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# 1 Soil pH moderates the resistance and resilience of C and N cycling to 2 transient and persistent stress

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## 10 Abstract

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

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

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

## 32 **1. Introduction**

33 Soil ecosystems are highly complex and subject to various types of stresses that influence the ability  
34 of soil to deliver ecosystem services. Depending on the duration, stresses are often classified as  
35 transient (short-term and discrete) and persistent (long-term and continuous) (Shade et al., 2012).  
36 Copper (Cu) contamination due to excessive use of Cu-based fungicide and fertilisers could cause long-  
37 lasting and adverse effects on microbial functions and soil fertility (Ballabio et al., 2018). Climate  
38 extremes, such as drought and heat waves, could cause negative fluctuations in soil ecosystems  
39 (Bardgett and Caruso, 2020). Copper (Cu) and heat have been widely used as experimentally  
40 representative transient and persistent stresses in soil ecosystems (Griffiths et al., 2001; Shu et al.,  
41 2019; Zhang et al., 2010). It is imperative to understand soil functional stability under various stresses,  
42 which typically is quantified as a combination of resistance (initial response to stress) and resilience  
43 (recovery to a stable state) (Griffiths and Philippot, 2013).

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

46 cycling (Schimel et al., 2007). Many heterotrophic microbial communities with large species diversity  
47 can decompose diverse C compounds and are involved in C mineralization (Schimel and Schaeffer,  
48 2012). Ammonia oxidizers, including bacteria (AOB) and archaea (AOA), are responsible for ammonia  
49 oxidation where ammonia is oxidised to hydroxylamine and then nitrite (Kuypers et al., 2018).  
50 Denitrifying bacteria, such as *nirK*- and *nirS*- harbouring bacteria, produce nitrite reductase to reduce  
51 nitrite ( $\text{NO}_2^-$ ) to nitric oxide (NO) which is a key reaction in denitrification (Kuypers et al., 2018). Given  
52 that microorganisms play a paramount role in regulating soil C and N cycling, it is logical that the  
53 resistance and resilience of C and N functions are governed by the underpinning microorganisms. For  
54 example, C mineralization exhibited approximately 2 times greater resilience to Cu ( $1 \text{ mg Cu soil g}^{-1}$ )  
55 than ammonia oxidation (Shu et al., 2021). This trend was also found in the resilience of 16S rRNA and  
56 AOB to Cu, suggesting a direct role of microbial populations to the pertaining functional resilience (Shu  
57 et al., 2021). The soil microbial community structure has been reported to be the central factor  
58 governing the resilience of microbial functions to heavy metal contamination (Jiang et al., 2020).  
59 Strong links between microbial community composition and soil multifunctionality's resistance to  
60 drought were also found in a meta-analysis on 59 dryland ecosystems (Delgado-Baquerizo et al., 2017).  
61 In contrast, discrepancies between microbial communities and pertaining functional  
62 resistance/resilience have been reported. For example, the process of denitrification was found to be  
63 recover more rapidly after heat and drought stress than denitrifying gene copy numbers (*narG* and  
64 *nosZ*) (Fikri et al., 2021). The loss of biodiversity was found to have no significant impacts on the  
65 resistance or resilience of denitrification and nitrification (Wertz et al., 2007). These results indicate  
66 that there is more than microbial community structure governing soil functional resistance and  
67 resilience. Potential contributors could be soil physicochemical properties, such as soil pH and redox  
68 potential (Griffiths and Philippot, 2013).

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

71 of ammonia oxidizers (Gubry-Rangin et al., 2015) and changes in the protonation of ammonia (NH<sub>3</sub>),  
72 which serves as the direct substrate for ammonia oxidizers (Suzuki et al., 1974). Soil pH was also  
73 reported to have a significant negative relationship to the ratio of N<sub>2</sub>O/N<sub>2</sub>, which indicates that pH  
74 may affect soil denitrification through its effect on the community structure, transcription and activity  
75 of denitrifiers due to different sensitivity of denitrifiers to soil pH variation (Herold et al., 2018; Liu et  
76 al., 2010; Qu et al., 2014).

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

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

96 their underpinning microbial communities to persistent (Cu) and transient (heat) stresses as these two  
97 stresses are representative threats (i.e., heavy metal pollution, and global warming) to soil. Our  
98 previous study demonstrated that the concentration of 1 mg Cu soil g<sup>-1</sup> could have a marked negative  
99 impact on microbial communities (Shu et al., 2021). Considering the bioavailability of Cu could be  
100 highly dependent on soil pH, here a high (1 mg Cu soil g<sup>-1</sup>) and low (500 µg Cu soil g<sup>-1</sup>) concentration  
101 of Cu were chosen as persistent stresses. Soils were incubated for 56 days under transient and  
102 persistent stresses to simulate expected impacts from heat (40 °C warming) and Cu (high and low  
103 concentration). Changes in C mineralization, denitrification, ammonia oxidation, gene abundances  
104 underpinning the processes, immediately after heat- and Cu- induced stress and during subsequent  
105 recovery over 56 days were measured. The soil microbial community structures of general bacteria  
106 (16S rRNA T-RFLP) and specific AOB (bacterial *amoA* T-RFLP) were analysed. We measured the  
107 resistance/ resilience of C and N processes and the corresponding microbial community abundances  
108 following the perturbation of Cu and heat over 56 days.

109 This experiment tests the hypothesis that functional resistance/resilience of C and N cycling process  
110 varies in soils of different pH. This will be due to the direct impacts of pH on microbial communities,  
111 which will impact their immediate response (resistance) and recovery (resilience) of biochemical  
112 cycles following a stress. When pH is optimal for the functions and communities, a greater  
113 corresponding resistance and resilience will occur. With increases in pH, we hypothesised the  
114 resistance and resilience of functions and microbial communities to Cu (especially for the higher  
115 concentration) would increase, as Cu bioavailability decreased. According to the “insurance  
116 hypothesis”, soils with greater microbial diversity are expected to contain organisms with a broader  
117 array of environmental tolerance ranges and maintain functioning even if others fail (Yachi and Loreau,  
118 1999). We hypothesised the highest resistance and resilience of functions and communities to heat in  
119 the neutral soil where there is a greater chance that heat tolerant taxa are present in a more diverse  
120 microbial community.

## 121 **2. Material and methods**

### 122 **2.1 Soil sampling**

123 Soils were collected from three treatment plots (pH 4.5, 6.0 and 7.5) of the long-term pH trial at  
124 Craibstone, Aberdeen, UK. The actual pH values measured by H<sub>2</sub>O for these plots at the time of  
125 sampling were 4.9, 6 and 7.1, respectively. Plots have been maintained at target pH's since 1961 by  
126 additions of aluminium sulphate or calcium carbonate (Herold et al., 2012). The soil is a free-draining  
127 sandy loam, Humic Entic Podzols (World Reference Base) classified locally as the Countesswells series.  
128 The plots follow an eight-year crop rotation (winter wheat, potatoes, spring barley, swedes, spring oat,  
129 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  
130 cm) soil was collected from the third-year grass ley in August 2016.

### 131 **2.2 Resistance and resilience**

132 The resistance and resilience assay followed the method described by Shu et al. (2021). Four replicates  
133 of each pH soil were imposed by either a stress (heat, low Cu or high Cu) or were unstressed, resulting  
134 in the factorial combination of three soil pH treatments and four stresses (3 pHs × 4 stresses × 4  
135 replicates = 48 microcosms). For Cu-stressed samples, 220 g soil (dry-weight equivalent) was amended  
136 with 2.2 ml of either 0.79 M CuSO<sub>4</sub>·5H<sub>2</sub>O or 1.57 M CuSO<sub>4</sub>·5H<sub>2</sub>O to reach a concentration of 500 µg Cu  
137 soil g<sup>-1</sup> (low Cu) or 1 mg Cu soil g<sup>-1</sup> (high Cu). The same volume of sterile water was added to soils (220  
138 g) to create the heat-stressed and unstressed (control) samples. The heat- stressed soils and the rest  
139 of soils were incubated at either 40 °C or 20 °C, respectively, for 16 hours. All samples were then  
140 stored at 20 °C for the remaining 56 days without any addition of C source.

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

145 as nitrite-N after incubating 10 g soil with 50 ml solution (0.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> + 10 mM NaClO<sub>3</sub>) for 24  
146 hours (Shu et al., 2021). Denitrification was estimated as N<sub>2</sub>O after incubating 20 g soil with 20 ml  
147 solution (25 mM glucose +3.57 mM KNO<sub>3</sub>) with the presence of 10% (v/v) acetylene for 5 hours (Shu  
148 et al., 2021). Soil physicochemical properties (*i.e.*, available N, SOC, TN, DOC, actual pH) were  
149 determined by the methods in Carter and Gregorich (2007).

### 150 **2.3 DNA extraction, PCR, and T-RFLP**

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

153 To analyse total bacterial community in the unstressed treatments, 10 folds diluted DNA were  
154 amplified using labelled 16F27 and 1392R primers (Lane, 1991) following the PCR conditions described  
155 in Table S1. Each amplification was conducted in 15-μl reaction mix containing 0.3 U Platinum Taq  
156 DNA Polymerase (Invitrogen, UK), 1.5 μl of buffer, 1 U *Hha I* (Promega, UK), 3 mM MgSO<sub>4</sub>, 3.75 mM  
157 dNTPs, 10 μg BSA, and 6 pM of each primer.

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

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



## 168 **2.4 qPCR**

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

## 175 **2.5 Data analysis**

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

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

$$179 \quad f(t) = \frac{\text{Stressed indicator } (t)}{\text{Unstressed indicator } (t)} \times 100$$

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

$$182 \quad \text{Resilience} = \int_1^{56} f(t)dt / (56 - 1)$$

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

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

## 194 **3. Results**

### 195 **3.1 Effects of soil pH on soil properties and functions**

196 A pH increases from 4.9 to 7.1 in the unstressed control soil resulted in greater concentrations of  
197 dissolved organic carbon (DOC) ( $P < 0.05$ ) (Table S2), but less ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) ( $P$   
198  $< 0.05$ ). Microbial biomass carbon (MBC) concentration in the soil at pH 4.9 was significantly ( $P < 0.05$ )  
199 lower than other soils. There was no significant difference in total nitrogen (TN) or soil organic carbon  
200 (SOC).

201 In the unstressed control soils at day 1, C mineralization and ammonia oxidation were greatest in the  
202 pH 7.1 soil, which were  $38 \mu\text{g C g}^{-1} \text{h}^{-1}$  and  $58 \mu\text{g N g}^{-1} \text{g}^{-1} \text{h}^{-1}$ , respectively (Figure 1). C mineralization ( $P$   
203  $< 0.001$ ,  $R^2 = 0.91$ ), and ammonia oxidation ( $P < 0.001$ ,  $R^2 = 0.99$ ) significantly increased with increases  
204 in soil pH (Figure 1). However, the relationship between denitrification and soil pH was quadratic ( $P <$   
205  $0.001$ ,  $R^2 = 0.98$ ) where denitrification rate reached its peak  $582.96 \text{ ng N g}^{-1} \text{h}^{-1}$  in the soil at pH 6  
206 (Figure 1 and S1).

### 207 **3.2 The effects of soil pH on functional resistance and resilience**

208 The two-way interactions pH\*stress, pH\*day, and stress\*day were significant ( $P < 0.001$ ) for C  
209 mineralization (Table 1). C mineralization dropped by 20-41% on day 1 after high Cu contamination  
210 for all pHs (Table S3 to S5). The resilience of C mineralization to high Cu was significantly ( $P < 0.05$ )  
211 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

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

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

226 Denitrification was significantly ( $P < 0.001$ ) impacted by the three-way interaction of pH\*stress\*day  
227 (Table 1). One day following Cu, denitrification significantly ( $P < 0.05$ ) decreased in all the soils (Table  
228 S3 to S5). The soil at pH 7.1 was significantly ( $P < 0.05$ ) more resilient to low (48% of control) and high  
229 (23% of control) concentration of Cu compared to other soils (Figure 2). Heat significantly decreased  
230 denitrification by 72% (pH 4.9), 82% (pH 6), and 92% (pH 7.1) (Figure 2). Obvious recoveries were  
231 observed after 56 days following heat in all soils where the soil at pH 6 (74% of control) exhibited  
232 significantly ( $P < 0.05$ ) greater resilience than the soil at pH 4.9 (65% of control).

### 233 **3.3 The effects of soil pH, stress, and day on microbial communities**

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

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

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

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

258 The abundance of *nirS* was significantly affected by three major factors pH ( $P < 0.001$ ), stress ( $P <$   
259  $0.001$ ), and day ( $P < 0.01$ ), but not their interactions (Table 1). In the unstressed soils at day 1, the

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

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

## 271 **4. Discussion**

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

### 280 **4.1 The effects of soil pH on functions, microbial abundance, and soil properties**

281 C mineralization significantly ( $P < 0.05$ ) increased when soil pH was towards neutral (Figure 1), which  
282 was consistent with a previous research on the same experimental site (Meharg and Killham, 1990).  
283 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

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

## 317 **4.2 The effects of soil pH on the resistance and resilience of functions and** 318 **microbial communities**

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

334 Ammonia oxidation was significantly ( $P < 0.05$ ) more resistant and resilient to Cu in the soil at pH 7.1  
335 compared to the soils at pH 4.9 and 6 (Figure 2). Similarly, the abundance of AOB was resistant and  
336 resilient to Cu in the pH 7.1 soil (Table S5). It is possible that decreased Cu-bioavailability due to the  
337 absorbance of  $\text{Cu}^{2+}$  to oxyhydroxides at higher pH, resulted in attenuated negative impacts of Cu on  
338 the activity and abundance of AOB (Degryse et al., 2009). Since the decrease in free  $\text{Cu}^{2+}$  activity is  
339 between 3- and 15-fold per unit pH increase (Degryse et al., 2009), the toxicity of Cu to bacteria in pH  
340 4.9 soil could be 6- to 30- times greater than that in pH 7.1 soil. The higher proportion of Cu-free niches  
341 in neutral soil allows AOB to colonise, reproduce, and thus recover from Cu. Additionally, the AOB  
342 community structure was clearly and significantly separated by pH (Figure 4). There is a possibility that  
343 higher pH favours the growth of Cu-tolerant AOB which are also functionally active. One example is  
344 the *Nitrosospira* lineage which is metal-tolerant and has been proven to markedly contribute to the  
345 recovery of nitrification to Zinc contamination (Mertens et al., 2009). We acknowledge the importance  
346 of AOA in ammonia oxidation, but AOA have been proven to be less versatile than AOB in a changing  
347 environment (Aigle et al., 2020). Considering AOA are more abundant than AOB in acidic soils (Gubry-  
348 Rangin et al., 2010), the greater resilience of ammonia oxidation in neutral soil may mainly be  
349 attributed to AOB. We suggest future studies on AOA, commammox and their transcriptional activities  
350 will help explain the impacts of soil pH on the response of ammonia oxidation to contrasting stress.

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



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

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

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

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

## 388 **5. Conclusion**

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

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408 PDH conceptualization (supporting); methodology (supporting); supervision (supporting); reviewing  
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