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Copper distribution among physical and chemical fractions in a former vineyard soil

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INTRODUCTION. – The Copper (Cu) contamination of vineyard soils due to the heavy use of copper-based fungicides is a well known problem (PROBST *et al.*, 2008; FERNÃNDEZ-CALVIÑO, 2008). Since Cu is scarcely mobile in soils, it tends to accumulate in surface horizons (PIETRZAK and MCPHAIL, 2004) even after land use change (FERNÃNDEZ-CALVIÑO, 2008). This Cu accumulation is well beyond the natural background concentrations of metal normally found in soils (22-55 mg kg^{-1} depending on the nature of the parent material) (BAKER and SENFT, 1995). Adsorption is a key process responsible for accumulation of heavy metals in soil and regulates their concentration in solution, which is also influenced by inorganic and organic ligands (BRADL, 2004; VIOLANTE *et al.*, 2008).

Cu in soils may occur in several forms that are partitioned between the solution and the solid phases. Cu bioavailability and phytotoxicity is closely related to its distribution in different chemical forms. Exchangeable Cu may be readily mobilized to the soil solution, with negative effects for plants and soil organisms. On the other hand, Cu phytoavailability can be reduced by Cu binding to soil organic matter (BOLAN and DURAISAMY, 2003). Cu distribution in the different chemical forms depends on several soil properties, such as pH, redox potential, cation exchange capacity (CEC), texture, soil organic matter (SOM), as well as Mn and Fe oxides content (MCLAREN *et al.*, 1983; SIMS, 1986). Cu fractionation studies have shown that this metal exists in soils predominantly as organically bound, in residual, precipitated, or acid-soluble form (BERTI and JACOBS, 1996; ALVA *et al.*, 2000). The most important interfaces involved in Cu adsorption in soils are Fe and Mn oxides, SOM, sulfides and carbonates (JENNE, 1968).

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The sequential extraction procedure reported by ALVA *et al.* (2000) involves the use of two labile extractants (KNO_3 and H_2O) for readily soluble Cu determination, NaOH for organically bound and Na_2EDTA for precipitated form. Other extraction procedures were those recommended by TESSIER *et al.* (1979) and by the Community Bureau of Reference (BCR) (URE *et al.*, 1993). The procedure discriminates the water soluble, the $MgCl_2$ exchangeable, the carbonate, the Fe and Mn and the organic matter bound fractions.

Physical fractionation is based on the concept that SOM associated with particles of different size and different mineralogical composition differs in its structure and function (CHRISTENSEN, 1992). As well, Cu associated with particles of different size differs in its stability and accessibility, e.g. clay-sized particles provide a large surface area and numerous reactive sites where SOM and metals can be sorbed by strong ligand exchange and polyvalent cation bridges (SPOSITO *et al.*, 1999), thus stabilizing the metal. Cu distribution in different soil fractions can therefore mirrors differences in Cu availability and in its environmental impact.

As reported by CHEN and MA (2001), the amount of trace elements extracted by the commonly used digestion methods might depends on their chemical nature, their origin (anthropogenic or natural), soil properties and element mass fractions. Accurate determination of heavy metals in soil has to consider the importance of the digestion step in releasing elements associated with soil matrix; the comparison of different digestion procedures can be therefore useful in order to evaluate the effectiveness of methods to detect Cu concentrations. The analysis using aqua regia digestion is reported by the ISO standard (1995) procedure and has been widely adopted in Europe. The aqua regia digestion is recommended for heavy metal content determination in soils polluted from anthropogenic sources (BECKETT, 1989). This digestion is considered effective for measuring trace element in soils and it is frequently used to estimate the maximum element availability to plants (USEPA, 1986). Moreover, hydrogen peroxide can be added to enhance the destruction of organic matter, as suggested by Italian legislation (G.U. n. 248, 21, 1999). The $H_2SO_4 + HNO_3$ digestion is also used for trace element analysis in sediments. The sulfuric – nitric acid mixture, after evaporation of the acids, allows the dissolution of a wide range of mineral mixtures.

The soil chosen for this study was cultivated with vineyard for at least 30 years, during which 3-4 treatments with $CuSO_4$ were performed each year. In 1983 the land use changed and the vineyard was replaced with annual crops. After the land use change the herbaceous crops presented pain symptoms, possibly attributable to Cu phytotoxicity (MARTIN *et al.*, 2003). To assess the extent of Cu pollution accumulated in the topsoil and provide an assessment of the long-term potential toxicity effects of pollution, is important to analyze both heavy metal concentration and its distribution into forms of variable phytotoxicity.

In this work we hypothesized that, extracting selectively the metal or analyzing its concentration in different soil fractions could highlight Cu interactions with other soil components such as clay minerals, organic matter and soil solution, and, therefore, forecast its mobility and retention, as well as its availability to plants (ALVA *et al.*, 2000; JARADAT *et al.*, 2006). Cu distribution in different soil chemical and physical fractions was detected with different methods, with the aim: I) to identify soil chemical and physical fractions more important in Cu retention; II) to reveal if pain symptoms shown by herbaceous crops were attributable to phytotoxicity of available Cu forms; III) to compare the effectiveness of different methodological approaches in detecting Cu concentration and distribution in soil.

MATERIALS AND METHODS – *Site description and soil sampling.* – The studied soil, located in Pellengo di Bibiana (Torino, Italy, N: 4962501; E: 365760, 406 m a.s.l.), is a *Dystric Eutrudept*, coarse-loamy over sandy-skeletal soil, pH 5, CEC 9,8 $cmol_+ kg^{-1}$, total organic C 2%. In October 2007 four soil samples were collected at the depth 5-20 cm in an homogeneous 1 ha field, where annual crops showed pain symptoms (yellowing, necrosis, reduced or absent growth). Further details are reported in MARTIN *et al.* (2003).

Soil samples were sieved (<2 mm) and stored at 4°C prior to analyses. Samples were dried in an oven at 105°C to constant weight to obtain the water content. All reported values are based on the dried weight of the samples.

Soil total Cu content. – The modified aqua regia digestion method followed the protocol suggested by Italian legislation (D.M.13 September 1999, G.U. n. 248, 21 October 1999). 1,5 ml H_2O_2, 4,5 ml HCl and 1,5 ml HNO_3 were added to 0,5 g dry soil. Hydrogen peroxide was used to enhance organic matter destruction. A blank with the same aliquots of reagents was also prepared.

The sulfuric-nitric digestion was performed by adding to 0,5 g of soil a mixture of 5 ml H_2SO_4 + 5 ml HNO_3 (Carlo Erba reagents). After digestion each aliquot was quantitatively transferred to a volumetric flask and diluted with milliQ water to 100 ml. A blank with the same aliquots of reagents was prepared.

Total Cu concentration in the extracts and blanks was measured on a Flame Atomic Absorption spectrometry (Perkin Elmer 4000, AA flame, detection limit 20 $\mu g\ l^{-1}$).

With both methods soil digestion was performed on dry (at 100°C for 24 hours) and moist samples (at 15% moisture content), in order to detect the impact of soil moisture in Cu determination.

Chemical fractionation. – The sequential procedure reported by ALVA *et al.* (2000) was used to fractionate total Cu into exchangeable, water extractable (sorbed), organically bound, and precipitate forms. Exchangeable and sorbed Cu represent the readily soluble forms, which are the most phytotoxic. Precipitate Cu includes those forms more strongly retained by soil 2 g soil sample in three replicates were weighed into 50-mL centrifuge tubes, and 25 ml each of following reagents were sequentially added and shaken for the time specified for each extractant: 0,5M KNO_3 for 16 h (exchangeable); three extractions in deionized water for 2 h each (sorbed); 0,5M NaOH for 16 h (organically bound); 0,05 M Na_2EDTA for 6 h (precipitate). At the end of each extraction period, the soil suspension was centrifuged for 15 min and filtered through Whatman n. 42 filter paper. The concentration of Cu was measured in each filtrate using Atomic Absorption spectrometry (Perkin Elmer 4000, AA flame, detection limit 20 µg l^{-1}). Residual Cu was calculated by the difference between total Cu and the sum of other forms.

The procedure modified from TESSIER *et al.* (1979) involves the sequential extraction of 2,0 g of soil samples (in dry weight) with 25 ml deionized water (30 min shaking, 120 rpm) for water soluble fraction, with 8 ml 0,5 M $MgCl_2$ (30 min shaking, 120 rpm) for exchangeable fraction, with 20 ml 0.04 M NH_2OH x HCl in 25 % HOAc pH 2 (6 h shaking, 120 rpm) for Fe and Mn oxides bound fraction, and with 3 ml 0,02 M HNO_3 and H_2O_2 at 30 % (2 h shaking, 120 rpm) plus 5 ml 3,2 M NH_4OAc in 20% (v/v) HNO_3 after cooling for organic matter bound fraction. At the end of each extraction period, the soil suspension was centrifuged for 15 min and filtered through Whatman n. 42 filter paper. The concentration of Cu was measured in each filtrate using Atomic Absorption spectrometry (Perkin Elmer 4000, AA flame, detection limit 20 µg l^{-1}). Residual Cu was calculated by the difference between total Cu and the sum of other forms.

Physical fractionatio. – For physical fractionation, soil samples were dispersed by low-energy sonication and the particle size fractions were separated by a combination of wet sieving and centrifugation, as described by STEMMER *et al.* (1998). 25 g soil was dispersed in 80 ml of cooled distilled water by a probe-type ultrasonic disaggregator (50 J s^{-1} for 3 min). The coarse and medium particle size fraction (>250 µm) and the fine particle size fraction (250–63 µm) were separated by manual wet sieving with maximum 700 ml of cooled distilled water. During the sieving process, the slurry was gently stirred to achieve complete disruption of the macro-aggregates. Silt-sized particles (63-2 µm) were separated from the clay fraction (<2 µm) by four centrifugation steps at 270 g for 2 min and at 15°C. Between each centrifugation the pellets were re-suspended in water and centrifuged again to purify the silt fraction. The combined supernatants were centrifuged at 5500 g to obtain clay-sized particles (2-0,1 µm).

On these fractions Cu was determined with H_2SO_4+HNO_3 mixture, as described for total Cu.

Statistical analysis. – All determinations were carried out in triplicate. Data were tested for normality with the Shapiro-Wilk statistics. Significance of the differences between mean values was calculated by the univariate ANOVA. Fisher's LSD Post Hoc test was performed to evaluate the differences between Cu fractions obtained with the different procedures. Statistical analyses were performed using the Statistica 6,0 software package (Statsoft, Tulsa, USA).

RESULTS – *Soil total Cu content.* – The total Cu content determined with H_2SO_4 + HNO_3 and aqua regia methods did not show significant differences (Figure 1). Cu concentration assessed on average around 478 ppm. Soil moisture content negatively affected Cu detection (Figure 1). On average a 21% lower Cu concentration was found on moist samples.

Chemical fractionation. – The two chemical fractionation procedures showed different results (Figure 2). The procedure by ALVA *et al.* (2000) showed the largest concentration of Cu into organically bound form (241 ppm), whereas the sum of sorbed and KNO_3 extractable Cu (readily soluble) accounted for 19 ppm. As relative percentage, the 52% of Cu was organically bound, 19% was precipitated and the 4% was readily soluble. The residual Cu was the 25% of the total. The procedure modified from TESSIER *et al.* (1979) showed the largest concentration of Cu in the fraction bound to Fe and Mn oxides (53%), whereas the fraction bound to organic matter accounted for 27% only. The readily available fraction was lower than 32 ppm (2,6% and 4,2% of the total respectively for the water soluble and the $MgCl_2$ extractable Cu). The residual fraction accounted 14% of the total.

Physical fractionation. – Considering the relative distribution of Cu in particle size fractions, more than 92% was found in fractions

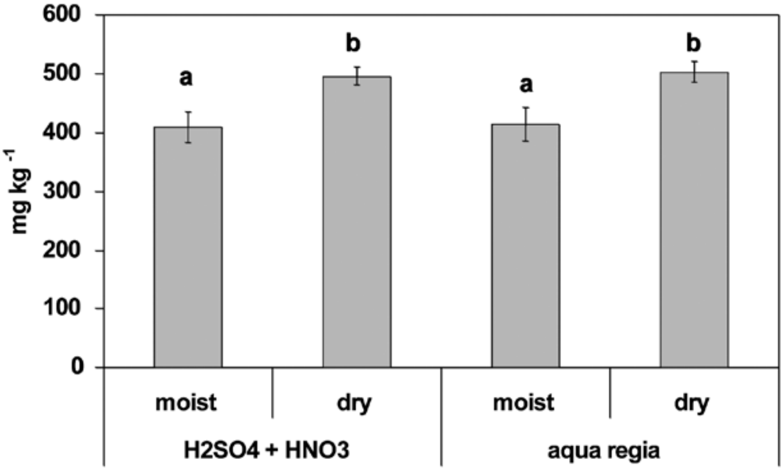

Fig. 1. – Total Cu determined with sulphur-nitric (H_2SO_4 + HNO_3) and aqua regia methods. Error bars represents standard errors. Different letters indicate significant differences in the concentration of Cu (Fisher LSD Post Hoc test).

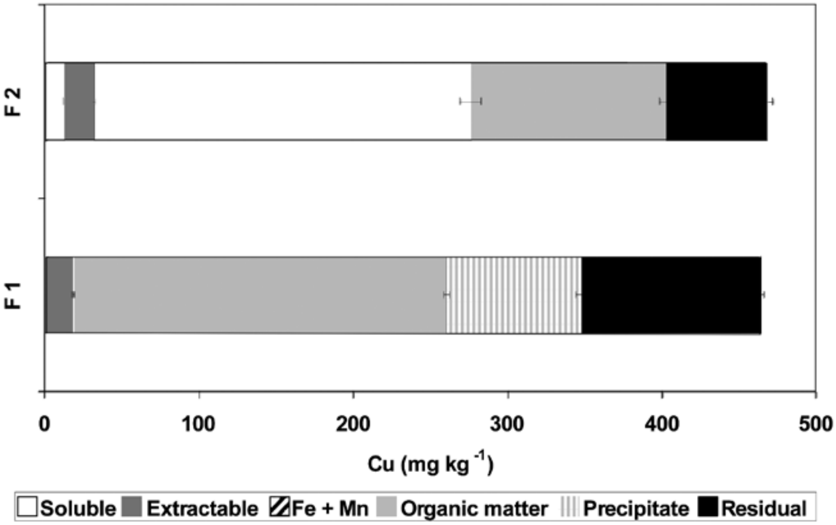

Fig. 2. – Concentrations of Cu in the chemical forms obtained with the two fractionation procedures (F1 – from Alva et al., 2000; F2 – modified from Tessier et al., 1979). Error bars represent standard errors.

smaller than 63 μm (Table I). Absolute values of Cu were similar in the silt-sized and clay-sized fractions, which accounted for 220 ppm of Cu each. The remaining 33 ppm of Cu were found in the sand-sized fractions (Figure 3).

DISCUSSION. – After 24 years from land use change from vineyard to herbaceous plants, the Cu concentration in soil was still high, also in comparison with other polluted soil (ARIAS *et al.*, 1998; ALVA *et al.*, 2000; SABIENË and BRAZAUSKIENË, 2004; PROBST *et al.*, 2008). The large Cu concentration in soil was the result of several years of heavy metal additions in small doses through pesticides and fertilizers during

TABLE 1. – *Percentage distribution of particle size fractions and Cu in soil fractions. Standard errors are reported in parenthesis.*

	Percentage distribution			
	Coarse Sand	**Fine Sand**	**Silt**	**Clay**
	250-2000 μm	63-250 μm	2-63 μm	0,1-2 μm
Particle size	14,7 (0,00)	23,0 (0,01)	48,9 (0,02)	13,4 (0,03)
Cu	1,5 (0,1)	5,4 (0,2)	46,7 (0,5)	46,4 (0,2)

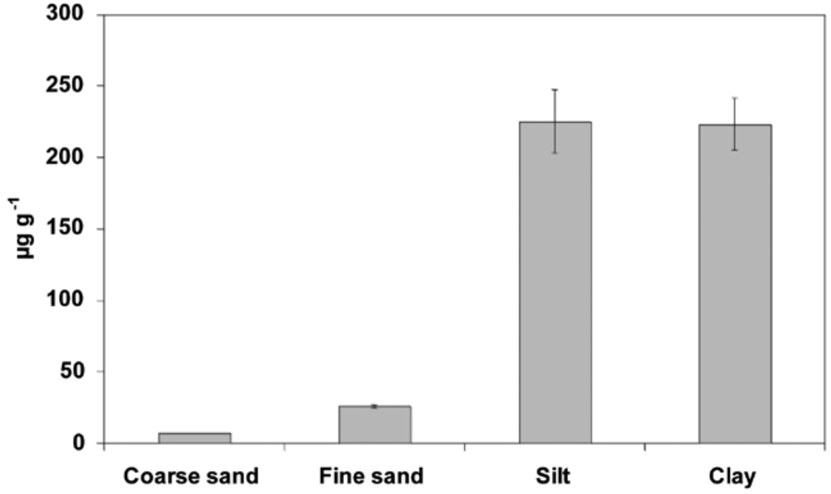

Fig. 3. – Concentration of total Cu into particle size soil fractions. Coarse sand: 250-2000 µm; fine sand: 63-250 µm; silt: 2-63 µm; clay: 0.1-2 µm. Error bars represents standard errors.

the vineyard cultivation. In fact, although the annual contribution of Cu is very small, its cumulative effects over several years can be substantial and persistent over years (ALVA *et al.*, 2000).

Since different acid digestion methods applied to soil samples can release a different amount of elements from this matrix, it is critical to compare different digestion methods to determine elemental mass fractions in soils. The Cu concentrations measured with $H_2SO_4 + HNO_3$ and with modified aqua regia methods were not significantly different, demonstrating the efficacy of both methods in Cu mineralization, for this soil. Most pollutant inputs are not silicate-bound, thus strong acid digestion, not involving dissolution of silicates by hydrofluoric acid, is adequate for this "*pseudototal*" analysis of Cu (SABIENĖ and BRAZAUSKIENĖ, 2004). Differently, the moisture content negatively affected Cu determination, with both methods. PETERSEN *et al.* (2004) found a similar effect in soil systems incubated under variable moisture contents, with the highest concentrations of Cu found in dry soil. The authors suggested that Cu retention in dry soils is much less pronounced than in soils with higher moisture contents. Hence, we suppose that an interference of soil water with digestion reactions occurred also in our study, with a resulting decrease of Cu detection. Moreover, it is reasonable to hypothesize that drying soil at 100°C might induce a modification in Cu association in soil which makes Cu more easily extractable from dry soil samples.

The results of the two chemical fractionation procedures were strongly different, mainly considering the soluble fraction and that bound to SOM. The procedure by ALVA *et al.* (2000) involves the extraction in H_2O after that in KNO_3, which removed the greatest part of Cu soluble in water. On the other hand our procedure, modified from TESSIER *et al.* (1979), involves the use of Mg^{2+} for the extractable fraction which, being bivalent, has a greater cation exchange capacity than K^+, in addition to the complexing ability of chloride. The readily available Cu extracted in $H_2O + MgCl_2$ was therefore much greater than that obtained with $KNO_3 + H_2O$. Also the fraction of Cu bound to SOM differed strongly between the two procedures. This is because the procedure by ALVA *et al.* (2000) involves an alkaline extraction immediately after the extraction of the readily soluble Cu, whereas TESSIER (1979) modified procedure involves an acid extraction only after that of the fraction bound to Fe and Mn oxides. We suppose that the different extractant capacity, as well as the shift in the sequential steps, might have caused the observed differences between these results. BHATTACHARYYA *et al.* (2008) also found Cu to be mostly associated with amorphous Fe and Mn minerals. Excluding this fraction, several works reported a large concentration of Cu in forms associated to the SOM (SAHA *et al.*, 1991; ALVA *et al.*, 2000; YIN *et al.*, 2002). BRADL (2004) reported Mn oxide and SOM the most likely to bind Cu in a non-exchangeable form and absorption maxima among soil constituents decrease in the order Mn oxide > organic matter > Fe oxide > clay mineral. SOM can strongly impact the relative distribution of Cu in the different chemical forms and therefore its availability. In particular, SOM exhibits a large number and variety of functional groups and high CEC values, which results in enhanced heavy metal retention ability mostly by surface complexation, ion exchange, and surface precipitation (KALBITZ and WENNRICH, 1998; NEAL and SPOSITO, 1986). Likewise, LUND *et al.* (1981) through X-ray absorption spectroscopy demonstrated that Cu form inner-sphere complexes with soil humic acid. In the case of non specific absorption (ion exchange) Cu bound to SOM might be considered as potentially bio available, whereas specific absorption (including chemisorbed inner-sphere complexes) might be an effective mechanism of Cu retention in soil.

From physical fractionation results, Cu resulted to be associated with silt and clay, suggesting stronger interactions with these small fractions. Clays are known for their ability to effectively remove heavy metals by specific adsorption and cation exchange as well as metal oxy-hydroxides (CRAWFORD *et al.*, 1993). Sorption isotherms indicate preferential adsorption of Cu onto SOM associated with the clay fraction of the soil (WU *et al.*, 1999). In addition, it is known that SOM associated with clay is more stable than that associated with larger fractions, due to SOM chemical characteristics, the increase in spatial inaccessibility and the adsorption of organic compounds on mineral surfaces (VON LÜTZOW *et al.*, 2007).

We can therefore hypothesize: I) the stability of Cu associated to organic matter-clay complexes, which can significantly contribute to lower concentration of the heavy metal in a readily soluble form and; II) the contribution of Fe and Mn oxides, as well as humified SOM, in stabilizing Cu in less available forms.

Cu in soil solution exists primarily in a form complexed with soluble organic matter (BRADL, 2004) and Cu non specifically adsorbed to SOM can be potentially available to soil solution through cation exchange mechanisms. In our study, the percentage of Cu in readily soluble forms varied between 4 and 6%, depending on the extractant. Several studies have shown a very low content of Cu in readily soluble forms (MCLAREN and CRAWFORD, 1973; HICKEY and KITTRICK, 1984; SAHA *et al.*, 1991; BERTI and JACOBS, 1996). However, this form of Cu is reported to be highly phytotoxic and negatively correlated with plants growing parameters, even at concentrations lower than 2,5 mg kg^{-1} (ALVA *et al.*, 2000). Pain symptoms shown by herbaceous crops could therefore be attributable to the presence of available Cu forms, still phytotoxic after more than 20 years from land use change.

In conclusion, the relative distribution of different chemical forms of Cu appears to depend on the fractionation procedure employed. In fact, depending on the type of extractant and the step sequence, different amounts of Cu were found to be associated with SOM or readily soluble Cu. Anyway, besides the intrinsic limitations of the sequential extractions, the largest part of Cu in soil bound with Fe and Mn oxides, humified SOM and clay-SOM complexes, which have potentially a high retention capacity for heavy metals in soil. At the same time, the persistence of pain symptoms in crops after more than 20 years from land use change suggests a release of Cu from less available to phytotoxic forms, still in the long term.

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ABSTRACT. – In a former vineyard soil, copper (Cu) concentration was detected in different soil chemical and physical fractions, with the aim: I) to identify soil fractions more important in Cu retention; II) to reveal if pain symptoms shown by herbaceous crops were attributable to phytoxicity of available Cu forms; III) to compare the effectiveness of different methodological approaches in detecting Cu concentration and distribution. A comparison of aqua regia and H_2SO_4 + HNO_3 mixture as soil digestion procedures, in moist and dry soil samples, was performed. Both methods were valid in Cu determination, giving comparable results for this soil. Differently, a 21% lower Cu

concentration was found in moist samples with both methods. Two sequential procedures were used to chemically fractionate total Cu. The first one separated exchangeable, sorbed, organically bound, and precipitated forms. The second one separated water soluble, exchangeable, Fe and Mn and organic matter bound fractions. Despite the intrinsic differences of the sequential extractions, Cu concentrated mostly in the Fe and Mn and organically bound forms. Cu distribution in particle-size fractions revealed a high concentration of this heavy metal in silt and clay. From the results obtained we confirm the essential role of soil organic matter (SOM) in decreasing Cu toxicity, in particular through the adsorption of the metal on humified SOM and clay-SOM complexes. Pain symptoms shown by herbaceous crops could be attributable to the presence of available Cu forms, still phytotoxic after more than 20 years from land use change.