Respiration parameters determined by the ISO-17155 method as potential indicators of copper pollution in vineyard soils after long-term fungicide treatment

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26 **ABSTRACT**

27 This study seeks to determine the impact of copper-based fungicides on the respiration of
28 vineyard soils. The ISO-17155 is an international standard recommended for monitoring soil
29 quality by the evaluation of the effects of pollutants on soil microbial activity. Respiration curves
30 and derived parameters [i.e. basal respiration (R_B), substrate-induced respiration (R_S), lag time
31 (t_{lag}), growth rate (μ), time to the peak maximum ($t_{peakmax}$), respiratory-activation quotient (Q_R),
32 and the cumulative O_2 consumption (C_R)] were determined from 95 vineyard soils that covered a
33 wide range of Cu contents. Statistical analyses showed that most of the variance of the ISO-
34 17155 parameters was due to soil pH and organic C content, but not to the Cu pollution. When
35 the parameters were expressed as a function of soil organic C content, the effect of soil Cu
36 content was found to be significant on R_S and $t_{peakmax}$ but not on R_B and C_R . The results indicated
37 that threshold values of total (Cu_T) and exchangeable (Cu_{EX}) contents indicative of soil Cu
38 pollution cannot be established. However, adequate management practices resulting in soil
39 organic C contents $> 2\%$ and pH > 5.5 are recommended for preserving vineyard soil quality.

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41 **Keywords:** vineyards; copper fractionation; soil respiration; ISO 17155; polluted soils

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45 **1. INTRODUCTION**

46 Changes in land use to viticulture have lead to heavy accumulations of pesticides in soils,
47 especially Cu-based fungicides, resulting in Cu contents significantly higher than in the natural
48 background of soils. This longstanding problem is has been detected in vineyards worldwide
49 (Komárek et al., 2010). Because of the human and environmental health hazards that may arise
50 from this pollution, setting soil-Cu limits and adequate management practices are necessary to
51 reach sustainability in this type of crop.

52 High Cu contents can harm soil microbial communities, which are the main agents
53 responsible for long-term sustainability of soil ecosystems (Nannipieri et al., 2003). Some
54 studies have evaluated the effects of Cu on enzymatic activities (Fernández-Calviño et al.,
55 2010a), microbial community structure (Dell'Amico et al., 2008; Lejon, et al., 2008; Viti et al.,
56 2008; Fernández-Calviño et al., 2010b), and bacterial community tolerance (Díaz-Raviña et al.,
57 2007; Fernández-Calviño et al., 2011) in vineyard soils. However, the establishment of limits for
58 Cu accumulation using microbial indicators is difficult because the confounding effects of soil
59 factors such as texture, pH, and organic matter content (Fernández-Calviño et al., 2011).

60 Basal respiration (R_B) is generally used to evaluate the steady rate of respiration in soil
61 originated from organic matter turnover, whereas the measurement of the substrate-induced
62 respiration (R_S) is a means of estimating metabolically active soil microbial biomass (Anderson
63 and Domsch, 1978). In this sense, both parameters are recognized as soil-quality indicators and
64 they have been commonly used as endpoints to assess the effects of heavy-metal pollution
65 (Brookes, 1995). Nevertheless, ecotoxicologically, it is more difficult to evaluate the pollution
66 effects in natural field soils than in artificially spiked soils because, in the former, neither an
67 appropriate control soil (basal level) nor a gradient of metal concentration is usually available
68 (Giller et al., 1998).

Moreover, little information is available on non-target effects of the fungicide-Cu pollution on field-soil microbial activity and little research has been done following standardized methods to determine the harmful effects of this pollution on soil respiration. The ISO-17155 is an international standard for determining soil microbial biomass activity using respiration curves (ISO, 2002), which may provide different parameters and indications for monitoring soil pollution and disturbance. However, a detailed analysis of soil respiration according to this standard method has been poorly reported to evaluate soil fertility (Hall et al., 2006), soil pollution with polycyclic aromatic hydrocarbons (Gong et al., 2005) and with inorganic and organic substances (Hund-Rinke and Simon, 2008; Bartling et al., 2009). In fact, only one of these studies deals with Cu-polluted soils (Wilke et al., 2005).

Therefore, the present work examines 95 field soils from vineyards after long-term treatment with Cu-fungicides that cover a wide range of total and exchangeable Cu contents. The aim was to calculate the parameters of the respiration curves determined by the ISO-17155 standard method in order to test the potential of the resulting parameters as indicators of this type of Cu pollution.

2. MATERIALS AND METHODS

2.1. Soil Samples

Samples were collected from 95 vineyards from 6 different wine-production areas in the NW Iberian Peninsula. The soils of the study area are dominated by *Regosols* and the climate is characterized by mild annual mean temperatures (10-15°C) together with low to moderate annual rainfall (700-1500 mm). Several soil sub-samples (0-20 cm depth) were collected with a soil auger for an overall amount of 2 kg (8-10 sub-samples) on each vineyard. Soil sub-samples were subsequently mixed into a single composite soil sample. The samples were air dried and sieved to 2 mm. Air drying as a pre-treatment to standardize moisture levels prior to rewetting, pre-incubation, and soil-respiration measurements is believed not to change the main conclusions

concerning the impact of heavy-metal pollution on soil biological functioning and quality (Bandick and Dick, 1999; Hinojosa et al., 2004).

2.2. Physico-chemical and chemical properties of soils

Sand (2-0.05 mm), silt (0.05-0.002 mm) and clay (<0.002 mm) proportions were determined by wet sieving (size fractions >0.05 mm) and using the international pipette method for all others. The pH in water (pH) and 0.1 M KCl (pH_{KCl}) were measured at a soil:water ratio of 1:2.5 after 10 and 120 min, respectively. Soil organic carbon (C) was determined on a ThermoFinnigan 1112 Series NC elemental analyser. The effective cation-exchange capacity (eCEC) was estimated as the combination of exchangeable-base cations (K, Na, Ca, and Mg) extracted with 0.2 M NH₄Cl plus exchangeable aluminium (Al) extracted with KCl 1M. Total Cu content (Cu_T) was determined by microwave digestion (HNO₃, HF, HCl) and Cu measurement with an atomic absorption spectrometer (Thermo Solar M series). Exchangeable copper (Cu_{EX}) was extracted from soils with 1 M NH₄OAc (pH 7.0). Table 1 shows the mean values, standard deviation and range of the main properties of the soil samples used in this work.

2.3. Soil respiration

Soil respiration was determined according to the International Standard ISO 17155 guidelines (ISO, 2002). Moist soil samples (60% water-holding capacity) were pre-incubated in glass bottles for 3 days inside a thermostatic chamber (20 ±1°C) in order to avoid undesirable fluxes in the early respiration measurements. Then the sensor-data loggers (OxiTop, WTW, Weilheim, Germany) were adjusted to the bottle heads and the O₂ consumption was measured at 1-h intervals until constant respiration rates in order to determine basal respiration (R_B). After R_B measurement, 0.2 g of a substrate mixture [80 g glucose, 13 g (NH₄)₂SO₄ and 2 g KH₂PO₄] per gram of soil organic matter were added and then O₂ consumption was measured hourly until the respiration rate declined. The substrate-induced respiration (R_S) was calculated as the average of

the values during a stable period (3-6 h) before the exponential increase of the respiration rate. Following the ISO 17155 guidelines, different parameters were calculated by a non-linear regression fit of accumulated data during the exponential phase.

The growth constant (μ) is the rate constant during the exponential increase. The time from the substrate addition until the exponential growth start is the lag time (t_{lag}), whereas the time to the peak maximum ($t_{peakmax}$) is the time from substrate addition to the maximum rate of induced respiration. The cumulative O_2 consumption (C_R) corresponds to the total area bounded by the soil-respiration rate curve to the time axis from the substrate addition to the $t_{peakmax}$. The respiratory-activation quotient (Q_R) is calculated by dividing the basal respiration rate by the substrate-induced respiration rate (R_B/R_s).

From R_s data recorded when using glucose as C substrate, the content of soil microbial biomass C (MBC) can be calculated using Eq. (1) of Anderson and Domsch (1978) and assuming the 1:1 ratio of CO_2 evolution per O_2 uptake during the R_s phase, i.e. between the glucose addition and before exponential microorganism growth (Dilly, 2001).

$$\mu g \text{ MBC } g^{-1} \text{ soil} = (40.04 \times \mu l \text{ CO}_2 (R_s) g^{-1} h^{-1}) + 0.37 \quad (1)$$

2.4. Statistical analyses

Correlation coefficients between respiration parameters and other soil characteristics were analysed by calculating the regression equation and the simple linear-correlation coefficients using a matrix of data corresponding to the whole data set of samples analysed ($n = 95$). Differences in soil-respiration parameters among soils grouped according to their Cu content were tested by means of a one-way ANOVA and, in the cases of significant F statistic, the Fisher least-significant difference (LSD) test was used to separate the means. Student's t-test was used to differentiate the general characteristics between soils designated as polluted and non-polluted

according to the ISO 17155 guidelines. The level of significance used was 95%. All statistical analyses were performed using SPSS v. 17.0 for Windows.

3. RESULTS

3.1. *Respiration parameters established from the respiration curves*

Figure 1 shows an example of the respiration curves drawn according to the guidelines of the International Standard ISO 17155. The mean values plus standard deviation and the range of respiration parameters are summarized in Table 2.

3.1.1. *Basal respiration*

The mean value of R_B in the soils studied was $3.0 \pm 1.8 \mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$ (range 0.5 to $9.5 \mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$; Table 2). The soil with the highest value presented 5.5% C, pH 6.7 and 139 mg kg^{-1} of Cu_T , whereas the soil with the lowest R_B presented 1.1% C, pH 4.7 and 76 mg kg^{-1} of Cu_T . The R_B value showed significantly positive correlations with C, pH, and eCEC (Table 3) but was not significantly correlated with Cu_{EX} or Cu_T (Table 3). The highest correlation was found with C content, followed by soil pH. The multiple-regression analysis between R_B and soil characteristics resulted in Eq. (2), where only significant factors were included. The most important variable was organic C, explaining 55% of R_B variance, followed by pH_{KCl} and Cu_T , which explained 11% and 3%, respectively.

$$R_B = -(1.84 \pm 0.54) + (0.83 \pm 0.10) C + (0.67 \pm 0.13) \text{pH}_{KCl} - (1.87 \cdot 10^{-3} \pm 0.66 \cdot 10^{-3}) \text{Cu}_T \quad (2)$$

$$R^2 = 0.69; p < 0.001$$

3.1.2. *Substrate-induced respiration*

The mean value of R_S was $24.0 \pm 22.7 \mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$ (range 0.4 to $92.5 \mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$) (Table 2), while the highest value was found in a soil with 5.4% C, pH 5.3, and 79 mg kg^{-1} of

Cu_T, and the lowest R_S was found in a soil with 1.1% C, pH 4.4, and 255 mg kg⁻¹ of Cu_T. Like R_B, the R_S value showed significantly positive correlations with C, soil pH, and eCEC (Table 3), but was not significantly correlated with Cu_{EX} or Cu_T. The highest correlation was found with C, followed by soil pH (Table 3). The multiple-regression analysis (Eq. 3) showed that organic C was the most important variable, explaining 40% of R_S variance, whereas pH_{KCl} and eCEC explained only 6% and 3%, respectively:

$$R_S = -(47.84 \pm 11.02) + (8.53 \pm 1.49) C + (12.29 \pm 2.91) \text{pH}_{\text{KCl}} - (0.69 \pm 0.32) \text{eCEC}$$

(3)

$$R^2 = 0.51; p < 0.001$$

According to these R_S values, the mean MBC content in soil was 722 ± 682 µg g⁻¹, showing a wide range (from 13 to 2780 µg g⁻¹). The multiple-regression analysis showed the Eq. (4) where only significant factors were included. Organic carbon explained 39% of MBC variance, while pH_{KCl} and eCEC explained only 8% and 2%, respectively.

$$\text{MBC} = -(1437.93 \pm 331.33) + (256.48 \pm 44.71) C + (369.37 \pm 87.47) \text{pH}_{\text{KCl}} - (20.76 \pm 9.71) \text{eCEC}$$

(4)

$$R^2 = 0.50; p < 0.001$$

3.1.3. Microbial growth-rate constant

The mean microbial growth rate (µ) was 0.07 ± 0.02 h⁻¹ (range 0.02 to 0.12 h⁻¹; Table 2). The highest value was exhibited by a soil with 1.5% C, pH 4.3, and 46 mg kg⁻¹ of Cu_T, whereas the lowest was found in a soil with 2.7% C, pH 6.9, and 83 mg kg⁻¹ Cu_T. The µ value showed significant negative correlations with organic C, pH, and eCEC (Table 3). Organic C and pH explained 28% and 4% of µ variance, respectively. The multiple-regression analysis provided Eq. (5):

$$\mu = (0.109 \pm 0.010) - (0.007 \pm 0.001) C - (0.005 \pm 0.002) \text{pH} \quad (5)$$

$$R^2 = 0.32; p < 0.001$$

3.1.4. Lag time

The mean value of the lag time (t_{lag}) was 29 ± 7 h (range 15 to 44 h; Table 2). The highest value was found in a soil with 1.1% C, pH 4.7, and 76 mg kg^{-1} of Cu_T . The lowest value was found in a soil with 6.9% C, pH 4.7, and 346 mg kg^{-1} of Cu_T . The t_{lag} value was negatively correlated with soil pH and organic C (Table 3). The multiple-regression analysis gave Eq. (6), in which pH explained 14% of t_{lag} variance:

$$t_{lag} = (43.9 \pm 4.0) - (2.81 \pm 0.72) \text{ pH} \quad (6)$$

$$R^2 = 0.14; p < 0.001$$

3.1.5. Time until peak of maximum respiration rate

The mean value of time from substrate addition until peak of maximum respiration rate ($t_{peakmax}$) was 60 ± 14 h (range 33 to 99 h; Table 2). The highest value was found in a soil with 1.4% C, pH 5.0, and 203 mg kg^{-1} of Cu_T , whereas the lowest $t_{peakmax}$ was found in a soil with 5.4% C, pH 5.3, and 79 mg kg^{-1} of Cu_T . The $t_{peakmax}$ value showed a significantly positive correlation with Cu_{EX} , and a negative correlation with C and soil pH (Table 3). The multiple-regression analysis showed the Eq. (7), where organic C and Cu_{EX} explained 14% and 4% of $t_{peakmax}$ variance, respectively.

$$t_{peakmax} = (66.9 \pm 3.2) - (3.82 \pm 1.01) C + (0.50 \pm 0.21) \text{ Cu}_{EX} \quad (7)$$

$$R^2 = 0.18; p < 0.001$$

3.1.6. Respiratory-activation quotient

The mean value of Q_R was 0.47 ± 0.79 (range 0.03 to 5.18; Table 2). The highest value was exhibited by a soil with 2.0% C, pH 4.8, and 350 mg kg^{-1} of Cu_T , whereas the lowest Q_R was found in a soil with 1.0% C, pH 5.3, and 49 mg kg^{-1} of Cu_T . The Q_R was positively correlated

with silt and clay contents, and negatively correlated with C, pH, and eCEC (Table 3). The multiple-regression analysis showed Eq. (8), where the sand content explained 11% of Q_R variance, whereas C and Cu_{EX} explained 9% and 4%, respectively:

$$Q_R = (2.13 \pm 0.38) - (0.024 \pm 0.006) \text{ Sand} - (0.171 \pm 0.055) C + (0.027 \pm 0.012) Cu_{EX} \quad (8)$$

$$R^2 = 0.24; p < 0.001$$

3.1.7. Cumulative O_2 consumption

The cumulative respiration (C_R) was $44 \pm 29 \text{ mg } O_2 \text{ g}^{-1}$ (range 5 to $124 \text{ mg } O_2 \text{ g}^{-1}$; Table 2), with the highest value in a soil with 2.8% C, pH 6.1, and 94 mg kg^{-1} of Cu_T . The lowest C_R was found in a soil with 1.5% C, pH 4.3, and 46 mg kg^{-1} of Cu_T . The C_R showed significant positive correlations with pH, pH_{KCl} , eCEC, and C (Table 3). The multiple-regression analysis gave Eq. (9), in which pH_{KCl} and C explained 38% and 6% of C_R variance, respectively:

$$C_R = -(37.7 \pm 11.1) + (14.3 \pm 2.7) pH_{KCl} + (6.4 \pm 2.0) C \quad (9)$$

$$R^2 = 0.44; p < 0.001$$

3.2. Cu-accumulation effect on respiration parameters

The respiration data show a low effect of Cu contents on some respiration parameters (R_B , $t_{peakmax}$ and Q_R) or no detectable effects on other parameters (R_S , μ , t_{lag} and C_R). On the other hand, the variance of most of the parameters was explained largely by soil organic C content. Thus, as a means of distinguishing the effect of organic C from Cu effect, the values of R_B , R_S , C_R , and MBC were standardized to relative values expressed with respect to the unit of soil organic C. With this standardization, the confounding influence of organic C was minimized and the parameters studied were better isolated (Li et al., 2009). Table 4 shows the correlations between soil characteristics and the respiration parameters expressed with respect to soil organic C (R_B/C , R_S/C , C_R/C and MBC/C).

The R_S/C and MBC/C values were negatively affected by Cu_{EX} , while R_B/C and C_R/C values were negatively correlated with Cu_T ; whereas, the soil pH was positively correlated with R_S/C , C_R/C and MBC/C . To elucidate the effects of Cu fractions on soil respiration, the soils studied were grouped according to Cu_T and Cu_{EX} contents. Seven different intervals were used and the differences in mean values from different groups were compared (ANOVA test via LSD analysis). Figure 2 shows mean values and statistical differences between groups.

Regarding Cu_T , although the values of μ , t_{lag} , and $t_{peakmax}$ tended to increase at higher Cu contents, no significant differences appeared between groups. The R_B/C and C_R/C values were significantly reduced over 50 mg kg^{-1} of Cu_T , but in both cases significant differences disappeared as Cu_T contents increased. The R_S/C and MBC/C values became significantly lower in soils with $Cu_T > 200 \text{ mg kg}^{-1}$ than in soils with $Cu_T < 50 \text{ mg kg}^{-1}$. The mean values of Q_R were higher (although not significantly so) for $Cu_T > 200 \text{ mg kg}^{-1}$.

According to the Cu_{EX} groups, no significant differences were found between the lowest and the highest contents for R_B/C , μ , t_{lag} , Q_R , C_R/C , and MBC/C . However, as observed with Cu_T , the values of Q_R were higher (although not significantly so) for Cu_{EX} contents over 10 mg kg^{-1} . The values of R_S/C and MBC/C decreased as Cu_{EX} increased, being significantly lower in soils with $Cu_{EX} > 12.5 \text{ mg kg}^{-1}$. Finally, $t_{peakmax}$ values were significantly higher in soils with $Cu_{EX} > 10 \text{ mg kg}^{-1}$.

3.3. Polluted soils according ISO-17155

The threshold values proposed by the ISO-17155 standard method for the respiration parameters indicative of soil pollution are: $Q_R > 0.3$, $t_{lag} > 20 \text{ h}$, $t_{peakmax} > 50 \text{ h}$. From the 95 samples analysed in the present study, these three parameters were over the threshold values in only 27 soils. When the parameters were considered individually, 30 soils were found with $Q_R > 0.3$, 84 soils with $t_{lag} > 20 \text{ h}$, and 67 soils with $t_{peakmax} > 50 \text{ h}$.

The soils classified as polluted according to the threshold values from ISO-17155 guidelines presented an organic C content lower than 2%, $\text{pH} < 5.6$, $\text{pH}_{\text{KCl}} < 4.5$, Cu_{EX} content between 0.4 and 25.5 mg kg^{-1} and Cu_{T} content between 46 and 428 mg kg^{-1} . To check for differences in general characteristics between polluted and non-polluted soils, Student's t-test was performed. The percentage of sand, organic C content, pH, pH_{KCl} , and eCEC were significantly higher in the non-polluted than in polluted soils, whereas silt, clay, and Cu_{EX} were significantly lower in non-polluted soils (Table 5).

4. DISCUSSION

4.1. Effect of Cu fractions on respiration parameters

The respiration parameters of the soils studied depended mainly on soil organic C and pH. The dependence of microbial activities on soil organic C is well known, this being the main factor that regulates soil respiration (Pell et al., 2006). The positive effect of pH in soil respiration has also been reported in previous works with similar soil organic matter content (Rousk et al., 2009). Both factors, pH and organic C, could be masking the Cu-pollution effect in the present study as the regression analyses showed.

When the organic C effect was removed, the pH effect also disappeared and a negative effect on R_{B}/C was detected over 50 mg kg^{-1} of Cu_{T} . The same effect of Cu_{T} was observed on R_{R}/C values, although the pH effect was still present. When the R_{S} and MBC values were normalized to organic C contents, although the pH effect persisted, a negative correlation of Cu_{EX} with R_{S}/C and MBC/C was found. Furthermore, both standardized parameters decreased progressively as the Cu_{T} and Cu_{EX} contents increased, implying that they could be used to set Cu limits: 200 mg kg^{-1} for Cu_{T} and 12.5 mg kg^{-1} for Cu_{EX} . The t_{peakmax} values were significantly correlated with Cu_{EX} , but also with organic C and pH. When soil samples were grouped according to Cu_{EX} contents, the values of t_{peakmax} became significantly higher over 10 mg kg^{-1} of Cu_{EX} . The parameters μ , t_{lag} , and Q_{R} were not significantly related to Cu contents, but they were

highly correlated with organic C and soil pH. For these parameters the differences between Cu_T or Cu_{EX} groups were not significant, although higher Q_R values were observed in soils with $Cu_T > 200 \text{ mg kg}^{-1}$ and $Cu_{EX} > 10 \text{ mg kg}^{-1}$.

4.2. Respiration parameters as potential indicators of Cu pollution

Our results in relation with Cu contents agreed partially with those found by Wilke et al. (2005), which found that R_B , Q_R , and t_{lag} were less sensitive to Cu-pollution. However, they observed a significant effect on R_S , μ , and $t_{peakmax}$, while C_R reacted as the most sensitive parameter. The combination of these previous findings and our results may suggest that in the present study Q_R , t_{lag} , and μ can be ruled out as potential indicators to establish Cu-content limits. The suitability of R_B and C_R as endpoints had some shortcomings: the low consistency of differences with increasing Cu_T contents, no differences between Cu_{EX} groups and, in the case of C_R , the persistent pH effect even when the organic C effect was removed. The reduction of C_R values over 50 mg kg^{-1} of soil Cu_T agrees with the inhibition effect on this parameter of around 40 mg kg^{-1} reported by Wilke et al. (2005).

The parameter $t_{peakmax}$ was positively and directly related to Cu_{EX} , but the effects of organic C and pH were also present. Despite a clear increase of $t_{peakmax}$ over 10 mg kg^{-1} of Cu_{EX} , no differences were found for this parameter between Cu_T groups. When the organic C effect was removed in the R_S parameter, it was negatively correlated with Cu_T but positively with soil pH. Also, R_S values significantly declined by over 200 mg kg^{-1} of Cu_T and over 12.5 mg kg^{-1} of Cu_{EX} . The negative effect in the R_S parameter of around 190 mg kg^{-1} of total Cu in soil has been found elsewhere (Wilke et al., 2005).

The limit of 200 mg kg^{-1} of total Cu was on the order of those proposed previously: around 100 mg kg^{-1} (Díaz-Raviña et al., 2007), 150 mg kg^{-1} (Bååth, 1992; Viti et al., 2008; Fernández-Calviño et al., 2010a), or 250 mg kg^{-1} (Rajapaksha et al., 2004). However, these values should be interpreted with caution. In an acidic soil spiked with Cu, Rajapaksha et al.

(2004) showed that over 250 mg kg⁻¹ of Cu, the bacterial growth decreased sharply, whereas the fungal growth increased. In vineyard soils with wide ranges of both pH (4.2-7.3) and total Cu (2-1120 mg kg⁻¹), no differences between bacterial and fungal growth were found as function of Cu content in soil (Fernández-Calviño et al., 2011a). This discrepancy was attributed to the much higher effect of pH than Cu on bacterial and fungal growth. Similarly the microbial community structure was much more affected by pH than by Cu contents (Fernández-Calviño et al., 2010b; de Boer et al., 2012). The confounding effect of pH on the evaluation of Cu toxicity was also found when enzymatic activities of the soil were tested (Wang et al., 2009; Fernández-Calviño et al., 2010a; Lebrun et al., 2012). Even when a specific end-point such as pollution-induced community tolerance (PICT) was used to study the direct effect of Cu contents in vineyard soils, the confounding effects of pH or organic C remained and the establishment of Cu tolerance was problematic (Fernández-Calviño et al., 2011a).

4.3. Polluted soils according to ISO-17155 thresholds

A major advantage of the ISO-17155 standard method is that it can identify which soils were polluted according the threshold values of the respiration parameters, i.e. which soils present functional problems. The analysis of our data indicated that in all soils designated as polluted, the pH values were below 5.6 (pH_{KCl} <4.5) and the organic C contents were lower than 2%. However, no differences were found respect to total Cu contents between soils designated as polluted and non-polluted. It is well known that soil organic matter (Senesi et al., 1989; Arias et al., 2002) and pH play a key role in Cu availability in soils (Sanders, 1982; Spark, 1997), and this is especially true in the vineyards soils studied, where pH governs Cu-organic matter binding (Fernández-Calviño et al., 2010c). Moreover, pH and organic C have a direct effect on soil microorganisms by controlling nutrient availability, microbial biomass, and the fungal and bacterial growth (Paul, 1984; Fountaine et al., 2003; Aciego Pietri and Brookes, 2008). In addition, bacteria are not well adapted to highly acidic soils (Fernández-Calviño et al., 2011b)

and their growth capacity is very low (Rousk et al., 2009), hampering the recovery of bacterial communities after any alteration (i.e. Cu pollution).

Since the introduction of a Cu limit in the soils studied was difficult, it might be more useful to establish management practices for vineyards which can avoid adverse effects on soil microbial activity. The results of the present work showed that when pH values were higher than 5.6 ($\text{pH}_{\text{KCl}} > 4.5$) or the organic C content was higher than 2%, pollution effects were not detected independently of soil Cu contents. Therefore, in acidic soils devoted to vineyards after long-term treatment with fungicides, the organic C content should be higher than 2% and the pH higher than 5.5 in order to avoid adverse effects of Cu accumulation on soil microbial activity.

Conclusions

The respiration measurements in 95 vineyard soils covering a wide range of Cu contents provided a variety of parameters to be used as potential indicators of Cu-pollution. Regression analyses showed that the variance of most of these parameters was explained to a great extent by soil organic C and pH, which probably masked the Cu-pollution effect. When the influence of organic C was removed, the higher Cu contents significantly affected R_S and t_{peakmax} , while the effect on R_B and C_R proved inconsistent. Parameters such as μ , t_{lag} , and Q_R were not significantly related to soil Cu contents. The values at which changes in the respiration parameters became evident were $\text{Cu}_T > 200 \text{ mg kg}^{-1}$ and $\text{Cu}_{\text{EX}} > 10 \text{ mg kg}^{-1}$. The establishment of threshold limits for Cu were difficult due to confounding effects of pH and organic C, since they govern Cu availability and microbial communities in soils. However, from the evaluation of 27 soil samples designated as polluted (according to the ISO-17155 guidelines), organic C contents $> 2\%$ and a $\text{pH} > 5.5$ would be recommendable for vineyards in acidic soils in order to avoid adverse effects of Cu accumulation from fungicides applications.

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386 **References**

387 Aciego Pietri JC, Brookes PC. Relationships between soil pH and microbial properties in
388 a UK arable soil. *Soil Biol Biochem* 2008;40:1856-61.

389 Anderson JPE, Domsch KH. A physiological method for the quantitative measurement of
390 microbial biomass in soils. *Soil Biol Biochem* 1978;10:215-21.

391 Arias M, Soto B, Barral MT. Copper sorption characteristics on mineral-humic acid
392 substrates. *Agrochimica* 2002;46: 155-64.

393 Bååth E. Measurement of heavy metal tolerance of soil bacteria using thymidine
394 incorporation into bacteria extracted after homogenization-centrifugation. *Soil Biol Biochem*
395 1992;24:1167-72.

396 Bandick AK, Dick RP. Field management effects on soil enzyme activities. *Soil Biol*
397 *Biochem* 1999;31:1471-79.

398 Bartling J, Kotzerke A, Mai M, Esperschütz J, Buegger F, Schlöter M, Wilke BM.
399 Microbial community structure and function during abnormal curve development of substrate-
400 induced respiration measurements. *Chemosphere* 2009;77:1488-94.

401 Brookes PC. The use of microbial parameters in monitoring soil pollution by heavy
402 metals. *Biol Fertil Soils* 1995;19:269-79.

403 de Boer TE, Taş N, Braster M, Temminghoff EJM, Röling WFM, Roelofs D. The
404 influence of long-term copper contaminated agricultural soil at different pH levels on microbial
405 communities and springtail transcriptional regulation. *Environ Sci Technol* 2012;46:60-8.

406 Dell'Amico E, Mazzocchi M, Cavalca L, Allievi L, Andreoni V. Assessment of bacterial
407 community structure in a long-term copper-polluted ex-vineyard soil. *Microbiol Res*
408 2008;163:671-83.

409 Díaz-Raviña M, Calvo de Anta R, Bååth E. Tolerance (PICT) of the bacterial
410 communities to copper in vineyards from Spain. *J Environ Qual* 2007;36:1760-4.

411 Dilly O. Microbial respiratory quotient during basal metabolism and after glucose
412 amendment in soils and litter. *Soil Biol Biochem* 2001;33:117-27.

413 Fernández-Calviño D, Soler-Rovira P, Polo A, Díaz-Raviña M, Arias-Estévez M, Plaza
414 C. Enzyme activities in vineyard soils long-term treated with copper-based fungicides. *Soil Biol*
415 *Biochem* 2010a;42:2119-27.

416 Fernández-Calviño D, Martín A, Arias-Estévez M, Bååth E, Díaz-Raviña M. Microbial
417 community structure of vineyard soils with different pH and copper content. *Appl Soil Ecol*
418 2010b;46:276-82.

419 Fernández-Calviño D, Soler-Rovira P, Polo A, Arias-Estévez M, Plaza C. Influence of
420 humified organic matter on copper behaviour in acid polluted soils. *Environ Poll*
421 2010c;158:3634-41.

422 Fernández-Calviño D, Arias-Estévez M, Díaz-Raviña M, Bååth E. Bacterial pollution
423 induced community tolerance (PICT) to Cu and interactions with pH in long-term polluted
424 vineyard soils. *Soil Biol Biochem* 2011a;43:2324-31.

425 Fernández-Calviño D, Rousk J, Brookes PC, Bååth E. Bacterial pH-optima for growth
426 track soil pH, but are higher than expected at low pH. *Soil Biol Biochem* 2011b;43:1569-75.

427 Giller KE, Witter E, McGrath SP. Toxicity of heavy metals to microorganisms and
428 microbial processes in agricultural soils: A review. *Soil Biol Biochem* 1998;30:1389-414.

429 Gong Z, Alef K, Wilke BM, Mai M, Li P. Assessment of microbial respiratory activity of
430 a manufactured gas plant soil after remediation using sunflower oil. *J Hazard Mat*
431 2005;B124:217-23.

432 Hall NM, Kaya B, Dick J, Skiba U, Niang A, Tabo R. Effect of improved fallow on crop
433 productivity, soil fertility and climate-forcing gas emissions in semi-arid conditions. *Biol Fertil*
434 *Soils* 2006;42:224-30.

435 Hinojosa MB, Carreira JA, García-Ruiz R, Dick RP. Soil moisture pre-treatment effects
436 on enzyme activities as indicators of heavy metal contaminated and reclaimed soils. *Soil Biol*
437 *Biochem* 2004;36:1559-68.

438 Hund-Rinke K, Simon M. Bioavailability of contaminants in soil via respiration and
439 nitrification tests. *Environ Pollut* 2008;153:468-75.

440 ISO, International Organization for Standardization. Soil quality - Determination of
441 abundance and activity of soil microflora using respiration curves. International Standard ISO
442 17155; 2002.

443 Komárek M, Čadkova E, Chrástný V, Bordas F, Bollinger J.C. Contamination of
444 vineyard soils with fungicides: A review of environmental and toxicological aspects. *Environ Int*
445 2010;36:138-51.

446 Lebrun JD, Trinsoutrot-Gattin I, Vincelas-Akpa M, Bailleul C, Brault A, Mougin C,
447 Laval K. Assessing impacts of copper on soil enzyme activities in regard to their natural
448 spatiotemporal variation under long-term different land uses. *Soil Biol Biochem* 2012;49:150-6.

449 Lejon DPH, Martins JMF, Lévêque J, Spadini L, Pascault N, Landry D, Milloux MJ,
450 Nowak V, Chaussod R, Ranjard L. Copper dynamics and impact on microbial communities in
451 soils of variable organic status. *Environ Sci Tech* 2008;42:2819-25.

452 Li YT, Rouland C, Benedetti M, Li FB, Pando A, Lavelle P. Microbial biomass, enzyme
453 and mineralization activity in relation to soil organic C, N and P turnover influenced by acid
454 metal stress. *Soil Biol Biochem* 2009;41:969-77.

455 Miguéns T, Leirós MC, Gil-Sotres F, Trasar-Cepeda C. Biochemical properties of
456 vineyard soils in Galicia, Spain. *Sci Total Environ* 2007;378:218-22.

457 Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G. Microbial
458 diversity and soil functions. *Eur J Soil Sci* 2003;54:655-70.

459 Paul EA. Dynamics of organic matter in soils. *Plant Soil* 1984;76:275-85.

Pell M, Stenström J, Granhall U. Soil Respiration. In: Bloem J, Hopkins DW, Benedetti A, editors. Microbiological Methods for Assessing Soil Quality. Wallingford: CABI Publishing; 2006. p. 117-126.

Rajapaksha RMCP, Tobor-Kaplon MA, Bååth E. Metal toxicity affects fungal and bacterial activities in soil differently. *Appl Environ Microb* 2004;70:2966-73.

Rousk J, Brookes PC, Bååth E. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. *Appl Environ Microb* 2009;75:1589-96.

Sanders JR. The effect of pH upon the copper and cupric ion concentrations in soil solutions. *J Soil Sci* 1982;33:679-89.

Senesi N, Sposito G, Holtzclaw KM, Bradford GR. Chemical properties of metal-humic acid fractions of a sewage sludge-amended aridisol. *J Environ Qual* 1989;18:186-94.

Spark KM, Wells JD, Johnson BB. Sorption of heavy metals by mineral-humic acid substrates. *Aust J Soil Res* 1997;35:103-12.

Viti C, Quaranta D, De Philippis R, Corti G, Agnelli A, Cuniglio R, Giovannetti L. Characterizing cultivable soil microbial communities from copper fungicide-amended olive orchard and vineyard soils. *World J Microb Biot* 2008;24:309-18.

Wang QY, Zhou DM, Cang L. Microbial and enzyme properties of apple orchard soil as affected by long-term application of copper fungicide. *Soil Biol Biochem* 2009;41:1504-9.

Wilke BM, Mai M, Gattinger A, Schlöter M, Gong P. Effects of fresh and aged copper contaminations on soil microorganisms. *J Plant Nutr Soil Sc* 2005;168:668-75.

Figure 1

Respiration parameters in the respiration curves. R_B , basal respiration rate; R_S , substrate-induced respiration; μ , growth rate; t_{lag} , lag time; $t_{peakmax}$, time to the peak maximum.

Figure 2. Mean respiration parameters (with respect to unit soil organic C) grouped according to the content of total copper (Cu_T) and exchangeable copper (Cu_{EX}) fractions. R_B/C ($\mu\text{g O}_2 \text{ mg}^{-1} \text{ C h}^{-1}$), R_S/C ($\mu\text{g O}_2 \text{ mg}^{-1} \text{ C h}^{-1}$), C_R/C ($\text{g O}_2 \text{ mg}^{-1} \text{ C}$), and MBC/C ($\text{mg g}^{-1} \text{ C}$). Values within each Cu fraction followed by the same letter are not significantly different ($p < 0.05$)

Table 1

Mean values, standard deviation (SD), and range of some main properties of the 95 soils used in this work

	Mean	SD	Maximum	Minimum
Sand (%)	59	12	77	23
Silt (%)	25	9	56	13
Clay (%)	16	4	34	8
C (%)	2.5	1.3	6.9	0.3
pH	5.4	0.9	7.9	4.0
pH _{KCl}	4.6	1.0	7.2	3.3
eCEC (cmol _c kg ⁻¹)	8.6	8.5	42.4	2.3
Cu _{EX} (mg kg ⁻¹)	5.9	6.1	30.3	0.1
Cu _T (mg kg ⁻¹)	176	166	1120	11

pH, pH in water; pH_{KCl}, pH in 0.1M potassium chloride; eCEC, effective cation-exchange capacity; C, organic carbon; Cu_{EX}, exchangeable Cu; Cu_T, total Cu.

Table 2

Parameters determined from respiration curves according to ISO 17155 guidelines

	Mean	SD	Maximum	Minimum
R_B ($\mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$)	3.0	1.8	9.5	0.5
R_S ($\mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$)	24.0	22.7	92.5	0.4
μ (h^{-1})	0.07	0.02	0.12	0.02
t_{lag} (h)	29	7	44	15
t_{peakmax} (h)	60	14	99	33
Q_R (dimensionless)	0.47	0.79	5.18	0.03
C_R ($\text{mg O}_2 \text{ g}^{-1}$)	44	29	124	5

R_B , basal respiration rate; R_S , substrate-induced respiration; μ , growth rate; t_{lag} , lag time; t_{peakmax} , time to the peak maximum; Q_R , respiratory activation quotient; C_R , cumulative O_2 consumption.

Table 3

Correlation coefficients between respiration parameters and general characteristics of soils

	R _B	R _S	μ	t _{lag}	t _{peakmax}	Q _R	C _R
Sand						-0.337**	
Silt						0.265**	
Clay						0.329**	
C	0.738**	0.630**	-0.528**	-0.299**	-0.370**	-0.321**	0.519**
pH	0.580**	0.475**	-0.397**	-0.376**	-0.243*	-0.304**	0.557**
pH _{KCl}	0.661**	0.561**	-0.402**	-0.367**	-0.286**	-0.312**	0.615**
eCEC	0.541**	0.379**	-0.347**			-0.221*	0.550**
Cu _{EX}					0.238*		
Cu _T							

pH, pH in water; pH_{KCl}, pH in 0.1M potassium chloride; eCEC, effective cation-exchange capacity; C, organic carbon; Cu_{EX}, exchangeable Cu; Cu_T, total Cu; R_B, basal respiration rate; R_S, substrate-induced respiration; μ, growth rate; t_{lag}, lag time; t_{peakmax}, time to the peak maximum; Q_R, respiratory activation quotient; C_R, cumulative O₂ consumption.

Table 4

Correlation coefficients between general characteristics of soils and some respiration parameters expressed with respect to soil organic carbon (R_B/C , $\mu\text{g O}_2 \text{ mg}^{-1} \text{ C h}^{-1}$; R_S/C , $\mu\text{g O}_2 \text{ mg}^{-1} \text{ C h}^{-1}$; C_R/C , $\text{g O}_2 \text{ g}^{-1} \text{ C}$; MBC/C , $\text{mg g}^{-1} \text{ C}$).

	R_B/C	R_S/C	C_R/C	MBC/C
Sand				
Silt				
Clay				
pH		0.295**	0.213*	0.295*
pH_{KCl}		0.292**		0.292*
eCEC				
Cu_{EX}		-0.252*		-0.253*
Cu_T	-0.208*		-0.227*	

pH, pH in water; pH_{KCl} , pH in 0.1M potassium chloride; eCEC, effective cation-exchange capacity; Cu_{EX} , exchangeable Cu; Cu_T , total Cu; R_B , basal respiration rate; R_S , substrate-induced respiration; C_R , cumulative O_2 consumption; MBC, microbial biomass carbon.

Table 5

Significance of general characteristics between soils designated as polluted and non-polluted according to the threshold values from ISO 17155

	Signification	Student's t-test
Sand	0.006	2.007
Silt	0.047	-1.476
Clay	0.049	-1.938
C	0.000	5.183
pH	0.000	4.602
pH _{KCl}	0.000	4.994
eCEC	0.000	3.304
CuEX	0.001	-1.063
CuT	0.343	0.347

pH, pH in water; pH_{KCl}, pH in 0.1M potassium chloride; eCEC, effective cation-exchange capacity; C, organic carbon; Cu_{EX}, exchangeable Cu; Cu_T, total Cu.

HIGHLIGHTS

- Soil-respiration parameters measured according to ISO-17155 were used to monitor soil quality in Cu-polluted soils.
- The study includes 95 vineyard soils.
- Threshold limits for Cu are difficult to establish due to confounding effects of pH and organic C.
- To preserve quality of these vineyard acidic soils, organic carbon >2% and pH >5.5 are recommended.