Influence of copper on soil microbial biomass and biodiversity in some NSW soils.

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Abstract
Copper is one of the essential elements for all living organisms; however it becomes toxic at concentration exceeding certain limits. Eight surface soils (0-15 cm) including one Ferrosol (Robertson), two Tenosols (Catombal, Windsor), two Kurosols (Somersby, Box Hill), one Sodosol (Pine), one Chromosol (Brinsley) and one Kandosol (Lucerne), were collected from mainly pasture sites in NSW. The soils had different physico-chemical properties and there were some differences between the sites in climatic conditions. Copper was added as $Cu(NO_3)_2.\ 4H_2O$ salt to 1 kg oven-dry equivalent soils at six different levels (0, 60, 150, 300, 600, 1500 mg Cu kg^{-1} dry soil) in polythene bags and samples were incubated at room temperature. Soil microbial biomass carbon (MBC) was estimated by the chloroform-fumigation (CF) extraction method, and substrate utilization patterns determined by the Biolog method were used to assess the functional diversity of the microorganisms in these soils after 45 and 270 days of incubation. The extractable Cu in soils was determined using EDTA and $CaCl_2$ solutions at each sampling time. After 45 days of incubation, soil microbial biomass and diversity in soils were significantly affected from 300 mg Cu kg^{-1} soil in all studied soils. However after 270 days of incubation, significant effects were observed at all Cu concentration in all the studied soils. Soil properties such as pH, organic carbon and clay content affected the Cu toxicity to soil microbes.

Key Words
Microbial biomass, diversity index, richness, evenness.

Introduction

There have been increasing concerns in Australia and world wide about heavy metal contamination of soil by urban wastes and by-products of rural, industrial, and agricultural activities. The effective toxicity of heavy metals to soil ecosystems depends not only on their total metal concentrations and soil properties, but also, and perhaps more importantly, on their biochemical speciation and available form (Singh 2002). Heavy metals affect growth, morphology and metabolism of microorganisms in soils, through functional disturbance, protein denaturation or the destruction of the integrity of cell membranes (Leita *et al*. 1995). Copper is one of the essential elements for all living organisms; however it becomes toxic at concentration exceeding certain limits. It is required for the functioning of more than 30 enzymes, all of which are either redox catalysts or dioxygen carriers (Wright and Welbourn 2002). Soil bacteria and fungi have mechanisms for transporting Cu into cells. Copper transfer between proteins protects the organism from the toxic effects of inappropriate Cu binding and to deliver the metal to the correct enzymes (Pufahl *et al*. 1997).

Major sources of Cu entering into the environment are extraction from its ore (mining, milling, and smelting), agricultural activities, and waste disposal (Ross 1994). Soils have become contaminated with Cu by deposition of dust from local sources such as foundries and smelters, as well as from direct application of fungicides and sewage sludge. Olszowy *et al*. (1993) observed 466 mg Cu kg^{-1} soil in Australian urban soils, where Tiller (1992) observed 360 mg kg^{-1} soil at one of the Sydney contaminated site. The National Environmental Protection Measure has set the Ecological Investigation Levels (EILs) for soil Cu concentration at 100 mg kg^{-1} (Interim Urban) in Australia in 1999 as trigger for remediation. Generally, heavy metals in soil exist in soluble and exchangeable forms and as organic and inorganic complexes. Different forms of heavy metals have different mobilities and, therefore, different bioavailabilities. Adsorption and desorption strongly affect bioavailability of heavy metals and therefore toxicity in soil. However, measured availability of metal ions also depends on pH, ionic strength of the extractant, contact time between metal ion and soils and on the competition with other metals ions as well as presence of inorganic or organic ligands (Renella *et al*. 2004). In the soil solution, Cu^{2+} ions compete with more abundant soil cations such as Ca^{2+} and Na^{+} for exchange sites.

Several authors have studied effects of Cu on soil microbial communities. Kandeler *et al*. (1996) observed that microbial biomass, enzyme activity, and functional diversity of soil communities decreased with increasing Cu pollution at 100 mg kg^{-1} Cu in three soils from Austria. Microbial biomass and metabolic quotient were also found to decrease with 112-182 mg Cu kg^{-1} soil in the UK by Khan and Scullion (2000). However, to our knowledge no such research has been carried out on Australian soils, which commonly containing variable charge minerals and have relatively lower organic matter content. In this study, the microbial biomass and diversity were measured in some Cu spiked soils from NSW.

Materials and methods

Soil sampling

Eight surface soils from Windsor, Somersby, Robertson, Catombal, Brinsley, Pine, Lucerne and Box Hill in New South Wales were used for this study. The soils represent a range of physico-chemical properties and climatic zones of NSW and were collected down to a depth of 10-15 cm, avoiding major roots as much as possible. At each site, 5-7 samples were taken from different places which were bulked to obtain a representative sample for the site. The samples were sieved through a 2 mm sieve except for the Robertson soil, which was sieved through a 3.75 mm sieve due to its high moisture content. Copper was added as $Cu(NO_3)_2$. $4H_2O$ salt to 1 kg oven-dry equivalent soils at six different levels (0, 60, 150, 300, 600, 1500 mg Cu kg^{-1} dry soil) in polythene bags and samples were incubated at room temperature. Copper was added in dilute solution to obtain a homogenous distribution in soil. Microbial biomass and microbial diversity of soils were measured after 45 and 270 days of incubation. The extractable Cu in soils was determined using EDTA (Clayton and Tiller 1979) and $CaCl_2$ (Oliver *et al*. 1999) solutions at each sampling time.

Soil chemical analysis

Subsamples of the sieved soils were air-dried for the analyses of physical and chemical properties. Standard laboratory methods as described by Banu *et al.* (2004) were used to measured soil pH, soil moisture, electrical conductivity, particle size distribution and cation exchange capacity. Total C and N were determined using finely ground (<250 μm) subsamples by the dry combustion method using a LECO CHN 1000 analyser. Since there was no $CaCO_3$ in any of the samples studied, the total C represents the organic C contents of the samples. Free iron in samples was determined by the citrate-bicarbonate-dithionate method (Mehra and Jackson 1960).

Soil Microbiological analysis

Soil microbial biomass carbon (MBC) was measured by the chloroform-fumigation extraction method as described by Vance *et al.* (1987) and microbial diversity was evaluated using the Biolog Ecoplates method (Yan *et al.* 2000). EcoPlates were incubated at 30° C and the colour intensity in the wells was measured as absorbance at 590 nm using a microplate reader (Labsystem, Multiskan, Ascent) after 120 h of incubation. The average well colour development (AWCD) in each Biolog plate was determined and was used to evaluate microbial functional diversity, the Shannon diversity index (H′), substrate richness (S) and substrate evenness (E) were calculated (Zak *et al*. 1994).

Statistical analyses

MBC, H′, S and E values for each soil were analyzed by one-way analysis of variance (ANOVA), and significant differences were determined by student t tests using JMP software (SAS Institute, 2000, version 4). Linear regressions were used to test relationships between various soil and microbial properties. For the ease of discussing the results, the treatments are called according to their respective concentrations Cu60, Cu150, Cu300, Cu600, and Cu1500 representing Cu concentrations 60, 150, 300, 600, and 1500 mg Cu kg^{-1} soil, respectively.

Results

Soil analysis

Relevant physical and chemical properties of the soil are given in Table 1. The Robertson soil contained the highest moisture (50%) and clay (33%), organic C (5.96%) and total N (0.45%), free iron (7.06%), and cation exchange capacity (133.5 $mmol_c$/kg), whereas the Brinsley soil had the lowest moisture content (5%), organic C (1.38%) and total N (0.07%). The Box Hill soil had the lowest pH (4.88), clay (10%), and cation exchange capacity (36.5 $mmol_c$/kg). The highest EC was in the Brinsley soil (359 μS/cm) and the lowest percentage of free iron (0.31%) in the Lucerne soil. Organic C in the studied soils

was positively correlated with clay ($r^2 = 0.63$), moisture content ($r^2 = 0.92$), rainfall ($r^2 = 0.73$), total N ($r^2 = 0.61$), and free iron ($r^2 = 0.76$).

Soil microbial biomass

Soil MBC was measured 45 and 270 days after adding Cu to the soils. Analysis of variance of microbial biomass carbon showed no significant differences between Cu60, Cu150, Cu300 and the control in all soils except the Lucerne soil after 45 days of incubation ($LSD_{0.05} = 55.3$, n = 144) (Figure 1). Highly significant decreases in MBC were observed with Cu600 and Cu1500 treatments in comparison to the control after 45 days of incubation in all soils except Catombal and Pine soils (Figure 1). After 270 days of incubation MBC in all Cu treatments showed significant decreases compared to the control in all soils except Lucerne ($LSD_{0.05} = 32.8$, n = 144). In Lucerne soil, significant differences in MBC were observed in Cu300, Cu600 and Cu1500 compared to control.

Table 1. Relevant physico-chemical properties of the soils used in this study .

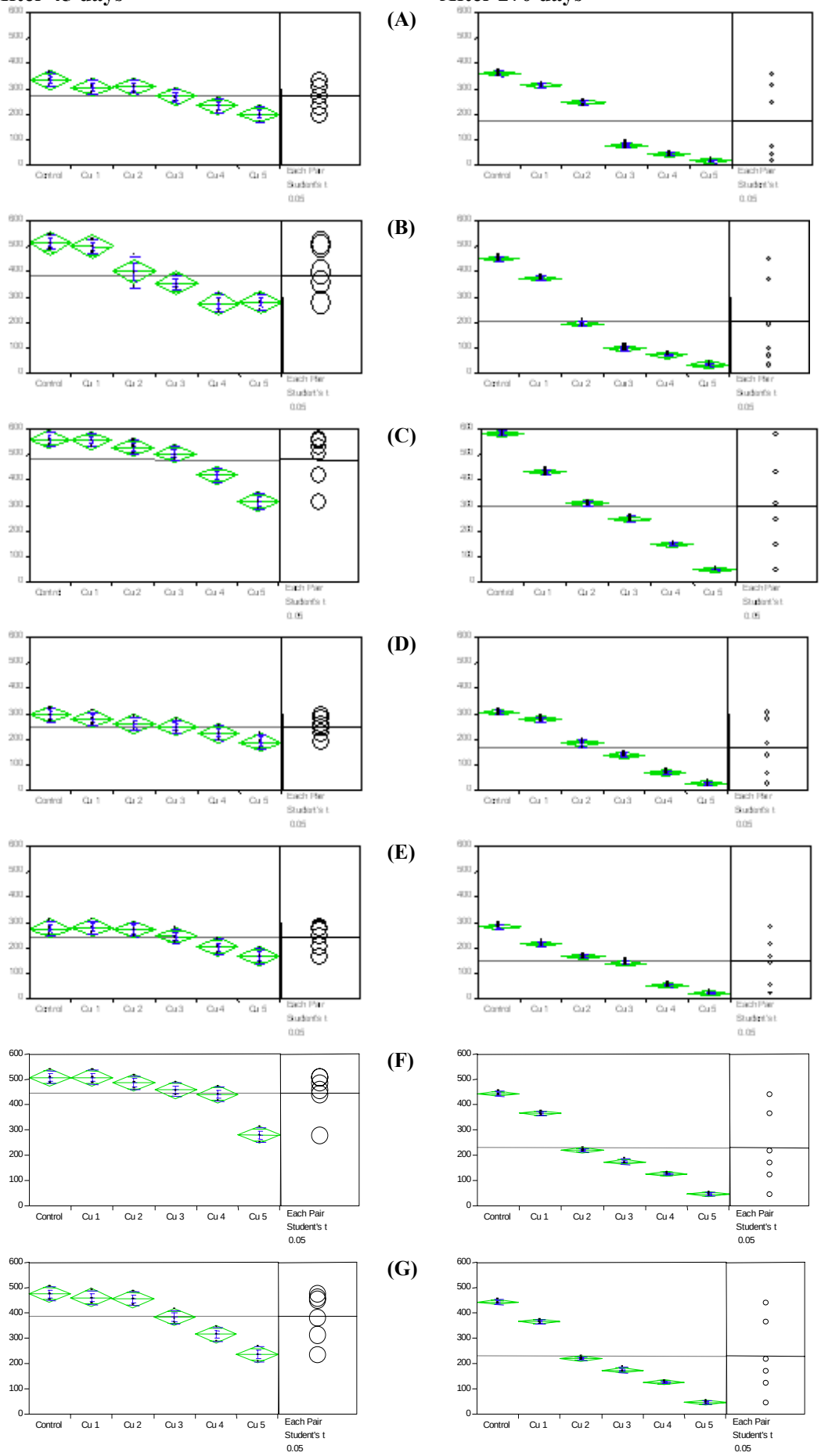
Soil name	Soil pH (1:5 water)	Gravimetric moisture (%)	EC (µS/cm) (1:5)	Sand / Silt / Clay (%)			Total C (%)	Total N (%)	Free iron (%)	CEC ($mmol_c$ /kg)
Windsor	6.98	10.8	230	75	12	13	2.15	0.16	1.07	94.1
Somersby	5.78	23.0	93	75	8	17	3.84	0.13	2.02	36.2
Robertson	5.50	49.8	123	30	37	33	5.96	0.45	7.06	133.5
Catombal	6.18	6.0	132	58	28	14	2.01	0.15	0.80	79.1
Brinsley	5.34	5.0	359	56	24	20	1.38	0.07	0.85	70.7
Pine	5.43	17.5	86	69	15	16	2.16	0.28	1.42	64.9
Lucerne	5.13	20.0	186	62	18	20	3.18	0.27	0.31	76.9
Box Hill	4.88	17.5	111	77	13	10	2.16	0.17	0.40	36.5

Soil microbial diversity

Average well colour development (AWCD) of four groups of substrates decreased gradually with increasing levels of Cu after 45 days of incubation, but the decrease was not statistically significant with Cu60 and Cu150 treatments in all soils (Figure 2). The amino acids substrate group was the most utilized group in Box Hill, Brinsley, Robertson, Somersby and Windsor soils, whereas the carbohydrates group was most utilized in Lucerne, Pine and Catombal soils. The carboxylic acids group was the least utilized group in all soils. At the second sampling (i.e. 270 days of incubation) AWCD dropped significantly even with the Cu60 concentration in all soils compared to control. Amino acids and carboxylic acids groups were the least utilized groups by soil microbial communities of the studied soils after 270 days of incubation. No colour developed in the wells that contained these two substrate groups in most of the soils, particularly with Cu1500, except Robertson, Lucerne and Pine soils.

H′ values in all treatments were significantly different from each other in Robertson, Catombal, Brinsley, Pine, and Lucerne soils at both sampling times except few observations. For example, after 45 days of incubation, no significant decreases were observed between Cu60 and Cu150 treatments in all soils except the Robertson soil, and between Cu150 and Cu300 treatments in the Pine soil. After 270 days of incubation, H′ values showed highly significant decreases with all Cu treatments than control ($LSD_{0.05} = 0.15$, n = 144). However, H′ did not significantly decrease in Cu150 and Cu300 treatments of Catombal, Box Hill and Lucerne soils. The highly significant decreases of H′ values were observed with increased level of Cu and the incubation time. Substrate evenness (E) values were significantly decreased in all Cu treatments after 45 days of incubation except the Cu60 treatment in Somersby, Lucerne and Box Hill soils ($LSD_{0.05} = 0.03$, n = 144).

After 45 days **After 270 days**



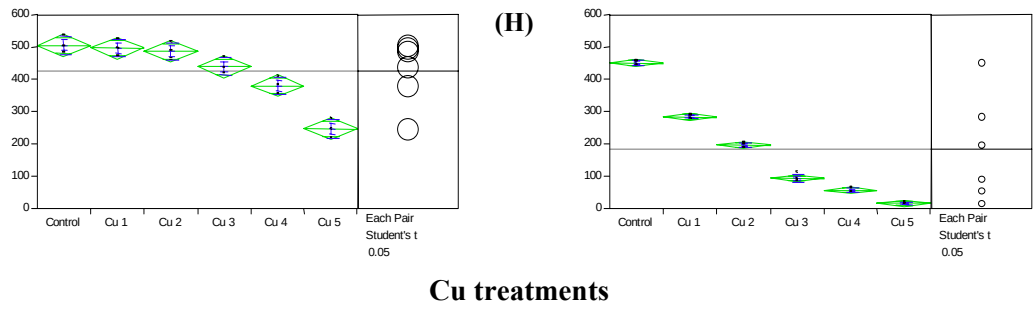


Figure 1. Effects of Cu treatments on microbial biomass carbon (mg kg^{-1}) in the studied soils after 45 and 270 days of incubation with different Cu concentrations. Cu 1 = 60, Cu 2 = 150, Cu 3 =300, Cu 4 = 600 and Cu 5 = 1500 mg kg^{-1} soil, respectively. A= Windsor, B= Somersby, C= Robertson, D= Catombal, E= Brinsley, F= Pine, G= Lucerne, and H= Box Hill.

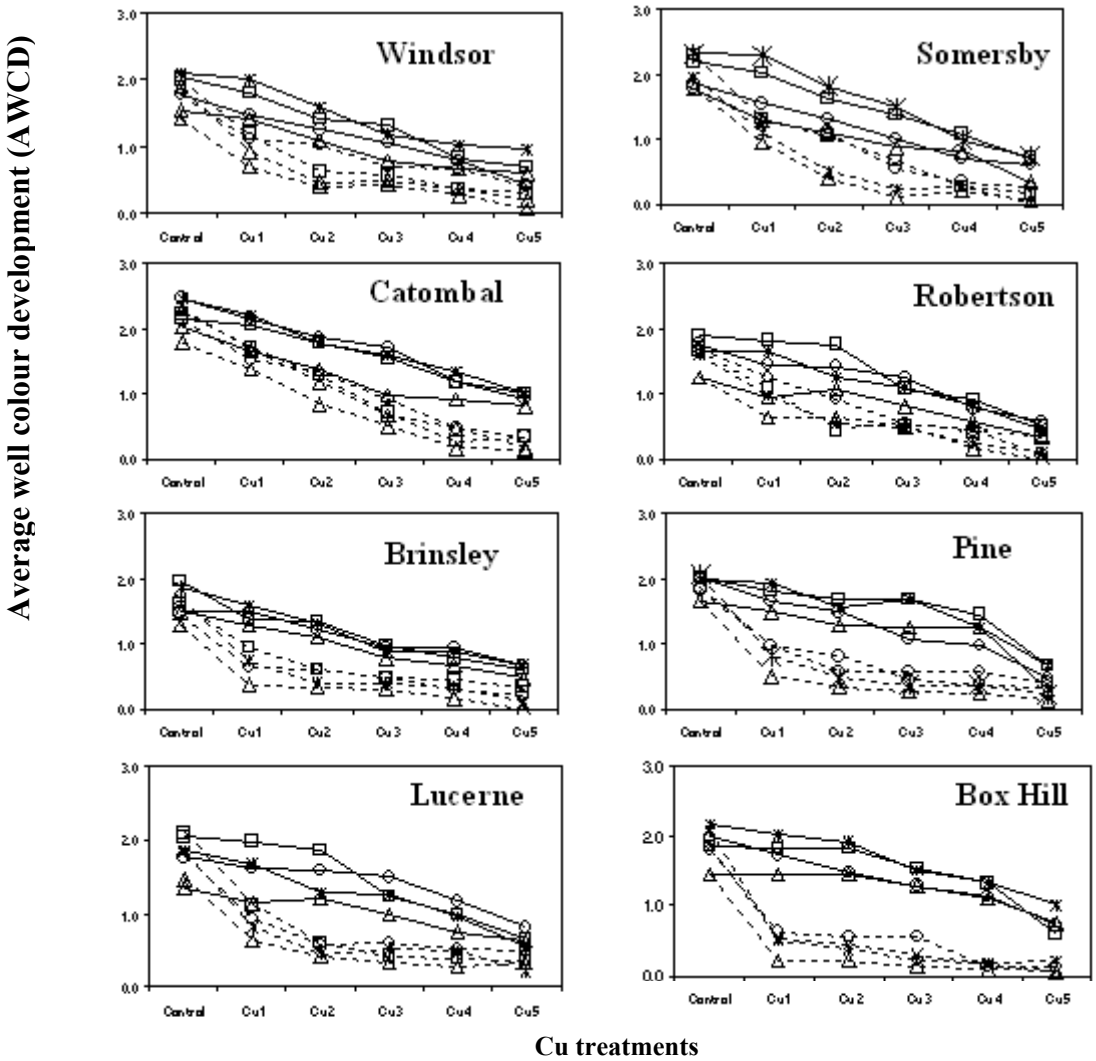


Figure 2. Substrate utilization patterns after 45 (—) and 270 (…) days of incubation in Cu spiked soils. □ = carbohydrates group, Δ = carboxylic acids group, * = amino acids group, and o = miscellaneous substrate group.

Extractable Cu in the soils

The EDTA- and $CaCl_2$-extractable Cu was measured in soils after 270 days of incubation to be between 314.4 and 382.2 mg kg^{-1}. Box Hill soil had the maximum amount of EDTA-extractable Cu (382.2 mg kg^{-1}) and the Lucerne soil had the least amount of Cu (314.4 mg kg^{-1}). Extractable Cu in all treatments was significantly different from each other ($LSD_{0.05}$= 12.6) in the studied soils. EDTA-extractable Cu showed no significant differences ($LSD_{0.05}$= 9.2) between Box Hill and Pine soils, between Pine and Catombal

soils, between Catombal, Pine and Somersby soils, and between Brinsley, Lucerne and Windsor soils. The $CaCl_2$-extractable Cu in soils was between 14.2 and 240.0 mg kg^{-1}. The Box Hill soil had the maximum amount of $CaCl_2$-extractable Cu (119.7 mg kg^{-1}) and the Brinsley soil had the least amount of Cu (57.0 mg kg^{-1}). $CaCl_2$ extractable Cu in all treatments showed significant differences between all treatments ($LSD_{0.05}$= 3.9). A highly positive correlation was observed between EDTA- and $CaCl_2$-extractable Cu (r = 0.90).

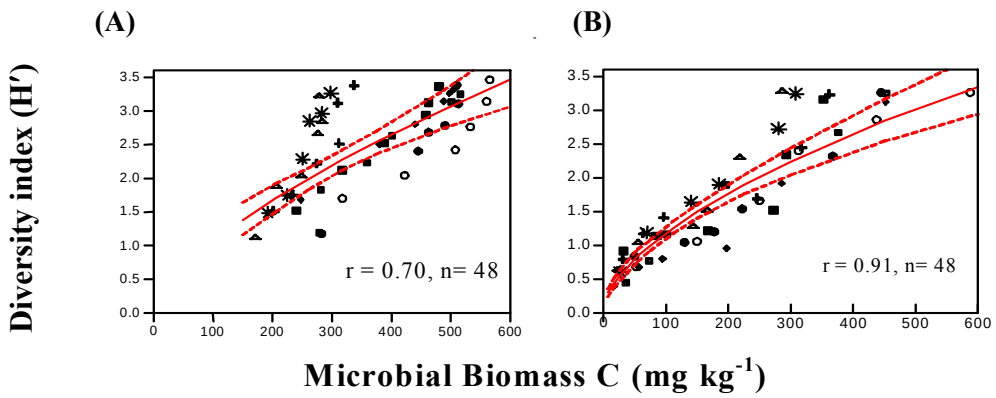


Figure 3. The relationships between soil microbial biomass C and diversity index (H′) of Cu contaminated soils after 45 days (A) and after 270 days (B) of incubation. Windsor (+), Somersby (□), Robertson (o), Catombal (∗), Brinsley (Δ), Pine (•), Lucerne (■), and Box Hill (◊) soils. Solid lines represent least square regression fit to the data points and broken lines represent 95% confidence limit of the model.

Discussion

This present study showed a consistent decrease in the microbial biomass and diversity with increasing Cu concentrations which is consistent with past studies. The effects of Cu on MBC were significantly low with below 300 mg Cu kg^{-1} concentrations after 45 days of incubation. Renella *et al.* (2002) stated that a single pulse of metals (e.g. 300 mg Cu salts kg^{-1} soil) caused only small changes (5-12%) in the biomass C measurement in a short-term (up to 50 days) laboratory incubation study. In this study, the greatest reduction in biomass and diversity was found in the Box Hill soil and the lowest reduction was observed in the Robertson soil at the highest Cu concentration (1500 mg Cu kg^{-1}). The Box Hill soil had the lowest pH and the highest EDTA extractable Cu (88%) among the studied soils which could be responsible for adverse effects on soil microbial biomass in this soil. Brookes and McGrath (1984) found that microbial biomass was approximately half in a soil with 415 mg kg^{-1} EDTA-extractable Cu than a soil with 12 mg kg^{-1} EDTA-extractable Cu. EDTA extractable Cu was lowest (85% of the total) in the Robertson soil and the microbial population was least affected. Therefore higher correlation between availability and effects on microbial population may be due to have the highest CEC (133.5 $mmol_c$ kg^{-1}), highest free iron (7.06%) and clay (33%) content in this soil compared to other soils. McGrath *et al.* (1995) reported that heavier soils with near neutral soil pH and/or containing organic matter may reduce the toxic effects of heavy metals to soil microbes, by binding the metals and making them less available. Kandeler *et al.* (2000) found that living and dead soil bacteria were mainly associated with the silt and clay fractions, whereas fungi and their exoenzymes, involved in the decay of complex organic compound, were associated with the particulate organic matter in the coarse sand fraction.

High Cu concentrations and longer incubation time had a strong influence on microbial diversity in all soils, but the effects on diversity index and substrate evenness values were much more pronounced compared to substrate richness values ($LSD_{0.05}$ = 1.3). A smaller effect on substrate richness values may be expected, since some microorganisms are more susceptible to certain toxic xenobiotics than the others. In general, microorganisms differ in their sensitivity to metals exposure to high levels of metal(s) will result in death of cells due to disruption of essential functions, and to more gradual changes in population sizes could be due to changes in viability or completive ability (Ross 1994). Pennanen *et al.* (1996) observed that 100-200 mg Cu kg^{-1}concentration was a threshold level for most toxic effects to microbial population around a primary smelter. On the other hand, Bååth *et al.* (1998) reported 1000 mg Cu kg^{-1} soil concentration was a threshold value for a pollution effect on phospholipid fatty acid pattern. A more recent investigation by Yao *et al.* (2003) revealed that the microbial substrate utilization was significantly

affected due to heavy metal pollution of paddy soils around a smelter in China where threshold value for Cu contamination was found between 34.8 to 146.7 mg kg^{-1}soils. Compared to other soils, microbial diversity in Windsor soil showed more sensitivity to Cu contamination at the early stage of incubation. The decrease in microbial diversity both in substrate richness and evenness ($LSD_{0.05} = 0.05$) in Cu spiked soils could be due to the lack sufficient tolerance microbial species to the stress imposed.

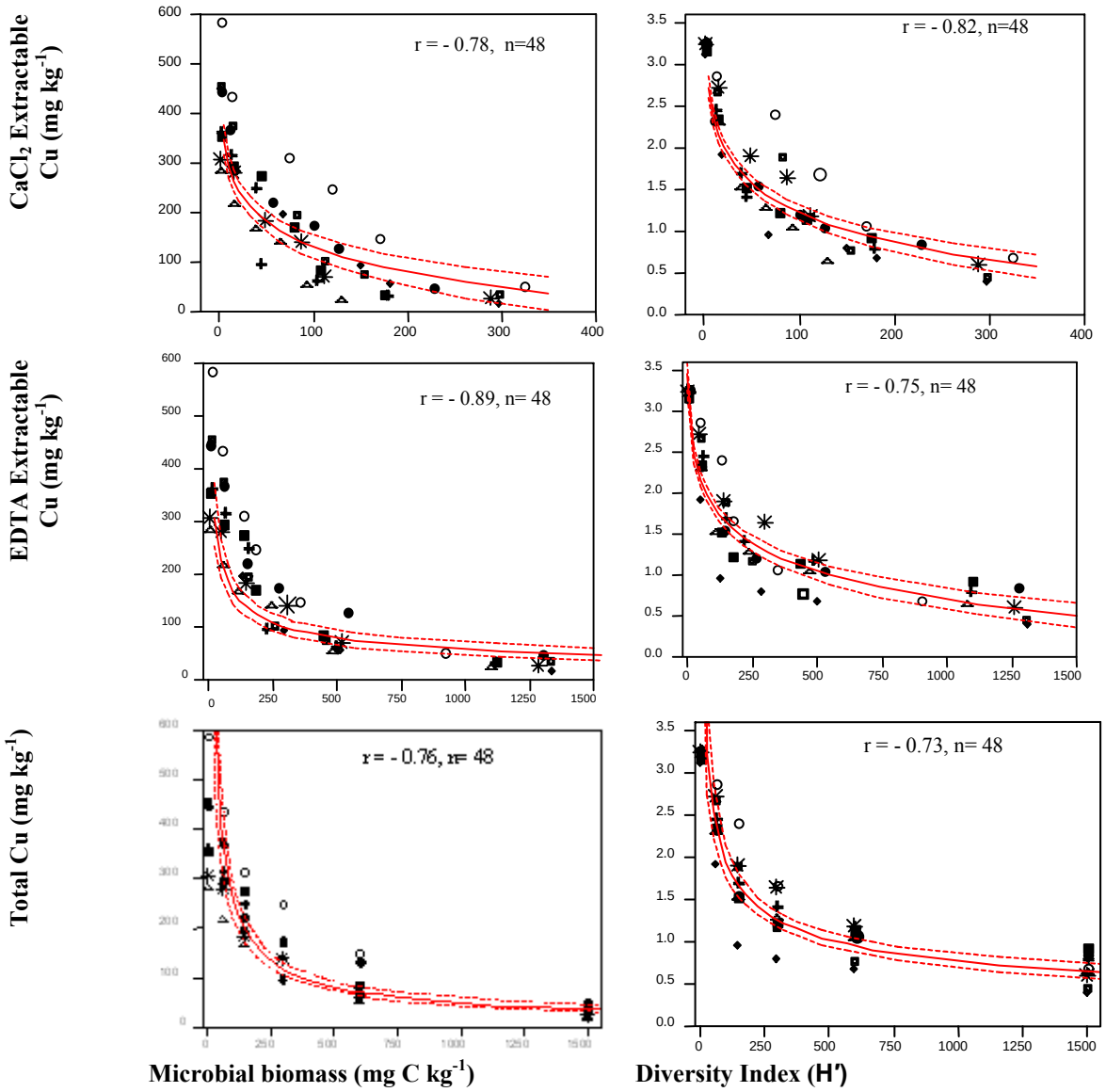


Figure 4. The relationships between soil microbial biomass C (mg kg^{-1}) and diversity index (H') with extractable ($CaCl_2$, EDTA) and Total Cu (mg kg^{-1}) in Cu spiked soils. Windsor (+), Somersby (□), Robertson (○), Catombal (∗), Brinsley (Δ), Pine (●), Lucerne (■), and Box Hill (◊) soils. Solid lines represent least square regression fit to the data points and broken lines represent 95% confidence limit of the model.

The purpose of measuring extractable heavy metal was to evaluate potential mobility and bioavailability of Cu. The $CaCl_2$ extractant is capable of extracting metal ions from exchangeable and solution phases (Clayton and Tiller 1979), whereas EDTA extractant is moderately dilute, weak acid which is capable of extracting metals from soluble organo-metallic substances (Oliver *et al*. 1999). In this study, microorganisms were exposed to higher available Cu with longer incubation time. The contact time between metal and soil is a critical factor in determining metal bioavailability. It has been reported that when heavy metal is added to soil, availability of the metals appears to increase with time, and establishment of equilibrium levels may be very slow (Holtan-Hartwig *et al*. 2002). To reach an equilibrium levels of metal addition in soil usually takes time, and hence to affect a biological system of an organisms. Availability of Cu associated with organic phase has increased in our study may be due to effect of metals on soil microbial biomass and activity. Soil microorganisms are significantly involved in

mobilization-immobilization processes of toxic elements in soils, but their mechanisms are not completely known yet (Leita *et al*. 1995). Microorganisms play an important role in decomposition of organic matter, transfer and mobility of toxic elements in soils (Welsch and Norvell 1997). Frankenberger *et al.* (1995) described the release of elements by bio-oxidation, bio-reduction, biosorption or methylation processes. If Cu is associated with soil microorganisms, they may be released by decomposition of soil microbial biomass which could have been elevated the extractable Cu in soils after 270 days of incubation. In some cases, microorganisms are able to alter metal availability in their vicinity through localized acidification of the environment, or production of compounds which complex metals. Renella *et al.* (2002) stated that when soil is incubated under optimal conditions soil organisms may have mediated some biochemical processes that can contribute to change the chemical status of the added metals. It is commonly observed that the bioavailability, chemical extractability, ad decomposition rate of soil organic contaminants decreases with time since initial exposure (Barrow 1998; Martinez 2003; McLaughlin 2001). In this study, $CaCl_2$, EDTA extractable and total Cu showed strong negative correlation with MBC and diversity index values (Figure 4) where the relationships found highly significant and equal. Therefore, $CaCl_2$, EDTA-extractable and total Cu in soils showed nonsignificant relationship between them.

Conclusions

The result of this study demonstrated that long term Cu contamination adversely affected the microbial biomass and diversity in the soils studied. Soil properties such as pH, organic carbon and clay content affected the Cu toxicity to soil microbes. A smaller reduction in microbial biomass and diversity due to Cu toxicity were observed in soils with higher organic C, total N and clay content. Conversely a significant reduction in microbial biomass and diversity were observed due to Cu toxicity in low soil pH. $CaCl_2$- and EDTA-extractable Cu and total Cu in soils showed negative correlations with MBC and diversity of microbial population in the studied soils.

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