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# **Copper Accumulation in Vineyard Soils: Distribution, Fractionation and Bioavailability Assessment**

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Additional information is available at the end of the chapter

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## **1. Introduction**

Metals are chemically very reactive in the environment, which results in their mobility and bioavailability to living organisms. Metals can be present in all environmental compartments as different species, with the TMs associated with different ligands, but never being irreversibly transformed or metabolized, and in those meaning metals are different from organic compounds. People can be exposed to high levels of toxic metal by breathing air, drinking water, or eating food that contains it. As a consequence, metals get into the human body by different routes - by inhaling, over skin, and ingestion of contaminated food. The issue of toxicity is usually merely a matter of quantity, with the range varying for each element.

### **1.1. Why we need to study trace metals in soils?**

Soil is an important compartment of the environment in which anthropogenic loading of trace metals puts ecosystems and their inhabitants at a health risk. Repeated use of metal-enriched chemicals, fertilizers, and organic amendments such as sewage sludge as well as wastewater may cause contamination at a large scale. So far, it is believed that most soils in Europe have not been significantly enriched in trace metals by anthropogenic activity. This is changing as livestock production expands, fertilizer application increases, and biosolids and effluent applications to agricultural soils become more common. Accumulation of trace metals in soil has potential to restrain the soil functions, cause toxicity to plants, and enter the food chain.

Many chemical reactions are responsible for the behaviour of TMs in soils, but the most important processes that control their bioavailability and mobility are precipitation-dissolution, adsorption-desorption, and complexation. The ability of soils to adsorb metal ions from aqueous solution is of special interest and has consequences for both agricultural issues such as soil fertility and environmental questions such as remediation of polluted soils and waste deposition [1].

Metal-soil interaction is such that when metals are introduced at the soil surface, their mobilisation does not occur to any great extent unless the metal retention capacity of the soil is overloaded, or metal interaction with the associated waste matrix enhances mobility [2]. The most important interfaces involved in TMs transformation in soils are mineral groups commonly found in soil: aluminosilicates, oxides and organic matter. Through their surface electrochemical properties, these soil minerals control adsorption, transformation, and release behaviour of chemical constituents (e.g. nutrients and contaminants) to water and soil solution [3]. Furthermore, soil-surface electrochemical properties vary between soil types and depend on factors such as parent material, climate, and vegetation.

So that, full understanding and prediction of chemical behaviour of an element in the terrestrial environment is possible only by identification of all forms in which that element can be found in soil under different environmental conditions. Copper is one of the major toxic metals, and a highly reactive one, as well. Elevated levels of Cu in agricultural soils result from the use of Cu-containing compounds to control plant diseases and from application of manure or sewage sludge. These applications may lead to gradual accumulation of Cu in the soil and thereby increase Cu toxicity toward crop and beneficial microorganisms. In this article, the actual risk of high concentrations of copper and its mobility in vineyard soils is reviewed considering sources, chemical processes in soil and biogeochemical behaviour of copper as well as impact on agroecosystem and environment in general.

## 1.2. Sources and behaviour of copper in soils

Copper occurs in the Earth's crust at concentrations between 25-75 mg kg<sup>-1</sup>, with the abundance pattern that shows the tendency for the concentration in mafic igneous rocks (60-120 mg kg<sup>-1</sup>) and argillaceous sediments (40-60 mg kg<sup>-1</sup>), but it is rather excluded from the carbonate rocks (2-10 mg kg<sup>-1</sup>) [4]. Values for soil contents generally range worldwide from 1 to 140 mg kg<sup>-1</sup> depending on the nature of the soil parent material.

In soil solids and solution copper occurs almost exclusively as the divalent cation Cu<sup>2+</sup>, and the reduction of Cu<sup>2+</sup> to Cu<sup>+</sup> and Cu<sup>0</sup> is possible under reducing conditions. As a chalcophile, copper associates with sulfide in the very soluble minerals, Cu<sub>2</sub>S and CuS. Being very reactive in soil, copper is found in all matrix components. Most of the colloidal soil material (clay minerals, oxides of Mn, Al i Fe, and organic matter) adsorb copper strongly, and increasingly so as the pH is raised [5]. For copper, specific adsorption, which is not significant for the most of metal ions, seems to play a more important than nonspecific adsorption. Amorphous and crystalline oxides of Fe and Al easily adsorb Cu<sup>2+</sup>, regardless the excess of alkali metals in the solution [6]. However, the most important sink for Cu is soil organic matter, and its complexation with organic matter is one of the most efficient mechanisms of Cu<sup>2+</sup>

retention in soil [7]. This restricts Cu bioavailability, but also considerably reduces the risks of phytotoxicity of the accumulated anthropogenic input and its vertical migration. Organically complexed  $\text{Cu}^{2+}$  is bound more tightly than any other divalent transition metal and of low lability these complexes results in limiting copper bioavailability. This prevents copper mobility and its transport through soil to underground, and reduces substantially the risk of the groundwater contamination.

### 1.3. Anthropogenic inputs and copper contamination of cultivated soils

Trace elements in general enter an agroecosystem through both natural and anthropogenic processes. The latest includes TEs inputs through use of agrochemicals, farm manure, biosolids and composts, industrial and municipal waste, irrigation, and wet and/or dry deposits. Being widely used, copper is a common metal pollutant released to environment as a result of man's activities. Copper is an essential nutrient, but in excess in soils it becomes toxic to plants and some micro-organisms, disrupting nutrient-cycling and inhibiting the mineralisation of essential nutrients such as nitrogen and phosphorus. Some species accumulate copper. Toxic effects on fish and other aquatic organisms have also been observed. For humans, excess amount of this trace metal can have serious health effects.

Elevated levels of copper in agricultural soils result from the use of Cu-containing compounds to control plant diseases and from application of manure or sewage sludge. Increased concentration of Cu in soils under long-term production of grapevine, citrus and other fruit crops have been recorded in numerous studies. The Bordeaux mixture, an efficient agent for prevention of vine «Downy Mildew», *Plasmopara viticola*, has been routinely used in Europe since the end of the 19th century with its concentrations and the number of treatments depending on weather conditions, infection intensity and vineyard management. The century-old practice of using Cu-sulphates and other copper containing fungicides to protect grapevine, but also other agricultural crops, in temperate and tropic climatic regions, resulted in significant Cu accumulation in soils [8]. Most of the copper accumulated in leaves and soil by spraying will be retained in topsoil through the biological cycle and tillage [9-11]. Comparison of copper contents of 110 to 1500 mg kg<sup>-1</sup> with its usual content in agricultural soils (20 – 30 mg kg<sup>-1</sup>) points to their connection with such practice [12]. Copper can be either a micro nutrient or a toxic element which depends on the copper concentration. Determination of the total content of metals in soils is an important step in estimating the hazards to the vital roles of soil in the ecosystem, and also in comparison with the quality standards in terms of the effects of pollution and sustainability of the system.

From the ecotoxicological point of view, it is equally important to determine the bioavailability of Cu accumulated in vineyards, i.e., the fraction of the total metal content in soil that can be utilized by biota [13]. It depends on the soils properties, temperature, water content and aeration, and also on plant species. The toxicity of copper is essentially observed in acid soils, but not in calcareous soils and for copper contents as high as those reported in vineyard soils. A number of authors have found positive correlation between copper retention and pH [14, 15] and sum of bases or exchangeable calcium [16]. The bioavailability of copper has also been reported to decrease when the cation exchange capacity or the level of organic

matter increases [5, 15]. Various electrolytes such as water, buffered or unbuffered salt solutions, chelating agents, diluted acids or a mix of these reagents can be used to estimate the bioavailability of copper in soils (reviews given by [17, 18]).

#### 1.4. Fractionation and bioavailability of copper in soils

The concepts of «bio-availability» and «bio-accessibility» were introduced to express whether the actual concentration of a toxic element would have effects on organisms [19]. The main challenge that comes out from the assessment of loads of trace and toxic metals is the methodology of determination or prediction of the trace element content in a soil that results in toxicity [20]. Trace element mobility and bioavailability is determined by their transfer between the soil solid phase and the soil solution [21], and trace element in soils can be divided into inert and the potentially toxic labile fraction [22]. Thus, the impact of trace metals on soil and the surrounding environment in most cases cannot be predicted simply by measuring their total concentration. This is because only soluble and mobile fractions have the potential to leach or to be taken up by plants, and enter the food chain. Furthermore, Cu content in the plants usually does not well correspond to the total soil copper content [23].

Water-soluble and exchangeable copper fractions are considered to be bioavailable; copper complexed with oxide, carbonate and organic matter are potentially bioavailable fractions; and mineral fraction is considered to be non-bioavailable [24]. However, fractionation does not provide information about species of metals in soil. Metal speciation is one of the most important properties that determine the behavior and toxicity of metals in the environment. Chemical speciation of an element refers to its specific form characterized by a different isotopic composition, molecular structure, and electronic or oxidation state [25]. Speciation is the process of identification and determination of different chemical and physical forms of elements present in a sample [26]. TMs speciation is determined by their reactivity and physical and chemical properties of the soil. Metals are very reactive in the environment and can relatively easy change form in soil, making their speciation non stable. Among many chemical processes that are involved in the transformation of TEs in soils, precipitation-dissolution, adsorption-desorption and complexation are the most important in controlling their bioavailability and mobility. Metals that occur in cationic forms have a higher ability of binding to negatively charged soil colloids, and are thus less bioavailable, but more easily accumulate in soil, unlike the anionic forms that are mainly present in soil solution and are more bioavailable, but are more readily leached from the soil. Formation of complexes with soil organic matter, adsorption onto the surfaces of clays and Fe and Mn oxides regulate the behavior of copper in the soil [23]. Thus, copper bioavailability in soil depends on soil pH, redox potential, CEC, amount and nature of organic matter and soil minerals [27].

Chemical extractions are used for soil copper bioavailability predictions, often in comparison with its content in plants [23]. Copper is not readily mobile in plants and root concentration is considered to be a good indicator of the plant copper content. It has been assumed that the factors affecting metal fractionation and bioavailability in soil include root-induced pH changes, metal binding by root exudates, root-induced microbial activities and root depletion as a consequence of plant uptake [28]. In the root developing zone, rhizosphere,

processes that control the mobility, transformation and toxicity of metals in soil are under direct influence of plant roots and may differ from those in bulk soil. Thus, root activities can considerably modify TM speciation in the rhizosphere. Metal plant root adsorption is determined by ionization of negatively charged binding sites for metal on root surfaces. Cupric ions bind to a specific carrier on the root cell plasmalemma surface [29] and plant uptake depends on the available copper in the soil and the nutritional status of a plant [30]. Soil pH determines aqueous metal speciation, affecting metal sorption and desorption on the solid phase [31], but plant roots as well. As the degree of biotic ligands ionization increases with pH, the metal ion root adsorption increases. Vice versa, the adsorption capacity of the plant root for cupric ion decreases with decreasing solution pH. Rhizosphere pH modifications by plant are a known occurrence and root zone alkalization may decrease the exposure of plant roots to copper by promoting formation of organic complexes and reducing copper solubility throughout rhizosphere area [32]. Furthermore, plant may directly decrease copper bioavailability near roots by excretion of metal-binding compounds that can complex the free cupric ions [33].

In this article, the actual risk of high concentrations of copper and its mobility in vineyard soils is reviewed considering sources, chemical processes in soil and biogeochemical behaviour of copper as well as impact on agroecosystem and environment in general.

## **2. Case study: Spatial distribution of copper concentrations in vineyard soils of Croatia: Wine-growing subregion of Plesivica**

Elements inherited from the bedrock are partitioned within the soil through specific processes. Besides the parent material characteristics, geomorphology and landscape features contribute greatly to the variability of elements distribution. The topography is especially important, since it affects water infiltration and drainage on the one hand and soil erosion on the other. This variability is additionally enhanced in cultivated soils, especially in mountain regions and on sloping terrains where erosion processes are more expressed [34].

Steep southern slopes of the mountains in north-western Croatia have been used for centuries to grow vine and produce wine (Figure 1). Wine quality is almost always associated with the location, which means with the specific natural viticultural environment where soil is one of the major factors: firstly in terms of soil physical properties, and secondly in terms of soil inorganic chemistry [35, 36].

Although the systems of vineyard planting and maintenance in north-western Croatia have been changing with time and modern plantations prevail nowadays, parcels under traditional cultivation and old, almost forgotten, cultivars can still be found. Therefore, the aim of this study was to explore the distribution and retention pattern of copper concentrations in vineyard soil and, and to study the copper speciation and its distribution within five operationally defined fractions to assess its bioavailability and possible downward movement.



**Figure 1.** Grapevine plantation in the study region (Site Lokosin dol, Plesivica wine-producing subregion, Croatia, photo M. Romić)

## 2.1. Study area

Research was carried out on different wine-producing subregions in Croatia, in the wider Zagreb region: the wine-growing subregion of Plesivica (approximate coordinates: latitude,  $45^{\circ} 42'$  and longitude,  $15^{\circ} 37'$ ). The area of Žumberak, which is a mountainous territory located to the west of Zagreb, is bordered to the north and west by Slovenia, and to the south by Kupa River (Figure 3). Soils are developed on Pannonian sediments. These sediments consist of limy marls, sands, sandstones, conglomerates and breccias [37-39]. The consistency of those sediments, solid Triassic dolomite as bedrock and periodic streams led to fast erosion and filling in valleys and formation of amphitheater-shaped valleys. Namely, those landscape shapes were formed by the distinct climatic oscillations and pulsation between glacial and stadial periods, along with constant tectonic activity and elevation of terrain [39]. The landscape reflects the features of the Dinaric, so numerous formations characteristic of the karst are found on highly dissected limestone terrains. As the Zumberacka Mt. piedmont spreads perpendicularly to the mountains, some slopes are firmly interlinked by ridges, forming well protected, amphitheatre shaped, vineyard areas in the wine-growing subregion of Plesivica.

Anthropogenic vineyard soils, classified as Aric Anthrosols [40], have been developed on Tertiary sediments and Pleistocene loams. Owing to exceptional geomorphological and agro-ecological conditions, these locations have been occupied almost exclusively by vineyards for many decades.

The climate of the wider area is humid and the average annual rainfall is 836 mm. The mean annual temperature is 10.3 °C, ranging from -0.6 °C (January) to 20.4 °C (July).

To investigate the spatial variability of surface soils, 67 soil samples were taken at the nodes of a square grid at intervals of 1 km (Figure 3). The samples were defined as composite samples made up of 10 increments collected from the soil upper 10 cm in a cross pattern, with a 5 m distance between increments (Eijkelkamp soil sampling kit used). Site descriptions were registered at the time of sampling to record the sample location in relation to vineyard characteristics and major environmental features.

Three soil profiles were then located according to the landscape feature: profile 1 (Aric Anthrosols) was dug in the vineyard plot down to the parent material (80 cm depth) at the 258 m asl, profile 2 (Aric Anthrosols) was located in vineyard plot at 231 m asl, and profile 3 (Colluvic soil) was located in the meadow at the foot of the hill 200 m asl, with the altitude difference of 28 % between P1 and P2, and 13 % between P-2 and P-3. Table 1 reports the selected physical and analytical features of soil profiles, and Figure 2 shows a sequence of horizons.


Profile / Horizon	Depth cm	pH <sub>H2O</sub>	CaCO <sub>3</sub> %	Corg %	Exchangeable cations (cmol <sub>(+)</sub> kg <sup>-1</sup> )				CEC cmol <sub>(+)</sub> kg <sup>-1</sup>	Sand	Silt	Clay	
					Ca <sub>ex</sub>	K <sub>ex</sub>	Mg <sub>ex</sub>	Na <sub>ex</sub>					
P1	P	0 - 45	8.2	48.8	2.6	24.1	0.63	2.14	0.09	24.0	11	64	26
	C	45 - 100	8.5	52.1	0.9	15.5	0.16	1.32	0.03	13.2	14	65	21
P2	PI	0 - 35	8.2	47.7	3.1	25.9	0.72	2.22	1.02	26.1	11	63	27
	PII	35 - 90	8.4	47.3	1.8	26.9	0.31	2.30	0.11	26.6	8	59	33
	C	90 - 130	8.5	51.0	0.5								
P3	Aa	0 - 10	7.9	41.0	3.6	32.6	0.18	2.30	0.05	31.1	15	68	17
	Ap	10 - 30	8.2	46.2	2.4	29.1	0.16	1.64	0.03	28.6	13	64	23
	I	30 - 50	8.2	33.9	1.8	28.2	0.19	1.64	0.05	30.5	9	59	32
	II	50 - 80	8.3	34.2	0.8	25.2	0.17	1.64	0.09	26.6	9	56	35
	Cg	80 - 150	8.4	44.2		22.0	0.20	1.64	0.25	20.9	11	58	31

**Table 1.** Selected physical and chemical features of soil profiles

## 2.2. Chemical analysis

Soil samples were air-dried, sieved at 2-mm, and subjected to the following analyses: pH in a 1:5 soil/water ratio (MettlerToledo MPC 227 pH- meter), soil organic carbon (SOC) by sulfochromic oxidation [41], calcium carbonate (CaCO<sub>3</sub>) by the volumetric calcimeter method after HCl attack, and effective cation exchange capacity (CEC) using BaCl<sub>2</sub> solution. Particle





<b>Profile 1</b>	
Coordinates (m) (Gaus-Krueger projection)	y 5 063 845, x 5 551 878
Altitude	258 m
Slope	28.1 %
Land use	Vineyard, >60 years old
Soil type	Aric Anthrosol (FAO, 1990)
Horizons	Ap 0-45 cm, heavy silt loam, C 45-100 cm, silt loam
<b>Profile 2</b>	
Coordinates (m) (Gaus-Krueger projection)	y 5 063 758, x 5 551 884
Altitude	231 m
Slope	13.2 %
Land use	Vineyard, recently abandoned
Soil type	Aric Anthrosol (FAO, 1990)
Horizons	Ap I 0-40 cm, heavy silt loam, Ap II 40-90 cm, heavy silt loam, C 90-130 cm, silt loam
<b>Profile 3</b>	
Coordinates (m) (Gaus-Krueger projection)	y 5 063 523, x 5 551 872
Altitude	200 m
Slope	level
Land use	Meadow
Soil type	Calcaric Regosol (FAO, 1990)
Horizons	Aa 0-10 cm, silt loam, Ap 10-30 cm, heavy silt loam, A I 30-50 cm, heavy silt loam, A II 50-80 cm heavy silt loam, Cg 80-120 cm, heavy silt loam

**Figure 2.** Soil profiles description (Site Lokosin dol, Plesivica wine-producing subregion, Croatia, photo M. Romic)

size distribution was determined by the pipette method after disaggregation in sodium pyrophosphate (HRN ISO 11277:2004). Soil samples were also digested in aqua regia [42] with the microwave technique on a MARSXpress system (CEM).

Copper concentrations in soil digests were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) on a Vista MPX AX (Varian). All concentrations were calculated on the basis of dry weight of samples (105 °C, 24 h). Quality control procedure consisted of reagent blanks, duplicate samples and several referenced soil and sediment samples with similar matrix from the inter-laboratory calibration program [43]. Maximum allowable relative standard deviation between replicates was set to 10 %.

Two other methods were used for evaluating soil available copper: DTPA extraction [44] and calcium chloride extraction [45].

DTPA extraction: 10 g of soil were extracted with 20 ml DTPA 0.005 M + TEA 0.1 M + CaCl<sub>2</sub> 0.01 M for 2 h at 20 °C under stirring (Heidolph PROMAX 2200 used), prior to being filtered.

Calcium chloride extraction: 0.5 g of soil were extracted with 50 ml of CaCl<sub>2</sub> 0.01 M for 2 h at 20 °C under stirring, prior to being filtered.

### 2.3. Metal fractionation

The selective sequential dissolution procedure was employed to divide metals into five solid-phase fractions [46, 47]. Chemical reagents and the experimental conditions applied are

summarized in Table 2. In this procedure, 0.5 g of each sample was weighted into 50 ml polyethylene centrifuge tube and the extractions were carried out directly in the tubes, except in the last step where Teflon tubes for microwave digestion were used. At each extraction step, after shaking and equilibration, solid-liquid separation was achieved by centrifugation at 3500 rpm (2000 x g) for 10 min (centrifuge Sigma 3-15 used). The supernatant was filtered through S&S 583 filter paper and placed to an acid-washed polyethylene tube. The solid residue was washed three times successively with 5 ml of ethanol and the liquid was discarded leaving the residue soil for the next step. The supernatant obtained at each step was analysed for metals using inductively coupled plasma optical emission spectroscopy (ICP-OES) on a Vista MPX AX (Varian). Single element standards were prepared for each extraction in the same solution as the extracting agent to minimise matrix effects. Blanks were used for background correction and other sources of error.

Fraction	Abbreviation	Procedure
Exchangeable	EXCH (F1)	0.1 M Mg(NO <sub>3</sub> ) <sub>2</sub> pH=7, 10 ml, room temperature, shake for 2 h
Associated with carbonates	CARB (F2)	1M CH <sub>3</sub> COONa + CH <sub>3</sub> COOH (pH = 5.0), 10 ml, room temperature, shake for 6 h
Associated with Fe/Mn oxides	Fe/Mn OX (F3)	0.04 M NH <sub>2</sub> OH.HCl in 25 % HOAc, 25 ml, water-bath at 90° for 3 h
Associated with organic matter	ORG (F4)	0.1 M K <sub>4</sub> P <sub>2</sub> O <sub>7</sub> , 25 ml, shake for 24 h
Residual	RESID (F5)	4 M HNO <sub>3</sub> , 50 ml, microwave digestion

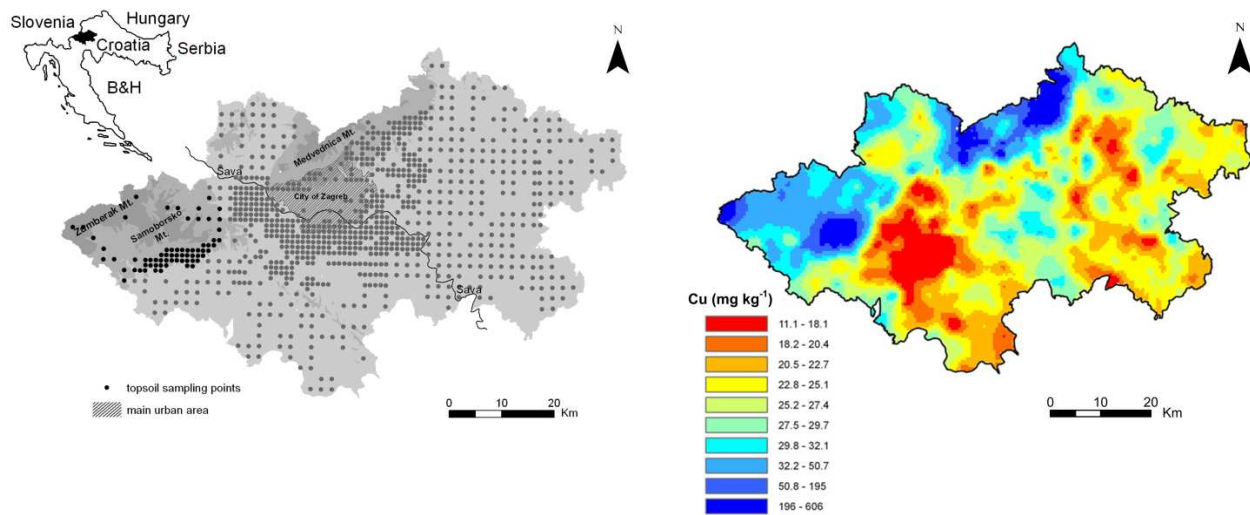
**Table 2.** The selective sequential dissolution procedure; chemical reagents and the experimental conditions.

## 2.4. Metal statistical analysis and data management

Linear regression: Relations between extractible copper in soils and soil properties were analyzed by simple and multiple linear regression [48]. General conditions for model application were verified after parameter determination. Thus, residuals should follow normal distribution, the assumption of homoscedasticity of variable variances should be proven, and they should be independent of one another. Distribution normality was tested using the Shapiro-Wilk test, while the other hypotheses were checked visually after the graphs were drawn. Details given by [49]. All soil data were incorporated into the GIS database.

## 3. Copper concentrations in surface vineyard soil

Total copper concentrations in vineyard soils ranged from 30 to 700 mg kg<sup>-1</sup>, while total copper in 88 % samples exceeded the maximum tolerant concentration under the Croatian regulation of 100 mg kg<sup>-1</sup> [50] (Figure 3).



**Figure 3.** Study area, surface soil sampling scheme and interpolated map of total surface soil copper concentrations [51]

The amount of copper built up in the surface vineyard soil was estimated on a *per hectare* basis taking into account the total copper concentration and the weight of 10 cm thick surface layer of soil, assuming a bulk density of 1.5 (Table 3). Approximate plantation age (with within-decade precision) was estimated for most plots, and a detailed history for some plots: time of the first and possibly second deep ploughing, time of vineyard restoration or supplementary planting, common growing practice in the past, and thereby also approximate frequency of copper fungicide applications. More than 64 % vineyards are more than 40 years old and it is assumed that the same percent of all plots received similar annual amounts of copper, ranging from 2 to 5 kg ha<sup>-1</sup>. This certainly allows only an estimation of the overall copper input into soil throughout the vineyard history. The upper layer of plots planted with about a century old vines exhibited high copper contents.

Location <sup>1</sup>	Cu <sub>TOT</sub> (mg kg <sup>-1</sup> )	Cu <sub>DTPA</sub> (mg kg <sup>-1</sup> )	Cu <sub>CaCl2</sub> (mg kg <sup>-1</sup> )	Cu <sub>TOT</sub> /ha (kg ha <sup>-1</sup> ) <sup>2</sup>
351	363	136	1.15	491
352	154	48	0.63	208
353	380	142	1.17	514
354	389	121	0.74	525
355	181	56	0.73	244
356	166	54	0.55	224
357	655	368	1.45	884
358	586	296	1.96	790
359	369	135	0.94	498
360	565	296	1.35	763
361	633	340	2.08	855
362	641	228	1.68	866

Location <sup>1</sup>	Cu <sub>TOT</sub> (mg kg <sup>-1</sup> )	Cu <sub>DTPA</sub> (mg kg <sup>-1</sup> )	Cu <sub>CaCl2</sub> (mg kg <sup>-1</sup> )	Cu <sub>TOT</sub> /ha (kg ha <sup>-1</sup> ) <sup>2</sup>
363	336	196	1.24	454
364	385	152	1.15	519
365	263	101	0.82	355
366	667	316	1.36	901
367	615	332	1.56	830
368	419	143	1.19	565
369	494	166	1.47	667
370	244	95	0.93	330
371	519	282	1.15	701
372	134	47	0.42	181
373	199	71	0.52	268
374	623	348	1.84	841
375	35	4.32	0.10	47
376	389	140	1.26	525
377	442	138	0.96	597
379	252	104	0.92	340
380	30	808	0.93	40
381	531	198	1.17	717
382	305	117	1.04	412
383	453	199	1.35	612
384	360	131	1.2	486
385	79	21.2	0.21	107
386	398	146	1.36	537
387	324	120	0.94	437
390	51	8.28	0.21	69
391	74	11.8	0.11	100
392	228	89	0.83	307
395	533	170	1.46	720
396	627	254	1.66	847
397	32	12.4	0.10	43
398	43	10.6	0.21	58
399	610	180	1.98	823
401	691	278	1.93	933
402	427	154	1.35	576

<sup>1</sup>Location: All the data available from the GIS database

<sup>2</sup>Cu/ha (kg ha<sup>-1</sup>) Calculated from the data on total Cu (mg kg<sup>-1</sup>) in the top 10 cm assuming a bulk density of 1.5

**Table 3.** Total Cu, extractable Cu and total amount of Cu per ha in the upper layer (10 cm) of vineyard soils (n=67) (Romic et al., 2004)

Determination of the total metal content in soils is an important step in assessing the hazards to the vital roles of soils in the ecosystem, but also in comparing them with quality standards referring to the effects of contamination and system sustainability. However, the mobility and availability of soil copper are governed by the processes of dynamic equilibrium, and not only by its total concentration [52]. This study shows that the mobile ( $\text{CaCl}_2$ -extractable) fraction of copper in vineyard soils amounted only to 0.2 – 3.1 % of its total content. As Cu accumulation in the studied soils is restricted to surface layer the risk of Cu phytotoxicity for grapevine is small, since grapevine develops most of its roots at a depth  $>30$  cm, depending on the soil type and the profile depth. Material suspended by erosion, however, carries away also a part of the applied copper, and redistribution of this material depends on a number of factors (relief, size and shape of the drainage basin, etc.). Halamic et al. [53] applied factor analysis in geochemical investigations of stream sediments in drainage basins in the Mt. Medvednica region, which also includes part of the studied area, without determining correlation of copper concentrations with any lithological unit, so they assumed anthropogenic influence, mostly grapevine production. Ribolzi [54] carried out the research in the Mediterranean drainage basins of wine-growing regions in France with the aim to characterize copper forms in suspended material and recorded an average total concentration as high as  $245 \text{ mg Cu kg}^{-1}$ , but Brun et al. [55] reported the maximum of  $250 \text{ mg kg}^{-1}$  of copper in vineyard soils of the Mediterranean part of France.

### 3.1. Correlation between total, extractable Cu and soil properties

In the total copper concentration extracted with aqua regia, DTPA-extractable copper amounted to 12-81 % and  $\text{CaCl}_2$ -extractable to 0.2-3.1 %. Both DTPA- and  $\text{CaCl}_2$ -extractable copper were largely explained by the total copper concentrations, as confirmed by their high correlation coefficient ( $R = 0.899$  and  $R = 0.896$ ). They were also highly correlated to each other ( $R = 0.763$ ) (Table 4).

	$\text{Cu}_{\text{total}}$	$\text{Cu}_{\text{DTPA}}$	$\text{Cu}_{\text{CaCl}_2}$	$\text{pH}_{\text{H}_2\text{O}}$	Org. C	CEC	$\text{CaCO}_3$
$\text{Cu}_{\text{total}}$	1	0.899***	0.896***	0.388**	0.601***	0.277*	0.314**
$\text{Cu}_{\text{DTPA}}$		1	0.763***	0.321**	0.395***	0.084 <sup>ns</sup>	0.236 <sup>ns</sup>
$\text{Cu}_{\text{CaCl}_2}$			1	0.392***	0.734***	0.257*	0.341**
$\text{pH}_{\text{H}_2\text{O}}$				1	0.121 <sup>ns</sup>	0.315**	0.587***
Org. C					1	0.339**	0.173 <sup>ns</sup>
CEC						1	0.130 <sup>ns</sup>
$\text{CaCO}_3$							1

ns - not significant

\* Correlation significant at  $p < 0.05$

\*\* Correlation significant at  $p < 0.01$

\*\*\* Correlation significant at  $p < 0.001$

**Table 4.** Correlation matrix, upper triangle

Highly significant positive correlation was also determined between total copper and organic matter in soil, and a weaker correlation, but still significant, between total copper and cation exchange capacity and carbonate content (Table 4). In the case of extractable copper, a significant correlation was determined between  $Cu_{DTPA}$  and organic carbon content and pH, but not between cation exchange capacity and carbonate content. Significant correlation was recorded between all the analyzed soil properties and  $Cu_{CaCl_2}$ .

### 3.2. Linear regression of total and extractable copper and selected soil properties

To establish the relation between copper fractions after particular extractions (aqua regia, DTPA and  $CaCl_2$ ) and soil properties that may affect their behavior in soil and availability to plants, the multiple linear regression analysis was done. The model included those variables for which correlation probability  $p < 0.05$  was determined [48]. For the regression model of aqua regia extracted copper, this condition was met by the following properties:  $Cu_{DTPA}$ ,  $Cu_{CaCl_2}$  and cation exchange capacity (CEC).

For total copper, the regression model explains 92 % of total variance (Table 5). The largest contribution to the variance in regression was that of  $Cu_{DTPA}$ .

Variable explained:  $Cu_{TOT}$

Source of variation	Degree of freedom	Sum of squares	F	Pr > F
CuDTPA	1	301395	101,8	0.0000
CuCaCl <sub>2</sub>	1	192403	65,0	0.0000
CEC	1	30384	10,3	0.0021
Total	63	2319596		

Regression equation:  $Cu_{TOT} = -63.42 + 0.956 * Cu_{DTPA} + 170.9 * Cu_{CaCl_2} + 3.761 * CEC$

$R^2 = 0.92$

**Table 5.** Linear regression of total copper ( $Cu_{TOT}$ ) as a function of DTPA-extractable ( $Cu_{DTPA}$ ),  $CaCl_2$ -extractable ( $Cu_{CaCl_2}$ ) and cation exchange capacity (CEC).

Concentration of total copper in vineyard topsoil went up with an increase in the cation exchange capacity. Square root transformation (SQRT) was applied to the regression model of DTPA-extractable copper, whereby the model conditions were satisfied, and the transformed variables  $SQRT(Cu_{DTPA})$  were predominantly dependent on  $Cu_{TOT}$ . As this relation was already determined, it was omitted from the model. Two other properties met the condition of correlation probability  $p < 0.05$ : organic matter content (Org-C) and  $CaCl_2$ -extractable copper ( $Cu_{CaCl_2}$ ), and they were included into the model. For  $Cu_{DTPA}$ , the regression model explains 85 % of total variance (Table 6).

Variable explained: SQRT (Cu <sub>DTPA</sub> )				
Source of variation	Degree of freedom	Sum of squares	F	Pr > F
CuCaCl <sub>2</sub>	1	685.7	240.0	0.0000
Org-C	1	44.54	15.59	0.0002
Total	63	1224		

Regression equation:  $\text{SQRT}(\text{Cu}_{\text{DTPA}}) = 5.534 + 9.400 \cdot \text{Cu}_{\text{CaCl}_2} - 1.326 \cdot \text{Org-C}$   
 $R^2 = 0.853$

**Table 6.** Linear regression of DTPA-extractable (Cu<sub>DTPA</sub>), as a function of CaCl<sub>2</sub>-extractable (Cu<sub>CaCl<sub>2</sub></sub>) and soil organic matter (Org. C)

Two parameters were included into the regression model of CaCl<sub>2</sub>-extractable copper: organic matter content (Org-C) and pH, and the model explains 62 % of total variance (Table 7). Concentrations of CaCl<sub>2</sub>- extractable copper mainly depend on pH, which relation was also confirmed by this investigation. However, since these are predominantly alkaline soils, this relation is not as strong as in the case of soils with a more varying pH [55].

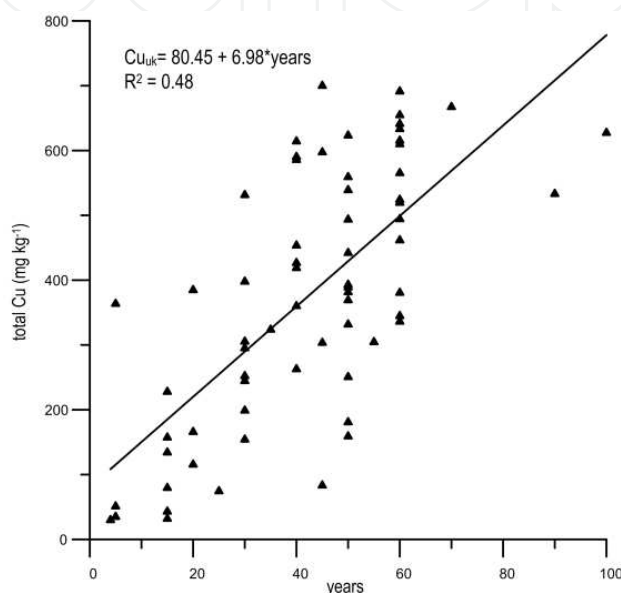
Variable explained: Cu <sub>CaCl<sub>2</sub></sub>				
Source of variation	Degree of freedom	Sum of squares	F	Pr > F
Org-C	1	8.30	83.37	0.0000
pH	1	1.62	16.23	0.0002
Total	64	17.34		

Regression equation:  $\text{Cu}_{\text{CaCl}_2} = -2.673 + 0.379 \cdot \text{Org-C} + 0.337 \cdot \text{pH}$   
 $R^2 = 0.621$

**Table 7.** Linear regression of CaCl<sub>2</sub>-extractable copper as a function of soil organic matter (Org. C) and pH

Over 65 % of vineyard plots under study were more than 40 years old, and some have been continuously cultivated for more than 100 years. It is assumed that the same percent of parcels received a similar annual amount of copper, ranging from 2 to 5 kg ha<sup>-1</sup>. This, naturally, does not allow an exact estimate of the overall copper input into soil throughout the vineyard history. Vineyard age parameter was not therefore included in the multiple linear regression model. There is, however, strong statistical evidence that an increase in vineyard age is related to the increase of expected total copper content (Figure 4). According to historical documents, the vine downy mildew infection started spreading in the vineyards of northwestern Croatian in 1882, and the Bordeaux mixture application became indispensable during the wine boom period at the end of the 19th century. Recognizing the benefits, wine-growers often did not observe the recommended concentrations and application times, and a large number of treatments, as many as 8 to 14, were often applied at positions exposed to disease attacks. However, numerous other factors, such as scattering during applications, washing off the leaves by rain, input of treated plant residues into soil, tillage and erosion,

make it difficult to establish the relation between vineyard age and accumulated copper. In France, for example, the Bordeaux mixture has been used since 1855, and it was found that after several decades of its continuous application the soil total copper reached a concentration of as much as  $1.0 \text{ g kg}^{-1}$  [56]. Research done by Deluisa et al. [11] on 43 plots in a humid region of northern Italy revealed an average copper accumulation in topsoil of  $297 \text{ mg kg}^{-1}$ . Moolenaar and Beltrami [57] have calculated that organic protection of grapevine, which implies exclusive use of the Bordeaux mixture, can result in an increase of soil copper concentration up to  $600 \text{ mg kg}^{-1}$  after 100 years.



**Figure 4.** Vine age as a function of total soil copper

Diethylenetriaminepentaacetic acid (DTPA) is a potent synthetic chelating agent, and the method of extraction with DTPA was developed for the purpose of determining zinc, iron, manganese or copper deficiency in neutral and carbonate soils [58]. Haq and Miller [59] reported negative results of the DTPA test, which they explained by their failure to determine sufficiently significant relations between concentrations of metals (copper and manganese) extracted from soil and those found in the tested plants. Mention should be also made of the research done by O'Connor [60], who gave a number of comments on the DTPA test, based also on non-significant correlation between DTPA-extractable metals in soil and their concentrations in plants. Regardless of the above considerations, DTPA is the most widely used agent for extraction of "available" cadmium, copper, nickel and zinc, and thereby also the most standardized one [44, 61, 62]. Starting from the fact that the data on total copper content reveals very little about its bio-availability, such strong correlation between copper extracted with aqua regia and DTPA actually indicates that neither the latter extraction method is suitable for assessing copper availability to plants.

Merry et al. [63] in vineyard soils recorded 25-35 times higher contents of copper, lead and arsenic, originating from plant protection agents, than their common values in uncontami-



nated soils; they also determined a strong correlation between total and DTPA-extractable copper ( $0.93 < r < 0.96$ ). Also Brun et al. [55] found that the regression model  $Cu_{DTPA}$  explained 90 % of total variance in vineyard soils, its largest part referring to total copper content.

When cation exchange capacity was included into the model, it was found that the DTPA-extractable copper decreased with increasing cation exchange capacity.

Soil extraction 0.01 M  $CaCl_2$  is the method that was increasingly used in the last decade for soil testing to determine soil fertility and the behavior of nutrients and contaminants in the soil. The capabilities of instrumental chemical analysis have improved to such an extent, even in the last few years, that it become possible to determine very low concentrations of nutrients and pollutants in soil extracts [45]. The advantage of this method for determining metal concentrations in soil is that the concentration of electrolytes stays practically constant and metal concentrations reflect the difference in binding strength or solubility between soils. The extractant is an unbuffered solution and therefore the measured metals reflect their availability at the pH of the soil.

The best criterion of the efficiency of the method for determining the soil bioavailable fraction is the high correlation between the Cu content observed in plants grown in situ, at least for neutral to acid soils [55].

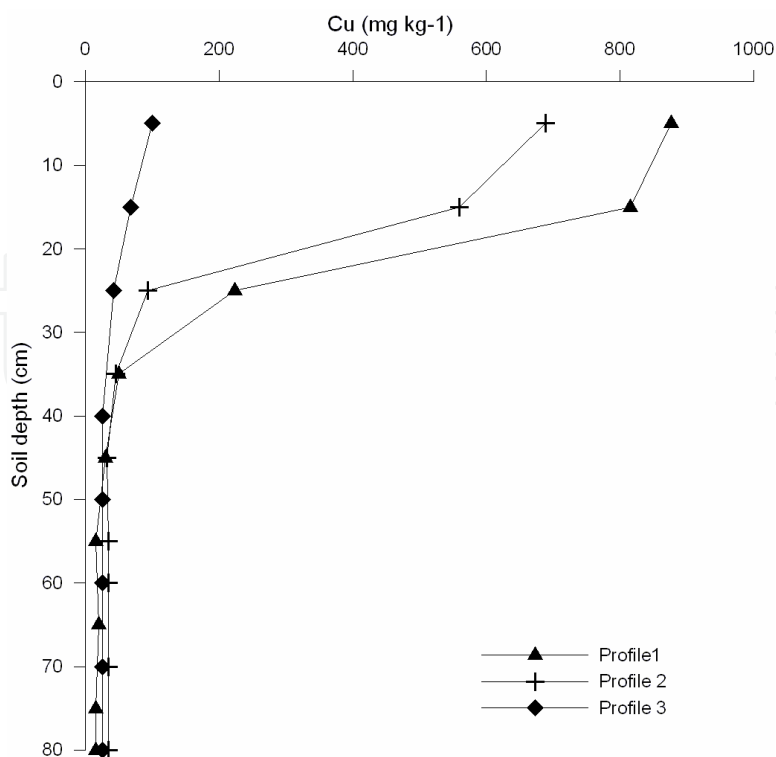
### 3.3. Vertical distribution of total copper in soil profiles

High copper concentrations were found in vineyard soils down to 20 cm depth (to 800 mg  $kg^{-1}$  in profile 1, and to 500 mg  $kg^{-1}$  in profile 2) (Figure 5). Profile 1 is situated at a higher altitude, erosion is more pronounced, and the anthropogenic horizon is less thick. Marl appears already at 45 cm depth, so that the root zone extends into horizon C as well, thus opening the transport routes of water, dissolved substances and solid particles deeper into the profile. In profile 2, the anthropogenic horizon is much thicker, while total copper concentrations are lower down to 30 cm depth. Erosion material was deposited at the base of the slope, so that as much as 100 mg  $kg^{-1}$  of copper was found in the topsoil of colluvial soil, to which no copper agents for plant protection had ever been directly applied. Accumulation of copper in colluvial soil (profile 3) was recorded down to 30 cm depth, that is, over the entire depth of the humus-accumulative horizon. Uniform copper concentrations of  $<25$  mg  $kg^{-1}$  were found at greater depths.

Land use for agriculture causes great changes in the natural properties of soil. Translocation of soil by tillage may be the key reason for redistribution of soil particles within the profile and over the entire site, while erosion due to tillage is especially present in hilly landscapes [64]. Tillage and homogenization of several natural horizons alter both morphological and physicochemical characteristics of soil.

### 3.4. Sequential extraction

Results of the five-step sequential extraction are presented in Figure 6, which shows the distribution of exchangeable (Exch), carbonate-associated (Carb), Fe-Mn oxides-bound (Fe-Mn

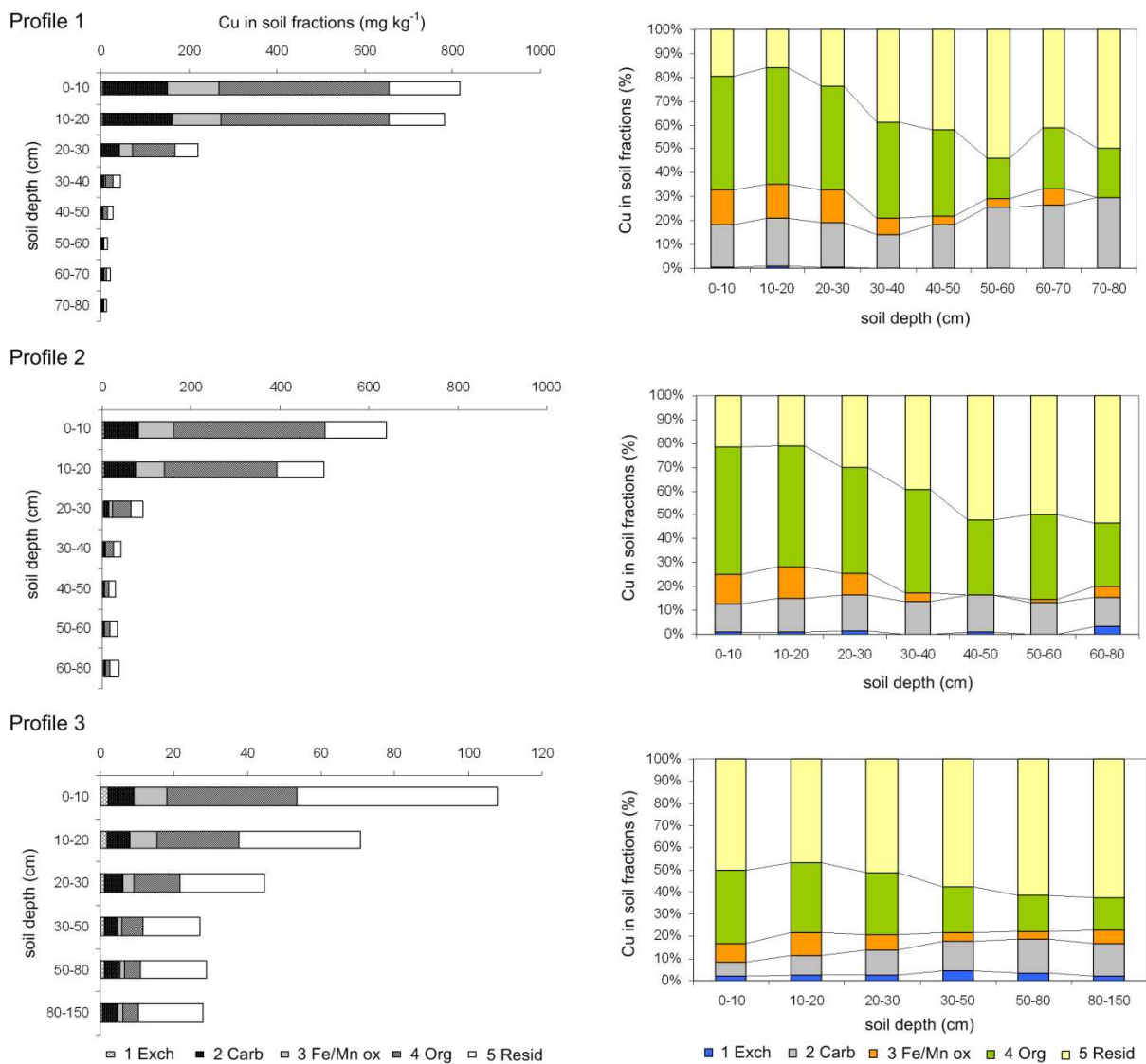


**Figure 5.** Vertical distribution of total copper within the soil profiles

ox), organic-bound (Org) and residual (Resid) copper found in the soil samples. These five metal fractions were separated in soil samples from 10-cm profile layers of Aric Anthrosols and master horizons of Calcaric Regosols. The data were used to calculate the relative error (RE %), which for most metals amounted to  $\pm 10$  %.

In the surface 10 cm of profile 1, 877 mg kg<sup>-1</sup> of copper was determined by extraction in *aqua regia*. The sum of copper fractions separated by selective sequential extraction is slightly lower and amounts to 816 kg<sup>-1</sup> (RE = 7 %). As much as 47 % of total copper was bound in the organic fraction, 20 % in the residual fraction, and 18 % and 16 % in the reductive and carbonate fractions, respectively. Down to 30 cm depth, the share of copper in exchangeable fraction was <1 %, whereas it was not detected in deeper layers (Figure 6). However, copper distribution per fractions changes with the profile depth. As the total concentration decreases to background values, the share of copper in residual fractions increases, since complexing ability decreases with the reduced amount of organic matter.

Considering the high content of the organic fraction (54 % in the 0–10 cm layer and 27 % at 60–80 cm depth), a very probable mechanism is translocation through complexation with soil organic matter. However, copper is certainly not translocated in the same way with surface eroded material as vertically through the profile depth. Eroded material deposited at the base of hillsides under vineyards is richer in silt, and copper is mostly strongly bound in the residual fraction. In the surface 10 cm of soil of the profile 1, 20 % of total copper content is bound in the residual fraction. In the profile 2, copper content in the residual fraction was increased to 21 %, while it amounted to 50.3 % in colluvial soil (profile 3).



**Figure 6.** The distribution of exchangeable (Exch), carbonate-associated (Carb), Fe-Mn oxides-bound (Fe-Mn ox), organic-bound (Org) and residual (Resid) copper in soils

Copper distribution per fractions in profile 2 resembled that in the preceding profile. High copper concentrations decrease with depth, the profile being deeper as well. Copper content of <1 % was found in the exchangeable fraction, and that of 3 % at 60–80 cm depth. It is obvious that copper translocation occurs within the profile, but this is also the zone where most of the vine roots develop.

Although no copper fungicides had ever been directly applied to the area on which profile 3 was dug, as much as 100 mg Cu kg<sup>-1</sup> was determined in topsoil by extraction in aqua regia. Background concentrations were reached at 30 cm depth, and copper was predominantly bound in the residual fraction (47 %–63 %). There was a significant share of the organic fraction, but it decreased with depth from 33 % to 15 %. The content of exchangeable copper was higher than in vineyard soils – 2 % to 5 %.

Research has shown that soil type is the main factor of accumulation and distribution of both natural and anthropogenic concentrations of heavy metals. In natural profiles, the indigenous element distribution is generated by long-term pedogenesis [65]. In the case of cultivated soils, their characteristics in time and space change in dependence on the ecological environment and land use and management, whereby also their production capacity and environmental impact get changed [66, 67]. When growing woody crops, and thus also grapevine, soil is homogenized to a greater depth, first by deep ploughing to xx cm depth before the setting up of the plantation, and then by regular tillage. This somewhat disrupts the morphogenetic soil properties, changes the sequence of genetic horizons, and often deepens the active part of the profile. Such changes are naturally reflected in the distribution of elements, changes in their mobility and bioavailability. Selective sequential extraction was used in this research to determine the way and strength of binding and retention of heavy metals in soil, which under certain conditions enables the estimation of potential mobility and bioavailability.

The spatial variability of trace metals in agricultural surface soils of the wider Zagreb area has shown that the application of agrochemicals has caused high accumulation of copper and zinc [51, 68]. This especially applies to vineyard soils, but also to orchard and vegetable garden soils. The presence of a buffer material, such as carbonate, can be particularly important in the retention of heavy metals. The trace metal retention capacity of silty soils with high carbonate content can be as high as, or higher than, the retention capacity of certain clayey soils [69].

In soils and sediments that were receiving high concentrations of copper (along with other metals) for at least 6 years, Hickey and Kittrick [70] established that about 28 % copper was bound in the organic fraction. It is also in the soils treated with waste sludge or stable manure rich in copper that most of this metal is bound in the organic fraction [71, 72]. Affinity of humic and fulvo acids to copper sorption was, among others, reported by Senesi et al. [73] while McLaren et al. [74], pointed to the importance of soluble copper chelates in soil solution. In soils of lighter texture, poorer in organic matter, the added copper is initially retained in the exchangeable fraction, whereafter it is translocated into the carbonate fraction [75]. Incubation in the laboratory experiment revealed that translocation of copper to more stable fractions was much slower in texturally light, but acid, vineyard soil, also poor in organic matter [76]. However, Flores-Velez et al. [10] report that in the case of sandy acid vineyard soils the selective sequential extraction procedure was not selective enough to specify the form of copper. The same authors report that copper in anthropogenic vineyard soil, originating from Cu-fungicides, was concentrated in the coarse organic fraction (plant residues) and in the mineral colloid fraction. In soils or sediments deficient in organic matter, a larger part of copper was bound to Fe and Mn oxides that it was found in this research. Thus, Szarek-Gwiazda and Mazurkiewicz-Boron [77] found that 40.2-54.1 % of total copper in fluvial sediment was bound to Mn oxides and amorphous Fe hydroxides, and only about 10 % to the organic fraction. These authors maintain that Fe(III) and Mn(IV)-oxides can occur either as coats on detritus particles, as cement between them or as pure concretions.

Their ability to adsorb and control heavy metal distribution between the solution and the matrix has been thoroughly explained in scientific literature (e.g., [78]).

Romic et al. [49] did not establish, either by individual correlations or by factorial analysis in the vineyard soils of NW Croatia, the importance of the contents of clay, Fe and Mn oxides or cation exchange capacity for copper sorption in soil, though some authors stress the importance of these fractions [74]. In their investigations, the relation between the content of metals and soil properties was assessed on the basis of their total contents. In this research, correlations were determined between copper fractions and the selected soil properties: significant correlation was recorded between the contents of organic C and ORG and RESID fractions, or its total content, whereas no significant correlation was found between copper in the said fractions and cation exchange capacity, or total carbonates. This corroborates the reports that the distribution of copper of anthropogenic origin among fractions depends prevalently on soil organic matter.

Finally, the use of copper containing fungicides is allowed in the organic agriculture by the European Union regulation, and the official guidelines for soil copper content are usually derived from the total soil copper content. However, these guidelines should be modified according to the soil properties, such as pH and organic matter content, which will affect the Cu solubility, and consequently its bioavailability. Furthermore, soil copper thresholds should be confirmed with toxicological data obtained for biota (e.g. plants, microorganisms, invertebrates). Above mentioned implies that site-specific guidelines should developed for the risk assessment of soil copper toxicity.

#### 4. Summary

Increased anthropogenic inputs of trace metals in soils have received considerable attention since they can enter the food chain by different ways. Soils receiving repeated applications of fungicides, pesticides or manure exhibit high concentrations of extractable metals, especially copper. From the commercial aspect, wine-growers are now showing increasing interest in the effects of soil composition, its fertility and texture upon wine quality. Special importance is laid on the influence of soil geochemical characteristics, including accumulation of certain toxic elements, on grape and wine quality. A variety of factors, both spatial and temporal, affect the grape quality, many of them being specific exactly to the given wine-growing site. Grape growing conditions and enological potential have been created, among other factors, also by landscape characteristics: soil, climate and topography. These factors are much less changeable than biological (cultivar, stock) or human (ampelotechnics, vinification) factors and for this reason the concept of *viticultural terroir* is based on the simple relationship between soil and wine. Since *terroir* is defined as an interactive ecosystem, it is very difficult to evaluate scientifically its contribution to plant capacity to accumulate bioactive phytochemicals good for human health.

Soil is a factor of the natural environment and its effect on wine quality and grape composition is highly complex, since it affects mineral nutrition of grapevine, water uptake, as well

as rooting depth and rhizosphere temperature. Land use for agriculture causes great changes in the natural properties of soil. Translocation of soil by tillage may be the key reason for redistribution of soil particles within the profile and over the entire site, while erosion due to tillage is especially present in hilly landscapes. Tillage and homogenization of several natural horizons alter both morphological and physicochemical characteristics of soil, which affect metal behaviour in soil – plant system.

The mobility and bioavailability of metals depends not only on its total concentration in soil but also on soil properties, metal characteristics and environmental conditions. Trace metals are present in soil in various forms: water soluble, exchangeable, carbonate associated, oxide associated, bound on organic matter and residual forms. Obviously, various metal forms have different mobility. Water soluble and exchangeable fractions are readily released to the environment, whereas the residual fractions are immobile under natural conditions. The most common observation in majority of studies dealing with sequential or partial extraction of soil trace elements, including copper as well, is that they are not completely specific to metals or chemical phases. The complexity of bioavailability phenomenon comes out from an array of matrix-related, species-related and metal-related issues.

Plant copper uptake occurs predominantly from the soil solution, indicating that soil characteristics that determine copper solubility, will also determine the copper phytoavailability. However, roots can alter the chemical mobility and thus the bioavailability of copper in the rhizosphere. Remediation of contaminated vineyard soils is an issue of debate and it should be focused on growing practice and vineyard management more than application of the remediation techniques per se (i.e. phytoextraction). Periodical monitoring of soil quality indicators is a required wine-growing practice. If systematically collected information is associated to a location, then it can be useful to all participants in the complex process of grape and wine production, wine marketing and consumption. Use of advanced analytical and information technologies may significantly improve the production and contribute to rational utilization of resources.

## Author details

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