**Effects of copper fungicide residues on the microbial function of
vineyard soils**

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Abstract

The use of copper-based fungicides leads to an accumulation of copper (Cu) in vineyard soils, potentially causing adverse effects to the microbial function and fertility of the soil. This study used a soil microcosm approach to assess the effects of Cu accumulation on microbial function in vineyard soils. Surface soil samples were collected from 10 vineyards and a number of un-impacted reference sites in each of three different viticultural regions of Australia. The field collected soils were transferred to microcosms and maintained for up to 93 days in the laboratory at 20-22°C and 60 % of their maximum water holding capacity. The microbial function of the soils was indicated by measuring phosphomonoesterase, arylsulfatase, urease, and phenol oxidase activity. In general, the vineyard soils had greater concentrations of Cu and lower enzyme activities than in the reference soils, although a weak negative relationship between Cu and enzyme activity could only be found for phosphomonoesterase activity. The results show that soil physical-chemical properties (i.e. organic carbon, pH) are greater determinants of soil enzyme activity than increased soil Cu concentration at the Cu concentrations present in vineyard soils.

Key words: copper fungicide, vineyard, soil, microbial function, enzyme activity, microcosm

1. Introduction

Copper-based fungicides (e.g. copper sulphate, copper oxychloride, copper hydroxide) are amongst the most widely used pesticides, particularly in vineyards and orchards. The regular use of Cu-based fungicides results in an accumulation of Cu in surface soils ([Wightwick et al. 2010a](#)). For instance, Cu concentrations in the surface soils of vineyards with histories of Cu-based fungicide use have typically been reported to range from 130 to 1280 mg/kg in European vineyards, and from 24 to 159 mg/kg in Australian vineyards ([Wightwick et al. 2008](#); [Komárek et al. 2010](#)). Applications of Cu-based fungicides have been linked to reductions in microbial function and changes in the structure of microbial communities of vineyard and orchard soils ([Ranjard et al. 2006](#); [Diaz-Ravina et al. 2007](#); [Wang et al. 2009](#); [Fernández-Calviño et al. 2010](#)). Agricultural ecosystems rely on soil microbial communities to facilitate key soil functions, such as nutrient cycling and litter decomposition, so any negative effects on soil microbial communities caused by Cu-based fungicides could have significant implications for the long-term fertility of agricultural soils ([Brussaard et al. 2007](#); [Komárek et al. 2010](#)).

The reported toxic effect concentrations (thresholds) of Cu causing adverse effects on soil microbial activity/function vary greatly between different soils and studies ([Bååth 1989](#); [Giller et al. 1998](#); [Oorts et al. 2006b](#); [Broos et al. 2007](#); [Giller et al. 2009](#)). For instance, Broos et al. (2007) reported that the Cu concentration causing a 50 % effect (EC₅₀) on substrate induced respiration (SIR) and substrate induced nitrification (SIN) varied from ~300 to > 1500 mg/kg total Cu, and from ~ 100 to > 1500 mg/kg total Cu, respectively in 12 vastly different Australian soils. Differences in effect

concentrations across different soils have been found to be related to the same soil factors known to influence the bioavailability of Cu in soil, including pH, organic carbon, clay content, and cation exchange capacity (CEC) (Oorts et al. 2006b; Broos et al. 2007; Fernández-Calviño et al. 2009; Wightwick et al. 2010c).

Most studies investigating the effects of Cu on soil microbial function have used ‘artificially’ contaminated soils, by either using spiked soils in the laboratory or by setting up experimental field plots where Cu is applied and incorporated into the soil (Bååth 1989; Giller et al. 2009). The use of such ‘artificially’ contaminated soils to assess the risk of Cu-based fungicide use to microbial function has a number of limitations. Firstly, the availability (and toxicity) of Cu is known to be greater in spiked soils compared with field soils (Oorts et al. 2006a). Secondly, in spiking experiments, large doses of Cu tend to be added in single applications, whereas in vineyards the total Cu concentration of the soil increases gradually over many years due to the repeated applications of relatively small doses of Cu-based fungicide. In the former case, large singular applications of Cu are likely to have a more drastic one-off impact on the abundance and composition of soil microbes, which in turn may adversely impact on the ability for the soil microbial community to recover from the Cu stress. Conversely, repeated applications of relatively small doses of Cu-based fungicides may have a relatively minor immediate impacts on the microbial community (from which microbial communities are able to recover), and increase total soil Cu concentrations gradually (providing plentiful opportunity for the soil micro-organisms to adapt to the increasing Cu), together producing microbial communities more tolerant to Cu than the microbial communities in ‘artificially’ contaminated soils (Giller et al. 1998; Brandt et al. 2010; Mertens et al. 2010).

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95 There have been relatively few studies surveying microbial function in actual ‘real-
96 world’ soils from farms where Cu-based fungicides have been used ([Merrington et al.](#)
97 [2002](#); [Bunemann et al. 2006](#); [Wang et al. 2009](#); [Fernández-Calviño et al. 2010](#);
98 [Komárek et al. 2010](#)). However, utilising ‘real-world’ soils in risk assessment studies
99 also presents challenges, particularly when comparing soils from different properties
100 and regions, because soil microbial function is influenced by regional and site specific
101 climatic conditions (e.g. rainfall) and farm management practices (e.g. irrigation
102 regimes, cover cropping) ([Giller et al. 1998](#)). Moreover, compared to spiked soil
103 experiments, the field soils have mostly likely received inputs of other, unknown
104 pesticides. These factors can make it challenging to determine whether differences in
105 microbial function are due to the accumulated Cu or whether they are due to site
106 factors such as climate, management practices, and the use of other pesticides. One
107 approach to overcome this is to conduct soil microcosm experiments ([Burrows and](#)
108 [Edwards 2002](#); [Schaffer et al. 2008](#)), whereby soils are collected from different sites
109 in the field and then allowed to equilibrate for a period of time under common
110 environmental conditions. This can enable a more real-world approach to assessing
111 the risks of Cu-based fungicide use to soil microbial function. This study used such a
112 microcosm approach to determine the effects of accumulated Cu-based fungicide
113 residues on the microbial function of field-collected vineyard soils from three
114 different viticultural regions of Australia. Specifically, we used soil enzyme activities
115 to indicate soil microbial function by measuring phosphomonoesterase (phosphorous
116 cycle), urease (nitrogen cycle), phenol oxidase (carbon cycle) and arylsulfatase (sulfur
117 cycle) activities.

118

2. Materials and Methods

2.1 Site selection and sampling

Soil samples were collected in May 2009 (autumn) from 10 vineyards in each of three viticultural regions of Australia: the south-east, South Australia (SA), the Murray Valley, Victoria (Vic), and the Grampians, Vic regions. These regions were selected to represent viticultural regions with distinctly different soil properties and therefore likely to have different Cu adsorption characteristics. In each region the vineyards surveyed were generally within a 10 km radius of each other. The vineyards in the south-east, SA and Murray Valley regions had received Cu-based fungicide inputs for at least 40 years, typically for 70 - 80 years, and some for more than 100 years. In the Grampians region, one of the vineyards had reportedly not received any Cu-based fungicide inputs, four of the vineyards had received Cu-based fungicide inputs for between 10 and 25 years, two for about 40 years, and three for more than 100 years. The majority of the vineyards were drip irrigated, with the exception of four of the vineyards in the Murray Valley region which were furrow irrigated. Six of the vineyards in the Grampians region were irrigated with recycled municipal water. The vineyards surveyed were cultivating grapes for wine production with the exception of five of the vineyards in the Murray Valley region which were cultivating for dried grape production.

In each vineyard, surface soil samples (5 cm depth) were collected from 10 different sampling locations according to a grid based sampling scheme ([Wightwick et al. 2008](#)). At each sampling location soil samples were collected from the under vine area and from the mid row area between two vine rows. The 10 sub-samples were combined to obtain a composite under vine and a composite mid row sample for each

vineyard. Also, at each vineyard a composite soil sample was collected from an area at least 15 m outside the edge of the vineyard to act as a reference soil, i.e. a soil with similar properties to those collected from the vineyard but which had not received any (or little) artificial inputs of Cu. In some instances vineyard sites were sufficiently close to each other that a single reference site was designated for more than one vineyard. A total of 81 soil samples were collected, comprising 30 under vine soils, 30 mid row soils, and 21 reference site soils. Following collection, each soil sample was gently passed through a 4 mm sieve and mixed well. On collection in the field, and following sieving, the soil samples were either sealed in plastic buckets or in polyethylene zip-lock bags and stored at room temperature ($< 20^{\circ}\text{C}$).

2.2 Soil microcosms

The effects of fungicides on soil microbial processes can either be tested in the field or in the laboratory. Field trials may be hampered by high variability in soils and climate, poor reproducibility and extremely high costs, and as with many laboratory studies, are often restricted to examining the effects of a single, large dose of chemical on a single soil. Soil microcosms represent an alternative approach that addresses the issues of variability and reproducibility whilst being inexpensive and easy to handle under controlled conditions. In this study, the soil microcosm method was based on published studies by [Chen and Edwards \(2001\)](#), [Burrows and Edwards \(2002\)](#), and [Warman & Munroe \(2010\)](#), modified to remove plants, insects and other organisms, and to use inexpensive plastic tubes instead of more expensive plant pots.

The microcosms were tubes made from 12 cm lengths PVC pipe (38 mm internal diameter). One end of the tube was covered with a square of permeable landscaping

169 fabric (Weedgard™, Gardman Ltd, UK) (approx 15 cm x 15 cm) secured using a
170 rubber band, and then sieved soil was loosely packed into the microcosm tubes to a
171 depth of approx 10 cm. The microcosm tubes were then placed on a bed of washed
172 river sand in plastic trays (40 cm x 30 cm). The trays were covered with 50 mm
173 square wire mesh (plastic coated) to support the microcosm tubes in an upright
174 position. Each of the 81 soil samples was randomly assigned a position on the trays.
175 Three microcosms were constructed for each of the 81 soil samples, and these three
176 replicates positioned next to each other in a row at the designated position for their
177 parent soil on the tray. The microcosm trays were set-up on racks within a controlled
178 temperature laboratory and were maintained at 20-22°C with a natural light:dark cycle
179 of 8:16 hours.

181 Prior to commencing the microcosm experiment the initial moisture content of the soil
182 samples was determined gravimetrically by drying a sub-sample at 60°C. The
183 maximum water holding capacity was also determined by saturating a sub sample (~5
184 g) of soil suspended in a fine mesh, allowing it to drain for 48 h, weighing wet, then
185 drying at 60°C to constant weight. Each of the soil microcosms were then
186 gravimetrically moistened up to 60 % of their WHC by gently adding the required
187 volume of ultra pure water (Millipore, Molsheim, France). For the duration of the
188 experiment the moisture content of the soils was maintained within 50 – 60 % of their
189 WHC by weekly additions of ultra pure water as required. One microcosm tube for
190 each soil was destructively sampled after 27, 54, and 93 days of incubation. The
191 contents of the microcosms were gently mixed, transferred to and sealed in a
192 polyethylene zip lock bag, and stored at 4°C until further processing. A sub sample
193 was taken and the moisture content determined by the oven drying to constant weight.

194

195 2.3 Enzyme activity assays

196 Soil enzyme activities were chosen over other more commonly used measures of
197 microbial function (e.g. respiration, nitrification) as they are generally more sensitive
198 to elevated concentrations of metals and environmental changes, enable more specific
199 measures of microbial activity that are relevant to the function of agricultural soils
200 (i.e. related to specific nutrient cycles in the soil), and the assays are relatively cost-
201 effective and rapid which enabled us to assess a greater number of sites and samples.
202 The trade-off using this approach being that the use of the soil enzyme activity
203 approach does not allow a distinction between the effect of Cu on the
204 size/composition of the soil microbial community (which influences the production of
205 enzymes) and the direct inhibitory effect of Cu on the enzymes.

206

207 Enzyme activity tests were conducted on the soils sampled after 27, 54, and 93 days
208 of incubation using published methods ([Sinsabaugh et al. 2000](#); [Tabatabai 1994](#)), the
209 main exception being that the assays were conducted without the use of pH buffers.
210 This latter was done to provide a measure of ‘actual’ enzyme activity so as to
211 investigate the microbial function of the soils in their ‘natural state’. The choice was
212 made as the alteration of pH through the use of buffers can create an unnatural soil
213 solution environment. Firstly, pH influences the solubility and composition of organic
214 matter in soil which is of significance as it is known that enzymes form complexes
215 with organic matter which can enhance their stability and protect them from inhibitory
216 effects from Cu ([Speir and Ross 2002](#); [Tabatabai and Dick 2002](#)). Secondly, pH
217 influences the speciation of Cu (i.e. free Cu^{2+} 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).

Phosphomonoesterase (E.C 3.1.3) and arylsulfatase (E.C 3.1.6.1) assays were based on the methods described in Tabatabai (1994), with some minor modifications. In short, 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 used instead of the buffer solution. The samples were incubated at 37°C for 1 h. 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).

The urease (EC 3.5.1.5) assay was based on the methods described by Tabatabai (1994) and Sinsabaugh et al. (2000). Nine ml of ultra pure water and 1 ml of 200 mM urea was added to 1 g (dry weight equivalent) of soil in a 15 ml centrifuge tube and mixed using a vortex mixer for 10 - 15 seconds. The samples were incubated for 1 hour at 37°C and then centrifuged to stop the reaction. Duplicate 200 µL aliquots of clear supernatant were then transferred into a 96-well microplate. The concentration of ammonium released was measured colorometrically using the salicylate-cyanurate method (Reardon et al. 1966; Sinsabaugh et al. 2000) and measuring absorbance at 610nm using a microplate reader (Sinsabaugh et al. 2000). This method is not sensitive to Cu interference (Pym and Milham 1976). Urease activity was expressed

as $\mu\text{g}/\text{NH}_4$ produced/g dry soil/h, adjusted for absorbance of negative and substrate controls.

The phenol oxidase (EC 1.14.18.1) assay was based on the methods described by [Pind et al. \(1994\)](#) and [Williams et al. \(2000\)](#). Two grams of soil (dry weight equivalent) was weighed into a 15 ml centrifuge tube, 10 ml of ultra pure water was then added and mixed using a vortex mixer for 10 - 15 seconds to create a soil suspension. A 3 ml aliquot of the suspension was then transferred into a separate 15 ml centrifuge tube and 2 ml of 10 mM 3,4-dihydroxy-L-phenylalanine (L-DOPA) (Sigma-Aldrich) added and mixed using a vortex mixer. The tubes were incubated at 37°C for 5 mins and then centrifuged for 5 mins to stop the reaction ([Pind et al. 1994](#); [Williams et al. \(2000\)](#)). Negative control samples were processed by taking a separate 3 ml aliquot of the soil suspension and then adding 2 ml of ultra pure water water, substrate controls comprised of 3 ml of MilliQ water and 2 ml of 10 mM L-DOPA. Four-hundred microliter aliquots of clear supernatant were then transferred (in duplicate) to a 96-well microplate and absorbance measured at 460 nm. Phenol oxidase activity was expressed as μmol of 2,3-dihydroindole-5,6-quinone-2-carboxylate (diqc) formed/g dry soil/min. The concentration of diqc was calculated based on the molar absorbancy coefficient for diqc of 3.7×10^4 ([Mason 1948](#)) and adjusted for the absorbance of the negative and substrate controls.

For each set of microcosms analysed, 10 % of the samples were randomly selected for duplicate analysis. The coefficient of variation (CV %) for the duplicate samples (mean, 95 % confidence interval) were: phosphomonoesterase (21, 14–29); arylsulfatase (23, 11–35); urease (82, 32–113); phenol oxidase (28, 16–41).

2.4 Concentrations of Cu and other elements in the soils

Total metals and major elements were determined with 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). Sample extracts were analysed by Inductively Coupled Plasma Emission Spectrometry (ICP-ES) (SPECTROFLAME-EOP; Spectro Analytical Instruments GMBH, Kleve, Germany) to quantify the total concentrations of elements (mg/kg) in the soil. Each of three standard reference soils were processed in triplicate being; ISE 967 and ISE 990 from the Wageningen Evaluating Programs for Analytical Laboratories (WEPAL) (Wageningen University, Netherlands) and an internal reference soil used in our laboratory (SCL 1000-6). Recoveries of Cu from the reference soils were within 20 % of the expected value. Recoveries for P and S were within 10 % of the expected value and for the most part recoveries of Ni, Pb, Cd, and Cr were within 20 % of the expected value. Recoveries for B (119 – 163 %), Na (111 – 130 %), and K (58 – 112 %) were poorer. Ten percent of the samples were processed in duplicate with of the variation between the duplicate readings being <10 % for all elements with the exception of B (up to 33 %) and Ca (up to 21 %).

Soluble Cu was extracted from the under vine, mid-row and reference 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) elements in the soil. Four samples of each of four different standard reference soils were processed (WEPAL soils, ISE 967, ISE 968 and ISE 990; and SCL 1000-6). Where higher reliability consensus values were available for the WEPAL reference soils (only for K

and Mg) the recoveries of the elements were within 10 % of the stated value, indicating that the 0.01 M CaCl₂ soil extractions provided acceptable extraction efficiency (Wightwick et al. 2010b). Recoveries of Cu and other trace elements were generally within 20 % of the values published by Wightwick et al. (2010a), with the exception of Al (66 – 80 % recovery) and Fe (42 – 144 % recovery). Ten percent of the samples were processed in duplicate with CV % of the duplicate readings being <10 %, with the exception of B on three occasions (14 %, 17 %, and 46 %) and Zn on one occasion (25 %).

2.5 Physical-chemical properties of the soils

Soil pH (H₂O and CaCl₂) and electrical conductivity (mS/cm) were determined in a 1:5 soil/water suspension. The total organic carbon (TOC) and nitrogen (TN) content (%) of the soil samples was measured with a LECO CNS element analyser (LECO, St. Joseph, MI, USA). 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).

2.6 Data analysis

Data analysis was conducted in GenStat 12th edition (VSN International Ltd) and SPSS Version 17.0 (XL Statistics, Chicago, Illinois). When assessing for differences between physical-chemical properties, metal concentrations and enzyme activity of

soil samples between regions and soil types (i.e. under vine, mid row, reference site soils), data did not conform to the assumptions of homogeneity and homoscedasity required for parametric statistical methodologies, therefore the non-parametric Kruskal-Wallis test was used to assess for an overall difference, with post hoc differences assessed using the Mann-Whitney test. Where necessary the Bonferroni correction was used to address the problem of a family wise error in multiple comparisons. The backwards stepwise approach to multiple regression was used to determine soil factors (concentrations of metals/elements, physical-chemical soil properties) that were significantly contributing to activity of the four different enzymes. Unless otherwise stated, significance was tested at the 95 % confidence level ($\alpha = 0.05$) for all analysis.

3. Results and Discussion

3.1 Physical chemical properties of soils

This study collected vineyard soils from three different viticultural regions of Australia. So, not surprisingly, the physical-chemical properties of soils varied between regions, in particular pH, clay, total organic carbon (TOC) and cation exchange capacity (CEC) (Supplementary material, Table S-1). In all regions, TOC and total N concentrations were highly correlated ($R^2 = 0.95$) and significantly lower in the under vine and mid row vineyard soils compared to the reference site soils. In the Grampians region, EC and pH were significantly higher in the vineyard soils compared to the reference. Concentrations of total P were significantly higher in the

vineyard soils compared to the reference soils in both the Grampians (mean total P; vineyard 401 mg/kg, reference 258 mg/kg) and Murray Valley (mean total P mg/kg; vineyard 711 mg/kg, reference 410 mg/kg) regions.

The size and function of soil microbial communities as well as at the activity of the enzymes they produce is known to be strongly influenced by physical-chemical soil properties (Taylor et al. 2002; Oorts et al. 2006b; Broos et al. 2007; Fernández-Calviño et al. 2010). The strong influence of TOC, pH and particle size on soil enzyme activity observed in this study was expected as the organic matter content of the soil dictates the size of the microbial biomass and therefore the amounts of enzymes produced (Tabatabai and Dick 2002; Taylor et al. 2002). Soil pH influences the size and composition of the microbial community as well as the rate of enzymatic reactions (Tabatabai and Dick 2002; Taylor et al. 2002; Rousk et al. 2010). It is also known that enzymes form complexes with organic matter and clay minerals in soil which can stabilise and protect them from degradation so that they remain active for longer (Speir and Ross 2002; Tabatabai and Dick 2002). Phosphorous (P) and nitrogen (N) are major soil nutrients and as such were also expected to have a significant influence on the microbial biomass and hence on soil enzyme activity.

3.2 Concentrations of metals in the soils

Concentrations of total Cu in vineyard soils ranged from 19 to 162 mg/kg with concentrations being highest in the south east, SA region, followed by the Murray Valley and Grampians regions (Figure 1; Supplementary material, Table S-3(a)). Across all regions, the concentrations of Cu in the under vine and mid row soils were statistically similar ($P = 0.55$), and the under vine and mid row vineyard soils had

significantly higher Cu concentrations than the reference soils in the south east, SA and Murray Valley regions (Figure 1). There was no significant difference between total Cu concentrations in under vine and reference soils in the Grampians region (Figure 1). In general, the concentrations of other metals (B, Cd, Cr, Pb and Zn) in the under vine soils were similar to the concentrations in both the mid row and reference soils (Supplementary material, Table S-3(a)). However, in the Murray Valley region the concentrations of total Cd in both the under vine (mean 0.21 mg/kg) and mid row soils (mean 0.20 mg/kg) were significantly higher than the concentrations in the reference soils (mean 0.10 mg/kg).

The CaCl_2 extractable Cu concentrations were generally at least two orders of magnitude lower than total Cu concentrations, and never more than 0.2 mg/kg Cu. In under vine soils, mean CaCl_2 extractable Cu was in the Murray Valley region (0.26 mg/kg) was higher than in the Grampians regions (0.22 mg/kg) and south east, SA region (0.13 mg/kg; Supplementary material, Table S3(b)). The mean CaCl_2 extractable Cu concentrations in the reference site soils were marginally lower than under vine soils (south east, SA, 0.12 mg/kg; Murray Valley 0.14 mg/kg; Grampians: mean 0.14 mg/kg). There were no significant differences in CaCl_2 extractable concentrations of other metals (Al, B, Cd, Cr, Mn, Ni, Pb, Zn) between the vineyard and reference site soils (Supplementary material, Table S3(b)).

The concentrations of total and CaCl_2 extractable Cu were within the range previously reported in Australian vineyard soils (Pietrzak and McPhail 2004; Wightwick et al. 2008; Wightwick et al. 2010c). Copper concentrations were generally higher in the under vine and mid row soils compared to the reference site soils (Supplementary

material, Table 3(a)). In the Grampians region the total Cu concentrations of soils in vineyards which have more than 30 years of Cu-based fungicides use (mean total, Cu 54 mg/kg) were significantly higher than soils from vineyards reporting less than 30 years of copper fungicides use (mean total Cu, 32 mg/kg). It is worth noting that in some instances unexpectedly elevated Cu concentrations were detected in reference site soils, perhaps suggesting that they had received anthropogenic inputs of Cu, mostly likely from spray drift. That the Cu concentrations in the under vine and mid row soils were similar was an unexpected observation, since it has previously been reported that the Cu concentration in mid row soils are lower than in under vine soils (Pietrzak and McPhail 2004; Wightwick et al. 2006). This observation may be the result of clearing, cultivation and/or re-development of vineyards causing lateral re-distribution of accumulated Cu in surface soils (Wightwick et al. 2006). The availability of metals in soils increases with decreasing soil pH due to a decrease in the number of negatively charge binding sites (Sauve et al. 2000), so the higher concentrations of CaCl₂ extractable Cu, Al, Mn and Zn in the Grampians region was not unexpected due to the acidic nature of the Grampians region soils.

3.3 Soil enzyme activities

The moisture content of the soils when collected in the field ranged from 1 – 28 % (mean 11 %), and was highest in the south east, SA region (8 – 28 %, mean 19 %), followed by the Grampians (1 – 25 %, mean 9 %) and Murray Valley (2 – 6 %, mean 4 %) regions. The large variation in the moisture content of the soils in the field re-affirmed the need to adopt the laboratory based microcosm approach to control environmental conditions, allow the resident microbial communities to incubate under

common conditions, and thus enable more valid comparisons between different sites and regions.

Length of incubation (i.e. 27, 54 and 93 days) had a significant effect on most enzyme activities, except that of arylsulfatase activity. Specifically, there was not a significant difference in phosphomonoesterase and urease enzyme activity after 93 days were significantly lower than the 27 and 54 day samples. Phenol oxidase activity was significant higher at 27 days compared to 54 days, and to a lesser extent 93 days ($P = 0.09$). Because 27 days may not have been long enough for the soil samples to equilibrate under common conditions, and phosphomonoesterase and urease enzyme activities were found to be lower in the 93 day samples, the 54 day samples have been used for further data analysis.

Significant differences between regions were found for all enzyme activities except urease. Phosphomonoesterase activity was highest in the Grampians region followed by the south east, SA and Murray Valley regions (Figure 2; Supplementary material, Table S-4). Arylsulfatase activity was significantly higher in south east, SA region, whilst phenol oxidase activity was significantly higher in the Murray Valley region (Figure 2; Supplementary material, Table S-4). When data is pooled across all regions, significantly lower activities were recorded for phosphomonoesterase, urease and arylsulfatase activity in vineyard soils than in the reference soils. Phenol oxidase activity did not differ significantly between vineyard and reference soils (Figure 2; Supplementary material, Table S-4). In the case of phosphomonoesterase and arylsulfatase, activities were lower in the under vine soil compared with the mid row soil (Supplementary material, Table S-4). In the south east, SA region significantly

lower activities were found in the vineyard than in reference soils for arylsulfatase and phosphomonoesterase and to a lesser extent for urease ($P = 0.08$) (Figure 2). In the Grampians region, significantly lower activity in vineyard relative to reference soils was only found for phosphomonoesterase activity, and in the Murray Valley region only for urease activity (Figure 2).

The overall aim of this study was to test the hypothesis that long-term Cu-based fungicide use adversely affects the microbial function of vineyard soils. If this is the case, then it might be expected that that enzyme activities would be lower in Cu-enhanced vineyard soils compared with reference soils. The results from this study are generally consistent with the tested hypotheses, with enzyme activity in vineyard soils being significantly lower than in the reference soils when data from all regions was considered. Phenol oxidase activity, however is an exception, although the lack of significant differences may be due to the considerably higher variation (site to site variation) in the results from this assay (Figure 2). Similarly, that the trend of enzyme activities being lower in the vineyard soils was not as apparent in the Murray Valley region may be due to the activities of the enzymes (including in the reference soils) generally being much lower compared with the other regions (Figure 2), and thus contributing to higher variation in the data. That differences in most of the enzyme activities between vineyard and reference soils were not found in the Grampians region is perhaps not unexpected as this was also the case with the total Cu concentrations in the soil. Whilst the activity of enzymes is generally related to the size of the microbial biomass, a reduction in enzyme activity does not necessarily mean a change in the size and composition of the microbial community, because other factors can interfere with the enzyme itself, e.g. elevated concentrations of Cu in soil

solution can react with the enzyme-substrate complex thus reducing the rate of the enzyme mediated process (i.e. non-competitive inhibition) (Speir and Ross 2002; Tabatabai and Dick 2002).

3.4 Influence of Cu and physical-chemical soil properties on soil enzyme activities

3.4.1 Phosphomonoesterase

No relationships were observed between phosphomonoesterase activity and either total Cu or CaCl₂ extractable Cu concentration (Figure 3). Microbial activity, and therefore enzyme activity in soil is known to increase with increasing soil organic matter content (Taylor et al. 2002). Moreover, it is known that organic matter plays a key role in influencing the bioavailability of Cu in soil (Stevenson and Fitch 1981; Sauve et al. 1997; Strawn and Baker 2008). To account for the influence of differing organic matter contents of the soils, phosphomonoesterase activity was expressed per unit of organic carbon. This produced a weak relationship ($R^2 = 0.14$) with total Cu concentration (Figure 3). However, a stronger negative relationship was observed when total Cu was expressed as the percentage (%) of the organic carbon content of the soil; this relationship could explain 40% of the variation in phosphomonoesterase activity across all regions, albeit that the correlations were markedly different between regions (south east SA, $R^2 = 0.72$; Murray Valley, $R^2 = 0.30$; Grampians region, $R^2 = 0.16$) (Figure 3). Using multiple stepwise (backwards) linear regression analysis it was found that 91% of the variation in phosphomonoesterase activity could be explained by TOC, pH, CaCl₂ extractable Mn, total Cu, total P and clay content of the soil (Table S-2). Within this regression, TOC and pH could explain 84 % of the variation in the phosphomonoesterase activity. When the regions were analysed

separately, 81 to 87 % of the variation in phosphomonoesterase activity could be explained by variables included in the multiple regressions (Table S-2).

3.4.2 Arylsulfatase

No significant relationships between arylsulfatase and total or CaCl_2 extractable Cu, were found in soils within or between different regions. However, when the total Cu concentration was expressed as the percentage of the organic carbon content of the soil a relatively weak relationship with arylsulfatase could be found (Figure 4). The relationship with total Cu as percent of organic carbon was stronger in the south east, SA ($R^2 = 0.34$) compared to the Murray Valley ($R^2 = 0.22$) and Grampians region ($R^2 = 0.06$). Using multiple regression analysis, 74 % of arylsulfatase activity could be explained by TOC, CaCl_2 extractable Mn and total Ca (Table S-2); within this regression, 59 % of the variation could be attributed to TOC. The regression relationships were poorer in the Grampians ($R^2 = 0.34$) and Murray Valley regions ($R^2 = 0.49$). In the south east SA and Grampians regions total Ca and/or exchangeable Ca were identified as important variables in explaining arylsulfatase activity.

3.4.3 Urease

A significant relationship between urease activity and total Cu could only be found in the Murray Valley ($R^2 = 0.19$) (Figure 5). There were no significant relationships with CaCl_2 extractable Cu. When total Cu was expressed as the percentage of the organic carbon content of the soil a relatively weak relationship with urease could be found ($R^2 = 0.14$) (Figure 5). This relationship was stronger in the Murray Valley region ($R^2 = 0.39$) compared to the south east, SA ($R^2 = 0.12$) and Grampians regions ($R^2 = 0.06$). Using multiple regression analysis, TOC and total N could explain 41 % of the

variation in urease activity, with TOC being the most significant contributor (38 %). When the regions were analysed separately, 49 to 56 % of the variation in urease activity could be explained by variables included in the multiple regressions (Table S-2).

3.4.4 Phenol oxidase

Phenol oxidase activity generally increased with increasing total Cu concentration, however a significant relationship was only found for the soils from the south east, SA region ($R^2 = 0.29$) (Figure 6). In the Grampians region a weak relationship was found between increasing CaCl_2 extractable Cu and decreasing phenol oxidase activity ($R^2 = 0.14$). Using multiple regression analysis, 56 % of the variation in phenol oxidase activity could be explained by total Ni, total N, total Ca, total K and total Pb; within this regression Ni, N and Ca accounted for 50 % of the variation (Table S-2). When the regions were analysed separately 65 to 82 % of the variation could be explained by variables included in the multiple regressions (Table S-2).

This study provides little evidence to indicate that elevated Cu concentrations are responsible for the decreases in soil enzyme activity in vineyard soils. Rather, the data shows that soil enzyme activity was largely driven by physical-chemical soil properties in the studied soils, principally TOC, pH and particle size, perhaps because the total Cu concentrations in the vineyard soils studied were generally below concentrations reported to have drastic effects on soil microbial function (Bååth 1989; Giller et al. 1998; Wyszowska et al. 2005; Broos et al. 2007; Chaperon and Sauve 2007; Speir et al. 2007; Wang et al. 2007; Floch et al. 2009; Fernández-Calviño et al. 2010). For instance, 90 % of the vineyard soils in this study contained less than 115

mg/kg total Cu, and none of the soils contained more than 162 mg/kg total Cu, whereas, [Broos et al. \(2007\)](#) reported the minimum Cu concentration causing a 50 % effect on substrate induced respiration (SIR) was 316 mg/kg total Cu. [Fernandez-Calvino et al. \(2010\)](#) suggest 200-250 mg/kg total Cu to be the threshold causing adverse effects to soil enzyme activities (phosphatase, dehydrogenase, β -glucosidase) in vineyards soils.

Overall, the results from this study are broadly consistent with findings from the two similar field-oriented studies assessing the effects of Cu-based fungicide residues on soil enzyme activities reported in the literature ([Wang et al. 2009](#); [Fernández-Calviño et al. 2010](#)). For instance, [Fernandez-Calvino et al. \(2010\)](#) determined enzyme activities in soils collected from 145 different vineyard sites in North-West Spain with known elevated Cu concentrations (due to Cu-based fungicide use); whilst Wang et al. (2009) collected soil samples from apple orchards in China that had received Cu-based fungicide inputs for 5, 15, 20, 30 and 45 years. In both of these studies, despite clear evidence of Cu accumulation in the studied soils, the authors also found it challenging to elucidate any negative effects of Cu on soil enzyme activities due to the strong confounding influence of soil properties, i.e. TOC, pH. That it appears that regular long-term Cu-based fungicide use may inhibit phosphomonoesterase activity in soils is a warning that the continued use of these fungicides may cause lasting adverse effects to P cycling in agricultural soils. Phosphomonoesterase plays an important role in the conversion of unavailable organic P to plant available inorganic forms of P ([Tabatabai 1994](#)). This is important in an Australian context where the soils in the main agricultural areas are naturally low in P ([Young and Young 2001](#)). Long-term disruption of P cycling in agricultural soils is likely to result in the need to

apply more P based fertilisers to meet plant/crop requirements due to the loss of ecosystem service provide by soil micro-organisms.

5. Conclusions

Data from this study has shown that enzyme activities in vineyard soils with elevated Cu concentrations were generally lower than in soils from 'clean' reference sites. However, there is little evidence to indicate that elevated Cu concentrations alone are responsible for the decreases in soil enzyme activity. Rather the data shows that soil enzyme activity in the studied soils (where 90 % of the vineyard soils contained total Cu concentrations less than 115 mg/kg) are being driven mostly by physical-chemical soil properties, principally TOC, pH and particle size. The data does, however, indicate the elevated Cu concentrations in the studied soils are causing some inhibition of phosphomonoesterase activity. This is an early warning that the continued use of Cu-based fungicides may, at some point cause long-term harm to microbial mediated P cycling processes in vineyard soils. The challenge is now for industry and government to formulate appropriate policy decisions on the future management of Cu-based fungicide use; this should consider the assessment of alternative fungicides compounds to ensure that they will pose a lower environmental risk than Cu-based compounds prior to more wide spread adoption.

6. Acknowledgements

This study was supported by the Co-operative Research Centre for Contamination Assessment and Remediation of the Environment (CRC CARE) and the Department of Primary Industries Victoria, Australia (Project # 06889). We would like to thank

laboratory staff in the inorganic chemistry section at DPI Werribee, Anitha Kunhikrishnan and Kerrie Bell (University of South Australia) for assistance with chemical analysis of the soil samples, as well as Andrew Brastrup for assistance with collection of samples in the field. We also thank the participating grape growers for so kindly enabling us to collect soil samples from their vineyards.

Appendix. Supplementary material

Supplementary material related to this article can be found online at: ([details to be added later by journal](#)).

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Figure captions

Figure 1 Concentrations of total Cu in vineyard and reference site soils. Values are mean $\pm$ standard deviation. All regions, vineyards n = 60 and reference n = 21. Individual regions n = 20 for vineyards; reference soils, south east, SA n= 8, Grampians n = 7, Murray Valley n = 6

Figure 2 Enzyme activity of vineyard and reference site soils from the different regions (after 54 days incubation). Phosphomonoesterase (a), arylsulfatase (b), urease (c), phenol oxidase (d). All regions, vineyards n = 60 and reference n = 21. Individual regions n = 20 for vineyards; reference soils, south east, SA n= 8, Grampians n = 7, Murray Valley n = 6. For each regional grouping of soils, * denotes significant difference from reference soils ($P \leq 0.05$)

Figure 3 Relationship between the phosphomonoesterase activity and Cu concentrations of the soils. Phosphomonoesterase and total Cu (a); phosphomonoesterase and CaCl_2 extractable Cu (b); phosphomonoesterase/organic carbon and total Cu (c); phosphomonoesterase and total Cu as percentage of organic carbon (d). A linear regression (dotted line) is fitted where a significant relationship ($P < 0.05$) was found using data from all soils (n = 81). Data from south-east, SA is represented by hollow circles, Grampians by solid squares, and Murray Valley by grey triangles

Figure 4 Relationship between the arylsulfatase activity and Cu concentrations of the soils. Arylsulfatase and total Cu (a); arylsulfatase and total Cu as percentage of organic carbon (b). A linear regression (dotted line) is fitted where a significant relationship ($P < 0.05$) was found using data from all soils ($n = 81$). Data from south-east, SA is represented by hollow circles, Grampians by solid squares, and Murray Valley by grey triangles

Figure 5 Relationship between the urease activity and Cu concentrations of the soils. Urease and total Cu (a); urease and total Cu as percentage of organic carbon (b). A linear regression (dotted line) is fitted where a significant relationship ($P < 0.05$) was found using data from all soils ($n = 81$). Data from south-east, SA is represented by hollow circles, Grampians by solid squares, and Murray Valley by grey triangles

Figure 6 Relationship between the phenol oxidase activity and Cu concentrations of the soils. Phenol oxidase and total Cu (a); phenol oxidase and CaCl_2 extractable Cu (b). Data from south-east, SA is represented by hollow circles, Grampians by solid squares, and Murray Valley by grey triangles

815 **Figure 1**

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825 **Figure 3**

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Figure 4

Figure 5

Figure 6

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Title:

Effects of copper fungicide residues on the microbial function of vineyard soils

Date:

2013-03-01

Citation:

Wightwick, A. M., Salzman, S. A., Reichman, S. M., Allinson, G. & Menzies, N. W. (2013). Effects of copper fungicide residues on the microbial function of vineyard soils. ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH, 20 (3), pp.1574-1585. <https://doi.org/10.1007/s11356-012-1114-7>.

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