Enzyme activities in vineyard soils long-term treated with copper-based fungicides

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ABSTRACT

Copper-based fungicides have been applied in vineyard soils for a long time, which has resulted in increasing soil Cu concentration. However, information relating to non-target effects of these fungicides on microorganisms of these soils is scarce. The aim of this study was to determine the potential enzyme activities of vineyard soils in relation to Cu content and evaluate the potential risks of long-term application of Cu-based fungicides. For this purpose, a wide range of soil samples, having different total, exchangeable and bioavailable Cu contents, were collected from six regions of quality wines located in the NW Iberian Peninsula, and the activity of dehydrogenase, β-glucosidase, urease and phosphatase were measured. Overall, the results obtained indicate adverse effects of Cu on dehydrogenase, β-glucosidase and phosphatase activities and an inconsistent effect on urease activity. Threshold Cu concentrations at which changes in the enzyme activities became evident were 150-200 mg total Cu kg^{-1} and 60-80 mg bioavailable Cu kg^{-1}.

Keywords: Vineyards; Copper fractionation; Enzyme activities; Soil degradation

1. Introduction

Since the end of the 19th Century the vineyard soils have received high amounts of copper-based fungicides to prevent or treat fungal diseases such as downy mildew (*Plasmopara viticola*). Consequently, in many regions devoted to vineyards the prolonged use of these fungicides has led to a significant input of Cu into the soil. Since Cu is scarcely mobile, it tends to accumulate in surface horizons (Fernández-Calviño et al., 2008). Thus, excessively high accumulation of total Cu in the topsoil, far in excess of the natural background metal concentrations normally found in soils, have been observed in traditionally wine-producing countries (Magalhães et al., 1985; Parat et al., 2002; Vavoulidou et al., 2005; Mirlean et al., 2007; Rusjan et al., 2007; Komárek et al., 2008; Wightwick et al., 2008; Fernández-Calviño et al., 2009a; Toselli et al., 2009), as well as in soils cultivated with vegetable crops receiving long-term application of Cu fungicides (Josanidia, 1994; Schramel et al., 2000; Loland and Sing, 2004; Van Zwieten et al., 2004).

The presence of high Cu levels can cause harmful effects on soil microorganisms, which are the main agents responsible for long-term sustainability of soil ecosystems, since they control the breakdown of organic matter and, hence, the net fluxes and amounts of soil carbon and nutrients through decomposition, mineralization, and immobilization processes (Nannipieri et al., 2003). There is, therefore, concern about the effect of Cu on soil microbial communities and their activity.

Many studies employing different methods have shown that several aspects of microbial presence including number, mass, activity and composition can, potentially, be negatively affected by a high Cu content (Bååth, 1989; Brookes, 1995; Wuertz and Mergueay, 1997; Giller et al., 1998; Viti et al., 2008). Enzyme activities can be potentially useful for detecting changes in soil quality, since they underpin nutrient cycling and function as indicators of altered microbial community caused by environmental impact, such as that due to heavy

54 metal pollution (Kandeler et al., 1996). Some of these studies, performed generally in soils
55 contaminated artificially under laboratory conditions with single and extreme doses of Cu,
56 have showed that enzymes are highly sensitive to Cu (Chaperon and Sauvé, 2007). However,
57 studies of soils long-term treated with Cu-based chemicals are scarce (Dell'Amico et al.,
58 2008).

59 Galicia, located in the temperate humid zone (NW Spain), has suffered a long tradition of
60 intensive grapevine with frequent use of Cu-based chemicals. A previous study of 170
61 vineyards, representatives of the seven winegrowing regions that cover a large area of land in
62 Galicia (33,273 ha), showed that high amounts of Cu (average values above 150 mg total Cu
63 kg^{-1}) were accumulated in the topsoil (Fernández-Calviño et al., 2009a). Recently, the
64 microbial communities of some vineyard soils of this area have been characterized with
65 particular regard to the estimation of biochemical indices (Miguéns et al., 2007), tolerance of
66 bacterial communities to Cu (Díaz-Raviña et al., 2007) and microbial community structure
67 determined by means of the PLFA pattern (Fernández-Calviño et al., submitted). However, a
68 detailed study concerning the potential enzymatic activity of these vineyard soils as affected
69 by different Cu content has not been conducted yet.

70 The aim of this work was to examine the activity of several enzymes (dehydrogenase, β-
71 glucosidase, urease and phosphatase) in a wide range of soil samples, a total of 145, collected
72 from six vineyard areas in NW Iberian Peninsula, as well as the potential of these
73 measurements as indicators of Cu pollution in vineyard soils. Taking into account the high
74 number of samples to process, enzymatic assays were chosen because they are sensitive,
75 rapid, inexpensive and representative of the potential metabolic capacity of the soil
76 (dehydrogenase is an intracellular enzyme catalyzing oxidoreduction reactions of organic
77 compounds and β-glucosidase, urease and phosphatase are extracellular enzymes implied in
78 the C, N and P cycles).

2. Materials and methods

2.1. Soil Samples

The study was conducted on a total of 145 vineyard-devoted soils distributed in 6 regions of quality wines, five located in Galicia (Monterrei, M, n = 36 samples; Rías Baixas, B, n = 24 samples; Ribeira Sacra, S, n = 18 samples; Ribeiro, R, n = 24 samples; Valdeorras, V, n = 25 samples) and one in the north-west of Portugal (Vinhos Verdes, VV, n = 18 samples). Composite samples, formed by several soil sub-samples (0-20 cm) collected from each plot and sieved to < 2 mm, were used for all subsequent analyses. Information about soil locations, sampling procedure and methods for determination of the physical and chemical characteristics (texture, pH, total C, total N, effective cation exchange capacity, total Cu and exchangeable Cu) have been described previously (Fernández-Calviño et al., 2009a). The potentially available Cu was obtained from soil after extraction with 0.02 M Na_2-EDTA and 0.5 M NH_4Ac (pH 4.65) according to Lakanen and Erviö, 1971 (Cu_{EDTA}) and with 0.005 M DTPA, 0.01M $CaCl_2$ and 0.1M TEA according to Lindsay and Norwell, 1978 (Cu_{DPTA}).

Physical and chemical properties varied between vineyards areas (Fernández-Calviño et al., 2009a; Table 1). The texture in most of the vineyard soils was mainly dominated by the sand fraction (mean 57±12 %; range 23-77 %) versus silt (mean 27±9 %; range:13-56 %) and clay (mean 17±6; range 7 - 40 %) fractions. The mean total C was 2.5±1.5 g kg^{-1} (range: 3-86 g kg^{-1}) and the mean total N content 2.1±1.0 g kg^{-1} (range: 0.4-7.0 g kg^{-1}). The soil pH and effective cation exchange capacity (eCEC) were also variable; water pH ranged from 4.0 to 7.9, pH in KCl range from 3.3 to 7.2, whereas eCEC varied between 2.3 and 42.4 $cmol_c$ kg^{-1}. Mean value of total copper content (Cu_T) in all studied samples was 164±114 mg kg^{-1} (range 25-666 mg kg^{-1}). The magnitude of Cu_T range varied in the different regions and followed the order: RS (260±120 mg kg^{-1}) soils = R (248±130 mg kg^{-1}) soils >V (174±88 mg kg^{-1}) soils >RB (139±122 mg kg^{-1}) soils >VV (103±42 mg kg^{-1}) = M (100±48 mg kg^{-1}) soils (Table 1).

Mean value of Cu_{EX} was 5.1±5.3 mg kg^{-1} (range: 0.1-30.3 mg kg^{-1}). Mean values of Cu_{EDTA} and Cu_{DPTA} were 60.4±46.49 mg kg^{-1} (range 1.2-215 mg kg^{-1}) and 52.6±45.8 mg kg^{-1} (range 1.0-220.7 mg kg^{-1}), respectively. The different Cu concentrations showed a high interdependence of Cu_T, exchangeable Cu and potentially bioavailable Cu. Cu_T content was strongly correlated with Cu_{DPTA} ($r=0.957$, $P<0.05$) and Cu_{EDTA} ($r=0.880$, $P<0.05$) and in a less extend with Cu_{EX} ($r=0.719$, $P<0.05$). Cu_{DPTA} and Cu_{EDTA} were also correlated with Cu_{EX} ($r=0.693$, and $r= 0.655$, respectively; $P<0.05$) and they were highly correlated to each other ($r=0.918$, $P<0.05$).

2.2. Enzyme activities

The present study was focused on the characterization of the potential soil enzyme activity of this wide range of vineyard soils in relation to soil Cu content. Therefore the measurement of different enzyme activities, an oxidoreductase – dehydrogenase – that gives information about overall activity of soil microbial communities and three hydrolases –β-glucosidase, urease and phospatase– that are involved in the soil cycles of C, N and P, respectively, were measured. The criteria for selecting enzyme bioassays were their importance in nutrient cycling and the simplicity of the assays. Prior to enzymes assays field soil samples were moistened with deionized water until to 60% of the water-holding capacity (WHC) and then incubated at 25 ºC for 7 days, as recommended by previous experiments with polluted soils (Hinojosa et al., 2004b).

Dehydrogenase activity was determined in 1 M Tris-HCl buffer (pH 7.5) by reduction of 2-*p*-iodo-nitrophenyl-phenyltetrazolium chloride (INT) to iodo-nitrophenyl formazan (INTF) and subsequent colorimetric quantification at 490 nm, following the method of García et al. (1993).

Urease activity was determined using the method described by Nannipieri et al. (1980)

in 0.1 M phosphate buffer at pH 7. In particular, 2 ml of buffer and 0.5 ml of 1M urea were added to 0.5 g of soil sample. The concentration of NH^{+}_{4} released after incubation at 30ºC (urease) for 90 min was determined spectrophotometrically at 520 nm.

Phosphatase and β-glucosidase activities were determined using *p*-nitrophenyl phosphate disodium (0.115 M) and *p*-nitrophenyl-b-d-glucopyranoside (PNG, 0.05 M) as substrates, respectively (Masciandaro et al., 1994). These assays are based on the release and detection of *p*-nitrophenol (PNP). Two ml of 0.1 M maleate buffer (pH 6.5 for both phosphatase and β-glucosidase activities) and 0.5 ml of substrate were added to 0.5 g of sample and incubated at 37ºC for 90 min. The reaction was stopped by cooling rapidly to 2ºC for 15 min; 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were then added and the mixture centrifuged at 2000*g* for 5 min. To stop the β-glucosidase assay, trishydroxymethyl aminomethane was used according to Tabatabai (1982). The amount of PNP was determined using a spectrophotometer at 398 nm (Tabatabai and Bremner, 1969).

2.3. Data analyses

To facilitate comparison of data between soils collected from different winegrowing regions, the average values and standard deviation (mean ± SD) were calculated using all soil samples from same region. Differences in enzyme activities among winegrowing regions as well as differences in enzyme activities among soils grouped according their Cu content were tested by means of one-way ANOVA and, in the cases of significant F statistic, the Fisher least Significant Difference (LSD) test was used to separate the means. Assumptions of analysis of variance (homoscedasticity and normality of data) were tested and assured by using transformed data sets when necessary. Correlations coefficients between enzymes activities and other soil factors (general soil properties and different Cu fractions) were analyzed by calculating the regression equation and the simple linear correlation coefficients

using a matrix of data corresponding to the whole data set of samples analyzed (n = 145 samples). All statistical analyses were performed using SPSS v. 17.0 for windows.

3. Results

3.1. Soil enzyme activities

The overall mean values plus standard deviation (mean ± SD) and the range (minimum and maximum values) of potential enzyme activities analyzed in the 145 soils, grouped in the six different vineyard regions, are summarized in Table 2. The more relevant correlations between these measurements and other soil properties are shown in Fig. 1.

3.1.1. Dehydrogenase activity

The mean value of dehydrogenase activity in the studied soils was 1.31±0.91 µg INTF g^{-1} h^{-1} (range 0.03 to 4.53 µg INTF g^{-1} h^{-1}). The highest values were exhibited by VV (2.00±0.98 µg INTF g^{-1} h^{-1}) and RB (1.87±1.14 µg INTF g^{-1} h^{-1}) soils, followed by V (1.28±0.65 µg INTF g^{-1} h^{-1}) and M (1.13±0.68 µg INTF g^{-1} h^{-1}) soils and finally RS (1.01±0.61 µg INTF g^{-1} h^{-1}) and R (0.79±0.75, µg INTF g^{-1} h^{-1}) soils showed the lowest values (Table 2). The dehydrogenase activity showed significantly positive correlations with soil pH ($r = 0.673$, $P< 0.01$ and $r = 0.712$, $P< 0.01$ for pH_{water} and pH_{KCl}, respectively), eCEC ($r = 0.353$, $P< 0.01$) and clay content ($r = 0.213$, $P< 0.05$), and was negatively correlated with Cu_T, Cu_{EX}, Cu_{EDTA} and Cu_{DPTA} ($r = -0.244$, $P< 0.01$, $r = -0.195$, $P< 0.01$, $r = -0.230$, $P< 0.01$ and $r = -0.285$, $P< 0.01$, respectively).

3.1.2. β-glucosidase activity

Mean value of β-glucosidase activity was 0.51±0.44 µmol PNP kg^{-1} h^{-1} (range 0.02 to 2.52 µmol PNP g^{-1} h^{-1}), the highest values being exhibited by VV (0.85±0.41 µmol PNP g^{-1} h^{-}

179 $^{-1}$), RB (0.77±0.50 µmol PNP g^{-1} h^{-1}) and RS (0.73±0.62 µmol PNP g^{-1} h^{-1}) soils followed by
180 M (0.45±0.29 µmol PNP g^{-1} h^{-1}) soils, whereas R (0.27±0.20) and V (0.21±0.11) exhibited
181 the lowest values (Table 2). The β-glucosidase activity showed significant positive
182 correlations with C content (r = 0.680, P< 0.01), N content (r = 0.639, P <0.01), pH_{KCl} (r =
183 0.332, P< 0.01), pH_{water} (r = 0.276, P< 0.01) and eCEC (r = 0.319, P< 0.01) and significant
184 negative correlation with Cu_{EX} (r = -0.192, P< 0.01).
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186 *3.1.3. Urease activity*
187 The mean value of urease activity was 0.61±0.44 µmol NH_4^+-N g^{-1} h^{-1} (range 0.01 to
188 2.29 µmol NH_4^+-N g^{-1} h^{-1}), the highest values being exhibited by RS (0.95±0.68 µmol NH_4^+-
189 N g^{-1} h^{-1}), VV (0.78±0.37 µmol NH_4^+-N g^{-1} h^{-1}) and M (0.70±0.39 µmol NH_4^+-N g^{-1} h^{-1}) soils
190 and the lowest by R (0.56±0.42 µmol NH_4^+-N g^{-1} h^{-1}), V (0.46±0.26 µmol NH_4^+-N g^{-1} h^{-1}) and
191 RB (0.31±0.22 µmol NH_4^+-N g^{-1} h^{-1}) soils. The urease activity showed significant positive
192 correlations with Cu_{EDTA} (r = 0.239, P< 0.01) and Cu_{DPTA} (r = 0.195, P< 0.01) and was
193 negatively related to soil pH (r = -0.342 for both pH_{water} and pH_{KCl}, P < 0.05) and eCEC (r = -
194 0.316, P< 0.01).
195
196 *3.1.4. Phosphatase activity*
197 The mean value of phosphatase activity was 2.86±1.76 µmol PNP g^{-1} h^{-1} (range 0.54
198 to 10.04 µmol PNP g^{-1} h^{-1}), the highest values being exhibited by VV (4.51±1.44 µmol PNP
199 g^{-1} h^{-1}) soils, followed by M (3.32±1.63 µmol PNP g^{-1} h^{-1}), RS (3.19±2.44 µmol PNP g^{-1} h^{-1})
200 and RB (3.03±1.39 µmol PNP g^{-1} h^{-1}) soils, whereas V (1.85±0.82 µmol PNP g^{-1} h^{-1}) and R
201 (1.44±0.82 µmol PNP g^{-1} h^{-1}) showed the lowest values (Table 2). The phosphatase activity
202 was positively correlated with C content (r = 0.553, P < 0.01) and N content (r = 0.514, P<
203 0.01), and negatively correlated with Cu_{EX} (r = -0.372, P< 0.01).

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205 *3.2. Effect of Cu accumulation on enzymatic activities*

206 It is well known that standardized microbial indices such as relative values that are
207 expressed with respect to unit soil organic C were regarded as more reliable indicators than
208 pure microbial parameters expressed as absolute values, because the confounding influence of
209 soil organic C is minimized and the studied parameter thus better isolated (Li et al., 2009). In
210 addition, our results showed that the soil C was significantly correlated with some enzymes
211 measured (the factor could explain 46% and 31% of variability of β-glucosidase and
212 phosphatase activity, respectively, Figs. 1.B and 1.C); therefore, in order to distinguish the
213 effect of soil C from metal toxicity, the values of all soil enzymatic activities were expressed
214 as percentage of organic C.

215 The different Cu fractions were negatively correlated with dehydrogenase, β-
216 glucosidase, and phosphatase (Table 3); however, other factors, mainly soil pH, had also a
217 significantly effect on the potential enzymatic activities, including urease (Table 3). In order
218 to facilitate the data interpretation and elucidate the Cu effects of Cu pollution on soil
219 microorganisms, the soils were grouped according the magnitude of Cu accumulation (Cu_T,
220 Cu_{DPTA}, Cu_{EDTA}, Cu_{EX}) in 7 different intervals. For each group, the mean ± SD values of
221 different potential enzyme activities (dehydrogenase, β-glucosidase, urease and phosphatase)
222 were determined. The threshold Cu concentrations of Cu_T, Cu_{DPTA}, Cu_{EDTA} and Cu_{EX} at which
223 changes in the potential enzyme activities became evident were determining by plotting the
224 different enzyme activities values versus metal concentration (Fig. 2-5). The figures showed a
225 large effect of Cu concentrations on dehydrogenase and phosphatase, a little effect on β-
226 glucosidase and an inconsistent effect on urease activity.

227

228

4. Discussion

4.1. Enzymatic activities: general pattern

As previously reported, information concerning biochemical properties of vineyard soils is still scarce. To the best of our knowledge only one study with a reduced set of samples (n=15) and without evaluating soil Cu contents have been previously performed in two winegrowing regions (R and RS) of the NW of Iberian Peninsula (Miguéns et al., 2007). The values reported in this previous work were found in the range of biochemical properties obtained in the present study. However, the higher number of samples in our study (n=145) that covered 35.600 km^2 of vineyards surface and 6 regions of quality wines (M, RB, RS, R, V and VV) increased the power. Overall, all enzymatic activities were very low and much lower than the usual reference values reported for acid soils under climax vegetation of the same area (Leirós et al., 2000; Trasar-Cepeda et al., 2000a). As suggested by the significant relationships between soil enzyme activities and general soil properties, the low organic matter content and the Cu accumulation in soils are the main factors responsible for the low levels of biochemical activity. These results indicate that long-term viticulture activity have reduced considerably the quality of these soils in the NW Iberian Peninsula, being a land use that causes an intense degradation of the agricultural soils.

4.2. Correlation among enzymatic activities

A significant positive relationship between β-glucosidase and phosphatase activities was observed ($r = 0.608$, $P< 0.01$) and, in a less extent, between urease with both phosphatase ($r = 0.395$, $P< 0.01$) and β-glucosidase ($r = 0.316$, $P< 0.01$) activities, which emphasized the interdependence of the activities associated with the biochemical cycles of C (β-glucosidase), P (phosphatase) and N (urease), especially for C and P cycles. In contrast, dehydrogenase activity, which is considered as an indicator of general microbial activity in soils, only

showed a positive correlation with β-glucosidase activity ($r = 0.394$, $P < 0.01$). Significant positive correlations among all enzymes were also detected by several authors in both undisturbed and disturbed soils soils, being phosphatase the enzyme that better correlates with other enzymes (Bonmatí et al., 1991; Tate el a., 1991; Perucci, 1992; Trasar-Cepeda et al., 2000b; Hinojosa et al., 2004a). Likewise, positive correlations between soil enzymes and total C, total N and other soil properties related with available nutrients (eCEC, pH), and negative correlations with Cu content have been previously observed by other authors in Cu-contaminated soils (Kuperman and Carreiro, 1997; Hinojosa et al., 2004a; Li et al., 2009; Wang et al., 2009), suggesting that the soil enzymatic activities are mainly limited by edaphic factors such as soil pH or availability of C and nutrients as well as by the presence of this toxic metal.

4.3. Enzymatic activities: effect of Cu accumulation

The effects of Cu_T on soil potential enzyme activities (Fig. 2; Table 3) depended on the soil enzyme considered. In particular, dehydrogenase, β-glucosidase and phosphatase activities tended to decrease with increasing the total Cu content, whereas an inconsistent effect of Cu pollution was observed for the urease activity. Phosphatase was the enzyme more affected by the Cu concentrations, followed by dehydrogenase (Fig 2; Table 3). These results agree with those reported by Madejón et al. (2001), who found that phosphate was the most sensitive in evaluating soil pollution, whereas β-glucosidase and urease were less affected by metals. Other authors, however, observed that phosphatase was less sensitive in tracing heavy metal effects than urease, β-glucosidase or dehydrogenase (Dick, 1997; Kuperman and Carreiro, 1997; Lee et al., 2002; Hinojosa et al., 2004a). Our data also showed that 200-250 mg Cu_T kg^{-1} seem to be the threshold concentration at which changes in dehydrogenase, β-glucosidase and phosphatase became evident, although for the latter an effect was even

279 observed at lower concentrations (150-200 mg kg^{-1}). These threshold values were slightly
280 higher than those reported previously for bacterial community tolerance to Cu (Díaz-Raviña
281 et al., 2007, threshold values around 100 mg Cu kg^{-1}), which might suggest a lower sensitivity
282 of soil enzymes versus other microbial parameters that are more specific for detecting Cu
283 toxicity. The results of this study are consistent with findings of Viti et al. (2008), who
284 observed that Cu levels up to 145 mg Cu kg^{-1} could be a risk to microorganisms from olive
285 orchard and vineyard soils treated with copper fungicides.

286 The estimated threshold concentrations, however, must be interpreted with caution
287 since the effects of soil pollution on potential enzymes activities are complex, particularly
288 under field conditions, where the interactive effects of numerous factors affecting soil
289 potential enzymatic activity could be influencing the results. Studies of several authors have
290 showed that the response of different enzymes to the same pollutant may vary greatly
291 (Madejón et al., 2001; He et al, 2003; Trasar-Cepeda et al. 2000b). The effect of pH on soil
292 microorganisms can not be discarded when the impact of soil pollution by heavy metals is
293 evaluated, particularly when different soils with a wide range of pH are considered. It is well-
294 known that soil pH is determinant for the composition of microbial communities since
295 completely different strains/species of microbes are predominant at different pHs (Lauber et
296 al., 2009; Rousk et al., 2010) and that these microbial modifications induced by soil pH
297 should be reflected on the level of soil enzymatic activities (Kandeler et al., 1996). In
298 addition, soil pH affects markedly Cu^{2+} availability (Fernández-Calviño et al., 2009b) and
299 therefore it can play an indirect role in the impact of metal on microbial communities. It the
300 present study, soil pH decreased with pollution level since mean pH values in first 4 intervals
301 (<50 to 150-200 mg Cu_T kg^{-1}) ranged from 5.4±1.0 to 5.7±0.7 whereas in last 3 intervals
302 (200-250 to >300 mg Cu_T kg^{-1}) ranged from 4.9±0.4 to 5.1±0.7. Dehydrogenase activity and
303 in a less extent β-glucosidase were positively related with soil pH, suggesting that acidity

suppressed potential enzyme activities (Table 3); therefore, low enzyme activities observed at highest levels of pollution can be also partly due to low pH values. Our data clearly indicated that pH is a confounding factor in determining Cu toxicity in these vineyard soils, being very difficult to separate the pH effect from that of metal.

Some authors have suggested that dehydrogenase determination is not a valid assay parameter for estimating the microbial activity of Cu-contaminated soils or recently amended with Cu-contaminated sewage sludges (Chandler and Brookes, 1991), meanwhile other authors found that it can be successfully used to evaluate Cu toxicity (Chaperon and Sauvé, 2007). The development of formazan color in the dehydrogenase assay using INT has been previously reported to be inhibited by soluble-Cu content over 1 mg l^{-1} (Obbard, 2001). However, this concentration was never exceeded in the samples of the present study since our assay was buffered to a pH value of 7.5, and under such conditions the Cu mobility is highly reduced. Similar to Cu_T, an adverse Cu effect on dehydrogenase, β-glucosidase and phosphatase activities and an inconsistent effect on urease activity were observed with increasing Cu_{EX} and Cu_{EDTA} and Cu_{DPTA} fractions (Fig. 3-5). The threshold concentrations of bioavailable Cu (Cu_{DPTA} and Cu_{EDTA}) for altered soil enzymes were quite similar, about 60-80 mg kg^{-1}, regardless of the soil extractant. Estimates were also obtained for exchangeable Cu (Cu_{EX}), but in this case the estimated threshold concentrations, around 7.5-10 and 10-12.5 mg kg^{-1}, seem to be less accurate, since fluctuations in soil enzymes induced by accumulation of Cu_{EX} were less consistent than those observed for the Cu_T, Cu_{DPTA} and Cu_{EDTA}. This is in general agreement with previous results reported by Chaperon and Sauvé (2007), who found that toxicity thresholds for enzyme activities based on dissolved metals seem to be more variable than those based on total metals. In most studies concerning the effect of Cu pollution on soil microorganisms, the total Cu content rather than the exchangeable or potentially available fractions were determined.

When combining all the information on the effects of Cu pollution on soil enzymes (Fig. 2-5), the data clearly showed that soil enzymes in vineyard soils were altered by Cu accumulation and that the sensitivity of measured enzyme to detect this long-lasting Cu effect followed the order phosphatase > dehydrogenase > β-glucosidase >> urease. The intracellular enzyme and two extracellular enzymes associated with the C and P cycles examined in this study responded similarly to the addition of Cu-based fungicides, decreasing their activities, whereas urease, an extracellular enzyme associated with the N cycle, was inconsistently affected or even stimulated. As a whole, the results obtained indicate that phoshatase, dehydrogenase and β-glucosidase activities (but not urease) can be potentially used for detecting the effect of Cu pollution on microbial communities. Taking into account the sensitivity of different enzyme assays as well as the influence of pH on their measurements, we consider that the best indicator for Cu metal toxicity is the phosphatase activity. The data also indicated that, on the basis of soil enzyme assays, an alteration of soil functionality seem to be observed in vineyard soils with more than 150-200 mg kg^{-1} of total Cu and 60-80 mg kg^{-1} of bioavailable Cu. Further studies are necessary in order to analyze other microbial parameters based on mass, activity and diversity of soil microorganisms and determine if Cu accumulation at concentrations higher than these Cu levels could be a threat to soil quality and productivity of vineyard soils.

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FIGURE CAPTIONS

Fig. 1. Relevant relationships between soil enzyme activities and the rest of soil properties analyzed for the whole set of vineyard soil samples from NW Iberian Peninsula (n = 145 samples).

Fig. 2. Box plot representation of (A) dehydrogenase, (B) β-glucosidase, (C) urease and (D) phosphatase activities, expressed with respect to unit soil organic C, grouped according the content of total Cu (Cu_T, mg kg^{-1}). The box bounds show the 25 and 75 percentiles, and the errors bars 90 and 10 percentiles. The continuous and discontinuous lines inside the boxes represent medians and means, respectively. Outliers are represented as black dots. Average values with the same letter in each figure indicate no significant differences between plots ($P < 0.05$).

Fig. 3. Box plot representation of (A) dehydrogenase, (B) β-glucosidase, (C) urease and (D) phosphatase activities, expressed with respect to unit soil organic C, grouped according the content of exchangeable Cu (Cu_{EX}, mg kg^{-1}). The box bounds show the 25 and 75 percentiles, and the errors bars 90 and 10 percentiles. The continuous and discontinuous lines inside the boxes represent medians and means, respectively. Outliers are represented as black dots. Average values with the same letter in each figure indicate no significant differences between plots ($P < 0.05$).

Fig. 4. Box plot representation of (A) dehydrogenase, (B) β-glucosidase, (C) urease and (D) phosphatase activities, expressed with respect to unit soil organic C, grouped according the content of bio available Cu extracted with DPTA (Cu_{DPTA}, mg kg^{-1}). The box bounds show the 25 and 75 percentiles, and the errors bars 90 and 10 percentiles. The continuous and discontinuous lines inside the boxes represent medians and means, respectively. Outliers are

represented as black dots. Average values with the same letter in each figure indicate no significant differences between plots ($P < 0.05$).

Fig. 5. Box plot representation of (A) dehydrogenase, (B) β-glucosidase, (C) urease and (D) phosphatase activities, expressed with respect to unit soil organic C, grouped according the content of bioavailable Cu extracted with EDTA (Cu_{EDTA}, mg kg^{-1}). The box bounds show the 25 and 75 percentiles, and the errors bars 90 and 10 percentiles. The continuous and discontinuous lines inside the boxes represent medians and means, respectively. Outliers are represented as black dots. Average values with the same letter in each figure indicate no significant differences between plots ($P < 0.05$).

Table 1

Mean (± standard deviation) of selected physico-chemical and chemical properties of vineyard soils at the studied winegrowing regions: Monterrei (M), Rías Baixas (RB), Ribeira Sacra (RS), Ribeiro (R), Valdeorras (V), Vinhnos Verdes (VV). *Fernández-Calviño et al., 2009.

	M	RB	RS	R	V	VV
Sand (%)	53±14	63±7	60±11	60±8	46±10	57±13
Silt (%)	32±11	21±6	27±9	22±6	31±6	25±11
Clay (%)	16±4	16±2	13±3	18±4	24±8	18±3
pH_{water}	4.9±0.6	6.6±0.7	5.0±0.6	4.9±0.6	5.6±0.4	5.7±0.4
pH_{KCl}	4.1±0.6	5.8±0.8	4.1±0.7	4.1±0.7	4.5±0.5	4.8±0.4
C (g kg^{-1})	22±12	35±9	34±20	21±15	14±7	32±12
N (g kg^{-1})	1.6±0.8	2.7±0.7	2.6±1.3	1.8±1.2	1.6±0.6	2.7±0.9
eCEC ($cmol_c$ kg^{-1})	3.9±1.9	21.0±8.4	3.5±0.8	5.4±2.9	5.6±1.4	6.4±2.0
Cu_T (mg kg^{-1})	100±48	139±122	260±120	248±130	174±88	103±42
Cu_{EX} (mg kg^{-1})	1±1	6±2	5±4	12±7	5±4	2±1
Cu_{DPTA} (mg kg^{-1})	31±20	42±47	93±64	80±46	58±35	23±13
Cu_{EDTA}(mg kg^{-1})	44±30	33±22	98±63	89±45	77±46	31±18

pH, pH in water; pH_{KCl}, pH in potassium chloride; eCEC, effective cation exchange capacity; C, organic carbon; N, nitrogen; Cu_T, total Cu; Cu_{EX}, exchangeable Cu; Cu_{DPTA}, bioavailable Cu extracted with DTPA; Cu_{EDTA}, bioavailable Cu extracted with EDTA.

Table 2

Mean (± standard deviation) and minimum-maximum values of enzyme activities of vineyard soils at the studied winegrowing regions: Monterrei (M), Rías Baixas (RB), Ribeira Sacra (RS), Ribeiro (R), Valdeorras (V), Vinhnos Verdes (VV). Different letters in columns denote significant differences between regions ($P < 0.05$).

	M	RB	RS	R	V	VV
Dehydrogenase	1.13±0.68b	1.87±1.14a	1.01±0.61bc	0.79±0.75c	1.28±0.65b	2.00±0.98a
	(0.12-3.66)	(0.31-4.31)	(0.07-2.10)	(0.03-3.06)	(0.46-2.69)	(0.67-4.53)
β-glucosidase	0.45±0.29b	0.77±0.50a	0.73±0.62a	0.27±0.20bc	0.21±0.11c	0.85±0.41a
	(0.02-1.24)	(0.23-2.52)	(0.19-2.28)	(0.02-0.85)	(0.03-0.45)	(0.32-1.81)
Urease	0.70±0.39b	0.31±0.22e	0.95±0.68a	0.56±0.42bcd	0.46±0.26de	0.78±0.37abc
	(0.01-1.71)	(0.01-0.72)	(0.01-2.29)	(0.01-1.36)	(0.08-1.20)	(0.01-1.48)
Phosphatase	3.32±1.63b	3.03±1.39b	3.19±2.44b	1.44±0.82c	1.85±0.82c	4.51±1.44a
	(0.66-6.41)	(1.20-5.91)	(0.41-10.04)	(0.54-4.11)	(0.84-3.86)	(2.38-7.61)

Dehydrogenase activity (μg INTF g^{-1} h^{-1}), β-glucosidase activity (μmol PNP g^{-1} h^{-1}), Urease activity (μmol NH_4^+ g^{-1} h^{-1}), Phosphatase activity (μmol PNP g^{-1} h^{-1})

Table 3
Correlation coefficients between potential enzymatic activities, expressed with respect to soil organic C, and general soil characteristics.

	Sand	Silt	Clay	pH_{water}	pH_{KCl}	eCEC	Cu_T	Cu_{EX}	Cu_{DTPA}	Cu_{EDTA}
Dehydrogenase	--	--	0.319**	0.381**	0.339**	--	-0.278**	-0.175*	-0.287**	-0.194*
β-glucosidase	--	--	--	0.271**	0.293**	--	-0.214**	-0.205*	-0.184*	--
Urease	--	--	--	-0.368**	-0.435**	-0.370**	--	0.164*	--	--
Phosphatase	-0.224**	0.246**	--	-0.178*	-0.257**	-0.338**	-0.392**	-0.382**	-0.340**	-0.300**

*p<0.05; **p<0.01

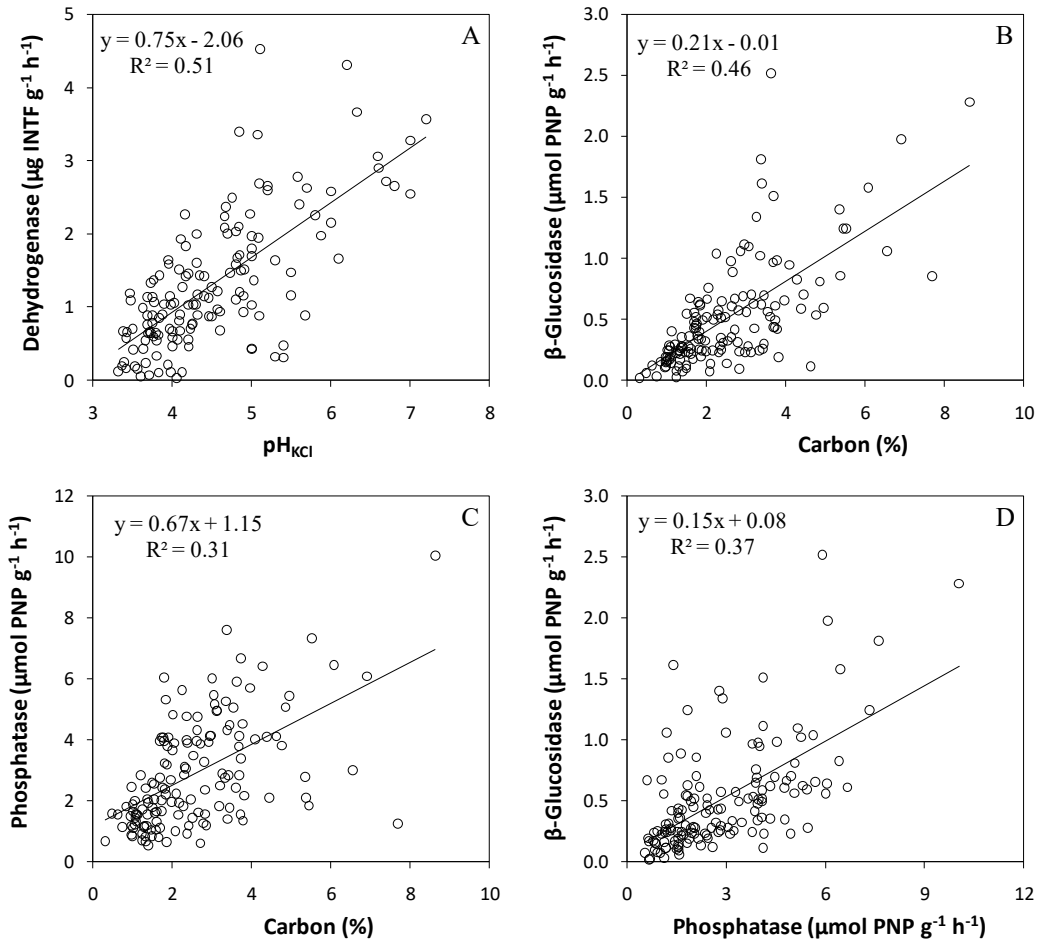
y = 0.75x - 2.06
R² = 0.51
A
Dehydrogenase (µg INTF g-1 h-1)
pHKCl
y = 0.21x - 0.01
R² = 0.46
B
β-Glucosidase (µmol PNP g-1 h-1)
Carbon (%)
y = 0.67x + 1.15
R² = 0.31
C
Phosphatase (µmol PNP g-1 h-1)
Carbon (%)
y = 0.15x + 0.08
R² = 0.37
D
β-Glucosidase (µmol PNP g-1 h-1)
Phosphatase (µmol PNP g-1 h-1)

Fig. 1.

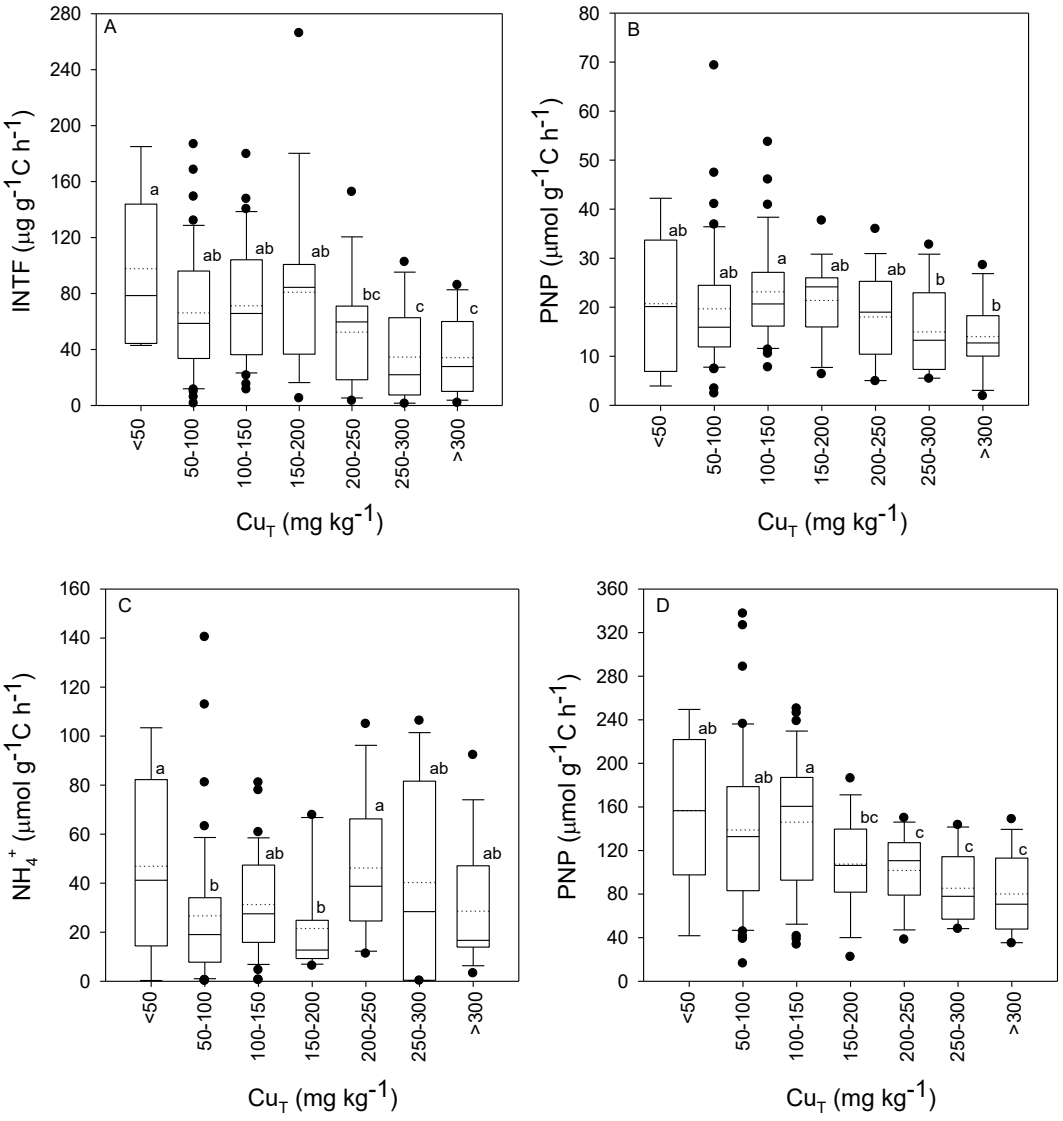
A
INTF (μg g-1C h-1)
a
ab
ab
ab
bc
c
c
<50
50-100
100-150
150-200
200-250
250-300
>300
CuT (mg kg-1)
B
PNP (μmol g-1C h-1)
ab
ab
a
ab
ab
b
b
CuT (mg kg-1)
C
NH4+ (μmol g-1C h-1)
a
b
ab
b
a
ab
ab
CuT (mg kg-1)
D
PNP (μmol g-1C h-1)
ab
ab
a
bc
c
c
c
CuT (mg kg-1)

Fig. 2.

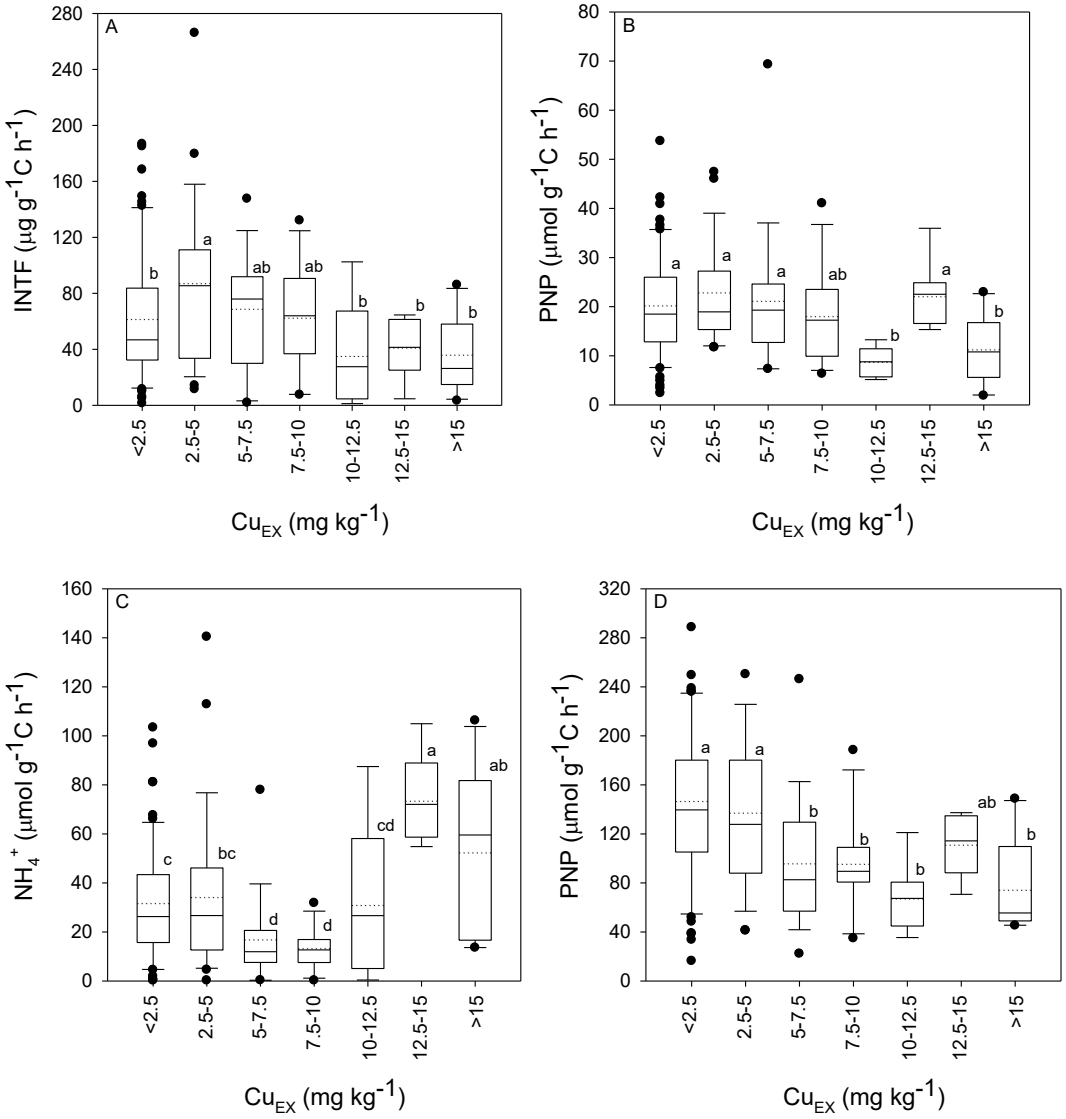
A
INTF (µg g-1C h-1)
B
PNP (µmol g-1C h-1)
C
NH4+ (µmol g-1C h-1)
D
PNP (µmol g-1C h-1)
<2.5
2.5-5
5-7.5
7.5-10
10-12.5
12.5-15
>15
CuEX (mg kg-1)

Fig. 3.

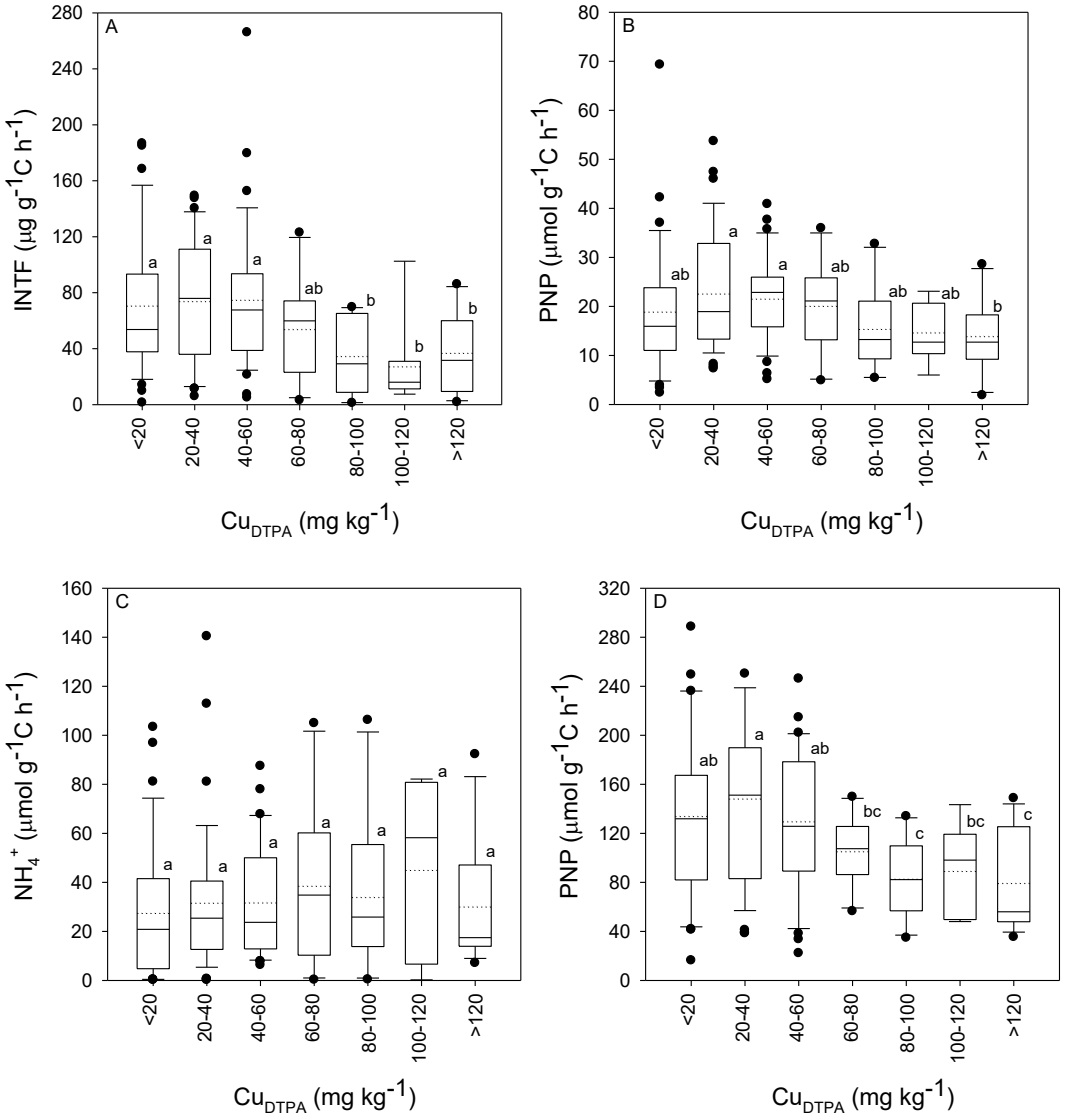
A
INTF (µg g-1C h-1)
B
PNP (µmol g-1C h-1)
C
NH4+ (µmol g-1C h-1)
D
PNP (µmol g-1C h-1)
<20
20-40
40-60
60-80
80-100
100-120
>120
CuDTPA (mg kg-1)

Fig. 4.

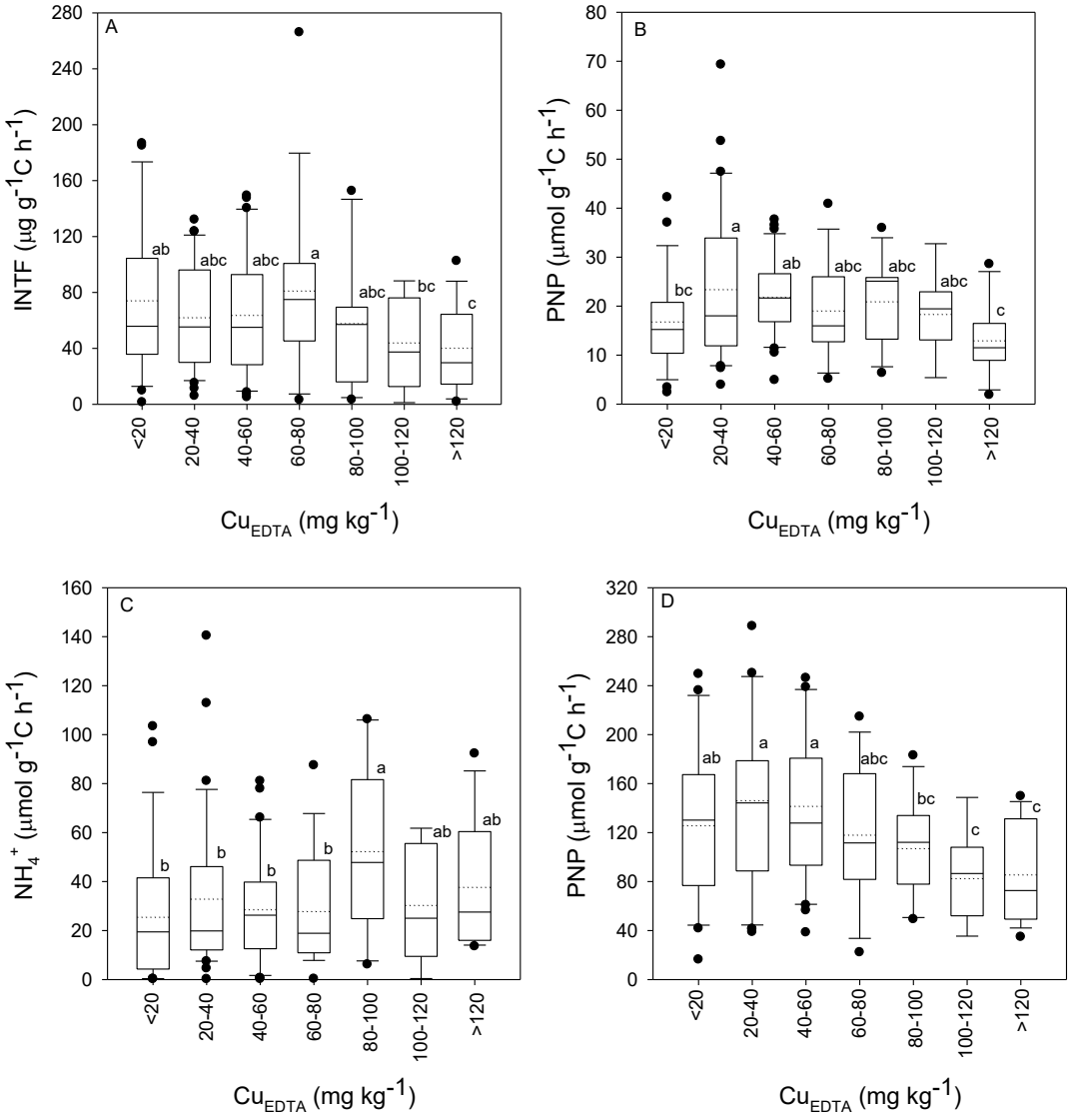
A
INTF (µg g-1C h-1)
ab
abc
abc
a
abc
bc
c
B
PNP (µmol g-1C h-1)
bc
a
ab
abc
abc
abc
c
C
NH4+ (µmol g-1C h-1)
b
b
b
b
a
ab
ab
D
PNP (µmol g-1C h-1)
ab
a
a
abc
bc
c
c
<20
20-40
40-60
60-80
80-100
100-120
>120
CuEDTA (mg kg-1)

Fig. 5.