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Prof. Dr. Ellen Kandeler

The Importance of Soil Microorganisms and Cover Crops for Copper Remediation in Vineyards

Dissertation

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Kathleen Allison Mackie
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Examination committee

Supervisor and Reviewer: Prof. Dr. Ellen Kandeler

Co-reviewer: Prof. Dr. Torsten Müller

Additional Examiner: Prof. Dr. Rainer Joergensen

Vice Dean and Head of Committee: Prof. Dr. Thilo Streck

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Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation selbständig angefertigt, nur die angegebenen Quellen und Hilfsmittel benutzt und inhaltlich oder wörtlich übernommene Stellen als solche gekennzeichnet habe. Ich habe noch keinen weiteren Promotionsversuch unternommen.

Stuttgart, den 23.07.2014 Kathleen A. Mackie

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1 Summary

The historical use of copper fungicides, as a plant protection agent, has moderately polluted agricultural topsoils across Europe. Organic agriculture, in particular, continues to be limited to the use of copper fungicides due to a lack of permitted alternative plant protection agents. In recent years, the effects of copper accumulation in the soil have been observed. Studies on the negative effects of copper in agricultural soils show a decrease in ecosystem services, which rely on macro- and micro-organisms. Thus, there is the question of how to remediate copper polluted crop fields. Although this topic has more recently been investigated in the laboratory, currently, there are no experiments available in the field. Viticulture is one of the largest perennial crops in Europe that utilize copper fungicides. Therefore, this dissertation was designed to investigate copper remediation strategies in vineyards, in order to best understand potential solutions for a growing problem, as well as their effect on ecosystem services. Understanding the reaction of and support by soil microorganisms will help determine which strategy has the best potential. The main project was implemented using two field experiments, each of which analyzed copper availability, microbial abundance, function and community composition to determine the overall outcome of copper remediation.

The dissertation is presented in four papers. The first paper is a review on copper in vineyards, which focused specifically on cutting-edge remediation strategies currently being studied. This paper also provided information on knowledge gaps in the literature. The second paper showed the spatial distribution of copper and soil microorganisms at the plot scale, providing a better understanding of copper and microbial distribution as well as a foundation for subsequent papers. The third paper analyzed copper phytoextraction by single species and mixed species cover crop plots and the microbial community that may support it. The fourth paper was

aimed at observing the ability of biochar and biochar-compost to immobilize copper and improve ecosystem services.

The field experiment in paper two was conducted in Baden Württemberg, Germany on the WINO Biolandbau vineyard planted with *Vitis vinifera* Trollinger. The field experiments utilized in papers three and four were conducted on a *V. vinifera* Pinot noir vineyard in Canton Wallis, Switzerland at the Ithaka Institute. Both sites were managed organically with cover crops grown between the vine rows. The studies utilized classic soil biological methods (enzyme activities, microbial C and N, ergosterol) and modern molecular techniques (quantitative polymerase chain reaction (qPCR) of 16S rRNA and taxa specific bacteria genes and phospholipid fatty acid analysis (PLFA)) as well as determination of chemical soil properties and copper fractions.

The review found that microbially assisted phytoextraction, using plants and bacteria to actively extract copper, is most promising. However, only pot experiment results had been conducted and plant potential in the field had yet to be seen. It was clear within the literature that one technique was not sufficient, but that vineyard remediation requires a holistic approach including sustainable soil management, proper plant selection, increasing biodiversity and abundant soil microorganisms. The second paper concluded that areas of high copper were also areas of low ergosterol content, phosphatase, arylsulfatase, and invertase activities. Thus, copper was negatively affecting fungal abundance and microbial functions. The third paper, regarding copper phytoextraction, saw that the amount of total plant biomass production determined which cover crop species were most effective at overall copper removal per hectare. A maximum annual removal rate of 0.033 kg Cu ha^{-1} y^{-1} by summer oat and winter vetch was measured, which is significantly below the current application rate. Copper concentration did not influence above- or below-ground properties, suggesting that soil microorganisms may have become tolerant to the copper levels at this site. Microbial abundance, activity or community structure of soil microorganisms were neither associated with increased copper

phytoextraction nor plant composition. Microorganisms were instead driven by seasonal fluxes and resource pools. The fourth paper observed that biochar, biochar-compost and compost did not immobilize copper in a temperate vineyard. Instead the cover crops took up significant amounts of copper over two growing seasons, decreasing soil copper concentrations. Compost and biochar-compost significantly increased soil microbial activity and abundance, likely due to increased substrate availability, while biochar did not. Additionally, each treatment was characterized by a specific microbial community composition. Compost and biochar-compost increased the relative abundance of *Firmicutes*, while control and biochar increased *Acidobacteria, Gemmatimonadetes* and *Actinobacteria*. *Actinobacteria* were prevalent at site, thriving despite the moderate Cu content and displaying capacities for assimilating labile C.

In conclusion, neither phytoextraction nor biochar offered potential relief from copper accumulation in temperate topsoils. A high biomass producing cover crop has the most potential for copper removal; however, *in situ* results had very low removal rates. Compost and biochar-compost addition to soil provided the most benefits for soil microorganisms. Soil microorganisms at the Swiss vineyard showed tolerance to copper in the soil, likely due to the presence of cover crops and/or compost. This thesis is the first to show the practicability of current remediation strategies in the field and has provided a foundation for further research, including microbially assisted phytoextraction, as well as the role of cover crops and compost addition within the vineyard.

2 Zusammenfassung

Die historische Nutzung von Kupferfungiziden als Pflanzenschutzmittel hat zu einer moderaten Belastung der landwirtschaftlichen Oberböden in Europa geführt. Besonders in der biologischen Landwirtschaft werden kupferbasierte Fungizide aus Mangel an alternativen Pflanzenschutzmitteln weiterhin eingesetzt. In den letzten Jahren wurden Effekte der Kupferanreicherung in Böden beobachtet. Studien zu den negativen Auswirkungen von Kupfer in landwirtschaftlichen Böden zeigen eine Verringerung der Ökosystemdienstleistungen, welche auf Makro- und Mikroorganismen beruhen. Daher stellt sich die Frage, wie kupferbelastete Anbauflächen saniert werden können. Obwohl diese Fragestellung in jüngerer Zeit in Laborversuchen untersucht wurde, stehen derzeit keine Studien mit Feldexperimenten zur Verfügung. Der Weinbau ist eine der größten Dauerkulturen Europas, in der kupferbasierte Fungizide eingesetzt werden. Daher wurde diese Dissertation zur Untersuchung von Kupfersanierungsstrategien in Weinbergen konzipiert, um ein besseres Verständnis für potentielle Lösungen dieses wachsenden Problems sowie für deren Effekte auf die Ökosystemdienstleistungen zur erlangen. Das Verständnis der Reaktion von und der Unterstützung durch Mikroorganismen wird helfen, die potentiell beste Lösungsstrategie zu bestimmen. Das Hauptprojekt wurde mit zwei Feldexperimenten umgesetzt, wovon jedes die Kupferverfügbarkeit, mikrobielle Abundanz, Funktion und Gemeinschaftsstruktur analysierte, um das Gesamtergebnis der Kupfersanierung zu bestimmen.

Diese Dissertation wird in vier wissenschaftlichen Veröffentlichungen präsentiert. Die erste wissenschaftliche Veröffentlichung ist ein Review über Kupfer in Weinbergen, das besonders auf derzeit untersuchte, innovative Sanierungsstrategien fokussiert ist. Diese Publikation bietet zudem Informationen über die derzeitigen Wissenslücken in der Literatur. Die zweite wissenschaftliche Veröffentlichung zeigt die räumliche Verteilung von Kupfer und Bodenmikroorganismen auf der Plotskala und bietet sowohl ein besseres

Verständnis für die Verteilung von Kupfer und Mikroorganismen als auch das Fundament für nachfolgende Publikationen. Die dritte wissenschaftliche Veröffentlichung untersuchte die Phytoextraktion von Kupfer durch einzeln und gemischt gepflanzte Zwischenfruchtarten und die mikrobielle Gemeinschaft, die dies unterstützen könnte. Die vierte wissenschaftliche Veröffentlichung zielte darauf ab, die Eignung von Biokohle und Biokohle-Kompost zur Immobilisierung von Kupfer und zur Verbesserung der Ökosystemdienstleistungen zu betrachten.

Das Feldexperiment der zweiten Veröffentlichung wurde in Baden-Württemberg, Deutschland, im WINO Bioland Weinberg mit einem *Vitis vinifera* Trollinger Bestand durchgeführt. Die Feldversuche der dritten und vierten Veröffentlichung wurden auf einem *V. vinifera* Pino noir Weinberg im Kanton Wallis, Schweiz, am Ithaka Institut durchgeführt. Beide Weinberge wurden biologisch bewirtschaftet und mit Zwischenfrüchten zwischen den Rebenreihen begrünt. Die Studien nutzten klassische bodenbiologische Methoden (Enzymaktivitäten, mikrobieller C und N, Ergosterol) und moderne molekularbiologische Techniken (quantitative Polymerase-Kettenreaktion (qPCR) von 16S rRNA und taxaspezifischen Bakteriengenen und Phospholipidfettsäurenanalyse (PLFA)) sowie die Bestimmung chemischer Bodeneigenschaften und Kupferfraktionen.

Das Review zeigte, dass die mikrobiell unterstützte Phytoextraktion, welche Pflanzen und Bakterien zur aktiven Kupferextraktion nutzt, am vielversprechendsten ist. Allerdings lagen dieser Erkenntnis nur Topfexperimente zu Grunde und das Pflanzenpotential im Feld konnte noch nicht abgeschätzt werden. Durch die Literaturstudie wurde deutlich, dass einzelne Techniken nicht ausreichen, sondern eine Weinbergsanierung einen holistischen Ansatz benötigt, der Bodenmanagement, geeignete Pflanzenauswahl sowie gesteigerte Biodiversität und Abundanz von Mikroorganismen beinhaltet. Die zweite Veröffentlichung zeigte, dass in Bereichen mit viel Kupfer gleichzeitig geringe Ergosterolgehalte, Phosphatase-, Arylsulfatase- und Invertaseaktivitäten auftraten. Folglich hatte Kupfer negative Auswirkungen auf die Pilzabundanz und mikrobielle Funktionen.

Die dritte Publikation, in Bezug auf die Phytoextraktion von Kupfer, stellte fest, dass die Gesamtpflanzenbiomasse bestimmte, welche Zwischenfruchtarten am effektivsten für den Kupferentzug pro Hektar waren. Es wurde eine maximale jährlich Entzugsrate von 0,033 kg Cu ha^{-1} y^{-1} durch Sommerhafer und Winterwicken gemessen, die deutlich unter der derzeitigen Applikationsrate liegt. Die Kupferkonzentrationen beeinflussten die über- und unterirdischen Bodeneigenschaften nicht, was darauf hindeuten könnte, dass Bodenmikroorganismen tolerant gegenüber dem Kupferniveau auf dieser Fläche geworden sind. Die mikrobielle Abundanz, Aktivität und Gemeinschaftsstruktur der Bodenmikroorganismen waren weder mit dem steigenden Kupferentzug durch Phytoextraktion noch mit der Zusammensetzung der Pflanzengesellschaft assoziiert. Die Mikroorganismen wurden stattdessen von jahreszeitlichen Veränderungen und Nährstoffressourcen beeinflusst. Die vierte Publikation fand heraus, dass Kupfer in einem Weinberg mit gemäßigtem Klima nicht von Biokohle, Biokohle-Kompost oder Kompost immobilisiert wird. Stattdessen nahmen die Zwischenfrüchte signifikante Mengen an Kupfer über zwei Wachstumsperioden auf und verringerten so die Kupferkonzentrationen im Boden. Kompost und Biokohle-Kompost erhöhten die mikrobielle Aktivität und Abundanz im Boden signifikant, wahrscheinlich durch eine gesteigerte Substratverfügbarkeit, während Biokohle dies nicht tat. Zusätzlich war jede Behandlung durch eine spezifische mikrobielle Gemeinschaftszusammensetzung charakterisiert. Kompost und Biokohle-Kompost erhöhten die relative Abundanz von *Firmicutes*, während die Kontrollbehandlung und die Biokohle *Acidobakterien*, *Gemmatimonadetes* und *Actinobakterien* förderten. *Actinobakterien* waren auf der Fläche vorherrschend und gediehen trotz moderater Kupfergehalte und zeigten Vermögen zur Assimilation labilen Kohlenstoffs.

Schlussendlich boten weder Phytoextraktion noch Biokohle potentielle Abhilfe gegen Kupferanreicherungen in Oberböden des gemäßigten Klimas. Eine Zwischenfrucht mit hoher Biomasseproduktion hat das größte Potential für den Kupferentzug; allerdings waren die *in situ* Entzugsraten sehr gering. Die Zugabe von Kompost und Biokohle-Kompost zum Boden bot den größten Nutzen für

Bodenmikroorganismen. Im Schweizer Weinberg zeigten sich die Bodenmikroorganismen tolerant gegenüber dem Kupfer im Boden, wahrscheinlich durch die Anwesenheit von Zwischenfrüchten und/oder Kompost. Diese Dissertation ist die erste, die die Praktikabilität derzeitiger Sanierungsstrategien im Feld aufzeigt und liefert ein Fundament für weitere Forschungen, die die mikrobiell unterstützte Phytoextraktion, und der Rolle von Zwischenfrüchten und Kompostzufuhr in Weinbergen beinhalten.

3 General Introduction

3.1 Soil Remediation

Heavy metal pollution is currently a worldwide problem to which soil remediation may be a solution. Copper is one of the more common metal polluters within the field of agriculture, particularly in viticulture where copper fungicides are used as plant protection agents (Maier et al., 2000). Farmers began to use copper fungicides in the late 1800s (McBride et al., 1981) and its continued use led the European Union to restrict application amounts to 6 kg copper fungicides ha^{-1} y^{-1} (European Commission, 2007). Most recently, soil copper concentrations in European vineyards ranged from 2 – 1,500 mg Cu kg^{-1} soil, depending on its management history (Mackie et al., 2012). This is problematic when concentrations are above 140 mg Cu kg^{-1} soil, as further metal addition, for example sewage sludge, is then prohibited due to risk (Council of the European Communities, 1986). Normal soil copper concentrations are between 5 – 20 mg Cu kg^{-1} soil (McBride et al., 1981). The accumulation of copper in vineyards, specifically in the topsoil and decreasing with depth and distance from the vine row, is hazardous to macro- and micro-organisms (McBride et al., 1981; Mackie et al., 2012). A thorough overview of copper pollution in vineyards and its impact on organisms can be read in Chapter 5.

Theoretically, remediating soil copper in vineyards should reduce copper concentrations and diminish its negative effects on organisms while allowing crops to continue to grow. This allows the farm to remain economically viable, while simultaneously achieving remediation goals, which is particularly necessary in vineyards, where grapevines are perennial. Possible methods, including soil mixing and various phytoextraction strategies, and their feasibility are discussed in-depth in Chapter 5. In each case, however, successful soil remediation should be composed of two parts, the ability to reduce the targeted pollutant and the ability to improve soil conditions (Epelde et al., 2009; Gómez-Sagasti et al., 2012). The two methods

used in this thesis are phytoextraction and stabilization, the most common forms of soil remediation.

3.2 Phytoextraction

Phytoextraction is the process of actively removing metals from the topsoil by accumulating them in plant shoots and potentially plant roots (Wenzel, 2009). Subsequently, the plant biomass, shoots and/or roots, is harvested from the remediation site and can be either burned or added to copper deficient sites (Kidd et al., 2009). This method requires specific plant species, which can take up the target metal in large amounts. Most recently, crops that can accumulate moderate amounts of copper, while producing large amounts of biomass in a short period of time have been the focus of laboratory research (Kidd et al., 2009). This is because most hyperaccumulator plants are low biomass and slow growing plants, which result in a long remediation period (Kidd et al., 2009). Currently, potential plant species have only been observed within the laboratory and greenhouse, with the exception of Poschenrieder et al. (2001), who analyzed wild plant species at a malachite outcropping in Spain. Therefore, the ability of high biomass producing plant species to accumulate copper *in situ* is unknown. The benefit of phytoextraction is that it is the only methods that reduces accumulation in the topsoil permanently, while remaining environmentally sensitive by keeping soil structure and the crop field intact.

3.3 Stabilization

Stabilization, on the other hand, is the process of immobilizing the metal with the use of either plants or absorbent materials and, therefore, inhibiting the metal from further affecting organisms. This method does not decrease the amount of total copper within the topsoil, but instead reduces the content of bioavailable copper. Although often attempted with plant species, i.e. phytostabilization, biochar has also been seen to adsorb pollutants, including copper, reducing its accessibility and minimizing toxin-induced stress to microorganisms and fauna (Namgay et al., 2010; Beesley and Dickinson, 2011; Buss et al., 2012; Ippolito et al., 2012). Biochar is made

by burning biomass without oxygen, also called pyrolysis, to produce a lightweight, porous charcoal (Brewer and Brown, 2012). The most critical factor for the solubility of copper in soil solution is a low pH and low dissolved organic carbon (DOC) (Hinojosa et al., 2010). Despite often initially increasing DOC, biochar has been observed to increase soil pH and adsorb copper compounds, reducing extractable copper concentrations (Brennan et al., 2014). Brennan et al. (2014) observed a reduction in maize shoot copper content with the addition of biochar, independent of biochar feedstock. Adsorbing copper in soil by use of biochar could reduce toxicity and improve plant growth and soil microorganism abundance when copper removal is not possible.

3.4 Microbial Ecosystem Services

Simultaneous to copper removal or immobilization is improving microbial ecosystem services (Epelde et al., 2014). In this case, this means returning the soil to its previous or an improved status of fertility and function. Soil microorganisms, in particular, are valuable indicators of ecosystem services. They are highly sensitive, thus they respond quickly to changes in their environment, and they have the ability to integrate many environmental factors (Gómez-Sagasti et al., 2012; Epelde et al., 2014). For example, increases in available substrate lead to increases microbial biomass. Soil microorganism abundance also indicates the ability to regulate soil organic matter decomposition as well as overall soil vigor (Epelde et al., 2014). Therefore, monitoring the abundance, functional activity and diversity of soil microorganisms provides insight into the broader success of remediation strategies.

Moreover, microbial biomass also reacts to increases and reductions in soil copper concentration. By moving through the cell membrane, copper alters cell transcription, translation and division, damaging cell DNA and denaturing proteins (Maier et al., 2000). Specifically, functional activity and microbial community composition can be modified by copper pollution to less active and more Gram-bacteria communities (Kandeler et al., 1996; Dell'Amico et al., 2008). Therefore, when copper becomes less available, soil microorganisms are better able to increase

microbial biomass, maintain functional activity and sustain a diverse microbial community. Additionally, soil microorganisms can play an important role in the fate of copper in the environment, affecting mobility and availability (Gadd, 2004). The presence of specific soil microorganisms can assist plants in taking copper up by chelating with copper compounds, this microbial characteristic has inspired the most recent remediation method called microbially assisted phytoextraction (Kidd et al., 2009; Wenzel, 2009; Rajkumar et al., 2010; Mackie et al., 2012).

Understanding the interaction between above- and below-ground organisms can improve our knowledge of remediation potential and ecosystem services as well as the immediate effects of above- or below-ground environmental changes (Wardle et al., 2004). This interaction, however, is very complicated. Plants provide available substrate for soil microorganisms through root exudates (Bardgett et al., 1998; Castaldi et al., 2009). Soil microorganisms, on the other hand, help to decompose dead plant material as well as provide a supply of available nutrients to the plants (Wardle et al., 2004). Epelde et al. (2010) observed that soil microorganisms significantly influenced plant biomass production by providing nutrients to the plants in an otherwise nutrient poor and polluted environment. In contrast, Castaldi et al. (2009) found that pea rhizodeposits, in comparison to wheat, increased microbial enzyme activity, suggesting that the rhizodeposits from pea were more available to soil microorganisms than other plant species. Understanding which soil microorganisms and plant species work together, will provide information on who is responsible for certain ecosystem services and the mechanisms that are at play.

Just as plants interact with soil microorganisms, so does the application of biochar. Soil microbial abundance has been observed to increase significantly in the presence of biochar (Lehmann et al., 2011). Gomez et al. (2014) observed biochar increase microbial abundance and activity, as well as modify the microbial community, by providing more available organic carbon substrates. Biochar may also provide a niche habitat for soil microorganisms, increasing abundance by providing pore space that is protected from predators (Joseph et al., 2010; Lehmann

et al., 2011). This is because not only does biochar have a high sorption capacity, but also a high cation exchange capacity and large surface area (Atkinson et al., 2010). This allows biochar to bind with cations and anions, increasing the availability of macro-nutrients (Atkinson et al., 2010). Moreover, it has been suggested that biochar addition will increase soil microbial diversity, especially within contaminated soils (Beesley et al., 2011).

3.4.1 Potential enzyme activity

Microbial abundance alone does not represent ecosystem services, however, as it does not indicate the functionality of the community (Nannipieri et al., 2003). Therefore, functional activity needs to be analyzed separately. Soil microorganisms are involved in 80 – 90% of the processes in soils, such as organic matter decomposition, nutrient cycling of carbon, nitrogen, phosphorus and sulfur, and pollutant detoxification (Maier et al., 2000; Nannipieri et al., 2003). Investigating the functioning of soil microorganisms relies on the activity of extracellular enzymes (Nannipieri et al., 2012). Enzymes are proteins, which break down complex compounds into more soluble smaller compounds (Burns, 2002). Enzyme activity analyses allow the observation of the regulation of elemental cycles and the availability of nutrients (Epelde et al., 2014). It is important to note that one potential enzyme activity analysis does not represent an entire nutrient cycle, but instead a part, which can signify the condition of the cycle (Burns, 2002; Nannipieri et al., 2012). Enzyme production is connected to many environmental factors, such as substrate concentrations, soil nutrients and pollutants. Low enzyme activity could indicate a lack of substrate or suppression due to an overabundance of product. Moreover, in the case of alkaline phosphatase and arylsulfatase activities, their decrease could also signify metal pollution, as they are considered valuable biological indicators for copper pollution (Fernández-Calviño et al., 2010; Hinojosa et al., 2010). Copper can inhibit cell membrane activity as well as processes of oxidation and reduction (Maier et al., 2000). On the other hand, unvarying enzyme activities could indicate functional stability of the soil microbial community (Nannipieri et al., 2003).

3.4.2 Soil microbial community

Knowledge on the diversity and structure of soil microbial communities can give insight into which environments microbial groups can succeed. For example, a dominant group at a polluted site indicates pollution tolerance of that group, while others may dominate under specific plant species that successfully take up copper. Although microbial groups may only perform specific functions, it is necessary that the community remains diverse so that the potential for withstanding environmental changes, such as copper pollution, is high. Consequently, to study microbial community structure both standard and molecular techniques can be employed. Phospholipid fatty acids (PLFAs) as well as quantitative polymerase chain reaction (qPCR) analyses yield results that are considered indicators of soil microbial diversity and community composition in metal polluted soils (Pennanen et al., 1996; Epelde et al., 2010). PLFA amounts give insight into higher levels of microbial groups and qPCR provides taxa specific bacterial gene copy number abundances, while both measure total bacterial abundance. Although it is not known which function each bacterial group conducts, in association with other analyses it is possible to begin to link microbial diversity to microbial functions in soil. Moreover, these analyses provide understanding of the structural diversity of soil microorganisms in copper polluted as well as remediated sites (Nannipieri et al., 2003; Epelde et al., 2014).

4 Objectives

Copper fungicides are frequently used plant protection agents in agriculture. Their application, however, has led to moderate to high copper pollution levels within agricultural topsoils. Particularly the use of copper fungicides in vineyards is of interest because the organic viticulture sector in Europe, which is restricted to using only copper fungicides, is growing. The goal of this thesis is three-fold; First, to fully understand the implications of copper fungicide use in vineyards, including distribution and impact; Second, to apply known copper remediation strategies in the field to understand their applicability and efficiency *in situ*; and, Third, to understand the interaction and impact that these remediation strategies had on soil microbial properties and the ecosystem services they provide. These studies are some of the first that show *in situ* phytoextraction results as well as the *in situ* capabilities of biochar in a temperate vineyard soil. Moreover, the microbiological and molecular techniques used to define the soil microbial community are state of the art and have been infrequently used in concert, as is done within this thesis.

The first study in this thesis is a review of copper remediation in vineyards. It discusses in-depth the current scientific knowledge of copper in vineyards as well as the remediation strategies currently available within the literature. The goal of the review was to synthesize the literature on copper in vineyards and determine opportunities for further research. Moreover, it provides a succinct summary of current state of the art remediation methods. This study is one of the first papers to review comprehensively copper remediation in vineyards.

The second study focused on the plot scale distribution of soil copper content and soil microorganisms within an organic vineyard using a 4 m × 5 m sampling grid. It was hypothesized that areas of high copper would indicate areas of low microbial activity as well as influence the distribution of the microbial community. Therefore, three distances from the vine row were analyzed at uniform spacing across the grid.

The objective of this study was to identify the effects of copper distribution on the distribution of microbial and fungal abundance, functional activity (phosphatase, invertase, xylanase, urease, arylsulfatase) and community structure as represented by PLFAs.

The third study used an *in situ* random block designed phytoextraction field experiment to investigate the ability of summer (*Avena sativa* [Oat], *Trifolium incarnatum* [Crimson clover], *Chenopodium* [Goosefoot]) and winter (*Vicia villosa* [Hairy vetch], *Secale cereale L.* [Rye], *Brassica napus L. partim* [Rape]) cover crops, including a mixed species treatments, to extract copper from an organic vineyard soil and the microbial communities that may support it. The goal was to observe the plant-microbial interactions of copper phytoextraction, laying a foundation for future microbially assisted phytoextraction research. As with the second study, ecosystem services, such as nutrient cycling and soil vigor, were monitored using microbial biomass and enzyme activities. Moreover, PLFAs were analyzed in order to identify bacterial groups that may be supporting successful copper phytoextraction.

As copper fungicides continue to be used and the true potential of phytoextraction was not yet clear, the fourth study focused on the ability to immobilize copper within the vineyard topsoil. The amendments used to immobilize copper were hardwood biochar and biochar-compost. Biochar-compost, a co-composted product, was used to reduce negative effects of pure biochar previously observed within temperate regions. This study used a block design with four treatments (control, biochar, compost, biochar-compost). It was hypothesized that biochar and biochar-compost would reduce exchangeable copper fractions, thus reducing plant copper accumulation and increasing microbial abundance. Additionally, it was hypothesized that compost and biochar-compost would further enhance soil microbial abundance, enzyme activities as well as community composition and diversity using PLFAs and taxa specific qPCR.

5 Remediation of copper in vineyards: A mini-review

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K.A. Mackie[a], T. Müller[b] & E. Kandeler[a]

[a]Institute of Soil Science and Land Evaluation, Soil Biology Section, University of Hohenheim, Emil-Wolff-Strasse 27, 70599 Stuttgart, Germany

[b]Institute of Crop Science, University of Hohenheim, Fruwirthstrasse 20, 70599 Stuttgart, Germany

Abstract

Viticulturists use copper fungicide to combat Downy Mildew. Copper, a non-degradable heavy metal, can accumulate in soil or leach into water sources. Its accumulation in topsoil has impacted micro and macro organisms, spurring scientists to research *in situ* copper removal methods. Recent publications suggest that microorganism assisted phytoextraction, using plants and bacteria to actively extract copper, is most promising. As vineyards represent moderately polluted sites this technique has great potential. Active plant extraction and chelate assisted remediation extract too little copper or risk leaching, respectively. However, despite interesting pot experiment results using microorganism assisted phytoextraction, it remains a challenge to find plants that primarily accumulate copper in their shoots, a necessity in vineyards where whole plant removal would be time consuming and financially cumbersome. Vineyard remediation requires a holistic approach including sustainable soil management, proper plant selection, increasing biodiversity and microorganisms.

5.1 Introduction

The goal of this review is to investigate and summarize the most important literature available on copper use and remediation in vineyards. The resulting document can be used to identify potential for improvement and opportunities for further research. Viticulture is an important and established crop on practically every continent. This review focuses on the influence of copper use in vineyards and the status and potential of remediating vineyards where copper accumulation is, or borders on, toxic. In viticulture, grape vines are plagued yearly by "Downy Mildew," *Plasmopara viticola*, triggering the use of copper fungicide by farmers. Over time copper has accumulated in the topsoil, reaching toxic levels, causing plant stress and reducing soil fertility (McBride et al., 1981). Downy Mildew is problematic when weather is moist and wet. Under rainy or windy conditions, particles are expelled from the ground onto the vine canopy, where they subsequently contaminate open stomata (Salinari et al., 2006). Consequently, grape leaf gas exchange declines and plant stress increases during the ripening stage, reducing grape quality and grape sugar content (Jermini et al., 2010b; Jermini et al., 2010a).

Plants, soil, and their interface in this complex system will be discussed. To date reviews on heavy metal remediation have not focused on copper or vineyards specifically (Wenzel, 2009; Jadia and Fulekar, 2009; Rajkumar et al., 2010; Baker et al., 1994; Kidd et al., 2009; McGrath et al., 2001). Two vineyard specific reviews have recently appeared, but neither addresses microbially assisted phytoextraction (Komárek et al., 2010; Pietrzak and Uren, 2011).

5.2 Copper

Copper as a heavy metal

Copper is a common metal pollutant (Maier et al., 2000). As an essential heavy metal it is by definition necessary for organism functions, but it is also potentially toxic. Uncontaminated soils generally have <20 mg Cu kg^{-1} soil, but when copper is

present in parent rock and natural minerals as much as 100 mg Cu kg^{-1} is possible (McBride et al., 1981; Wightwick et al., 2006). Copper can be either immobile or mobile in the soil. Immobile copper is non-bioavailable and can be adsorbed or precipitated into the soil matrix. Copper is predominately cationic, naturally attracted to negatively charged clay minerals, anionic salts, organic matter, hydroxides, phosphorus, and sulfate and therefore producing complexes (Maier et al., 2000; Moolenaar, 1998). Although not bioavailable, newly formed complexes can prevent enzyme reactions or stimulate new reactions with enzyme-substrate complexes (Hinojosa et al., 2010). The optimal environment for sustaining immobility and preventing plant/organism uptake is with high cation exchange capacity (CEC), high pH, and high organic matter (OM) content (Maier et al., 2000). High CEC encourages metals to bind with soil aggregates, depending on the OM and clay content of the soil (Maier et al., 2000). High pH enhances the dissociation of organic acids and, therefore, the formation of complexes with metals, altering metal speciation and reducing bioavailability (Maier et al., 2000; Selim and Amacher, 2001).

When the opposite properties are observed, i.e. low pH, low CEC, and little organic matter, copper can be mobile and potentially bioavailable. Bioavailability is defined as the portion of a pollutant in the soil that is available for uptake by soil organisms and/or plants (Hinojosa et al., 2010). Dissolved organic carbon (DOC), or dissolved organic matter, also increase the potential mobility of copper, especially to groundwater and plant roots (Moolenaar, 1998). Low pH is often considered the most critical factor influencing the solubility of copper in soil solution (Maier et al., 2000; Hinojosa et al., 2010; Selim and Amacher, 2001; Nachtigall et al., 2007). Chemical speciation also plays a large role and toxicity decreases in this order: $Cu(OH)^{+}/Cu(OH)_2$, $CuCO_3$, $CuHCO_3^{+}$, $Cu(CO_3)_2^{2-}$, chloro-complexes (Komárek et al., 2010). Copper fractions do not correlate to total copper and should be measured separately (Maier et al., 2000; Ristic et al., 2006).

Copper in Vineyards

The first commonly applied copper solution, "Bordeaux mixture" ($CuSO_4$), was used in France in 1885 (McBride et al., 1981). Pierre Marie Alexis Millardet, a French botanist, observed that copper sulfate, which had been effective for many years in preventing passersby from eating grapes along the road, was also efficient in combating Downy Mildew (McBride et al., 1981; Moolenaar, 1998). It is now known that Cu creates complexes in pathogens, destroying cell proteins and enzyme functions (Spencer-Phillips et al., 2002). Although the original application rate is not known, it can be speculated from recent restrictions that >8 kg ha^{-1} y^{-1} was used. In much of the world $CuSO_4$ is no longer recommended for use as it is found to be too concentrated and toxic for workers and the environment. Instead, copper hydroxide ($Cu(OH)_2$) and copper oxychloride ($Cu_3Cl_2(OH)_4$), both less concentrated, are extensively sold and applied by grape cultivators. In 1995, copper solutions represented 20% of global fungicide sales (Spencer-Phillips et al., 2002).

Anthropogenic sources have increased the soil copper load. Almost all organic wineries use copper fungicides because it is currently the only organic option to fight fungi. Worldwide, 121 825 ha of organic grapes are cultivated, with Europe managing 100 000 ha (IFOAM, 2009). Despite strict certification, copper use has created a dispute over the sustainability of organic production (Russo, 2008; FAO, 2009). Its toxic characteristics have prompted governments to set copper limits. The Netherlands has banned copper use, while the European Union permits only 6 kg ha^{-1} y^{-1} in agriculture (Wightwick et al., 2008; European Commission, 2007). Some countries have no restrictions in place, such as Australia and the United States (Wightwick et al., 2008; R. Mazur, personal communication, August 26, 2011). Despite difficulties in calculating specific soil toxicity, as total concentrations rarely correlate to plant available concentrations, boundaries between nontoxic to toxic and toxic to critical total copper levels have been defined; in Australia and New Zealand >60 mg Cu kg^{-1} soil, in the Netherlands >36 mg Cu kg^{-1} and in the European Community 50 and 140 mg Cu kg^{-1}, limits for sewage sludge addition, are often used

as indicators of toxicity (Maier et al., 2000; Pietrzak and McPhail, 2004; Council of the European Communities, 1986; BBodSchV, 1999). Due to the above stringent toxicity guidelines, most groups that set organic standards have copper limits below those required by their national governments; in Germany 3 kg ha^{-1} y^{-1}, in Australia 8 kg ha^{-1} y^{-1}, and in the United States it is prohibited (BIOLAND, 2011; Wightwick et al., 2008; National Organic Program, 2000). This illustrates their awareness of the problem despite its widespread use. Although organic farmers are big users of copper fungicide, conventional farmers also use it, depending on the region; at rates of 1 to 2 kg ha^{-1} y^{-1} in Europe, 0 to 20 kg ha^{-1} y^{-1} in Australia and up to 65 kg ha^{-1} y^{-1} in Brazil, in order to supplement other chemicals (Nachtigall et al., 2007; Pietrzak and McPhail, 2004). Up to 90% of wine growers in Australia apply Cu, contributing to the increase in copper levels in agricultural soils (Pietrzak and McPhail, 2004).

Fungicidal spraying contributes more copper to soils than any other agricultural activity (McBride et al., 1981). Copper is sprayed directly onto the vine canopy rather than over the entire vineyard area in a light mist using a radial blowing sprayer or, in steep regions, by hand. These methods, however, do not prevent the spray from drifting or falling onto the soil below. (Such machines are now available, but are expensive and are currently the exception in vineyards.) Therefore, containment of copper spray is low, resulting in non-point source pollution. This is problematic because copper cannot be degraded or destroyed (Maier et al., 2000). Although copper is found to be “one of the least mobile of the trace elements,” soluble fractions are always present (McBride et al., 1981). The total copper concentration is in fact distributed over all possible solid, liquid, and biotic forms and these have different effects on the environment (Maier et al., 2000). Also, copper applied anthropogenically is more potentially available to plants than naturally occurring copper (Fernández-Calviño et al., 2009a).

Historical and current applications have resulted in copper accumulation in the soil and total copper quantities have been measured in vineyards worldwide (Table 5.1).

Table 5.1. Total copper and extractable copper values, including method, in vineyards around the world.

Location	Soil Type	Total Copper (mg kg^{-1})	Method for Extractable Copper Fraction	Extractable Copper Fraction (mg kg^{-1})	Source
Europe					
Czech Republic	Various types	6	EDTA Calcium chloride	11 – 81 0.43 – 0.70	(Komárek et al., 2008)
France & Mosel, Germany	--	60 – 360	--	--	(Fründ et al., 2007)
Burgundy, France	Calcaric cambisol and leptosol	57 – 332	--	--	(Parat et al., 2002)
Burgundy & Beaujolais, France	Rendosol and brunisol	17; 34	--	--	(Dousset et al., 2007)
Beaujolais, France	Acidic sandy	323	--	--	(Flores-Vélez et al., 1996)
Champagne, France	--	100 – 1 500	--	--	(Besnard et al., 2001)
Hérault, S. France	Calcareous sandstone-slate and alluvial deposits	31 – 251	EDTA DTPA Ammonium acetate Calcium chloride	2.3 – 95.1 1.5 – 82.1 0.6 – 29.7 0.10 – 9.24	(Brun et al., 1998)
Hérault, S. France	Calcareous sandstone-slate and alluvial deposits	20 – 251	EDTA DTPA Ammonium acetate Calcium chloride	3.1 – 95.1 2.4 – 82.1 1.6 – 29.7 0.10 – 9.24	(Brun et al., 2001)
Reims, France	Calcaric cambisols	248 – 519	--	--	(Besnard et al., 2001)
Roujan, France	Various types	22 - 398	Calcium chloride EDTA	0 – 40 4 - 176	(Chaignon et al., 2003)
Bari, Italy	Alkaline loam	93	DTPA	9.6	(Provenzano et al., 2010)
Piedmont, Italy	Acidic soils	251 – 372	KNO_3 (Alva et al., 2000)	9 – 30	(Dell'Amico et al., 2008)
Torino, Italy	Dystric Eutrudept	478	KNO_3	19	(Lagomarsino et al., 2010)
Italy	Various types	9 – 945	DTPA Calcium chloride	0.5 – 418 0 – 0.90	(Deluisa et al., 1996)
Galicia, Spain	Sandy loam to loam granite and schist	41.5 – 583	Ammonium acetate EDTA	0.4 – 23.3 6.3 – 212.6	(Fernández-Calviño et al., 2008b)
Galicia, Spain	Sandy loam to loam granite and schist	61 – 434	--	--	(Fernández-Calviño et al., 2008c)
Galicia, Spain	Sandy loam to loam granite and schist	25 – 272	Ammonium acetate EDTA	0.1 – 2.5 1 – 141	(Fernández-Calviño et al., 2008a)
Rioja, Spain	Various types	22.5 – 66.8	--	--	(Herrero-Hernández et al., 2011)
NW Spain, N Portugal	Various types	25 – 666	Ammonium acetate	0.1 – 30.3	(Fernández-Calviño et al., 2009a)
NW Spain, N Portugal	Various types	100 - 268	Ammonium acetate DTPA EDTA	1 – 12 23 – 93 31 – 98	(Fernández-Calviño et al., 2010)
N Portugal	--	8 – 574	--	--	(Pessanha et al., 2010)

Table 5.1. continued… Total copper and extractable copper values, including method, in vineyards around the world.

Location	Soil Type	Total Copper (mg kg^{-1})	Method for Extractable Copper Fraction	Extractable Copper Fraction (mg kg^{-1})	Source
S Portugal	Calcareous loam	2	Olsen, 1972	14 - 46	(Magalhaes et al., 1985)
Serbia	Neutral soils	24 – 432	EDTA Ammonium acetate	5.1 – 92 0 – 25	(Ristić et al., 2006)
Slovenia	Various types	87 – 120	Calcium chloride	0.04 – 0.06	(Rusjan et al., 2007)
Slovenia	Calcaric leptosol and regosol, anthrosol	65 – 99	--	--	(Rusjan et al., 2006)
Asia					
Taichung, Taiwan	Acidic sandy loam	9.1 – 100	--	--	(Lai et al., 2010)
Oceania					
Australia	Various types of acidic soil	22 – 228	$MgCl_2$	0 – 4.3	(Pietrzak and McPhail, 2004)
Australia	Various types	6 – 223	--	--	(Wightwick et al., 2008)
Australia	Various types	1 – 223	Calcium chloride	0 – 0.94	(Wightwick et al., 2010)
Marlborough, New Zealand	Silt loam to clay loam	50 - 150	--	--	(Robinson et al., 2006)
New Zealand	Various types	1 – 259	--	--	(Morgan and Taylor, 2004)
Africa					
Stellenbosch, South Africa	--	10 – 20	--	--	(Eijsackers et al., 2005)
Americas					
Brazil	Acidic dystrophic lithic udorthent and humic dystrudept	1 355 – 1 381	DTPA Mehlich III Calcium chloride	~375 – 550 ~850 – 1 000 ~1 – 15	(Nachtigall et al., 2007)
Brazil	Ferrasols, podzols, arenosols	36 – 3 215	Calcium chloride Ammonium acetate EDTA	0.2 – 5 7.3 – 606 19 – 1 264	(Mirlean et al., 2007)
New York, USA	Alton gravelly loam	87 – 142	--	--	(Taschenberg et al., 1961)

It has been suggested that areas with greater humidity and precipitation, such as Brazil or Champagne, France, exhibit higher copper concentrations than dry environments, due to higher copper use (Komárek et al., 2010; Fernández-Calviño et al., 2009b; Deluisa et al., 1996; Morgan and Taylor, 2004). However, even regions with low total copper levels can be labeled with either “warning” or “critical” alerts. These values, however, do not describe the distribution throughout the vineyard or their uptake by fauna or flora.

Copper accumulates in the topsoil and decreases with depth, eventually returning to natural levels between 40-60cm (Ristic et al., 2006; Pietrzak and McPhail, 2004; Deluisa et al., 1996; Magalhaes et al., 1985; Besnard et al., 2001; Brun et al., 1998; Flores-Vélez et al., 1996; Rusjan et al., 2007; Komárek et al., 2008). Depth and quantity of copper accumulation do, however, increase in more tropical climates, such as Brazil (Mirlean et al., 2007). One study from Ristic et al. (2006) suggests that copper accumulation at depth decreases with increasing sand content, allowing copper to be more mobile and to leach out; however copper reduction at depth is seen in many soils with and without sand. Another characteristic of copper in vineyards is that it accumulates within rather than between the rows; copper is sprayed on the vines, under which the soil is left largely undisturbed (see Figure 5.1A) (Wightwick et al., 2006; Pietrzak and McPhail, 2004; Komárek et al., 2008; Mirlean et al., 2007). Depending on the age of the vineyard and technical operations within the vineyard, i.e. removal of foliage or distribution of organic matter, the difference between copper quantities within and between the rows can be more or less significant (Mirlean et al., 2007). Plowing between rows is a common soil management technique in vineyards to reduce weed and plant growth under the vines. It is also thought to affect the distribution of copper in the vineyard. Generally, tilling can be as deep as 50–60 cm, resulting in lower copper concentrations, due to mixing, than in untilled areas (Ristic et al., 2006; Mirlean et al., 2007; Paoletti et al., 1998). Provenzano et al. (2010) observed that copper levels were the same in the topsoil and at 40cm depth when tillage was practiced. However, as most vineyards commonly utilize tillage as a practice, copper

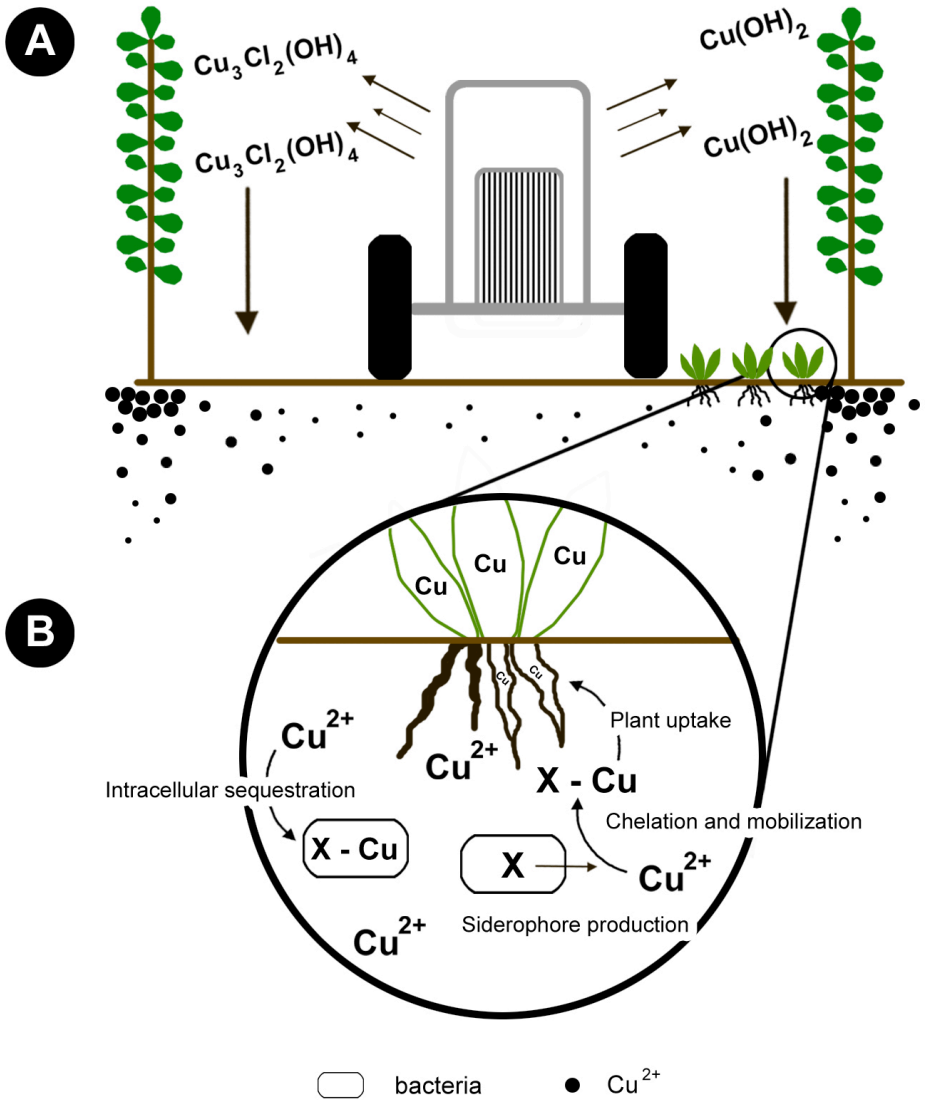

Figure 5.1. A) Copper spraying and subsequent copper distribution in a vineyard. B) Microbially assisted phytoextraction, where bacteria use siderophores to chelate copper ions and enhance plant metal uptake.

distribution in a completely untilled vineyard is yet to be investigated. Copper treated wooden posts, which support vine cordons, have also been found to leach copper into the surrounding soil, but untreated or lacquered wood posts can prevent it (Robinson et al., 2006). Knowledge of specific vineyard management and soil sample placement is important in vineyard research.

Some research indicates that as vineyards age they are subjected to long periods of Cu application, enhancing Cu accumulation (Morgan and Taylor, 2004; Rusjan et al., 2007; Fernández-Calviño et al., 2008a). Fernández-Calviño et al. (2008a) found that abandoned vineyards had the highest levels of total copper when compared to young and old vineyards. Pietrzak and McPhail (2004), however, did not see any difference between young and old vineyards in Australia with respect to total copper concentration, and found that vineyard age does not play a role in how much copper is used each year, suggesting that perhaps even less is applied in older vineyards, which produce fewer grapes. However, Morgan and Taylor (2004) suggest this result is due only to soil erosion from vineyard sites rather than from a true reduction in copper use or accumulation. CEC has also been shown to increase with vineyard age, but not with depth, which suggests that as a vineyard ages and more copper accumulates, it becomes less available (Rusjan et al., 2007).

In vineyards, most of the copper complexes with organic matter and clay soil fractions (Besnard et al., 2001; Flores-Vélez et al., 1996; Parat et al., 2002; Lagomarsino et al., 2010). Copper is found in all soil fractions in different forms; its affinity for organic matter and clay particles has been verified. In this way copper indirectly prevents the complete decomposition and mineralization of organic matter (McBride et al., 1981; Moolenaar, 1998; Parat et al., 2002). It has also been observed that as copper concentrations increase, the fraction bound to organic matter also increases, illustrating a pattern similar to that of vineyard age and copper concentration (Fernández-Calviño et al., 2008a). Within the clay fraction copper demonstrated an affinity for Fe (oxyhydroxides) (Morgan and Taylor, 2004; Parat et al., 2002; Lagomarsino et al., 2010). Morgan and Taylor (2004) identified the largest copper fraction in vineyards as copper residuals, with organically bound copper closely followed by Fe bound copper. On the other hand, Pietrzak and McPhail (2004) measured greater fractions of active copper fractions in the topsoil, where water soluble, sorbed and exchangeable fractions represented up to 50%, and organically bound forms only 25%. They also saw that sandy soils had less

active and more organically bound copper. In general, however, analysis of organic matter and clay fractions gives a good estimate of where copper can be found.

Vineyard soil pH is generally neutral (Wightwick et al., 2006; Nachtigall et al., 2007). Acidic pH between 4 and 6 can be found in specific regions, such as Spain and Brazil (Fernández-Calviño et al., 2008a). Under neutral pH copper is largely immobilized and therefore likely to accumulate in the root zone. Grapevines have deep rooting systems; so far, copper has not affected them (Wightwick et al., 2008). But topsoil could become toxic for cover crops and young, newly planted vines with shallow root systems. Toxicity depends on the form and concentration of copper as well as soil texture, and copper lowers soil pH, enhancing its solubility (Hinojosa et al., 2010). Copper can also influence the quantity of organic carbon in vineyard soil. When copper concentrations are high organic carbon also increases (Besnard et al., 2001; Flores-Vélez et al., 1996; Parat et al., 2002; Fründ et al., 2007). Fründ et al. (2007) determined that copper levels >180 mg Cu kg^{-1} increase organic carbon and decrease microbial biomass. Ristic et al. (2006) found the same to be true for bioavailable copper.

Copper also affects sediments in watersheds. As vineyards are often planted on steep hillsides to increase the amount and duration of sunshine, erosion is common, especially when cover crops are not planted between rows to stabilize the soil (Kosmas et al., 1997). Sediments in a Galician (Spain) river valley were found to have higher copper levels than nearby vineyard soils (422 mg kg^{-1}; 204 mg kg^{-1}, respectively) (Fernández-Calviño et al., 2008b). The portion of potentially bioavailable copper was almost 50% of total copper measured and was bound mainly to organic matter in the sediments, as seen in soils (Fernández-Calviño et al., 2008b). However, in another river valley Fernández-Calviño et al. (2008c) found that copper levels did not exceed 209 mg kg^{-1}. Differences in the two studies may be due to their different locations and upstream land uses. Highest total copper observed in sediments was found downstream from an area with the largest percentage of vineyards (Fernández-Calviño et al., 2008c). As in vineyard soils,

copper release from sediments increased in acidic environments. Water samples showed no correlation to copper in vineyards in Spain, but in Champagne, France basin water had 53.6 μg Cu L^{-1}, higher than the LC_{50} for many aquatic species (Banas et al., 2010). The authors suggested storm water basins as a deterrent.

The effect of landscape or topography on copper levels is unclear. Rusjan et al. (2007) found that amongst plains, plateaus and terraces, copper levels were highest on terraces. Deluisa et al. (1996), in a comprehensive study of mountainous and plain regions in northern and southern Italy, found that the mountain zone had higher copper accumulation. They attributed this to differences in soil type and precipitation, the latter encouraging greater use of copper. However, in Australia Pietrzak and McPhail (2004) did not see a difference in copper application in Victoria (Australia) between humid regions and drier regions. They suggest, "slope, exposure, amount of sun received, surrounding vegetation, wind breakers" are factors in controlling mildew outbreaks. According to them the source of copper and organic matter content largely determine copper distribution. Other authors have suggested that erosion, leaching and plowing are the main determinants. Differences between findings may be due to vineyard age, but this information was not available.

Environmental Impact of Copper

Total copper has induced mal effects on organisms with as little as 55 mg kg^{-1} (Jänsch and Römbke, 2009). To better estimate potential complications bioavailable copper must also be analyzed. Measuring bioavailable copper directly is not possible; bioavailable fractions can be approximated by sequential or single-step extraction methods. Thus, fractions can only be labeled as exchangeable, soluble, complexed, etc. The exchangeable fraction is considered to be the most similar to bioavailable copper. Suggested extraction methods include ethylene diamine tetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), ammonium acetate, calcium chloride and ammonium nitrate (Komárek et al., 2008; VDLUFA, 2006; Marschner, 2003; Chaignon et al., 2003; Song et al., 2004; BBodSchV, 1999).

DTPA was developed to chelate many heavy metals, not only copper, and could be an inconsistent indicator of the effect of Cu on yield (McBride et al., 1981). The EDTA Cu fraction tends to correlate with total Cu, not to plant available Cu, explaining why this extraction can lead to an overestimation of plant available Cu (Mirlean et al., 2007; Fernández-Calviño et al., 2008b; Song et al., 2004). $CaCl_2$ can predict copper levels in wild plants from acidic to neutral soils, but in alkaline soils it can underestimate copper (Brun et al., 1998; Komárek et al., 2008; Mirlean et al., 2007). The literature does not recommend a particular method, but governments often require specific analyses, such as ammonium nitrate in Germany. As the various methods may be extracting different parts of the same source, multiple analytical methods in comparison with plant copper values may better clarify site-specific contamination.

On the macro-scale, plants and animals as well as ecosystem processes are impacted by copper pollution (McBride et al., 1981; Moolenaar, 1998). Plant toxicity reduces root growth, increases atrophy, and causes chlorosis (Moolenaar, 1998; Magalhaes et al., 1985). In vineyards plant toxicity depends on rooting depth. Grapevines root deeply within the soil in search of water. Therefore, vines do not often display toxicity, while grasses and shallow rooting plants do (Magalhaes et al., 1985). Copper accumulation can, however, affect the development of new vine shoots (Coïc and Coppent, 1989; Komárek et al., 2010; Romeu-Moreno and Mas, 1999). As plants age copper in vine leaves accumulates (Pessanha et al., 2010). Angelova et al. (1999) observed, however, that copper in grapevine leaves decreased substantially when copper spraying was halted and leaves were washed by precipitation before harvest. Pessanha et al. (2010) did not observe any significant increase in grape, wine, or must copper levels, despite an increase in leaves, a finding supported by Lai et al. (2010). However, Provenzano et al. (2010) measured increased copper levels in berries with copper applications in Italy, but no phytotoxicity in grape leaves was detected. They also observed higher accumulation in white chardonnay grapes (5mg kg^{-1}) than in traditional red Italian varieties (1.7mg kg^{-1}), but not at levels hazardous to human health. In their study copper concentration remained constant throughout

the seasons, which contrasts with the leaf research of Angelova et al. (1999), perhaps due to differences in spraying frequency and duration. Angelova et al. also analyzed grape berries and found that vineyards in an industrial area had significantly higher copper levels than those in non-industrial areas, suggesting that atmospheric deposition was a significant contributor rather than uptake from the soil.

Brun et al. (1998) detected greater Cu in aboveground biomass of wild Asteraceae plants, known hyperaccumulators (Prasad and Freitas, 2003), than in Poaceae plants, which had high Cu in the root mass. Copper found in roots is mainly concentrated in the fine roots (Brun et al., 1998; Angelova et al., 1999). Brun et al. (2003) indicated that the Asteraceae species showed signs of chlorosis at copper levels equal to or greater than 200 mg Cu kg^{-1}, inducing delayed growth and low seed set. One species of Poaceae, *Dactylis glomerata*, however, was completely tolerant of copper. The effects of copper contaminated vineyard soils on other crop plants have provided a better understanding of copper distribution in plants. In general, neither maize nor tomatoes accumulated high levels of copper in their shoots (Chaignon et al., 2003; Brun et al., 2001). Brun et al. (2001) and Marschner (2003) suggest that the aerial parts of a plant do not indicate soil bioavailable Cu, but that the roots show the first signs of contamination. These results were seen in calcareous soils but not acidic soils where copper accumulation was much higher, suggesting that pH is a determinant in the speciation and activity of copper.

Earthworms play a key role in soil fertility, assisting in litter decomposition; they increase water-holding capacity with burrow tunnels and increase root penetration by loosening soil. While earthworms are also potential copper immobilizing agents, digesting copper and accumulating it in their tissue, they are also fatally affected by high copper quantities (McBride et al., 1981; Hinojosa et al., 2010; Udovic and Lestan, 2010). In 1984 copper chloride was seen to greatly suppress cocoon production with as little as 65 mg Cu kg^{-1} in sandy loam soils and 130 mg Cu kg^{-1} in sandy soils (Ma). Paoletti et al. (1998) found that earthworm abundance, species

number, and biomass were greatly reduced by both copper (140-210 mg Cu kg^{-1}) and soil tillage in vineyards. Copper oxychloride has been shown in lab studies to decrease earthworm-burrowing rates and increase copper body content, influencing growth and survival (Eijsackers et al., 2005). The authors saw mal effects with vineyard soils (14.4 mg Cu kg^{-1}) and increasing in spiked soils (55.4 mg Cu kg^{-1}). They believe this indicates a decrease in sustainable soil quality because only low numbers of big and old earthworms were observed (Eijsackers et al., 2005).

On a micro-scale copper "severely decreases the functional diversity of the soil microbial community and impairs specific pathways of nutrient cycling" (Kandeler et al., 1996). Microorganisms can interact with heavy metals in three ways: through sequestration and immobilization, enhancement of solubility, or potential harm due to toxicity (Maier et al., 2000). By crossing through the cell membrane, copper alters cell transcription, translation, and division, damaging cell DNA, denaturing proteins, and disturbing microbial biomass and communities (Maier et al., 2000).

Dell'Amico et al. (2008) found that, when comparing a high copper site (372 mg kg^{-1}) to a lower copper site (215 mg kg^{-1}) in acidic soils in Piedmont, Italy, the high copper site had higher exchangeable Cu and fewer fast growing, r-strategist bacteria, i.e. lower bacterial populations. Both sites contained copper tolerant bacteria, but the high copper site had more gram-negative bacteria, while the low copper site had more gram-positive bacteria. Bacteria have also been shown to increase community tolerance to copper (Díaz-Raviña et al., 2007). Known copper resistant bacteria are *Pseudonomas*, *Xantomonas* and *Escherichia coli* (Silver and Phung, 1996). *Pseudonomas* is the only bacteria that visually displays (by turning blue) copper storage in high copper environments (Silver and Phung, 1996).

Copper can affect soil microorganism enzyme activity, an aspect of biological health, cell membrane activity, and the processes of oxidation and reduction (Maier et al., 2000; Moolenaar, 1998; Hinojosa et al., 2010; White, 2009). It has been found to inhibit arylsulfatase, acid phosphatase, protease, urease, β-Glucosidase, and

cellulase at varying levels of toxicity (Hinojosa et al., 2010). Phosphatase and arylsulfatase show the highest sensitivity to copper (Hinojosa et al., 2010). In vineyards, copper has been shown to reduce levels of dehydrogenase, β-glucosidase, and phosphatase, and to inconsistently affect urease, sometimes even increasing its activity at moderate copper levels (Fernández-Calviño et al., 2010). This suggests that copper decreases enzymes associated with the C and P cycles.

5.3 Remediation

Remediation strategies

Remediation efforts for heavy metals can be achieved in five ways: immobilization through pH alterations or OM addition, removal, sequestration, active mixing, and plant uptake or phytoextraction (Pietrzak and Uren, 2011; Maier et al., 2000).

Altering pH to immobilize copper, called phytostabilization, is often suggested in the literature (Kidd et al., 2009; Flores-Vélez et al., 1996; Robinson et al., 2006; Fernández-Calviño et al., 2008a). This method requires continuous addition of lime, however, and is only a temporary solution, as copper will increase with continued use. As for OM addition, Brandt et al. (2008) found that manure addition to copper laden soils reduces free Cu^{2+} ion activity, but increases copper bioavailability. Copper can actually form bioavailable complexes with dissolved organic matter (DOM) derived from manure. Nachtigall et al. (2007) studied whether or not poultry litter would reduce Cu availability; it did not. However, other studies have had contradictory results and recommended manure to reduce Cu mobility (Besnard et al., 2001; Flores-Vélez et al., 1996; Fernández-Calviño et al., 2008a; Lejon et al., 2008).

Second, removal through acid washing and incineration are not practical, financially or logistically, for large areas of land that continue to be utilized for crops. Third, sequestration through exopolymers has only been achieved under laboratory conditions and is, therefore, not viable for large tracts of land. Fourth, active mixing

of top and deep soils to dilute Cu contamination destroys soil structure and is also not be possible directly within vinerows where Cu is highest.

Fifth, phytoextraction, uptake of soil heavy metals by plants, seems most feasible (Jadia and Fulekar, 2009). After sufficient growth and metal accumulation in roots and shoots, plants are harvested (Kidd et al., 2009). In general, phytoextraction is non-invasive; i.e. it retains soil fertility, is inexpensive, and utilizes the natural ecosystem to help control anthropogenic contamination. Phytoextraction is suggested for low to moderately contaminated environments, which vineyards often are (Jadia and Fulekar, 2009; Kidd et al., 2009). Vegetation cover also helps reduce erosion and maintain nutrients within the soil (Flores-Vélez et al., 1996). Morlat (2003) found that cover crops encourage vine roots to grow deeper by leaving less room for them in the upper soil and resulting in improved juice quality. However, cover crops are labor intensive to harvest. Three types of phytoextraction exist; no addition, chelate addition, and microorganism addition.

Plant selection

Plant selection is a key element of phytorextraction. Hyperaccumulator plants have the ability to take up more copper (>20 mg kg^{-1}), particularly in their shoots (McGrath et al., 2001; Reuter and Robinson, 1997), than other plants and still remain physiologically unaffected by toxicity (Rajkumar et al., 2010; Kidd et al., 2009). They are technically defined as plants that can accumulate more than 0.1% copper in leaf dry matter (Baker et al., 1994). Hyperaccumulating plants have also been shown to support a higher fraction of metal resistant microorganisms in the rhizosphere when compared to non-accumulators (Kidd et al., 2009). Several hyperaccumulating species are found in the *Brassicaceae* family (Kidd et al., 2009). Unfortunately, hyperaccumulators often have low biomass and slow growth. Usually ruderal or rosette plants growing along the ground, they are not an efficient option in field-contaminated systems. High biomass crops that are not necessarily hyperaccumulators, but which take up high levels of trace metals, are now being researched for their potential (Kidd et al., 2009). There have also been suggestions

that genes for metal hyperaccumulation should be transferred to more productive host plants (Baker et al., 1994), but this has not yet been pursued.

In a contaminated vineyard it is critical to choose plant types that accumulate large amounts of copper in their shoots, or aboveground biomass. In the narrow two-meter space between vine rows it is difficult to remove both roots and shoots without specialized, and currently non-existent, machinery. The second most important factor is to choose plants with relatively high biomass so that a greater amount of copper can be removed in fewer cuttings. Table 5.2 indicates plants tested for copper removal. Removal rates are not given, as there are no field trials. Should these methods prove successful and sufficient copper is accumulated in the plants, what should then be done with the harvested biomass? Pyrolysis, anaerobic decomposition and burning of plant biomass after which copper is phytomined, are most often cited in the literature (Kidd et al., 2009).

Phytoextraction

Phytoextraction without addition requires hyperaccumulator plants (Poschenrieder et al., 2001). Physiologically, plants have the ability to increase the solubility of metals, both through their roots and in the surrounding rhizosphere. Plant techniques to increase solubility include “acidification/alkalinisation, modification of soil redox potential, exudation of metal chelants and organic ligands” (Wenzel, 2009;Kidd et al., 2009). The amount and structure of root exudates (Quartacci et al., 2009) and the reducing capacity of roots play a large role (Kidd et al., 2009). Root exudates release metal chelating compounds to mobilize metals (McGrath et al., 2001). Vineyards represent a practical environment for this method, as copper is bioavailable in the root zone (Kidd et al., 2009). The role of microorganisms is difficult to assess because plants and microorganisms cooperate closely (Kidd et al., 2009).

Table 5.2. Plants selected for copper removal around world.

Location	Experiment Type	Soil Type and Origin	Plant	Approach	Copper accumulated in shoots mg kg^{-1}	Copper accumulated in roots mg kg^{-1}	Source
Europe							
Hérault, S. France	Greenhouse pot experiment	Calcareous sandstone-slate and alluvial deposits	*Zea mays* cv. Gaucho	No addition	7.14 – 17.2	23.4 - 584	(Brun et al., 2001)
Hérault, S. France	Field	Calcareous sandstone-slate and alluvial deposits	Wild species	No addition; *Dactylis glomerata* *Poa annua* *Andryala integrifolia* *Hypochoeris radicata* *Senecio vulgaris* *Sanguisorba minor* *Rumex acetosella* *Allium polyanthum* *Rubia peregrina*	 3.54 – 12.2 10.9 – 11.4 9.44 – 26.6 8.48 – 21.6 14.7 – 19.8 9.01 – 11.3 9.78 – 32.7 3.80 – 10.2 4.53 – 16.0	 -- -- 4.56 – 18.00 7.40 – 16.5 14.9 – 28.3 9.81 – 13.1 -- -- --	(Brun et al., 1998)
Roujan, France	Pot experiment	Variable soil samples	*Lycopersicon esculentum* cv. St Pierre	No addition	4.1 – 13.9	19 - 189	(Chaignon et al., 2003)
Portugal	Greenhouse pot experiment	$CuSO_4$ amended soil	*Brassica juncea*	Addition of microorganisms; *Achromobacter xylosoxidans*	~2 – 8	~30 – 180	(Ma et al., 2009)
Serbia	Outdoor pot experiment	Clay loam / Vineyard	*Brassica napus*	Addition of Chelators; EDDS EDTA	 38.6 – 316.4 34.2 – 52.0	 78.1 – 244.4 202.1 - 390.6	(Zeremski-Škorić et al., 2010)
Slovenia	Soil column experiment	Sandy loam / Vineyard	*Brassica rapa*	Addition of Chelators; Citric acid EDDS DTPA EDTA	 11.28 37.81 24.28 21.71	 -- 	(Kos and Leštan, 2004)
Spain	Soil and plant collection from 15 sites	Malachite outcrops	+32 species	No addition; *Sedum sediforme* *Hirschfeldia incana* *Hyparrhenia hirta* *Brachypodium retusum* *Echium vulgare* *Foeniculum vulgare* *Alyssum maritimum* *Psoralea bituminosa*	 406 355 10 34 32 16 25 11	 639 97 202 124 99 51 30 32	(Poschenrieder et al., 2001)
United Kingdom, Chile, China	Greenhouse pot experiment	Factory and mine tailings	*Silene vulgaris* *Elsholtzia splendens*	No addition; *Silene vulgaris* *Elsholtzia splendens*	 0.77* 0.60*	 0.75* 0.74*	(Song et al., 2004)

Table 5.2. continued… Plants selected for copper removal around world.

Location	Experiment Type	Soil Type and Origin	Plant	Approach	Copper accumulated in shoots mg kg^{-1}	Copper accumulated in roots mg kg^{-1}	Source
South America							
Brazil	Greenhouse pot experiment	Inceptisol and Mollisol / Vineyard	*Avena sativa*	Addition of microorganisms; *Pseudomonas putida A1* *Stenotrophomonas maltophilia A2* *Acinetobacter calcoaeticus A6*	~1 150 – 1 200 mg kg^{-1} ~1 100 – 1 200 mg kg^{-1} ~1 100 – 1 300 mg kg^{-1}		(Andreazza et al., 2010a)
Asia							
Zhejian Province, China	Greenhouse pot experiment	Alluvial sandy loam / Agriculture	*Elsholtzia splendens*	Addition of Chelators; Glucose citric acid	 ~85 ~75	 ~440 ~400	(Chen et al., 2006)
Zhejian Province, China	Greenhouse pot experiment	Alluvial sandy loam / Agriculture	*Trifolium repens*	Addition of Chelators; Glucose citric acid	 ~50 ~25	 ~320 ~360	(Chen et al., 2006)

*log transformed data

Poschenrieder et al. (2001) found that the following species had high shoot/root ratios indicating copper resistance and high potential for phytoextraction: *Alyssum maritimum* (>1.5), *Hirschfeldia* (>6), *Reseda lutea* (>1.5), *Reseda phyteuma* (>1.5), *Ononis natrix* (>6), and *Sparitum junceum* (>1.5). In particular, the authors suggest *R. phyteuma* for use in vineyards and orchards because it can grow in soils with as much as 950 mg kg^{-1} copper while extracting as much as 51 mg Cu kg^{-1} DM in its shoots (Poschenrieder et al., 2001). However, its low biomass limits this plant's efficiency.

Unfortunately, there is no published literature on natural phytoextraction in vineyards and the use of plants alone has not resulted in efficient outcomes. Because successful phytoremediation depends on the accessibility of copper in the field (Chen et al., 2006), there are two possible methods for increasing copper availability; adding chelators or using microorganisms.

Chelate assisted phytoextraction

Chelate assisted phytoremediation consists of adding an acid amendment to the entire contaminated area, inducing an acidic environment in which the copper becomes more bioavailable. Examples are EDTA, citric acid, glucose, or DTPA (McGrath et al., 2001; Chen et al., 2006). Chen et al. (2006) conducted a laboratory experiment with contaminated agricultural soils. They found that both chelators used, citric acid and glucose, promoted the availability and uptake of copper, especially by a hyperaccumulator plant. They also found that the chelators did not negatively affect soil microorganisms, a potential problem due to leaching. Treatments with plants also had higher microbial diversity than those without plants (Chen et al., 2006).

Kos & Leštan (2004) investigated the ability of four chelators to assist plants in removing copper from contaminated vineyard soils. The authors found that all but citric acid increased copper mobility, although plant uptake did not increase in the same proportion. EDDS was found to be the most successful. However, this method was also found to induce copper leaching (Kos and Leštan, 2004). The authors suggested that horizontal permeable barriers be installed to decrease

environmental degradation (Kos and Leštan, 2004). This would, however, be a costly investment requiring extensive soil upheaval in vineyards and is therefore not practical. Zeremski-Škorić et al. (2010) also looked at vineyard soils to identify differences in EDTA and EDDS chelate assisted phytoextraction by *Brassica napus*. The authors found that EDTA was inefficient while EDDS had potential for copper extraction. However, large amounts of EDDS induced growth suppression in the plants.

In conclusion, EDDS assisted experiments in vineyard soils were most successful. However, unlike Chen et al. (2006), other authors have observed leaching (McGrath et al., 2001; Kos and Leštan, 2004; Zeremski-Škorić et al., 2010). According to Wenzel (2009), due to the high cost of chelators and their unavoidable tendency to induce copper leaching it is not recommended in sites connected to groundwater. As groundwater is always present, chelate assisted phytoextraction is not suggested for vineyard sites.

Microbially assisted phytoextraction

Microorganisms aid in environmental processes such as mobility and availability of heavy metals in soil (Gadd, 2004). "Metal mobilization can arise from a variety of leaching mechanisms, complexation by metabolites and siderophores, and methylation" (Gadd, 2004). Microbially assisted phytoextraction is potentially a suitable tool for efficient remediation in vineyards. Bacteria that produce siderophores are exploited due to their capacity to mobilize metals, especially Fe, but also Cu, and to make metals more bioavailable for plants uptake (Wenzel, 2009; Rajkumar et al., 2010; Kidd et al., 2009; Gadd, 2004; Boukhalfa and Crumbliss, 2002). This method could be considered a "co-cropping component" (Wenzel, 2009) because the bacteria are added to the soil with the plants.

Metal resistant siderophore-producing bacteria are commonly found in areas of iron deficiency where their function is to make iron more accessible to plants (Van Loon, 2009). In heavy metal polluted environments the same bacteria can also chelate other non-ferrous metals, assisting plants in taking up more metal in a shorter period of time (see Figure 5.1B) (Rajkumar et al., 2010). Over 500

siderophore compounds have been identified from bacteria occurring naturally in the rhizosphere (Boukhalfa and Crumbliss, 2002), which increases the likelihood of success because they should already be present in the soil. At least one study has shown that contaminated environments can even stimulate these bacteria, either because they are an integrated part of siderophore production or because pollution restricts Fe availability and pushes the bacteria into an iron deficient, and thus a siderophore necessary, environment (Rajkumar et al., 2010).

There are two plant strategies to accumulate metals; plants (dicots and monocots, except Poaceae) rely on reducing agents or acidification of the surrounding environment; or, plants (Poaceae) release siderophores (Rajkumar et al., 2010). Siderophores act as chelators and as solubilizing vehicles by taking metals into the bacterial cell membrane, reducing their charge and releasing them into the cell (Rajkumar et al., 2010). The siderophore can be destroyed or recycled (Rajkumar et al., 2010). In order to implement this method, bacteria resistant to toxins must be isolated and then inoculated into the particular environment in which they are resistant (Andreazza et al., 2010c).

Currently, only one study has attempted this method in a copper contaminated vineyard soil and to date only in the laboratory. "Three copper resistant bacterial isolates from oatmeal rhizosphere (*Pseudomonas putida* A1; *Stenotrophomonas maltophilia* A2 and *Acinetobacter calcoaceticus* A6) were used for the stimulation of copper phytoextraction" (Andreazza et al., 2010a). Copper uptake in the plant shoots was improved with the isolates (see Table 5.2 for figures). Overall, the bacteria assisted oats in taking up more copper than it naturally would. The authors suggest this method for the removal of copper from polluted sites (Andreazza et al., 2010a). Thereafter, they also isolated *Pseudomonas* sp. strain NA, a copper resistant bacteria, from a vineyard soil and investigated how it reduces copper from Cu(II) to Cu(I), which increases availability to ATPases and the cell (Andreazza et al., 2010b). It had greater than 50% efficiency in reducing Cu (II) using mainly a copper reductase enzyme and its efficiency increased with greater total copper levels. This suggests that siderophores and specifically the

copper reductase enzyme boost copper bioavailability also to plants. As total copper increased and reduction percent increased, however, the bacterial biomass decreased. Therefore, more copper was reduced despite fewer bacteria. Copper reduction occurred between 20°C and 35°C with pH between 5 and 7, which suggests that reduction is possible over a range of temperatures if other factors, i.e. pH and pollutant concentration, are suitable (Andreazza et al., 2010c).

Other studies have suggested different enzymes or bacteria to enhance phytoextraction, such as histidine/proline, a Cu chelator in the xylem sap of *Brassica carinata* (Kidd et al., 2009). Plant growth promoting rhizobacteria (PGPR) have also been used to encourage plant growth, increasing metal uptake indirectly (Kidd et al., 2009; Ma et al., 2009). Uptake in roots or shoots remains unclear. These methods have not been tested in vineyards, but they provide interesting results and should be reviewed more closely as another method for combining bacteria and plants in copper removal.

A disadvantage of this method for wineries, as is the case for all phytoextraction techniques, is that it requires time to achieve Cu reduction in the soil and labor to remove the copper enriched plant mass (Wenzel, 2009). This is especially true if most of the copper is held in the roots. Also, since to date it has been demonstrated only in the laboratory, there remains the question of feasibility *in situ*.

5.4 Conclusions & Outlook

Robinson et al. (2009) consider phytoextraction to be "unsuitable for most, if not all, sites due to low-extraction rates and problems caused by site heterogeneity, the limited rooting depth of plants and the presence of contaminant mixtures." The available literature does not wholly support this statement. First in vineyards copper contamination is low to moderate. Second copper resides in the topsoil, making it suitable for shallow depth remediation. Third vineyards represent a single contaminant site where only copper is found in high levels. Finally, site heterogeneity can be accommodated through improvements in

methods and new ideas that could be integrated into vineyard systems over time. These include integrated pest management with increased biodiversity. Downy Mildew originates in the soil. Encouraging diverse plant life to create a barrier between the ground and the grapevines could further suppress fungal growth and also enhance dynamic microbial communities. When a complex system is diverse it adapts more easily to changes in toxicity, climate, soil type, etc. Focus on field experiments to define both true applicability and true resource competition in contaminated environments is also necessary (Wenzel, 2009). Pot experiments cannot replicate field environmental factors such as rooting density, compaction, and soil heterogeneity.

If no remediation techniques prove workable in vineyard ecosystems there are no options other than restricting copper to levels can be removed through the afore-mentioned strategies or banning copper use completely. The former would require a much more precise application of copper. Precision application is already being utilized in certain parts of Europe and can be achieved with software programs indicating when to most effectively apply copper, including vitimeteo.at and agromefeo.de, the "Coptimizer," the "Environmental Impact of an Organic Viticulture Indicator," and others (Kuflik et al., 2009; Fragoulis et al., 2009; Pellegrini et al., 2010).

As alternative options for organic winegrowers are not yet effective (Heibertshausen et al., 2007) and the climate is changing, increasing potential for infection from plant pathogens (Salinari et al., 2006), a holistic remediation approach is necessary. Phytoremediation can be a sustainable technique creating zero waste, while improving soil quality (Haferburg and Kothe, 2010). Plant biodiversity, enhancement of microorganisms, specifically siderophore production, and reduced copper use, should be reinforced. Specifically, multidisciplinary relationships in the rhizosphere should be the focus of further research.

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6 Long-term copper application in an organic vineyard modifies spatial distribution of soil microorganisms

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K.A. Mackie[a], T. Müller[b], S. Zikeli[c], & E. Kandeler[a]

[a]Institute of Soil Science and Land Evaluation, Soil Biology Section, Unversity of Hohenheim, Emil-Wolff-Strasse 27, 70599 Stuttgart, Germany

[b]Institute of Crop Science, University of Hohenheim, Fruwirthstrasse 20, 70599 Stuttgart, Germany

[c]Co-ordination for Organic Farming and Consumer Protection, University of Hohenheim, Fruwirthstrasse 14 – 16, 70599 Stuttgart, Germany

Abstract

Organic viticulturists utilize copper to prevent and reduce downy mildew (*Plasmopara viticola*) within the vineyard. Being a heavy metal, copper either builds up in the soil or is leached into the groundwater or taken up by living organisms. Therefore, its use impacts the environment. In organic farming there are currently no copper substitutes available and, therefore, it is necessary to understand the depth of damage that copper is inflicting on soil microbial communities over the long-term. Here a field-scale grid, 4 m by 5 m, was analyzed within a 17 year practicing organic vineyard in Southwestern Germany. Copper fractions, enzyme analyses (phosphatase, arylsulfatase, invertase, urease, xylanase), fungal analyses (ergosterol, fungal PLFA), bacterial analyses (bacterial PLFA), and microbial biomass were measured and spatial distribution maps were interpolated. Readily available and exchangeable copper fractions were higher within the vine rows and lower between them. Total copper ranged from 43 mg kg^{-1} to 142 mg kg^{-1}, which is above prevention levels for Germany. In areas of high copper, a negative effect on total carbon, ergosterol, as well as phosphatase and invertase enzyme activities was observed. Tillage practices were found to be more important than copper for the distribution of carbon, nitrogen and xylanase activity within the vineyard.

6.1 Introduction

Viticulture is an important perennial cropping system in Europe. In certain regions, however, these crops are blighted by downy mildew, *Plasmopara viticola*, a mold that in turn stresses the vine and reduces grape quality (Salinari et al., 2006; Jermini et al., 2010a). Copper-based fungicides have been used to combat *P. viticola* in different production systems worldwide for more than a century. Today, synthetic fungicides play a major role in conventional farming; these fungicides replace copper products and, thus, reduce this input of the heavy metal in vineyard soils, although copper-based fungicides continue to be applied in restrained amounts. In organic farming, copper-based fungicides are still the only effective method permitted to treat grapes against *P. viticola*. As organic methods are becoming more common, especially in Europe, where 100,000 ha of grapes are under organic management (IFOAM, 2009), the total use of copper-based fungicides remains important even though the maximum amount of copper input per year is limited to a maximum of 6 kg y^{-1} ha^{-1} according to the EU Regulation on Organic Production and Labeling of Organic Products (European Commission, 2007). As copper is known to accumulate within topsoil following fungicidal sprays (Pietrzak and McPhail, 2004; Rusjan et al., 2007) and can never be degraded (McBride et al., 1981), its potential to have adverse eco-toxicological effects on the environment is large. In environments, such as vineyards, where pH is often above neutral, copper is immobile, accumulating over years of use. However, soluble fractions are always present (McBride et al., 1981) and both low organic matter content (OM) and low cation exchange capacity (CEC) often found in vineyards encourage copper mobilization (Maier et al., 2000). When copper becomes mobile and more available to organisms, it stresses macro fauna (such as earthworms), microorganisms and their enzyme activities and, in high amounts, becomes toxic to plants, thereby disturbing essential elemental cycles (McBride et al., 1981; Moolenaar, 1998; Paoletti et al., 1998; Maier et al., 2000; White, 2009; Hinojosa et al., 2010).

The focus of this study was the influence of copper use in grape cultivation on the spatial abundance and function of soil microorganisms. Copper is said to reside in the topsoil and decrease with distance from the vine (Wightwick et al., 2006; Komárek et al., 2008; Mackie et al., 2012). The question remains, how does copper affect soil quality within the vineyard system and where is its impact greatest? Soil microbial abundance and activity are heterogeneously distributed within the soil matrix at different scales ranging from millimeters to meters (Berner et al., 2011). This distribution is affected by the carbon/nitrogen ratio, the chemical composition, the porosity, the location of organic substrates and plant biomass, the grade of humification, the water content, the pH, and the heavy metal content of the soil (Buscot and Varma, 2005). Investigation of meso-scale (field-scale) distribution can be helpful in understanding multivariable interactions, which are difficult to observe on a larger scale (Philippot et al., 2009; Berner et al., 2011; Keil et al., 2011). Heavy metals, particularly, can modify spatial distribution of microbial abundance and activity by altering soil characteristics and reducing microbial biomass (Kandeler et al., 1996). In vineyard soils, copper has been found to diminish the rate of ammonification, which signifies an alteration in bacterial presence and/or functions (McBride et al., 1981). However, field knowledge regarding copper impacts specifically on soil microorganisms is limited (Dell'Amico et al., 2008; Wang et al., 2009; Brandt et al., 2010; Fernández-Calviño et al., 2010) and, as these communities maintain essential processes and support soil fertility, their upkeep is vital for viticultural and other agricultural systems. We expected that even in areas of low to moderate copper pollution, such as vineyards, soil microorganisms and enzymes involved in the nitrogen (N), phosphorus (P) and sulfur (S) cycling will be compromised. Therefore, it was the goal of this study to investigate meso-scale distribution of copper, soil properties and microorganisms within an organic vineyard and to identify the interactions between copper accumulation and the soil eco-system. We hypothesized that the spatial distribution of the activity (i.e. enzyme activity) and abundance of soil microorganisms is correlated with the spatial distribution of copper in the soil, thus, (i) areas of high copper will indicate areas of low soil microbial activity and (ii) copper distribution will influence the spatial

distribution of the microbial community composition (i.e. phospholipid fatty acid composition) in the soil.

6.2 Materials & Methods

Study area

Samples were taken from an organic vineyard in Brackenheim, Baden Württemberg, Germany (49°5'43.53"N, 9°2'57.93"E). The site has an elevation of approximately 230 m, an average precipitation of 650 mm and an average temperature between 7°C and 14°C. The vineyard was established in 1988 and has been under certified organic management since 1993. The soil type sampled on the site is an Umbric Leptosol. It is planted with Trollinger vines. Various species of grass were growing between each row. The site has a southern exposure and a slope of approximately 35 %. Harvest residues (leaves, wood chips, pomace), mixed with an addition of wheat straw, were spread homogenously over the vineyard during a period of five years. Each year every second row is plowed. The last plowing event was in April of 2009, more than one year before soil samples were taken.

Since 1993, based on organic certification standards in Germany, the vineyard manager has sprayed a maximum of 3 kg y^{-1} ha^{-1} of copper solution on the grape vines (BIOLAND, 2011). Both copper oxychloride ($Cu_3Cl_2(OH)_4$) and copper hydroxide ($Cu(OH)_2$) have been used.

Soil Sampling

Soil samples were taken in April 2010 prior to any copper solution treatment for the 2010 season. Figure 1 illustrates the sampling pattern chosen based on maximal coverage of the plot and statistical accuracy. Each box represents 0.25 m by 0.25 m. There were a total of 59 sampling points, each either 0, 50 or 100 cm from the grape vine row. Within a row grape vines are 1 m apart, while the rows are separated by a distance of 2 m. The right interrow was plowed in 2009.

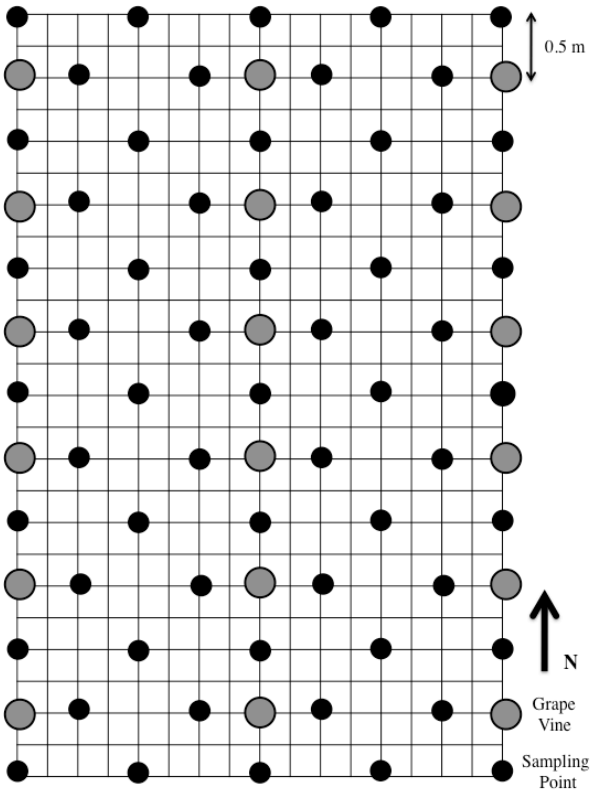

Figure 6.1. Sampling grid. Each box within the grid represents 25 cm x 25 cm within a total area of 24 m^2 (4 m x 6 m). Numbers represent sampling points with distinct distances from the vine row (0 cm, 50 cm or 100 cm).

Soil cores (5.5 cm diameter) were taken to a depth of 10 cm. Samples were kept in a cooler and placed in a 4°C refrigerator after sampling for a maximum of 5 days. The soil was sieved through 2 mm mesh metal sieves and was stored in a -20°C freezer until further analyses. Samples for copper analyses were air-dried.

Plant Sampling

Grass samples were taken on the same day as the soil samples. A 30 cm diameter metal ring was used to mark the area directly around the sampling spot. All living aboveground biomass within the ring was harvested and placed in plastic bags. Directly after sampling, the plant material was cut and dried in aluminum foil trays in an oven at 60°C for 48 hours and at 105°C for 10 hours to completely dry the samples.

Soil Analyses

Total copper (Cu_T) was extracted by Aqua regia (HNO_3 + HCl) extractant (DIN ISO 11466, 1995). Ammonium nitrate extractable copper (Cu_{NH4NO3}) was extracted with ammonium nitrate (NH_4NO_3) and was chosen because it may provide a good indication of copper that is not complexed (Ettler et al., 2007; DIN ISO 19730, 2008). As an indicator of bioavailable copper, the DTPA exchangeable copper fraction (Cu_{DTPA}) was analyzed using diethylenetriamine pentaacetic acid extractant ($CaCl_2$+DTPA) (VDLUFA, 2011a). DTPA is an effective extractant for neutral and slightly alkaline soils and is used for extracting ligand-bound metals within the soil (Brun et al., 1998; Ettler et al., 2007). All methods were finally analyzed with an atomic absorption spectrophotometer.

pH was determined with a glass electrode in a suspension of 4 g soil in 10 ml 0.01 M $CaCl_2$. Total carbon (C_T) and nitrogen (N_T) were analyzed by dry combustion (element analyzer, Elementar Analysensysteme GmbH, Germany) using dried and finely ground samples. Organic carbon (C_{org}) and calcium carbonate were quantified by a carbon analyzer (Leco C/N 2000, St. Joseph, USA). Water content was determined after drying 1 g soil at 60°C in an oven for 72 hours.

Ergosterol was extracted according to Djajakirana et al. (1996) to determine living fungal biomass. Aliquots (2 g) of moist soil were shaken with ethanol and analyzed using a High Performance Liquid Chromatograph autosampler (Beckmann Coulter, System Gold 125 Solvent Module). Phospholipid Fatty Acids (PLFA) were extracted according to Frostegård et al. (1993) and Bardgett et al. (1996), where 4 g of soil were taken from each sample. Samples were shaken with Isooctan and measured with a gas chromatograph autosystem XL (Perkin Elmer, MA, USA). Nomenclature and division of PLFA's into bacteria and fungi was based on Kandeler et al. (2008), where total bacteria PLFA's ($PLFA_{bacteria}$) are represented by 16:1ω7, 18:1ω7/18:1ω9t, cy17:0, cy19:0, i15:0, a15:0, i16:0, and i17:0, and 18:2ω6 represents fungal PLFA's ($PLFA_{fung}$). The chloroform fumigation extraction (CFE)

method of Vance et al. (1987) was used to measure microbial (C_{mic})(N_{mic}) and extractable (C_{ext})(N_{ext}) carbon and nitrogen, respectively, using a fresh soil weight of 10 g and analyzed using the water analyzer DimaTOC 100 (Dimatec, Essen, Germany). This extraction method includes fine roots and the microorganisms associated with them (Mueller et al., 1992). The following enzyme analyses were read using a spectrophotometer (UV-1601 Spectrophotometer). Arylsulfatase, measured according to Schinner et al. (1996) using 1 g soil as suggested for marginal soils, was analyzed to assess the mineralization of organic sulfur compounds. Xylanase was measured based on the method of Schinner and von Mersi (1990) using 0.3 g soil. Invertase was analyzed based also on the method of Schinner and von Mersi (1990) using 0.3 g soil. Urease was measured based on the method of Kandeler and Gerber (1988), where 1 g of soil was used. Phosphatase is a component of soil enzymes that removes phosphate from its substrate: it has both alkaline (bacterial) and acid (plant and bacterial) types. In this study the alkaline phosphatase was measured using the method of Schinner et al. (1996) and 0.3 g soil.

Plant analyses

Dried plant material was finely milled and underwent microwave digestion in nitric acid following the method of VDLUFA (2011b). To determine the Cu content of the plant biomass the method of VDLUFA (2011c) was used and analyzed with inductively coupled plasma optical emission spectrometry (ICP-OES).

Statistical analysis

SAS 9.2 (SAS Institute Inc., North Carolina, USA) was used to conduct statistical analysis. Requirements for ANOVA, i.e. normal distribution and homogeneity of variance, were fulfilled by all variables. A two-way factorial ANOVA was conducted for the factors tillage, distance from vine and their interaction. In the case that the interaction was significant the p-value was given. Arithmetical means and standard errors were calculated for each variable based on tillage (vine row, left and right interrows) and distance from the vine row (0 cm, 50 cm and 100 cm). Significant differences between were calculated using a Tukey post hoc test ($p<0.05$). Sample

correlation analyses were based on Pearson's Correlation with a significance given if $p<0.05$ and an $|r|>0.50$.

Geographic information system software ArcGIS 9.3 (ESRI Deutschland GmbH, Germany) was utilized to create interpolated maps of selected variables. These maps illustrate trends within the data, but do not indicate significant correlations. They provide a visual demonstration of the values obtained in the analyses and indicate trends that are further explored with above classical statistical methods. Kriging interpolation method was used and the lowest root mean square map was chosen. The foundation values and choices for each map can be seen in supplemental material (Table S.6).

6.3 Results

Chemical properties and distribution

Table 1 shows means and significant differences ($p<0.05$) based on tillage and distance from the vine row. Cu_T (Table 6.1) ranged from 43 to 142 mg kg^{-1}, where the vine row had significantly higher values than the interrows. Cu_T (Fig. 6.2.D), Cu_{DTPA} (Fig. 6.2.E) and Cu_{NH4NO3} (Table 6.1) were significantly higher in the vine rows, while the interrows had a significantly lower mean (Table 6.1). LHowever, the map shows (Fig. 6.2.D) that Cu_T was amassing in the upper slope. Cu_{DTPA} had even significantly lower values 100 cm away from the vine when compared to both 50cm away and in the vine row. Cu_{NH4NO3} was the only copper variable that was significantly influenced by the interaction between tillage and distance from the vine. Cu_{DTPA} was significantly negatively correlated with C_T ($p=0.0001$, $r=-0.50$). Cu_{NH4NO3} was not correlated to any other variable.

Table 6.1. Two-way ANOVA for carbon, nitrogen, pH, water content, copper, plant dry weight, and copper in plant dry weight per area. Two-way ANOVA is shown as affected by tillage, distance from the vine row and their interaction. Arithmetical mean ± standard errors (where n ranges from 56 to 59), including values for samples never tilled (vine row), tilled one year ago (right interrow) and tilled two years ago (left interrow), as well as, 0 cm, 5 cm and 100 cm distance from the vine row. Within one variable, different letters (a, b, c) indicate significant differences (Tukey test $p<0.05$). The interaction, Tillage*Distance from vine, is represented either by non-significant (ns) results or significant p-value ($p<0.05$) results.

	Tillage			Distance from vine			Tillage *Distance
Variable	Vine row	Left interrow	Right interrow	0 cm (vine row)	50 cm	100 cm (middle of interrow)	p-value (p<0.05)
Total Carbon (%)	4.56 ± 0.07 a	5.92 ± 0.12 b	4.95 ± 0.08 c	4.56 ± 0.07 a	5.44 ± 0.15 a	5.37 ± 0.16 a	0.0317
Organic Carbon (%)	1.86 ± 0.06 a	3.25 ± 0.13 b	2.16 ± 0.07 c	1.86 ± 0.06 a	2.68 ± 0.16 a	2.70 ± 0.16 a	0.0268
Microbial Carbon (µg g^{-1})	441 ± 20 a	567 ± 24 b	485 ± 22 a	441 ± 21 a	529 ± 20 a	518 ± 34 a	ns
Extractable Carbon (µg g^{-1})	194 ± 7 a	274 ± 12 b	218 ± 10 a	194 ± 6.7 a	242 ± 12.2 a	252 ± 12 a	ns
Total Nitrogen (%)	0.22 ± 0.01 a	0.35 ± 0.02 b	0.26 ± 0.01 c	0.22 ± 0.009 a	0.3 ± 0.02 a	0.3 ± 0.02 a	0.0235
Microbial Nitrogen (µg g^{-1})	26.9 ± 2.2 a	34.9 ± 2.23 a	31.2 ± 2.95 a	26.9 ± 2.2 a	33.1 ± 2.1 a	32.8 ± 3.8 a	ns
Extractable Nitrogen (µg g^{-1})	18.4 ± 1.3 a	26.3 ± 1.5 b	21.8 ± 1.3 a	18.4 ± 1.3 a	23.2 ± 1.1 a	25.5 ± 2.1 a	ns
pH	7.3 ± 0.06 a	7.4 ± 0.06 a	7.4 ± 0.06 a	7.3 ± 0.06 a	7.3 ± 0.05 a	7.4 ± 0.07 a	ns
Calcium Carbonate (%)	2.7± 0.03ab	2.7 ± 0.04 a	2.8 ± 0.03 b	2.7 ± 0.03 a	2.8 ± 0.03 a	2.7 ± 0.05 a	ns
Water Content (%)	9.8 ± 0.64 a	12.5 ± 0.37 b	11.4 ± 0.39 ab	9.8 ± 0.64 a	12 ± 0.39 a	11.8 ± 0.4 a	ns
Total Copper (mg kg^{-1})	87 ± 6.0 a	66 ± 4.2 b	65 ± 4.7 b	87 ± 6 a	68 ± 4.1 b	61 ± 4.7 b	ns
DTPA Cu (mg kg^{-1})	15.3 ± 0.44 a	8.8 ± 0.42 b	8.7 ± 0.41 b	15.3 ± 0.44 a	9.5 ± 0.34 b	7.5 ± 0.33 c	ns
Ammonium Nitrate (mg kg^{-1})	0.27 ± 0.01 a	0.20 ± 0.01 b	0.20 ± 0.01 b	0.27 ± 0.01 a	0.20 ± 0.01 a	0.19 ±0.02 a	0.0070
Plant Dry Matter (kg m^{-2})	168 ± 15.5 a	309 ± 29.4 b	159 ± 13.8 a	168 ± 16 a	241 ± 30 a	223 ± 19 a	0.0013

Table 6.1. continued…Two-way ANOVA for copper in plant dry weight per area.

	Tillage			Distance from vine			Tillage*Distance
Variable	Vine row	Left interrow	Right interrow	0 cm (vine row)	50 cm	100 cm (middle of interrow)	p-value ($p<0.05$)
Plant Copper (mg m^{-2})	1.76 ± 0.43 a	1.98 ± 0.18 a	1.33 ± 0.17 a	1.76 ± 0.43 a	1.54 ± 0.17 a	1.80 ± 0.22 a	

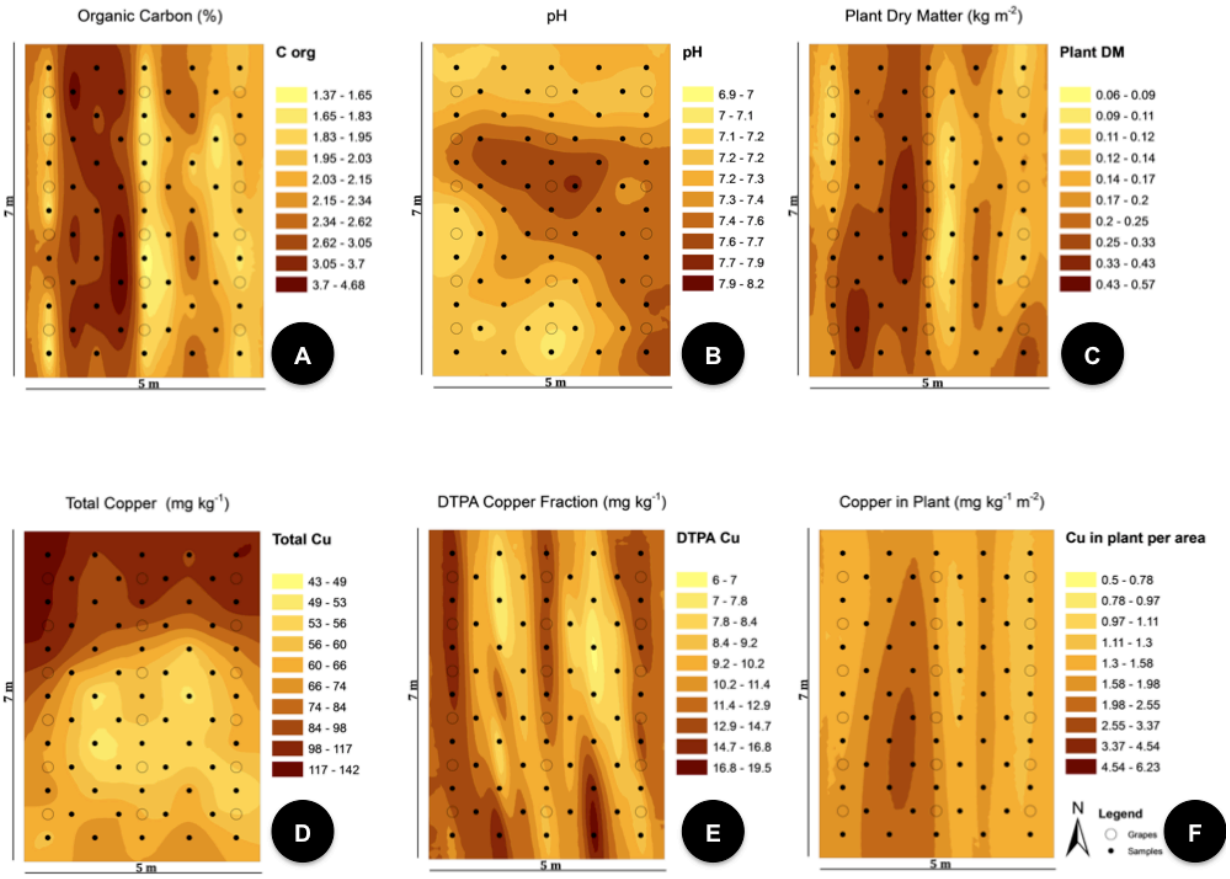

Figure 6.2. Soil organic carbon, soil pH, plant dry matter, soil total copper, soil DTPA copper fraction, and copper in plant interpolation maps where high quantities are illustrated with a dark brown coloration and low values with a light yellow color. The North arrow also indicates the upslope.

In Fig. 6.2.A, it can be seen that the left interrow had a significantly higher content of C_{org} than the right interrow and that in the interrows the C_{org} content was significantly higher than within the vine row. C_T, C_{mic} and C_{ext}, as well as N_T and N_{ext}, were significantly higher in the left interrow compared with the right interrow and the vine row. Although none of the C or N variables were significantly different based on distance from the vine, C_T, C_{org} and N_T were all significantly influenced by the interaction between tillage and distance from the vine. C_{org} was positively correlated to C_T ($p=0.0001$, $r=0.98$) as well as N_T ($p=0.0001$, $r=0.83$), C_{mic} ($p=0.0001$, $r=0.61$) and plant dry matter ($p=0.0001$, $r=0.63$).

pH values (Fig. 6.2.B) did not show any significant differences between interrows, vine rows or along the slope, the values were fairly uniform, ranging from neutral to slightly alkaline (Table 6.1). Mean water content of the soil was significantly lower within the vine rows and highest within the left interrow. Apart from this, water only showed a correlation to arylsulfatase ($p=0.0001$, $r=0.53$) and C_{org} ($p=0.0001$, $r=0.50$)

Plant dry matter (Fig. 6.2.C) demonstrated significantly higher plant biomass in the left interrow, in concert with C_{org} ($p=0.0001$, $r=0.63$) and C_T ($p=0.0001$, $r=0.63$). Copper concentration of the plant indicated no trend (Table 6.1). When the copper was calculated for plant biomass per area (Fig. 6.2.F), however, a different trend was observed, where it was highest between the vine rows, but this was not significant. It was not correlated to Cu or any other variable.

Microbial distribution

Bacterial and fungal communities

Table 2 displays the means and significant differences ($p<0.05$) based on tillage and distance from vine row for each variable. $PLFA_{bacteria}$ (Fig. 6.3.A) and $PLFA_{fungal}$ (Fig. 6.3.B) were both concentrated within the left interrow, with a significantly lower mean within the vine row and right interrow. $PLFA_{fungal}$ also trended to be higher in the lower slope when compared to the upper slope. Ergosterol (Fig. 6.3.C), however, was distributed evenly based on the lower and upper slope. $PLFA_{bacterial}$, fungal/bacterial ratio and ergosterol were all significantly influenced by the interaction between tillage and distance from the vine. Ergosterol was significantly positively correlated with $PLFA_{fungal}$ ($p=0.0001$, $r=0.66$), invertase ($p=0.0001$, $r=0.71$) and arylsulfatase ($p=0.0001$, $r=0.54$). Ergosterol was significantly negatively correlated to total copper ($p=0.0001$, $r=-0.72$), but not to the copper fractions.

Table 6.2. Two-way ANOVA for enzymes, fungi and bacteria. Two-way ANOVA is shown as affected by tillage, distance from the vine row and their interaction. Arithmetical mean ± standard errors (where n ranges from 56 to 59) are shown, including values for samples never tilled (vine row), tilled one year ago (right interrow) and tilled two years ago (left interrow), as well as, 0 cm, 5 cm and 100 cm distance from the vine row. Within one variable, different letters (a, b, c) indicate significant differences (Tukey test $p<0.05$). The interaction, Tillage*Distance from vine, is represented either by non-significant (ns) results or significant p-value ($p<0.05$) results.

	Tillage			Distance from vine			Tillage*Distance
Variable	Vine row	Left interrow	Right interrow	0 cm (vine row)	50 cm	100 cm (middle of interrow)	p-value (p<0.05)
Bacterial PLFA (nmol g^{-1})	34.6 ± 1.5 a	50.4 ± 2.1 b	39.4 ± 1.5 a	34.6 ± 1.5 a	45.4 ± 2.1 a	44 ± 2.2 a	0.0048
Fungal PLFA (nmol g^{-1})	2.6 ± 0.19 a	4.9 ± 0.36 b	3.4 ± 0.25 a	2.6 ± 0.19 a	4.2 ± 0.33 a	4.1 ± 0.42 a	ns
Fungal/Bacterial Ratio	0.09 ± 0.003 a	0.11 ± 0.01 b	0.09 ± 0.01 ab	0.09 ± 0.003 a	0.1 ± 0.006 a	0.1 ± 0.008 a	0.0085
Ergosterol (µg g^{-1})	4.77 ± 0.42 a	6.12 ± 0.60 b	6.35 ± 0.46 b	4.77 ± 0.42 a	6.71 ± 0.46 b	5.47 ± 0.55 a	<0.0001
Invertase (µg Glucose Equivalent g^{-1} 3 h^{-1})	5005 ± 347 a	6024 ± 363 b	6486 ± 300 b	5005 ± 347 a	6919 ± 202 b	4964 ± 347 a	ns
Xylanase (µg Glucose Equivalent g^{-1} 24 h^{-1})	1712 ± 85 a	2825 ± 161 b	1841 ± 128 a	1712 ± 85 a	2324 ± 179 a	2346 ± 182 a	ns
Urease (µg N g^{-1} 2 h^{-1})	93 ± 5.8 a	119 ± 6.3 b	128 ± 5.5 b	93 ± 5.8 a	128 ± 4.9 a	117 ± 7.5 a	0.0044
Phosphatase (µg phenol g^{-1} 3 h^{-1})	816 ± 45 a	1337 ± 50 b	1088 ± 45 c	816 ± 45 a	1223 ± 56 a	1213 ± 48 a	<0.0001
Arylsulfatase (µg 4-Nitrophenol g^{-1} h^{-1})	98.0 ± 4.2 a	107 ± 4.2 b	117 ± 5.4 c	97.2 ± 4.1 a	113 ± 4 a	114 ± 7.2 a	ns

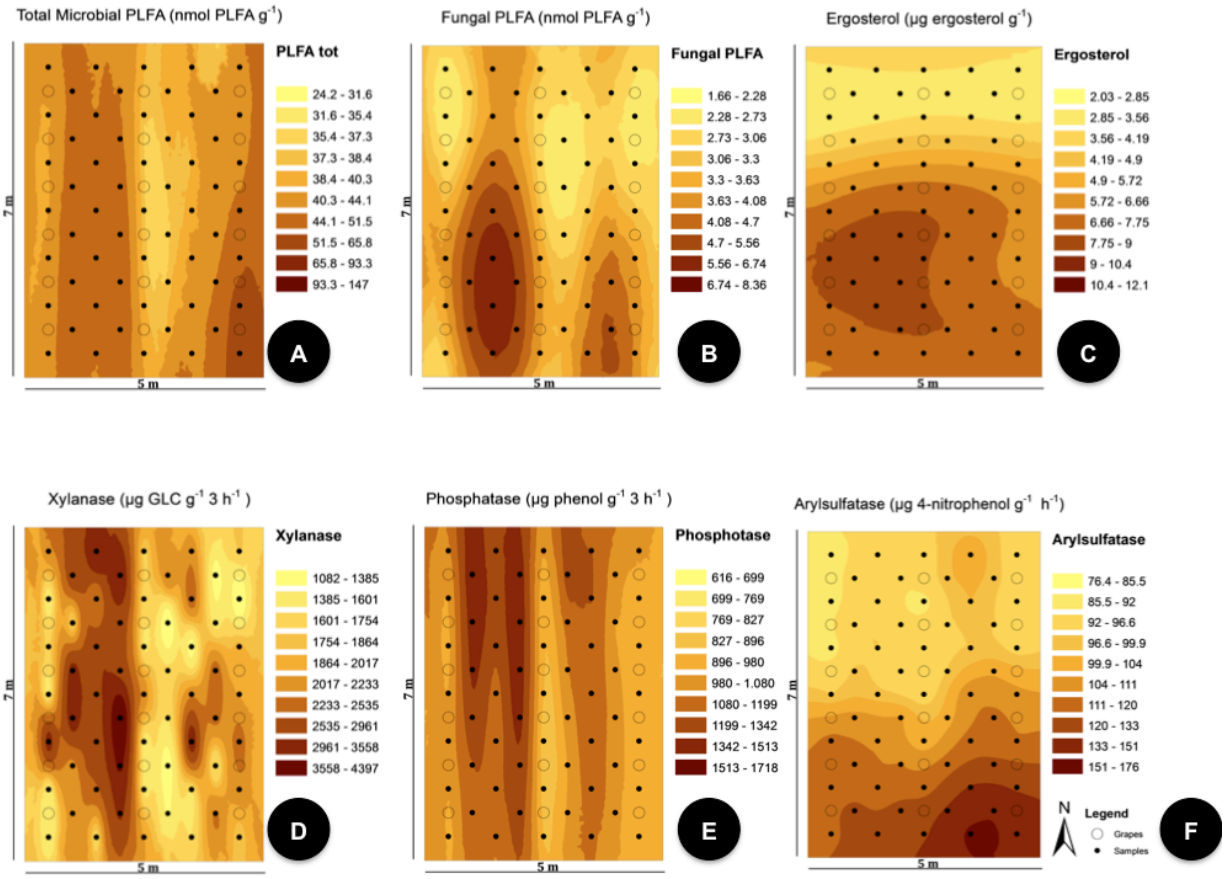

Figure 6.3. Total microbial PLFA's, fungal PLFA's, ergosterol, xylanase, phosphatase, and arylsulfatase interpolation maps where high quantities are illustrated with a dark brown coloration and low values with a light yellow color. The North arrow also indicates the upslope.

Enzyme activity

Fig. 6.3 also represents the enzymes involved in C, P and S turnover in the soil. Xylanase (Fig. 6.3.D) had a significantly higher activity in the left interrow than in the right interrow. There was a positive correlation between xylanase and C_T ($p=0.0001$, $r=0.78$), C_{org} ($p=0.0001$, $r=0.78$), C_{mic} ($p=0.0001$, $r=0.53$), phosphatase ($p=0.0001$, $r=0.72$), and plant dry matter ($p=0.0001$, $r=0.60$). However, there was no correlation with different Cu fractions or Cu_T. Invertase (Table 6.2) exhibited a slight tendency towards the lower slope and was significantly negatively correlated with Cu_T ($p=0.0001$, $r=-0.52$).

The activity of arylsulfatase (Fig. 6.3.F) was concentrated in the lower slope of the grid, which was in contrast to Cu_T. Although not immediately evident in Fig. 6.3.F, the mean of arylsulfatase became significantly higher in the interrows, especially the right interrow (Table 6.2).

Urease (Table 6.2) was significantly more concentrated in the interrows than within the vine rows, but had a fairly uniform spatial coverage. It was positively correlated with N_T (p=0.0001, r=0.55) and phosphatase (p=0.0001, r=0.73). Phosphatase (Fig. 6.3.E) was also significantly higher within the interrows, and highest on the left side. Phosphatase had a negative correlation with Cu_{DTPA} (p=0.0001, r=-0.49). It also had a positive correlation with N_T (p=0.0001, r=0.75), C_T (p=0.0001, r=0.79), C_{org} (p=0.0001, r=0.78), and C_{mic} (p=0.0001, r=0.67). Both urease and phosphatase were significantly influenced by the interaction between tillage and distance from the vine row.

6.4 Discussion

Spatial distribution of soil microorganisms related to copper treatment

Although Cu was hypothesized to modify the spatial distribution of soil microbial activity and composition, not all results could be explained by Cu contamination. Results showed that a second factor, tillage, also played a role in influencing the spatial placement of microorganisms, which follow carbon substrate availability. Cu_T concentration in the vineyard site was highest at the upper slope of the grid with maximum of 142 mg kg^{-1}. Comparing results from our vineyard to those from other studies in the literature, it appears that the copper content was at the lower end of the observed range (Mackie et al., 2012). It is, however, between the "warning" (50 mg kg^{-1}) and "critical" (140 mg kg^{-1}) classification used by the Council of the European Communities (EC) (1986) when discussing whether sewage sludge can be added to farm land. This classification has become the standard within the literature when referring to critical pollution levels due to an absence of heavy

metal limitations in European legislation. Cu_T is also above the prevention level for clayey soils (60 mg kg^{-1}) given by the German Legislation for Soil Protection (BBodSchV, 1999). Although these values do not always refer to bioavailability, our study showed a significant negative correlation between total copper values and ergosterol and invertase and similar negative trends with arylsulfatase and $PLFA_{fungal}$.

Cu_{NH4NO3} and Cu_{DTPA} were highest within the vine rows, but were also present across the plot, probably due to wind drift or leaf litter input. Representing the extractable fraction, Cu_{NH4NO3} did not correlate to any other variable. Similar values (Ammonium nitrate method) were also found in a hops field in Bavaria, where copper use is also a common practice (Schramel et al., 2000). The exchangeable fraction, Cu_{DTPA}, in our study was greater than the extractable fraction (Cu_{NH4NO3}). Brun et al. (1998) found Cu_{DTPA} values between 1.5 and 71.4 mg kg^{-1} in French Mediterranean vineyards and these values were highly correlated with Cu_T. Here, only the Cu_{DTPA} and Cu_{NH4NO3} were significantly correlated to each other. Similarly to Brun et al., however, pH was not found to play a significant role on Cu in our study. Nevertheless, Cu_T, Cu_{DTPA} and Cu_{NH4NO3} were all significantly lower in samples taken farther from the vine row (Table 6.1).

C_{mic} in vineyards has been shown to have values between ~150 – 750 µg C_{mic} g^{-1} DM, suggesting that our site was representative and neither high nor low (Kostov and Van Cleemput, 2001; Goulet et al., 2004; Lejon et al., 2008; Probst et al., 2008; Okur et al., 2009; Peregrina et al., 2010). C_{mic} values were significantly lower in the vine row and right interrow than the left interrow. This could be an indication that a low carbon content under the vines is limiting microbial substrates. Although it was expected that C_{mic} would have a negative correlation with Cu, as suggested by other studies (Parat et al., 2002; Wang et al., 2009), this was not observed in our study. This suggests that in the left interrow, where more plant biomass was growing due to a longer interval from tillage and likely replenished soil organic matter, the substrate was more favorable for the development of soil microorganisms. This is

supported by the positive correlation of C_{org} with plant dry matter and C_{mic} (see section 3.2). Bardgett and Saggar (1994) and Khan and Scullion (2000) studied metal contamination, including Cu, and how it affects soil characteristics. With a comparable C_{mic} to our study, Khan and Scullion's C_{mic} tended to decrease with more contamination, which was also seen here, although not supported statistically. Bardgett and Saggar (1994) suggest that microorganisms in soils polluted with heavy metals use more energy on maintenance and use substrates inefficiently in the production of biomass. However, due to a lack of negative correlation, it is not clear here whether heavy metal is additionally playing a role in the quantity of microbial biomass.

$PLFA_{bacteria}$ (Fig. 6.3.A) exhibited a similar trend, significantly lower activity under the vine rows and in the right interrow, but no relationship was found with any form of Cu. A study in China based on Cu contaminated paddy soils showed a negative correlation between Cu, both total and bioavailable, and $PLFA_{bacteria}$ (Ge and Zhang, 2011). In that study, Cu_T ranged from 67 – 2712 mg kg^{-1}, while $PLFA_{bacteria}$ ranged from 5.8 – 27.8 nmol g^{-1}. In another experiment, assessing resistance to high Cu exposure of Cu adapted and non-adapted bacterial communities, Brandt et al. (2010) found that neither community changed in structure, size or function after five years. This could suggest that microbial populations are being affected by other soil properties, but not by copper. This is reinforced by the fact that $PLFA_{bacteria}$ was significantly affected by tillage and the interaction between tillage and distance from the vine.

Ergosterol and $PLFA_{fungal}$ both amassed in the lower half of the study site slope, where Cu_T was lower. These trends, as well as the significant negative correlation between ergosterol and Cu_T, suggest that the fungal community is more sensitive to the Cu_T concentration rather than copper fractions, where no signifcant negative correlation was found. Pennanen et al. (1996) also found that high Cu_T levels decrease fungal population and Ge and Zhang (2011) concluded that fungal populations were more affected by Cu than bacterial populations. Copper fungicides

are expected to inhibit fungi, albeit on plants (Rajapaksha et al., 2004). The ergosterol levels within this study were comparably lower than those found by Khan and Scullion (2000), where with similar amounts of Cu and pH, levels ranged from 9.8 to 12.5 mg kg^{-1}.

When Cu influenced enzyme activity, a correlation could be established for Cu_{DTPA} and Cu_T, but not for Cu_{NH4NO3}. Invertase, although not previously found to have a specific sensitivity to Cu or other metals, was negatively correlated to Cu_T (Hinojosa et al., 2010). It was also consistent with fungal trends seen in Fig. 6.3, which is reasonable because invertase is often associated with yeasts, a member of the fungal kingdom, to catalyze the breakdown of sucrose (Moneke et al., 2008). Invertase appears to be highly variable, as some studies have found it to be stimulated by Cu, while others have observed slight reductions in activity, but not always with significant correlation (Fu et al., 2009; Wang et al., 2009; Kenarova and Radeva, 2010). Arylsulfatase has also shown a sensitivity to Cu_T levels rather than to Cu fractions. This is supported when observing the high arylsulfatase activity in the lower slope of Fig. 6.3.F. This was not expected, as arylsulfatase is known to be a biological indicator for heavy metal contamination of plants (Hinojosa et al., 2010). However, the fact that arylsulfatase has responded to these lower levels of Cu_T is in line with the sensitivity of this enzyme. Arylsulfatase was significantly lower within the vine row when compared to the interrows, but surprisingly not due to tillage, as the right interrow was significantly higher than the left. Arylsulfatase activities in this study, however, were higher than those measured by Kandeler et al. (1996) and Kandeler et al. (2000) at multi-contaminated, including Cu.

In our study, phosphatase displayed a contrasting map to that of Cu_{DTPA}. Wang et al. (2008) and Fernández-Calviño et al. (2010) have identified phosphatase as having specific sensitivity to Cu, especially Cu_{NH4NO3} and Cu_T. Here phosphatase was the only enzyme negatively correlated to Cu_{DTPA}. Phosphatase activity levels in our study were much higher than those measured in the multi-contaminated soils by Kandeler et al. (1996). Our study indicated an even lower Cu threshold for

phosphatase (43 – 142 mg Cu_T kg^{-1}, 10 - 19 mg Cu_{DTPA} kg^{-1}) than that found by Fernández-Calviño et al. (2010) (150 – 200 mg Cu_T kg^{-1}, 60 – 80 mg bioavailable Cu kg^{-1} (DTPA and EDTA)). Although the ranges for phosphatase activities cannot be easily compared because a different method of analysis was used, we found a negative correlation to exchangeable Cu at a lower Cu_T range than found in their study. Wang et al. (2009) observed apple orchards with similar Cu_T values, but on luvisols, and found that phosphatase levels were reduced to a much greater extent than were other enzymes. This suggests that for phosphatase soil type and/or texture might be of less importance when confronted with the impacts of copper. However, in the experiment of Wightwick et al. (2012), phosphatase was found to be highly dependent only on soil chemical factors, such as C_{org} and pH. The authors conclude that, although the Cu levels were higher than those on reference soils, physical and chemical factors play a much larger role in determining soil enzyme activities in vineyards. The findings here also support positive correlations with C and N, as well as a significant influence of tillage and the interaction between tillage and distance from the vine, demonstrating the interdependence between C, P and N cycles and the influence of vineyard management for specific enzymes. However, no enzymes had a correlation to pH, making it difficult to distinguish between Cu and substrate and pH influences as previously alluded to in the literature (Fernández-Calviño et al., 2009, 2010; Wightwick et al., 2012). This study shows, nonetheless, that Cu did contribute to a decrease in phosphatase, which may indicate restricted P availability to plants.

Urease, a catalyst for the breakdown of urea to carbon dioxide and ammonia, was positively correlated with both N_T and C_{mic}, but was not influenced by Cu. Fernández-Calviño et al. (2010) found copper fungicide to have variable effects on urease. Wightwick et al. (2012) conducted a microcosm experiment from ten vineyards, where only one vineyard showed a significant effect of Cu_T on urease activity. Both studies had lower Cu_T levels than in our study, suggesting that Cu_T was too low to cause a marked change in urease production. Ge and Zhang (2011) showed that urease activity was up to four times higher in low Cu (150 – 300 mg Cu_T

kg^{-1}) areas when compared to high Cu (>700 mg Cu_T kg^{-1}) areas. This suggests that there may be a Cu level, that once surpassed, is more likely to affect enzyme activity, and that our site was still below this level.

Copper content of plants

Neither Cu_{DTPA} nor Cu_T was significantly correlated with Cu uptake by the plants between the vine rows. Cu in non-accumulating plants ranges from 5 to 20 mg Cu kg^{-1} (Schulze et al., 2005). Deficiency occurs with < 5 mg kg^{-1} and by one account toxicity takes place at > 40 mg Cu kg^{-1} (Schulze et al., 2005). Another author found that >20 mg Cu kg^{-1} is toxic to plants (McBride et al., 1981). The Cu within grass biomass in this study was found to be in the normal range, which suggests that bioavailable Cu fractions were not being transported to the leaves. This could indicate either that the Cu is being sequestered within the roots or that the plants are not taking up the copper due to other factors. When the Cu in plants was calculated per area, however, it can be seen that there tended to be more Cu held in the plants in the left interrow largely due to the fact that there was a greater amount of plant biomass. Critical Cu values are difficult to determine because they are dependent on soil type and crops being grown, therefore, real toxicity levels for grapevines may be different than noted here (McBride et al., 1981).

Spatial distribution of soil microorganisms due to tillage practice

The second factor, which has modified the spatial distribution of both chemical and microbial properties of the vineyard site, is tillage. C_T, C_{org}, C_{mic}, C_{ext}, N_T, $N_{ext,}$ and plant dry matter values (Table 6.1, Fig. 6.2.A, 6.2.C) indicated highest concentrations within the left interrow. The positive correlation between C_{org} and plant dry matter suggests that C_{org} represented the living root material, but it also reflects the cultivation methods used by the proprietor, i.e. that every second row is plowed once per year (section 2.1). In the short term, minimum or reduced tillage allows soil C_{org} to accumulate in the uppermost centimeters of the soil (Kandeler et al., 1999). This can be seen in the left interrow, which was last plowed in 2008. Soil tillage, on the other hand, interrupts plant growth, incorporates plant biomass and

effectively reduces the soil carbon pool (right interrow, plowed in 2009). Depending on depth, tillage can homogenize and dilute carbon quantity by mixing and redistributing top- and sub-soils (Kandeler et al., 1999; Soane et al., 2012). This reduction in substrate availability can slow the mineralization of organic matter within the tillage area (Buscot and Varma, 2005). As topsoil samples were taken in the present study it is possible that the C_{org} values represent C dilution due to more recent plowing in the right interrow. In general, vineyards have been observed to have the lowest average C_{org} stocks in the upper 20 cm of soil when compared with crop fields, grasslands, alpine meadows, and orchards/gardens (Buscot and Varma, 2005). C_T and N_T in this study are comparable to levels in other vineyards in Europe (Parat et al., 2002; Fernández-Calviño et al., 2009). Parat et al. (2002) and Fründ et al. (2007) identified a significant positive correlation between C_{org} accumulation in coarse particles and Cu_T in vineyard soils. However, in this study, C_{org} had only a slight negative correlation with Cu_{DTPA} and no relationship to Cu_T. C_T, on the other hand, was significantly negatively affected by Cu_{DTPA}. This suggests that not only tillage patterns affect C turnover, but also metal pollution. Fig. 6.2 and Fig. 6.3 show that the Cu_{DTPA} pattern was in contrast to the C contents and also supported by significantly higher Cu values below the vine in contradiction to the low C values found there. In addition, the interaction of tillage and distance from the vine significantly affected both C_T and C_{org}, further supporting the complex influences at work.

Xylanase showed no correlation to Cu, but a positive correlation with C_T, C_{org}, C_{mic} and $PLFA_{bacterial}$. In general, where there were low C contents, there was also low xylanase activity, suggesting a limitation due to substrate, as xylanase represents the degradation of hemi-cellulose. Fig. 6.3.D and Table 6.2 also illustrate the effects of tillage on xylanase. The left interrow, which had higher levels of C_{org}, C_T and plant dry matter, also had higher xylanase activity, which has been observed previously by Kandeler et al. (1999).

6.5 Conclusions & Outlook

This study is one of the first to illustrate the spatial distribution of soil microorganisms and soil enzyme activities within a vineyard after long-term copper application on agricultural soils. The trends displayed in the organic vineyard identify copper loading and subsequent speciation below the vines, predominately in the DTPA exchangeable fraction, and a total copper concentration that is considered low to moderate pollution levels, as defined by the EC, and requiring copper prevention by German definition. Both the bacterial and fungal communities expressed trends in which there was greater activity between the vine rows than within them, in contrast to the spatial distribution of copper. Fungal communities were observed to be more affected by total copper, however, than other copper fractions or chemical, biological and physical characteristics. Enzyme activity did not display a clear pattern. However, trends showed a negative relationship to both total (arylsulfatase, invertase) and exchangeable (phosphatase) copper patterns. These trends were seen at much lower copper levels than those discussed previously within the literature. Changes in tillage patterns were also observed to have modified C and N variables, $PLFA_{bacterial}$, enzyme activities within the carbon cycle, and phosphatase activity and, therefore, in these cases it is difficult to separate the effects of copper and environmental impacts.

Due to the complexity of the system it is necessary to conduct more controlled experiments to better understand the influence of copper and physical and chemical factors, such as C substrate availability. One suggestion to do this would be to compare both non-tilled and tilled vineyards to assess the impacts of modifying soil structure and C pools. It has also been shown that cover crops are not yet negatively affected by Cu accumulation. Are mycorrhiza playing an important function in sequestration on roots? Would copper tolerant species offer significant uptake rates within cover crops? Would biosensor analysis provide better insight into bioavailable Cu fractions? It is clear that if copper continues to accumulate within the soil that it would be difficult if not impossible to alleviate, implying that there is

an increasing possibility of Cu toxicity in plants in the future. It would also be beneficial to understand exactly which bacterial and fungal species are present in a low polluted site, as well as their evolution over time. This study has well exhibited the copper, soil microorganism and soil enzyme trends within an organic vineyard and reinforces the need for additional research and insight into the topic.

6.6 Acknowledgements

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6.7 Supplementary Material

Table S.6. ArcGIS kriging interpolation maps model choices.

ArcGIS Interpolation	Model	Semivar./Covar.	Anisotropy	Neighbors to include	Include at least	Root mean square
Organic carbon	Exponential	Covar.	Yes	16	11	0.5505
pH	Pentaspherical	Semivar.	No	11	6	0.2492
Plant DM	Circular	Covar.	Yes	16	11	5.718
Total copper	Circular	Semivar.	No	15	10	15.81
DTPA copper	Rati. Quad.	Semivar.	Yes	15	10	2.975
Plant Cu per m^2	Gaussen	Semivar.	Yes	20	16	0.998
Bacterial PLFA	Tetraspherical	Covar.	Yes	20	11	16.53
Fungal PLFA	Hole-Effect	Covar.	Yes	20	16	1.11
Ergosterol	Gaussen	Semvar.	Yes	20	6	1.863
Xylanase	Rati. Quad.	Covar.	Yes	8	3	599.6
Phosphatase	Pentaspherical	Semivar.	Yes	13	8	214.9
Arylsulfatase	Circular	Semivar.	No	21	16	15.86

7 The importance of cover crops and soil microorganisms for phytoextraction of copper from a moderately contaminated vineyard

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K.A. Mackie[a], H.P. Schmidt[b], T. Müller[c], & E. Kandeler[a]

[a]Institute of Soil Science and Land Evaluation, Soil Biology Section, Unversity of Hohenheim, Emil-Wolff-Strasse 27, 70599 Stuttgart, Germany

[b]Ithaka Institute, La Place 92, 1966 Ayent, Switzerland

[c]Institute of Crop Science, University of Hohenheim, Fruwirthstrasse 20, 70599 Stuttgart, Germany

Abstract

We investigated the ability of summer (*Avena sativa* [Oat], *Trifolium incarnatum* [Crimson clover], *Chenopodium* [Goosefoot]) and winter (*Vicia villosa* [Hairy vetch], *Secale Cereale* L. [Rye], *Brassica napus* L. partim [Rape]) cover crops, including a mixed species treatment, to extract copper from an organic vineyard soil *in situ* and the microbial communities that may support it. Clover had the highest copper content (14.3 mg Cu kg^{-1} DM). However, it was the amount of total biomass production that determined which species was most effective at overall copper removal per hectare. The winter crop rye produced significantly higher amounts of biomass (3532 kg DM ha^{-1}) and, therefore, removed significantly higher amounts of copper (14920 mg Cu ha^{-1}), despite less accumulation of copper in plant shoots. The maximum annual removal rate, a summation of best performing summer and winter crops, would be 0.033 kg Cu ha^{-1} y^{-1}. Due to this low annual extraction efficiency, which is less than the 6 kg Cu ha^{-1} y^{-1} permitted for application, phytoextraction cannot be recommended as a general method of copper extraction from vineyards. Copper concentration did not influence aboveground or belowground properties, as indicated by sampling at two distances from the grapevine row with different soil copper concentrations. Soil microorganisms may have become tolerant to the copper levels at this site. Microbial biomass and soil enzyme activities (arylsulfatase and phosphatase) were instead driven by seasonal fluxes of resource pools. Gram+ bacteria were associated with high soil moisture, while fungi seemed to be driven by extractable carbon, which was linked to high plant biomass. There was no microbial group associated with the increased phytoextraction of copper. Moreover, treatment did not influence the abundance, activity or community structure of soil microorganisms.

7.1 Introduction

Due to the long history of application and continued use of copper containing fungicides in agriculture, copper (Cu) has accumulated within these topsoils (McBride et al., 1981; Mackie et al., 2012). Moderate levels of Cu have been shown to negatively affect macro-organisms, such as earthworms and plants, specifically in biomass and seed set, as well as organic matter decomposition (Moolenaar, 1998; Paoletti et al., 1998; Brun et al., 2003; Hinojosa et al., 2010). It severely decreases the functional diversity of the soil microbial community, impairs specific pathways of nutrient cycling and impacts soil fertility indicators at amounts as low as 140 mg Cu kg^{-1} (Kandeler et al., 1996; Fernández-Calviño et al., 2010; Hinojosa et al., 2010; Mackie et al., 2013). For these reasons, the European Union has set a limit on the amount of copper fungicide permitted for use in agriculture at 6 kg ha^{-1} y^{-1} (European Commission, 2007). However, as there are currently no viable alternatives in organic agriculture (Heibertshausen et al., 2006; La Torre et al., 2007) and as the potential for infection from plant pathogens increase with climate change (Salinari et al., 2006), Cu fungicides have not been prohibited and may even increase in the future.

In response to these negative effects, one possible solution is Cu removal through *in situ* accumulation by plants. Phytoextraction is the use of (hyper)accumulator plants to remove metals/metalloids from the environment by taking them up into their shoots and subsequently removing them from the contaminated area (Wenzel, 2009). It is a low cost, environmentally sensitive method, which displaces Cu from the environment, but does not require full soil removal impractical in perennial agriculture and/or large tracts of land (Gómez-Sagasti et al., 2012; Meier et al., 2012a).

Particular microorganisms prefer specific plants and plant species support and encourage associated microorganisms (Terry and Bañuelos, 2000; Wardle et al.,

2004; Castaldi et al., 2009; Narula et al., 2009; Epelde et al., 2010; Haferburg and Kothe, 2010). The most recent mechanism for enhancing phytoextraction is inoculating the soil with bacteria producing siderophores, which assist in chelating Cu, suggesting that microorganisms may play a significant role in successful phytoextraction (Haferburg and Kothe, 2010; Rajkumar et al., 2010). Phytoextraction, with and without inoculated microbial assistance, has been successfully investigated in laboratories and greenhouses (Poschenrieder et al., 2001; Brun et al., 2003; Kos and Leštan, 2004; Song et al., 2004; Chen et al., 2006; Meier et al., 2012b; Ma et al., 2009; Zeremski-Škorić et al., 2010; Andreazza et al., 2011). However, phytoextraction of Cu has seldom been monitored in the field (Poschenrieder et al., 2001; Clemente et al., 2005; Brej and Fabiszewski, 2006).

The aim of this study was to investigate the *in situ* relationship between microorganisms and plants within Cu contaminated topsoil, identify the practicability of phytoextraction and monitor ecosystem services, such as soil health and nutrient mineralization, using microbial biomass, enzyme activity and phospholipid fatty acids (PLFAs) (Epelde et al., 2014). Enzyme activities are consistent biological indicators of heavy metal pollution and PLFA patterns have been seen to change quickly with changing soil metal concentration in as little as two weeks (Frostegård et al., 1996; Hinojosa et al., 2010; Ge and Zhang, 2011). Additionally, PLFAs identify microbial groups, which may indicate whether such groups naturally support increased phytoextraction of specific plants *in situ*. This project focused particularly on vineyards, a representative system of fruit production where Cu is most often applied. In vineyards with sufficient water, i.e. central Europe, cover crops grown between the vine rows have been observed to increase desirable properties in soil and vine performance (Morlat and Jacquet, 2003; Guerra and Steenwerth, 2012). Therefore, phytoextraction has the potential to improve grape production and soil fertility in vineyards, while removing Cu from the topsoil. We investigated whether (i) the efficiency of phytoextraction depends on plant species, plant community composition, distance from vine row, and growing season, (ii) if effective phytoextraction is associated with a microbial

community structure, and (iii) if diverse plant communities will mitigate the negative influence of Cu on soil microorganisms.

The plants chosen within the present study were a mixture of Cu adapted plant species with high biomass production known from laboratory research as well as common vineyard cover crop species in central Europe not yet researched for Cu removal potential (Poschenrieder et al., 2001; Kos and Leštan, 2004; Andreazza et al., 2010; Haferburg and Kothe, 2010). Moreover, diverse plant systems, in comparison to monoculture systems, have been seen to reduce the impact of pollution by increasing microbial diversity and activity (Yang et al., 2007). Therefore, a treatment consisting of a mixture of plant species has also been added.

7.2 Materials & Methods

Study site and experimental setup

This field experiment was designed specifically to understand the *in situ* potential of cover crop plant species to accumulate Cu and was established at the Ithaka Institute in Canton Wallis, Switzerland (46°16'N, 7°24'E) in the spring of 2012. The study site is a vineyard planted with Pinot noir (*Vitis vinifera L.*) with a southeastern exposure at an elevation of 760 - 780 m a.s.l. The site has a mean annual precipitation of 550 mm and an average temperature of 11.4°C. The soil is predominately calcaric Leptosol with a bulk density of 1.34 g cm^{-3} and 47% gravel (> 2 mm). It has a pH_{CaCl2} of 7.5, C_T of 37 g kg^{-1}, N_T of 4.1 g kg^{-1}, and a total microbial biomass of 694 µg C_{mic} g^{-1} soil. The vines are planted at a distance of 1 m and a row width of 3 m with an adapted Mosel arch training system. At their maximum, vine plant height from July until October averages 2.50 m. The Ithaka Institute manages the vineyard organically; however, for this field experiment the use of copper fungicides was excluded during the trial period so that the samples would not be superficially contaminated. Plant and compost derived solutions (100 L compost tea ha^{-1}, 100 g NU-Film ha^{-1}, 2 kg stinging nettle ha^{-1}, 1 kg horsetail ha^{-1}, and 100 g sage ha^{-1}) and sodium bicarbonate used were sprayed to stimulate the plants natural

defenses and protect against *Oidium* and *Peronospora*, respectively. The initial total Cu in soil was 135 mg Cu_T kg^{-1} soil (95.9 kg Cu_T ha^{-1}), while the exchangeable Cu fraction was initially 48.6 mg Cu_{DTPA} kg^{-1} soil (34.5 kg Cu_{DTPA} ha^{-1}). Soil mass was calculated using soil depth, bulk density and fraction of coarse material (>2 mm) in order to calculate kg soil Cu ha^{-1}. The site was superficially tilled (8 cm) before seeding in April 2012.

We designed four summer (2012) treatments followed successively by four winter (2012/2013) treatments. The summer crops were seeded in April 2012 and harvested in August 2012; a) *Avena sativa* (Oat) 12 g m^{-2}, b) *Trifolium incarnatum* (Clover) 3 g m^{-2}, c) *Reseda luteola* (Reseda) 0.3 g m^{-2}, and d) a mixture of treatments 1, 2 and 3 (Summer Mix) at 4, 1 and 0.1 g m^{-2}, respectively. Unfortunately, although a drought tolerant species of *Reseda* was chosen, the species did not germinate well in all plots and *Chenopodium album* L., *Chenopodium hybridum* L. and *Chenopodium fielfolium* Sm. (Chenopodium) spontaneously took over. Therefore, Chenopodium was harvested and sampled in both treatments c and d. The winter crops were seeded in September 2012 and harvested in May 2013; a) *Vicia villosa* (Hairy vetch) 10 g m^{-2}, b) *Secale Cereale* L. (Rye) 20 g m^{-2}, c) *Brassica napus* L. partim (Rape) 0.6 g m^{-2}, and d) a mixture of treatments 1, 2 and 3 at 3.3, 6.7 and 0.2 g m^{-2}, respectively (Winter Mix). The treatments were set up in a random block design, where each plot had an area of 36 m^2 and spanned three vine rows. The treatments were replicated five times. Plant and soil samples were taken over three sampling dates: June 2012 (normal mowing date for summer cover crop), August 2012 (extended mowing date and plant senescence), and May 2013 (normal mowing date for winter cover crop). As there is a buffer time between the summer crop harvest and winter crop seeding, two dates were chosen to assess whether the extended time could achieve higher extraction rates. As summer crop seeding has to be done within a specific time window, there was no possibility to extend the winter crop harvest and only one date was chosen. Samples were taken on either side of the middle vine row, reducing edge effects, at both 70 cm and 120 cm away from the vine row in order to represent high Cu areas and high plant biomass areas, respectively (S.7).

Plant sampling and analyses

Plant samples were taken directly above and surrounding the soil corer with the guidance of a plastic ring (∅=30.5 cm) and were removed prior to soil sampling. All living above ground biomass was cut and placed directly into paper bags. The samples were placed in a 60°C oven for at least 48 h after which they were weighed for dry mass (DM).

Dried plant material was finely milled (SM1 Schneidemühle, Retsch GmbH, Germany) and underwent microwave digestion in nitric acid following the method of VDLUFA (2011a). To determine the Cu content of the plant biomass the method of VDLUFA (2011b) was used and analyzed with inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer, USA).

Soil sampling and analyses

Four soil cores (∅=5.5 cm), at a depth of 10 cm, from each plot, were taken to generate a plot bulk sample. The cores were taken following a random sample design and in order to exclude surface plant residues, the top one-centimeter was removed from each soil core. Samples were kept in a cooler and placed in a 4°C refrigerator after sampling. The soil was sieved at 2 mm and homogenized. Finally, the samples were stored in a -20 °C freezer for further analyses. Samples for Cu analyses were air-dried after sieving.

Total copper (Cu_T) was extracted by Aqua regia (HNO_3 + HCl) extractant (DIN ISO 11466, 1995). As an indicator of bioavailable copper, the DTPA extractable copper fraction (Cu_{DTPA}) was analyzed using diethylenetriamine pentaacetic acid extractant ($CaCl_2$+DTPA) (VDLUFA, 2011c). DTPA is an effective extractant for neutral and slightly alkaline soils and is used for extracting ligand-bound metals within the soil (Brun et al., 1998; Ettler et al., 2007). All analyses of extractants were done with an atomic absorption spectrophotometer. pH was determined with a glass electrode in a suspension of 4 g soil in 10 ml 0.01 M $CaCl_2$. Soil water content was determined

gravimetrically by drying samples at 105°C for 24 h. Samples dried at 105°C were finely ground and weighed to 0.060 g to be analyzed for total carbon (C_T) and nitrogen (N_T) using dry combustion (element analyzer, Elementar Analysensysteme GmbH, Germany).

The chloroform fumigation extraction (CFE) method of Vance et al. (1987) was used to estimate microbial (C_{mic}, N_{mic}) and to measure extractable organic carbon (EOC) and nitrogen (ETN), using a fresh soil weight of 10 g. Chloroform fumigated and non-fumigated samples were extracted with 40 ml 0.5 M K_2SO_4 on a shaker (250 U min^{-1}) for 30 min. After being centrifuged at 4400 g for 30 min a 1:4 dilution of the supernatant was analyzed using a TOC-TNb Analyzer Multi-N/C 2100S (Analytik Jena, Germany). 1 M HCl was added to the sample dilutions before measurement to remove small amounts of inorganic C. Since only visible roots were removed prior to fumigation and extraction, it cannot fully be excluded that cholorform labile C and N was contaminated by C and N derived from fine roots remaining in the soil sample (Mueller et al., 1992). The estimation of C_{mic} and N_{mic} used k_{ec} factors of 0.45 and 0.54, respectively (Joergensen, 1996).

The following enzyme analyses were measured using a spectrophotometer (UV-1601 Spectrophotometer, Shimadzu, Germany). Arylsulfatase, measured according to Schinner et al. (1996), was analyzed to assess the mineralization of organic sulfur compounds. One g soil, as suggested for marginal soils, was mixed with acetate buffer and 4-nitrophenysulfate and incubated for 1 h at 37°C. Samples were then mixed with water, filtered and sodium hydroxide was added before analysis. Phosphatase is a component of soil enzymes that removes phosphate from its organic substrate. Whereas soil microorganisms exclusively produce alkaline phosphatases, either soil microorganisms or plants can produce acid phosphatases. In this study the alkaline phosphatase was measured using the method of Schinner et al. (1996). Soil, 0.3 g, was mixed with borate buffer and phenylphosphate-disodium salt and incubated for 3 h at 37°C. Samples were filtered and mixed with

2,6-dibromchinon-chloromid, the color was allowed to develop for 30 min before analyzing.

Four g of soil were taken for lipid extraction and fractionation following the alkaline methylation method according to Frostegard et al. (1991). The resulting phospholipid fatty acid (PLFA) methyl ethers (MEs) were dissolved in Isooctan and measured by gas chromatograph (Auto System XL, PerkinElmer, USA) using an HP-5 capillary column, a flame ionization detector and helium as the carrier gas. FAMEs were identified using their retention time based on fatty- and bacterial-acid methylester-mix. Quantification was calculated with the use of an internal FAME standard, which had been added before methanolysis. Nomenclature and division of PLFAs into bacteria and fungi was based on Kandeler et al. (2008), Frostegård and Bååth (1996), and Zelles (1999). Gram+PLFAs ($PLFA_{gram+}$) were represented by i15:0, a15:0, i16:0, and i17:0. Gram- ($PLFA_{gram-}$) were represented by cy17:0 and cy19:0. Total bacteria PLFAs ($PLFA_{bacteria}$) were represented by the sum of $PLFA_{gram-}$, $PLFA_{gram+}$ and 16:1ω7. Fungal PLFAs 18:2ω6 represented ($PLFA_{fungal}$).

Statistics

The R statistical program was used for all statistical analyses (R Core Team, 2013). Initial sampling in April 2012 was tested for significant differences using a one-way ANOVA with treatment as a fixed factor. As treatment was never found to be significant, the average mean was taken of each variable and given as baseline soil data (section 2.1.; C_T, N_T, C_{mic}, Cu_T, Cu_{DTPA}) and not taken as a co-variable in the statistical analysis. To analyze differences between a normal mowing date and an extended date during the summer crop, June 2012 and August 2012 were tested using a three-way ANOVA, where treatment, distance and date were fixed factors; a time series analysis was used. The same statistical analyses were used in order to compare overall differences between summer and winter crops, June 2012 and May 2013, with the exception that date was now considered crop season. June 2012 was selected, instead of August 2012, because it had green aboveground vegetation and was the normal mowing date, both of which were also true of May 2013. Block and

column were used as fixed factors in every case. When significant differences were observed, Tukey's Honest Significant Distance test was performed so that differences could be specified. Significance was tested for $P < 0.05$ in all cases.

In order to determine how microbial communities were affected by date, treatment and distance from the vine row, multivariate statistical analysis (MANOVA) was conducted. Individual PLFAs (see above section 2.6) were used for the MANOVA analysis. When significant results were obtained, individual PLFAs were normalized and principal components analysis (PCA) was performed. The PLFA loadings for the first two axes were examined to determine which PLFAs were most strongly associated with each axis. The scores of the first two PCs were then correlated with soil chemical properties and plant properties measured to see which were significantly correlated with each PC. Significance was tested for $P < 0.05$ in all cases.

7.3 Results

Aboveground vegetation and copper uptake

Summer crop

Plant copper content

Plant shoots contained varying concentrations of Cu, ranging in mean from 8.4 to 14.3 mg Cu kg^{-1} DM between June 2012 and August 2012 (Fig. 7.1a). Treatment was highly significant (Table 7.1). Clover shoots had the highest Cu content (14.3 mg Cu kg^{-1} DM), which was significantly more than summer mix, oat and chenopodium. Summer mix shoots had the second highest Cu content (11.8 mg Cu kg^{-1} DM), significantly more than chenopodium. Plant Cu content increased significantly from June 2012 to August 2012.

Plant shoot biomass and plant copper removal

Plant shoot biomass was only significantly influenced by the interaction between distance from the vine and plant treatment (Table 7.1). The treatments had biomass ranging from 311 – 1,476 kg DM ha^{-1}, where oat-120 cm (1,476 kg DM ha^{-1}) was

significantly higher than all other treatments and distances (Fig. 7.1b). There was a negative correlation between plant biomass and plant Cu content (r=-0.59, p<0.001).

Plant Cu removal was significantly influenced by the interaction between plant treatment and distance from the vine row (Table 7.1). Oat at 120 cm (13,620 mg Cu ha^{-1}) was found to be significantly better at Cu removal when compared to all other treatments and distances (3,357 – 4,983 mg Cu ha^{-1}), with the exception of clover-120 cm (8,477 mg Cu ha^{-1}), which was not significantly different from any treatment (Fig. 7.1c). Cu removal had a significant positive correlation with plant shoot biomass (r=0.84, p<0.001).

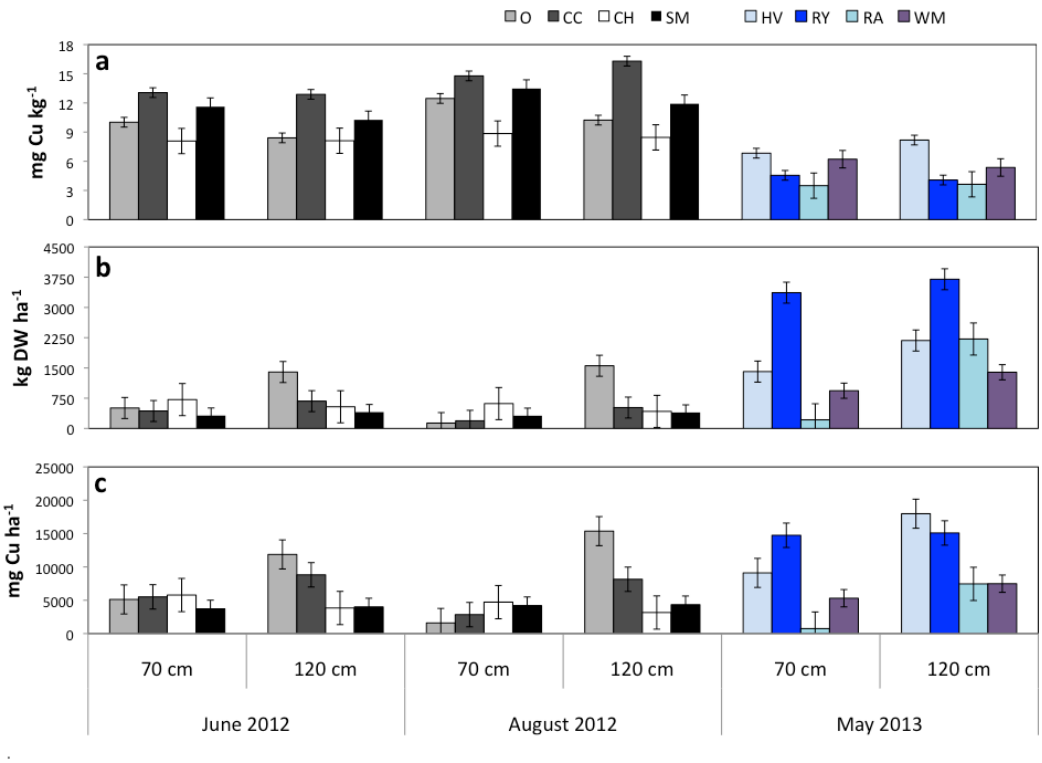

Figure 7.1. Plant copper content (a), plant shoot biomass (b) and copper removal (c) during the summer crops (June and August 2012) and winter crop (May 2013) at a distance of 70 cm and 120 cm from the vine row. Where O is oat, CC is clover, CH is chenopodium, SM is summer mix, HV is hairy vetch, RY is rye, RA is rape, and WM is winter mix. Mean ± SE is shown.

Table 7.1. Statistical significance of the three-way ANOVA. Treatment, distance and date, including their interactions for each variable, during the summer crop (June and August 2012) is shown. Treatment*Date, Distance*Date and Treatment*Distance*Date were not significant in any case. Significance is shown only for a P<0.05 and n.s. is not significant.

	Treatment (3 d.f.)		**Distance (1 d.f.)**		**Date (1 d.f.)**		**Trt*Dist (3 d.f.)**	
	F-value	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**
Plant Cu content	21.69	<0.0001	n.s.	n.s.	19.00	0.0001	n.s.	n.s.
Plant shoot biomass	5.63	0.0051	15.4	0.0007	n.s.	n.s.	8.35	0.0007
Plant Cu removal	5.01	0.0085	19.45	0.0002	n.s.	n.s.	7.87	0.001
C_{mic}	n.s.	n.s.	n.s.	n.s.	9.94	0.0031	n.s.	n.s.
Arysulfatase	n.s.	n.s.	11.02	0.0023	n.s.	n.s.	n.s.	n.s.
Phosphatase	n.s.	n.s.	17.48	0.0002	n.s.	n.s.	n.s.	n.s.
EOC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ETN	n.s.	n.s.	9.36	0.0045	n.s.	n.s.	n.s.	n.s.
Cu_T	n.s.	n.s.	34.97	<0.0001	20.98	<0.0001	n.s.	n.s.
Cu_{DTPA}	n.s.	n.s.	50.81	<0.0001	7.10	0.0112	n.s.	n.s.
N_{mic}	n.s.	n.s.	n.s.	n.s.	4.91	0.0326	n.s.	n.s.
$PLFA_{bacteria}$	n.s.	n.s.	11.56	0.0019	n.s	n.s.	n.s.	n.s.
$PLFA_{fungal}$	n.s.	n.s.	n.s.	n.s.	7.84	0.0079	n.s.	n.s.

Table 7.1. continued… Statistical significance of the three-way ANOVA.

	Treatment (3 d.f.)		Distance (1 d.f.)		Date (1 d.f.)		Trt*Dist (3 d.f.)	
	F-value	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**
$PLFA_{Gram+}$	n.s.	n.s.	16.10	0.0004	16.35	0.0002	n.s.	n.s.
$PLFA_{Gram-}$	n.s.	n.s.	5.01	0.0326	n.s.	n.s.	n.s.	n.s.
Soil moisture	n.s.	n.s.	10.26	0.0031	472.52	<0.0001	n.s.	n.s.

Winter crop

Plant copper content

Plant shoot Cu content was significantly influenced by plant treatment and crop season (Table 7.2). The summer crop treatments were generally significantly higher than those of the winter crop treatments, regardless of distance from the vine row. Hairy vetch Cu content was significantly higher than other winter treatments, but it was significantly lower than the highest summer crops (Fig. 7.1a).

Plant shoot biomass and plant copper removal

The interaction between plant treatment and crop season significantly influenced plant shoot biomass (Table 7.2). Rye had significantly higher biomass than all other cover crops. Hairy vetch was also significantly higher than the summer crops (Fig. 7.1b). Distance was significant for both crop seasons; treatments grew more at 120 cm than at 70 cm.

When comparing rates of plant Cu removal, treatment, crop season and distance were significant (Table 7.2). Winter crops (10,930 mg Cu ha^{-1}) removed significantly more Cu than summer crops (6,247 mg Cu ha^{-1}) (Fig. 7.1c). Overall, there was also significantly higher Cu removal at 120 cm than at 70 cm from the vine rows. The average means of summer mix and winter mix (5,125 mg Cu ha^{-1}) were significantly lower than the means of oat and hairy vetch (11,018 mg Cu ha^{-1}) and clover and rye (11,039 mg Cu ha^{-1}).

Table 7.2. Statistical significance of the three-way ANOVA. Treatment, distance and crop season, including their interactions for each variable, between the summer crop (June 2012) and the winter crop (May 2013) are shown. Treatment*Distance and Treatment*Distance*Crop Season were not significant in any case. Significance is shown only for a P<0.05 and n.s. is not significant.

	Treatment (3 d.f.)		**Distance (1 d.f.)**		**Crop Season (1 d.f.)**		**Trt*Crop Season (3 d.f.)**		**Dist*Crop Season (1 d.f.)**	
	F-value	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**
Plant Cu content	6.90	0.0016	n.s.	n.s.	316.62	<0.0001	29.91	<0.0001	n.s.	n.s.
Plant shoot biomass	6.45	0.0025	11.39	0.0026	64.69	<0.0001	7.34	0.0009	n.s.	n.s.
Plant Cu removal	4.70	0.0106	7.40	0.0122	22.15	<0.0001	n.s.	n.s.	n.s.	n.s.
C_{mic}	n.s.	n.s.	6.22	0.0182	13.77	0.0006	n.s.	n.s.	n.s.	n.s.
Arysulfatase	n.s.	n.s.	27.41	<0.0001	33.55	<0.0001	n.s.	n.s.	n.s.	n.s.
Phosphatase	n.s.	n.s.	42.80	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
EOC	n.s.	n.s.	7.47	0.0103	74.56	<0.0001	n.s.	n.s.	10.84	0.0022
ETN	n.s.	n.s.	n.s.	n.s.	14.10	0.0006	n.s.	n.s.	n.s.	n.s.
Cu_T	n.s.	n.s.	52.65	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Cu_{DTPA}	n.s.	n.s.	64.11	<0.0001	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N_{mic}	n.s.	n.s.	6.03	0.0199	19.10	0.0001	n.s.	n.s.	n.s.	n.s.
$PLFA_{bacteria}$	n.s.	n.s.	15.07	0.0006	14.77	0.0005	n.s.	n.s.	n.s.	n.s.
$PLFA_{fungal}$	n.s.	n.s.	10.33	0.0031	40.29	<0.0001	n.s.	n.s.	6.04	0.0186

Table 7.2. continued… Statistical significance of the three-way ANOVA.

	Treatment (3 d.f.)		Distance (1 d.f.)		Crop Season (1 d.f.)		Trt*Crop Season (3 d.f.)		Dist*Crop Season (1 d.f.)	
	F-value	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**	**F-value**	**P-value**
$PLFA_{Gram+}$	n.s.	n.s.	22.46	0.0001	6.29	0.0171	3.31	0.0315	n.s.	n.s.
$PLFA_{Gram-}$	n.s.	n.s.	10.04	0.0034	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Soil moisture	n.s.	n.s.	11.53	0.0019	537.66	<0.0001	n.s.	n.s.	n.s.	n.s.

Soil chemical properties

Summer crop

C_T, N_T, and ETN were significantly higher at 70 cm than at 120 cm from the vine row over the summer crop season (June and August 2012) (Table 7.1, 7.3). EOC was not significantly influenced by any factor. Carbon and nitrogen were not correlated to any aboveground plant characteristics.

Cu_T and Cu_{DTPA} were significantly influenced by distance from the vine row (Table 7.1, 7.3). At 70 cm Cu concentration was higher than at 120 cm from the vine row. Cu_T and Cu_{DTPA} were significantly positively correlated to each other ($r=0.91$, $p<0.001$). Cu_T was significantly positively correlated with C_T and N_T ($r=0.75$ and $r=0.69$, respectively, $p<0.001$), as was Cu_{DTPA} ($r=0.72$ and $r=0.73$, respectively, $p<0.001$). Cu_{DTPA} was also significantly correlated to EON ($r=0.51$, $p<0.001$).

Winter crop

C_T and N_T were significantly higher at 70 cm than at 120 cm (Table 7.2, 7.3). EOC, however, was influenced by the interaction between crop season and distance from the vine row. In May 2013, EOC was significantly higher at 70 cm (221 µg EOC g^{-1}) than at 120 cm (166 µg EOC g^{-1}). EOC was also significantly higher in May 2013 than in June 2012. ETN significantly differed by both distance and crop season. ETN content was significantly higher at 70 cm (29.4 µg ETN g^{-1}) than at 120 cm (21.1 µg ETN g^{-1}) and higher in May 2013 than in June 2012.

Cu_T and Cu_{DTPA} were only significantly influenced by distance from the vine row, where values at 70 cm were higher than at 120 cm (Table 7.2, 7.3).

Table 7.3. Carbon, nitrogen, extractable organic carbon (EOC), extractable total nitrogen (ETN), total soil copper, DTPA exchangeable copper, microbial nitrogen, and bacterial and fungal PLFAs. Mean concentrations ± SE.

		June 2012		August 2012			May 2013	
	Treatment	70 cm from vine row	120 cm from vine row	70 cm from vine row	120 cm from vine row	Treatment	70 cm from vine row	120 cm from vine row
C %	Oat	5.0 ± 0.4	3.8 ± 0.3	6.0 ± 0.4	4.7 ± 0.4	Hairy vetch	4.7 ± 0.8	3.8 ± 0.2
	Clover	4.6 ± 0.4	4.0 ± 0.1	5.8 ± 0.4	5.2 ± 0.3	Rye	4.6 ± 0.4	3.7 ± 0.3
	Chenopodium	4.7 ± 0.6	3.9 ± 0.3	5.4 ± 0.3	4.3 ± 0.5	Rape	5.0 ± 0.3	4.0 ± 0.3
	Summer mix	4.5 ± 0.3	3.7 ± 0.3	5.6 ± 0.3	5.2 ± 0.4	Winter mix	4.8 ± 0.3	3.5 ± 0.1
N %	Oat	0.51 ± 0.1	0.39 ± 0	0.50 ± 0	0.40 ± 0	Hairy vetch	0.45 ± 0.1	0.37 ± 0
	Clover	0.46 ± 0	0.41 ± 0	0.52 ± 0	0.50 ± 0	Rye	0.46 ± 0	0.38 ± 0
	Chenopodium	0.46 ± 0	0.41 ± 0	0.48 ± 0	0.37 ± 0.1	Rape	0.47 ± 0	0.40 ± 0
	Summer mix	0.46 ± 0	0.39 ± 0	0.50 ± 0	0.47 ± 0	Winter mix	0.46 ± 0	0.37 ± 0
EOC μg g^{-1}	Oat	132 ± 29	121 ± 16	133 ± 30	115 ± 14	Hairy vetch	228 ± 31	168 ± 27
	Clover	107 ± 6.2	133 ± 13	130 ± 25	102 ± 14	Rye	200 ± 20	159 ± 13
	Chenopodium	125 ± 27	118 ± 20	121 ± 32	102 ± 11	Rape	243 ± 26	178 ± 14
	Summer mix	156 ± 13	131 ± 15	141 ± 31	98.7 ± 27	Winter mix	211 ± 15	160 ± 6
ETN μg g^{-1}	Oat	19.8 ± 6.3	19.2 ± 3.6	30.6 ± 11	17.5 ± 3	Hairy vetch	38.0 ± 5.9	23.7 ± 4.3
	Clover	18.5 ± 2.8	18.0 ± 1.6	28.0 ± 4.2	14.9 ± 2.7	Rye	31.3 ± 4.6	21.5 ± 2.4
	Chenopodium	23.1 ± 6.5	18.4 ± 5	22.0 ± 3.6	14.3 ± 2.3	Rape	38.5 ± 3.7	25.4 ± 2.7
	Summer mix	33.6 ± 5.2	21.9 ± 5.1	26.1 ± 2.4	19.9 ± 1.8	Winter mix	32.5 ± 2.4	22.4 ± 1.2

Table 7.3. continued… Mean concentrations ± SE.

		June 2012		August 2012			May 2013	
	Treatment	70 cm from vine row	120 cm from vine row	70 cm from vine row	120 cm from vine row	Treatment	70 cm from vine row	120 cm from vine row
Cu_T mg kg^{-1}	Oat	150 ± 13	116 ± 9.7	179 ± 12	136 ± 13	Hairy vetch	162 ± 15	122 ± 4.9
	Clover	137 ± 14	115 ± 8.2	135 ± 45	132 ± 8.7	Rye	140 ± 9.6	115 ± 8.7
	Chenopodium	135 ± 12	108 ± 9.1	146 ± 13	127 ± 11	Rape	151 ± 9.7	120 ± 11
	Summer mix	140 ± 8.2	104 ± 7.6	150 ± 6.5	123 ± 5.9	Winter mix	131 ± 5.8	105 ± 6.6
Cu_{DTPA} mg kg^{-1}	Oat	58.6 ± 5.3	43.6 ± 5.7	64.8 ± 4.9	43.0 ± 4.1	Hairy vetch	59.1 ± 7.2	38.4 ± 3.3
	Clover	51.8 ± 6.7	39.2 ± 2.1	54.7 ± 7.4	43.1 ± 4.1	Rye	50.3 ± 3.8	36.0 ± 3.1
	Chenopodium	49.1 ± 6.5	34.9 ± 3	53.7 ± 5.6	40.8 ± 4.3	Rape	51.4 ± 2.3	36.0 ± 3.9
	Summer mix	54.5 ± 4.1	36.8 ± 3.1	55.5 ± 2.7	42.3 ± 2.8	Winter mix	47.3 ± 1.0	34.9 ± 2.4
N_{mic} µg g^{-1}	Oat	99.1 ± 11	163 ± 39	151 ± 26	135 ± 16	Hairy vetch	233 ± 25	159 ± 20
	Clover	146 ± 32	128 ± 19	113 ± 18	114 ± 14	Rye	200 ± 9.7	169 ± 14
	Chenopodium	158 ± 32	142 ± 38	124 ± 20	95.7 ± 7.9	Rape	214 ± 10	184 ± 16
	Summer mix	200 ± 18	139 ± 15	145 ± 37	94.9 ± 15	Winter mix	223 ± 14	175 ± 4.9
$PLFA_{bacterial}$ nmol g^{-1} DM	Oat	51.7 ± 3.0	44.7 ± 4.3	64.8 ± 3.6	51.3 ± 4.0	Hairy vetch	70.4 ± 8.1	61.1 ± 7.9
	Clover	55.8 ± 3.5	46.1 ± 3.6	60.3 ± 2.6	58.9 ± 4.5	Rye	60.0 ± 3.5	49.0 ± 3.2
	Chenopodium	54.1 ± 6.9	62.8 ± 19	61.8 ± 5.8	52.8 ± 3.8	Rape	74.2 ± 4.8	58.8 ± 4.0
	Summer mix	56.7 ± 1.8	56.0 ± 6.8	61.8 ± 1.9	55.2 ± 3.4	Winter mix	63.3 ± 3.6	48.6 ± 1.2
$PLFA_{gram+}$ nmol g^{-1} DM	Oat	32.2 ± 1.9	27.5 ± 2.7	40.6 ± 2.1	32.3 ± 2.3	Hairy vetch	40.9 ± 4.6	39.5 ± 8.3
	Clover	35.2 ± 2.3	28.5 ± 2.1	37.9 ± 1.3	36.9 ± 2.5	Rye	35.8 ± 2.0	29.2 ± 1.9
	Chenopodium	33.9 ± 4.3	44.9 ± 17	38.7 ± 3.4	32.7 ± 2.4	Rape	44.3 ± 2.8	35.3 ± 2.4
	Summer mix	35.4 ± 1.3	34.9 ± 4.3	39.5 ± 1.1	34.7 ± 2.2	Winter mix	37.7 ± 2.0	29.0 ± 0.6

Table 7.3. continued... Mean concentrations ± SE.

		June 2012		August 2012			May 2013	
	Treatment	70 cm from vine row	120 cm from vine row	70 cm from vine row	120 cm from vine row	Treatment	70 cm from vine row	120 cm from vine row
$PLFA_{gram-}$ nmol g^{-1} DM	Oat	4.7 ± 0.3	4.3 ± 0.3	5.7 ± 0.3	4.6 ± 0.3	Hairy vetch	5.5 ± 0.6	5.4 ± 1.3
	Clover	5.0 ± 0.2	4.4 ± 0.3	5.1 ± 0.2	5.3 ± 0.4	Rye	4.7 ± 0.2	4.0 ± 0.3
	Chenopodium	4.9 ± 0.6	4.8 ± 0.7	5.3 ± 0.5	4.6 ± 0.3	Rape	5.8 ± 0.3	4.8 ± 0.3
	Summer mix	5.3 ± 0.1	5.2 ± 0.6	5.2 ± 0.1	4.7 ± 0.3	Winter mix	4.9 ± 0.3	3.9 ± 0.1
$PLFA_{fungal}$ nmol g^{-1} DM	Oat	6.1 ± 1.0	5.7 ± 0.9	6.2 ± 0.7	5.0 ± 0.4	Hairy vetch	13.2 ± 1.6	7.8 ± 1.5
	Clover	8.7 ± 1.1	6.6 ± 0.9	6.0 ± 0.5	6.2 ± 0.6	Rye	10.2 ± 0.7	7.8 ± 0.4
	Chenopodium	6.6 ± 1.4	7.0 ± 1.2	6.0 ± 0.7	5.3 ± 0.6	Rape	12.3 ± 0.8	11.3 ± 1.4
	Summer mix	7.0 ± 0.4	7.5 ± 1.0	5.8 ± 0.3	6.0 ± 1.4	Winter mix	11.9 ± 1.5	8.5 ± 0.3

Soil microbial abundance and function

Summer crop

In June and August 2012, microbial abundance only differed by date (Table 7.1). C_{mic} and N_{mic} decreased significantly from June 2012 to August 2012 (Fig. 7.2a, Table 7.3). C_{mic} and N_{mic} were significantly correlated to both EOC and ETN (r=0.69, r=0.67 and r=0.60, r=0.60, respectively, p<0.001).

Microbial enzyme activities of arylsulfatase and phosphatase were significantly influenced by distance from the vine row; 70 cm was significantly higher than 120 cm from the vine row (Table 7.1, Fig. 7.2b and 7.2c). Arylsulfatase was significantly correlated to C_{mic} (r=0.54, p<0.001) as well as EOC (r=0.54, p<0.001).

Winter crop

Microbial abundance increased significantly from June 2012 to May 2013 (Fig. 7.2a, Table 7.3). It was also significantly influenced by distance (Table 7.2). C_{mic} and N_{mic} were significantly higher at 70 cm than at 120 cm.

Arylsulfatase activity was also significantly higher at 70 cm than 120 cm (Fig. 7.2b, Table 7.2). Activity increased significantly from June 2012 to May 2013. Phosphatase activity did not increase in May 2013 and 120 cm remained significantly lower than 70 cm (Fig. 7.2c).

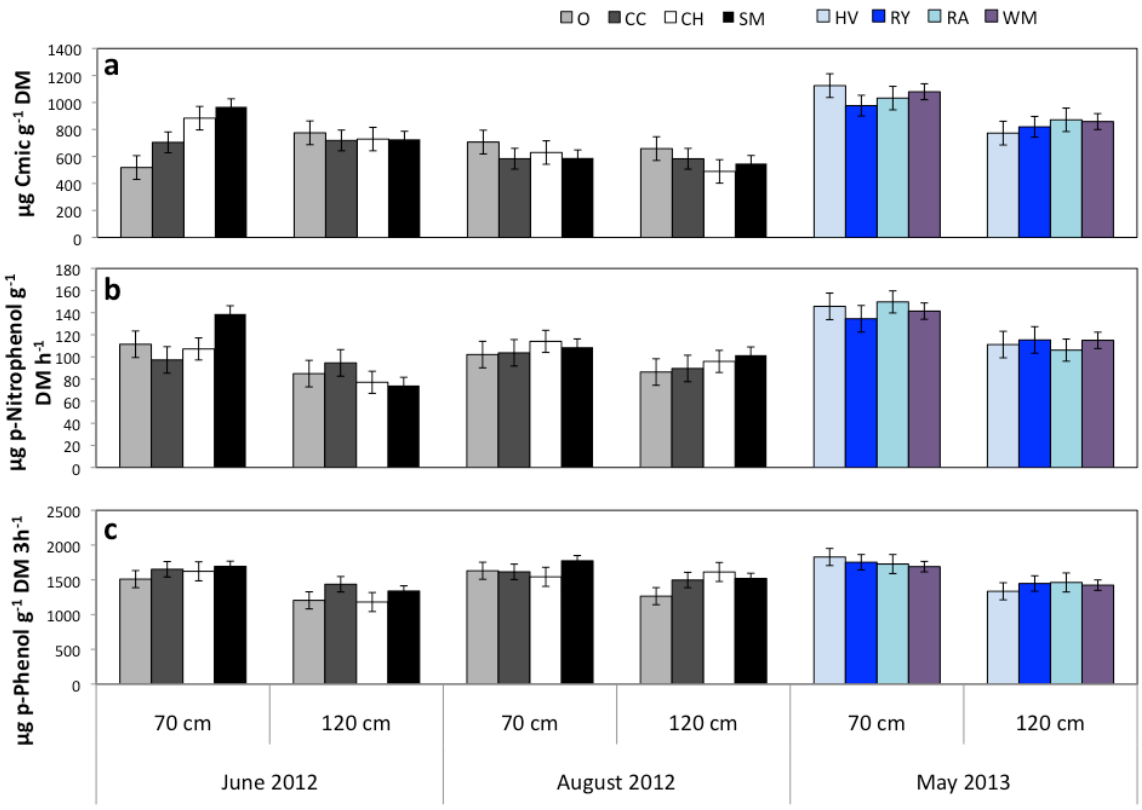

Figure 7.2. Microbial biomass (a), arylsulfatase (b) and phosphatase (c) during summer crops (June and August 2012) and winter crop (May 2013) at a distance of 70 cm and 120 cm from the vine row. Where O is oat, CC is clover, CH is chenopodium, SM is summer mix, HV is hairy vetch, RY is rye, RA is rape, and WM is winter mix. Mean ± SE is shown.

Microbial community

Summer crop

Bacterial PLFAs were significantly influenced by distance from the vine row (Table 7.1). $PLFA_{bacteria}$, $PLFA_{gram+}$ and $PLFA_{gram-}$ were all higher at 70 cm than at 120 cm. $PLFA_{bacteria}$ and $PLFA_{gram+}$ also increased from June 2012 to August 2012 (Table 7.3). $PLFA_{bacteria}$ was positively correlated to C_{mic} ($r=0.51$, $p<0.001$), C_T ($r=0.58$, $p<0.001$), and N_T ($r=0.55$, $p<0.001$). $PLFA_{gram+}$ was not correlated to any other variable. $PLFA_{gram-}$, on the other hand, was positively correlated to C_T ($r=0.61$, $p<0.001$) and N_T ($r=0.54$, $p<0.001$). $PLFA_{fungal}$ was the only fraction not influenced by distance

from the vine and was significantly higher in June 2012 than in August 2012. $PLFA_{fungal}$ was significantly positively correlated to C_{mic} ($r=0.71$, $p<0.001$), EOC ($r=0.69$, $p<0.001$) and ETN ($r=0.54$, $p<0.001$).

Winter crop

All measured PLFAs were significantly higher in May 2013 than in June 2012, with the exception of Gram- bacteria (Table 7.3). In addition, distance from the vine row was highly significant, where bacterial PLFAs were higher at 70 cm than at 120 cm (Table 7.2). The interaction between treatment and crop season was significant for $PLFA_{gram+}$, where Rape in May 2013 had significantly higher abundance than Oat in June 2012. For $PLFA_{fungal}$ there was a significant interaction between distance and crop season, where abundance was higher at 70 cm from the vine than at 120 cm in May 2013 as well as greater than both distances in June 2012.

Multivariate analyses

The multivariate analysis of the bacterial and fungal PLFAs indicated that date ($F=45.98$, $P<0.0001$) and distance ($F=6.20$, $P<0.0001$) from the vine row were highly significant. To take a deeper look into how the PLFAs separated, a PCA was performed (Fig. 7.3). The first two principal components (PCs) together accounted for 81% of the PLFA variance for the entire year. May 2013 is clearly separated from August 2012 and June 2012 by both PC1 and PC2. PC2 separates August and June 2012. When the scores of the PCs were correlated with abiotic soil as well as plant properties, it can be seen that PC1 was significantly positively correlated to EOC and plant biomass, and negatively correlated to plant Cu content (Table 7.4). PC2 was significantly positively correlated to soil moisture. The loadings for each PC indicated that PC1 was mainly associated with the PLFA fungal marker, while PC2 was mainly associated with gram-positive bacteria and eubacteria, which can be Gram+ and Gram- (Table 7.4).

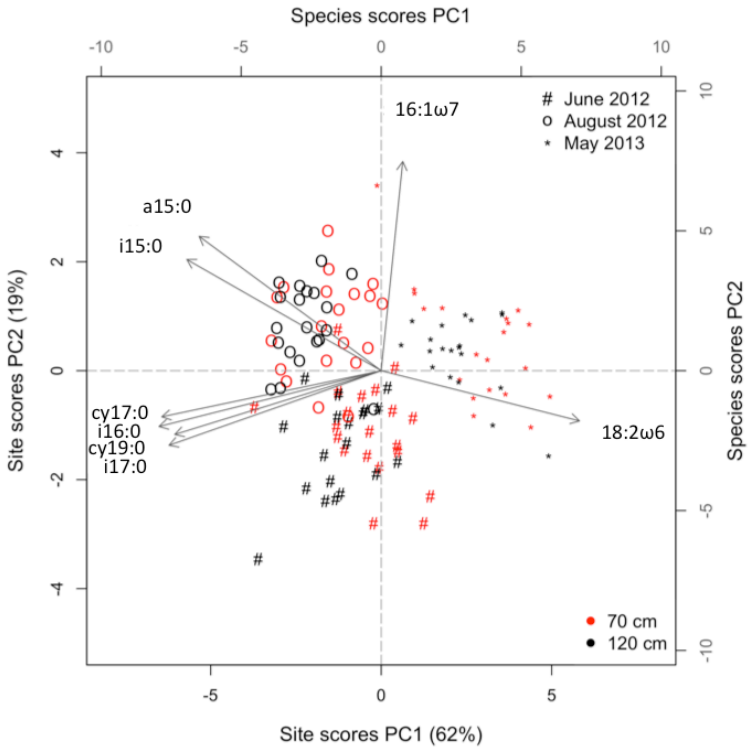

Figure 7.3. Principal component analysis (PCA) of phospholipid fatty acids (PLFAs) in June 2012, August 2012 and May 2013. Together PC1 and PC2 account for 81% of the variation amongst the PLFAs. The loadings (species scores) for the PLFAs are shown by the vectors.

Table 7.4. Correlations of scores of principal component analysis for microbial communities (PLFA data) with abiotic properties and plant characteristics for summer and winter crops combined. Significant correlations ($P<0.05$, $r>0.50$) with axes PC1 and PC2 are shown. Properties in italics indicate negative correlations.

PLFAs		Abiotic parameter	
PC1	PC2	PC1	PC2
18.2ω6	i15.0	EOC	Soil moisture
	a15.0	Plant biomass	
	16.1ω7	*Plant Cu content*	

7.4 Discussion

Aboveground vegetation and copper uptake

Copper uptake

This study is one of few that have sampled plants growing *in situ* for phytoextraction purposes (Clemente et al., 2005; Brej and Fabiszewski, 2006; Clemente et al., 2006). Although there is a clear distinction between the maximum plant shoot Cu content, the amount of Cu accumulated per plant shoot DM, achievable by the treatments, the quantities suggested in the literature were much higher than observed in this study. Oat has been previously researched by Andreazza et al. (2010) in a greenhouse experiment with two long-term vineyard soils (pH: 6-6.3, 140-200 mg Cu_T kg^{-1} soil). The control treatment showed that oat shoots contained approximately 55 mg Cu kg^{-1} DM, nearly five times the amount measured in our field study. *Reseda*, although not shown due to its sporadic germination, was roughly five times lower than suggested by the literature (51 mg Cu kg^{-1} DM) (Poschenrieder et al., 2001). In 2010, Zeremski-Škorić et al. (2010) conducted a pot experiment of *B. napus* (pH: 7.2, 250 mg Cu kg^{-1} soil) and observed a shoot content of 16.6 mg Cu kg^{-1} DM, approximately four times greater than in our study. These comparisons demonstrate that field results are less optimistic than those observed in the laboratory. This could be because the Cu levels were higher in the lab experiments as well as suboptimal environmental factors in the field, such as less available water.

Cu_T and Cu_{DTPA} were distinguished by distance from the grapevines, where higher levels of Cu were nearest to the vine rows. Plant Cu content, however, was not significantly differentiated by distance. It was also, therefore, not affected by Cu quantity. Only treatment played a significant role in determining how much Cu plants were able to amass. This was also seen by Brej and Fabiszewski (2006), where in their field experiment only plant species, rather than heavy metal quality, played a role in plant Cu concentration.

Summer crop

Summer crops significantly increased their plant Cu content from June to August 2012, showing that the longer the investigated plants grew, the more Cu plants accumulated per DM. Despite this they did not significantly increase their biomass over time, tending more towards a decrease in biomass with plant senescence. Moreover, high Cu accumulating plants were not high biomass producers, as plant biomass was negatively correlated to plant Cu content. Therefore, although clover had the highest Cu content, followed closely by summer mix, oat had the highest plant biomass at a distance of 120 cm. Biomass production significantly influenced Cu removal, the amount of Cu removed from the soil per area, more than plant shoot Cu content. This is because biomass and Cu removal are significantly positively correlated. At 120 cm from the vine row, Oat attained the most successful summer Cu removal rate. Andreazza et al. (2010) estimated that oats could remove approximately 175,000-200,000 mg Cu ha^{-1} for a normal vineyard soil, assuming a biomass of 4000 kg DM ha^{-1}. In our study, oat produced 898 kg DM ha^{-1}, which was much lower than assumed by Andreazza et al. (2010), most likely due to variations in fertilization, plant competition and water availability. These lower biomass results, compared to the lab study, led to less Cu removal than estimated in the literature. However, more biomass production during this period of the year would have been impractical for the winegrower. This is because growing oat and rye requires an increased input of water and fertilizer on the part of the winegrower. Moreover, the increased Cu content over time did not have any significant effect on Cu removal over time. This indicated that the normal mowing date (June 2012) was sufficient for Cu extraction because there was not a significant increase in Cu removal during the two-month time extension.

Winter crop

Although summer crops were more successful in accumulating Cu in their plant shoots, winter crops were significantly better at producing higher amounts of plant biomass. Rye was the most successful treatment compared to all others in June 2012 and May 2013. This was possible despite an unusually long 2012/2013 winter,

probably because more water was available in May 2013 as well as higher activity of microorganisms in the spring when the crops began to grow vigorously. Therefore, the winter crops were significantly more successful in removing Cu. These results support current practice, where cover crops are grown over winter due to more available water and less competition with grapevines (Steenwerth and Belina, 2008; Celette and Gary, 2013).

Copper phytoextraction potential

Distance from the grapevine was significant for plant biomass, which was higher at a distance of 120 cm compared to 70 cm. This is in contrast to the patterns displayed by Cu_T and Cu_{DTPA}, suggesting either that the plants grew better in lower Cu soil, which affected overall potential for Cu removal, or that the plants avoided direct competition with the grapevines. As Cu was not negatively correlated to plant biomass, plant Cu content or Cu removal, it could be assumed that resource competition, especially for sunlight, was the major influencing factor. As biomass has a large overall effect on which plant species can be used to efficiently remove Cu from soil *in situ*, both species chosen, as well as seeding placement, has an influence on Cu phytoextraction (Kidd et al., 2009; Bhargava et al., 2012). When calculating the annual removal rate of summer plus winter crops, the best-case scenario is when oat (approx. 15,000 mg Cu ha^{-1}) and hairy vetch (approx. 18,000 mg Cu ha^{-1}) are based on growth 120 cm from the vine row; then there is the potential to remove 0.033 kg Cu ha^{-1} y^{-1}. These results indicate that phytoextraction will not be a sufficient method for removing Cu in this vineyard soil. Initially, there was 95.9 kg Cu ha^{-1} in the soil to a depth of 10 cm; in addition, 4 kg Cu ha^{-1} can be applied annually in Switzerland (Bio Suisse Standards, 2012). An extraction rate of 0.033 kg Cu ha^{-1} y^{-1} will not balance the amount applied nor remove a significant amount of Cu from the soil. It might be that when additionally removing the plant roots, as the literature suggests (Poschenrieder et al., 2001; Andreazza et al., 2010; Zeremski-Škorić et al., 2010), more Cu could be harvested. However, this would hardly be feasible in practice.

Soil properties, microbial abundance and activity

Impact of plants on soil microorganisms

This study did not find any interaction between plants and microbial properties. In contrast to the results aboveground, those belowground varied only by distance from the vine row and date and showed no correlation to properties aboveground. There was also no beneficial impact from a diverse plant system as hypothesized. Kulmatiski and Beard (2011), who also did not see any influence of plants on the microbial community in a short-term experiment, suggested that plants leave a long-term microbial legacy in the soil, which may take years to adjust. In a four year study in Germany, Habekost et al. (2008) also found little evidence to show a change in soil microbial community due to plant diversity, concluding that there is a significant time-lag belowground when aboveground modifications are made.

Impact of copper on soil microorganisms

In spite of the fact that high C and N pools coincided with high Cu areas, indicated by their positive correlations and likely due to the bond between Cu and organic matter, microbial biomass and microbial function, i.e. enzyme activity, were either also high in these areas or were not negatively affected. Although Wightwick et al. (2013a) showed that vineyards had higher Cu (approx. 100 mg Cu kg^{-1}) and lower enzyme activity (100-400 µg p-nitrophenol/phosphatase g^{-1} h^{-1}) than in reference soils, and Mackie et al. (2013) showed that Cu (approx. 140 mg Cu kg^{-1}) negatively affected phosphatase activity (813 µg phenol g^{-1} 3 h^{-1}), this study showed neither low values nor a negative correlation of enzymes to Cu quantities. This could be because the relative difference in Cu concentration was lower in this study or because of different soil types, which affect pH, carbon and Cu speciation.

PLFAs were also not influenced by Cu in this study, suggesting that soil microorganisms are regulated by resource pools for survival and not negatively by environments of pollution. Therefore, chemical properties may be a more important factor for microorganisms than heavy metals are a deterrent, particularly in

environments of low to moderate contamination (Zhang et al., 2006; Wightwick et al., 2013). Zhang et al. (2006) suggests that this is due to overall resistance and resilience over time. This is supported by Brandt et al. (2010), where the authors showed that entire bacterial communities could develop a Cu tolerance without changing their structure and that these communities could even withstand a small increase in Cu pollution. It can be concluded that transformations resulting from changes in Cu quantity and aboveground vegetation species occur over a period longer than one year. As there are neither vineyards available that are completely uncontaminated nor vineyards that have significant Cu gradations within one area, it is difficult to say at which Cu level toxicity begins occurring. Brun et al. (2003) suggested that the plant toxicity limit is hit when contamination is greater than 250 mg Cu_T kg^{-1}, while Mackie et al. (2013) saw microbial function impaired when contamination was 140 mg Cu_T kg^{-1}. It can be conjectured that varying environmental factors, plant presence, nutrient availability, and microbial communities could change this limit at different sites.

Seasonal variations

It was observed that soil microorganism abundance changed over the seasons. During the summer crop season, C_{mic} and N_{mic} were significantly lower in abundance than in May 2013. These observations could be explained by a significant positive correlation between extractable organic nutrients and microbial biomass and arylsulfatase. As water and nutrients increased so did the microorganisms.

Seasonal shifts were also observed in the microbial community, as represented by PLFAs and differentiated by PCA. The fact that different groups of the microbial community were more associated with different environmental parameters, which coincided with crop season, suggests that there was a change in dominance over the year (Regan et al., 2014). $PLFA_{fungal}$ had a strong association with EOC and plant biomass, indicating an indirect reliance on nutrient pools most likely provided by root exudates (Khalid et al., 2007). Habekost et al. (2008) also found that PLFAs increased in abundance due to the quality and abundance of resources. Although

there was no treatment specific response from the microbial community, the presence of high plant biomass seemed to indirectly drive the abundance of the fungal community. Gram+ bacteria, on the other hand, exhibited a strong relationship to soil moisture, implying that Gram+ bacteria are more influenced by physical drivers than by nutrient resources. This supports observations made by Regan et al. (2014) and Lennon et al. (2012), where decreasing environmental stress factors, such as increases in soil moisture and extractable nutrients, increase fungal and bacterial abundance. Lennon et al. (2012) additionally observed that specific taxonomic groups responded differently to changes in a moisture gradient, where Gram+ bacteria, in particular Actinobacteria, were found to have a wet moisture optimum and a narrow niche. Soil moisture and extractable nutrients supported the abundance of microorganisms, while likely soil moisture and microorganisms supported plant biomass production, all of which were at their peak in May 2013. This is relevant because further investigations in microbially-assisted phytoextraction will require the success of soil microorganisms and plants together and this has been shown to vary by season.

Microbially assisted copper phytoextraction potential

Current literature shows that Cu phytoextraction could be increased by approximately 200% with the inoculation of siderophore producing bacteria (Andreazza et al., 2010). Although this would be a marked improvement, in this study it would imply that, at best, 0.066 kg Cu ha^{-1} could be removed in one year with the assistance of inoculated microorganisms. This continues to be a removal rate well below the amount of Cu applied annually. *In situ* research is needed to see whether these high biomass species, due to the important role of biomass in overall Cu accumulation potential, will significantly increase their Cu uptake in the presence of inoculated bacteria. Moreover, the time of inoculation should be investigated, as this study highlighted that environments with considerable soil moisture and available resources, which are seasonal, increase microbial abundance as well as plant biomass. It would benefit research to use field results of inoculation

experiments to model potential results before applying it on a greater scale, as suggested by Brej and Fabiszewski (2006).

7.5 Conclusion

When considering overall Cu removal capabilities, the plants with the most potential in a Wallisian vineyard were oat, hairy vetch and rye. As hypothesized, plant species played a significant role in the effectiveness of phytoextraction. The maximum removal rate, at 120 cm from the vine row would be 0.033 kg Cu ha^{-1} DM y^{-1}. These quantities were achievable mostly because of the plants' high biomass production and not their shoot Cu content. Winter cover crops, in particular, achieved the highest removal rates because more water was available, more nutrients were accessible and microorganisms were more active and abundant. Nevertheless, these *in situ* removal rates were too low to make phytoextraction of Cu in vineyards feasible, especially as Cu continues to be applied. Contrary to our hypothesis that plant diversity would increase microbial abundance and diversity, mixed plots had no significant influence on soil microbial properties.

In this study, soil chemical properties and seasonal variations were more influential than the negative impact of Cu. Microbial abundance and activity were clearly regulated by C and N pools despite the fact that these were also areas of high Cu. This suggests that organic matter and nutrient contents are masking or reducing the negative effects of heavy metals. Two different drivers, soil moisture and extractable carbon, divided microbial community structure into groups based on fungi and Gram+ bacteria. Abundance of the microbial community (C_{mic}, PLFAs) increased at the same time as biomass production of plants, in May 2013. This indicates that the time of planting, in this case spring, has potential for future microbially-assisted phytoextraction experiments in the field.

7.6 Acknowledgements

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7.7 Supplementary Material

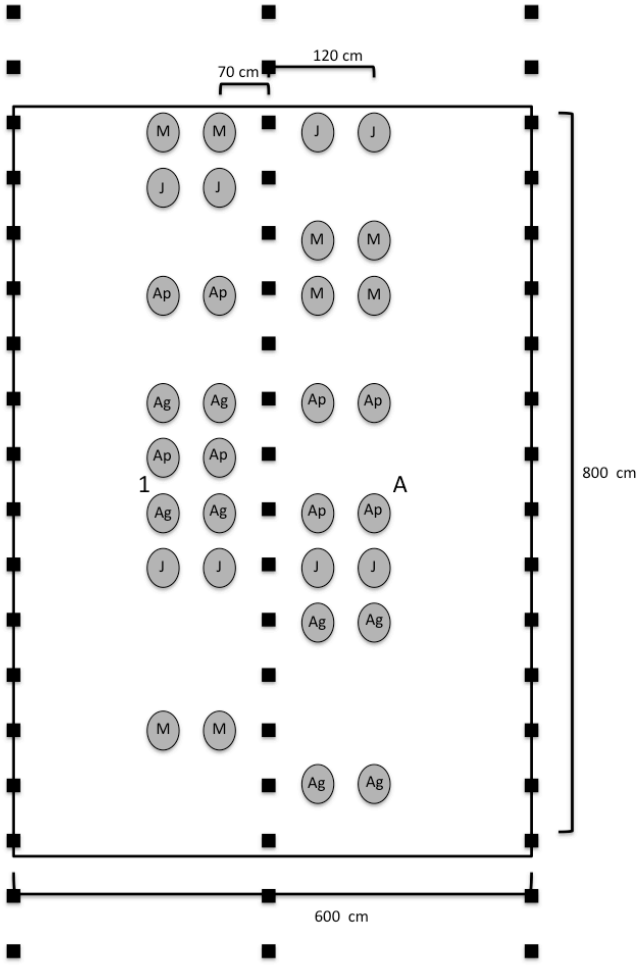

Figure S.7. Example plot A, where n represents a grape vine, which were planted at a distance of 1 m and a row width of 3 m. ¢ represents a randomly designed sampling point at 70 cm and 120 cm from the middle vine row. Sampling dates are represented by Ap (April), J (June), Ag (August), and M (May).

8 The effects of biochar and compost amendments on copper immobilization and soil microorganisms in a temperate vineyard

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K.A. Mackie[a], S. Marhan[a], Ditterich, F.[a], H.P. Schmidt[b], & E. Kandeler[a]

[a]Institute of Soil Science and Land Evaluation, Soil Biology Section, Unversity of Hohenheim, Emil-Wolff-Strasse 27, 70599 Stuttgart, Germany

[b]Ithaka Institute, La Place 92, 1966 Arbaz, Switzerland

Abstract

The use of copper (Cu) fungicides in agriculture has led to Cu accumulation in European topsoils. This study is the first to investigate the *in situ* efficacy of biochar and biochar-compost as Cu immobilizers, reducing Cu uptake by plants and increasing microbial abundance and activity, in a temperate vineyard topsoil (0 – 10 cm). After application of biochar, compost and biochar-compost in April 2011, plant and soil samples were taken in November 2011, April 2012, August 2012, and November 2012. Similar amounts of exchangeable Cu fractions (Cu_{DTPA}) in all treatments showed that there was no significant effect on Cu immobilization in soil. In contrast, cover crops grown between vine rows were observed to take up a significant amount of Cu (38.7 mg Cu kg^{-1}), reducing soil Cu concentrations over time. Treatments with biochar and/or compost initially increased total carbon, with compost and biochar-compost additionally increasing extractable organic carbon in soil. Compost and biochar-compost significantly increased microbial biomass, phospholipid fatty acids (PLFAs), enzyme activities (phosphatase, arylsulfatase) and bacterial taxa abundances (*Actinobacteria*, *α-Proteobacteria*, *β-Proteobacteria*, *Firmicutes*, *Gemmatimonadetes*). A high abundance of Gram+ *Actinobacteria* in all treatments suggested that they are adapted to heavy metals, likely due to their specific cell membrane structures. Additionally, each treatment was characterized by a specific microbial community composition. Compost and biochar-compost increased the relative abundance of *Firmicutes*, while control and biochar increased *Acidobacteria, Gemmatimonadetes* and *Actinobacteria*. In conclusion, biochar and/or compost were not viable Cu remediation options, but compost and biochar-compost provided ecosystem services by reinforcing the microbial community.

8.1 Introduction

Agricultural use of copper (Cu) fungicides has led to a high baseline level of Cu in many European soils, which persists and further accumulates in the topsoil with current management practices (McBride et al., 1981; Mackie et al., 2012). Increasing Cu concentration in soils has negative effects (Mackie et al., 2012); reducing the abundance of earthworms (Paoletti et al., 1998) and biomass of plants (Brun et al., 2003), decreasing phosphatase, β-glucosidase, dehydrogenase, and arylsulfatase enzyme activities (Fernández-Calviño et al., 2010; Mackie et al., 2013) and abundance of bacteria and fungi (Ge and Zhang, 2011), and impairing nutrient cycles (Hinojosa et al., 2010).

A relatively recent agricultural amendment, biochar, may immobilize Cu and reduce its bioavailability to macro- and micro-organisms. Biochar is made by pyrolysis to produce a lightweight, porous charcoal (Brewer and Brown, 2012; Glaser and Birk, 2012). Feedstock can range from woody materials to straw to manure, while pyrolysis production can differ in both temperature and duration, producing a range of products that identify as biochar (EBC, 2012; Brewer and Brown, 2012). Despite these differences, biochar has been observed to improve soil fertility and increase microbial biomass, water holding capacity, nutrient retention, and carbon sequestration (Beesley et al., 2011; Lehmann et al., 2011; Brewer and Brown, 2012). Biochar has also been seen to adsorb pollutants, including Cu, reducing its accessibility and minimizing toxin-induced stress to microorganisms and plants (Namgay et al., 2010; Beesley and Dickinson, 2011; Buss et al., 2012; Ippolito et al., 2012).

Most biochar experiments have been completed on marginal soils in tropical and subtropical environments (Jeffery et al., 2011). Of the few that have been conducted in temperate regions, some have shown biochar has no benefits for crop yield, soil nutrients or microorganisms and highly variable effects on microbial enzyme

activity (Bailey et al., 2011; Quilliam et al., 2012). These effects have been proven tempered when biochar is activated with compost at the beginning of the composting process (Borchard et al., 2012; Beesley et al., 2013; Schulz et al., 2013). Compost, including its combination with biochar, has been seen to increase carbon, nitrogen and phosphorus in soils and stabilize soil aggregates, as well as stimulate microorganisms (Sizmur et al., 2011; Schulz et al., 2013). Moreover, compost-biochar blends have been observed to increase Cu sorption and reduce plant Cu content (Sizmur et al., 2011; Borchard et al., 2012).

The aim of the present study was to verify the *in situ* ability of biochar and biochar-compost to immobilize Cu in soil, thus reducing Cu uptake by plants and increasing microbial abundance and activity. This is one of the first studies to investigate biochar in the field over time using a range of microbial abundance, functional activity and diversity analyses. Plant and soil samples were taken four times over an entire year, from 8 to 20 months after initial application of amendments. We hypothesized that in comparison to the unamended soils (i) biochar and biochar-compost would retain more Cu, thus reducing Cu uptake by plants, that (ii) biochar would increase soil microbial abundance, and that (iii) biochar-compost would increase the abundance, activity and diversity of microorganisms.

8.2 Materials & Methods

Study site and treatment

This study is part of a larger field experiment exploring the effects of biochar on vine growth and grape quality that was established by the Ithaka Institute in Canton Wallis, Switzerland (46°16'N, 7°24'E) in the late spring of 2011 (Schmidt et al., 2014). The study site is a vineyard planted with Pinot noir (*Vitis vinifera L.*) with a southeastern exposure at an elevation of 820 m a.s.l. The site has a mean annual precipitation of 550 mm and an average temperature of 11.4°C. The parent material is a calcareous schist and the soil has been classified as a Haplic Regosol (WRB) with a pH of 7.7 and 47% gravel and 53% fine soil (26% clay, 32% silt, 42% sand). The

Ithaka Institute manages the vineyard organically, including the use of Cu fungicides (Table S.8.1). A consistent green cover between the vine rows composed of 50% *Lotus corniculatus*, 22% *Medicago lupulina*, 25.4% *Trifolium spp.*, 2% *Anthyllis vulneraria*, 0.1% *Hippocrepis comosa*, and 0.5% mix of various herbs was installed at the beginning of the trial.

A block design was created for four treatments, with five replicates each (Figure S.8). The field site was 960 m^2 and each plot had an area 44 m^2, which spanned four vine rows. The treatments were applied once in April 2011; 1) control, 2) biochar (8 t DM ha^{-1}), 3) compost (55 t ha^{-1}), and 4) biochar-compost (63 t ha^{-1}, where 8 DM t biochar were added to the compost at the beginning of the composting process). After manual spreading of the different substrates, each with a particle size < 5 mm, onto the topsoil (vine rows included), the soil was superficially tilled into the interrows with a tiller to 7 – 10 cm depth. Cover crops were sown directly after adding amendments in April 2011 and since has neither been mowed nor plowed (Schmidt et al., 2014).

Compost and biochar characterization

The biochar was produced from 80% varied hardwood and 20% varied coniferous wood chips. Pyrolysis took place in a "Schottdorf"-type reactor (Carbon Terra GmbH, Augsburg, Germany) at 750°C in a 36 hour cycle. The particle size distribution of the biochar was < 5 mm. All physical and chemical properties were well within the thresholds of the European Biochar Certificate (EBC, 2012) and are summarized in Table 8.1. The compost was produced mainly from cow, horse and chicken manures and straw following a standard protocol for professional aerobic quality composting (Kompostforum-Schweiz, 1998). The piles were turned each day for the first three weeks and every three days for the last four weeks. Piles were watered when necessary (daily water content determination). Before the application date, substrates were tested for phytoxicology using a closed cress test (Fuchs, 2000) showing no effects of plant growth inhibition. The main

characteristics of the mature compost and biochar-compost substrate are summarized in Table 8.2.

Table 8.1. Analytical parameters of the biochar used for the biochar and the biochar-compost treatments. Analytical methods following the European Biochar Certificate (EBC, 2012).

Parameter	in dry matter
Density kg m^{-3}	269
Specific surface (BET) m^{-2} g	144
Ash 550 °C mass-%	16.3
Hydrogen mass-%	1.31
Carbon mass-%	75.8
Nitrogen mass-%	0.43
Oxygen mass-%	6.2
Carbonate CO_2 mass-%	1.83
Organic carbon mass-%	75.3
H/C_{org} (molar)	0.21
O/C (molar)	0.06
pH	9.5
Electric conductivity µS cm^{-1}	578
Phosphorous mg kg^{-1}	810
Magnesium mg kg^{-1}	2,580
Calcium mg kg^{-1}	20,500
Potassium mg kg^{-1}	9,030
Sodium mg kg^{-1}	1,700
Iron mg kg^{-1}	8,230
Silica mg kg^{-1}	35,400
Sulfur mg kg^{-1}	730

Table 8.2 Analytical parameters of the compost and the biochar-compost used in the compost and biochar-compost treatments. Analysed by Eurofins, Germany, following the guidelines of the Bundesgütegemeinschaft Kompost (2006), trace elements analyzed using DIN EN ISO 11885.

Parameter	Compost	Biochar-Compost
Density g l^{-1} OS	770	620
Dry matter mass % FM	79.8	74.6
Organic matter mass % DM	27.8	33.2
Salt content g KCl kg^{-1} DM	13.4	13.7
Total organic carbon (TOC) mass % DM	13.6	21.4
C/N ratio	13	17
pH (1:10 $CaCl_2$)	7.2	7.2
Nitrate-N ($CaCl_2$) mg N kg^{-1} DM	1,128	878
Ammonium-N ($CaCl_2$) mg N kg^{-1} DM	41	0.4
Total nitrogen g N kg^{-1} DM	12.8	12.1
Mineral nitrogen g N kg^{-1} DM	1.17	0.89
Phosphorus as P_2O_5 mass % DM	0.85	0.74
Potassium as K_2O mass % DM	1.1	1.18
Magnesium mass % DM	0.74	0.65
Calcium (total) mass % DM	6.91	6.83
Lead mg kg^{-1} DM	15	15
Cadmium mg kg^{-1} DM	<0.1	<0.1
Copper mg kg^{-1} DM	36	32
Nickel mg kg^{-1} DM	25	24
Zinc mg kg^{-1} DM	130	130

Plant sampling and analyses

Cover crop plant samples were taken on four sampling dates: November 2011 (autumn), April 2012 (spring), August 2012 (summer), and November 2012 (autumn). All living above ground plant biomass was cut directly above the soil corer and placed directly into paper bags. The samples were placed in a cooler until returning to the laboratory, where they were placed in a 60°C oven for at least 48 h. Dried plant material was finely milled and underwent microwave digestion in nitric

acid following the method of VDLUFA (2011a). Cu content of the plant biomass was analyzed with inductively coupled plasma optical emission spectrometry (ICP-OES) (Spectro GmbH, Germany) following the method of VDLUFA (2011b).

Soil sampling and analyses

Soil samples were taken at the same dates as the plant samples. Four soil cores (∅ = 5.5 cm), at a depth of 10 cm, were taken from each plot to generate a plot bulk sample. Samples were randomly taken between the two middle vine rows, to reduce edge effects. In order to exclude surface plant residues, the top one-centimeter was removed from each soil core. Samples were kept in a cooler and placed in a 4°C refrigerator after sampling. The soil, including biochar and/or compost particles, was sieved (2 mm) through metal mesh and homogenized. Finally, the samples were stored in a -20°C freezer until further analyses. Soil samples for Cu analyses were air-dried.

Total Cu (Cu_T) was extracted by Aqua regia (HNO_3 + HCl) extractant (DIN ISO 11466, 1995). As an indicator of bioavailable Cu, the DTPA exchangeable Cu fraction (Cu_{DTPA}) was analyzed using diethylenetriamine pentaacetic acid extractant ($CaCl_2$+DTPA) (VDLUFA, 2011c). Five grams soil was added to 50 ml DTPA solution and shook for one hour. All analyses of extracts were done with an atomic absorption spectrophotometer (PerkinElmer, USA). pH was determined with a glass electrode in a suspension of 4 g soil in 10 ml 0.01 M $CaCl_2$ after a period of one hour contact time. Soil water content was determined gravimetrically by drying samples at 105°C for 24 h. Samples dried at 105°C were finely ground and weighed to approximately 0.06 g to thereafter be analyzed for total carbon (C_T) and nitrogen (N_T) using dry combustion. Cation exchange capacity (CEC) was analyzed with ICP-OES using Vista Pro equipped with a Scott type spray chamber and operated at 1.1 kW (DIN ISO 11260, 2011).

The chloroform fumigation extraction (CFE) method of Vance et al. (1987) was used to determine microbial biomass carbon (C_{mic}), nitrogen (N_{mic}) and extractable organic carbon (EOC) and extractable total nitrogen (ETN). A soil weight of 10 g was fumigated under vacuum with ethanol-free chloroform in a desicator for 24 h. After removing the chloroform, fumigated, as well as not fumigated replicates, were extracted with 40 ml 0.5 M K_2SO_4 on a horizontal shaker (250 U min^{-1}) for 30 min. After being centrifuged at 4400 g for 30 min a 1:4 dilution of the supernatant was analyzed using a TOC-TNb Analyzer Multi-N/C 2100S (Analytik Jena, Jena, Germany). 40 µl 1 M HCl was added to the sample dilutions before measurement to remove inorganic C. Since only visible roots were removed prior to fumigation and extraction, it cannot fully be excluded that cholorform labile C and N was contaminated by C and N derived from fine roots remaining in the soil sample (Mueller et al., 1992). The estimation of C_{mic} and N_{mic} was done using k_{eC} 0.45 and k_{eN} 0.54 extraction factors, respectively (Joergensen, 1996). Ammonium (NH_4^+) and nitrate (NO_3^-) were measured colorimetrically in extracts from non-fumigated samples with an Autoanalyzer III (Bran & Luebbe, Norderstedt, Germany).

The following soil enzyme potential activites were measured using a UV-1601 spectrophotometer (Shimadzu Corp., Japan). Arylsulfatase activity, measured according to Schinner et al. (1996), where one g soil was mixed with acetate buffer (0.5 M, pH 5.8) and 4-nitrophenysulfate and incubated for 1 h at 37°C. Samples were then mixed with water, filtered and sodium hydroxide (0.5 M) was added before analysis. Invertase activity was analyzed based on the method of Schinner and von Mersi (1990). Saccharose substrate (50 mM) and acetate buffer (2 M, pH 5.5) was added to 0.3 g soil and incubated for 3 h at 50°C. Samples were filtered, diluted with water and the color reaction was conducted using Na_3CO_3, potassium cyanide and potassium ferrocyanide. After a 15 min incubation at 100°C, ammonium iron (III) sulfate solution was added, the samples were analyzed after 45 min. Alkaline phosphatase activity was measured using the method of Schinner et al. (1996). Soil, 0.3 g, was mixed with borate buffer (1 M, pH 10) and phenylphosphate-disodium salt (0.1 M) and incubated for 3 h at 37°C. Samples were filtered and mixed with 2,6-

dibromchinon-chloromid, the color was allowed to develop for 30 min before analyzing.

Soil fungal biomass was determined by extracting ergosterol using the method of Djajakirana et al. (1996). Two g of soil was suspended in 50 ml of ethanol in dark brown bottles and shaken for 30 min at 250 U min^{-1} followed by centrifugation in 50 ml tubes at 4400 g for 30 min. An aliquot of 10 ml was transferred into a test tube and evaporated in a vacuum rotary evaporator at 50°C. The dry extract was dissolved in 1 ml methanol and percolated through a syringe filter (cellulose-acetate, 0.45 µm pore size) into brown HPLC vials. Extracts were measured using a HPLC Autosampler equipped with a 250 mm x 4.6 mm Spherisorb ODS II 5 µm column (Beckmann Coulter, System Gold 125, USA). Detection was conducted using a UV-detector at a wavelength of 282 nm.

Four g of soil were taken for lipid extraction and fractionation following the alkaline methylation method according to Frostegård et al. (1991). The resulting phospholipid fatty acid (PLFA) methyl ethers (MEs) were dissolved with isooctan and measured by gas chromatograph using an Auto System XL (PerkinElmer, USA) using an HP-5 capillary column, a flame ionization detector and helium as the carrier gas. FAMEs were identified using their retention time based on fatty- and bacterial-acid methylester-mix (Sigma-Aldrich, St. Louis, USA). Quantification was calculated with the use of an internal FAME standard, which had been added before methanolysis. Nomenclature and division of PLFAs into bacteria and fungi was based on Kandeler et al. (2008), Frostegård and Bååth (1996) and Zelles (1999). PLFAs related to Gram+ bacteria ($PLFA_{gram+}$) were represented by i15:0, a15:0, i16:0, and i17:0. Gram- bacteria ($PLFA_{gram-}$) were represented by cy17:0 and cy19:0. Total bacteria PLFAs ($PLFA_{bacteria}$) were represented by the sum of $PLFA_{gram-}$, $PLFA_{gram+}$ and 16:1ω7. 18:2ω6 represented fungal PLFAs ($PLFA_{fungal}$).

DNA was extracted from 0.3 g soil using the FastDNA Spin Kit for soil (BIO101, MP Biomedicals, USA). The extracted DNA was quantified using a Nanodrop ND-2000 spectrophotometer (Thermo Scientific, USA). For quantitative PCR (qPCR) measurements, samples were diluted to a target concentration of 5 ng DNA μl^{-1} with ultra-pure water. The quantification of the abundances of 16S rRNA gene and *Actinobacteria*, *Acidobacteria*, *α-* and *β-Proteobacteria*, *Firmicutes*, and *Gemmatimonadetes* taxa was carried out with an ABI prism 7500 Fast System (Applied Biosystems, USA) SYBR Green as the detection system (Lopez-Gutierrez et. al, 2004; Fierer et al., 2005; Philippot et al., 2009). For each reaction a mixture of 0.75 µL each of forward and reverse primers, 4.125 µL ultra-pure water, 0.375 µL T4gp32, 7.5 µL SYBER Green, and 1.5 µL DNA (5 ng μL^{-1}) template was used. Standard curves were obtained with serial dilutions of a known amount of plasmid DNA containing fragments of the respective genes. Two no template controls were run for each qPCR assay and all obtained no or negligible values. Table S.8.2 lists the primers, thermal cycling conditions and efficiencies. Absolute abundances are represented by copies per gram soil, while relative abundances are represented by dividing the absolute abundance of the specific taxa by 16S rRNA gene abundance.

Statistical analyses

The R statistical program was used for all statistical analyses (R Core Team, 2013). To analyze differences between the addition of compost and biochar to soil a three-way ANOVA was tested, where compost, biochar and date were fixed factors; a time series analysis was used. Block was used as fixed factor. When significant differences were observed, Tukey's HSD-test was performed so that differences could be specified. Significance was tested for $P < 0.05$ in all cases. Correlations were carried out using Pearson's Correlation, significance was tested for $P < 0.001$ and $|r| > 0.50$. Significance was tested for $P < 0.05$ in all cases.

8.3 Results

Chemical properties

Seasonal variation

Cu_T and Cu_{DTPA} decreased significantly from the beginning of the field experiment until the end (Fig. 8.1a, b; Table 8.3), with values ranging from 125 – 130 mg Cu_T kg^{-1} soil and 30-31 mg Cu_{DTPA} kg^{-1} soil in November 2011 and April 2012 and from 96 – 107 mg Cu_T kg^{-1} soil and 22-23 mg Cu_{DTPA} kg^{-1} soil in August 2012 and November 2012. As is expected, plant biomass increased during the spring and summer months (April and August 2012). Furthermore, Cu accumulation by plant shoots was greatest in November 2012 (39 mg Cu kg DW) (Fig. 8.1c). EOC, however, was highest in April 2012, decreasing into August and November of 2012 (Fig. 8.2b). On the other hand, ETN, ammonium and nitrate were highest in August 2012 (Table 8.4). All extractable and mineralized forms of C and N were greatest at the same time as the peak growth of plant biomass (Table 8.4).

Treatments

Biochar did not affect soil Cu_T or Cu_{DTPA} contents or plant properties; plant Cu content, biomass and Cu removal (Fig. 8.1; Table 8.4). Biochar significantly increased C_T content (2.07%) in the topsoil compared to the control (1.77%) (Fig. 8.2a), but did not significantly influence any other measured chemical soil properties (Fig. 8.2b; Table 8.4).

Similar to biochar, compost and biochar-compost neither influenced Cu_T or Cu_{DTPA} in soils nor plant Cu content, plant biomass or plant Cu removal (Fig. 8.1; Table 8.3, 8.4). However, compost and biochar-compost amendments significantly increased C_T (2.15% and 2.37%, respectively), EOC (84.9 and 90.4 µg g^{-1} soil), N_T (0.25% and 0.25%), ETN (11.1 and 9.44 µg g^{-1} soil), and ammonium (2.81 and 2.94 µg g^{-1} soil) contents and tended to increase nitrate content in soil (Fig. 8.2; Table 8.4). Soil

moisture and $CEC_{effective}$ were also significantly increased by the presence of compost and biochar-compost (Table 8.3, 8.4).

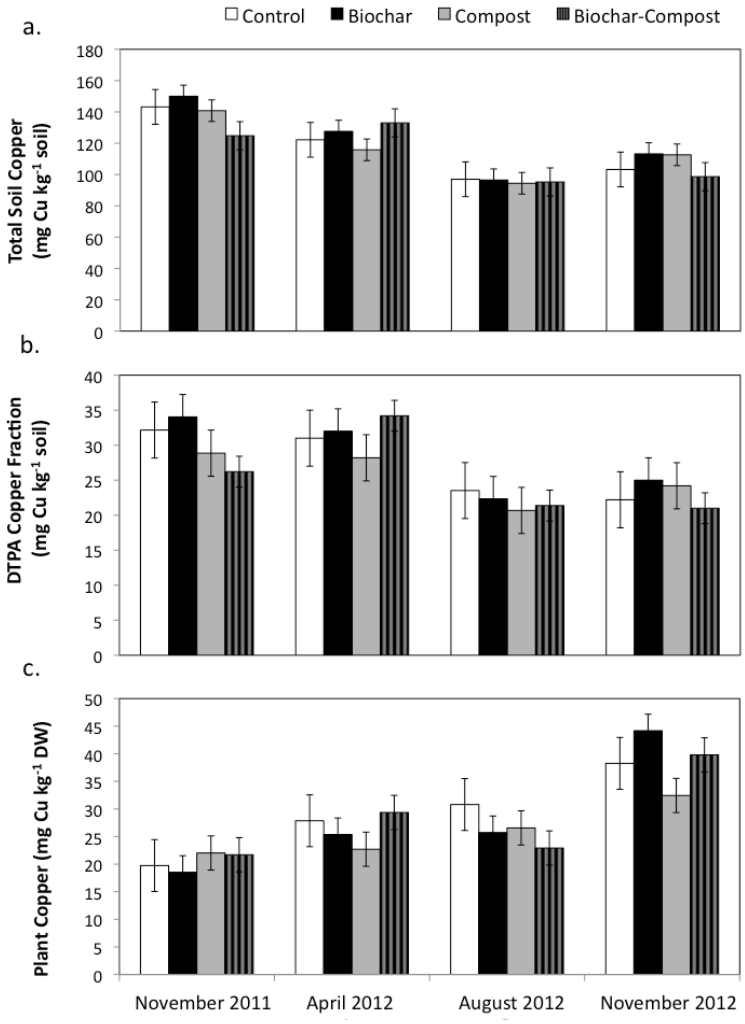

Figure 8.1. Total soil copper (a), DTPA copper (b) and plant copper content (c) from November 2011 until November 2012. Control, biochar, compost and biochar-compost amendment means ± SE are shown.

Microbial properties

Seasonal variation

Microbial biomass (C_{mic}, N_{mic}), bacterial PLFAs ($PLFA_{bacterial}$, $PLFA_{gram-}$, $PLFA_{gram+}$) and invertase activity were highest at the beginning of the study (November 2011)

Table 8.3. Statistical significance of the three-way ANOVA. Compost, biochar and date, including their interactions for each variable. Biochar × Date, Biochar × Compost and Biochar × Compost × Date were not significant in any case. Significance is shown by $^{+}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and n.s. is not significant.

	Biochar (1 d.f.)	**Compost (1 d.f.)**	**Date (3 d.f.)**	**Compost*Date (3 d.f.)**
	F/P-value	**F/P-value**	**F/P-value**	**F/P-value**
Cu_T	n.s.	n.s.	19.63***	n.s.
Cu_{DTPA}	n.s.	n.s.	10.22***	n.s.
Plant Cu Content	n.s.	n.s.	16.39***	n.s.
Plant Biomass	n.s.	n.s.	20.85***	n.s.
Plant Cu Removal	n.s.	n.s.	9.42***	n.s.
C_T	12.87**	19.00***	20.56***	n.s.
N_T	n.s.	34.44***	51.0***	n.s.
EOC	n.s.	53.20***	13.44***	n.s.
ETN	n.s.	36.97***	11.97***	n.s.
Ammonium	n.s.	13.21**	14.40***	n.s.
Nitrate	n.s.	6.39+	36.02***	n.s.
Soil moisture	n.s.	14.61**	52.45***	n.s.
$CEC_{effective}$	n.s.	9.22**	9.30***	n.s.
C_{mic}	n.s.	36.32***	12.55***	3.37*
N_{mic}	n.s.	34.71***	8.93***	3.44*
$PLFA_{bacteria}$	n.s.	81.51***	4.56***	3.49*
$PLFA_{Gram+}$	n.s.	104.88***	4.27***	3.96*
$PLFA_{Gram-}$	n.s.	126.13***	10.55***	7.08*
16S	n.s.	26.97***	3.14*	n.s.
$PLFA_{fungal}$	n.s.	7.19**	11.37***	n.s.
Ergosterol	n.s.	6.92**	n.s.	n.s.
Arylsulfatase	n.s.	17.03***	8.54***	n.s.
Phosphatase	n.s.	37.49***	n.s.	n.s.
Invertase	n.s.	n.s.	13.01***	3.11*
Actinobacteria	n.s.	17.10***	5.17**	n.s.
Acidobacteria	n.s.	n.s.	n.s.	n.s.
α-Proteobacteria	n.s.	10.96**	n.s.	n.s.
β-Proteobacteria	n.s.	11.78**	n.s.	n.s.
Firmicutes	n.s.	209.23***	n.s.	n.s.

	Biochar (1 d.f.)	**Compost (1 d.f.)**	**Date (3 d.f.)**	**Compost*Date (3 d.f.)**
	F/P-value	**F/P-value**	**F/P-value**	**F/P-value**
Gemmatimonadetes	n.s.	8.90**	n.s.	n.s.
***Actinobacteria* %**	n.s.	4.76*	n.s.	n.s.
***Acidobacteria* %**	n.s.	11.93**	2.82+	n.s.
***α-Proteobacteria* %**	n.s.	n.s.	n.s.	n.s.
***β-Proteobacteria* %**	n.s.	n.s.	n.s.	n.s.
***Firmicutes* %**	n.s.	123.18***	3.45+	3.35+
***Gemmatimonadetes* %**	n.s.	14.91**	6.01**	n.s.

and significantly decreased over time (Fig. 8.2, 8.3; Table 8.3). 16S rRNA gene copy abundance, representing total bacterial abundance, *Actinobacteria* and arylsulfatase activity was highest in August 2012, concurrently with extractable and mineralized forms of N and plant biomass (Fig. 8.4b, 8.3a). This was also true of $PLFA_{fungal}$, which significantly increased in April and August 2012 (Table 8.4). Season did not significantly influence phosphatase activity, ergosterol content or abundances of bacterial taxa (*α*- and *β-Proteobacteria*, *Gemmatimonadetes*, *Firmicutes*, and *Acidobacteria*) (Fig. 8.3b, 8.4; Table 8.4).

Treatments

Biochar did not influence the abundance of microbial biomass (C_{mic}, N_{mic},), PLFAs ($PLFA_{bacterial}$, $PLFA_{gram+}$, $PLFA_{gram-}$, $PLFA_{fungal}$), absolute bacterial gene abundance (16S rRNA, *α*- and *β-Proteobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Acidobacteria*, and *Actinobacteria*), ergosterol, or enzyme activities (Fig. 8.2, 8.4; Table 8.3, 8.4).

Microbial biomass (C_{mic}, N_{mic}) and bacterial PLFAs ($PLFA_{bacterial}$, $PLFA_{gram+}$, $PLFA_{gram-}$) showed a significant interaction between compost and date; specifically compost-amended treatments were significantly greater in November 2011 and decreasing over time (Table 8.3). Compost and biochar-compost amendments also had significantly more copy numbers of 16S rRNA than biochar and control (Table 8.3). Compost addition to soil significantly increased bacterial taxa abundance, with the

exception of *Acidobacteria*. Moreover, compost and biochar-compost increased the relative abundance of *Firmicutes* (2.43 and 2.33%, respectively) compared to the control (1.22%). However, compost treatments decreased the relative abundances, in comparison to non-compost treatments (biochar and control), for *Acidobacteria* (52% to 65%, respectively), *Gemmatimonadetes* (1.7% and 2.2%) and

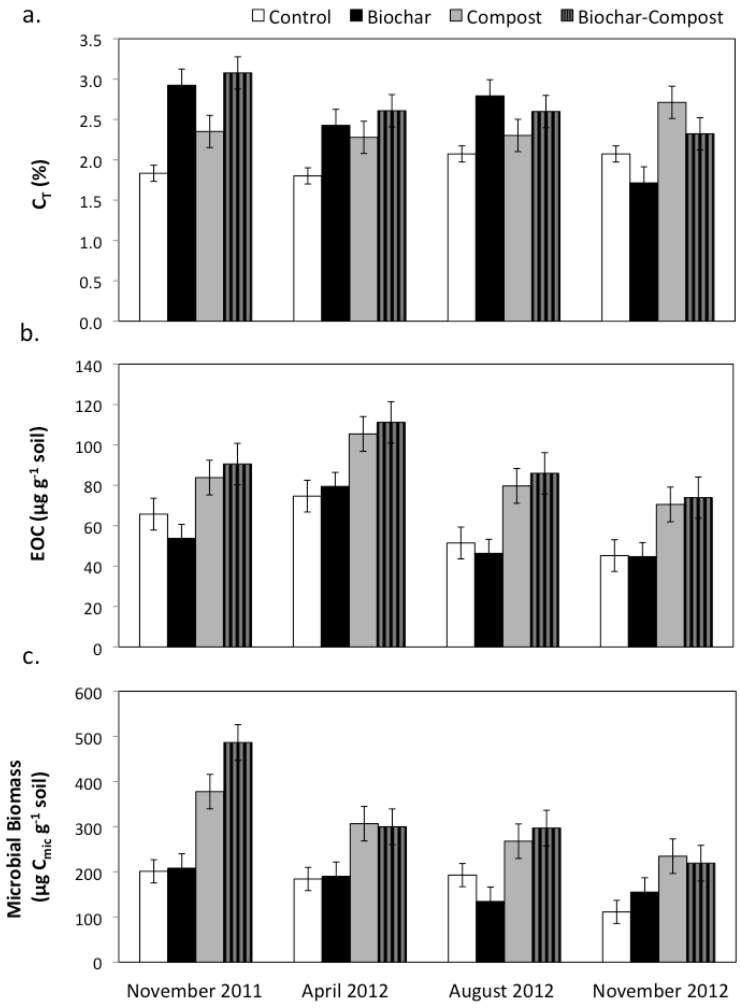

Figure 8.2. Total carbon (a), extractable organic carbon (b) microbial biomass carbon (c) from November 2011 until November 2012. Control, biochar, compost and biochar-compost amendment means ± SE are shown.

Actinobacteria (38% and 44%, respectively), where (Table 8.3). $PLFA_{fungal}$ (2.9 and 3.4 nmol g^{-1} DM, respectively) and ergosterol (3.3 and 3.7 µg g^{-1} DM, respectively)

content were highest in compost and biochar-compost compared to biochar and control treatments (mean of 1.25%, 2.6 nmol g^{-1} DM and 2.8 µg g^{-1} DM) (Table 8.3). The addition of compost significantly increased the potential activities of arylsulfatase and phosphatase, but not invertase (Fig. 8.3, Table 8.3).

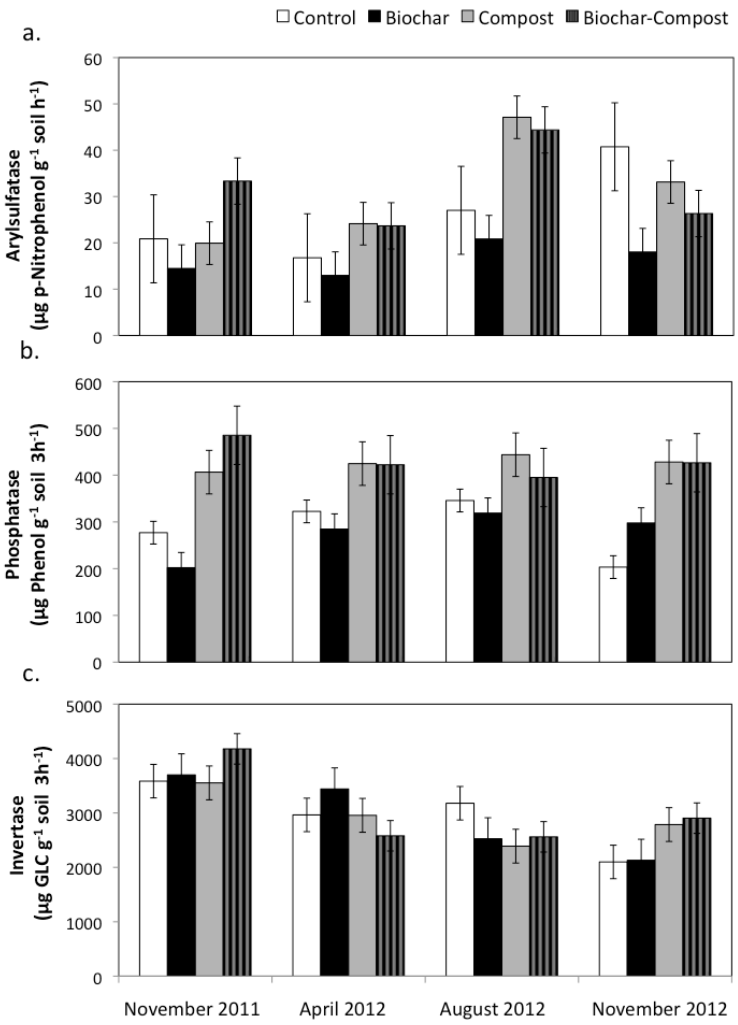

Figure 8.3. Arylsulfatase (a), phosphatase (b) and invertase (c) potential activity from November 2011 until November 2012. Control, biochar, compost and biochar-compost amendment means ± SE are shown.

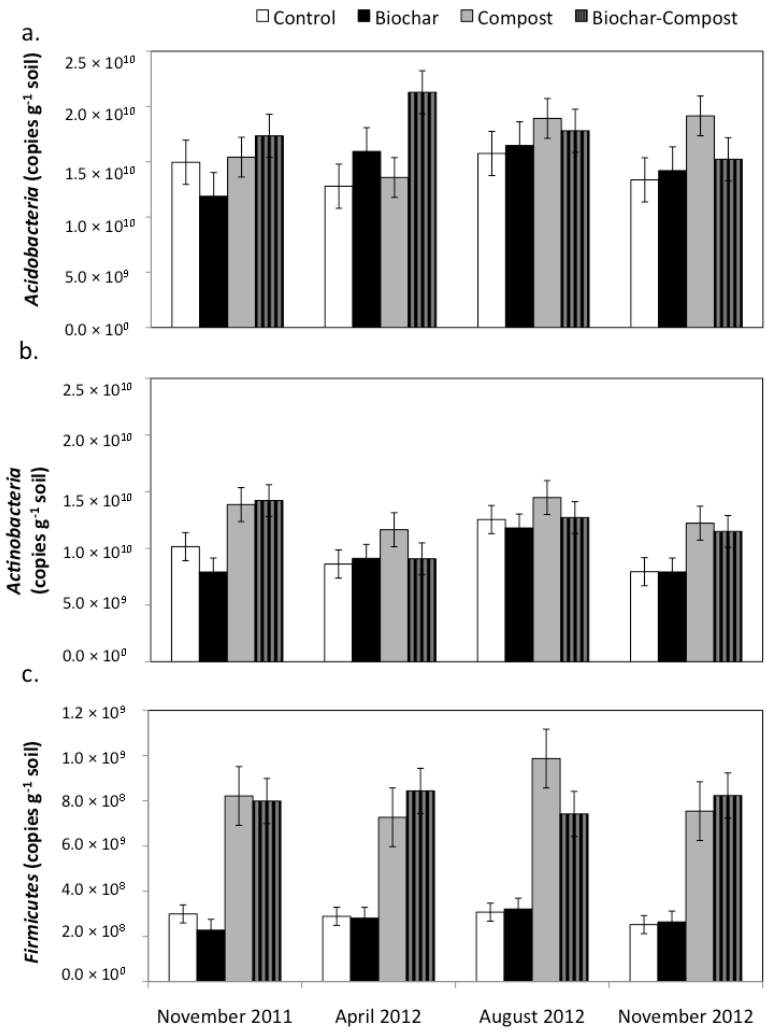

Figure 8.4. *Acidobacteria* (a), *Actinobacteria* (b) and *Firmicutes* (c) gene copy numbers from November 2011 until November 2012. Control, biochar, compost and biochar-compost amendment means ± SE are shown.

Table 8.4. Plant biomass, plant copper removal, nitrogen, extractable organic nitrogen, microbial biomass N, bacterial and fungal PLFAs, 16S rRNA, ergosterol, and taxa specific bacteria. Mean concentrations ± SE.

Variable	Treatment	November 2011	April 2012	August 2012	November 2012
Plant Biomass	Control	1,383 ± 210	2,428 ± 360	3,570 ± 560	585 ± 326
kg DM ha^{-1}	Biochar	1,225 ± 275	2,480 ± 531	1,795 ± 401	596 ± 298
	Compost	998 ± 197	2,922 ± 188	2,240 ± 495	814 ± 364
	Biochar-Compost	1,299 ± 329	3,010 ± 175	2,475 ± 999	1,271 ± 578
Plant Copper Removal	Control	26,939 ± 4190	67,502 ± 15,405	107,745 ± 26,012	18,240 ± 10,185
mg Cu ha^{-1}	Biochar	24,791 ± 7809	65,224 ± 18,269	48,654 ± 15,176	26,154 ± 14,014
	Compost	20,578 ± 3038	67,684 ± 10,239	57,666 ± 12,498	30,035 ± 14,679
	Biochar-Compost	29,562 ± 9473	89,554 ± 13,139	62,412 ± 31,241	54,571 ± 25,188
Total Nitrogen mg g^{-1}	Control	1.4 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	2.1 ± 0.1
	Biochar	1.4 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	2.1 ± 0.1
	Compost	1.8 ± 0.1	2.8 ± 0.1	2.7 ± 0.1	2.6 ± 0.1
	Biochar-Compost	1.8 ± 0.1	2.8 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
Extractable Total Nitrogen	Control	4.9 ± 2	3.5 ± 1	8.4 ± 1	2.0 ± 1
µg ETN g^{-1} DM	Biochar	1.1 ± 1	4.7 ± 2	7.4 ± 2	2.7 ± 1
	Compost	10.8 ± 2	9.2 ± 2	18.3 ± 5	5.9 ± 1
	Biochar-Compost	10.0 ± 2	8.4 ± 2	13.8 ± 1	5.5 ± 2
Ammonium	Control	2.3 ± 0.4	1.9 ± 0.1	3.5 ± 0.8	1.7 ± 0.1
µg g^{-1} DM	Biochar	2.0 ± 0.2	2.0 ± 0.2	3.3 ± 0.5	1.8 ± 0.2
	Compost	3.0 ± 0.5	2.6 ± 0.3	4.5 ± 1.3	3.7 ± 1.2
	Biochar-Compost	3.3 ± 0.5	4.6 ± 2.5	5.4 ± 1.5	3.6 ± 1.2

Table 8.4. continued… Mean concentrations ± SE.

Variable	Treatment	November 2011	April 2012	August 2012	November 2012
Nitrate	Control	3.9 ± 1.1	1.9 ± 0.2	15.8 ± 4.9	3.0 ± 0.4
µg g^{-1} DM	Biochar	2.7 ± 0.4	2.0 ± 0.4	12.4 ± 2.8	3.0 ± 0.6
	Compost	7.1 ± 1.2	2.9 ± 0.8	22.8 ± 5.6	4.8 ± 1.6
	Biochar-Compost	6.5 ± 1.2	2.7 ± 0.7	15.3 ± 2.6	4.5 ± 0.8
Gravimetric Soil Moisture %	Control	18.3 ± 1	14.5 ± 1	16.2 ± 1	21.2 ± 0
	Biochar	19.6 ± 1	14.7 ± 1	18.2 ± 1	20.3 ± 1
	Compost	19.6 ± 1	15.8 ± 0	18.9 ± 1	23.0 ± 1
	Biochar-Compost	23.0 ± 1	16.2 ± 1	18.1 ± 1	22.3 ± 1
$CEC_{effective}$	Control	20.4 ± 0.4	19.1 ± 0.1	19.0 ± 0.3	19.5 ± 0.6
cmol kg^{-1}	Biochar	21.9 ± 1.6	18.9 ± 0.2	19.3 ± 0.5	19.6 ± 0.6
	Compost	20.9 ± 0.7	19.2 ± 0.6	19.3 ± 0.8	20.7 ± 0.7
	Biochar-Compost	21.9 ± 0.5	19.9 ± 0.3	19.9 ± 0.4	20.1 ± 0.6
Microbial Biomass N	Control	25.7 ± 6	29.0 ± 4	28.6 ± 5	14.3 ± 1
µg N_{mic} g^{-1}	Biochar	27.5 ± 7	27.7 ± 5	19.7 ± 3	22.1 ± 3
	Compost	53.5 ± 11	44.5 ± 4	41.2 ± 6	36.4 ± 6
	Biochar-Compost	73.3 ± 5	47.3 ± 8	46.0 ± 6	33.5 ± 5
$PLFA_{bacterial}$	Control	17.6 ± 3	13.3 ± 2	18.6 ± 2	14.6 ± 1
nmol g^{-1} DM	Biochar	17.0 ± 2	16.7 ± 2	18.5 ± 2	14.8 ± 1
	Compost	29.2 ± 4	23.1 ± 2	22.5 ± 3	23.2 ± 2
	Biochar-Compost	33.2 ± 2	27.2 ± 4	23.6 ± 1	24.2 ± 2

Table 8.4. continued… Mean concentrations ± SE.

Variable	Treatment	November 2011	April 2012	August 2012	November 2012
$PLFA_{gram+}$	Control	9.6 ± 1	6.8 ± 1	10.6 ± 1	8.6 ± 1
nmol g^{-1} DM	Biochar	9.4 ± 1	8.8 ± 1	10.7 ± 1	8.9 ± 1
	Compost	18.6 ± 3	13.8 ± 1	14.4 ± 2	14.6 ± 1
	Biochar-Compost	21.2 ± 2	17.0 ± 3	14.6 ± 0	15.4 ± 2
$PLFA_{gram-}$	Control	1.3 ± 0.2	1.1 ± 0.1	1.6 ± 0.2	0.98 ± 0.1
nmol g^{-1} DM	Biochar	1.2 ± 0.1	1.4 ± 0.2	1.6 ± 0.1	0.97 ± 0.1
	Compost	2.4 ± 0.4	2.0 ± 0.1	2.1 ± 0.3	1.7 ± 0.1
	Biochar-Compost	2.8 ± 0.3	2.4 ± 0.3	2.1 ± 0.1	1.8 ± 0.2
16S rRNA	Control	$2.4 \times 10^{10} \pm 2 \times 10^{9}$	$2.3 \times 10^{10} \pm 4 \times 10^{9}$	$2.8 \times 10^{10} \pm 8 \times 10^{9}$	$1.6 \times 10^{10} \pm 3 \times 10^{9}$
copies g^{-1}DM	Biochar	$1.7 \times 10^{10} \pm 2 \times 10^{9}$	$2.7 \times 10^{10} \pm 6 \times 10^{9}$	$3.0 \times 10^{10} \pm 5 \times 10^{9}$	$1.9 \times 10^{10} \pm 2 \times 10^{9}$
	Compost	$4.2 \times 10^{10} \pm 8 \times 10^{9}$	$3.0 \times 10^{10} \pm 2 \times 10^{9}$	$3.6 \times 10^{10} \pm 7 \times 10^{9}$	$3.0 \times 10^{10} \pm 4 \times 10^{9}$
	Biochar-Compost	$4.4 \times 10^{10} \pm 5 \times 10^{9}$	$3.5 \times 10^{10} \pm 4 \times 10^{9}$	$3.7 \times 10^{10} \pm 7 \times 10^{9}$	$2.8 \times 10^{10} \pm 4 \times 10^{9}$
$PLFA_{fungal}$	Control	1.8 ± 0.2	2.8 ± 0.3	2.9 ± 0.4	2.3 ± 0.1
nmol g^{-1} DM	Biochar	1.6 ± 0.2	3.8 ± 0.7	3.2 ± 0.7	2.0 ± 0.2
	Compost	2.1 ± 0.3	3.9 ± 0.3	2.7 ± 0.5	2.8 ± 0.3
	Biochar-Compost	2.2 ± 0.1	4.1 ± 0.7	3.8 ± 0.4	3.5 ± 0.6
Ergosterol	Control	3.1 ± 0.5	2.3 ± 0.3	3.3 ± 0.6	2.3 ± 0.1
µg g^{-1} DM	Biochar	2.9 ± 0.6	2.7 ± 0.4	3.1 ± 0.6	2.9 ± 0.3
	Compost	4.5 ± 1.0	3.3 ± 0.4	3.4 ± 0.6	2.9 ± 0.3
	Biochar-Compost	4.6 ± 0.2	3.0 ± 0.6	3.5 ± 0.5	3.5 ± 0.5

Table 8.4. continued… Mean concentrations ± SE.

Variable	Treatment	November 2011	April 2012	August 2012	November 2012
α-Proteobacteria	Control	$1.1 \times 10^9 \pm 3 \times 10^8$	$9.1 \times 10^8 \pm 3 \times 10^8$	$1.0 \times 10^9 \pm 2 \times 10^8$	$6.2 \times 10^8 \pm 7 \times 10^7$
copies g^{-1}DM	Biochar	$8.4 \times 10^8 \pm 2 \times 10^8$	$8.4 \times 10^8 \pm 2 \times 10^8$	$9.2 \times 10^8 \pm 1 \times 10^8$	$7.8 \times 10^8 \pm 5 \times 10^7$
	Compost	$1.6 \times 10^9 \pm 4 \times 10^8$	$1.2 \times 10^9 \pm 1 \times 10^8$	$1.9 \times 10^9 \pm 4 \times 10^8$	$1.1 \times 10^9 \pm 3 \times 10^8$
	Biochar-Compost	$1.4 \times 10^9 \pm 2 \times 10^8$	$1.2 \times 10^9 \pm 2 \times 10^8$	$1.2 \times 10^9 \pm 2 \times 10^8$	$1.4 \times 10^9 \pm 2 \times 10^8$
β-Proteobacteria	Control	$2.3 \times 10^9 \pm 2 \times 10^8$	$1.7 \times 10^9 \pm 5 \times 10^8$	$2.8 \times 10^9 \pm 1 \times 10^9$	$1.3 \times 10^9 \pm 1 \times 10^8$
copies g^{-1}DM	Biochar	$1.4 \times 10^9 \pm 3 \times 10^8$	$1.8 \times 10^9 \pm 3 \times 10^8$	$2.7 \times 10^9 \pm 9 \times 10^8$	$1.4 \times 10^9 \pm 10 \times 10^7$
	Compost	$3.2 \times 10^9 \pm 8 \times 10^8$	$2.4 \times 10^9 \pm 4 \times 10^8$	$2.1 \times 10^9 \pm 4 \times 10^8$	$2.1 \times 10^9 \pm 5 \times 10^8$
	Biochar-Compost	$2.9 \times 10^9 \pm 4 \times 10^8$	$1.9 \times 10^9 \pm 3 \times 10^8$	$2.4 \times 10^9 \pm 5 \times 10^8$	$2.5 \times 10^9 \pm 3 \times 10^8$
Gemmatimonadetes	Control	$4.8 \times 10^8 \pm 6 \times 10^7$	$5.1 \times 10^8 \pm 7 \times 10^7$	$5.6 \times 10^8 \pm 6 \times 10^7$	$4.5 \times 10^8 \pm 3 \times 10^7$
copies g^{-1}DM	Biochar	$3.6 \times 10^8 \pm 4 \times 10^7$	$5.0 \times 10^8 \pm 6 \times 10^7$	$4.9 \times 10^8 \pm 7 \times 10^7$	$4.9 \times 10^8 \pm 6 \times 10^7$
	Compost	$5.5 \times 10^8 \pm 5 \times 10^7$	$4.6 \times 10^8 \pm 3 \times 10^7$	$6.6 \times 10^8 \pm 9 \times 10^7$	$6.8 \times 10^8 \pm 8 \times 10^7$
	Biochar-Compost	$4.7 \times 10^8 \pm 5 \times 10^7$	$6.4 \times 10^8 \pm 9 \times 10^7$	$5.9 \times 10^8 \pm 5 \times 10^7$	$5.9 \times 10^8 \pm 7 \times 10^7$

8.4 Discussion

Copper bioavailability

Biochar, compost and biochar-compost did not affect Cu bioavailability in the present experiment. This is in contrast to other studies, including that of Beesley et al. (2014), who measured a reduction in pore water Cu in the presence of biochar and compost amended soil. Biochar was previously found to adsorb Cu, while compost's high organic matter content attracted Cu (Hua et al., 2009; Navel and Martins, 2014). On the other hand, Debela et al. (2012) had similar results as seen in this study. After analyzing Cu, Cd and Zn for their adsorption to wood biochar, the authors only found that biochar was successful in absorbing Cd and Zn. The lack of immobilization in the present study could potentially be due to the type of feedstock used. This study utilized a high temperature hardwood biochar, which, although shown to immobilize Cu in the lab, was less successful than gasification coke and avian litter biochar-compost blend (Uchimiya et al., 2010; Borchard et al., 2012; Uchimiya et al., 2012). Although Kolodynska et al. (2012) (manure biochar) and Ippolito et al. (2012) (pecan shell biochar) observed that only at pH >8 there was a significant decrease in metal adsorption due to the precipitation or formation of hydroxide complexes of carbonates and phosphates, it may be that the alkalinity of the vineyard soil diminished the immobilization by biochar. Field experiments often have results, which are not seen in the lab and in this study a neutral soil pH in addition to organic matter, either as biochar, compost or plant biomass, may have led to similar levels of immobilization across all plots (Smolders et al., 2012; Ruyters et al., 2013). That ability of organic matter to bind with Cu is discussed in section 4.2. Ippolito et al. (2012) observed, however, that at Cu concentrations below approx. 1,000 mg Cu kg^{-1} biochar the differences between pH values became much less significant for biochar adsorption. This suggests that, in addition to pH inhibition at this site, Cu pollution levels in vineyards may be generally too low to induce metal adsorption by biochar. As most studies have only analyzed pure

biochar and biochar-compost in the laboratory and Beesley et al. (2014) only analyzed a soil with 2,940 mg Cu kg^{-1} soil, it is not yet possible to confirm this.

Additionally, treatments did not significantly reduce plant Cu content or overall plant Cu removal. This is likely because Cu_{DTPA} content, i.e. plant available Cu fraction, was not reduced by biochar or compost. Moreover, it could indicate that the cover crops were receiving sufficient amounts of nutrients and water without the help of biochar and compost. This can be seen in plant biomass, an important factor significantly correlated to Cu removal ($P < 0.001$, $r = 0.88$), which was not increased by treatments. Schmidt et al. (2014) conducted grapevine analyses on the same field trial as the present study and also found that there were no biochar or compost effects on grapevine growth or health. In fact, in contrast to our hypothesis that biochar and biochar-compost would reduce Cu uptake by plants, cover crops significantly increased their uptake of Cu over time, independent of treatment. Thus, while Cu_T and Cu_{DTPA} significantly decreased from November 2011 until November 2012, plant Cu content simultaneously significantly increased. It can be assumed that soil Cu content was reduced because plant Cu content increased over time. This suggests that long-term (> 20 mo) cover crop growth could be a possible phytoremediation option. However, the maximum removal rate achieved was 0.11 kg Cu ha^{-1} in August when plant biomass was highest. As this amount of Cu removal is lower than that which is applied per year, phytoremediation cannot be considered an efficient method to decrease soil Cu content. Moreover, the overall loss of Cu_T was more than the uptake by cover crops. This could mean that plant roots are additionally immobilizing Cu at a much greater amount than aboveground biomass, which has been reported previously (Brun et al., 2001; Chaignon et al., 2003) or that soil erosion due to the vineyard's slope has removed significant amounts of Cu_T, as it resides in the upper soil horizons (Kosmas et al., 1997).

Microbial abundance and activity

Despite our initial hypothesis, that the addition of biochar would increase microbial abundance by reducing soil Cu content and providing a niche habitat, biochar had no

significant effects on soil microbial abundance or activity in this study. This result was also observed by Castaldi et al. (2011) and Rutigliano et al. (2014) in a field study, where 14 months after initial hardwood biochar addition no differences were observed. After 3 months, however, significant differences in microbial biomass, respiration and enzyme activity were seen in both studies and the authors concluded that biochar had a priming effect on soil microorganisms. Biochar had provided easily consumable carbon resources initially, derived from the production process (Smith et al., 2010), after which the remaining carbon was recalcitrant. As the present study did not include sampling shortly after biochar application, this hypothesis cannot be confirmed. However, this study does affirm that biochar does not provide a long-term (> 8 months) benefit to soil microorganisms. This is further supported by the fact that biochar did not increase EOC, a potentially available C-resource for soil microorganisms.

In contrast to biochar and according to our hypothesis, compost and biochar-compost amendments significantly increased microbial abundance and enzyme activities. This observation has been supported throughout the literature and it has been concluded that compost enhances microbial abundance (microbial biomass, PLFAs) and enzyme activities (alkaline phosphatase, β-glucosidase, leucine aminopeptidase, urease, arylsulfatase) (Ros et al., 2006; Galvez et al., 2012; Pardo et al., 2014). Compost addition also increased fungal biomass. Whereas ergosterol content did not change over the sampling period, $PLFA_{fungal}$ abundance increased in April and August 2012 together with plant biomass, suggesting that fungi containing this biomarker are influenced by root exudates. The influence of substrates on the fungal biomarker is supported by the correlation between $PLFA_{fungal}$ and C_T and N_T ($P < 0.001$, $r = 0.51$ and 0.68, respectively), whereas ergosterol was only significantly correlated to C_{mic} ($P < 0.001$, $r = 0.67$) and $PLFA_{bacterial}$ ($P < 0.001$, $r = 0.74$).

That compost enhances microbial properties may be due to the increase of substrate availability. This is underlined by the significant correlation between C_{mic} ($P < 0.001$,

r = 0.53), bacterial PLFAs (P < 0.001, r = 0.62) and phosphatase activity (P < 0.001, r = 0.58) and EOC as well as comparable low C_{mic} values in the control and biochar plots (Probst et al., 2008; Galvez et al., 2012; Mackie et al., 2012; Beesley et al., 2014). Moreover, Cu binding with organic matter, in this case compost, can moderate its impact on soil microorganisms (Lejon et al., 2010). The "protective effect toward bacteria" is due to the release of dissolved organic ligands, which reduce Cu bioavailability and subsequently metal stress (Navel and Martins, 2014). Compost has been observed to increase soil resiliency to Cu and promote microbial activity (Kostov and Van Cleemput, 2001). Although Cu_{DTPA} was not reduced by compost in this study, this mechanism could still be at play as Cu_{DTPA} is one exchangeable fraction and does not completely represent all of the bioavailable Cu in soil. Moreover, it is possible that compost simply provided necessary substrates for bacteria to tolerate Cu, as their main Cu strategy is efflux pumps, which require large amounts of energy (Klein and Lewinson, 2011). However, Kostov and Van Cleemput (2001) and Smolders et al. (2012) agree that the addition of organic matter has the potential to reduce the impact of Cu, but does not guarantee it even in soil with a neutral pH. This study found that microbial and chemical benefits from compost did not remain stable over time. C_{mic}, bacterial PLFAs, invertase activity and EOC significantly decreased over the sampling period in the compost treatments. This is likely because microorganisms degraded substrates initially provided, reducing the resource pool over time. Repeated application of compost each year could potentially preserve the positive effects of compost on soil microbial properties.

Microbial community composition

Actinobacteria were found to be one of the dominant bacterial groups in this vineyard soil. As *Actinobacteria* are Gram+, their thicker peptidoglycan layer increases the surface area for binding metals, supporting metal resilience and increasing metabolic activity in polluted sites (Gremion et al., 2003; Hema et al., 2014; Zhan and Sun, 2014). Multivariate analysis, however, confirmed that the treatments had significantly different community compositions.

Compost and biochar-compost significantly increased the absolute abundances of PLFAs and all bacterial taxa, with the exception of *Acidobacteria*. In particular, the relative abundance of *Firmicutes* increased. *Firmicutes* are copiotrophs, or *r*-strategists, which are predominately present during the thermophilic stage of the composting process. Therefore, it is likely that this bacterial group was added with the compost, increasing the bacterial community already present in soil and remaining abundant over time (Yamamoto et al., 2009; Neher et al., 2013). This is additionally supported by the fact that only *Firmicutes* was correlated to EOC ($P < 0.001$, $r = 0.57$) provided by compost amendments. In contrast, control and biochar treatments had higher relative abundances of *Acidobacteria*, *Gemmatimonadetes* and *Actinobacteria*. *Gemmatimonadetes* and *Acidobacteria* are Gram-, slow growing oligotrophs, adapted to substrate poor environments and, therefore, less reliant on easily available substrates provided by compost (Naether et al., 2012). *Actinobacteria*, on the other hand, are expected to increase their relative abundance when C resources are more recalcitrant (Lazcano et al., 2012; Partanen et al., 2010). This has been observed in biochar experiments (Prayogo et al., 2013; Gomez et al., 2014; Rutigliano et al., 2014; Watzinger et al., 2014). Despite higher relative abundance, however, control and biochar treatments had significantly lower absolute *Actinobacteria* abundances than compost amendments in November 2011 and 2012. They increased to similar absolute abundances in April and August 2012, coinciding with high plant biomass. This suggests that *Actinobacteria* may profit from easily available C provided during plant growth. Moreover, August was also a period of increased mineralized N levels, which is significantly correlated to *Actinobacteria* in this study ($P < 0.001$, $r = 0.50$) and other (Philippot et al., 2009). The role of substrate availability on *Actinobacteria* in this study, in contradiction to the literature, could be because these Cu tolerant bacteria are more competitive at this site, allowing them to take over the processes of other bacteria. It is also possible that certain species within the *Actinobacteria* taxa, which are more driven by labile C, became more abundant.

Despite changes in composition, absolute abundances of most taxa, except *Actinobacteria*, were not modified over time, despite a decrease in total microbial biomass. Zhan and Sun (2014) also observed consistent bacterial abundances over time on a Chinese Cu mine soil. The authors concluded that bacterial groups are less sensitive to environmental changes and that lack of variation represented a stable and resilient microbial community. Other possibilities are that species within each taxon changed, but that total taxa abundance remained stable or perhaps the volume and/or cell size of the bacteria (as measured by C_{mic} and PLFAs) decreased with less available substrate, but DNA within each cell remained intact.

8.5 Conclusion

Biochar, compost and biochar-compost did not immobilize exchangeable soil Cu content at this vineyard. Treatments did not reduce plant Cu content or the exchangeable Cu fraction in soil. Cover crops, however, were found to take up significant amounts of Cu within two growing seasons, reducing soil Cu. In general, the treatments increased C_T, with compost and biochar-compost additionally increasing extractable organic C. The ability of these amendments to sequester C can only be determined with longer-term sampling. Compost and biochar-compost were shown to increase soil microbial abundance and functional activity for as long as 20 months following initial application. This is beneficial for ecosystem services, such as nutrient cycling, however, these microbial properties decreased over time. *Actinobacteria* was found to be Cu tolerant as well as linked to available substrates. Moreover, microbial community composition was modified by treatment. Additionally, there is no indication that the addition of biochar to the compost process has added benefit for the soil. In conclusion, neither biochar nor compost or biochar-compost has improved Cu remediation. Compost is recommended to enhance ecosystem services, but should be reapplied annually in order to resupply substrates.

8.6 Acknowledgements

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8.7 Supplementary Material

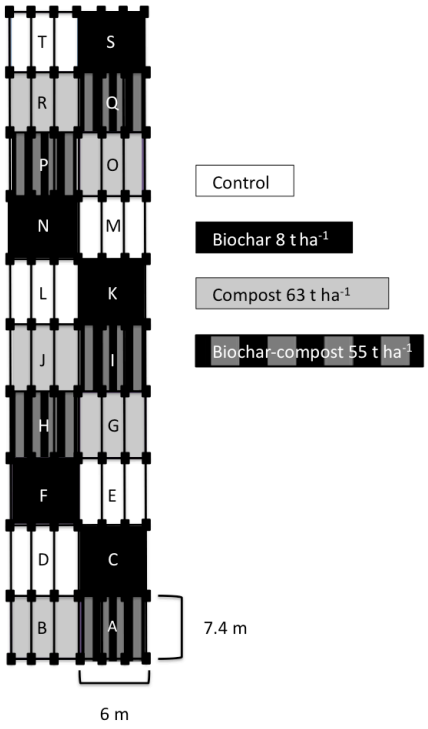

Figure S.8. Blocked design, including five replicates of biochar, biochar-compost, compost and a control. Vertical lines represent vine rows.

Table S.8.1. Plant protection spraying plan 2012. Plant extracts consists of 50% H_2O, 26% compost tea, 11.9% milk, 10% plant extracts, 2% algae extract, and 0.1% nu-film. Copper solution consists of 96.5% H_2O, 3.2% Sodium bicarbonate (4.6 kg), 0.26% Kocide-Opti (Cu), and 0.1% nu-film. Sodium bicarbonate solution consists of 96.8% H_2O, 3.1% sodium bicarbonate and 0.1% nu-film.

Date/Treatment	Plant extracts	Copper solution	Sodium bicarbonate solution
25.05.12	✔		
06.06.12	✔		
14.06.12	✔		
27.06.12	✔		
07.07.12		✔	
18.07.12		✔	
26.07.12		✔	
14.08.12			✔

Table S.8.2. Conditions, protocols and efficiencies of qPCR analyses.

Gene	Primer*	Thermal profile**	No. of cycles	Efficiency mean (%)	Reference
16S rRNA	314F 534R	95°C – 10 m 95°C – 15 s, 60°C – 30 s, 72°C – 30 s, 75°C – 30 s	1 35	102%	(Lopez-Gutierrez et al., 2004)
Acidobacteria	Acid31 Eub518	95°C – 10 m 95°C – 15 s, 55°C – 30 s, 72°C – 30 s, 76°C – 30 s	1 35	99%	(Philippot et al., 2009)
Actinobacteria	Act920F3 Act1200R	95°C – 10 m 95°C – 15 s, 61.5°C – 30 s, 72°C – 30 s, 76°C – 30 s	1 35	94%	(De Gregoris et al., 2011)
α-Proteobacteria	Eub338 Alfa685	95°C – 10 m 95°C – 15 s, 60°C – 30 s, 72°C – 30 s, 79°C – 30 s	1 35	81%	(Fierer et al., 2005)
β-Proteobacteria	Eub338 Bet680	95°C – 10 m 95°C – 15 s, 55°C – 30 s, 72°C – 30 s, 80°C – 30 s	1 35	88%	(Philippot et al., 2009)
Firmicutes	Lgc353 Eub518	95°C – 10 m 95°C – 15 s, 60°C – 30 s, 72°C – 30 s, 79°C – 30 s	1 35	98%	(Fierer et al., 2005)

Gemmatimonadetes	Gem440 Eub518	95°C – 10 m 95°C – 15 s, 58°C – 30 s, 72°C – 30 s, 78°C – 30 s	1 35	91%	(Philippot et al., 2009)

*Primer concentration was 10 pmol μl^{-1}

**Additionally, a 60°C to 95°C step was added to each run to obtain the denaturation curve specific for each amplified sequence.

References

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9 General Conclusions

9.1 Effects of Copper Pollution on Soil Microorganisms

Wightwick et al. (2013b) recently concluded in an Australian case study that there is currently a low ecological risk with regards to copper pollution. The authors expect that available Cu concentration will only first become a risk in 2030-2040. In Europe, however, Michaud et al. (2007) has already seen the negative effects of Cu accumulation at an old French vineyard site, where vineyard production has been abandoned and other crops sown. These crops are producing lower yield and showing signs of chlorosis. This suggests that although direct effects may not always be observed, with continued use or change in crop, negative effects will become more pronounced. Moreover, chapters 5 and 6 of this thesis showed that moderate concentrations of copper in European vineyard topsoils currently do have negative effects on soil microorganisms. This was found to be particularly true for their functional activity (alkaline phosphatase, arylsulfatase and invertase activities). In particular, it was seen that the spatial distribution of these enzyme activities as well as ergosterol were in contrast to that of total and exchangeable copper and at concentrations much lower than previously observed. The results of chapters 5 and 6 were the basis for hypothesizing that reducing copper concentration and immobilizing copper compounds would increase microbial abundance and activity. Moreover, the review concluded that microbially assisted phytoextraction is currently the most promising copper remediation strategy in viticulture. This conclusion suggested that soil microorganisms may not only be affected by copper pollution, but may also play a role in its remediation in soils. Additionally, it was clear in chapter 5 that field experiments were absent from the literature and necessary for understanding remediation practicality. Chapters 7 and 8 used these results to examine potential solutions for copper pollution in agriculture.

9.2 Copper Remediation Strategies

The phytoextraction field experiment (chapter 7) investigated the practicality of phytoextraction. Moreover, it looked at the above- and below-ground interactions that support phytoextraction with the intention to create a foundation for further research in microbially assisted phytoextraction. As hypothesized, some plant species were more successful than others. It was observed that oat, rye and hairy vetch were able to remove significantly more amounts of copper than clover, chenopodium, rape, or mixed species plots. However, the maximum annual amount of copper removed (0.033 kg Cu ha^{-1} y^{-1}) was much lower than the annual amount of copper applied (3 kg Cu ha^{-1} y^{-1}). Recently, Malagoli et al. (2014) also tested the ability of two plant species (*Festuca rubra*, *Sinapsis alba*) to accumulate copper from vineyard soils in a pot experiment. The authors concluded that *F. rubra* had the most potential, likely removing 0.06 – 1.3 kg ha^{-1} y^{-1} in the field with three cuttings annually. As with the present study, these removal rates are too low, especially at a time when copper fungicides continue to be applied. Therefore, phytoextraction was not successful in removing significant amount of copper from the soil and cannot be considered a practical remediation method in this vineyard.

The biochar field experiment (chapter 8) explored an alternative strategy to reduce the negative effects of copper pollution, immobilization with biochar. However, neither biochar nor biochar-compost was found to significantly reduce copper availability to plants. The adsorption capacity of biochar previously observed in the laboratory was not detected in the field, suggesting that either soil copper concentrations were too low or that the soil pH was too high. Moreover, in contrast to our hypothesis, cover crops, regardless of treatment, took up significant amounts of copper over two growing seasons. The cover crops succeeded in removing a maximum of 0.1 kg ha^{-1} over the sampling period. As suggested above, however, these extraction rates are well below current soil concentrations and annual application rates. Immobilization with biochar was, therefore, unsuccessful in

significantly reducing copper availability to plants and cannot be considered a copper remediation strategy in temperate vineyards.

9.3 Effects of Copper Remediation on Soil Microorganisms

Despite the conclusions of chapters 5 and 6, that microbial functional activity and abundance are reduced in moderately contaminated vineyards, chapters 7 and 8 saw no influence of copper concentration on soil microorganisms. This is largely in contrast to our hypotheses that copper would negatively affect soil microorganism abundance, activity and diversity. All field experiments in this thesis had average total copper concentrations ranging from 100 - 150 mg Cu_T kg^{-1} soil. Therefore, it could be that modifying copper speciation with organic matter enhanced the copper tolerance of soil microorganisms in the final studies. Copper is attracted to soil organic matter, which was provided both by cover crops (chapter 7) and compost (chapter 8) in this study (Brunetto et al., 2014; Navel and Martins, 2014). It is also possible that the experiment design of the final studies (chapters 7, 8) resulted in a much lower relative difference in copper concentration, as there was no longer a spatially defined grid sampling scheme. Although this had no effect on the success of the remediation strategies, it suggests that the negative effects of copper on soil microorganisms may lie at a finer scale.

Soil microorganisms were instead mainly influenced by the availability of substrate. This thesis demonstrated that substrate could be provided by cover crops (chapters 6, 7) or compost addition (chapter 8). In the Swiss vineyard system, soil microorganisms did not seem to differentiate by substrate origin, i.e. specific plant species, but instead only its availability. Moreover, microbial groups, obtained with PLFAs, were not found to be associated with specific plant species. Instead, the same community was found across all treatments. This underlines the generality of the above- and below-ground and plant – microbial interactions in this vineyard soil. It would be beneficial, however, to conduct further experiments with a fallow plot added. This would provide clearer results on what ecosystem services are provided by the presence of year round cover crops in a copper polluted system. Season also

played an indirect role in substrate availability by modifying plant growth as well as physical soil properties. Seasonal changes in soil moisture were observed to increase Gram+ bacteria, a microbial group that has a narrow soil moisture niche. In contrast to specific plant species, compost addition did amend microbial community composition. In chapter 8, it was observed that the addition of compost and biochar compost significantly increased all microbial abundances, with the exception of *Acidobacteria*. Specifically, *Firmicutes* increased in relative abundance with compost addition, identifying these bacterial taxa as r-strategists as well as predominant bacteria during the composting process. Control and biochar plots, on the other hand, increased the relative abundance of *Acidobacteria*, *Actinobacteria* and *Gemmatimondates*. These bacteria were better able to assimilate more complex carbons. Additionally, *Actinobacteria* increased in absolute abundance in the control and biochar plots at the same time as plant biomass increased. *Actinobacteria* were one of the most predominant bacteria in this soil, showing characteristics of being copper tolerant, as seen in other studies (Gremion et al., 2003; Hema et al., 2014; Zhan and Sun, 2014). Their role in assimilating easily available substrates, not previously observed in the literature, suggests that they had a competitive advantage over other bacterial groups. Microbial functional activity also responded to increases in available substrate, with only the results of the chapter 6 showing a direct effect from higher copper concentrations.

This thesis has shown the benefit of executing field experiments, as they provide *in situ* results and quantifiable conclusions on the practicality and feasibility of copper remediation. In conclusion, neither copper phytoextraction nor immobilization with biochar was found to be an efficient method for remediating copper in temperate vineyards. There is evidence, however, that cover crops and compost may be providing an indirect service to and stimulating soil microorganisms, allowing them to tolerate copper polluted environments. It is recommended to continue to monitor these methods in the field in order to better understand their interactions.

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Curriculum Vitae

Personal Information

Name:	Kathleen Allison Mackie
Birth Date:	November 22nd, 1983
Birth City:	Lexington, Kentucky, USA
Address:	Alte Dorfstrasse 75, 70599 Stuttgart
E-Mail:	katie.mackie@gmail.com
Family Status:	Married

Secondary Education

August 1998 - June 2002	Connelly School of the Holy Child, Potomac, MD, USA Final grade: 3.8 (American GPA) Graduation: June 2002

Undergraduate Education

August 2002 - May 2006	Bachelor of Science: Geology, Honors College College of Charleston, SC, USA Final grade: 3.65 (American GPA), Cum laude Graduation: May 7th, 2006 Focus: Hydrogeology Minor: Environmental Studies Thesis: Consequences of Sea Level Rise on Wetlands

Graduate Education

October 2008 - October 2010	Master of Science: Environmental Protection and Agricultural Food Production University of Hohenheim, Stuttgart, Germany Final grade: 3.4 (American GPA) Graduation: October 29th, 2010 Focus: Soil Biology Thesis: Long-term Application of Copper in Organic Vineyard Modifies Spatial Distribution of Soil Microorganisms
January 2011 – October 2014	Doctoral student University of Hohenheim, Institute for Soil Science and Land Evaluation, Section for Soil Biology Final Grade: Summa cum laude Dissertation: “The Importance of Soil Microorganisms and Cover Crops for Copper Remediation in Vineyards” Carl-Zeiss-Stiftung PhD Scholar (June 2011-June 2014)

Professional Experience

June - August 2006	Hydrogeology Intern, U.S. Forest Service, Durango, CO, USA
September 2006 - April 2007	Winery Apprentice, Weingut Leo Hillinger GmbH, Jois, Austria
May - November 2007	Hydrology Technician, U.S. Forest Service, Durango, CO, USA
April - August 2008	Sommelier Assistant, Restaurant Eve, Alexandria, VA, USA
September - November 2009	Student Assistant, Tropenzentrum, Uni Hohenheim
August - October 2010	Student Assistant, Co-ordination for organic agriculture and consumer protection, Uni Hohenheim

August 2013 – June 2014	Student Assistant, English correction and German to English translation for the Food Security Center, Uni Hohenheim
July 2014 – present	Scientific Assistant, lab analyses, literature review, manuscript preparation

Volunteer Work

January 2009 – present	F.R.E.S.H. – Food Revitalization and Eco-gastronomic Society of Hohenheim; a society of students focused on stimulating dialogue about the way we produce, eat and enjoy food, as well as providing an arena to produce and eat organic, local food on campus.
May 2009 – October 2009	Co-Founder of the Student Garden and Treasurer
October 2009 – October 2010	2. Chair
October 2010 – March 2011	1. Chair
October 2009	2nd EU Organic Congress, Representative for Slow Food's Youth Food Movement
October 2010, 2012	Terra Madre Conference, Slow Food's Youth Food Movement Delegate

Language	Mother tongue: English Other: German (advanced), Spanish (elementary)
Computer knowledge	Microsoft Office, ArcGIS 9.3, Pathfinder 2.90/3.10, SAS 9.2, R statistical software 3.0.2.

Stuttgart, Kathleen A. Mackie

Publications & Presentations

Peer-reviewed Journal Articles

Mackie, K.A., Müller, T., Kandeler, E., 2012. Remediation of copper in vineyard – A mini Review. Environmental Pollution 167, 16-26.

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Poster Presentations

Mackie, K.A., Müller, T., Zikeli, S., Kandeler, E., 2010. Long-term copper application in an organic vineyard modifies spatial distribution of soil microorganisms. European League of Life Sciences Student Conference: "Food and the Environment; production, quality and planning," Copenhagen, Denmark.
Award winner of the Helmut Aurenz scholarship

Mackie, K.A., Müller, T., Zikeli, S., Kandeler, E., 2011. Long-term copper application in an organic vineyard modifies spatial distribution of soil microorganisms. Enzymes in the Environment, Bad Nauheim, Germany.

Award winner of Federation of European Microbiological Societies (FEMS) Young Scientists Meeting Grant; award winner "Best Poster Award 2011" from Soil Biology & Biochemistry

Mackie, K.A., Kassemeyer, H-H., Müller, T., Zikeli, S., Kandeler, E., 2011. Doctoral perspectives on phytoremediation of copper in vineyards. Deutschen Bodenkundlichen Gesellschaft 2011, Berlin, Germany.

Mackie, K.A., Schmidt, H-P., Müller, T., Kandeler, E., 2012. Biochar as a copper stabilizer in vineyards. Biochar Workshop, University of Wageningen, Wageningen, Netherlands.

Mackie, K.A., Schmidt, H-P., Müller, T., Kandeler, E., 2012. Biochar and compost as soil management options in vineyards. EuroSoil Conference 2012, Bari, Italy.

Mackie, K.A., Schmidt, H-P., Müller, T., Kandeler, E., 2013. Effects of biochar on soil microbial properties of a copper polluted vineyard. Deutschen Bodenkundlichen Gesellschaft 2013, Rostock, Germany.

Oral Presentations

Mackie, K.A., Schmidt, H-P., Kandeler, E., 2012. Biochar as a copper stabilizer in vineyards. Deutsche Bodenkundlichen Gesellschaft Kommission III Conference, University of Hohenheim, Stuttgart, Germany.

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