Characteristics of N\(_2\) and N\(_2\)O Fluxes from a Cultivated Black Soil: A Case Study through In Situ Measurement Using the \(^{15}\text{N}\) Gas Flux Method

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Abstract: The magnitudes and source partitioning of soil dinitrogen (N\(_2\)) and nitrous oxide (N\(_2\)O) emissions are not well documented, yet. To address both issues for black soil subject to a typical cool temperate climate, soil N\(_2\)O and N\(_2\) fluxes following the basal application event of an ammonium-based fertilizer (labeled by \(^{15}\text{N}\)) for maize were simultaneously measured in situ by using the \(^{15}\text{N}\) gas flux (\(^{15}\text{N}\)FG) method. During the two-month field experiment, the measured N\(_2\) and N\(_2\)O fluxes cumulated to 1.61 \(\pm\) 0.47 and 0.12 \(\pm\) 0.01 kg N ha\(^{-1}\), respectively, showing N\(_2\)O to N\(_2\O\) plus N\(_2\) ratios (\(r_{\text{N}_2\text{O}}\)) of 0.02-0.31 (0.15 on average). Temperature was identified as a key factor regulating the total soil N\(_2\) fluxes (\(r^2 = 0.27, p < 0.01\)), despite the N\(_2\) fluxes originated from nitrate denitrification related to dissolved organic carbon concentrations (\(r^2 = 0.39, p < 0.01\)). Differently, both temperature and soil moisture jointly accounted for 85% and 74% of the variances in the N\(_2\)O fluxes and the \(r_{\text{N}_2\text{O}}\) values, respectively (\(p < 0.01\)). Moreover, the process(es) other than autotrophic nitrification and heterotrophic denitrification could be of substantial importance for the soil N\(_2\O\) emissions. Our findings emphasized the importance of temperature in regulating N\(_2\) emissions from black soil and the possible site- and/or time specificity of a soil factors-based parametrization of \(r_{\text{N}_2\text{O}}\). In addition, this study implicates that labeling a nitrogen substrate of nitrification while using the \(^{15}\text{N}\) enrichment of N\(_2\)O is necessary to more accurately quantify total soil N\(_2\O\) fluxes in situ by using the \(^{15}\text{N}\)FG approach even though further confirmation in future studies is still needed.

Keywords: N\(_2\) flux; N\(_2\O\) flux; N\(_2\O\)/(N\(_2\O\) + N\(_2\)) ratio; nitrification; denitrification; \(^{15}\text{N}\) gas flux method

1. Introduction

Nitrogen (N), an essential element for life, is of great concern in crop production and has numerous environmental and ecological consequences [1]. With the world’s growing population, the use of fertilizer N increased to 109 Tg N year\(^{-1}\) in 2019 and is expected to increase in the future decades [2,3]. However, more than 50% of applied N fertilizer in cropland is unutilized by crops, thus resulting in lower N use efficiency, particularly in developing countries [4,5]. This low N use efficiency frequently results from soil denitrification, ammonia (NH\(_3\)) volatilization, runoff, and leaching in croplands [6]. As a major pathway of fertilizer N loss, soil denitrification can convert nitrate (NO\(_3^−\)) and nitrite (NO\(_2^-\)) to nitric oxide (NO), nitrous oxide (N\(_2\O\)), and dinitrogen (N\(_2\)) under anaerobic or microsite-anaerobic conditions [7]. However, the incomplete denitrification process releases N\(_2\O\) as an intermediate product, which contributes to climate warming and stratospheric ozone depletion [8,9]. Denitrification is both a source and sink of N\(_2\O\), with the N\(_2\O\) emission from this process being the net result of gas production and consumption (reduction of N\(_2\O\) to N\(_2\)). In field soil conditions of uplands, N\(_2\) may dominate among the gas.
species being released due to denitrification while showing high spatiotemporal variability under different case-specific soil environments [10–12]. For example, Chen et al. [13] found that, based on the gas-flow-soil-core (GFSC) technique, up to 90% of the total gaseous N loss due to denitrification in the maize-wheat rotation cropping system of North China was attributed to N2 emission. Li et al. [14] reported that soil N2 emissions accounted for up to 10% of the applied fertilizer N in the maize cropping system of Japan. Notwithstanding this, complete denitrification to N2 is often overlooked as this gas is not reactive [15]. Nevertheless, a better understanding of soil N2 emission and its driving factors in agricultural ecosystems is an essential prerequisite for reducing the loss of available N and developing effective N2O mitigation strategies (as N2 is the sole sink for N2O).

In the past half-century, there have been extensive studies on soil N2O emissions, including field observations [16,17], reviews [18,19], and meta-analyses [20,21], in which the magnitude of soil N2O emission has been well understood. Nevertheless, the process-based source partitioning of soil N2O emission is not well known. Likewise, advancement in soil N2 emissions has been considerably slow due to the inherent difficulty in their measurement [15]. Currently, soil N2 emissions from terrestrial ecosystems are measured in situ via the acetylene inhibition technique (AIT) and the 15N gas flux method (15NGF) [22]. However, a growing number of studies have demonstrated that the AIT may cause an unpredictable underestimation of soil N2 emissions because of its distinct shortcomings, e.g., the catalytic decomposition of NO in the presence of oxygen and the incomplete inhibition of N2O reduction to N2 [22–24]. In contrast to the AIT, the 15NGF method can overcome these shortcomings. It measures soil N2 emissions by detecting increased 15N-labeled N2 and N2O in the chamber headspace, which encloses a designated plot of soil amended with highly enriched 15N-labeled fertilizer [25]. Moreover, the 15NGF method can discriminate sources of soil N2O, i.e., nitrification and denitrification, by measuring the enrichment of N2O, NH4+, and NO3− pools [26]. These advantages indicate that the 15NGF method is the most suitable technique to directly measure in situ soil N2 fluxes of terrestrial ecosystems. Nevertheless, only a few studies have been conducted under field conditions, most likely due to the requirement for homogenous 15N labels and the high cost [15,25]. Even for the existing field studies, only some cases have explored the magnitude of soil N2 emissions from fertilized croplands, whereas the rest have focused on manipulated experiments in various ecosystems (reviewed in Table S2). The lack of soil N2 emission data measured in situ has undoubtedly led to large uncertainties in estimating soil N2 losses at regional or global scales [23,24]. Therefore, more research using the 15NGF method is urgently required to measure in situ N2 losses from fertilized agricultural soils.

It is generally recognized that microorganism-mediated N2 emissions are affected by soil abiotic and biotic factors such as temperature, moisture and carbon (C), and N substrate availability [27,28]. Soil temperature, not only as a proximal regulator, directly affects soil N2 emissions through altering the kinetic reaction, but also as a distal regulator, exerts a positive influence on soil N2 emissions by enhancing the soil respiration rate and thus depleting oxygen (O2) concentrations [27,29]. Zistl-Schlingmann et al. [30] found that soil temperature had the highest predictive power for N2 emissions potentially due to the decreasing O2 availability with the increasing soil respiration rate at a higher temperature. It has been well established that complete soil denitrification, with higher N2 emissions, is an anaerobic process [7,23], which is often associated with higher soil moisture. This is largely attributed to the fact that soil moisture can indirectly modulate soil N2 emissions by reducing O2 concentrations in soil pores and enhancing the input of substrates (C and N) into the microsites of denitrification [31–33]. However, the influences of N substrate availability (e.g., NO3−) on soil N2 emissions are inconsistent, with stimulatory or inhibitory effects [13,34]. More specifically, N2 is the main product of soil denitrification when the supply of C substrate, e.g., dissolved organic carbon (DOC), is adequate [35,36]. Otherwise, C substrate limitation occurs, causing incomplete denitrification, with N2O being produced as the main product [37]. These indicate that the responses of soil N2 emissions to soil NO3− or DOC availability strongly depend on the DOC:NO3− ratio [38]. Furthermore, a large
share of studies has found that amendment of crop straw or organic manure to soils does not necessarily decrease soil $\text{N}_2\text{O}$ emissions though reducing the ratio of $\text{N}_2\text{O}$ to $\text{N}_2$ plus $\text{N}_2$ ($R_{\text{N}_2\text{O}}$) [21,34,39,40]. One possibility is that the decomposition of organic amendments results in relatively low C:N ratios [41] that are not very conducive to the reduction of $\text{N}_2\text{O}$ to $\text{N}_2$ [38]. In addition to the aforementioned factors, fertilization events are expected to play a key role in driving soil $\text{N}_2$ emissions [14,33,42]. The application of synthetic N fertilizer, as a common management practice in agriculture, can rapidly increase the available N contents in soils, thereby affecting the microbial activity and resulting in high gaseous N emissions from nitrification and denitrification. Soil $\text{N}_2\text{O}$ emissions induced by fertilization events, showing increases in orders of magnitude over short durations, have been well documented in numerous previous studies [43–45]. However, information from field in situ measurements on how N fertilization affects soil $\text{N}_2$ emissions is still lacking. However, field $\text{N}_2$ emissions due to N fertilization and the effects of regulating factors are critical in the attempt to reduce soil N losses so as to improve fertilizer N use efficiency while mitigating $\text{N}_2\text{O}$ emissions.

Maize (Zea mays L.) planted under intensive fertilization management is one of the three major cereal crops in China. Chernozem with a mollic horizon (black soils), which is one of the dominant soil groups in the northeastern China, covers an important maize cultivation area of the country, contributing to 26% of national maize production (2013–2017 data from the National Bureau of Statistics of China). As a result of the increasing degradation of the black soils, a large amount of N fertilizers is applied to maintain fertility and boost crop yields, thereby ensuring food security [46]. Such high N application also attracts many environmental concerns, such as substantial gaseous N losses and N leaching into groundwater and surface waters [6,47]. A recent research in this black soil region reported that 23% of fertilizer N was lost via gaseous N emissions or leaching [48]. Although some studies have investigated gaseous N emissions from local maize cropping systems, a wealth of these studies have focused on soil $\text{N}_2\text{O}$ emissions while field studies assessing possible changes in soil $\text{N}_2$ emissions due to N fertilization are rather scarce [49–52]. In this case study, we conducted a two-month field experiment in a long-term (12 years) continuous no-tillage maize cropping system, in which the soil $\text{N}_2$ and $\text{N}_2\text{O}$ fluxes following the fertilization event of an ammonium-based fertilizer were measured in situ using the $^{15}\text{N}$GFF method. Our objectives were (a) to characterize and quantify the fertilization-induced dynamical soil $\text{N}_2\text{O}$ and $\text{N}_2$ fluxes, as well as $R_{\text{N}_2\text{O}}$, (b) to explore key regulating factors for the fluxes of both gases and the $R_{\text{N}_2\text{O}}$, and (c) to partition the sources of $\text{N}_2\text{O}$ and $\text{N}_2$ emissions.

2. Materials and Methods

2.1. Study Site

The experimental site is located at the Lishu Conservation Tillage Research and Development Station of the Chinese Academy of Sciences (43°19′ N, 124°14′ E) in northeastern China. The region is subject to a cool temperate, sub-humid continental monsoon climate. The multiple-year mean annual precipitation is 614 mm, of which 75% occurs from June to September. The mean annual temperature is 6.9 °C, with monthly means ranging from −13.5 °C in January to 23.7 °C in July. The soil is classified as a mollisol [53]. Compared to red soil, purplish soil, and so on, mollisol, i.e., black soil, is subject to a cool temperate climate and usually more fertile [54]. Some soil properties of the 0–20 cm depth are presented in Table 1 [55]. The cultivation regime in this region is overwhelmingly dominated by the continuous single cropping system of maize, with maize sown in late April and harvested in mid-October each year.
Table 1. The main soil properties in the 0–20 cm soil layer.

| Sand (%) | Silt (%) | Clay (%) | SOC (%) | TN (%) | BD (g cm$^{-3}$) | pH (H$_2$O) |
|----------|----------|----------|---------|--------|-----------------|-------------|
| 24.8     | 47.7     | 27.5     | 1.1     | 0.12   | 1.45            | 7.1         |

SOC: soil organic carbon; TN: total N; BD: bulk density.

2.2. Experimental Design and $^{15}$N Application

Our two-month case study campaign was conducted from 28 April to 22 June 2019 in three replicated field plots (8.7 m × 30 m each) of the long-term no-tillage experimental trial (since 2007) with the conventional fertilization practice and mulching of 100% maize straw [55]. These selected plots were laid out in a completely randomized block design (Figure 1). Table 2 summarized their field management practices. After harvest, the maize straw was applied to evenly cover the soil surface. In the following year, the straw was cut into 30–70 cm pieces prior to the maize sowing. All fertilizers were once incorporated basally for each plot by banding in a ~ 10 cm deep furrow for each row of maize.

![Figure 1. Field layout for experimental plots, $^{15}$N labeling and soil/gas sampling. The dark grey area indicates the plots selected in this study. Four squares in each plot represent the stainless steel frames used to measure N gas fluxes and sample soil.](image)

Table 2. Field cropping managements in this study.

| N Fertilizer (kg N ha$^{-1}$) | P Fertilizer (kg P ha$^{-1}$) | K Fertilizer (kg K ha$^{-1}$) | Straw Mulching (t ha$^{-1}$ yr$^{-1}$) | Tillage (Yes/No) | Irrigation (Yes/No) |
|-------------------------------|-------------------------------|-------------------------------|--------------------------------------|------------------|---------------------|
| 240                           | 48                            | 91                            | 7.5                                  | No               | No                  |

*Chemical fertilizers, with urea, phosphorus pentoxide and potassium oxide to provide nitrogen (N), phosphorus (P), and potassium (K), respectively, were basally applied for maize.

One week before this experiment, four square stainless steel frames (with an area of 30 cm × 30 cm for each) were randomly inserted into the soil to a depth of 10 cm in
each plot (Figure 1). Two frames were used to measure N gas fluxes, and the other two were used to sample soil. Given that the hydrolysis of urea leads to $^{15}$N tracer loss via ammonia volatilization, thus impeding obtaining a more accurate detectable $^{15}$N-N$_2$ signal, labeled ammonium sulfate ([($^{15}$NH$_4$)$_2$SO$_4$]) was used to simulate relative real condition in the field. The solution of ([($^{15}$NH$_4$)$_2$SO$_4$] fertilizer (98 atom %) was applied to the soil within the frames at a rate equivalent to 240 kg N ha$^{-1}$, thereby mimicking the rate of N fertilizer in the long-term field trial. To achieve uniform distribution, a tracer solution of 180 mL was added via 180 injections of equal volume (1 mL for each) at three different depths (4, 8, and 12 cm). A plastic board (29.5 cm × 15 cm) perforated with 12 holes was employed to guide and position the injection microsites (Figure 1). The injected depth between any two adjacent holes was different to ensure homogeneously horizontal and vertical distribution of the $^{15}$N tracer [56]. The injection was performed using a custom-made lateral opening needle (length: 15 cm and outer diameter: 7 mm) connected to a syringe.

2.3. Gas Sampling

We measured the fluxes of N$_2$ and N$_2$O using a static opaque chamber-based method [14,57,58]. The frame mentioned above was fitted with a square groove of 3 cm depth, which was mounted with a sampling chamber (30 cm length × 30 cm width × 3 cm height, and headspace volume 2700 cm$^3$), providing a gastight seal when filled with water in the groove (Figure 1). The chamber was wrapped with a layer of Styrofoam and aluminum foil to minimize temperature changes within the chamber headspace during the gas sampling in sunny daytime. To obtain a detectable $^{15}$N-N$_2$ signal, a 6-h chamber enclosure time was employed for the gas sampling. This enclosure time was jointly determined by the sensitivity of the isotope detection instrumentation for N$_2$ in air samples, the expected soil N$_2$ flux levels as referred to measurements using the AIT method, and the conclusions of previous investigations on chamber enclosure time (Table S2) [52,59,60]. If a chamber is enclosed for a shorter time period, the $^{15}$N-N$_2$ signal would be undetectable in this study region. On each sampling occasion, an ambient air sample ($t_0 = 0$ h) near the sampling chamber was collected immediately before closing the chambers. A 70 mL gas sample from the chamber headspace was collected after a chamber was enclosed for 6 h ($t_1 = 6$ h). The gas samples were collected using a polypropylene syringe fitted with three-way stopcocks. Duplicate gas samples (21 mL each) in each syringe were injected into two pre-evacuated (<100 Pa) septum-capped 20 mL glass vials (for determining the $^{15}$N abundances of N$_2$O and N$_2$) after flushing the dead volume of the three-way stopcock with ~2-mL gas sample. Slight positive pressure was maintained to prevent atmospheric gases from leaking into the vials and contaminating the sample [61]. Another 25 mL gas sample in each syringe was injected into a pre-evacuated screw-cap 12 mL glass vial to determine N$_2$O concentration. In general, flux measurements were performed daily for 3–5 days following the $^{15}$N tracer application and/or rainfall events, and every other day during the other periods. On each sampling day, the flux measurements were performed between 9:00 a.m. and 3:00 p.m.

2.4. Analysis of N$_2$O and Flux Calculation

The N$_2$O concentrations of the gas samples stored in the 12-mL glass vials were measured using a gas chromatograph equipped with an electron capture detector (Agilent GC 6820, Shanghai, China) and the DN-CO$_2$ method [62]. Before analyzing gas samples, air reference standards (0.32 µmol mol$^{-1}$) were repeatedly measured, showing precision of 0.147 µmol mol$^{-1}$ as 1 standard deviation (SD) or 0.46% as the coefficient of variation (CV). The measured N$_2$O concentrations were calculated by applying a calibration curve, with a set of standard N$_2$O concentrations varying between 0.32 and 9.93 µmol mol$^{-1}$.

The $^{15}$N content of the N$_2$O in each-20 mL vial was analyzed by an IsoPrime 100 continuous-flow isotope ratio mass spectrometer (CF-IRMS) (IsoPrime Ltd., Cheadle Hulme, UK) interfaced with an IsoPrime trace gas analyzer (TG). The ion currents for $m/z$ 44, 45, and 46 ($^{44}$N$_2$O, $^{45}$N$_2$O, and $^{46}$N$_2$O, respectively) and the molecular mole ratios $^{45}$R (i.e., the $^{45}$N$_2$O/$^{44}$N$_2$O molar ratio) and $^{46}$R (i.e., the $^{46}$N$_2$O/$^{45}$N$_2$O molar ratio) in both the ambient
(t₀ = 0 h) and enriched (t₁ = 6 h) air samples were measured. The precision of ⁴⁵R and ⁴⁶R was 1.79 × 10⁻⁶ and 1.87 × 10⁻⁶, respectively, as 1 SD for 27 repeated analyses of dry ambient air.

Using the measured ⁴⁵R and ⁴⁶R for the air samples, the ¹⁵N enrichments of the N₂O (α_{soil}N₂O) in atom % and ¹⁵N₂O fluxes (F_{N₂O}^¹⁵) were calculated as described in Text S1 [63]. The N₂O fluxes (F_{N₂O}) and their method detect limits (DL) were calculated from the differences in the N₂O concentrations between t₁ and t₀ and the precision (1 SD) of N₂O analysis ((Text S2) [62]).

2.5. Analysis of ¹⁵N₂ and N₂ Flux Calculation

The method and procedures used for ¹⁵N₂ analysis in the present study mainly followed those of Yang et al. [64] and Liu et al. [60]. Briefly, the ¹⁵N abundance of N₂ in each air sample was determined using the aforementioned CF-IRMS with a modified TG. Using a tight-gas syringe, a gas sample of 700 µL was taken from each 20-mL vial. After flushing the pre-loop dead volume (~20 µL) using a 100 µL gas sample from the syringe, the remaining 600 µL gas sample was fully injected manually into the sample loop (50 µL). After purification, the sample was analyzed, and the ion currents for (⁴⁵N₂, ⁴⁶N₂) were quantified as

\[\left(\frac{1}{m/z} \text{28, 29, and 30} \right) (\text{²⁸N₂, ²⁹N₂, and ³⁰N₂, respectively}) \text{ and the molecular mole ratios } ²⁹R \text{ (i.e., } ²⁹N₂/²⁸N₂ \text{ molar ratio}) \text{ and } ³⁰R \text{ (i.e., } ³⁰N₂/²⁸N₂ \text{ molar ratio)} \text{ in both ambient (t₀ = 0 h) and enriched (t₁ = 6 h) air samples were measured. The precision (1 SD) of 68 ambient air analyses was 3.05 × 10⁻⁷ for ²⁹R and 1.53 × 10⁻⁶ for ³⁰R.}

Following the classic approach (Text S3) [65,66]), the atomic ¹⁵N enrichment in the N₂ of each enriched sample exclusively due to NO₃⁻ reduction in denitrification was quantified as ¹⁵X_N or α_D, using the measured ²⁹R, ³⁰R, ⁴⁵R, and ⁴⁶R. The calculated ¹⁵X_N or α_D and the directly measured ²⁹R or ³⁰R were involved in the calculation of the NO₃⁻-originated N₂ fluxes (F_{N₂}^N). Following the modified approach ((Text S4) [64]), the atomic ¹⁵N enrichment of the N₂ (α’) in the enriched and ambient air samples was determined and then used to calculate ¹⁵N₂ fluxes. The ¹⁵N₂ fluxes were divided by the ¹⁵X_N to provide F_{N₂}^n or by the ¹⁵N enrichment of N₂O from soil (i.e., α_{soil}N₂O, in atom %) to determine the total soil N₂ flux (F_{N₂}^T) due to the reduction of the soil N₂O originated from NO₃⁻ reduction and other N transformation processes. As described in Text S2, the maximum method DL corresponding to each F_{N₂}^n observation at the 95% confidence interval (CI) was estimated following the function presented by Liu et al. [60]; and the maximum method DL corresponding to each F_{N₂}^T measurement was calculated using the method of Friedl et al. [25].

2.6. Auxiliary Measurements

Rainfall data were monitored closely by meteorological stations. During the gas flux measurements, the soil temperature at a 10 cm depth was measured from the vicinity of the chamber frames using mercury thermometers. One or two composite soil samples (0–20 cm) were collected from the two frames in each replicated plot. Soil sampling was performed daily on the first 3 days following the N tracer application and/or rainfall events and every 2–4 days during the other periods. Soil moisture was measured after oven-drying 20 g of fresh soil for 24 h at 105 °C. The moisture content was converted to water-filled pore space (WFPS) using a theoretical soil particle density of 2.65 g cm⁻³ and a BD of 1.45 g cm⁻³. To determine the concentration and ¹⁵N enrichment of soil NH₄⁺ and NO₃⁻, 20 g of fresh soil was extracted by shaking in 100 mL of 1 M KCl solution at room temperature for 1 h, after which the soil extract was filtered. Another 20 g of fresh soil was extracted to quantify DOC by shaking in 100 mL of deionized water and then centrifuging at 85.83 g s⁻¹ for 5 min. The supernatant was filtered through a polyethersulfone membrane with a 0.45-µm pore size (Merck Millipore, Darmstadt, Germany). Filtered extracts were analyzed for NH₄⁺, NO₃⁻, and DOC by a colorimetric continuous flow analyzer (San + +, Skalar Analytical B.V, Breda, The Netherlands).
To measure the $^{15}$N enrichment of NH$_4^+$ (αNH$_4^+$, in atom %) and NO$_3^-$ (αNO$_3^-$, in atom %), the diffusion method was used according to the procedure described by Dannenmann et al. [67]. In brief, NH$_3$ from 30 mL filtered extracts amended with 0.25 g MgO was trapped on acidified ashless filter papers (Whatman GF/A, Springfield, UK). All residual NH$_4^+$ in MgO-added filtered extracts must be removed using over-acidified filter papers to avoid the effect of residual NH$_4^+$ on the measurement of the $^{15}$N enrichment of NO$_3^-$ . Thereafter, 0.25 g Devarda’s alloy was amended to reduce NO$_3^-$ to NH$_4^+$. The $^{15}$N content of nitrogen captured on the dried filter paper was analyzed using the aforementioned CF-IRMS coupled with an automated C/N elemental analyzer (vario MICRO cube, Hanau, Germany).

In addition, soil cores were sampled half-weekly on average from the ambient areas (fertilized basally with urea) of the field plots during the experimental period, stored, and transported at a temperature of approximately 4 °C to the lab in Beijing. And then the GFSC technique was used to measure the total soil N$_2$ fluxes from the intact soil cores following the procedures described by Wang et al. [33].

2.7. Source Partitioning of N$_2$O and N$_2$ Emissions

Based on measured $\alpha_{\text{soil}}$N$_2$O, αNH$_4^+$, and αNO$_3^-$, the relative contributions (%) of heterotrophic denitrification of NO$_3^-$ ($C_d$) or autotrophic nitrification ($C_n$) to the total N$_2$O fluxes were calculated following Equations (1) and (2), assuming that other N$_2$O sources were negligible [26].

$$C_d = 100(\alpha_{\text{soil}}\text{N}_2\text{O} - \alpha\text{NH}_4^+)/ (\alpha\text{NO}_3^- - \alpha\text{NH}_4^+)$$

(1)

$$C_n = 100 - C_d$$

(2)

Using the N$_2$ fluxes calculated by using the classic and modified approaches (Texts S3 and S4), the relative contributions (%) of NO$_3^-$ reduction ($K_d$) or other processes ($K_o$) to the total N$_2$ fluxes were estimated by following Equations (3) and (4).

$$K_d = 100 F_{\text{N}_2}/F_T$$

(3)

$$K_o = 100 - K_d$$

(4)

The respective relative contributions (%) of the amended fertilizer N ($^{15}$C$_m$) or soil N (${^6}$C$_m$) to the total fluxes of N$_2$O (m = 1) and N$_2$ (m = 2) were calculated, following Equations (5)–(7) in which $k$ denotes the $^{15}$N fraction in the applied fertilizer N ($k = 0.98$ in this study).

$$fC1 = 100F_{\text{N}_2}^{15}/(kF_{\text{N}_2})$$

(5)

$$fC2 = 100F_T^{15}/(kF_{\text{N}_2}^T)$$

(6)

$${^6}C_m = 100 - fC_m$$

(7)

2.8. Data Process and Statistical Analysis

If not specified, data were reported as the mean ± standard error of three or six spatial replicates. Linear, nonlinear, or multiple regression analyses were performed to test the dependences of N$_2$O and N$_2$ fluxes, as well as $R_{N_2}$O, against soil temperature, moisture, and/or concentrations of NH$_4^+$, NO$_3^-$, and/or DOC. The level of $p < 0.05$ was regarded as statistically significant at 95% CI. Statistical analyses were performed with the SPSS 19.0 software package (SPSS China, Beijing, China). Experimental data were graphically plotted using the Origin 8.0 (OriginLab, Northampton, MA, USA) software package.
3. Results

3.1. Soil Factors

Soil temperature gradually increased from 8.4 to 22.3 °C (average: 15.3 °C) during the experimental period (Figure 2a). Soil moisture in WFPS was primarily controlled by rainfall, varying between 66.2% and 85.9% with an average of 72.9% (Figure 2b). The soil NH$_4^+$ and NO$_3^-$ concentrations prior to the $^{15}$NH$_4^+$-labeled fertilizer application were approximately 3.0 and <15.4 mg N kg$^{-1}$ dry soil (d.s.), respectively. The soil NH$_4^+$ concentrations increased to 87.7 mg N kg$^{-1}$ d.s. in the four days after fertilization (DAF) then decreased gradually after that to <10 mg N kg$^{-1}$ d.s. (Figure 2c). The soil NO$_3^-$ concentrations increased from 8.9 to 63.1 mg N kg$^{-1}$ d.s. within 30 DAF, they decreased slightly then tended to stabilize in the range of 39.3–53.8 (average: 45.0) mg N kg$^{-1}$ d.s. The soil DOC concentrations varied between 15.5 and 32.8 (average: 23.9) mg C kg$^{-1}$ d.s. during the observation period (Figure 2d).

![Figure 2. Temporal dynamics of (a) soil (10 cm) temperatures, (b) daily rainfall and soil (0–20 cm) moisture in water-filled pore space (WFPS), and concentrations of (c) soil (0–20 cm) ammonium (NH$_4^+$) and nitrate (NO$_3^-$), and (d) dissolved organic carbon (DOC) following the basal application of ammonium sulfate for maize. Each vertical bar indicates the standard error of three or six spatial replicates. The data were obtained following the basal ammonium fertilization for maize from 28 April to 22 June 2019.](image)

3.2. $N_2O$ and $N_2$ Fluxes and $R_{N_2O}$

The maximum method DL of $F_{N_2O}$ at the 95% CI was 0.01–0.15 μg N m$^{-2}$ h$^{-1}$. All the measured $N_2O$ fluxes were higher than their corresponding maximum method DLs (Figure 3a). For $F_{N_2}$, a half of the measured data (about 58%) were above their corresponding method DLs at the 95% CI (Figure S1a). Regarding the $F_{N_2}^{24}$, about 66% of the data were
above their corresponding maximum method DLs (95% CI) that fell in a range of 29 to 155 μg N m\(^{-2}\) h\(^{-1}\) (Figures 3b and S1b).

Based on the dynamical variations in the total soil fluxes of both gases, i.e., \(F_{\text{N}_2\text{O}}\) and \(F_{\text{N}_2}\), the two-month experimental period following the basal fertilization event could be split into three phases, which were the early, mid, and late phase at 1–17, 18–44 and 45–60 DAF, respectively (Figure 3a,b). The soil \(N_2O\) fluxes increased gradually from 2 to 11 μg N m\(^{-2}\) h\(^{-1}\) during the early phase, and thereafter tended to remain at a relatively stable and high level of 9 to 13 μg N m\(^{-2}\) h\(^{-1}\) except for an emission peak (20 μg N m\(^{-2}\) h\(^{-1}\)) at about 30 DAF (Figure 3a). Then, the \(N_2O\) fluxes decreased gradually to 3 μg N m\(^{-2}\) h\(^{-1}\) during the late phase. In contrast, a fluctuating decrease in the total soil \(N_2\) fluxes was observed, declining from 192 to 23 μg N m\(^{-2}\) h\(^{-1}\) during the early phase (Figure 3b). But the soil \(N_2\) fluxes showed a gradually increasing trend during the other two phases, growing from 30 to 280 μg N m\(^{-2}\) h\(^{-1}\), except for a spike of 460 μg N m\(^{-2}\) h\(^{-1}\). It was somewhat surprising that there were no sharply abrupt peak emissions of \(N_2O\) and \(N_2\) during the early phase immediately after the ammonium amendment (Figure 3a,b). Over the whole two-month experimental period, the total soil \(N_2\) and \(N_2O\) emissions cumulated to 1.61 ± 0.47 and 0.12 ± 0.01 kg N ha\(^{-1}\), respectively, indicating that \(N_2\) dominated emissions of the two nitrogenous gases in our case.

The \(R_{\text{N}_2\text{O}}\) values showed a similar trend as that of the \(N_2O\) fluxes (Figure 3a,c). Its values increased rapidly from 0.02 to 0.31 during the early phase, varied from 0.11–0.30 during the mid-phase, and then decreased gradually from 0.14 to 0.03 during the late phase, with a mean of 0.15 during the entire experimental period (Figure 3c).

During the whole observation period, the dynamical total fluxes of both gases or their ratios were significantly affected by a single soil factor. As shown in Table 3, soil moisture, temperature, and ammonium concentration could independently explain 57%, 64%, and 31% of the variance in the \(N_2O\) fluxes (\(F_{\text{N}_2\text{O}}\)), respectively (\(p < 0.01\)), while temperature
alone might have exponentially accounted for 27% of the variance in the total soil $N_2$ fluxes ($F_{N_2}^{T}$) ($p < 0.01$). Soil moisture and temperature could also explain 55% and 44% of the variance in the $R_{N_2O}$ values, respectively ($p < 0.01$). At the same time, soil temperature and moisture in combination could explain up to 85% or 74% of the variance in total soil $N_2O$ fluxes or $R_{N_2O}$ ($p < 0.01$). In addition, soil DOC concentration was found to be a key regulating factor for the $NO_3^-$-originated $N_2$ fluxes ($F_{N_2}^{T}$) during the whole experimental period ($p < 0.01$, Table 3).

| Variable ($y$) | Regression Function | $n$ | $r^2$ | $p$ |
|----------------|---------------------|-----|------|-----|
| $F_{N_2O}$     | $y = 0.58WFPS - 34.19$ | 22  | 0.57 | $<0.01$ |
|                | $y = -0.18T_s^2 + 5.75T_s - 34.27$ | 34  | 0.64 | $<0.01$ |
|                | $y = -0.10[NH_4^+] + 11.40$ | 22  | 0.31 | $<0.01$ |
|                | $y = 0.29WFPS - 0.16T_s^2 + 5.03T_s - 49.73$ | 22  | 0.85 | $<0.01$ |
| $F_{N_2}^T$    | $y = 19.58e^{0.11T_s}$ | 34  | 0.27 | $<0.01$ |
| $F_{N_2}^F$    | $y = 2.11[DOC] - 12.91$ | 22  | 0.39 | $<0.01$ |
| $R_{N_2O}$     | $y = 0.012WFPS - 0.76$ | 22  | 0.55 | $<0.01$ |
|                | $y = -0.0037T_s^2 + 0.11T_s - 0.66$ | 34  | 0.44 | $<0.01$ |
|                | $y = 0.008WFPS - 0.0028T_s^2 + 0.083T_s - 1.04$ | 22  | 0.74 | $<0.01$ |

WFPS, $T_s$, $[NH_4^+]$, and [DOC] represent soil moisture in water-filled pore space (%), soil temperature ($^\circ C$), and concentrations of soil ammonium (mg N kg$^{-1}$ d.s.) and dissolvable organic carbon (mg C kg$^{-1}$ d.s.), respectively. $n$, $r^2$, and $p$ present number of observations, determination coefficient, and probability to accept a null hypothesis (representing a significance level), respectively. Data used to obtain the regression functions were observed during the period given in the footnotes of Figure 2.

3.3. Contributions of Nitrogen Sources and Processes to Gas Emissions

After the application of the $^{15}$NH$_4^+$-labeled fertilizer, the $^{15}$N enrichment (in atom % excess) of the NH$_4^+$ pool reached 93.5%, which was close to the theoretical value (96.4%) (Figure 4a). It was hypothesized that autotrophic nitrification of the fertilized NH$_4^+$ and the following heterotrophic denitrification of NO$_3^-$ as the nitrification final product would mainly contribute to soil N$_2$O emission. However, our data supported this hypothesis only during the early phase when the observed $^{15}$N enrichment of N$_2$O remained between the $^{15}$N abundances of NH$_4^+$ and NO$_3^-$ (Figure 4a,b). The contribution of autotrophic nitrification almost linearly increased from 34% to 53% on the first 6 DAF, slightly declined to 47% on the next 6 d, and then soon dropped down to 4–9% on 13–17 DAF, while the contribution of heterotrophic denitrification of NO$_3^-$ accounted for 47–66% during the initial two weeks and then over 80% on the rest days of the early phase. The measured $^{15}$N enrichments of N$_2$O during the mid and late phases were almost fully lower than the $^{15}$N abundances of NO$_3^-$ while being lower than those of NH$_4^+$ (Figure 4a), suggesting that another N pools (e.g., organic N that could be oxidized by heterotrophic nitrification whereby N$_2$O could also be produced) with natural abundance were also responsible for the soil N$_2$O emission, though the value was not quantifiable in this experiment.

The direct contributions of NO$_3^-$ reduction in heterotrophic denitrification to the measured total soil N$_2$ emissions during the two-month experiment period varied within a huge range (Figure 5). As Table S1 shows, the variation was mainly regulated by temperature or soil concentration of NH$_4^+$ alone ($p < 0.01$), or jointly by multiple soil factors. In particular, temperature and concentrations of NH$_4^+$ and NO$_3^-$ in a linear combination accounted for 65% of the variance in these dynamical contributions ($p < 0.01$). In the first three weeks following the basal ammonium application, the contribution of NO$_3^-$ denitrification almost linearly increased from around 40% to near 90%. It then declined almost linearly to about 20% in the next three weeks, remaining at this low level until the end of the experiment. For the cumulated total soil N$_2$ emission during the entire experimental
period, the NO$_3^-$-originated emissions directly accounted for no more than 50% (Figure 5), suggesting that application of the modified approach may be necessary when using the $^{15}$NGF method to measure N$_2$ fluxes from upland soils.

**Figure 4.** $^{15}$N enrichment of nitrous oxide (N$_2$O), ammonium (NH$_4^+$), and nitrate (NO$_3^-$) pools after $^{15}$N labeling of field soil NH$_4^+$ (a) and contributions of autotrophic nitrification (C$_n$) or heterotrophic denitrification (C$_d$) to N$_2$O emissions (b). The observational period and meaning of each error bar are found in the footnotes of Figure 2.

**Figure 5.** Contributions of nitrate reduction in denitrification (Kd) to the total soil N$_2$ emissions. The observational period and meaning of each error bar are found in the footnotes of Figures 2 and 3, respectively.

For the total N$_2$O or N$_2$ emissions during the two-month experimental period, the contributions of amended fertilizer or soil N were similar between the two gases and dynamically varied greatly (Figure 6). Although these dynamical contributions of fertilizer N to the emissions of either gas were regulated significantly by soil temperature, or concentrations of NH$_4^+$ or DOC alone, up to 85% of their temporal variance could be explained...
by temperature, and concentrations of NH$_4^+$ and NO$_3^-$ in a linear combination ($p < 0.01$, Table S1). As Figure 6 illustrates, the amended NH$_4^+$-based fertilizer showed relatively smaller contributions during the early 3 DAF, compared to soil N (42–46% versus 54–58%), but became a dominant contributor (48–73%) during the 4–28 DAF. Then, its contributions almost linearly decreased to about 18% during the following two weeks, and then increased to and remained at around 23% until the end of the experiment. Broadly speaking, the total N$_2$O or N$_2$ emissions of the former half experimental period were mainly attributed to the added fertilizer; but those of the latter half period were overwhelmingly contributed by soil N. During the entire two-month experiment period, the fertilizer- and soil-originated N emitted in forms of either N$_2$O or N$_2$ exhibited a share ratio of approximately 4:6.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Contributions of ammonium-based fertilizer ($^1$C$_1$, $^1$C$_2$) or soil ($^1$C$_1$, $^1$C$_2$) nitrogen (N) to (a) nitrous oxide (N$_2$O) and (b) dinitrogen (N$_2$) emissions from a cultivated black soil subjected to a cool temperate climate. The observational period and meaning of each error bar are found in the footnotes of Figures 2 and 3, respectively.

4. Discussion

4.1. N$_2$ Fluxes

For a long time, soil N$_2$ emissions have been one of the most uncertain components of the terrestrial N cycle, despite their importance in regulating ecosystem N availability and mitigating emissions of environmentally unfriendly N-trace gas, e.g., NO and N$_2$O [1,23]. One challenge is the high spatiotemporal variability in quantifying soil N$_2$ fluxes [23,24], which closely relates to soil temperature, soil moisture, and substrate availability (e.g., NH$_4^+$, NO$_3^-$, and DOC), as demonstrated in previous studies [27,28]. It is well known that N fertilization usually elevates soil N availability, as was also shown by the soil NO$_3^-$ concentrations in our study (Figure 2c), and thus it promotes soil N$_2$ emissions [36]. Therefore, fertilization events are often considered to be hot moments, in which there is a brief and disproportionally high biogeochemical response to the combined effects of multiple factors [68].

In our study, we observed a fertilization-induced increase in total soil N$_2$ fluxes ($F_{N_2}$) during the early phase (Figure 3b), which is in line with the result from a recent investigation of Ding et al. [69]. However, the soil N$_2$ fluxes during the early phase following the N fertilization event did not increase as much as expected as in previous studies conducted in croplands [14,33] or grasslands [42], where the timing of fertilizer application was synchronized with high soil temperature. It has been considered that soil temperature is a major trigger of the biogeochemical responses [44]. This was supported by our observations of total soil N$_2$ fluxes in the early and mid-phases, during which the mean soil temperature was 10.9 and 17.2 °C, respectively, which were remarkably lower than those during the late phase, during which the soil temperature was 21.0 °C on average (Figures 2a and 3b).
This was reinforced further by the fact shown in our study that the observed total soil N\textsubscript{2} fluxes exponentially correlated with soil temperature during the entire experiment period (\(p < 0.01\), Table 3). Such a finding on the role of temperature in soil N\textsubscript{2} fluxes aligns with those of laboratory and field studies [13,70,71].

Soil moisture is another major trigger of biogeochemical response, particularly in terms of affecting denitrification [23,44]. Moisture of 60\% WFPS is often considered to be the threshold above which denitrification starts to occur [72]. Accordingly, a high moisture level is expected to make denitrification proceed more completely and thus cause higher soil N\textsubscript{2} emissions [27,28]. In our study, however, the observed total soil N\textsubscript{2} fluxes in the mid-phase were not higher than in the other two phases, even though the moisture in this phase was occasionally as high as 80\% WFPS (Figures 2b and 3b). This result is in contrast with previous findings that showed higher soil N\textsubscript{2} fluxes accompanied with higher moisture contents [31,33]. This discrepancy could be attributed to the fact that in our case soil moisture was not a limiting factor for the total soil N\textsubscript{2} fluxes (\(r^2 = 0.01, p = 0.59\)). The absence of soil moisture among the key factors regulating soil N\textsubscript{2} fluxes in our study (Table 3) agreed with a recent study by Sgouridis and Ullah [58], who reported that higher soil N\textsubscript{2} fluxes were not observed under higher soil moisture conditions.

Additionally, in our study, the availability of substrates (i.e., DOC as electron donors and NO\textsubscript{3}\textsuperscript{−} as an electron acceptor for denitrification) in the mid-phase with relatively lower soil N\textsubscript{2} fluxes was similar to that in the late phase with relatively higher soil N\textsubscript{2} fluxes (on average with DOC of 23.2 versus 19.9 mg C kg d.s. and NO\textsubscript{3}\textsuperscript{−} of 44.4 versus 47.2 mg N kg d.s.) (Figure 2c,d, and Figure 3b). This similarity indicated that soil NO\textsubscript{3}\textsuperscript{−} or DOC availability did not limit the total soil N\textsubscript{2} fluxes (Table 3). In our study, the effects of soil moisture and substrate availability on the total soil N\textsubscript{2} fluxes might have been limited by the low temperature mostly below 20 °C (Figure 2a). Similar results were also reported in a previous study which indicated that soil NO\textsubscript{3}\textsuperscript{−} availability limitation masks the importance of soil moisture on soil N\textsubscript{2} fluxes [58].

However, DOC availability was found to be a key regulatory factor if the N\textsubscript{2} fluxes resulted from NO\textsubscript{3}\textsuperscript{−} denitrification (\(F_{N_2}^{\text{NO_3}}\)), instead of \(F_{N_2}^{\text{N}}\), were taken into account (Table 3). According to the measured DOC and NO\textsubscript{3}\textsuperscript{−} concentrations (Figure 2c,d), near 70\% of the observed DOC to NO\textsubscript{3}\textsuperscript{−}-N molar ratios during the experimental period were less than 1.0. This fact indicated that carbon availability was a limiting factor for the soil N\textsubscript{2} production through NO\textsubscript{3}\textsuperscript{−} denitrification that theoretically requires DOC to NO\textsubscript{3}\textsuperscript{−}-N molar ratios of at least 1.5. Given the sufficient NO\textsubscript{3}\textsuperscript{−} supplies (Figure 2c), the \(F_{N_2}^{\text{NO_3}}\) fluxes therefore were significantly sensitive to changes in the DOC availability (Table 3). Different to \(F_{N_2}^{\text{N}}\), the observed \(F_{N_2}^{\text{NO_3}}\) fluxes were significantly sensitive to changes in temperature (Table 3). This finding could be supported by the fact that the \(F_{N_2}^{\text{NO_3}}\) to \(F_{N_2}^{\text{N}}\) ratios were smaller than 40\% during the latter half experimental period (Figure 5). This fact indicated that nitrification might have indirectly contributed to the total soil N\textsubscript{2} emissions substantially by producing N\textsubscript{2}O as one of its by-products and thus providing this gas as the electron acceptor for the last step of denitrification process to form N\textsubscript{2}. This is because that nitrification as an oxidation process was more sensitive to a temperature change than NO\textsubscript{3}\textsuperscript{−} denitrification as a reduction process. Such a different sensitivity could be shown by larger temperature coefficients of nitric oxide fluxes (mainly from nitrification under field conditions) as compared to N\textsubscript{2}O fluxes (mainly from both nitrification and denitrification) [73].

Furthermore, the soil NO\textsubscript{3}\textsuperscript{−} concentrations did not decrease but actually increased slightly in the late phase, even though there were relatively higher total soil N\textsubscript{2} fluxes (Figures 2c and 3b). It is well known that soil NO\textsubscript{3}\textsuperscript{−} concentration is a net result of NO\textsubscript{3}\textsuperscript{−} production and consumption [74]. According to the data presented in Figures 2c and 3b, the soil NO\textsubscript{3}\textsuperscript{−} mass in the 0–20 cm soil layer showed a much higher increment than the cumulated total soil N\textsubscript{2} fluxes (approximately 42 versus 0.8 kg N ha\textsuperscript{−1}) during the late phase. These results suggested that the total soil N\textsubscript{2} emission as the pathway through NO\textsubscript{3}\textsuperscript{−} consumption were much less than the NO\textsubscript{3}\textsuperscript{−} production from nitrification in our case.
The soil N\textsubscript{2} fluxes observed in this study (on average 112 \textmu g N m\textsuperscript{-2} h\textsuperscript{-1}) were even far lower than the means of the lower boundaries for the flux ranges in cropland, grassland, forest, and peat/marsh ecosystems (262–23,059, 976–10,586, 902–17,492, and 593–2741 \textmu g N m\textsuperscript{-2} h\textsuperscript{-1}, respectively), which were measured in situ by other studies through using the \textsuperscript{15}NGF method (Figure 3b, Table S2). Over the entire experimental period, the observed total soil N\textsubscript{2} emissions accounted for approximately 0.67\% of fertilizer N. This fertilizer N loss rate via N\textsubscript{2} emission was comparable with those (0.5–0.75\%) of black soil in the USA, which was measured in a maize-barley cropping system [57]. These results suggest that cultivated upland black soils may be characterized by low fertilizer N loss through N\textsubscript{2} emissions. However, this needs to be confirmed by further studies. Additionally, black soils are all subject to cool climates, which have been warming faster than other warmer climate zones [8]. Thus, warming-induced soil N\textsubscript{2} emissions are likely to exacerbate fertilizer N loss and thereby further reduce fertilizer N use efficiency of black soils. But experimental evidence needs to be provided by further studies to test this hypothesis.

### 4.2. N\textsubscript{2}O Fluxes

In our study, the observed peak flux of about 20 \textmu g N m\textsuperscript{-2} h\textsuperscript{-1} (Figure 3a) was comparable with the lower end of the multiyear peak fluxes (24–97 \textmu g N m\textsuperscript{-2} h\textsuperscript{-1}) reported for another maize field in the same region [75]. However, our soil N\textsubscript{2}O fluxes (2–20 \textmu g N m\textsuperscript{-2} h\textsuperscript{-1}, Figure 3a) were lower than those (24–439 \textmu g N m\textsuperscript{-2} h\textsuperscript{-1}) of a black soil cultivated with maize in Indiana, US [76]. The intra- and inter-annual variations in the soil N\textsubscript{2}O fluxes of both previous studies were largely attributed to the fluctuations in soil moisture and temperature [75,76]. This aligns with our finding that the dynamic soil N\textsubscript{2}O fluxes could be well described by the combination of soil temperature and moisture (Table 3). Therefore, the lower mean annual air temperature (6.9 versus 19 \textdegree C) and annual precipitation (614 versus 970 mm) at our study site might have potentially accounted for the lower soil N\textsubscript{2}O fluxes compared to those in the study of Omonode and Vyn [76], except for the effects of long chamber enclosure time discussed later.

In contrast to the fertilizer-induced abrupt peak events observed for soil N\textsubscript{2}O fluxes in numerous other studies [43–45], we observed that soil N\textsubscript{2}O fluxes increased slowly during the early phase following the fertilizer application (Figure 3a). The soil environmental conditions in our study, particularly the soil temperature and soil moisture conditions, slowed down the stimulation of fertilizer N on microbial processes producing N\textsubscript{2}O. Our N\textsubscript{2}O fluxes during this early phase exhibited significantly a linearly negative relationship with concentrations of NH\textsubscript{4}\textsuperscript{+} (\textit{r}\textsuperscript{2} = 0.54, \textit{p} < 0.01) and a linearly positive relationship with soil concentration of NO\textsubscript{3}\textsuperscript{-} (\textit{r}\textsuperscript{2} = 0.90, \textit{p} < 0.01), indicating that nitrification contributed substantially to the emissions of this gas (Figures 2c and 3a). It is usually considered that optimal temperature and soil moisture for nitrification, whereby N\textsubscript{2}O is produced as a by-product, are 25–35 \textdegree C and 30–60\%, respectively [77,78]. The lower soil temperature (8–15 \textdegree C) than 25 \textdegree C and the higher moisture (66–77\%) than 60\% WFPS during the early phase were not very favorable for nitrification (Figure 2a,b). It is usually considered that the maximum N\textsubscript{2}O production in either denitrification or nitrification occurs when soil moisture falls within 55–85\% WFPS [79] and higher soil moisture contents stimulate the reduction of N\textsubscript{2}O to N\textsubscript{2} in denitrification [80]. As Figure 4b illustrates, denitrification dominated the N\textsubscript{2}O emissions during the early phase. Although the soil moisture (>60\% WFPS) was favorable, the other soil conditions (e.g., soil temperature of 8–15 \textdegree C) were not very favorable for N\textsubscript{2}O production in denitrification which requires optimums of 25 \textdegree C for temperature and > 1.25 for the DOC/NO\textsubscript{3}\textsuperscript{-}-N ratio [81]. Hence, the stimulatory effect of amended N on N\textsubscript{2}O emissions in the early phase following fertilization was limited not only in terms of N\textsubscript{2}O production by nitrification but also by denitrification. In addition, the significant relationship (negative) between N\textsubscript{2}O fluxes and the concentrations of soil NH\textsubscript{4}\textsuperscript{+} (Table 3) indicated that nitrification dominated the emissions of this gas during the entire study period when the organic N mineralization might have been weaker than the autotrophic nitrification, as suggested by the \textsuperscript{15}N enrichments of NH\textsubscript{4}\textsuperscript{+} (Figure 4a).
Total soil N₂O emissions are well known to be composed of N₂O produced by different microbial processes [23]. In our investigation, in addition to autotrophic nitrification and denitrification, other processes such as heterotrophic nitrification were found to contribute substantially to the soil N₂O emissions, especially in the mid and late phases (Figure 4a). Similar results from cultivated black soils in the same region were observed in previous laboratory studies, which showed contributions of heterotrophic nitrification at approximately 25–49% [49,50]. These latest findings are challenging the idea that heterotrophic nitrification principally occurs in grassland and forest soils and is usually negligible in agricultural soils [82,83]. There is compelling evidence that heterotrophic nitrification closely relates to low pH, low BD, and high SOC contents or high C/N ratios [84]. However, Elriss et al. [85] recently found that low temperatures would also be favorable for heterotrophic nitrification. This might have accounted for our results from the cultivated black soil subject to low temperature (ranging from 12–22 °C with a mean of 18 °C) during the mid and late phases (Figure 2a). To the best of our knowledge, our field study is the first to confirm the conclusion from previous laboratory studies that heterotrophic nitrification plays a substantial role in producing N₂O in cultivated black soils [49,50].

Furthermore, our finding that the contributions of different N₂O pathways were dynamically variable during the experimental period (Figure 4) is in good agreement with previous observations for a forest soil, wherein the contribution of each process varied in different seasons [86]. The dynamical variation could be primarily attributed to the variable soil environmental factors (Figure 2), including temperature, moisture, and availability of N substrates (e.g., NH₄⁺ and NO₃⁻) [87]. For instance, the amended NH₄⁺ enhanced the contribution of autotrophic nitrification to soil N₂O emissions during the early phase when autotrophic nitrification might have played a key role in the production and emission of N₂O, even though the low temperature and high moisture (Figure 2a,b and Figure 4) did not favor complete autotrophic nitrification to produce NO₃⁻ [23,88]. Subsequently, substantial contributions of heterotrophic nitrification to soil N₂O emissions were implicated to be enhanced during the other two phases (Figure 4a). This was largely a consequence of the reductions in the relative contributions of autotrophic nitrification and heterotrophic denitrification to soil N₂O emissions, especially during the late phase when the magnitude of NH₄⁺ oxidized to NO₃⁻ was quite low due to the low NH₄⁺ concentrations and the high soil moisture (67–73% WFPS), as indicated by the concentrations of NO₃⁻ and its ¹⁵N enrichment (Figure 2b,c and Figure 4a). Meanwhile, the soil N₂ emissions increased with the increasing soil temperature in the late experimental phase (Figures 2a and 3a,b), implying that the heterotrophic denitrification was complete, with more N₂O consumed, thus reducing the contribution of heterotrophic denitrification to soil N₂O emissions. This was shown by the relationship between soil N₂O emissions and temperature (Table 3). Similar results were also reported previously by other studies [86,89]. Also, the increase in soil temperature during the late phase might have promoted the contribution of heterotrophic nitrification to soil N₂O emissions [88]. Moreover, the contribution of the soil N pool to the N₂O emissions (C1) was found to increase to over 60% since 30 DAF, possibly due to the enhancement of heterotrophic nitrification (Figure 6a). However, our data were not adequate enough to quantify contributions of heterotrophic nitrification in the early phase and those of autotrophic/heterotrophic nitrification and heterotrophic denitrification in the other two phases (Figure 4). Therefore, further field studies with improved experimental designs are needed to improve the understanding of soil N₂O emissions and thus support the development of effective mitigation strategies for black soils.

4.3. Implications of R₉₅O for Estimating Soil N₂ Emissions

A mean of observed R₉₅O in combination with field-measured soil N₂O emissions has often been used to estimate soil N₂ emissions at site, regional or global scales due to the inherent difficulty in direct N₂ measurement [90,91]. However, existing studies showed that temporal variations in R₉₅O were driven by the changes in factors affecting N₂O and N₂ emissions, such as climatic/weather variables [13,92], soil properties [33,35], and
management practices [58]. Similarly, in this study, the variations in the measured \( R_{\text{N}_2\text{O}} \) values were evidently regulated by soil temperature and moisture (Table 3). In addition, there are limited \( R_{\text{N}_2\text{O}} \) data measured via non-AITs [23,24]. In these regards, a mean of measured \( R_{\text{N}_2\text{O}} \) may induce great uncertainties in estimating soil \( N_2 \) emissions at site, regional or global scales. Recently, a study suggested that temporal \( R_{\text{N}_2\text{O}} \) values during a full maize season could be parametrized as a multivariate function of soil factors including soil temperature, moisture and concentrations of soil \( \text{NO}_3^- \) and \( \text{DOC} \) [33]. Differently, in the present study, \( R_{\text{N}_2\text{O}} \) was shown to be better described jointly by soil temperature and moisture (Table 3), indicating that the previously reported parametrization of \( R_{\text{N}_2\text{O}} \) may be site-specific [33]. Thus, more studies on the quantitative relationship between \( R_{\text{N}_2\text{O}} \) and environmental variables are needed to improve estimates of terrestrial \( N_2 \) emissions.

4.4. Methodological Uncertainties of Measured Fluxes

Although recent advances in analytical instrumentation and flux calculations have facilitated the application of the \( ^{15}\text{NGF} \) method to obtain in situ soil \( N_2 \) fluxes across a range of ecosystems, there is still some uncertainties induced from, e.g., non-homogenous \( ^{15}N \) labeling, the mathematical approach to calculate the total \( N_2 \) fluxes, and long chamber enclosure time [59,64,66,93].

The basic assumption for the application of the \( ^{15}\text{NGF} \) method is that the denitrifying pool as a single source of both \( N_2 \) and \( N_2O \) is uniformly labeled by the \( ^{15}N \) isotope, which is crucial for the calculation of \( N_2 \) fluxes by using the non-equilibrium equations (Equations (S3.1)–(S3.7) in Text S3) as the classic approach [25,94]. Spatial heterogeneity in the labeled \( N \) pool is well known to result in overestimation of the \( ^{15}N \) enrichment of the denitrifying pool \( \left(^{15}X_N\right) \), and thereby underestimate \( N_2 \) flux [95]. In the present study, the measured \( ^{15}N \) abundances of the soil \( \text{NH}_4^+ \) pool were slightly less than the calculated theoretical values on the early 1–3 DAF; but they became similar on the 4 DAF (Figure 4a). These data indicated that there was a non-homogeneous mixing of the added \( ^{15}\text{NH}_4^+ \) tracer with the soil \( \text{NH}_4^+ \) on the four days after the soil \( \text{NH}_4^+ \) pool was labeled, despite our effort to uniformly add the \( ^{15}N \) tracer. The non-homogeneity was further confirmed by variations of soil \( \text{NH}_4^+ \) concentrations measured during the early experimental days (Figure 2c). A similar problem also encountered by Sgouridis et al. [39]. Actually, it is impossible to completely mix the added \( ^{15}N \) tracer with the soil \( \text{NH}_4^+ \) in intact soils in situ. Nevertheless, previous trial studies have suggested that the error of the \( N_2 \) fluxes, induced by the non-homogeneity, is likely lower as compared to those caused by the analytical errors and that the measured soil \( N_2 \) fluxes may be acceptable in terms of measurement accuracy even though the denitrifying pool is less-uniformly labelled [96,97]. Furthermore, our \( ^{15}X_N \) data during the experimental period ranged between 57.0% and 97.5%, with an average of 80.8% that was close to the \( ^{15}N \) enrichment of the labeled \( \text{NH}_4^+ \) pool (Figure 4a and Figure S2). This suggests that coupled nitrification-denitrification might have been a major pathway of the \( N_2 \) production [98]. Moreover, the temporal variation of \( ^{15}X_N \) during the study period might have been partially due to nitrification and denitrification with different rates occurring in various microsites, resulting in changing enrichment of \( ^{15}N \) in the \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) pools over time. In most cases, the error induced by the temporal variation of \( ^{15}X_N \) is perhaps negligible in estimations of soil \( N_2 \) fluxes, particularly at higher \( ^{15}N \) enrichment levels, as used in this study [99].

In a large body of previous studies, the non-equilibrium equations (Equations (S3.1)–(S3.7) in Text S3) as the classic approach are extensively applied for determining the \( \text{NO}_3^- \)-originated \( N_2 \) fluxes [25]. This approach usually assumes that \( N_2 \) is produced only from the reduction of \( \text{NO}_3^- \)-derived \( \text{N}_2\text{O} \) [94]. In our study, the \( \text{NO}_3^- \)-originated \( N_2 \) fluxes calculated based on the measured \( ^{29}R \) and \( ^{30}R \) through using the classic approach and the modified approach (Texts S3 and S4) were almost completely identical (Figure S3). This finding suggests that both approaches are applicable in terms of determining the \( \text{NO}_3^- \)-originated \( N_2 \) fluxes. However, \( \text{N}_2\text{O} \) from either denitrification or other processes such as nitrification is a free substrate that can be reduced by denitrifiers to \( N_2 \) [64,100,101]. It has
been found that N$_2$O produced in aerobic microsites can diffuse into anaerobic microsites or that aerobic microsites can become anaerobic ones if O$_2$ is depleted by nitrification or organic matter decomposition [98,102], to facilitate N$_2$ production from the reduction of N$_2$O from non-denitrification processes. Hence, it could be more reasonable to determine total soil N$_2$ fluxes by using the modified approach (Equations (S4.1)–(S4.3) and (S4.5) in Text S4) based on the $^{15}$N enrichment of N$_2$O [64]. As Figure S1a,b,d,e illustrate, the NO$_3^{-}$-originated N$_2$ fluxes on average accounted for only 46% (ranging between 6% and 109%) of the total N$_2$ fluxes, suggesting a possible enigma of the soil N balance due to being irrespective of the reduction of N$_2$O derived from non-denitrification to N$_2$ [103]. This result indicated that labeling N substrates of nitrification while using the $^{15}$N enrichment of N$_2$O would be necessary to more accurately determine total soil N$_2$ fluxes by using the $^{15}$NGF method. Such an indication could be supported at least to some extent by the total N$_2$ fluxes measured via using the GFSC technique [33] for the intact soil cores sampled from the field plots during the full experimental period (Figure S1c,f). These GFSC-measured N$_2$ fluxes showed a similar dynamical trend as compared to those determined by the modified approach based on the $^{15}$N enrichment of N$_2$O (Figure S1b,c,e,f).

Due to the limits of high background atmospheric $^{14}$N-$^{15}$N concentration (~78%) and the lower instrumental sensitivities of $^{15}$N-$^{15}$N analysis, longer chamber enclosure times (e.g., 1 to 20 h) are employed to measure N$_2$ fluxes in situ by using the $^{15}$NGF method (see Table S2). However, non-linear increases in the $^{15}$N-N$_2$ enrichments within a chamber headspace may occur due to distortion of the soil gas concentration gradient during a relatively long enclosure time, thus underestimating the N$_2$ fluxes to some extent; and so does N$_2$O [60,104]. A recent study indicated that downward subsoil diffusion of N$_2$O and N$_2$ in a soil profile could also be one of the key causes for the non-linearity during a long enclosure [93]. Contrasting to N$_2$, an increase of storage N$_2$O in soil, induced by distortion of the gas concentration gradient, may enhance the opportunity of N$_2$O reduction to N$_2$ [59,93]. In these regards, our study determining each gas flux with only two samples over a 6-h enclosure and with the linearity assumption might have underestimated the soil N$_2$O and N$_2$ fluxes. Moreover, the long chamber enclosures at mid-day with a relatively high temperature might have been favorable for reducing more stored N$_2$O to N$_2$, thus resulting in negative errors on soil N$_2$O fluxes. Apart from the long chamber enclosure time, the addition of N tracer might have triggered an overestimation of either N$_2$O or N$_2$ fluxes as a non-homogeneous tracer distribution could increase the N substrate availability and soil moisture around the injection microsites and thus create hot spots of denitrification activity [56,59]. In further studies using the $^{15}$NGF method to simultaneously measure soil N$_2$ and N$_2$O emissions, therefore, it is essential to increase the sample number over a long enclosure time to capture the non-linearity for either gas and to improve the homogeneity of the $^{15}$N tracer addition so as to reduce the uncertainties of the measured gas fluxes.

5. Conclusions

In agricultural ecosystems, reliable estimations of field dinitrogen gas (N$_2$) emissions are essential for reducing the loss of available nitrogen (N) via reductive nitrous oxide (N$_2$O) conversion and developing effective N$_2$O mitigation strategies. Here, our observations of fluxes in situ from a cultivated upland black soil through $^{15}$N-labeling of an applied ammonium fertilizer showed a low fertilizer N loss through N$_2$ emissions during a two-month period following the basal fertilization event. This was caused largely by the fact that the low temperature limited the nitrification-originated N$_2$O as the direct substrate of N$_2$ production in the last step of denitrification and thus inhibited the total soil N$_2$ fluxes ($F_{N_2}$) while the low availability of dissolvable organic carbon limited the N$_2$ fluxes originated from nitrate denitrification ($F_{N_2}$). This finding implies that, in the future, warming-induced soil N$_2$ emissions may lead to more fertilizer N loss from black soils while reducing fertilizer N use efficiency. It is noticeable that the responses of N$_2$O and N$_2$ emissions from the investigated black soil to environmental factors were different from those reported previously for other soils. Accordingly, we conclude that soil
variables-based parametrization of $R_{N_2O}$ obtained from a given soil may be site- and/or time specific and thus may not be widely applicable for estimation of field $N_2$ fluxes from other soils by using their $N_2O$ fluxes measured in situ. As this study implicates, other processes such as heterotrophic nitrification might have contributed substantially to the $N_2O$ emissions from black soil during a short term following ammonium fertilization. But further studies are still needed to clarify the roles of the processes other than autotrophic nitrification and heterotrophic denitrification during a full cropping season, a full year or even longer periods for a black soil or for other soils. This study also implicates that labeling nitrogen substrate(s) of nitrification while using the $^{15}N$ enrichment of $N_2O$ is necessary to more accurately quantify total soil $N_2$ fluxes in situ by applying the $^{15}N$ gas flux ($^{15}NGF$) method. When using the $^{15}NGF$ method to simultaneously measure soil $N_2$ and $N_2O$ fluxes, however, it is essential to increase the sample number over a long enclosure time for capturing the non-linearity for either gas so as to reduce the flux uncertainties of both gases.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12101664/s1, Text S1: Calculations for $^{15}N$ enrichment of $N_2O$ and $^{15}N_2O$ fluxes; Text S2: Detection limits of N2O and N2 fluxes; Text S3: Using a classic approach to calculate nitrate-originated soil N2 fluxes; Text S4: Using a modified approach to calculate total N2 fluxes from both nitrate and non-nitrate pools; Figure S1: Dinitrogen (N2) fluxes measured by different approaches for a cultivated black soil subject to a cool temperate climate; Figure S2: Temporal dynamics of $^{15}XN$ and aD (in atom %) as the $^{15}N$ enrichment of soil nitrate pool undergoing denitrification; Figure S3: Comparison between nitrate-originated dinitrogen ($N_2$) fluxes ($F_n$) calculated by using the classic and modified approaches; Table S1: Regressions for the contributions of nitrate reduction in denitrification ($K_d$) to the total $N_2$ fluxes and the contributions of fertilizer ($C_1$ or $C_2$) to nitrous oxide (N2O) and dinitrogen (N2) emissions against soil factors, respectively; Table S2: Soil dinitrogen ($N_2$) fluxes measured in situ in cropland, grassland, and other ecosystems using the $^{15}N$ gas-flux method. References [105–123] are cited in the supplementary materials.

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