N_2O isotopomers and N_2:N_2O ratio as indicators of denitrification in ecosystems

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Introduction

The world is experiencing climate change and variability due to increased greenhouse gas (GHG) emissions. The main GHG's of concern are nitrous oxide (N_2O), carbon dioxide (CO_2) and methane (CH_4). Agriculture contributes approximately 14% of the world's GHG emissions. Nitrous oxide is one of the key GHG and ozone (O_3) depleting gas, constituting 7% of the anthropogenic greenhouse effect. On a molecular basis, N_2O has a 310- and 16-fold greater global warming potential than each of CO_2 and CH_4, respectively, over a 100-year period. Nitrous oxide can be produced through both chemical and biochemical pathways. They occur during denitrification (the stepwise conversion of nitrate (NO_3^-) to nitrogen gas (N_2) and during nitrification by ammonia-oxidizing archea (bacteria) during the oxidation of hydroxylamine (NH_2OH) to nitrite (NO_2^-) which is then reduced to N_2O and N_2 by nitrifier denitrification or heterotrophic denitrification (Fig. 1).

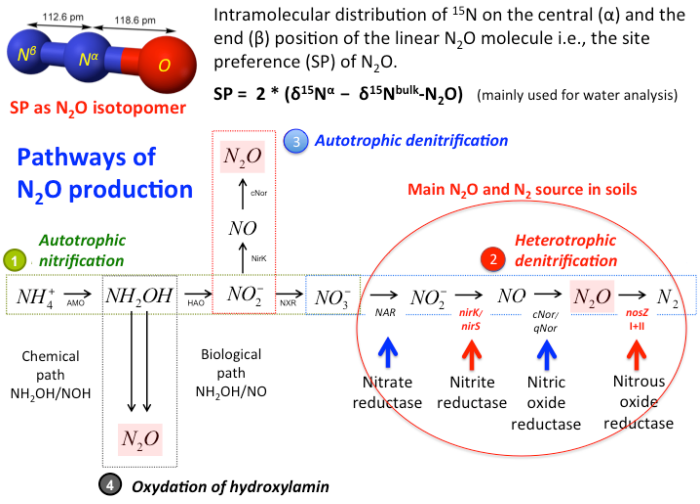

Figure 1. Site preference (SP) as N_2O isotopomer and main pathways of N_2O production

The conversion of N_2O to N_2, which depends on the abundance and expression of the *nosZ* gene of bacteria in sediments (Ligi et al., 2014), and is influenced by soil pH. The disruption of this step results in incomplete denitrification meaning N_2O emissions rather than di-nitrogen (N_2) (Zaman et al., 2012).

Apportioning N_2O to its source processes is still a challenging task. However, the use of methods involving stable isotope labeled ^{15}N precursors offers the best opportunity for quantifying both N_2O and N_2 products and also to identify their microbial source. The N_2O site-specific ^{15}N signatures from bacterial denitrification and the NH_2OH to N_2O pathway of nitrification are clearly different, making this signature a potential tool for identifying the bacterial or other source of N_2O (Sutka et al., 2006). The majority of past studies have been dedicated to the analysis of the $\delta^{15}N$ and $\delta^{18}O$ isotopomers ($d^{15}N^{\alpha}$ and $d^{15}N^{\beta}$) in relation to the production of N_2O, with only a limited number of studies being dedicated to the analysis of dissolved N_2O in groundwater (Well et al., 2012).

Only a limited number of studies have quantified N_2 fluxes and calculated the N_2:N_2O ratio (an important indicator of the denitrification process), the latter varying from 0.8 to 55 in typical agricultural soils, from 0.3 to 261 in soils under natural vegetation, and from 3 to 250 in wetlands and recently flooded soils (Butterbach-Bahl et al., 2013). The objective of our study was to quantify N_2O and N_2, and their isotopic signatures in wetland.

Methods

The analysis of stable isotope abundances ($\delta^{15}N$, $\delta^{18}O$, $\delta^{13}C$) of atmospheric gases present in trace amounts (specifically N_2O and CH_4) can be used with the coupling of a pre-concentration unit interfaced with a gas-chromatograph isotope ratio mass spectrometer (IRMS) (Fig. 2). For the isotopologue signatures of N_2O in water, i.e. $\delta^{18}O$ ($\delta^{18}O$–N_2O), the average $\delta^{15}N$ ($\delta^{15}N^{bulk}$–N_2O) and $\delta^{15}N$ from the central N position ($\delta^{15}N^{\alpha}$), can be analyzed after cryo-focusing using IRMS. Thus, in Mander et al., (2014), the analysis was conducted using a Delta XP IRMS, which allowed simultaneous detection of mass to charge ratio (m/c) 30, 31 for N_2O fragments and m/c 44, 45, and 46 for the intact N_2O molecules. The IRMS was connected to a modified Precon (Thermo−Finnigan, Bremen, Germany) equipped with an auto-sampler (model Combi-PAL CTCAnalytics, Zwingen, Switzerland).

^{15}N site preference (SP; ‰) was obtained as:

$$SP = 2 * (^{15}N^{\alpha} - ^{15}N^{bulk} - N_2O)$$

The isotopologue ratios of a sample (Rsample) were expressed as the deviation from the $^{15}N/^{14}N$ and $^{18}O/^{16}O$ ratios of the reference standard materials (Rstd), atmospheric N_2 and standard mean ocean water (SMOW) respectively:

$$\delta X = (Rsample/Rstd - 1) * 1000$$

where X = $^{15}N^{bulk}$ – N_2O, $^{15}N^{\alpha}$, $^{15}N^{\beta}$ or ^{18}O. Typical analytical precision was 0.6%, 0.9% and 0.9% for $\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$ and $\delta^{18}O$ respectively. The detection limit for N_2O-N was 1.5 ppb.

In Fig. 2, we show a N_2 flux measurement system, one which is an upgraded prototype of the systems used by Butterbach-Bahl et al. (2002).

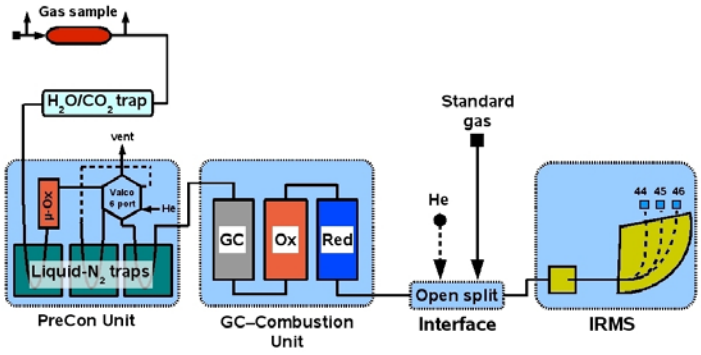

Figure 2. The instrument couplings used for the substance specific determination of N, O and C stable isotope abundances ($\delta^{15}N$, $\delta^{18}O$, $\delta^{13}C$) of atmospheric trace gases (specifically N_2O and CH_4) in a natural abundance range

Results and Discussion

In a study conducted on a horizontal subsurface flow (HSSF) in a constructed wetland (CW) designed specifically for municipal wastewater treatment (Mander et al., 2015), the isotopologue signatures of N_2O in the HSSF water suggest that the main source of N_2O was denitrification. This is supported by high SP values and a significant positive correlation between the $\delta^{18}O$-N_2O vs SP-N_2O (Fig. 3B). In comparison with the results from groundwater (Koba et al., 2009; Well et al., 2012) or sediments (Mothet et al., 2013), the results from the HSSF water are in good agreement. For example, the values of a $\delta^{18}O$-N_2O vs $\delta^{15}N^{bulk}$–N_2O plot are somewhat higher than those values observed in groundwater by Koba et al. (2009), though they are still in the range of presumable denitrification process values (Fig. 3A).

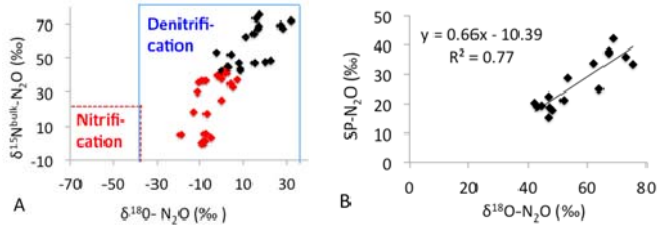

Figure 3. Isotopologue relations in the HSSF bed of a CW for wastewater treatment of a schoolhouse. Red diamonds, data from Koba et al. (2009); black diamonds, data from Mander et al. (2015). Presumed borders of nitrification and denitrification are adapted from Koba et al. (2009)

The reduction of N_2O to N_2 leads to an increase in SP-N_2O and $\delta^{18}O$-N_2O in the residual N_2O. In groundwater this has a wide range for both $\delta^{18}O$-N_2O and SP-N_2O (Well et al., 2012). Moreover, this leads to a close correlation between both "signatures", where the slope of SP-N_2O vs. $\delta^{18}O$-N_2O has been shown to vary between 5 (Koba et al., 2009) and 0.8 (Well et al., 2012). It should be noted that the lower values were obtained in groundwater with intense denitrification. These values are typical for denitrifying groundwater, and they result from isotopologue values of initially produced N_2O by bacterial denitrifiers with SP-N_2O values below 0‰ (Sutka et al., 2006) and $\delta^{18}O$-N_2O values below 30 m, with a subsequent increase in $\delta^{18}O$-N_2O and SP-N_2O in the residual N_2O during the progressive reduction to N_2. In the current study, the same pattern was evident, together with a close correlation of $\delta^{18}O$-N_2O and SP-N_2O (slope of 0.67), although there was a large range of SP values (Fig. 3B). A similar range of SP values were found by Mothet et al. (2013) for river (0–10‰) and lagoon sediments (-7 to 25‰), whereas there was a significant correlation between the nitrate reduction rate and the site preference values.

There have been recent advances in metagenomics, facilitated through high throughput sequencing, but these advances have not yet been utilized for N_2O emission studies. Possible reasons for this situation appear to include the argument that it is not necessary to understand which specific members of the denitrifier or ammonia oxidizer communities are actually present (and/or active) in response to, for example, a change in environmental conditions (Butterbach-Bahl et al., 2013).

Nonetheless, some relationships have been found between the functional genes of denitrifiers and dinitrogen fixers. Also, it appears that there is a causal relationship between the N_2O flux and the abundance of *nosZ* genes - which control N_2O reduction to N_2. These relationships provide incentive for further investigations at the molecular level (Fig. 4). Similar "causal" relationships also appear to occur between several functional denitrification genes, *nirK*, *nirS* and *nosZ* and environmental variables in wetland ecosystems (Ligi et al., 2014).

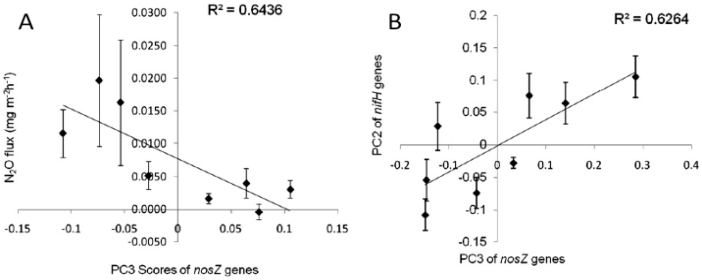

Figure 4. (A) Relationship between N_2O flux rate and the composition of the denitrifying community, as estimated from T-RFLP data of nosZ genes. (B) Relationship between the denitrifying (PC3 of nosZ genes) and N_2-fixing communities (PC2 of nifH genes). Error bars are one standard deviation of the mean. Adapted from Singh et al 2011

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