Nitrification is the primary source for NO in N-saturated subtropical forest soils: results from in-situ ^{15}N labeling

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Abstract

Rationale: Acidic subtropical forest soils that receive high atmospheric nitrogen (N) deposition have been identified as important sources of nitric oxide (NO). The relative importance of major processes producing NO is unclear.

Methods: To partition NO sources, we conducted an *in situ* tracing experiment with $^{15}NH_4NO_3$ and $NH_4^{15}NO_3$ in well-drained acid soils of an N-saturated subtropical forest in Chongqing, Southwest China.

Results: In the $^{15}NH_4NO_3$ treatment, the ^{15}N signature of NO emitted from the foot of the hillslope (Lower site) was similar to that of the NH_4^+ pool, indicating predominant autotrophic nitrification for NO formation. In the $NH_4^{15}NO_3$ treatment, the ^{15}N enrichment of NO was smaller than that of the NO_3^- pool, suggesting minor contribution of denitrification to NO production (~15%).

Conclusions: Nitrification is the main process responsible for NO emissions, even in monsoonal summers when soil water filled pore space values are relatively high.

1 Introduction

High atmospheric nitrogen (N) deposition in forest ecosystems introduces large amounts of inorganic N (NH_4^+ and NO_3^-) to the soil, potentially causing elevated N leaching [1] and provoking large emissions of nitrogen gases (NO, N_2O and N_2) [2-4]. In soils, nitric oxide (NO) is mainly formed as a by-product of autotrophic bacterial or archaeal NH_3 oxidation [5], or as an intermediate of denitrification [6], which reduces NO_3^- to N_2 via NO and N_2O under anaerobic conditions. In the conceptual Hole-In-The-Pipe (HIP) model, Firestone and Davidson [6]

proposed that the partitioning of NO to nitrification and denitrification is mostly determined by soil moisture. Optimum soil water-filled pore space (WFPS) for nitrification are normally at 30-60% [7,8]. NO is also produced through nitrifier denitrification (i.e. the dissimilatory reduction of nitrite (NO_2^-) to NO), or during direct conversion of organic N by heterotrophs [9], which is still not fully understood. If NO_2^- is released during nitrification or denitrification, NO also may be produced from nitrosylation, i.e. the reaction of NO_2^- with organic N [10,11], or in acid soils, through disproportionation of nitrous acid (HNO_2) [12]. Aerobic and anaerobic processes can take place simultaneously in aggregated soil, providing spatially disconnected microsites for oxidative and reductive processes. This makes it difficult to predict NO formation or consumption from bulk soil conditions. Pathways of N_2O turnover have been investigated in laboratory studies, using acetylene (C_2H_2) inhibition of autotrophic nitrification [13,14]. However, C_2H_2 catalyzes the autoxidation of NO in the presence of oxygen [15], and is therefore not suited to study NO partitioning.

^{15}N labeling of soil N pools has been widely used to apportion N_2O and N_2 to soil biogenic processes [16-19]. So far, only two studies have applied ^{15}N tracing for *in situ* NO source partitioning, both in semi-arid soils [20,21]. Both studies indicated that nitrification of NH_4^+ and abiotic decomposition of NO_2^- are the major processes responsible for NO formation in dry soils. By contrast, subtropical forest soils with monsoonal climate, receiving large volumes of precipitation in summer, are characterized by relatively high moisture contents over prolonged periods. In a recent study in an N-saturated, subtropical forest, Kang et al [4] reported *in situ* NO emissions amounting to 48.4 µg N m^{-2} h^{-1} in a wet summer and 88.4 µg N m^{-2} h^{-1} in a dry

summer. These values are comparable with NO emissions reported from fertilized agricultural soils [22]. In a laboratory incubation experiment with soil cores from the N-saturated temperate Höglwald forest in Germany, a mean NO flux of 213 µg N m^{-2} h^{-1} was reported, even increasing to 600 µg N m^{-2} h^{-1} at lower water tension [23]. Although laboratory experiments without plants may overestimate the NO emission potential, these findings indicate that forest soils with high atmospheric N deposition may be a substantial source of NO. However, little is known about the relative importance of the different pathways of NO formation and consumption in forest soils. We hypothesized that unlike in arid or semi-arid soils, where abiotic reactions contribute significantly to NO production upon rewetting [21], biogenic processes such as nitrification and denitrification may be the main source for NO formed in relatively moist subtropical forest soils. To determine the quantitative importance of nitrification and denitrification for NO emissions from soils of N-saturated subtropical forests, we applied a novel ^{15}N tracing technique during an intensive field campaign.

2 Materials and Methods

2.1 Site description

The labeled experiment was carried out in a coniferous-broadleaf mixed subtropical forested catchment, which is called TieShanPing (TSP) catchment. TSP is located about 25 km northeast of Chongqing City, SW China (29038'N 104041'E, Figure 1A). The region has a monsoonal climate with a mean annual temperature of 18.2°C and a mean annual precipitation

of 1028 mm [24]. The average annual atmospheric inorganic nitrogen (N) deposition has increased from 40 to 60 kg N ha^{-1} yr^{-1} in recent years [1]. The dominant vegetation is Masson pine (*Pinus massoniana*) with an understory of grass and shrubs. The soils on the well-drained hillslope (HS) of the forest are loamy yellow mountain soils (classified as Haplic Acrisols; WRB, 2014) with low pH_{H2O} (3.8 - 4.0). Because of fast decomposition of organic matter, the organic horizons are thin (0-4 cm).

In the summer of 2015, we conducted a short-term *in situ* labeling experiment in a 4.6 ha sub-catchment of TSP. We established two experimental sites on a northeast facing hillslope, one on the top of the hillslope (Upper site) and one in the foot of the hillslope (Lower site, Figure 1B). Soil moisture and organic matter contents are higher at the Lower than at the Upper sites, because the hillslope experiences considerable interflow over the Bt horizon following rain episodes [25].

2.2 Experimental design, sample collection and sample analysis

At each site, three replicated experimental blocks were set up, each with three adjacent 1.2 m x 1.2 m plots. Labeling treatments were assigned randomly to the three plots in each block (Figure 1B). To focus on N turnover in the soil and reduce the influence of plant roots uptake in a short period, all experimental plots only contained some ground vegetation without big trees. To avoid ^{15}N cross contamination between adjacent plots, plywood boards were inserted ∼3cm deep into the soil to separate adjacent plots. ^{15}N tracer was applied on June 23, 2015, as either $^{15}NH_4NO_3$ ($^{15}NH_4$, 99 atom% ^{15}N), or $NH_4^{15}NO_3$ ($^{15}NO_3$, 98 atom% ^{15}N). The

third plot was only received deionized water as a Reference. The total ^{15}N dose was 1 kg ^{15}N

ha^{-1} for the ^{15}NH$_4$NO$_3$ and NH$_4$^{15}NO$_3$ treatments. The N addition amounts accounted for less

than 4% of the annually deposited atmospheric N at TSP (40–60 kg N ha^{-1}) [1], thus leading to a

minimum fertilization effect. The minimum fertilization effect was confirmed by the fact that

the soil KCl-extractable NH$_4$$^+$ and NO$_3$$^-$ concentrations did not change appreciably after adding

the tracer (see Results). At each labeled plot, 7.2 L of solution (equal to 5 mm precipitation)

with ^{15}N tracers dissolved in deionized water was sprayed on the surface of the soil. After the

addition of ^{15}N tracer, we sprayed an additional 0.72 L deionized water on the ground

vegetation to wash away the intercepted tracer. The reference plot received the same volume

of deionized water as the labeled treatment, in total 7.92 L. The even spraying of the solutions

was completed in 0.5 h.

NO fluxes were measured 0.5, 7, 26, 50, 95, 144, 174 and 219 h after label addition by means

of opaque dynamic chambers, described in detail by Kang et al [4] (Figure S1, supporting

information). A 40 cm × 40 cm frame was inserted into the soil at each plot prior to N addition

to serve as an airtight base for the dynamic chambers. The same frames were used as a base for

NO collectors [26], in which NO was oxidized to NO$_2$ by CrO$_3$, which was trapped subsequently

as NO$_3$$^-$ in a H$_2$O$_2$/NaOH solution. After removing excess H$_2$O$_2$ by manganese dioxide (MnO$_2$)

and neutralizing the trapping solution by hydrogen chloride (HCl), NO$_3$$^-$ in the trapping solution

was converted to N$_2$O using a modified denitrifier method [27] for the ^{15}N abundance analyzing

by PreCon-GC/IRMS system (ThermoFinnigan, Bremen, Germany). A detailed description of

the field trapping method is given in the Supporting Information (*Section 2*; Figure S2) together with methodology and computational procedures (*Section 1*).

Simultaneously to NO, N_2O was sampled at all plots using static chambers and analyzed for ^{15}N by PreCon-GC/IRMS with a precision of 0.2‰; results, presented in Yu et al [19], are included here for comparison (Figure S4, supporting information).

While measuring the NO flux and collecting NO for ^{15}N analysis, soils from the O/A and AB horizons were sampled, and extracted immediately on site in 1 M KCl. Extracts were frozen and analyzed later for NH_4^+ and NO_3^- concentration and ^{15}N abundance. The atom% ^{15}N of NO_3^- in the KCl extracts was determined using the modified denitrifier method [27]. The atom% ^{15}N in NH_4^+ was determined after its quantitative conversion to N_2O using a chemical method [28] in which NH_4^+ is oxidized to NO_2^- by hypobromite (BrO^-), followed by reduction of NO_2^- to N_2O using azoimide (HN_3). The soil KCl-extractable $^{15}NH_4^+$ and $^{15}NO_3^-$ values were recently published in Yu et al [19] and used to partition N_2O sources (Figure S4, supporting information). Here, we used the same KCl-extractable $^{15}NH_4^+$ and $^{15}NO_3^-$ data to partition NO sources by an end-member mixing model [29] (Eq. 1). The soil pH was measured in a soil-to-water suspension (1:2.5) using an ORION SA720 electrode pH meter and an Orion ROSS Ultra pH Electrode (Thermo Fisher Scientific, Waltham, MA, USA). Total nitrogen (TN) and organic carbon (TOC) were determined using a CHN analyzer (CHN-1000, LECO, St Joseph, MI, USA). The soil physicochemical properties are presented in Table 1.

2.3 Calculations and statistics

Assuming that NO is produced through either denitrification or nitrification, the partitioning of NO production to denitrification and nitrification was calculated based on an end-member mixing model [29] as

$$a_m = d \times a_d + (1\text{-}d) \times a_n \qquad\qquad (1)$$

where a_m is the ^{15}N atom% of the produced NO, a_d the ^{15}N atom% of NO_3^- (electron acceptor for denitrification), a_n is the ^{15}N atom% of NH_4^+ (substrate for autotrophic nitrification), d is the fraction of the NO emission due to denitrification, and 1-d is the fraction of NO due to autotrophic nitrification.

Due to the technical failure, the NO_x analyzer was malfunctioning during the labeling experiment, so the NO flux had to be estimated using multiple linear regressions with flux data as well as soil moisture and temperature. Since the airtight bases for dynamic chambers in the fertilized plots were taken away after the labeling experiment, being used for subsequent field NO flux measurements in other sites, we measured NO flux and soil moisture and temperature only in the non-labelled reference plots (D-plots in Figure 1B) during a four-day period right after the labelling experiment. Separate models that refer to the relationship between NO flux and soil moisture and temperature were set up for the Upper and the Lower sites by multiple regression in Minitab 16.0 (for details see Results; Figure S3, supporting information). Significant tests for differences in soil properties and NO fluxes at the Upper and the Lower sites were performed, using the two-sample *t*-test (Minitab 16.0) at $p < 0.05$.

3 Results and Discussion

3.1 NO emission rates, measurements and modeling

NO emission rates were measured in the non-labelled reference plots immediately after the labeling experiment. Rates were positively correlated with soil temperature and negatively correlated with soil water-filled pore space (WFPS) for both Upper and Lower sites (Eq. 2 for Upper site and Eq. 3 for Lower site). Multiple linear regression models with soil moisture and temperature explained 79% and 91% of the variability in observed NO flux at the Upper and the Lower sites, respectively (Eqs. 2 and 3). NO flux had varied similarly with soil moisture during previous measurement campaigns on the hillslope covering two summers [4]. The observed and simulated NO fluxes estimated from the model were comparable at the Upper and Lower sites (Figure S3, supporting information), suggesting that equations 2 and 3 could be applied to calculate NO flux using soil WFPS and temperature.

$$F_{Upper} = 13.8 - 78.4 \ WFPS + 1.4 \ ST \qquad (2)$$

Adjusted coefficient of determination: R-Sq(adj) = 79.4%, n=11

$$F_{Lower} = 244 - 599 \ WFPS + 7.5 \ ST \qquad (3)$$

Adjusted coefficient of determination: R-Sq(adj) = 91.5%, n=11

where F_{Upper} and F_{Lower} are NO flux ($\mu g \ N \ m^{-2} \ h^{-1}$) at the Upper and the Lower sites respectively, WFPS water-filled pore space, and ST soil temperature (^{o}C).

Since the amount of N added in the labeling experiment was small relative to the native soil N pool, and soil NH_4^+ and NO_3^- concentrations were not affected significantly (Figure S4,

supporting information) [19], the NO flux in the labeled plots is expected to follow the same relationship with soil temperature and WFPS as found in the reference plots. The negligible fertilization effect by N addition at the two sites, is also supported by the similarity in N_2O emission rates at treated and reference plots (Figure S4, supporting information) [19]. The estimated NO flux was significantly larger at the Lower than at the Upper site ($p < 0.001$) (Figure 3). The opposite response was observed for N_2O emission by Yu et al [19], who attributed this to larger N_2O reduction activity of denitrifiers, leading to more complete denitrification at the Lower site with its wetter soils. The Lower site also held significantly larger soil organic C and N pools in the top soil ($p < 0.05$, Table 1). Pool dilution approaches presented by Yu et al [19] suggested that, in addition to the deposited NH_4^+, considerable amounts of NH_4^+ were released from the native soil N pool. Larger NO emissions at the Lower than the Upper site were also found during measurement campaigns covering two summers [4]. Therefore, the higher N mineralization activity may contribute to the observed larger NO flux at the Lower than the Upper site.

3.2 ^{15}N abundances of NO

In the $^{15}NH_4^+$ treatment, the ^{15}N in the soil NH_4^+ pool decreased from ~12 to ~0.4 atom% within the first 95 h after labeling at both the Upper and Lower sites (Figures 2A and 2C) [19]. Simultaneously, the atom% ^{15}N of NO_3^- increased from natural abundance to 7% - 10% within the first 50 h after label application, followed by a decrease due to dilution with NO_3^- derived from non-labeled NH_4^+. The rapid increase of ^{15}N abundance in the NO_3^- pool of both soils

indicated efficient nitrification of added $^{15}NH_4^+$, despite the low soil pH (Table 1). At the Upper site, ^{15}N enrichment in NO was less than in any of the two measured mineral N pools (Figure 2A). Assuming homogeneity of the ^{15}N-labeled pool, less ^{15}N enrichment in a product than in its putative substrate pool points to dilution by sources with depleted ^{15}N [30]. Low ^{15}N atom% in NO emitted in the $^{15}NO_3^-$ treatment at the Upper site (Figure 2B) indicates that denitrification contributed only marginally to NO emission. Therefore, denitrification is unlikely to explain the low ^{15}N abundance of NO in the $^{15}NH_4^+$ treatment. Heterotrophic nitrification, i.e. the direct conversion of non-labeled, native organic N to NO_3^-, is another potential source of ^{15}N-depleted NO. Recently, heterotrophic nitrification has been suggested to be the predominant pathway of NO_3^- and N_2O production in acid subtropical soils, based on *ex situ* ^{15}N labeling studies with homogenized soils [31,32]. In addition Zhang et al [33] found that N input in carbon-abundant acidic forest soils may stimulate soil heterotrophic nitrification. In the $^{15}NH_4^+$ treatment, less ^{15}N enrichment in NO than in the two mineral N pools at the Upper site (Figure 2A) would be alternatively attributed to the dilution of NO produced from autotrophic nitrification of non-labeled NH_4^+.

The ^{15}N atom% in NO released in the $^{15}NH_4^+$ treatment at the Lower site was greater than at the Upper site (Figures 2A and 2C). Here, ^{15}N enrichment of NO was similar to that of NH_4^+ over time (Figure 2C), indicating that NO was predominantly produced through autotrophic nitrification. In the $^{15}NH_4^+$ treatment, the variability of ^{15}N atom% in N_2O over time followed that in NO_3^- (Figures 2A and 2C). However, ^{15}N enrichment in N_2O was less than in NO_3^-, indicating that a minor N_2O portion was due to nitrification [19].

In the $^{15}NO_3^-$ treatment, the ^{15}N atom% in NO_3^- decreased gradually with time at both sites, probably due to leaching of added $^{15}NO_3^-$. The importance of NO_3^- leaching is supported by the significant recovery of ^{15}N in the deeper AB horizon (Figure S5, supporting information) [19]. Significant loss of labelled NO_3^- from the soil was also observed in a recent study at the same site [34]. In addition to leaching, the atom% ^{15}N in NO_3^- also declined due to dilution by NO_3^- produced from nitrification of non-labeled NH_4^+, which showed stable atom% ^{15}N close to natural abundance throughout the experiment (Figures 2B and 2D). The ^{15}N enrichment in NO was only slightly above that of NH_4^+, while being significantly less than in NO_3^-, confirming nitrification as the main source of NO in this treatment. Conversely, the ^{15}N enrichment of N_2O was similar to that of NO_3^- (Figures 2B and 2D). The significantly different ^{15}N enrichment between NO and N_2O in the $^{15}NO_3^-$ treatment thus points to different prevailing source processes for NO and N_2O production. While denitrification is the dominant source for N_2O production [19], we show here that nitrification is the main source of NO.

3.3 NO source partitioning

We calculated the contribution of denitrification to NO production by an end-member mixing model [29] on the basis of ^{15}N enrichments in NO and NO_3^- in the $^{15}NO_3^-$ treatment (Eq. 1). The contribution of denitrification to NO production at the Upper site averaged 14% (ranging from 5% to 35%). At the Lower site, the average value was 16% (range: 11 - 23%), slightly varying with WFPS (Figure 3). The WFPS values at both sites ranged from 45% to 60% throughout the labeling experiment (Figure 3). This is the common range of soil moisture where

nitrification is found to produce NO dominantly in forest soils [8,35]. In our study, at both sites, more than 80% of the emitted NO was derived from nitrification (Figure 3). This is the first time that NO sources have been quantified for N-saturated acidic subtropical forest soils by *in situ* [15]N tracing.

Chemical decomposition of NO_2^- may have contributed to NO emission, since the soil is acidic [36]. Compared with dry soil with higher pH, which tends to accumulate NO_2^- and releases NO in emission pulses following rewetting of soil [37], subtropical forest soils are less likely to accumulate NO_2^-. Due to high water availability in these subtropical forest soils, biological NH_3 and NO_2^- oxidation are balanced, and anoxic microsites may provide effective sinks for NO_2^- by nitrifier denitrification. In addition, incorporation of NO_2^- into soil organic matter has been reported [38]. Therefore, in the relatively moist TSP soils, the chemical decomposition of NO_2^- to NO is expected to be less pronounced than in dry soils upon rewetting.

4 Conclusions

Nitrification, primarily autotrophic nitrification, is the predominant pathway of NO production in acidic, N-saturated subtropical forest soil in SW China, even during monsoonal summers as long as the WFPS values are below 60%. By contrast, N_2O is primarily derived from denitrification.

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Table 1. Physicochemical parameters of soils sampled from O/A (~0-5 cm) and AB (~5-15 cm) horizons at Upper and Lower sites on the hillslope in TieShanPing forested catchment, Chongqing, SW China

Location	Horizon	Silt[a] (%)	Clay[a] (%)	Sand[a] (%)	Bulk Density[a] (kg m^{-3})	pH$_{H2O}$	TOC pool (kg m^{-2})	TN pool (kg m^{-2})	C/N
Upper	O/A	n.a.	n.a.	n.a.	750	3.8	3.54	0.19	18.7
	AB	57	30	14	1410	3.8	2.12	0.14	15.9
Lower	O/A	n.a.	n.a.	n.a.	760	4.0	4.86	0.28	17.3
	AB	51	19	31	1430	3.9	3.56	0.24	14.7

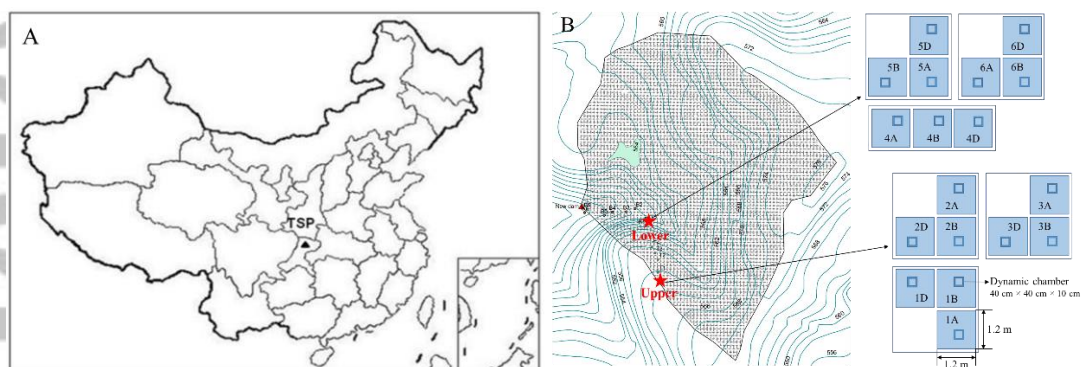[a]data from Sørbotten et al. (2017) [25]; n.a.: not available

Figure 1. (A) The TieShanPing Forested Catchment, SW China. (B) Location and experimental plots at two sites (Upper and Lower sites). Treatments: A = $^{15}NH_4NO_3$, B = $NH_4{}^{15}NO_3$, D = Reference.
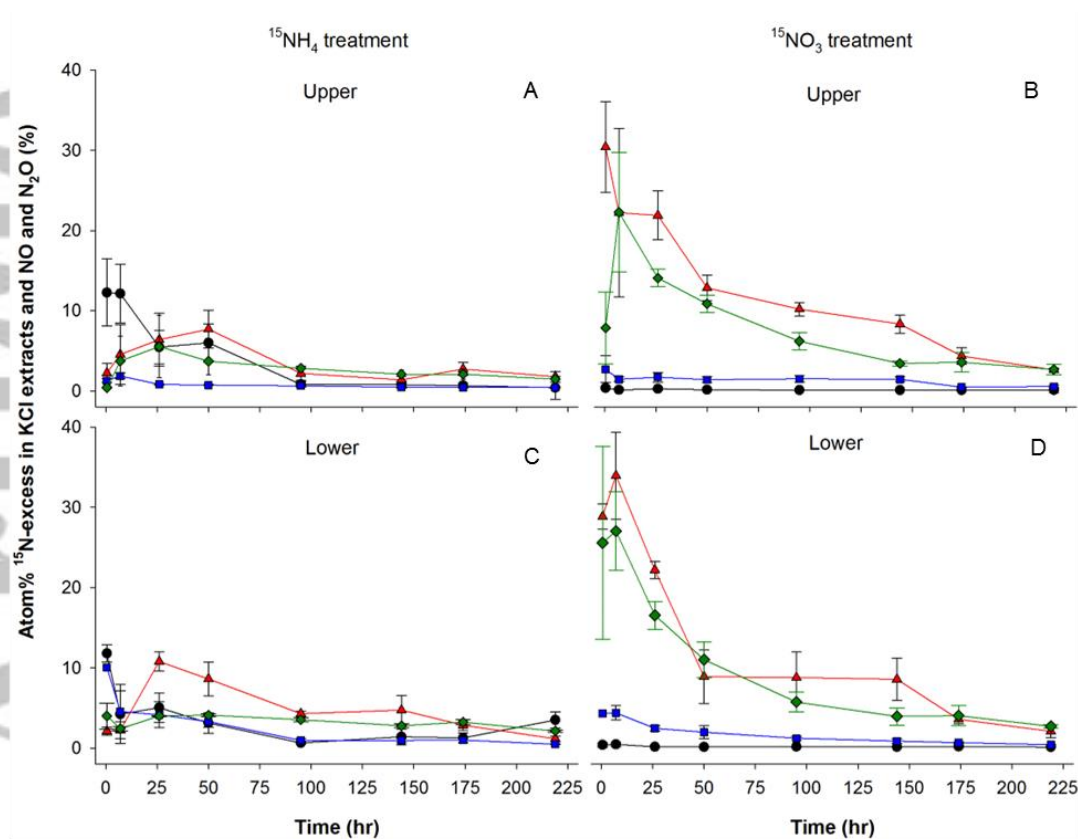
Figure 2. Atom% ^{15}N-excess of NH_4^+ and NO_3^- in KCl extracts of soils from O/A horizon, and emitted NO and N_2O in the $^{15}NH_4$ (panels A & C) and $^{15}NO_3$ (panels B & D) treatments at the Upper and the Lower sites. Values are means and standard errors (n=3). The data of atom% ^{15}N in N_2O was taken from Yu et al (2017) [19] for comparison.
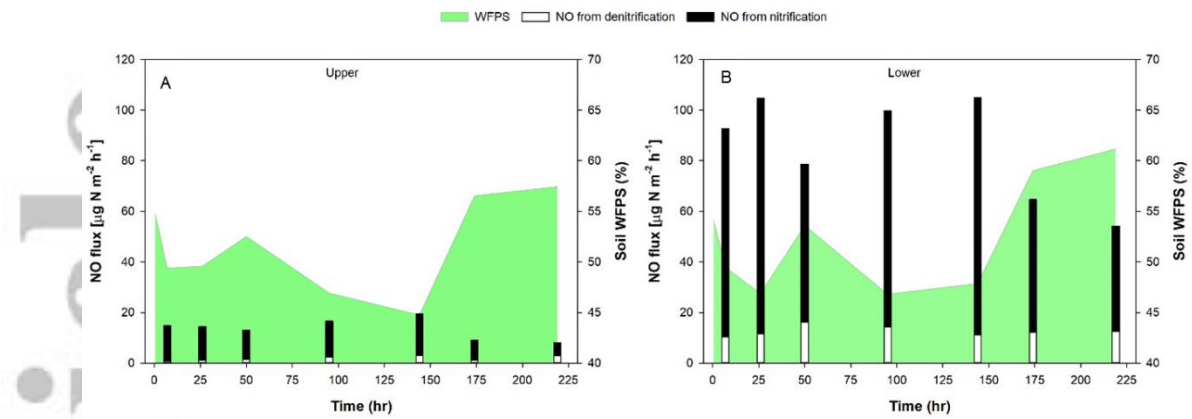
Figure 3. Mean partitioning (n=3) of NO flux to nitrification and denitrification at the Upper (A) and Lower (B) site. The shaded area indicates water-filled pore space (WFPS) (n=3).