Role of microbial communities in conferring resistance and resilience of soil carbon and nitrogen cycling following contrasting stresses

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Abstract

Soils frequently experience environmental stresses that may have transient or persistent impact on important ecosystem services, such as carbon (C) and nitrogen (N) cycling. Microbial

25 communities underpin resistance (the ability to withstand a
26 stress) and resilience (the ability to recover from a stress) of
27 these functions. Whilst functional stability and resilience have
28 been studied extensively, the link to genetic stability is missing.
29 In this study, the resistance and resilience of C mineralization,
30 ammonia oxidation and denitrification, their associated gene
31 abundances (16S rRNA, bacterial *amoA*, *nirK*, *nirS*, *nosZ-I* and
32 *nosZ-II*) and bacterial community structures (T-RFLP 16S rRNA)
33 were compared in two managed soils for 28 days after stressing
34 the soils with either a persistent (1 mg Cu soil g^{-1}) or a transient
35 (heat at 40 °C for 16 h) stress. The average resistance of C
36 mineralization to Cu was 60%, which was significantly greater
37 than the resistance of ammonia oxidation (25%) and
38 denitrification (31%) to Cu. Similarly, the average resilience of
39 C mineralization to Cu was 52%, which was significantly greater
40 than the resilience of ammonia oxidation (12%) and
41 denitrification (18%) to Cu. However, this pattern was not
42 significant after heat stress, indicating the critical role of
43 different stressors. Changes in total bacterial community
44 structure rather than abundance of 16S rRNA reflected the
45 responses of C mineralization to Cu and heat. Both Cu and heat
46 significantly decreased functional gene abundance (*amoA*, *nirK*,
47 *nirS*, *nosZ-I* and *nosZ-II*), however, significant recovery of

48 denitrifying gene abundance was observed after 28 days
49 following heat. There was lack of constant relationships
50 between functional and genetic stability, highlighting that soil
51 physiochemical properties, the nature of the stressor, and
52 microbial life history traits combine to confer functional
53 resistance and resilience. Genetic responses on their own are
54 therefore inadequate in predicating changes to soil functions
55 following stresses.

56 **Keywords**: microbial community, mineralization, denitrification,
57 ammonia oxidation, stresses, sustainability

58 1. Introduction

59 Soil ecosystems are undergoing unprecedented disturbances
60 from anthropogenic activities and climate extremes. A key
61 challenge is how to maintain soil sustainability under a situation
62 where severity and frequency of anthropogenic stresses are
63 increasing [1]. It is not just the ability of an ecosystem to
64 withstand these stresses (resistance), but their ability to
65 recovery (resilience) over time that is important to
66 understanding soil ecosystem sustainability, as appreciated in a
67 wide body of research [2,3]. Stresses are often classified as
68 transient or persistent depending on their duration [4].

Transient stresses are usually abrupt and intense and are associated with climate extremes, such as droughts and heat waves [1]. Although transient stresses are short-term, their effects to soil ecosystem could be detrimental. Heat stress is a typical transient stress because it gives soils a short shock in temperature with minimal impact to other soil physicochemical properties [5]. For example, a heat stress (50 °C) could diminish soil microbial biomass by 60% and beta-glucosidase activity by 50% [6]. By contrast, the effects of persistent stresses (e.g. heavy metal contamination) are long-lasting and continuous [4]. With the excessive uses of Cu-based pesticides and Cu-contaminated manure, agricultural soils are continuously exposed to the accumulation of Cu toxicity [7]. The long-term use of Cu-based pesticides has been reported to cause adverse effects on soil enzyme activities and organisms [7]. Microorganisms in forest soils which are adjacent to a metal smelter are also facing deleterious impact from increased metal pollution [8].

Stresses on soils affect the extraordinary diversity of microbial communities and their capacity to drive carbon (C) and nitrogen (N) fluxes between the atmosphere and terrestrial ecosystems, and in regulating soil nutrient availability [1,9]. Many aerobic

91 heterotrophic microbial communities with large species
92 diversity are responsible for the largest flows of C in soil,
93 including C mineralization and storage [10]. In contrast,
94 denitrification and nitrification which are the important parts of
95 N cycling that usually involve specific N-transforming
96 microorganisms. Ammonia oxidation where ammonia is
97 oxidized to hydroxylamine using ammonia monooxygenase (the
98 rate limiting step of nitrification) is carried out by aerobic
99 ammonia-oxidizing bacteria (AOB) and archaea (AOA) [11]. AOA
100 belong to the class *Nitrososphaeria* within the phylum
101 *Thaumarchaeota* [11]. AOB are limited to a small number of
102 genera *Nitrosomonas*, *Nitrosospira* (*Betaproteobacteria*) and
103 *Nitrosococcus* (*Gammaproteobacterial*) [12]. Although AOA and
104 AOB could coexist and contribute to ammonia oxidation in soil,
105 AOB have been found to play a crucial role driving ammonia
106 oxidation in managed grassland and arable soils [13,14]. In
107 contrast, complete denitrification where nitrate is reduced to
108 dinitrogen (N_2) involves a series of enzymes reactions and
109 diverse microbial communities which are phylogenetically more
110 heterogeneous than ammonia oxidizers [15]. Denitrifying
111 bacteria have been identified in over 50 genera with over 125
112 different species and are estimated to represent 10-15% of
113 bacteria populations in water, soil and sediment [16]. Among

114 denitrifying bacteria, *nirK*- and *nirS*- harbouring bacteria
115 produce nitrite reductase to reduce nitrite (NO_2^-) to nitric oxide
116 (NO), and *nosZ*- containing bacteria generate nitrous oxide
117 reductase to catalyse nitrous oxide (N_2O) reduction to
118 dinitrogen [12]. Therefore, *nirK*, *nirS* and *nosZ* are important
119 genes to determine the production of N_2O and N_2 during
120 denitrification.

121 According to the insurance hypothesis, high diversity insures
122 against declines in ecosystem function because many species
123 provide a greater guarantee that some will maintain their
124 function if others are killed or inhibited by stresses [17]. Thus
125 species richness or diversity is typically considered a key
126 attribute associated with resistance and resilience [17]. Several
127 studies have aimed to capture the relationship between
128 functional resilience and biodiversity. Griffiths et al. (2000) [18]
129 found that loss of biodiversity impaired the resistance of
130 decomposition to a spike in Cu. In contrast, a more recent study
131 focused on the stability of both general (e.g. substrate-induced
132 respiration) and specific (e.g. nitrification and denitrification)
133 functions to heavy metal contamination and herbicides, and
134 found bacterial diversity had no significant effect on the
135 stability of these functions [19]. A weak association between

136 microbial functions and microbial diversity was also reported in
137 soils following transient stresses. Selmants et al. (2016) [20] did
138 not found a significant relationship between bacterial richness
139 and the response of microbial respiration to warming in a forest
140 soil. Likewise, the resilience of multifunctionality in a forest soil
141 to drought was found to be not related to the bacterial diversity
142 [21]. These contrasting findings suggested that soil functional
143 resistance/resilience may not only be related to total microbial
144 diversity, but also to specific microbial communities pertaining
145 to the function.

146 Griffiths and Philippot (2013) [3] reviewed 51 studies on
147 microbial resistance and resilience in soil systems, of which
148 most focused on C processes (e.g. C mineralization/ respiration),
149 and only 3 studied both C and N processes (e.g. denitrification
150 and nitrification). Fewer of these studies investigated the soil
151 microbial communities, and even fewer explored the specific
152 communities underlying measured functions which could be
153 sensitive indicators of resilience [21]. Although the research
154 interest in soil sustainability has boomed, there is a lack of
155 evidence of the linkage of functions and underpinning microbial
156 communities involving both C and N processes to stresses,
157 particularly in relation to transient stresses typical of climatic

158 variability and persistent stresses typical of pollution. Our
159 objectives were (1) to compare the responses of selected C and
160 N processes to experimental heat or Cu stresses, (2) to
161 determine the response of bacterial communities to these
162 experimental stresses, and (3) to identify any relationships
163 between microbial communities and the corresponding
164 functional resilience.

165 To do this we examined two soils of similar texture and climate,
166 but under different management practices that altered C
167 composition. Changes in C mineralization, ammonia oxidation,
168 denitrification, functional gene abundance relating to these
169 processes and bacterial community structure were measured
170 immediately after heat- and Cu- induced stresses and during
171 subsequent recovery over 28 days. Sampling encompassed fast
172 alterations to microbial community structure that can occur in
173 1 day [22], up to 28 days of incubation where a range of studies
174 have demonstrated temporal dynamics to heat stress decrease
175 [5,6,23]. Microbial communities and functions were speculated
176 to recover from heat due to the short-term exposing time, but
177 not recover from Cu because of its long-lasting effects. C
178 mineralization was hypothesized to be more resistant and
179 resilient than denitrification and ammonia oxidation to the

same stress, reflecting different degrees of heterogeneous phylogeny and functional redundancy. We hypothesized that changes in C and N processes were positively correlated to the changes in underlying bacterial abundances. In particular, the positive links between ammonia oxidation and bacterial *amoA* were expected to be the strongest in these managed soils.

2. Materials and Methods

2.1 Soil samples

Two soils were collected in Aberdeenshire in north-east Scotland. The climate was temperate oceanic with the average temperature ranging from 9 to 16 °C during June to August, and the average temperature ranging from 0 to 6 °C during December to February. The annual average rainfall was 753 mm with 64 mm during June to August and 62 mm during December to February. The soils were sampled according to two land uses: a managed grassland soil at Craibstone (57° 11' 19'' N, 2° 12' 43'' W) and an arable soil at Insch (57° 20' 36'' N, 2° 36' 20'' W) in January 2015 when the soils had received no chemical treatment in the month preceding soil sampling. At each sample site, three surface soil samples (0–20cm in depth) approximately 10 kg in weight were randomly collected from

different locations at the same site and were mixed thoroughly to form a representative sample of that soil. The litter layer, visible fauna, and vegetation were removed before the soils were passed through a 4 mm metal sieve at field water content and stored at 4 °C.

For each soil, four replicates were measured for soil characteristics before subjecting to stresses. Soil physicochemical properties were measured following standard methods [24]. Soil microbial biomass carbon (MBC) was analysed by chloroform fumigation. Soil mineral N (NO_3^--N and NH_4^+-N) was extracted by shaking with 2 M KCl for 1 hour and analysed using a continuous flow analyser (Skalar San++ 4800, Netherlands). Dissolved organic carbon (DOC) was analysed using a TOC analyser (Dohrmann DC-80, UK). Total N and organic C were analysed using a Thermo Scientific Flash 2000 elemental analyser. Soil pH was determined in 0.01M $CaCl_2$ at a ratio of 1:2 (soil: solution). Soil characteristics and their WRB (World Reference Base) classification are presented in Table 1.

2.2 Resistance and resilience assay

Soil moisture of sieved soils at field water content was adjusted to 60% water-filled pore space (WFPS) and incubated for 7 days

222 at 20 °C prior to analysis. This allowed for mineralization of
223 nutrients released during sieving and the equilibration of soil
224 moisture. Our selection of stresses follows standard
225 approaches used in a large body of previous research exploring
226 soil resilience [5,23,25]. For each soil, aliquots were exposed to
227 either a heat or Cu stress or were unstressed as a control, with
228 four replicates for each soil and stress. Each aliquot contained
229 250 g dry-weight equivalent of soil loosely packed to a bulk
230 density of 1.1 g cm^{-3} in a 500 ml pot (89 mm diameter, 80.4 mm
231 height). The Cu- perturbed soils were amended with 2.5 ml of
232 1.57 M $CuSO_4.5H_2O$ to obtain a concentration of 1 mg Cu soil g^{-1}.
233 Cu concentration above 0.17 mg g^{-1} could result a significant
234 loss of bacterial biodiversity [26], thus we expected 1 mg Cu g^{-1}
235 could be a strong stress for these soils. Both heat- and un-
236 stressed soil had 2.5 ml of sterile distilled water added. Because
237 at the sampling period the average soil temperature ranges
238 from 0 to 6 °C, a temperature of 40 °C could induce a large
239 transient stress. This temperature was documented to
240 significantly impair key functions in temperature soils [5]. The
241 heat- stressed soils were incubated at 40 °C for 16 h, in a sealed
242 pot to prevent any water loss, while both Cu- and un- stressed
243 soil were incubated at 20 °C for 16 h. After 16 h, all pots were
244 then incubated at 20 °C for the remainder of the resilience assay.

Soil C mineralization, potential ammonia oxidation, and potential denitrification were measured at intervals of 1, 3, 7, 14, and 28 days after the stress was imposed. At each sampling time, each aliquot of soil was gently mixed, and subsamples were taken for analysis (described below). Subsamples were also collected and stored at -80 °C for later analysis of functional gene abundance and microbial community structure (described below).

2.3 Soil C mineralization

Soil C mineralization was measured as the short-term decomposition of organic C compounds. A stock solution to mimic root exudate containing 86.8 mM fructose, 86.8 mM glucose, 43.4 mM sucrose, 130.2 mM succinic acid, 130.2 mM malic acid, 86.8 mM arginine, 173.6 mM serine and 173.6 mM cysteine was prepared and filter- sterilised [27]. Two g soil was mixed with 120 µl of this solution to give a concentration of 3 mg C soil g^{-1} and stored in an air-tight gas vial (22 ml) over 24 hours at 20 °C, prior to measurement of CO_2-C concentration on a gas chromatograph (Agilent 7890A, UK) with a thermal conductivity detector (Agilent Technologies, UK).

2.4 Potential ammonia oxidation

Potential ammonia oxidation (referred to here as ammonia oxidation) was determined by the chlorate inhibition method [28]. Briefly, 50 ml solution (0.5 mM $(NH_4)_2SO_4$ + 10 mM $NaClO_3$, pH 7.2) was added to 10 g soil. The samples were then shaken on a rotary shaker (180 rpm) at 20 °C for 24 h, with the resulting slurry being centrifuged at 10,000 g min^{-1} for 10 min. Nitrite-N concentration was determined in 0.4 ml supernatant amended with 0.1 ml sulphanilamide, 0.1 ml naphthylethylene diamide and 2.4 ml H_2O at λ=543 nm on a spectrophotometer (Agilent 8453 UV-visible Spectroscopy System, UK).

2.5 Potential denitrification

Potential denitrification (referred to here as denitrification) was determined following anaerobic incubation of soil samples in the presence of 10% (v/v) acetylene (C_2H_2) [29]. A 20 ml solution of 25 mM glucose + 3.57 mM KNO_3 was mixed with 20 g soil in a 200 ml flask, followed by flushing with helium at 600 ml min^{-1} for 2 min. A 20 ml headspace sample was then removed from each flask by syringe and replaced with 20 ml C_2H_2 to give a headspace concentration of 10% (v/v) C_2H_2. The flasks were shaken on a rotary shaker (150 rpm) at 20 °C for 5 h. Gas

samples (22 ml) for analysis of N_2O-N concentration were collected at 5 h, stored in pre-evacuated 22 ml vials and analysed on a gas chromatograph (Agilent 7890A, UK) equipped with an electron capture detector (Agilent Technologies, UK).

2.6 Soil DNA extraction

DNA of the soil stored at -80 °C was extracted by a phenol-chloroform method [30]. Briefly, 1 g of soil was suspended in 2 ml of solution (0.12 M Na_2HPO_4 + 1% SDS) which contained 1×10^6 copies of a mutated reference gene to determine extraction efficiency [31]. One ml of the resulting slurry was further bead-beaten for 4.5 mins at 30 Hz (TissueLyser, Qiagen, Hilden, Germany). The slurry was then centrifuged at 5,000 rpm for 10 mins, and the aqueous phase was transferred to new 96-well microplates (Qiagen, Hilden, Germany), where the soil DNA was purified with phenol/ chloroform/ isoamyl alcohol, and precipitated with isopropanol/ sodium acetate, and treated with polyvinylpolypyrrolidone. The extracted DNA was stored at -80 °C for later analysis.

2.7 PCR and T-RFLP of 16S rRNA

Bacterial DNA was amplified from 1:10 diluted DNA extracts. The primers used were 16F27 with labelled 8'-FAM (AGA GTT

307 TGA TCC TGG CTC AG) and 1392R (ACG GGC GRT GTG TAC A)
308 [32]. Fourteen µl 'master mix' which contained 0.3 U DNA
309 polymerase (Platinum Taq DNA Polymerase High Fidelity,
310 Invitrogen, UK), 6 pM each primer, 1.5 µl of 10X High Fidelity
311 Buffer (Invitrogen), 1 U *Hha* I (Promega, UK), 3.75 mM dNTPs, 3
312 mM $MgSO_4$ and 10 µg bovine serum albumin (BSA) was digested
313 at 37 °C for 40 min. One µl template was combined with 'master
314 mix' to form 15 µl final reaction mix. PCR was performed with a
315 hot start step of 94 °C for 4.5 mins, followed by 35 cycles of
316 94 °C for 30 s, 57 °C for 30 s and 68 °C for 90 s; cycling was
317 completed by a final extension period of 68 °C for 10 mins. PCR
318 products showed equal intensity after visualization by agarose
319 gel electrophoresis (1.5%) with 1X Tris/Borate/EDTA as the
320 buffer.

321 Three µl fluorescent PCR products were digested with 0.5 µl *Alu*
322 I restriction enzyme (Promega, UK) at 37 °C. One µl one to ten
323 diluted digests were mixed with 0.25 µl 1200 LIZ dye Size
324 Standard (Life Technologies, UK) and 8.75 µl formamide (Life
325 Technologies, UK). Samples were analysed by an ABI 3730
326 automated sequencer (Thermo Fisher Scientific, UK). Peaks of
327 resulting electropherograms were analysed by GeneMapper

(Applied Biosystems, CA, USA) and processed following Deng et al. (2010) [30].

2.8 qPCR for quantification of functional genes and total bacteria

For relative qPCR, 16S rRNA, *amoA*, *nirK, nirS*, *nosZ-I* and *nosZ-II* gene copy numbers were quantified including amplification of the added reference target as described in Daniell et al. (2012) [31]. Amplification was performed on a LightCycler 480 Sybr Green qPCR (Roche, UK). qPCR for functional gene (bacterial *amoA*, *nirK*, *nirS*, *nosZ-I*), total bacterial (16S rRNA) and the reference fragment abundance were performed in 20 µl reaction mixtures containing 10 µl SYBR Green 1 Master Mix (Applied Biosystems, UK); 0.5 µl of BSA, 1 µl of 10 pM of each primer, 2 µl template DNA (10-fold dilutions of soil nucleic acid extracts) and DNase-free water. In the reaction mixtures of the *nosZ-II*, each primer was increased to 2 µl with the proportional decrease of water. Standard curves were obtained using serial dilutions of linearized plasmids containing cloned *amoA*, *nirK*, *nirS*, *nosZ-I*, *nosZ-II*, 16S rRNA genes and the reference fragment. No-template controls gave null or negligible values. The primers and thermal cycling conditions for amplification of target genes are described in Table S1.

2.9 Data analysis

All statistical analysis were performed using R 3.5.2 [33] except for analysis of similarity (ANOSIM) which was conducted using Primer 7 (PRIMER-E limited, Auckland, New Zealand).

Stability (%) was calculated as the reduction in biological functions of the stressed soil compared with the unstressed control at day t [23]:

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

Resistance was defined as stability measured at day 1 after perturbation, while resilience was estimated as the integrative stability after day 1 up to 28 days following stress [27].

$$Resilience = \int_{1}^{28} f(t)dt/(28-1)$$

The mean and standard deviation of resistance and resilience was estimated using all possible combinations of stressed and unstressed soils.

One-way analysis of variance (ANOVA) followed by a post hoc Tukey's test was used to detect the differences of

367 physicochemical properties between soils. Two-way ANOVA
368 was performed to determine the significance of stress, time and
369 their interaction on functions, the copy number of genes as well
370 as the functional and genetic stability in each soil. Pearson
371 correlations of functional and genetic stability were performed
372 to detect relationships between functions and the underlying
373 bacteria communities.

374 For each soil, 16S rRNA T-RFLP data was normalized by Hellinger
375 transformation and Bray-Curtis dissimilarity matrices were
376 constructed under the “vegan” package [34]. Ordinations were
377 produced using non-metric multidimensional scaling (NMDS)
378 and significant effects of stress, time and their interactions on
379 the bacterial community structure were tested using a two-way
380 PERMANOVA. A one-way ANOSIM on Bray-Curtis resemblance
381 matrix in each soil was performed to determine pair-wise
382 comparison between different treatments. A separate NMDS
383 on Bray-Curtis distance of bacterial communities in the
384 unstressed soils was performed to distinguish bacterial
385 community structure influenced by soil properties.

3. Results

3.1 Resistance and resilience of soil functions to Cu and heat stress

Over the 28 days incubation the unstressed Craibstone soil exhibited 7.0 µg C g^{-1} h^{-1} C mineralization, 14.8 µg N g^{-1} d^{-1} ammonia oxidation and 356.9 ng N g^{-1} h^{-1} denitrification on average (Table S3). These were significantly (P < 0.001) decreased by Cu as well as heat (Table S2). The unstressed Insch soil exhibited 13.0 µg C g^{-1} h^{-1} C mineralization, 13.0 µg N g^{-1} d^{-1} ammonia oxidation and 280.8 ng N g^{-1} h^{-1} denitrification on average, which were also significantly (P < 0.001) decreased by Cu and heat (Table S2).

For both soils, functional stabilities in response to Cu significantly (P < 0.001) varied by the measured function (Table 2). C mineralization in the Insch soil was significantly (P < 0.05) more resistant and resilient to Cu compared to denitrification and ammonia oxidation (Figure 1). C mineralization in the Craibstone soil was also significantly (P < 0.05) more resilient to Cu in comparison to denitrification and ammonia oxidation in this soil (Figure 1).

The stability to heat varied significantly ($P < 0.001$) between functions in the Craibstone soil (Table 2), where C mineralization was significantly ($P < 0.05$) more resistant and resilient compared to ammonia oxidation, and was significantly ($P < 0.05$) more resilient than denitrification (Figure 1). In the Insch soil, the stability to heat did not vary significantly between functions (Table 2), and there were also no differences in resilience between functions (Figure 1).

3.2 Resistance and resilience of bacterial abundances to Cu and heat stress

In both soils, applying stress had a significant ($P < 0.05$) impact on the abundance of genes (Table S2). One day after Cu stress, the gene copy number of 16S rRNA, *amoA*, *nirS*, *nirK*, *nosZ-I* and *nosZ-II* in both soils significantly ($P < 0.05$) decreased (Table S4 and S5). One day following heat stress, significant ($P < 0.05$) decreases in genetic abundance were observed in *amoA*, *nirS*, *nirK*, *nosZ-I* and *nosZ-II* (Table S4 and S6).

The genetic stability to Cu significantly ($P < 0.01$) varied by the measured genes and time (Table 2). 16S rRNA was significantly ($P < 0.05$) more resilient to Cu compared to bacterial *amoA* in

both soils (Figure 2). There was no significant difference of the resilience to Cu between denitrifying genes in both soils.

Similarly, the stability of gene abundance to heat significantly ($P < 0.001$) varied with gene identity and time (Table 2). The abundance of 16S rRNA was significantly ($P < 0.05$) more resistant to heat than bacterial *amoA* and all measured denitrifying genes in both soils (Figure 2). 16S rRNA was also significantly ($P < 0.05$) more resilient to heat than *amoA* in both soils. Among denitrifying genes, *nirS* and *nosZ-I* were significantly ($P < 0.05$) more resilient than *nirK* and *nosZ-II* in the Craibstone soil; *nirK* was significantly ($P < 0.05$) more resilient than *nirS* in the Insch soil (Figure 2).

3.3 Changes in bacterial community structure

Non-metric multidimensional scaling (NMDS) demonstrated a clear separation of the bacterial community structure by soil (Figure S1). Applying heat and Cu stress markedly shifted the community structure away from the unstressed control (Figure 3). PERMANOVA results showed significant ($P < 0.001$) effects of stress, time and their interactions on the bacterial community structure in both soils (Table 3). Compared to the unstressed control, Cu did not significantly ($P < 0.05$) shift the

447 bacterial community structure until 14 days after stress in both
448 soils (Table S7 and S8 and Figure 3). The bacterial community
449 structure did not recover from Cu stress in either soil. In both
450 soils, heat significantly (P < 0.05) altered the bacterial
451 community structure away from that of the control just one day
452 after the stress. Over time, the difference of the bacterial
453 community structure between heat stressed and unstressed
454 was smaller though still significantly (P < 0.05) different (Figure
455 3 and Table S7 and S8).

456 **3.4 Relationships between functional and genetic stability**
457 **to Cu and heat**

458 The relationships between functional and genetic stability
459 varied between soils, stresses and time (Table 4). There was no
460 significant correlation between the stability of C mineralization
461 and 16S rRNA across 5 time points in the Craibstone soil under
462 either Cu or heat stress. However, a significant relationship was
463 detected in the Insch soils after 1, 7 and 28 days following Cu,
464 and after 1 and 14 days following heat stress.

465 When soils were under Cu stress, the stability of ammonia
466 oxidation was significantly positively correlated with *amoA* at
467 day 7 in the Craibstone soil (r =0.615, P < 0.05), whereas no

468 significant relationship was found in the Insch soil over 28 days.
469 There was a significant positive correlation between the
470 stability of ammonia oxidation and *amoA* (r =0.729 $P < 0.01$) in
471 the Craibstone soil after 3 days following heat stress. However,
472 a significant ($P < 0.05$) negative correlation between the
473 stability of ammonia oxidation and *amoA* was found in the Insch
474 soil after 14- and 28-days following heat.

475 The stability of denitrification was not significantly correlated
476 with *nirS* in the Craibstone soil regardless of stresses, but
477 significantly positively correlated with *nirS* in the Insch soil after
478 14 days following Cu (r =0.631, $P < 0.01$). Significant ($P < 0.05$)
479 positive correlations between the stability of denitrification and
480 the stability of *nirK*, *nosZ-I* and *nosZ-II* were also found in the
481 Craibstone soil after 7 days following Cu. In the Insch soil,
482 denitrification was significantly ($P < 0.05$) positively correlated
483 with *nirK* and *nosZ-I* after 1 day following Cu. After one day
484 following heat stress, the stability of denitrification was
485 significantly positively correlated with *nosZ-II* in the Craibstone
486 soil (r =0.710, $P < 0.001$), and *nirK* in the Insch soil (r =0.578, $P <$
487 0.05).

4. Discussion

4.1 The resistance and resilience of soil functions to Cu and heat

We verified our hypothesis that C mineralization will be more stable than denitrification and ammonia oxidation under Cu stress (Figure 1). However, this pattern became less significant after heat stress was imposed on soil. The results challenge previous conclusions that processes carried out by the general microbial community (e.g. C mineralization) are more stable than those carried out by narrower and specialised microbial populations (e.g. ammonia oxidation) [18,25]. Differences of functional resistance and resilience varied with the nature of the imposed stress. Heat, a transient stress is relatively discrete and intense having the potential to shift resource supplies and alter biomass, whereas Cu as a persistent stress is continuous and likely to increase in intensity through time [2]. It is likely that the soil microbial communities respond differently to these contrasting stresses (discussed below).

4.2 Resistance and resilience of microbial communities to Cu and heat stress

Compared to the unstressed control, the bacterial community structure shifted significantly ($P < 0.05$) just one day after heat, although the copy number of 16S rRNA remained relatively stable (Figure 2 and 3). Similarly, distinct responses of genetic structure and abundance of microbial communities to warming (5 °C above ambient) have been observed previously [35]. Microorganisms have evolved appropriate life-strategies to survive in stressful situations. For example, *Actinobacteria* increase their synthesis of ribosomes when stressed by desiccation, and then grow quickly as soon as conditions become more favourable for nutrient acquisition [36]. In our two soils, heat tolerant bacteria, such as *Actinobacteria* may have employed this "preparedness strategy" to accelerate their cell division and increase their biomass [36]. The competition between tolerant and sensitive species following a stress would allow the remaining tolerant species to be detected in higher densities in the absence of their competitors [37], resulting in an altered community structure. After 28 days, the difference between heat- and un- stressed soil was smaller (Table S7 and S8) which is in accordance with heat being an abrupt, intense but short-term stress. Removing heat would have given time for the microbial communities to resuscitate and reconstruct to another stable state.

In contrast, the bacteria community structure remained unchanged until 14 days after Cu addition in both soils (Figure 3), complying with the nature of Cu as a gradual but persistent stress [2]. Bacterial communities can adapt to Cu stress by gathering the required resistance genes through horizontal gene transfer, mainly mediated by plasmids or transposons [38]. However, the transfer of genetic information and expression is energy-consuming [38]. Lack of C and energy supplies possibly caused restricted recovery of bacterial from Cu in these two soils (Figure 3 and Table S7 and S8). The response of the bacterial community structure showed a strong time-dependence (Figure 3 and Table S7 and S8) that could have resulted from Cu diffusion through soil pores and aggregates and microbial evolutionary adaptation allowing Cu tolerant bacteria to prevail in the continuous stressful condition [39]. Considering this incubation experiment lasted only 28 days it may not have been possible to detect the recovery of bacterial communities following the decrease of Cu bioavailability which could be resulted from the formation of complexes with inorganic or organic ligands or association with mineral colloids [40].

552 Denitrifying genes were not completely resistant to heat but a
553 significant recovery in gene copy number was observed after 28
554 days following heat (Figure 2), in agreement with a previous
555 study demonstrating a low resistance but high resilience of
556 denitrifying genes to drought [41]. A significant recovery
557 following heat may be a result of a resuscitation of dormant
558 denitrifiers supported by the flush of available C and N after the
559 heat-induced breakdown of links between soil aggregates and
560 organic-mineral components [42]. The recovery of *nosZ*-
561 harbouring denitrifiers (Figure 2) in particular could help the
562 completion of denitrification where N_2 rather than N_2O would
563 be the end product, which could alleviate the excessive
564 emission of N_2O from denitrification after recurring heat waves
565 [15]. The stability to heat varies with genes (Table 2 and S6).
566 Such a different response of denitrifiers to the changes of
567 environment could be due to their phylogenetic and
568 physiological variation [43]. Cu has an extensive involvement in
569 the denitrification pathway, such as a co-factor in Cu-containing
570 nitrite reductase (*nirK*) and nitrous oxide reductase (*nosZ*) [12].
571 Therefore, soils that are Cu deficient could result in incomplete
572 denitrification and a rise in N_2O emission [44]. However, in this
573 study the gene copy number of all measured denitrifying genes
574 were significantly decreased by 1 mg Cu g^{-1}, (Table S4),

575 suggesting this concentration is lethal to denitrifying bacteria.
576 Indeed, the Cu concentration in our study largely exceeds
577 previously reported ideal concentration (150–200 μg g^{-1}) to
578 maximize the activity of *nirS*- and *nosZ*- harbouring bacteria
579 [45].

580 In this study, we mainly focused on AOB because both soils are
581 under management, thus AOB are likely to play a dominant role
582 in ammonia oxidation [13,14]. Moreover, these soils remained
583 slightly acid (pH 4.95 and 5.30 for Craibstone and Insch,
584 respectively) which are within the preferred soil pH range of
585 AOB *Nitrosospira* clusters 2 [46]. We found that AOB were
586 vulnerable to these stresses (Figure 2). High sensitivity of AOB
587 to increasing temperature [41] and Cu contamination [47] were
588 also reported. The bacterial membrane is permeable to ions
589 which increases the contact of AOB to Cu^{2+} through the pore
590 water [47]. Additionally, constructing a Cu-tolerant ammonia
591 oxidizing community requires long-term exposure and highly
592 depends on the native composition [48], thus very little
593 recovery was seen in AOB abundance over the time frame of
594 this study. The detrimental impact on bacterial *amoA* copy
595 number following heat could be a result of the narrow
596 thermodynamic trait in AOB [49].

4.3 Relationships between microbial communities and functions under stress

In contrast to the hypothesis that functional stability was positively correlated to the underlying genetic stability, the relationship between functional and genetic stability was not constant and varied between soils, stresses and time (Table 4). In the Craibstone soil, the stability of C mineralization was not significantly correlated with 16S rRNA under either stress. This is consistent with previous studies that have showed no significant relationship between bacterial diversity and microbial respiration under the stress of a mixture of heavy metals and the herbicide Siduron [19] and no significant relationship was found between CO_2 fluxes and bacterial abundance under a 5.2 °C warmer condition [20]. However, a significant correlation between C mineralization and 16S rRNA under either stress in the Insch soil was observed (Table 4), suggesting that soil native bacterial composition (Figure S1) and physicochemical properties (Table 1) could play an important role in determining this relationship. AOB have been reported to play a dominant role in controlling ammonia oxidation in a N-rich grassland soil and an arable soil [13,14]. However, in our two managed soils ammonia oxidation was not consistently

correlated with the stability of bacterial *amoA* (Table 4). Our result aligns with a recent study which found no significant correlation between nitrification and the abundance of any nitrifier group (AOB, AOA and nitrite oxidizers) [49]. The decoupling between the response of ammonia oxidation and bacterial *amoA* to the stresses could also reflect that not all the qPCR-quantified *amoA* are active [50]. We acknowledge that AOA could also contribute to ammonia oxidation in a stressed environment. Therefore, we suggest future studies on the responses of AOA, AOB and their transcriptional activities will help to explain the response of ammonia oxidation to contrasting stress. Denitrification was not correlated with any of these measured denitrifying genes constantly over 28 days, which could be a result of competition within functionally similar taxa for the same niche, such as the competition between *nirS* and *nirK*, and between *nosZ-I* and *nosZ-II* [51].

We offer the following explanations for the lack of constant relationship between functional and genetic stability over time. Firstly, soil native microbial composition (Figure S1) and physicochemical properties (Table 1) could determine the direction of the relationship between functions and genetic abundance as two soils exhibited contrasting relationship

641 between functional and genetic stability (Table 4). Secondly,
642 applying stress had significant effects on shifting microbial
643 communities (Table 3) which varied between transient and
644 persistent stresses (Figure 3). Thus, the nature of the stress
645 could produce an increasingly strong deterministic selection on
646 microbial assemblage towards a stable successional stage [52].
647 This could explain why microbial communities responded
648 distinctly to Cu and heat (Figure 3). Thirdly, microbial growth
649 and substrate utilization are phylogenetically deeply conserved.
650 Low microbial abundance may not necessarily lead to low
651 substrate utilization because of high overlap in substrate
652 utilization among species [53]. In our study C mineralization,
653 ammonia oxidation and denitrification were determined as
654 microbial potential usages of certain added substrates. Lastly,
655 the strategies for microbes to survive under unfavourable
656 conditions could vary, such as shifting from an active to a
657 dormant state and reversible transition between “non-growing”
658 and “growing” status [54]. These transitions could reverse
659 because intensity and persistence of the imposed stress could
660 vary over time. However, the DNA captured in our study cannot
661 discriminate between metabolically active and inactive bacteria,
662 especially the inactive microbes that could compose a large
663 fraction of the extracted DNA [54]. Testing this hypothesis

664 requires further studies combining both RNA and DNA based
665 microbial fingerprinting technologies to detect the changes of
666 total and active microbial communities [50].

667 Interestingly, the resistance and resilience of C mineralization
668 to Cu were both higher than to heat, which is in accordance with
669 the response of the bacterial community structure to these
670 stresses (Figure 1 and 3). This indicates that changes in the
671 bacterial community structure may play a more important role
672 than bacterial abundance in conferring the resistance and
673 resilience of C mineralization to Cu and heat. For instance,
674 Naveed et al. (2014) found that *Acidobacteria* were tolerant
675 while *Proteobacteria* were vulnerable to a gradient of Cu
676 contamination [26]. Our two soils are slightly acidic so they may
677 favour the growth of *Acidobacteria*. Thus, the increases in the
678 proportion of *Acidobacteria* to *Proteobacteria* may enhance the
679 resistance and resilience of C mineralization to Cu without
680 altering total bacterial abundance. During microbial succession,
681 the presence of rare species can have a preponderant role in
682 determining functional resistance and resilience [55]. Griffiths
683 et al. (2004) [56] used progressive and irradiated-reinoculated
684 methods to manipulate the microbial community, finding that
685 the stability of decomposition of plant residues to Cu and heat

686 was not consistent between these two methods of reducing
687 microbial diversity. This suggests that future studies applying
688 DNA-sequencing techniques could explain to which extent rare
689 taxa play in conferring functional stability under environmental
690 change.

691 **Conclusion**

692 Relatively higher resistance of C mineralization to Cu than to
693 heat was related to the bacterial community structure rather
694 than the copy number of 16S rRNA, implying rare taxa may play
695 a paramount role in developing stability in the microbial
696 community and securing functional resistance and resilience.
697 The functional genes we targeted here (i.e., *amoA*, *nirK*, *nirS*,
698 *nosZ-I* and *nosZ-II*) were not resistant to either Cu or heat.
699 However, there was a significant recovery of denitrifying genes'
700 copy number after heat. The degree of recovery from heat may
701 vary from genes encoding nitrite reductase and nitrous oxide
702 reductase which could alter the production ratio of N_2O to N_2,
703 and thus contribute to lowering net emission of N_2O under
704 warmer climate and during heat waves. The relationship
705 between functional and genetic stability was not constant,
706 which could be a result of interaction of soil physicochemical
707 properties, the nature of stressor and microbial life history

strategy. C mineralization was found to be significantly more resistant and resilient to Cu than ammonia oxidation and denitrification. However, this difference was not significant when soil was under heat stress. This challenges previous findings that a broad function such as C mineralization is more stable than narrower functions involved in N cycling under various stressors. Thus, our study suggests that functional resilience is tightly stress-dependent which is not merely reflected by the diversity of microbial communities carrying out the functions. Such differences in stress responses of the soil C and N processes and their underlying microbial communities contribute to understanding of soil buffering capacities against environmental challenges.

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Table 1 Soil physicochemical properties. Mean (n = 4) ± standard deviation. Values in columns sharing the same letter do not differ significantly ($P < 0.05$) as determined by the post hoc Tukey's test.

Soil	Classification	Sand	Silt	Clay	pH	DOC	NO_3^{-1}	NH_4^+	MBC	TN	TC
Craibstone	Humic Podzol	58±5a	23±2a	19±2a	4.95±0.06b	970.21±76.82b	39.57±0.81b	0.47±0.04b	116.61±26.75b	0.30±0.00b	4.07±0.04b
Insch	Dystric Cambisol	54±4a	24±3a	22±3a	5.30±0.00a	457.64±13.72a	23.90±0.17a	0.37±0.04a	34.93±6.37a	0.21±0.00a	2.25±0.04a

Note: TN- total nitrogen (%); SOC- soil organic carbon (%); MBC- microbial biomass carbon (µg C soil g^{-1}); pH was measured by 0.01 M $CaCl_2$ extraction; NH_4^+- the amount of ammonia N (µg N soil g^{-1}); NO_3^-- the total concentration of nitrate (µg N soil g^{-1}); DOC- dissolved organic carbon (µg C soil g^{-1}).

Table 2 Two-way ANOVA significance testing of the functional and genetic stability to Cu and heat for the Craibstone and the Insch soil. DF is the degree of freedom (n = 4). *, **, * following F value represents significance at 0.05, 0.01, and 0.001 level.**

				Craibstone				
	Functional stability to Cu				**Functional stability to Heat**			
	DF	Sum of Squares	Mean Square	F value	DF	Sum of Squares	Mean Square	F value
Functions	2	108732	54366	293.54***	2	28190	14095	110.03***
Day	4	4475	1119	6.04***	4	24984	6246	48.76***
Functions*Day	8	19725	2466	13.31***	8	17153	2144	16.74***
	Genetic stability to Cu				**Genetic stability to Heat**			
	DF	Sum of Square	Mean Square	F value	DF	Sum of Square	Mean Square	F value
Genes	5	29498	5900	3.86**	5	615329	123066	50.52***
Day	4	51865	12966	8.48***	4	164285	41071	16.86***
Genes*Day	20	103869	5193	3.40***	20	296361	14818	6.08***
				Insch				
	Functional stability to Cu				**Functional stability to Heat**			
	DF	Sum of Squares	Mean Square	F value	DF	Sum of Squares	Mean Square	F value
Functions	2	35553	17777	94.32***	2	7669	3834	2.17
Day	4	8390	2098	11.13***	4	135531	33883	19.13***
Functions*Day	8	19343	2418	12.83***	8	49594	6199	3.50***
	Genetic stability to Cu				**Genetic stability to Heat**			
	DF	Sum of Squares	Mean Square	F value	DF	Sum of Squares	Mean Square	F value
Genes	5	95872	19174	7.48***	5	146640	29328	10.38***
Day	4	75053	18763	7.32***	4	210027	52507	18.58***
Genes*Day	20	82552	4128	1.61*	20	211266	10563	3.74***

Table 3 Two-way PERMANOVA on Bray-Curtis distance of Hellinger transformed T-RFLP 16S rRNA in each soil. DF is the degree of freedom (n = 4).

Craibstone						
Parameter	DF	Sum of Squares	Mean Square	F value	R^2	P value
Stress	2	4.266	2.133	59.522	0.425	< 0.001
Day	4	1.378	0.344	9.611	0.137	< 0.001
Stress*Day	8	2.770	0.346	9.662	0.276	< 0.001
Insch						
Parameter	DF	Sum of Squares	Mean Square	F value	R^2	P value
Stress	2	1.973	0.986	31.156	0.308	< 0.001
Day	4	0.769	0.192	6.070	0.120	< 0.001
Stress*Day	8	2.242	0.280	8.852	0.350	< 0.001

Table 4 Pearson correlations between stability of different indicators to Cu and heat at five time points after stress in the Craibstone and Insch soil, respectively (n = 4). *, ** and * means significant correlation at *P* < 0.05, 0.01, and 0.001.**

Correlation in the Craibstone soil	**Cu**					**Heat**				
	Day1	Day3	Day7	Day14	Day28	Day1	Day3	Day7	Day14	Day28
C mineralization ~ 16S rRNA	-0.218	-0.325	0.255	0.189	-0.141	-0.179	-0.031	-0.255	0.430	0.287
Ammonia oxidation ~ amoA	0.382	0.140	0.615*	0.032	-0.388	0.000	0.729**	-0.633**	-0.430	0.080
Denitrification ~ nirS	0.462	-0.357	0.401	0.079	-0.099	0.109	-0.377	-0.254	-0.065	0.042
Denitrification ~ nirK	0.145	0.226	0.553*	0.393	-0.131	0.291	-0.263	-0.478	-0.303	-0.118
Denitrification ~ nosZ-I	-0.174	-0.149	0.782***	0.426	0.140	-0.059	-0.596*	-0.121	-0.565*	0.305
Denitrification ~ nosZ-II	0.239	-0.156	0.547*	0.494	0.098	0.710**	-0.312	-0.372	-0.612*	0.284
Correlation in the Insch soil	**Cu**					**Heat**				
	Day1	Day3	Day7	Day14	Day28	Day1	Day3	Day7	Day14	Day28
C mineralization ~ 16S rRNA	-0.550*	-0.274	0.528*	0.161	-0.700**	-0.599*	0.418	0.041	-0.625*	-0.403
Ammonia oxidation ~ amoA	-0.106	-0.274	0.246	0.412	-0.294	0.000	0.000	0.000	-0.590*	-0.723**
Denitrification ~ nirS	0.300	-0.312	-0.196	0.631**	0.095	0.214	-0.309	-0.265	-0.032	0.037
Denitrification ~ nirK	0.529*	-0.359	-0.434	0.427	0.327	0.578*	-0.260	-0.442	-0.051	0.200
Denitrification ~ nosZ-I	0.779***	-0.507*	-0.330	0.306	-0.194	0.473	-0.580*	-0.599*	0.236	-0.214
Denitrification ~ nosZ-II	0.476	-0.339	-0.373	0.492	0.235	0.199	-0.543*	-0.273	0.493	0.198

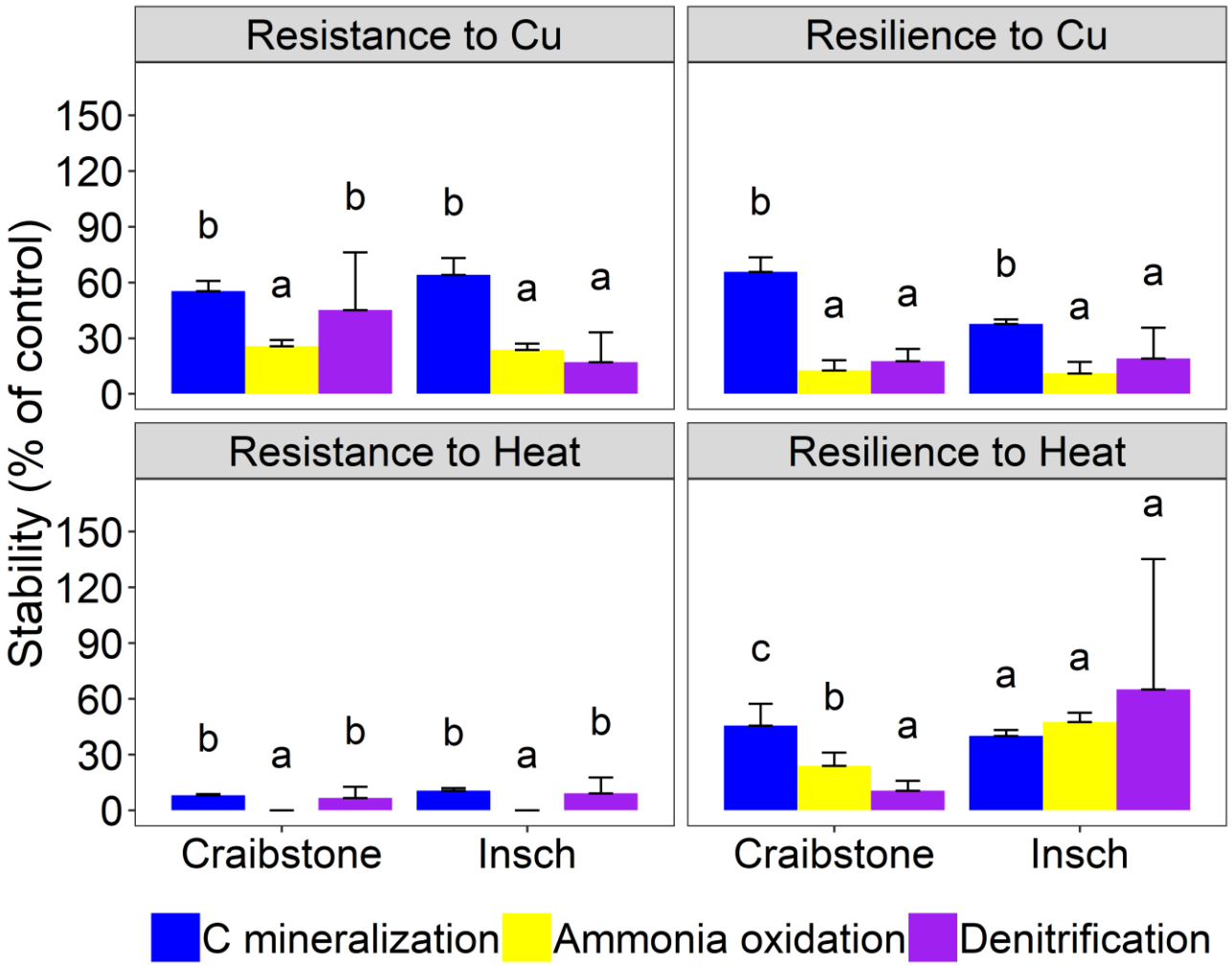

Figure 1 **Resistance and resilience of C mineralization, ammonia oxidation and denitrification to Cu and heat in the Craibstone and the Insch soil. Means (n = 4) and standard deviation (error bar). Significant (*P* <0.05) differences are indicated by different lowercase letters within the same resistance or resilience in the same soil.**

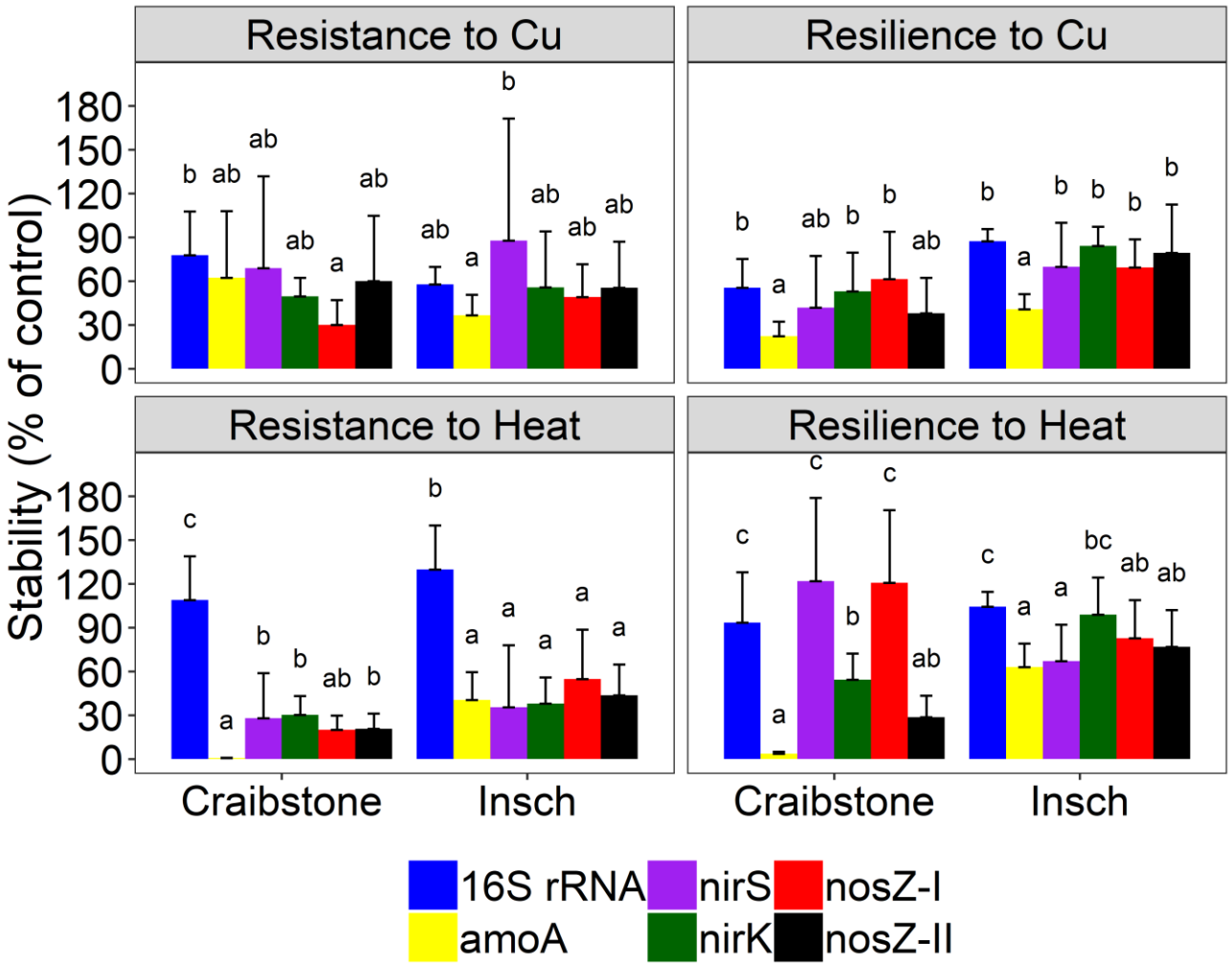

Figure 2 **Resistance and resilience of gene abundance to Cu and heat in the Craibstone and the Insch soil. Means (n = 4) and standard deviation (error bar). Significant ($P < 0.05$) differences are indicated by different lowercase letters within the same resistance or resilience in the same soil.**

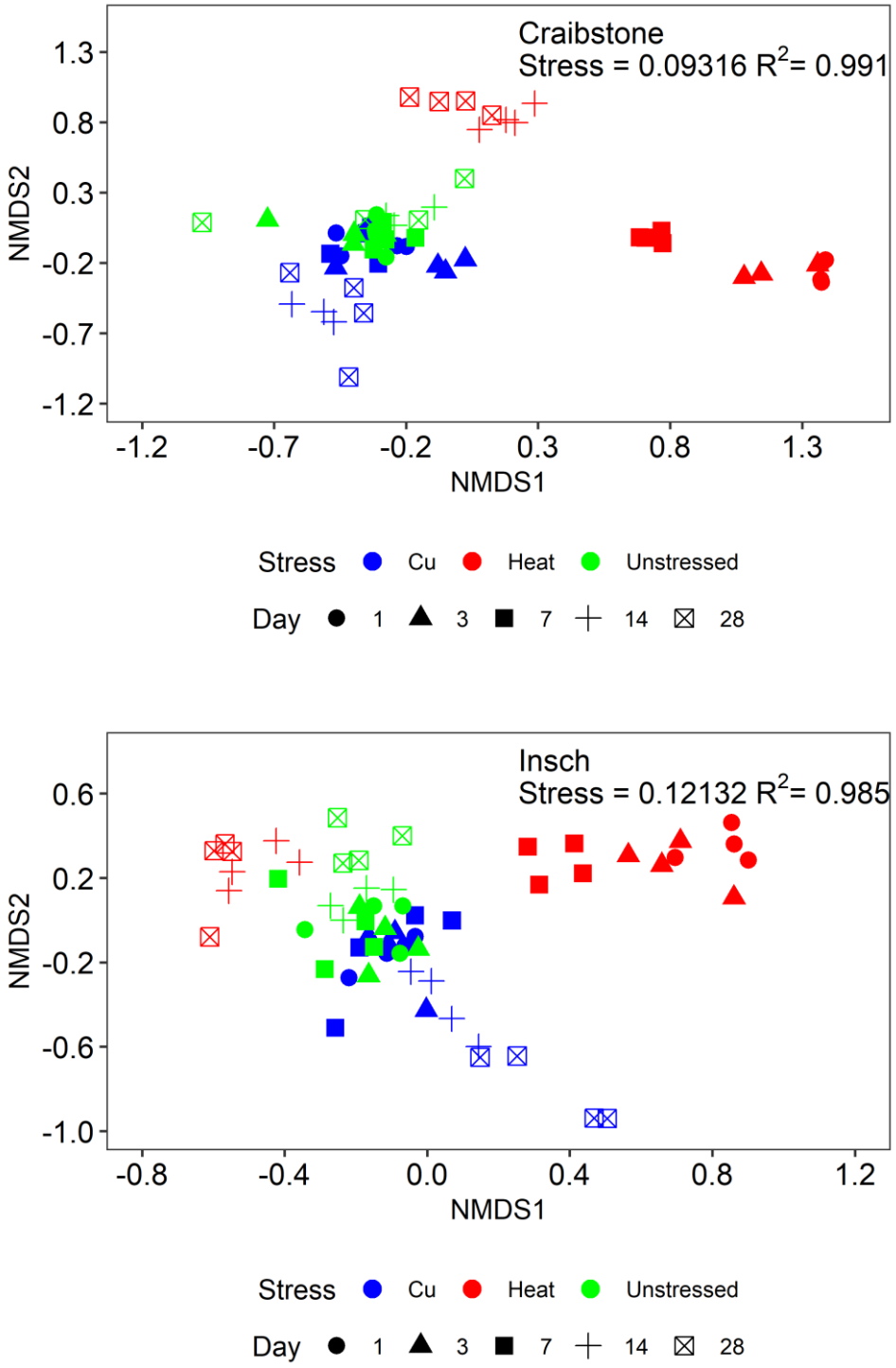

Figure 3 **The bacterial community structure of two soils at 1, 3, 7, 14 and 28 days after being stressed with either Cu or heat. For each soil, individual non-metric multidimensional scaling (NMDS) was measured based on Bray-Curtis distance analysis of Hellinger transformed 16S rRNA T-RFLP data (n = 4). Different symbols represent samples at different time and under different stresses.**