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The effects of copper hydroxide, captan and trifloxystrobin fungicides on soil phosphomonoesterase and urease activity

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Abstract

There is increasing community awareness of the potential environmental risks posed by Cu-based fungicide use, which is placing increasing pressure on governments and industry to undertake risk minimisation action. However, if there is going to be a widespread move away from the use of Cu-based fungicides, logically there needs to be assurance that the alternatives pose a lower environmental risk. To that end, this study compared the effect of copper hydroxide, captan and trifloxystrobin on soil enzymatic (phosphomonoesterase and urease) activity. Compared to an untreated control, copper did not inhibit either enzyme activity, even at the highest dose used in the study (156 mg/kg). At their respective high doses, captan (96 mg/kg) and trifloxystrobin (144 mg/kg) did not cause inhibition of phosphomonoesterase activity, but did inhibit urease activity. Consequently, the results from this study suggest that the copper hydroxide alternatives, captan and trifloxystrobin, do not pose a short term risk to P cycling processes in soil, although the results do suggest that these two are more toxic than copper hydroxide to N cycling processes in soil. Moreover, captan and trifloxystrobin compounds are unlikely to pose a long-term risk to soil microbial function as they are unlikely to persist in soil at concentrations found to cause an adverse effect on urease activity. Nonetheless the potential disruption to N cycling processes needs to be recognised and consideration given to limiting the annual applications of these fungicides, particularly around the timing of repeat fungicide applications, to prevent accumulation of the fungicides in surface soils.

Keywords Key words: copper, captan, trifloxystrobin, soil, microbial function, enzyme activity, microcosm

Introduction

Fungicides are widely used in horticultural productions systems, in particular vine and orchard crops, to control fungal diseases such as downy mildew (*Plasmopara viticola*), grey mould (*Botrytis cinerea*) and black spot (*Diplocarpon rosae*). Agricultural best practices include adoption of preventative management strategies to control the spread of diseases and this typically involves the regular application of fungicides throughout the growing season whether or not fungal infection is present (McConnell et al. 2003). Regular use of Cu-based fungicides in Australian vineyards has led to an increase in the concentrations of Cu in the surface soils (Wightwick et al. 2010a,b), albeit that the proportion of available Cu is relatively low.

There is increasing community awareness of the potential environmental risks posed by Cu-based fungicide use, which is placing increasing pressure on governments and industry to undertake risk minimisation action. For instance, organic agriculture standards currently limit annual Cu use to a maximum of 8 kg/ha (OIECC 2009). Moreover, whilst Cu-based fungicide compounds have recently being re-registered for use by the European Commission, member states are required to take steps to ensure that the rate and number of Cu-based fungicide applications are the minimum to achieve desired effects (EC 2009). However, if there is going to be a widespread move away from the use of Cu-based fungicides, logically there needs to be assurance that the alternatives pose a lower environmental risk, rather than a well intentioned change in agrochemical use leading to a transfer in risk rather than an intended reduction in risk.

There are currently 47 different fungicides (active ingredients) registered for use in Australian vineyards representing 16 different chemical classes (Wightwick et al. 2012). Like Cu, the majority of these fungicides are designed as preventative sprays to protect against disease infection before it occurs. Many current use synthetic fungicides were developed in the 1940 – 50's and are based on 'old fungicide chemistry'; these have broad-spectrum multi-site modes of action and are applied at high application rates (1.5 – 3.5 kg/ha), e.g. the dithiocarbamates and phthlamides (Russell 2005). Other fungicides developed since 1970 are based on 'modern fungicide chemistry'; these have specific modes of action, greater activity and are applied at relatively low application rates (0.13 – 0.25 kg/ha; Russell 2005).

There have been few studies of the impact of fungicides on soil enzyme activity, with contradictory effects reported. For instance, one alternative to Cu-based fungicides, mancozeb, has been reported

to both cause a reduction in populations of fungi and actinomycetes and an inhibition of nitrification, as nwell as to stiumlate bacterial populations (Hussain et al. 2009; Lo 2010). The effect of captan on soil microbial activity has been more widely studied and has generally been reported to cause pronounced effects on soil microbial activity and N dynamics (Chen and Edwards 2001; Chen et al. 2001; Hussain et al. 2009), including adverse effects such as a reduction in populations of fungi, nitrifying and N_2 fixing bacteria as well as inhibition of biochemical processes including nitrification rates and activities of the enzymes nitrogenase, dehydrogenase, cellulose and phosphatase and in some instances urease (Chen and Edwards 2001; Chen et al. 2001; Hussain et al. 2009; Piotrowska-Seget et al. 2008). In contrast, there has been a paucity of studies investigating the effects of 'modern' strobilurin fungicides to microbial activity, although azoxystrobin has been reported to reduce dehydrogenase activity (Lo 2010).

Most studies investigating the effects of pesticides 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 pesticide is applied and incorporated into the soil (Bååth 1989; Giller et al. 2009). The use of such 'artificially' contaminated soils has a number of limitations. Firstly, the availability (and toxicity) of fungicides may be greater in spiked soils compared with field soils (Oorts et al. 2006). Secondly, in spiking experiments, large doses of chemical tend to be added in single applications, which may have a 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 stress. Conversely, repeated applications of relatively small doses of fungicides in vineyards may increase total chemical concentration of the soil gradually over many years. This may have a relatively minor immediate impacts on the microbial community (from which microbial communities are able to recover), and provide plentiful opportunity for the soil micro-organisms to adapt to the chemicals, producing microbial communities more tolerant to chemicals than the microbial communities in 'artificially' contaminated soils (Giller et al. 1998; Brandt et al. 2010; Mertens et al. 2010).

Using 'real-world' soils in risk assessment studies also presents challenges, particularly when comparing soils from different properties and regions, because soil microbial function is influenced by regional and site specific climatic conditions (e.g. rainfall) and farm management practices (e.g. irrigation regimes, cover cropping; Giller et al. 1998). Moreover, compared to spiked soil experiments, the field soils have mostly likely received inputs of other, often unknown chemicals. These factors can make it challenging to determine whether differences in observed enzyme activity

are due to the accumulated fungicide or whether they are due to site factors such as climate, management practices, and the use of other pesticides. One approach to overcome this is to conduct soil microcosm experiments (Burrows and Edwards 2002; Schaffer et al. 2008), whereby soils are collected from different sites in the field and then allowed to equilibrate for a period of time under common environmental conditions. This can enable a more real-world approach to assessing the risks of a fungicide on soil microbial function. To enable better comparison of the extent and nature of effects of Cu and alternative fungicide compounds on soil microbial activity, research needs to incorporate these fungicides within common experiments utilising consistent soil types and methodologies. This will enable a fairer comparison of the relative risks thus producing information that will be more useful to decision-makers in industry and policy. To that end, the aim of this study was to determine the effect of applications and the relative risks of copper hydroxide and two synthetic organic compounds; captan (representing 'old' fungicide chemistry) and trifloxystrobin (representing 'modern' fungicide chemistry) on soil enzymatic activity.

Materials and methods

Test soil

A bulk surface soil sample (0 – 5 cm depth) (approximately 10 kg) was collected from an 'uncontaminated' reference location (reserve grassland) at Drysdale in Victoria, Australia (38° 10' S, 144° 34' E). Following collection, the soil was gently passed through a 4 mm sieve and mixed. A sub-sample of the bulk soil was dried and ground to < 2 mm for analysis of total metal concentrations and selected physical-chemical soil properties according to the methods described in Wightwick et al (2010c). In short, the soil was a sandy clay loam of $pH_{(1:5water)}$ 5.8, 5.8% organic matter, 3.1% organic carbon, electrical conductivity (EC) 0.1dS/cm, and cation exchange capacity (CEC) 8.5 cmol/kg. The soil contained <0.1 mg/kg (dry weight (d.w.)) Cu, along with 8.9, 0.8, 7.4, 2.1, 14 and 71 mg/kg (d.w.) total As, Cd, Cr, Ni, Pb and Zn. The bulk soil was not analysed for a broader range of contaminants for financial reasons, and also because it was considered unlikely to be contaminated with fungicides as there is no agricultural and/or industrial activity within 2km of the site.

Prior to being used in experiments, aliquots of the bulk soil were moistened up to 40 % of the maximum water holding capacity (WHC) by the addition of deionised water (Millipore, Molsheim,

France). The soil was then allowed to incubate in a plastic bucket for 7 days at 20-22^{o}C, during which time additions of deionised water were used to maintain the soil at 40 % WHC. For each of the two experiments, portions of the pre-incubated bulk soil were divided into a series of 500 g (dry weight equivalent) aliquots which were randomly assigned to the different experimental fungicide negative control treatments.

Treatment of soil with fungicides

Three fungicides were used in this study: stabilised technical grade copper (II) hydroxide (Sigma-Aldrich, St Louis, MO, USA), analytical grade trifloxystrobin (methyl (*E*)-methoxyimino-((*E*)-α-[1-(α,α,α-trifluoro-m-tolyl)ethylideneaminooxy]-o-tolyl)acetate) and captan (*N*-(trichloromethylthio) cyclohex-4-ene-1,2-dicarboximide) (both from Fluka Analytical; ≥ 99.5%; Sigma-Aldrich, St Louis, MO, USA). Copper hydroxide was selected to represent a traditional Cu-based fungicide that has been in use for over 50 years in many parts of the world. Captan was selected to represent an alternative fungicide compound with a broad-spectrum multi-site mode of toxic action that has been in use for 50 – 60 years. Trifloxystrobin is a modern alternative fungicide compound, with a specific mode of action, and has been in use for the last 10 – 15 years.

Two separate experiments were conducted to assess the effect of the three fungicides on the enzyme activity of the soil. In both experiments, treated soils were compared to a control soil which did not receive fungicide treatments. Application rates were determined based on the typical mixing rates (i.e. g/100 L) for grapes stated on product labels for the fungicide formulations, and recommended application rates (i.e. L/ha) for dilute spraying of grapevines (CRCV 2005). It was also assumed that all of the fungicide applied ends up on the ground and becomes incorporated into the top 5 cm of surface soil.

Experiment 1: Single application of fungicide at an environmentally realistic soil concentration. In this case, the soil was exposed to low nominal application rates of captan and trifloxystrobin (1.0 and 0.1 mg/kg); and a medium application rate of copper hydroxide (16 mg/kg). The Cu applied represented a rate equivalent to a full growing season's worth of fungicide applications, based on five fortnightly applications. However, there were no statistically significant differences in phosphomonoesterase and urease activity between any of the treatments in Experiment 1 (Figures 1(a) and 2(b)). Therefore, a further experiment was conducted using higher application rates of fungicides.

Experiment 2: Single application of fungicide and high soil concentration. In this case, the soil was exposed to medium and high rates of copper hydroxide (16 and 156 mg/kg), captan (10 and 96 mg/kg) and trifloxystrobin (1.4 and 144 mg/kg). The high dose was in part chosen to represent an extreme, worst case scenario and, in part, to ensure that some degree of toxic effect would be observed in the experiment.

All of the fungicide compounds have low solubility in water making addition to the soil as an aqueous solution impractical, therefore a carrier was required. For captan and trifloxystrobin the required quantity (dose) of fungicide was first dissolved in a small volume (< 5 ml) of acetone (≥ 99.9%; Sigma-Aldrich, St Louis, MO, USA) before being made up to 50 ml with deionised water. Copper hydroxide has low solubility in acetone, so the required quantity of copper hydroxide was firstly mixed with 10 g of fine dry washed quartz sand (OECD 2000). The captan and trifloxystrobin solutions and the copper hydroxide sand mixture were then added to the respective aliquot of moist soil (40 % WHC). To ensure consistency of treatment, 10 g of sand was also added to the captan, trifloxystrobin and control treatment soils. For the same reason, the appropriate volumes of acetone were added to the treatments so that each soil received the same quantity of acetone. The final acetone concentration in the treatment soils was < 1 %. The soil samples were then thoroughly mixed and additional volumes of ultra pure water added to bring each of the soil treatments up to 60 % WHC. Aliquots (~ 40 g) of each treated soil were then transferred into 65 ml amber glass jars to create 15 replicates for each chemical/control treatment. These samples were then incubated in the dark at 20 – 22^{o}C, loosely covered to minimise evaporation losses whilst ensuring air exchange. For each treatment, five replicates were sampled on days 1, 3 and 7 following fungicide application. The soil was transferred from the amber glass jars to plastic zip-lock bags and stored at 4^{o}C until further processing. The microbial function of the soil samples was determined by measuring the activity of the phosphomonoesterase and urease enzymes. A short 7 day exposure period was used in this study because both captan and trifloxystrobin degrade rapidly in soil (half-lives < 10 days). Thus the magnitude of any adverse effects to soil microbial function is likely to be greatest in the short-term.

Enzyme activity assays

Soil enzyme activities were chosen over other more commonly used measures of microbial function (e.g. respiration, nitrification) as they are generally more sensitive to elevated concentrations of metals and environmental changes, enable more specific measures of microbial activity that are

relevant to the function of agricultural soils (i.e. related to specific nutrient cycles in the soil), and the assays are relatively cost-effective and rapid which enabled us to assess a greater number of sites and samples. The trade-off using this approach being that the use of the soil enzyme activity approach does not allow a distinction between the effect of fungicide on the size/composition of the soil microbial community (which influences the production of enzymes) and the direct inhibitory effect of fungicide on the enzymes.

Phosphomonoesterase (E.C 3.1.3) and urease (EC 3.5.1.5) enzyme activity tests were conducted 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. This 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 and protect them from inhibitory effects from Cu (Speir and Ross 2002; Tabatabai and Dick 2002). Secondly, pH 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 activity was expressed as μg *p*-nitrophenol/g dry soil/h and urease activity as $\mu g/NH_4$ produced/g dry soil/h.

Data analysis

Statistical analyses were conducted using GenStat version 12.1.0.3278 (VSN International Ltd). When assessing for differences between fungicides treatments, data did not conform to the assumptions of homogeneity and homoscedasity required for associated with 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. Unless otherwise stated, statistical significance for all data analysis was at the 95 % confidence level ($\alpha = 0.05$).

Results and Discussion

Several studies have indicated that the long-term use of Cu-based fungicides may disrupt P cycling in receiving soils (Fernández-Calviño et al. 2010; Wang et al. 2009). In comparison, this study's high dose experiment (Experiment 2) found that captan and trifloxystrobin did not cause inhibition of phosphomonoesterase activity, even under worst-case application scenarios (Figures 1(b)). Rather, the high application rate of trifloxystrobin caused a significant increase in phosphomonoesterase activity 1 day after application, although this effect was not apparent in the Day 3 and Day 7 samples (Figure 1(b)). This may be an indication of a direct toxic effect on soil micro-organisms resulting in a short-term increase in enzyme concentration due to their release from dying cells or be related to a stimulation of microbial growth due to an additional carbon source provided by applied trifloxystrobin. On the other hand, the results from this study did show that the medium and high application rates of captan and trifloxystrobin caused disruption to N cycling in the soil because urease activity was inhibited by captan and trifloxystrobin (Figure 2(b)). The high application rate of captan had the greatest effect on urease activity causing 40 – 70 % inhibition of enzyme activity compared to the untreated control soils for all sampling days. The medium application rate of captan caused some inhibition (~ 10 %) on Day 7 only. Urease activity for both the medium and high application rate of trifloxystrobin was significantly lower than the untreated control soil (Figure 2(b)) on Days 3 and 7. However, unlike captan the was no evidence of increased severity of this effect with increased application rate as both medium and high applications cause inhibition of urease activity in the range of 15 – 25 %. Moreover, the medium and high treatments used in this study in effect represented the additive effect of multiple applications of these fungicides and because these synthetic organic fungicide compounds are readily degraded in soil, it is unlikely that captan and trifloxystrobin will ever be present at these concentrations in the soils of vineyards, and so captan and trifloxystrobin pose a low risk to phosphomonesterase and urease activity in vineyard soils.

There have been few reported studies on the effect of captan and trifloxystrobin on phosphomonoesterase and urease activity with which to compare the results from this study. Unlike the present study, there has been some reported inhibition of phosphomonoesterase by captan (Chen et al. 2001; Piotrowska-Seget et al. 2008). However, this was observed at much higher application rates (≥ 125 mg/kg) than used in the present study. Captan has previously been reported to cause adverse effects to the N dynamics of soil (Chen and Edwards 2001; Chen et al. 2001; Hussain et al. 2009; Uyanoz et al. 2005). This is consistent with the inhibition of urease activity observed in this

study. Furthermore, varying responses of captan towards urease have been reported in the literature. Like this study, Uyanoz et al. (2005) reported strong inhibition of urease activity at concentrations of captan ≥ 100 mg/kg, whereas Chen et al. (2001) reported that urease was stimulated in response to 125 mg/kg captan.

This study used a single soil type and methodology, so it is challenging to compare the relative risks of these different fungicides, particularly as enzyme activity is influenced greatly by physical-chemical soil properties (Taylor et al. 2002), and it is known that the toxicity of pesticides can vary widely across different soils (Bending et al. 2007; Hussain et al. 2009; Lo 2010; Wightwick et al. 2010a). For instance, Bending et al. (2007) reported that applications of azoxystrobin, tebuconazole, and chlorothalonil affected the size and structure of microbial communities in a soil with low organic matter but not in a comparable soil with high organic matter. Moreover, the interactive effects of Cu on the toxicity of captan and trifloxystrobin to soil microbial functions are not known. The toxicity of the alternative synthetic organic fungicide compounds may be increased if there are additive or synergistic effects with Cu. Finally, exposure to prolonged Cu stress may alter the susceptibility of soil microbial communities to the additional stress caused by applications of these synthetic organic fungicide compounds. Whilst outside of the scope of the current study, this should be the subject of further research as vineyard soils receiving these fungicide compounds will most likely already have elevated concentrations of Cu in the surface soils.

Conclusions

The results from this study suggest that the copper hydroxide alternatives, captan and trifloxystrobin, do not pose a short term risk to P cycling processes in soil, although the results do suggest that captan and trifloxystrobin are relatively more toxic than copper hydroxide to N cycling processes in soil. Moreover, captan and trifloxystrobin compounds are unlikely to pose a long-term risk to soil microbial function as they are unlikely to persist in soil at concentrations found to cause an adverse effect to urease activity. Nonetheless the potential disruption to N cycling processes needs to be recognised and consideration given to limiting the annual applications of these fungicides, particularly around the timing of repeat fungicide applications, to prevent accumulation of the fungicides in surface soils. To some extent viticultural industries are already self-regulating this with industry guidance recommending restrictions on annual applications and consecutive sprays of captan and trifloxystrobin to prevent fungicide resistance (AWRI 2010).

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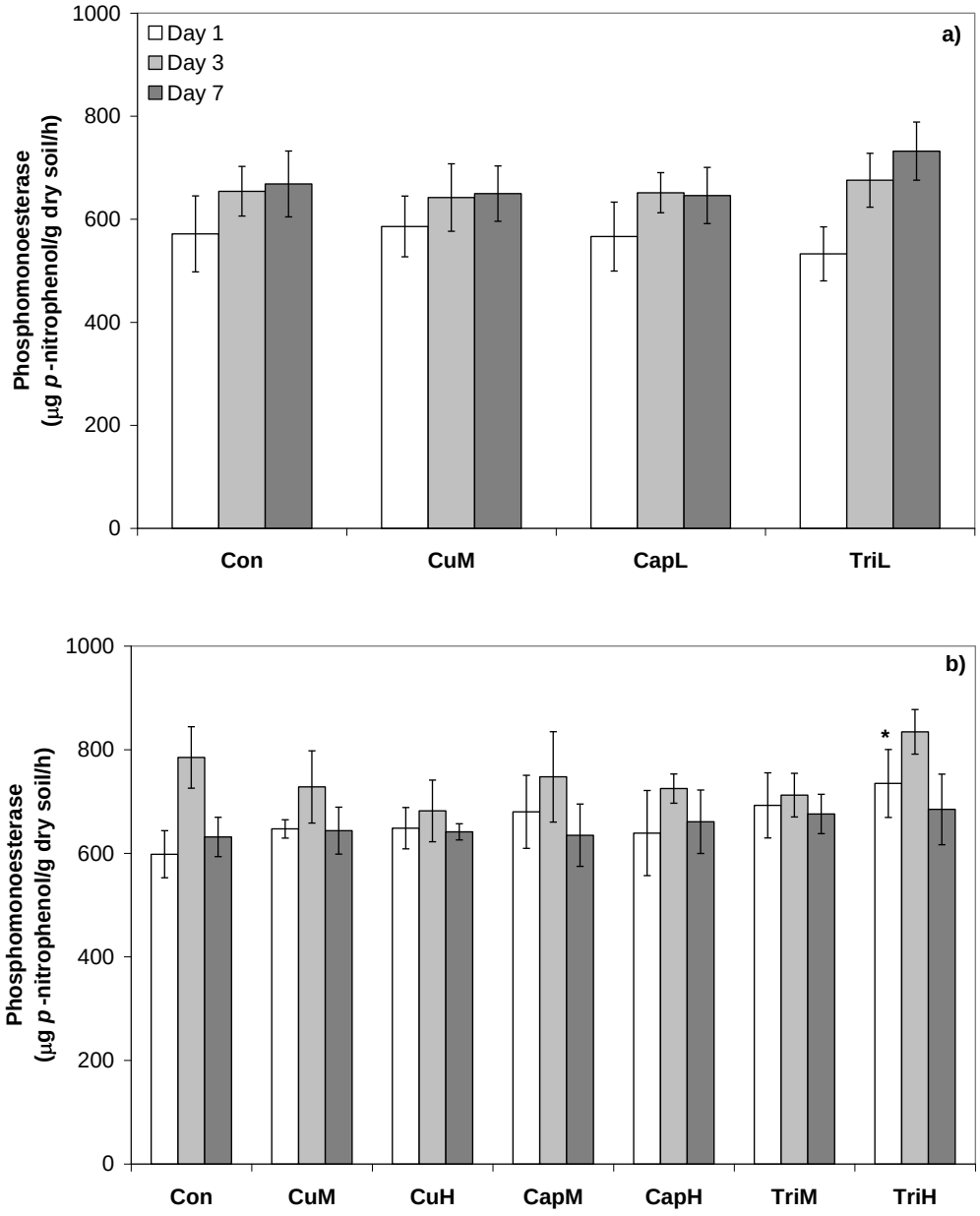

Figure 1 Phosphomonoesterase activity in the fungicide treated soils: (a) Experiment 1 and (b) Experiment 2. Error bars are means ± standard deviation (n = 5). Significant difference ($P \leq 0.05$) from the untreated control soil is denoted by * for Day 1 samples (Con = Control; Cu = Cu; Cap = Captan; Tri = Trifloxystrobin; L = low application rate; M = medium application rate; H = high application rate.)

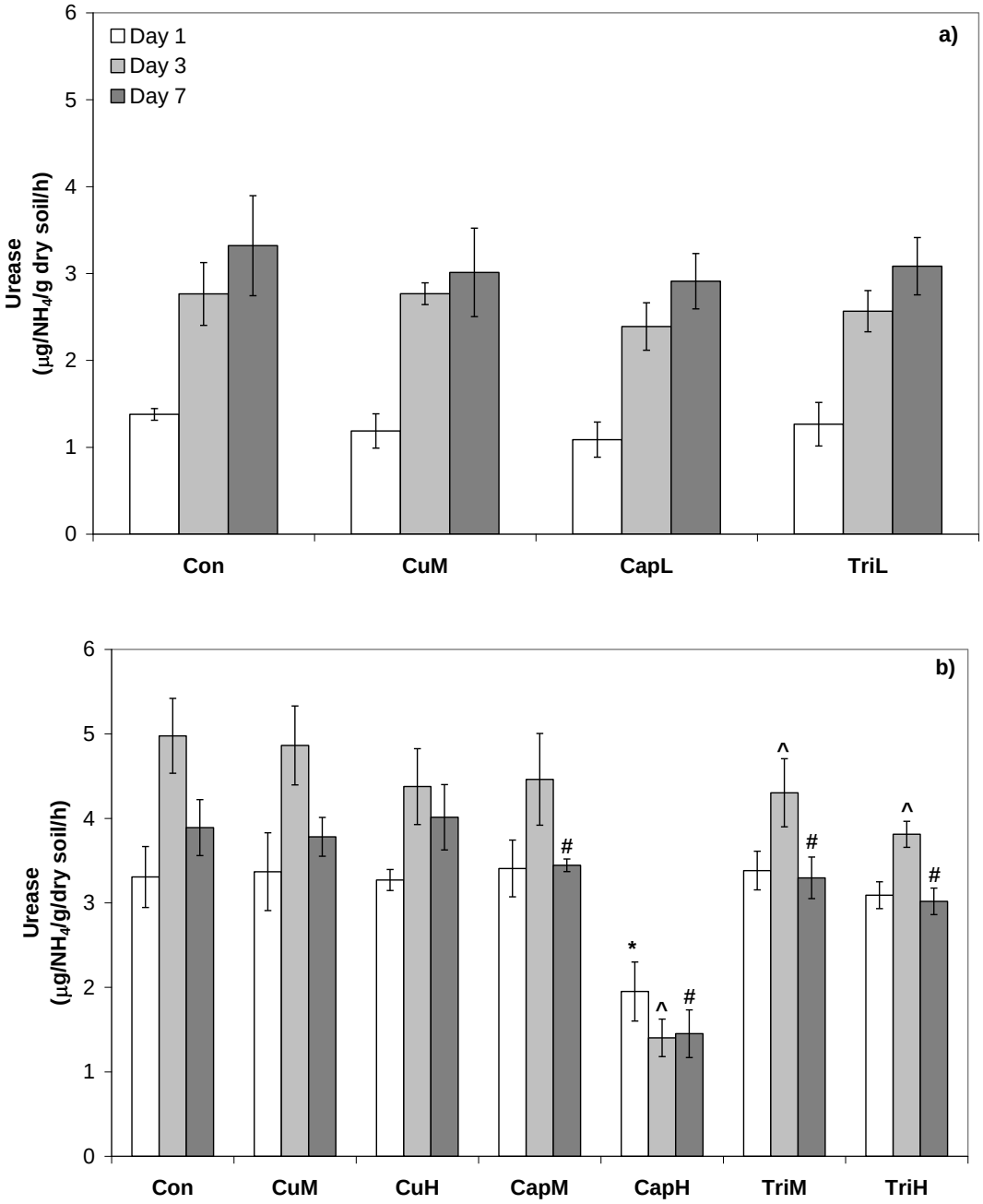

Figure 2 Urease activity in the fungicide treated soils: (a) Experiment 1 and (b) Experiment 2. Error bars are means ± standard deviation (n = 5). Significant difference ($P \leq 0.05$) from the untreated control soil is denoted by * for Day 1 samples, ^ for Day 3 samples, and # for Day 7 samples. (Con = Control; Cu = Cu; Cap = Captan; Tri = Trifloxystrobin; L = low application rate; M = medium application rate; H = high application rate).