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Effect of different Cu based fungicides on microbial biomass, microbial community structure and bacterial community tolerance to Cu

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ABSTRACT:

The effect of Cu on three different microbial endpoints was studied comparing pure Cu salts and commercial Cu fungicides. Cu additions caused similar dose-response curves of substrate induced respiration (SIR) decreases regardless of Cu source, i.e. the use of pure Cu salts to estimate the effect of Cu fungicides on bacterial biomass using SIR may be useful. Phospholipid fatty acid (PLFA) analysis showed that Cu source was more relevant for the bacterial community structure than Cu concentration; thus, the use of Cu salts to infer the effects of Cu fungicides on microbial community structure via PLFA analysis isn´t recommended. Pollution induced community tolerance (PICT) to Cu analysis showed that the use of pure Cu salts may overestimate Cu effects if Cu salt additions modified the soil pH. Therefore, the use of pure Cu salts to estimate the Cu fungicides effects on soil microbes isn´t correct for PLFAs analyses, not suitable for PICT at high Cu concentrations, while allowed for SIR although not recommendable.

Keywords: commercial Cu fungicides, pure salt copper, PLFA, SIR, PICT

1. Introduction

One of main sources of Cu in agricultural soils is the use of Cu-based fungicides (Epstein et al, 2001; Merrigton et al, 2002). In vineyard soils, these fungicides are used against diseases such as mildew. Consequently, high Cu accumulations are commonly found in vineyard-devoted soils (Komarek et al, 2008). Copper accumulated in soils may be toxic for soil microbial communities (Lebrun et al, 2012; Mackie et al, 2013), which may be affected by Cu pollution by many ways, including reductions in biomass and changes in microbial community structure (Tang et al., 2019). Also, soil pollution by Cu can induce increases in the bacterial community tolerance to Cu (Berg et al., 2012; Fernández Calviño et al., 2012). Laboratory and field experiments clearly show effects of Cu additions to soils on microbial biomass (Bogomolov et al., 1996; Knight et al., 1997), microbial community structure (Frostegård et al., 1993) or bacterial community tolerance to Cu (Díaz-Raviña and Bååth, 1996; Brandt et al., 2010). However, these experiments, as most of the experiments on the effects of heavy metals on microbial communities, were performed using pure Cu salts such as $Cu(NO_3)_2$ or $CuSO_4$. Adding pure Cu salts to soil may highly reduce soil pH and thus affect microbial communities not only by the toxicity of Cu, but also by changing the soil pH and thereby increasing Cu availability in the soil (Degryse et al., 2009). Therefore, the use of pure Cu salts in the experiments may lead to wrong conclusions since pure Cu salts are not generally used as fungicide in wine growing practice.

In a previous study comparing the effects of Cu on soil microbial activity (respiration, and bacterial and fungal growth; Vázquez-Blanco et al., 2020) important differences on the effects of pure Cu salts and commercial Cu fungicides were found. Generally, the negative effects of Cu were higher using pure Cu salts than using commercial Cu

fungicides. However, effects of pure Cu salts and commercial Cu fungicides differed depending on the endpoint used. Thus, the general bacterial growth response pattern as function of Cu concentrations was similar for all Cu sources, with only differences in the magnitude of Cu toxicity. On the other hand, the general basal soil respiration and fungal growth response pattern were significantly different between pure Cu salts and commercial Cu fungicides. Therefore, similar studies should be extended to other endpoints in order to have a more general view about possible differences in their response.

The aim of this work thus was to study the toxicity of pure copper salts [$Cu(NO_3)_2$ and $CuSO_4$] and commercial Cu fungicides (used by farmers in vineyards), on different aspects of the microbial community in soil: on microbial biomass (using substrate induced respiration, SIR), microbial community structure (using the phospholipid fatty acid (PLFA) pattern) and finally on the bacterial community tolerance to Cu (using the ^3H leucine incorporation method).

2. Materials and methods

2.1 Copper sources

Six different Cu sources were used, four commercial Cu-based fungicides [Ridomil Gold plus (RGP), Covicampo bordeless (BM), ZZ-Cuprocol (ZZ) and Oxicol-50 (OX)] and two pure copper salts [Copper Nitrate ($Cu(NO_3)_2$) and Copper Sulfate ($CuSO_4$)]. The characteristics of the six Cu sources were previously described in Vázquez-Blanco et al. (2020), and shown in Table S1 (Supplementary material).

2.2 Experimental design

The same vineyard soil and spiking procedure used previously by Vázquez-Blanco et al. (2020) was used in the present work. The soil was developed over schist and presents a loam texture (50% of sand, 37% of silt and 13% of clay), is slightly acid (pH 6.6), presents a total carbon content of 18 g kg^{-1} and a total Cu concentration of 29 mg kg^{-1}. The soil spiking with Cu procedure was as follows: dry soil was weighted in 64 polypropylene jars (50 g on each one), rewetted until 30% of water holding capacity to recover the microbial activity, and incubated at 22 ºC for 7 days. Then soil samples were spiked with Cu (in solutions) using the four types of commercial fungicides and the 2 pure Cu salts. Each Cu source was added to the soil (in duplicate) in 5 concentrations: 2, 4, 8, 16 and 32 mmol kg^{-1}. Also, 4 soil subsamples were used as controls without Cu additions, resulting in 64 soil microcosms with a final soil moisture of 60% of the water holding capacity. Subsamples were incubated with lids at room temperature, aerated frequently, and the moisture content was maintained at a constant level throughout the incubation period by adding water if necessary. Substrate induced respiration (SIR) was measured after 91 incubation days, PLFA analysis was performed after 30 and 110 days, while bacterial community tolerance to Cu and pH were estimated after 40 and 45 days respectively.

The addition of increasing Cu concentrations to the soil caused clear pH dose-response curves (decreasing soil pH as Cu concentration increased) for pure Cu salts, while changes in pH induced by increasing Cu concentrations of commercial Cu fungicides became negligible (Table S2, supplementary material; Vázquez-Blanco et al., 2020).

2.3. Substrate induced respiration (SIR)

We estimated the soil microbial biomass using the substrate-induced-respiration (SIR) method of Anderson and Domsch (1978). Briefly, 50 mg of glucose and talcum (4:1) were added to 2 grams of soil weighed in 20 mL glass vials, and then mixed by shaking

100 the vials with hand during 10 seconds. After 30 minutes, the headspace atmosphere was
101 purged with air and the vials sealed with crimp caps. Then, soil samples were incubated
102 for 2-4 hours at 22°C and finally the CO_2 in the head space was determined using a gas
103 chromatograph equipped with a methanizer and a flame ionization detector (FID).
104
105 *2.4. Phospholipid fatty acid (PLFA) analysis*
106 The microbial community structure was determined by phospholipid fatty acid (PLFA)
107 methodology (Frostegård et al., 1993a). Briefly, lipids were extracted from soil with a
108 chloroform:methanol:citrate buffer mixture (1:2:0.8 (v/v/v)) and separated into neutral
109 lipids, glycolipids and phospholipids using a pre-packed silica column. Then
110 phospholipids were subjected to a mild alkaline methanolysis and the fatty acid methyl
111 esters were identified by gas chromatography (flame ionization detector) by the relative
112 retention times of the fatty acids, using methyl nondecanoate (19:0) as internal standard.
113
114 *2.5. Bacterial community tolerance to Cu* (PICT)
115 Cu tolerance of the soil bacterial community was measured essentially according to Bååth
116 (1992) and Díaz-Raviña et al. (1994) with the modifications of Fernandez-Calviño and
117 Bååth (2016). Bacterial suspensions were extracted from soil samples using the
118 homogenization/centrifugation method. Then, in a 2 mL micro-centrifugation tube, 1.35
119 mL of bacterial suspension were mixed with 0.15 mL of 7 different concentrations of Cu
120 as $CuSO_4$ (between 3 x 10^{-3} and 3 x 10^{-7} M Cu final concentration) plus a control with
121 distilled water. Bacterial growth was then measured on each micro-centrifugation tube
122 using ^{3}H leucine (Leu) incorporation into bacteria (Bååth et al., 2001).
123 The bacterial community tolerance to copper was estimated as $logIC_{50}$, the logarithm of
124 the added concentration that resulted in 50% inhibition of growth. $LogIC_{50}$ was calculated

using a logistic model, $Y = c/[1-e^{b(X-a)}]$, where Y is the measured level of Leu incorporation, X is the logarithm of the concentration of Cu added to the bacterial suspension, a is the $logIC_{50}$, c the bacterial growth rate without added Cu, and b a slope parameter indicating the inhibition rate. A higher value of $logIC_{50}$ indicates a higher community tolerance, while a lower value indicates that Cu is more toxic to the community. The Pollution Induced Community Tolerance (PICT) was estimated as $\Delta logIC_{50}$ normalizing the effect of Cu pollution to the control subtracting $logIC_{50}$ in the unpolluted controls for each polluted soil sample.

2.6. Bacterial community tolerance to pH

The bacterial community tolerance to Cu was measured according Fernández-Calviño and Bååth (2010). Essentially it is the same procedure as for Cu tolerance, but instead 0.15 mL of Cu solutions, buffers with different pH values were used (4.0, 5.0, 6.0, 7.0 and 8.0). The results were fitted to the cardinal pH model (Rosso et al., 1995), to estimate bacterial community optimum pH after incubation with different Cu sources and concentrations. $A = A_{max}(pH - pH_{min})(pH - pH_{max})/[(pH - pH_{min})(pH - pH_{max}) - (pH - pH_{opt})^2]$ where A is the bacterial growth rate and pH is the pH of the bacterial suspension using the different buffers. pH_{min}, pH_{opt} and pH_{max} are the minimum, optimum and maximum pH for bacterial growth, and A_{max} is the bacterial growth at pH_{opt}.

2.7 Statistics

Mean ± SD values of the different analyzed variables are shown (n=3). Concentrations of all the individual PLFAs data, expressed as mole percentage and logarithmically transformed, were subjected to principal component analysis (PCA) to elucidate the main differences in the PLFA patterns. Correlation coefficients to different PC axes and soil

150 variables were calculated as simple correlations. SPSS 23.0 (IBM, Armonk, NY, USA)
151 was used for all statistical calculations.
152
153 **3. Results**
154 *3.1. Microbial biomass (SIR) response to Cu pollution*
155 Substrate induced respiration (SIR) decreased as a function of Cu concentrations after 91
156 days incubation. For the lowest Cu dose (2 mmol kg^{-1}), SIR decreased more than 5 % for
157 all copper sources, while for the highest dose, SIR decreased more than 40% for all copper
158 sources. Differences between Cu sources were thus minor, with no clear differences
159 between commercial Cu fungicides and pure Cu salts.
160
161 *3.2. Changes in the microbial community structure*
162 After 30 incubation days, PC1 explain 42% of the variance in the PLFA pattern, while
163 PC2 explain 21% (Fig 2a, b). The samples formed three different groups. Pure copper
164 salts, $Cu(NO_3)_2$ and $CuSO_4$, were grouped close to the control; another group was
165 Bordeaux mixture and Oxicol-50 samples, mainly differentiating along PC2. The last
166 group was composed by ZZ-cuprocol and RGP, with highest scores along PC1. Thus, the
167 type of Cu source seemed to be more important than the Cu concentrations of each sample
168 early after adding Cu.
169 After 110 incubation days, 24% and 19% of the variance in the PLFA pattern was
170 explained by PC1 and PC2, respectively but there were no clear grouping of samples in
171 relation to Cu sources or concentrations (Figure S3, supplementary material). However,
172 after 110 days scores along PC3 (explaining 14% of the variation) correlated to the
173 logarithm of added Cu ($r=-0.73$; $P<0.05$), irrespective of Cu source (Fig. 2c, d). For PC1
174 the most relevant PLFAs were (loadings within parenthesis): a15:0 (0.93), i15:0 (0.78),

i14:0 (0.78), i16:0 (0.74), i17:0 (0.67), 14:0 (0.65), 18:1ω9 (-0.75) and 18:1ω7 (-0.73), while for PC3 relevant PLFAs were br18 (0.81), 10Me17 (0.77), 10Me16b (0.75), br17 (0.69), cy17:0 (-0.65) and 16:1ω7 (-0.61).

3.3. Bacterial community tolerance to Cu as function of Cu source

For all Cu sources, there was a clear dose response curve between Cu concentration and bacterial community tolerance to Cu (Δ logIC_{50}), i.e, higher Cu concentrations caused increased bacterial community tolerance to Cu (Figure 3). However, the magnitude of these increases was different for the different Cu sources, especially for higher Cu concentrations. For lower Cu doses (2 and 4 mmol kg^{-1}), the increases in log IC_{50} were similar for all Cu sources. For higher doses (8, 16 and 32 mmol kg^{-1}) bacterial community tolerance to Cu increases were higher for pure Cu salts than for commercial Cu fungicides. Bacterial communities polluted with Cu $(NO_3)_2$ salt, greatly increase their tolerance to Cu. ΔLog IC_{50} for Cu $(NO_3)_2$ was 2.8 for 32 mmol kg^{-1} Cu dose, meaning that bacterial community tolerance to Cu increased more than 600 times at the highest concentration. In the case of $CuSO_4$, maximum Δlog IC_{50} was 2.5, equivalent to 300 times higher bacterial community tolerance to Cu for the highest Cu dose (32 mmol kg^{-1}) than for the control. For the highest Cu dose (32 mmol kg^{-1}), bacterial community tolerance to Cu increased 1.3 log units for CB, 1.9 for RGP, 2.0 for ZZ and 2.2 for OX. Thus, bacterial community tolerance to Cu for commercial copper sources increased around of 20 to 160 times for the highest Cu dose.

3.4. Optimum pH for bacterial community growth

In the control soil, the optimum pH was 6.6 (Figure 4). For all Cu sources, bacterial communities had optimum pH for growth around this values for 2, 4 and 8 mmol Cu kg^{-1} concentrations. For higher doses (16 and 32 mmol kg^{-1}), different Cu sources showed different results. Bacterial communities from soils polluted with pure Cu salts decreased optimum pH for growth to 4.5 for $Cu(NO_3)_2$ and 5.4 for $CuSO_4$ using 32 mmol Cu kg^{-1} Cu. The RGP copper source has a similar behaviour to pure copper salts, but a lower decrease, with an optimum pH for growth of 5.0 for the highest Cu dose. Bordeaux mixture (BM), ZZ and OX did not decrease optimum pH at highest two Cu doses.

4. Discussion

Results confirm that the effect of Cu on soil microbes may change as function of Cu source, but the magnitude of these changes vary among the different endpoint used, as was previously shown for bacterial and fungal growth (Vázquez-Blanco et al., 2020). These differences were previously attributed to modification in soil pH when Cu is polluted with pure Cu salts, which may increase Cu toxicity (Degryse et al., 2009) or directly affect microbial communities (Rousk et al., 2009).

Substrate-induced respiration (SIR) was affected by Cu concentrations, with low variations depending on Cu source, i.e. microbial biomass decreased due Cu pollution regardless of the type of copper source. In a previous work (Vázquez-Blanco et al, 2020), we observed that there is a difference between commercial copper sources and pure copper salts for bacterial and fungal growth. Bacterial growth decreased for both types of copper sources, although there was a greater decrease in pure copper salts. Fungal growth also decreased for commercial Cu sources, while for pure Cu salts and highest

concentrations fungal growth increased (Vázquez-Blanco et al., 2020). Due these opposing effects, it is possible that the general effect on microbial biomass became similar for all Cu sources. Also, Ramos-Vásquez and Zúñiga-Dávila (2008) determined that CO_2 production by microorganisms does not vary depending on pH. Since SIR basically is a respiration measure, the effect on SIR may be a pure effect of Cu. Therefore, the use of pure Cu salts to estimate the effect of Cu fungicides on microbial biomass via SIR determinations may be correct, with only minor overestimations of Cu toxicity.

The addition of RGP and ZZ altered the PLFA pattern in the short time (30 days) compared to the other sources, most likely because they present organic compounds in their chemical composition: metalaxyl-M and propylene glycol, respectively. Metalaxyl-M is biodegradable by soil microorganisms (Baker et al, 2010) and presents low toxicity to bacterial communities (Bermúdez-Couso et al, 2013). In the case of propylene glycol, it is also easily biodegradable by bacterial communities (French et al, 2001). Organic compounds may be an alternative source of carbon for bacterial growth, favouring growth of microorganisms with high C degradation capacity, which will change the PLFA pattern.

An effect of Cu concentrations on the PLFA pattern was only found in PC3 after 110 incubation days, accounting for only a minor part of the total variance. This is a surprising result since in previous works using only a pure Cu salt ($CuSO_4$), changes in the PFLA pattern was dependent of the Cu dose (Frostegård et al, 1993b). The highest loadings for individual PLFAs for PC3 after 110 incubation days were, however, attributed, among others, to PLFAs such as br17:0, cy17:0, 15:0, 17:0 or 10Me18:0, which are indicative of soil pollution by heavy metals (Frostegård et al., 1993b; Bååth et

al., 1998), suggesting that there were real effects on the PLFA pattern. Similar low effect of different Cu concentrations on the PLFA pattern was previously shown in field soils, where other factors such as pH or organic matter content were more important (Fernández-Calviño et al., 2010). Previous works also suggest that the PLFA pattern depended on pH variations (Deng et al., 2009; Rousk et al., 2010). After 110 incubation days, PC3 was also correlated with pH ($r=0.62$; $P<0.05$). This was attributed to PLFAs such as i16:0, 16:1ω5, 18:1ω7 and cy19:0, which all are indicative of changes in soil pH (Rousk et al., 2010). These results suggest that the PLFA methodology is not always adequate to study the effects of Cu on soil microorganisms when other potential confounding factors are variable (pH, or Cu source). Therefore, to infer toxicity using PLFA as an endpoint variable from studies with pure Cu salts may lead to wrong conclusions.

Bacterial community tolerance to Cu (PICT) data follow the general trend found in previous works, i.e. increases with increases in Cu pollution (Fernández-Calviño et al, 2011; Pennanen et al, 1996; Berg et al, 2012; Díaz-Raviña et al, 94; Díaz-Raviña and Bååth, 1996). However, important differences were found between pure copper salts and copper-based fungicides for high Cu doses. Pure copper salts increased bacterial community tolerance to Cu more than 600 times for $Cu(NO_3)_2$ and more than 310 times for $CuSO_4$, while commercial copper sources increased PICT between 20 to 160 times for 32 mmol kg^{-1} of Cu. This may be due to the fact that pure Cu salts have greater impact on soil pH (Table S2, supplementary material; Vázquez-Blanco et al, 2020), because decreases in soil pH cause increases in Cu availability and, therefore, in toxicity (Brand et al, 2010; Berg et al, 2012). These results indicated that the use of Cu salts to estimate the effect of Cu fungicides on bacterial community tolerance to Cu may overestimate the toxic effects when high concentrations of Cu induce high decreases in soil pH.

Pure copper salts caused a decrease in optimum pH for growth of the bacterial community, while copper based fungicides in most cases did not change optimum pH much. If we compare optimum pH with data of soil pH obtained in a previously work for the same soil, Cu sources and Cu concentrations (Vázquez-Blanco et al, 2020) a high correlation was found ($r=0.745$; $P<0.05$; Figure 5), agreeing with previous findings by Fernández-Calviño et al. (2011). This is because bacterial communities are under selective pH pressure, adapting to an optimal pH for growth close to the soil pH (Fernández-Calviño and Bååth, 2010). There is, therefore, a double adaptation, one because of the copper pollution and the other due to changes in soil pH caused by pure copper salts. Both these selection pressure will change the community composition. This fact may mask direct Cu effects when studying impacts of Cu on bacterial community related variables, like PLFA, using pure Cu salts instead Cu fungicides.

5. Conclusions

The use of pure Cu salts to study potential effects of Cu fungicides on soil microbes may lead to wrong conclusions because results may differ between experiments performed with pure Cu salts and commercial Cu fungicides. However, the quantitative and qualitative magnitude of these differences largely varied as function of the endpoint used. Thus, SIR experiments used as a bacterial biomass endpoint, showed similar results for pure Cu salts and commercial Cu fungicides. The results of the present work showed that the use of pure Cu salts isn´t recommended when PLFA methodology when is used to estimate the effect of Cu fungicides on soil microbes, showing also that this methodology isn't sensitive for Cu pollution evaluation. In the case of PICT (bacterial community tolerance to Cu), the use of Cu salts to estimate the effect of Cu fungicides on bacterial

community tolerance to Cu presented a similar pattern than when commercial Cu fungicides were used. However, their use may overestimate the effects of Cu when high concentrations of Cu induce high decreases in soil pH.

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419 Tables

420 **Table S1.** Characteristics of commercial cu-based fungicides and pure salt copper (Vázquez-Blanco et al., 2020)

Name	Provider	Description	CAS no	Chemical formula
Ridomil Gold plus (RGP)	Syngenta Agro SA O Porriño (SP)	Wettable powder 60% w/w copper hydroxide, 5% w/w metalaxyl-M, 10-30% w/w silica <10% w/w diatomaceous earth	70630-17-0 20427-59-2 7631-86-9 61790-53-2	$Cu_2(OH)_3Cl$ $C_6H_3(CH_3)_2[N(C_3H_5O_2)(C_4H_7O_2)]$ SiO_2
Covicampo bordeless (BM)	Agrides SA Paterna (SP)	Wetteable powder 20% WP bordeaux mixture	8011-67-0	$CuSO4_2.3Cu(OH)_2$
ZZ-Cuprocol (ZZ)	Syngenta Ago SA O Porriño (SP)	Concentrated suspension 50-70% w/w Copper oxychloride [ISO] 1-10% w/w Propylene glycol [USP:JAN]	1332-40-7 57-55-6	$CuCl_2.3Cu(OH)_2$ $C_2H_6O_2$
Oxicol- 50 (OX)	Insecticidas MAFA SL Castellón (SP)	Wettable powder 50% w/w Copper oxychloride [ISO]	1332-40-7	$CuCl_2.3Cu(OH)_2$
Copper Nitrate	Panreac Barcelona (SP)	Solid Copper(II) Nitrate 3-hydrate	10031-43-3	$Cu(NO_3)_2.3H_2O$
Copper Sulfate	Panreac Barcelona (SP)	Solid Copper(II) Sulfate 5-hydrate	7758-99-8	CuSO4 5H2O

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423 **Table S2.** Soil pH in water in response to Cu additions using 6 different Cu sources.

424 (adapted from Vázquez-Blanco et al., 2020)

Cu Source	**Cu concentration (mmol kg^{-1})**	**pH**
Control soil	0	6.6
$Cu(NO_3)_2$	2	6.3
$Cu(NO_3)_2$	4	6.2
$Cu(NO_3)_2$	8	6.0
$Cu(NO_3)_2$	16	5.6
$Cu(NO_3)_2$	32	4.8
$CuSO_4$	2	6.5
$CuSO_4$	4	6.4
$CuSO_4$	8	6.0
$CuSO_4$	16	5.8
$CuSO_4$	32	5.0
BM: Covicampo bordelés	2	6.6
BM: Covicampo bordelés	4	6.5
BM: Covicampo bordelés	8	6.6
BM: Covicampo bordelés	16	6.5
BM: Covicampo bordelés	32	6.6
OX: (Oxicol-50)	2	6.7
OX: (Oxicol-50)	4	6.6
OX: (Oxicol-50)	8	6.5
OX: (Oxicol-50)	16	6.5
OX: (Oxicol-50)	32	6.4
ZZ: (ZZ-Cuprocol)	2	6.6
ZZ: (ZZ-Cuprocol)	4	6.6
ZZ: (ZZ-Cuprocol)	8	6.6
ZZ: (ZZ-Cuprocol)	16	6.5
ZZ: (ZZ-Cuprocol)	32	6.6
RGP: (Ridomil Gold plus)	2	6.8
RGP: (Ridomil Gold plus)	4	6.7
RGP: (Ridomil Gold plus)	8	6.6
RGP: (Ridomil Gold plus)	16	6.5
RGP: (Ridomil Gold plus)	32	6.5

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Figures

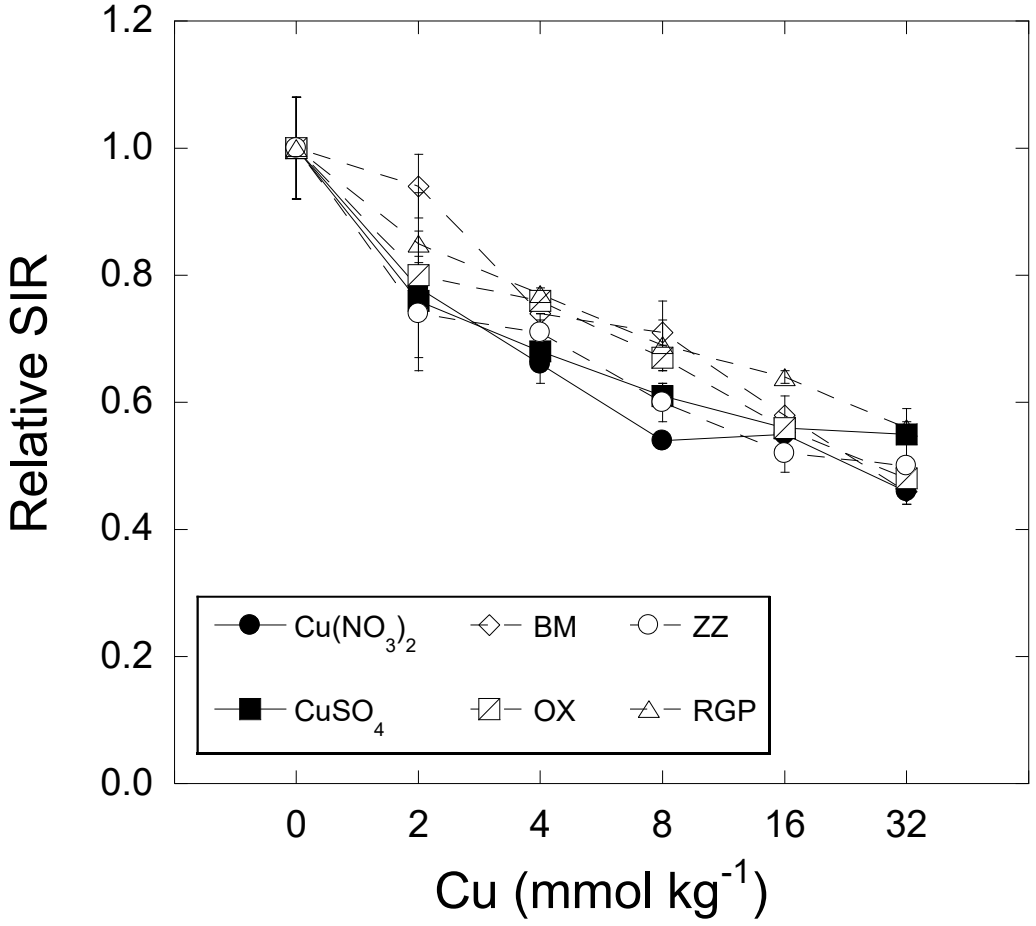


Fig 1. Relative Substrate Induced Respiration (SIR) after 91 incubation days in response to Cu additions using 6 different Cu sources. BM (Covicampo bordeless), OX (Oxicol-50) ZZ (ZZ-Cuprocol), RGP (Ridomil Gold plus). Bars denote SD (n=2).

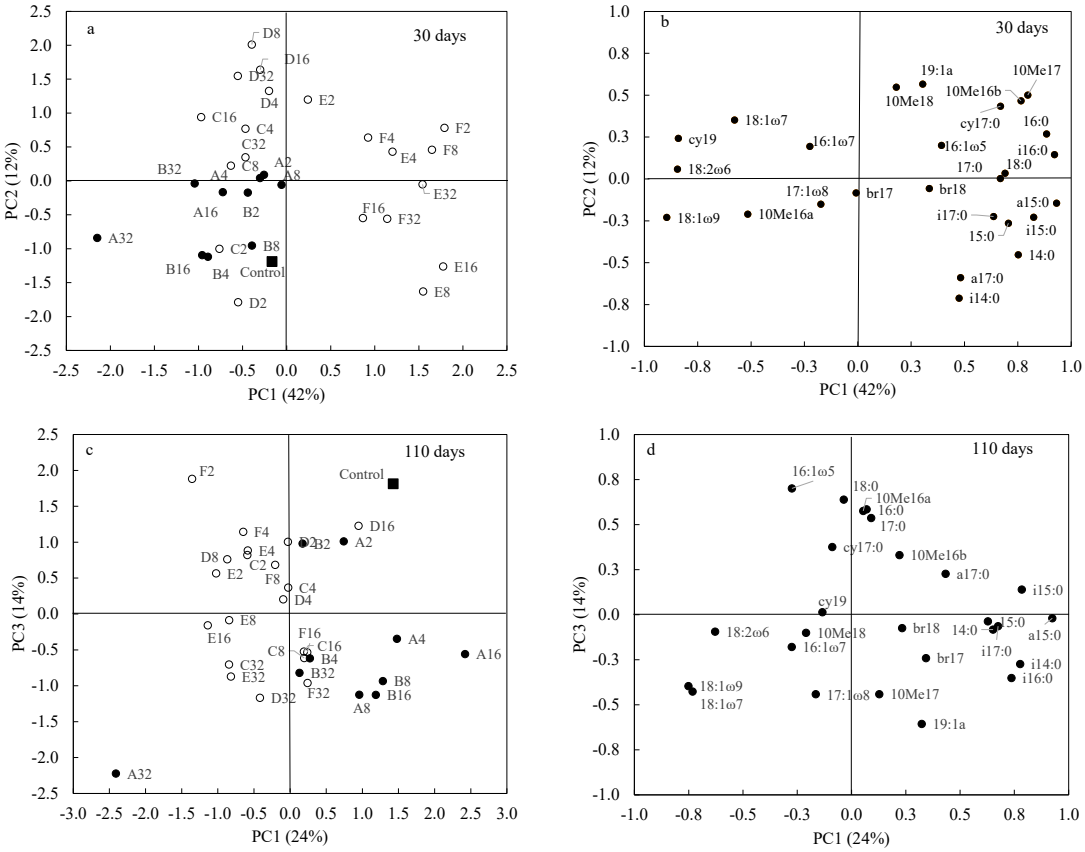


Fig 2. Principal component analysis of the PLFA pattern (after different incubation days) in a vineyard soil polluted with 6 copper sources: $Cu(NO_3)_2$ (A), $CuSO_4$ (B), BM (Covicampo bordeless) (C), OX (Oxicol-50) (D); ZZ (ZZ-Cuprocol) (E), and RGP (Ridomil Gold plus) (F). Numbers (0, 2, 4, 8, 16 and 32) indicate the Cu concentration in the soil (mmol kg^{-1}). (a, b) Scores of the different soil samples; (c, d) loadings of the different PLFAs.

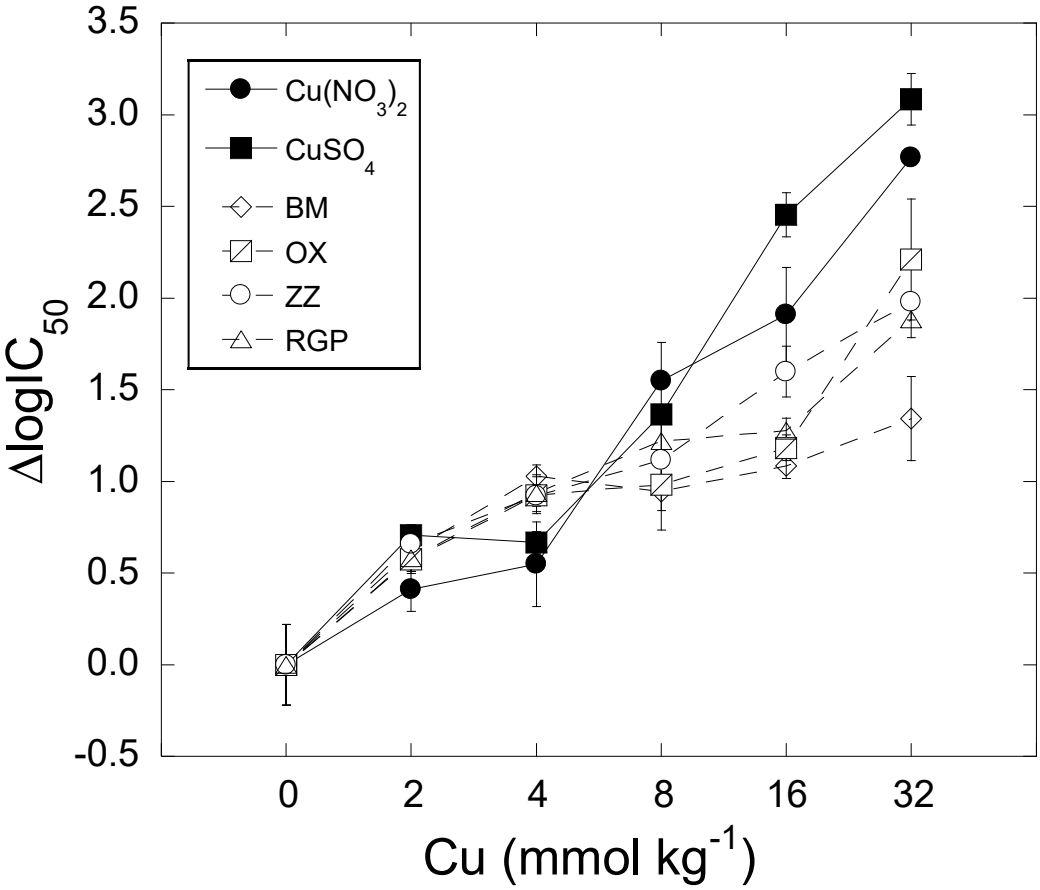


Fig 3. Increase in bacterial community tolerance to Cu ($\Delta logIC_{50}$) in response to soil Cu pollution with 6 copper sources after 40 incubation days: ($Cu(NO_3)_2$, $CuSO_4$, BM (Covicampo bordeless), OX (Oxicol-50), ZZ (ZZ-Cuprocol) and RGP (Ridomil Gold Plus).

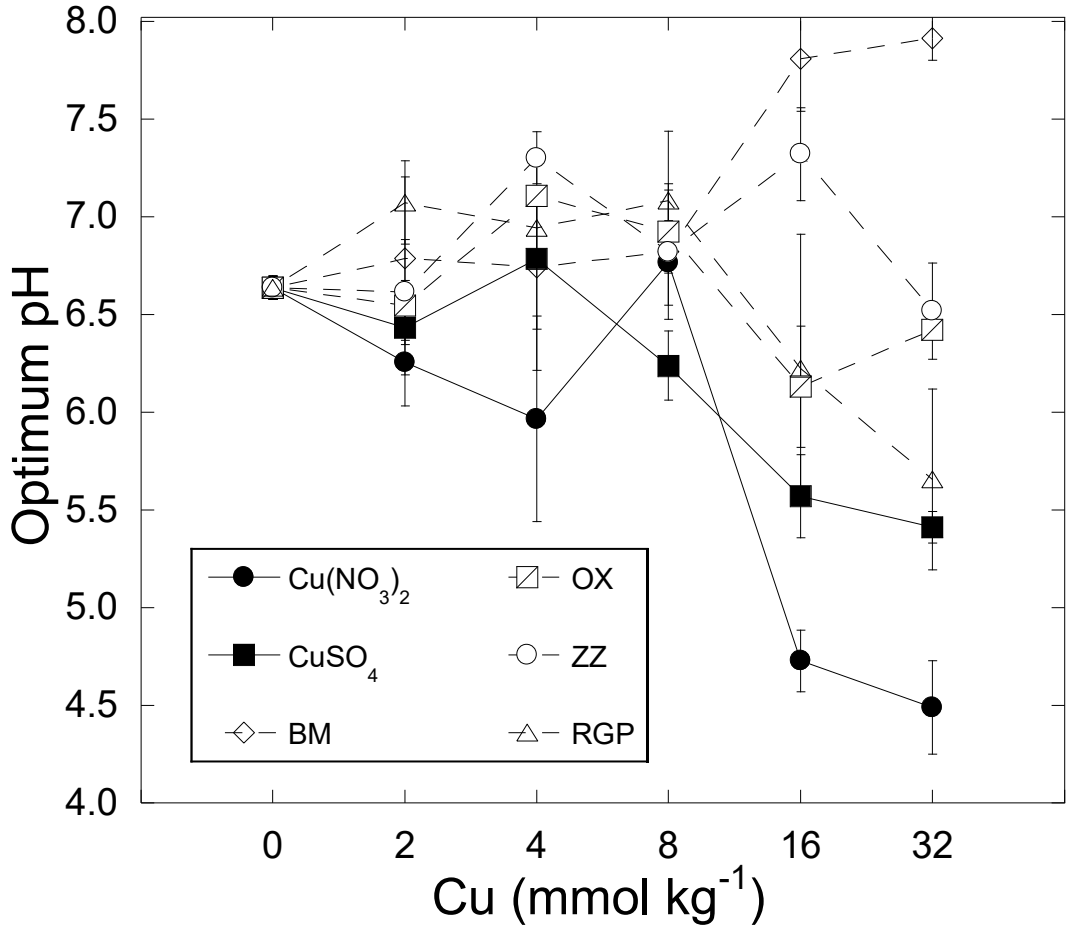
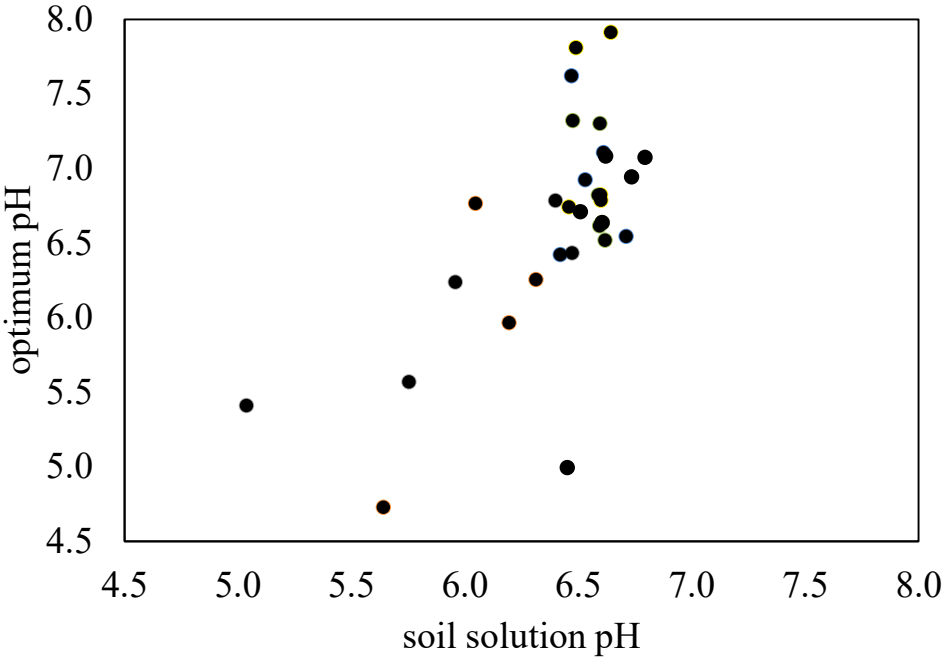


Fig 4. Relationship between optimum pH of bacterial communities and soil Cu concentration after 45 incubation days. The soil of a two years old vineyard was polluted with 6 copper sources ($Cu(NO_3)_2$, $CuSO_4$, BM (Covicampo bordeless), OX (Oxicol-50), ZZ (ZZ-Cuprocol) and RGP (Ridomil Gold Plus).



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472 **Figure 5.** Optimum pH of soil bacterial communities as function of the soil solution pH

473 caused by soil spiking with different Cu sources and concentrations

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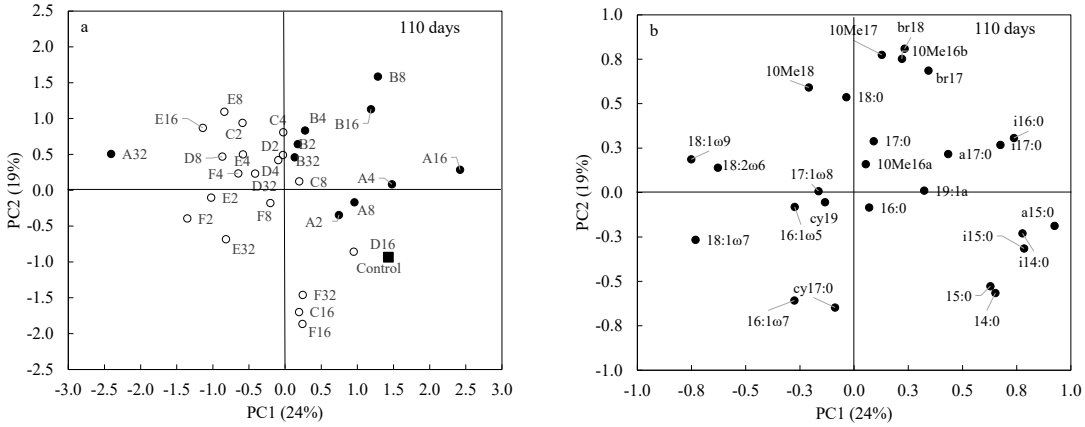

Fig S3. Principal component analysis of the PLFA pattern (after 110 incubation days) in a vineyard soil polluted with 6 copper sources: $Cu(NO_3)_2$ (A), $CuSO_4$ (B), BM (Covicampo bordeless) (C), OX (Oxicol-50) (D); ZZ (ZZ-Cuprocol) (E), and RGP (Ridomil Gold plus) (F). Numbers (0, 2, 4, 8, 16 and 32) indicate the Cu concentration in the soil (mmol kg^{-1}). (a) Scores of the different soil samples; (b) loadings of the different PLFAs.