Copper toxicity in soils under established vineyards in Europe: a survey

Authors: Stefan Ruyters^{*(1)}, Peter Salaets⁽¹⁾, Koen Oorts⁽²⁾ and Erik Smolders⁽¹⁾

Address: ⁽¹⁾Department of Earth and Environmental Sciences, Division of Soil and Water Management, KU Leuven, Kasteelpark Arenberg 20, 3001 Heverlee, Belgium

⁽²⁾Arche (Assessing Risk of Chemicals), Stapelplein 70, box 104, 9000 Ghent, Belgium

*Corresponding author

Department of Earth and Environmental Sciences, Division of Soil and Water Management, KU Leuven, Kasteelpark Arenberg 20, 3001 Heverlee, Belgium

email address: stefan.ruyters@lessius.eu

Tel: +32 (0)15/30 55 93 Fax: +32 (0)15/30 55 99

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

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 CuCl₂. 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 CuCl₂. 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.

Key words: copper; vineyard; toxicity; fungicide; soil; risk assessment

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 unploughed vineyard soils, whereas ploughing 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 6m 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 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 Cu²⁺ 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, ageing times in such soils can now be >150 years whereas the factor 2 was derived based on ageing 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 ($<100 \text{ mg kg}^{-1}$) 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 ($< 4\text{mm}$) 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 3M and >1 h boiling at 140°C) and analysed 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 0.01M CaCl₂ 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 analyser 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 cm³ soil cores. After one 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 CuCl₂ (50 g Cu l⁻¹) at 5 doses above control (100, 200, 400, 800, 1600 mg Cu kg⁻¹) one 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 KH₂PO₄ and 100 mg N kg⁻¹ soil as KNO₃ one 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 deionised 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 reduce evaporation. Plants were grown in a growth room as described above. As soon as 70% of the seedlings in the control treatments emerged, i.e. after 5-10 days of growth, emergence was determined within each pot and seedlings were thinned to give a total of five evenly spaced representative specimens of the plants in the pots. All soils within a vineyard site and all spiked treatments of the corresponding control soil were thinned at the same day. After an additional 14 days of growth (i.e. 14 days after the day of >70% emergence), shoot biomass above the soil surface was removed. The dry mass of the shoots was determined after oven drying at 70 °C for at least 16 h.

2.4. Microbial assays

The test performed in this study is the ISO 14238-1 (1997) soil quality test in which ammonia is added to the soil before incubation. The Potential Nitrification Rate (PNR, $\text{mg NO}_3^- \text{-N kg}^{-1} \text{ day}^{-1}$), which is the nitrate production rate at unlimited substrate ($\text{NH}_4^+ \text{-N}$) availability, was measured after 3 days incubation. Therefore, moist vineyard, control and spiked soils were incubated at 20 °C during 7 days prior to toxicity testing. At the start of the test 100 $\text{mg NH}_4^+ \text{-N kg}^{-1}$ fresh soil was added using a stock solution containing 15 $\text{mg NH}_4^+ \text{-N mL}^{-1}$ (NH_4Cl). Soil samples were incubated at 20 °C covered with a perforated lid to ensure aerobic conditions. The soil $\text{NO}_3^- \text{-N}$ was measured colorimetrically (Skalar, SA40) in a centrifuged soil extract (2g subsample, 20ml KCl 1N, 2 h end-over-end shaking, $n = 2$) 0 and 3 days after substrate addition as described by Smolders et al. (2001). The PNR was calculated as the increase of the $\text{NO}_3^- \text{-N}$ concentration in soil at day 3 compared to the average $\text{NO}_3^- \text{-N}$ concentration ($n=2$) at day 0 ($\text{mg NO}_3^- \text{-N kg}^{-1}$ fresh soil day^{-1}).

2.5. Invertebrate toxicity test

Chronic toxicity tests with *Enchytraeus albidus* (*E. albidus*) were performed following OECD guideline 220 (OECD, 2004). *E. albidus* is one of the largest species of the genus *Enchytraeus* (adults reach 15–40 mm). All test species were maintained in laboratory cultures. Cultures of *E. albidus* were bred in moist soil (50% OECD soil, 50% natural garden soil), at 20 °C, kept in the dark, and fed once a week with finely ground and autoclaved rolled oats according to Römcke and Moser (2002). The test duration of the test is 6 weeks. The endpoint for toxicity testing is the reproduction (juveniles per container).

Ten adult animals, with clearly visible clitellum, were added to 125 ml plastic containers containing 30 g moist soil. Six replicate test containers were used per soil sample, for the control soil 10 replicates were used. The containers were closed with plastic lids to prevent loss of moisture and placed in an incubation chamber with the following incubation conditions: 16 h/8 h cycle day/night at 20 °C. Once a week containers were opened to ensure aerobic conditions and a small amount of crushed oat meal was added as food. Pots were sprayed with water to ensure moist conditions in the pot. After 3 weeks, surviving adults were counted and removed from the containers. In this study the number of surviving adults is not used as a toxicity measure, but as the validity criterion of this assay (at least 70% in the control soils, i.e. 7 adults per container). After another 3 weeks incubation, including weekly feeding except for the first week, juveniles were counted by extraction and staining with 1% Bengalrose ethanol solution as described in the OECD guideline 220 (2004). The bright pink coloured juvenile enchytraeids were isolated by sieving over 250 and 160 µm and manually counted using a magnifying glass.

2.6. Data analysis

Dose-response curves for each assay of the spiked soils were calculated based on added Cu concentrations (measured concentration minus the background concentration of the control soil) and included all replicates of the response. Because dose-response relationships were established using a log-scale, an arbitrary small non-zero value of 0.1 mg Cu kg⁻¹ was assigned to the control soils. Dose-response curves were fitted by the log-logistic model (Doelman and Haanstra, 1989) presented below using the Marquardt method (SAS 9.3):

$$y = \frac{c}{1 + \exp(b(x - a))} \quad (\text{Eqn. 1})$$

with y = response variable, c = response in the control soil, b = slope parameter, x = logarithm of the added Cu concentration and a = logarithm of the ED50 value, i.e. the dose at which 50% inhibition is found. The ED50 and ED10 (10% inhibition) values and their 95% confidence interval, expressed as added Cu dose (mg Cu kg⁻¹), are predicted from the appropriate parameters of the curve. For the vineyard sites, linear regression between dose (added Cu) and response was used to test for significant ($p < 0.05$) effects within each vineyard site, since no dose-response curves could be fitted with the above equation. The Dunnett test was performed to detect significant different responses in soil samples compared to the corresponding control soil ($p < 0.05$; SAS 9.3). Pearson correlations of the responses (plant growth, root length, PNR and *E.albidus* reproduction) with soil variables (soil total Cu, eCEC, %N, %OC and pH) in the vineyard sites were calculated (SAS 9.3, proc CORREL).

3. Results

3.1. Soil properties

Copper concentration in the control soils ranged from 23 to 105 mg Cu kg⁻¹ dry soil, the eCEC from 7 to 29 cmol_c kg⁻¹ and the pH from 5.7 to 7.7 (Table 1 and Supplementary Information, Table 1). The maximal total soil Cu concentration in the vineyard samples ranged from 435 to 689 mg Cu kg⁻¹ dry soil (Table 1). Differences in eCEC, pH and organic matter content are observed in each set of soils collected from the same vineyard. In general, the eCEC in the control soil was lower than the average eCEC within the corresponding vineyard soil samples. In contrast, the eCEC in the vineyard soil samples of site 3 (21 cmol_c kg⁻¹) was lower than that in the control soil sample (29 cmol_c kg⁻¹). Correlation analysis revealed that soil total Cu was unaffected ($p>0.05$) by either eCEC, soil organic C or pH within the samples of any site with the exception of the positive correlation with eCEC at site 4 (Table 1). Analysis of total soil Cu in spiked soils showed that most soils had been dosed by, on average for all sites and bioassays, 30% more than the nominal values (details not shown). All toxicity data below are expressed on measured rather than on nominal values.

Table 1: Overview of selected properties of the six vineyard sites. The soil total Cu concentration in the control soil adjacent to the vineyard is given and the soil total Cu concentration in the corresponding vineyard site is given as a range. Texture, pH, organic carbon (OC), nitrogen (N) and eCEC refer to the control soil. Pearson correlations between soil total Cu and either soil organic carbon (%OC) or eCEC within the soil samples of each site are given; bold values are statistically significant at $p < 0.05$. A detailed overview of the soil properties of each soil sample of each site can be found in Supplementary Information Table 1.

site	Country	Soil total Cu	Soil total Cu	Texture			pH (CaCl ₂)	OC (%)	N (%)	eCEC (cmol _c kg ⁻¹)	Correlation coefficient r	
		Control soil (mg Cu kg ⁻¹)	Vineyard (mg Cu kg ⁻¹)	Sand (%)	Clay (%)	Loam (%)					Cu versus OC	Cu versus eCEC
1	France	23	201-689	58.1	15.4	20.7	6.8	2.1	0.17	17.2	0.84	0.50
2	Italy	105	194-448	44.0	8.9	19.9	7.3	9.1	0.22	16.1	0.46	0.54
3	Germany	65	276-516	14.5	27.4	46.1	7.3	5.9	0.40	29.2	-0.66	0.39
4	Germany	74	142-513	32.3	20.6	38.6	6.6	3.8	0.38	11.2	0.80	0.86
5	Germany	105	159-435	43.8	22.8	34.5	6.7	2.1	0.24	7.3	0.20	0.65
6	Italy	66	155-455	41.9	14.7	36.1	7.5	5.6	0.08	15.3	0.66	0.64

3.2. Biological responses

The tomato seedling shoot yield in the *control soil samples* for the six sites ranged between 0.82 and 2.64 g dry weight per pot and the barley root length after 4 days growth in the control soil samples between 28 and 108 mm (Table 2). The PNR in the control soil samples ranged between 8 and 23 mg NO₃⁻-N kg⁻¹ d⁻¹ (Table 2). The *E. albidus* reproduction (number of juveniles) ranged from 1891 to 5311 juveniles (Table 2). Differences in response among control soils can be due to nutritional or textural differences of the soils. The validity criterion for tomato growth (>14 shoots emerged within the first 10 days of incubation) and *E.albidus* (>7 surviving adults after 3 weeks incubation) was met for the control soils of all sites (details not shown).

Table 2: Response of the four bioassays in the control soil of each of the six sites (standard deviation in brackets, n=4 (tomato growth), n=3 (barley root length), n=2 (PNR), n=10 (*E. albidus* reproduction)).

Site	Tomato yield (g pot ⁻¹)	Barley Root Length (mm)	PNR (mg NO ₃ ⁻ -N kg ⁻¹ d ⁻¹)	<i>E.albidus</i> reproduction (Number of Juveniles)
1	0.95 (0.05)	55 (1)	8 (1)	1891 (1420)
2	1.88 (0.07)	80 (16)	18 (2)	2731 (746)
3	2.64 (0.18)	79 (12)	23 (0.3)	5311 (490)
4	2.60 (0.16)	108 (3)	9 (1)	4443 (532)
5	1.43 (0.06)	77 (5)	10 (1)	2889 (396)
6	0.82 (0.04)	28 (9)	8 (1)	4484 (673)

Table 3: The toxicity of added Cu in spiked soils (ED50 values and 95% confidence interval) and in vineyard soil samples. The effect of Cu on the response is indicated (p<0.05, linear regression analysis, ‘-’ a significant negative effect, ‘+’ a significant positive effect). The expected response (relative to control, 95% confidence interval in brackets) in the vineyard is calculated at the highest added Cu in the vineyard sample ($C_{\max \text{ corr}}$, i.e. assuming equal availability of added soil Cu) and is based on the dose-response curve of the corresponding spiked soils. The measured response (with standard deviation, n=4 (tomato growth), n=3 (barley root elongation), n=2 (PNR), n=6 (*E. albidus* reproduction)) in the highest contaminated vineyard soil sample is given. See Eqns. (3) and (4) for calculation details.

	Site	Spiked		Vineyard			
		effect	ED50	effect	$C_{\max \text{ corr}}$	Expected	Measured
		(p<0.05)	(mg Cu kg ⁻¹)	(p<0.05)	(mg Cu kg ⁻¹)	response (%)	response (%)
Tomato growth	1	-	752 [631; 896]	+	503	64 [49; 78]	358 (7)
	2	-	771 [671; 886]		323	85 [71; 99]	95 (6)
	3	-	828 [341; 2011]	-	615	54 [16; 92]	42 (10)
	4	-	469 [429; 512]	+	223	91 [76; 107]	117 (4)
	5	-	426 [370; 491]	+	134	90 [77; 104]	211 (33)
	6	-	190 [173; 209]	+	313	22 [9; 35]	129 (4)
Barley Root elongation	1	-	538 [408; 709]	+	546	50 [28; 71]	139 (5)
	2	-	240 [120; 480]		329	42 [8; 76]	122 (13)
	3	-	937 [755; 1162]		561	84 [55; 112]	92 (39)
	4	-	441 [412; 471]	-	272	77 [69; 85]	80 (6)
	5	-	432 [326; 571]		175	78 [59; 98]	113 (14)
	6	-	638 [240; 1694]	+	334	70 [14; 127]	229 (25)
PNR	1	-	341 [275; 423]	+	484	33 [13; 53]	164 (30)
	2	-	Unbounded		321	81 [64; 98]	80 (1)
	3	-	1039 [854; 1265]	-	641	72 [56; 89]	61 (1)
	4	-	600 [504; 714]	+	204	97 [78; 115]	167 (8)
	5	-	340 [301; 383]	+	119	94 [80; 108]	144 (0.1)
	6	-	274 [173; 436]	+	304	47 [24; 69]	187 (17)

<i>E. albidus</i> reproduction	1	-	571 [376; 868]	+	562	50 [0; 153]	178 (57)
	2	-	505 [136; 1876]		330	56 [14; 98]	88 (9)
	3	-	474 [311; 1723]	-	544	49 [29; 69]	70 (5)
	4	-	512 [371; 707]	-	292	62 [40; 83]	37 (20)
	5	-	617 [495; 770]		192	94 [55; 132]	94 (22)
	6	-	532 [350; 811]		382	67 [11; 123]	49 (21)

Mean biological responses (relative to the mean response of the corresponding soil) to soil Cu in all *spiked soil samples* are displayed in Figure 1. Linear regression showed significant reductions of the response ($p < 0.05$) with increasing Cu concentrations for all soils and all toxicity assays. The ED50 values ranged from 190 to 828 mg Cu kg⁻¹ for tomato yield, from 240 to 937 mg Cu kg⁻¹ for barley root elongation, from 274 to >2175 mg Cu kg⁻¹ for PNR and from 474 to 617 mg Cu kg⁻¹ for *E. albidus* reproduction (Table 3). This means that the ED50 values in spiked soils are generally in the range of Cu concentrations encountered in the vineyard sites.

Overall, multiple regression of the average relative responses of all *vineyard soil samples* of all sites with average soil parameters (total soil Cu, pH, eCEC, %OC, %N) only showed a significant negative relation with pH for tomato yield and a positive relation with %OC for PNR, whereas no relation with soil total Cu, eCEC, %N was observed for any bioassay. In the vineyard of site 3 (tomato yield, PNR and *E. albidus* reproduction) and site 4 (barley root elongation and *E. albidus* reproduction; Table 3) linear regression revealed a significant decrease ($p < 0.05$) of the response with increasing Cu concentrations. In all other cases, effects of Cu were absent or even positive. In none of the sites a significant progressive decrease of biological responses with increasing Cu concentration was observed within the vineyard transect ($p > 0.05$) when excluding the control soil.

Figure 1: Dose-response relationships for the four biological assays in vineyard soil samples (grey triangles) and corresponding spiked soils of each site (black circles). Error bars denote standard deviations. Curves are based on average response data including all spiked soils.

4. Discussion

4.1. Copper concentrations in vineyard soils

Previous studies in vineyard top soils have reported the enrichment of soil with Cu ranging from 77 up to 3200 mg Cu kg⁻¹ as summarized by Komarek et al. (2010) and Wightwick et al. (2008). In this study, we reported maximal Cu concentrations from 349 to 689 mg Cu kg⁻¹, largely exceeding the European thresholds for Cu in soil ranging from 20-200 mg Cu kg⁻¹ (SCHER, 2009). Within a vineyard, soil Cu concentrations varied largely, as given in Table 1, illustrating the heterogeneous spreading of the Cu contamination (Wightwick et al., 2010; Komarek et al., 2010). Strawn and Baker (2008) showed that Cu in a vineyard soil was mainly bound to soil organic matter, most likely resulting from decay of organic debris, which was sprayed with Cu fungicides. However, total soil Cu did not significantly increase with increasing organic matter content (%OC) in the present set of soils, either when analyzing data per site or in the whole dataset ($p > 0.05$). This is in contrast to the study of Parat et al. (2002) who found a correlation between soil Cu and organic carbon. The absence of a significant effect of organic matter content on total Cu concentration in soil in the present study is potentially related to the few number of soils in the analysis, since correlations are generally positive in the 6 sites (Table 1).

4.2. Correction of copper concentrations in vineyard soil samples due to a different eCEC

Several studies have shown the discrepancy of Cu toxicity between spiked soils and field contaminated or aged soils (e.g. Renella et al., 2002; Lock and Janssen, 2003; Smolders et al., 2009; Smolders et al., 2012; Oorts et al., 2006). This is ascribed to the difference in metal bioavailability, a combination of effects such as the source of the metal (as Cu²⁺ salt or incorporated in minerals or soil amendments), ageing time (years versus weeks) and confounding

factors in spiked soils (salinity and acidification in metal salt spiked soils). For microbial processes, adaptation of the soil microbial community to the metal contamination might also explain part of the observed discrepancy (Giller et al., 1998; Mertens et al, 2006; Ruyters et al., 2010). Differences in soil properties, such as eCEC, between the spiked control soils and corresponding vineyard soil samples might also contribute. Toxicity at a given soil Cu concentration decreases with increasing soil eCEC, because Cu bioavailability decreases (Smolders et al., 2009). The eCEC in the vineyard soil samples is, in general, larger than that in the corresponding control soil (Table 1 and Supplementary Information Table 1). This means that the lack of toxicity in the vineyard sites may be related to larger eCEC compared to that in the control soil used for Cu spiking. In other words, before quantifying the discrepancy in response between spiked and field contaminated soils, a correction factor for differences in Cu bioavailability because of differences in eCEC should be used in order to normalize the added soil Cu in the vineyard soil samples to the eCEC of the corresponding control soil sample. Empirical bioavailability models of Cu, which describe the relation between toxicity and eCEC, have been calibrated to different soils (SCHER 2009). Such models have the form:

$$\log(\text{ED}_{50}) = a + b \times \log(\text{eCEC}) \quad (\text{Eqn. 2})$$

With ED_{50} in mg Cu kg^{-1} and eCEC in $\text{cmol}_c \text{ kg}^{-1}$, ‘a’ an intercept value and ‘b’ a slope specific for the bioassay. A slope $b=1$ effectively means that toxicity is proportional to eCEC. The slopes are 0.96 for tomato growth, 0.68 for barley root elongation, 1.09 for PNR and 0.58 for *E. albidus* which is assumed similar to *Eisenia fetida* (SCHER, 2009). Equation (2) is used to normalize added Cu concentrations in the vineyard soil sample (C) using the eCEC as:

$$C_{\text{corr}} = C \times \left[\frac{\text{eCEC}_{\text{controlsoil}}}{\text{eCEC}_C} \right]^b \quad (\text{Eqn.3})$$

With $eCEC_{\text{control soil}}$ the value of the control soil, $eCEC_C$ the value of the vineyard soil sample with Cu concentration C and b the slope in Eqn 2. For example, if the $eCEC$ in a vineyard soil sample with 500 mg added Cu kg^{-1} is 1.5 fold that of the control soil, the C_{corr} is reduced to 339 mg added Cu kg^{-1} when assessing effects on tomato, but 380 mg Cu kg^{-1} when assessing effects on root elongation.

4.3. Difference of copper toxicity between spiked soils and vineyard sites

Below, several analyses of these data are reported in order to show the difference of the Cu toxicity in spiked soils and the vineyard sites. The response in a vineyard soil sample predicted from the corresponding spiked soils, using the parameters from Eqn. 1, is

$$\text{Expected response (\%)} = \frac{c}{1 + \exp(b \times (\log(C_{\text{corr}}) - a))} \quad (\text{Eqn. 4})$$

Differences in expected and measured response are related to the ageing or source of Cu and not due to a different $eCEC$, since C_{corr} (see 4.2) is used. Expected and measured responses in the vineyard soil samples are plotted against the corresponding soil total Cu concentration in Figure 2. The difference between measured and expected response for each vineyard soil sample significantly increased ($p < 0.05$) with increasing total soil Cu concentration (details not shown), showing that, overall, the expected response was lower than the measured response. This illustrates lower adverse effect or even stimulating effect with increasing total soil Cu in the vineyard soil samples compared to spiked samples. The large scatter among the responses of the vineyard soil samples in Figure 2 illustrates that the biological responses in the vineyard soil samples are affected by other soil variables than total soil Cu, whereas the opposite is true in spiked soils.

Figure 2: The measured response in vineyard soil samples (grey triangles) and the response predicted from toxicity tests on spiked soils at corresponding added Cu (black circles) (Y-axis) in relation to soil total Cu (X-axis), taking into account difference in eCEC between soils (Equation 4). Average values are shown with standard deviation for the measured responses. Error bars of the expected responses are constructed with the 95% confidence intervals. In general, the measured response is larger than the corresponding expected response based on the spiked soils.

Table 3 shows the expected response at vineyard soil sample with the highest Cu concentration of each vineyard site ($C_{\max}$) and the corresponding measured responses. Significantly higher toxicity in the vineyard sites compared to the spiked soils, i.e. lower measured response than expected response, was only detected in one case (*E. albidus* reproduction, site 4), whereas significant lower toxicity (higher response) was found in 11 cases out of 24 (Table 3). On average among all sites and assays, the measured response in vineyard samples was a factor 2.1-fold larger (standard deviation 1.5) than the expected response at equal corrected Cu added, the factor being the ratio of the observed and expected response at the highest soil Cu of each vineyard site. (Table 3). However, the largest factor difference for each bioassay in each vineyard site is not necessarily found at $C_{\max}$. The largest factor difference for each vineyard site and bioassay was, on average, 2.4 (standard deviation 1.8). Another expression denoting the general difference in toxicity is the Leaching/Ageing factor (L/A factor), defined as the difference in total soil metal concentration between field and spiked soils to elicit the same adverse effect (e.g. 10% inhibition; Smolders et al., 2009). In the absence of toxicity in the field samples (as found here), the added $C_{\max \text{ corr}}$ was used as a conservative estimate for the 10% effect level in the field. The L/A factors for each bioassay are summarized in Table 4. These L/A factors are in agreement with the L/A factors of a field gradient that was gradually contaminated with Cu containing wood preservation products (Hygum soil, SCHER, 2009). On average, the L/A factor was 3.2 and this is an underestimate of the true value because no toxicity is identified (Table 4). This value shows that, on average, 3.2 times larger Cu might be dosed in vineyards than in spiked soils to obtain 10% inhibition and is larger than the factor of 2.0, which is proposed in the generic risk assessment of Cu in soil (SCHER, 2009). These leaching/ageing effects were apparently more pronounced in vineyards than assumed before in the risk assessment of Cu in soil. Metal concentrations in these vineyard

soils are indeed well above the PNEC values for these specific vineyard soils calculated according to the EU Risk Assessment on Cu (103 - 126 mg Cu kg⁻¹, details not shown).

Table 4: Overview of the L/A factors calculated for each bioassay among all vineyard sites. These factors are minimal L/A factors, calculated as the ratio of the highest added Cu concentration in each vineyard site ($C_{\text{max corr}}$) and the ED10 value in the spiked soils, since no dose response curves and ED10 values were obtained for the vineyard sites.

Site	Tomato yield	Barley Root Length	PNR	<i>E. albidus</i> reproduction
1	>2.1	>4.7	>4.1	>1.7
2	>1.0	>14.5	>3.1	ND
3	>4.2	>1.4	>1.6	ND
4	>0.8	>1.5	>0.7	>6.5
5	>0.8	>1.4	>0.8	>0.7
6	>3.9	>5.5	>8.1	>2.0

ND: not determinable since the ED10 value in the corresponding spiked soil was lower than the lowest spiked dose, resulting in unreliable, large L/A factors

Previous studies detected negative effects in vineyard soils on plant growth (Brun et al., 2001; Chaignon and Hinsinger, 2003 and Michaud et al., 2007), microbial processes (Fernandez-Calvino et al., 2010) and earthworms (Eijsackers et al., 2005) most likely due to increased soil Cu (ranging from 200-1000 mg Cu kg⁻¹). As a precautionary approach, it is important to also focus here on the limited number of cases where significant effects in the vineyard have been found. A significant negative effect of Cu on tomato yield ($p < 0.05$, Dunnett test) was observed in the vineyard soil samples of site 3 (measured response 67-42% of the response of the control soil), which was comparable to the spiked soils with similar Cu concentration. However, tomato yields in all vineyard soil samples of site 3 are significantly lower than that of the control soil and no significant decrease of the tomato yield with increasing soil Cu concentrations was found within all the vineyard samples. The plant yield might be ascribed to lower soil N and OC content

compared to the control soil (40% decrease compared to the control soil, Supplementary Information Table 1) as these soil variables were correlating more to tomato yield than total soil Cu (r is 0.97 and 0.84, respectively). A similar analysis can be made for PNR (72-46% of the response of the control soil, correlated to lower pH ($r=0.99$) and OC content ($r=0.99$)) and for *E. albidus* reproduction (70-60% of the response of the control soil, correlated to lower N content ($r=0.87$)) in this vineyard site. In soil samples of site 4, significantly shorter barley root lengths ($p<0.05$) compared to the control soil were observed, effects being moderate (minimally 70 % of the response of the control soil). In the highest Cu contaminated vineyard soil sample, effects were similar as in spiked soils (Table 3). However, at lower Cu contamination in the vineyard, barley root length was similar to that of the highest contaminated vineyard soil sample and was even lower than the root length of the corresponding spiked soils. Therefore, this decrease is unlikely causally affected by Cu and might be ascribed to a higher pH in the vineyard soil samples (negatively correlated to the root length; $r=0.85$) or to soil texture differences. The *E. albidus* reproduction in samples of vineyard site 4 was significantly reduced ($p<0.05$) compared to that of the control soil at 290 and 440 mg added Cu kg⁻¹. However, no significant Cu toxicity ($p>0.05$) to *E. albidus* reproduction was observed at 418 mg added Cu kg⁻¹, again suggesting that other factors besides Cu only explain the negative effects. The negative effects may again be attributed to differences in soil pH which is more negatively correlated to *E. albidus* reproduction ($r=0.60$) than total soil Cu ($r=0.58$) between the control soil and the vineyard soil samples rather than to soil Cu concentrations only.

4.4. Conclusion

In conclusion, this survey did not identify significant effect of soil Cu on biological responses in vineyard samples, clearly in contrast with that in corresponding spiked soils at equivalent total Cu and even corrected for differences in eCEC between vineyard soil samples and spiked samples. Significant differences in toxicity to added Cu between field contaminated vineyard soils and spiked soils were observed, augmenting to a factor 3.2 on average among all sites and bioassays as calculated above. This is likely due to a decreased Cu bioavailability in vineyard soils compared to freshly added Cu as in spiked soils. Copper added during Cu fungicide applications might become less bioavailable due to incorporation into organic plant residues (e.g. Cu sprayed vine leaves). Such non-labile Cu is known to be present in sewage sludge (Donner et al., 2011), however the transformation of Cu in soils amended with Cu due to the use of Cu fungicides deserves dedicated chemical studies.

5. Acknowledgments

This study was funded by the European Union Copper Task Force. The authors would like to thank Lionel Ley and Alain Koeberle (INRA Colmar, France), Valérie Sappin-Didier (INRA Bordeaux, France), Barbara Agabiti, Enzo Mescalchin and colleagues (Istituto Agrario San Michele, Italy), Philippe Hinsinger, Patrick Andrieux and colleagues (UMR INRA-IRD-SupAgro, Montpellier, France), Beatrice Marin, Marie Ponthieu and colleagues (Université de Reims-Champagne-Ardenne, France), Christoph Emmerling and Raimund Schneider (University of Trier, Germany), René Ruyters and Helmut Scherf for assistance in finding and sampling contaminated vineyards. In addition, we would like to thank all farmers for their participation in this study, access to their vineyards and their assistance with sampling. Karla Moors and Kristin

Coorevits (Division Soil and Water Management, KU Leuven) are acknowledged for assistance in determining soil properties.

The proprietary owner of the data presented in this article is the European Union Copper Task Force. These data cannot be used for non-academic purposes. Permission to use this data for the approval of plant protection products containing copper is only granted on production of a Letter of Access provided by the European Union Copper Task Force.

6. References

Baroux J. Toxicity of copper on ammonification phenomena in soils of Bordeaux vineyards. *Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences Serie D* 1972; 275: 499-502.

Brun LA, Maillet J, Hinsinger P, Pepin M. Evaluation of copper availability to plants in copper-contaminated vineyard soils. *Environmental Pollution* 2001; 111: 293-302.

Chaignon V, Hinsinger P. A biotest for evaluating copper bioavailability to plants in a contaminated soil. *Journal of Environmental Quality* 2003; 32: 824-833.

Chhabra RJ, Pleysier J, Cremers AC. The measurement of the cation exchange capacity and exchangeable cations in soils: a new method. *Proceedings of the International Clay Conference* 1975; Mexico, Applied Publishing Ltd.

Delas J. Bonds between copper and organic matter in a podzol developed on sand of Landes and accidentally enriched in copper. *Annales Agronomiques* 1967; 18: 17-20.

Diaz-Ravina M, de Anta RC, Baath E. Tolerance (PICT) of the bacterial communities to copper in vineyards soils from Spain. *Journal of Environmental Quality* 2007; 36: 1760-1764.

Doelman P, Haanstra L. Short-Term and Long-Term Effects of Heavy-Metals on Phosphatase-Activity in Soils - an Ecological Dose-Response Model Approach. *Biology and Fertility of Soils* 1989; 8: 235-241.

Donner E, Howard DL, de Jonge MD, Paterson D, Cheah MH, Naidu R, et al. X-ray Absorption and Micro X-ray Fluorescence Spectroscopy Investigation of Copper and Zinc Speciation in Biosolids. *Environmental Science & Technology* 2011; 45: 7249-7257.

EC (European Commission) regulation 473/2002 amending Annexes I, II and VI to Council Regulation (EEC) No 2092/91 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs, and laying down detailed rules as regards the transmission of information on the use of copper compounds; 2002

Eijsackers H, Beneke P, Maboeta M, Louw JPE, Reinecke AJ. The implications of copper fungicide usage in vineyards for earthworm activity and resulting sustainable soil quality. *Ecotoxicology and Environmental Safety* 2005; 62: 99-111.

Fernandez-Calvino D, Soler-Rovira P, Polo A, Diaz-Ravina M, Arias-Estevéz M, Plaza C. Enzyme activities in vineyard soils long-term treated with copper-based fungicides. *Soil Biology & Biochemistry* 2010; 42: 2119-2127.

Giller KE, Witter E, McGrath SP. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. *Soil Biology and Biochemistry* 1998; 30: 1389-1414.

ISO 14238. Soil quality - Biological methods - Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes. G. International Organization for Standardization, Switzerland 1997.

ISO 11277. Soil quality - Determination of particle size distribution in mineral soil material - Method by sieving and sedimentation. G. International Organization for Standardization, Switzerland 1998.

ISO 11269-1 and 2. Soil quality - Determination of the effects of pollutants on soil flora – Part 2: Effects of chemicals on the emergence and growth of higher plants. G. International Organization for Standardization, Switzerland 2005.

Komarek M, Cadkova E, Chrastny V, Bordas F, Bollinger JC. Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. *Environment International* 2010; 36: 138-151.

Lehnart R, Michel H, Lohnertz O, Linsenmeier A. Root dynamics and pattern of 'Riesling' on 5C rootstock using minirhizotrons. *Vitis* 2008; 47: 197-200.

Lock K, Janssen CR. Influence of aging on copper bioavailability in soils. *Environmental Toxicology and Chemistry* 2003; 22: 1162-1166.

Mertens J, Springael D, De Troyer I, Cheyns K, Wattiau P, Smolders E. Long-term exposure to elevated zinc concentrations induced structural changes and zinc tolerance of the nitrifying community in soil. *Environmental Microbiology* 2006; 8: 2170-2178.

Michaud AM, Bravin MN, Galleguillos M, Hinsinger P. Copper uptake and phytotoxicity as assessed in situ for durum wheat (*Triticum turgidum durum* L.) cultivated in Cu-contaminated, former vineyard soils. *Plant and Soil* 2007; 298: 99-111.

Mirlean N, Roisenberg A, Chies JO. Metal contamination of vineyard soils in wet subtropics (southern Brazil). *Environmental Pollution* 2007; 149: 10-17.

OECD guideline220: Enchytraeid Reproduction Test, OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems. OECD Publishing 2004.
doi: 10.1787/9789264070301-en

Oorts K, Bronckaers H, Smolders E. Discrepancy of the microbial response to elevated copper between freshly spiked and long-term contaminated soils. *Environmental Toxicology and Chemistry* 2006; 25: 845-853.

Oorts K. Copper. In: Alloway B, editor. *Heavy Metals in Soils* (third edition). Dordrecht: Springer; 2012. p. 367-394

Parat C, Chaussod R, Leveque J, Dousset S, Andreux F. The relationship between copper accumulated in vineyard calcareous soils and soil organic matter and iron. *European Journal of Soil Science* 2002; 53: 663-669.

Provenzano MR, El Bilali H, Simeone V, Baser N, Mondelli D, Cesari G. Copper contents in grapes and wines from a Mediterranean organic vineyard. *Food Chemistry* 2010; 122: 1338-1343.

Renella G, Chaudri AM, Brookes PC. Fresh additions of heavy metals do not model long-term effects on microbial biomass and activity. *Soil Biology & Biochemistry* 2002; 34: 121-124.

Rombke J, Moser T. Validating the enchytraeid reproduction test: organisation and results of an international ringtest. *Chemosphere* 2002; 46: 1117-1140.

Ruyters S, Mertens J, Springael D, Smolders E. Stimulated activity of the soil nitrifying community accelerates community adaptation to Zn stress. *Soil Biology and Biochemistry* 2010; 42: 766-772.

SCHER. Voluntary risk assessment report on copper and its compounds, environmental part. 2009 (http://echa.europa.eu/chem_data/transit_measures/vrar_en.asp).

Smolders E, Brans K, Coppens F, Merckx R. Potential nitrification rate as a tool for screening toxicity in metal-contaminated soils. *Environmental Toxicology and Chemistry* 2001; 20: 2469-2474.

Smolders E, Oorts K, Lombi E, Schoeters I, Ma YB, Zrna S, et al. The Availability of Copper in Soils Historically Amended with Sewage Sludge, Manure, and Compost. *Journal of Environmental Quality* 2012; 41: 506-514.

Smolders E, Oorts K, van Sprang P, Schoeters I, Janssen CR, McGrath SP, et al. Toxicity of Trace Metals in Soil as Affected by Soil Type and Aging after Contamination: Using Calibrated Bioavailability Models to Set Ecological Soil Standards. *Environmental Toxicology and Chemistry* 2009; 28: 1633-1642.

Strawn DG, Baker LL. Speciation of cu in a contaminated agricultural soil measured by XAFS, mu-XAFS, and mu-XRF. *Environmental Science & Technology* 2008; 42: 37-42.

Wightwick AM, Mollah MR, Partington DL, Allinson G. Copper fungicide residues in Australian vineyard soils. *Journal of Agricultural and Food Chemistry* 2008; 56: 2457-2464.

Wightwick AM, Salzman SA, Reichman SM, Allinson G, Menzies NW. Inter-Regional Variability in Environmental Availability of Fungicide Derived Copper in Vineyard Soils: An Australian Case Study. *Journal of Agricultural and Food Chemistry* 2010; 58: 449-457.

7. Supplementary Information

Table 1: Overview of soil properties of the control (gray shaded) and vineyard soils (with increasing soil Cu) of the 6 gradients. Means and standard deviations of two analytical replicates (SD: standard deviation). Soil pH refers to the value before liming the soil for toxicity testing.

Site	Soil Cu	pH		eCEC		N		OC		
	mg Cu kg ⁻¹	SD		SD	cmol _c kg ⁻¹	SD	%	SD	%	SD
Site 1	23	0.2	7.38	0.01	17	1.22	0.17	0.01	2.09	0.09
	201	11	5.93	0.03	10	0.05	0.20	0.02	2.62	0.23
	425	32	6.83	0.03	22	0.35	0.26	0.03	3.77	0.11
	531	25	6.78	0.11	14	0.27	0.20	0.01	2.84	0.09
	689	65	6.88	0.004	23	1.26	0.28	0.01	4.03	0.08
Site 2	105	6	7.35	0.02	16	1.19	0.22	0.01	9.09	0.09
	194	6	7.41	0.03	18	0.14	0.25	0.01	11.18	0.01
	272	7	7.46	0.01	20	0.88	0.26	0.003	10.95	0.03
	425	8	7.34	0.00	35	0.50	0.37	0.03	9.79	0.004
	448	18	7.17	0.15	17	1.42	0.25	0.002	11.80	0.04
Site 3	65	2	7.22	0.01	29	0.03	0.40	0.01	5.87	0.04
	276	15	7.33	0.01	21	0.50	0.22	0.01	5.11	0.06
	335	7	7.22	0.02	21	1.57	0.21	0.001	5.07	0.10
	360	3	7.28	0.04	22	0.83	0.21	0.01	5.02	0.01
	418	1	7.34	0.01	21	0.48	0.19	0.04	4.47	1.20
	516	18	7.33	0.003	21	1.27	0.11	0.15	2.74	3.86

Table 1 continued

Site	Soil Cu		pH		eCEC		N		OC	
	mg Cu kg ⁻¹	SD		SD	cmol _c kg ⁻¹	SD	%	SD	%	SD
Site 4	74	21	5.73	0.00	11	0.90	0.38	0.02	3.48	0.02
	142	1	6.65	0.04	17	1.34	0.35	0.02	3.45	0.10
	304	10	6.75	0.00	19	0.65	0.43	0.04	4.24	0.19
	364	11	6.71	0.01	20	0.03	0.44	0.001	4.58	0.08
	491	39	6.76	0.01	19	0.49	0.42	0.04	4.06	0.001
	513	37	6.76	0.01	23	0.68	0.52	0.002	5.61	0.32
Site 5	105	5	6.50	0.01	7	2.15	0.24	0.002	2.08	0.15
	159	9	6.36	0.03	20	0.29	0.40	0.01	4.72	0.21
	237	10	6.93	0.005	16	0.89	0.51	0.01	5.80	0.04
	321	34	6.87	0.03	19	0.69	0.37	0.02	3.74	0.02
	376	16	6.83	0.00	20	1.14	0.40	0.01	4.02	0.16
	435	47	6.87	0.01	19	0.47	0.39	0.01	3.93	0.07
Site 6	66	3	7.67	0.01	15	0.03	0.08	0.02	5.85	0.20
	155	14	7.55	0.02	15	0.75	0.10	0.02	6.94	0.10
	187	12	7.58	0.01	17	1.24	0.11	0.01	6.63	0.12
	328	38	7.41	0.01	18	0.37	0.30	0.02	8.65	0.03
	389	17	7.45	0.01	18	0.39	0.21	0.01	7.11	0.05
	397	3	7.37	0.01	15	0.69	0.25	0.03	7.58	0.18
	455	50	7.33	0.01	19	0.41	0.24	0.01	7.34	0.05