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Can pH amendments in grazed pastures help reduce N_2O emissions from denitrification? – The effects of liming and urine addition on the completion of denitrification in fluvial and volcanic soils

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ABSTRACT

Soil pH plays a critical role in determining the overall rate of several important processes in the agricultural nitrogen cycle. During denitrification, the activity of nitrous oxide reductase (N_2O-R) is reduced at low pH. This effect has led to suggestions that soil pH adjustment via liming to enhance the activity of this enzyme might be a viable agricultural greenhouse gas mitigation strategy by enhancing the reduction of N_2O in the soil to climatically inert N_2. We assessed the effect of liming on the apparent activity of N_2O-R by measuring the denitrification end products, N_2O and N_2, in a series of short-term anaerobic incubations. We compared a weakly-buffered fluvial soil and a well-buffered, volcanic soil under different incubation temperatures and in the presence or absence of a ~600 kg ha^{-1} cow urine-N amendment. Our results indicated that the liming effect was heavily modulated by soil type, temperature, and urine amendment. Liming (at rates of 1.5 and 3.0 t ha^{-1} for the volcanic soil and at rates of 5 and 10 t ha^{-1} for the fluvial soil) caused pH increases of between 0.43 and 1.25 pH units. The highest reductions in N_2O in the fluvial soil occurred when the 1.5 t ha^{-1} rate was used in the fluvial soils under urine addition and at the higher temperature. The combined flux of $N_2O + N_2$ did not change with liming. However, an interaction of soil type and urine amendment caused large differences in the partitioning of the denitrification end-products between N_2O and N_2 – an effect that overwhelmed the relatively modest effects of liming. When the soils were amended with urine-N, the resulting denitrification gases from the volcanic soil were mostly in the form of N_2O (60–77%), whereas in the fluvial soil the denitrification products were mostly in the form of N_2 and a much smaller portion were in the form of N_2O (11–45%). Nevertheless, we found liming-induced enhancements of N_2O-R of 15–20% ($P < 0.05$) in urine-amended, fluvial soil. We suggest some possible mechanisms that would explain such large differences in the $N_2O/(N_2O + N_2)$ product ratio.

1. Introduction

In intensively grazed livestock farming animal excreta in the form of dung and urine comprise a major part of soil's nitrogen (N) input (de Klein et al., 2003), in quantities normally in excess of what is assimilated by plants. The resulting surplus N is subject to loss via three major mechanisms: volatilisation, leaching, and denitrification (Ball et al., 1979; Bolan et al., 2004; Monaghan et al., 2007). Nitrous oxide (N_2O) emitted from pastoral soils in New Zealand is most likely to be derived from denitrification, rather than nitrification (Saggar et al., 2009). Research directed towards mitigating N_2O emissions from pastoral agriculture has mainly focused on inhibiting nitrification (Monaghan et al., 2013), with the joint aim of both reducing emissions of N_2O from nitrification and minimising the production of nitrate, the initial substrate for denitrification. However, nitrification inhibitors are not 100% effective especially when soil temperatures

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are high (Kelliher et al., 2008), and moreover, their commercial use has been discontinued due to export sensitivities associated with the detection of dicyandiamide traces in New Zealand dairy exports (Archer, 2013). It is therefore worthwhile to investigate other approaches for N_2O mitigation, including methods to minimise the N_2O emissions from denitrification.

The reduced denitrifying activity of soils under low pH has been understood for several decades, but it is not clear that a universally optimal pH exists for denitrification (Simek and Cooper, 2002). The advent of the acetylene inhibition technique has allowed estimation of the relative production rates of the denitrification end-products, N_2O and N_2, and there is now a general acceptance that the product ratio, defined here as $N_2O/(N_2O + N_2)$, is also larger under acidic conditions (Liu et al., 2010; Saggar et al., 2013). In the highly fertilised soils of China, pH has been identified as the master variable controlling the product ratio (Qu et al., 2014). The increased amounts of fertiliser-N usage and animal urine deposition associated with agricultural intensification leads to an acidification of soil and pH levels that are increasingly suboptimal for N_2O-R activity.

The greater pH sensitivity of N_2O-R, relative to other denitrifying reductase enzymes, has previously thought to have been because the enzyme is located in the periplasm of the cell – a poorly buffered region in Gram-negative bacteria (Simek et al., 2002). Recent work has indicated that it is the translation or assembly of N_2O-R, rather than transcriptions rates of the encoding gene, which is sensitive to pH (Liu et al., 2010). However, the effect of pH on soil microbial ecology is multifarious. Fungal communities tend to predominate at lower pH and denitrifying fungi have been reported to be responsible for an increasing portion of the N_2O flux as soil becomes more acidic (Chen et al., 2014). Since fungi are not known to synthesise N_2O-R, this may be an additional reason for elevated N_2O fluxes at low pH.

The possibility that pH-manipulation of soils through liming is a viable N_2O mitigation strategy has been raised several times previously (Clough et al., 2003; Zaman et al., 2007; Galbally et al., 2010; Bakken et al., 2012), but its effect on the total flux of N_2O from the soil is not clear. Both nitrification and denitrification can be enhanced at higher pH (Simek and Cooper, 2002) and therefore the mitigation benefit of reducing the $N_2O/(N_2O + N_2)$ product ratio must be weighed against the increased bulk N_2O emissions that might result from a stimulation of either of these processes. Additionally, since the practice of liming is a known source of carbon dioxide (CO_2) emissions (De Klein et al., 2006), this must ultimately be incorporated into the cost-benefit of lime as a mitigation strategy.

The influence of liming on N_2O production and consumption from denitrification is likely to vary according to soil temperature, the level and type of N input, and the buffering capacity of the soil. Temperature affects the various N transformations in a differential manner. Quite different Q_{10} values (i.e. the net change in gas production per 10 °C increase in temperature) for N_2O production compared with N_2O consumption have been reported recently (Phillips et al., 2014).

The extent to which liming changes N_2O losses will vary according to soil type. For example, volcanic soils with high allophane content have a high buffering capacity and require more lime to produce a given pH increase compared with poorly buffered, fluvial soils.

In intensively grazed pasture soils, the majority of the N_2O emitted is derived from the N deposited as animal urine that causes very high local N inputs of typically ~600 kg ha^{-1} (Selbie et al., 2015) in patches that cover 2–5% of area after each grazing (Moir et al., 2011). In addition to the marked increase in N content, urine deposition causes large changes to the physical and chemical properties of the affected soil including a marked but temporary elevation in pH due to urea hydrolysis (of up to 3–5 days), increases in the soil moisture content and increases in the available carbon (C) (Selbie et al., 2015). Therefore, urine presence is likely to heavily modulate a soil's response to lime. We might expect this effect to be particularly pronounced in volcanic soils due to the highly pH-dependent nature of their cation exchange capacity (Holland and During, 1977).

Here, we investigated how liming influences N_2 and N_2O emissions from a volcanic and a fluvial soil under anaerobic conditions, and compare these effects at different temperatures, with and without an amendment of dairy cow urine. Our hypotheses were:

1. The liming-induced pH increases will lower the N_2O production;
2. The liming-induced pH increases will increase the overall rate of the denitrification end products, N_2O and N_2;
3. The liming-induced pH increases will lower the product ratio (irrespective of the changes in the bulk production rates of N_2O or N_2;
4. This liming effect on N_2O and N_2 production will be modulated by soil type, incubation temperature and the presence of urine.

Thus to test these hypotheses we investigated the response of denitrifiers to interacting effects of temperature, soil and urine-amendment; second, utilizing a methodology that achieves simultaneous, automated and direct N_2O and N_2 measurements, incorporating an extended pH adjustment period to allow the soils to equilibrate to the liming treatments.

2. Methods and materials

2.1. Soils

The soils from the permanent pastures of two farms were used in this study. One soil was a weakly-buffered, non-allophanic fluvial sandy loam from a farm near Massey University, Palmerston North (40° 22′ 60″ S, 175° 36′ 37″ E) while the other was a strongly buffered, allophanic volcanic silt loam from a farm near Hamilton, New Zealand (40° 46′ 40″ S. 175° 18′ 46″ E). The fluvial soil is classified as a weathered fluvial recent soil in the New Zealand Soil Classification System (Hewitt, 1992). It is a well-drained soil derived from slowly accumulating alluvium and has low allophane content (Cowie and Rijkse, 1977), and is hereafter referred to as the fluvial soil. The volcanic soil is classified as a Typic Orthic Allophanic soil. It is a well-drained soil derived from largely volcanic alluvium and has high allophane content (Singleton, 1991), and is hereafter referred to as the volcanic soil. Relevant properties of each of these soils are given in Table 1.

Field fresh soil samples (0–100 mm depth) were collected by taking multiple replicate cores from randomly assigned locations in each farm. A total quantity of 10–12 kg was collected from each farm, and the soil was bulked, sieved to 4 mm, and stored at 4 °C.

Table 1
Relevant properties of soil used in this study.

Property	Soil	
	Fluvial	Volcanic
Soil texture class	Sandy loam	Silt loam
Cation exchange capacity ($cmol_c\ kg^{-1}$)	13.0	35.6[a]
Organic C content ($mg\ g^{-1}$)	44.6	62.7
Bulk density ($g\ cm^{-3}$)	1.06	1.10

[a] Volcanic CEC values from Srinivasan et al. (2013).

2.2. Lime treatments

Field replicate sieved soil samples of both soils were initially treated with Ravensdown Fine Lime (Ravensdown, Christchurch, New Zealand) at four rates of liming (0, 5, 10, and 20 t lime ha^{-1}) to change the pH by 1–1.5 units. The fine lime has a high neutralising value (85–90% calcium carbonate content) and a small particle size (>95% less than 100 micron) compared with normal agricultural lime (>95% less than 2 mm). However, these rates caused excessive pH increases in the fluvial soil. Thus the two new liming rates of 1.5 and 3.0 t ha^{-1} were created to achieve pH adjustments of 0.4–1.3 pH units. Liming rates for subsequent laboratory incubations were 0.0, 1.5 and 3.0 t ha^{-1} for the fluvial soil and 0.0, 5.0 and 10.0 t ha^{-1} for the volcanic soil. The liming pre-incubation period continued for 180 days. The treated soils were thoroughly mixed twice weekly till the pH levels of the soils had stabilised. Soil moisture was maintained at desired gravimetric moisture contents of 35% and 50% for the fluvial and volcanic soils respectively.

2.3. Experimental design

The effect of liming on the rate of N_2O and N_2 production was measured during anaerobic incubations of the two contrasting soils under combinations of experimental conditions including the presence or absence of a cattle urine amendment and two different incubation temperatures (10 °C and 15 °C, intended to represent typical soil temperatures for New Zealand soils). The effects of soil type, urine presence/absence, and incubation temperature were assessed in a partial factorial design, and only certain combinations of incubation temperature and urine amendment could be tested in a single incubation due to a need to keep the incubation at one fixed temperature and a maximum number of vial positions on the sample rack fixed at 25 (including calibration standards).

The experimental design for the anaerobic incubations is illustrated in Table 2.

2.4. Anaerobic incubations in a N_2-free atmosphere

Twenty g dry weight equivalent of each soil was placed in 0.125 L serum vials (Sigma Aldrich, Part No. 98334, Milwaukee, WI, USA) and wetted to saturation, either with deionised water or with freshly collected cattle urine.

Immediately following wetting of the soil, the vials were capped with PTFE/butyl rubber septa (Grace Discovery, Part No. 95584, Auckland, New Zealand) with the butyl rubber surface facing down and lightly greased with silicone vacuum grease. They were then evacuated to 0.06 kPa and refilled with ultra-pure helium (99.9999%) to atmospheric pressure (100.7 kPa). The evacuation and backfill with helium was repeated a further two times. Generally, less than 1 h had passed between the imposition of anoxia and the analysis of the first vial so that the opportunity for the denitrifiers to develop enzymes was minimised.

Using exactly the same evacuation and refilling procedure, vials containing helium blanks, and N_2 standards of 500 ppm and 2000 ppm were also prepared.

Table 2
The experimental design: Two soils (Fluvial, volcanic) were incubated at two temperatures (10 °C, 15 °C) with 2 amendment conditions (no urine/added urine 600 kg Urine-N ha^{-1} equivalent) and three liming treatments (No Lime, Lime 1, Lime 2). This design resulted in 24 possible treatment combinations. Three experimental replicates were prepared for each of the 24 possible treatment combinations. Liming rates were different for each soil (refer to Table 3).

Experimental factor	Treatments
Soil type	Fluvial, Volcanic
Temperature	10 °C, 15 °C
Amendment	No Urine, Added Urine
Liming treatment	No Lime, Lime 1, Lime 2

2.5. Measurement of the denitrification end-products, N_2O and N_2

Analysis of vial headspaces was achieved using an automated sampling and GC analysis system, referred to below as the Denitrification Dynamics Gas Chromatograph (DDGC) and described fully by McMillan et al. (2014). The DDGC was designed and assembled following Molstad et al. (2007), but with slight modifications. The GC system was a Shimadzu 2010 Plus (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with an Electron Capture Detector (ECD) for N_2O and CO_2 and a Thermal Conductivity Detector (TCD) for N_2, O_2 and CO_2.

The autosampling system was a Gilson GX-271 LH (Gilson, Luton, UK) that was set up to allow sampling from a rack submersed in a temperature-controlled bath. The rack can hold up to 25 Serum Vials (0.125 L) and 44 Exetainers (0.0125 L). Each vial was measured 12–15 times at intervals of ~4.2 h resulting in incubation periods of 50–67 h.

The system can reliably measure concentrations of N_2 down to a concentration of ~1500 ppmv and N_2O to a concentration of ~400 ppbv. In contrast to earlier work by Butterbach-Bahl et al. (2002), who also measured N_2O by headspace replacement with helium, we did not seek to avoid all N_2 contamination. Contaminating sources of N_2 include diffusion through the vial septa and introduction via the sampling needle of the autosampler. Our approach was to carefully measure and account for these contamination sources. These were quantified satisfactorily and accounted for when calculating the biological production of N_2. These and other methodological details relevant to this study are outlined in the Online Supplementary material.

Nitric oxide (NO) is also a gaseous product of both nitrification and denitrification and can account for a small percentage (0.15%–0.75%) of emissions following addition of fertiliser-N (Skiba et al., 1993). However, NO is rapidly consumed during denitrification (Skiba et al., 1993; Menendez et al., 2006; Saggar et al., 2013). The current study did not measure NO production since our principal focus was on the final step in the denitrification pathway —conversion of N_2O to N_2.

2.6. Soil pH and mineral N analysis

Soil pH and the concentration of ammonia-N and (nitrite + nitrate)-N were measured at several occasions during each incubation, usually at 24 or 48 h intervals. These measurements were made on a parallel set of incubation vials (Exetainers, Labco, High Wycombe, United Kingdom) that were a tenth of the volume of the vials used for the gas measurements (0.0125 L versus 0.125 L). Accordingly, all soil additions and their amendments were identical to those of the gas vials, except for being scaled by a factor of 0.1, and were incubated in the same water bath as the gas vials. At each of these samplings the soil from the vial was extracted and analysed for soil pH and mineral N (nitrite + nitrate and ammonium) using standard analytical techniques (Blakemore et al., 1987). Using this analytical technique, nitrate-N is reduced to nitrite-N and detected photometrically. Accordingly, this also includes the native soil nitrite. For convenience, we refer to this (nitrite + nitrate)-N pool as nitrate-N for the remainder of this manuscript.

2.7. Data analysis

Raw peak areas of the analysed gases from the DDGC were converted to headspace concentrations based on calibrations with certified gravimetric gas standards ("Alpha" Standards, Specialty Gases, BOC, New Zealand).

We then calculated the amount of each gas evolved per g of dry soil by accounting for the distribution of the gas between its dissolved and gaseous phases, the leakage of N_2 into the vial due to diffusion through the septa and introduction from the autosampler needle injection, the dilution of gas due to the sampling and helium backfilling of the vial headspace, as described in Molstad et al. (2007) (and also described fully in the Online Supplementary material).

We compared accumulated headspace gases at three stages during the incubation. The earliest stage, 5 h, is intended to represent the gas production rates of the soil before it has been substantially affected by the incubation conditions. The subsequent stages (20 h and 40 h) are intended to characterise the response of the denitrifiers during the later stages of the incubation conditions but before there is significant depletion of substrate for N_2O and N_2 formation. Our hypotheses are tested at the 40 h stage when there is an optimal balance between the growing concentration of the headspace gases (which improves the signal-to-noise ratio) and the need to keep the incubation time short to minimise incubation-related artefacts.

Statistical analysis was conducted using analysis of variance (ANOVA) and linear regression functions in R (R Core Team, 2013). We used one way ANOVAs with the liming rate as the predictor to test hypotheses A (liming decreases N_2O), B (liming increases denitrification) and C (liming decreases the product ratio) within each combination of soil/temperature and urine amendment. We used a four-factor linear regression model to test hypothesis 4 (that the liming response is modulated by soil type, temperature, and urine amendment). We used the R package, MASS (Venables and Ripley, 2002) to determine the best formulation of these linear regression models, employing the Aikake Information Criterion (Akaike, 1974). The response variables were either log transformed, or logit transformed in the case of the product ratio, to stabilise the variance (Warton and Hui, 2010).

3. Results

3.1. Effect of liming treatment and urine amendment on soil pH

The soil pH response to liming was markedly different between the two soils (Fig. 1). The volcanic soil had a greater pH initially, and required an addition of 5 and 10 t ha^{-1} of lime to cause a change in pH of 0.43 and 0.79 pH units, respectively. A much smaller amount of lime was required to cause equivalent pH changes in the fluvial soil due to its lower cation exchange capacity (Table 1), and only 1.5 t ha^{-1} and 3.0 t ha^{-1} of lime was needed to increase pH by 0.68 and 1.25 pH units, respectively.

The soil pH fluctuated during the course of the incubation. The volcanic soil pH increased markedly immediately following lime addition by up to 1 pH unit and then decreased by ~0.25 units over the subsequent 180 days. Six months after liming the soils, we found that the volcanic soil had increased in pH by 0.08–0.09 pH units per each t ha^{-1} of lime applied. By contrast, the fluvial soil increased by 0.42 and 0.45 pH units per each t ha^{-1} of lime for the two smaller liming rates used for this soil (1.5 and 3.0 t ha^{-1}) (Table 3).

The urine amendment in the experimental treatments caused an additional large increase in the soil pH (Table 3), but narrowed the pH differences between the limed and unlimed soil. On average the addition of urine increased the pH of the volcanic soil by 0.73 and narrowed the pH range among liming treatments from 0.81 to 0.41. The same amount of urine N addition increased the pH of the fluvial by 1.52 pH units and narrowed the range from 0.94 to 0.24.

3.2. Evolution of N_2O during the incubations

Patterns of N_2O production were very similar among replicates indicating a high degree of experimental precision, but showed substantial variation due to the experimental treatments (Fig. 2). The greatest amounts of N_2O were produced in the urine-amended volcanic soil at 15 °C where accumulated amounts approached 15 mg N_2O-N kg $soil^{-1}$. The three factor linear regression model indicated that soil type had the greatest effect on net N_2O production. The effects of temperature, urine addition, and liming treatment were secondary and varied according to the soil type.

The time course of N_2O production varied substantially among the various soil/treatment combinations. In the urine-amended fluvial soil, net N_2O production was slow at the initial stages of incubation, increased markedly between 20 h and 30–35 h and then slowed, completely halted and even began to decrease during the later stages of the incubation, presumably due to increased activity of N_2O-reductase. However in the urine-amended volcanic soil, urine-amendment stimulated net N_2O production such that the N_2O accumulated exponentially throughout the incubation.

The effect of liming was most pronounced in the urine-amended fluvial soil (Fig. 3). Higher rates of net N_2O production occurred in the warmer (15 °C) incubations relative to the cooler incubations (10 °C), and in most cases the rates increased by roughly 50% with the 5 °C increase in temperature.

The liming effect of N_2O production was dependent on the other effects (soil type, incubation temperature, and urine amendment). Under urine-amended conditions at 10 °C, liming reduced N_2O production in both soils, but most markedly in the fluvial soil. Under urine-amended conditions at 15 °C, liming reduced N_2O production in the fluvial soil only. In the fluvial soil, significant differences were also observed between the two levels of liming, while in the volcanic soil, no differences were observed between the two liming levels.

3.3. Evolution of N_2 during the incubations

The biotic production of N_2 for the two soils under the temperature and urine-amended treatments is shown in Fig. 4. The larger variation among replicates (denoted by the error bars) reflected the greater signal-to-noise ratio in the N_2 measurement (relative to the N_2O measurement).

The sensitivity of N_2 production to the treatments differed between the soils. The fluvial soil responded positively to both temperature and the urine application whereas the volcanic soil was much less responsive to these experimental treatments. Slow linear rates of N_2 accumulation occurred for the urine-free fluvial soils, but N_2 accumulation was much more rapid and positively curvilinear for the urine-amended fluvial soil. N_2 production rates were more similar and approximately linear for all treatments of the volcanic soil. N_2 production was greatest for the urine-amended fluvial soil, particularly in the 15 °C incubation when accumulated amounts exceeded 10 mg N kg (dwt $soil)^{-1}$ after 40 h. The effect of either temperature or urine amendment was not nearly so pronounced in the volcanic soil, and the production rates were broadly similar across different treatments.

Lime addition increased N_2 production only in the urine-amended fluvial (at both temperatures), and may have had some effect in the unamended volcanic – inhibitory at 10 °C and

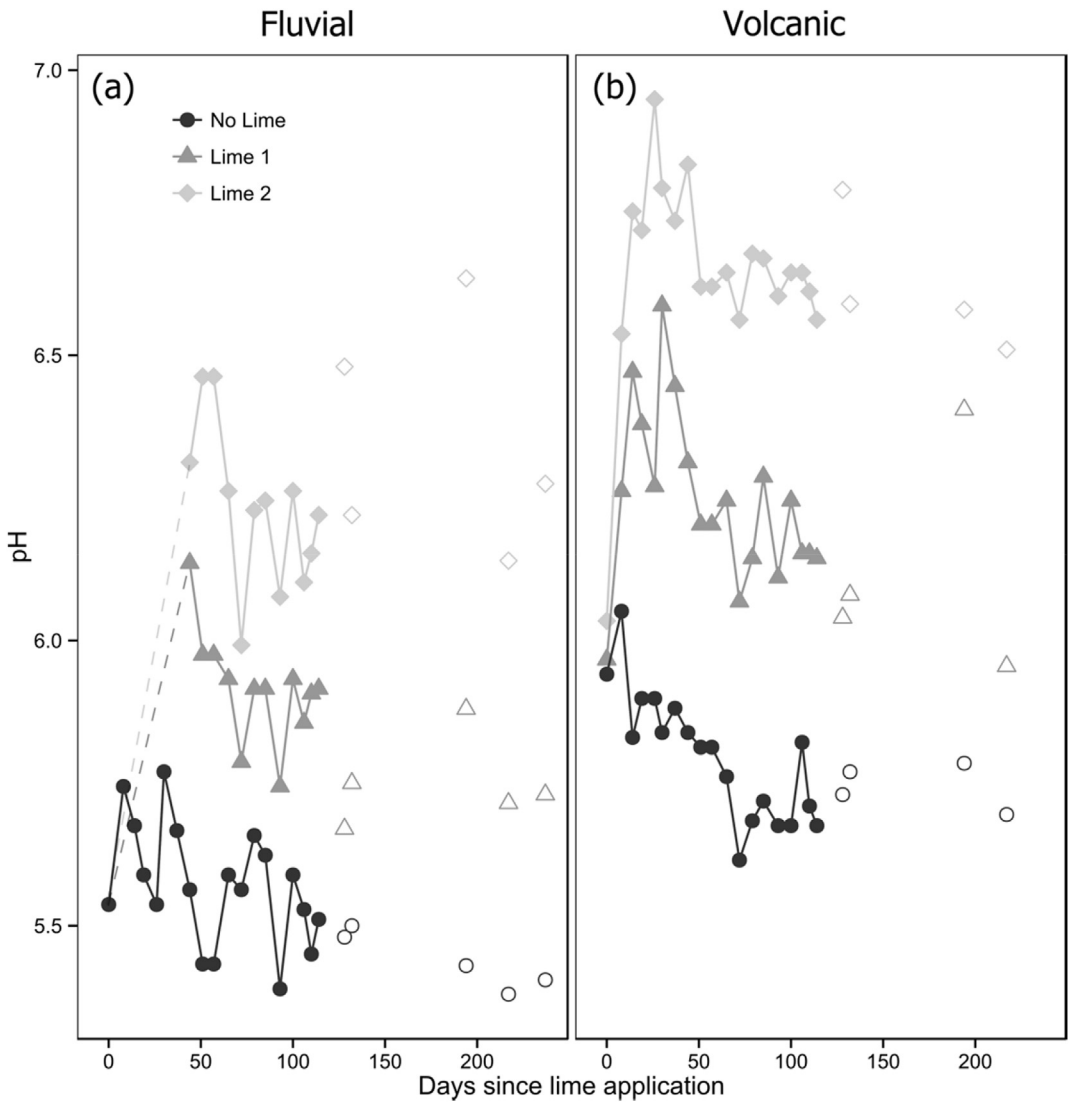

Fig. 1. Changes in soil pH following liming treatment. Lime 1 corresponds to a liming rate of 1.5 t ha^{-1} for the fluvial soil and 5 t ha^{-1} for the volcanic soil. Lime 2 corresponds to a rate of and 3.0 t ha^{-1} for the fluvial soil and 10 t ha^{-1} for the volcanic soil. Solid symbols indicate pH measurements made during liming equilibration period. Open symbols indicate the pH of the same soils measured immediately after the liming equilibration period and immediately before the incubations. Dotted lines indicate that initial liming rate for fluvial soil was excessive and dilution of liming effect was required to achieve target pH (see text).

Table 3
Effect of liming treatments on soil pH over the pH equilibration period (180 days).

Soil	Fluvial			Volcanic		
Liming rate (t ha^{-1})	0.00	1.50	3.00	0.00	5.00	10.00
Start pH	5.10	5.10	5.10	5.75	5.75	5.75
Final pH	5.41	5.78	6.35	5.74	6.18	6.55
Δ pH	0.31	0.68	1.25	−0.01	0.43	0.79
Δ pH/tonne lime/ha	–	0.45	0.42	–	0.09	0.08
Final pH after Urine addition	7.24	7.38	7.48	6.69	6.89	7.09

stimulatory at 15 °C – but these differences were not statistically significant.

The effect of temperature, urine addition, and liming on the relative production of N_2O and N_2 as well as the overall rate of denitrification during the anaerobic incubations are presented as time series in Fig. 5 and the accumulated amounts at 40 h are provided in Table 4. The overall rate of denitrification was most strongly influenced by urine addition to the soil. However, the two soils exhibited strikingly different partitioning of the gaseous N products: the fluvial soil produced mostly N_2 (product $ratio_{40\ h}$ = 0.13–0.17) whereas the volcanic soil produced an approximately even mixture of N_2O and N_2 (product $ratio_{40\ h}$ = 0.47–0.55).

The soils exhibited marked differences in how they partitioned N_2O and N_2 in response to increasing temperature (Fig. 5). Under warmer conditions, the fluvial soil tended to partition more towards N_2 whereas the volcanic soils partitioned more towards N_2O. This indicates that Q_{10} values for N_2O and N_2 formation are not fixed, but vary according to soil type. In a recent study, Q_{10} values were estimated for these processes in the fluvial soil at a higher temperature (19–35 °C) than that used in this study (10–15 °C) where it was found that the Q_{10} for N_2O formation (2.0) was higher than the Q_{10} for N_2 formation (1.4) (Phillips et al., 2014).

3.4. Change in soil mineral-N concentrations

In the unamended treatments, the ammonium-N concentrations remained very low (<5 mg ammonium-N kg^{-1}) during the entire incubation (data not shown). The urine addition caused a

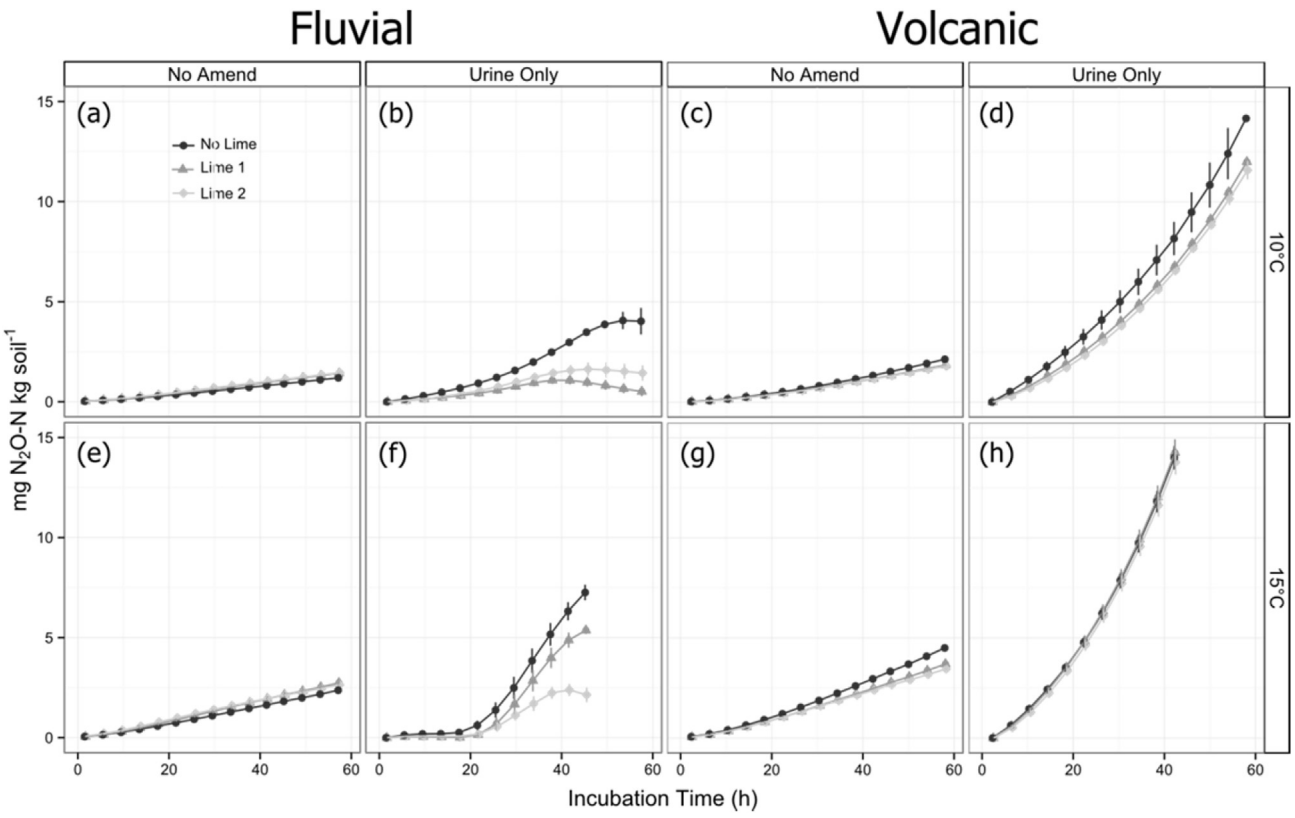

Fig. 2. N_2O production at different liming rates in a fluvial soil (a, b, e, f) and a volcanic soil (c, d, g, h) at 10 °C (a–d) and 15 °C (e–h) with either no amendment ("No Urine") or urine addition ("Urine Amended"). Error bars represent standard error of the mean (n = 3). Liming rates were different for each soil (see text).

pronounced increase in the ammonium-N content with pre-incubation concentrations ranging between 317 and 367 mg N kg^{-1} (Fig. 6), indicating that just over half of the 600 mg kg^{-1} of applied urine N was converted to ammonium-N. In the 10 °C incubations, for which a full set of ammonium-N measurements were obtained, these concentrations increased on average by 124 mg ammonium-N kg^{-1}, or in relative terms, by 39%.

The initial soil nitrate-N (includes nitrite-N as well) concentrations were higher in the volcanic soil, and in the unlimed treatments, suggesting a slightly greater rate of nitrification in the limed treatments prior to the anaerobic incubations (Fig. 7). The soil nitrate-N concentration was generally unaffected by the high amounts of ammonium-N associated with the addition of urine, since nitrification would have been limited by oxygen availability in these anaerobic incubations. However, the addition of urine greatly stimulated the disappearance of nitrate with rates of consumption varying between 0.9 and 1.6 mg NO_3-N kg^{-1} h^{-1}, while concentrations in the unamended treatments remained more or less static. Rates of nitrate-N disappearance were remarkably similar among liming treatments and were not more than 10% different from each other. Interestingly, the net amount of nitrate-N disappearance in urine-amended treatments was greatly in excess of the N gases formed, implying a sink for nitrate – in addition to denitrification.

3.5. *Effect of lime on accumulated amounts of N_2O and $N_2O + N_2$ at three time points during the incubation*

We calculated the net accumulation of gaseous N products at three stages of the incubation: 5 h, 20 h, and 40 h (indicated on Fig. 5) and determined the response to liming within each soil/temperature/urine-amendment combination (Table S3) (the effect of all treatment factors is discussed in Section 3.6 below). The significance of the differences between the mean accumulated amounts of N_2O and total denitrification end-products ($N_2O + N_2$) from limed and unlimed treatments was tested using one-way ANOVAs followed by the Tukey's Honest Significant Difference test to test for difference between the means (Table S2).

In the fluvial soil, liming strongly reduced N_2O accumulation at 5, 20 and 40 h (23–92% reduction) under urine-amended conditions, but liming caused moderate increases (by 17–33%) in N_2O accumulation under unamended conditions. In the volcanic soil, liming caused moderate reductions in N_2O accumulation (12–45% reduction) under all conditions (Fig. 3, Table S2).

Responses of N_2O accumulation to liming after 40 h are shown in Fig. 8. At 10 °C, lime addition caused a slight but significant elevation (20–26% increase) from the low rates of N_2O production found in the unlimed urine-free fluvial soil (Fig. 8a). However, in the urine-amended fluvial soil, liming caused a statistically significant 48–64% decrease in accumulated N_2O (Fig. 8a). At 15 °C in the fluvial soil, liming caused increases in N_2O accumulation of 18–20% under urine-free conditions, and decreases of 26–63% under urine-amended conditions (Fig. 8c).

Liming caused smaller relative reductions of N_2O (14–21%) in the volcanic soil except for the 15 °C urine-amended treatment, where no significant change occurred (Fig. 8b and d). This finding confirmed Hypothesis 1 (that liming decreases N_2O accumulation) for the fluvial soil for urine-amended soils but did not support Hypothesis 1 for urine-free soils, where N_2O either increased slightly or remained constant with liming.

The total N gas ($N_2O + N_2$) evolved at 40 h was consistently higher in the urine-amended treatments compared to the

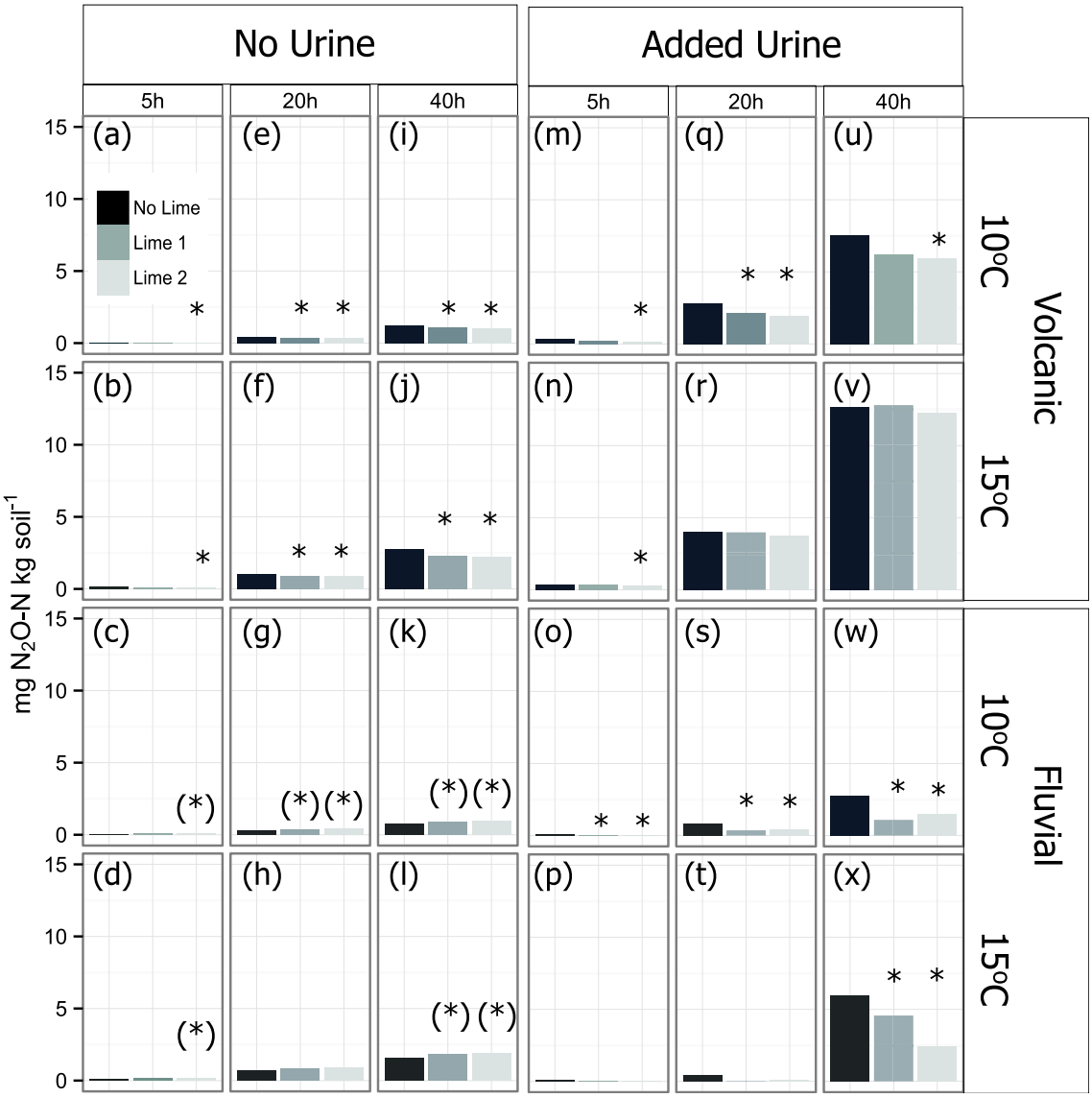

Fig. 3. Accumulated N_2O at 3 different stages during the incubation (5 h, 20 h and 40 h) from two soils (Volcanic and Fluvial) incubated at two temperatures. Left panels (a) to (l) show amounts under unamended conditions and right panels (m) to (x) show N_2O amounts under urine-amended conditions. Asterisks over grey bars (lime treatments) indicate significant reductions in N_2O production from control treatments (black bars). Asterisks in parenthesis indicate statistically significant ($P < 0.05$) increases in accumulated N_2O in response to liming.

unamended treatments (Fig. 9). The fluvial soil was more responsive to temperature increase than the volcanic soil. Liming did not affect the total N gas evolved (Tukey's Honest Significant Difference, $P < 0.05$) for any combination of soil/temperature/amendment combination. This finding leads us to reject Hypothesis 2 that liming induced pH increases lead to greater rates of denitrification.

Like the metrics discussed above, the product ratio at 40 h varied with soil type, temperature, and amendment; and any variation according to liming treatment, while sometimes statistically significant, was of secondary importance (Fig. 10). In the fluvial soil, the product ratio was similar or higher in the unamended-compared with the urine-amended soil (Fig. 10a and c). By contrast, in the volcanic soil, the product ratio was substantially lower for the unamended soils than that of the amended soil (Fig. 10b and d). The liming treatment caused a significant decrease only in the urine-amended fluvial soil (at both temperatures) (Fig. 10a and c). This leads us to accept Hypothesis 3 (that liming decreases the product ratio) for the fluvial urine-amended soil but reject it under urine-free conditions, or under any conditions in the volcanic soil.

The instantaneous N_2 production rates at 40 h were of interest because they most closely relate to the activity of N_2O-R at this time point. These values varied widely as a function of soils type, temperature and urine amendment but only vary slightly due to liming (Fig. 11). The only statistically significant ($P < 0.05$) response for liming was in the fluvial soil at 10 °C in which a slight enhancement of the N_2 production rate occurred between the unlimed and limed treatments (Fig. 11a).

3.6. Combined influence of all experimental factors on the rate of N gas production and the product ratio

We used the R package MASS (Venables and Ripley, 2002) to fit linear models of the predictive factors (soil type, temperature, urine amendment and the measured soil pH of the differently limed soils)

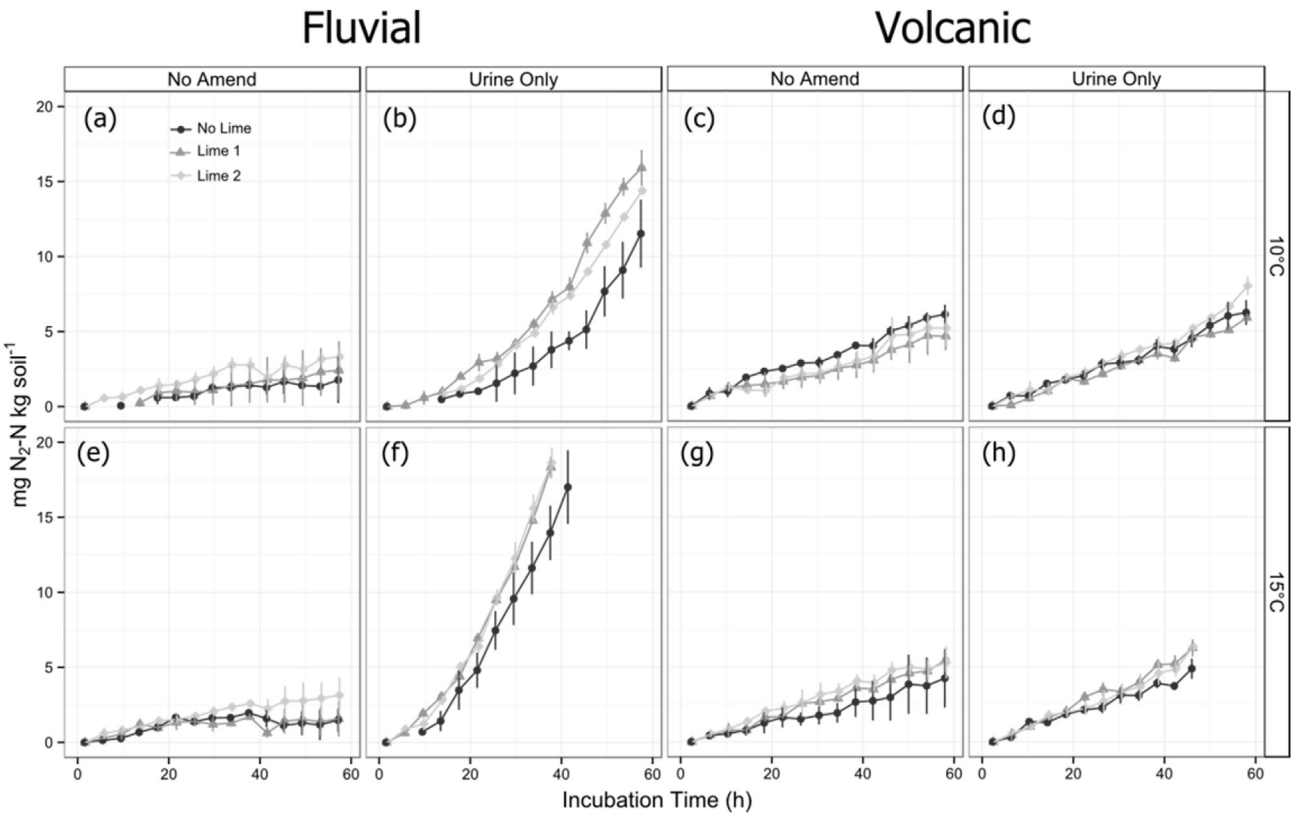

Fig. 4. N_2 production at different liming rates in a fluvial soil (a, b, e, f) and a volcanic soil (c, d, g, h) at 10 °C (a–d) and 15 °C (e–h) with either no amendment ("No Urine") or urine addition ("Urine Only"). Error bars represent standard error of the mean (n = 3). Liming rates were different for each soil (see text).

to the instantaneous production rate of the following response variables: N_2O, N_2, $N_2O + N_2$ and the product ratio, $N_2O/(N_2O + N_2)$. Each predictor and its interactions with other predictors were introduced in a stepwise manner and Aikake's Information Criterion was used as a metric to determine the best model (Akaike, 1974). The coefficients for the main effects and two-way interactions, as well as the overall goodness of fit for each soil, are provided in Table S3.

Overall, this method of selecting models was most successful for explaining N_2O production (adjusted R^2 values ranged from 0.75 to 0.94), where urine amendment, temperature, and soil type not only acted main effects but also interacted with each other. Soil pH effects due to liming were not a significant factor for predicting N_2O production in this analysis. However, there were significant interactions between pH and urine amendment at 5 and 40 h, and between pH and temperature at 20 h.

N_2 production could not be predicted well in this 4-way analysis with any single main effect and the only significant term in any of the three time stages was the three way interaction between soil type, urine amendment and temperature at 40 h.

The product ratio was most strongly predicted by soil type, but only in the later stages of the incubation. Temperature had a statistically significant but small main effect. pH was not a main factor nor did it interact with other variables.

The statistical testing within each soil type/temperature/urine combination (Table S3) indicated that liming had significant effects on the accumulated amounts of the denitrification gases at 5, 20, and 40 h. We conclude from the four factor regression analysis above that liming-induced pH increases were much less important than the effect of urine addition and temperature increase for these two soils. However, it was clear that in the cases where the liming affected either N_2O or total denitrification, this effect interacted with the influence of urine addition, incubation temperature and/or soil type. This leads us to support Hypothesis 4, that the liming effect was modulated by the effect of these three other variables.

4. Discussion

The effect of liming on the activity of N_2O-R was assessed in this study by directly measuring the gaseous end products of denitrification in a series of anaerobic incubations. By performing the incubations in a N_2-free atmosphere we were able to observe the biological production of N_2 together with the production of N_2O thus avoiding the well-known artefacts associated with the acetylene inhibition method (Groffman et al., 2006). This experiment was conducted under highly controlled conditions of temperature, water content, and pH using homogenised and sieved soil, and without the presence of plants and animals. The gas production rates presented here indicate the relative responses of N_2O-R activity to liming. We have imposed conditions optimal for denitrification in the anaerobic incubations, which do not take into account shifting oxic–anoxic conditions. However, our aim was to assess the response of the denitrification-based N_2O production and consumption in isolation from N_2O produced under oxic conditions.

Since the anaerobic conditions prevented the ammonium conversion to nitrate via nitrification, we were also able to determine how urine addition modified the soil denitrification without the interfering effects of large amounts of urine-derived N.

Under field conditions, the soil is likely to oscillate, temporally, between oxic and anoxic states. Spatially, the soil will be a mosaic of anaerobic and aerobic microsites. Both sources of oxygen status

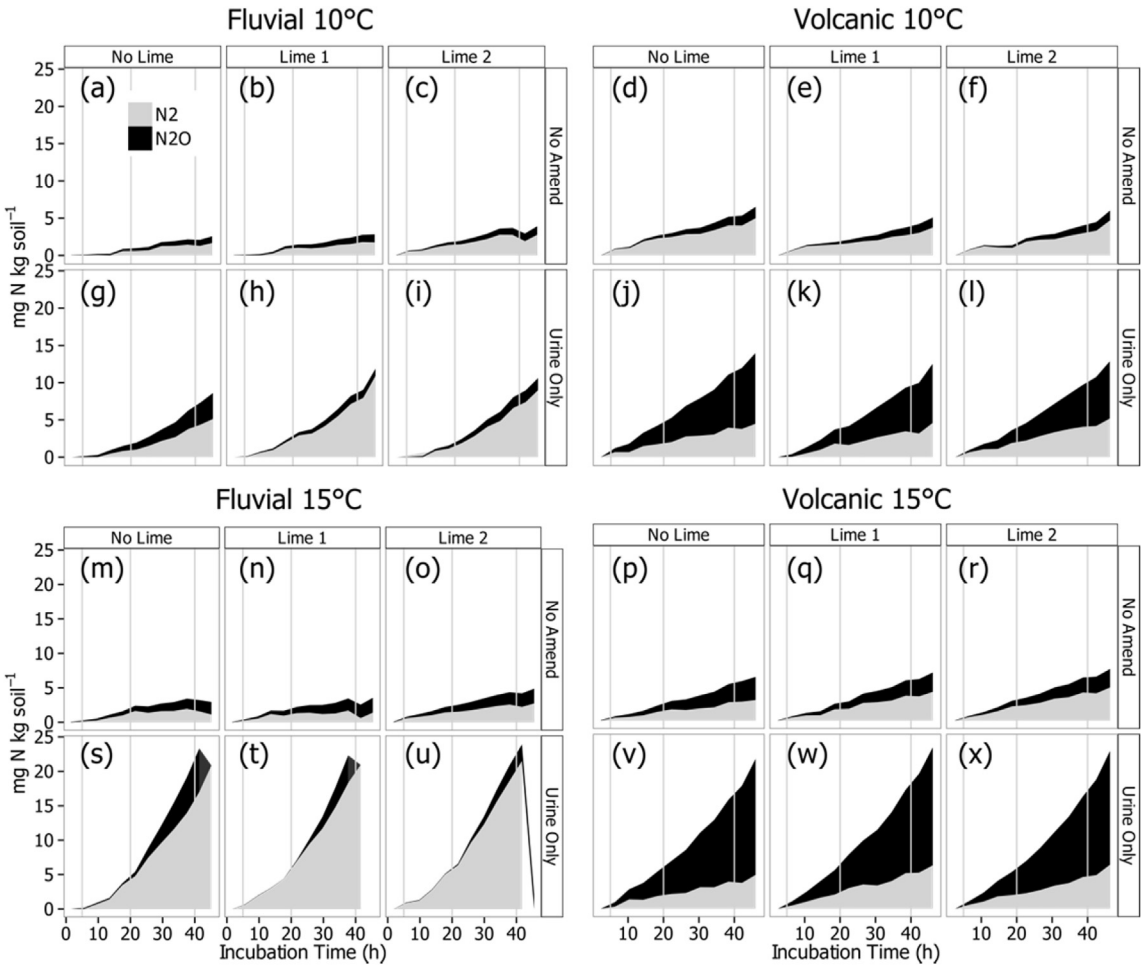

Fig. 5. N_2 and N_2O production in the fluvial and volcanic soils at 10 °C and 15 °C. The vertical extent of the grey band relative to the total height (grey plus black) represents the $N_2O/(N_2O + N_2)$ product ratio at any point during the incubation. The grey vertical lines indicate the time periods at which liming differences were statistically tested.

Table 4
Denitrification (total accumulated $N_2O + N_2$) and the product ratio calculated at 40 h during the anaerobic incubations.

Soil	Temperature	Amendment	Accumulation of denitrification end products, $N_2O + N_2$ (mg N kg soil^{-1})			Product ratio $N_2O/(N_2O + N_2)$		
			No lime	Lime 1	Lime 2	No lime	Lime 1	Lime 2
Fluvial	10 °C	No Urine	2.24 ± 0.6	2.71 ± 0.5	3.15 ± 0.92	0.35 ± 0.09	0.34 ± 0.06	0.31 ± 0.09
		Urine	7.15 ± 1.76	8.61 ± 0.57	8.72 ± 0.38	0.39 ± 0.1	0.13 ± 0.02	0.17 ± 0.03
	15 °C	No Urine	3.53 ± 0.17	2.87 ± 0.39	4.22 ± 0.55	0.45 ± 0.02	0.65 ± 0.09	0.45 ± 0.06
		Urine	21.41 ± 0.72	24.64 ± 0.38	22.4 ± 0.45	0.28 ± 0.01	0.19 ± 0.01	0.11 ± 0.01
Volcanic	10 °C	No Urine	5.29 ± 0.48	4.05 ± 0.6	3.99 ± 0.23	0.23 ± 0.02	0.27 ± 0.04	0.26 ± 0.02
		Urine	11.31 ± 1.06	9.59 ± 0.35	9.99 ± 0.41	0.67 ± 0.11	0.65 ± 0.04	0.6 ± 0.05
	15 °C	No Urine	5.61 ± 0.17	6. ± 0.1	6.17 ± 0.17	0.49 ± 0.02	0.38 ± 0.01	0.36 ± 0.01
		Urine	16.57 ± 0.67	17.97 ± 0.58	17.12 ± 0.33	0.77 ± 0.04	0.71 ± 0.03	0.72 ± 0.02

variability produce a complex series of interactions between nitrification and the various steps of denitrification. Our goal was not to attempt to simulate these complex environmental patterns that occur in this environment, but rather to focus on two particular steps in the denitrification pathway – production and consumption of N_2O under completely anaerobic conditions.

We were surprised to observe a large discrepancy between the amount of soil nitrate-N consumed (Fig. 7) and the amount of N gases formed (Fig. 5). At 40 h, 48 ± 8 mg of nitrate-N (average ± 1 SD across all amended treatments) had disappeared from the vials but only 14 ± 6 had evolved as either N_2O or N_2. This discrepancy implied that an additional sink for nitrate-N was operating in the incubated soil. A likely candidate for this sink is the process of nitrate ammonification (also called dissimilatory nitrate reduction to ammonium or DNRA) (Tiedje et al., 1982; Baggs, 2011). This process is known to be active under highly reduced conditions, and favoured at elevated pH (Schmidt et al., 2011) as was the case in these anoxic, urine-amended incubations.

Large increases in ammonium-N occurred during the incubations (Fig. 6) and could be attributed to urea hydrolysis, which can occur under anaerobic conditions if the urease enzyme has already been synthesised (Mccarty and Bremner, 1991). However, the increase in ammonium-N is also consistent with DNRA converting the non-denitrified nitrate-N into ammonium-N. Although,

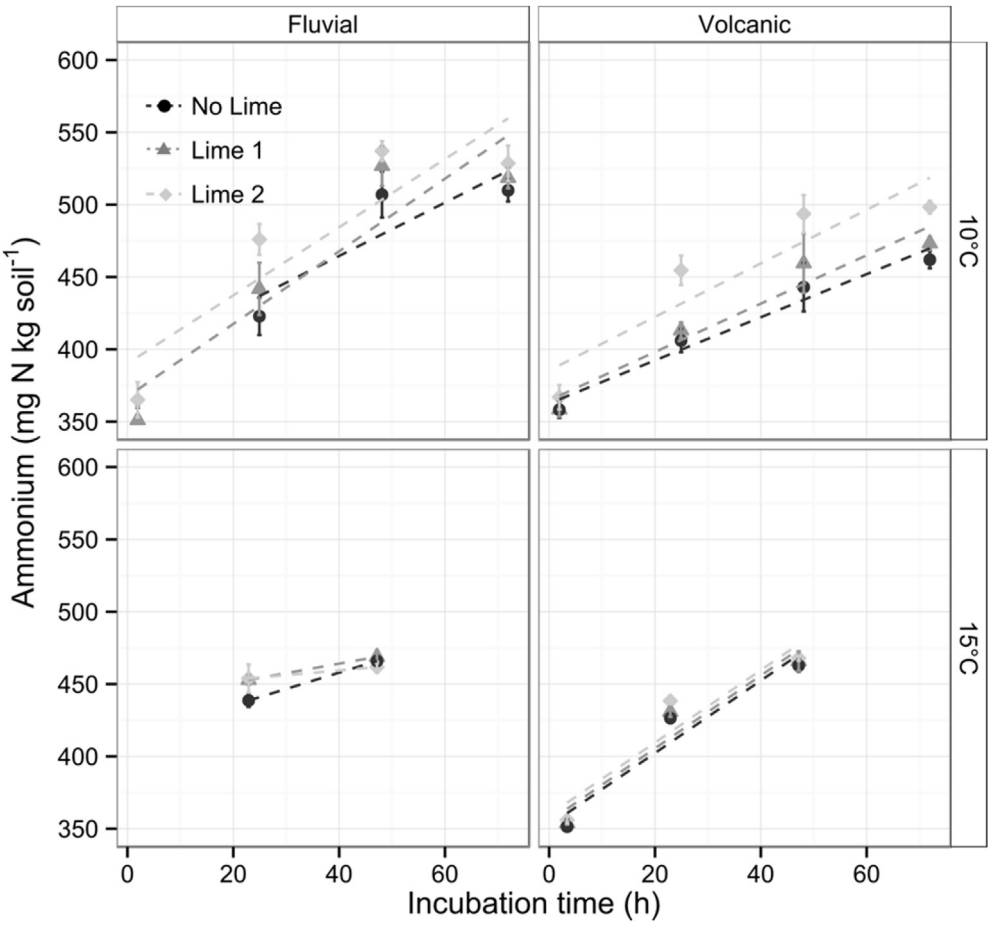

Fig. 6. . Changes in soil ammonium concentrations in urine-amended soil during the incubation (concentration of ammonium in unamended soils remained below 5 mg ammonium-N kg soil^{-1} throughout the incubations and are not shown in this figure).

DNRA is a plausible explanation for the excess consumption of nitrate-N, the quantification of DNRA, and its importance relative to urea hydrolysis and denitrification, was beyond the scope of the present study, but certainly deserving of future investigations.

Regardless of uncertainties about the dynamics of mineral N, this study provided constraints on the extent to which liming soils can reduce N_2O production. We demonstrated that lime addition can decrease N_2O production by significant amounts, but most substantially in the fluvial soil and only under conditions of urine amendment (where much larger fluxes of N_2O occur). Our findings here concur with Zaman et al. (2007), who found that liming caused a reduction in the product ratio of ~0.2 in an allophanic urine-amended soil. While liming significantly reduced N_2O production for both soils, the effect was most marked in the urine-amended fluvial soil, confirming our first hypothesis for this soil.

The partitioning of the denitrification end-products, N_2O and N_2, following urine deposition is important for determining the denitrification component of N_2O flux. While the response to liming was subtle, the large difference in the product ratio between the two soils was striking and deserves further investigation. It is of particular interest to explore whether the higher product ratio found in the volcanic soil under urine-amended conditions is related to its allophanic nature. The addition of urine had a greater effect on soil pH than liming, so any pH-dependent processes are likely to respond sharply to the urine amendment.

The denitrification product ratio can either be expressed as $N_2O/(N_2O + N_2)$, or more simply just as N_2O/N_2, and this quantity has been investigated for almost 60 years. In an analysis of twelve studies, primarily conducted in the laboratory, Rochester (2003) found a negative exponential relationship between the product ratio N_2O/N_2 and pH. This can be recalculated as an approximately linear decrease in the $N_2O/(N_2O + N_2)$ product ratio used that we use in the present manuscript as a function of pH. The product ratios found at 5, 20 and 40 h for each of the liming and urine treatments exhibit very different pH dependencies for the two soils. In the fluvial soil there is a decrease in the $N_2O/(N_2O + N_2)$ product ratio (PR) with increasing pH ($PR = -0.095 \times pH + 0.86$, $r^2 = 0.29$, $P < 0.001$) broadly consistent with the trend derived from the product ratio/pH dependencies found in the meta-analysis by Rochester (2003) ($PR = -0.22 \times pH - 0.22$, $r^2 = 0.56$, $P < 0.001$) (Fig. 12a). By contrast, the volcanic soil exhibits a markedly different pH dependency — the $N_2O/(N_2O + N_2)$ product ratio increases sharply with increasing pH ($PR = 0.21 \times pH - 0.99$, $r^2 = 0.42$, $P < 0.001$) (Fig. 12b). However the this positive trend is mostly driven by the influence of urine increasing the pH in the volcanic soils so the increase in PR might be driven by the factors associated with the urine addition itself rather than a simple pH dependency.

The reductions in N_2O observed in the fluvial soil corresponded with increases in N_2 production, providing a strong indication that liming enhanced the activity of N_2O-R. The interaction between the volcanic soil and the urine addition that caused the product ratio to be so high could have resulted either from chemical factors, or from some aspect of the soil microbial ecology. However, in either case,

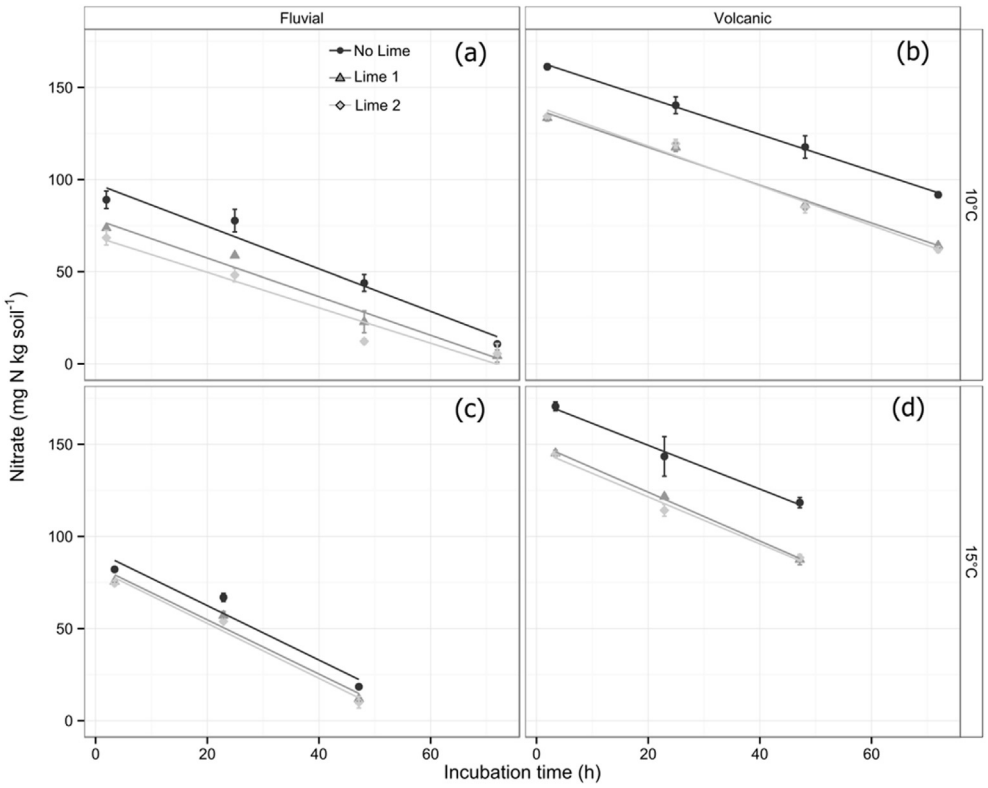

Fig. 7. Changes in soil nitrate (includes soil nitrite as well) from unamended and urine-amended soil measured over the course of the incubation. Solid lines and symbols indicate unamended treatments and dashed lines and open symbols indicate urine-amended treatments.

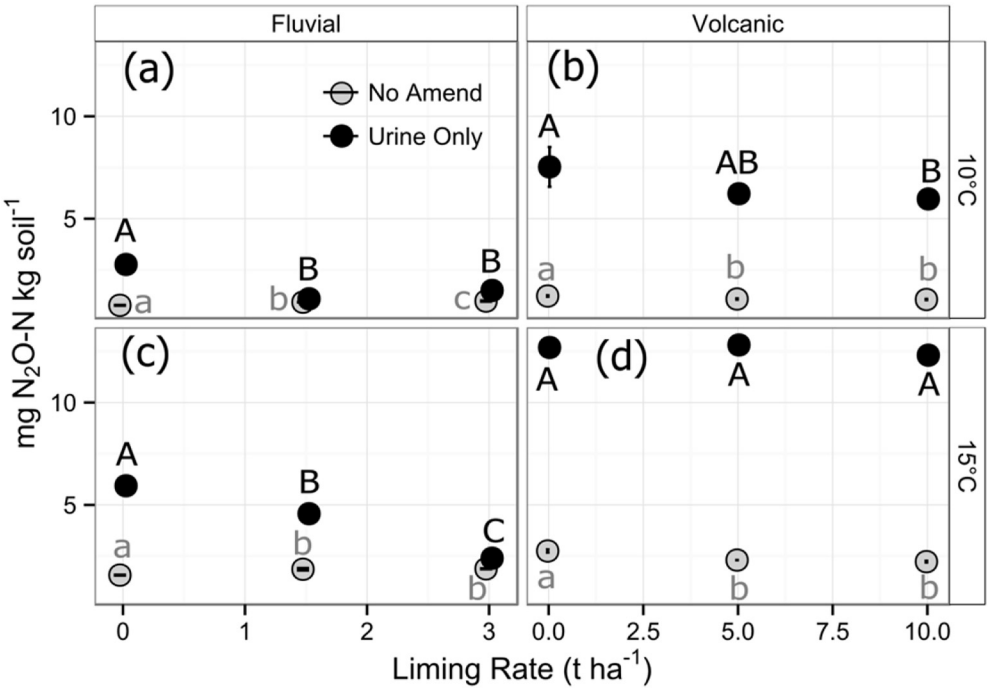

Fig. 8. Accumulated N_2O (average ± 1 SD) measured at 40 h as a function of liming rate in fluvial soil (left panels) and volcanic Soil (right panels) at incubation temperatures of 10 °C (upper panels) and 15 °C (lower panels). Significant differences between means due to liming alone within each soil/temperature/amendment treatment are indicated by dissimilar letters (Tukey's Honest Significant Difference, $P < 0.05$; lower case letters refer to unamended treatments and upper case letters refer to urine-amended). Note the difference in x scales.

the high allophane content of the volcanic soil is most probably responsible. Copper, an essential co-factor for N_2O-R (Pomowski et al., 2011), is strongly sorbed by allophane–humic complexes through coordination with exposed hydroxyl groups (Yuan et al., 2002). Moreover, this sorption increases with increasing pH (Yuan et al., 2002). It is likely that in the volcanic soil, N_2O-R was limited by the availability of copper due to this sorption and as a result the consumption of N_2O was greatly diminished.

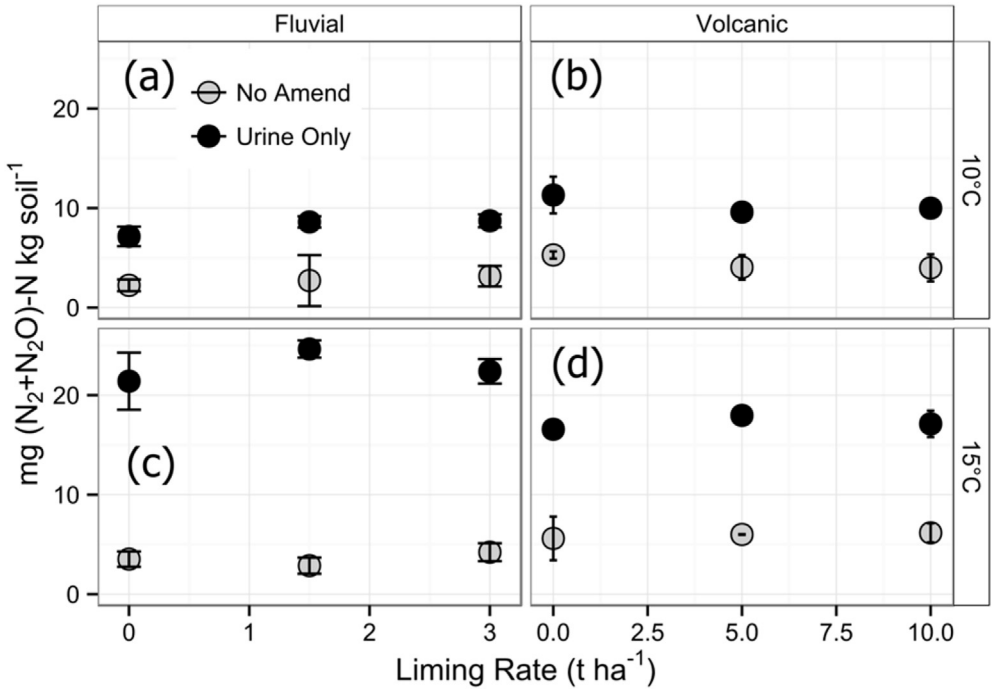

Fig. 9. Accumulated N gases ($N_2O + N_2$) measured at 40 h as a function of liming rate in fluvial soil (left panels) and volcanic soil (right panels) at incubation temperatures of 10 °C (upper panels) and 15 °C (lower panels). No significant differences between means due to liming alone within each soil/temperature/amendment group were found. Note the difference in x scales.

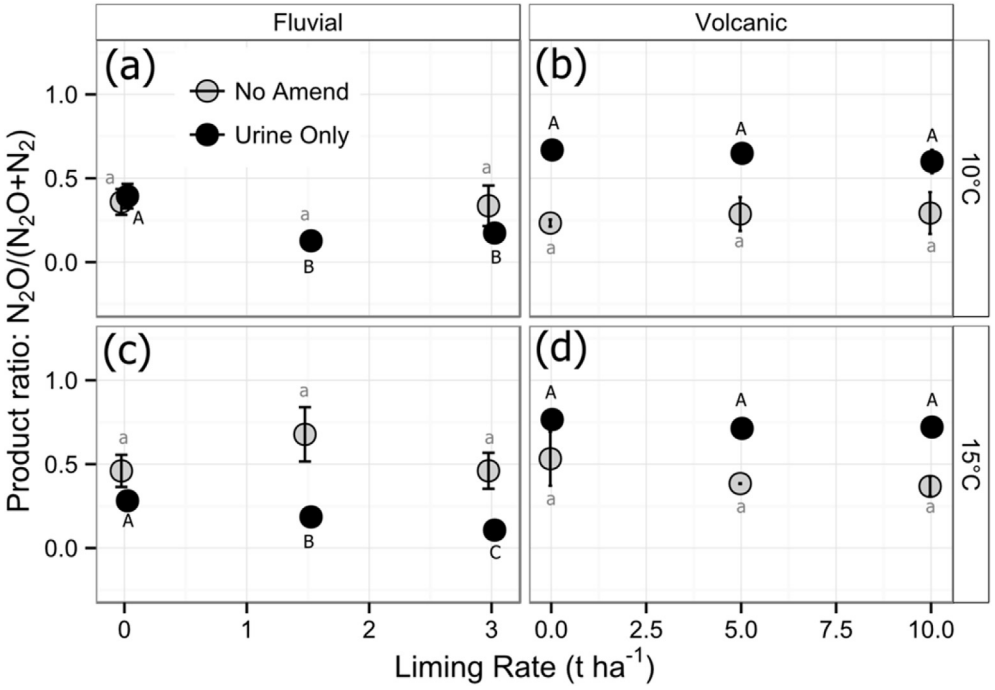

Fig. 10. The product ratio ($N_2O/(N_2O + N_2)$) measured at 40 h as a function of liming rate. Error bars represent SD (n = 3). Symbols sharing the same letter within each panel) are not significantly different from each other (Tukey's Honest Significant Difference, $P < 0.05$; lower case letters refer to unamended treatments and upper case letters refer to urine-amended treatments). Note the difference in x scales representing the different liming rates required for each soil.

It is also possible that N_2O may have been limited by C availability. The high allophane content of volcanic soil is more likely to immobilise organic C (Saggar et al., 1994). Thus, the enhanced response of N_2O-R activity to liming in the urine-amended fluvial soil is possibly due to an increased ability of the denitrifiers to readily access the C provided by the urine. Previous work demonstrated that N_2O-R activity in this particular fluvial soil is stimulated by C addition (McMillan et al., 2014). This explanation is less compelling than the possibility of copper limitation because the simple sugars that provide the C source for denitrification are not

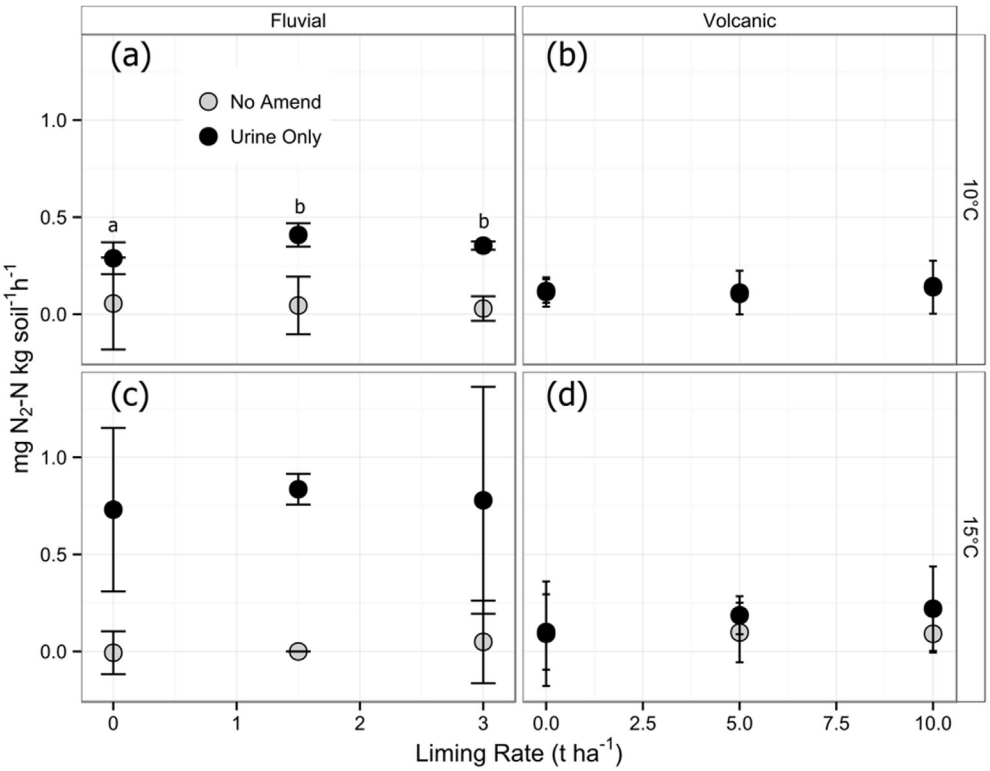

Fig. 11. N_2 Production rates at 40 h. Error bars are 1 SD. The different letters shown in top left panel indicate significantly different rates due to lime for the urine-amended fluvial soil at 10 °C. For all other combinations of soil, urine amendment, and temperature there were no significant differences due to liming (Tukey's Honest Significant Difference, $P < 0.05$).

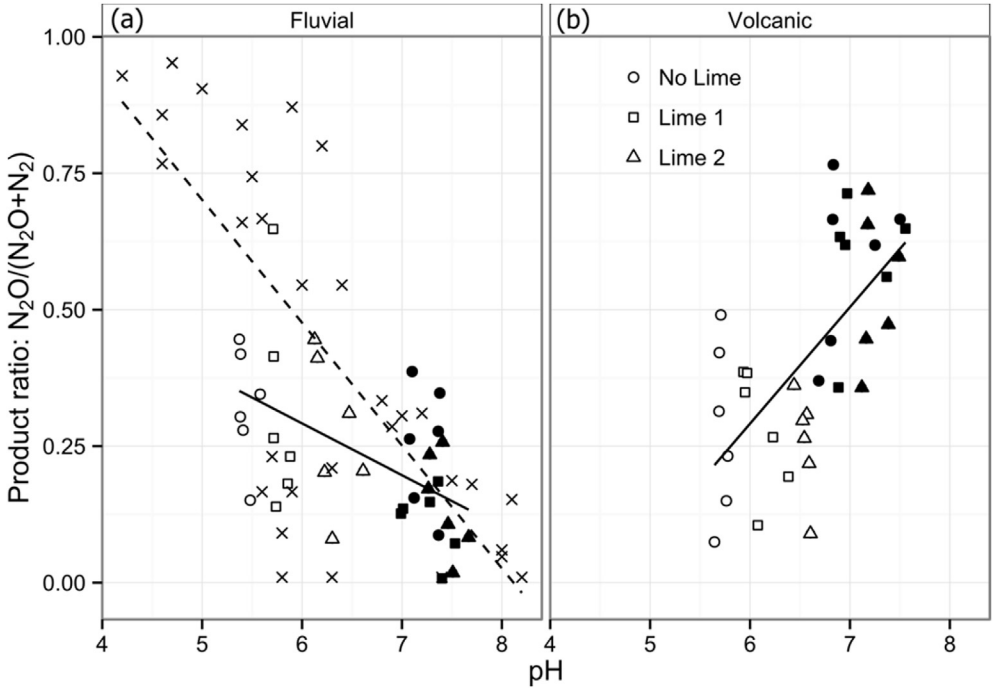

Fig. 12. Product ratio $N_2O/(N_2O + N_2)$ as a function of pH. The circles, squares and triangles represent all experimental treatments at 5 h, 20 h and 40 h. Open symbols indicate treatments with no urine amendment and closed symbols indicate treatments with urine addition. The solid lines are linear regression fits to the pooled data for each soil. The black crosses are PR-pH data from a number of previous studies collated in the meta-analysis by Rochester (2003). The dashed line is the linear fit to the meta-analysis data. Regression statistics provided in text.

strongly charged as cations, and interaction of sugars with the allophanic surface would presumably play a smaller role than that of copper.

Alternatively, the difference in urine-amended product ratios may have a microbial basis. The product ratio of allophanic versus fluvial soils was investigated by Jha et al. (2012), where it was found that a similar fluvial soil (also a fluvial fine sandy loam) had a product ratio of 0.2, whereas a heavier allophanic silt loam (Otorohanga series) had a product ratio of 0.9. In a subsequent study the abundance of genes responsible for the formation of N_2O, *nirS* and *nirK* was found to be slightly less in the fluvial soil (5.5×10^8 gene copies) compared with the volcanic soil (7.5×10^8 gene copies), whereas the abundance of the gene responsible for the consumption of N_2O, *nosZ*, was higher in the fluvial soil (8.6×10^6 gene copies) compared with the volcanic soil (2.7×10^7 gene copies) (Morales et al., 2015).

Insufficient evidence exists to support this latter explanation for this current study, although a parallel study measuring denitrifier gene abundance is underway to verify whether our observed variation in the product ratio was due to smaller quantity of *nosZ* genes. However, it has recently been shown that the product ratio is not directly connected to mRNA transcription rates of *nosZ*, and the decrease in activity of N_2O-R at low pH was postulated to be post-transcriptional phenomenon (Liu et al., 2010).

While we are not able definitively to explain the reason for the different product ratios between the two soils, we have established that liming-induced enhancement of N_2O-R activity is feasible for some soils under anaerobic laboratory conditions. The next logical step would be to confirm that this liming would reduce N_2O emission in the field. Here, it would be crucial to determine the source of N_2O under such conditions so that the effects of lime on nitrification and denitrification can be properly established. The measurement of natural isotopomer ratios (site preference of ^{15}N) of emitted N_2O using laser spectroscopy is a promising emerging methodology to attack this question (Köster et al., 2013).

5. Conclusion

We tested the effects of liming soil on the production and ratio of the denitrification end-products, N_2O and N_2. Liming decreased N_2O production under some conditions, with the greatest proportional reductions occurring in the urine amended fluvial soil. However, this was not associated with an increase the overall production rate of the combined denitrification end-products, $N_2O + N_2$. Accordingly, the $N_2O/(N_2O + N_2)$ product ratio decreased with liming-induced pH changes in the fluvial soil.

The effect of lime was heavily modulated by urine amendment, temperature, and soil type. The fluvial soil was more responsive to lime than the volcanic soil when treated with urine. Urine amendment sharply increased the production of N gases in both soils, but the ratio of N_2O to $(N_2O + N_2)$ was much larger for the volcanic soil (60–77%) than for the fluvial soil (11–45%).

The viability of lime as a mitigation strategy must account for the CO_2 emissions associated with lime manufacture and application. This study provided an upper limit to the effect of liming on denitrification N_2O emissions (16–64% depending on soil type, temperature, urine amendment, and liming rate) and indicated that the strategy is potentially viable with a fluvial soil. Future studies should investigate the precise mechanism by which liming enhances the consumption of N_2O and determine how this effect compares with other liming induced alterations to the N cycle.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.10.013.

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