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Soil pH moderates the resistance and resilience of C and N cycling to transient and persistent stress

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Abstract

The resilience of microbial functions like carbon (C) and nitrogen (N) cycling to stress is likely heavily dependent on pH. Past research, however, has been limited to laboratory manipulations or a pH gradient resulting from differences in soil mineralogy. In this study, soils were collected from a >50-year field trial where plots have been maintained at pH 4.9, 6 and 7.1. We selected copper (Cu) and heat to represent persistent and transient stresses, respectively. Changes in C mineralization, ammonia oxidation, denitrification, and gene (16S rRNA, *nirK*, *nirS* and *amoA*) abundance were immediately measured after heat- (40 °C for 16 hours) and Cu- (500 μg Cu soil g^{-1} or 1 mg Cu soil g^{-1}) induced stresses, during subsequent recovery over 56 days, and compared to an unstressed control. Higher soil pH significantly increased C mineralization (by 217%), ammonia oxidation (by 617%), and the gene abundances of 16S rRNA (by 77%), *nirK* (by 976%) and *nir*S (by 997%). Soil pH had a significant ($P < 0.001$) selection effect on the phylotypes of bacterial communities and ammonium oxidising

bacteria (AOB). Ammonia oxidation was significantly ($P < 0.05$) more resistant and resilient to both Cu stresses in the pH 7.1 soil. C mineralization in the soil at pH 7.1 was significantly ($P < 0.05$) more resilient to low Cu than the soil at pH 4.9. Correspondingly, significantly ($P < 0.001$) distinct bacterial communities were present in these soils, indicating that bacterial composition triggered by the adaptation and tolerance to stress is a central factor governing functional resilience. Denitrification in the pH 7.1 soil was significantly ($P < 0.05$) more resilient to low and high Cu, compared to the soil at pH 4.9. Similarly, the abundances of *nirS* and *nirK* genes were greater in the higher pH soil. Although soil pH directly affects Cu but not heat stress, our results indicated that neutral soils harboured greater resilience of C and N cycling to both Cu (persistent) and heat (transient) stresses.

Keywords: soil pH, microbial community, stability, nutrient cycling, Cu, Heat

1. Introduction

Soil ecosystems are highly complex and subject to various types of stresses that influence the ability of soil to deliver ecosystem services. Depending on the duration, stresses are often classified as transient (short-term and discrete) and persistent (long-term and continuous) (Shade et al., 2012). Copper (Cu) contamination due to excessive use of Cu-based fungicide and fertilisers could cause long-lasting and adverse effects on microbial functions and soil fertility (Ballabio et al., 2018). Climate extremes, such as drought and heat waves, could cause negative fluctuations in soil ecosystems (Bardgett and Caruso, 2020). Copper (Cu) and heat have been widely used as experimentally representative transient and persistent stresses in soil ecosystems (Griffiths et al., 2001; Shu et al., 2019; Zhang et al., 2010). It is imperative to understand soil functional stability under various stresses, which typically is quantified as a combination of resistance (initial response to stress) and resilience (recovery to a stable state) (Griffiths and Philippot, 2013).

Resistance and resilience will affect the capacity of soil microorganisms to support a plethora of biogeochemical functions that influence soil ecosystems services, such as carbon (C) and nitrogen (N)

cycling (Schimel et al., 2007). Many heterotrophic microbial communities with large species diversity can decompose diverse C compounds and are involved in C mineralization (Schimel and Schaeffer, 2012). Ammonia oxidizers, including bacteria (AOB) and archaea (AOA), are responsible for ammonia oxidation where ammonia is oxidised to hydroxylamine and then nitrite (Kuypers et al., 2018). Denitrifying bacteria, such as *nirK*- and *nirS*- harbouring bacteria, produce nitrite reductase to reduce nitrite (NO_2^-) to nitric oxide (NO) which is a key reaction in denitrification (Kuypers et al., 2018). Given that microorganisms play a paramount role in regulating soil C and N cycling, it is logical that the resistance and resilience of C and N functions are governed by the underpinning microorganisms. For example, C mineralization exhibited approximately 2 times greater resilience to Cu (1 mg Cu soil g^{-1}) than ammonia oxidation (Shu et al., 2021). This trend was also found in the resilience of 16S rRNA and AOB to Cu, suggesting a direct role of microbial populations to the pertaining functional resilience (Shu et al., 2021). The soil microbial community structure has been reported to be the central factor governing the resilience of microbial functions to heavy metal contamination (Jiang et al., 2020). Strong links between microbial community composition and soil multifunctionality's resistance to drought were also found in a meta-analysis on 59 dryland ecosystems (Delgado-Baquerizo et al., 2017). In contrast, discrepancies between microbial communities and pertaining functional resistance/resilience have been reported. For example, the process of denitrification was found to be recover more rapidly after heat and drought stress than denitrifying gene copy numbers (*narG* and *nosZ*) (Fikri et al., 2021). The loss of biodiversity was found to have no significant impacts on the resistance or resilience of denitrification and nitrification (Wertz et al., 2007). These results indicate that there is more than microbial community structure governing soil functional resistance and resilience. Potential contributors could be soil physicochemical properties, such as soil pH and redox potential (Griffiths and Philippot, 2013).

Soil pH is a major driver affecting C and N cycling and microbial communities (Fierer and Jackson, 2006; Liu et al., 2010). In N cycling, soil pH could strongly influence ammonia oxidation via the diversification

of ammonia oxidizers (Gubry-Rangin et al., 2015) and changes in the protonation of ammonia (NH_3), which serves as the direct substrate for ammonia oxidizers (Suzuki et al., 1974). Soil pH was also reported to have a significant negative relationship to the ratio of N_2O/N_2, which indicates that pH may affect soil denitrification through its effect on the community structure, transcription and activity of denitrifiers due to different sensitivity of denitrifiers to soil pH variation (Herold et al., 2018; Liu et al., 2010; Qu et al., 2014).

The growth of bacteria and fungi (e.g., the ratio of fungi to bacteria) could also be shaped by soil pH, resulting in a shift in C use efficiency and C mineralization (Rousk et al., 2009). For instance, Zhang et al. (2016) investigated 24 soil samples in a transect from north to south China and found a positive correlation between the resistance of C mineralization to Cu (100 mg Cu soil kg^{-1}) and soil pH (pH ranged from 4.5 to 8.5). Another study also suggested that the effects of particular microbial taxa on multifunctionality resistance could be controlled by altering soil pH (Delgado-Baquerizo et al., 2017). However, both studies collected soils from different regions of contrasting mineralogy, thus the results cannot rule out that soil type (e.g., physical structure) played a more important role than soil pH per se. Although pH can be manipulated on a single soil in the laboratory, acute short-term change could create artefacts with the microbial community having insufficient time to adapt. pH impacts on soil biological processes have been explored in longer-term liming experiments, however, the research has been limited to two pH levels (Vishwanath et al., 2022).

To overcome this potential problem, we used soils from a long-term established field experiment where all soils were the same type which allowed us to study soil pH specifically, including the long-term impacts to the microbial community driven directly by pH and indirectly by the impact of pH on plant growth. The experimental site was established since 1961 and has been studied previously. It was reported that denitrifying bacteria abundance (Herold et al., 2018) as well as denitrification rate (Herold et al., 2012) varied with soil pH. With such knowledge of pH impacts on microbial processes, here we explored how soil pH regulated the resistance and resilience of C and N cycling processes and

their underpinning microbial communities to persistent (Cu) and transient (heat) stresses as these two stresses are representative threats (i.e., heavy metal pollution, and global warming) to soil. Our previous study demonstrated that the concentration of 1 mg Cu soil g^{-1} could have a marked negative impact on microbial communities (Shu et al., 2021). Considering the bioavailability of Cu could be highly dependent on soil pH, here a high (1 mg Cu soil g^{-1}) and low (500 µg Cu soil g^{-1}) concentration of Cu were chosen as persistent stresses. Soils were incubated for 56 days under transient and persistent stresses to simulate expected impacts from heat (40 °C warming) and Cu (high and low concentration). Changes in C mineralization, denitrification, ammonia oxidation, gene abundances underpinning the processes, immediately after heat- and Cu- induced stress and during subsequent recovery over 56 days were measured. The soil microbial community structures of general bacteria (16S rRNA T-RFLP) and specific AOB (bacterial *amoA* T-RFLP) were analysed. We measured the resistance/ resilience of C and N processes and the corresponding microbial community abundances following the perturbation of Cu and heat over 56 days.

This experiment tests the hypothesis that functional resistance/resilience of C and N cycling process varies in soils of different pH. This will be due to the direct impacts of pH on microbial communities, which will impact their immediate response (resistance) and recovery (resilience) of biochemical cycles following a stress. When pH is optimal for the functions and communities, a greater corresponding resistance and resilience will occur. With increases in pH, we hypothesised the resistance and resilience of functions and microbial communities to Cu (especially for the higher concentration) would increase, as Cu bioavailability decreased. According to the "insurance hypothesis", soils with greater microbial diversity are expected to contain organisms with a broader array of environmental tolerance ranges and maintain functioning even if others fail (Yachi and Loreau, 1999). We hypothesised the highest resistance and resilience of functions and communities to heat in the neutral soil where there is a greater chance that heat tolerant taxa are present in a more diverse microbial community.

2. Material and methods

2.1 Soil sampling

Soils were collected from three treatment plots (pH 4.5, 6.0 and 7.5) of the long-term pH trial at Craibstone, Aberdeen, UK. The actual pH values measured by H_2O for these plots at the time of sampling were 4.9, 6 and 7.1, respectively. Plots have been maintained at target pH's since 1961 by additions of aluminium sulphate or calcium carbonate (Herold et al., 2012). The soil is a free-draining sandy loam, Humic Entic Podzols (World Reference Base) classified locally as the Countesswells series. The plots follow an eight-year crop rotation (winter wheat, potatoes, spring barley, swedes, spring oat, and 3 years ley grass with no re-sowing). Within the plots at pH 4.9, 6.0 and 7.1, 10 kg surface (0-20 cm) soil was collected from the third-year grass ley in August 2016.

2.2 Resistance and resilience

The resistance and resilience assay followed the method described by Shu et al. (2021). Four replicates of each pH soil were imposed by either a stress (heat, low Cu or high Cu) or were unstressed, resulting in the factorial combination of three soil pH treatments and four stresses (3 pHs × 4 stresses × 4 replicates = 48 microcosms). For Cu-stressed samples, 220 g soil (dry-weight equivalent) was amended with 2.2 ml of either 0.79 M $CuSO_4.5H_2O$ or 1.57 M $CuSO_4.5H_2O$ to reach a concentration of 500 µg Cu soil g^{-1} (low Cu) or 1 mg Cu soil g^{-1} (high Cu). The same volume of sterile water was added to soils (220 g) to create the heat-stressed and unstressed (control) samples. The heat- stressed soils and the rest of soils were incubated at either 40 °C or 20 °C, respectively, for 16 hours. All samples were then stored at 20 °C for the remaining 56 days without any addition of C source.

Subsamples were taken at 1, 7, 14, 28 and 56 days following stresses, for analysis of functions (C mineralization, denitrification, and ammonia oxidation) and gene abundance and microbial community structure. C mineralization was measured as CO_2 after 24 hours following mixing 2 g soil with a 120 µl solution of organic C compounds (Shu et al., 2021). Ammonia oxidation was determined

as nitrite-N after incubating 10 g soil with 50 ml solution (0.5 mM $(NH_4)_2SO_4$ + 10 mM $NaClO_3$) for 24 hours (Shu et al., 2021). Denitrification was estimated as N_2O after incubating 20 g soil with 20 ml solution (25 mM glucose +3.57 mM KNO_3) with the presence of 10% (v/v) acetylene for 5 hours (Shu et al., 2021). Soil physicochemical properties (*i.e.*, available N, SOC, TN, DOC, actual pH) were determined by the methods in Carter and Gregorich (2007).

2.3 DNA extraction, PCR, and T-RFLP

DNA extraction and purification from 1 g soil were performed by a phenol-chloroform method with the addition of 1×10^6 copies of a mutated reference gene *Spike* (Daniell et al., 2012).

To analyse total bacterial community in the unstressed treatments, 10 folds diluted DNA were amplified using labelled 16F27 and 1392R primers (Lane, 1991) following the PCR conditions described in Table S1. Each amplification was conducted in 15-µl reaction mix containing 0.3 U Platinum Taq DNA Polymerase (Invitrogen, UK), 1.5 µl of buffer, 1 U *Hha I* (Promega, UK), 3 mM MgSO4, 3.75 mM dNTPs, 10 µg BSA, and 6 pM of each primer.

Ten folds diluted DNA in the unstressed treatments were amplified for analysis of *amoA* using the labelled primers *amoA* 4F (Webster et al. 2002), and *amoA* 2R (Rotthauwe et al., 1997) following the PCR program (Table S1). Fourteen µl of a 'master mix' contained 0.3 U Expand High Fidelity Enzyme mix (Roche, UK), 5 pM each primer, 1.5 µl of Expand High Fidelity Buffer with 15 mM $MgSO_4$ (Roche, UK), 6.25 mM dNTPs and 10 µg BSA.

T-RFLP was conducted following the method in Langarica-Fuentes et al., (2018). Three µl PCR products were digested with restriction enzyme using *Msp I* for *amoA* and Alu *I* for 16S rRNA, respectively. One µl 10 folds diluted digests were mixed with 1200 LIZ dye Size Standard and formamide (Life Technologies, UK), and then was subjected to an ABI 3730 sequencer (Thermo Fisher Scientific). Peaks were analysed in GeneMapper as described in Deng et al. (2010).

2.4 qPCR

Bacterial 16S rRNA, *amoA*, *nirK*, and *nirS* genes were quantified by qPCR (Daniell et al., 2012). Each amplification was performed in 20 µl reaction mixtures containing 10 µl SYBR Green 1 Master Mix (Applied Biosystems, UK), 0.5 µl of 0.3 µg μl^{-1} of BSA, 1 µl of 10 pM of each primer, 2 µl of 10 folds diluted DNA. Standards were generated by serial dilutions of linearized plasmids containing cloned *nirK*, *amoA*, *nirS*, and 16S rRNA gene PCR products (Langarica-Fuentes et al., 2018). The PCR conditions and primers are illustrated in Table S1.

2.5 Data analysis

All statistical analyses were carried out using R 4.0.3 (R Core Team 2018).

Stability was estimated as the change in functions of the stressed soil compared with the corresponding unstressed soil at day t (Zhang et al., 2010):

$$f(t) = \frac{Stressed\ indicator\ (t)}{Unstressed\ indicator\ (t)} \times 100$$

Resistance was the stability measured at 1 day, while resilience was the stability after 1 to 56 days following stress (Shu et al., 2019).

$$Resilience = \int_{1}^{56} f(t)dt/(56-1)$$

A three-way ANOVA was performed to determine the effects of soil pH, stress and time on the functions and gene abundances. A one-way ANOVA followed by a Tukey honestly significant difference test was carried out to detect the effect of soil pH on the resistance and resilience of functions to different stresses. Regression was conducted in the unstressed soil (collected at day 1) to estimate the relationships between soil pH and functions as well as gene abundance.

Non-metric multidimensional scaling (NMDS) on Bray-Curtis distance of AOB ("Hellinger" transformed abundance of bacterial *amoA* T-RFLP) and total bacterial community ("Hellinger" transformed abundance of 16S rRNA T-RFLP) were carried out under the "vegan" package (Oksanen et al., 2019). A one-way ADONIS pairwise comparison on Bray-Curtis distance was conducted to test the effect of soil pH on the structure of total bacteria and AOB in the unstressed soils using the package "pairwiseAdonis" (Martinez Arbizu, 2020).

3. Results

3.1 Effects of soil pH on soil properties and functions

A pH increases from 4.9 to 7.1 in the unstressed control soil resulted in greater concentrations of dissolved organic carbon (DOC) ($P < 0.05$) (Table S2), but less ammonium (NH_4^+) and nitrate (NO_3^-) ($P < 0.05$). Microbial biomass carbon (MBC) concentration in the soil at pH 4.9 was significantly ($P < 0.05$) lower than other soils. There was no significant difference in total nitrogen (TN) or soil organic carbon (SOC).

In the unstressed control soils at day 1, C mineralization and ammonia oxidation were greatest in the pH 7.1 soil, which were 38 µg C g^{-1} h^{-1} and 58 µg N g^{-1} $g^{-1}h^{-1}$, respectively (Figure 1). C mineralization ($P < 0.001$, $R^2 = 0.91$), and ammonia oxidation ($P < 0.001$, $R^2 = 0.99$) significantly increased with increases in soil pH (Figure 1). However, the relationship between denitrification and soil pH was quadratic ($P < 0.001$, $R^2 = 0.98$) where denitrification rate reached its peak 582.96 ng N g^{-1} h^{-1} in the soil at pH 6 (Figure 1 and S1).

3.2 The effects of soil pH on functional resistance and resilience

The two-way interactions pH*stress, pH*day, and stress*day were significant ($P < 0.001$) for C mineralization (Table 1). C mineralization dropped by 20-41% on day 1 after high Cu contamination for all pHs (Table S3 to S5). The resilience of C mineralization to high Cu was significantly ($P < 0.05$) greater in the soil at pH 4.9 and pH 7.1 than at pH 6 (Figure 2). One day after the low Cu addition, C

mineralization significantly ($P < 0.05$) decreased by 22% in the pH 4.9 soil. The resilience of C mineralization to low Cu was greatest in the soil at pH 7.1 with a stability of 62% of control (Figure 2). Heat significantly ($P < 0.05$) decreased C mineralization by 70% (pH 4.9), 45% (pH 6), and 38% (pH 7.1), respectively (Table S3 to S5 and Figure 2). The resilience of C mineralization to heat was greatest in the soil at pH 7.1 (93% of control).

The three-way interaction pH*stress*day was significant ($P < 0.001$) for ammonia oxidation (Table 1). One day after applying either Cu or heat stress, ammonia oxidation significantly ($P < 0.05$) decreased in all soils (Table S3 to S5). The resistance of ammonia oxidation to Cu, regardless of Cu concentration, increased with increases in soil pH with the greatest stability found in soil at pH 7.1 (Figure 2). Similarly, ammonia oxidation in soil at pH 7.1 was significantly ($P < 0.05$) more resilient to both Cu stresses compared to other pH soils. Ammonia oxidation in the soil at pH 4.9 and pH 6 did not fully recover from Cu addition but recovered from heat during the experiment. Especially, the pH 6 soil showed significantly ($P < 0.05$) greater resistance (64% of control) and resilience (93% of control) to heat compared to other soils (Figure 2).

Denitrification was significantly ($P < 0.001$) impacted by the three-way interaction of pH*stress*day (Table 1). One day following Cu, denitrification significantly ($P < 0.05$) decreased in all the soils (Table S3 to S5). The soil at pH 7.1 was significantly ($P < 0.05$) more resilient to low (48% of control) and high (23% of control) concentration of Cu compared to other soils (Figure 2). Heat significantly decreased denitrification by 72% (pH 4.9), 82% (pH 6), and 92% (pH 7.1) (Figure 2). Obvious recoveries were observed after 56 days following heat in all soils where the soil at pH 6 (74% of control) exhibited significantly ($P < 0.05$) greater resilience than the soil at pH 4.9 (65% of control).

3.3 The effects of soil pH, stress, and day on microbial communities

As main factors, pH ($P < 0.001$), stress ($P < 0.001$) and day ($P < 0.05$) had significant impact on the abundance of 16S rRNA gene copies (Table 1). However, the abundance of 16S rRNA genes was not

significantly influenced by any of the interactions. In the unstressed (control) soils at day 1, there was no significant relationship between the abundance of 16S rRNA gene copy count and soil pH (Figure 1). The abundance of 16S rRNA genes was resistant to both Cu stresses in all the soils since no significant changes were found one day after the stresses (Table S3 to S5). The abundance of 16S rRNA genes significantly ($P < 0.05$) dropped after 56 days following the addition of high and low Cu in the soil at pH 4.9 (Table S3). The abundance of 16S rRNA was resistant and resilient to heat in all the soils because no significant change in the abundance were found during 56 days.

The two-way interactions of pH*stress, and stress*day had significant ($P < 0.001$) impacts on the abundance of bacterial *amoA*. In the unstressed soil at day 1, the abundance of *amoA* in the pH 7.1 soil was significantly ($P < 0.05$) smaller than that in the pH 4.9 soil (Figure 1), resulting in a significant negative ($P < 0.01$, $R^2 = 0.62$) relationship between the abundance of *amoA* and soil pH (Figure 1). Compared to the unstressed control, the abundance of *amoA* in the pH 4.9 soil significantly ($P < 0.05$) decreased one day after the application of low Cu (decreased by 20%), high Cu (decreased by 74%), and heat (decreased by 41%) (Table S3). However, the abundance of *amoA* was highly resistant to the applied stress in the soil at pH 7.1 and 6 (Table S4 to S5). After 56 days following the addition of high and low Cu, the abundance of *amoA* significantly ($P < 0.05$) dropped in the soil at pH 4.9 and 6.

The abundance of *nirK* was not significantly affected by any interactions between pH, stress, and day (Table 1). It was highly resistant and resilient to all the applied stresses because no significant changes were found between the stressed and unstressed soil at all pHs (Table S3 to S5). In the unstressed soils at day 1, the abundance of *nirK* was significantly ($P < 0.05$) greater in the soil at pH 7.1 (6.89×10^7 copies g^{-1}) than the one in the soil at pH 4.9 (6.41×10^6 copies g^{-1}) (Figure 1). The abundance of *nirK* significantly ($P < 0.01$, $R^2 = 0.54$) increased with soil pH (Figure 1).

The abundance of *nirS* was significantly affected by three major factors pH ($P < 0.001$), stress ($P < 0.001$), and day ($P < 0.01$), but not their interactions (Table 1). In the unstressed soils at day 1, the

gene abundance of *nirS* was significantly ($P < 0.05$) greater in the soil at pH 7.1 (4.07×10^7 copies g^{-1}) than other soils (Figure 1), leading to a significantly positive ($P < 0.01$, $R^2 = 0.66$) relationship with soil pH (Figure 1). The abundance of *nirS* was highly resistant to Cu and heat in all soils since no significant changes in the abundance were found one day after imposing stresses (Table S3 to S5). After 56 days following the addition of high and low Cu, the abundance of *nirS* significantly ($P < 0.05$) dropped in the soil at pH 4.9 and 6. By contrast, the abundance of *nirS* was not significantly affected by the addition of Cu in the soil at pH 7.1.

Significantly ($P < 0.001$) distinct bacterial community structures were observed at different soil pH with the biggest difference between soils at pH 7.1 and pH 4.9 (Figure 3). AOB community structures were significantly ($P < 0.001$) separated by soil pH with the most obvious differences between the soils at pH 6 and pH 4.9 (Figure 4).

4. Discussion

Previous studies focusing on the influence of pH variation have mainly relied on geological gradients (Wang et al., 2019; Zhang et al., 2016) or short-term laboratory manipulation of pH that imposes its own stress (Liu et al., 2020) In contrast, soil for this experiment was sampled from a controlled experimental site which reflected the impacts of long-term agricultural management (liming and fertilization) on soil pH and the impacts on soil functional resilience. Of the three pHs examined here, our study found that the optimal pH varies with C and N processes. Generally, the soils at pH 7.1 and pH 6 exhibited greater resilience of C and N processes to Cu and heat stresses compared to those at pH 4.9.

4.1 The effects of soil pH on functions, microbial abundance, and soil properties

C mineralization significantly ($P < 0.05$) increased when soil pH was towards neutral (Figure 1), which was consistent with a previous research on the same experimental site (Meharg and Killham, 1990). The bacterial community structure varied significantly ($P < 0.001$) between soil pHs (Figure 3) with the

284 smallest MBC in soil at pH 4.9 (Table S2). This could be the result of slow microbial growth at lower pH
285 (Rousk et al., 2011). The intracellular pH is between 6 to 8 in most microorganisms, thus any external
286 pH more extreme than these values will likely stress and influence the structure and composition of
287 microbial communities (Wang et al., 2020). Considering heterotrophic bacteria and fungi mainly
288 contribute to C cycling (Schimel and Schaeffer, 2012), it is not surprising that C mineralization was
289 lowest in the pH 4.9 soil due to its smallest concentration of microbial biomass (Figure 1).

290 Ammonia oxidization significantly increased with soil pH (Figure 1). Higher pH soils have also
291 previously been shown to favour ammonia oxidation (Baggs et al., 2010). The community structure of
292 AOB were distinct between soil pHs with increasing variation at higher pH (Figure 4) and their
293 abundances were significantly different between different pHs although surprisingly the abundance
294 was higher at low pH (Figure 1), corroborating that distinct phylotypes of AOB are shaped by soil pH
295 (Nicol et al., 2008) and AOB can survive in a wide range of pH (Hayatsu et al., 2017). AOB and
296 nitrification rates did not respond in the same way probably due to 1) not all the measured abundance
297 were functionally active; 2) the DNA-based approach could include relic DNA (i.e., extracellular DNA
298 of necromass), which could elucidate around 40% of prokaryotic DNA and obscure the relationships
299 between microbial abundance and functioning (Philippot et al., 2021); 3) there were possible
300 contributions from comammox bacteria and AOA to nitrification (Prosser et al., 2019).

301 The relationship between soil pH and denitrification was quadratic with the highest rate in pH 6 soil
302 (Figure 1), suggesting that pH 6 is close to the optimal condition for denitrification in this soil above or
303 below which denitrification rate is lowered (Herold et al., 2012). Compared to the soil at pH 4.9, the
304 abundances of *nirK* and *nirS* were significantly ($P < 0.05$) greater in the soil at pH 7.1 (Figure 1), which
305 was in agreement with a previous study on the same site that both *nirK* and *nirS* abundance increased
306 with soil pH ranging from pH 4.2 to 6.6 (Herold et al., 2018). The variation of denitrifiers at different
307 pH could be the result of a direct selection of denitrifier communities (Herold et al., 2018), and also
308 an indirect selection via changes in DOC availability. Bárta et al., (2010) found a high positive

correlation between DOC concentration and *nirK* abundance, implying that when the concentration of DOC is low it leads to the starvation of denitrifiers and a decrease in denitrification. In this study, higher concentration of DOC in the pH 7.1 soil than the pH 4.9 soil was observed (Table S2), corroborating that DOC could be an indirect driver for the differences in denitrifier abundances between soil pHs. One reason that DOC in neutral soils was higher could be greater aboveground biomass and increased C return into soil via C exudates through roots or residue retention (Paradelo et al., 2015). Additionally, acidic soils are interconnected with higher mobility of toxic Al^{3+} forms, which can precipitate DOC making it unavailable for biological processes (Bárta et al., 2010).

4.2 The effects of soil pH on the resistance and resilience of functions and microbial communities

C mineralization in the soil at pH 7.1 was significantly ($P < 0.05$) more resistant and resilient to heat than the soil at pH 4.9 (Figure 2), which was accompanied by significantly ($P < 0.001$) distinct bacterial community structures (Figure 3) rather than bacterial abundance (Figure 1). Similarly, ammonia oxidation was significantly ($P < 0.05$) more resistant and resilient to heat in the pH 6 soil than at the other pH levels (Figure 2), which was followed by their significantly ($P < 0.001$) different AOB community structures (Figure 4) instead of significant changes in AOB abundance (Figure 1). This phenomenon corroborated that microbial composition triggered by the adaptation and tolerance to stress is a paramount factor governing functional resilience (Jiang et al., 2020). Resilience being due to microbial communities, rather than microbial abundance, could be a reason for high functional redundancy if not all present species are functionally active (Allison and Martiny, 2008). A recent meta-analysis, which assembled 32 studies, revealed that ecological communities with more functionally redundant species are more likely to display higher resilience following disturbance relative to those with fewer functionally redundant species (Biggs et al., 2020). Moreover, rare species have been found to contribute disproportionately more to overall community functions to counteract environmental disturbances (Liang et al., 2020).

Ammonia oxidation was significantly ($P < 0.05$) more resistant and resilient to Cu in the soil at pH 7.1 compared to the soils at pH 4.9 and 6 (Figure 2). Similarly, the abundance of AOB was resistant and resilient to Cu in the pH 7.1 soil (Table S5). It is possible that decreased Cu-bioavailability due to the absorbance of Cu^{2+} to oxyhydroxides at higher pH, resulted in attenuated negative impacts of Cu on the activity and abundance of AOB (Degryse et al., 2009). Since the decrease in free Cu^{2+} activity is between 3- and 15-fold per unit pH increase (Degryse et al., 2009), the toxicity of Cu to bacteria in pH 4.9 soil could be 6- to 30- times greater than that in pH 7.1 soil. The higher proportion of Cu-free niches in neutral soil allows AOB to colonise, reproduce, and thus recover from Cu. Additionally, the AOB community structure was clearly and significantly separated by pH (Figure 4). There is a possibility that higher pH favours the growth of Cu-tolerant AOB which are also functionally active. One example is the *Nitrosospira* lineage which is metal-tolerant and has been proven to markedly contribute to the recovery of nitrification to Zinc contamination (Mertens et al., 2009). We acknowledge the importance of AOA in ammonia oxidation, but AOA have been proven to be less versatile than AOB in a changing environment (Aigle et al., 2020). Considering AOA are more abundant than AOB in acidic soils (Gubry-Rangin et al., 2010), the greater resilience of ammonia oxidation in neutral soil may mainly be attributed to AOB. We suggest future studies on AOA, commammox and their transcriptional activities will help explain the impacts of soil pH on the response of ammonia oxidation to contrasting stress.

Denitrification was significantly ($P < 0.05$) more resilient to Cu in the soil at pH 7.1 than pH 4.9 (Figure 2). Consistently, the abundances of *nirS* and *nirK* were greater in the pH 7.1 soil (Figure 1), suggesting that denitrifiers could confer the resistance/resilience of denitrification to Cu. The soil at pH 7.1 which harboured greater abundances of denitrifiers are more likely to contain species that can cope with Cu and maintain functioning even if others fail (Yachi and Loreau, 1999). Given that Cu is a co-factor in Cu-containing nitrite reductase (CuNIR) which is encoded by *nirK*, a deficiency in Cu could inhibit the expression of *nirK* and the transport of nitrite reductase to the periplasm (Pacheco et al., 2022).

Neither low nor high Cu had a significant impact on the abundance of *nirK* in this study, confirming the level of Cu provided was not a limiting factor to the growth and activity of *nirK*.

The response of denitrification to temperature variation was reported to be bell-shaped, with a temperature optimum (25 – 35 °C) beyond which activity progressively declined (Braker et al., 2010). Our results further proved that stressing soil briefly at 40 °C severely impaired denitrification (Figure 2). Despite the smaller abundances of *nirK* and *nirS*, denitrification was significantly ($P < 0.05$) more resistant to heat in the soil at pH 4.9 than in the other pH soils. This could be attributed by denitrifying fungi which possess copper-containing nitrite reductase gene (*nirK*) (Aldossari and Ishii, 2021). Fungal denitrification plays an essential role in N cycle especially in acidic soils because those soils usually have a higher fungal to bacterial ratio as well as higher redox potential, increasing the potential of denitrifying fungi (Aldossari and Ishii, 2021). Indeed, it has been reported that acidic environments promote fungal denitrification (Thomson et al., 2012). Some denitrifying fungi seem more tolerant to elevated temperature than bacteria because their ability to produce heat shock proteins and chaperones to assist in the repair of functional structure after heat stress (Xu et al., 2017). Future consideration of denitrifying fungi may help to explain the greater resistance of denitrification to heat in the acidic soil.

Our study demonstrated greater resilience of C and N processes to heat than to Cu (Figure 2), which is attributed to our heat application being a more transient stress with short and acute effect (Shu et al., 2019). Although soil pH has no direct impact on heat, the effect of pH on functional and microbial resilience to heat may mostly be attributed to selective effects of soil pH on microbial taxa. For example, the rise in soil pH was reported to have a positive effect on the abundance of specific bacterial classes (e.g., Gitt-GS-136) that have been found to promote the resistance of multifunctionality to drying-wetting cycles (Delgado-Baquerizo et al., 2017). In our study, the soil microorganisms had been exposed to this range of pH since 1961. Such a long-term exposure allows microbial acclimatisation to the environment through local adaptation and horizontal transfer (Epelde

et al., 2015). In order to survive in acidic conditions, bacteria may change their physiological properties, such as cellular structure, membrane permeability, and resistance mechanism (Epelde et al., 2015). These physiological changes can have severe impacts when the cells encounter other types of stresses (e.g., heat and Cu). Therefore, functional resistance and resilience may be a result of both physiological evolution of individual organisms and shifts in the community structure.

5. Conclusion

Soil pH has significant impacts on C and N functions and underpinning bacterial communities. The resilience of ammonia oxidation and denitrification to Cu, regardless of its concentration, were greatest in the soil at pH 7.1, which was attributed to the lower bioavailability of Cu at this pH and the selection of Cu-tolerant functionally active species. The soil at pH 7.1 exhibited significantly greater resilience of C mineralization to heat, potentially through the recovery of heat-tolerant species as distinct bacterial community structures rather than bacterial abundance were found in the soil at different pH. Our results showed that the acidic soils were particularly more susceptible to additional perturbations (e.g., metal contamination and temperature variation). This implies that land management such as precision liming and balanced fertilization to keep soil pH neutral can enhance C and N cycling in soils subject to environmental stresses, especially for Cu contaminated soil.

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TJD conceptualization (supporting); methodology (supporting); reviewing and editing (supporting).

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