Environ Sci Pollut Res (2014) 21:3254–3263
DOI 10.1007/s11356-013-2271-z

RESEARCH ARTICLE

Comparative tolerance of *Pinus radiata* and microbial activity to copper and zinc in a soil treated with metal-amended biosolids

Paramsothy Jeyakumar · Paripurnanda Loganathan · Christopher W. N. Anderson · Sivalingam Sivakumaran · Ronald G. McLaren

Received: 17 March 2013 / Accepted: 22 October 2013 / Published online: 12 November 2013

Abstract A study was conducted to evaluate the effects of elevated concentrations of copper (Cu) and zinc (Zn) in a soil treated with biosolids previously spiked with these metals on *Pinus radiata* during a 312-day glasshouse pot trial. The total soil metal concentrations in the treatments were 16, 48, 146 and 232 mg Cu/kg or 36, 141, 430 and 668 mg Zn/kg. Increased total soil Cu concentration increased the soil solution Cu concentration (0.03–0.54 mg/L) but had no effect on leaf and root dry matter production. Increased total soil Zn concentration also increased the soil solution Zn concentration (0.9–362 mg/L). Decreased leaf and root dry matter were recorded above the total soil Zn concentration of 141 mg/kg (soil solution Zn concentration, >4.4 mg/L). A lower percentage of Cu in the soil soluble+exchangeable fraction (5–12 %) and lower Cu^{2+} concentration in soil solution (0.001–0.06 μM) relative to Zn (soil soluble+exchangeable fraction, 12–66 %; soil solution Zn^{2+} concentration, 4.5–4,419 μM) indicated lower bioavailability of Cu. Soil dehydrogenase activity decreased with every successive level of Cu and Zn applied, but the reduction was higher for Zn than for Cu addition. Dehydrogenase activity was reduced by 40 % (EC_{40}) at the total solution-phase and solid-phase soluble+exchangeable Cu concentrations of 0.5 mg/L and 14.5 mg/kg, respectively. For Zn the corresponding EC_{50} were 9 mg/L and 55 mg/kg, respectively. Based on our findings, we propose that current New Zealand soil guidelines values for Cu and Zn (100 mg/kg for Cu; 300 mg/kg for Zn) should be revised downwards based on apparent toxicity to soil biological activity (Cu and Zn) and radiata pine (Zn only) at the threshold concentration.

Keywords Bioavailability · Bio accumulation factor · Dehydrogenase activity · EC_{50} · Metal toxicity · Radiata pine · Rhizosphere · Soil metal fractions

Responsible editor: Zhihong Xu

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Introduction

Pinus radiata (radiata pine) is one of the world's most commercially important conifers, and its planting covers a total global area now exceeding 4.3 million hectares. It is grown extensively as an exotic timber species in several countries, such as New Zealand, Australia, Chile, South Africa and Spain (Sutton 1999; Toro and Gessel 1999; Putoczki et al. 2007; Sevillano-Marco et al. 2009). Radiata pine forestry has a very important place in the economy of New Zealand and constitutes 89.5 % of the national plantation forestry land area (approximately 1.6 million ha) (NZFOA 2009).

Soil cultivated for pine forestry production is often low in essential nutrients and deficiencies can limit plant growth (Mosquera-Losada et al. 2010). One strategy to improve the fertility of pine forestry soils is to apply biosolids at rates of up to 6.7 t/ha/year (NZWWA 2003). Biosolids application to pine forestry soils is often considered to be a relatively safe option for disposal of this waste. This is because the biosolids can improve and maintain productivity of soils by means of increasing organic matter content, aggregate stability, porosity, water infiltration rate and simulate plant growth (Wang et al. 2006). Further, there is little risk of contaminants from the biosolids entering the human food chain under this type of land use. However, due to repeated surface applications of biosolids, it has been reported that heavy metal bioavailability may be increased in the litter-rich surface soil, potentially leading to some degree of movement of these heavy metals into the underlying mineral soil, and eventually into groundwater (McLaren et al. 2007; Su et al. 2008). If these lands are converted to crop production in the future, then heavy metals accumulated in the forest soil may represent an unacceptable risk to the food chain (McLaren et al. 2010).

Many studies have attempted to investigate the toxicity effect of Cu and Zn on pines grown in metal-contaminated soils or in solution culture (Hartley et al. 1999; Gratton et al. 2000; Egiarte et al. 2009). Metal accumulation and movement in pine forest soils that have been amended with biosolids and in the litter layer beneath pine trees have been studied in recent trials (McLaren et al. 2007; Su et al. 2008; Egiarte et al. 2009; Mosquera-Losada et al. 2009). However, in each of these studies, no toxic effect of Cu and Zn on pine growing in biosolids-amended soil could be quantified. We believe this is because the metal levels tested were not sufficiently high, and not representative of long-term biosolids amendment strategies to forestry soil. For example, Fuentes et al. (2007a) showed that soils amended with low levels of Cu and Zn contaminated biosolids (44 and 122 mg/kg soil, respectively) had no adverse effect on pine growth. Similarly, Ferreiro-Dominguez et al. (2012) showed that forest soils in Northwest Spain fertilised with biosolids did not induce a phytotoxic effect on pines when the total soil Cu concentration ranged from 1.9 to 5.2 mg/kg. Under the same field conditions, Rigueiro-Rodríguez et al. (2012) demonstrated that total soil Zn at a rate of 35–45 mg/kg had no adverse effect on pines. Because these studies did not show Cu and Zn toxicity to pines, it was not possible to determine the critical levels for Cu and Zn toxicity. In order to determine the threshold levels of Cu and Zn toxicity to pine, higher concentrations of Cu and Zn spiked biosolids should be used.

Soil microorganisms are also affected by the presence of high concentrations of metals in soil (Giller et al. 1998), but the threshold of phytotoxicity may not be at the same metal concentration level as for plants. However, the comparative effect of Cu and Zn toxicity on microorganisms and pine is lacking in scientific literature. Alloway (1995) stated that the microorganisms are more vulnerable to Cu and Zn toxicity than higher plants because they have less well developed homeostatic defence mechanisms. Therefore, in order for regulatory agencies to set guidelines for the safe disposal of metal-contaminated biosolids to pine forest land, threshold heavy metal toxicity levels both to plants and soil microorganisms need to be assessed. Jeyakumar et al. (2010) studied the effect of incorporating Cu (12–226 mg/kg soil) and Zn (25–686 mg/kg soil) spiked biosolids into soils on poplar plant growth and soil microbial activity in a pot trial conducted for 147 days. They found that Cu was not phytotoxic to poplar, whereas Zn caused phytotoxicity at a total soil Zn concentration beyond 141 mg/kg. All rates of Cu and Zn addition decreased soil microbial activity, and Cu was identified as a more toxic metal than Zn to soil microorganisms.

This paper presents the results of a study that was conducted to determine the bioavailability of Cu and Zn in soils amended with biosolids, and the effect of Cu and Zn on growth and metal uptake by radiata pine, and on soil microbial activity using elevated concentrations of the metals. A key objective of this work was to quantify critical levels of Cu and Zn in soil that can be considered toxic to radiata pine and soil microbial activity so that more reliable environmental threshold guidelines can be defined.

Materials and methods

Treatments and design

One-year-old radiata pine (*P. radiata* D. Don) clones (96004) collected from ArborGen plant nursery, New Zealand were used in a controlled plant growth unit experiment. Radiata pine was planted in pots containing 13 kg of biosolid-amended recent soil (“Dystric Fluventic Eutrudept”) classified as “Manawatu fine sandy loam” (Hewitt 1998). Soil was amended with biosolids in three separate levels of Cu or Zn which were previously spiked with metal sulphate salts ($CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$), as explained by Jeyakumar et al. (2008). The control treatment utilised biosolids not amended with metals. There were six metal-spiked treatments with the following total metal concentrations (in milligrams per kilogram soil): Cu 48 (designated Cu1), 146 (Cu2), 232 (Cu3), and Zn 141 (Zn1), 430 (Zn2), 668 (Zn3). The Cu and Zn concentration of the control treatment (designated both Cu0 and Zn0) was 16 and 36 mg/kg, respectively. These seven treatments were replicated three times in a randomised complete block design (RCBD). Soil moisture content of the pots was maintained at 80 % “pot field capacity” and the temperature of the plant growth unit was regulated at a minimum of 9 ±5 °C (night) and a maximum of 18±6 °C (day). The experiment was concluded over 312 days. At harvest, plant shoots, roots, and rhizosphere and bulk soil samples from each pot were collected. The following soil parameters were measured: total soil Cu and Zn concentration; Cu and Zn fractionation using a sequential extraction procedure; total soil solution Cu and Zn concentration and metal speciation; the cations Ca^{2+}, Mg^{2+}, K^+, Na^+, and NH_4^+; the anions SO_4^{2-}, Cl^-, and NO_3^-; dissolved organic carbon; pH; and dehydrogenase activity in rhizosphere and bulk soils. Radiata pine needle and root dry matter (DM) yields, and root and needle Cu and Zn concentration were also measured.

Plant harvest and soil sampling

Plant aerial biomass was collected from each pot 312 days after the initiation of the experiment. The bulk soil was separated from rhizosphere soil and root, and care was taken not to loosen rhizosphere soils from the root ball of the plant. The rhizosphere soil attached to the roots was separated by vigorously shaking the roots inside a bag (Liu et al. 2008). Bulk and rhizosphere soil were split into three equal size sub-samples. One sub-sample was used for soil pH measurement and soil solution extraction. The second sub-sample was transferred into a sterilised container at 28 °C for measurement of dehydrogenase activity. The third sub-sample was air dried and sieved through a 1-mm stainless steel sieve for total metal analysis and fractionation. Root and needles were separately dried (at 60 °C) and ground using a Cyclotech 1093 Plant Mill equipped with a stainless steel blade. The dry weight of the harvested material was recorded and the ground plant material was stored for chemical analysis.

Chemical analysis

Soil pH was measured (soil/water 1:2.5 *w*/*w*) using a Eutech Instruments Cyber Scan pH 310 meter. Soil solution was extracted from each subsample by centrifuging the moist soil at 11953*G* and 48 °C for 30 min. The resulting solutions were filtered through a 0.45-mm filter and analysed for pH, Cu and Zn concentration, and base cation and anion concentration. The Cu concentration was determined by graphite furnace atomic absorption spectrometry (Analyst 600, Perkin Elmer); Zn, Mg, Na, K, and Ca concentrations were determined by flame atomic absorption spectrometry (FAAS: GBC Avanta∑). The soil solution SO_4^{2-} and Cl^- concentrations were determined by ion chromatography, and NO_3^- and NH4+ by an automated analysis technique (Tecator 1983). Dissolved organic carbon was measured using a Shimadzu TOC-5000 analyzer (Wu et al. 1990). The Windermere humic aqueous model (WHAM; Centre for Ecology and Hydrology 2002) was used to define the speciation of Cu and Zn in the soil solution. The soil solution cations (Ca^{2+}, Mg^{2+}, K^+, Na^+, and NH_4^+), anions (SO_4^{2-}, Cl^-, and NO_3^-), dissolved organic carbon concentration, and pH were used as the model inputs. The total soil Cu and Zn concentration was measured using a wet digestion method developed by Kovács et al. (2000). A sequential extraction technique developed by Tessier et al. (1979) and modified by McLaren and Clucas (2001) was used to determine the solid-phase Cu and Zn metal fractionation in the soil. Root and needle samples (0.4 g) were digested separately using 10 mL of 65 % HNO_3. The bio-concentration factor (BCF), defined as a ratio of metal concentration in plant shoots to metal concentration in soil, was calculated to assess the ability of the plants to uptake Cu and Zn from the soil and to translocate these metals to shoots.

Certified reference materials were used to ensure the accuracy of the measurements. For total metal analysis, river sediment sample US NBS-SRM-1645 and a sewage sludge BCR CRM 145R sample from the Commission of the European Community were used. The measured mean Cu and Zn concentration for these reference materials were 101–104 % of the expected values. For metal fractionation, internal standards (Hort 1 and 2) were used as standard reference material, and the concentration of metal associated with each fraction was found to be within 92–104 % of the expected mean values. Blanks used in all analysis showed only <0.2–0.9 % of the sample concentration, and were used to correct the unknown sample values. A Wageningen plant standard (2004-04, no. 2) was used as a standard reference material to verify the accuracy of the analytical procedure from radiata pine biomass. Analytical derived metal concentrations were from 95 to 104 % of the reported value.

Dehydrogenase activity

The effect of Cu and Zn on the biological activity of soil was assessed by determining dehydrogenase activity in the soil (Taylor et al. 2002; Mills et al. 2006). The method of Chander and Brookes (1991) was used to quantify dehydrogenase activity. Briefly, 5 g of field moist soil was mixed with 3 mL of 3 % 2,3,5-triphenyl tetrazolium chloride and 0.1 g $CaCO_3$, and incubated for 24 h at 28 °C. Triphenyl formazan (TPF) formed in the reaction was extracted with methanol and the concentration was measured by absorbance at 485 nm using a spectrophotometer (DU-640; Beckman, Krefeld).

Data analysis

Significant differences among treatments ($n=3$) for all measured parameters were tested by the "Analysis of variance" (one-way ANOVA) procedure and Duncan multiple range test at the 95 % confidence level ($P=0.05$) using SAS® 9.1.2 statistical software (SAS Institute Inc 2004). In addition, a set of simple correlation analyses relating the soil dehydrogenase activity with the metal concentrations in the solid-phase fraction and the soil solution species was performed using SigmaPlot 10 (Systat Software Inc. 2006) curve-fitting software. The dehydrogenase activities in the metal-spiked treatments were calculated as a percentage of activity of the control treatment. The equations used for the curve fitting were described by Jeyakumar et al. (2010).

Results and discussion

Dry matter yield and metal concentration in the plants

Increased Cu levels had no significant effect on either needle or root DM yield (Table 1). However, the concentration of Cu in needles and roots for all Cu treatments was significantly increased with every level of Cu applied. The needle Cu concentration at all levels of Cu (4.2–7.3 mg/kg) was higher than the critical Cu concentration of 2.1–2.3 mg/kg, generally considered the limit for Cu deficiency, but below the phytotoxic level of 40 mg/kg for juvenile radiata pine plants (Boardman et al. 1997).

Increasing the soil Zn concentration decreased needle and root DM yields beyond the Zn1 treatment (total soil Zn> 141 mg/kg; Table 1). In roots, a much greater DM yield reduction was observed between the Zn1 and Zn2 treatments (from 33.7 to 3.1 g) than was observed for needles. Plant needles at the Zn2 and Zn3 treatments showed severe drying and yellowing. The DM yield reduction and severe damage of needles caused by the Zn2 and Zn3 treatments (≥430 mg/kg) could be well deduced from the severe yellowing and wilting of the pine needles and indicate Zn toxicity at high levels of Zn addition. Serious reductions in the needle chlorophyll concentration (manifest as red needles) may lead to reduced C fixation and thus reduced growth (Ivanov et al. 2011). The data in the current work do not allow pinpointing of the exact cause of the yellowing and reddish coloration in pine needles. However, it is likely that the reduction is largely caused by the Zn-induced impairment of nutrient transfer to needles, in particular N, Mg and Fe which play a key role in chlorophyll synthesis and functioning (Adriaensen et al. 2006). This was probably due to some degree of root damage (Wang et al. 2009) and reduction in root formation (Castiglione et al. 2007) as a consequence of direct contact with a high concentration of bioavailable Zn. The concentration of Zn in the needles and roots for all Zn treatments was significantly increased with every successive level of Zn applied. The needle Zn concentration above the Zn1 treatment was much higher than the phytotoxic level of 200 mg Zn/kg DM defined by Boardman et al. (1997) for juvenile radiata pines. This explains the lower DM yields obtained for the Zn2 and Zn3 treatments. The severe DM reduction above the total soil Zn concentration of 430 mg/kg (Zn2 treatment) tends to support the maximum permissible soil Zn concentration of 300 mg/kg set by NZWWA (2003).

The BCF was much lower for Cu (0.03–0.26) than for Zn (0.8–1.5; Table 1). The needle/root concentration ratio was also lower for Cu than for Zn suggesting that the translocation of Cu from roots to needles was lower than that for Zn. Copper transport to aboveground plant tissues is generally restricted. Arduini et al. (1996) found during an experiment with 2-week-old seedlings of two pine species, with a range of Cu concentration in solution culture (0.012–5 μM), that a maximum 16.5 % of the total root Cu concentration was accumulated in the shoot and concluded that the root was an effective barrier to Cu translocation. They also reported that X-ray microanalysis of root tip sections confirmed that Cu was strongly accumulated in the cell walls of the cortex, where its concentration sharply reduced from outer to inner cell layers. Bücking and Heyser (1994) conducted a study to determine the influence of exposure to five different EDTA extractable Zn concentrations ranging from 0.3 to 3,000 μM for 28 days on *Pinus sylvestris* root and shoot Zn concentrations. They observed an increase in “shoot to root Zn concentration ratio” (1.5–1.8) at low external Zn concentrations but at high external Zn concentrations, this ratio decreased drastically (0.1) as observed in our study (Table 1). Although Jeyakumar et al. (2010) also reported that the BCF values were higher for Zn than Cu for poplar (Cu=0.1–0.8 and Zn=5.5–10), their values were much higher than the corresponding values for radiata pine in the current study. This is probably due to the higher translocation and accumulation rate of metals in poplar leaves than radiata pine. Poplar is known to be a shoot metal accumulator plant (Dos Santos Utmazian and Wenzel 2007; Castiglione et al. 2009).

Table 1 Effect of Cu and Zn on total soil metal concentration, soil pH, radiata pine metal concentration and DM yield

Treatment	Total soil metal conc.		pH	Dry matter (g)		Metal conc. (mg/kg)		Conc.ratio	BCF
	Solid[a] (mg/kg)	Solution (mg/L)		Needle	Root	Needle	Root	Needle: Root	
Cu0	16	0.03 c	6.1 a	72.3 a	34.8 a	4.2 d	10 d	0.42 a	0.26 a
Cu1	48	0.27 b	5.8 ab	67.5 a	32.7 a	5.3c	65 c	0.08 b	0.11 b
Cu2	146	0.37 b	5.7 b	65.6 a	35.6 a	6.3 b	166 b	0.04 b	0.04 c
Cu3	232	0.54 a	5.7 b	65.0 a	39.6 a	7.3 a	267 a	0.03 b	0.03 c
Zn0	36	0.9 c	6.1 a	72.3 a	34.8 a	39 d	45 d	0.85 a	1.08 ab
Zn1	141	4.4 c	5.5 b	68.1 a	33.7 a	205 c	550 c	0.37 b	1.45 a
Zn2	430	89 b	4.8 c	33.5 b	3.1 b	341 b	2,464 b	0.14 c	0.79 b
Zn3	668	362 a	4.7 c	12.6 c	1.5 b	1,000 a	5,033 a	0.20 c	1.50 a

Values in columns followed by different letters are significantly different ($P \leq 0.05$)

BCF bioconcentration factor

[a] Original biosolid-treated soil

Metal speciation in soil solution phase

The total metal concentrations in soil solution obtained in the present study indicate that increased total soil metal concentrations have increased soil solution metal concentrations (Table 1). When the soil solution Cu speciation was investigated using the WHAM speciation model, the Cu^{2+} concentrations (in micromolar) in soil solution were 0.001, 0.008, 0.040 and 0.060 for the Cu0, Cu1, Cu2 and Cu3 treatments, respectively. The free copper ion concentration contributed a maximum 1 % of the soil solution Cu (Table 2). The low Cu^{2+} concentration for all treatments is attributed to about 99 % of Cu in soil solution being complexed to dissolved organic carbon (DOC). This is because Cu forms very strong complexes with DOC through chelation with constituent functional groups such as carboxylic acids, amines and phenols (Altun and Köseoĝlu 2005). Furthermore, the stability of these complexes is higher than Zn (Pandey et al. 2000). Strobel et al. (2001) reported that Cu belongs to a group of elements that have strong interactions with DOC in the pH range 4–7. In our study, the pH values for all Cu treatments ranged from 5.7 to 6.1, and therefore we expect Cu in soil solution to have strongly complexed to DOC. As there are no published data available on soil solution Cu speciation and its relationship to pine growth, hydroponic studies are used to explain the Cu^{2+} concentration effect on pine growth. As hydroponic solutions have no dissolved organic matter for complexation of Cu, approximately all Cu in hydroponic solution is expected to exist as Cu^{2+} and therefore the total Cu concentration in hydroponic solution can be assumed to be the Cu^{2+} concentration. Van Tichelen et al. (2001) carried out a trial with ectomycorrhizal (ECM)-inoculated Scot pine seedlings grown in hydroponics solution and reported that even at a Cu concentration of 47 μM, shoot Cu concentration was <25 mg/kg. In another study, Fuentes et al. (2007b) conducted an experiment with seedlings of several Mediterranean woody species (*Pinus halepensis*, *Pistacia lentiscus*, *Juniperus oxycedrus* and *Rhamnus alaternus*) in a hydroponic culture for 12 weeks. They reported that increasing the nutrient solution Cu concentration from 0.047 to 1 μM Cu increased the pine biomass DM, but at 4 μM Cu the shoot and root biomass were decreased by 27 and 33 %, respectively, as compared to the 1 μM Cu treatment. In the present trial with radiata pine, the maximum Cu^{2+} concentration in soil solution was 0.06 μM showing that even at an elevated treatment level (Cu3), bioavailable Cu was low. Even though the Cu^{2+} concentrations were low for all treatments, the Cu concentration in needle was above the critical level for Cu deficiency in radiata pine. Therefore, the plants were taking up Cu from soil Cu fractions other than Cu^{2+}, and there must be other factors influencing the Cu uptake. Zhao et al. (2006) studied Cu toxicity in tomato plants in 18 European soils and found that free Cu^{2+} activity alone was a poor predictor of the Cu concentrations in tomato roots and shoots. They concluded that bioavailability in soil depended on the combined effects of Cu speciation, interaction with protective ions such as H+, and Cu resupply from the solid phase. In a greenhouse study with ryegrass, Luo and Christie (1997) similarly concluded that shoot Cu concentration may not depend solely upon labile soil solution Cu concentration, and that uptake is also influenced by solid phase Cu fractions.

The soil solution Zn^{2+} concentrations (in micromolar) for the Zn0, Zn1, Zn2 and Zn3 treatments were 4.5, 41, 983 and 4, 419, respectively; and contributed 32–80 % of total Zn in the soil solution (Table 2). Fuentes et al. (2007b) also examined the effect of Zn on pine seedlings (*P. halepensis*) in hydroponic culture and reported that by increasing the solution Zn concentration from 0.073 to 25 μM Zn, pine seedling DM was increased. However, at 100 μM Zn, shoot and root biomass was decreased by 35 and 24 %, respectively relative to the 25 μM Zn concentration. Considering these experimental results along with data from the present trial with radiata pines, we can hypothesise that the Zn treatment above the Zn1 level may be phytotoxic to the pine seedlings. This confirms the observed effect on DM yield and needle Zn concentration resulting from an increasing Zn concentration in the soil. The critical phytotoxic level of Zn in soil solution for the current study was determined by fitting a curve that relates DM content to the Zn^{2+} concentration in soil solution (Fig. 1). Assuming that 10 % DM yield reduction is an indicator of Zn toxicity (Reichman et al. 2001), Fig. 1 derives a critical Zn^{2+} concentration for Zn toxicity of 62 μM. In

Table 2 Percentage of metal (M) species in soil solution (organic M calculated by subtracting the total concentration of inorganic species from the corresponding total metal concentration)

	M^{2+}	$MHCO_3$	MSO_4	MCl	Total	Organic M
Cu0	0.01 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.01 (0.00)	99.99 (1.89)
Cu1	0.20 (0.02)	0.01 (0.00)	0.07 (0.01)	0.00 (0.00)	0.28 (0.3)	99.72 (1.34)
Cu2	0.75 (0.10)	0.03 (0.01)	0.22 (0.02)	0.00 (0.00)	1.00 (0.12)	99.00 (2.01)
Cu3	0.73 (0.10)	0.02 (0.00)	0.28 (0.03)	0.00 (0.00)	1.04 (0.10)	98.96 (1.64)
Zn0	31.65 (1.44)	0.09 (0.01)	5.73 (0.24)	0.02 (0.00)	37.48 (1.23)	62.52 (1.08)
Zn1	61.15 (1.51)	0.04 (0.01)	16.79 (0.64)	0.15 (0.02)	78.14 (1.51)	21.86 (0.86)
Zn2	72.24 (1.47)	0.01 (0.00)	27.92 (1.03)	0.19 (0.02)	100.36 (2.02)	0.00 (0.00)
Zn3	79.83 (1.54)	0.01 (0.00)	21.36 (1.12)	0.20 (0.03)	101.41 (2.10)	0.00 (0.00)

Values are means ± standard error, *n* =3 (in parenthesis)

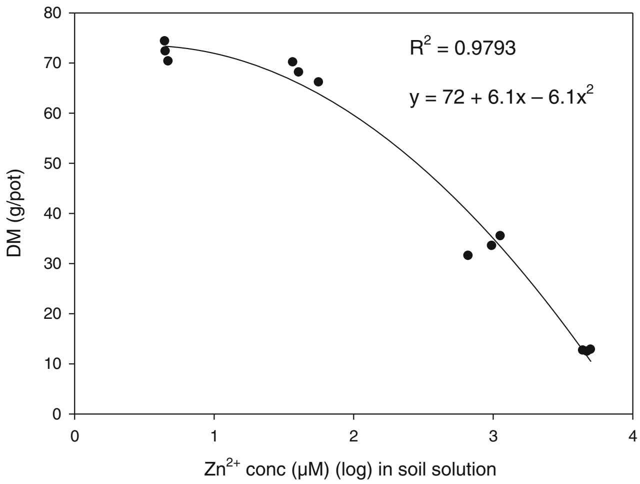


Fig. 1 Relationship of radiata pine needle DM with the Zn^{2+} concentration (in micromolar) in soil solution

previous hydroponics experiments, 40 μM Zn has been shown to cause a reduction in root elongation for grasses which are sensitive to Zn stress (Al-Hiyaly et al. 1988), while 76 μM Zn reduced the growth as well as the N and P uptake capacity of Zn-sensitive ECM (*Suillus bovinus*) associated with pine seedlings (Adriaensen et al. 2004). The critical Zn concentration for Zn toxicity in this study also agrees with our previous study with poplar (Jeyakumar et al. 2010) where 88.5 μM Zn^{2+} caused a reduction in DM yield of leaves.

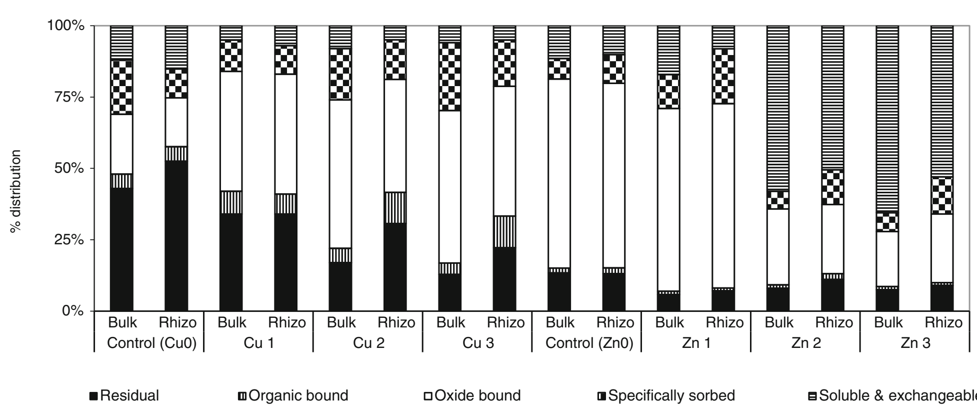
The considerable percentage of $ZnSO_4$ species (6–28 %) observed in soil solution is due to the use of sulphate salts of the metals in the spiking of biosolids (Jeyakumar et al. 2008). Metal carbonate and chloride concentrations were negligibly small for both metals at all levels.

Metal fractionation in the soil solid phase

The percentage distribution of Cu and Zn in the various soil solid-phase fractions was measured to determine the fate of added metals and to explain the bioavailability and potential phytotoxicity of Zn and Cu derived from the biosolids (Fig. 2). Further, the degree of bioavailability associated with non-bioavailable fractions can be better interpreted by means of expressing fractionation in percentage. Copper ions generally have a strong affinity with soil organic matter (SOM; Stevenson 1991). Therefore, organic fraction in the soil can be the most important factor in determining Cu bioavailability (del Castilho et al. 1993). However, Zn ions have less affinity with SOM than Cu (McLaren and Clucas 2001).

When the soil Cu level was low, the residual fraction was the largest contribution to total soil Cu (43 and 34 % for the Cu0 and Cu1 treatments, respectively), followed by oxide-bound Cu fraction (21–42 %) and specifically sorbed Cu (19 and 11 % for Cu0 and Cu1 treatments, respectively). However, as the Cu level increased, the relative contribution of the various fractions changed. The residual fraction significantly reduced (13–17 %), whereas the percentage of Cu associated with the oxide fraction (52–54 %) increased at higher Cu levels (Cu2 and Cu3). The increase in organic bound Cu was from 5 to 11 %, as the Cu levels increased. The soluble+exchangeable Cu fraction for the control treatment contributed 12 % to total soil copper, but in the biosolids-amended soils (from Cu1 to Cu3), was reduced to 5–8 % of the total Cu concentration. The differences were not significant among the Cu treatments. These results show that increasing the level of Cu changed the relative percentage of Cu among the non-bioavailable Cu fractions, but did not significantly change the percentage of Cu associated with the bioavailable soluble+exchangeable fraction in the soil. However, the absolute concentration of soluble+exchangeable Cu in the solid-phase increased with an increase in Cu levels (2, 2, 11 and 13 mg/kg soil for the Cu0, Cu1, Cu2 and Cu3 treatments, respectively). The increase in the percentage of Cu in the oxide fractions is probably due to strong sorption of the added Cu to the oxide constituents of the soil. Perhaps with time the sorbed Cu may move to the residual fraction by diffusion into lattice structures (McBride 1991), and this would lead to an increased percentage of copper associated with the residual fraction as observed in the native soil (Cu0 treatment).

Fig. 2 Percentage distribution of Cu and Zn fractionation in rhizosphere and bulk soils amended with biosolids under radiata pine



Huang et al. (2008) conducted an experiment with ECM-inoculated Chinese pine seedlings grown in a brown soil amended with Cu (25–400 mg/kg soil), and reported that the percentage of Cu associated with the non-labile soil Cu fractions increased with increasing total soil Cu concentration (carbonate fraction from 23 to 57 %, oxide fraction from 24 to 31 % and residual fraction from 11 to 23 %), but the percentage of Cu associated with the soluble+exchangeable fraction was reduced (from 8 to 1 %). Consistent with these results, in our study the percentage of soluble+exchangeable Cu concentration reduced from 12 % for the no-Cu treatment to 5 % for the highest Cu treatment. Our data infers that the increasing amount of Cu added to the soil was mainly associated with non-labile forms.

The relative distribution of the Zn fractions was different to that of Cu (Fig. 2). The percentage of organic-bound Zn was much lower (1–2 %) than that for Cu (5–11 %). For low levels of total soil Zn (Zn0 and Zn1), the percentage of oxide-bound metal was higher (64–66 %) than for the high levels of soil Zn (19–26 %), whereas at the higher rates (Zn2 and Zn3) the Zn was mostly present in the soluble+exchangeable fraction (58–66 %). The bioavailability of Zn as a proportion of the total Zn in soil, up to the Zn1 level, was low, as indicated by the relatively low contribution of the soluble+exchangeable fraction to total soil Zn (12–17 % up to Zn1). But at higher rates (Zn2 and Zn3) added Zn significantly increased Zn availability and caused toxicity to the plant tissues. The absolute concentrations of soluble+exchangeable Zn in the Zn0, Zn1, Zn2 and Zn3 treatments were 4, 32, 249 and 437 mg/kg, respectively. Reduced plant DM content and increasing needle Zn concentration as a function of Zn amendment is consistent with these results. The lower soil pH range (4.4–4.8) for the Zn2 and Zn3 treatments relative to the Zn0 and Zn1 treatments (pH 5.6–6.1) could have reduced the sorption of Zn to soil organic matter and minerals through a reduction in net negative charge on soil components (McBride 1991). This would have led to a higher percentage of Zn in the soluble+exchangeable fractions at higher levels of Zn treatment. The rhizosphere soils did not show any significant difference in Zn fractionation relative to the bulk soil. This may be due to poor separation of rhizosphere soil from bulk soil because the root density in the pots was very high.

The lower soil solution and soluble+exchangeable solid-phase Cu concentrations relative to the corresponding Zn concentrations as also found for metals amended biosolids by McLaren and Clucas (2001) and soils (Kunito et al. 2001) explain the lack of apparent phytotoxicity of Cu, but obvious phytotoxicity of Zn to radiata pine growth in the current study. These observations are in agreement with those made by Jeyakumar et al. (2010) for poplar under similar growth conditions.

Dehydrogenase activity

The rhizosphere soils did not show any significant difference in dehydrogenase activity relative to the bulk soil. Even though Cu was not found to be toxic to plants, it was found to be toxic to soil microorganisms at all levels of Cu addition, as observed from the decrease in soil dehydrogenase activity with increasing Cu concentration (Fig. 3).

The effect of Zn on soil microorganisms was the same as that for Cu; increasing the rate of Zn decreased soil dehydrogenase activity at all levels of Zn addition. However, Zn addition also reduced plant growth (Table 1). A reduction in dehydrogenase activity affected by both Cu and Zn was also found by Jeyakumar et al. (2010) for similar concentrations of total Cu and Zn in soil. The decrease in dehydrogenase activity relative to the control was greater for Zn than Cu, as has also been found by others (Kunito et al. 2001; Broos et al. 2007). This is because of the much higher increase in bioavailable Zn (soil solution and exchangeable Zn) compared to Cu which was largely complexed by DOC and soil organic matter. However, toxicity per unit soil solution or exchangeable Cu was greater for Cu as explained in the following paragraph.

The dehydrogenase activity data were correlated with solution-phase and soluble+exchangeable solid-phase metal concentrations. The concentration of metal in the solution-phase and in the soluble+exchangeable solid-phase that corresponded to a 50 % reduction in dehydrogenase activity (EC_{50}) was determined. For Cu, the EC_{50} values were not able to be determined because there were insufficient data points at high Cu concentrations. Therefore, EC_{40} values (Cu concentration corresponding to 40 % reduction in dehydrogenase activity which is equivalent to 60 % activity in Fig. 4) were calculated. The EC_{40} values for solution-phase and for soluble+exchangeable solid-phase Cu were 0.5 mg/L and 14.5 mg/kg, respectively. The EC_{50} values for solution-phase and for soluble+exchangeable solid-phase Zn were 9 mg/L and 55 mg/kg, respectively (Fig. 4). The EC_{40} values for corresponding species for Zn were 5 mg/L and 33 mg/kg, respectively. The lower EC values for Cu than for Zn indicate that Cu is more toxic for microbial activity. This may suggest that the intra- and extracellular enzymes functioning in the microorganisms is more sensitive to excessive Cu than Zn despite the higher soil solution concentrations of the latter (Hartikainen et al. 2012).

Where relative toxicity of Cu and Zn is ascribed to the total metal concentration in soil, Zn has a greater toxic effect on soil microbial activity than Cu (Fig. 3). This statement is validated through consideration of soil dehydrogenase activity as a function of Cu and Zn at the same total soil metal concentration. Comparison of TPF for the Cu2 (146 mg Cu/kg) and Zn1 (141 mg Zn/kg) treatment shows that microbial activity was lower

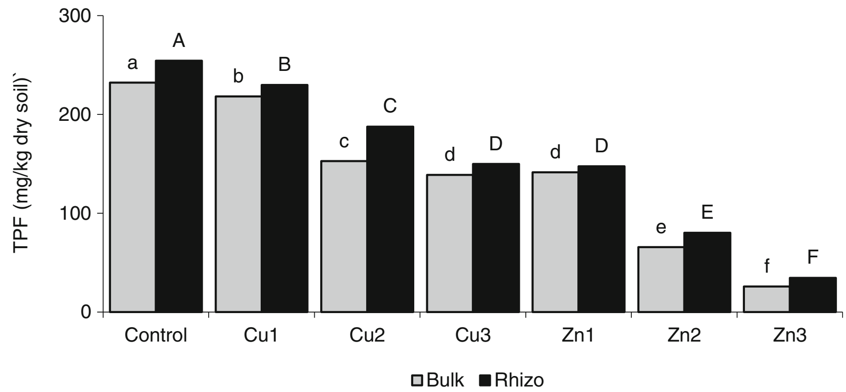


Fig. 3 Dehydrogenase activity (TPF) in bulk and rhizosphere soils at different levels of Cu and Zn. *Bars with different letters* are significantly different ($P \leq 0.05$). *Simple letters* indicate the variance among the bulk soils and *capital letters* are the rhizosphere soils

for Zn than Cu. However, when the metal concentration is expressed in terms of bioavailable soil metal (soil solution and the soluble+exchangeable fraction), the toxicity of Zn was lower than that of Cu (quantified as a higher EC_{40} value of Zn than Cu; Fig. 4). This difference illustrates variations in bioavailability between Cu and Zn relative to total soil metal concentrations. This differential effect highlights the importance of carefully interpreting results of the toxicity of Cu and Zn on microbial activity. Broos et al. (2007) estimated the Cu and Zn EC_{50} concentration values for substrate-induced nitrification and respiration activities in 12 soils previously amended with Zn (5–9,100 mg/kg) and Cu (3–5,880 mg/kg) that were collected in fields from various parts of Australia. They concluded that when the EC_{50} values were based on a total metal concentration, there was a strong 1:1 relationship between EC_{50} values for Cu and Zn across all soils, indicating equal microbial toxicity of these metals in soil. However, when EC_{50} values were based on the soil solution concentration, Cu generally was more toxic (lower EC_{50}) than Zn as observed in the current study. These results indicate the importance and reliability of interpreting the toxic effects of metals in terms of their bioavailability as measured by metal concentration in soil solution rather than the total metal concentration associated with soil particles.

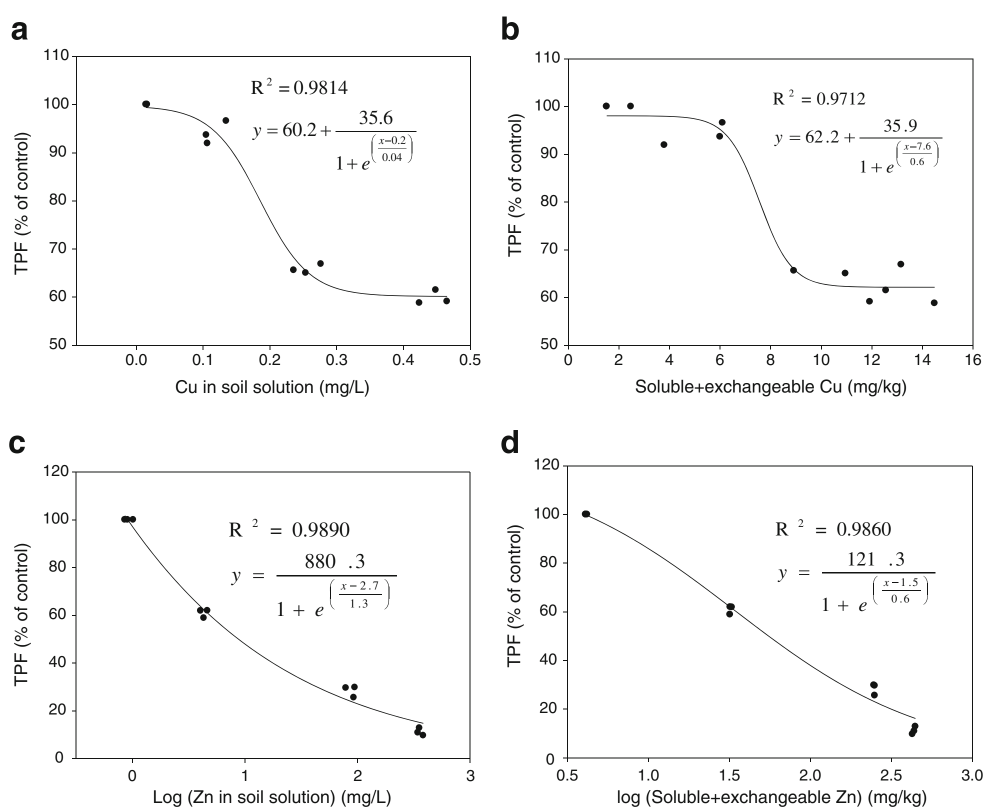


Fig. 4 Relationship of dehydrogenase activity (TPF) with **a** soil solution Cu, **b** soluble+exchangeable Cu, **c** soil solution Zn and **d** soluble+exchangeable Zn

Conclusion and recommendations

An increasing amount of Cu applied to soil in the form of Cu-amended biosolids increased the concentration of Cu in radiata pine needle, but had no effect on needle and root DM production, even at the highest total soil concentration of 232 mg/kg. However, the effect of Zn was varied when an increasing amount of Zn was applied to soil in the form of Zn-amended biosolids. A severe phytotoxic effect was observed when the total soil Zn concentration exceeded 141 mg/kg. Cu was not phytotoxic because the Cu^{2+} concentration in soil solution was extremely low (0.06 μM) due to most of the solution Cu being complexed to dissolved organic matter (approximately 99 %). The phytotoxic effect of Zn was due to a high Zn^{2+} concentration in soil solution (41–4419 μM, 61–80 % of soil solution Zn). The critical concentration value for phytotoxicity was calculated to be 62 μM Zn^{2+}. In addition, soil metal fractionation showed that the total Cu concentration in the soluble+exchangeable Cu fraction (2–13 mg Cu/kg soil; 5–12 % of total Cu) was significantly lower than that for Zn (4–437 mg Zn/kg; 12–66 % of total Zn) suggesting that the bioavailable solid-phase Zn may have also contributed to Zn phytotoxicity to radiata pine. The lower bioavailability of Cu resulted in a lower BCF value for Cu (0.03–0.26) compared with that for Zn (0.8–1.5). Translocation of Cu from root to needle was also lower than that for Zn as indicated by the lower needle/root ratio for Cu relative to Zn.

Soil microbial activity was inhibited by both Cu and Zn at all levels of metal addition. The rate of reduction relative to the control was greater for Zn than for Cu. EC_{40} for Cu for solution-phase and soluble+exchangeable solid-phase was calculated to be 0.5 mg/L and 14.5 mg/kg, respectively. For Zn, the corresponding EC_{50} values were 9 mg/L and 55 mg/kg.

In summary at similar rates of total soil metal concentration, Cu was not phytotoxic to radiata pine, but Zn was. Both Cu and Zn showed the same toxicity trend to soil microbial activity. With respect to commercial pine forestry, we propose that the current recommended limits for the total Cu and Zn concentration in New Zealand soil (100 mg/kg for Cu; 300 mg/kg for Zn) are too high for soil microbial activity. With respect to phytotoxicity, we recommend that the total Zn concentration limit in soils be revised downwards as the present limit exceeds the upper critical toxicity level to radiata pine.

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