₄NO₃ (100 µg N g⁻¹ soil) with water. After 12-30 h equilibrium, amber jars were capped for 2 h with open-top closures and silicone septa and then headspace gas was withdrawn and injected into the 1 L PVC jar for the measurement of N₂O concentration and isotopocule abundance using the QCL system as described above. Measurements were made on three replicates and four times. To test the impacts of organic substrates on the sources of soil N₂O fluxes, glucose, cellulose, and chicken manure were added into soil at a rate of 2 mg C g⁻¹ soil and soil moisture was adjusted to 70% WFPS. During the 10 d incubation, amber jars were periodically capped for 2 h and then headspace gas sample was withdrawn and injected into the 1 L PVC jar for the measurement of N₂O concentration and isotopocule abundance using the QCL system. Measurements were made on three replicates.

2.4. Field experiment

The QCL system was deployed adjacent to an agricultural research field cultivated with corn (*Zea mays*) located in Holly Springs, North Carolina in May 17-20 and June 14-17, 2012. The field received about a total of 20 mm and 45 mm precipitation during May 14-16 and June 12-13, respectively, according to a local weather station report. The soil is fine-loamy, siliceous, thermic Typic Kandiudults with 2-6 percent slope. Fertilizer of 21-0-0 ammonium sulfate was applied at 180 kg N ha⁻¹. The QCL system was housed in an air conditioned trailer and monitored remotely via cellular data network.

A dynamic flow-through soil chamber was used to collect soil accumulation of N₂O every 30 min. The soil chamber was constructed in two parts, a 20 cm diameter PVC flux ring and a PCV cap. The flux ring had a beveled edge and was permanently driven into the ground at a

height of 2.54 cm above the soil surface. The chamber cap was placed over the flux ring for soil gas measurements and had a total height of 13 cm. The closed chamber had a total volume of 3900 cm³ and a surface area approximately 314 cm². The cap had two one-way sampling ports, one inlet connected to a 2.1 m high tower adjacent to the chamber and the other outlet directly linked to the QCL system. An ambient air measurement was also made using a 2.1 m tower located 10 m away from the chamber. For the 15 min. of gas accumulation in the chamber, the two sampling ports in the chamber cap were closed, and the QCL system directly sampled ambient air from the 2.1 m tower located 10 m away from the chamber. During 15 min. gas measurement, a continuous gas flow was created from the 2.1 m tower through the chamber to the QCL system via an automatic valve, so the gas built up in the chamber could be analyzed for $R^{15}N_{total}$ and ρ_{air} over time. Due to the pressure change by the automatic valve, the first five data points of low pressure were not used, while the system pressure was well maintained at a constant level of 50 Torr for the remaining time. After 15 min. of sampling, $^{14}N^{14}N^{16}O$ reading was back to the background level, and thus the gas accumulated in the chamber was completely replaced by fresh ambient air. The field measurements were conducted for 72 h twice from 12:00 pm May 17 to 12:00 pm May 20 and from 12:00 pm June 14 to 12:00 pm June 17, respectively. An example of $^{14}N^{14}N^{16}O$, $^{14}N^{15}N^{16}O$, and $^{15}N^{14}N^{16}O$ readings of gas sample in the chamber during 180 sec and the corresponding linear regression for estimating $R^{15}N^{\alpha}_{sample}$ and $R^{15}N^{\beta}_{sample}$ are given in Fig. 2.

2.5. Data analysis

For laboratory experiments, analysis of variance (ANOVA) of a completely randomized block design with repeated measures (SAS 9.3, SAS Institute Inc. Cary, NC) was used to assess

if the soil water and organic treatments significantly ($P \leq 0.05$) affected the SP, $\delta^{15}\text{N}^{\text{Bulk}}$, and $\delta^{18}\text{O}$ of the N_2O produced from soils. Data sets were tested for normal distribution and homoscedasticity.

3. Results and discussion

3.1. N_2O of known isotopic composition

By testing standard gases of known N_2O isotopocule, our results showed that $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, and $\delta^{18}\text{O}$ measurements by the Keeling plot method were independent of N_2O concentrations as low as 0.44 ppm (Supplementary Table S1). Also, since the Keeling plot method had generally lower coefficient of variation (CV) than the averaging method, the isotopocule abundances estimated by the Keeling plot method were more accurate than the method using the average values as mentioned above. However, standard errors and the % differences from the known value for gas samples with N_2O concentrations > 0.7 ppm were smaller than those < 0.7 ppm regardless of calculation methods. Air inflow could quickly dilute the sample N_2O in the 1 L jar and therefore at < 0.7 ppm, N_2O concentration was insufficient to provide accurate data for obtaining precise isotopic abundances. In a field chamber, however, large chamber volume resulted in a slower return to atmospheric concentrations of N_2O and longer counting times of sample gases. Consequently, accurate isotopic ratios could be obtained at lower concentrations than in the 1-L jars. As illustrated in Fig. 1 and Fig. 2, N_2O spike duration was much longer (~ 60 sec) in a field chamber than in a laboratory microcosm (~ 20 sec) despite the fact that N_2O concentration in the field chamber was one third of that in the microcosm. Nonetheless, Keeling plot calculation method can provide reliable estimates of $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, and $\delta^{18}\text{O}$ at N_2O spike duration > 20 sec (equivalent to > 0.7 ppm N_2O concentrations in a 1-L jar).

3.2. Denitrifying bacterial and fungal cultures

Not only did bacteria and fungi differ in N₂O production rates, but also in the SP of N₂O (Table 1). The ¹⁵N SP of N₂O from the fungal sources ranged from 18.4 to 28.6 ‰, which was greater than the SP of bacterial sources that ranged from -12.8 to -4.6 ‰ ($P < 0.05$). One possible explanation is that fungi and bacteria differ in the preferential cleavage of the N-O bond in symmetric intermediates, e.g., hyponitrite ⁻ONNO⁻, resulting in different depletion or enrichment of ¹⁵N at α or β sites (Schmidt et al., 2004; Toyoda et al., 2015; Toyoda et al., 2002). It should be mentioned that our estimates of ¹⁵N SP in N₂O for bacterial and fungal denitrification are consistent with other studies that denitrifying fungi produced N₂O of SP value between 15.8-36.7 ‰, while slightly negative SP values have been reported for bacterial denitrifiers (Maeda et al., 2015; Rohe et al., 2014; Sutka et al., 2008; Sutka et al., 2006; Toyoda et al., 2005). Further, $\delta^{15}\text{N}^{\text{bulk}}$ and $\delta^{18}\text{O}$ were significantly different between bacterial and fungal sources ($P < 0.05$).

3.3. Soil incubation microcosms

In both ORG and CON soils, N₂O fluxes peaked at 24 hours, and were significantly greater at 80-100% WFPS compared to those at 40-60% WFPS ($P < 0.05$) (data not shown). Data for 40% WFPS were not reported since N₂O fluxes were too low to reliably estimate isotopocule abundance. Contrary to fluxes, SP in N₂O at 60% WFPS, 25.44 and 28.73‰ in ORG and CON respectively, was significantly greater than the SP at 80-100% WFPS ranged from 7.13 to 16.96 ‰ ($P < 0.05$) (Table 2). Our results also agree with others (Well et al., 2008; Well et al., 2006) that SP (13.9-29.8 ‰) determined for soils at 30-50% WFPS appeared to be greater than the SP (8.6-15.3‰) at 75-85% WFPS.

At 80-100% WFPS, SP in CON (13.9-17.0 ‰) was also significantly greater than the SP in ORG (7.1-8.6 ‰) ($P < 0.05$). At high soil moisture levels, oxygen-limiting conditions may restrain the contribution of autotrophic nitrifiers to N₂O emissions, and instead favor bacterial and fungal denitrifiers for N₂O production. As such, the SP of N₂O emitted from soil would depend on the relative contribution of bacterial and fungal denitrifiers. For example, the SP would reduce when the contribution of bacterial denitrifiers increased, because SP of N₂O produced by denitrifying bacteria was considerably lower (Decock and Six, 2013). On the contrary, the SP of N₂O would increase when denitrifying fungi contributed more to N₂O. The greater SP of N₂O from CON than that from ORG suggested that fungal denitrifiers were more dominant in CON. Such inference derived from the SP of N₂O was consistent with our previous finding, made using the aid of antibiotics, that fungi contributed more to soil N₂O production in CON than ORG (Chen et al., 2014).

During the 10-d incubation, only the first two d for soils amended with glucose and manure and d 7 and 8 for soils amended with cellulose and manure generated > 0.7 ppm N₂O in a 1 L jar required for reliable estimates of SP (data not shown). Therefore, comparisons were made between glucose and manure for d 1 and 2 and between cellulose and manure for d 7 and 8 (Table 3). Both ORG and CON soils showed significantly greater SP in glucose (14.8-18.1‰) than manure (6.7-9.0 ‰) ($P < 0.05$). However, the SP of N₂O emitted from manure-amended ORG and CON soils (8.6-11.8 ‰) was greater than that from cellulose-amended soils (1.0-4.9 ‰) ($P < 0.05$). The differences in SP between different treatments suggested that biological sources of N₂O varied with the organic C complexity. By comparing glucose vs. cellulose and winter pea of low C:N ratio vs. switchgrass of high C:N ratio, Chen et al. (2015a) demonstrated that complex organics (e.g., cellulose and switchgrass) likely promoted fungal denitrification

over bacterial denitrification under oxygen-limited conditions. While a large portion of organic C in manure is generally less labile than glucose, there are soluble organics in manure (e.g., amino acids, mono-carbohydrates) that are comparable to or even more labile than glucose. Decomposition of these labile C compounds in manure took place during the first few d of incubation and perhaps promoted bacterial denitrification more than that of glucose. Chen et al. (2015) showed that the relative importance of fungal denitrification over bacterial denitrification decreased over time as the decomposition of cellulose proceeded. At the present study, the SP of N₂O from cellulose-amended soil could not be reliably estimated during the first few d of incubation because the concentration was less than 0.7 ppm of N₂O. Therefore, inferences regarding dynamic impacts of cellulose on biological sources of N₂O could not be made.

It should be noted that the SP of N₂O emitted from manure-amended ORG soil kept relatively stable over time, but significantly increased for manure-amended CON soil, suggesting that fungal denitrifiers might contribute more to N₂O emissions as decomposition of manure-organic C in CON proceeded. This different response between ORG and CON soils was likely due to the greater activity of fungal denitrifiers in CON as discussed above.

3.4. Soil chamber in the field

The N₂O fluxes in the soil chamber were monitored continuously for two periods of 72 h starting at 12:00 pm, May 17 and June 14, respectively. Flux rates changed over time during both monitoring periods (Fig. 3). The SP of N₂O also showed temporal variations in the range of -18.9‰ and 2.2 ‰ throughout the monitoring periods. In addition, the variation of $\delta^{15}\text{N}^{\text{bulk}}$ and $\delta^{18}\text{O}$ were from -45.4 to 31.0 ‰ and 27.5 to 30.4 ‰, respectively. The negative SP of N₂O on average suggested that soil N₂O emissions originated from bacterial denitrification and/or

nitrifier denitrification. Rainfalls occurred for 2 to 3 d continuously prior to the monitoring on May 17 and June 14 and therefore surface soil was almost saturated. The field results aligned well with the results of an early study (Chen et al., 2015b), showing that bacterial contribution to N_2O increased as soil moisture content increased towards saturation. The field results were also supported by the observations of decreasing SP as soil WFPS increased (Table 2).

So far, a few monitoring of soil N_2O isotopocules in field chambers has been reported using gas chromatograph-isotope ratio mass spectrometer. SP of N_2O was found to change substantially under field conditions. For example, SP of N_2O varied between -6 and 14.4 ‰ in a no-till soybean field (Smemo et al., 2011) and between 4.0 and 24.7 ‰ in irrigated rice paddy (Yano et al., 2014). A large change, up to ~ 120 ‰ was also found between measurements of short-time intervals (e.g., 4 h) (Kato et al., 2013). Using the QCL system with closed-chambers, Yamamoto et al. (2014) measured SP of N_2O emitted from a potato field and showed that SP varied from 7 to 34 ‰ during 13 d. Nevertheless, the fact that our field SP data under near soil saturation were within the forecast range of SP based upon bacterial and nitrifier denitrification indicates that in-situ, real-time monitoring with the QCL system can be reliable for identifying biological sources of soil N_2O emissions.

4. Conclusions

The Keeling plot calculation method provides reliable estimates of isotopocule abundance of N_2O in gas samples containing atmospheric N_2O as the background. It allows for the QCL system with high temporal resolution to track biological sources of soil N_2O emissions generated in microcosms and field chambers. Soil moisture contents and organic amendments significantly shifted the biological sources of soil N_2O emissions. As soil moisture increased towards

saturation, bacterial and/or nitrifier denitrification became the dominant sources, whereas fungal denitrification and/or nitrification were likely the major sources of soil N₂O emissions at 60% WPFS. Despite that temporal changes in soil N₂O emission and isotopocule abundance in field was more complex than in the laboratory microcosm, relatively consistent and negative SP of N₂O in field also supported the dominance of bacterial and/or nitrifier denitrification for soil N₂O emissions under near soil saturation conditions. Further, biological sources of soil N₂O emissions (e.g., bacterial vs. fungal denitrification) could change rapidly with environmental conditions.

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414 Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. *Microbiology and*
415 *molecular biology reviews* 61, 533-616.

416

417 Table 1. Flux rates, ^{15}N isotope site preference (SP), $\delta^{15}\text{N}^{\text{Bulk}}$, and $\delta^{18}\text{O}$ of N_2O produced from fungal and bacterial cultures. Data are
 418 means $\pm$ standard errors (n = 9).

Microbial cultures	SP	$\delta^{15}\text{N}^{\text{Bulk}}$	$\delta^{18}\text{O}$	N_2O Flux ($\mu\text{mol h}^{-1} \text{ flask}^{-1}$)
Fungi				
<i>Fusarium neocosmosporiellum</i> Con-0506	21.73 $\pm$ 2.22	-22.64 $\pm$ 9.49	29.41 $\pm$ 0.25	0.05-16.27
<i>Aspergillus terreus</i> Org-4110	19.60 $\pm$ 0.46	-36.28 $\pm$ 1.87	29.50 $\pm$ 0.38	0.57-4.09
<i>Aspergillus sydowii</i> Org-4511	23.22 $\pm$ 1.21	-35.13 $\pm$ 2.19	29.33 $\pm$ 0.33	0.44-13.07
<i>Trichoderma asperellum</i> Suc-6412	19.05 $\pm$ 0.81	-32.74 $\pm$ 1.64	29.93 $\pm$ 0.26	0.54-2.51
<i>Aspergillus oryzae</i> For-7014	28.55 $\pm$ 0.76	-36.63 $\pm$ 2.67	29.91 $\pm$ 0.54	0.80-22.92
<i>Penicillium pinophilum</i> Con-1718	19.89 $\pm$ 1.23	-39.78 $\pm$ 0.59	28.56 $\pm$ 0.10	0.56-5.11
<i>Trichoderma hamatum</i> Suc-5023	22.53 $\pm$ 1.89	-37.77 $\pm$ 0.63	29.42 $\pm$ 0.21	0.06-2.46
<i>Penicillium brasilianum</i> Suc-5826	18.36 $\pm$ 0.37	-34.14 $\pm$ 0.80	29.53 $\pm$ 0.17	0.36-11.91
Bacteria				
<i>Ochrobactrum anthropi</i> ATCC BAA-749	-4.57 $\pm$ 1.65	13.75 $\pm$ 3.69	34.00 $\pm$ 0.75	1.19-588.37
<i>Alcaligenes faecalis</i> ATCC 8750	-6.43 $\pm$ 1.05	13.85 $\pm$ 1.38	34.03 $\pm$ 0.28	1.20-583.63
<i>Pseudoalteromonas haloplanktis</i> ATCC 14393	-9.66 $\pm$ 1.48	59.88 $\pm$ 2.75	41.06 $\pm$ 0.43	10.49-2053.12
<i>Actinoplanes missouriensis</i> ATCC 31681	-10.70 $\pm$ 0.64	64.91 $\pm$ 6.61	41.06 $\pm$ 0.94	857.21-7486.79
<i>Pseudomonas stutzeri</i> ATCC 53819	-10.20 $\pm$ 0.88	31.76 $\pm$ 5.10	36.03 $\pm$ 1.22	19.81-662.47
<i>Paracoccus pantotrophus</i> ATCC 35512	-12.83 $\pm$ 1.36	46.43 $\pm$ 3.57	38.78 $\pm$ 0.80	4.99-2429.76

419

420

421 Table 2. Flux rates, ^{15}N isotope site preference (SP), $\delta^{15}\text{N}^{\text{Bulk}}$, and $\delta^{18}\text{O}$ of N_2O produced from soils at 60-100% water-filled pore
 422 space (WFPS). Data are means $\pm$ standard errors (n=12).

Soil	WFPS	SP	$\delta^{15}\text{N}^{\text{Bulk}}$	$\delta^{18}\text{O}$	N_2O Flux ($\text{nmol g}^{-1} \text{ soil h}^{-1}$)
ORG	60%	25.44 $\pm$ 2.43 a [#]	-5.18 $\pm$ 1.62 a	33.47 $\pm$ 0.64 a	11.29-41.70
	80%	7.13 $\pm$ 0.97 b	-6.87 $\pm$ 5.41 a	32.21 $\pm$ 0.82 a	163.77-1099.80
	100%	8.58 $\pm$ 0.97 b	-5.58 $\pm$ 6.07 a	32.71 $\pm$ 0.85 a	201.24-1147.61
CON	60%	28.73 $\pm$ 3.55 a	-7.88 $\pm$ 1.95 a	33.33 $\pm$ 0.38 a	5.14-40.30
	80%	16.96 $\pm$ 0.95 b	-17.12 $\pm$ 2.44 b	31.49 $\pm$ 0.32 a	110.78-280.72
	100%	13.90 $\pm$ 0.71 b	-15.66 $\pm$ 2.22 ab	31.47 $\pm$ 0.26 a	130.76-309.16

423 [#] Different letters within each column indicate significant differences of mean values at $P<0.05$.
 424

425 Table 3. Flux rates, ^{15}N isotope site preference (SP), $\delta^{15}\text{N}^{\text{Bulk}}$, and $\delta^{18}\text{O}$ of N_2O produced from soils subjected to glucose, cellulose and
426 chicken manure additions. Data are means $\pm$ standard errors (n= 6, i.e., three replicates and two times).

Soil	Time	Treatment	SP	$\delta^{15}\text{N}^{\text{Bulk}}$	$\delta^{18}\text{O}$	N_2O Flux (nmol g ⁻¹ soil h ⁻¹)
ORG	Day 1 & 2	Glucose	14.79 $\pm$ 1.66 a [#]	41.70 $\pm$ 7.70 a	39.16 $\pm$ 1.25 a	129.36-1017.57
		Manure	8.94 $\pm$ 1.07 b	45.54 $\pm$ 3.23 a	39.51 $\pm$ 0.43 a	504.92-704.47
	Day 7 & 8	Cellulose	1.03 $\pm$ 1.28 a	37.01 $\pm$ 2.54 a	37.19 $\pm$ 0.50 a	230.44-231.79
		Manure	8.61 $\pm$ 2.68 b	18.68 $\pm$ 0.99 b	35.27 $\pm$ 0.27 b	138.64-191.72
CON	Day 1 & 2	Glucose	18.11 $\pm$ 0.61a	16.99 $\pm$ 0.71 a	35.78 $\pm$ 0.33 a	275.40-754.52
		Manure	6.70 $\pm$ 0.68 b	42.62 $\pm$ 1.03 b	38.21 $\pm$ 0.14 b	269.60-1090.82
	Day 7 & 8	Cellulose	4.91 $\pm$ 1.48 a	32.40 $\pm$ 2.36 a	36.34 $\pm$ 0.70 a	42.05-61.41
		Manure	11.79 $\pm$ 1.46 b	32.15 $\pm$ 1.37 a	36.19 $\pm$ 0.23 a	56.49-87.56

427 [#] Different letters within each column indicate significant differences of mean values at $P<0.05$.

Figure Captions

Fig. 1.

(A) An example of 112-second $^{14}\text{N}^{14}\text{N}^{16}\text{O}$, $^{14}\text{N}^{15}\text{N}^{16}\text{O}$, and $^{15}\text{N}^{14}\text{N}^{16}\text{O}$ readings of N_2O in a 1-L jar of ambient N_2O mixed with 60 mL 90 ppm N_2O standard gas (CB09715 from EMPA). Inner plot shows considerable fluctuation of $^{14}\text{N}^{15}\text{N}^{16}\text{O}$ and $^{15}\text{N}^{14}\text{N}^{16}\text{O}$ readings with fairly stable $^{14}\text{N}^{14}\text{N}^{16}\text{O}$ readings when $^{14}\text{N}^{14}\text{N}^{16}\text{O}$ readings are close to the background level.

(B) Corresponding linear regression of $R^{15}\text{N}^a$ (or $R^{15}\text{N}^b$) with ρ_{air} using 112 points generated from $^{14}\text{N}^{14}\text{N}^{16}\text{O}$, $^{14}\text{N}^{15}\text{N}^{16}\text{O}$, and $^{15}\text{N}^{14}\text{N}^{16}\text{O}$ readings.

Fig. 2.

(A) An example of 180-second $^{14}\text{N}^{14}\text{N}^{16}\text{O}$, $^{14}\text{N}^{15}\text{N}^{16}\text{O}$, and $^{15}\text{N}^{14}\text{N}^{16}\text{O}$ readings of N_2O in the soil chamber, which had been closed for 15 minutes before gas measurement.

(B) Corresponding linear regression of $R^{15}\text{N}^a$ (or $R^{15}\text{N}^b$) with ρ_{air} using 180 points generated from $^{14}\text{N}^{14}\text{N}^{16}\text{O}$, $^{14}\text{N}^{15}\text{N}^{16}\text{O}$, and $^{15}\text{N}^{14}\text{N}^{16}\text{O}$ readings.

Fig. 3.

(A, B) Soil N_2O flux rates, (C, D) ^{15}N isotope site preference (SP), (E, F) $\delta^{15}\text{N}^{\text{Bulk}}$, and (G, H) $\delta^{18}\text{O}$ of N_2O measured by the QCL system every 30 min for 72 h twice from 12:00 pm May 17 to 12:00 pm May 20 and from 12:00 pm June 14 to 12:00 pm June 17, respectively, in the soil chamber located in an agricultural field.

450 Fig. 1

(A)

(B)

451
452

453 Fig. 2
 (A)

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