Contingent Effects of Liming on N₂O-Emissions Driven by Autotrophic Nitrification

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Liming acidic soils is often found to reduce their N₂O emission due to lowered N₂O/(N₂O + N₂) product ratio of denitrification. Some field experiments have shown the opposite effect, however, and the reason for this could be that liming stimulates nitrification-driven N₂O production by enhancing nitrification rates, and by favoring ammonia oxidizing bacteria (AOB) over ammonia oxidizing archaea (AOA). AOB produce more N₂O than AOA, and high nitrification rates induce transient/local hypoxia, thereby stimulating heterotrophic denitrification. To study these phenomena, we investigated nitrification and denitrification kinetics and the abundance of AOB and AOA in soils sampled from a field experiment 2–3 years after liming. The field trial compared traditional liming (carbonates) with powdered siliceous rocks. As expected, the N₂O/(N₂O + N₂) product ratio of heterotrophic denitrification declined with increasing pH, and the potential nitrification rate and its N₂O yield (Y_N₂O: N₂O-N/NO₃⁻-N), as measured in fully oxic soil slurries, increased with pH, and both correlated strongly with the AOB/AOA gene abundance ratio. Soil microcosm experiments were monitored for nitrification, its O₂-consumption and N₂O emissions, as induced by ammonium fertilization. Here we observed a conspicuous dependency on water filled pore space (WFPS): at 60 and 70% WFPS, Y_N₂O was 0.03-0.06% and 0.06–0.15%, respectively, increasing with increasing pH, as in the aerobic soil slurries. At 85% WFPS, however, Y_N₂O was more than two orders of magnitude higher, and decreased with increasing pH. A plausible interpretation is that O₂ consumption by fertilizer-induced nitrification cause hypoxia in wet soils, hence induce heterotrophic nitrification, whose Y_N₂O decline with increasing pH. We conclude that while low emissions from nitrification in well-drained soils may be enhanced by liming, the spikes of high N₂O emission induced by ammonium fertilization at high soil moisture may be reduced by liming, because the heterotrophic N₂O reduction is enhanced by high pH.

Keywords: soil pH, nitrification, N₂O-reductase, N₂O, moisture
INTRODUCTION

Although soils are buffered by ion exchange reactions and weathering of minerals (Chadwick and Chorover, 2001), they become gradually acidified by cultivation, primarily due to ammoniacal nitrogen fertilization and loss of base cations (von Uexküll and Mutert, 1995; Guo et al., 2010). Soil acidification is commonly counteracted by carbonate addition, either as frequent low doses or as infrequent heavy dressings (Shaaban et al., 2014; Goulding, 2016). Crop plants display substantial variation in acid tolerance (Goulding, 2016), and even cultivars within one species may vary in their tolerance to soil acidity (Kochian et al., 2004). Thus, the target pH for sustaining high yields will depend on the crop, with the minimum given by its tolerance to acidity and the maximum by the micronutrient availability of the soil at elevated pH (White and Robson, 1989). Liming may also serve other purposes than securing an agronomic minimum pH for crop production. Heavy dressings of CaO (or Ca(OH)$_2$) reduce soil erosion by improving the structure of heavy clay soils (Ulén and Etana, 2014), while the effect on crop growth appears variable, possibly due to reduced manganese availability at high pH in some soils (Blomquist et al., 2018).

Liming beyond the minimum for crops has also been proposed as a means of reducing N$_2$O emissions, since soil acidification leads to high N$_2$O/N$_2$ product ratios of denitrification under standardized anoxic incubations. This was first observed by Wijler and Delwiche (1954), and corroborated by subsequent investigations, although the reason remained obscure (Cahel and Simek, 2011). Recent studies of transcription and enzyme kinetics have provided compelling evidence that this is a post-transcriptional phenomenon: the making of functional N$_2$O-reductase is increasingly impaired with increasing proton concentrations (Liu et al., 2014).

Numerous field and microcosm experiments have been conducted to determine if N$_2$O emission can be reduced by increasing soil pH, be it by carbonates (Shaaban et al., 2014, 2015, 2018; Oo et al., 2018), or biochar (reviewed by Cayuela et al., 2014). Although the investigations generally corroborate the hypothesis, there are also cases where N$_2$O emissions were either unaffected or even increased in response to elevated soil pH (see review by Qu et al., 2014). One reason for the variable effect of liming on N$_2$O emissions from soils could be the production of N$_2$O by ammonia oxidizing organisms. The three known groups of ammonia oxidizing organisms are the ammonia oxidizing bacteria (AOB), the ammonia oxidizing archaea (AOA) and the recently discovered comammox group (Daims et al., 2015), which oxidizes NH$_3$ all the way to NO$_3^-$ While little is known about the ecology and N$_2$O production of comammox, the biochemistry and ecophysiology of AOB and AOA and their N$_2$O production have been subject to intense studies. AOA are frequently found all the way to NO$_3^-$ (Daims et al., 2015), which oxidizes NH$_3$ to NO$_2^-$ and NO$_3^-$, hence potentially inducing denitrification, either by heterotrophic or nitrifying organisms (Kool et al., 2011a,b; Huang et al., 2014). Isotope tracing data have been taken to suggest that nitrifier denitrification is more significant than heterotrophic denitrification in soils (Zhu et al., 2013; Wrage-Mönning et al., 2018), but the validity of this has been challenged recently by Bakken and Frostegård (2017), who claim that the isotope tracing method cannot be used to distinguish between the two pathways. This distinction is important in the context of pH management because the net production of N$_2$O by heterotrophic denitrification is strongly dependent on pH, while that by nitrifier denitrification is not: denitrification by nitrifying organisms produces pure N$_2$O regardless of pH, because they lack the gene for N$_2$O reductase (nosZ) (Klotz and Stein, 2011).

While increasing soil pH was expected to reduce the N$_2$O emission from denitrification by enhancing the synthesis of functional N$_2$O reductase, we hypothesized that it would have the opposite effect on N$_2$O emission from nitrification, both by increasing its N$_2$O yield (mol N$_2$O per mol NO$_3^-$ produced) and by enhancing the potential nitrification rate. The latter would lead to high oxygen consumption after fertilization with ammonium, hence increasing the risk for hypoxia and denitrification if the soil moisture content is high. Such nitrification induced denitrification would be a strong N$_2$O source, hypothetically enhanced by increasing the soil pH, unless effectively counteracted by enhanced synthesis of N$_2$O reductase at high pH. To shed light on the potential stimulation of N$_2$O emission from nitrification after liming, we conducted a series of laboratory incubations with soils sampled over a period of 2 years from a field trial, in which carbonates were compared with siliceous rock powders as agents to increase the soil pH (Nadeem et al., 2015). The motive for testing powdered siliceous rocks was to explore the possibility of using carbonate-free minerals, thus avoiding the emission of carbonate-CO$_2$. In addition, we describe the effect of the rock powders on the AOB/AOA ratio as a function of increasing soil pH.

While corroborating the well-known effect of pH-increase on the synthesis of functional N$_2$O reductase (hence reducing N$_2$O emission from denitrification), the strictly oxic incubations demonstrated that the rate of nitrification and its N$_2$O yield increased with soil pH. We further demonstrate that nitrification-induced denitrification increases with soil moisture, and provide evidence that oxygen consumption by nitrification induces heterotrophic denitrification, rather than nitrifier-denitrification. The results are important because they explain why the effect of liming on the N$_2$O emission induced by ammonium fertilization depends on the soil moisture content: at very high soil moisture content, ammonium fertilization may cause high N$_2$O emission by inducing heterotrophic denitrification, and this emission can be reduced by liming. At modest/low moisture content, ammonium fertilization induces modest...
N$_2$O emission from nitrification alone, and this is enhanced by liming.

**MATERIALS AND METHODS**

**Experimental Site and Soil Sampling**

Soils were sampled from a field trial established in autumn 2014, to test the effect of finely ground siliceous minerals as alternatives to liming. The trial is situated on the research farm of the Norwegian University of Life Sciences (NMBU) at Ås (59° 49' N, 10° 47' E, 75 m a.s.l) in southeast Norway. Mean annual temperature is 7.7°C and mean annual precipitation is 1,083 mm (Hansen and Grimnes, 2015). The field had been under crop rotation including leys since 1953, and the soil (clay loam) had not been limed since 1970. The field trial consists of six treatments: two types of calcareous lime (calcite and dolomite), and three types of siliceous rock powders (olivine, norite, and larvikite) and an untreated control. Each treatment was replicated four times and randomly distributed in two blocks (same blocks as the previous crop rotation experiment). Soil pH and content of soil organic matter measured prior to liming is given in Supplementary Table 1. Materials were applied during autumn 2014 at a rate of 30 t ha$^{-1}$ (siliceous rock powders and calcite) and 23 t ha$^{-1}$ (dolomite). The siliceous minerals were Olivine: Blueguard 63, particle size <63 µm, from the company Sibelco Nordic AS Norway; Larvikite: rock cutting dust from Lundhs AS Norway, which was further ball milled and wet sieved to achieve a particle size <63 µm; Norite: waste material from titanium enrichment process (cyclone removal of particles), from Titania AS Norway, particle size <300 µm (~50% W/W <63 µm); Calcite obtained as a suspension of colloidal particles (0.4–1.5 µm) from OMYA-Hustadmarmor AS; and Dolomite, obtained as an agricultural lime from Franzefoss A/S Norway. The mineral materials were mixed evenly into the upper 20 cm of the soil by plowing after adding half of the material, then harrowing after adding the second half. Winter wheat was sown in late autumn 2014, but failed to establish, and the field was plowed again in the late spring of 2015. barley (Hordeum vulgare, cultivar Sunita) was sown on June 5 as a cover crop together with a grass mixture (20% Phleum pratense, 25% Lolium perenne, 25% Festuca pratensis, 20% Festuca arundinacea schreb, 10% Poa pratensis L), fertilized with 100 kg N ha$^{-1}$ (mineral fertilizer, NPK 22:3:10). The cover crop was harvested after 8 weeks. Ley growth was poor in the year of establishment, but improved in subsequent years. The ley was fertilized with 270 kg N ha$^{-1}$ N$_2$O as NPK or CAN, split into 120 kg N in spring, 90 kg N after the 1st harvest and 60 kg N after the 2nd harvest. Soil samples were collected in 2015 and 2016 (during summer and late autumn), at 15, 21, and 27 months after liming. A composite sample (2–10 cm) was taken from each replicate plot by pooling at least 6 auger cores per plot. The soil samples were immediately transferred to the laboratory, sieved (2 mm), and stored in plastic bags at 4°C, until used for analyses and laboratory incubations (1–5 days after collection). To determine soil pH, 10 g soil were thoroughly dispersed in 25 mL 0.01 M CaCl$_2$ (hand shaken). The slurry was left to settle overnight, then shaken again, and finally left to settle for 15 min before measuring the pH.

**Nitrification and Denitrification Kinetics**

**Soil Slurry Experiments**

Soil slurries were used for potential nitrification and denitrification experiments. Equivalents of 4 g dry weight soil were transferred to 120 ml serum flasks and dispersed in 40 ml 1 mM NH$_4$Cl (for nitrification) or KNO$_3$ solution (for denitrification). All flasks were equipped with Teflon coated magnetic stirrers, and crimp-sealed with Teflon-coated silicone septa for nitrification experiments (to avoid inhibition of nitrification), and butyl rubber septa for denitrification experiments.

To ensure low initial NO$_3^-$ concentrations in the nitrification assay, the soils were first flooded with deionized water and drained by vacuum in 500 ml filter funnels (Millipore) with 0.45 µm filters, then transferred to the vials (4 g dry weight equivalents). The nitrification assay was performed at 23°C, while shaking the flasks horizontally on a reciprocal shaker (125 rpm) to ensure that fully oxic condition were maintained throughout the incubation. To measure N$_2$O, the bottles were removed from the shaker once or twice per day and placed for a short period in a temperature-controlled water bath (23°C) of the incubation robot (Molstad et al., 2007). The robot sampled the headspace (~1 mL) with a hypodermic needle, to determine O$_2$ and N$_2$O concentrations. After each sampling, an equal volume of He was pumped back to the headspace to keep the flask pressure at ~1 atm. We used the new version of the robot, described by Molstad et al. (2016), equipped with an Agilent GC-7890A gas chromatograph with three detectors (FID, TCD, ECD) for determining the concentrations of O$_2$, N$_2$, N$_2$O, and CO$_2$, and with a chemiluminescence detector (Teledyne NO/NO$_3^-$ analyzer mod 200E) for detection of NO. After each gas analysis, the flasks were returned to the reciprocal shaker. Oxygen concentrations in the headspace were monitored throughout, and pure oxygen was added when O$_2$ concentration fell below 16 vol% in the headspace, thus maintaining O$_2$ concentration between 15 and 20 vol%.

Denitrification kinetics were measured by frequent sampling from the headspace of 120 ml serum flasks containing 4 g dry weight soil and 40 ml of 1 mM KNO$_3$. The flasks were placed in the water bath of the incubation robot, and the slurries were stirred continuously (400 rpm). Before the incubation, all flasks were washed with He by repeated evacuation and filling. The final He overpressure was released by using a syringe without plunger containing water. The observed gas kinetics were corrected for dilution and leakage as outlined by Molstad et al. (2007). Soil pH for each replicate sample was measured at the beginning and end of the incubation, and the average of these two values was used for further calculations.

**NO$_3^-$ and NO$_2^-$ Measurements**

Nitrification rates were determined from the accumulation of NO$_3^-$ and NO$_2^-$ in the soil slurries over time. Once per day, 0.5 ml of the soil slurry was removed with a syringe and centrifuged at 10,000 g for 15 min at 4°C. NO$_3^-$ and NO$_2^-$ concentrations in the supernatant were determined immediately by colorimetry using a microplate reader (Infinite F50, TECAN Austria GmbH) at 540 nm. Both NO$_3^-$ and NO$_2^-$ were measured by Griess reaction (Keeney and Nelson, 1982),
with NO$_3^-$ being converted to NO$_2^-$ by vanadium chloride (Doane and Horwath, 2003).

**Calculations**

Nitrification rates were calculated from NO$_2^-$ + NO$_3^-$ accumulation, while denitrification rates were calculated as the sum of NO, N$_2$O and N$_2$ accumulation. N$_2$O emission potentials from each process were estimated by calculating the N$_2$O yield as N$_2$O/(NO$_2^-$ + NO$_3^-$) for nitrification and the N$_2$O production index ($I_{N2O}$) for denitrification. The N$_2$O index of denitrification was calculated as described in Liu et al. (2010).

$$I_{N2O} = \frac{\int_0^T N_2O \, dt}{\int_0^T (N_2O + N_2) \, dt}$$

where N$_2$O (t) is the accumulated flux of N$_2$O at any time t, N$_2$ (t) is the accumulated flux of N$_2$ at any time, and T is the time when a certain amount of NO$_3^-$-N kg$^{-1}$ soil was recovered as gaseous N (NO$_2^-$, NO, N$_2$O, and N$_2$). $I_{N2O}$ was calculated for two time periods, T$_{10}$ and T$_{25}$; i.e., the time point when 10 and 25 µmol NO$_3^-$, respectively, had been denitrified to gaseous N.

**Incubation Experiments With Remolded Soil**

To study nitrification and denitrification under more realistic conditions than in slurries, we conducted a series of incubations with remolded soils.

Aerobic incubation was used to study nitrification (and its N$_2$O emission): 10 g of sieved soil from each plot (15 months after liming) was added to 120 ml serum flasks, packed to a bulk density of 1 g cm$^{-3}$ and adjusted to 60% water filled pore space (WFPS). To induce nitrification, the soil was amended with a concentrated (NH$_4$)$_2$SO$_4$ solution equivalent to 110 mg N kg$^{-1}$ soil by carefully distributing the solution throughout the soil volume, using a syringe with a long needle. The flasks were incubated with air in the headspace at 20°C for 150 h, and O$_2$ consumption and N$_2$O production were monitored by the robotized incubation system described above. Nitrification was estimated from NO$_3^-$ and NO$_2^-$ accumulation in parallel offline incubation flasks covered with aluminum foil, which were subsampled periodically for determination of NO$_3^-$ and NO$_2^-$ concentrations (0.5 g soil was transferred to Eppendorf tubes with 1 ml deionized water, shaken, and centrifuged). The experiment was repeated at higher WFPS values (70 and 85%) with soils collected 27 months after liming to quantify the net N$_2$O emissions under conditions supporting coupled nitrification-denitrification, i.e., the reduction of NO$_3^-$ or NO$_2^-$ from nitrification by denitrification.

Anaerobic incubation of remolded soil was used to study denitrification: Prior to incubation, the soils were flooded with 2 mM KNO$_3$ solution and then drained, as described by Liu et al. (2010). After drainage an equivalent of 20 g dry weight soil was loosely packed into 120 ml serum flasks. Headspace gas was replaced with He. Headspace gasses were monitored as described above.

**SSU rRNA Gene Sequence Analysis**

DNA was extracted according to the protocol of Lim et al. (2016). To determine the relative proportion of AOB vs. AOA, we conducted amplicon sequencing of the SSU rRNA gene from the microbial communities in the different field plots as sampled December, 2016. Briefly, the V4 region of the 16S rRNA gene was amplified with the 515f (5′-GTGYCAGCMGGCCGCGTAA-3′) and 806rB (5′-GGACTACNVGGGTWTCTAAAT-3′) primers (Aprill et al., 2015; Parada et al., 2016) by following the Earth Microbiome Project protocol1, and amplicons were sequenced on an Illumina MiSeq instrument using a 600 cycle kit, v3 (2 × 300 bp paired-end reads). A total of 36 samples were sequenced, which includes field plots that were sequenced in triplicate (Supplementary File 1) as technical replicates. The number of sequence reads per sample ranged from 81,276 to 457,377 with a mean of 299,296 and standard deviation of 99,835. The demultiplexed FASTQ files were obtained from the sequencer and analyzed in the statistical software R$^2$ using the packages DADA2 (Callahan et al., 2016) and Phylsoseq (McMurdie and Holmes, 2013). Taxonomy of the resultant Amplicon Sequence Variants (ASVs) was established by reference to the Silva SSU rRNA database release 132 (Yilmaz et al., 2014). We then identified ammonia-oxidizers by searching for the term “nitroso” among all levels of the taxonomic hierarchy, whereby we determined that the ammonia-oxidizing Archaea consisted solely 42 ASVs in 2 classes of the phylum *Thaumarchaeota*, *Nitrosophaerida* and “Group_1.1c”. Similarly, we identified ammonia-oxidizing Bacteria as consisting of 77 ASVs within the family *Nitrosomonadaceae* within the phylum *Gammaproteobacteria*. The abundances of these ASVs in the different samples were tallied to obtain the relative abundance of AOB and AOA and thus to determine the AOB/AOA ratio. To identify nitrite oxidizing bacteria, we similarly searched for their taxonomic descriptors and identified *Nitrospira*, *Nitrobacter*, and *Candidatus* Nitrotoga as present in the dataset. A total of 20 Nitrosira ASVs were detected while only one sample contained one Nitrobacter ASV and another sample contained a single *Candidatus* Nitrotoga ASV.

1http://www.earthmicrobiome.org/emp-standard-protocols/16s/

| Treatment | Soil pH$_{CaCl_2}$ | ΔpH |
|-----------|--------------------|-----|
| Control   | 5.01 ± 0.05        | –   |
| Larvikite | 5.02 ± 0.04        | 0.01 ± 0.01 |
| Norite    | 5.12 ± 0.04        | 0.11 ± 0.02 |
| Olivine   | 5.17 ± 0.03        | 0.16 ± 0.03 |
| Dolomite  | 5.55 ± 0.04        | 0.58 ± 0.04 |
| Calcite   | 6.62 ± 0.06        | 1.57 ± 0.08 |

The table shows the average soil pH$_{CaCl_2}$ (n = 14 ± SE) throughout the 2 years after incorporation of lime and minerals, and the average increase in pH by each mineral treatment relative to the control (ΔpH). Pairwise t-tests for difference between mineral treatments and control (ΔpH) showed significant effects (p < 0.05) for all minerals except Larvikite. Fluctuations in pH and ΔpH throughout the 2 years are shown in Supplementary Figure 1.
FIGURE 1 | Denitrification in soil slurries. NO and N$_2$O are shown as measured, while N$_2$ is the cumulative production (measured values corrected for sampling loss, see Molstad et al., 2007). Plotted values are means and standard errors of four field-replicates for each treatment, sampled 27 months after mineral applications in the field experiment. Samples taken 15 months after mineral treatment showed very similar response to soil pH whereby the N$_2$O production index (I$_{N_2O}$) declined with soil pH. I$_{N_2O}$ for individual soil samples in both experiments are shown in Supplementary Figure 4.
Accession Numbers
The sequence data analyzed in this study is available for download at the Sequence Read Archive (SRA) under BioProject accession PRJNA541961, which corresponds to BioSample accessions SAMN12414160 to SAMN12414195 and SRA accessions SRS5197145 to SRS5197180.

RESULTS
Soil pH
Average soil pH measured in the field plots throughout the 26 months after application of lime and siliceous minerals are shown in Table 1. The pH increased 1.57 units in the calcite treatment, 0.58 in the dolomite, 0.16 in the olivine and 0.11 in the norite treatment. Larvikite had no significant effect on the soil pH (p = 0.12 for pairwise t-test of larvikite versus control). The calcite effect decreased over time by 0.5 units (Supplementary Figure 1).

Denitrification Kinetics in Anoxic Slurry Incubations
The gas kinetics in anoxic slurries of soils sampled 27 months after incorporation of lime and rock powder (Figure 1) showed transient accumulation of both NO and N₂O which clearly declined in magnitude with increasing soil pH, more dramatically so for N₂O than for NO. Stable plateaus of N₂, reached after ~60 h indicated depletion of N oxides since the levels largely accounted for the initial amount of NO₃⁻-N present in the flasks (~14 mmol N kg⁻¹; the soil contained ~4 mmol NO₃⁻-kg⁻¹). Table 2 summarizes the maximum denitrification rates, maximum NO and N₂O accumulation, and the N₂O index (Iₙ₂₀) for incubation experiments carried out with soils sampled 15, 21, and 27 months after liming. Denitrification rates were lowest in samples taken during winter 2015 (the year of ley establishment), and largest in summer of the first production year, after which they decreased again in the following winter. Denitrification rates exhibited a slight but statistically significant increase with soil pH (Supplementary Figure 3), while the transient accumulation of NO declined with pH. The influence of soil pH on the N₂O/(N₂O + N₂) ratio of denitrification was examined by plotting the N₂O index (Iₙ₂₀) across pH, revealing a marked decrease with increasing pH (Supplementary Figure 4).

Nitrification and N₂O Yield in Oxic Slurry Incubations
Figure 2 shows the kinetics of oxygen consumption, transient accumulation of NO⁻⁻ and the accumulation of N₂O + NO⁻⁻ in oxic slurries of soils sampled in December 2015 and 2016 (15 and 27 months after incorporation of lime and siliceous minerals). Similar kinetics were observed for soils sampled 21 months after liming and Table 3 summarizes essential variables for all three sampling dates. The oxygen consumption rate was largest in calcite and dolomite treated soils, followed by olivine.

| Treatment | Denitrification rate μmol N kg⁻¹ h⁻¹ | Max NO μmol N kg⁻¹ | Max N₂O μmol N kg⁻¹ | Iₙ₂₀ |
|-----------|-----------------------------------|--------------------|---------------------|-----|
| December 2015 (15 months) | | | | |
| Control | 69 ± 3 | 46 ± 6 | 1749 ± 351 | 0.22 ± 0.04 |
| Larvikite | 63 ± 3 | 41 ± 3 | 906 ± 185 | 0.11 ± 0.03 |
| Norite | 60 ± 5 | 47 ± 4 | 1390 ± 428 | 0.20 ± 0.07 |
| Olivine | 69 ± 6 | 45 ± 7 | 1460 ± 451 | 0.19 ± 0.05 |
| Dolomite | 90 ± 6 | 28 ± 2 | 247 ± 77 | 0.03 ± 0.01 |
| Calcite | 91 ± 7 | 16 ± 1 | 5 ± 1 | <0.01 |
| July 2016 (21 months) | | | | |
| Control | 376 ± 18 | 40 ± 4 | 5724 ± 665 | 0.33 ± 0.04 |
| Larvikite | 354 ± 29 | 40 ± 1 | 5384 ± 853 | 0.26 ± 0.05 |
| Norite | 409 ± 20 | 43 ± 3 | 5886 ± 1454 | 0.22 ± 0.02 |
| Olivine | 398 ± 9 | 38 ± 3 | 2844 ± 832 | 0.18 ± 0.04 |
| Dolomite | 424 ± 15 | 35 ± 3 | 224 ± 54 | 0.10 ± 0.03 |
| Calcite | 413 ± 7 | 27 ± 2 | 111 ± 74 | 0.01 ± 0.00 |
| December 2016 (27 months) | | | | |
| Control | 204 ± 15 | 39 ± 8 | 3629 ± 748 | 0.42 ± 0.04 |
| Larvikite | 191 ± 17 | 35 ± 11 | 3201 ± 893 | 0.34 ± 0.06 |
| Norite | 207 ± 12 | 35 ± 4 | 3627 ± 539 | 0.44 ± 0.08 |
| Olivine | 255 ± 23 | 26 ± 4 | 1421 ± 870 | 0.18 ± 0.10 |
| Dolomite | 263 ± 26 | 27 ± 4 | 265 ± 102 | 0.04 ± 0.01 |
| Calcite | 293 ± 33 | 18 ± 2 | 97 ± 60 | <0.01 ± 0.00 |

Denitrification rates (μmol N kg⁻¹ soil h⁻¹) are given as average values (n = 4, ±SE) for the first 50 h of incubation. Maximum amounts of NO and N₂O are given as μmol N kg⁻¹ soil. The N₂O index (Iₙ₂₀) is a proxy for the propensity of N₂O emission from denitrification calculated by equation (1) for the period until the total amount of gaseous N (NO + N₂O + N₂) accumulation reaches the specific limit indicated by the subscript (Iₙ₂₀*: 25 μmol N-gas vial⁻¹ = 6.3 mmol N-gas kg⁻¹ soil). Rates and Iₙ₂₀ for individual soil samples in both experiments are shown in Supplementary Figures 3, 4, respectively.
FIGURE 2 | Nitrification in oxic soil slurries. Oxygen consumption (cumulative), and production of N₂O nitrite and nitrate oxic soil slurries. Plotted values are the means and standard errors of four field replicates of each mineral treatment. Plotted gas kinetics are from two incubations experiments with soil collected from the field December 2015 (Panel A) and 2016 (Panel B) (15 and 27 months after application of minerals).

(p < 0.0005), whereas larvikite and norite treatments were indistinguishable from the control.

The NH₄⁺ oxidation rates (measured as NO₂⁻ + NO₃⁻ accumulation) increased linearly with pH (Figure 3). NH₄⁺ was evidently not depleted during the incubation, except for the calcite-amended soil sampled 15 months after mineral application (Figure 2: the NO₃⁻ plateaus reached after 60–90 h match the initial amounts of NH₄⁺, which was 1.4 mmol NH₄⁺ kg⁻¹). Transient accumulation of NO₂⁻ was substantial in the high pH soils (calcite > dolomite), whereas it was marginal in the other treatments and was found to increase exponentially with pH (Supplementary Figure 4). The calculated N₂O yield (Y_N₂O) ranged from 0.09 to 0.34% and was significantly higher in the calcite and dolomite treatments than in the other treatments.
Assuming that the oxidation of 1 mole of NH₃ to NO₃⁻ consumes 2 moles of O₂, the measured nitrification accounted for 20–90% of the measured oxygen consumption rate (consistently highest for calcite).

**Anoxic Incubations of Remolded Soil**

For loosely remolded soil sampled 27 months after liming and incubated under fully anoxic conditions, denitrification gas kinetics exhibited similar N gas accumulation patterns as observed in the anoxic soil slurries. Calcite and dolomite treated soils accumulated less N₂O than any other treatment (Figure 4). Denitrification rate, maximum N₂O and N₂O index (Y N₂O) in remolded soil experiments are shown in Table 4. Denitrification rates were calculated for the period 0–50 h of incubation, and the N₂O index for the time point when 500 μmol N kg⁻¹ soil was recovered as NO + N₂O + N₂-N.

**Oxic Incubations of Remolded Soils**

The O₂ consumption rates, NH₄⁺ oxidation rates, maximum NO₂⁻ concentrations, and the N₂O yields for these incubations are all summarized in Table 5, and the N₂O kinetics is shown in Figure 5A. The O₂ consumption rate increased gradually with increasing soil moisture content: the average O₂ consumption rates (all treatments) were 75, 106, and 153 μmol O₂ kg⁻¹ soil h⁻¹ at 60, 70, and 85% WFPS, respectively. The higher O₂ consumption rate at 70% than at 60% WFPS likely reflects a higher concentration of available organic carbon: soils used for the 60% WFPS experiments were sampled in December 2015, while the 70 and 85% WFPS experiments used soil samples taken in December 2016, when the ley was fully established. This is confirmed by oxygen consumption rates measured in oxic soil slurries (Table 3): the average rates for the December samples were 230 and 274 μmol O₂ kg⁻¹ soil h⁻¹ in 2015 and 2016, respectively, i.e., a 20% increase from 2015 to 2016.

The percentage of O₂ consumption theoretically accounted for by NH₃ oxidation (assuming 2 mol O₂ consumed per mol NH₄⁺ oxidized) varied between 20 and 40% at 60% WFPS, which is somewhat lower than in the soil slurries. For the 85% WFPS treatment, much lower percentages were calculated, reflecting that NO₃⁻ consumption by denitrification resulted in grossly underestimated nitrification rates.

On average, the estimated N₂O yield (Y N₂O) at 70% WFPS was twice as high as at 60% WFPS (0.088 versus 0.043%, Table 5). The Y N₂O values for 70% WFPS are uncertain, however, since they are based on the assumption that nitrification rates were the same as at 60% WFPS. The 85% WFPS treatment differed markedly from the other two moisture levels in that O₂ consumption rates were larger, rates of NO₂⁻ + NO₃⁻ accumulation were lower, and N₂O production rates were two orders of magnitude higher. The apparent N₂O yields calculated for the initial 75 h (Table 5) were high, ranging from 2 to 15%, and approximately twice as high if calculated for the first 45 h of incubation, except for calcite, for which the N₂O production was low compared to the others, and more constant throughout. The pH response of the N₂O production rate in the 85% WFPS treatment differed fundamentally from those of the lower WFPS treatments (Figure 5B): while the N₂O production rates increased with pH at 60 and 70% WFPS, they declined sharply at 85% WFPS.

### Table 3 | Rates of O₂ consumption, ammonium oxidation, N₂O production, NO₂⁻ accumulation and N₂O yield (Y N₂O) in oxic slurry incubations.

| Treatment | O₂ μmol kg⁻¹ h⁻¹ | NH₄⁺ oxid. μmol N kg⁻¹ h⁻¹ | N₂O prod. μmol N kg⁻¹ h⁻¹ | max NO₂⁻ μmol N kg⁻¹ | Y N₂O (%) |
|-----------|------------------|-----------------------------|-----------------------------|----------------------|-------------------|
| **December 2015 (15 months)** | | | | | |
| Control   | 176 ± 19         | 41 ± 6                      | 0.04 ± 0.01                 | 25 ± 7               | 0.09 ± 0.01       |
| Larvikite | 181 ± 8          | 61 ± 2                      | 0.05 ± 0.01                 | 31 ± 3               | 0.08 ± 0.01       |
| Norite    | 168 ± 16         | 51 ± 5                      | 0.05 ± 0.01                 | 36 ± 5               | 0.10 ± 0.00       |
| Olvine    | 238 ± 20         | 69 ± 6                      | 0.07 ± 0.01                 | 110 ± 66             | 0.10 ± 0.01       |
| Dolomite  | 276 ± 23         | 86 ± 9                      | 0.14 ± 0.01                 | 471 ± 133            | 0.14 ± 0.01       |
| Calcite   | 340 ± 57         | 165 ± 28                    | 0.25 ± 0.04                 | 2687 ± 550           | 0.15 ± 0.01       |
| **July 2016 (21 months)** | | | | | |
| Control   | 189 ± 22         | 32 ± 3                      | 0.04 ± 0.00                 | nd††                 | 0.12 ± 0.00       |
| Larvikite | 198 ± 47         | 31 ± 3                      | 0.05 ± 0.01                 | nd                   | 0.15 ± 0.00       |
| Norite    | 207 ± 24         | 38 ± 4                      | 0.05 ± 0.01                 | nd                   | 0.14 ± 0.01       |
| Olvine    | 198 ± 20         | 49 ± 4                      | 0.10 ± 0.01                 | nd                   | 0.19 ± 0.01       |
| Dolomite  | 249 ± 9          | 77 ± 3                      | 0.22 ± 0.01                 | 423 ± 78             | 0.28 ± 0.03       |
| Calcite   | 269 ± 53         | 99 ± 2                      | 0.35 ± 0.04                 | 1461 ± 135           | 0.34 ± 0.06       |
| **December 2016 (27 months)** | | | | | |
| Control   | 213 ± 26         | 24 ± 5                      | 0.04 ± 0.01                 | 1 ± 1                | 0.14 ± 0.01       |
| Larvikite | 216 ± 27         | 31 ± 5                      | 0.05 ± 0.00                 | 17 ± 12              | 0.16 ± 0.02       |
| Norite    | 246 ± 13         | 39 ± 2                      | 0.06 ± 0.00                 | 23 ± 7               | 0.13 ± 0.01       |
| Olvine    | 281 ± 25         | 44 ± 4                      | 0.07 ± 0.01                 | 71 ± 22              | 0.15 ± 0.01       |
| Dolomite  | 343 ± 11         | 64 ± 5                      | 0.10 ± 0.00                 | 411 ± 107            | 0.16 ± 0.01       |
| Calcite   | 347 ± 22         | 84 ± 7                      | 0.13 ± 0.01                 | 1563 ± 66            | 0.18 ± 0.03       |

Data were averaged over the entire incubation period. Results for three experiments with soil sampled 15, 21, and 27 months after incorporation of lime and rock powders. Presented values are means of four field replicates and standard error. Maximum NO₂⁻ concentrations for individual field plot samples plotted against soil pH are shown in Supplementary Figure 4. †NH₄⁺ oxid., ammonium oxidation rate; N₂O prod., N₂O production rate. ††nd, not detected.
AOA, AOB, and Nitrite Oxidizer Abundances

The relative abundance of AOA-, AOB-, and NOB-SSU in the overall bacterial community, as indicated by SSU rRNA amplicon sequencing, are shown in Figure 6. AOA identified in the samples consisted of 42 ASVs in the phylum Thaumarchaeota including the genera Candidatus Nitrosotalea, Nitrososphaera and Nitrocosmicus, as well as undescribed taxa within the phylum (Supplementary File 1). The identified AOB consisted of 77 ASVs from the family Nitrosomonadaceae within the phylum Proteobacteria and consisted of the genera Nitrosomonas and Nitrosospira, as well as several uncharacterized genera (Supplementary File 1). We also identified SSU rRNA genes affiliated to the phylum Nitrospira and the genus Nitrospira, which are nitrite oxidizers (Supplementary File 1 and Supplementary Figure 8). Regression analyses showed that the relative abundances of AOB and NOB increased significantly with soil pH ($p < 0.001$ for both), while AOA declined with pH ($p = 0.02$), and the AOB/AAO abundance ratio increased with pH ($p < 0.001$). SSU rRNA identified as Nitrospira may include comammox, i.e., organisms that oxidize ammonium to nitrate (Daims et al., 2015), but the ability of a given Nitrospira to carry out comammox as opposed to only nitrite oxidation may only be discerned by analysis of their functional genes (Daims et al., 2015). Hence, our SSU rRNA analysis cannot discern whether the Nitrospira ASVs detected are simple NOB or comammox.

DISCUSSION

It has become increasingly clear that the reason why emissions of N$_2$O from soils increase with acidity (Wang et al., 2018) is that the synthesis of functional N$_2$O reductase is impeded by low pH (Liu et al., 2014). Therefore, liming of acidic soil should reduce N$_2$O emissions and would justify the use of high doses of lime/biochar to mitigate these emissions, hence reducing the climate forcing that occurs as a result of fertilizer use in crop production. In theory, this beneficial effect of liming on climate forcing could be outweighed by emission of carbonate-CO$_2$,
FIGURE 4 | Gas kinetics of denitrification in remolded soil under anoxic conditions. Plotted values are the means and standard error of four field replicates. Soil samples were collected in December 2016 (27 months after application of minerals).
Nevertheless, the emission of carbonate-CO$_2$ has been contested (Hamilton et al., 2007; Page et al., 2009). The weathering of siliceous minerals will increase the pH of acidic soils, but our results so far are discouraging: while the carbonate materials caused a substantial increase in soil pH, the weathering rate is proportional to the surface area, but the cost would be prohibitively high (van Noort et al., 2018).

N2O emission from denitrification typically occurs during hypoxic/anoxic spells and is caused by transiently high soil moisture, high oxygen consumption rates, or both. The N$_2$O emissions during such "hot events," or in "hot spots" if localized in microsites with high oxygen consumption rates (Schlüter et al., 2019) depends on the enzyme kinetics: early/efficient synthesis of active N$_2$O reductase secures a low N$_2$O/(N$_2$ + N$_2$O) product ratio, hence minimizing emission of N$_2$O, whereas delayed synthesis will lead to a high N$_2$O product ratio and emission. Since the synthesis of functional N$_2$O reductase is severely impeded/delayed by low pH (Liu et al., 2014),...
the emission of N$_2$O will decrease with increasing soil pH. Anoxic soil incubations are commonly used to mimic anoxic spells, and such experiments have invariably shown that the transient accumulation of N$_2$O is controlled by pH (Čuhel and Šimek, 2011; Raut et al., 2012; Qu et al., 2014). The present result is no exception: increasing the pH by liming clearly lowered the N$_2$O/(N$_2$O + N$_2$) product ratio during anaerobic incubations of both soil slurries (Figure 1 and Table 2) and remolded soil (Figure 4). Thus, our findings corroborate previous investigations of denitrification as affected by pH. Our main aim, however, was to investigate the effect of liming on both denitrification and nitrification, and their contribution to N$_2$O emission.

Based on Hink et al. (2018) we hypothesized that increasing the pH by using lime or siliceous minerals would increase the potential nitrification rates in the soil, and selectively stimulate bacterial (AOB) over archaeal ammonia oxidizers (AOA). We further hypothesized that the N$_2$O yield of nitrification (Y$_{N2O}$) would increase with soil pH (as observed previously by Markved et al., 2007), because AOB have an inherently higher Y$_{N2O}$ than AOA (Hink et al. 2017a). The SSU rRNA gene quantification corroborated the hypothesis regarding selective stimulation of AOB (Figure 6). The effect of soil pH on AOA and AOB abundances has also been studied by Hu et al. (2013), who observed that the qPCR-determined ratio of AOA/AOB decreased with increasing soil pH, and hence the inverse of that ratio, or
the AOB/AOA ratio increases, which supports our findings (Figure 6). The AOA taxa included several candidate genera as well as a number of uncharacterized taxa, suggesting that there remains considerable diversity to be explored among AOA, whereas for the AOB, all the ASVs were from the Nitrosomonadaceae and mostly affiliated with named genera, although most were apparently uncultivated phyotypes such as "mle1-7," "MND1," etc. (Supplementary File 1). In addition, the slurry experiments confirmed the effect on nitrification rates and N₂O yields (Figure 3). The soil slurry experiments were designed to secure oxic conditions, thus eliminating denitrification as a source of N₂O. This is important, because it means that the N₂O produced in these experiments largely reflects the inherent N₂O production by the ammonia oxidation pathways. A similar increase in abundance of the nitrite-oxidizing *Nitrospira* (Supplementary Figure 8) with increasing pH was observed by Rousk et al. (2010), although in that study the r² between pH and *Nitrospira* relative abundance was 0.16 with P = 0.20.

We further hypothesized that the enhancement of nitrification by liming would increase the risk that the oxygen consumption by nitrification creates hypoxic/anoxic microsites in soil, thus inducing heterotrophic denitrification. This would be aggravated by high soil moisture content (Schurgers et al., 2006). The results with remolded soils shed some light on this (Table 5): at low soil moisture content (60 and 70% WFPS), Y₂N₂O was lower than in the soil slurries, but increased with soil pH (as in the soil slurries) thus suggesting that nitrification was the main dominating process. At high soil moisture content (85% WFPS), the apparent Y₂N₂O was 2–3 orders of magnitude higher than in the low moisture (60 and 70% WFPS) experiments, plausibly because oxygen consumption by nitrification along with high WFPS resulted in hypoxic/anoxic microsites and hence denitrification. This N₂O-production by denitrification could be expected to be promoted by high soil pH because of the higher nitrification rate (providing NO₂⁻ and NO₃⁻ for denitrification), but the opposite was the case; the N₂O production rates clearly declined with soil pH. Our interpretation of this finding is that, while denitrification was induced at all pH levels, the N₂O/(N₂ + N₂O) product ratio declined with increasing soil pH due to more efficient synthesis of functional N₂O reductase at high pH (Liu et al., 2014). This necessarily implies that heterotrophic denitrification, rather than *nitrifier denitrification* must have been dominating, because ammonia oxidizing bacteria lack the gene for N₂O-reductase. A recent study of the electron flow to denitrification in ammonia oxidizing bacteria support this interpretation, showing that although the electron flow to nitrite- and nitric oxide reductase in these organisms increased in response to hypoxia, it never amounted to more than 1.2% of the total electron flow (Hink et al., 2017b). This finding lends little support to the common notion that *nitrifier denitrification* is a
strong source of N₂O in soils under partially hypoxic conditions (Zhu et al., 2013; Mushinski et al., 2019). As argued elsewhere (Bakken and Frostegård, 2017), the evidence for significant N₂O production by nitrifier denitrification is in fact rather weak. The notion emerged as a result of circumstantial evidence (co-occurrence, and selective inhibition of ammonia oxidation), and later by employing ¹⁸O/¹⁵N tracing to differentiate between N₂O from nitrifier denitrification and heterotrophic denitrification (Wrage-Mönning et al., 2018). The differentiation is based on the assumption that nitrite produced by ammonia oxidizers is exclusively reduced by the ammonia oxidizers themselves, because heterotrophs prefer nitrate. This is highly improbable, however: a fraction of the denitrifying heterotrophs carrying nitrite reductase lack nitrate reductase (Lycus et al., 2017), and those with nitrate reductase will reduce external nitrite along with nitrate because nitrite is a free intermediate that is reduced outside the cytoplasmic membrane. This, together with the fact that heterotrophic denitrifiers grossly outnumber ammonia oxidizing organisms, and that nitrifiers allocate miniscule fractions of the electron flow to denitrification (Hink et al., 2017a), suggests the opposite: under hypoxic conditions the nitrite produced by ammonia oxidizers is more likely to be reduced by heterotrophs than by ammonia oxidizers themselves.

Our results are important because they explain why increasing the pH of acidic soils (by lime or biochar) has a contingent effect on their N₂O emission. Highly variable effects may be expected for systems/conditions where N₂O emission is driven by high ammonium oxidation rates such as urine patches (Clough et al., 2004; Carter, 2007; Khan et al., 2011), while consistent reduction of the N₂O emission can be expected for systems/conditions where heterotrophic denitrification is dominant. Laboratory experiments may be designed to isolate nitrification- and denitrification driven N₂O emissions, but in agronomically realistic field experiments, the contributions of nitrification and denitrification merge and fluctuate (Russenes et al., 2016), being further modulated by a plethora of other factors (Butterbach-Bahl et al., 2013; Saggar et al., 2013; Rochette et al., 2018). As a result, the net effect of pH management on N₂O emissions in single field experiments is to some extent anecdotic, and the evaluation of pH management as a mitigation option should be based on ensembles of well-designed field experiments. Unfortunately, most of the existing emission data are from field experiments designed to test other factors than pH. Nevertheless, a recent meta-study by Wang et al. (2018), which used emission data from 117 studies worldwide, demonstrated an overall reduction of N₂O emission by increasing soil pH, creating a basis for recommending liming for mitigating N₂O emissions from acidified soils.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

**AUTHOR CONTRIBUTIONS**

SN: experimental design and writing, did the laboratory work, and analyses of kinetics. LB: experimental design and writing. ÅF: molecular analyses and writing. JG: bioinformatic analyses and writing. PD: experimental design and writing. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.598513/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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