1 **Influence of increasing soil copper concentration on the susceptibility**
2 **of phosphomonoesterase and urease to heat disturbance**

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24

25 **Abstract**

26 Long-term exposure to elevated copper (Cu) concentrations may affect the ability of
27 soil microbes to withstand additional transient disturbances, such as heat. Bulk surface
28 soil samples collected from three south east Australian locations were spiked with a
29 series of Cu concentrations ranging from 0 to 1000 mg/kg. To determine the effect of
30 increasing soil Cu concentrations on phosphomonoesterase and urease activity
31 following a simulated heat disturbance, aliquots of each of the Cu treated soils were
32 exposed to 60°C for 24 hours (perturbed group), and phosphomonoesterase and urease
33 activity measured after 1, 2 and 7 days. Without heat disturbance, the Cu
34 concentration causing significant inhibition of enzyme activity ranged from 50 to >
35 1000 mg/kg added Cu for phosphomonoesterase activity and 500 to > 1000 mg/kg
36 added Cu for urease activity. The Cu pre-exposure concentration increased the
37 susceptibility of phosphomonoesterase activity but not urease activity to the heat
38 disturbance. However, this did not follow a clear dose response relationship and the
39 effects were not lasting. The response differed according to soil type, with decreased
40 resistance to heat found at concentrations ranging from 100 mg/kg added Cu in the
41 silty loam soil to > 1000 mg/kg added Cu in the clay loam soil. The results from this
42 study suggest that, at the concentrations of Cu typically reported in agricultural soils,
43 Cu stress is unlikely to cause lasting alterations to the susceptibility of soil enzymes to
44 a single moderate heat disturbance.

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48 **Key words:** Copper; Soil; Enzyme activity; Functional stability; Resilience; Heat

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52 **Introduction**

53 Agricultural ecosystems rely on soil microbial communities for key soil functions
54 responsible for maintaining long-term soil fertility (e.g. nutrient cycling and litter
55 decomposition). Soil microbial communities in agro-ecosystems are subjected to
56 many short-term disturbances, such as wetting-drying cycles, cultivation, and drastic
57 changes in temperature resulting from fires, as well as longer-term stresses, such as
58 persistent pesticide and metal contamination. Healthy soil microbial communities
59 should be functionally stable and thus be able to withstand and recover from the short-
60 term disturbances (such as a heat wave) (Brussaard et al. 2007). On the other hand
61 Longer term stressors (such as contamination) may have adverse effects on the
62 fitness of soil microbial communities and reduce their ability to withstand (show
63 resistance) and recover (show resilience) from short-term stressors caused by
64 agricultural practices and climatic conditions (Brussaard et al. 2007).

65
66 Copper based fungicides have been used in vineyards throughout the world for many
67 decades to prevent downy mildew (*Plasmopara viticola*) outbreaks which can occur
68 under warm humid conditions following rain. For example, in Victoria (Australia)
69 90% of grape growers apply copper based fungicides, with annual rates ranging from
70 0 to 35 kg/ha (Pietrazk and McPhail 2004). Historically copper fungicides have been
71 applied as a mixture of copper sulphate and lime ($\text{CuSO}_4 \cdot 3\text{Cu}(\text{OH})_2 \cdot 3\text{CaSO}_4$)
72 (Bordeaux Mixture), however now copper oxychloride ($\text{CuCl}_2 \cdot 3\text{Cu}(\text{OH})_2$) and copper
73 hydroxide ($\text{Cu}(\text{OH})_2$) are the main forms applied (Pietrazk and McPhail 2004).
74 Although these copper-based fungicides are effective for disease control in vineyards,
75 their use can result in an accumulation of copper (Cu) in surface soils (Wightwick et
76 al. 2008). The concentration of Cu reported to cause a 50% effect (reduction) (EC_{50})
77 on substrate induced respiration (SIR) is variable, ranging from 316 to 1585 mg Cu
78 (total) /kg soil (Broos et al. 2007), but comparable with Cu concentrations in
79 European vineyard soils with a history of copper based fungicide use (130 to 1280
80 mg/kg; Komárek et al. 2010; Wightwick et al. 2008). Similar increases have occurred
81 in stone fruit, pome fruit, and citrus orchard soils where copper-based fungicides have
82 been used (110 – 1500 mg/kgCu; Tiller et al. 1981; Merrington et al. 2002). Natural
83 background copper concentrations in soils are typically less than ~20 mg/kgCu, but
84 may be as high as 100 mg/kg depending on the parent soil material (NEPC 1999;
85 Sauve 2002).

86
87 In recent years, several studies have investigated whether exposure of soil microbial
88 communities to persistent Cu contamination induces a decreased ability to withstand
89 additional stressors/disturbances, such as heat, wet-drying cycles, salinity, other
90 toxicants, with varying results (Brandt et al. 2010; Deng et al. 2009; Dussault et al.
91 2008; Philippot et al. 2008; Tobor-Kaplon et al. 2006; Tobor-Kaplon et al. 2005). One
92 theory is that microbes exposed to a persistent stress, such as Cu, will be more
93 susceptible to an additional stress (Brussaard et al. 2007). This hypothesis is
94 supported by the work of Tobor-Kaplon et al. (2005) who found that microbial
95 activity in Cu contaminated soils showed lower stability when exposed to increasing
96 lead and salt compared with uncontaminated soils. Dussault et al. (2008) also reported
97 that the normal activity pattern of protease activity following heat stress was altered in
98 Cu contaminated soils compared with 'uncontaminated' soils. An alternative
99 hypothesis is that soil microbes exposed to a persistent stress are more stable and
100 exhibit greater resilience to additional stress due to the development and dominance
101 of tolerant organisms (Brussaard et al. 2007). For example, Philippot et al. (2008)
102 found that microbial activity in Cu contaminated soil exposed to a secondary mercury
103 stress recovered more rapidly than a soil without pre-exposure to Cu. One reason for
104 the conflicting results may be related to the severity of the pre-exposure to Cu. For
105 instance, where recovery from the additional stress was reported, microbial
106 communities were pre-exposed to 50 to 82 mg/kg total Cu (Brandt et al. 2010;
107 Philippot et al. 2008). Whereas, where adverse effects on the ability for the microbial
108 communities to recover from the additional stress were observed the pre-exposure
109 concentration was higher (114 to 250 mg/kg total Cu) (Dussault et al. 2008; Tobor-
110 Kaplon et al. 2005). In addition to the extent of Cu stress, differences in soil
111 characteristics may also influence the ability for microbial communities to withstand
112 combined Cu and other stressors. For example, it has been reported that the ability for
113 soil microbes to withstand stressors increases with increasing soil organic matter
114 content as these soils are able to support larger more diverse microbial communities
115 (Girvan et al. 2005; Gregory et al. 2009). However, to date detailed studies have yet to
116 adequately evaluate the effect of varying Cu pre-exposure concentrations on the
117 ability for soil microbes to withstand secondary stressors within common soils.

118

During the first ten years of this century, south eastern Australia endured one of its longest running periods of below average rainfall and above average temperatures on record. For instance, by the final week of January 2009, the state of Victoria had endured one of the most severe and prolonged heatwaves on record, with the temperature in Melbourne, the state's capital, above 43°C in the shade for three consecutive days for the first time since records began. By 2030, annual average temperatures in Victoria are expected to increase by around 0.6 to 1.2°C. Warming is likely to be greatest in spring and summer, with the number of hot days (>35°C in the shade) increasing in both frequency and intensity (DSE 2008). This in turn increases the risk of wild fires in both natural and agro-ecosystems, risks that are often controlled by pre-emptive use of deliberately lit, low intensity fires (controlled burns). The objective of this study was to determine whether a transient heat disturbance in the presence of a persistent stressor (increased soil Cu concentrations) has adverse effects on the ability of soil microbial activity. More specifically, the relative susceptibility of two soil enzyme activities, phosphomonoesterase (phosphorous cycling) and urease (nitrogen cycling) to persistent Cu stress and heat disturbance was tested for three distinctly different soils. These enzymes were chosen as they are indicators of two nutrient cycles of importance to the fertility of agricultural soils.

137

138 **Materials and methods**

139 *Collection of bulk soil samples*

A bulk surface soil sample (0 – 5 cm depth) was collected from an uncontaminated reference location adjacent to farming land in each of the Grampians (Soil A) (37°9' S, 142°51' E), Murray Valley (Soil B) (34°19' S, 142°11' E), and south-east, South Australia (Soil C) (37°18' S, 140°49' E) viticultural regions of Australia. The collection sites were selected to represent soils with different physical-chemical properties and from different climates (Table 1). The soil samples were then stored in plastic buckets at room temperature (< 20°C) until further processing. Following collection, the soil samples were passed through a 2 mm sieve and mixed to homogenise each sample. Physical-chemical properties of the three bulk soil samples are shown in Table 1. Soil pH and electrical conductivity (mS/cm) were determined in a 1:5 soil/water suspension (Rayment and Higginson, 1992). The total organic carbon (TOC) and nitrogen (TN) content (%) of the soil samples was measured with a LECO CNS element analyser. Samples for TOC were treated with sulfurous acid prior to

analysis to remove any inorganic carbon present (Nelson and Sommers 1996). Cation exchange capacity (CEC) (cmol/kg) was determined by extraction with 1 M ammonium acetate (pH 7.0) with pre-treatment to remove soluble salts (Sumner and Miller 1996). The proportion of sand, silt and clay was determined using the micropipette method with sample pre-treatment to remove carbonates, soluble salts and organic matter (Gee and Bauder 1986; Miller and Miller 1987).

Copper spiking and aging of soils

Each of the three bulk soils were spiked with copper (II) hydroxide (stabilised tech; Sigma-Aldrich, St Louis, MO, USA) to achieve final targeted added Cu concentrations of 0 (unspiked control), 50, 100, 200, 500, and 1000 mg/kg, such that tested concentrations were representative of the increase in soil Cu concentrations observed in Australian vineyard soils (Wightwick et al. 2008) and the concentration of Cu reported to cause significant effects on substrate induced respiration (SIR) (Broos et al. 2007). The soil for each treatment was spiked by weighing 300 g (dry weight) soil into a 2 L plastic bucket and adding ultra pure water (Millipore, Molsheim, France) to bring the soil moisture up to 40 % of the maximum water holding capacity (WHC). Copper hydroxide has low solubility in water (2.9 mg/L, pH 7, 25°C) making Cu addition as a solution impractical. Therefore the required small amount of dry copper hydroxide was first mixed with 10 g of fine dry washed quartz sand before being thoroughly mixed with the pre-moistened soil (OECD 2000). Spiking the soils at six Cu concentrations for each of the three soil types produced 18 bulk soils (Cu treatments) for testing. It has been reported that the response of soil microbes to increasing Cu concentration differs between freshly spiked soils and field contaminated soils (Oorts et al. 2006). Therefore, following spiking with Cu the bulk treatment soils were subjected to a series of wetting and drying cycles over a period of 90 days to simulate field aging of the Cu contamination. This was also to allow time for the soil microbial communities to adapt to the increased Cu conditions thus more closely representing field aged soils.

The aging process applied was based on the methodology used by Chaperon and Sauve (2007). Following spiking with Cu, the bulk test soils were stored in sealed containers at 20 - 22°C for three days to equilibrate. Ultra pure water was then added to the test soils to bring them up to 90 % of their maximum WHC. These test soils

were then stirred and allowed to air-dry at room temperature (20 - 22°C) in unsealed containers for approximately 30 days. The wetting and drying cycle was repeated a further three times. On one hand, wetting-drying brings a condition that more resembles field aged soils, but on the other hand can have unpredictable effects on the behaviour of soil enzymatic activities (Rao et al. 2003). Arguably, air-drying and concomitant rewetting, especially when done several times, can be a stress as severe as the other two studied herein. Although this complicates the work, the wetting-drying stress is accounted for in this ecotoxicological experiment because all exposure data is compared back to control soils which have received the same wetting-drying cycles.

After the final drying cycle each bulk test soil (Cu treatment) was ground with a mortar and pestle to break up the aggregates, passed through a 2 mm stainless steel sieve, and well mixed before being loosely packed into PVC tubes (12 cm long with 38 mm internal diameter) with one end covered with a square of permeable landscaping fabric (Weedgard™, Gardman Ltd, UK) (approx 15 cm x 15 cm) secured using a rubber band. The filled tubes were then supported upright within plastic trays (40 cm x 30 cm) which contained a bed of washed sand. A volume of ultra pure water sufficient to wet the soils to 110 % of the maximum WHC was added to leach out excess salts and unbound Cu ions to more closely represent a field weathered soil. The test soils were spiked with concentrations of Cu 25 % greater than the target concentrations to allow for expected losses due to this leaching process, as has been reported by others (Chaperon and Sauve 2007). The test soils were then allowed to incubate for at least two weeks at 20-22°C and maintained at 60 % maximum WHC prior to being used in the experiments. Care was taken to ensure that all treatments and controls were subjected to the same ‘aging’ processes.

Heat disturbance experiment

The heat disturbance experiment was based on the methodology described by Dussault et al. (2008). For each bulk test soil, 18 sub-samples (15 – 20 g) were transferred into 65 ml amber glass jars. In this way, 324 sub-samples were created for testing with each Cu treatment replicated 18 times (i.e. three soils × six Cu treatments × 18 sub-samples). For each Cu treatment, the 18 replicates were further assigned to one of two groups: perturbed (the nine sub-samples to be exposed to the heat

disturbance) and unperturbed (the remaining nine sub-samples). Samples in the perturbed and unperturbed groups were then further randomly assigned such that three separate samples could be collected on each of three sampling days (days 1, 2 and 7) post disturbance for each group.

Samples in the unperturbed treatment group were stored at 20 – 22°C for 24 hours. Samples in the perturbed group were exposed to a heat disturbance by placing them in an oven at 60°C for 24 hours. This temperature can easily be reached in topsoils exposed to a light grass fire, and represents the temperature at which most proteins will be denatured (DPI 2011). Following the heat disturbance the perturbed soil samples were allowed to cool to 20 – 22°C. Thereafter, all samples were re-adjusted to 60 % of the maximum WHC and maintained at 20 - 22°C for the remainder of the experiment. Three samples of perturbed and unperturbed soils were collected from each of the 18 Cu treatments sampled on day 1 (immediately after disturbance), day 2 and day 7 after disturbance. Each of these samples was measured for microbial activity by determining the activity of the phosphomonoesterase and urease enzymes.

Concentrations of Cu in spiked soils

For total Cu determination, bulk tests soils (including the unspiked control soils) were digested using a microwave assisted aqua-regia method (closed-vessel) using 0.5 g of dried soil (< 2 mm) and 5 mL of reverse aqua regia (3HNO₃ : 1HCl) according to the method of Zarcinas et al (1996). Sample extracts were analysed by inductively coupled plasma emission spectrometry (ICP-ES) to quantify the total concentration (mg/kg) of Cu in the soil. Soluble Cu was extracted from the soil samples based on the 1:10 0.01 M CaCl₂ extraction method using 4 g dry soil and 40 ml of 0.01 M CaCl₂ (Wightwick et al. 2010b). Sample extracts were analysed by ICP-ES to quantify the concentration of 0.01 M CaCl₂ extractable (mg/kg) Cu in the soil. For both the total and CaCl₂ extractable Cu determinations, the standard reference soil ISE 967 (Wageningen Evaluating Programs for Analytical Laboratories (WEPAL), Wageningen University, Netherlands) and two randomly selected samples were processed in duplicate. The recovery of Cu from the standard reference soil was 103 % and 113 % of the stated WEPAL value for total Cu and 102 % and 104 % of the values published by Wightwick et al. (2010b) for CaCl₂ extractable Cu. The

coefficient of variation (CV %) for the duplicate samples was 6 and 16 for total Cu and 1 and 5 for CaCl₂ extractable Cu.

Enzyme activity assays

Enzyme activity tests were conducted on perturbed and unperturbed soils based on published methods (Sinsabaugh et al. 2000; Tabatabai 1994), the main exception being that the assays were conducted without the use of pH buffers. The latter was done to provide a measure of ‘actual’ enzyme activity so as to investigate the microbial function of the soils in their ‘natural state’. The choice was made as the alteration of pH through the use of buffers can create an unnatural soil solution environment. Firstly, pH influences the solubility and composition of organic matter in soil which is of significance as it is known that enzymes form complexes with organic matter which can enhance their stability (Speir and Ross 2002; Tabatabai and Dick 2002). Secondly, pH influences the speciation of Cu (i.e. free Cu²⁺ activity) in soil solution and therefore may alter the inhibitory effects of Cu on the enzyme/s. The buffer constituents can also act as ligands to reduce Cu availability (Dussault et al. 2008).

The phosphomonoesterase (E.C 3.1.3) assay, which is based on the use of p-nitrophenylphosphate as the substrate, as described in Tabatabai (1994) was used with several modifications. For instance the assays were conducted using 15 mL plastic centrifuge tubes with the contents mixed using a vortex mixer for 10 – 15 seconds. In addition, 4 mL of ultra pure water was added to 1g soil (dry weight equivalent) instead of the buffer solution, so that the enzyme activities were determined at natural soil pH. Samples were incubated at 37°C for 1h after which time 1m 0.5M CaCl₂ and 4ml of 0.5M NaOH were added. Colour development in the samples was determined by transferring 300 µL aliquots of clear supernatant into 96-well microplates and measuring absorbance at 400 nm using a microplate reader (UVM340, ASYS Hitech GmbH, Nordstrasse, Eugendorf). Enzyme activity was expressed as µg p-nitrophenol/g dry soil/h, adjusted for the background absorbance of a negative control sample (Tabatabai 1994).

286 The urease (EC 3.5.1.5) assay was based on the methods described by Tabatabai
 287 (1994) and Sinsabaugh et al. (2000). Nine ml of ultra pure water and 1 mL of 200 mM
 288 urea (99.0 – 100.5%, Sigma-Aldrich) were added to 1 g (dry weight equivalent) of
 289 soil in a 15 mL centrifuge tube and mixed using a vortex mixer for 10 - 15 seconds.
 290 The samples were incubated for 24 hours at 20 - 22°C and then centrifuged to stop the
 291 reaction. Duplicate 200 µL aliquots of clear supernatant were then transferred into a
 292 96-well microplate. The concentration of ammonium released was measured
 293 colorimetrically using the salicylate-cyanurate method (Reardon et al. 1966;
 294 Sinsabaugh et al. 2000) and measuring absorbance at 610nm using a microplate reader
 295 (Sinsabaugh et al. 2000). This method is not sensitive to Cu interference (Pym and
 296 Milham 1976). Urease activity was expressed as µg/NH₄ produced/g dry soil/h,
 297 adjusted for absorbance of negative and substrate controls.

298

299 *Calculation of resistance, resilience and functional stability*

300 For each of the 18 Cu treatment soils (i.e. three soils × six spiked Cu concentrations),
 301 the initial resistance of enzyme activity (EA) to the heat disturbance was determined
 302 using the day 1 samples and calculated as the percent change in activity in the heat
 303 perturbed sample compared to the unperturbed sample (i.e. no heat) (Eq 1.). The
 304 resilience of EA to the heat disturbance (i.e. ability to recover) was calculated in the
 305 same manner as using the day 2 and then day 7 samples.

$$306 \text{ Change (\%)} = \frac{(EA(\text{unperturbed}) - EA(\text{perturbed}))}{(EA(\text{unperturbed}))} \times 100 \quad (1)$$

307

308 *Data analysis*

309 Statistical analyses were conducted using GenStat version 12.1.0.3278 (VSN
 310 International Ltd). To determine significant effects of soil type and Cu treatments,
 311 data were analysed by ANOVA and *t*-tests to determine significant differences.
 312 Where data did not conform to assumptions for parametric statistical methodologies
 313 the Kruskal-Wallis one-way ANOVA and Mann-Whitney U tests were used to
 314 determine significant differences. Where necessary the Bonferroni correction was
 315 used to address the problem of a family wise error in multiple comparisons. Unless
 316 otherwise stated, statistical significance for all data analysis was at the 95 %
 317 confidence level ($\alpha = 0.05$).

318

319 **Results**

320 *Effect of Cu on enzyme activities*

321 The measured concentrations of total Cu in the spiked test soils were generally within
322 20 % of the nominal added Cu concentration (Table 2). Represented as a proportion of
323 the total Cu concentration, CaCl₂ extractable Cu in the spiked soils was greater in
324 Soils A (0.33 % $\pm$ 0.11 %) and B (0.34 % $\pm$ 0.11 %) compared to Soil C (0.15 % $\pm$
325 0.05 %; Table 2). The pH of the Cu spiked test soils was within 0.2 pH units of the
326 untreated control soil for each of the three different soils.

327

328 In the day 1 Cu spiked test soils (unperturbed samples), phosphomonoesterase activity
329 in the unspiked control soils was greatest in Soil A, followed by Soil B and Soil C
330 (Table 2). Urease activity was greatest in Soil C, followed by Soil A, and Soil B.
331 Phosphomonoesterase activity in Soil A was stimulated by increasing Cu
332 concentration, with activity in the 500 mg/kg Cu treatment significantly higher than
333 the unspiked control soil. Increasing Cu concentration caused a decrease in
334 phosphomonoesterase activity in both Soil B and C. The magnitude of this adverse
335 effect was greatest for Soil B where activity was significantly lower (60 – 75%
336 inhibition) than the unspiked control for all Cu treatments. For Soil C, activity was
337 significantly lower (30 – 60 % inhibition) than the unspiked control in all but the 50
338 mg/kg Cu treatment. A significant stimulatory effect on urease activity was found at
339 the 200 mg/kg Cu treatment for Soil A, whilst the 1000 mg/kg Cu treatment caused
340 significant inhibition (50 %) compared to the unspiked control. In Soil B, urease
341 activity was significantly lower than the unspiked control soil in the 500 and 1000 Cu
342 treatments (70 – 85 % inhibition). No significant differences were found between the
343 Cu treatments for urease activity in Soil C.

344

345 *Effect of heat on enzyme activities*

346 Whilst there was some variation in enzyme activity for each of the three soils types
347 between days (Fig. 1) this was generally minimal and both the perturbed and
348 unperturbed samples generally followed a similar pattern. For instance,
349 phosphomonoesterase activity reduced in response to the heat perturbation, with each
350 of the three soils responding somewhat differently. For Soil C, phosphomonoesterase

activity was reduced immediately following heat perturbation and this activity did not recover after 7 days. Soil A also did not show recovery after 7 days, although unlike Soil C a significant reduction in phosphomonoesterase was not observed until 2 days after the heat perturbation. Phosphomonoesterase activity in Soil B was relatively stable following the heat perturbation, with similar activities found in both perturbed and unperturbed soils at day 7. Urease activity was relatively unaffected by the heat perturbation with similar activities observed for both perturbed and unperturbed samples (the exception being day 7 for Soil C where the perturbed samples were significantly lower than the unperturbed samples).

Effect of increasing Cu stress on susceptibility of enzyme activities to heat

In Soil A phosphomonoesterase activity in the 100, 500 and 1000 mg/kg Cu treatments were significantly less resistant (day 1 samples) to the heat perturbation than the unspiked control soil (Fig. 2). No such significant differences could be found for resilience at days 2 and 7. In Soil B, phosphomonoesterase activity in the 200 mg/kg treatment was significantly less resistant than the unspiked control soil; similarly all Cu treatments showed decreased resilience at day 2. However it is noted that this day 2 effect was influenced by the fact that, unlike days 1 and 7, enzyme activity in the unspiked control soil did increase following heat perturbation. Therefore this result should be viewed with caution particularly given that the change in phosphomonoesterase activity in the Cu treatment soils following the heat perturbation was relatively small (i.e. generally < 20 %; Fig. 2). In Soil C, resistance and resilience to the heat perturbation for the Cu treatment soils did not differ significantly from the unspiked control soil. This was also the case for urease activity for Soils B and C (Fig. 3), Soil A being the exception where, unlike the rest of the treatment soils, enzyme activity increased in the 1000 mg/kg Cu treatment soil following heat perturbation. However, there was considerable variation between replicates for urease activity (Fig. 3), particularly for Soil B, which influenced our ability to identify the effects of increasing Cu concentration.

Discussion

Effect of Cu on enzyme activities

Increased concentrations of Cu in soil can inhibit the activity of soil enzymes via two main mechanisms. Firstly, by causing a reduction in the microbial biomass in the soil

385 resulting in a decrease in the total amount of enzyme produced. Secondly, by reacting
386 with the enzyme-substrate complex thus reducing the rate of the enzyme mediated
387 process (i.e. non-competitive inhibition; [Speir and Ross 2002](#); [Tabatabai and Dick](#)
388 [2002](#)). The susceptibility of enzyme activities to Cu stress in different soils is
389 expected to be influenced by physico-chemical soil properties (e.g. pH, TOC) due to
390 their role in controlling the size and diversity of microbial communities, the
391 availability of Cu, and the stability of enzymes and in soil ([Tabatabai and Dick 2002](#);
392 [Taylor et al. 2002](#); [Wightwick et al. 2010c](#)). It is generally expected that soils with
393 higher organic matter contents will support larger and more diverse microbial
394 communities and thus be more resilient to stressors than soils with lower organic
395 matter contents ([Girvan et al. 2005](#); [Gregory et al. 2009](#)). This may explain why Cu
396 had a greater inhibitory effect on enzyme activities in the low TOC content Soil B
397 (TOC 0.8%) compared with Soils A and C (TOC > 3.5 %), however there is
398 insufficient data to confirm if such a correlation exists.

399
400 The availability and toxicity of Cu in soil is expected to increase with decreasing pH,
401 TOC, CEC and clay content ([Broos et al. 2007](#); [Kim et al. 2008](#); [Wightwick et al.](#)
402 [2010c](#)). The results for phosphomonoesterase and urease activity appear to agree with
403 this hypothesis in that the inhibition of enzyme activity was more pronounced in soils
404 where CaCl₂ extractable Cu was greater, for example Soil B (0.34 % of total Cu)
405 compared with Soil C (0.15 % of total Cu). It was expected that the enzyme activities
406 would also have been significantly inhibited in Soil A as CaCl₂ extractable Cu (0.33
407 % of total Cu) was similar to that of Soil B. That this, for the most part, was not the
408 case indicates that some other mechanism/s prevented the inhibition of the enzyme
409 activities. The CaCl₂ extraction method provides an indication of the availability of
410 Cu in soil. However it may overestimate biological availability as it measures more
411 than just free Cu ions (i.e. Cu²⁺) which are considered the most biologically available
412 form of Cu in soil ([Peijnenburg et al. 2007](#)). It is therefore possible that the
413 inconsistent result for Soil A may be due to an overestimation of Cu bioavailability,
414 given the relatively high TOC content (5.44 %) of Soil A and that free Cu activity is
415 strongly influenced by organic matter content ([Sauve et al. 1997](#)). Alternatively, it is
416 known that enzymes form complexes with organic matter and clay minerals in soil
417 which can stabilise and protect them from degradation so that they remain active for
418 longer ([Speir and Ross 2002](#); [Tabatabai and Dick 2002](#)). It is therefore possible that

the combination of both the high clay mineral (80% of particles being clay and silt) and TOC (5.44 %) content of Soil A have stabilised the phosphomonoesterase and urease enzymes, thus protecting them from Cu inhibition. This hypothesis also provides another explanation as to why the inhibition of enzyme activities with increasing Cu was greatest in Soil B, as this was a very coarse textured soil (85% sand), low in TOC (0.8 %). However, it is important to highlight that due to the preliminary nature of this study there is insufficient data to confirm the theories around the influence of soil properties. It should also be noted that whilst, for the most part, Cu did not inhibit enzyme activities in Soil A, that enzyme activities were stimulated at elevated Cu concentrations may be an early indicator of adverse effects of Cu to the soil microbial communities, i.e. an increase in soil enzyme activity can occur due to the release of enzymes from dead/dying microbial cells following exposure to a stressor, which remain active in soil for a period time (Tabatabai and Dick 2002).

Effect of heat on enzyme activities

Regardless of the Cu concentration, urease activity did not appear to be directly effected by heat, whereas phosphomonoesterase activity generally decreased and did not recover 7 days following the heat perturbation. Although it is recognised that phosphomonoesterase activity may have shown greater recovery over a longer time period, i.e. > 7 days. This study also indicated that the response of the enzyme activities following the disturbance varied according to soil type, with data indicating that enzyme activities in Soil B showed a greater ability to withstand the heat perturbation compared to the other soils. It might be expected that Soil B would be more susceptible to the heat perturbation due to the inherently low TOC content and therefore likely relatively small microbial biomass (as reflected in the low enzymes activities). For instance, based on a 15 field collected soils, Gregory et al. (2009) found a relationship ($R^2 = 0.42$) between increasing soil organic matter content and increased resilience of soil microbial activity (as indicated by SIR) following heat perturbation. A limitation to the study presented in this manuscript is that the experiments did not include a measure of microbial biomass, thus it is not possible to confirm if the low enzyme activities measured for Soil B were related to the size of the microbial population.

453

454 That the low TOC content Soil B was quite resilient to the heat stress suggests that
455 other factors may be influencing susceptibility to heat perturbation. This may be
456 related to the climatic conditions in the locations from which these soils were
457 collected. For instance, Soil B was collected from a region which experiences a warm
458 semi-arid climate with frequent summer heat waves (i.e. daytime maximum
459 temperatures $> 35^{\circ}\text{C}$) (Table 1), whilst Soil A and Soil C were collected from cooler
460 regions with higher rainfall. Thus, it may be that the soil microbial communities in
461 Soil B were pre-adapted to extreme heat conditions due to living in this hostile
462 environment. Further detailed investigations are needed to test this hypothesis.

463

464 *Effect of increasing Cu stress on susceptibility of enzymes activities to heat*

465 The Cu pre-exposure concentration did not alter the susceptibility of urease to the heat
466 perturbation (although this observation may well be a function of the inconsistency
467 and high variability in the response of urease activity to the heat disturbance). This
468 variable response was exacerbated for Soil B where the inherently low activity (0.07
469 $\mu\text{g}/\text{NH}_4/\text{g}$ dry soil/h) contributed to the inability to obtain reliable measures of urease
470 activity. The results from this study suggest that urease is not a robust measure to
471 assess the combined effect of Cu and heat stress. Data from this study did, however,
472 indicate that Cu pre-exposure concentration altered the susceptibility of
473 phosphomonoesterase to the heat perturbation. However, this did not follow a clear
474 dose response relationship. Rather where adverse effects were shown (Soils A and B)
475 the data indicates that when a threshold was reached a similar, not increasing,
476 response was found at higher Cu concentrations. Further studies are needed to
477 determine if this holds true at high Cu pre-exposure concentrations, albeit the 1000
478 mg/kg Cu treatment is already much higher than the concentrations of Cu typically
479 reported in agricultural soils (Komárek et al. 2010; Wightwick et al. 2010a). Clear
480 effects of altered susceptibility to heat perturbation with increasing Cu concentration
481 were only found for the initial resistance. This suggests that for the studied soils, Cu
482 stress up to 1000 mg/kg does not induce lasting susceptibility to heat disturbance.
483 However, in this study only a single 24 hour heat perturbation was applied, so it is not
484 known whether Cu stress would alter susceptibility to heat over more prolonged
485 and/or repeated heat disturbances.

486
487 The response of phosphomonoesterase to Cu and heat differed according to soil type
488 with decreased resistance to heat found at concentrations ranging from 100 mg/kg Cu
489 in Soil A to > 1000 mg/kg Cu in Soil B. Exposure to Cu did not result in an
490 inhibitory effect on phosphomonoesterase in Soil A, but it did increase the
491 susceptibility to the heat. This supports the theory that exposure to persistent stressors
492 can make them more vulnerable to additional stressors (Brussaard et al. 2007), as has
493 been reported by others (Dussault et al. 2008; Tobor-Kaplon et al. 2005). The
494 alternate theory is that exposure to persistent stressors produces more resilient
495 microbial communities due to the development and dominance of tolerant organisms
496 (Brussaard et al. 2007). There was no evidence of decreased susceptibility to heat with
497 increased Cu pre-exposure concentration from this study with which to support this
498 theory. However, that susceptibility of phosphomonoesterase to heat in Soil C did not
499 alter with increasing Cu pre-exposure concentration, may well be related to the fact
500 that, unlike soil A, regardless of heat, increased Cu did inhibit phosphomonoesterase
501 activity, and thus may have induced a more tolerant microbial community. Increased
502 Cu also inhibited phosphomonoesterase activity in Soil B, although in this case there
503 is some evidence of increased susceptibility to heat, perhaps due to the fact that
504 without the heat Cu had a more dramatic adverse effect on phosphomonoesterase
505 activity. The results from this study are consistent with those reported from other
506 studies in that generally the ability of soil microbial functions to withstand additional
507 disturbances/stresses has not been adversely effected where the microbial
508 communities were pre-exposed to < 100 mg/kg Cu (Brandt et al. 2010; Philippot et al.
509 2008; Tobor-Kaplon et al. 2006). Like this study, adverse effects on the ability to
510 withstand additional stressors have been reported where microbial communities were
511 pre-exposed to Cu concentrations > 100 mg/kg (Dussault et al. 2008; Tobor-Kaplon et
512 al. 2005). Further studies are need to elucidate the reasons behind the different
513 responses between the three different soil types, as this response is likely to be
514 complicated by physical-chemical soil properties (e.g. TOC, pH) which influence the
515 size and diversity of soil microbial communities as well as the climatic conditions at
516 the locations that the soils were collected from.

517

518 **Conclusions**

This study sought to determine a dose-response relationship between increased Cu pre-exposure concentration and altered susceptibility of phosphomonoesterase and urease activity to heat. Little could be concluded from the urease results, whilst for the most part the susceptibility of phosphomonoesterase was not altered by increased Cu concentrations up to 1000 mg/kg Cu. Where the Cu pre-exposure concentration did induce increased susceptibility to heat there was no clear dose response relationship within or between the three different soils assessed. The reasons for the variable response in the different soils are likely to be related to physical-chemical properties of the soils and the climatic conditions in locations these soils were collected and the direct effect of Cu on soil microbial communities. The results from this study suggest that at the concentrations of Cu typically reported in agricultural soils, Cu stress is unlikely to cause lasting alterations to the susceptibility of urease and phosphomonoesterase activity to a single heat disturbance. Whether this holds true over more prolonged/repeated heat disturbances, as would be expected to occur over the summer period in agro-ecosystems, should be the subject of further research.

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680 **Figure Captions**

681 **Figure 1** Enzyme activity response to the heat perturbation in the unspiked
682 control soils; (a) phosphomonoesterase; (b) urease. Soil A is
683 represented by hollow circles, Soil B by grey triangles, and Soil C by
684 solid squared; solid lines represent unperturbed and dashed lines
685 represent heat perturbed samples. Error bars are $\pm$ standard deviation
686 from the mean ($n = 3$).

687
688 **Figure 2** Percentage of change in phosphomonoesterase activity (relative to the
689 unperturbed sample) 1, 2 and 7 days after heat perturbation. Soil A (a),
690 Soil B (b), Soil C (c). Error bars are mean $\pm$ standard deviation ($n = 3$).
691 For each soil, a significant difference ($P \leq 0.05$) from the unspiked
692 control soil (0 mg/kg added Cu) is denoted by * for day 1 and ^ for day
693 2.

694
695 **Fig. 3** Percentage of change in urease activity (relative to the unperturbed
696 sample) 1, 2 and 7 days after heat perturbation. Soil A (a), Soil B (b),
697 Soil C (c). Error bars are mean $\pm$ standard deviation ($n = 3$). For each
698 soil, a significant difference ($P \leq 0.05$) from the unspiked control soil
699 (0 mg/kg added Cu) is denoted by ^ for day 2.

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Figure 2

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Figure 3

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713 **Table 1** Selected physical-chemical soil properties for the tests soils and climate

714 information from the bulk soil sampling sites

	Soil A	Soil B	Soil C
Soil type	Silt loam	Loamy sand	Clay loam
pH (H ₂ O)	5.8	8.6	7.8
Cation exchange capacity (cmol/kg)	12.3	12.9	24.7
Total organic carbon (%)	5.44	0.80	3.66
Total nitrogen (%)	0.40	0.09	0.34
Electrical conductivity (dS/cm)	0.12	0.12	0.23
Sand (%)	20	85	31
Silt (%)	54	7.3	32
Clay (%)	26	7.7	37
Total Cu (mg/kg)	26	5	54
CaCl ₂ extractable Cu (mg/kg)	<0.20	<0.20	<0.20
Mean annual maximum temperature (°C) ^a	19.0	23.7	20.3
Mean annual rainfall (mm) ^a	591	285	573

715 ^a Data from (BOM, 2010)

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726 **Table 2** Concentrations of Cu and enzyme activities in the Day 1 Cu spiked test soils (unperturbed samples (no heat disturbance); n = 3)

Nominal added Cu concentration (mg/kg)	Total Cu concentration (mg/kg)	Measured added Cu concentration (mg/kg)	CaCl ₂ extractable Cu concentration (mg/kg)	Phosphomonoesterase			Urease		
				(µg <i>p</i> -nitrophenol/g dry soil/h)			(µg/NH ₄ produced/g dry soil/h)		
				Mean	SD	CV%	Mean	SD	CV%
<i>Soil A</i>									
0	26	0	<0.20	117	16.4	14	0.43	0.08	18
50	92	66	<0.20	122	37.3	31	0.43	0.08	18
100	147	122	0.33	149	30.4	21	0.42	0.04	9
200	222	196	0.68	149	28.2	19	0.64*	0.07	10
500	586	561	1.83	206*	13.0	6	0.51	0.05	10
1000	1130	1110	5.57	113	10.8	10	0.19*	0.02	11
<i>Soil B</i>									
0	5	0	<0.20	63.7	0.6	1	0.07	0.01	18
50	58	53	0.28	24.6*	3.9	16	0.07	0.01	20
100	91	86	0.34	21.8*	1.4	7	0.05	0.02	47
200	175	170	0.65	19.7*	1.3	7	0.03	0.002	8
500	489	484	1.24	14.8*	0.6	4	0.02*	0.001	4
1000	954	949	2.00	15.7*	1.2	8	0.01*	0.01	102
<i>Soil C</i>									
0	54	0	<0.20	100	14.1	14	0.99	0.12	12
50	115	61	0.24	74.0	15.2	21	0.89	0.17	19
100	156	102	0.31	66.0*	6.4	10	1.1	0.24	22
200	272	218	0.42	58.0*	13.6	23	1.1	0.34	31
500	555	501	0.66	56.5*	13.8	24	0.82	0.26	31
1000	1250	1190	1.05	37.7*	8.4	22	0.94	0.01	1

727 * denotes significant difference ($P \leq 0.05$) from respective unspiked control soil for each soil

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