Suitability of Potential Nitrification Rate Determined by Shaken Slurry Method to Capture the Contingent Effect of Liming of Two Tropical Soils

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Authors' contributions

This work was carried out in collaboration between both authors. Author KKMBM managed the analyses of the study, performed the statistical analysis, and wrote the first draft of the manuscript. Author DWS designed the study, wrote the protocol, and revised the manuscript. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Potential nitrification rate (PNR) provides maximum soil nitrification rate under optimal incubation conditions, but optimum conditions may vary for different nitrifier species. This study investigated whether the estimates from PNR and nitrifying potential (NP) assays reflect the effect of sudden changes in soil pH as with liming on nitrifying activity. Soil samples were collected from intensively cultivated and uncultivated lands representing soil orders Alfisol and Ultisol. A sub-sample of each cultivated soil was treated with $CaCO_3$ (1.8 g/kg). Cultivated soil with and without liming and uncultivated soil from each soil types were incubated at 30 °C maintaining moisture content at 0.25 (v/v) for 2 weeks. PNR of soil was measured with shaken slurry method using P-buffer at pH 7 and

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modified P-buffer with adjusted pH to match soil pH, separately. NP of each soil was determined in a 21 days static soil incubation. Soil type and land use had significant effects ($P<0.001$) on PNR. Ultisol had significantly higher ($P<0.05$) PNR than Alfisol, and uncultivated soils had significantly higher ($P<0.05$) PNR than cultivated soils, irrespective of the P-buffer used. Liming yielded significant differences ($P<0.05$) in PNR only when pH adjusted P-buffer was used. Treatment effects on NP was significant only in Ultisol. Results suggest that PNRs may be biased towards certain groups of nitrifiers and PNR measured using modified P-buffer and percent increase in NO_3^- production due to external application of NH_4^+ are better indicators of potential nitrifying activity than traditional PNR measured using P-buffer at pH 7 and NP.

ABBREVIATIONS

PNR	*: Potential Nitrification Rate*
NP	*: Nitrifying Potential*
P-Buffer	*: Phosphate Buffer*
NH_3	*: Ammonia*
NO_2	*: Nitrite*
NO_3	*: Nitrate*
N	*: Nitrogen*
AOB	*: Ammonia Oxidizing Bacteria*
AOA	*: Ammonia Oxidizing Archaea*
NOB	*: Nitrite Oxidizing Bacteria*
Comammox	*: Complete Ammonia Oxidizer*
EC	*: Electrical Conductivity*
MPN	*: Most Probable Number Method*

1. INTRODUCTION

Analyzing the microbiological properties of soils is very challenging because soils harbor diverse groups of microorganisms that respond differently to different environmental conditions [1,2]. Therefore, based on how we handle the samples and the conditions introduced through the analytical procedures, there can be biases introduced, favoring certain groups of organisms over others, affecting the accuracy and precision of the data generated [1,2]. Nitrification is a biologically mediated essential transformation in soil where ammonia (NH_3) is sequentially oxidized to nitrite (NO_2^-) and nitrate (NO_3^-). This conversion changes the mobility of nitrogen (N), and NO_3^- is the most susceptible form of N for losses from the soil system via leaching and denitrification [3]. Understanding the dynamics of nitrification in agricultural soils is important for efficient management of N-fertilizers, minimizing the losses of reactive N to the environment [3]. However, studying this process and the members involved in it is challenging, mainly due to the high diversity of microorganisms involved in it, and the differences in their sensitivity to environmental conditions such as pH, temperature, NH_3 concentration, O_2 levels, and moisture content [4-6].

A diverse group of microorganisms are involved in nitrification, including lithoautotrophs and heterotrophs, of which lithoautotrophs are the predominant players [5-8]. In agricultural soils, nitrification is mainly carried out by lithoautotrophic bacteria that gain energy from the oxidation of NH_3 (ammonia oxidizing bacteria – AOB) or NO_2^- (nitrite oxidizing bacteria – NOB) [4,7,8]. Moreover, ammonia oxidizing archaea (AOA) are also important contributors to nitrification and are widespread in soil environments [4,5,9,10]. In addition, complete ammonia oxidizer (Comammox) type bacteria have also been discovered as important contributors to nitrification in environmental samples [11]. The optimal growth conditions for these different groups of microorganisms who are involved in the nitrification process vary widely [4]. Thus, the composition and activity of nitrifying communities would vary across soils based on inherent soil properties and agronomic practices that affect soil conditions [8,9,10,12]. In Sri Lanka, differences in the abundance and activity of AOB and NOB based on land-use, cultivation practices, and soil type have been reported by Nawarthna et al. [12].

Substrates required for lithoautotrophic nitrifiers are NH_4^+/NH_3, CO_2, and O_2, and most of the time, the availability of NH_4^+/NH_3 limits both the rate of nitrification and the size of the resultant nitrifier populations in many soils [4]. Some, but not all, cultured AOB and AOA are sensitive to high levels of their substrate, NH_4^+/NH_3 [4,5]. Both ammonia and nitrate oxidation are optimal under neutral to slightly alkaline pH levels, but still lithoautotrophic nitrification has been confirmed in soils with pH values from 3.0 up to 10.0, and possible organisms have been isolated [4,5].

Out of the several available methods to determine terrestrial nitrification rates, potential nitrification rate (PNR) assays have been used

for many decades as a rapid method [6]. The shaken soil slurry method by Hart et al. [13] is the most common, low resource consuming, and convenient PNR assessment method, and therefore, it is a popular technique used in the studies on nitrifiers. A search in Google Scholar for research articles that present work on potential nitrification rate assays following the method described by Hart et al. [13] with or without modifications revealed about 60 publications since 2020. Further, the publication by Hart et al. in 1994 [13] on "Nitrogen mineralization, immobilization, and nitrification" had over 1450 citations by June 2024 in Google Scholar. This short-term shaken slurry incubation measures the maximum rates of nitrate production under optimal, non-substrate limiting conditions over a period of one to two days, but there are some drawbacks in this technique [6]. The technique uses a phosphate buffer (P-buffer) at pH 7 to buffer the soil slurry, avoiding acidification as the nitrification progresses because acidification of the media has a negative feedback effect on nitrification. Further, the soils are incubated at 28 °C to 30 °C temperature facilitating the growth of the mesophilic group. Shaking speed and the volume of the flasks would affect the oxygenating rate of soil slurry, which is another important factor that affects diverse groups of nitrifiers differently.

Nitrifying potential (NP) is another laboratory-based estimate of nitrification that is used to compare nitrifying activity across soils with different properties or to compare the effects of different management practices [14]. One main difference between NP and PNR assays is that NP is a static analysis and PNR is a kinetic analysis. In the NP assay, the rate of nitrate formation over a period of 21 days is assessed with and without an exogenous supply of NH_4^+ [14]. When NH_4^+ is not supplied externally, then the activity of nitrifiers would be limited first by the supply of the nitrifiable substrate. When NH_4^+ is supplied, the activity and size of the nitrifying population would increase in soil until another factor or a combination of factors limits their activity [15]. The measurement obtained with the NH_4^+ supply is considered as nitrifying potential. If acetylene like inhibitor is not used then the estimate of nitrifying activity represents the collective nitrification from both lithoautotrophic and heterotrophic type nitrifiers [15]. Measuring nitrifying activity in agricultural soils with exogenous NH_4^+ addition provides information on how fast NH_4^+ applied through fertilizers would be converted to NO_3^- in a given soil with matching moisture and temperature conditions used in the assays [15].

Drastic changes in soil pH could change the community structure of soil nitrifiers, shifting the dominance of different groups of nitrifiers better adapted to the new environmental conditions [5,8]. Zhang et al. [5] observed that applying CaO as a liming material to an acidic soil increased the abundance of AOB significantly during the first week. They further observed that when N-fertilized soils were freshly amended with CaO, only autotrophic growth of AOB was observed after seven days, but not after 30 days when growth of AOA was observed [5]. Whether the drastic changes in pH in a short-term due to liming like activities would be reflected in potential nitrifying activity is not clearly established.

Our study aims to assess whether the changes in soil pH, which would have an impact on the activity and community structure of soil nitrifiers, are detected in potential nitrifying activity estimates from PNR and NP assays. Here we have used two contrasting soil types and a liming treatment to capture the effect of changing pH and differences in nitrifying communities. We determined PNR with soil buffered at its original soil pH and at pH 7 to assess whether the modification of the pH of the buffer used in shaken slurry method would change our observations on nitrification potential of soil. We hypothesized that using P-buffer set at original soil pH (modified P-buffer) would result in a PNR different from the PNR measured when we use the P-buffer set at pH 7. We also hypothesized that PNR measured using the modified P-buffer would capture the short-term drastic changes in soil pH due to liming and also would correlate well with the NP of the soils.

2. MATERIALS AND METHODS

2.1 Soil Sampling, Incubation and Basic Soil Characterization

Soil samples were collected from *Seethaeliya* and *Mahailuppallama,* which belong to WU3 and DL1 agro-ecological regions of Sri Lanka, respectively. The dominant soil orders in the two regions are Ultisol and Alfisol, respectively [16,17]. According to the local classification, these soils are classified under the great soil groups Red Yellow Podzolic (RYP), Reddish Brown Earth (RBE), respectively. Disturbed soil samples were collected at a depth of 1-20 cm

from a fallowed crop field in the Government Seed Potato Farm, *Seethaeliya* (6°58'22.84"N 80°47'60.0"E) located in the Upcountry Wet-zone, and from an adjacent uncultivated area (6°57'6.05"N 80°47'56.08"E). In the same manner, soil was collected from a fallowed crop field in the Field Crop Research and Development Institute at *Mahailuppallma* (8°5'57.3"N 80°26'29.90"E) located in the Low country Dry-zone and from an adjacent uncultivated land (8°5'52.764"N 80°26'31.812"E). Then soil samples were transported to the University of Peradeniya for laboratory analysis. Before starting incubation, field-fresh soil was passed through a 4 mm sieve. For basic soil characterization, a sub-sample from each soil was air dried and passed through the 2 mm sieve. Initial soil pH was measured in soil: 1M KCl suspension of 1:2.5 using EUTECH CON500® pH meter, and electrical conductivity (EC) in soil: water suspension of 1:5 using EUTECH 510® EC meter. Organic carbon content was determined using modified Walkley and Black method [18] and nitrate concentrations of soil samples were measured using the rapid colorimetric method described by Cataldo et al. [19]. Soil samples were analyzed for total N and soil texture using standard protocols [20]. The abundance of ammonia oxidizers in field-moist soils were determined using the most probable number (MPN) method as described by Schmidt and Belser [14].

Then soil samples were arranged for the incubation study. Field moist soil was pre-incubated for 2 weeks before applying treatments [2]. Then soil pH of a sub-sample of two cultivated soils was altered using $CaCO_3$ applied at 1.8 g/kg rate. One kilogram of each soil (cultivated soil with and without liming and uncultivated soil from each Ultisol and Alfisol types) was transferred to plastic trays separately and volumetric moisture content of soil was adjusted to 0.25 (v/v), which was maintained throughout the incubation period. Soils were incubated at 30 °C for 2 weeks under dark condition. At the end of the incubation period subsamples were collected to perform PNR assay and NP immediately.

2.2 Assessing PNR and NP

The PNR of incubated soils was determined by the shaken slurry method, which was previously described by Hart et al. [13]. The standard protocol for this method uses a P-buffer set at pH 7. We have prepared a modified P-buffer for each soil by adjusting the pH of the P-buffer to original pH of the soil. Uncultivated, cultivated, and pH-adjusted cultivated Ultisol had pH levels of 4.16, 4.24, and 5.09, respectively. The pH levels of uncultivated, cultivated, and pH-adjusted cultivated Alfisol were 6.13, 5.45, and 7.35 respectively. Therefore, six modified P-buffers were prepared to be used with each soil. The PNR was performed with five replicates per soil. Method in brief, for each sample, 10 g of soil (dry weight equivalent) was placed into a 250 ml Erlenmeyer flask, and a 45 ml aliquot of standard or modified P-buffer containing 1.5 mM $(NH_4)_2SO_4$ was added. Flasks were shaken at 180 rpm on an orbital shaker, and aliquots were removed at 4, 20 and 24 hours after incubation, and analyzed for NO_3^--N colorimetrically as described by Cataldo et al. [19]. The PNR was calculated using readings from 4 to 24 hours and expressed as mg NO_3^--N kg^{-1} of dry soil day^{-1}.

The NP of incubated soil samples was measured using the method described by Schmidt and Belser [14]. Initial NO_3^--N in soils before using in NP was analyzed colorimetrically as described by Cataldo et al. [19]. Then 100 g moist soil was taken into a beaker and 2 mL of $(NH_4)_2SO_4$ solution was added to supply 75 mg N kg^{-1} soil. The moisture content of the soils was adjusted with water to obtain a moisture factor of 1.23±0.01. A duplicate of the same soil was prepared without adding $(NH_4)_2SO_4$ and brought to the same moisture level. Four replicates were used in the study. The soils were incubated for 21 days at 25 °C maintaining the initial moisture level throughout. At the end of the incubation, the NO_3^--N concentration of soil samples was measured using the rapid colorimetric method described by Cataldo et al. [19] and the amount of NO_3^--N formed during incubation was calculated and expressed as mg NO_3^--N kg^{-1} of dry soil. Nitrate formed under NH_4^+ supply was considered as the NP. The percent increase in NO_3^--N formation due to NH_4^+-N supply was also calculated.

2.3 Data Analysis

The statistical design used for both experiments was completely randomized design. All statistical analysis was carried out using PASW statistics 18 software. Two data sets were tested for normality and homogeneity of variances using the Shapiro-Wilk test and Leven's test respectively. Then three-way ANOVA and one-way ANOVA followed by Post-hoc test were conducted for PNR and two-way ANOVA was

conducted for NP data. After that correlations among measured parameters were analyzed using the Pearson correlation test at P=0.05.

3. RESULTS AND DISCUSSION

3.1 The Effect of Land use and Soil Type on Nitrifiers

Alfisol is the predominant soil type found in the low country dry zone of Sri Lanka, and the relatively more weathered Ultisols are commonly found in the upcountry wet zone of the country [16,17]. Cropping patterns and agronomic practices implemented on Ultisols and Alfisols are also different. In annual cropping systems in the upcountry wet zone of Sri Lanka, farmers cultivate three to four crops per year with high nutrient inputs. Most farmers cultivate potato and high value temperate vegetable crops by applying animal manures at least once a year, in addition to chemical fertilizers. In contrast, only one or two crops per year is being cultivated to coincide with rainy seasons in the low country dry zone of Sri Lanka. The upland areas in the dry zone are cultivated with tropical field crops like maize, chili, and legumes, often using only chemical fertilizers. Ultisols are more acidic than Alfisols and therefore, farmers practice liming when using Ultisols for crop production [16,17]. We also observed that Alfisols used in the present study were less acidic, had lower organic carbon and total N contents, and had higher EC and clay contents than the Ultisols (Table 1). The characteristics of the soils used in the present study were different based on soil type as well as land-use (Table 1). Major differences between the two soil types (Ultisol and Alfisol) were observed with respect to soil pH, EC, organic carbon, and texture. Land-use affected the abundance of ammonia oxidizers in the Ultisol. Incubating soils after applying $CaCO_3$ resulted in a range of soil pH levels (Table 2).

The diversity of soil nitrifiers is influenced by environmental conditions such as pH, temperature, NH_3 concentration, O_2 levels, and moisture content [4,5,6,8,21]. Therefore, the responsiveness of nitrifying communities in the soils we studied to liming may not be consistent [8]. De Boer and Kowalchuk [22] reported that nitrification rates of the AOB isolates they have studied decreased dramatically as pH decreased, indicating sensitivity of soil nitrifiers to pH. Therefore, considering inherent soil fertility characters and differences in soil management and agronomic practices considerable dissimilarity in microbial communities can be expected between Ultisols and Alfisols, and also between cultivated and uncultivated soils of each soil type [12,23]. The differences in the abundance of AOB in the soils used in our study (Table 1) also support this idea. Although the community composition of nitrifiers was not

Table 1. Characteristics of the soils collected from two land uses (cultivated and uncultivated) under Ultisol and Alfisol soil orders

Parameter	Ultisol		Alfisol	
	Cultivated	Uncultivated	Cultivated	Uncultivated
Soil pH	4.24±0.03	4.16±0.02	5.45±0.02	6.13±0.03
Electrical conductivity (dS m^{-1})	0.25±0.01	0.24±0.01	0.34±0.02	0.30±0.01
Organic carbon (%)	1.7±0.2	2.2±0.4	0.9±0.1	1.1±0.1
NO_3^- concentration (mg kg^{-1})	19.25±0.20	16.12±0.13	20.06±0.09	17.98±0.15
Total N (%)	0.28±0.02	0.37±0.01	0.14±0.01	0.17±0.01
Abundance of ammonia oxidizers (Log$_{10}$Cells g^{-1} dry soil)	2.7±1.0	4.0±0.5	3.7±1.9	4.7±2.5
Soil texture	Sandy loam	Sandy loam	Sandy clay loam	Sandy clay loam

Values are presented as Average ± SD (SD=Standard Deviation)

Table 2. The pH levels of soils at the end of two weeks incubation

| Parameter	Ultisol			Alfisol		
	Uncultivated soil	Cultivated soil		Uncultivated soil	Cultivated soil	
	- CaCO$_3$	- CaCO$_3$	+ CaCO$_3$	- CaCO$_3$	- CaCO$_3$	+ CaCO$_3$
Soil pH	4.16±0.02	4.24±0.01	5.09±0.01	6.13±0.02	5.45±0.02	7.35±0.01

assessed to species level, based on the observations on differences in soil characteristics and previous literature on factors driving community dynamics of nitrifiers [4,5,8] we can conclude that the soils collected from cultivated and uncultivated land uses under Alfisols and Ultisols should have different nitrifying populations. Therefore, it warrants our requirement of diverse initial nitrifying communities to assess the suitability of using PNR and NP assays to detect the changes in nitrifying activity in response to drastic changes in soil pH.

3.2 The Trends of PNR and NP

The PNR was significantly affected ($P<0.001$) by the soil type (i.e., Ultisol vs. Alfisol) and the soil treatment (i.e., uncultivated, cultivated without $CaCO_3$ treatment and cultivated with $CaCO_3$ treatment) but not by the P-buffer type (i.e., P-buffer at pH 7 or P-buffer with adjusted pH) used in the assay ($P=0.699$). Further, except for the interaction effect between soil treatment and the P-buffer type other interactions between the main grouping factors were significant ($P<0.001$). In both soil types, uncultivated soil had significantly highest PNR ($P<0.05$) when measured using P-buffer at pH 7

(Fig. 1). Adjusting P-buffer pH yielded significant differences ($P<0.05$) in PNR estimates in uncultivated soil and cultivated soil without $CaCO_3$ treatment for both soil types. In Ultisol, adjusting P-buffer pH resulted in a higher PNR, whereas in Alfisol, the trend was opposite. The effect of $CaCO_3$ application to the cultivated soil on nitrifying activity was captured in PNR estimates obtained with pH adjusted P-buffer but not with P-buffer at pH 7 in both soil types (Fig. 1).

The potential nitrifying activity estimates from NP analysis did not follow the same trend as the results from the PNR assay (Fig. 2). The soil treatment effect on NP was significant only in Ultisol but not in Alfisol. In Ultisol, the highest NP was observed in the cultivated soil treated with $CaCO_3$ and the lowest was observed for the uncultivated soil (Fig. 2). Further, the application of $CaCO_3$ has increased NP significantly ($P<0.05$) only in Ultisol (Fig. 2). However, the increase in NO_3^- production in response to NH_4^+ supply in the NP assay was significantly affected by the soil type and soil treatment (Fig. 3). The percent increase in NO_3^- production with the external supply of NH_4^+ was higher in cultivated soil than uncultivated soil for both soil types (Fig. 3).
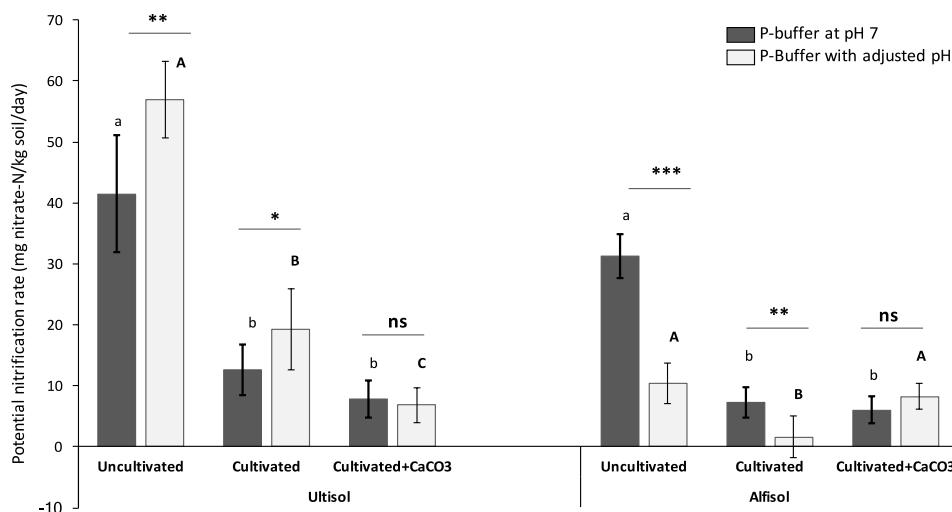

Fig. 1. The estimated potential nitrifying activity using potential nitrification rate assay with (PNR) shaken slurry method in an uncultivated soil and a cultivated soil with and without being treated with $CaCO_3$ for two soil types (Ultisol and Alfisol). The PNR was measured using P-buffer at pH 7 or adjusted to match the pH of the soil (dark color and light color bars, respectively) over a period of 24 hours

*Significance of the pH of P-buffer on PNR estimate is reflected by probability level (P<0.001 = ***, P<0.01 = **, P<0.05 = * and P>0.05 = ns) indicated above each soil treatment. Mean comparison was performed for PNR of three soil treatments measured using a given P-buffer for each soil type separately. Means followed by the same letter are not significantly different (P>0.05). Error bars indicate standard deviation. ns=not significant*
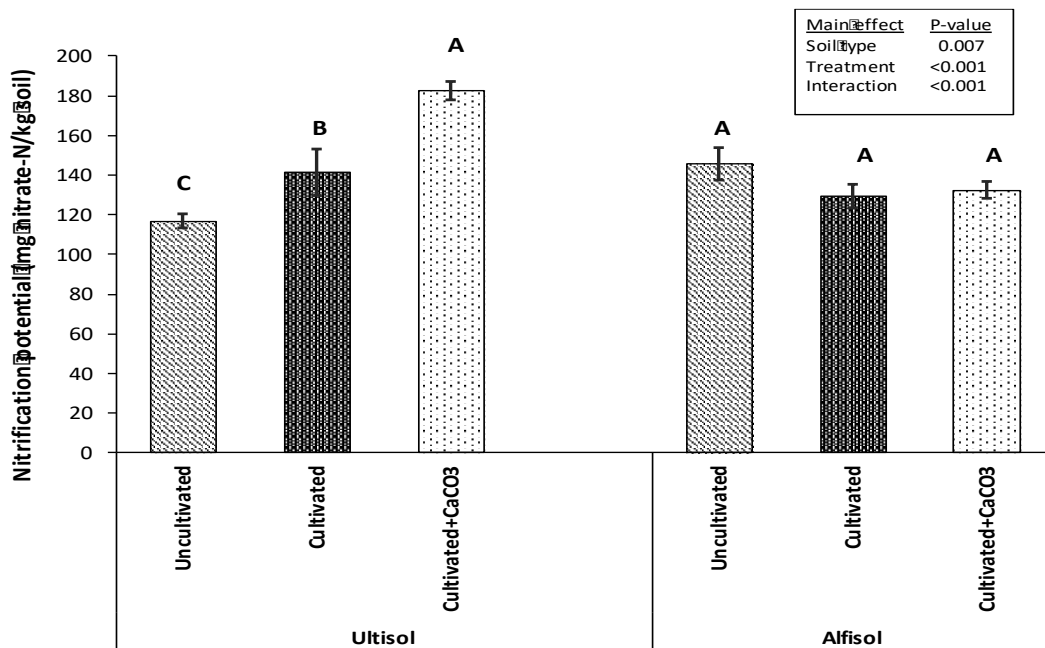
Fig. 2. The nitrifying potential (NP) estimated by measuring NO_3^- produced in 21 days after applying NH_4^+ to soil in an uncultivated soil and a cultivated soil treated with and without $CaCO_3$ for two soil types (Ultisol and Alfisol)
The means followed by same letter for a given soil type are not significantly different (P>0.05). Error bars indicate standard deviation
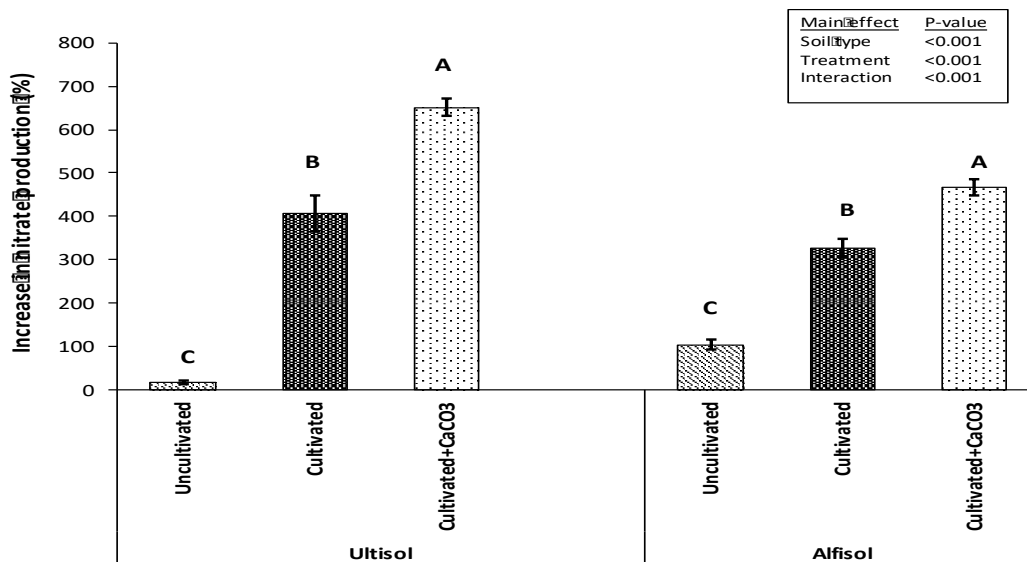
Fig. 3. The increase in NO_3^- production over 21 days period in response to external supply of NH_4^+ in an uncultivated soil and a cultivated soil treated with and without $CaCO_3$ for two soil types (Ultisol and Alfisol).
The means followed by same letter for a given soil type are not significantly different (P>0.05). Error bars indicate standard deviation.

The percent increase in NO_3^- production showed significant negative correlations with NP measured without adding NH_4^+ to the soil and PNR measured with P-buffer at pH 7 (Fig. 4).

The NP measured without adding NH_4^+ to the soil had a significant positive correlation with PNR measured with P-buffer at pH 7 ($r2= 0.98$, $P<0.001$). Soil pH, NP measured with external

supply of NH_4^+ and PNR measured using pH adjusted P-buffer did not have significant correlations (P>0.05) among each other or with NP measured without adding NH_4^+ to soil, PNR measured with P-buffer at pH 7 or percent increase in NO_3^- production in response to NH_4^+ supply in NP assay.

The PNR depends on the abundance and potential activity of the members of the soil nitrifying community [14,15]. The assay seeks to characterize the nitrifying population in non-limiting substrate and oxygen settings using a brief incubation period of one to two days that effectively prevents increases in the standing population during the experiment [14,24]. Yao et al. [25] reported PNR ranging from 1.2 to 31.7 mg NO_3^--N kg^{-1} dry soil day^{-1} for acidic soils cultivated with tea [25]. Dias and Dandeniya [23] observed that PNR was significantly higher in Ultisol (71±0.94 mg NO_3^--N kg^{-1} dry soil day^{-1}) than in Alfisol (50±5.20 mg NO_3^--N kg^{-1} dry soil day^{-1}) they used in their study.

The physiological diversity of ammonia oxidizers and their differential responses to environmental conditions remain the biggest challenges for using PNR to estimate potential nitrifying activity in soils [5,6]. The maximum specific growth rate, cell specific activity, inhibitory ammonia and nitrite concentrations, affinity for ammonia and their responsiveness to changes in pH differ among nitrifying species [6,22]. Hence, the optimum conditions provided during the PNR assay may not facilitate a maximum nitrification rate for all nitrifiers. Therefore, the shaken slurry method could be bias towards the lithoautotrophic AOB and the PNR may not reflect the activity of nitrifiers *in situ* [6]. Irrespective of this limitation, PNR assay using shaken slurry method is widely used in soil microbiology research [5,23,26,27]. Significant correlations between PNR and the abundance and/or community structure of AOB have been detected in many agricultural soils [5,26,27]. Drawbacks in using PNR arise mainly when studying nitrification in acidic soil environments where the dominance of fast growing AOB is absent because nitrification under high NH_4^+ supply is often associated with AOB than AOA [5,8,27].
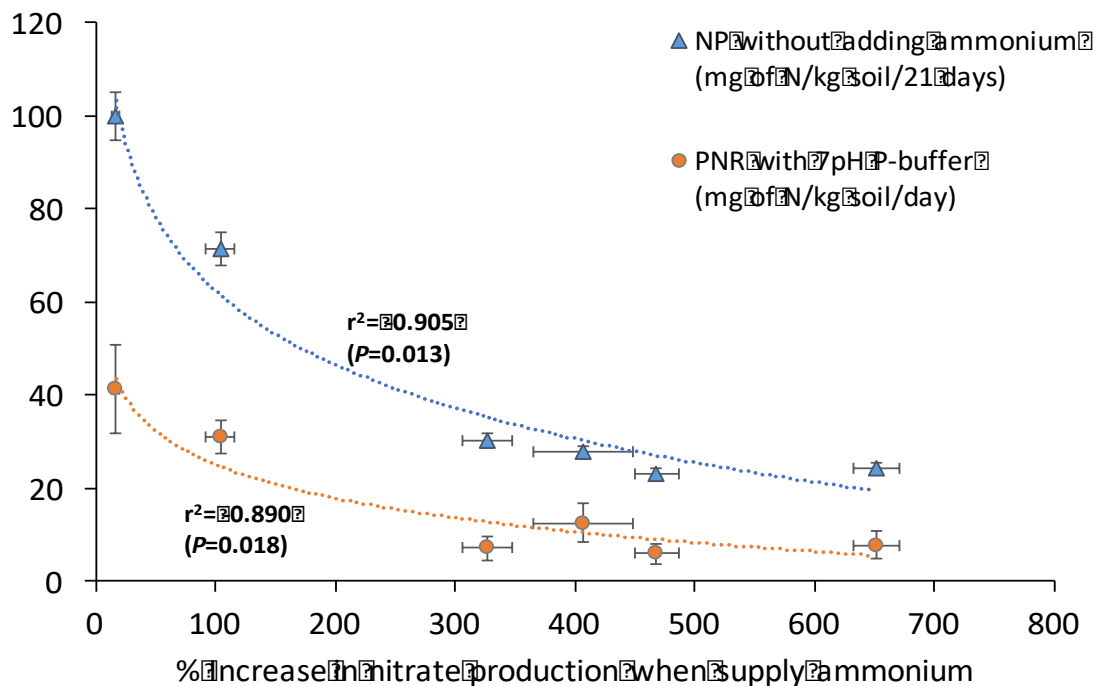
Fig. 4. Correlations between percent increase in nitrate production when NH_4^+ is added to soil and nitrifying activity measured as nitrifying potential (NP) under zero application of NH_4^+ to soil (blue triangles) and potential nitrification rate (PNR) measured using P-buffer at pH 7 (orange circles). Error bars indicate standard deviation

In contrast to PNR, which is a kinetic type assay, NP is determined using a static incubation of soil for a period of 21 days, during which there is a chance of changing the population size of soil nitrifiers and drastic shifts in the dominance of different groups of nitrifiers [24]. Further, the estimated nitrate production in NP is a net-production because denitrification, immobilization, and mineralization like other transformations that decide nitrate levels in soil continues over the relatively long incubation period [14,15]. We observed that the NP assay provided a lower estimate of nitrification than PNR (Fig. 1 and Fig. 2). This is as expected because in the NP assay, the oxygen diffusivity is limited by the presence of anaerobic microsites similar to those that happen under field conditions [15], and some amount of NO_3^- produced may be immobilized because of diffusional constraints [28] and denitrified during 21-day incubation period [27]. Further, N-fertilizer induced nitrification may induce oxygen consumption leading to hypoxia, discouraging lithoautotrophic nitrifiers and encouraging heterotrophic nitrifiers, especially in wet soils with 70% water filled pore spaces [27]. The nitrifying ability of the heterotrophic nitrifiers are much lower than the lithoautotrophs [27]. But in the PNR assay, soil is oxygenated with continuous shaking of the slurry avoiding oxygen limitation for the aerobic nitrifiers [13]. Although both PNR and NP are used to study the potential nitrifying activity of soils, the two approaches provide different information about soil nitrification and may not necessarily correlate well with each other [15]. However, both methods provide important information on potential changes in soil nitrifying activity in response to a sudden increase in NH_4^+ concentration in soil as happens with N-fertilizer application [13,14].

In our study, PNR measured using P-buffer at pH 7 had a strong positive correlation with the net NO_3^- produced during soil incubation without adding NH_4^+ to soil in NP assay. But NP measured by supplying NH_4^+ to the soil did not correlate with PNR. When NH_4^+ is not supplied, the activity of nitrifiers is first limited by the substrate availability [14]. Under this condition, the initial population size and the activity of nitrifies would be more important than the changes in their population size to decide the amount of NO_3^- produced during the incubation [8,24]. Because the population growth will be limited by the substrate limitation.

According to our results, the differences in nitrifying activity based on land use and soil types were more reflected in PNR than in NP (Fig. 1 and Fig. 2). Both the abundance of AOB and PNR were higher in uncultivated soils than in cultivated soils used in our study (Table 1 and Fig. 1). But interestingly, NP showed an opposite trend in the Ultisol and no difference based on land use was observed in the Alfisol (Fig. 2). However, the increase in NO_3^- production in response to NH_4^+ supply was more sensitive to land use than NP, and it was significantly higher in cultivated soil than in uncultivated soil for both Alfisol and Ultisol (Fig. 3). Organic carbon and total N contents in Ultisol were higher than Alfisols for both land use types (Table 1). Therefore, the amount of NH_4^+ liberated through mineralization in Ultisol should be higher than that in Alfisol. Further, the external supply of NH_4^+ may have stimulated the inherent N mineralization in soil, increasing substrate availability for nitrifiers. In the calculation of the percent increase in NO_3^- production in response to NH_4^+ supply, the amount of NO_3^- produced from NH_4^+ added through mineralization is removed and therefore, the values reflect the response of the community to external supply of NH_4^+ [14]. The results indicate that although uncultivated soils may have nitrifiers with high potential activity, the members that respond to a sudden increase in NH_4^+ levels by increasing population growth would be high in cultivated soils. Also, the results indicate the important link that might be there between mineralization and nitrification processes in soil for determining the dynamics of nitrifiers. Conducting a detailed analysis of changes in the community structure of nitrifiers coupled with $^{13}CO_2$-SIP-DNA and NP assays could be considered in a future study to explain these observations [5].

Previous studies suggest that the PNR assay may be biased towards lithoautotrophic bacteria, and therefore there is a close link between PNR estimates and AOB abundance [5,6,27]. It has been shown that pH and exposure to high NH_4^+ concentrations are the main factors that would decide on the dominance of AOB over AOA [5,8,27] It is interesting to note that in our study, the PNR or NP did not have significant correlations with soil pH, and acidic Ultisols had higher PNR rates than slightly acidic Alfisols.

3.3 The Effect of Liming on PNR and NP

Evolution aided by the adaptations of microorganisms and the selection of the best fitting members would shape the community structures in the long run [4,5,8,27]. As a result, it is not surprising that the soil nitrifying

communities are different among different land uses and soil types [8]. In our study, PNR largely and NP to some extent were capable of capturing the difference in potential nitrifying activity, which is determined by the community composition of nitrifiers, as affected by soil type and land use (Fig. 1 and Fig. 2).

PNR estimated following the original protocol that used P-buffer at pH 7, and the NP measured with NH_4^+ supply, were not sensitive to the changes that happen with the modification of soil pH due to liming (Fig. 1). However, PNR measured using modified P-buffer with adjusted pH, and the percent increase in NO_3^- production due to external supply of NH_4^+ indicate changes in nitrifying activity in soils due to liming (Fig. 1). Further, the PNR determined using two P-buffers resulted in statistically similar estimates only when used with the soil treated with $CaCO_3$. This may be because pH of the P-buffer used in standard protocol may be more similar to the modified pH in soil due to $CaCO_3$ application. As we have hypothesized PNR measured using the modified P-buffer captured the short-term drastic changes in soil pH due to liming. However, we did not observe a positive correlation between PNR measured using P-buffer with adjusted pH and NP. The trends seen with the results from these two measurements using soils treated with and without $CaCO_3$ were similar for Alfisol but not for Ultisol. In Ultisol, treating soil with $CaCO_3$ decreased PNR when measured using P-buffer with adjusted pH (Fig. 1) but boosted the percent increase in NO_3^- production over 21 days in the NP assay (Fig. 3). As we have hypothesized, using P-buffer set at original soil pH (modified P-buffer) resulted in a PNR different from the PNR measured with the P-buffer set at pH 7. The results confirm that traditional PNR and only considering NP may not be suitable to detect the contingent effect of liming in soils.

In a microcosm study conducted by Teutscherova and team [24] to determine the effect of $CaCO_3$ induced soil pH change on net nitrification, a significant increment in net nitrification was not observed until day 11, even with optimum NH_4^+ supply. Authors attributed this observation to the low activity of nitrifiers at the beginning of the experiment [24]. Che et al. [8] also explained that the low nitrification rates they have observed for Jiangxi soil regardless of increasing pH with $CaCO_3$ with or without NH_4^+ supply may be due to the low abundance of activity of both AOB and AOA. Therefore, the success of using NP and PNR to determine the

contingent effects due to liming would be influenced by the diversity and functionality of the nitrifying community in a soil [5,8,24]. Thus, soil to soil variability in results generated for similar types of treatments could be expected.

In most of the crop cultivations, the first dose of N-fertilizer is applied within a few weeks after liming. In cultivating potatoes in Ultisols in the upcountry of Sri Lanka, farmers apply both organic manures and chemical fertilizers only a few days apart from liming. Understanding the contingent effects of liming on nitrifiers is therefore important for managing N-fertilizer use efficiency. Although $^{13}CO_2$-SIP-DNA and ^{15}N based techniques can be used to estimate nitrifying activity with higher accuracy than PNR and NP assays, such methods are costly and require special laboratory conditions and equipment. Our study indicated that PNR can be used with modifications to assess short-term changes in nitrifying activity.

4. CONCLUSION

Both PNR and NP determination captured the differences in nitrifying activity as influenced by soil type and land-use. In here, the PNR determined following the standard protocol using P-buffer at pH7 was more sensitive than NP to detect the differences in soil nitrifying activity between land uses. However, these two measurements were not sensitive to the changes that might happen to nitrifying community with the application of liming materials in the short-run (i.e., two weeks after the application of $CaCO_3$). Instead, PNR determined using modified P-buffer with pH adjusted to match soil pH and the percent increase in NO_3^- production due to external application of NH_4^+ as calculated based on NP assay have captured the differences in potential nitrifying activity due to liming. The differences in PNR estimates obtained when using two different P-buffers (i.e., P-buffer at pH 7 and P-buffer modified to match soil pH) highlight that traditional PNR should be biased towards certain groups of nitrifiers, mainly based on their adaptations to different pH levels. Hence, the results from traditional PNR and NP assays conducted to capture short-term changes in nitrifying activity should be treated with caution. We conclude that PNR measured using P-buffer modified to match the original soil pH, and determining the percent increase in NO_3^- production due to external application of NH_4^+ are better determinations of potential nitrifying

activity than traditional PNR measured using P-buffer at pH 7 and NP.

Our results from the NP assay suggest that there is an important link between mineralization and nitrification processes in soil, shaping the dynamics of soil nitrifiers. A detailed analysis of changes in the community structure of nitrifiers in short term in response to $CaCO_3$ application and the relationships between PNR and NP assays with the community shifts should be studied using a polyphasic approach, including molecular techniques to explain the observations in future studies.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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