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# Copper toxicity in soils under established vineyards in Europe: A survey

Stefan Ruyters [a,*], Peter Salaets [a], Koen Oorts [b], Erik Smolders [a]

[a] Department of Earth and Environmental Sciences, Division of Soil and Water Management, KU Leuven, Kasteelpark Arenberg 20, 3001 Heverlee, Belgium
[b] Arche (Assessing Risk of Chemicals), Stapelplein 70, Box 104, 9000 Ghent, Belgium

## HIGHLIGHTS

- Survey of Cu contamination and its effects on plants, microbes and invertebrates
- Increased copper concentrations are observed in established vineyard soils.
- Pronounced copper toxicity is not observed in vineyard soils.

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## ABSTRACT

Copper (Cu) containing fungicides have been used for more than one century in Europe on agricultural soils, such as vineyard soils. Total Cu concentrations in such soils can exceed toxicological limits that are commonly derived using artificially spiked soils. This study surveyed Cu toxicity in vineyard soils with reference to soils spiked with $CuCl_2$. Soil was collected in six established European vineyards. At each site, samples representing a Cu concentration gradient were collected. A control (uncontaminated) soil sampled nearby the vineyard was spiked with $CuCl_2$. Toxicity was tested using standard ecotoxicity tests: two plant assays (*Lycopersicon esculentum* Miller (tomato) and *Hordeum vulgare* L. (barley) growth), one microbial assay (nitrification) and one invertebrate assay (*Enchytraeus albidus* reproduction). Maximal total Cu concentrations in the vineyard sites ranged 435–690 mg Cu $kg^{-1}$, well above the local background (23–105 mg Cu $kg^{-1}$). Toxicity in spiked soils (50% inhibition) was observed at added soil Cu concentrations from 190 to 1039 mg Cu $kg^{-1}$ (mean 540 mg Cu $kg^{-1}$) depending on the assay and the site. In contrast, significant adverse effects were only found for three bioassays in vineyard samples of one site and for two bioassays in another site. Biological responses in these cases were more importantly explained by other soil properties than soil Cu. Overall, no Cu toxicity to plants, microbial processes and invertebrates was observed in vineyard soil samples at Cu concentrations well above European Union limits protecting the soil ecosystem.



## 1. Introduction

Since about 1850, Cu containing fungicides have been used to protect crops from fungal infections such as downy mildew (*Plasmopara viticola*). Intensive and long term use of these fungicides has increased soil Cu concentrations and this is likely most pronounced in vineyards (Komarek et al., 2010). Currently, EC regulation 473/2002 (European Commission, 2002) restricts the annual dose of applied Cu to 6 kg Cu $ha^{-1}$, which corresponds to an annual accumulation of about 5 mg Cu $kg^{-1}$ soil in the top 10 cm assuming no losses. Such a sustained application since >150 years would have increased soil Cu concentrations to 750 mg Cu $kg^{-1}$ in unplowed vineyard soils, whereas plowing the first 30 cm after removing old vines (every 30–50 years) would yield a topsoil Cu concentrations of about 250 mg Cu $kg^{-1}$. Measured Cu concentrations in vineyard soils range from 77 up to 3200 mg Cu $kg^{-1}$ (Komarek et al., 2010; Wightwick et al., 2008; Mirlean et al., 2007) and are above legislative limits affecting the sustainability and potentially the productivity of these agroecosystems (Komarek et al., 2010). For example, the predicted no effect concentration (PNEC) of Cu in soils in the EU, estimated in the EU Risk Assessment on Cu ranges from 20 to 200 mg Cu $kg^{-1}$ depending on soil properties (Smolders et al., 2009; SCHER, 2009) showing that Cu concentrations are above the European legislative limits in many European vineyards. Copper toxicity to vines is unlikely since Cu is mainly present in the top layer, well above the root system of vines (Provenzano et al., 2010). Roots of vines are generally found up to 6 m deep, however, the root length density is generally largest at a depth of 0.4–0.8 m (Lehnart et al., 2008). However, soil Cu might become toxic to other agricultural crops when land use changes. Michaud et al. (2007) reported increased Cu uptake by durum wheat cultivated in former vineyard soils contaminated with up to 1000 mg Cu $kg^{-1}$ and chlorosis of plants growing on these soils was visually observed. These symptoms were more pronounced in calcareous soils than in acid soils, likely due to Cu induced Fe deficiency (Michaud et al., 2007). Brun et al. (2001) and Chaignon and

* Corresponding author. Tel.: +32 15 30 55 93; fax: +32 15 30 55 99.
*E-mail address:* stefan.ruyters@lessius.eu (S. Ruyters).

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Hinsinger (2003) showed increased Cu concentrations in roots of crops grown on vineyard soils containing up to 400 mg Cu $kg^{-1}$, whereas no increase was observed in the shoots. To our knowledge, crop yield decline in arable land on a former vineyard has not yet been reported. Toxic effects of Cu on the microbial communities in vineyard soils have been observed. Enzyme activities in soil were affected at and above total concentrations of 150–200 mg Cu $kg^{-1}$ soil (Fernandez-Calvino et al., 2010) and nitrification was impaired in soils contaminated up to 380 mg Cu $kg^{-1}$ (Baroux, 1972). Evidence for increased Cu tolerance of the microbial community in response to the Cu contamination has also been reported in such soils (Diaz-Ravina et al., 2007).

Ecological thresholds of Cu in soil are based on laboratory studies using soils artificially spiked with increasing Cu concentrations. The PNEC value referred to above was derived with data of soils freshly spiked with $Cu^{2+}$ salts and finally corrected by a so-called leaching/aging (L/A) factor. That factor refers to the larger total Cu required to elicit equal effects in aged soils as in freshly spiked soils and was set as 2 for Cu. This factor was based on numerous comparisons between field contaminated, aged spiked soils and freshly spiked soils. It is unclear if the same factor also applies to the vineyard soils. First, aging times in such soils can now be >150 years whereas the factor 2 was derived based on aging times of 1.5 years (and only one soil aged for >70 years). Second, it is possible that the largest Cu concentrations are found in vineyards where soil organic matter content is largest (Delas, 1967), given that most Cu enters the soil attached to or incorporated in the organic matrix. Increasing soil organic matter content in soil may offset total Cu toxicity, as is well known from studies in sewage sludge amended soils (Smolders et al., 2012).

The objective of this study is to survey Cu toxicity to plants, microbial processes and invertebrates using standardized bioassays and to compare Cu toxicity in Cu contaminated vineyard sites with the Cu toxicity in corresponding freshly spiked soils. We hypothesize that differences in Cu toxicity between the vineyard soils and spiked soils are more pronounced than tested before in the EU Risk Assessment of Cu in soil (SCHER, 2009).

## 2. Materials and methods

### 2.1. Soil sampling and soil properties

Several vineyards where Cu containing fungicides have been used or are still used were sampled in Europe in January–March 2011 and samples of six of these were selected where soil Cu concentrations were most pronounced (site 1 in France, sites 3, 4 and 5 in Germany and sites 2 and 6 in Italy). At each site, four to six soil samples with increasing Cu concentrations were collected in the vineyard and a control soil with low Cu concentration (<100 mg $kg^{-1}$) was collected outside the vineyard. A portable XRF (Omega XPD 4300, VM Vision International) was used to identify the location with the largest soil total Cu concentration and these were generally found in vineyards with old vines (>30 years). Soils were collected with a spade from the top 10 cm as the concentration strongly decreased with increasing depth. Soils were stored air-dried after sieving (<4 mm) prior to toxicity testing.

A soil subsample was dried at 105 °C and ground before determining soil properties. Total Cu concentrations in soil (mg Cu $kg^{-1}$ dry soil) were determined in duplicate by boiling aqua regia extraction (0.2 g soil in 2 ml aqua regia, 2 h boiling at 140 °C; subsequent addition of 3 ml HCl 3 M and >1 h boiling at 140 °C) and analyzed with inductively coupled plasma-optical emission spectroscopy (ICP-OES, Perkin Elmer Optima 3300 DV). Oorts (2012) showed that aqua regia Cu is typically less than 10% different from total Cu content determined with for example XRF, which makes aqua regia Cu a good estimate of total Cu. The soil pH was measured in a 0.01 M $CaCl_2$ extract (1/5 solid/liquid ratio) (Smolders et al., 2012). The effective CEC (eCEC, $cmol_c\,kg^{-1}$) was determined using the unbuffered AgTU method (Chhabra et al., 1975) and silver concentrations in the extracts were measured with ICP-OES. Total organic carbon and total nitrogen was measured using the oxidative digestion method (C/N analyzer EA1110). Particle size distribution (texture) of the control and largest contaminated vineyard soil sample was determined according to ISO, 11277 guidelines (1998). Water content of the control soils at pF 2 (100 cm suction) was determined by the sandbox method using 100 $cm^3$ soil cores. After 1 week saturation, samples were allowed to equilibrate for 14 days at pF 2.0. All data are reported in Table 1 and Supplementary information Table 1.

### 2.2. Soil spiking and incubation of spiked and vineyard soil samples

The uncontaminated (control) soil samples were spiked with $CuCl_2$ (50 g Cu $l^{-1}$) at 5 doses above control (100, 200, 400, 800, 1600 mg Cu $kg^{-1}$) 1 week before the start of the toxicity tests creating the Cu spiked soils. Dose confirmation of spiking was performed by boiling aqua regia digestion (in duplicate) as described above. The water content of the spiked samples and that of vineyard samples was adjusted to maximally 70% of pF 2. All soil samples were manually mixed with a spoon after amendments and incubated for 7 days at 20 °C prior to toxicity testing. The toxicity test of spiked and vineyard soil samples was always performed simultaneously for each soil sample of the corresponding site to exclude variance beyond difference in Cu contamination history. Differences in soil pH within samples of the same site were adjusted by adding $Ca(OH)_2$ simultaneously with soil wetting for soil samples of sites 1 and 4. This resulted in a pH of 6.2 in the soil sample of site 1 with 201 mg Cu $kg^{-1}$. For site 4, pH of the control soil and all spiked soils only increased with 0.2 units to 5.9, whereas the pH is 6.7 on average in the other samples of that site.

### 2.3. Plant toxicity tests

Fertilizers were added to the soil samples at a rate of 50 mg P $kg^{-1}$ soil as $KH_2PO_4$ and 100 mg N $kg^{-1}$ soil as $KNO_3$ 1 week before the start of the test, i.e. simultaneously with Cu spiking for the spiked soils and simultaneously with soil wetting for the vineyard soil samples. Tests were performed in a growth chamber with the following growing conditions: 16 h/8 h cycle day/night, 20 °C during light hours and 16 °C during night time and 70% humidity. Water loss of the pots was restored daily with deionized water. The barley root elongation assay (BRE) is based on ISO 11269-1 (2005). The endpoint of this assay is the length of the plant roots after 4 days incubation in soil. Pre-germinated summer barley (*Hordeum vulgare* L.) seeds with radicle smaller than 2 mm in length were used for this assay. For each soil sample (control, spiked and vineyard) three replicates were included (90 g fresh soil per replicate). Per pot (diameter 26 mm, depth 105 mm) 3 seeds were planted, the soil surface was covered with polyethylene beads to reduce evaporation and the pots were placed in a growth chamber. After 4 days of growth, intact roots were washed out of the soil matrix and the length of the longest root on each plant was recorded using a calibrated paper resulting in 3 root length values per pot. The average root length of each pot was calculated resulting in 3 replicates per soil treatment. The plant growth assay is based on ISO 11269-2 (2005). Two endpoints are recommended in this guideline, i.e. the number of seedlings that emerge per pot and the shoot yield of the plants. In this study the number of seedlings that emerge per pot is the validity criterion of this assay (at least 70% in the control soils, i.e. 14 plants/pot). The endpoint for toxicity testing is the dry shoot yield (g) of the plants. Tomato (*Lycopersicon esculentum* Miller) was selected for the test. For each soil sample (control, spiked and vineyard) four replicates were included (550 g fresh soil per replicate). Twenty uniform, undressed seeds were sown in each plastic pot (top internal diameter 85 mm, depth 92 mm), the soil surface was covered with polyethylene beads to