Effect of copper on extracellular enzyme activities in apple orchard soils

Candidate: Michael Mitterer

Supervisors: Prof. Tanja Mimmo, Prof. Luciano Cavani, Dr. Giulio Genova

1. Introduction

Soil is a dynamic, living, natural body that plays an important role in the earth`s biosphere. Soil represents a medium for plant growth by supplying water, essential nutrients, physical support, and oxygen to roots (Doran and Parkin, 1994).

Soil contamination by heavy metals has raised concerns on soil quality in the past and still today. Both geogenic/lithogenic (Alloway, 2012; Marrugo-Negrete, Pinedo-Hernández and Díez, 2017) and anthropogenic (Lu *et al.*, 2012; Hou *et al.*, 2020) sources are responsible for heavy metal contamination of soils. Since the discovery of the antifungal activity of copper (Cu) compounds in 1885 by the French scientist Pierre-Marie Alexis Millardet, Bordeaux mixture (Cu sulfate) and Cu oxychloride (Jones and Jarvis, 1981) have been used against a wide range of fungal diseases (Lamichhane *et al.*, 2018). This has led to the accumulation of Cu in topsoil layers in both agroecosystems apple orchards (Genova *et al.*, 2021) and vineyards (Brunetto *et al.*, 2016). Numerous studies reported an increase of Cu concentrations worldwide after long term Cu use (Mirlean, Roisenberg and Chies, 2007; Wightwick *et al.*, 2008; Genova *et al.*, 2021).

Soil biological properties of soils are an early and responsive indicator to changes in land use and environmental contaminations (Doran and Parkin, 1994). Intensive agricultural management practices significantly affect soil biological properties such as enzyme activities (Fernández-Calviño *et al.*, 2010; Genova *et al.*, 2021). Enzyme activities play an important role in soil nutrient cycling rendering nutrients available to plants and microorganisms (Dick *et al.*, 2011). To our knowledge, information concerning the effect of Cu on soil biological properties of apple orchard is still scarce. To the best of our knowledge two previous studies evaluated

the effect of Cu on enzymatic activities in vineyard soils (Fernández-Calviño *et al.*, 2010; Wightwick *et al.*, 2013), due to the long time application of Cu based fungicides on these soils (Brunetto *et al.*, 2016). More specifically, information relating the effects of Cu on enzyme activities in orchard soils is lacking. To our knowledge there is only one study which assessed the effect of Cu in apple orchard soils, due to long term Cu accumulation (Wang, Zhou and Cang, 2009). In this study significant negative relationship was found between soil microbial biomass and soil Cu concentrations (total and $CaCl_2$-extractable soil Cu). Furthermore, major findings include that soil acid phosphatase in orchards was negatively influenced by high Cu concentrations, while urease (involved in N-cycling) and invertase (involved in C-cycling) activities are mainly affected by other soil chemical properties.

In South Tyrol, apple orchard soils show high concentrations of Cu in topsoil layers, inferring to their long-lasting vineyard land-use history (Genova *et al.*, 2021). Land use changes (from grassland and arable crops to permanent crops) occurred in the past within different timings (Tasser, Ruffini and Tappeiner, 2009; Genova *et al.*, 2021), explaining the high variability in Cu concentrations found in these soils. Especially vineyards contributed to the accumulation of Cu, whereas apple orchards contributed less (Genova *et al.*, 2021). However, by now no study assessed the toxic effect of Cu on enzyme activities on these soils. This study aimed at assessing the impact of long-term Cu accumulation in apple orchards in the Adige/Etsch, Passiria/Passeier and Venosta/Vinschgau Valley, South Tyrol, Italy on soil biological quality evaluating soil enzyme activities involved in the C- (exoglucanase, ß-glucosidase, exochitinase), N- (protease), P- (acid-phosphatase) and S- (arylsulfatase) cycling and quantity of DNA. In particular, considering a Cu gradient in 21 sampling sites in South Tyrol we aimed at assessing the correlation of Cu and important soil chemical properties (soil organic matter (SOM), pH in water, soil texture, zinc (Zn)) with soil enzyme activities and soil DNA applying linear regression models. Further, we aimed at determining an ecotoxicological threshold value of soil Cu concentration for enzyme activities.

2. Material and Method

2.1. Study area

This study covered agricultural soils cultivated with apples on the floors and sidehills of the Venosta/Vinschgau, Passiria/Passeier and Adige/Etsch valley, in the Province of Bolzano/Bozen, South Tyrol, Italy.

South Tyrol is located in the north-east of Italy and borders to Switzerland and Austria. It is on the southern site of the main alpine ridge. The area has a typical continental climate with low annual precipitation (450-850 mm). Mean air temperature in apple and grape growing sites is between 9.9 °C (Silandro/Schlanders), 12.3 °C (Bolzano/Bozen) and 10 °C (Bressanone/Brixen) (Hydrographic Office South Tyrol). From a topographic point of view, South Tyrol has a large elevation gradient, from 200 to 3900 m a.s.l. South Tyrol is Europe's largest, continuous apple growing region with over 18.000 ha. South Tyrol produces half of all apples in Italy and every tenth apple in the European Union comes from South Tyrol. Apples are grown from 200 m a.s.l. up to 1000 m a.s.l, from Salorno/Salurn to Malles/Mals (Venosta/Vinschgau Valley). Today 10 % are cultivated organically, whereas 90 % are cultivated with integrated pest management practices (South Tyrol Apple Consortium, 2019).

The prevalent soil types at hillsides are Leptosols and Cambisols. The soils in the valley floors are gleyic Cambisols, (partially calcaric) Fluvisols or Gleysols (Grashey-Jansen, 2010), with pH values around 6-7.3 for apple orchards (slightly acid to neutral) (Della Chiesa, Genova, Balotti, *et al.*, 2019)

2.2. Soil sampling and storage

The study was conducted on 21 apple-devoted soils in South Tyrol (Italy) (Figure 1.1). Sampling was performed in June 2020. Soil samples were taken from conventionally/integrated (n= 13 sites) and organically managed orchards (n= 8 sites). In each apple orchard soil samples were taken from the root zone of 5 adjacent trees in 3 rows (n= 15 samples) with a hand auger (Eijkelkamp Auger Edelman, ∅ 7 cm). Soil samples for chemical analysis were the result of mixing all 5 soil subsamples of one row (n= 3 per site). Samples were taken from the topsoil (0-20 cm). Big soil particles were crushed and pieces of plant material, roots and stones were removed. In addition, soil moisture was measured underneath each tree by TDR HydroSense II and averaged.

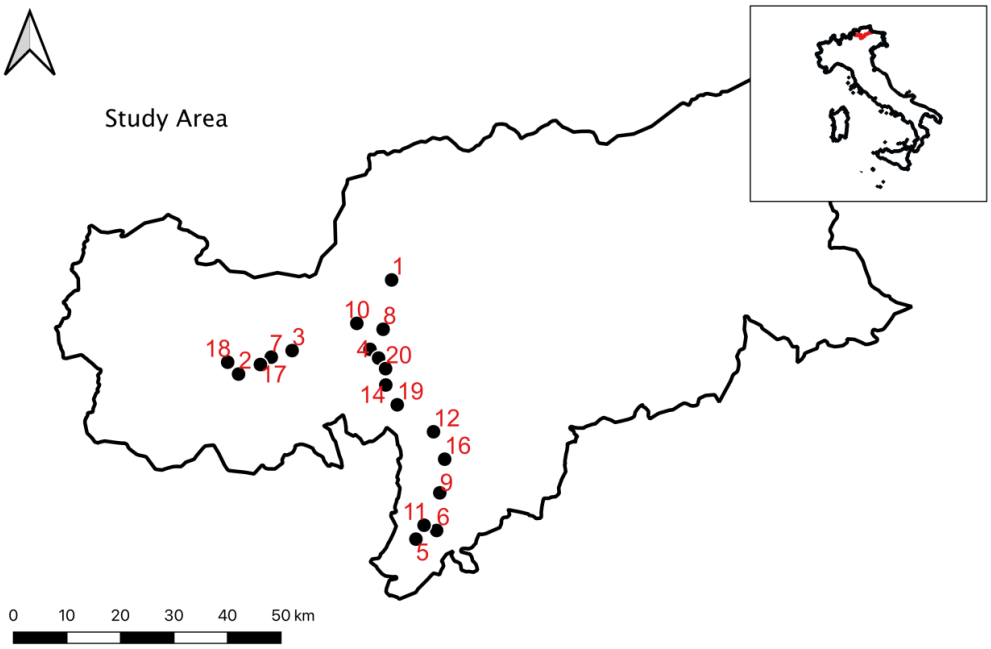

Figure 1.1 Study area, black dots represent sampling sites

Soil samples for DNA extraction and enzymatic analysis were maintained cool during transport to the lab. Soil samples for DNA extraction and enzymatic analysis were stored at -21 °C and then after thawn at 4°C for analysis. Enzymatic analysis was performed on each sample (n= 15 per site). Soil samples for chemical analysis (n= 3 per site) were air dried at ambient temperature and sent to a soil testing laboratory. Analysis was carried out by a routine soil testing laboratory (Ecorecycling Felderer) according to standardized procedures.

2.3. Soil chemical analysis

The soil type was determined by feel test (Thien, 1979) according to the German classification (AD-HOC AG Boden, 2005). pH was determined in 0.01 M $CaCl_2$ solution and deionized water with a calibrated pH-meter, at a soil to solution ratio of 1:2.5. Electrical conductivity (EC) was analyzed using a conductometer. Total carbon (TC), total nitrogen (TN) and total inorganic carbon (TIC) was assessed using an elemental analyzer (Thermo Scientific FlashEA® 1112 analyzer). All soil samples for TC and TN analysis were dried at 1400 °C prior to analysis. Soil samples for TIC were dried at 130 °C and determined with

phosphoric acid. The ammonium lactate method according to Egner-Riehm (K_{AL}, pH 3.75) (Egnér, Riehm and Domingo, 1960) was used to determine reserve Ca, Mg, K, P, B, Fe, Al content in soil (the suffix "res" is used to refer to this method). The plant available fraction of Mg, Fe, Cu, Mn, Zn and B was analyzed using 0.01 M $CaCl_2$ and 0.002 M DTPA (diethylenetriaminepentaacetic acid) solution, at a soil to solution ratio of 1:8 (subsequently CAT is used to refer to this method) (VDLUFA, 1991). Water soluble fraction of Na, P, K, Mg was determined in deionized water (soil to solution ratio 1:5). Elemental analysis of solution was conducted through ICP-AES for both reserve, water soluble and plant available fraction.

2.4. Enzyme assay

The potential hydrolytic enzyme activity of cellobiohydrolase ("exoglucanase") (Schwarz *et al.*, 1987; Clayssens *et al.*, 1989), acid phosphatase ("phosphatase") (Neumann, 1948; Grange, 1978), β-1,4-glucosidase (Dick, Opoku-Gyamfua and DeMarco, 1990; Sirová, Adamec and Vrba, 2003), ß-*N*-acetylglucosaminidase ("exochitinase") (Frouz *et al.*, 2003; Sirová, Adamec and Vrba, 2003), arylsulfatase (Chiba *et al.*, 1998) and leucine aminopeptidase ("protease") (Sirová, Adamec and Vrba, 2003) was measured fluorometrically according to Deltedesco et al. (2020) with minor modifications. Briefly, soil samples were analyzed 1-3 weeks after soil sampling. Soil suspension was prepared by placing 1g soil into a 150 mL beaker adding 100 mL of sodium acetate buffer (100 mM adjusted to pH 5.5 with acetic acid) to the soil. The soil suspension was homogenized using an ultrasonicator (Fisherbrand™, Schwerte, Germany) for 1 min at 35% amplitude. The soil suspension was withdrawn to a black micro plate well by continuous stirring. 200 mL were transferred to a black microplate well, having 3 replicates for each sample. Fluorometrically labelled (Methylumbelliferyl (MUF) and Amino-methylcoumarin (AMC)) substrates were used for the measurement of enzyme activities. The potential activity of N- acetyl-glucosaminidase ("chitinase" EC 3.2.1.52), cellobiohydrolase ("cellulase" EC 3.2.1.91), β-1,4-glucosidase ("cellulase" EC 3.2.1.21), acid phosphatase ("phosphatase" EC 3.1.3.2), arylsulfatase ("sulfatase" EC 3.1.6.1) and Leucin-aminopeptidase ("protease" EC 3.4.21) was determined by using 4-methyl-umbelliferyl-N-acetyl-β-D-glucosamine (CAS No. 37067-30-4), 4- methyl-umbelliferyl-β-D-cellobioside (CAS No.72626-61-0), 4-Methylumbelliferyl β-D-glucopyranoside (CAS No. 18997-57-4), 4-methylumbelliferly-phosphate (CAS No.3368-04-5), 4-Methylumbelliferyl sulfate potassium salt (CAS No. 15220-11-8) and L-leucine-7-amido-4-methyl coumarin (CAS No. 62480-44-8) as substrates respectively. 50 µL of substrate were pipetted into micro plate wells with 200 µL

of soil suspension. To set the calibration different concentrations of MUF and AMC were used. MUF was used for phosphatase, cellulase, sulfatase and chitinase activities, whereas AMC was used for calibration of protease activity. A soil specific calibration curve was developed for each assay. Buffer control, substrate control and sample control were used to adjust measured sample enzyme activities. The micro plate was than incubated for 3 hours at 30 °C in the dark. Relative fluorescence was measured at 365 nm extinction and 450 nm emission with a fluorescence spectrophotometer (Tecan Infinite F200 Fluorometer, Werfen, Austria).

2.5. DNA extraction

DNA extraction was accomplished with a DNA extraction kit according to the manufactures protocol (DNeasy PowerSoil Kit, Qiagen, Hilden, Germany). Stones, plant debris and roots were removed from soil samples before extraction. Briefly for DNA extraction, 0.25 g soil was added to PowerBead tubes provided by the manufacturer. DNA has been extracted from soil by beat beating the soil in lysis buffer. In a cleanup step lysate was then subjected to inhibitor removal, eliminating humic acid content leaving highly pure DNA. Purified lysate was then mixed with a DNA binding solution and passed through a silica spin filter membrane. The silica-bound DNA was washed in a two-step washing regime. The quantity of DNA (ng/µL) was measured using Qubit 4 Fluorometer (Fisherbrand™, Schwerte, Germany). DNA extraction measures the DNA of bacteria, fungi, nematodes, arthropods in soil.

2.6. Data analysis

Statistical analyses were performed using the open-source software R (version 4.0.5., 2021). Linear regression was performed in R software with the function “lm_model” to describe the value of the outcome variable Y (enzyme activity) based on more input predictor variables X (Cu_{CAT}, Zn_{CAT}, pH_{H2O}, SOM and soil texture). Shapiro Wilk test was performed to check if the dataset of the predictor variables is normally distributed. Cu_{CAT} and Zn_{CAT} data were log transformed before performing linear regression. All other soil chemical properties were not log transformed. Soil textural classes (categorical variable) were transformed into metric variables by assigning dummy variables (0,1) to the different classes (sandy loam and loamy sand). The input predictor variables were chosen based on previous literature that seem to influence enzyme activities. Diagnostic plots were created to check whether the assumptions made by the linear regression model are met or not (residuals vs fitted, normal Q-Q, scale location, residuals vs leverage). One site was excluded from arylsulfatase activity due to high

activities. In addition, added variable plots ("avPlots") were created in R to show the correlation between an independent variable and dependent variables, conditional on other independent variables. In addition, added variable plots ("avPlots") were created in R to show the correlation between an independent variable and dependent variables, conditional on other independent variables.

A simple linear regression ("lm_model") was used to assess the impact of Cu_{CAT} (independent variable) on quantity of DNA (dependent variable). For the ecotoxicological threshold value of soil Cu concentration for enzyme activities, enzyme activities were grouped according to their bioavailable Cu content. Difference between groups was tested by a non-parametric Kruskal Wallis test and in case of significant p value ($p < 0.05$) a Dunn's test was used to separate the means.

3. Results

3.1. Soil chemical analysis

The selected soils were quite different in terms of physical and chemical properties. The SOM values ranged from 2 to 8.75 %. The pH values in H_2O varied widely from 5.2 to 7.9 and ranged from slightly acidic to slightly alkaline. Average Zn_{CAT} concentration was 23.75 ± 16.54 mg/kg, with values ranging from 3.85 to 68.80 mg/kg soil. Average Cu_{CAT} concentration in soils was 108.29 ± 156.97 mg/kg soil, with values ranging from 8.18 to 641.08 mg/kg soil. Reserve Cu (Cu_{res}) concentrations ranged from 1.26 to 406.83 mg/kg soil with an average value of 53.69 ± 88.38 mg/kg.

3.2. Enzyme activities

Enzyme activities with min, mean, median, standard deviation, max and skewness values are shown in Table 1.1. The exoglucanase activity in the soils varied from 13.76 to 377.05 nmol $g^{-1}h^{-1}$. Mean value of exoglucanase activity was 132.11 ± 72.65 nmol $g^{-1}h^{-1}$ while ß-glucosidase activity ranged from 24.97 to 632.98 nmol $g^{-1}h^{-1}$. The mean value of ß-glucosidase activity in the studied soils was 289.37 ± 114.82 nmol $g^{-1}h^{-1}$. The exochitinase activity varied from 16.81 to 613.81 nmol $g^{-1}h^{-1}$. The mean value of exochitinase activitiy was 165.48 ± 83.41 nmol $g^{-1}h^{-1}$. The phosphatase activity in soils ranged from 75.5 to 1816.6

$nmol\ g^{-1}h^{-1}$ and protease activity ranged from 11.57 to 324.71 $nmol\ g^{-1}h^{-1}$. Mean phosphatase activity in soils was 783 ± 269.05 $nmol\ g^{-1}h^{-1}$. Mean protease activity was 82.06 ± 47.87 $nmol\ g^{-1}h^{-1}$. Arylsulfatase activity varied from 1.355 to 176.168 $nmol\ g^{-1}h^{-1}$ in soils with mean of 57.78 ± 35.14 $nmol\ g^{-1}h^{-1}$.

Table 1.1 Values of min, mean, median, standard deviation (sd), max and skewness for exoglucanase, ß-glucosidase, exochitinase, phosphatase, protease and arylsulfatase activity assessed in the 21 sampling sites (n= 315 samples)

Variable	min	mean	median	sd	max	skewness
Exoglucanse (nmol g-1h-1)	13.76	132.11	114.06	72.65	377.05	1.03
B_Glucosidase (nmol g-1h-1)	24.97	289.37	265.00	114.82	632.98	0.77
Exochitinase (nmol g-1h-1)	16.81	165.48	148.27	83.41	613.81	1.65
Phosphatase (nmol g-1h-1)	75.50	782.99	754.71	269.05	1,816.61	0.25
Protease (nmol g-1h-1)	11.57	82.06	69.18	47.87	324.71	1.95
Arylsulfatase (nmol g-1h-1)	1.35	57.78	55.07	35.14	176.17	0.99

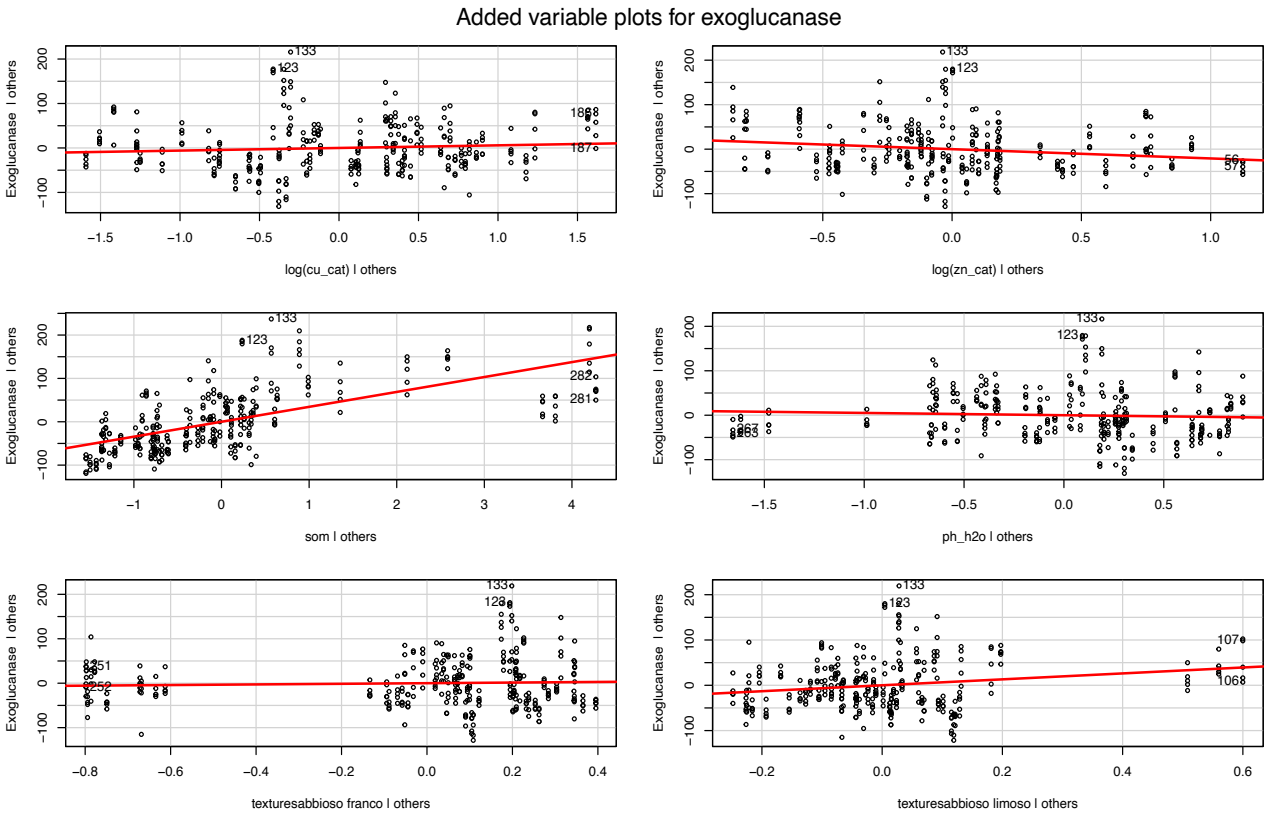

Figure 1.2 Correlation between the independent variables Cu_{CAT}, Zn_{CAT}, SOM, pH_{H2O}, sandy loam and loamy sand (n= 63 samples) and exoglucanase activity (n= 315 samples) (dependent variable) assessed in the 21 sampling sites conditional on the other independent variables graphically shown as added variable plots

The multiple linear model for exoglucanase activity showed significantly positive correlation with soil textural class sandy loam ($p < 0.001$) and SOM ($p < 0.001$) and significantly negative correlation with Zn_{CAT} ($p < 0.01$) and no significant correlation with pH_{H2O}, loamy sand and Cu_{CAT} ($p > 0.1$). The R^2 for the model resulted 0.4236. Added variable plots for exoglucanse activity are shown in Figure 1.2.

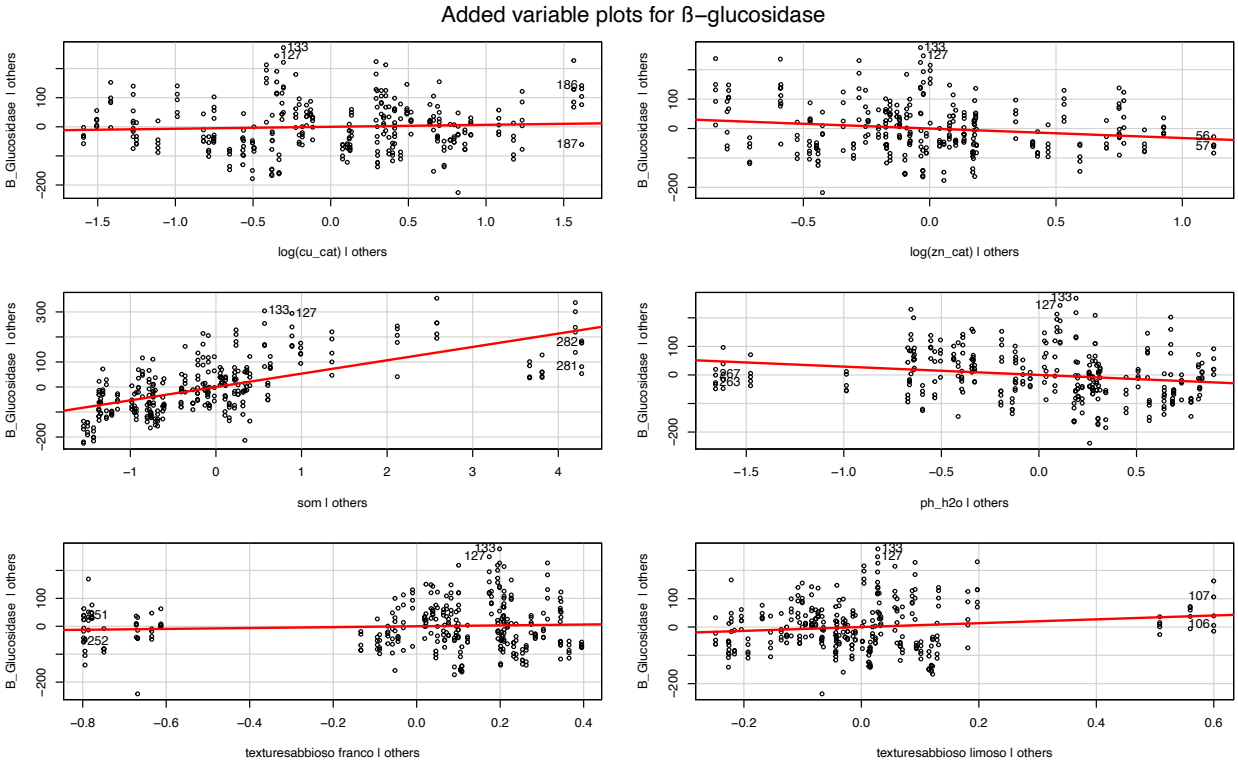

Figure 1.3 Correlation between the independent variables Cu_{CAT}, Zn_{CAT}, SOM, pH_{H2O}, sandy loam and loamy sand (n= 63 samples) and ß-glucosidase activity (dependent variable) determined in the 21 sampling sites (n= 315 samples) conditional on the other independent variables graphically shown as added variable plots

ß-glucosidase activity was positively related to SOM ($p < 0.001$) and soil textural class sandy loam ($p < 0.05$) and negatively related to Zn_{CAT} ($p < 0.01$) and pH_{H2O} ($p < 0.001$). No correlation was found with Cu_{CAT} and soil textural class loamy sand ($p > 0.1$). Correlations between ß-glucosidase activity and the above-mentioned soil properties showed a R^2 of 0.4594. Added variable plots for ß-glucosidase activity are shown in Figure 1.3.

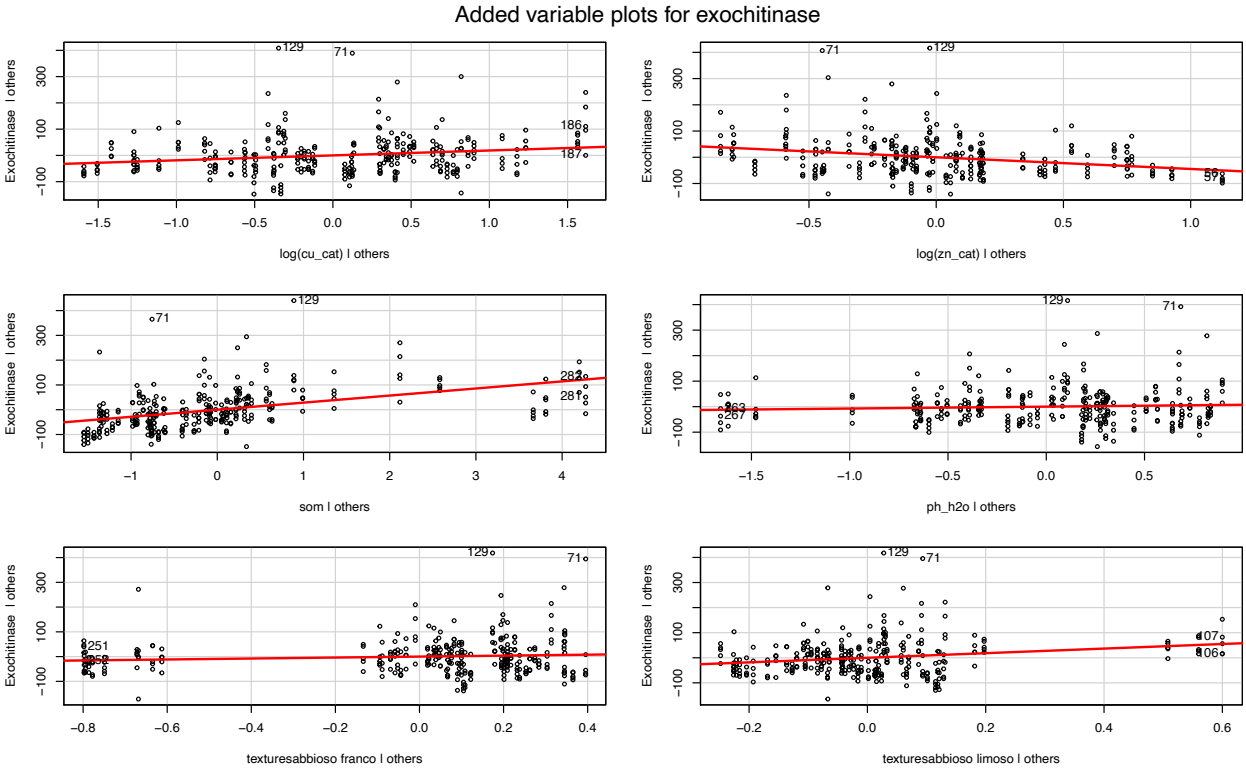

Figure 1.4 Correlation between the independent variables Cu_{CAT}, Zn_{CAT}, SOM, pH_{H2O}, sandy loam and loamy sand (n= 63 samples) and the dependent variable exochitinase activity (n= 315 samples) assessed in the 21 sampling sites, conditional on the other independent variables graphically shown as added variable plots

For exochitinase activity a significantly positive relationship with Cu_{CAT} ($p < 0.001$), SOM ($p < 0.001$) and sandy loam ($p < 0.001$) was observed. The analysis showed a significantly negative relationship for Zn_{CAT} ($p < 0.001$) and no significant relationship with pH_{H2O} and loamy sand ($p > 0.1$). R^2 for the exochitinase model resulted in 0.2586. Added variable plots for exochitinase activity are shown in Figure 1.4.

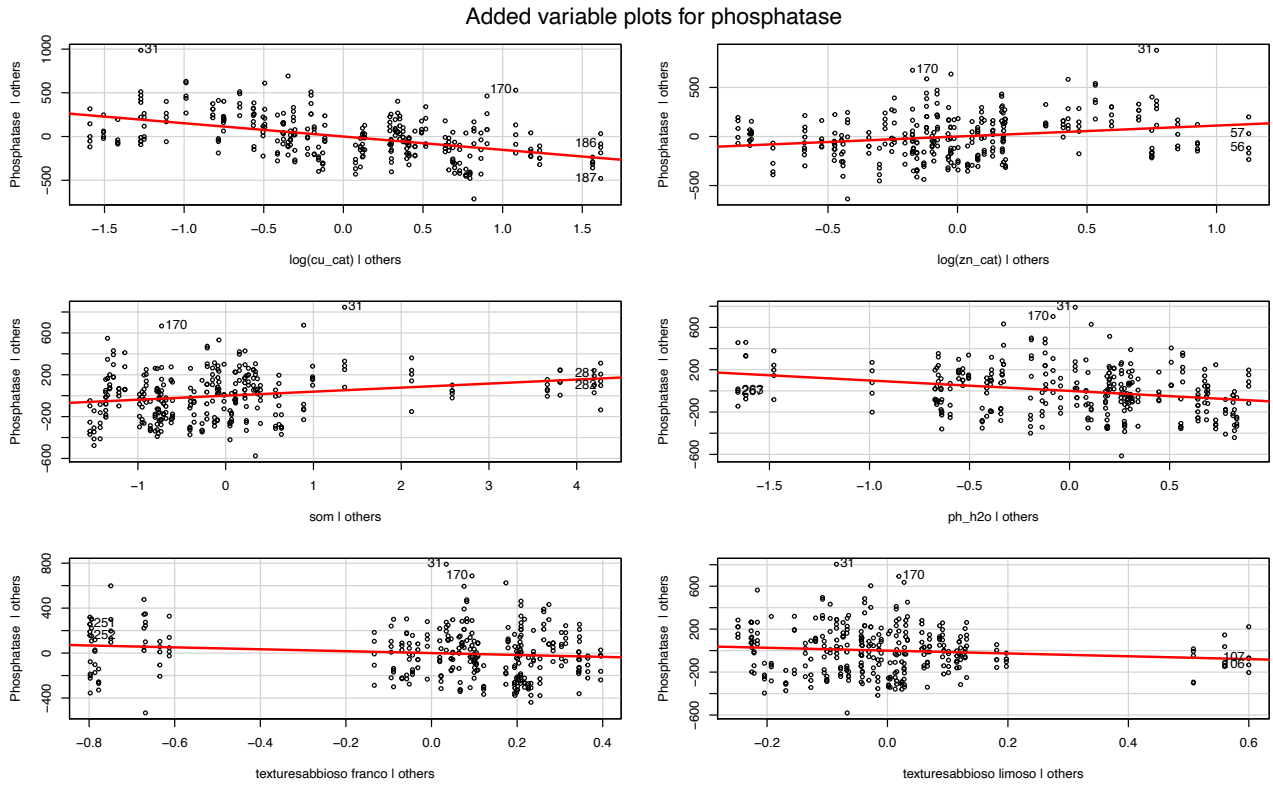

Figure 1.5 Correlation between the independent variables Cu_{CAT}, Zn_{CAT}, SOM, pH_{H2O}, sandy loam and loamy sand (n= 63 samples) and phosphatase activity (n= 315 samples) (dependent variable after controlling for the presence of the other independent variables) determined in the 21 sampling sites graphically shown as added variable plots

Phosphatase activity showed significantly positive correlation with Zn_{CAT} ($p < 0.001$), SOM ($p < 0.001$), while significantly negative correlation has been shown for Cu_{CAT} ($p < 0.001$), pH_{H20} ($p < 0.001$) and soil textural classes sandy loam ($p < 0.1$) and loamy sand ($p < 0.05$). R^2 for phosphatase activity was 0.3651. Added variable plots for phosphatase activity are shown in Figure 1.5.

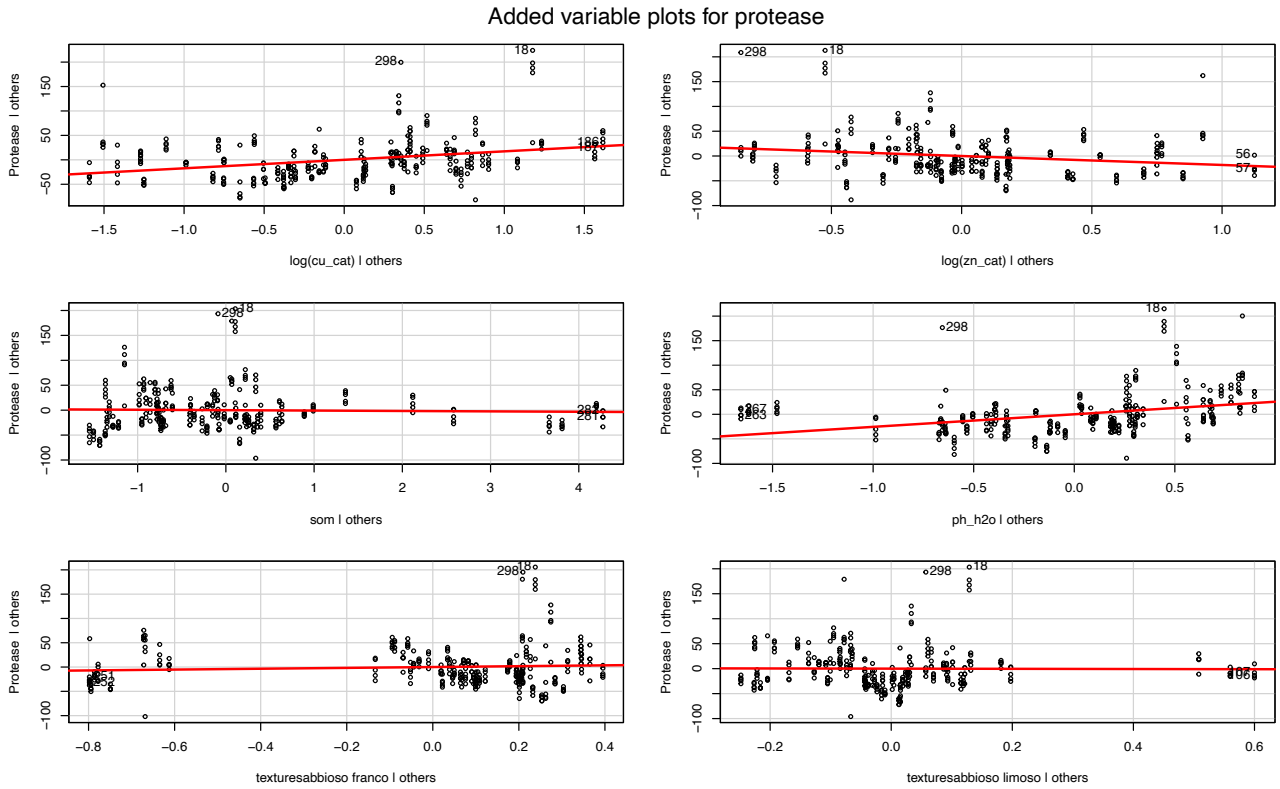

Figure 1.6 Correlation between the independent variables Cu_{CAT}, Zn_{CAT}, SOM, pH_{H2O}, sandy loam and loamy sand (n= 63 samples) and protease activity (n= 315 samples) (dependent variable) assessed in the 21 sampling sites, conditional on the other independent variables graphically shown as added variable plots

The linear model for protease activity showed significantly positive correlation with Cu_{CAT} ($p < 0.001$) and pH_{H2O} ($p < 0.001$) while significantly negative correlation for Zn_{CAT} ($p < 0.01$). No relationship has been shown for SOM and soil textural classes sandy loam and loamy sand ($p > 0.1$). The coefficient of determination (R^2) for this model was 0.2511. Added variable plots for protease activity are shown in Figure 1.6.

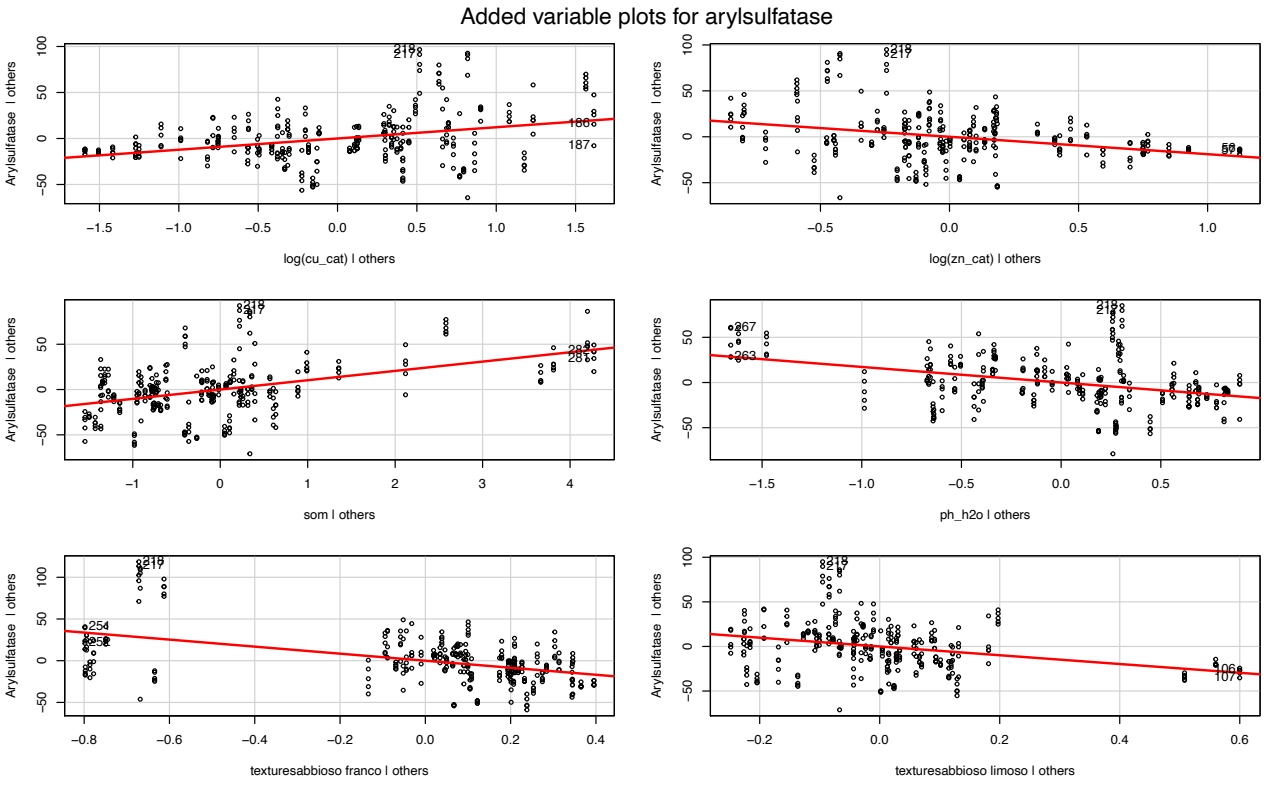

Figure 1.7 Relationship between the independent variables Cu_{CAT}, Zn_{CAT}, SOM, pH_{H2O}, sandy loam and loamy sand (n= 60 samples (20 sampling sites), site No. 9 excluded) and the dependent variable arylsulfatase activity (n= 300 samples (20 sampling sites), site No. 9 excluded), conditional on the other independent variables, graphically represented as added variable plots

Arylsulfatase activity showed significantly positive relationship with Cu_{CAT} ($p < 0.001$), SOM ($p < 0.001$), while it showed significantly negative relationship with Zn_{CAT} ($p < 0.001$), pH_{H2O} ($p < 0.001$) and soil textural classes sandy loam ($p < 0.001$) and loamy sand ($p < 0.001$). R^2 for arylsulfatase activity was 0.5571. Added variable plots for arylsulfatase activity are shown in Figure 1.7.

The effect of Cu on enzyme activities can be summarized as follows: it has a significantly negative impact on phosphatase activity, while significantly positive effect of Cu on protease, arylsulfatase and exochitinase activity were determined.

3.3. DNA extraction

The DNA Qubit quantity in soils varied from 1.37 ng/µL to 57.60 ng/µL. Average DNA concentration in soils was 24.94 ± 9.42 ng/µL. Minimum, maximum, mean, median, standard deviation and skewness values for quantity of DNA per mg of soil and per µL of extractant after accomplishing DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) are reported in Table 1.2.

Table 1.2 Values of min, mean, median, standard deviation (sd), max and skewness for the quantity of DNA obtained from Qubit measurement "DNA (ng/µL)" and quantity of DNA per unit of soil "DNA (ng/mg)" assessed in the 21 sampling sites (n= 315 samples)

Variable	min	mean	median	sd	max	skewness
DNA (ng/mg)	0.55	9.97	9.18	3.76	23.01	1.2
DNA (ng/µl)	1.37	24.94	23.00	9.42	57.60	1.2

The linear model of the output of the Qbit for DNA in ng/µL showed no correlation with Cu_{CAT} ($p > 0.1$). R^2 for the whole model is 0.0003006. The equation obtained was DNA quantity = 0.1350 * log(Cu_{CAT}).

3.4. Ecotoxicological threshold value calculation

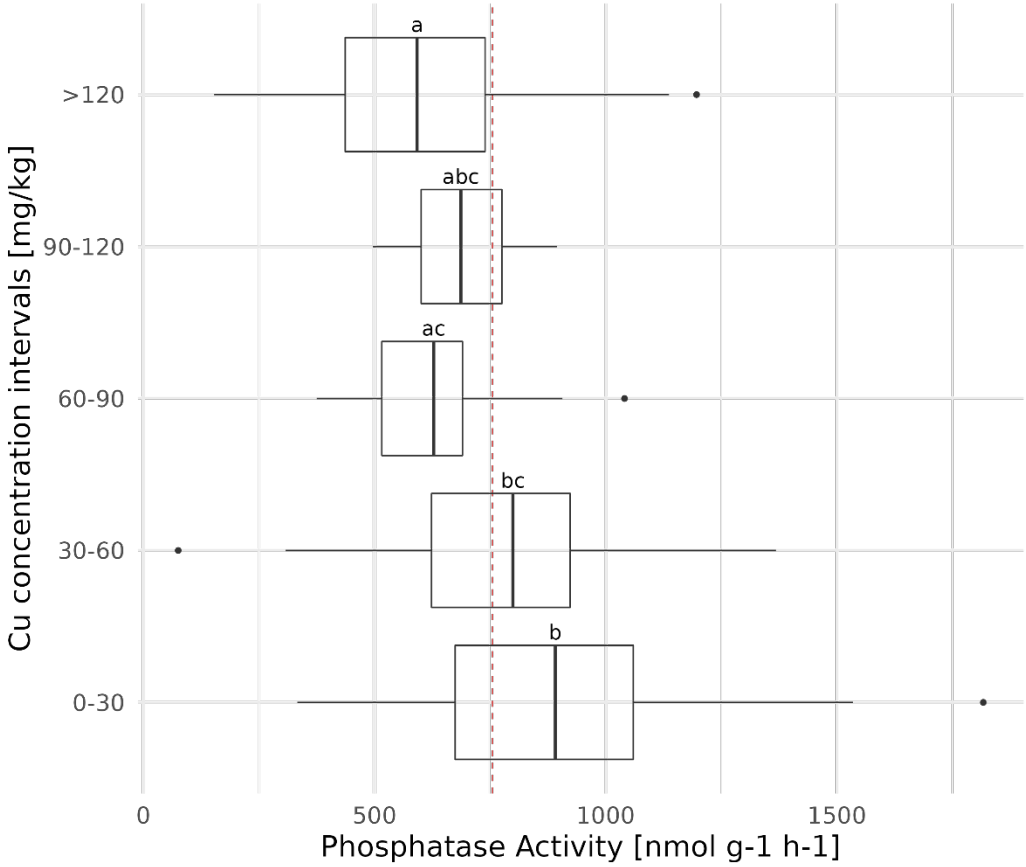

Figure 1.8 Boxplot representation of phosphatase activity grouped according to their Cu_{CAT} content. Red dotted line represents the overall median of all groups (754.71 nmol g-1 h-1), results of Dunn`s test are indicated with letters above boxplots, different letters indicate difference among groups (n(0-30)= 155 samples, n(30-60)= 70 samples, n(60-90)= 10 samples, n(90-120)= 15 samples, n(>120)= 65 samples)

Group 0-30 mg Cu_{CAT}/kg and group 30-60 mg Cu_{CAT}/kg soil differ significantly from group >120 mg Cu_{CAT}/kg ($p < 0.001$). All other groups did not show a clear difference ($p > 0.01$). However, an effect of Cu_{CAT} on phosphatase activity can be seen at values of Cu_{CAT} between 60 and 120 (Figure 1.8). In this range medians of groups are below the overall median, but the groups are not significantly different between each other.

4. Discussion

Our study area covered integrated and organic managed apple orchards on the valley floors and sidehills of the Venosta/Vinschgau, Passiria/Passeier and Adige/Etsch Valley in South Tyrol, which have undergone land use changes (from grassland and arable crops to permanent crops) that occurred in the past within different timings (Tasser, Ruffini and Tappeiner, 2009; Genova *et al.*, 2021) . The great variability of pH, SOM, soil texture and other soil chemical properties in our study area are due to different postglacial evolution, geomorphological processes and anthropogenic influences (Della Chiesa, Genova, Balotti, *et al.*, 2019). pH had an overall low variability (range from 5.2 to 7.9 measured in water), indicating the effect of liming used to control pH in intensive agriculture (Bogunovic *et al.*, 2017). The overall positive effect of SOM on enzyme activities (Figure 1.2-1.7) found in our study is in accordance with previous studies (Sinsabaugh *et al.*, 2008; Aponte *et al.*, 2021) and is based on significantly positive correlation of SOM on enzyme activities, found in the multiple regression model. Our data clearly suggest that all enzyme activities except protease activity, which showed no significant correlation with SOM (Figure 1.6), are positively correlated to SOM. These patterns resemble recent findings of Sinsabaugh et al. (2008) where organic matter did not significantly correlate with leucine aminopeptidase activity. It is well known that organic C is important in the growth of soil microorganisms (D'Ascoli *et al.*, 2006) and serves as substrate for enzymes (Dick *et al.*, 2011).

In soil Cu is mainly found in the topsoil layer (Genova *et al.*, 2021) and bound to soil organic matter (Hough, 2010; Brunetto *et al.*, 2016). It is hypothesized that in field situations SOM can mask the negative effect of Cu on many soil enzymes (D'Ascoli *et al.*, 2006), by forming stable complexes with SOM, thus reducing the bioavailable fraction of Cu found in soil (Laurent *et al.*, 2020).

In our study some apple orchards had relatively high Cu_{CAT} concentrations of up to 641.08 mg Cu_{CAT}/kg soil, inferring to their long-lasting vineyard land-use history (Genova *et al.*, 2021). The values obtained for available Cu in our study far exceed the reference values for available Cu, that are 4 mg kg^{-1} in our study area (Genova *et al.*, 2021). The bioavailable Cu obtained in our study ranged from 8.18 to 641.08 mg Cu_{CAT}/kg soil and are 2- to 160-fold higher than the reference values obtained by Genova et al. (2021). In vineyards $CuSO_4$ (Bordeaux mixture) has been long applied (Brunetto *et al.*, 2016), against downy mildew and other fungal and bacterial diseases (Lamichhane *et al.*, 2018), thus contributing mainly to the accumulation of Cu into topsoil layers, whereas apple orchard management practices contribute less to Cu

accumulation into topsoil layers (Genova *et al.*, 2021). Genova et al. (2021) estimated in our study area a linear accumulation rate of available Cu between 19.4 and 41.3 mg/kg·10 y^{-1}, while for apple orchards this value was between 2.8 and 3.6 mg/kg·10 y^{-1}. Copper accumulation in topsoil layers is related to agricultural pest management practices in vineyards and apple orchards with the use of Bordeaux mixture and Cu oxychloride (Jones and Jarvis, 1981) as well as fertilizers and manure (Alloway, 2012). High Cu_{CAT} values in our study were especially found in the valley floors and sidehills of the Adige/Etsch valley (data not shown). The conversion to permanent crops (vineyards and apple orchards) in the valley floors of the Adige/Etsch began earlier than in the Venosta/Vinschgau Valley (Genova *et al.*, 2021), explaining the high Cu concentrations found in some sites in this area. In the Venosta/Vinschgau Valley conversion to permanent crops started later, with no or little land dedicated to vine growing (Genova *et al.*, 2021).

In our case Cu_{CAT} had variable effects on enzyme activities, from no effect to an effect (negative to positive), depending very much on the enzyme studied (Figure 1.2-1.7). The linear model in our study revealed negative effect of bioavailable Cu on acid phosphatase activity, whereas positive effect on protease, arylsulfatase and exochitinase activity was observed.

In particular, acid phosphatase activities tended to decrease with increasing available Cu content (Cu_{CAT}) (Figure 1.5). These results agree with those reported by Fernández-Calviño et al. (2010) in vineyard soils exposed to gradual Cu accumulation. Similar effects of Cu on phosphatase activity in soils, long time exposed to Cu based fungicides, of apple orchards have been reported in a study of Wang, Zhou and Cang (2009). Copper can interact with serine and arginine residues in the phosphatase active site, therefore inhibiting acid phosphatase activity (Aponte *et al.*, 2020). Acid phosphatase is of particular importance due to their role in the soil P-cycle and it is an extracellular enzyme produced by a large number of soil microorganisms (Dick *et al.*, 2011). It is responsible for hydrolyzing organic P compounds to inorganic P (Megharaj *et al.*, 1999). Most frequently, P deficiency is related to limited P availability in soil and not to low P status. In South Tyrol P deficiency in apple orchards is rarely found (Della Chiesa, Genova, la Cecilia, *et al.*, 2019). A large proportion of apple orchards in South Tyrol exceed optimal values for bioavailable P and is due to the large scale use of P fertilizers (Della Chiesa, Genova, la Cecilia, *et al.*, 2019), probably limiting the negative effect Cu has on phosphatase activity. Optimal values for phytoavailable P are in the range of 120-200 mg P/kg soil measured with calcium acetate lactate (CAL) method (VDLUFA, 1991). P demand for

apple trees is low compared to N and K and is around 20 kg/ha for mature apple trees yielding 90 t/ha (Neilsen *et al.*, 2008). However further studies need to be undertaken to analyze whether low phosphatase activities in soil, due to high Cu concentrations, result in lower P levels in fruits and leaves of apple trees. It should be noted that P deficiency signs in commercial apple orchards occur mainly on acid soils (pH < 5.5), and are more evident in the growing seasons with cold springs, and dry and hot summers (Wojcik and Wojcik, 2007).

The effect of bioavailable Cu_{CAT} on DNA quantity can be seen as a useful estimate of the potentially ecotoxicological effect of Cu on soil microorganisms and extraction efficiency of DNA from soil. In our study quantity of DNA was not significantly correlated with increasing Cu content in soil. These results could be due to the selection pressure exerted by high Cu concentrations, gradually exposing soil living microorganisms, with the survival of metal tolerant microbes (Fagnano *et al.*, 2020). Quantitative DNA analysis is not a useful indicator for assessing the impact of Cu on community diversity but is generally used to see whether enough DNA has been extracted from soil. However quantitative DNA studies dealing with the impact of Cu on extraction efficiency from soil are scare and need further analysis.

In the present study we also calculated an ecotoxicological threshold value for enzymes which were significantly negative correlated to Cu in the linear regression model. This threshold value should give some indication at which level enzyme activities become impaired. Our data showed threshold values around 60 mg Cu_{CAT}/kg soil at which changes in phosphatase activity became evident (Figure 1.8). These results are consistent with those of Fernández-Calviño et al. (2010) for phosphatase activity, who reported values around 60-80 mg bioavailable Cu/kg soil to be critical for phosphatase activity. In a Cu spiked soil acid phosphatase activity was reduced by 20 to 30 % (depending on the soil and the field crops) when a dose of 600 kg Cu ha^{-1} or 150 mg Cu kg^{-1} respectively was applied to the soil, and was up to 55% under a dose of 1800 kg Cu ha^{-1} or 450 mg Cu kg^{-1} respectively (Wyszkowska, Kucharski and Kucharski, 2010; Karimi *et al.*, 2021). The comparability of this result to our results is low, since it is measuring acute toxicity on phosphatase activity and it is not taking into account the bioavailable fraction of Cu in soil. Threshold values in our study have only been calculated for phosphatase activity, as it was the only enzyme that responded negatively to Cu in soil. However, the estimated threshold value in our study must be interpreted with caution, since enzyme activities depend on many soil chemical properties and not only bioavailable Cu. The effect of SOM and pH

cannot easily be discarded. Furthermore, some groups in our study had larger input data, leading to weakly generic conclusions.

5. Conclusion

In the present study we focused on the influence of long-term Cu accumulation in apple orchards on soil extracellular enzymes. It can help authorities in deciding which future actions should be taken related to the use of Cu-based fungicides. It can help in decisions, also in combination with other studies, for further legislations in deciding if the use of Cu based fungicides should be further reduced. The knowledge about the ecotoxicological threshold value is also helpful to inform farmers about the impact of Cu. It delivers a threshold at which remediation strategies are beneficial to increase the overall efficiency of fertilizers applied.

Results of our study highlighted that Cu negatively affected only phosphatase activity, whereas protease, arylsulfatase and exochitinase activity were influenced positively. The results obtained indicate that phosphatase can be potentially used for assessing the effect of Cu pollution on enzyme activities in apple orchard soils. In our case phosphatase activity was the best indicator for Cu metal toxicity assessment. Impairment of phosphatase activity were detected at levels higher than 60 mg bioavailable Cu per kg soil. Our data also indicate that most soil enzymes respond positively to SOM, indicating a positive relationship between enzyme activity and SOM in soil. The quantity of DNA was not influenced by Cu content in soil, indicating that there was no influence of Cu on extraction efficiency of DNA from soil nor on the quantity of microorganisms.

In order to make data for threshold values more reliable, laboratory ecotoxicological experiments should be conducted. They can be used to construct dose response curves, which makes them an attractive tool for setting metal limits in soils.

6. References

AD-HOC AG Boden (2005) *Bodenkundliche Kartieranleitung*. Hannover.

Alloway, B. J. (2012) 'Sources of Heavy Metals and Metalloids in Soils', in *Heavy metals in soils*, pp. 11–50. doi: 10.1001/archderm.1978.01640230053014.

Aponte, H. *et al.* (2020) 'Alteration of enzyme activities and functional diversity of a soil contaminated with copper and arsenic', *Ecotoxicology and Environmental Safety*, 192(January), p. 110264. doi: 10.1016/j.ecoenv.2020.110264.

Aponte, H. *et al.* (2021) 'Enzyme activities and microbial functional diversity in metal(loid) contaminated soils near to a copper smelter', *Science of the Total Environment*, 779. doi: 10.1016/j.scitotenv.2021.146423.

Bogunovic, I. *et al.* (2017) 'Reducing sampling intensity in order to investigate spatial variability of soil pH, organic matter and available phosphorus using co-kriging techniques. A case study of acid soils in Eastern Croatia', *Archives of Agronomy and Soil Science*, 63(13), pp. 1852–1863. doi: 10.1080/03650340.2017.1311013.

Brunetto, G. *et al.* (2016) 'Copper accumulation in vineyard soils: Rhizosphere processes and agronomic practices to limit its toxicity', *Chemosphere*, 162, pp. 293–307. doi: 10.1016/j.chemosphere.2016.07.104.

Chiba, M. *et al.* (1998) 'Carrier-mediated entry of 4-methylumbelliferyl sulfate: Characterization by the multiple-indicator dilution technique in perfused rat liver', *Hepatology*, 27(1), pp. 134–146. doi: 10.1002/hep.510270122.

Della Chiesa, S., Genova, G., Balotti, A., *et al.* (2019) 'Farmers as data sources : Cooperative framework for mapping soil properties for permanent crops in South Tyrol (Northern Italy)', *Geoderma*, 342(June 2018), pp. 93–105. doi: 10.1016/j.geoderma.2019.02.010.

Della Chiesa, S., Genova, G., la Cecilia, D., *et al.* (2019) 'Phytoavailable phosphorus (P2O5) and potassium (K2O) in topsoil for apple orchards and vineyards, South Tyrol, Italy', *Journal of Maps*, 15(2), pp. 555–562. doi: 10.1080/17445647.2019.1633962.

Clayssens, M. *et al.* (1989) 'Fungal cellulase systems. Comparison of the specificities of the cellobiohydrolases isolated from Penicillium pinophilum and Trichoderma reesei', *Biochemical Journal*, 261(3), pp. 819–825. doi: 10.1042/bj2610819.

D'Ascoli, R. *et al.* (2006) 'Impact of river overflowing on trace element contamination of

volcanic soils in south Italy: Part II. Soil biological and biochemical properties in relation to trace element speciation', *Environmental Pollution*, 144(1), pp. 317–326. doi: 10.1016/j.envpol.2005.11.017.

Deltedesco, E. *et al.* (2020) 'Soil microbial community structure and function mainly respond to indirect effects in a multifactorial climate manipulation experiment', *Soil Biology and Biochemistry*, 142(January). doi: 10.1016/j.soilbio.2020.107704.

Dick, A. J., Opoku-Gyamfua, A. and DeMarco, A. C. (1990) 'Glycosidases of apple fruit: A multi-functional β-galactosidase', *Physiologia Plantarum*, 80(2), pp. 250–256. doi: 10.1111/j.1399-3054.1990.tb04404.x.

Dick, R. P. *et al.* (2011) *Methods of Soil Enzymology*.

Doran, J. W. and Parkin, T. B. (1994) 'Defining soil quality for a sustainable environment', in *Defining and assessing soil quality*. Available at: http://dx.doi.org/10.2136/sssaspecpub35.c1.

Egnér, H., Riehm, H. and Domingo, W. R. (1960) 'Untersuchungen über die chemische Bodenanalyse als Grundlage fur die Beurteilung des Nährstoffzustandes der Böden', in *Chemische Extraktionsmethoden zur Phosphor- und Kaliumbestimmung*.

Fagnano, M. *et al.* (2020) 'Copper accumulation in agricultural soils: Risks for the food chain and soil microbial populations', *Science of the Total Environment*, 734. doi: 10.1016/j.scitotenv.2020.139434.

Fernández-Calviño, D. *et al.* (2010) 'Enzyme activities in vineyard soils long-term treated with copper-based fungicides', *Soil Biology and Biochemistry*, 42(12), pp. 2119–2127. doi: 10.1016/j.soilbio.2010.08.007.

Frouz, J. *et al.* (2003) 'Changes in amount of bacteria during gut passage of leaf litter and during coprophagy in three species of bibionidae (Diptera) larvae', *Folia Microbiologica*, 48(4), pp. 535–542. doi: 10.1007/BF02931337.

Genova, G. *et al.* (2021) 'Copper and zinc as a window to past agricultural land-use', *Journal of Hazardous Materials*, (March), p. 126631. doi: 10.1016/j.jhazmat.2021.126631.

Grange, J. M. (1978) 'Fluorimetric assay of mycobacterial group-specific hydrolase enzymes J.', pp. 378–381.

Grashey-Jansen, S. (2010) 'Pedohydrological case study of two apple-growing locations in South Tyrol (Italy)', *Agricultural Water Management*, 98(2), pp. 234–240. doi:

10.1016/j.agwat.2010.08.012.

Hou, D. *et al.* (2020) 'Metal contamination and bioremediation of agricultural soils for food safety and sustainability', *Nature Reviews Earth & Environment*, 1(7), pp. 366–381. doi: 10.1038/s43017-020-0061-y.

Hough, R. L. (2010) 'Copper and Lead', *Trace Elements in Soils*, pp. 441–460. doi: 10.1002/9781444319477.ch18.

Hydrographic Office South Tyrol (2020) *Autonome Provinz Bozen - Südtirol | Abteilung | Home*. Available at: http://www.provinz.bz.it/hydro/index_d.asp (Accessed: 30 November 2020).

Jones, L. H. P. and Jarvis, S. C. (1981) 'The fate of heavy metals', *Chem. soil Process*.

Karimi, B. *et al.* (2021) 'Ecotoxicity of copper input and accumulation for soil biodiversity in vineyards', *Environmental Chemistry Letters*, 19(5), pp. 3525–3527. doi: 10.1007/s10311-021-01259-y.

Lamichhane, J. R. *et al.* (2018) 'Thirteen decades of antimicrobial copper compounds applied in agriculture. A review', *Agronomy for Sustainable Development*, 38(3). doi: 10.1007/s13593-018-0503-9.

Laurent, C. *et al.* (2020) 'Increased soil pH and dissolved organic matter after a decade of organic fertilizer application mitigates copper and zinc availability despite contamination', *Science of the Total Environment*, 709, p. 135927. doi: 10.1016/j.scitotenv.2019.135927.

Lu, A. *et al.* (2012) 'Multivariate and geostatistical analyses of the spatial distribution and origin of heavy metals in the agricultural soils in Shunyi, Beijing, China', *Science of the Total Environment*, 425, pp. 66–74. doi: 10.1016/j.scitotenv.2012.03.003.

Marrugo-Negrete, J., Pinedo-Hernández, J. and Díez, S. (2017) 'Assessment of heavy metal pollution, spatial distribution and origin in agricultural soils along the Sinú River Basin, Colombia', *Environmental Research*, 154(January), pp. 380–388. doi: 10.1016/j.envres.2017.01.021.

Megharaj, M. *et al.* (1999) 'Persistence and effects of fenamiphos on native algal populations and enzymatic activities in soil', *Soil Biology and Biochemistry*, 31(11), pp. 1549–1553. doi: 10.1016/S0038-0717(99)00078-4.

Mirlean, N., Roisenberg, A. and Chies, J. O. (2007) 'Metal contamination of vineyard soils in wet subtropics (southern Brazil)', *Environmental Pollution*, 149(1), pp. 10–17. doi:

10.1016/j.envpol.2006.12.024.

Neilsen, G. H. *et al.* (2008) 'Annual bloom-time phosphorus fertigation affects soil phosphorus, apple tree phosphorus nutrition, yield, and fruit quality', *HortScience*, 43(3), pp. 885–890. doi: 10.21273/hortsci.43.3.885.

Neumann, H. (1948) 'Some new substrates for determinations of phosphatase', *Experientia*, 4(2), pp. 74–75. doi: 10.1007/BF02155992.

Schwarz, W. H. *et al.* (1987) 'Activity staining of cellulases in polyacrylamide gels containing mixed linkage β-glucans', *Analytical Biochemistry*, 164(1), pp. 72–77. doi: 10.1016/0003-2697(87)90369-1.

Sinsabaugh, R. L. *et al.* (2008) 'Stoichiometry of soil enzyme activity at global scale', *Ecology Letters*, 11(11), pp. 1252–1264. doi: 10.1111/j.1461-0248.2008.01245.x.

Sirová, D., Adamec, L. and Vrba, J. (2003) 'Enzymatic activities in traps of four aquatic species of the carnivorous genus Utricularia', *New Phytologist*, 159(3), pp. 669–675. doi: 10.1046/j.1469-8137.2003.00834.x.

South Tyrol Apple Consortium (2019) 'Apple country South Tyrol', p. 21.

Tasser, E., Ruffini, F. V and Tappeiner, U. (2009) 'An integrative approach for analysing landscape dynamics in diverse cultivated and natural mountain areas', pp. 611–628. doi: 10.1007/s10980-009-9337-9.

Thien, S. J. (1979) 'A flow diagram for teaching texture-by-feel analysis', *Journal of Agronomic Education*, 8(1), pp. 54–55. doi: 10.2134/jae.1979.0054.

VDLUFA (1991) *Methodenbuch Band 1, Die Untersuchung von Böden*.

Wang, Q. Y., Zhou, D. M. and Cang, L. (2009) 'Microbial and enzyme properties of apple orchard soil as affected by long-term application of copper fungicide', *Soil Biology and Biochemistry*, 41(7), pp. 1504–1509. doi: 10.1016/j.soilbio.2009.04.010.

Wightwick, A. M. *et al.* (2008) 'Copper fungicide residues in Australian vineyard soils', *Journal of Agricultural and Food Chemistry*, 56(7), pp. 2457–2464. doi: 10.1021/jf0727950.

Wightwick, A. M. *et al.* (2013) 'Effects of copper fungicide residues on the microbial function of vineyard soils', *Environmental Science and Pollution Research*, 20(3), pp. 1574–1585. doi: 10.1007/s11356-012-1114-7.

Wojcik, P. and Wojcik, M. (2007) 'Response of mature phosphorus-deficient apple trees to

phosphorus fertilization and liming', *Journal of Plant Nutrition*, 30(10), pp. 1623–1637. doi: 10.1080/01904160701615509.

Wyszkowska, J., Kucharski, M. and Kucharski, J. (2010) 'Activity of β-glucosidase, arylsulfatase and phosphatases in soil contaminated with copper', *Journal of Elementology*, 15(1), pp. 213–226. doi: 10.5601/jelem.2010.15.1.213-226.