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SHORT COMMUNICATION

Hysteretic response of N_2O reductase activity to soil pH variations after application of lime to an acidic agricultural soil

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Abstract

N_2O contributes to increasing the greenhouse effect and is also involved in stratospheric ozone depletion. In soil and water, N_2O reductase catalyses the reduction of N_2O into the inert form N_2. N_2O reductase activity is known to be affected by acidic conditions and the application of liming materials to acidic soils is now proposed as a solution for mitigating soil N_2O emissions. During a one-year laboratory experiment, we studied the functioning of N_2O reductase after the application of calcium carbonates to an acidic soil with a very low capacity to reduce N_2O. The functioning of N_2O reductase was characterised through anaerobic incubations using the acetylene inhibition technique combined with a logistic model to determine the main enzyme functioning characteristics (latency, maximal rate). Both changes in soil pH and soil capacity to reduce N_2O were rapidly observed after the application of lime materials. The activity of N_2O reductase was observed to be efficient throughout the experiment even when the soil had returned to initial acidic conditions, revealing a hysteretic response of N_2O reductase to pH variations. Nevertheless, some signs of lower N_2O reductase activity over time were observed mainly after 200 days of applying lime materials. Altogether, these results suggest that, in this soil condition, the beneficial impact of the application of liming materials on N_2O emissions could last longer than this on soil pH.

Keywords Climate change mitigation · Soil · pH · Lime application · N_2O reductase · Logistic modelling

Introduction

Nitrous oxide (N_2O) has been identified as the third major greenhouse gas after carbon dioxide (CO_2) and methane (CH_4) as well as being involved in stratospheric ozone depletion (UNEP 2013). The Agriculture, Forestry and other Land Use sector is considered to be a significant source (about 23%) of these GHG emissions, especially that of N_2O with the increased use of fertilisers. Denitrification, generally considered as the main source of N_2O release, is a multistep process (Roy et al. 2021) starting from the reactive nitrate and changing to the inert form dinitrogen when complete. However, the denitrification process is often incomplete and the reactive intermediate forms NO and N_2O are released into the atmosphere (Wrage et al. 2001). The last step of the denitrification process, the reduction of N_2O into N_2, is currently the only known terrestrial pathway leading to the consumption of N_2O (Thomson et al. 2012). Different enzymes catalyse each step of the denitrification process. Periplasmic N_2O reductase (N_2OR) is the core catalytic enzyme of the N_2O consumption process. It is encoded by the *nosZ* gene and now recognised as a key environmental enzyme with high biotechnological potential (Zhang et al. 2019). Nevertheless, many organisms do not possess the *nosZ* gene (Pauleta et al. 2013). Moreover, with two metal centres, N_2OR requires the implementation of complex process units to be functional, i.e., for it to effectively catalyse the reduction of N_2O into N_2. Soil pH is now well recognised as affecting the functioning of N_2O reductase, which is impaired in acidic conditions (Qu et al. 2014). Several studies have reported a linear decrease in the $N_2O/(N_2O+N_2)$ ratio of denitrification

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in agricultural soils with increasing soil pH (Liu et al. 2010; Russenes et al. 2016).

Liming acidic soil is a long-established practice used to increase soil pH and globally ameliorate acidic soils (Holland et al. 2018), with calcium carbonate ($CaCO_3$) being the most commonly used liming material. Liming has been proposed as a potential lever for promoting nitrous oxide reduction and consequently mitigating soil N_2O emissions. Hénault et al. (2019) suggested a specific pH of 6.8 below which the N_2O reduction path is progressively inhibited. However, liming mobilizes natural resources, modifies different soil properties with agronomic and environmental impacts, and has an economic cost for farmers. Therefore, precise understanding of the underlying mechanisms (i.e., pH effect on N_2OR activity) and interactions between liming practices, soil pH and N_2O emissions is required to ensure the efficiency of this solution. The aim of this study is to provide a better understanding of the evolution of the pH effect on N_2OR in order to fully benefit from using liming to mitigate soil GHG emissions. Its main objective was therefore to study how, through their effect on soil pH, the addition of lime materials could impact the functionality of N_2O reductase on a cultivated soil over a 12 month-period following the application of lime materials.

Materials and methods

The soil was obtained from a crop field located at 47°15′38.369″ N; 4°11′18.953″ E on December 2020. Formed on a granitic substrate, this soil is composed of sand (61%), silt (24.5%) and clay (14.7%) and is classified as a brunisol (IGN 2022). At sampling, its pH was 5.6 and its gravimetric water content was 25% w/w ($\psi = 170$ hPa, pF = 2.22). Its total organic carbon content was 2.47% and CEC 130 meq kg^{-1}. Its initial characteristics relative to N_2O reduction were $r_{max} = 1.2$ and index = 115, typical of a soil barely able to reduce N_2O (Hénault et al. 2019). At sampling, twelve soil samples were taken randomly from a 2500 m^2 plot to a depth of 20 cm. The day of sampling, soil samples were sieved at 5-mm and mixed together. The composite obtained was divided into 9 containers of 6 L, to obtain 1 kg of fresh soil per container. The containers were closed and placed in a temperature control chamber (20 °C).

Three treatments were applied the day after soil sampling (day 0 of the experiment), replicated 3 times. Three containers, named MC, received calcium carbonate ($CaCO_3$) of marine origin marketed by Timac Agro (Calcimer®) with a neutralising value (NV) of 40. Three containers, named SC, received a synthetic $CaCO_3$ marketed by Carl Roth® with an NV of 52. The three other containers did not receive any liming products and were used as control. To target a pH of 6.8, 2.5 g kg^{-1} soil and 1.9 g kg^{-1} soil of the liming products were applied to the sieved soil for the MC and SC treatments respectively. Liming materials were incorporated in the soil using a metal spatula and homogenized manually for 7 min. The homogenization was also performed on the control.

The experiment included 2 types of incubation: (i) an aerobic one over about a one-year period, and (ii) periodical anaerobic incubations over short periods (168 h). The one-year aerobic incubation of the sieved soil was performed in the 6 L containers from which the headspace air was sampled and renewed about weekly over the 360 days. Gas samples were analysed on a Thermo Scientific™ TRACE™ 1310 GC fitted with an ECD detector for determining their N_2O concentration. A solution of KNO_3 (16.6 mg N-NO_3 in 40 g of water per kg of soil) was applied on day 34. Aliquots of 75 g of soil were periodically sampled (about monthly) from these containers (on days 33, 61, 96, 124, 194, 253, 292 and 348) to determine changes over time of the soil's pH (ISO 10390: 2005), exchangeable mineral nitrogen contents (Yang et al. 1998), and to study the N_2O reductase functionality.

The capacity of the soil to denitrify and reduce N_2O during denitrification was investigated under anaerobic conditions at 20 °C (ISO/TS 20,131; Hénault et al. 2001). Briefly, two soil samples of 25 g soil per container were sampled on days 33, 61, 96, 124, 194, 253, 292 and 348 and each placed in 333 mL flasks. Two series of incubations were then carried out, both with the addition of NO_3^- (25 mL KNO_3 solution 7 mM). The first series was incubated in the presence of acetylene (7 mL) to totally inhibit the reduction of N_2O into N_2 (Yoshinari et al. 1977; Ye et al. 2021), N_2O therefore being the final product of the denitrification process. The second series was incubated without the addition of acetylene, the fate of the N_2O produced being either its accumulation in the flask or its reduction into N_2, depending on the N_2O reductase functionality. The flasks were constantly shaken at 150 rpm in an orbital shaker over a period of one week. Their atmosphere was sampled after 0, 24, 48, 72, 96 and 168 h. The gas samples were analysed on an Agilent 990 micro-GC fitted with a PorapakQ column, a TCD detector and coupled with an autosampler designed by SRA Instruments.

Parameters describing the functioning of the denitrification process were calculated from the data collected during the anaerobic incubations of soil aliquots after random pairing. They dealt with both the total denitrification process (DN) and the consumption of N_2O (CN). Concerning DN, we used data obtained in the presence of acetylene. The parameters determined were (i) QDN_{168} (µg N g^{-1} soil), the quantity of denitrified nitrate at the end of the 168 h of incubation, i.e., the quantity of N-N_2O produced in the presence of acetylene, (ii) maxDN (µg N g^{-1} soil h^{-1}), the maximum denitrification rate observed during the reaction kinetics, and (iii) lagDN (h), the time necessary for maxDN to be reached. maxDN and lagDN were obtained after a logistic regression

(Putz et al. 2007) performed using xlstat®. The equations are given in Oneline Resource 1. As far as the consumption of N_2O (CN) was concerned, we determined two types of parameters. The first type of parameters (empirical) were deduced directly from the experimental points. These parameters were previously defined in Hénault et al. 2001 as r_{max}, t and index. r_{max} is the maximal ratio between the N_2O concentration in the flasks without acetylene and with acetylene, t is the time during which the N_2O concentration in the flasks without acetylene continued to increase, and the index is the multiplicative combination between r_{max} and t. The second type of parameters (mechanistic) were obtained with a logistic regression as described in Online Ressource 1. The effects of the (1) treatments (Control, SC, MC) and (2) incubation times were tested on the different parameters using an analysis of variance (ANOVA) followed by a post hoc Tukey test when appropriate. The hypothesis of linear changes over time of the different parameters was tested by applying the linear regression model ($p = 0.05$).

Results

Throughout the 1-year period of aerobic incubation, the soil pH of the control treatment slowly decreased from 5.6 to 4.65 (Fig. 1). The soil pH responded very quickly to the application of lime materials and reached the targeted pH of 6.8 during the first month following the application for both materials. This pH value was not maintained over time. A decrease was observed mainly during the first 3 months and reached 5.7 at the end of the experiment.

Throughout the aerobic incubation period, both nitrate and ammonium contents increased continuously and were not affected by the lime applications. N_2O emissions were higher in the control than in the limed treatment throughout the incubation period. Nevertheless, after the addition of nitrate, high N_2O emissions observed on the control were due to a hot spot for one replicate. Therefore, despite large differences, N_2O emissions between the control and the other treatments were not significantly different (Online Resource 2).

During the anaerobic incubations in the presence of acetylene, all the added nitrate was denitrified after 168 h of incubation for the 3 treatments in the month following the addition of lime materials (Fig. 2). The quantity of denitrified nitrate (QDN) slowly decreased over time for the control due to a significant decrease over time of maxDN, despite a small decrease of lagDN (Fig. 3). Whereas a significant small decrease of maxDN over time was observed for the soil samples that received the liming materials, no significant changes in QDN or in lagDN were observed in these soil samples.

N_2O consumption (CN) was strongly affected by the addition of the lime materials. For the control, it was less than 2% of the N_2O produced after day 33 and generally not detected. The parameters r_{max} (around 1), index (mean = 103) and t (mean = 160) and lagCN (mean = 166) were high while maxCN and QCN were not detected (Fig. 3; Online Resource 3).

After liming a strong capacity to reduce N_2O into N_2 in anaerobiosis conditions was observed for both materials. This capacity was reached less than 1 month after application and was conserved throughout the one-year experiment even

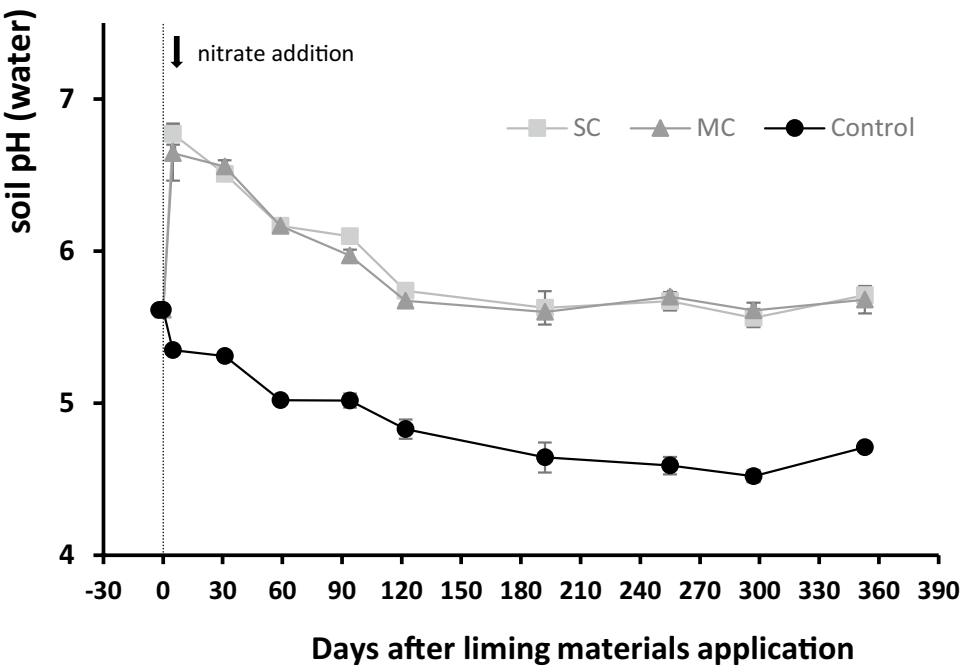

Fig. 1 Variation of soil pH following the application of liming materials (synthetic carbonates: SC ▫ and marine carbonates: MC ▲) in comparison with a control (●). The dashed vertical line represents the day of lime incorporation in soil. The arrow represents the day of application of the nitrate solution. Error bars represent the standard error ($n = 3$)

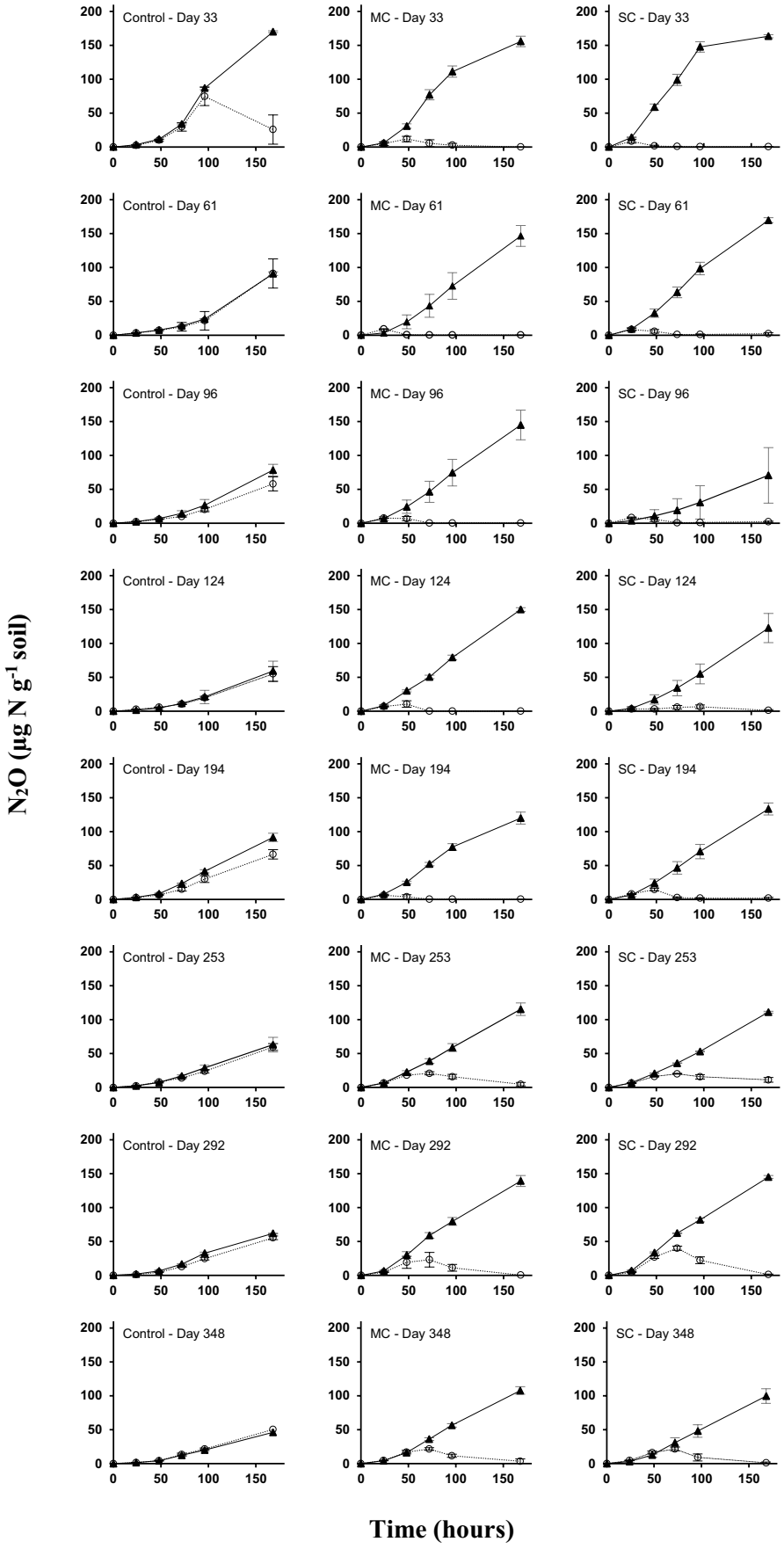

Fig. 2 N_2O production by soil samples in anaerobiosis conditions, with (—▲—) or without acetylene (C_2H_2·····○·····). The experiment includes 3 soil treatments, (i) control, (ii) addition of synthetic carbonates (SC) and (iii) addition of marine carbonates (MC). Error bars represent the standard error ($n = 3$)

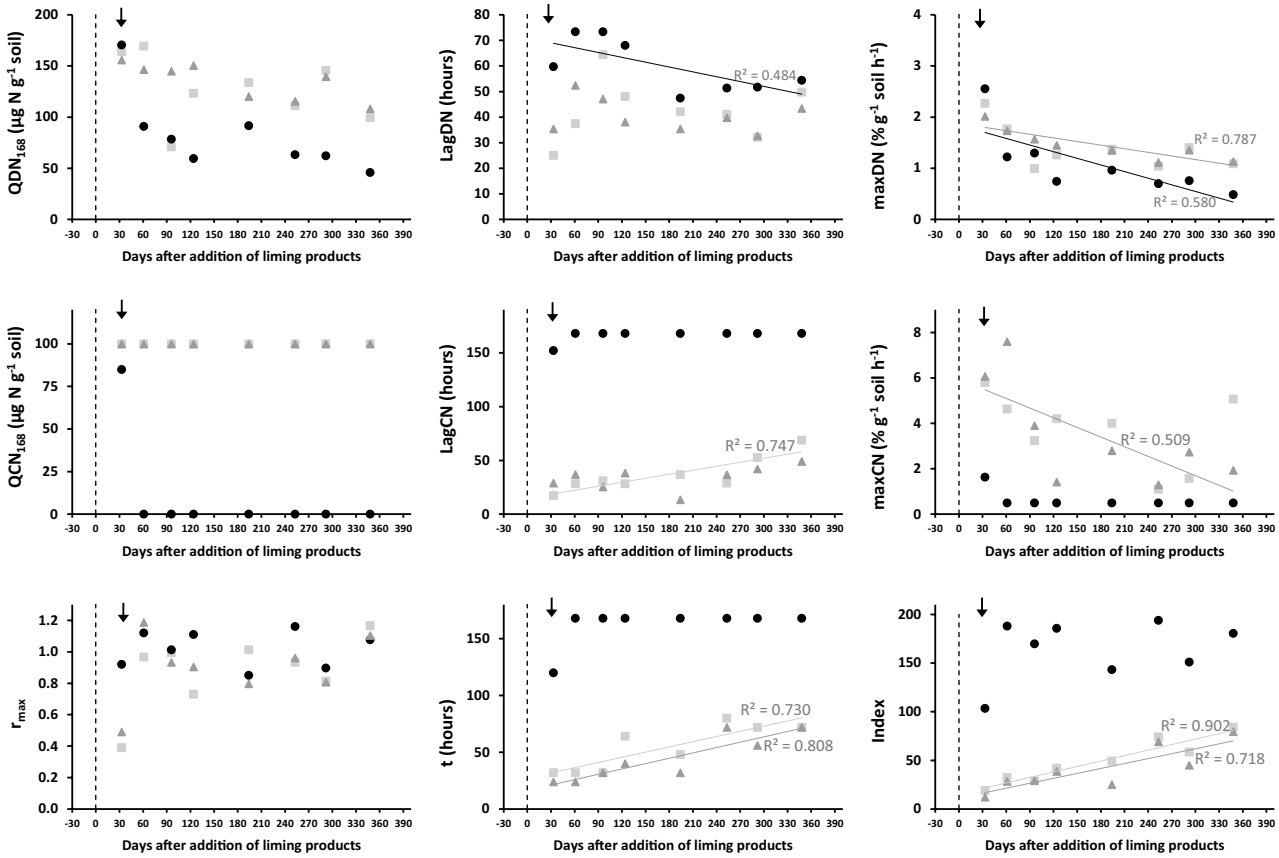

Fig. 3 Changes over time of the mechanistic N_2O reductase parameters (QDN—the quantity of denitrified nitrate -, maxDN—the maximum denitrification rate -, lagDN—the time necessary for maxDN to be reached-, QCN—the part of N_2O that has been consumed, maxCN—the maximum N_2O consumption rate, lagCN—the time necessary for maxCN to be reached -) and the empirical (r_{max}, t, index). The experiment includes 3 soil treatments, (i) control (●), (ii) addition of synthetic carbonates (SC ■) and (iii) addition of marine carbonates (MC ▲). The dashed vertical line represents the day of lime incorporation in soil. The arrow represents the day of application of the nitrate solution

when the soil pH had decreased below the value of 6.4. Nevertheless, some subtle changes in the capacity of the limed soil samples to reduce N_2O were observed over time. In particular, the time required for total N_2O consumption during anaerobic incubations, assessed by different complementary methods, had increased with the duration of aerobic incubation. After 194 days of aerobic incubation, more time was required for N_2O reduction to occur (Fig. 2; Online Resource 4). This evolution could also be detected through the changes over time of the mechanistic (lagCN and maxCN) and empirical parameters (t and index), (Fig. 3). Nevertheless, the empirical parameters r_{max} revealed a lime effect only in the month following lime application. Although $(lagCN)_{SC}$ had increased linearly over time, $(lagCN)_{MC}$ seemed to start increasing after 194 days after the liming materials were added. The empirical parameters t and index both increased linearly with the duration of the aerobic incubation.

Therefore, the remarkable extension of N_2O reductase activity after the pH returned to its initial state was nevertheless accompanied by a slow increase in the latency revealed by lagCN and t, suggesting a return to initial characteristics. This return to initial characteristics could be predicted more than one year after the soil pH returned to its initial value.

Moreover, the application of lime materials clearly increased maxCN. Nevertheless, the change of this parameter over time remained unclear at the end of this experiment, as a barely significant decrease was observed only for the MC treatment (Fig. 3).

Discussion

The rapid response observed of N_2O reductase to the application of the lime materials (less than one month), clearly suggests that the *nosZ* gene was initially present in the soil even if not clearly able to reduce N_2O. In addition to the concept that associates "one gene—one enzyme" (Beadle and Tatum 1941), our study applied the concept of enzyme functionality, implying that the presence of a gene

and the synthesis of its associated enzyme would not ensure the corresponding catalysis or, in other words, the presence of an enzyme would not ensure its functionality. This appears especially relevant for enzymes with a very complex structure such as N_2O reductase, a dimer, with each monomer composed of two domains (Rosenzweig 2000). One of the novel findings obtained during this study was the observation of a hysteretic effect of soil pH on N_2O reductase activity. N_2O reductase activity increased very rapidly with the increase of soil pH as a result of applying lime materials. Less than one month after the addition of liming materials, soil pH reached neutrality and N_2O reductase activity reached its highest rate and lowest latency, whereas N_2O reductase activity decreased very slowly after the soil pH rapidly returned to its initial state. As the N_2O reductase is a periplasmic enzyme sensitive to external pH, this observation was consistent with the association of: (i) the possibility of the presence of old cells undergoing negligible growth, with a mean generation time of 200 days growth as suggested by Borer and Or (2022), and (ii) cells that had first been capable of assembling the denitrification proteome at pH 7.0 and which indeed reduced N_2O at pH 6.11 (Bergaust et al. 2010). Thus, the functional pattern could be due to the presence of the gene and N_2O reductase whatever the soil pH (Wilks and Slonczewski 2007). However, the protein assembly conformed only at a pH higher than 6.8. Since bacteria can survive for long periods in soils, those that synthetize N_2O reductase at neutral pH will continue to reduce N_2O even in decreasing pH conditions, being progressively replaced by new bacteria with nonfunctional N_2O reductase synthetized in acidic conditions. The increasing nitrate concentrations over time could also have contributed to the decrease of maxCN and the increase of lagCN (Qin et al. 2017; Sánchez and Minamisawa 2019) observed for limed soil samples.

According to our knowledge, the mitigation of N_2O emissions due to liming had been demonstrated at the field scale in 12 studies out of 19 reporting N_2O emissions in limed conditions (Online Resource 5). While field experiments remain the most valuable demonstration of N_2O mitigation by the application of lime materials, laboratory studies can help to reveal the extent to which liming could be a generic means of mitigating N_2O emissions. Here, we developed a methodology for characterising N_2O reductase activity, described by mechanistic (QCN_t, lagCN and maxCN) and empirical (r_{max} and index) parameters and we demonstrated on one soil that the duration of N_2O activation by the application of lime materials can exceed the duration of soil neutrality. This suggests that increasing soil pH to about neutrality (6.8) would have positive environmental impacts (decrease of N_2O emissions) several months after the pH had returned to acidic conditions, consistent with the cumulated N_2O emissions observed over the year of aerobic incubation. This is especially important in the calculation of the complete lifecycle assessment of liming involving both N_2O mitigation and CO_2 emission, as the balance between these two gases largely depends on the duration of action of liming materials on N_2O emissions.

As quantitative values, both the empirical and mechanistic parameters, could potentially be used in mechanistic and semi-empirical models of soil N_2O emission. Although interesting simulations have already been performed with r_{max}, the mechanistic parameters lagCN and maxCN exhibited a finer resolution, with the potential for more precise modelling. As they suggest that N_2O reduction could occur at least one year after the soil pH returned to its initial state, we suggest calibrating with in situ N_2O fluxes in (i) variable soil conditions, for example for testing the impact of latency as a function of the time required and soil porosity on N_2O transfer, and in (ii) variable climatic conditions, since temperature is a major determinant in enzymology. Such calibrations will continue to improve their predictive uses.

Conclusions

The use of logistic modelling applied to data collected throughout a one-year laboratory experiment allowed capturing certain functional characteristics (latency, maximal rate) of N_2O reductase, a key enzyme for mitigating climate change. A hysteretic response of N_2O reductase activity to soil pH variations following the application of lime materials to an acidic soil was observed, suggesting that the mitigation of soil N_2O emission could last after soil pH returns to acidic conditions. On the quantitative level, the enzyme functional characteristics obtained provide interesting perspectives for N_2O emission modelling and N_2O mitigation prediction. We suggest to extend this study in (i) variable soil conditions, and in (ii) variable climatic conditions, since temperature is a major determinant in enzymology.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00374-023-01717-5.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflicts of interest The authors declare no conflicts of interest.

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