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Copper induces nitrification by ammonia-oxidizing bacteria and archaea in pastoral soils

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Abstract

Copper (Cu) is the main co-factor in the functioning of the ammonia monooxygenase (AMO) enzyme, which is responsible for the first step of ammonia oxidation. We report a greenhouse-based pot experiment that examines the response of ammonia-oxidizing bacteria and archaea (AOB and AOA) to different bioavailable Cu concentrations in three pastoral soils (Recent, Pallic, and Pumice soils) planted with ryegrass (*Lolium perenne* L.). Five treatments were used: control (no urine and Cu), urine only at 300 mg N kg^{-1} soil (Cu0), urine + 1 mg Cu kg^{-1} soil (Cu1), urine + 10 mg Cu kg^{-1} soil (Cu10), and urine + 100 mg Cu kg^{-1} soil (Cu100). Pots were destructively sampled at Day 0, 1, 7, 15, and 25 after urine application. The AOB/AOA *amoA* gene abundance was analyzed by real-time quantitative polymerase chain reaction at Days 1 and 15. The AOB *amoA* gene abundance increased 10.0- and 22.6-fold in the Recent soil and 2.1- and 2.5-fold in the Pallic soil for the Cu10 compared with Cu0 on Days 1 and 15, respectively. In contrast, the Cu100 was associated with a reduction in AOB *amoA* gene abundance in the Recent and Pallic soils but not in the Pumice soil. This may be due to the influence of soil cation exchange capacity differences on the bioavailable Cu. Bioavailable Cu in the Recent and Pallic soils influenced nitrification and AOB *amoA* gene abundance, as evidenced by the strong positive correlation between bioavailable Cu, nitrification, and AOB *amoA*. However, bioavailable Cu did not influence the nitrification and AOA *amoA* gene abundance increase.

1 | INTRODUCTION

Grazing livestock inefficiently utilize ingested nitrogen (N) (Kebreab et al., 2001). About 80% of ingested N is excreted with urine, and several studies have shown that a significant proportion of N can be lost from the soil via nitrate (NO_3^--N) leaching before it can be taken up by plants (Di & Cameron, 2002, 2016). Leached NO_3^--N can trigger environmental problems such as water and air pollution (Rex et al., 2021; Richards et al., 2021; Yu et al., 2019), and science-led strategies to improve NO_3^--N management are actively sought to decrease the impact of N losses.

Abbreviations:: AMO, ammonia monooxygenase; AOA, ammonia-oxidizing Archaea; AOB, ammonia-oxidizing bacteria; DW, dry weight; qPCR, quantitative polymerase chain reaction.

Nitrification is a naturally occurring process that describes the microbial oxidation of ammonia (NH_4^+-N) to NO_3^--N via nitrite (NO_2). This process is performed by ammonia-oxidizing bacteria and archaea (AOB and AOA) found in most soils (Carey et al., 2016; Prosser & Nicol, 2012). There are unique biological characteristics between AOB and AOA that are mostly influenced by environmental conditions (Lu et al., 2020; Prosser et al., 2020). For example, oxidizing bacteria have low affinity of NH_4^+, and several studies have reported AOB to be dominant in high NH_4^+ soils (Carey et al., 2016; Jia & Conrad, 2009). In contrast, AOA are dominant in low NH_4^+ conditions because of their high affinity to NH_4^+ (Nicol et al., 2008; Rütting et al., 2021; Ying et al., 2010). Both AOB and AOA possess the ammonia monooxygenase (AMO) enzyme encoded in the *amoA* gene, which is responsible for the first and often rate limiting step of NH_4^+ oxidation into hydroxylamine (NH_2OH) (Principi et al., 2009).

Previous studies demonstrated that the AMO enzyme contains a Cu-active site, making Cu a co-factor in the functioning of the enzyme (Hooper et al., 1997; McCarty, 1999). For example, Vandevivere et al. (1998) showed that application of 5 mg Cu L^{-1} as $CuSO_4$ improved NH_4^+-N oxidation in sewage sludge, while Wagner et al. (2016) found that increasing the Cu dose from 0.05 to 5 µg Cu L^{-1} significantly increased NH_4^+-N oxidation during water treatment. However, several studies presented contrasting results on the effect of Cu on NH_4^+-N oxidation (Loveless & Painter, 1968; Wagner et al., 2016). Cela & Sumner (2002) demonstrated that a water-extractable Cu concentration above 3.8 mg kg^{-1} severely inhibited nitrification in three soils. He et al. (2018) reported that a Cu concentration above 100 mg Cu kg^{-1} significantly reduced AOB *amoA* gene transcripts in an incubation study with a Fluvo-aquic soil (Shondong, China) relative to a control. Scientific literature, therefore, indicates that there is a lack of clear evidence on the relationship between Cu concentrations in soils and AOB/AOA functioning.

In a previous incubation experiment (Matse et al., 2022), we identified that Cu is an important trace element in the process of nitrification in three pastoral soils in New Zealand (Pumice, Pallic, and Recent soils). Our data demonstrated that reducing the Cu concentration in these soils negatively affected the nitrification rate in the soil. However, relative changes in the profile of ammonia nitrifiers were not determined. Therefore, to provide direct evidence of the significance of Cu on microbial nitrification processes, the current study was conducted to evaluate the effect of Cu on bacterial and archaeal population, and AOB/AOA *amoA* gene expression. In this study, *amoA* gene abundance has been used to quantify the AMO enzyme activity responsible for the first step of nitrification. To our knowledge, no study has previously explored the relationship between bioavailable Cu and AOB/AOA in pastoral soils.

Core Ideas

- Copper concentration 0.24–0.33 mg kg^{-1} increased nitrification rate for Recent and Pallic soils but inhibited above 6 mg kg^{-1}.
- Changes in nitrification rate in Recent and Pallic soils were positively correlated with AOB amoA gene abundance.
- AOA amoA gene was stimulated at higher Cu concentration in Recent and Pallic soils but not in Pumice soil.

The present study examines the response of bacterial and archaeal total population, and AOB/AOA *amoA* gene abundance, to different Cu concentrations in pastoral soils. The objective was to quantify the effect of bioavailable Cu on AOA/AOB and nitrification rate in the context of research programmes that are developing nitrification inhibitors for dairy pastoral systems. The aim of our work is to provide new insights into the relationship between Cu and ammonia nitrifiers in pastoral soils.

2 | MATERIALS AND METHODS

2.1 | Soil collection and characterization

Bulk topsoil samples of Recent, Pallic, and Pumice soil were sampled to a depth of 20 cm. The soils were representative of the Manawatu Recent soil, Pallic Firm Brown, and Orthic Pumice soil in terms of the New Zealand soil orders (Dystric Fluventic Eutrudept, Typic Dystrustept, and Typic Dystrustept, respectively, according to the U.S. Soil Taxonomy classification [Hewitt, 2010]). Recent soil was collected from the Dairy 1 farm located at Massey University (40°23′0.95″ S, 175°36′36.16″ E), Pallic soil was collected from the Canterbury region (43°34′13.15″ S, 171°55′47.33″ E), and Pumice soil was collected from a farm near Stratford in the Taranaki region (39°20′9″ S, 174°18′20″ E). Soil samples were sieved through <2-mm stainless steel sieve and divided into two portions. The first portion was air-dried for soil pH and cation exchange capacity determination. The second portion was stored fresh at <4 °C for less than a week to minimize any changes that might occur before use in the pot experiment described in this paper. Subsamples were analyzed for mineral N, moisture, and water-holding capacity. All soil chemical properties were analyzed using the

TABLE 1 Soil chemical characteristics

Parameter	Recent soil	Pallic soil	Pumice soil
Soil pH	5.8	5.2	5.8
NH_4^+-N (mg kg^{-1})	2.22	3.02	1.68
NO_3^--N (mg kg^{-1})	24.29	38.01	21.40
Bioavailable Cu (mg kg^{-1})	0.11	0.19	0.28
Exchangeable cations- (cmol$_c$ kg^{-1})			
Ca	4.5	3.5	3.4
Mg	1.15	0.58	0.76
K	0.44	0.46	0.33
Na	0.11	0.08	0.09
CEC	11	13	20
BS (%)	56	36	27
WHC (%)	31.5	45.9	80.6

Note. BS = base saturation; CEC = cation exchange capacity; WHC = water-holding capacity.

methods described by Matse et al. (2022). A summary of chemical parameters is presented in Table 1. These soils are the dominant soils in New Zealand under dairy pastoral system and were used in this study because they present contrasting soil properties.

2.2 | Treatments and application

This study was conducted using five treatments with three replicates of five sets in each soil: control (no urine and no Cu), urine-only at 300 mg N kg^{-1} (Cu0), urine + 1 mg Cu kg^{-1} (Cu1), urine + 10 mg Cu kg^{-1} (Cu10), and urine + 100 mg Cu kg^{-1} (Cu100). The applied Cu concentration ranges were selected based on conditions of deficiency, sufficiency, and toxicity with respect to the bioavailable Cu concentration in soil (Cela & Sumner, 2002). Hydrated copper sulphate ($CuSO_4$•$5H_20$) was used as the Cu source, with application rate calculated using the dry weight (DW) of the different soils to achieve the required concentrations. Field moist soil (0.5 kg) was spiked with the specified treatment and filled into each pot. Soil spiking and mixing were done as described by Ubeynarayana et al. (2021). Soils were incubated for 3 wk at 25 ˚C to equilibrate the Cu with the soil matrix. The soil was maintained at 70% water-filled pore space throughout the incubation period by weighing pots every after 2 d and maintaining the moisture level with deionized water.

Pots were transferred into the greenhouse at the Massey University Plant Growth Unit after 21 d of soil incubation and were arranged in a randomized complete block design. Perennial ryegrass (*Lolium perenne* L. 'Maxsyn NEA4') seeds (20 seeds pot^{-1}) were planted to model the dominant pasture cover for New Zealand dairy soils. Ryegrass was thinned to maintain 15 plants pot^{-1} after 7 d of germination. Ryegrass was then allowed to establish for 4 wk before urine application.

2.3 | Synthetic urine preparation and application

Synthetic urine was prepared using the formulation described by Clough et al. (1998): urea applied at 11.7 g L^{-1}, glycine at 2.90 g L^{-1}, $KHCO_3$ at 13.98 g L^{-1}, K_2SO_4 at 1.38 g L^{-1}, and KCl at 5.04 g L^{-1}. Four weeks after ryegrass establishment, a calculated amount of synthetic urine was applied at an equivalent rate of 300 mg N kg^{-1} to all treatments except the no-urine control. Daily watering was done after weighing each pot to ensure 70% water-filled pore space during the perennial ryegrass growth period. Average day and night temperatures were recorded over the time period from synthetic urine application to last harvest (Supplemental Figure S1).

2.4 | Plant and soil sampling

Destructive pot sampling was done in the lab at Day 0 (before urine application), and at Day 1, 7 15, and 25 after urine application (three replicates sampled at each time point). A total of 225 samples were collected throughout the experimental period (5 treatments × 3 replicates × 3 soils × 5 times). Plants were gently pulled from the soil by hand, and rhizosphere soil around the roots was removed by shaking before roots were washed several times using tap water. The ryegrass shoots and roots were separated using a set of stainless steel scissors (Matse et al., 2020). The DW of shoot and root biomass was recorded after oven-drying at 65 ˚C for 72 h. During sampling, the field moist soil from each pot was homogenized and subsampled into two parts. The first part was immediately stored at −20 ˚C for soil DNA extraction. The second part was kept at <4 ˚C for NH_4^+-N and NO_3^--N, and bioavailable Cu analysis.

2.5 | Soil analysis

Soil bioavailable Cu concentration was measured through extraction of 5 g field moist soil with 30 ml 0.05 $CaCl_2$ in an end-over-end shaker for 2 h. The resulting extraction was centrifuged at 1,100 g for 10 min, then filtered through Whatman 42 filter papers before analysis of bioavailable concentration Cu using microwave plasma atomic emission spectroscopy (4200 MP-AES, Agilent).

Soil mineral NH_4^+-N and NO_3^--N concentration was measured through extracting 5 g field moist soil with 30 ml, 2 M KCl in an end-over-end shaker for 1 h. The resulting extraction was centrifuged at 1,100 g for 10 min then filtered through Whatman 42 filter papers before analysis of soil mineral NH_4^+-N and NO_3^--N concentration using a Technicon autoanalyzer (Blakemore, 1987).

2.6 | Quality control measures

Two certified standard reference materials (SRM 2710a, Montana soil and SRM 1573a, tomato leaves) were analyzed during all sample runs. Recovery concentrations of the tomato leaves and Montana soil ranged from 92.4 to 98% and 93.4 to 98.6%, respectively, of certified Cu concentration values.

2.7 | Soil DNA extraction and qPCR

Dai et al. (2013) indicated that the ammonia oxidizer activities are peak approximately 15 d after treatment application. Therefore, soil sampling was done only on Day 1 and 15 for the microbial gene analysis. Soil DNA was extracted from 0.25 g of soil using the PowerSoil DNA Isolation Kit (MO BIO Laboratories) following the manufacturer's protocol. DNA quantity was confirmed by the ratio of absorbance at 260 and 280 nm using a NanoDrop ND-1000 (Thermo Fisher Scientific Inc.). The quality of the DNA was further confirmed using gel electrophoresis (1.5% TAE-agarose gel, tris-acetate-EDTA).

Quantitative polymerase chain reaction (qPCR) of AOB *amoA*, AOA *amoA*, bacterial 16S rRNA, and archaeal 16S rRNA was performed on a LightCycler 480II (Roche, software release 1.5.1.62 SP3) based on the fluorescence intensity during amplification using SsoFast EvaGreen Supermix (Bio-Rad Laboratories Inc.). Each DNA soil sample was analyzed in duplicate during the qPCR reaction. The primers used, sequences, and qPCR conditions are outlined in Supplemental Tables S1 and S2. The qPCR reaction mixture (total of 10 µl) contained 1 µl of 10-fold diluted DNA samples, 1× SsoFast EvaGreen Supermix, and 0.52 µl of each forward and reverse primer. The qPCR standard curves were performed using DNA samples with known concentration serially diluted ranging from 10^2 to 10^7. The qPCR efficiency for each primer pair ranged between 89 and 98%. Each DNA soil sample was analyzed in duplicate during the qPCR reaction. Each qPCR 96-plate run contained triplicates of nontemplate control samples for each primer pair. The copy of target gene per gram of soil was calculated according to Behrens et al. (2008).

2.8 | Statistical analysis

Analysis of variance (ANOVA) followed by Turkey's post hoc test was applied to determine significant differences between applied treatments means using Minitab (Version 19, Minitab Inc.).

3 | RESULTS

3.1 | Changes in bioavailable Cu concentration

There was a trend toward increasing bioavailable Cu concentration in soil as a function of the applied Cu (Figure 1). For the Recent soil, all Cu treatments (Cu1, Cu10, and Cu100) showed increased bioavailable Cu for all sampling days relative to the Cu0 treatment. For the Pallic soil, no clear changes were induced by the application of the Cu1 treatment relative to Cu0. The Cu10 and Cu100 treatments increased bioavailable Cu at all sampling days relative to Cu0. In the Pumice soil, the Cu1 treatment did not induce significant changes in bioavailable Cu relative to Cu0, while the Cu10 treatment showed a nominal increase with time. The Cu100 treatment increased bioavailable Cu across all samplings relative to the Cu0 treatment (Figure 1).

3.2 | Soil NH_4^+-N and NO_3^--N concentration

Copper had a variable effect on the NH_4^+-N concentration for the Recent and Pallic soil but no effect on the NH_4^+-N concentration for the Pumice soil (Figure 2). For the Recent soil, the Cu1 treatment reduced the NH_4^+-N concentration by a factor of 24 and 45% relative to the Cu0 treatment, at Days 1 and 7, respectively. There was a similar reduction for the Cu10 treatment, by 32 and 39% at Days 1 and 7, respectively. In the Cu100-treated soil, there was a nominal increase in NH_4^+-N relative to the Cu0 treatment, but this was only apparent on Day 1. For the Pallic soil, there was no significant change in the NH_4^+-N concentration induced by the Cu1 treatment at any sampling time relative to the Cu0 treatment. However, the Cu10 treatment reduced the NH_4^+-N concentration by a factor of 23 and 40% compared with Cu0 on Days 1 and 7, respectively (Figure 2). The Cu100 treatment was associated with an increase in NH_4^+-N concentration by factors of 18 and 106% relative to the Cu0 at Days 1 and 7, respectively. For the Pumice soil, there was no significant change in NH_4^+-N concentration associated with any treatment, with the exception of the Cu10 treatment on Day 7, which was significantly lower than the Cu0 treatment (Figure 2).

The NO_3^--N concentration was influenced by Cu treatment in all three soils. For the Recent soil, the Cu1 treatment increased the NO_3^--N concentration by 38 and 18% on both Days 1 and 7, respectively, relative to Cu0. The Cu10 treatment increased the NO_3^--N concentration by 145 and 48% (relative to Cu0) at Days 1 and 7, respectively (Figure 2). In

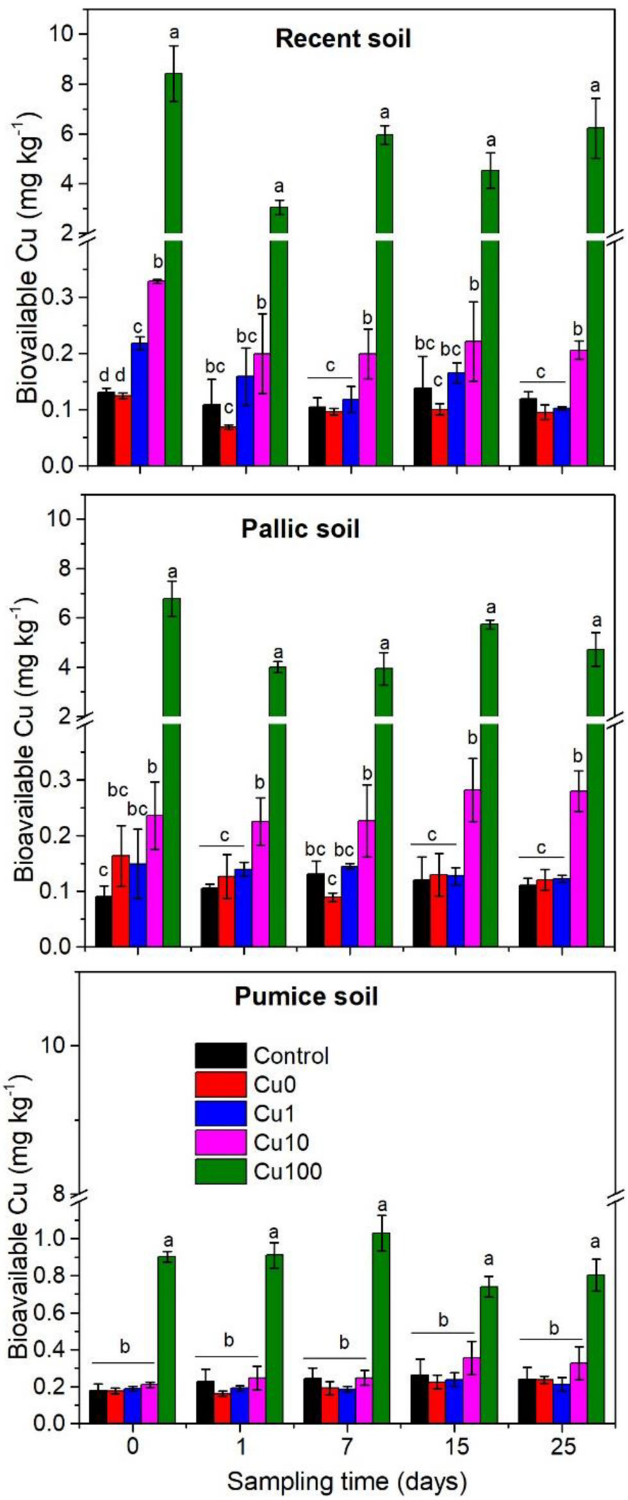

FIGURE 1 Bioavailable Cu concentration in Recent, Pallic, and Pumice soils as a function of treatments and sampling time. Error bars indicate standard deviation of mean ($n = 3$). Control = no urine and Cu; Cu0 = urine only at 300 mg N kg^{-1} soil; Cu1 = urine + 1 mg Cu kg^{-1} soil; Cu10 = urine + 10 mg Cu kg^{-1} soil; Cu100 = urine + 100 mg Cu kg^{-1} soil

contrast, the Cu100 treatment reduced the NO_3^--N concentration by factors of 27 and 31% at Days 1 and 7, respectively. For the Pallic soil, there was no significant effect of the Cu1 treatment on soil NO_3^--N concentration relative to the Cu0 treatment at any sampling time; however, the Cu10 treatment increased the NO_3^--N concentration by values of 205 and 37% at Days 1 and 7, respectively. The Cu100 treatment showed a nominal reduction in NO_3^--N concentration at Days 7 and 15 compared with Cu0. For the Pumice soil, there was a reduction in NO_3^--N concentration induced by the application of Cu1 and Cu10 at Days 7 and 15 (Figure 2). There was no consistent effect of the Cu100 treatment on soil NO_3^--N concentration in the Pumice soil relative to the Cu0 treatment.

3.3 | Bacterial and archaeal population in soil

The total bacterial and archaeal population in the soil was analyzed based on the abundance of 16S rRNA (Figure 3). There was no effect of the Cu1 treatment on bacterial population when compared to the Cu0 treatments in any of the three soils at either Day 1 or 15. However, the bacterial population in the Cu10 soil was increased by 1.6- and 3.3-fold in the Recent soil, and 1.4- and 1.5-fold in the Pallic soil, for Days 1 and 15, respectively relative to the Cu0 treatment. There was no significant increase in bacterial population that could be attributed to Cu10 and Cu100 for the Pumice soil.

The archaeal population in the control treatment was higher in all three soils relative to the Cu0 treatment irrespective of sampling time (Figure 3). Application of urine to the Cu1 and Cu10 treatments did not change the archaeal population relative to Cu0 treatments at any sampling time for any of the three soils. The Cu100 treatment showed higher archaeal population in the Recent and Pumice soil relative to the lower Cu treatments at both sampling times, although this increase was not apparent for the Pallic soil.

3.4 | AOB/AOA *amoA* gene abundance in soil

There were no significant differences in AOB *amoA* gene abundance between the Cu0 and Cu1 treatment for all three soils at all sampling times (Figure 4). However, for the Cu10 treatment, AOB *amoA* gene abundance increased 10.0- and 22.6-fold in the Recent soil and 2.1- and 2.5-fold in the Pallic soil relative to the Cu0 treatment on Days 1 and 15, respectively (Figure 4). This increase was not apparent for the Pumice soil. After application of the Cu100 treatment,

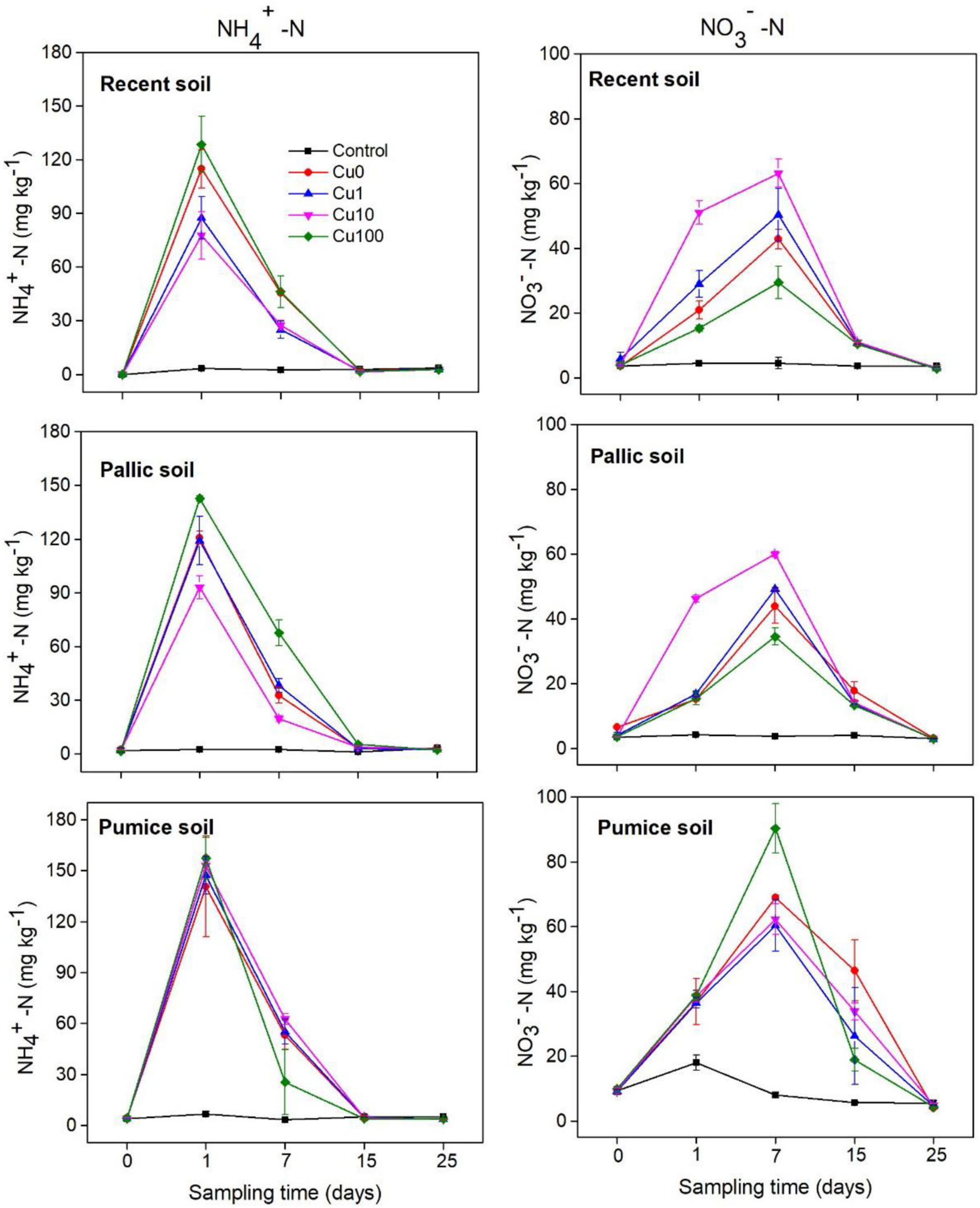

FIGURE 2 NH_4^+-N and NO_3^--N concentration for Recent, Pallic, and Pumice soils as a function of treatments and sampling time. Error bars indicate standard deviation of mean ($n = 3$). Control = no urine and Cu; Cu0 = urine only at 300 mg N kg^{-1} soil; Cu1 = urine + 1 mg Cu kg^{-1} soil; Cu10 = urine + 10 mg Cu kg^{-1} soil; Cu100 = urine + 100 mg Cu kg^{-1} soil

AOB *amoA* gene abundance reduced 7.4- and 1.3-fold in the Recent soil and 2.6- and 2.5-fold in the Pallic soil relative to the Cu0 on Days 1 and 15, respectively. For the Cu100 treatment, AOB *amoA* abundance increased 1.2- and 1.2-fold in the Pumice soil relative to the Cu0 treatment on Days 1 and 15, respectively.

Generally, there was an increase in AOA *amoA* gene abundance in the control treatment (relative to the urine treatments) in all three soils for both sampling times (Figure 4). There were no significant changes in AOA *amoA* gene abundance associated with the Cu1 or Cu10 treatments on Days 1 and 15 for all three soils relative to Cu0 treatment, but there was an increase in abundance for the Recent and Pallic soils at the Cu100 treatment level on Days 1 and 15 (Figure 4). There was no effect of Cu100 treatment on AOA *amoA* gene abundance for the Pumice soil.

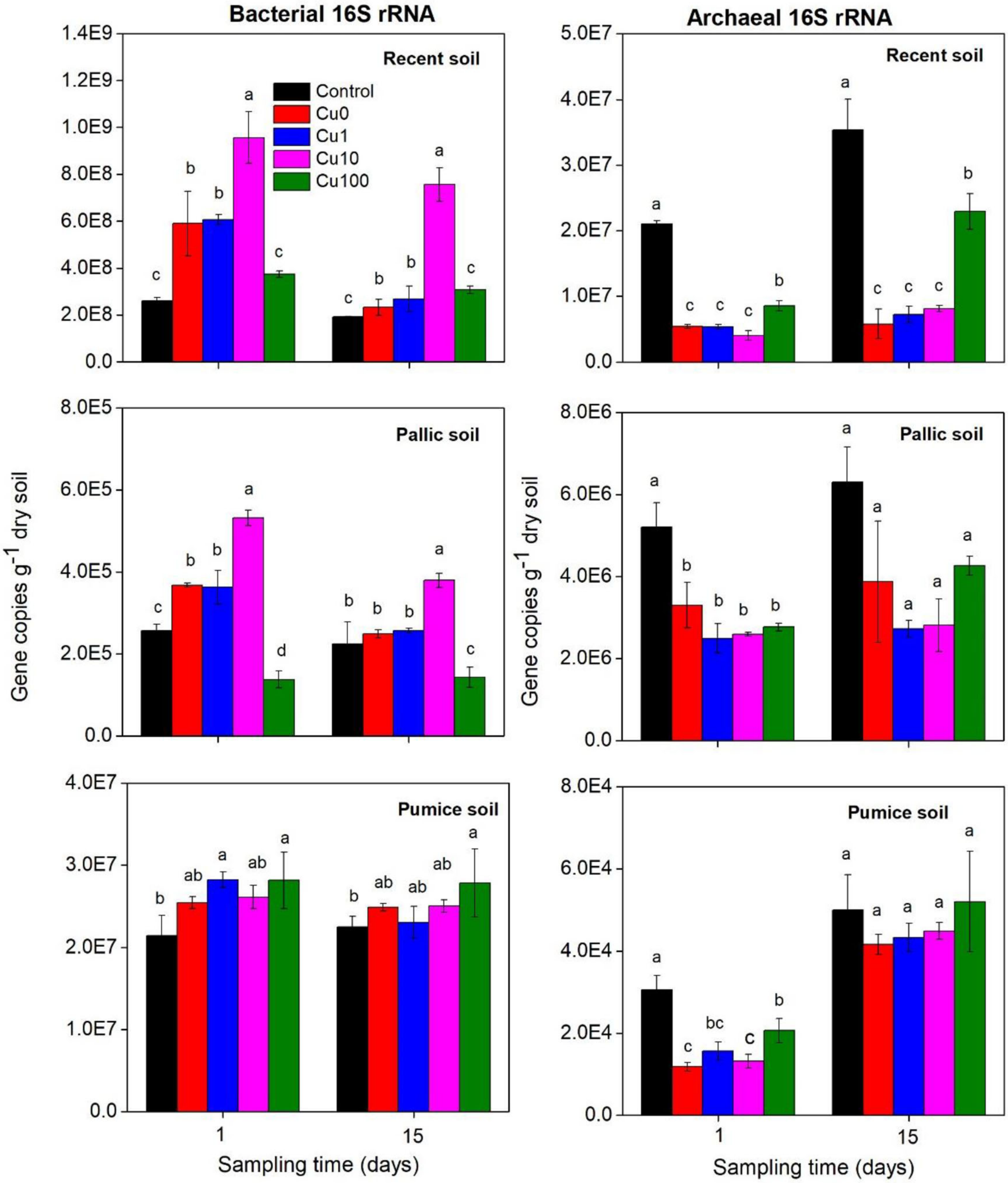

FIGURE 3 Abundance of bacterial and archaeal 16S rRNA for Recent, Pallic, and Pumice soils as a function of treatments and sampling time. Different lowercase letters in the same sampling day represent significant difference ($P < .05$). Vertical error bars represent standard deviation of mean ($n = 3$). Control = no urine and Cu; Cu0 = urine only at 300 mg N kg^{-1} soil; Cu1 = urine + 1 mg Cu kg^{-1} soil; Cu10 = urine + 10 mg Cu kg^{-1} soil; Cu100 = urine + 100 mg Cu kg^{-1} soil

3.5 | Perennial ryegrass growth

There was a trend toward increasing shoot DW as a function of time and treatment for all three soils. The Cu1 and Cu10 treatments increased the shoot DW by an average of 64 and 83%, respectively, for the Recent soil relative to Cu0 (Figure 5). For the Pallic and Pumice soils, there were no significant differences in mean DW between the Cu0, Cu1, and Cu2 treatments. An increase in shoot DW due to the Cu100 treatment relative to Cu0 was only recorded for the Recent soil on Day 25. There was a general trend for reduced DW associated with the Cu100 treatment relative to the lower Cu levels for the Pallic and Recent soils. The reduction due to Cu100 was not significant for the Pumice soil (Figure 5).

Root DW tended to increase for the Cu1 and Cu10 treated soils relative to the Cu0 treatment, but DW gains were mitigated by the Cu100 treatment (Figure 5). An increase in root DW due to the Cu1 treatment was recorded in all

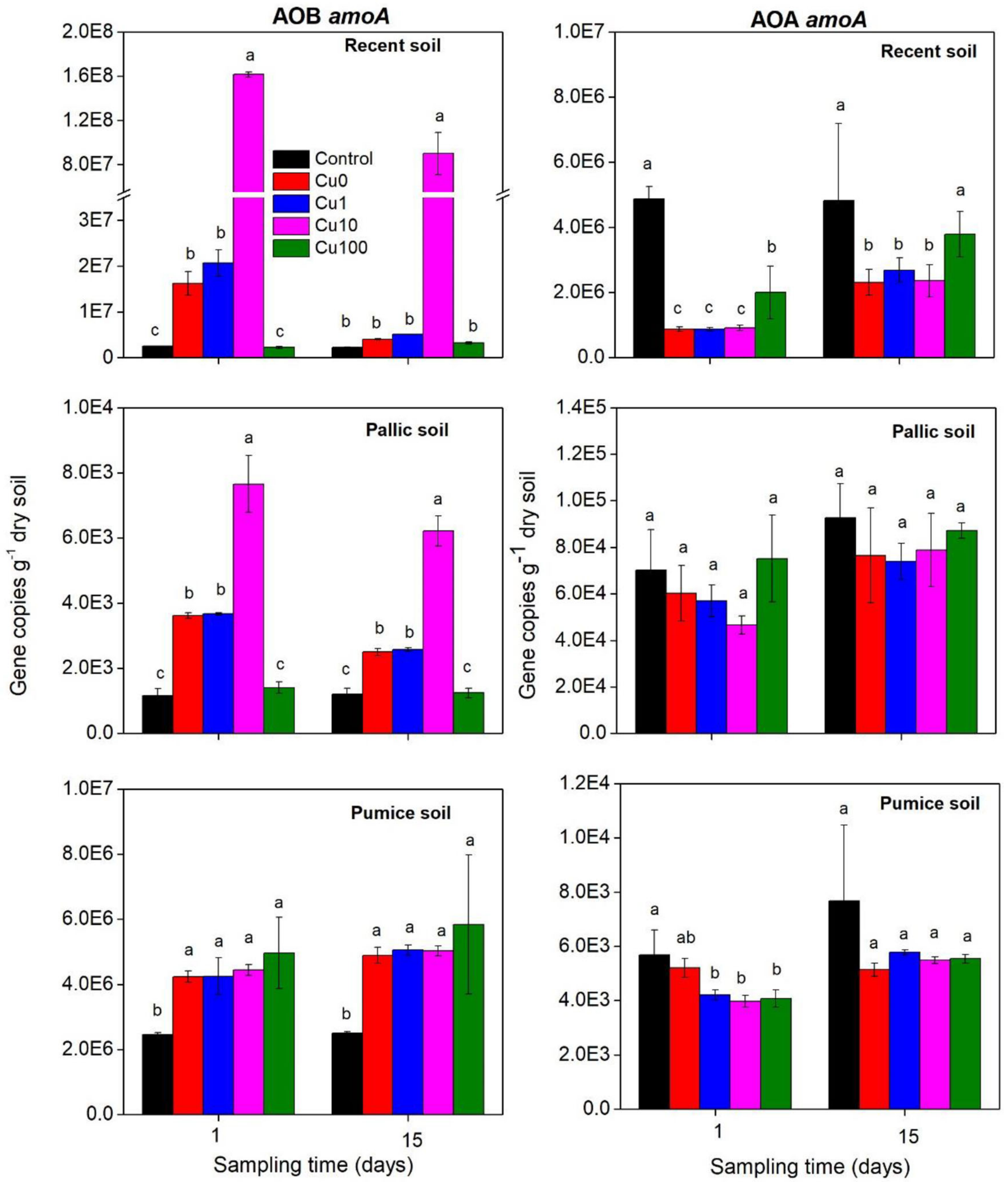

FIGURE 4 Abundance of ammonia-oxidizing bacteria and archaea (AOB/AOA) *amoA* gene for Recent, Pallic, and Pumice soils as a function of treatments and sampling time. Different small letters in the same sampling day represent significant difference ($P < .05$). Vertical error bars represent standard deviation of mean ($n = 3$). Control = no urine and Cu; Cu0 = urine only at 300 mg N kg^{-1} soil; Cu1 = urine + 1 mg Cu kg^{-1} soil; Cu10 = urine + 10 mg Cu kg^{-1} soil; Cu100 = urine + 100 mg Cu kg^{-1} soil

samplings for the Recent soil, with no clear changes for the Pallic and Pumice soils. There was a significant increase in root DW for the Cu10 treatment in the Recent soil after Day 1, but no changes were recorded for the Pallic and Pumice soils. There was a reduction in root DW induced by the Cu100 treatment for the Recent and Pallic soils relative to the Cu0 treatment; however, this effect was not apparent in the Pumice soil.

4 | DISCUSSION

4.1 | Dynamics of the NH_4^+-N and NO_3^--N

Following urine application to the soils of this study there was an increase in NH_4^+-N concentration that we attribute to rapid hydrolysis of urea in the urine by the urease enzyme (Cameron et al., 2013). Within 15 d after urine application,

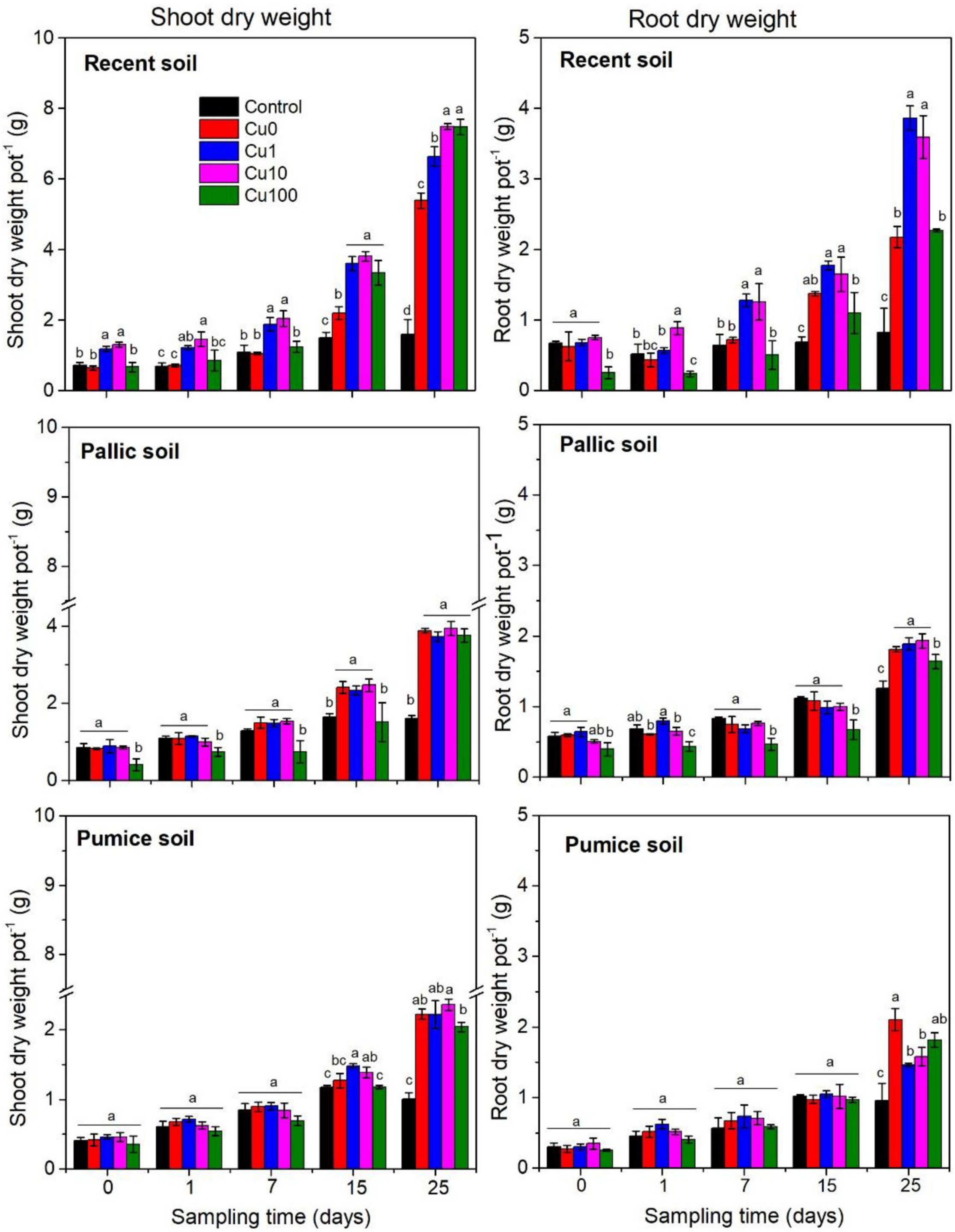

FIGURE 5 Shoot and root dry weight for the Recent, Pallic, and Pumice soils as a function of treatments and sampling time. Vertical error bars indicate standard deviation of mean ($n = 3$). Control = no urine and Cu; Cu0 = urine only at 300 mg N kg^{-1} soil; Cu1 = urine + 1 mg Cu kg^{-1} soil; Cu10 = urine + 10 mg Cu kg^{-1} soil; Cu100 = urine + 100 mg Cu kg^{-1} soil

there was rapid oxidation of NH_4^+-N to NO_3^--N, which resulted in accumulation of NO_3^--N in the soil (Figure 2). Our observations are consistent with results reported in previous studies (Duan et al., 2019; Hink et al., 2018; Williams & Haynes, 2000). The rapid nitrification rate observed in this study may have been influenced by optimal environmental conditions during this experimental period such as temperature, soil moisture and N availability (Cameron et al., 2013). We propose that the decline in NO_3^--N after 15 d is associated with N uptake by grass.

4.2 | Effect of Cu application on NH_4^+ oxidation to NO_3^-

Significant changes in the NH_4^+-N and NO_3^--N concentrations in soil were induced by Cu treatments, with the change varying between soils. The lower Cu treatment induced a reduction in NH_4^+-N in the Recent soil, whereas the Cu10 treatment induced a significant reduction in both the Recent and Pallic soils (Figure 2). The reduction in NH_4^+-N corresponded with an increase in NO_3^--N concentration in soil; this demonstrates that there was an increase in the oxidation of NH_4^+-N to NO_3^--N. We are able to correlate this increase in NO_3^--N with an increase in the bioavailable Cu concentration in both soils (Recent soil, $r = .937$, $P < .01$ [Supplemental Table S3] and Pallic soil, $r = .748$, $P < .05$ [Supplemental Table S3]). To further analyze the effect of the different Cu concentrations on changes in mineral N, the ratio of NO_3^--N/NH_4^+-N was calculated (Chen et al., 2021) (Figure 6). The ratio of NO_3^--N/NH_4^+-N quantified for the Cu10 treatment was greater in the Recent and Pallic soils, providing strong evidence that this treatment had a significant effect on nitrification rate in these two soils. Copper has been reported in various pure cell incubation and water treatment studies to play a significant role in ammonia oxidation (Gwak et al., 2020; Matse et al., 2022; Wagner et al., 2019). For example, Matse et al. (2022) reported that increasing the Cu concentration from 0.1 to 3 mg Cu kg^{-1} significantly increased the soil nitrification rate. Results from the current study therefore provide strong evidence that the change in NH_4^+-N in the Recent and Pallic soil was influenced by the Cu concentration in the soil. In the Pumice soil, there was no change in NH_4^+-N concentration induced by the Cu1 or Cu 10 treatments, suggesting that these Cu treatments did not effect the bioavailable Cu concentration in the Pumice soil.

Increasing the applied Cu concentration to 100 mg kg^{-1} (Cu100 treatment) induced a significant increase in bioavailable Cu concentration in all three soils (Figure 1), and this was associated with a higher NH_4^+-N concentration in the Recent and Pallic soils (Figure 2). The higher NH_4^+-N concentration corresponded with a lower NO_3^--N concentration, demonstrating that the high Cu level reduced nitrification in both soils, which may be due to Cu inducing toxicity to nitrifying microbes. The ratio of NO_3^--N/NH_4^+-N showed a reduction for the Cu100 treatment, providing evidence that this treatment had a toxicity effect to the nitrifying microbes (Figure 6). However, this reduction in nitrification and ratio of NO_3^--N/NH_4^+-N was not observed in the Pumice soil, where the absolute concentration of bioavailable Cu, while significantly greater than the control, was 6–8 times lower than in the Recent and Pallic soils. The difference in bioavailable Cu between the Pumice soil and the other two soils may be associated with differences in soil properties. The higher cation exchange capacity of the Pumice soil compared with the Recent soil and Pallic soils (Table 1) may have led to greater adsorption of added Cu through formation of organometal complexes reducing the concentration of Cu in soil solution (Gao et al., 1997; Rieuwerts et al., 1998). In our previous study (Matse et al., 2022), we reported that this Pumice soil was high in percentage Al and Fe oxides. These soil components may have complexed with Cu, reducing the bioavailable Cu concentration (Rieuwerts, 2007).

4.3 | Changes in microbial population and AOB/AOA *amoA* gene abundance

Our results show that bacterial populations were dominant in the Recent and Pumice soils, but that archaeal populations were dominant in the Pallic soil (Figure 3). The low soil pH of the Pallic soil (5.2) compared with the Recent (5.8) and Pumice soil (5.8) may have been one of the key factors that increased the dominance of AOA over AOB in this soil. Dominance of AOA over AOB under conditions of low soil pH has been reported in several other studies (Gubry-Rangin et al., 2010; Waggoner et al., 2021; Zhang et al., 2012). However, literature does not clearly describe whether numerical dominance at genomic level has an effect at the functional level.

An increase in the AOA population was observed when the NH_4^+-N concentration was low (control) (Figure 4). This trend has been observed in other studies (Di et al., 2010; Huérfano et al., 2022; Ouyang et al., 2017; Waggoner et al., 2021) and has been associated with the ability of AOA to thrive under conditions of low NH_4^+-N availability. In the present study, AOB abundance dominated at higher NH_4^+-N availability (Day 1). We associate this with the greater tolerance of AOB to high NH_4^+-N concentration, which may be inhibitory to AOA (Ouyang et al., 2017), or due to the greater competition for substrate by AOB.

In terms of the Cu effect across the different soils, our results show that the Cu10 treatment significantly increased AOB *amoA* gene abundance in both the Recent and Pallic soils relative to all other applied treatments. We attribute this to a beneficial increase in bioavailable Cu concentration in both of these soils. The greater AOB *amoA* abundance for the Recent and Pallic soils at the Cu10 treatment levels corresponded with a reduction in NH_4^+-N and significantly higher NO_3^--N recorded on Days 1 and 7 (Figure 2), demonstrating that nitrification in these soils was Cu limited. Our data provide strong evidence that bioavailable Cu plays a significant role in influencing AOB *amoA* abundance in soil through a correlation between bioavailable Cu and AOB *amoA* (Recent soil $r = .940$, $P < .01$ [Supplemental Table S3] and Pallic soil $r = .702$, $P < .05$ [Supplemental Table S4]). With respect to AOA *amoA* gene abundance, there were no significant changes associated with the application of the Cu1 and

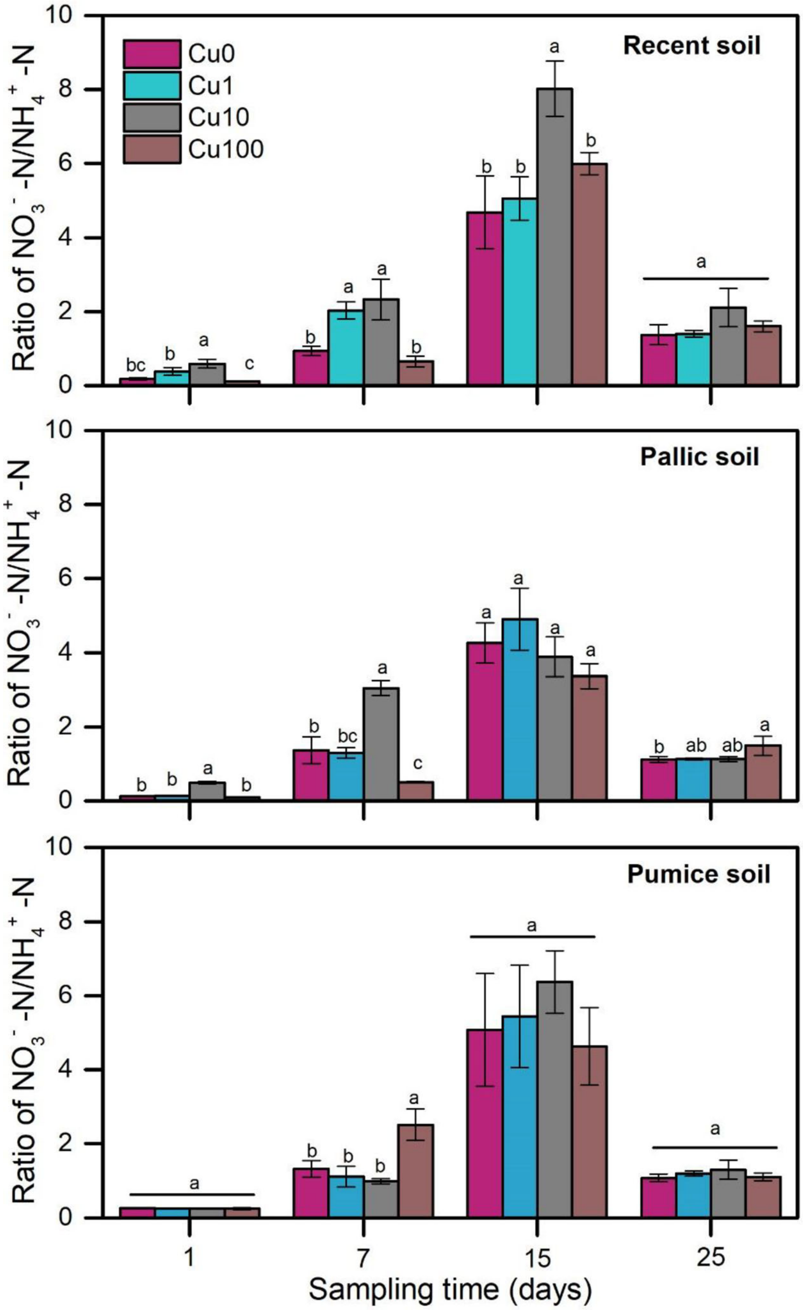

FIGURE 6 Ratio of NO_3^--N/NH_4^+-N for Recent, Pallic, and Pumice soils as a function of treatments and sampling time. Error bars indicate standard deviation of mean ($n = 3$). The control treatment was excluded because no urine was added in this treatment and Day 0 was before urine addition. Cu0 = urine only at 300 mg N kg^{-1} soil; Cu1 = urine + 1 mg Cu kg^{-1} soil; Cu10 = urine + 10 mg Cu kg^{-1} soil; Cu100 = urine + 100 mg Cu kg^{-1} soil

Cu10 treated soils relative to the Cu0 treatment. Therefore, we can conclude that the AOA *amoA* was not the dominant nitrifier responsible for nitrification rate at these Cu levels.

Soil Cu at the Cu100 treatment level inhibited AOB *amoA* abundance in the Recent and Pallic soils. The inhibition of Cu to AOB *amoA* in the present study was substantiated by the higher NH_4^+-N concentration and lower NO_3^--N in the Cu100 treatment (Figure 2), indicative of low nitrification taking place for this treatment. However, the toxicity effect of the Cu100 treatment was not observed in the Pumice soil. This was associated with the low bioavailable Cu concentration recorded in the Pumice soil (Figure 1) relative to the other two soils.

The AOA *amoA* gene showed greater abundance under the Cu100 treatment for both the Recent and Pallic soils. This behavior was reported by He et al. (2018), where the AOA *amoA* gene showed greater dominance in a high Cu environment (100 mg kg^{-1} added Cu) than the AOB *amoA* gene. The greater tolerance of AOA to higher Cu concentration is associated with greater cell wall membrane rigidity than AOB, making this barrier less permeable to ions (Kandler & König, 1998).

4.4 | Effect of treatments on perennial ryegrass growth

The presence of Cu in soil at the Cu1 or Cu10 treatment level increased ryegrass shoot and root dry weight in the Recent soil (Figure 5); we attribute this to the increase in bioavailable Cu in the soil (Figure 1). Copper is an important micronutrient in plant growth that is responsible for various metabolic processes and for the synthesis of chlorophyl (Rehman et al., 2019). Similar results were reported by Kumar et al. (1990), where application of 5 mg Cu kg^{-1} to soil resulted in a significant increase in shoot and root dry matter yield and increased N uptake by wheat (*Triticum aestivum* L.) plants. In the Pallic and Pumice soils, the Cu1 and Cu10 treatments did not increase shoot and root DW. This was because the concentration of Cu added in these treatments was insufficient to stimulate plant growth (no deficiency) or because the Cu concentration in soil was already at a level sufficient to support plant growth (sufficiency). Our data show that the Cu concentration in the Recent soil is deficient for plant growth.

The reduction in root growth for the Recent and Pallic soils (Figure 5) associated with the highest Cu treatment (Cu100) suggests this level of applied Cu was toxic to ryegrass. Previous studies reported plant Cu toxicity at a similar treatment level. Yan et al. (2006) reported that 100 mg kg^{-1} added Cu reduced average rice (*Oryza sativa* L.) grain yield and straw height by 17.37 and 13.74%, respectively, relative to a control treatment. Xu et al. (2006) also found that application of 100 mg Cu kg^{-1} significantly decreased rice growth and grain by 22.13 and 10.76%, respectively. Copper toxicity was more apparent in ryegrass roots than shoots, possibly due to the limited translocation of Cu from root to shoots reported by Bolan et al. (2003). However, no Cu-induced toxicity was apparent for the Pumice soil; we attribute this to the limited increase in bioavailable Cu concentration for this soil compared with the other two soils.

4.5 | Application of our findings to farming systems

In the current study, we have demonstrated that Cu is an important trace element not only for plant growth but also in soil processes such as nitrification. Bioavailable Cu plays a significant role in AMO activity, and soil concentrations can influence nitrification rate. Urine patches are recognized to be a main source of $NO_3{}^{-}$-N leaching in dairy systems, and we propose that understanding the relationship between Cu and ammonia oxidizers is important in examining possible ways to reduce $NO_3{}^{-}$-N leaching. This knowledge could be applied to the development of more effective nitrification inhibitors that may reduce N losses from dairy farming systems. Our findings are significant in the context of pastoral systems because dairy farms heavily supplement cows with Cu (López-Alonso & Miranda, 2020; Silva et al., 2022) or apply farm effluent containing Cu (Panagos et al., 2018). Such Cu inputs can lead to increasing levels of bioavailable Cu in established dairy soils. As demonstrated in this study, an increase in bioavailable Cu could possibly increase nitrification depending on the soil type.

Apart from the dairy systems, our results are also relevant to the horticulture industry, where several studies have reported increasing levels of Cu concentration in the soil (Mirlean et al., 2007; Pietrzak & McPhail, 2004) due to the accumulation of Cu in the soil from copper-fungicide sprays. For example, Pietrzak & McPhail (2004) reported that in Victorian vineyards, the total Cu concentration in some vineyards increased up to 250 mg kg^{-1} relative to a background concentration of <10 mg kg^{-1}. At such high concentrations, nitrification could potentially be inhibited, interfering with the nitrogen cycle and the natural break down of organic material.

5 | CONCLUSION

Our results demonstrate that a bioavailable Cu concentration of up to 0.33 mg kg^{-1} in the Recent soil and 0.24 mg kg^{-1} in the Pallic soil increased nitrification rate. However, a bioavailable Cu concentration above 6 mg Cu kg^{-1} proved toxic to nitrifying bacteria and reduced nitrification rate in both these soils. For the Pumice soil, a bioavailable Cu concentration of up 0.8 mg Cu kg^{-1} did not induce an increase in nitrification rate and AOB/AOA *amoA* gene abundance. Our results show that AOA *amoA* is more resistant to Cu than AOB *amoA* due to the greater abundance of AOA *amoA* at higher bioavailable Cu concentrations. Bioavailable Cu was the main factor in the Recent and Pallic soils influencing both nitrification and AOB *amoA* gene abundance as evidenced by the strong positive correlation between bioavailable concentration, nitrification, and AOB *amoA*. The results from this study will help expedite the development of new inhibitors to reduce $NO_3{}^{-}$-N leaching in pastoral dairy systems.

DATA AVAILABILITY STATEMENT

The analyzed data during this current study is not publicly available because it is part of the first author's doctoral thesis but can be made available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

Dumsane Themba Matse: Conceptualization; Formal analysis; Methodology; Writing – original draft. Paramsothy Jeyakumar: Conceptualization; Project administration; Supervision; Writing – review & editing. Peter Bishop: Conceptualization; Methodology; Resources. Christopher W N Anderson: Conceptualizing; Validation; Writing-review and editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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