# Technical Note: The effect of time since metal salts addition on the toxicity of Cu and Zn to wheat and microbial function

Heemsbergen DA, Warne MStJ, Broos K and McLaughlin MJ

Centre for Environmental Contaminants Research, CSIRO, PMB 2 Glen Osmond,
South Australia, Australia.

## Introduction

Processes of natural attenuation of metals (i.e. ageing) such as copper (Cu) and zinc (Zn) in soils can decrease the extractability and bioavailability of Cu and Zn over time and therefore lead to a substantial reduction in their toxicity to terrestrial species (Ma et al., 2006). Attenuation processes include adsorption and absorption of metals to organic matter, specific hydroxides, oxidation/reduction processes to less available forms and complexation in the water phase to nano-particles and dissolved organic matter. It is known that natural attenuation processes of Cu and Zn can be quite rapid, depending on the soil type (McBride, 2000). Han and Banin (2000) showed a dual phased adsorption for Cu where initially fast adsorption was followed by a slower phase that may last for an indefinite time period.

Current terrestrial ecotoxicology and ecological risk assessments are based almost exclusively on soils freshly spiked with metal salts in the laboratory, which only allow a limited equilibration time of generally one week. Some scientists have questioned if this limited equilibration time is sufficient for laboratory soils to resemble field aged soils (e.g. Amorim et al., 2005). Several studies have shown a discrepancy between toxicity of field aged soils and freshly spiked soils (Oorts et al., 2006; Smolders et al., 2004; Smit and Van Gestel, 1998; Lock and Janssen, 2003). In general, the freshly spiked metals were more bioavailable and therefore showed more toxic potential than metals in the field aged soils.

The Australian National Biosolids Research Program (NBRP) consisted of both field- and laboratory-based components which ran over three or four years depending on the site. One of the aims of the NBRP was to develop biosolids guidelines and soil quality guidelines for Cu and Zn. As the NBRP had phytotoxicity and microbial toxicity data available for a number of years, it was necessary to determine which years data should be used (i.e. immediately after metal application, after 1 year, after 2 year etc). This could be done by assessing changes in toxicity over time. In the field-sites, the crops grown were consistent with standard farmer practice in that region. This increased the number of species for which field-based toxicity data were generated but also decreased the number of sites at which the same crop was grown in any given year (Table 1). The most frequently grown field crop was wheat but even this was only grown in two or more years during the NBRP at three sites (Table 1). Therefore field-based phytotoxicity data could not reliably be used to determine if there were temporal changes in phytotoxicity. However, laboratory-based phytotoxicity tests that exposed bread wheat (*Triticum aestivum* L.) to Cu and Zn metal salts were conducted using freshly spiked soils from the NBRP field sites and the same spiked soils that were field-aged for 3 years and these could be used to assess ageing. Field-based microbial toxicity data was available over two years. Thus it was possible to determine whether ageing of Cu and Zn metal salts occurred in the Australian soils used in the NBRP in terms of toxicity to micro-organisms and plants. The aim of this study was therefore to determine whether ageing of Cu and Zn added to soils occurred and therefore which toxicity data should be used to derive soil quality guidelines.

## Methods

To test the effect of natural attenuation on phytotoxicity we performed wheat growth bioassays using soils from the NBRP field-sites that were freshly spiked with metal salts (T0) and that had been aged in the field for three years (T3). Details of the phytotoxicity test methods are provided in Warne et al. (*in press*). Each toxicity test consisted of eleven treatments: a control and ten increasing metal concentrations each conducted in duplicate. The toxicity tests were based on the OECD plant toxicity test method (OECD, 2000) in which *Triticum aestivum* L. (var. "Frame") was grown for 21 days under optimal conditions in the laboratory. The measure of toxicity was wheat shoot biomass after 21 days.

To test the effect of natural attenuation on microbial functioning we performed substrate induced respiration (SIR) and substrate induced nitrification (SIN) bioassays using soils from the NBRP field-sites that were freshly spiked with metal salts (T0) and that had been aged in the field for one and two years (T1 and T2). The microbial toxicity test methods are described in Broos et al. (2007).

The soil concentrations of Cu and Zn that caused a 10% reduction in plant growth (EC10) and a 20% reduction in SIN and SIR (EC20), their standard error and 95% confidence intervals were calculated by fitting a logistic distribution to the added total metal concentrations data using the method of Barnes et al. (2003). Added total metal concentrations were determined as the measured total concentration of each soil sample minus the average total metal concentration of all the controls for that site.

Differences between the toxicity of Cu or Zn in freshly spiked and field aged soils to microbial SIN, SIR and wheat were determined at the level of individual sites and across sites at the national level.

To assess temporal differences between EC10s at each site, multiple t-tests with Fisher-Bonferroni's correction were performed using the method of Satterthwaite (1946). The Fisher-Bonferroni's correction was used as when multiple statistical tests are conducted the probability of stating that there is a significant difference when there is not (i.e. a type I error), increases. The probability of making at least one type I error when multiple tests are conducted is calculated using the formula (Sankoh et al., 1997):

Probability of making at least one type I error $= 1 - (1 - \alpha)k$; (1)

where $\alpha$ is the level of significance used for each test (typically 0.05) and k is the number of tests repeated in the study. Therefore as the number of repeat statistical tests increases, the probability of making a type I error also increases. For example, in five tests the probability of finding at least one significant difference due to chance equals 0.2, or one in five, while in ten tests this probability increases to 0.40, which is approaching one in two. The Fisher-Bonferroni correction addresses this by making the critical p value (equal to or below which a significant difference is inferred) the normal value of 0.05 by the number of tests being conducted. Thus, if ten tests are conducted the critical p value is 0.005 (i.e. 0.05/10).

For the SIR data, we only tested significant changes of T0 and T2 data to reduce the number of t tests needed and prevent the critical p value decreasing to an unrealistic level due to the Fisher-Bonferroni's correction. If p values get too low, the differences in the EC20 values would have to be very large in order to be significantly different. For the SIN data the site specific analyses was not performed as changes in soil pH occurred between the sampling events and it is known that pH has a strong effect on SIN toxicity data (Broos et al., 2006; Smolders et al., 2004).

For the SIR, EC20 values were not always calculable due to the metals not exerting a marked effect. In such cases the highest tested concentration in the field trials was adopted as an unbounded no observed effect concentration. The NOECs do not have any measure of variability, which is required for the t-tests therefore no statistical comparisons could be made for sites that had NOEC values.

The effect of time across all sites was assessed using linear regression with groups on GenStat 8 (VSN International, Hertz, UK), with time being the group factor. The explanatory factor was the soil property which explained most of the variance in toxicity data found in the normalisation relationships for wheat developed by Warne et al. (*in press*) and for SIN and SIR developed by Broos et al. (2007). For wheat these were pH for Cu and CEC for Zn. For SIN this was soil pH for both metals. This type of analysis tests whether the group factor (i.e. time in this case) affects the gradient and the y-intercept of the relationships between toxicity and the explanatory factor(s). There were no statistically significant normalisation relationships for SIR EC10 and EC20 data for either metal. Therefore this approach could not be used and we resorted to analysis for differences at the site level.

The EC20 values generally have a smaller range between the lower and upper 95% confidence limits than EC10 data (data not shown). Therefore the differences between EC20 values can be smaller and significant differences still detected than if EC10 values were compared. In order to use the type of toxicity data in the comparisons for SIN and SIR EC20 data were used.

In the microbial toxicity data the number of sites that did not yield an EC20 value increased over time (i.e. T0 – T2). This meant that the normalisation relationships would be based on fewer sites at each time interval and as a result it would be increasingly difficult to determine if there were significant changes in toxicity over time. In cases where an EC20 was not available the highest tested concentration was used as a surrogate of the EC20 and used in the derivation of the normalisation relationships at each sampling time (i.e. T0, T1 and T2). The use of EC10 data, rather than EC20 data, would not have alleviated this problem, as generally if we obtained an EC10 value an EC20 was also obtained.

## Results and Discussion

### *Temporal changes in wheat toxicity*

Wheat toxicity data (EC10s) for the two time periods and the probability of the t tests comparing corresponding values are shown for Cu and Zn in Tables 2 and 3 respectively. Nine t-tests were conducted for the Cu dataset (Table 2), therefore the critical p value was 0.0056 (i.e. $0.05 \div 9$). There were two sites (i.e. Brennans and Cecil Plains) for which no EC10 value was available for the field-aged soils. For these two sites the highest soil concentration after three years field ageing did not cause a 10% inhibitory effect. Despite not having an EC10 value the situation at Brennans is consistent with ageing having occurred as at T0 a concentration of 590 mg/kg caused an EC50. The concentration at Cecil Plains is insufficiently high at T3 that nothing can be inferred regarding ageing of Cu. Of the nine sites where statistical comparisons could be made, only three sites (Avon, Bundaberg and Night Paddock) had significant ($p \leq 0.006$) differences in toxicity and in all three cases the toxicity of the field-aged soils was lower (i.e. the EC10 values were larger).

For Zn, all the sites had EC10 values for both the freshly spiked and field-aged soils, except for Wilsons which was lacking a field-aged toxicity value. The highest concentration in the field-aged Wilsons soil was essentially identical to the concentration that caused a 10% inhibition of wheat growth initially. But given the normal experimental error nothing can be inferred regarding the ageing of Zn at Wilsons. With the Wilsons field-aged toxicity value missing, only ten statistical comparisons could be made. As a result, the critical p values were 0.005 (i.e. $0.05 \div 10$). Thus, two sites (Spalding and Tintinara) showed a significant ($p < 0.0001$ for both) decrease in toxicity (i.e. EC10 values increased) while one site (Cecil Plains) showed a significant ($p < 0.001$) increase in toxicity (i.e. EC10 values decreased) for Zn. The remaining seven sites did not exhibit any significant ($p > 0.05$) change in toxicity over time.

While the above results of whether ageing occurs at individual sites are useful, it is less relevant in deriving soil and biosolids quality guidelines at the national level. Here, the results of the second analysis, where changes in phytotoxicity over time across all sites were examined, are more relevant. There were no significant ($p > 0.05$) differences in the slopes and y intercepts of the normalisation relationships between Cu EC10 values and pH at T0 and T3 (Figure 1). Similarly, there were no significant ($p > 0.05$) differences in slopes and y intercepts of the normalisation relationships between Zn EC10 and CEC at T0 and T3 (Figure 2). This indicates that although some individual sites exhibited changes in bioavailability of Cu and/or Zn, this did not significantly ($p > 0.05$) affect the normalisation relationships between soil properties and toxicity values at a national scale. Therefore at a national scale there was no ageing of Cu and Zn toxicity between the freshly spiked and field-aged (3 years) soils.

The T0 phytotoxicity tests consisted of spiking the soils with metal salts followed by a seven day incubation period, at which point seeds were introduced and exposed for 21 days. Thus the metal salts aged for seven days before introduction of the plants and then continued for the duration a total of 28 days. The fact that there was no change in phytotoxicity after three years ageing in the field at the national level indicates that the extent of ageing was the same in the freshly spiked and field-aged soils. It also means that the ageing of Cu and Zn was rapid and predominantly occurred within the first month of metal salt application.

*Temporal changes in microbial toxicity*

As stated previously the site-specific comparison for ageing could not be conducted for the SIN data therefore a comparison was made at the national level by comparing the relationships between pH and a combination of SIN EC20 and NOEC values over time. There were no significant ($p > 0.05$) differences in the slopes and y intercepts of the normalisation relationships between Cu EC20 and NOEC (SIN) values and pH at any time interval (Figure 3). Similarly, there were no significant ($p > 0.05$) differences in the slopes and y intercepts of the normalisation relationships between Zn EC20 (SIN) and pH at any time interval (Figure 4). Therefore at a national scale there was no decrease of Cu and Zn toxicity to SIN between the freshly spiked and field-aged (2 years) soils and hence no evidence of ageing.

The T0, T1 and T2 SIR log EC20 and log NOEC values for Cu and Zn at each site are presented in Figures 5 & 6 respectively. For Cu, four sites showed a consistent increasing trend in EC20 values over time, one site showed a consistent downward trend and seven sites showed no consistent trend in the EC20 values over time. Only for two of the sites (Flat Paddock, and Spalding) were the EC20 values at T2 significantly greater than T0 (Table 4). For Zn, one site showed a consistent increasing trend in their EC20 values over time, whereas two sites showed a consistent downward trend in EC20 values and eight sites showed no consistent trend in EC20 values over time (Figure 6). Spalding had a significantly higher EC20 at T2 than T0 indicating ageing at this site. Therefore at a national level there is no overall temporal change in the SIR values for Cu and Zn.

The T0 microbial SIN and SIR toxicity tests consisted of spiking the soils with metal salts followed within two weeks by the collection of soil samples. Samples were dried (40 ºC) and stored in airtight containers under ambient conditions until required. For SIR the soils were incubated for 14 days followed by six hours exposure to $^{14}$C glucose. Assuming natural attenuation processes were limited after drying of the soils, the effective natural attenuation time was a maximum of 29 days. For SIN soils were incubated for 14 days followed by 28 days exposure, giving an effective natural attenuation time of 56 days. The fact that there was no change in microbial toxicity after two years ageing in the field at the national level indicates that the extent of ageing was the same in the freshly spiked and field-aged soils for most soils. It also means that the ageing of Cu and Zn was rapid and predominantly occurred within the first two months of metal salt application.

*Chemical estimates of temporal change in toxicity*

Calcium chloride ($CaCl_2$) extractable metal concentrations were measured using the same soil samples that were used for the microbial toxicity testing. The $CaCl_2$ extractable metal concentrations varied with site and with the concentration of metal salt added. Despite this, at soil metal concentrations below 1000 mg/kg the percentage of extractable metal was generally low at T0; i.e. 10 – 40% for Zn and 1 – 25% for Cu

(Whatmuff et al., *in prep*). If it is assumed that the percentage of extractable metal on addition of the metal salts was 100% then the $CaCl_2$ extractable soil concentrations show that ageing is rapid, which is consistent with the inferences from the phytotoxicity and microbial toxicity results. At a few sites the $CaCl_2$ extractable fraction decreased over time (i.e. between T0 and T3) but the decrease was less than 10% (at concentrations below 1000 mg/kg Zn). Work by Donner (2006) examined ageing of Zn under laboratory conditions in soils from four NBRP sites. This found the vast majority of the ageing occurred within days with little subsequent ageing as evidenced by $CaCl_2$ extractable concentrations and toxicity to a genetically modified *lux*-marked bacterial biosensor, *Escherichia coli* HB101 pUCD607. These findings are consistent with other research that has shown that chemical ageing of Cu and Zn can be quite rapid and ageing rates depend on soil type (e.g. McBride, 2000).

### *Implications of the lack of temporal change*

As there was no temporal change in the toxicity of Cu and Zn to wheat and microbial SIN and SIR at a national level and changes were limited to only a few individual sites, it could be argued that toxicity data from all sites and all times could be used to derive soil and biosolids quality guidelines. However, the data obtained from each site at successive sampling times are not statistically independent (Warne et al., *in prep a*). Therefore, only toxicity data for the first wheat crop grown at each site (Table 1) and the first sampling for microbial SIN and SIR (i.e. T0) was used to develop relationships between phytotoxicity and soil physicochemical properties and used to derive soil and biosolids quality guidelines. These relationships for wheat are presented in Warne et al. (*submitted*) and for microbial function in Broos et al. (2007) and Warne et al. (*in prep b*).

## Conclusions

For a limited number of the NBRP sites there are changes in the phytotoxicity and hence bioavailability of Cu and Zn between freshly spiked and soil aged in the field for three years. However, in terms of deriving national guidelines for soils and biosolids changes in phytotoxicity (and bioavailability) at individual sites are not particularly relevant. There was no change in the normalisation relationships that explain wheat phytotoxicity and microbial toxicity using soil properties for freshly spiked and field-aged soils. In other words at the national level there was no change in the bioavailability of Cu and Zn at the NBRP sites over at least two years post application of metal salts. As data obtained from successive samples from the field-sites are not independent, only the toxicity data from the first wheat crop grown in each site and the first set of microbial toxicity data were used to derive relationships between toxicity and soil physicochemical properties and hence in the derivation of soil and biosolids quality guidelines.

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Table 1: Crops grown in the field sites of the National Biosolids Research Program

| Site | 2002 | 2003 | 2004 | 2005 | 2006 |
|---|---|---|---|---|---|
| Avon | Wheat | Wheat | Barley | Wheat | - |
| Brennans | - | Wheat | Canola | Wheat | - |
| Bundaberg | Sugar cane | Sugar cane | Sugar cane | Peanut | - |
| Cecil Plains | Sorghum | Cotton | Wheat | - | - |
| Dookie | - | Canola | Wheat | Barley | - |
| Dutson Downs | - | Triticale | Wheat | Barley | - |
| Esk | Eucalypt | Eucalypt | Eucalypt | Eucalypt | - |
| Flat Paddock | Pasture & Wheat | Pasture & Wheat | Pasture & Wheat | - | - |
| Kingaroy | Millet | Maize | Peanut | Sorghum & Wheat [1] | - |
| Lowood | Sorghum | Sorghum | - | - | - |
| Melton | - | Oats | Wheat | Barley | - |
| Mildura | - | - | Grapes | Grapes | Grapes |
| Night Paddock | Pasture & Wheat | Pasture & Wheat | Pasture & Wheat | - | - |
| Packenham | - | - | Pasture | Pasture | Pasture |
| Spalding | Wheat | Wheat | Barley | Barley | - |
| Tintinara | Canola | Wheat | Barley | Canola | - |
| Wilsons | - | Canola | Wheat | Canola | - |

[1] sorghum grown Nov 04 – Apr 05 and wheat grown Jun – Dec 05.

Table 2: Added total concentrations of copper that caused a 10% inhibition of wheat growth (EC10 21 day shoot biomass) (mg/kg added), the logarithm of the standard error and number of data points (n) used to derive each toxicity value in freshly spiked and field aged spiked (three years) soils and the probability that the EC10 values are significantly different (using student t tests). Fisher-Bonferroni correction for the nine t-tests results in a $p \leq 0.006$ being significant. Sites in bold have significant changes in toxicity over time.

| **Site** | **Spiked** | | | **Field aged** | | | **T test** |
|---|---|---|---|---|---|---|---|
| | **EC10 (mg/kg)** | **log SE** | **n** | **EC10[a] (mg/kg)** | **log SE** | **n** | ***p*** |
| **Avon** | **945** | **0.035** | **22** | **2764** | **0.131** | **24** | **0.003** |
| Brennans | 205 | 0.727 | 22 | >1194 | na | 23 | na |
| **Bundaberg** | **260** | **0.158** | **22** | **1004** | **0.025** | **22** | **0.002** |
| Cecil Plains | 3295 | 0.125 | 21 | >1012 | na | 22 | na |
| Dookie | 490 | 0.054 | 22 | 550 | 0.156 | 24 | 0.762 |
| Flat Paddock | 115 | 0.310 | 44 | 39 | 0.099 | 14 | 0.171 |
| Kingaroy | 810 | 0.094 | 22 | 868 | 0.099 | 21 | 0.818 |
| **Night Paddock** | **110** | **0.116** | **22** | **582** | **0.114** | **10** | **0.0003** |
| Spalding | 930 | 0.076 | 21 | 841 | 0.022 | 24 | 0.586 |
| Tintinara | 430 | 0.096 | 22 | 1623 | 0.279 | 24 | 0.063 |
| Wilsons | 465 | 0.108 | 22 | 946 | 1.685 | 22 | 0.856 |

[a] EC10 values with > mean that the EC10 value was greater than the highest measured soil concentration which is stated. na = not available.

Table 3: Added total concentrations of zinc (mg/kg added) that inhibit wheat growth by 10% (EC10 21 day shoot biomass), the logarithm of the standard error and number of data points (n) used to derive each toxicity value in freshly spiked and field aged spiked (three years) soils and the probability that the EC10 values are significantly different (student t test). Fisher-Bonferroni correction for the nine t-tests results in a $p \leq 0.005$ being significant. Sites in bold have significant changes in toxicity over time.

| **Site** | **Spiked** | | | **Field aged** | | | **T test** |
|---|---|---|---|---|---|---|---|
| | **EC10 (mg/kg)** | **log SE** | **n** | **EC10[a] (mg/kg)** | **log SE** | **n** | ***p*** |
| Avon | 755 | 0.073 | 22 | 2023 | 0.142 | 22 | 0.012 |
| Brennans | 275 | 0.045 | 22 | 284 | 0.095 | 23 | 0.053 |
| Bundaberg | 235 | 0.492 | 21 | 109 | 0.095 | 18 | 0.522 |
| **Cecil Plains** | **5855** | **0.063** | **21** | **1064** | **0.040** | **20** | **< 0.001** |
| Dookie | 965 | 0.743 | 22 | 930 | 0.058 | 24 | 0.983 |
| Flat Paddock | 250 | 0.181 | 44 | 431 | 0.094 | 12 | 0.264 |
| Kingaroy | 505 | 0.175 | 21 | 716 | 0.310 | 22 | 0.670 |
| Night Paddock | 275 | 0.096 | 22 | 1278 | 0.137 | 12 | 0.031 |
| **Spalding** | **620** | **0.087** | **22** | **1771** | **0.089** | **24** | **< 0.001** |
| **Tintinara** | **430** | **0.100** | **22** | **1995** | **0.133** | **23** | **< 0.001** |
| Wilsons | 335 | 0.041 | 22 | > 364 | na | na | na |

[a] EC10 values with > mean that the EC10 value was greater than the highest measured soil concentration which is stated. na = not available.

Table 4: Added total concentrations of copper (mg/kg added) that inhibit substrate induced respiration by 20% (EC20 SIR), the logarithm of the standard error and number of data points (n) used to derive each toxicity value in freshly spiked and field aged spiked (two years, T2) soils and the probability that the EC20 values are significantly different (student t test). Fisher-Bonferroni correction for the twelve t-tests results in a p ≤ 0.004 being significant. Sites in bold have significant changes in toxicity over time.

| **Site** | **Spiked** | | | **Field aged** | | | **T test** |
|---|---|---|---|---|---|---|---|
| | **EC20[a] (mg/kg)** | **log SE** | **n** | **EC20[a] (mg/kg)** | **log SE** | **n** | ***p*** |
| Avon | 345 | 0.107 | 26 | 45 | 0.571 | 34 | 0.14 |
| Brennans | 31 | 0.486 | 30 | 230 | 0.174 | 36 | 0.10 |
| Bundaberg | >555 | na | 19 | 351 | 0.285 | 20 | - |
| Cecil Plains | 385 | 0.204 | 21 | 596 | 0.081 | 24 | 0.40 |
| Dookie | 503 | 0.166 | 28 | 284 | 0.073 | 34 | 0.18 |
| Dutson Downs | 134 | 0.203 | 28 | > 449 | na | 34 | - |
| **Flat Paddock** | **111** | **0.182** | **29** | **2240** | **0.196** | **41** | **< 0.0001** |
| Kingaroy | 559 | 0.183 | 26 | 505 | 0.173 | 26 | 0.86 |
| Night Paddock | 421 | 0.100 | 29 | > 2440 | na | 37 | - |
| **Spalding** | **73** | **0.124** | **26** | **263** | **0.103** | **34** | **0.001** |
| Tintinara | 259 | 0.142 | 28 | 224 | 0.166 | 34 | 0.77 |
| Wilsons | 97 | 0.211 | 30 | 243 | 0.193 | 37 | 0.1 |

[a] EC10 values with > mean that the EC10 value was greater than the highest measured soil concentration which is stated. na = not available.

Table 5: Added total concentrations of zinc (mg/kg added) that inhibit substrate induced respiration by 20% (EC20 SIR), the logarithm of the standard error and number of data points (n) used to derive each toxicity value in freshly spiked and field aged spiked (two years, T2) soils and the probability that the EC20 values are significantly different (standard t test). Fisher-Bonferroni correction for the twelve t-tests results in a p ≤ 0.004 being significant. Sites in bold have significant changes in toxicity over time.

| **Site** | **Spiked** | | | **Field aged** | | | **T test** |
|---|---|---|---|---|---|---|---|
| | **EC20[a] (mg/kg)** | **log SE** | **n** | **EC20[a] (mg/kg)** | **log SE** | **n** | ***p*** |
| Avon | 486 | 0.216 | 28 | 1022 | 0.169 | 34 | 0.24 |
| Brennans | 260 | 0.075 | 30 | 189 | 0.085 | 36 | 0.31 |
| Bundaberg | 537 | 0.128 | 20 | > 425 | na | 20 | - |
| Cecil Plains | 583 | 0.216 | 23 | 1229 | 0.587 | 24 | 0.61 |
| Dookie | 1135 | 0.142 | 26 | 492 | 0.079 | 34 | 0.03 |
| Dutson Downs | > 2107 | na | 28 | 537 | 0.137 | 34 | - |
| Flat Paddock | 245 | 0.237 | 29 | 858 | 0.170 | 37 | 0.07 |
| Kingaroy | 931 | 0.135 | 26 | 432 | 0.306 | 26 | 0.33 |
| Night Paddock | 807 | 0.153 | 29 | > 3861 | na | 41 | - |
| **Spalding** | **134** | **0.189** | **28** | **806** | **0.189** | **32** | **<0.0001** |
| Tintinara | 546 | 0.128 | 26 | 567 | 0.099 | 34 | 0.92 |
| Wilsons | 550 | 0.089 | 30 | 257 | 0.121 | 35 | 0.04 |

[a] EC10 values with > mean that the EC10 value was greater than the highest measured soil concentration which is stated. na = not available.

Figure 1: Normalisation relationships between Cu EC10 (wheat growth) and soil pH for freshly spiked soils (T0) (Warne et al., *in press*) and the same soils that had been aged for three years in the field (T3). The normalisation equations were log EC10 Cu = 0.28* pH + 1.08 for T0 and log EC10 Cu = 0.30*pH + 1.19 for T3. The gradients and y-intercepts of the normalisation relationships were not significantly ($p > 0.05$) different at the different sampling times.

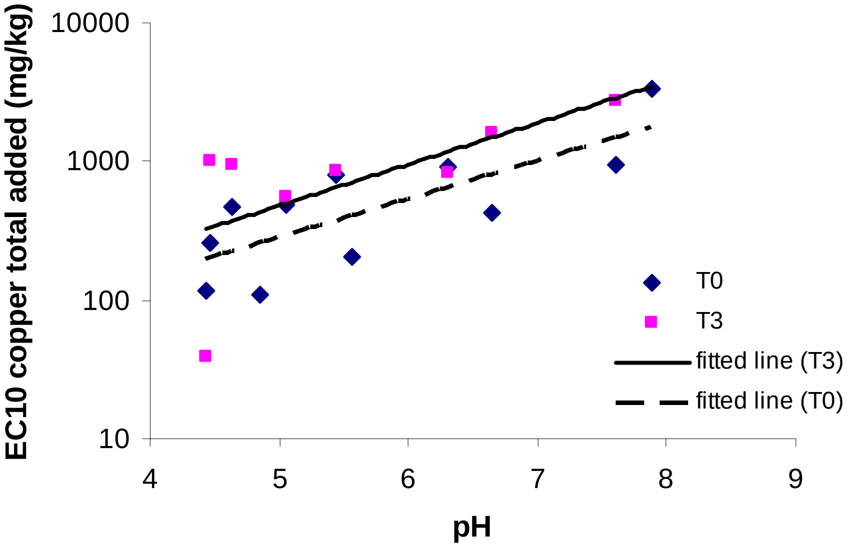


Figure 2: Normalisation relationships between Zn EC10 (wheat growth) and cation exchange capacity (CEC) for freshly spiked soils (T0) (Warne et al., *in press*) and the same soils that had been aged for three years in the field (T3). The normalisation equations were log EC10 Zn= 0.93 CEC + 1.76 for T0 and log EC10 Zn = 0.69 CEC + 2.16 for T3. The gradients and y-intercepts of the normalisation relationships were not significantly ($p > 0.05$) different at the different sampling times.

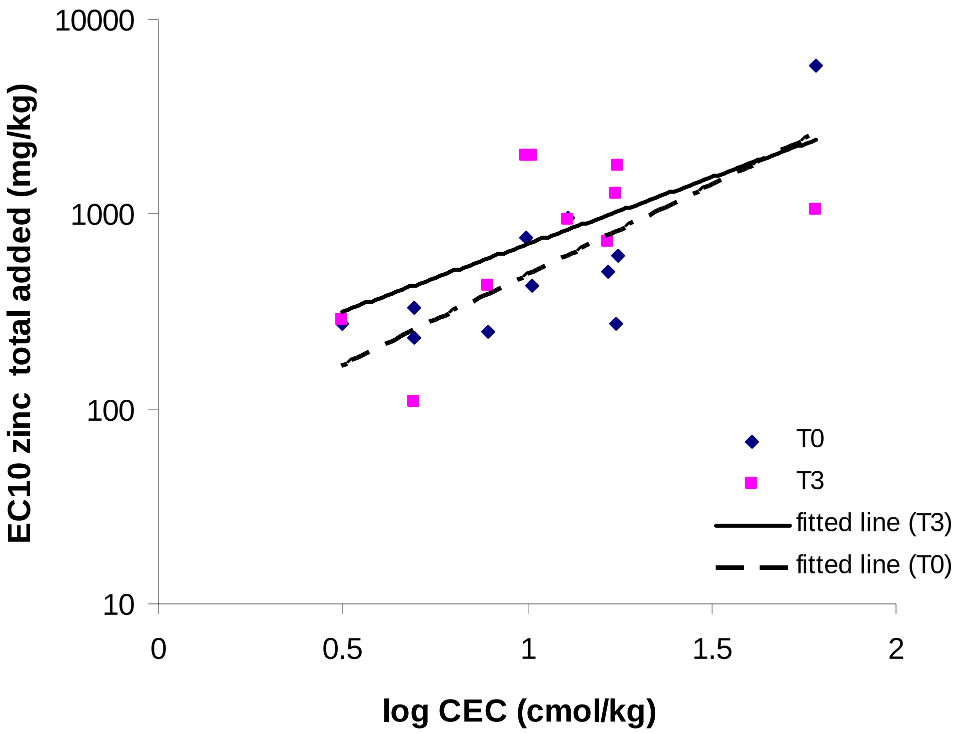


Figure 3: Normalisation relationships between Cu EC20 (substrate induced nitrification, SIN) and soil pH for freshly spiked soils (T0) (Broos et al., 2007) and the same soils that had been aged for one and two years in the field (T1 and T2). The normalisation equations were log Cu EC20 (SIN) = 0.43x + 0.23 $r^2 = 0.79$ for T0, log Cu EC20 (SIN) = 0.41x + 0.69 $r^2 = 0.62$ for T1, log Cu EC20 (SIN) = 0.33x + 0.68 $r^2 = 0.69$ for T2. The gradients and y-intercepts of the normalisation relationships were not significantly ($p > 0.05$) different at the different sampling times.

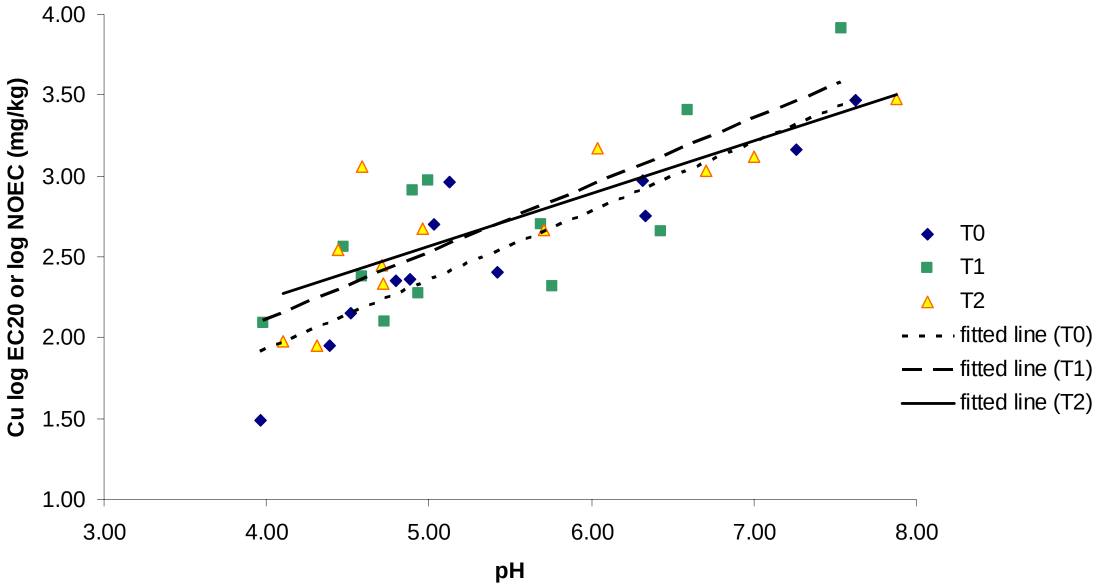


Figure 4: Normalisation relationships between Zn EC20 (substrate induced nitrification, SIN) and soil pH for freshly spiked soils (T0) (Broos et al., 2007) and the same soils that had been aged for one and two years in the field (T1 and T2). The normalisation equations were log Zn EC20 (SIN) = 0.47x - 0.004 $r^2$ = 0.74 for T0, log Zn EC20 (SIN) = 0.61x - 0.55 $r^2$ = 0.87 for T1, log Zn EC20 (SIN) = 0.44x + 0.42 $r^2$ = 0.65 for T2. The gradients and y-intercepts of the normalisation relationships were not significantly ($p > 0.05$) different at the different sampling times.

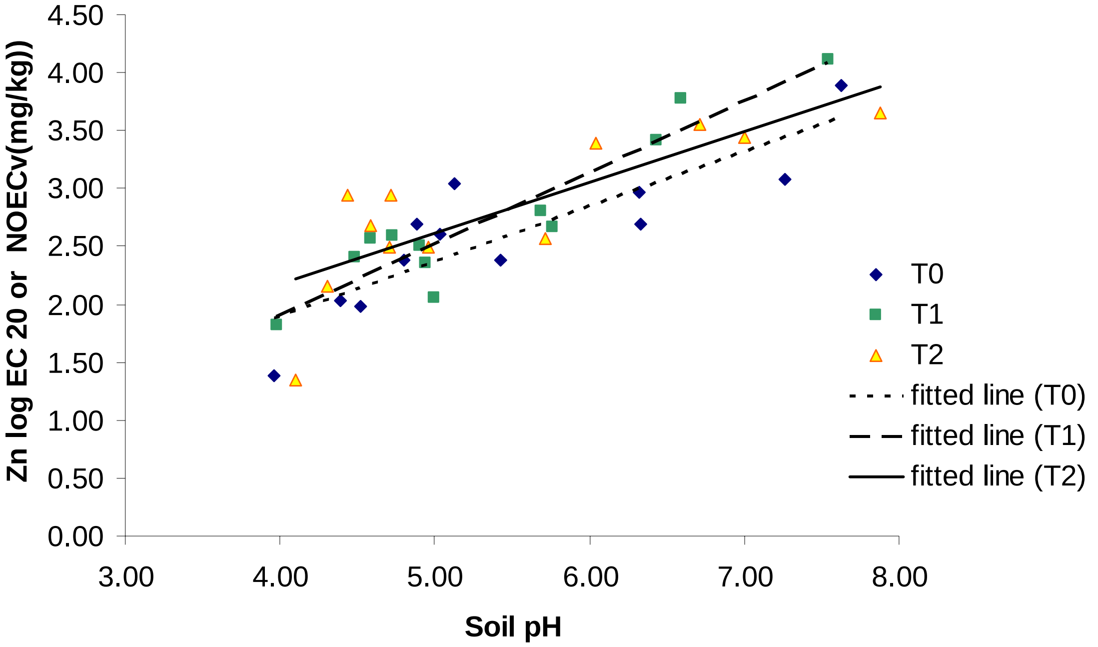
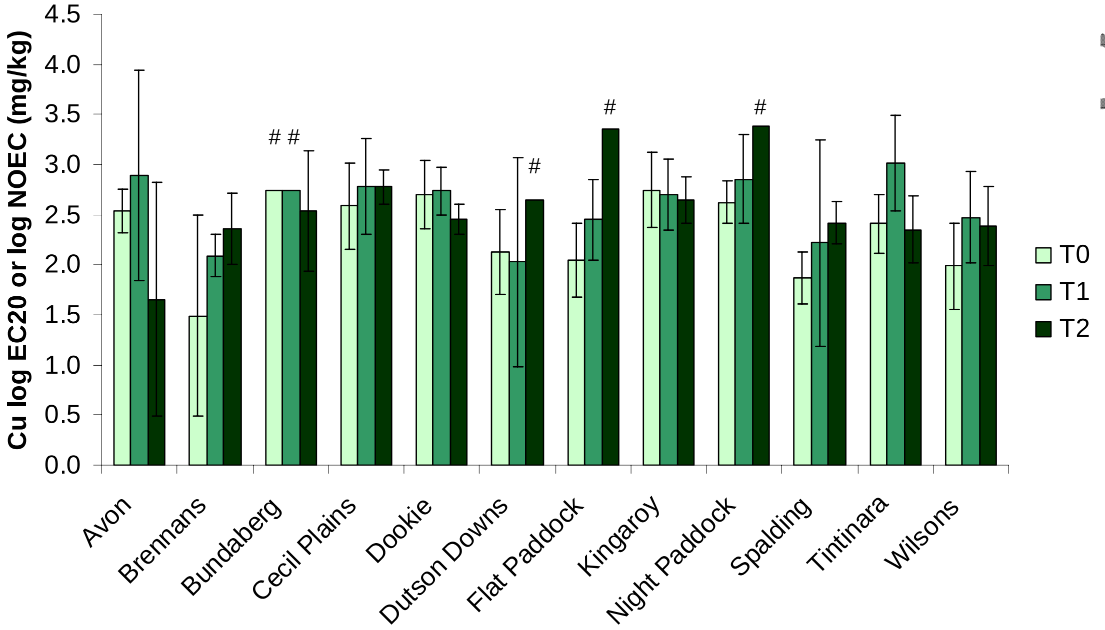
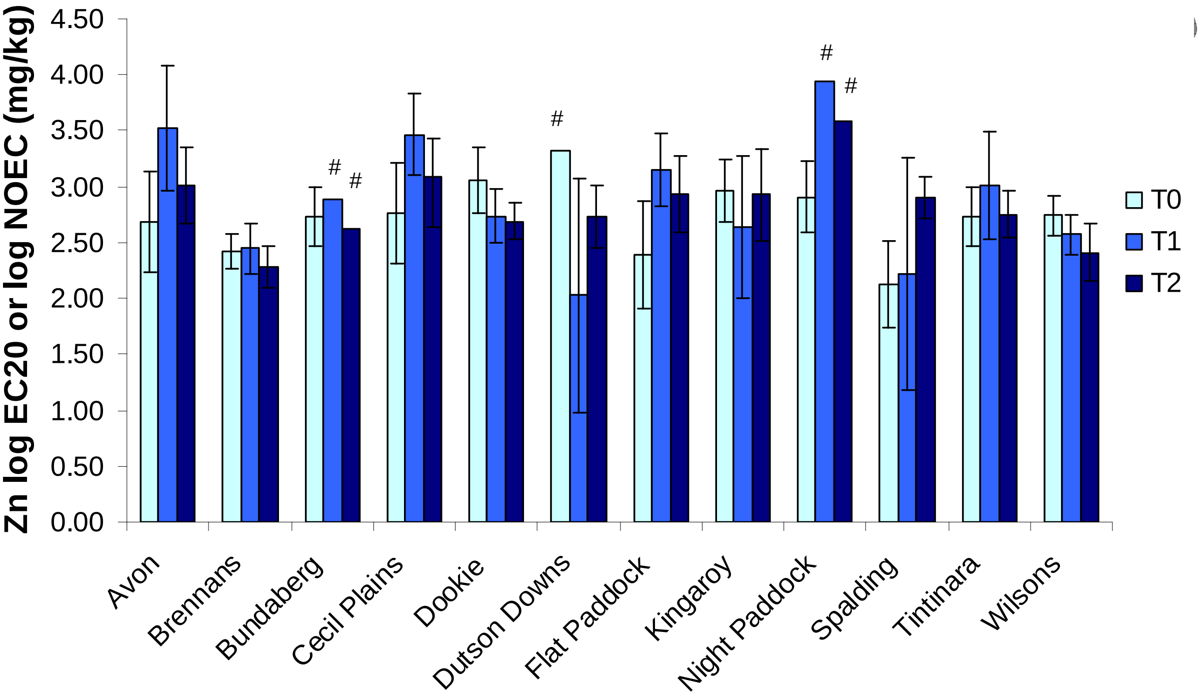


Figure 5: The logarithms of the copper concentrations that cause a 20% inhibition of substrate induced respiration (log EC20 (SIR)) or no observed effect concentration (log NOEC) at each site in freshly spiked (T0) soils and those aged in the field for one and two years (T1 and T2 respectively). Error bars are the 95% confidence limits.



# these values are log NOEC values.

Figure 6. The logarithms of the zinc concentrations that cause a 20% inhibition of substrate induced respiration (log EC20 (SIR)) or no observed effect concentration (log NOEC) at each site in freshly spiked (T0) soils and those aged in the field for one and two years (T1 and T2 respectively). Error bars are the 95% confidence limits.



# these values are log NOEC values.