

1 **Models for the field-based toxicity of copper and zinc salts to**
2 **wheat in eleven Australian soils and comparison to laboratory-**
3 **based models**

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1 Capsule

2 Field-based toxicity of Cu and Zn to wheat can be modelled using soil properties.

3 Laboratory based models should not be used to estimate toxicity in the field.

4

5 **ABSTRACT**

6 Laboratory-based relationships that model the phytotoxicity of metals using soil
7 properties have been developed. This paper presents the first field-based
8 phytotoxicity relationships. Wheat (*Triticum aestivum* L.) was grown at eleven
9 Australian field sites at which soil was spiked with copper (Cu) and zinc (Zn)
10 salts. Toxicity was measured as inhibition of plant growth at 8 weeks and grain
11 yield at harvest. The added Cu and Zn EC10 values for both endpoints ranged
12 from approximately 3 to 4760 mg/kg. There were no relationships between field-
13 based 8 week biomass and grain yield toxicity values for either metal. Cu toxicity
14 was best modelled using pH and organic carbon content while Zn toxicity was
15 best modelled using pH and the cation exchange capacity. The best relationships
16 estimated toxicity within a factor of two of measured values. Laboratory-based
17 phytotoxicity relationships could not accurately predict field-based phytotoxicity
18 responses.

19

20 **Key words** – Zinc, Copper, Wheat, Phytotoxicity, Soil Properties

21

INTRODUCTION

1
2 It has long been recognised that soil physicochemical properties are important
3 modifiers of the toxicity of contaminants (e.g. De Vries and Tiller, 1978;
4 Lexmond, 1980; Alloway, 1995) yet this knowledge has not widely been
5 incorporated into ecological risk assessments or the derivation of soil quality
6 guidelines. As a result very conservative values have tended to be adopted in order
7 to protect all or most terrestrial ecosystems.

8
9 Recently, a number of studies have converted this qualitative knowledge into
10 quantitative relationships. Thus, linear relationships, termed normalisation
11 relationships, have been developed that explain the variation in toxicity of As, Cu,
12 Zn and Ni to micro-organisms, plants and invertebrates (Rooney et al., 2006;
13 Smolders et al., 2003; Smolders et al., 2004; Oorts et al 2006; Broos et al., 2007;
14 EU, 2006; Song et al., 2006) and the uptake of Cd, Cu, Pb and Zn by plants (Nan
15 et al., 2002; Li et al., 2003; McLaughlin et al., 2006). These relationships permit
16 the normalisation of terrestrial toxicity and uptake data across soil types, and
17 allow soil quality guidelines to be site or soil specific, thus providing one
18 consistent level of protection at each site. This is a significant improvement on the
19 traditional approach, which applies a single guideline and therefore different
20 levels of protection to each site. The new approach should greatly reduce
21 unnecessary remediation or decontamination of sites and permit regulators and
22 proponents to concentrate on those sites that pose the greatest risk.

23

24 The authors are not aware of any field-based relationships for phytotoxicity
25 responses. It is worth noting that many have questioned the appropriateness of

1 laboratory phytotoxicity data for field situations. For example, Davies (1992)
2 found that pot experiments may not predict the toxicity of heavy metals to crops
3 grown in field conditions, while De Vries and Tiller (1978) found that data from
4 glasshouse-grown plants gave ‘completely erroneous’ indications of metal uptake
5 by field-grown plants.

6
7 Thus, while phytotoxicity normalisation relationships represent a significant
8 advance in environmental management, their reliance on laboratory-based toxicity
9 means that they may not be applicable to field situations. As a result, it is
10 important that predictions of laboratory-based normalisation relationships be
11 compared to field data.

12
13 This study reports findings from the Australian National Biosolids Research
14 Program (NBRP). The NBRP was established to quantify the potential human and
15 environmental benefits and risks of applying biosolids to agricultural land and to
16 develop soil and biosolids quality guidelines for metals (i.e. Cd, Cu and Zn) and
17 nutrients. The program is predominantly field-based, with 17 field sites in major
18 agricultural regions of Australia. Models of the toxicity of Cu and Zn to micro-
19 organisms (Broos et al., 2007) and risks of soil-grain transfer of biosolid Cd
20 (McLaughlin et al., 2006) have already been published. The aims of this paper
21 were to develop relationships based on soil physicochemical properties able to
22 model the field-based toxicity of Cu and Zn to bread wheat (*Triticum aestivum* L.)
23 and to determine how well laboratory-based relationships predict phytotoxicity in
24 the field.

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MATERIALS AND METHODS

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Plant sampling and preparation

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Eleven field sites from major agricultural regions of Australia were selected to provide a range of soil physicochemical properties (Table 1), with separate toxicity tests for Cu and Zn conducted for each site. Each test consisted of 12 duplicated treatments - 11 application rates of either CuSO₄ or ZnSO₄ and a control plot which received no metal addition. The concentrations of Cu and Zn added to the plots (minimum of 3m x 4m) were based on the results of laboratory-based toxicity tests (Warne et al., in press) and selected so that the effects on wheat at each site would range from no effect through to 100% lethality.

The metal salts were immediately incorporated into the soil to a depth of 10 cm using a rotary hoe or discs. Fertilizer was then applied to all plots according to local farmer practice.

Soil and crop data were collected from the sites for 3-4 years depending on the site. The crops grown were consistent with farmer practice in that region, resulting in 14 different crops being grown, but this paper reports only data for the most prevalent species - bread wheat (*Triticum aestivum* L.). Details of the sites and years when wheat was grown are shown in Table 2.

Plant samples were collected during each cropping season at 8 weeks post-sowing and at harvest. The 8-week sampling was used to assess early plant growth responses when plants might be more sensitive to metal toxicity, but also to

1 provide a sampling at a growth stage that was relatively similar to that used in
2 laboratory-based phytotoxicity assays.

3

4 At each sampling, the outer two rows of crops on each side of a plot were
5 discarded to avoid edge effects. At the eight week sampling, 20 wheat plants were
6 randomly selected from the central rows of each plot and the entire plant removed.

7 At harvest, two one-metre long rows from each plot were collected by cutting the
8 plants off at the soil surface and combined.

9

10 All plant samples were washed in a 0.1% solution of a phosphorus and metal-free
11 detergent in reverse osmosis water and then dried at 40 °C. For the harvest
12 samples, the grain was then separated from the stalks. The stalks were ground to <
13 2 mm. Stalk and grain samples were separately stored in sealed plastic vials prior
14 to analysis.

15

16 Total biomass of the sampled plants (g) was determined at the 8-week sampling,
17 while at harvest shoot yield and grain yield were calculated (t/ha) and grain size
18 was determined by measuring the mass of 100 dry grains. Grain protein content
19 was determined by ashing whole grains in a LECO C, N and S analyzer (Leco
20 Corporation, St. Joseph, Michigan, USA) at approximately 1200 °C and analyzing
21 for nitrogen. The percent protein in wheat grains was calculated by:

22

$$23 \quad \text{Grain Protein (\%)} = \text{grain nitrogen (\%)} \times 5.7 \quad (1)$$

24

25 *Soil sampling and preparation*

1 Within two weeks of metal salt application, four composite top soil samples (0-10
2 cm depth) were collected using a 5 cm diameter auger from each plot. In addition
3 soil samples were also collected from directly beneath the sampled plants at each
4 harvest. All soil samples were air dried at 40°C, ground, sieved < 2 mm and stored
5 in air-tight containers at room temperature prior to chemical analysis.

6 7 ***Soil properties***

8 The soil pH (0.01M CaCl₂) was measured in 50 mL aerated deionised water (1:5
9 soil/solution ratio) after shaking for 1 h and settling for 20 minutes then adding
10 1mL of 0.5M CaCl₂, shaking for 30 seconds and settling for another 20 minutes
11 (Rayment and Higginson, 1992). The cation exchange capacity (CEC, cmol_c kg⁻¹)
12 was measured in alcoholic 1M NH₄Cl at pH 8.5 for Avon, Spalding, Tintinara and
13 Cecil Plains and at pH 7.0 for all other soils (Rayment and Higginson, 1992).
14 Organic carbon (OC) was determined as the difference between total carbon and
15 carbonate carbon. Total carbon concentration was measured by ignition with a
16 Leco CNS elemental analyser (Leco, St. Joseph, MI, USA) and the carbonate
17 carbon was determined from pressure increases after addition of HCl to the soil in
18 closed containers (Sherrod et al., 2002). Clay content was determined using the
19 USDA particle size analysis method (USDA, 1996).

20 21 ***Metal analysis***

22 *Total soil digests.* A 0.5 g sample of soil was digested in reverse *aqua*
23 *regia*. Quality control was maintained by digesting certified reference samples
24 (two standard soils in duplicate), and four blank samples. After digestion, samples

1 were vortexed and passed through a Whatman No. 42 filter paper, discarding the
2 first few mL of filtrate (Whatman International, Maidstone, Kent, UK).

3

4 *Soil solution extraction.* A 20 to 30 g sample of soil was wetted to -5 kPa
5 in a syringe and allowed to equilibrate for 16 h before removal of pore water by
6 centrifugation (McLaughlin et al, 1997). Samples were centrifuged at 4,000 g for
7 45 min and then at 30,000 g for 45 min and the supernatant passed through a 0.45
8 μm filter. Electrical conductivity and pH were measured immediately after
9 filtration. Soil solutions were then diluted to 10 mL and acidified with 1 mL of
10 concentrated HNO_3 and stored at 4 °C until analyzed.

11

12 *CaCl₂ extraction.* A 25 mL aliquot of 0.01 M CaCl_2 was added to 5 g dry
13 soil and shaken end over end for 4 h. The pH of the soil was measured and then
14 the tubes were centrifuged at 1,300 g for 20 min and the supernatant passed
15 through a 0.45 μm filter. Samples were stored at 4°C until analyzed.

16

17 All aqueous extracts of soil and digest solutions were analyzed for concentrations
18 of Cu and Zn using ICP–OES.

19

20 *Calculations and Statistical analyses*

21 Background concentrations of Cu and Zn at each site were calculated using the
22 method of Hamon et al. (2004). All statistics were calculated with Sigstastat
23 Version 3.1.1 (Systat Software, Point Richmond, VA, USA).

24

1 Added metal concentrations (i.e. measured total metal concentration of each soil
2 sample minus the average metal concentration of the controls for that site) were
3 used to determine toxicity threshold values. The concentrations and the 95%
4 confidence intervals (95% CI) that caused a 10, 20 and 50% reduction (i.e. EC10,
5 EC20 and EC50) in plant biomass at eight weeks, shoot yield, grain yield, grain
6 protein content and 100 grain weight at harvest were calculated by fitting a
7 logistic distribution to the data (Barnes et al., 2003).

8
9 The toxicity data obtained from each site at successive sampling times are not
10 statistically independent. To overcome this, only the toxicity data for the first
11 wheat crop grown at each site (Table 2) were used to develop relationships
12 between phytotoxicity and soil physicochemical properties.

13
14 Normality of the toxicity data and soil properties was tested using the
15 Kolmogorov-Smirnov test with a significance level of 0.05. Parameters that were
16 not normally distributed were log transformed. This was sufficient to achieve
17 normality in all cases. The parameters used in the models were: log EC50 (grain
18 yield), log EC20 (grain yield), log EC10 (grain yield), pH (CaCl₂), log CEC
19 (cmol kg⁻¹), clay content (%), log OC (%), background Cu concentration and
20 background Zn concentration. The degree of collinearity of the soil properties was
21 determined using the Pearson Correlation Coefficient test. Only properties that
22 were not significantly ($p > 0.05$) correlated were combined in multiple linear
23 regressions (MLRs). Toxicity values were related to soil properties using simple
24 and multiple linear regression analysis. Constant variance of the toxicity data
25 about the regression plane was tested by determining the Spearman rank

1 correlation between the absolute values of the residuals and the observed values of
2 the dependent variable. Whether any particular datum exerted an unduly large
3 influence on the relationships was tested using Cooks distance test and the test for
4 leverage.

5

6 **RESULTS AND DISCUSSION**

7 *Soil physico-chemical properties in control soils*

8 Selected soil properties of the sites are presented in Table 1. Soil pH (CaCl_2)
9 ranged from 4.0 to 7.6 with 8 of the soils having a pH below 5.5. The OC content
10 ranged from 0.9 – 5.6%, with one markedly higher value. Clay content ranged
11 from 4 to 66 % and CEC ranged from 3 to 61 cmol/kg , with one site having a
12 markedly higher value for both parameters.

13

14 *Correlation between soil physicochemical properties*

15 The only significant ($p \leq 0.05$) correlations were between clay content and log CEC
16 and between clay content and background metal concentrations. These
17 combinations of properties were not used in the MLR analysis.

18

19 *Toxicity data*

20 Grain yield and 8-week plant biomass toxicity data are presented in this paper and
21 used to derive normalisation relationships. These were selected, as grain protein
22 content and 100 grain weight were not significantly ($p > 0.05$) affected by either Cu
23 or Zn (data not shown) and for shoot yield there was only data for 7 sites.

24

1 Toxicity data (EC10, EC20 and EC50 for each endpoint) were expressed using
2 total added, soil solution and CaCl₂-extractable metal concentrations in soil. These
3 sets of toxicity data for each combination of endpoint and metal concentration
4 measure were compared to determine which could best explain toxicity across the
5 sites. The measure of metal concentration that has the smallest variability in
6 toxicity values across the sites is deemed to be the best measure of the
7 bioavailable fraction (McLaughlin et al., 2000; Broos et al., 2007) and therefore is
8 the most appropriate to use in developing normalisation relationships. As an
9 example of these comparisons, the plots of Cu and Zn log EC50 values are
10 presented (Figures 1 and 2 respectively). There was a wide range of ECx values
11 across soils, irrespective of the concentration measure used. Overall, the range of
12 the EC50 values expressed as total metal concentrations were similar or smaller
13 than EC50 values expressed as soil solution or CaCl₂-extractable metal
14 concentrations. The same occurred for the EC10 and EC20 analysis (data not
15 shown).

16
17 A number of authors (Smolders et al., 2003; Smolders et al., 2004; Oorts et al.,
18 2006; Zhao et al., 2006; Broos et al., 2007) have also found extractable or soil
19 solution measurements were not useful predictors of plant and microbial toxicity
20 in soils and thus used added total metal concentrations to develop normalisation
21 relationships.

22
23 Given the results of the above comparison from the present study, combined with
24 results from other research (Smolders et al., 2003; Smolders et al., 2004; Oorts et
25 al., 2006; Zhao et al., 2006; Broos et al., 2007), that there is considerably more

1 total metal concentration data available and there is regulatory acceptance and
2 understanding of this concentration measure, we used toxicity data based on total
3 added metal concentrations.

4
5 The EC₁₀, 20 and 50 (grain yield) values for Cu ranged from approximately 130
6 to 1130, 155 to 1560 and 210 to 5700 mg/kg, respectively (Table 3). The
7 corresponding Zn toxicity values ranged from approximately 90 to 4760, 130 to
8 4775 and 260 to 4790 mg/kg, respectively (Table 4). The EC₁₀, 20 and 50 (8
9 week plant biomass) values for Cu ranged from approximately 3 to 1100, 36 to
10 1130 and 220 to 2070 mg Cu/kg respectively (Table 3). The corresponding Zn
11 toxicity values varied from approximately 17 to 2765, 75 to 3 120 and 160 to 12
12 900 mg Zn/kg, respectively (Table 4).

13
14 The differences between the highest and lowest toxicity value for each measure of
15 toxicity and endpoint were highly variable, varying between 5 to 330 fold for Cu
16 and 17 to 160 fold for Zn. These differences in toxicity across the sites could have
17 been due in part to different sensitivities of the various cultivars of wheat used
18 (Table 2), spatial and temporal differences in climatic conditions, differences in
19 soil properties and temporal variation in the bioavailability of the metal (i.e.
20 aging). Laboratory-based toxicity tests (unpublished data) have shown that there
21 were no significant ($p \leq 0.05$) differences in the sensitivity to either Cu or Zn of
22 three wheat cultivars (cv. Callingiri, cv. Dollarbird and cv. Frame) used in the
23 current study, so this factor is unlikely to be important in the variation in toxicity
24 values between sites. On the other hand, laboratory-based toxicity tests exposing
25 *T. aestivum* cv. Frame to Cu and Zn in the same soils as the present study found

1 differences in soil properties explained the majority of the variation in
2 phytotoxicity (Warne et al., in press). It is therefore likely that soil properties,
3 modified by effects of climatic conditions and variable metal aging, were the
4 major factors causing the wide variation in toxicity across sites.

5
6 Eight week plant biomass EC10, EC20 and EC50 values for both Cu and Zn were
7 not significantly ($p > 0.05$) correlated to the corresponding grain yield toxicity
8 values (based on results of both Pearson correlation coefficient and Spearman
9 rank order correlation tests), with the exception of the Zn EC50 value. This has
10 potential implications as many phytotoxicity studies use short-term growth
11 endpoints similar to the 8 week plant biomass used in the present study in hazard
12 and risk assessments, and they have been used to derive normalisation
13 relationships between phytotoxicity and soil physicochemical properties (e.g.
14 Rooney et al., 2006; Warne et al., in press). Thus, if this finding applies to other
15 datasets then such data may have little relevance to long-term plant responses.

17 *Development of predictive toxicity models*

18 All the significant ($p \leq 0.05$) and valid linear relationships for each combination of
19 metal and toxicity measure are presented in Table 5.

20
21 Only one significant ($p \leq 0.05$) relationship (eqn 1, Table 5) could be developed for
22 the Cu 8 week plant biomass data – for Cu log EC50. This was based on soil pH
23 and explained 54% of the variation in toxicity data. Normally relationships that
24 explain such a low proportion of the data variability would not be used, but as no
25 better relationships were available it could be used as an interim measure.

1
2 Four significant ($p \leq 0.05$) relationships were developed for the Cu grain yield
3 phytotoxicity data (eqns 3 to 6, Table 5). No statistically significant ($p \leq 0.05$)
4 relationships could be derived for Cu log EC50. For Cu log EC20 and log EC10
5 data, soil pH formed significant ($p \leq 0.05$) but relatively poor quality positive
6 relationships that are not reliable enough to use in predicting toxicity. The soil
7 properties that formed significant ($p \leq 0.05$) MLRs with Cu log EC20 and log
8 EC10 data were pH and log OC (eqns 4 and 6, Table 5). These relationships
9 explained 76% and 80%, respectively, of the variation in these endpoints (Table
10 5).

11
12 The relationships for Cu developed in the present study and those developed for *T.*
13 *aestivum* (20 d shoot growth) laboratory assays by Warne et al. (in press) all
14 contained soil pH but had different second parameters in the MLRs. The
15 laboratory-based relationships had either CEC or electrical conductivity as the
16 second parameter, while those from the present study had OC. The highest quality
17 laboratory-based relationship that involved log OC was only able to account for
18 approximately 60% of the variability (Warne et al., in press). Possible causes for
19 the increased importance of log OC in the field-based relationships are explored
20 later.

21
22 Only one significant ($p \leq 0.05$) relationship (eqn 2, Table 5) could be developed for
23 the Zn 8 week plant biomass data, and that was for Zn log EC50. This was based
24 on soil pH and it could explain 85% of the variation in toxicity data.

25

1 In contrast, 13 significant ($p \leq 0.05$) relationships (eqns 7 to 19, Table 5) were
2 developed for the Zn grain yield toxicity data. Positive simple linear relationships
3 (SLRs) for Zn were based on pH or log CEC (Table 5). The SLRs based on log
4 CEC (eqns 8, 13 and 17, Table 5) explained less than 50% of the variation in
5 toxicity data and thus were not suitable for predicting grain yield toxicity data.
6 The SLRs based on pH (eqns 7, 12 and 16, Table 5) explained between 53 and
7 61% of the variation in toxicity data. The addition of log OC or log CEC as an
8 additional parameter to pH led to an additional 6 and 13% of the variation in the
9 toxicity data being accounted for, respectively. It should be noted that the
10 probability associated with the log CEC parameter in the log EC₁₀ relationship
11 was 0.068 and thus strictly this relationship should not be used. However, when
12 models are based on small datasets (in this case 10 values) it is appropriate to use
13 higher levels of probability as the acceptance level for statistical significance
14 (Bailey, 1979). Relationships for Zn that explained more than 60% of the
15 observed variability (i.e. eqns 7, 10, 11, 14, 15 and 19, Table 5) can be used to
16 predict toxicity values with reasonable confidence.

17
18 The addition of background Cu and Zn concentrations to SLRs only increased the
19 variability accounted for in the Zn log EC₅₀ relationships (Eqns 9 and 10, Table
20 5). This suggests that the sensitivity of the wheat was not greatly affected by
21 background concentrations of Cu or Zn. This result is in direct contrast to the
22 findings of McLaughlin et al. (2001) who found that the sensitivity of micro-
23 organisms to Zn was inversely related to background Zn concentrations and Broos
24 et al (2007) who found that Zn background concentrations helped explain Zn
25 toxicity to microbial substrate induced respiration.

1
2 None of the above relationships had data points which influenced or leveraged the
3 relationships, based on the results of influence and Cook's distance statistical
4 tests. However, the OC, clay content and CEC data each contained one site which
5 was markedly different to the others. By including these data to develop
6 relationships it is assumed that data points lying between the extreme value and
7 the remainder of the data conform to the same relationships that were developed.
8 However, we have no data to support or disprove this assumption. It is also
9 possible that while the extreme values do not influence the relationships their
10 inclusion could lead to changes in the ability of the relationships to accurately
11 estimate phytotoxicity. From a regulatory point of view, under-estimation of
12 toxicity is of greater concern than over-estimation. Therefore the effect of
13 including and excluding the extreme values on the extent of under-estimation of
14 toxicity at the 11 sites was determined for the best relationships developed in the
15 present study. This was not necessary for the 8 week plant biomass relationships
16 as they were based on pH which had no extreme values. However, for the grain
17 yield the five best relationships contained either CEC or OC which had extreme
18 values. The average percent of underestimation by the relationships never
19 exceeded 25% and for three of the five relationships the underestimation was less
20 when the extreme sites were excluded. However, exclusion of the extreme values
21 would severely limit the range of soils to which the relationships could be applied.
22 Given the above and that a key intended use of these relationships is to normalise
23 toxicity data as part of a process of deriving soil and soil amendment quality
24 guidelines, it was decided to use relationships that included the extreme values.
25

1 Some or all of the soil properties (i.e. pH and OC for Cu and pH and log CEC for
2 Zn) featuring in the best statistical models of the present study also occur in the
3 laboratory-based phytotoxicity relationships developed in other studies for Cu and
4 Zn effects on plants and micro-organisms (Table 6). This suggests that there may
5 be a common set of easily-measured soil properties that can be used to predict Cu
6 and Zn toxicity to a diverse range of terrestrial organisms.

7
8 The relationships for Cu developed from the field experiments agree with well-
9 known reactions of this metal with soil. Increasing soil pH markedly increases Cu
10 sorption in soil to both mineral and organic matter phases (e.g. Smith, 1994;
11 Alloway, 1995), as well as increasing the movement of Cu from labile to non-
12 labile forms, often termed aging or fixation (Ma et al. 2006a, b). Organic carbon
13 forms organo-metallic complexes with Cu which decreases the bioavailability of
14 Cu to plants (e.g. Alloway, 1995). Thus, increasing soil pH and OC content would
15 be expected to decrease the toxicity of Cu and this was observed (Table 5).

16
17 The relationships predicting the toxicity of Cu to wheat developed by Warne et al.
18 (in press) from laboratory-based studies did not contain OC and it was argued that
19 this may be due to the limited range of OC values for soils that were used (i.e.
20 0.95 – 3.45% with one value of 5.7%). This was supported by Broos et al. (2007)
21 who also found OC was not a significant ($p>0.05$) modifier of Cu toxicity to
22 microbial substrate induced nitrification and respiration in fourteen Australian
23 soils that included all those used in the present study. Additionally Broos et al.
24 (2007) found that reanalysis of data from Smolders et al. (2004) and Oorts et al
25 (2006), after removing soils with OC concentrations greater than those found in

1 the Australian soils, resulted in OC no longer being an important factor explaining
2 the toxicity of Zn and Cu to nitrifying micro-organisms. While the field soils used
3 in the current study have essentially the same range and distribution of OC values
4 as those used by Warne et al. (in press) and Broos et al. (2007), OC was found to
5 be an important modifier of field-based Cu toxicity. A possible explanation for
6 this is that OC is involved with water retention and in field situations this is likely
7 to play a greater role than in the laboratory where optimal soil moisture is
8 generally maintained.

9
10 The significant ($p \leq 0.05$) relationships for Zn which include CEC are consistent
11 with well established mechanisms of Zn retention and/or fixation in soil (Crout et
12 al. 2006) as noted above for Cu. The use of CEC has a number of strengths and
13 limitations discussed in Warne et al (in press). If relationships that include log
14 CEC data are to be used to derive soil and soil amendment quality guidelines then
15 (i) a CEC method must be specified and only this method used to measure CEC
16 and implement the guidelines, or (ii) other CEC methods can be used providing
17 they have been calibrated against the recommended CEC method.

18
19 The quality of the field-based relationships developed in the present study are
20 similar to, or better than, the best laboratory-based relationships for Cu and Zn to
21 plants (Smolders et al., 2003; Rooney et al., 2006; Warne et al., in press); to
22 micro-organisms (Smolders et al., 2004); and Ni to plants, invertebrates and
23 micro-organisms (EU, 2006). It thus appears possible to develop high quality
24 relationships using field-based phytotoxicity values despite the increased
25 variability that is expected in such data compared to laboratory-based studies.

1

2 ***Predictive accuracy of the relationships***

3 In order to maximise the number of sites used to develop the relationships we did
4 not validate the relationships using independent datasets. Rather, we determined
5 the accuracy of the highest quality (based on adj r^2 values) relationships by
6 plotting measured field-based Cu EC10 and EC20 values against the
7 corresponding values calculated by the relationships for Cu and the corresponding
8 EC10, EC20 and EC50 values for Zn (Figures 3 & 4 respectively). The vast
9 majority (47 out of 55) of the predicted values were within a twofold margin of
10 error from the measured values, and in only 3 cases was the toxicity
11 underestimated by a factor greater than two. Thus, the developed field-based
12 relationships were relatively reliable predictors of the toxicity of Cu and Zn to
13 wheat in these field studies. This was unexpected given that the field-based
14 models were derived from phytotoxicity data from nine cultivars of *T. aestivum*
15 that were grown over 4 different seasons in very different climatic conditions.

16

17 The ability of the laboratory-based normalisation relationships developed by
18 Warne et al. (in press) to predict the toxicity of Cu and Zn to *T. aestivum* in the
19 field was tested by plotting EC50 and 10 values derived from the field
20 experiments against the corresponding values predicted by the laboratory-based
21 relationships. This was done for both 8 week plant yield and grain yield at harvest.

22 The laboratory-based relationships predicted the field-based 8 week plant biomass
23 data (Figures 5 and 6 for Cu and Zn respectively) considerably better than they
24 predicted grain yield data (data not shown). This most probably reflects these

1 endpoints having a more similar exposure duration and growth period (i.e. 21 and
2 56 days) compared to 6 months for the grain yield data.

3

4 The most important point to note from Figures 5 and 6 is that the range in toxicity
5 values for Cu and Zn were much greater under field conditions than in the
6 laboratory. In many instances the laboratory-based models significantly
7 underestimated Cu and Zn toxicity in the field. Points to the right of the lower 2
8 fold error line (Figures 5 and 6) show that the frequency of instances in which
9 laboratory-based models underestimated toxicity in the field increased as the
10 severity of the toxic effect being modelled decreased (i.e. EC10 > EC50). These
11 results indicate that the laboratory-based relationships for Cu and Zn toxicity to
12 wheat are clearly not suitable for predicting phytotoxicity responses for the same
13 plant species in the field.

14

15 The reasons for the much wider range of toxicity values found under field
16 conditions than predicted by the laboratory model are unknown. It may be that
17 effects of soil physicochemical conditions in modifying metal toxicity are
18 expressed less in controlled laboratory conditions, or that multiple stressors (e.g.
19 heat, moisture, disease, etc.) accentuate the effect of soil physicochemical
20 conditions in modifying toxicity. Irrespective of the reasons for the greater range
21 in toxicity found in the field soils than predicted by the laboratory models, in
22 relation to regulatory use of the information, overestimation of toxicity using
23 laboratory-based relationships is protective and therefore of little concern.

24 However, any underestimation of toxicity by the laboratory models is of concern.

25 Our data indicate that it is important to benchmark laboratory-based normalisation

1 relationships with field data to validate or recalibrate the relationships in situations
2 where they will be applied.

3

4 Normalisation relationships, such as those developed in the present study, do have
5 a number of limitations (Warne et al., in press) but can still play a significant role
6 in risk and hazard assessment by providing estimates of the toxicity of chemicals
7 when experimentally derived values are lacking. Through their ability to
8 normalise toxicity data they can also be used to derive site or soil specific SQGs.

9

10

CONCLUSION

11 Field-based phytotoxicity values based on total added metal concentrations were
12 either more accurate, or at least as accurate, a measure of bioavailability as those
13 based on soil solution and calcium chloride extractable metal concentrations.
14 Early growth toxicity data (e.g. 8 week plant biomass) may not be a suitable
15 surrogate for effects on grain yield at harvest. The variation in toxicity values
16 across the sites, irrespective of the measure of toxicity, was 5 - 330 fold for Cu
17 and 17 - 160 fold for Zn, indicating that soil properties strongly influenced
18 phytotoxicity. Multiple linear regression relationships incorporating soil properties
19 as variables were able to explain between 57 and 87% of the variation in field-
20 based toxicity of Cu and Zn. Copper toxicity could be modelled using soil pH and
21 organic carbon content while Zn toxicity could be modelled using the soil pH and
22 the cation exchange capacity. The addition of background zinc concentrations
23 proved useful for explaining additional variation in Zn EC50. The best
24 relationships for EC50, 20 and 10 for each metal all contained the same soil
25 parameters and were consistent with the known behaviour of Cu and Zn in soil,

1 which strengthens the case for using such relationships in predicting the risks of
2 metal contamination in different soils. Relationships between soil properties and
3 phytotoxicity data derived from laboratory studies could not accurately predict
4 field-based phytotoxicity responses. We recommend that relationships based on
5 field derived data be used to predict phytotoxicity risks in the field, rather than
6 using predictions based on laboratory-derived relationships.

7

8

ACKNOWLEDGEMENTS

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15 solution speciation and diffusive gradients in thin films measurement as an
16 indicator of copper bioavailability to plants. *Environmental Toxicology and*
17 *Chemistry* 25, 733-742.

1 Table 1: Details of the field trials and selected soil properties (dry weight basis).

Field site	Location ^a	Soil type	pH (0.01 M CaCl ₂)	Organic carbon (%)	Clay content (%)	CEC ^c (cmol _c kg ⁻¹)
Avon	SA	calcarasol	7.6	1.1	12.4	10
Brennans	WA	tenosols	5.4	0.9	4.0	3.2
Cecil Plains	Qld	vertosol	7.3	1.4	66	61
Dookie	Vic	dermosol	4.9	2.0	23	13.0
Dutson Downs	Vic	sodosol	4.0	5.6	5.0	11.6
Flat Paddock	NSW	chromosol	4.4	1.2	17	7.8
Kingaroy	Qld	ferrosol	5.0	1.8	41	16.5
Night Paddock	NSW	chromosol	5.1	3.4	24	17.4
Spalding	SA	chromosol	6.3	1.9	27	17.7
Tintinara	SA	sodosol	6.3	1.8	10	10.3
Wilsons	WA	tenosols	4.8	2.6	6.0	5.0

2 ^a SA = South Australia, NSW = New South Wales, QLD = Queensland, VIC = Victoria, WA = Western Australia. ^b B = biosolids, M = metal salts. ^c CEC = cation exchange

3 capacity. ^d these sites were not used to grow wheat for this study.

- 1 Table 2: Cropping seasons when wheat (*T. aestivum* L.) was grown in National
 2 Biosolid Research Program sites and the cultivars that were grown.

Site	Cropping seasons when wheat (including cultivar) was grown			
	1	2	3	4
Avon	Aroona	Y-C ^a		
Brennans	Callingiri		Callingiri	
Cecil Plains			Kennedy	
Dookie		Whistle		
Dutson Downs		C-D ^b		
Flat Paddock		DB ^c		
Kingaroy				Hartog
Night Paddock		DB		
Spalding	Aroona	Y-C		
Tintinara		Frame		
Wilsons		Callingiri		

3 ^a Yitpee-Crickoff cultivar; ^b Chara-Drysdale cultivar ^c Dollarbird cultivar.

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1 Table 3: The concentrations (mg/kg) of added Cu that reduced wheat grain yield
 2 and 8 week plant biomass by 50, 20 and 10% (EC50, EC20 and EC10) and 95%
 3 confidence limits in brackets for each site.

4

Field trial	Grain yield		
	EC50	EC20	EC10
Avon	1 147 (60 – 21 700)	1 138 (152 – 8 500)	1 133 (261 – 4 900)
Brennans	286 (196 – 420)	175 (94 – 330)	132 (55 – 317)
Cecil Plains	5 705 (640 – 51 200)	1 560 (577 – 4 200)	731 (300 – 1 780)
Dookie	476 (270 – 840)	228 (120 – 433)	148 (64 – 350)
Dutson Downs	649 (390 – 1 080)	385 (180 – 805)	284 (100 – 800)
Flat Paddock	212 (133 – 340)	156 (89 – 274)	150 (58 – 294)
Kingaroy	310 (230 – 413)	241 (152 – 382)	209 (112 – 390)
Night Paddock	3 170 (61 – 166 000)	1 316 (61 – 28 500)	787 (39 – 15 700)
Spalding	632 (93 – 4310)	603 (19 – 1200)	586 (0.8 – 434 000)
Tintinara	1 040 (634 – 1710)	752 (345 – 1 640)	622 (207 – 1 870)
Wilsons	1 760 (795 – 3900)	768 (290 – 2020)	473 (132 – 1 700)
	8 week plant biomass		
	EC50	EC20	EC10
Avon	2 070 (234 – 18 300)	35.7 (0.42 – 3 030)	3.32 (0.003 – 4 130)
Brennans	375 (141 – 996)	360 (0.5 – 256 000)	351 (0.02 – 6 × 10 ⁶)
Cecil Plains	1 154 (905 – 1 470)	792 (595 – 1 060)	635 (400 – 1 010)
Flat Paddock	315 (140 – 715)	168 (72 – 396)	117 (37 – 370)
Kingaroy	272 (227 – 328)	220 (170 – 283)	193 (137 – 272)
Night Paddock	526 (335 – 830)	232 (135 – 400)	144 (70 – 300)
Spalding	223 (133 – 375)	74.7 (31 – 182)	40 (11 – 136)
Tintinara	1 183 (1 100 – 1 250)	1 130 (1 000 – 1 265)	1 100 (900 – 1 330)
Wilsons	330 (170 – 650)	102 (38 – 275)	52 (14 – 190)

5

- 1 Table 4: The concentrations (mg/kg) of added Zn that reduced wheat grain yield
 2 and 8 week plant biomass by 50, 20 and 10% (EC50, EC20 and EC10) and 95%
 3 confidence limits in brackets for each site.

Field trial	Grain yield		
	EC50	EC20	EC10
Avon	4 789 (na) ^a	4 773 (na)	4 760 (na)
Brennans	263 (170 – 410)	134 (42 – 437)	91 (16 – 515)
Cecil Plains	4 560 (1 120 – 18 600)	3 000 (1 500 – 6 000)	2 351 (1 520 – 3 600)
Dookie	833 (555 – 1 250)	548 (300 – 1 000)	428 (205 – 900)
Dutson Downs	702 (236 – 2 100)	370 (110 – 1 260)	255 (50 – 1 280)
Flat Paddock	632 (330 – 1200)	453 (190 – 1 080)	374 (111 – 1 260)
Kingaroy	389 (246 – 615)	303 (190 – 480)	262 (15 – 655)
Night Paddock	1 320 (na)	1 315 (na)	1 312 (na)
Spalding	1 780 (1 100 – 2 780)	1 400 (920 – 2 100)	1 217 (626 – 2 360)
Tintinara	1 350 (515 – 3 500)	883 (190 – 4 100)	670 (95 – 5 000)
Wilson's	363 (220 – 600)	163 (82 – 325)	102 (39 – 268)
	8 week plant biomass		
	EC50	EC20	EC10
Avon	12 900 (1 000 – 170 000)	198 (4.7 – 8 260)	17.2 (0.04 – 7 580)
Brennans	270 (207 – 354)	195 (91 – 420)	161 (54 – 490)
Cecil Plains	3 900 (137 – 108 000)	3 120 (345 – 28 400)	2 765 (540 – 14 200)
Flat Paddock	182 (75 – 445)	75 (21 – 267)	45 (8.5 – 240)
Kingaroy	377 (307 – 463)	287 (223 – 369)	244 (163 – 366)
Night Paddock	1 120 (795 – 1 580)	785 (510 – 1 210)	640 (370 – 1 110)
Spalding	1 875 (1 180 – 2 990)	967 (483 – 1 935)	657 (267 – 1 615)
Tintinara	2 123 (865 – 5 220)	1 490 (600 – 3 720)	1 210 (390 – 3 750)
Wilson's	159 (110 – 229)	79 (38 – 165)	52 (19 – 143)

4 ^a na – not available

- 1 Table 5: Statistically significant ($p \leq 0.05$) linear regressions of toxicity for grain yield and 8 week plant biomass (8 wk pb) and selected soil
 2 properties for Cu and Zn values based on added metal concentrations (mg kg^{-1}). Standard errors of the coefficients are presented in parentheses.
 3 All equations had 10 degrees of freedom.

Equation no.	Metal	Toxicity endpoint	Toxicity measure	Regression Equations ^a	Adjusted r^2	Prob
1	Cu	8 wk pb	log EC50	$1.38 (0.43) + 0.23 (0.07) * \text{pH}$	0.54	0.015
2	Zn			$-0.16 (0.46) + 0.54 (0.08) * \text{pH}$	0.85	<0.0001
3	Cu	grain yield	log EC20	$1.61 (0.45) + 0.20 (0.08) * \text{pH}$	0.34	0.036
4				$0.63 (0.37) + 0.32 (0.057) * \text{pH} + 1.17 (0.29) * \log \text{OC}$	0.76	0.001
5			log EC10	$1.44 (0.40) + 0.20 (0.070) * \text{pH}$	0.42	0.0108
6				$0.56 (0.31) + 0.31 (0.048) * \text{pH} + 1.05 (0.24) * \log \text{OC}$	0.80	<0.001
7	Zn		log EC50	$1.39 (0.40) + 0.29 (0.07) * \text{pH}$	0.61	0.003
8				$2.07 (0.35) + 0.86 (0.31) * \log \text{CEC}$	0.40	0.021
9				$2.14 (0.29) + 1.06 (0.28) * \log \text{CEC} - 0.005 (0.002) * \text{CB Zn}$	0.57	0.014
10				$1.32 (0.25) + 0.20 (0.05) * \text{pH} + 0.72 (0.17) * \log \text{CEC} - 0.004 (0.001) * \text{CB Zn}$	0.87	< 0.001
11				$1.17 (0.34) + 0.23 (0.063) * \text{pH} + 0.53 (0.22) * \log \text{CEC}$	0.74	0.002
12			log EC20	$1.00 (0.50) + 0.33 (0.088) * \text{pH}$	0.56	0.005

13		$1.73 (0.41) + 1.04 (0.37) * \log \text{CEC}$	0.41	0.02
14		$0.32 (0.61) + 0.41 (0.094) \text{pH} + 0.81 (0.48) \log \text{OC}$	0.64	0.007
15		$0.73 (0.43) + 0.26 (0.080) * \text{pH} + 0.64 (0.28) * \log \text{CEC}$	0.70	0.003
16	log EC10	$0.77 (0.57) + 0.35 (0.10) * \text{pH}$	0.53	0.007
17		$1.53 (0.45) + 1.14 (0.41) * \log \text{CEC}$	0.40	0.021
18		$0.069 (0.71) + 0.44 (0.11) \text{pH} + 0.84 (0.36) \log \text{OC}$	0.59	0.012
19		${}^b 0.48 (0.51) + 0.27 (0.094) * \text{pH} + 0.70 (0.33) * \log \text{CEC}$	0.66	0.005

1 ^a OC = organic carbon content; CEC = cation exchange capacity; CB Zn = background zinc concentration. ^b the probability associated with the
 2 log CEC parameter is 0.068.

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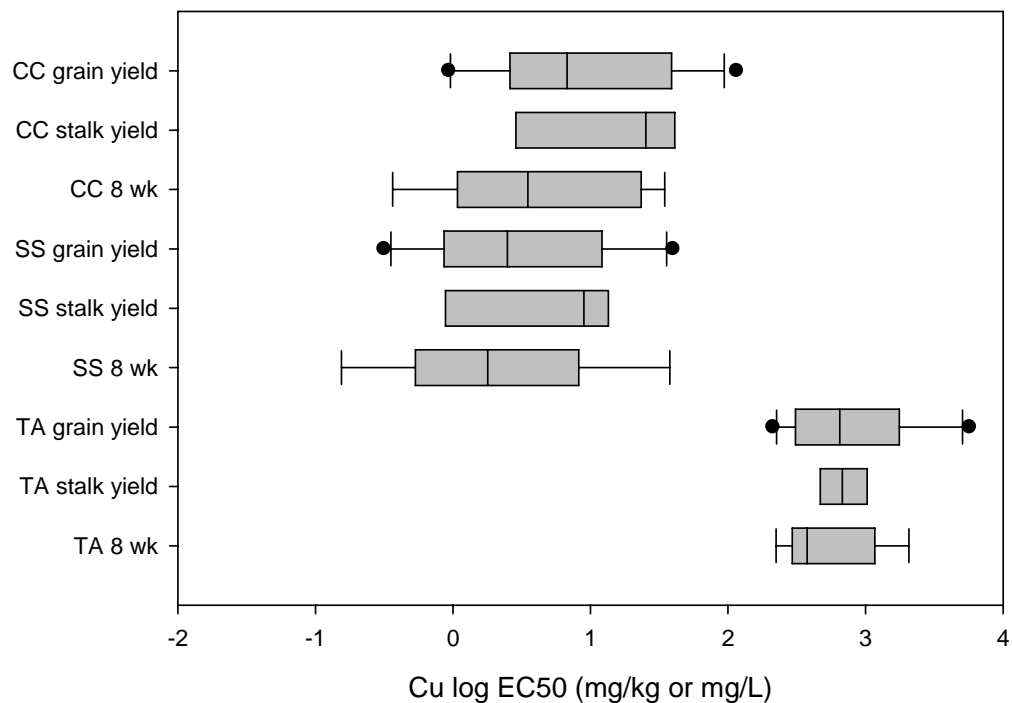
- 1 Table 6. Details of the test organism and soil physicochemical properties used in laboratory-based microbial and plant toxicity normalisation
 2 relationships.

Metal	Organism	Parameters	Reference
Cu	Tomato and Barley	1 st . Ca or CEC. 2 nd . Fe oxide, OC, clay content or pH	Rooney et al., 2006
	Microbial (substrate induced nitrification, SIN)	1 st . pH. 2 nd clay content or log CEC	Broos et al., 2007
	Microbial (substrate induced respiration, SIR)	Clay content	Broos et al., 2007
Zn	Bread wheat	1 st . pH. 2 nd . log CEC	Smolders et al., 2003
	Microbial (SIR)	Background Zn conc	Broos et al., 2007
	Microbial (potential nitrification rate, PNR)	1 st pH. 2 nd OC	Smolders et al., 2004
	Microbial (SIR)	1 st pH. 2 nd background Zn and log CEC	Smolders et al., 2004
	Microbial (maize residue respiration, MRR)	log CEC	Smolders et al., 2004

1 Figure 1: Box and whisker plots of the log EC50 values for total added (TA) Cu,
2 calcium chloride (CC) extractable Cu and soil-solution (SS) Cu on grain yield,
3 shoot yield and 8 week plant biomass (8 wk). The box spans the interquartile
4 range of the log EC50 values, with the vertical line within the box indicating the
5 median. Horizontal lines extending beyond the boxes indicate the 10th and 90th
6 percentiles and dots are log EC50 values outside the 10th and 90th percentiles.

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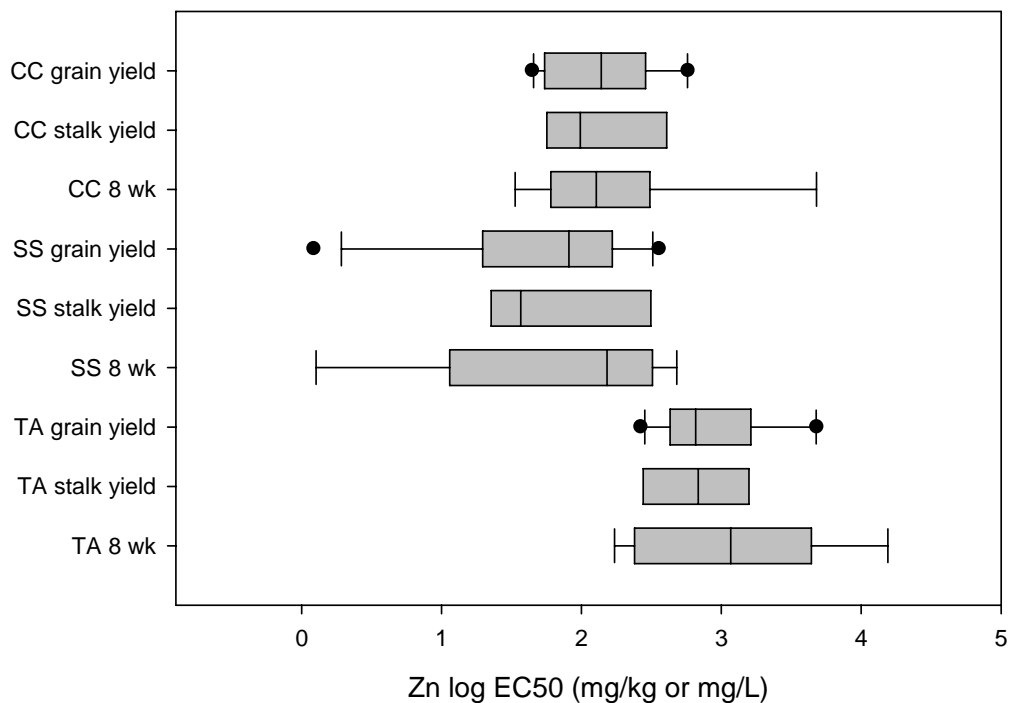
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1 Figure 2: Box and whisker plots of the log EC50 values for total added (TA) Zn,
 2 calcium chloride (CC) extractable Zn and soil-solution (SS) Zn on grain yield,
 3 shoot yield and 8 week plant biomass. The box spans the interquartile range of the
 4 log EC50 values, with the vertical line within the box indicating the median.
 5 Horizontal lines extending beyond the boxes indicate the 10th and 90th percentiles
 6 and dots are log EC50 values outside the 10th and 90th percentiles.

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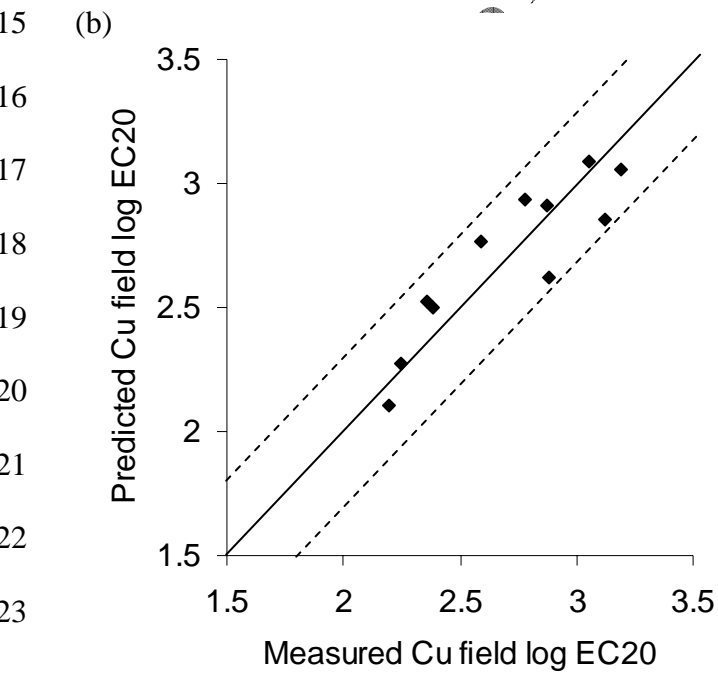
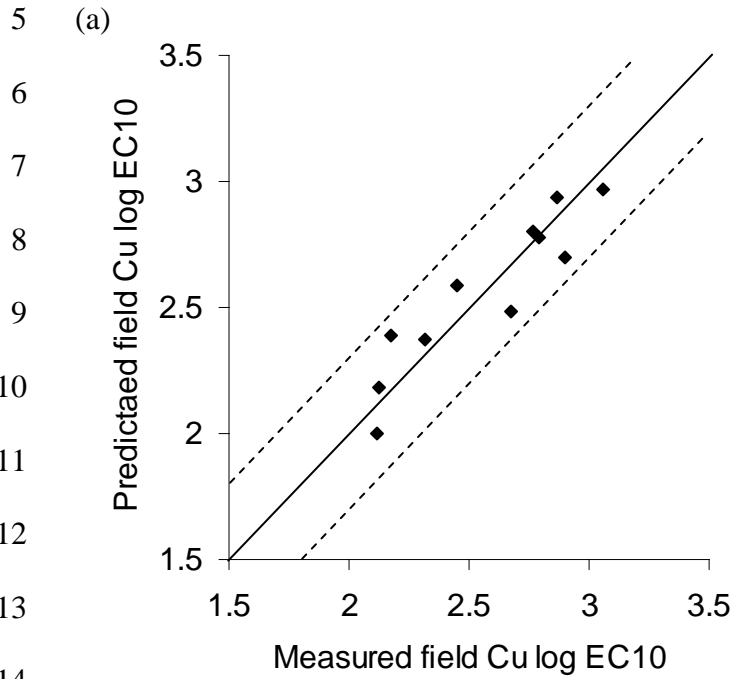
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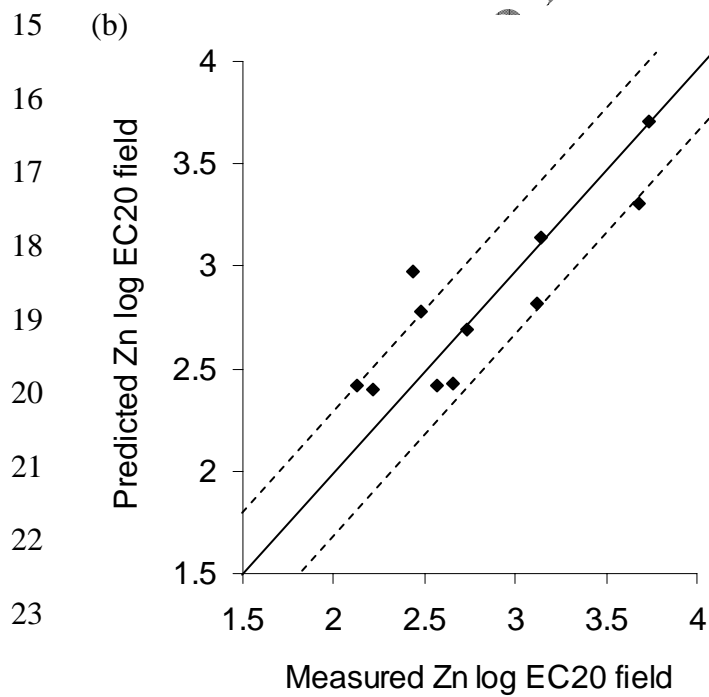
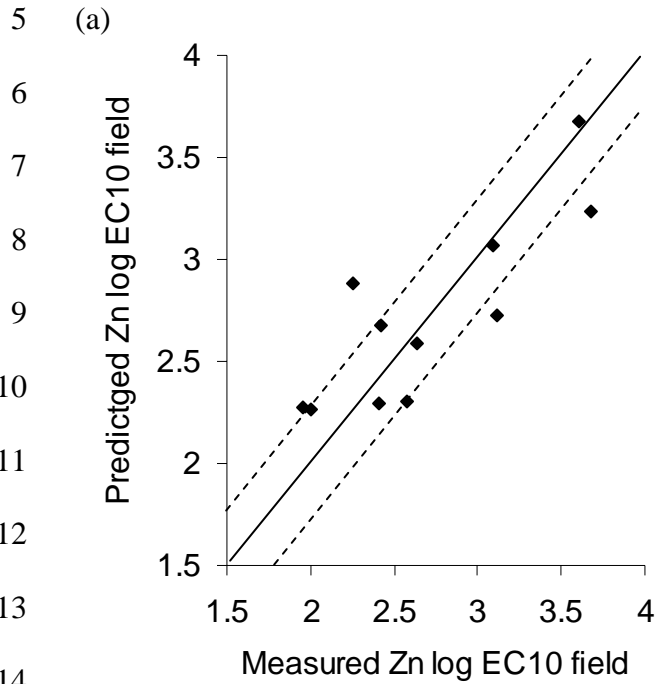
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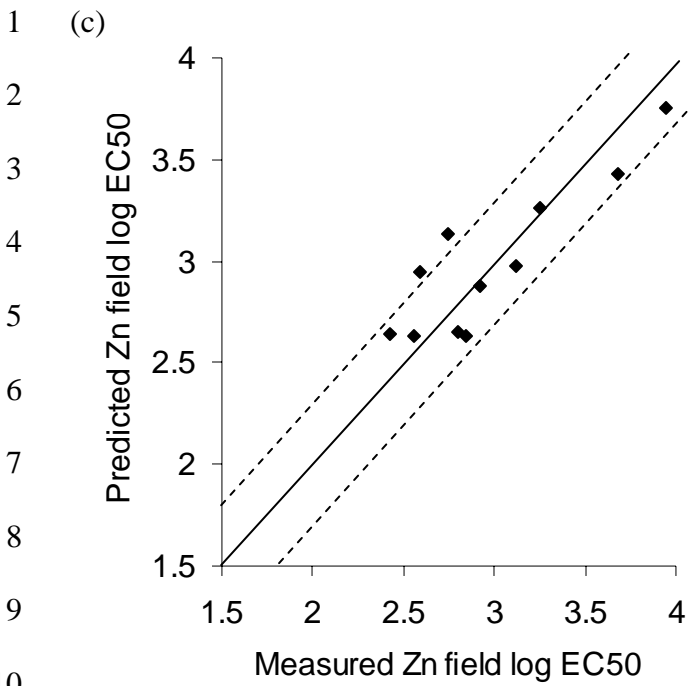
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1 Figure 3: Measured field-based Cu toxicity data (wheat grain yield) versus
2 predicted values using selected field-based relationships (a) log EC10 and (b) log
3 EC20. The solid line is a 1:1 slope and the dashed lines indicate a two fold error
4 from the solid line.



1 Figure 4: Measured field-based Zn toxicity data for wheat grain yield versus
2 predicted values using selected field-based relationships (a) log EC10, (b) log
3 EC20 and (c) log EC50. The solid line is a 1:1 slope and the dashed lines indicate
4 a two fold error from the solid line.





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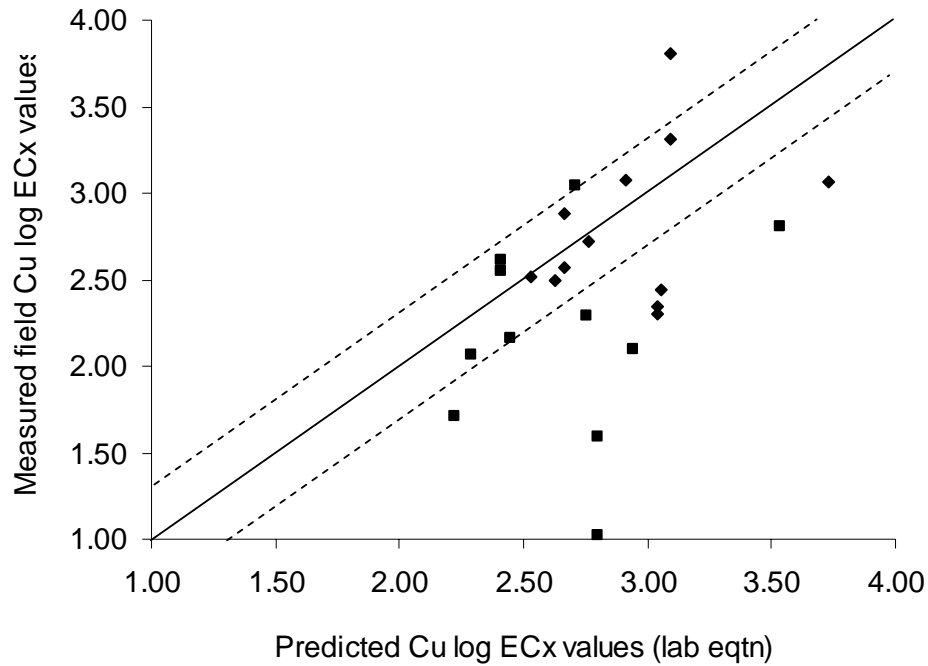
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1 Figure 5: Measured Cu toxicity values (8 week yield) for wheat grain yield versus
2 predicted values using selected laboratory-based relationships based on 20 day
3 plant growth for log EC50 (diamonds) and (b) log EC10 (squares). The solid line
4 is a 1:1 slope and the dashed lines indicate a two fold error from the solid line.

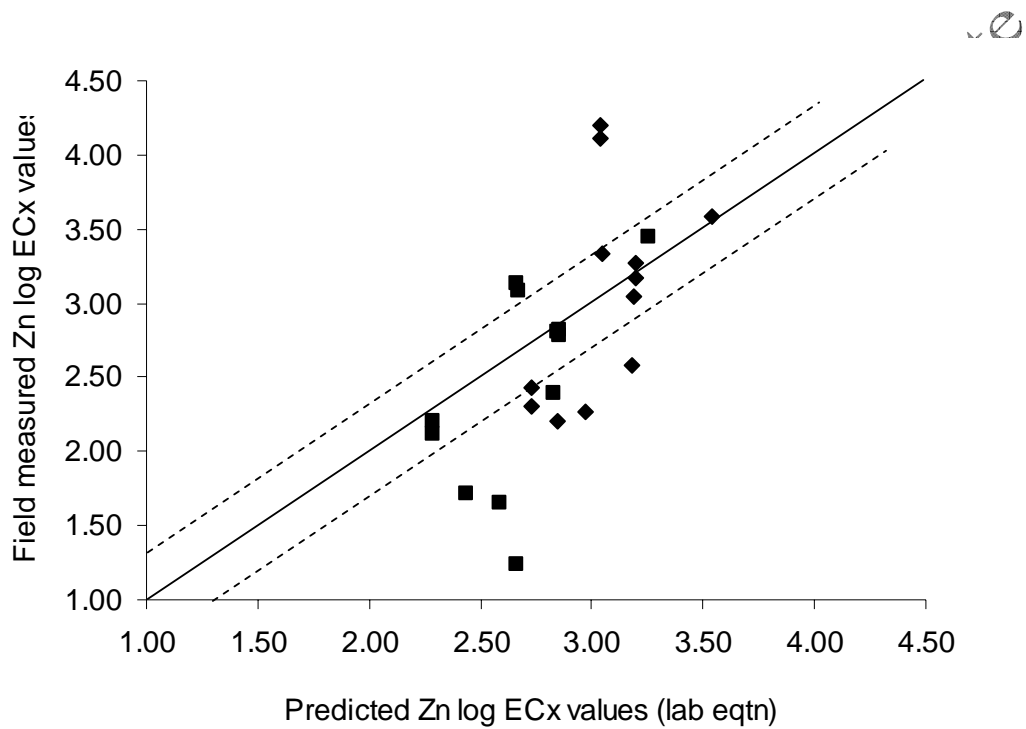
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1 Figure 6: Measured field-based Zn toxicity values (8 week yield) for wheat grain
2 yield versus predicted values using selected laboratory-based relationships based
3 on 20 day plant growth for log EC50 (diamonds) and (b) log EC10 (squares). The
4 solid line is a 1:1 slope and the dashed lines indicate a two fold error from the
5 solid line.



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