Deep N fertilizer placement mitigated N₂O emissions in a Swedish field trial with cereals

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Abstract Deep fertilizer placement is a proposed strategy to increase crop yield and nitrogen (N) use efficiency while decreasing nitrous oxide (N₂O) emissions from soil to atmosphere. Our objective was to test three fertilization depth orientations to compare overall N use efficiency, based on a 2-year field trial on a mineral soil cropped with cereals in Uppsala, Sweden. The field was fertilized with ammonium nitrate at a rate of 120 kg ha⁻¹ (2016) and 105 kg ha⁻¹ (2017) and a deep fertilizer placement (DP) at 0.20 m was compared to a shallow placement (SP) at 0.07 m and a mixed-depth placement (MP) where fertilizer was halved between the depths of 0.07 and 0.20 m, and a non-fertilized control (NF). In 2016, compared to SP, MP and DP increased N content in harvested grain by 3.6% and 2.5% respectively, and DP increased grain yield by 11% (P < 0.05). In both years, N₂O emissions were similar in DP and NF, whereas SP and MP emissions were similar but generally higher than those in DP and NF. Fertilizer-induced emission factors (EF) for the growing season of 2017 decreased with fertilizer placement depth and were 0.77 ± 0.07, 0.58 ± 0.03, and 0.10 ± 0.02 for SP, MP, and DP, respectively. Although deep N placement benefits are likely dependent on weather conditions and soil type, this strategy has a clear potential for mitigating N₂O emissions without adversely affecting yield.

Keywords Nitrous oxide · Deep N fertilization · Nitrogen use efficiency · Fertilizer N placement

Introduction

The intensification and expansion of agriculture is on a course for rapid increase as the Earth will need to support a projected additional two billion people by 2050 (United Nations 2019). The use of mineral nitrogen (N) fertilizer directly and indirectly contributes to the microbial production of the greenhouse gas (GHG) nitrous oxide (N₂O) via soil and water systems. The residence time of N₂O in the atmosphere is about 120 years and is 265 times more potent as a GHG compared to carbon dioxide (CO₂) on a 100-year time scale (Myhre et al. 2013). Atmospheric N₂O is either removed by a sink via microbial reduction or...
transported to the stratosphere and consumed in an ozone-depleting chemical reaction, making it one of the most dominant sources of ozone depletion (Ravishankara et al. 2009).

N\textsubscript{2}O is produced by two processes, nitrification and denitrification, which occur under oxic and anoxic conditions, respectively. The former process is primarily mediated by autotrophic bacteria from the genera Nitrosomonas and Nitrosospira, and strictly a source of N\textsubscript{2}O. The latter process, however, can be either N\textsubscript{2}O-consuming or N\textsubscript{2}O-producing. Though N\textsubscript{2}O is naturally emitted, the trend of increasing emissions is due to human activities, of which around 60\% comes from agriculture (Smith et al. 2014), inherently connected to the use of nitrogenous fertilizers. The near quarter-fold increase in atmospheric N\textsubscript{2}O since the industrial revolution is attributed to a widening use of mineral N fertilizer (Park et al. 2012).

Fertilization is vital for food security and cannot be excluded from crop production, necessitating a sharp focus on identifying fertilizer application strategies that can mitigate N\textsubscript{2}O emissions. While surface-applied fertilizer can lead to N losses from both ammonia (NH\textsubscript{3}) volatilization (Pan et al. 2016) and microbial nitrification and denitrification (Cameron et al. 2013), increasing fertilizer placement depth is a method for improving current agricultural practices, with potential to increase overall nutrient use efficiency (NUE).

Furthermore, temperature and moisture are major controls on soil N turnover, availability and mobility, affecting N losses via leaching and gaseous losses derived from nitrification and denitrification (Godde and Conrad 1999; Robinson 2002). Wet-dry cycles in soil induce pulses of N and carbon (C) mineralization upon re-wetting (Schimel 2018), of which the upper topsoil is most affected via rainfall events that mobilize fertilizer N. The amplitude of temperature and moisture variability decreases with increasing soil depth. Increasing fertilizer placement depth may be an effective method for keeping plant available N over longer periods with less rainfall due to more constant soil moisture conditions.

A deeper fertilizer placement may even improve crop growth over standard shallow or surface placements. Crop roots tend to proliferate around the area of the fertilizer grain, thus deeper placement can promote root length density and enhance N uptake (Lotfollahi et al. 1997; Li et al. 2009) as well as water utilization (Singh et al. 1976) from deeper soil layers. Crops can obtain more than two thirds of their nutrition from deeper layers in the soil profile when nutrient availability and/or water is limited in the topsoil (Kautz et al. 2013) and deep fertilization could improve plant growth, particularly during periods of little to no precipitation. On the contrary, and particularly under high water availability, deeper placements have been shown to both increase (e.g. Ke et al. 2018) and decrease (e.g. Grant et al. 2019) N leaching and the amount of mineral N in the soil layers below the fertilizer placement.

Previous studies have indicated that augmenting the residence time of the gas in the soil matrix can decrease the N\textsubscript{2}O:N\textsubscript{2} ratio, either by entrapment (Harter et al. 2016) or by lengthening the path of diffusion from the “source” of denitrification, i.e., location of the fertilizer grains to the soil surface (Clough et al. 1998). In studies where microbial N\textsubscript{2}O uptake was observed, it tended to be in cases where soil moisture limited gas diffusion through the soil matrix, particularly in the absence of mineral N (Chapuis-Lardy et al. 2007). Thus, with deeper placement of fertilizer, the distance for N\textsubscript{2}O diffusion from the fertilization layer to the soil surface would be increased, meaning a longer residence time and a potentially increased reduction of N\textsubscript{2}O to N\textsubscript{2} in the upper zone of the topsoil where no fertilizer N was placed. Furthermore, deep placement concentrates fertilizer-NH\textsubscript{4}\textsuperscript{+} into localized areas, stimulating methane (CH\textsubscript{4}) oxidation by soil methanotrophs and reducing CH\textsubscript{4} emissions (Bodelier et al. 2000a, b). Deeper root growth promoted by fertilizer placement increases the oxygen availability in the rhizosphere which is likely to enhance CH\textsubscript{4} consumption in deeper layers (Gilbert and Frenzel 1998; Kruger et al. 2001).

Previous field studies showed that deep fertilizer placement, compared to broadcast application, increased yields, improved NUE, and decreased N runoff (Mengel et al. 1982; Kelley and Sweeney 2007; Xia et al. 2016; Zhu et al. 2019). Regarding N\textsubscript{2}O emissions, however, results are rather contradicting: while deep N fertilizer placement effectively lowered N\textsubscript{2}O emissions in rice paddies (Gaihre et al. 2015; Wu et al. 2017) and field experiments comparing conservation tillage methods (Liu et al. 2006; Nash et al. 2012), other studies (e.g. Cai et al. 2002; Drury et al. 2006; Chu et al. 2007) found that N\textsubscript{2}O emissions were higher from deeper N placement compared to shallow
N placement. In terms of CH$_4$ emissions, deep N placement has been found to be a promising management practice with regard to CH$_4$ mitigation (Linquist et al. 2012). However, the studies summarized by Linquist et al. (2012) focusing on the impact of N fertilizer placement on CH$_4$ emissions have been conducted in rice systems, which were either continuously flooded or rainfed. Methane measurements under different fertilizer depth management under cereals are still scarce.

The local agronomic practice in central Sweden prescribes a sub-surface placement of fertilizer around 0.07 m during seeding, which in many studies is already considered a “deep” placement. In this study, 0.07 m depth of fertilizer placement was considered as a baseline in comparison to considerably deeper placements. We tested the effect of three different mineral N fertilizer placements representing a shallow (0.07 m), deep (0.20 m), and mixed placement (half at 0.07 m, half at 0.20 m) along with a non-fertilized control on crop growth, yield, and N$_2$O and CH$_4$ emissions on a conventionally farmed mineral soil in Central Sweden. We expected that the two deeper fertilizer placements (deep and mixed) would have a positive effect on overall N use efficiency, improve crop yield, and lower N$_2$O and CH$_4$ emissions (Linquist et al. 2012; Xia et al. 2016). The mixed placement could elucidate if crops benefited from two placement depths for both early and later plant growth stages, but also if N$_2$O and CH$_4$ emissions were affected by the presence of an overlaying unfertilized zone acting as a buffer or sink.

**Materials and methods**

**Site characteristics and experimental setup**

A 2-year experiment was established in the spring of 2016 in Säby (59° 83’ N, 17° 71’ E), near Uppsala, Sweden on a Eutric Cambisol that has been used as cropland for at least a century. The site has a silt loam texture in the topsoil and is composed of 21.2% clay, 55.7% silt, 23.1% sand, and 6.1 pH$_{H_2O}$. The climate is cold temperate with a mean annual air temperature of 5.5 °C and precipitation of 528 mm (Table 1), of which 215 mm occur during the growing season (May–August).

In May 2016 prior to planting we sampled soil from a 20 m long · 1.5 m deep pit running parallel to the experimental plots where 24 1.5 m-deep soil columns had been removed from the field. Total soil organic carbon (SOC) and total nitrogen (TN) concentrations were analysed via dry combustion (LECO CNS Analyser, LECO Corporation, St. Joseph, MI, USA) using bulked samples taken down to 1 m depth at 0.10 m intervals at three points along the length of the pit (Table 2).

In 2016, the field was sown at a rate of 238 kg ha$^{-1}$ with spring wheat (*Triticum aestivum* L. var. ‘Quarna’) and fertilized with ammonium nitrate at a rate of 120 kg N ha$^{-1}$. The following year spring barley (*Hordeum vulgare* L. var. ‘Makof’) was sown at a rate of 200 kg ha$^{-1}$ and fertilized with 105 kg N ha$^{-1}$ ammonium nitrate. The fertilizer used in both years was YaraBela AXAN (Yara International, Oslo, Norway). The plots were sown and fertilized simultaneously using a Combi drill with the ability to adjust fertilizer and seed depth (Spirit 400C Strip Drill, Väderstad, Sweden), with two available fertilizer outlets allowing for split-level placement in the same vertical plane. Seed row spacing was 0.125 m and the fertilizer was incorporated into one or two 0.05 m-wide bands (depending on the treatment) below the seedbed. The general agronomic practice for the area is to place seeds at approximately 0.05 m and fertilizer at 0.07 m depth, so that the sub-surface soil moisture will promote seed germination without the reliance on subsequent rainfall. In 2017, because sowing depth was shallow (≤ 0.03 m) and planting occurred before a period without rainfall, seed emergence was greatly delayed in many plant rows. Irrigation is rarely used in this area, but due to poor seed emergence, plots were irrigated once after sowing with an equivalent of 17 mm rainfall on June 22nd (Fig. 1). The fields are typically cultivated in the fall, but after the 2017 growing season, a 4 m wide × 64 m long strip where the chambers had been previously established during the growing season was left uncultivated to facilitate further GHG measurements. However, field conditions after fall cultivation, particularly after rainfall and subsequent accumulation of snow and ice and then initial melt, rendered the field inaccessible and the planned GHG measurements were unobtainable for much of the autumn and winter of 2017–2018.
The experimental setup followed a randomized block design with four repetitions of four treatments corresponding to three depths of fertilizer placement plus a non-fertilized control. Experimental plots were 4 x 20 m and consisted of an unfertilized control (NF), a shallow placement of fertilizer at 0.07 m (SP), a deep placement at 0.20 m (DP) and mixed placement (MP) where half of the fertilizer was placed at 0.07 m and the other half at 0.20 m.

**GHG flux measurements**

Immediately after the fields were seeded and fertilized, a 0.55 x 0.35 m steel frame with a water well welded to its top and a 0.10 m lip underneath was pressed into the soil in the middle of each plot. Frames were centered encompassing the same number of crop rows, and the first gas measurement was performed within 24 h. Plant number and seed emergence within frames was monitored and found to be consistent across all plots. Before harvest, frames were removed from the plots to avoid damage from agricultural equipment during combine harvesting and fall tillage. Static chamber measurements were performed by placing opaque polypropylene chambers (0.57 x 0.37 x 0.23 m) into the water-filled well on top of the frames. In the second year, each chamber was additionally equipped with a ventilation tube and a small battery-powered axial fan for air mixing within the chamber during sampling. When the chamber height became insufficient as crops grew taller, a riser, constructed from a similar plastic box as the chamber, but with the bottom removed, was added to the underside of the chamber to prevent crop damage and increase air movement during GHG measurements.

At each sampling occasion, chambers were closed for approximately 45 min and sampled five times at 10 min intervals beginning at time of closure. Air samples were collected using the flow-through method where air was circulated for one minute between the chamber, a 20 ml glass collection vial, and an air pump connected in a loop with tygon tubing. Air temperature inside the chamber was monitored during gas flux measurements. Thereafter, gas sample vials were stored at room temperature and analyzed within a week simultaneously for N₂O and methane (CH₄) concentration on a gas chromatograph (Clarus 500, Perkin Elmer, USA) equipped with an FID and ECD using an automatic headspace injector (Turbo Matrix 110, Perkin Elmer, USA). In the first year, eight gas flux measurements were performed during the growing season (between 18 May–27 July) timed to occur immediately following the initial fertilization and significant rainfall events. The following year, the measurement scheme was intensified so that ten measurements were performed within the first 2 weeks after sowing, two measurements per week during the subsequent 2 weeks, followed by weekly or biweekly measurements during the rest of the growing season. Measurements were timed to occur

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### Table 1
Mean air temperature (°C) and sum of precipitation (mm) during the growing season in 2016 and 2017 (May-August), and climate normal in Uppsala (1961–1990); data from Ultuna meteorological station

|                  | 2016 |       | 2017 |       | Climate normal (1961–1990) |
|------------------|------|-------|------|-------|---------------------------|
|                  | May–Aug | Annual | May–Aug | Annual | May–Aug | Annual |
| Temperature (°C) | 15.1  | 6.9   | 14.6  | 6.6   | 14.2  | 5.5   |
| Precipitation (mm) | 208  | 443   | 197  | 507   | 215  | 528   |

### Table 2
Total C (TC) and total N (TN) (%) along the soil profile sampled in spring 2016 prior to fertilization and sowing

| Soil depth (m) | TC %     | TN %     | n  |
|----------------|----------|----------|----|
| 0–0.10         | 2.83 ± 0.08 | 0.24 ± 0.005 | 3  |
| 0.10–0.20      | 2.66 ± 0.12 | 0.22 ± 0.010 | 3  |
| 0.20–0.30      | 1.43 ± 0.58 | 0.12 ± 0.050 | 3  |
| 0.30–0.40      | 0.66 ± 0.26 | 0.06 ± 0.030 | 3  |
| 0.40–0.50      | 0.39 ± 0.01 | 0.04 ± 0.002 | 2  |
| 0.50–0.60      | 0.35 ± 0.03 | 0.04 ± 0.004 | 3  |
| 0.60–0.70      | 0.38 ± 0.01 | 0.05 ± 0.001 | 3  |
| 0.70–0.80      | 0.33 ± 0.02 | 0.04 ± 0.003 | 3  |
| 0.80–0.90      | 0.64 ± 0.02 | 0.09 ± 0.004 | 3  |
| 0.90–1.00      | 0.65 ± 0.02 | 0.09 ± 0.003 | 3  |

Values are given as mean ± standard error. n = number of samples.
immediately following periods of rainfall or irrigation when possible. In addition, three measurements were done during a two-week period of spring thaw in April 2018, following the second cropping season. Due to logistical reasons, only the measurement period in 2017 includes the whole cropping season, while measurements are limited to eight occasions in 2016 and three occasions in the spring of 2018. Trends and significant differences between the N placements will be discussed for the cropping periods in 2016 and 2017.

**Biomass sampling and analysis**

Above-ground biomass was sampled by hand at harvest and twice mid-season, at stem elongation and at heading, approximately Zadok’s growth stage (ZGS) 32 and 52, respectively, in 2016. In 2017 plant biomass was collected at harvest and at booting, approximately ZGS 45. The biomass was collected by removing all above-ground crop biomass within a 0.5 × 0.5 m metal frame randomly placed at four locations within each plot. Grain biomass was measured both in the hand-harvested small plots and in a net plot of 34.8 m² in the center of each plot that was combine harvested. Collected biomass was dried, threshed at harvest, ground and analysed for N content on an organic elemental combustion instrument (LECO, USA). One to two days prior to each mid-season biomass collection, leaf chlorophyll was measured using a hand-held SPAD-502 m (Minolta Camera Co., Osaka, Japan). Four plants within four randomly chosen areas within each plot were selected, and four measurements were made on the first fully expanded leaf at the top of the selected plant. During SPAD measurements, sixteen plants within each plot were randomly chosen for measuring plant height.

**Soil measurements**

On gas sampling days, soil moisture was measured with a Theta probe (Delta-T Devices, Cambridge, UK) to a depth of 0.05 m at four locations both inside and outside the frames. Observed soil moisture was converted to water-filled pore space (WFPS). Soil temperature, as depicted in Fig. 1, was accessed from Table S1. NF = no fertilizer, SP = shallow fertilizer placement (0.07 m), MP = mixed placement of fertilizer (half at 0.07 m, half at 0.20 m), and DP = deep fertilizer placement (0.20 m). *Accessed on 13-Feb-2019 from Uppsala Funbo-Lövsta Lantmet climate station (http://www.ffe.slu.se/lm/LMHome.cfm?LMSUB=1)
a nearby climate station (Funbo-Lövsta) and was not measured at the field site. In the second year, plots were sampled to 0.40 m depth and soil cores were subdivided into 0.05 m depth increments to 0.30 m, and one at 0.30–0.40 m for analysis of mineral N content by 2 m potassium chloride (KCl) extraction followed by colorimetric determination on a segmented flow analyzer (SEAL AutoAnalyzer 3, Seal Analytical, UK). Composite soil samples were collected on three occasions from each plot, prior to fertilization, 39 days after fertilization and immediately following harvest.

Calculations and statistical analyses

The R-software R 3.4.4 (RStudio Team 2018) was used for statistical analyses. Differences between treatments, i.e. fertilizer placements, were investigated by repeated measures Anova, i.e. a linear mixed-effects model using the lme function \( \text{nlme package, Pinheiro et al.} \ 2019 \) with the repetitions as random factor and the log-likelihood maximized method “ML”. Analysis of variance was done with the Anova function \( \text{car package, Fox and Weisberg} \ 2019 \). Posthoc analysis was done by Tukey’s all pair comparisons and using the glht function \( \text{multcomp package, Hothorn et al.} \ 2008 \). Differences were regarded significant for \( P < 0.05 \). We used a linear regression to check for climate effects (e.g., WFPS) on \( \text{N}_2\text{O} \) and \( \text{CH}_4 \). In addition, a linear model consisting of per-plot mean WFPS, mid-season soil mineral N (0–0.20 m), and mean \( \text{N}_2\text{O} \) or \( \text{CH}_4 \) from 2017 to check for combined soil water and N effects on GHG emissions. Figures were made using ggplot from the \text{ggplot2 package (Wickham} \ 2016 \) and plot_grid from the \text{cowplot package (Wilke) 2019}. Nitrous oxide and \( \text{CH}_4 \) fluxes were determined from concentration increase or decrease inside the chambers and using the R package \text{gasfluxes (Fuss} \ 2019 \) using the “robust linear” flux calculation method. Cumulative GHG fluxes for the three measurement periods (2016 and 2017 cropping seasons and 2018 spring thawing period) were calculated by linear interpolation between the days when measurements were taken using the aggfluxes function from the aforementioned \text{gasfluxes R package (Fuss} \ 2019 \). The fertilizer-induced seasonal emission factor (EF), which evaluates the amount of \( \text{N}_2\text{O} \) emissions that result from anthropogenic N inputs into soils, was calculated over the growing season for all three fertilized treatments according to

\[
\text{EF}_{\text{N}_2\text{O}\text{-N}}[\%] = \left( \frac{\text{N}_2\text{O}_{\text{fert}}[\text{kg N ha}^{-1}]}{\text{N}_{\text{applied}}} - \frac{\text{N}_2\text{O}_{\text{unfert}}[\text{kg N ha}^{-1}]}{\text{N}_{\text{applied}}} \right) \times 100,
\]

with \( \text{N}_2\text{O}_{\text{fert}} = \) cumulative \( \text{N}_2\text{O} \) fluxes from fertilized treatment, \( \text{N}_2\text{O}_{\text{unfert}} = \) cumulative \( \text{N}_2\text{O} \) flux from unfertilized treatment, and \( \text{N}_{\text{applied}} = \) amount of applied fertilizer N. Yield-scaled \( \text{N}_2\text{O} \) emissions were calculated following Venterea et al. (2011):

\[
\text{N}_2\text{O}_{\text{yield}} = \frac{\text{N}_2\text{O}_{\text{fert}}[\text{kg N ha}^{-1}]}{\text{grain yield}[\text{kg N ha}^{-1}]}.
\]

The N surplus (potential N loss to the environment) was calculated for each treatment as the difference between N inputs (N in seed and fertilization) and outputs (N in harvested grains and straw, as well as N losses in the form of \( \text{N}_2\text{O} \)). The nitrogen use efficiency (NUE) gives an indication of resource efficiency (Quemada et al. 2020) and was calculated as

\[
\text{NUE}[\%] = \left( \frac{\sum \text{crop N outputs}[\text{kg N ha}^{-1}]}{\sum \text{N fertilizer inputs}[\text{kg N ha}^{-1}]} \right) \times 100.
\]

However, as this measure only concerns the fertilized treatments, we calculated the agronomic efficiency of N (AE_N), which is the ratio of yield to N supply (Lahda et al. 2005) and the recovery efficiency of N (RE_N), which is the ratio of plant N–N supply (Lahda et al. 2005; Dobermann 2005). Both AE_N and RE_N take the unfertilized control into consideration:

\[
\text{AE}_N[\text{kg kg}^{-1}] = \frac{\text{grain yield}_{\text{fert}} - \text{grain yield}_{\text{unfert}}}{\text{N}_{\text{applied}}},
\]

\[
\text{RE}_N[\%] = \frac{\text{plant N uptake}_{\text{fert}} - \text{plant N uptake}_{\text{unfert}}}{\text{N}_{\text{applied}}} \times 100.
\]
Results

Environmental conditions

The growing seasons in 2016 and 2017 were slightly warmer than normal and precipitation was slightly lower than normal (215 mm), particularly in the earlier part of the season of 2017 (Table 1; Fig. 1).

WFPS measured at 0.05 m ranged from 17 to 51% in 2016 with the lowest value in late May and the highest value in mid July. In 2017, observed WFPS was lower than in the previous year with the lowest value (9.6%) observed in late July and the highest value (65.7%) observed in mid September. We found no correlations between WFPS and soil mineral N content or N\textsubscript{2}O emissions in any of the measurement periods.

Greenhouse gas emissions

Nitrous oxide

Fertilizer placement depth affected cumulative N\textsubscript{2}O emissions during the two growing seasons in 2016 and 2017 (Fig. 2). Compared with the control NF, N fertilization resulted in an increase in cumulative N\textsubscript{2}O emissions, between 32–61% in 2016 and 10–70% in 2017. In 2016, cumulative N\textsubscript{2}O emissions were significantly highest in SP, and MP and DP were intermediates between that and NF and not significantly different from the other treatments.

During the more intense measuring period in 2017, average DP emissions were similar to those in NF, but significantly lower than in SP. Emissions from MP and SP did not differ significantly. Among the fertilized treatments, N\textsubscript{2}O emissions were significantly the lowest in DP and MP and highest in SP on 8 out of 22 occasions in 2017, primarily in the first third of the cropping season (mid-May to early July) during a period of the most vigorous crop growth and minimal precipitation (Fig. 1, Table S1). The average value (\pm SD) of measured N\textsubscript{2}O fluxes in 2017 was highest in SP and MP, 69.9 ± 49.1 and 56.9 ± 52.9 \(\mu\)g N\textsubscript{2}O–N m\textsuperscript{-2} h\textsuperscript{-1} respectively, and lowest in DP and NF, 44.9 ± 39.2 and 43.8 ± 37.9 \(\mu\)g N\textsubscript{2}O–N m\textsuperscript{-2} h\textsuperscript{-1} respectively. Across all treatments, the lowest measured flux occurred early in the growing season, within either the first two days (SP and MP) or shortly after seed emergence (NF and DP), around 1.5 weeks of measurements, and the highest fluxes measured were on August 1st. Individual NF fluxes ranged from −18.9 to 210.9 \(\mu\)g N\textsubscript{2}O–N m\textsuperscript{-2} h\textsuperscript{-1} and were never statistically higher than the fertilized plots. The lowest and highest measured fluxes among all treatments during this period were in MP, −10.6 and 400.1 \(\mu\)g N\textsubscript{2}O–N m\textsuperscript{-2} h\textsuperscript{-1} respectively. On 7 occasions, MP and SP were statistically highest but on a further 3 occasions MP was statistically lower than SP and
either similar to DP or an intermediate between the two treatments (Table S1). Fluxes of N\textsubscript{2}O in SP ranged from 3.7 to 291.9 \(\mu g\) N\textsubscript{2}O–N m\textsuperscript{–2} h\textsuperscript{–1}. Nitrous oxide fluxes in DP ranged from 0.8 to 174.9 \(\mu g\) N\textsubscript{2}O–N m\textsuperscript{–2} h\textsuperscript{–1} (Table S1).

When crop season cumulative N\textsubscript{2}O emissions were yield-scaled (Fig. 3), a consistent trend emerged among treatments. Fertilizer depth significantly affected yield-scaled N\textsubscript{2}O emissions in 2017 where the GHG measurement period was longer. Yield-scaled N\textsubscript{2}O emissions were lowest in NF both years, 0.10 and 0.35 g N\textsubscript{2}O–N kg grain\textsuperscript{–1} in 2016 and 2017, respectively. SP was highest in both 2016 (0.15 g N\textsubscript{2}O–N kg grain\textsuperscript{–1}) and 2017 (0.70 g N\textsubscript{2}O–N kg grain\textsuperscript{–1}). Among the fertilized treatments, DP yield-scaled emissions were the lowest, 0.11 and 0.40 g N\textsubscript{2}O–N per kg grain in 2016 and 2017, respectively, a reduction of 26 and 43\% compared to SP. MP reduced yield-scaled emissions by 9\% (0.14 g N\textsubscript{2}O–N kg grain\textsuperscript{–1}) and 25\% (0.52 g N\textsubscript{2}O–N kg grain\textsuperscript{–1}) in 2016 and 2017 compared to SP. Fertilizer-induced emission factors (EF) calculated for the 2017 cropping season also decreased with depth of fertilizer placement. The percentage of applied N that was directly emitted as N\textsubscript{2}O for SP, MP, and DP was 0.77 \(\pm\) 0.07, 0.58 \(\pm\) 0.03, and 0.10 \(\pm\) 0.02, respectively.

Cumulative N\textsubscript{2}O (Figure S1) and daily emissions (Figure S2) from the two-week spring thaw measurement period in spring 2018 were no longer affected by fertilizer placement, but comprised between 40 and 70\% of the cumulative emissions from the 2017 cropping season (see supplementary material). However, given the low number of observations, those results are less reliable. More frequent measurements over a longer period have to be made in order to make a concise statement about the impact of thaw conditions on N\textsubscript{2}O fluxes.

**Methane**

Methane fluxes were generally negative or very low in all treatments (Fig. 1). There was no statistical treatment differences in cumulative emissions in 2017 (Fig. 2) and a treatment effect was detected on only four different measurement occasions (Table S1), excluding the initial disturbance effect from planting and fertilization. The non-fertilized control had the highest uptake, with fluxes averaging (\(\pm\) SD) \(-5.5 \pm 19.1\) \(\mu g\) CH\textsubscript{4}–C m\textsuperscript{–2} h\textsuperscript{–1} and individual fluxes ranged from \(-76\) to \(33\) \(\mu g\) CH\textsubscript{4}–C m\textsuperscript{–2} h\textsuperscript{–1}. The average CH\textsubscript{4} flux in DP was \(-3.9 \pm 13.1\) \(\mu g\) CH\textsubscript{4}–C m\textsuperscript{–2} h\textsuperscript{–1} with highest and lowest measured fluxes \(-41.0\) and \(32.4\) \(\mu g\) CH\textsubscript{4}–C m\textsuperscript{–2} h\textsuperscript{–1}, respectively. NF and DP were generally lower than both SP and MP on dates with significant treatment differences (Table S1). In 2017 SP and MP had the highest average CH\textsubscript{4} emissions (\(\pm\) SD), \(-1.7 \pm 16.3\) and \(-2.0 \pm 20.0\) \(\mu g\) CH\textsubscript{4}–C m\textsuperscript{–2} h\textsuperscript{–1}, respectively. MP minimum and maximum values were \(-92\) and \(57\) \(\mu g\) CH\textsubscript{4}–C m\textsuperscript{–2} h\textsuperscript{–1}. Minimum and maximum fluxes in SP were \(-38\) and \(43\) \(\mu g\) CH\textsubscript{4}–C m\textsuperscript{–2} h\textsuperscript{–1}. Methane fluxes were significantly different among treatments in the latter part of the season, up to the final measurement in mid-September at the time of harvest (Table S1).

In the 2-week spring 2018 measurement period (Figure S2), no treatment differences were detected either cumulatively or on individual measurement dates (see supplementary material). However, there are too few observations from which to draw conclusions.

**Biomass and yield**

Fertilization increased N concentrations mid-season in the plant biomass and in harvested straw and grain for both growing seasons, observable during mid-season
SPAD readings, and after N analysis of collected biomass and harvested grain (Table 3). In 2016, there was no detectable treatment difference in SPAD values among fertilized plots, but mid-season biomass weight increased with fertilizer placement depth in the latter part of the growing season. Early plant height, an indication of accelerated maturation, when measured around the same time, was highest in NF (0.69 m), followed by SP and DP (0.67 and 0.66 m, respectively), and was significantly lowest in MP (0.64). N content in the first mid-season biomass during elongation was highest in both MP and DP (2.42 and 2.48%, respectively), but later biomass N fertilizer placement differences during heading were not observed. Additionally, in 2016, grain yield was increased by approximately 11% in DP compared to SP, and grain N content also increased in both MP and DP. In 2017, despite higher mid-season SPAD readings in DP and MP treatments compared to SP, no significant differences in grain yield or grain N content were observed among fertilized treatments in the second growing season.

Following the insignificant differences in 2017 grain yields and the higher yields in NF compared to the fertilized treatments, the agronomic efficiency $A_{EN}$ was negative for all fertilized treatments. However, due to the higher N contents in the grains and the straw in the fertilized treatments, the N recovery efficiency $R_{EN}$ was still low but above zero. They ranged from 2.3 in SP to 18.1% in DP (Table 4).

Table 3 Treatment effects on N concentration in crop biomass (dry matter) and harvested straw and grain, leaf relative chlorophyll content (SPAD-index), and harvest grain yield (15% water content) at respective Zadok’s growth stages in the 2016 and 2017 growing seasons

| Zadok stage | NF | SP | MP | DP |
|-------------|----|----|----|----|
| 2016        |    |    |    |    |
| Biomass N (%) |    |    |    |    |
| Elongation  | 1.74 ± 0.05c | 2.22 ± 0.01b | 2.42 ± 0.05a | 2.48 ± 0.07a |
| Heading     | 1.25 ± 0.05b | 1.63 ± 0.03a | 1.66 ± 0.04a | 1.52 ± 0.05a |
| Plant biomass (t ha$^{-1}$) |    |    |    |    |
| Elongation  | 4.06 ± 0.14b | 4.81 ± 0.20a | 4.64 ± 0.10a | 4.88 ± 0.13a |
| Heading     | 7.65 ± 0.25b | 8.20 ± 0.33ab | 8.44 ± 0.22ab | 8.78 ± 0.26a |
| Plant height (m) |    |    |    |    |
| Elongation  | 0.68 ± 0.005a | 0.66 ± 0.004 | 0.64 ± 0.005 | 0.66 ± 0.005b |
| Heading     | 0.70 ± 0.005 | 0.71 ± 0.005 | 0.70 ± 0.005 | 0.71 ± 0.006 |
| SPAD-index  |    |    |    |    |
| Elongation  | 49.4 ± 0.4b | 54.2 ± 0.3a | 54.0 ± 0.3a | 53.1 ± 0.3a |
| Heading     | 47.9 ± 0.5b | 53.6 ± 0.4a | 53.7 ± 0.3a | 54.0 ± 0.3a |
| Straw N (%) |    |    |    |    |
| Harvest     | 0.23 ± 0.02b | 0.31 ± 0.01a | 0.33 ± 0.02a | 0.33 ± 0.02a |
| Grain N (%) |    |    |    |    |
| Harvest     | 2.45 ± 0.02c | 2.76 ± 0.02b | 2.87 ± 0.02a | 2.83 ± 0.02a |
| Grain yield (kg ha$^{-1}$) |    |    |    |    |
| Harvest     | 4.18 ± 0.06c | 4.40 ± 0.12bc | 4.62 ± 0.09ab | 4.88 ± 0.1a |
| 2017        |    |    |    |    |
| Biomass N (%) |    |    |    |    |
| Bothing     | 1.49 ± 0.04 | 1.87 ± 0.06 | 1.87 ± 0.05 | 1.87 ± 0.03 |
| Plant biomass (t ha$^{-1}$) |    |    |    |    |
| Bothing     | 5.05 ± 0.32 | 5.21 ± 0.31 | 5.74 ± 0.38 | 5.66 ± 0.25 |
| Plant height (m) |    |    |    |    |
| Bothing     | 0.65 ± 0.008b | 0.63 ± 0.007b | 0.66 ± 0.008ab | 0.69 ± 0.008a |
| SPAD-index  |    |    |    |    |
| Bothing     | 57.6 ± 0.6b | 59.1 ± 0.5b | 60.1 ± 0.6a | 59.7 ± 0.5a |
| Straw N (%) |    |    |    |    |
| Harvest     | 0.52 ± 0.01b | 0.73 ± 0.03a | 0.72 ± 0.03a | 0.68 ± 0.04a |
| Grain N (%) |    |    |    |    |
| Harvest     | 2.11 ± 0.02b | 2.41 ± 0.03a | 2.43 ± 0.02a | 2.40 ± 0.02a |
| Grain yield (kg ha$^{-1}$) |    |    |    |    |
| Harvest     | 4.49 ± 0.12a | 3.87 ± 0.16a | 4.08 ± 0.28a | 4.36 ± 0.12a |

Values are reported as means ± standard errors. Different letters indicate statistical difference ($\alpha = 0.05$, Tukey’s HSD)

NF = no fertilizer, SP = shallow fertilizer placement (0.07 m), MP = mixed placement of fertilizer (half at 0.07 m, half at 0.20 m), and DP = deep fertilizer placement (0.20 m)
Table 4  Nitrogen balance components in the four experimental treatments for the 2017 cropping season

|                          | NF   | SP   | MP   | DP   |
|--------------------------|------|------|------|------|
| **N inputs and outputs** |      |      |      |      |
| Seeds                    | 5.3  | 5.3  | 5.3  | 5.3  |
| Fertilization            | 0    | 105  | 105  | 105  |
| Harvested grains         | -101.1 ± 9.2 | -97.0 ± 8.1 | -104.4 ± 14.9 | -111.1 ± 4.8 |
| Harvested straw          | -21.1 ± 3.3  | -27.5 ± 3.3  | -30.5 ± 2.8  | -30 ± 2.9  |
| N₂O loss                | -1.6 ± 0.1  | -2.7 ± 0.1  | -2.1 ± 0.3  | -1.7 ± 0.1  |
| N surplus               | -118.5 ± 5.4 | -16.9 ± 4.5  | -26.7 ± 6.8  | -32.5 ± 3.2  |
| NUE (%)                 | n.a.  | 119  | 128  | 134  |
| AE₅₃ (kg N ha⁻¹)        | n.a.  | -5.9 | -3.9 | -1.2  |
| RE₅₃ (%)                | n.a.  | 2.3  | 12.2 | 18.1  |
| **Soil mineral N content and changes** |      |      |      |      |
| Soil mineral N at sowing (0–0.25 m) | 37.4 ± 2.3 | 42.6 ± 0.9  | 42.4 ± 2.7  | 36.8 ± 3.9  |
| Soil mineral N at sowing (0.25–0.40 m) | 22.1 ± 1.6 | 30.5 ± 1.8  | 37.4 ± 6.5  | 34.0 ± 4.2  |
| Soil mineral N after harvest (0–0.25 m) | 25.0 ± 3.0 | 82.7 ± 17.1 | 54.0 ± 7.8  | 42.2 ± 1.6  |
| Soil mineral N after harvest (0.25–0.40 m) | 16.6 ± 2.6 | 31.6 ± 0.7  | 47.6 ± 20.5 | 37.3 ± 2.5  |
| Δ Soil mineral N (0–0.25 m) | -15.7 ± 6.2 | 40.4 ± 16.5 | 6.9 ± 10.0  | -1.9 ± 2.3  |
| Δ Soil mineral N (0.25–0.40 m) | -8.6 ± 3.4  | -1.7 ± 2.7  | -0.7 ± 26.0 | -4.8 ± 2.2  |
| Total increase in mineral N (0–0.40 m) | -24.3 ± 9.6 | 38.7 ± 13.9 | 6.2 ± 36.0  | -6.6 ± 4.7  |

The change in soil mineral N content was calculated from subtracting mineral N content measured one week before fertilization (at sowing) from soil mineral N content measured one week after harvest. Values are presented as mean ± standard error.

NF = no fertilizer, SP = shallow fertilizer placement (0.07 m), MP = mixed placement of fertilizer (half at 0.07 m, half at 0.20 m), and DP = deep fertilizer placement (0.20 m). NUE = N use efficiency, AE₅₃ = agronomic efficiency of N, RE₅₃ = recovery efficiency of N.

Soil mineral N profiles and N balance

Data from the 2017 soil mineral N profile measurements (Fig. 4) and resulting N balance (Table 4) indicate that mineral N content had decreased in the 0.25–0.40 m layer in all treatments (Table 4). For NF and DP, mineral N had even disappeared from the 0–25 cm layer during the growing season. SP resulted in a higher surplus of mineral N remaining in the system after harvest than in the other treatments (38.7 ± 13.9 kg N ha⁻¹). The mineral N content in the mid-crop season soil profile pinpoint more or less where the fertilizer grains had been placed; SP, which was placed at 0.07 m depth, was primarily found between 0.05 and 0.10 m. After harvest, it appears that the bulk of SP soil mineral N essentially remained in the soil, but had leached further down in the profile to 0.25 m. However, in the 0.25–0.40 m layer, mineral N content had decreased by 1.7 ± 2.7 kg N ha⁻¹. The MP treatment had its largest mid-season N content peak at 0.10 m, with a gradual decline in soil N content from 0.10 to 0.25 m. After crop harvest, the 0.10 m N content peak of MP had nearly halved, and another distinct N content peak was observed deeper in the soil profile at 0.40 m. Only a small fraction of the negative N surplus of 118.5 kg in the unfertilized control was explained by the decrease in soil mineral N (6.2 ± 36.0 kg N ha⁻¹) during the cropping season. Thus net N mineralization during the growing season would have been at least 100 kg explaining the weak fertilizer response of crop yield in the fertilized treatments (Table 4). Less soil mineral N was detected in DP after harvest than in the other fertilized treatments and more N was removed from the system through harvested straw and grains resulting in a higher nutrient use efficiency of the applied fertilizer N and a higher uptake of mineral N (Table 4). Similar to NF, mineral N in the 0–0.40 m layer decreased over the growing season.
**Discussion**

Effect of N placement on N\textsubscript{2}O emissions

Different depth placement of N fertilizer had a marked effect on N\textsubscript{2}O emissions. The reduction in N\textsubscript{2}O emissions from the DP treatment compared to both MP and SP was consistent with previous studies pointing out the connection between residence time of N\textsubscript{2}O in soil and uptake or reduction in the emission of N\textsubscript{2}O (Clough et al. 1998; Harter et al. 2016). Nitrous oxide emissions from DP were generally as low as those from the unfertilized plots, a trend consistent during both the abbreviated cumulative measurement period of 2016, and for both cumulative and individual measurements in 2017. We had expected that MP, which received half the amount of fertilizer at the same depth as SP to be an intermediate between the highest and lowest emitters, but that was not always the case. During both cropping seasons, the N\textsubscript{2}O emissions from MP plots were generally as high as those from SP, consistent with findings of Chapuis-Lardy et al. (2007). The higher concentration of mineral N in the upper topsoil of MP (Fig. 4) could explain why no significant reduction was achieved. Compared with SP, DP reduced cumulative N\textsubscript{2}O emissions by 18\% and 35\% during the GHG measurement periods during the first and second growing seasons, respectively. The fertilizer-induced N\textsubscript{2}O emissions decreased with placement depth and the calculated emission factors for SP, MP, and DP were 0.77 ± 0.07, 0.58 ± 0.03, and 0.10 ± 0.02, respectively. Similar to our findings, van Kessel et al. (2013) found in a metanalysis that N\textsubscript{2}O emissions were reduced when N fertilizers were placed at a depth ≥ 0.05 m. Moreover, they reported that deep fertilizer placement significantly reduced yield-scaled emissions in no tillage and reduced tillage systems in humid climates. This is similar to our findings, where yield-scaled emissions were lower from deep (DP) than from shallow (SP) placement. Gaihre et al. (2015) found that urea deep placement (0.07–0.10 m depth
placement) reduced N₂O emissions by up to 84% compared to surface broadcast application during the dry season and also increased rice grain yields by 13% in one season and gave similar yields in another season, despite a lower N application (Gaihre et al. 2018).

Generally, treatment differences were first detectable several weeks after fertilization. The strongest significant treatment effects on N₂O formation and emissions were recorded during the 3rd–5th week after sowing and fertilization, i.e., in the first third of the 2017 growing season. It can be assumed that vigorous plant growth and N uptake from soil influenced the decreased N₂O emissions in DP, but not MP, which still had high N₂O emissions.

The treatment effect on N₂O emissions largely disappeared during the latter two-thirds of the 2017 growing season (Fig. 1). This was during a time when chamber measurements were less frequent, so it is possible that some emission peaks and thus treatment effects were missed. On the other hand, in the final weeks of the 2017 growing season, N₂O fluxes from fertilized and non-fertilized plots were similar, showing that neither fertilizer depth placement nor crop utilization were important drivers for N₂O emissions at this stage when mineral N was largely utilized or had been translocated to a lower soil depth (Fig. 4). This assumption is somewhat supported by the higher WFPS observed towards the end of the experiment (41% in late August and 66% in mid-September). Apart from that, WFPS was rather low (25% on average) throughout the whole growing season in 2017. This leads to the assumption that nitrification rather than denitrification has been the major process of N₂O production. However, based on the data observed in this study, we did not find a correlation between N₂O emissions and WFPS in either of the years.

Effect of N placement on CH₄ emissions

Methane fluxes in 2017 were generally low and negative in all treatments with little differences between DP and NF. Moreover, there was lower CH₄ oxidation and consequently higher positive fluxes in the MP and SP treatment (Fig. 2). This is consistent with previous findings that have linked surface and shallow fertilizer N application to higher CH₄ fluxes (Bodelier 2011) as most CH₄ oxidation occurs in the upper (0–0.05 m) soil layer (Crill et al. 1994; Kruger et al. 2001). For rice fields, Linquist et al. (2012) reported reduced CH₄ emissions from urea deep placement as compared to broadcast application. The studies included in their meta-analysis mostly reported lower CH₄ emissions when N fertilizer was placed below the soil surface in continuous (Schutz et al. 1989), rainfed (Rath et al. 1999) and irrigated (Setyanto et al. 2000) water management. However, when comparing irrigated and rainfed rice systems, Setyanto et al. (2000) reported higher CH₄ emissions from the deep N placement under rainfed conditions. In general, a decreasing effect of deep N placement has been related to concentrated NH₄⁺ into localized areas, as well as increased O₂ availability in the rhizosphere, thus stimulating CH₄ oxidation and reducing overall emissions (Bodelier et al. 2000a, b; Gilbert and Frenzel 1998). By contrast, results from studies focussing on fertilizer placement revealed that N placement has no effect on CH₄ emissions in irrigated rice systems (Adviento-Borbe and Linquist 2016; Yao et al. 2017), upland soil under corn (Liu et al. 2006), or winter barley (Chu et al. 2007). In the study presented here, observed WFPS was comparatively low throughout the growing seasons, indicating that the soil water regime was the major driver of the low CH₄ emissions observed. Aside from the differences in the water regimes between the above-mentioned studies on rice cultivation and the results presented here, the definition of what is considered a deep placement is quite relative and varies between studies. For example, Schutz et al. (1989) and Yao et al. (2017) studied a placement depth of 0.20 and 0.10–0.15 m, respectively, which is comparable to the DP treatment presented in this study. By contrast, Rath et al. (1999) considered 0.05 m to be a deep placement, which is analogous to our SP treatment.

Biomass, N balance, and soil mineral N

Both cropping seasons had less than normal rainfall during the former part of the growing season (Table 1), which was a possible culprit for generally lower than normal yields. Spring wheat grain yield was 4.18–4.88 t ha⁻¹ in 2016 and spring barley grain yield was 3.87–4.49 t ha⁻¹ in 2017 (Table 3). In comparison, the average yield in Uppsala county for spring wheat in 2016 was 4.49 tons ha⁻¹, and barley in 2017 was 5.07 t ha⁻¹ (Jordbruksverket 2017, 2018).
However, in 2016, nearly half of the field had been overtaken by weeds halfway through the growing season. In 2017, the uneven and delayed seed emergence from shallow seed placement resulted in differing rates of plant maturation that ultimately led to high variation in both yields and average nutrient uptake in all treatments. Despite poor growth and high variation across all plots in 2017, an increase in NUE uptake in all treatments. Despite poor growth and high variation in both yields and average nutrient uptake in all treatments.

In 2017, the uneven and delayed seed emergence from shallow seed placement resulted in gaseous N losses, and a slight decrease in 0.25–0.40 m depth (−1.7 ± 2.7 kg N ha⁻¹). Similarly, the mineral N increased in the MP treatment, in which the fertilizer was placed at 0.07 and 0.20 m, at 0–0.25 m (6.9 ± 10.0 kg ha⁻¹) and decreased in 0.25–0.40 m depth (−0.7 ± 26.0 kg ha⁻¹), highlighting that N probably has leached further down the soil profile. Similar to NF, mineral N decreased in DP (−6.6 ± 4.7 kg ha⁻¹), which may be explained by the higher N uptake and, consequently, yield (Table 4).

Ke et al. (2018) moreover reported an increase in $RE_N$ under the deep placement treatment compared to the broadcast application. Considering the grain yield, the positive impact of fertilizer deep placement depended on the fertilizer type and significantly higher grain yields were found for sulphur-coated urea, but not when polymer-coated urea was used. Similarly, Guo et al. (2016) found that deep placement of controlled-release fertilizer has the potential to increase N uptake and NUE in maize cultivation.

In the study presented here, the N fertilizer rate was designed for higher yields than those obtained in 2017. The fact that harvested grain yield was highest under the control treatment suggested that N fertilization was not needed in 2017 or even counterproductive as shown by the high values for NUE and the negative values for $AE_N$, which indicate that application of exogenous N did not lead to an increase in yield (Table 4). In contrast to 2017, values for $AE_N$ were positive in 2016. However, they were still rather low and between 1.8 for SP and 5.8 kg grain kg⁻¹ applied N for DP. According to Dobermann (2005), common values for $AE_N$ are 10–30 kg grain kg⁻¹ applied N, with higher values in well-managed systems or at low N levels. For Europe, Lahda et al. (2005) reported an average $AE_N$ of 21.3 kg grain increase per kg N applied, given a similar average fertilization rate as used in this study in 2017 ($100 ± 13.9$ kg ha⁻¹).

In contrast to $AE_N$, the positive 2017 values for $RE_N$ (2.3, 12.2 and 18.1% for SP, MP and DP, respectively) indicate that, despite the very low yield, the plants were capable of acquiring the additional N in the grains and the straw. However, the obtained values for $RE_N$ are much lower than common values summarized by Dobermann (2005), which range between 30 and 50%, with up to 80% achieved in...
well-managed systems. Compared to $\text{RE}_N$ values for cereals, as summarized by Lahda et al. (2005), i.e. 10 and 70%, the efficiencies of the MP and DP treatment were at the lower range of this interval.

The positive impact of deep-placed fertilizer on N uptake and N efficiencies is strongly related to the higher soil moisture in deeper layers. The occurrence of favorable nutrient and soil moisture conditions, which are expected to stimulate root proliferation, is more probable in deeper layers. Therefore, deep placement has been shown to be a successful management strategy to reach this aim (Li et al. 2009). However, the adoption of this practice might involve additional labor and costs in terms of purchasing suitable equipment for placing the fertilizer at the correct depth, as well as increased fuel consumption as compared to broadcast application.

Conclusions

Increasing the fertilizer N placement depth has the potential to both improve crop N content and yield, but also mitigate fertilizer-induced $\text{N}_2\text{O}$ emissions, and to a smaller extent, increase methane oxidation. The GHG mitigation effect of deeper fertilizer placement was first detectable several weeks after fertilization. Deep-placed fertilizer N did not appear to have been exposed to a greater downward mobility likely because of smaller changes in soil moisture following precipitation at this depth. The benefits of increased depth placement of N are likely dependent on climate and soil type but could be a further step in precision farming and environmentally sustainable agriculture. However, further investigations are needed before deeper placement of fertilizer can be recommended as a sustainable farming practice as indicated by our study.

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