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REGULAR ARTICLE

# Effects of slurry acidification on soil $N_2O$ fluxes and denitrification

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## Abstract

**Background:** Reductions of ammonia volatilization resulting from slurry applications to intensively managed grassland may be achieved via slurry acidification. However, it remains uncertain if this may result in pollution swapping, that is, due to reduced ammonia volatilization and increased soil N availability, emission of nitrous oxide from soils may increase.

**Aims:** In this study, we compared control (no fertilizer) and slurry fertilized grassland treatments [not acidified (S) and acidified (AS)] to assess whether slurry acidification results in changes of soil N availability, denitrification potential and activity as well as soil fluxes of nitrous oxide.

**Methods:** The study was carried out in a montane grassland system in southern Germany, and parameters were followed over a 43-days period with continuous measurements of soil GHG fluxes and biweekly measurements of microbial and soil parameters preceding and following two fertilizing events.

**Results:** Over the entire observation period cumulative $N_2O$ emissions were significantly elevated for treatments receiving slurry applications, with differences between acidified and non-acidified slurry treatments being overall insignificant. Transcripts of the nirK type nitrite reductase showed significantly higher numbers in soils of the AS treatment. While soil potential denitrification rates (PDR) did not differ between treatments, there was a strong tendency of increased PDRs for the AS treatment.

**Conclusions:** Against expectation, we did not find that application of AS affects PDR or soil $N_2O$ emissions significantly, though in tendency higher rates of soil $N_2O$ emissions as well as higher potential denitrification rates were found in treatments receiving acidified slurry as compared to the slurry only treatment. Our results indicate that longer observation periods and given the significant spatial variability, higher numbers of replicates are needed, to finally assess if slurry application indeed results in increased soil denitrification activity, soil $N_2O$ production and soil-atmosphere $N_2O$ emissions.

KEYWORDS
methanogens, nitrite reducers, nitrous oxide reducers, potential denitrification, slurry acidification

## 1 | INTRODCTION

The application of animal excreta (urine + faeces) onto arable land is a farming practice that can be traced back to thousands of years (Wilkinson, 1982). The application of solid farmyard manure or liquid slurry to agricultural land and grassland can be beneficial to maintain soil fertility (Chen et al., 2018), but also has some serious environmental drawbacks as significant amounts of nutrients are also lost to the environment along hydrological and gaseous pathways (Hou et al., 2015; Wang et al., 2019). For example, manure or slurry management related to livestock production is globally the most important source for atmospheric $NH_3$, accounting for 80%–90% of global $NH_3$ emissions (Xu et al., 2019). Ammonia volatilization is related to serious environmental problems such as eutrophication and acidification of soils, rivers or lakes, and associated impacts on biodiversity (Sutton et al., 1998).

In order to reduce the emissions of $NH_3$, different national and international directives are in place (e.g., DEFRA, 2018; Guthrie et al., 2018; United Nations Economic Commission for Europe, 2015) which include practices to mitigate $NH_3$ losses from agricultural sources. Practices discussed to reduce $NH_3$ volatilization following slurry application to agricultural lands are dilution of slurries, addition of acids, salts and formalin, direct injection of slurry in the soil or timing of slurry/ manure spreading (Bussink & Oenema 1998; Emmerling et al., 2020; Hou et al., 2015; Seidel et al., 2017; Sommer & Jensen 1994; Velthof et al., 1990; Whitehead & Raistrick 1990). However, practices regarding incorporation of slurry into the soil and better timing of slurry applications in response to plant nutrient demands, for example, via split application, can be cost and time intensive for small farms. Therefore, in regions with intensive livestock production the acidification of slurry is discussed as a cost-effective measure to reduce $NH_3$ volatilization. Acidification pushes the balance between $NH_4^+$ and $NH_3$ in slurry towards $NH_4^+$, which cannot be volatilized, and which is not easily washed out of soil due to the high cation retention capacity of soils (Weil & Brady, 2017). However, if the application of acidified slurry to agricultural land and the resulting increase in soil $NH_4^+$ availability may finally result in increased soil emissions of the greenhouse gas $N_2O$, remains uncertain.

In Germany, about half the area is used for agricultural purposes, 30% of which is grasslands, covering an area of approximate 5 million hectares (BMELV, 2010). The largest grassland regions in Germany are the grassland belt in the alpine and pre-alpine area with over 1 million hectare, mainly used for fodder production. Those temperate grasslands provide important economic value through meat and milk production (Soussana & Lüscher, 2007) and offer essential ecosystem services regarding soil organic carbon (SOC) and nitrogen (N) storage and water retention in addition to their importance for biodiversity and recreation (Chan et al., 2006; Kremen, 2005).

Montane grasslands in the pre-alpine region are used for feed production, receiving usually 2–5 broadcast slurry applications during the growing season. N use efficiency is often rather low as high gaseous N losses due to $NH_3$ volatilization and denitrification do occur (Zistl-Schlingmann et al., 2019, 2020). Fertilized grasslands are not only significant sources for $NH_3$, but as well for the atmospheric greenhouse gas (GHG) nitrous oxide ($N_2O$). In a recent study it was estimated that $N_2O$ emissions from fertilized grasslands may account for 12% of the total anthropogenic $N_2O$ emissions in Germany (UBA, 2017).

Soil $N_2O$ emissions are mainly a result of the soil microbial processes of nitrification and denitrification (Butterbach-Bahl et al., 2013). Main drivers of soil $N_2O$ emissions are associated with the (1) availability of inorganic N substances ($NH_4^+$ and $NO_3^-$) and easily degradable carbon as substrates for nitrification and denitrification, (2) soil redox potential or oxygen supply, which controls if the oxidative process of nitrification or the reductive process of denitrification prevails and (3) soil microbial community composition (Ramirez et al., 2012). Many studies have shown that following slurry application, which results in increasing availability of $NH_4^+$ as well as of $NO_3^-$ (due to stimulated nitrification), soil respiration is stimulated, while soil moisture increases. Both factors may increase anoxic conditions in soil resulting in the subsequent formation of the potent greenhouse gas (GHG) nitrous oxide ($N_2O$) by denitrification (e.g., Bakken & Frostegård, 2017; Bakken et al., 2012; Dannenmann et al., 2008; Samad et al., 2016; ŠImek & Cooper, 2002).

Though the use of acids has proven to be a valid strategy to mitigate $NH_3$ losses due to slurry spreading (e.g., Emmerling et al., 2020; Kai et al., 2008; Park et al., 2018; Sindhöj et al. 2019), a limited number of studies have focused on the potential consequences of acidified slurry applications on $N_2O$ emissions in soil (e.g., Emmerling et al., 2020; Seidel et al., 2017). In a review on animal slurry acidification, Fangueiro et al. (2015) reported only two studies, one demonstrating $N_2O$ emissions approx. doubled in the first 2–3 weeks following the application of cattle slurry acidified with nitric acid (Velthof & Oenema 1993), while the other showed a 23% increase of $N_2O$ emissions within the first 60 days following the application of pig slurry acidified with sulfuric acid (Fangueiro et al., 2010). However, a few more studies were published after 2015 (e.g., Fangueiro et al., 2016; Seidel et al., 2017), while Emmerling et al. (2020) concluded on a limited of published studies that slurry acidification may result in an approximately 20% reduction of soil $N_2O$ emissions.

Hitherto published work on effects of slurry acidification on soil $N_2O$ emissions hardly reports associated changes on soil denitrification and microbial community composition and activity, which hampers a functional understanding of impacts of slurry acidification on soil N cycling. Therefore, the main objectives of the present study were to (1) determine the stimulating effect of broadcast slurry application on grassland soil $N_2O$ emissions and underlying microbial processes and abiotic soil properties, and (2) compare the effects of acid-treated and untreated cow slurry applications on those parameters. We hypothesized that application of acidified slurry leads to higher soil mineral N concentrations, higher soil potential denitrification rates (PDR) as well as increased activities of microbes which catalyze the conversion of nitrite to NO, resulting in higher $N_2O$ emissions as compared to the application of untreated slurry. To test our hypothesis, we determined in subdaily resolution soil $N_2O$ emissions from three replicated treatments [control (C), addition of non-acidified slurry (S) or addition of acidified slurry (AS)], measured in bi-weekly intervals soil inorganic N concentrations to a depth of 4 cm, microbial biomass, PDRs and

**TABLE 1** Properties of the topsoil (0–15) taken from the site at Garmisch–Partenkirchen

| Site | GAP |
|---|---|
| Annual mean Temperature (°) | 6.8 |
| Annual mean Precipitations (mm) | 1371 |
| Soil type | Calcaric Cambisol |
| Land management | Grassland |
| Carbon content (%) | 5.4 |
| Nitrogen content (%) | 0.54 |
| pH (0.01 $_M$ $CaCl_2$) | 6.2 |
| Soil texture | Silt loam |
| Sand/silt/clay (%) | 20/45/35 |

the activity of denitrifiers (and methanogens) at three selected time points.

## 2 | MATERIALS AND METHODS

### 2.1 | Site description

Experiments were carried out at a grassland site at Garmisch-Partenkirchen, Germany (47.47566 N, 11.06248 S, elevation 720 m asl). The site is a typical montane grassland at the foothill of the northern Alps and is part of the TERENO-SOILCan experimental network of sites (TERENO: http://teodoor.icg.kfa-juelich.de/ddp/index.jsp). The soil is a Calcaric Cambisol of silty clay loam texture (proportions of sand/silt/clay of 20/45/35%, respectively) with a topsoil (0-15 cm) soil organic carbon content of 5.4%, and a pH of ca. 6.2 (Pütz et al., 2016). For further site information see Table 1.

### 2.2 | Experimental and sampling design

The experiment was carried out in the period 24 March–6 May, 2020. With regard to amounts and timing, the first slurry application was carried out following local farmers practice to promote grass growth, while the second slurry application, about three weeks later, but still before the first cutting event, is optional and only occasional realized by farmers. In the frame of this treatment three treatments were investigated:

1. Control, that is, no slurry application (C),
2. application of non-acidified, untreated slurry (S),
3. application of acidified slurry (AS).

For each treatment four replicated plots (2 m × 2 m) were randomly selected at the grassland site and marked accordingly. The slurry was collected a few days prior to applications from a local farm practicing organic farming with a cattle diet dominated by fresh grass, hay and silage. Slurry was analyzed for Total Nitrogen (TN) according to DIN ISO 13878 by the independent Raiffeisen laboratory (Ormont, Germany). Slurry TN concentration was 2.3 g N $kg^{-1}$, including 1.5 g $NH_4^+$-N $kg^{-1}$. Slurry was applied manually to the soil surface, thereby mimicking the farmers practice of slurry broadcast spreader application on 31 March, 2020, at a rate of 70 kg N $ha^{-1}$, followed by second application of 20 kg N $ha^{-1}$ on 22 April, 2020. The second slurry application is rather unusual as it is too close to the first one and only done if storage capacity for slurry is limited. However, we decided to do so as the first application happened at the beginning of an extended, unusual long period without rainfall and as only a very weak effect of slurry application on soil $N_2O$ emissions could be detected, and that, only following an artificial rainfall event. It should be noted that the second slurry application was only 20 kg N $ha^{-1}$, whereas the first one was with 70 kg N $ha^{-1}$ 3.5 times higher. It should be noted that total spring-time slurry N application to grasslands in the study region varies between 60–100 kg N $ha^{-1}$, so that there was no over-supply of slurry N in our study.

Application rates were used to calculate $N_2O$ emission factors ($EF_{N2O}$). For slurry acidification sulfuric acid ($H_2SO_4$ 96% purity) was added as suggested by Eriksen et al. (2008) since it provides sulphur and prevents additional $NO_3^-$ application as compared to acidification with nitric acid ($HNO_3$), at approximately 7 g $L^{-1}$ which reduced the slurry pH from 7.4 to 5.3.

As weather conditions were unusually dry in the first 20 days (9 mm in total) of our study, including a drought period of ca. 10 days following the first slurry application, we simulated two rain events on 11 April (+ 60mm) and 12 April (+ 40mm). The water was applied with a watering can over a period of time of approximately 1 h. No ponding occurred during soil re-wetting. The amount of applied rainfall is approximately equal to amounts occurring due to heavy rainfalls during spring-time in our pre-alpine region, with such heavy rain events usually being observed at the end of drought periods. Simulated rainfall raised the soil moisture to approx. 80% WFPS, that is., favourable conditions for denitrification. Moreover, we simulated a moderate rain event by applying 10 mm water directly following the second application on April 22, 2020. Generally, it is recommended to apply slurry prior to upcoming moderate rain events to reduce $NH_3$ losses, while increasing soil nutrient availability. Soil temperature as well as soil moisture was continuously monitored in 5 cm depth as described by (Dannenmann et al., 2016).

Topsoil was destructively sampled 12 times over the experimental period. At each sampling date, surface soils were sampled using cylindrical soil corers (100 $cm^3$ volume and 4 cm height) at five randomly selected microsites per plot and pooled at the plot level for subsequent homogenization. Stones, roots and shoots were removed, and soils prepared for further analyses as described in the following.

### 2.3 | Basic soil properties

Soil pH was measured in a 1:2.5 (soil: water) suspension of 10 g soil in 25 mL of 0.01M $CaCl_2$ solution. For determination of soil moisture, the 100 $cm^3$ soil samples were weighted and gravimetric water content

was obtained by drying soil samples at 105°C for 24 h. Based on that, soil bulk density as well as Water-Filled Pore Space (WFPS) was calculated.

For determination of inorganic soil N concentrations, 50 g of soil was extracted with 100 mL 0.5 M $K_2SO_4$ (Wang et al., 2016). Soil extracts were analyzed for $NH_4^+$ and $NO_3^-$ using a spectrophotometer (Epoch, Biotek Instrument, Inc.) following the protocol as described in Hood-Nowotny et al. (2010). Values were used to calculate soil N intensities, that is, the time-weighted average of soil N concentrations over the observation period in g N d $kg^{-1}$ soil dry weight (SDW) (Burton et al., 2008; Yao et al., 2020).

The extraction of a further, chloroform-fumigated soil sample allowed the quantification of soil microbial biomass C and N. Both unfumigated and fumigated $K_2SO_4$ extracts were analyzed for TN and dissolved organic carbon (DOC) using a TOC/TN analyzer (Analytik Jena multi N/C 3100). Microbial biomass C and N were calculated as the difference in DOC or TN, respectively, between fumigated and non-fumigated soil extracts (Dannenmann et al., 2006; Vance et al., 1987) without using a conversion factor for extraction efficiency.

## 2.4 | Potential denitrification rates

For the determination of potential denitrification rates, 10 g of soil was analyzed using the equipment and protocol as detailed by Malique et al. (2019), which was adapted on basis of the protocol provided by Groffman et al. (1999). In short, soil subsamples were placed in septum-sealed bottles together with a media solution containing N- and C-substrate for denitrifiers (0.72 g $KNO_3$ and 0.5 g glucose per litre of ultra-pure water) and chloramphenicol (0.125 g chloramphenicol per litre of ultra-pure water) to inhibit bacterial growth and protein synthesis. The headspace air was evacuated three times and flushed with $N_2$, before acetylene ($C_2H_2$) was added to approximately 10% of the headspace volume to inhibit denitrification-based production of $N_2$. The formation of the end product $N_2O$ was calculated by sampling the headspace 0, 30, 60 and 90min after the introduction of $C_2H_2$ and analyzed using a gas chromatograph (SRI 8610C) equipped with an electron capture detector (Valco 140BN) for $N_2O$ detection.

## 2.5 | Molecular analysis of denitrifier activity

To quantify activity of denitrifiers, transcripts of the genes *nirK* and *nirS* (coding for the two bacterial nitrite reductases), *nosZ* (coding for the $N_2O$ reductase) and *mcrA* (coding for the methyl-coenzyme M reductase) were analyzed. For this, 5 g of soil was stored in Eppendorf tubes® and immediately frozen at −80°C using liquid $N_2$ directly after sampling. To limit the intensive work, three sampling dates were selected based on dynamics of other obtained data (8 April, 20 April and 23 April), that is, following the first fertilization event and before and after the second fertilization event, respectively. DNA and RNA were co-extracted from 0.5 g of frozen soil (Lueders et al., 2004; Töwe et al., 2011). The co-extracted DNA and RNA was dissolved in 50 $\mu$L of sterile diethyl pyrocarbonate (DEPC) water and the nucleic acids quality was estimated spectrophotometrically by the ratios of absorbance at 260–280 nm, and 260–230 nm (NanoDrop, ND-1000, Thermo Fischer Scientific, USA). RNA was purified from the co-extracts by DNA digestion (DNase I, Zymo Research) and column-clean up (Qiagen, Germany) following the manufacturert's instructions. Quality and purification of RNA was proven by an agarose gel electrophoresis and a PCR targeting the16S rRNA gene, to confirm total degradation of the DNA. The purified RNA was quantified by Ribogreen according to the manufacturers protocol (Quant-iT RiboGreen RNA Assay Kit; ThermoFischer). Before storing the purified RNA at −80°C, 5 $\mu$L of sample were used as template for cDNA synthesis by using the SuperScript IV VILO Master Mix kit (ThermoFisher). A negative control for the reverse transcriptase was included to ensure the purity of the synthetized cDNA. In addition, the cDNA quality was checked by PCR as described above. Afterwards, the cDNA was stored at −80°C for further quantitative Real-Time PCR (qPCR) assays. For qPCRs, a pre-experiment was conducted to determine the optimal cDNA dilution to avoid qPCR inhibition, which resulted in an optimal sample dilution of 1:16. Diluted cDNA samples and a negative control (only DEPC water) were used as template to perform qPCR for quantifying *nirK*, *nirS*, *nosZ* and *mcrA* transcripts. All genes were amplified using 1 × Power SYBR Green PCR Master Mix (Life Technologies Ltd, United Kingdom) to a final volume of 25 $\mu$L. Details on the primers and qPCR conditions are described in supplementary material (Table S1). Serial plasmid dilutions ($10^1$–$10^6$ gene copies $\mu L^{-1}$) specific for each gene were used for standard curve in each 96-well plate. The PCR efficiencies of the amplifications were calculated as $E = 10^{(-1/\text{slope})} - 1 \times 100\%$ (Töwe et al., 2010). The efficiencies were 80, 92, 91 and 90% respectively for *nirK*, *nirS*, *nosZ* and *mcrA*, with determination coefficients ($R^2$) of the standard curves above 0.99. The specificity of the amplified products was checked by melting curves of the amplicons and on 2% agarose gels.

## 2.6 | Soil GHG flux measurements

Soil $N_2O$, $CH_4$ and $CO_2$ fluxes were measured by the static chamber technique, thereby using an automated measuring system (Butterbach-Bahl et al., 1997, 1998). Each plot was equipped with one chamber (0.5 m × 0.5 m × 0.15 m height). During the closure time of 36 min, chamber air was sampled four times for 3 min and changes in chamber headspace gas concentrations were used to calculate gas fluxes. Following chamber head space gas sampling, chambers were opened for a period of 6 h. Hence, up to 3–4 flux measurements were obtained for every chamber per day without rainfall, as during rainfall periods chambers would open automatically. The chambers were covered with aluminium foil to prevent light intrusion during closure. The feed pump delivered an excess (≈ 100 mL $min^{-1}$) of sample air to a Picarro G2508 (PICARRO Inc., USA) analyzer, with the overflow being monitored using a mass flow meter. The analyzer determined dry mixing ratios for $N_2O$, $CH_4$ and $CO_2$. GHG flux rates were calculated based on the slope of the linear regression of the gas mixing ratio

versus time. For details on calculation procedures see Pihlatie et al. (2013).

### 2.7 | Statistical analyses

All results are given with the standard error representing the spatial variability of plots ($n = 4$). Levene's test for comparison of variances was applied for tested parameters. Consecutive one-way ANOVA or the corresponding non-parametric Kruskall–Wallis test, and when needed the Fisher post hoc test was applied. A two-way ANOVA was applied to test for treatment and sampling date effects on denitrification potential rates. Pearson's correlations were performed to link soil N concentrations and intensities with PDRs and $N_2O$ emissions. To account for repeated measurements, non-normality and different variances of trace gas fluxes, the non-parametric Friedman and paired sample Wilcoxon rank tests were applied to identify treatments differences. Origin2019b (Origin Lab Corporation, Northampton, USA) was used for statistical analyses as well as graphical display.

## 3 | RESULTS

### 3.1 | Soil temperature and soil moisture dynamics during the observation period

Temporal dynamics of soil temperature at 5 cm and moisture at 0–4 cm soil depth are shown in Figure 1A. Mean daily soil temperature values ranged from 2.9 to 12.0°C, with temperatures increasing over time across the observation period. The mean soil temperature for the entire observation period was 8.4°C with no statistical differences between treatments.

Daily mean topsoil moisture values varied from 43% to 78% WFPS. Highest soil moisture values were observed on April 14, that is, following the first two days with simulated rainfall. Lowest soil moisture values were observed on 25 April. The mean soil moisture values across the entire observation period for the C, S, and AS treatments were 65, 64, and 65% WFPS, respectively.

### 3.2 | Effects of slurry application on soil pH, soil inorganic N concentrations and soil microbial biomass

Slurry application did not affect topsoil pH values, which stayed constant over the observation period and ranged between 6.8–7.0. Soil $NH_4^+$ concentrations ranged from 1.0 to 22.5 $\mu$g N $g^{-1}$ SDW, with treatment mean values of 3.4 ± 2.1, 6.6 ± 3.3 and 10.0 ± 7.2 $\mu$g N $g^{-1}$ SDW for the C, S and AS, respectively. One week after the first slurry application, soil $NH_4^+$ concentrations were significantly higher in the AS as compared to the S treatment, indicating that $NH_3$ volatilization losses were lower if acidified slurry is applied. Mean soil $NO_3^-$ concentrations were approx. in the same magnitude as compared to soil $NH_4^+$ concentrations (Table 2). Peak soil $NH_4^+$ concentrations were observed directly following the first slurry application (S: 12.4 ± 4.7 $\mu$g N $g^{-1}$ SDW; AS: 22.5 ± 4.3 $\mu$g N $g^{-1}$ SDW) and rapidly decreased within 1 week to concentrations as observed for the control plots. Peak topsoil $NO_3^-$ concentrations occurred approximately 2 weeks following the first slurry application (Figure 2). Soil DON concentrations ranged from 12.5 to 57.2 $\mu$g N $g^{-1}$ SDW, with mean values of 19.6 ± 6.1, 19.3 ± 1.9 and 24.8 ± 11.0 $\mu$g N $g^{-1}$ SDW for the C, S and AS treatments, respectively. Application of acidified slurry leads to significantly higher DON concentrations compared to the other treatments on April 1 only, that is, after the first slurry application (Figure 2, Table 2).

For soil microbial biomass C and N no significant treatment effects were found. Mean values for microbial C varied in a range of 247–277 $\mu$g C $g^{-1}$ SDW and for microbial N in a range of 23–26 $\mu$g N $g^{-1}$ SDW (Figure S1, Table 2). The average C:N ratio of the microbial biomass was 10.5.

### 3.3 | Soil $N_2O$ and $CH_4$ fluxes and ecosystem respiration

Soil $N_2O$ fluxes from all treatment plots were < 5 $\mu$g N $m^{-2}$ $h^{-1}$ before slurry application and stayed as low for the rest of the observation period for plots of the control treatment (Figure 1B). Also, for treatments receiving slurry applications soil $N_2O$ fluxes stayed low, and only increased to maximum values of about 10 $\mu$g N $m^{-2}$ $h^{-1}$ following the first simulated rainfalls on 11 and 12 April. However, for the second slurry application on 21 April, immediately followed by another simulated rainfall of 10 mm, mean daily $N_2O$ fluxes for the AS treatment increased to 47 ± 24 $\mu$g N $m^{-2}$ $h^{-1}$, while $N_2O$ fluxes for the S treatment only increased to values of up to 27 ± 21 $\mu$g N $m^{-2}$ $h^{-1}$. Peaks of $N_2O$ fluxes for the AS and S treatments, though lower as the ones observed on April 22, were also observed following rainfall events (Figure 1B). Across the observation period, mean cumulative $N_2O$ fluxes were 3 ± 1, 37 ± 15, and 51 ± 25 g $N_2O$-N $ha^{-1}$ for the C, S and AS treatments, respectively (Table 2). Cumulative soil $N_2O$ fluxes for the S and AS treatments following the first slurry application were not significantly different from the control. In contrast, following the second slurry application, cumulative $N_2O$ fluxes from the slurry treatments were significantly higher as compared to the control. However, differences in cumulative soil $N_2O$ fluxes between AS and S remained insignificant in period II, though in tendency higher fluxes were observed for AS. The $EF_{N2O}$ for applied slurry N was 0.05% for the S and 0.06% for the AS treatments.

Results for soil respiration and $CH_4$ fluxes are provided in Table 2 and illustrated in Figure 3. Across the entire observation period, soil respiratory fluxes constantly increased from values <50 mg C $m^{-2}$ $h^{-1}$ to 80–200 mg C $m^{-2}$ $h^{-1}$. Slurry application led to a significant stimulation of ecosystem respiration as compared to the control, while slurry acidification had no additional effect as compared to S. Cumulative ecosystem respiration across the observation period ranged from 300 (C) to about 1000 (S and AS treatments) kg $CO_2$-C $ha^{-1}$, with significant

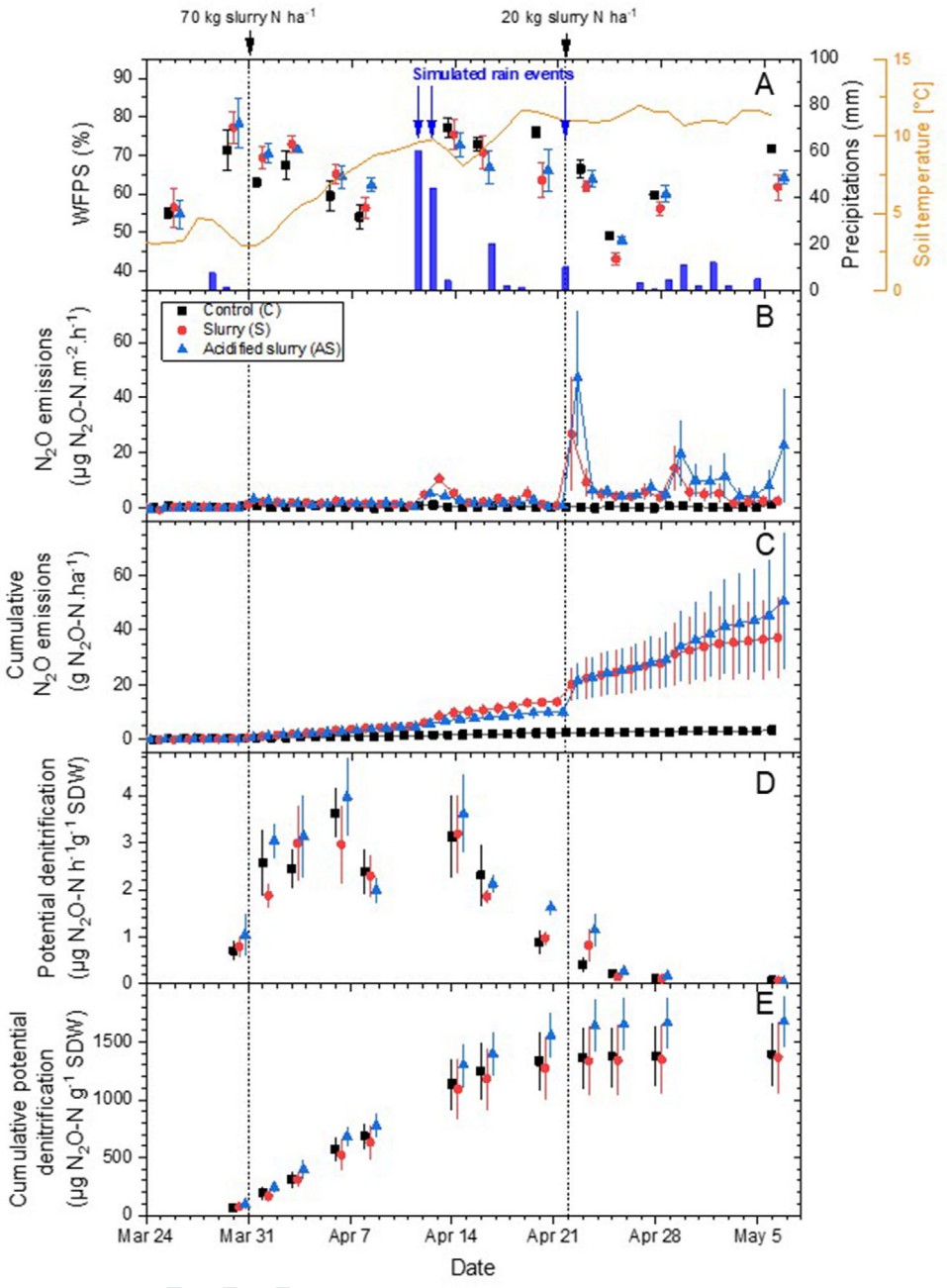


**FIGURE 1** Temporal variations of (A) the environmental parameters soil moisture and temperature (0-5 cm) and rainfall (incl. 3 days with simulated rainfall), (B) mean daily soil $N_2O$ emissions, (C) cumulative soil $N_2O$ emissions, (D) soil potential denitrification rates and (E) cumulative soil potential denitrification rates. Given are means ± SE ($n = 4$). The vertical dash lines show dates of slurry applications

higher values for the AS and S treatments as compared to the control (Table 2, Figure 3).

Regarding $CH_4$ fluxes, uptake as well as emissions were observed. While for the control treatment, soils were overall a sink for atmospheric $CH_4$ (mean flux 10 ± 0.5 $\mu$g $CH_4$-C $m^{-2}$ $h^{-1}$), we observed high net $CH_4$ peak emissions from the AS and S treatments in the first 2–3 days following slurry application, with mean daily $CH_4$ emissions on the day of slurry application reaching up to 4434 ± 1009 $\mu$g $CH_4$-C $m^{-2}$ $h^{-1}$ for the S treatment (Figure 3). However, at all other times $CH_4$ fluxes for AS and S were close to zero or even a net $CH_4$ uptake was observed. Cumulative $CH_4$ fluxes were significantly different between all treatments and changed from net uptake to net emissions in the following order: S (-109 ± 26 g $CH_4$-C $ha^{-1}$) < AS (163 ± 112 g $CH_4$-C $ha^{-1}$) < S (998 ± 971 g $CH_4$-C $ha^{-1}$) (Table 2, Figure 3).

## 3.4 | Soil potential denitrification rates

Results of potential denitrification rates (PDR) of soil are listed in Table 2 and illustrated in Figure 1D and E. Mean soil PDRs ranged

**TABLE 2** Mean values ± SE of topsoil (0–4 cm) $NH_4^+$, $NO_3^-$ and DON concentrations, microbial biomass C and N, soil $N_2O$ and $CH_4$ fluxes and ecosystem respiration as well respective cumulative values across the observation period (24 March–8 May, 2020) for the three investigated treatments. Different lowercase letters indicate significant treatment differences of mean values ($n = 4$)

| | Unit | Control (C) | Slurry (S) | Acidified Slurry (AS) |
|---|---|---|---|---|
| Soil-$NH_4^+$ | $\mu g\ N\ g^{-1}$ SDW | $3.4 \pm 0.6^a$ | $6.6 \pm 1.0^b$ | $10.0 \pm 2.1^c$ |
| Soil-$NO_3^-$ | $\mu g\ N\ g^{-1}$ SDW | $3.7 \pm 0.4^a$ | $6.6 \pm 1.0^b$ | $6.2 \pm 0.7^b$ |
| Soil-DON | $\mu g\ N\ g^{-1}$ SDW | $19.6 \pm 1.8^a$ | $19.3 \pm 0.6^a$ | $24.8 \pm 3.2^a$ |
| Microbial biomass C | $\mu g\ C\ g^{-1}$ SDW | $1057 \pm 39^a$ | $1011 \pm 39^a$ | $1001 \pm 30^a$ |
| Microbial biomass N | $\mu g\ N\ g^{-1}$ SDW | $123 \pm 4^a$ | $110 \pm 5^b$ | $105 \pm 4^b$ |
| $N_2O$ fluxes | $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$ | $0.3 \pm 0.1^a$ | $3.5 \pm 0.7^b$ | $4.8 \pm 1.2^b$ |
| Cumul. $N_2O$ fluxes | $g\ N_2O\text{-}N\ ha^{-1}$ | $3.3 \pm 1.4^a$ | $37.2 \pm 14.6^b$ | $50.9 \pm 24.9^b$ |
| Ecosystem respiration | $mg\ CO_2\text{-}C\ m^{-2}\ h^{-1}$ | $69.8 \pm 4.5^a$ | $91.7 \pm 6.7^b$ | $94.6 \pm 7.3^b$ |
| Cumul. ecosystem resp. | $kg\ CO_2\text{-}C\ ha^{-1}$ | $737 \pm 96^a$ | $968 \pm 178^{ab}$ | $999 \pm 121^b$ |
| $CH_4$ fluxes | $\mu g\ CH_4\text{-}C\ m^{-2}\ h^{-1}$ | $-10 \pm 0.5^a$ | $94 \pm 101^b$ | $114 \pm 17^b$ |
| Cumul. $CH_4$ fluxes | $g\ CH_4\text{-}C\ ha^{-1}$ | $-109 \pm 26^a$ | $989 \pm 971^b$ | $164 \pm 112^b$ |
| Pot. denitrification | $\mu g\ N\ g^{-1}\ h^{-1}$ | $1.6 \pm 0.4^a$ | $1.5 \pm 0.3^a$ | $1.8 \pm 0.4^a$ |
| Cumul. pot. denitrif. | $\mu g\ N\ g^{-1}$ SDW | $919 \pm 150^a$ | $887 \pm 146^a$ | $1091 \pm 176^a$ |

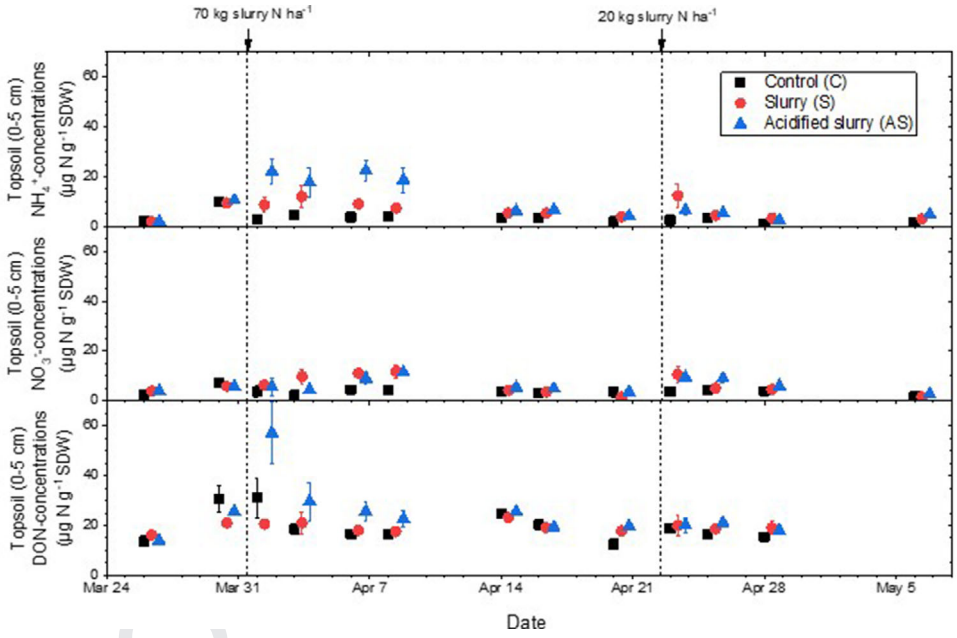


**FIGURE 2** Changes of mean ± SE ($n = 4$) topsoil (0–10 cm) $NH_4^+$ (top), $NO_3^-$ (middle), and DON (bottom) concentrations across the observation period for the different treatments. Dashed lines and arrows indicate dates of slurry applications to S and AS plots

from 0–4 $\mu g$ N $g^{-1}$ SDW $h^{-1}$ with lowest values being observed at the beginning and towards the end of the measuring period (Figure 1D). Mean PDR rates for the different treatments were 1.6 ± 0.4, 1.5 ± 0.3 and 1.8 ± 0.4 $\mu g$ N $g^{-1}$ SDW $h^{-1}$ for the C, S and AS treatments, respectively, without significant differences between treatments. However, mean soil PDRs rates for the AS treatment were at all sampling dates in tendency higher as compared to AS and C (Table S2). Cumulative soil PDR ranged from 1350–1675 $\mu g$ N $g^{-1}$ SDW $h^{-1}$) (Figure 1E).

## 3.5 | Activity of nitrite and $N_2O$ reducers as well as methanogens

Results of the analysis of *nirK*, *nirS*, *nosZ* and *mcrA* transcripts for the three selected time points are listed in Table 3. The transcript copies per $g^{-1}$ SDW for *nirK* varied from $1.87 \times 10^5$ to $2.11 \times 10^6$, for *nirS* from $6.4 \times 10^2$ to $3.0 \times 10^4$, for *nosZ* from $1.45 \times 10^5$ to $1.39 \times 10^6$ and *mcrA* from 0 to $2.73 \times 10^5$. Across all dates, only the number of transcripts of the *nirK* type nitrite reductase showed significantly higher numbers

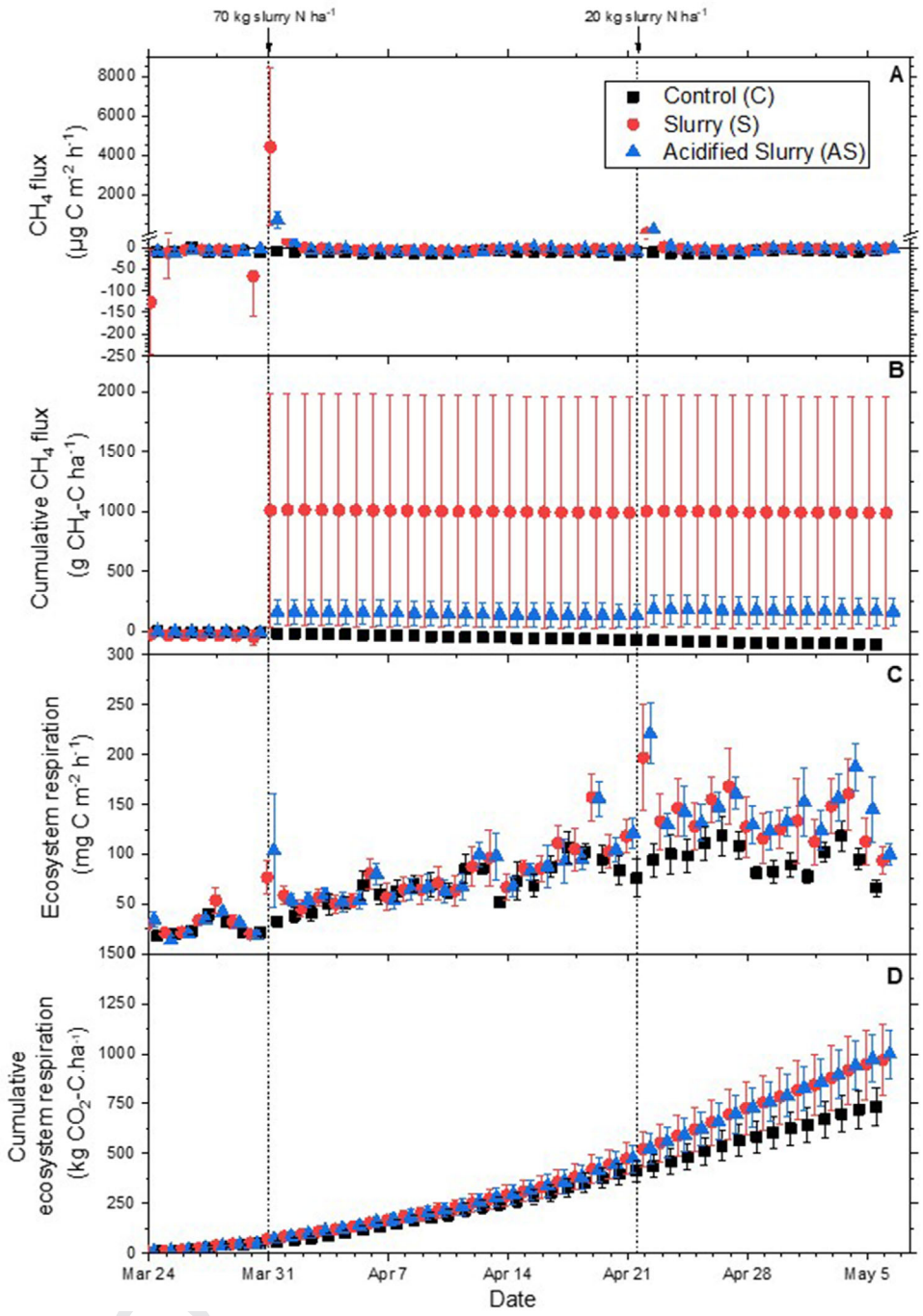


**FIGURE 3** Temporal variations of (A) mean daily $CH_4$ fluxes, (B) cumulative $CH_4$ fluxes, (C) ecosystem respiration and (D) cumulative ecosystem respiration. Given are means ± SE ($n = 4$). The vertical dash lines show dates of slurry applications

in soils of the AS treatment as compared to the C and S treatments and this only for the last two analyzed dates, that is, before and after the second slurry application. For other enzyme activities, results did not significantly differ between slurry treatments. For the single time points, the use of nonlinear mixed effect models did not show significant differences between treatments for the different transcripts as well as for the (*nirK*+*nirS*)/*nosZ* ratio (data not shown).

The null value reported for *mcrA* potential activity in the control plots suggests that no methanogens were active in non-amended soils. For the treated plots *mcrA* transcripts were slightly increased in the S treatment as compared to the AS.

## 3.6 | Correlation of GHG fluxes with measured soil nitrogen and microbial parameters

PDR were found to be correlated with soil $NH_4^+$ concentrations in the AS treatment, while for other treatments correlations with any

**TABLE 3** Transcript copy numbers for *nirK*, *nirS*, *nosZ* and *mcrA* genes for all treatments at three sampling dates ($n = 4$)

| | Reductases activity for each treatment (log transcript copies $g^{-1}$ soil dry weight) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *nirK* | | | *nirS* | | | *nosZ* | | | *mcrA* | | |
| Date | C | S | AS | C | S | AS | C | S | AS | C | S | AS |
| 08/04 | $5.30^{a}$ | $5.66^{a}$ | $5.62^{ab}$ | $3.30^{a}$ | $3.97^{a}$ | $3.83^{a}$ | $5.23^{a}$ | $5.67^{ab}$ | $5.32^{a}$ | n.a. | $5.05^{ab}$ | $4.75^{a}$ |
| 20/04 | $5.27^{a}$ | $5.42^{a}$ | $6.33^{bc}$ | $2.81^{a}$ | $2.83^{a}$ | $3.95^{a}$ | $5.16^{a}$ | $5.70^{ab}$ | $6.00^{ab}$ | n.a. | n.a. | $4.82^{a}$ |
| 23/04 | $5.61^{ab}$ | $5.56^{a}$ | $6.10^{bc}$ | $4.48^{a}$ | $4.31^{a}$ | $4.35^{a}$ | $5.89^{ab}$ | $6.14^{b}$ | $5.90^{ab}$ | n.a. | $5.44^{b}$ | $5.31^{ab}$ |
| Mean | 5.42 | 5.56 | 6.10 | 4.04 | 4.00 | 4.11 | 5.56 | 5.90 | 5.82 | n.a. | 5.11 | 5.04 |

Lower case letters indicate significant differences in transcript copy numbers between the different treatments ($p < 0.05$) for each of the enzymes individually. n.a. indicates a gene copy number of 0.

kind of measured soil N pools ($NH_4^+$, $NO_3^-$ or DON concentrations) remained insignificant (Figure S2). With regard to soil $N_2O$ fluxes, a significant correlation was only identified to $NO_3^-$ concentrations in the C treatment, while, for example, no correlation was found to PDRs. However, cumulative $N_2O$ fluxes were significantly positively correlated to cumulative PDRs for the control plots ($\rho = 0.95$), while this correlation remained insignificant for the S ($\rho = 0.85$) and AS ($\rho = 0.75$), treatments. Moreover, cumulative $N_2O$ fluxes were closely correlated with cumulative $CO_2$ fluxes (Figure S2).

A positive correlation was found for the ratio of *nir*(*S*+*K*):*nosZ* transcripts and $N_2O$ fluxes for control treatments only (Figure S3), while for the slurry fertilized treatments no significant correlation between $N_2O$ flux magnitude and gene copies for denitrification enzymes were found.

## 4 | DISCUSSION

### 4.1 | Effect of slurry acidification on soil $N_2O$ emissions and potential denitrification rates

Overall, as in many other experiments (e.g., Arias-Navarro et al., 2017; Clemens et al., 1999; Giles et al., 2012; Marton et al., 2015; Russenes et al., 2016; Yanai et al., 2003), we found significant spatial and temporal variations regarding soil $N_2O$ fluxes and soil potential denitrification rates, which hampered the assessment of treatment effects. Therefore, while in tendency the application of acidified slurry as compared to untreated slurry resulted in enhanced soil denitrification and soil $N_2O$ production, these differences were not statistically significant. Same applies to peak $CH_4$ fluxes following slurry application, which were in tendency as well lower if acidified slurry was applied.

Following the first application, soil $NH_4^+$ concentrations were significantly increased in the plots amended with acidified slurry (AS) as compared to the plots receiving untreated slurry (S), indicating a substantially smaller $NH_3$ volatilization due to slurry acidification. However, as $NH_3$ fluxes were not measured, this remains speculative. Even though soil $NH_4^+$ (but not $NO_3^-$) concentrations were elevated following the first slurry application, soil $N_2O$ fluxes were similar between the control and the S and AS treatments. As previously described, the first slurry application happened during a pronounced dry period without rainfall, so that the applied slurry may only be partially transported into the soil and thus not readily available for the soil microbiota. As the unusual dry weather conditions continued, we simulated rainfalls on 11th and 12th April, but even following this soil rewetting event, soil $N_2O$ emissions hardly increased, likely as most of the slurry N was either lost due $NH_3$ volatilization or plant N uptake. To ensure that slurry N is indeed washed into the soil, the second application was followed by a small, simulated rainfall event. This second slurry application resulted in a clear response and significant increase in $N_2O$ fluxes despite the slurry N application rate was 3.5-fold lower as compared to the first application. However, while in tendency soil $N_2O$ fluxes for AS were higher as for S, differences were not significant. Also, over the entire observation period the plots amended with acidified slurry showed 35% higher mean $N_2O$ fluxes as compared to the plots receiving untreated slurry, though also this difference was not statistically significant. However, in tendency increased $N_2O$ emissions from the AS treatment are in line with higher numbers of transcripts of the nitrite reductase gene *nirK* for AS as compared to S, suggesting that increased N availability due to slurry acidification and associated reductions in $NH_3$ volatilization (Hou et al., 2015) promoted the activity of denitrifiers in soil. Moreover, we found some indications that slurry acidification directly promoted denitrification as potential denitrification rates (PDRs) were also found to be stimulated in AS as compared to S soils, though the difference in mean rates was with +7.4% again statistically not significant. Overall, results for $N_2O$ fluxes, potential denitrification rates and nitrite enzyme activities, though not statistically different between both slurry treatments, point toward an increase activity of denitrifiers in soils receiving acidified slurry. Though our study only indicates that slurry acidification stimulates soil denitrification and associated N production, likely due to pronounced spatial variability of measured parameters and unusual low soil moisture conditions, which hampered nutrient transport into the soil, it may stimulate future studies to further explore the possibility that slurry acidification may under certain conditions, specifically in wetter soils, result in stimulated soil denitrification rates and $N_2O$ emissions as compared to applications of non-acidified slurry. Also, results of soil $NH_4^+$ concentrations need to be carefully addressed as soil sampling was performed on the first 4 cm of topsoil, that is, results are not suitable for mass balance or interpretation of the overall nitrogen use efficiency. However, it can be used comparatively as an indicator of the amount of

N that is retained in the slurry and not volatilized as $NH_3$. However, if slurry application is occurring extended dry periods, as in our study following the first slurry application, $NH_3$ volatilization will dominate the fate of applied N, so that even following soil re-wetting to up to 80% WFPS hardly stimulates soil $N_2O$ emissions (Figure 1B).

Despite a limited number of studies considered so far, the impact of slurry acidification on soil $N_2O$ emissions, few available studies point towards the same direction, that is, stimulation of soil $N_2O$ emissions if acidified instead of untreated slurry is applied (Velthof & Oenema, 1993). The latter authors reported low soil $N_2O$ fluxes following slurry application if no rainfall was simulated in conjunction with the slurry application. However, if slurry application was followed by simulated rainfall, $N_2O$ fluxes from grassland sites of up to 2 kg $ha^{-1}$ $N_2O$-N within 7 days were reported for plots receiving acidified slurry, while for those plots receiving untreated slurry, fluxes stayed with 0.1 kg $ha^{-1}$ $N_2O$-N per week rather low (Velthof & Oenema, 1993). The authors also indicated that soil type played a major role in modulating the response of grassland to slurry applications and that soils with a sandy texture hardly show any response to slurry application likely due to low organic matter contents, hampering microbial activity, and increased N leaching rates. In a study with soil mesocosms Fangueiro et al. (2010) reported an increase in cumulative $N_2O$ emissions over a 61-day incubation period by 23% for soils receiving acidified slurry as compared to soils receiving untreated slurry. The increase of soil $N_2O$ emissions is thus close to results of our field study (+35% in 43 days). These studies (Fangueiro et al., 2010; Velthof & Oenema, 1993) also mention that the type of slurry (e.g., pig or cattle), the acid used and the targeted slurry pH were found of importance in regulating soil $N_2O$ emissions.

However, other studies have pointed out that slurry acidification is an effective method for minimizing both $NH_3$ and $N_2O$ emissions. For instance, Park et al. (2018) showed that pig slurry acidification resulted in a delayed nitrification, reduced $NO_3^-$ leaching, and decreased $NH_3$ volatilization and soil $N_2O$ emissions. In the Park et al. (2018) study, the decrease in soil $N_2O$ fluxes was attributed to a delay of soil nitrification due to reductions in soil pH following slurry application and a promotion of $NH_4^+$-uptake by plants. However, the study of Fangueiro et al. (2010) shows, that even if $N_2O$ fluxes may be lower in soils treated with acidified slurry as compared to those with non-acidified slurry in the first 12 day, increased $N_2O$ emissions may be observed later as cumulative $N_2O$ fluxes over the full 61 day incubation period were higher for the acid treated slurry. This show that on basis of current knowledge the effect of slurry acidification on soil processes and soil $N_2O$ emissions cannot be generalized as possibly soil properties and composition of the microbial community, but also the technique by which slurry is applied (e.g., surface vs. injection) or the chemical used for acidification largely affects the response of the system to applications of acidified slurry. Therefore, we stay critical with regard to the results of a meta-analysis by Emmerling et al. (2020) who concluded that applications of acidified slurry may not result in stimulation of soil $N_2$ emission, specifically as details of the underlying studies, which were anyhow small in numbers (2–7), are not displayed in detail. However, there is compelling evidence, also observed in our study, that slurry acidification results in higher soil N availability, which is known to promote crop growth for example, of winter wheat, spring barley or maize (Birkmose & Vestergaard 2013; Fangueiro et al. 2015; Kai et al. 2008).

## 4.2 | Does slurry application affect denitrification product ratios?

While our study provides some evidence that slurry acidification stimulates soil denitrification activity and soil $N_2O$ emissions, we still have little evidence if, for example, slurry acidification affects as well the $N_2$:$N_2O$ ratio of denitrification. While we did not observe any change of soil pH due to the application of acidified slurry, very likely as the grassland soils in our study are still rich in carbonates, other studies did show that soil pH changes will affect the $N_2$:$N_2O$ product ratio of denitrification. For example, Dannenmann et al. (2008) showed that a pH decrease from 7.3 to 6.3 may result in a strong increase of the $N_2O$:$N_2$ emission ratio of a forest soil (Dannenmann et al., 2008). Thus, continuous application of acidified slurry, in conjunction with soil acidification due to nitrification and $NO_3^-$ leaching, may in the long-term result in a change of the $N_2O$:$N_2$ emission ratio in favour of $N_2O$.

Independent of acidification, we found evidence that in plots receiving slurry, the number of transcripts of the *nosZ* gene, coding for the enzyme which catalyzed the reduction of $N_2O$ to $N_2$, was higher as in control plots (Table 3). As transcripts of the *nosZ* gene proved to be a very good indicator of measured $N_2$ fluxes in a similar soil (Chen et al., 2015), this indicates that not only the denitrification activity is stimulated by slurry application, but that the stimulation of $N_2$ production may be higher as for $N_2O$. In a recent laboratory incubation study using comparable soil, Zistl-Schlingmann et al. (2019) concluded that $N_2$ losses are a far overlooked key component of N balance in montane grassland, accounting in their study for 31%–42% of the applied slurry-N, against 0.4%–1.0% of slurry-N lost in the form of $N_2O$. High $N_2O$ reductase activity as found in our study might thus explain the rather low $N_2O$ fluxes and $N_2O$ emission factors found. To successfully assess all N losses from the plant–soil–microbe system before and after slurry application, further experiments are needed, which also assess $N_2$ losses as well as $NH_3$ volatilization, N leaching and plant uptake. However, such experiments are complex and expensive, but would allow to fully trace the fate of applied N and to fully evaluate if acidification of slurry is indeed an environmental sound option to reduce the environmental footprint of slurry applications to grasslands.

## 4.3 | Interactions of slurry application with weather and vegetation development

The experiment took place a few days after the last snow melted, that is, at the start of the growing season with in tendency constantly rising temperatures. We assume that the marked observed changes in potential denitrification rates in all treatments, peaking a first time at the end of the first week in April, is mainly due to increased soil temperatures (Figure 1D). This interpretation is further supported by observed steady increases in ecosystem respiration (Figure 3, Table 2), which not

only points towards increasing plant growth, but also to increasing soil microbial activity. In contrast, we interpret the significant decline in PDRs from mid of April towards the end of measurements on May 6 and with values decreasing from about 3 to close to 0 $\mu$g $N_2O$-N $h^{-1}$ $g^{-1}$ SDW, as a sign of increasing competition for soil N between the soil denitrifier community and plants (Figure 1D). As plant biomass was quickly developing in our study, we assume in agreement with other studies, that plants were increasingly better competitors for the additional slurry N provided as the soil microbes (Cott et al., 2018; Dong et al., 2001; Giri et al., 2017; Laine et al., 1994; Malique et al., 2019; Marriott et al., 1988; Rummel et al., 2021). This interpretation also explains why PDRs did not rise following the second application, that is, after addition of substrate and following a simulated rainfall of 10 mm. Unfortunately, we did not sample shoot biomass, so that this interpretation cannot be supported by measured changes in plant aboveground biomass.

Unlike findings of Yao et al. (2020), the lack of correlation between $N_2O$ fluxes and soil N intensities in this study might be explained by the rather short observation period, the dry weather conditions for the first slurry application and sampling bias. In other words, during soil sampling of the topsoil (0–4 cm), part of the dried slurries might have been mixed with the soil, rising the N content from the soil extracts, while the dried slurry N may not have been available for soils microbial processing. Also, the fact that following the first slurry application no rain occurred for 11 days, so that we finally decided to simulate rainfalls (Figure 1), has likely affected our results. This is because slurry N is easily volatilized as $NH_3$, though somehow reduced if acidified, if not washed into soils by rain (Hou et al., 2015), so that treatments effects remained rather low. In contrast, for the second slurry application, which followed directly a simulated rainfall event of 10 mm, a pronounced increase in soil $N_2O$ fluxes as compared to the first slurry application was observed despite the total amount of slurry N was 3.5-fold lower than for the first application. The different weather conditions between the first and the second application highlight the importance of post application rainfall and soil moisture to increase the bioavailability of slurry N to plants and microbes. Consequently, the farmers practice of timing slurry application to a period with expected rainfalls is suitable to minimize N losses via ammonia volatilization, though it may promote denitrification and associated $N_2O$ production and emission.

## 5 | CONCLUSIONS

Several studies show that acidification of slurry results in significant reductions of $NH_3$ emissions. However, impacts of slurry acidification on soil $N_2O$ emissions have so far not systematically been investigated. The same is true for impacts on N leaching losses. Our data show, that slurry application may promote pollution swapping, that is, while reducing $NH_3$ emissions, our data indicate that acidified slurry may promote soil denitrification activity and soil $N_2O$ production. Though not statistically significant, owe found 35% higher cumulative soil $N_2O$ emissions and in tendency higher rates of potential denitrification for the acidified slurry treatment as compared to the treatment receiving non-acidified slurry. However, our study also shows, that longer measuring periods as well as studies for different soils may be needed, to finally conclude if slurry acidification technique may lead to a significant pollution swapping. Therefore, given the widespread change of slurry spreading practices by farmers, we call for a systematic assessment of effects of slurry acidification on pathways and magnitudes of environmental N losses and grassland productivity.

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### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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