MASTER´S THESIS/ MASTERARBEIT

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Copper uptake in an organic vineyard treated with different soil amendments- investigated in *Elymus repens, Poa angustifolia, Trifolium repens* and *Lumbricus terrestris*

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Abstract

Copper-based fungicides are inevitable in organic viticulture, since there are no alternatives known. Their use since the 18th century has contaminated vineyards worldwide. Although copper is considered to be an essential nutrient, high concentrations are toxic, especially to shallow rooted plants, and the soil fauna. Actions have to be taken to restore contaminated vineyards and save those important ecosystems.

Natural, organic soil amendments, like compost and biochar, can stabilise the coppers mobility and therefore prevent the metal to be taken up by plants. An organically managed vineyard in the Wachau region has been divided into various plots, treated with special soil amendments, to test the efficiency of various mixtures. In the frame of the KUSTAW project the copper-uptake of two plant species, a common grass and the white clover, and of a soil organism of great importance, the earthworm, has been investigated via atomic absorption spectroscopy and the distribution of this heavy metal within the plant and animal tissue has been observed with EDX (energy dispersive x-ray spectrometer).

Generally, one can state, that the clover takes up more copper than the grass, but the levels in the earthworm are the highest. Comparing the different plots, the bioavailability of copper is the lowest in the biochar amended soils, but among the investigated organisms, there are no striking differences between the different treatment-types, which could underline the thesis of a lowered biological uptake.

Zusammenfassung

Derzeit ist der biologische Weinbau auf den Gebrauch von kupferhaltigen Fungiziden angewiesen, da keine ökologischen Alternativen zur Pilzbekämpfung bekannt sind. Ihre Verwendung seit dem 18. Jahrhundert führte weltweit zur Kontamination von Weingärten. Kupfer zählt zu den essentiellen Nährstoffen, doch in hohen Konzentrationen ist es besonders für flachwurzelnde Pflanzen und Bodentiere giftig. Um die wichtigen Lebensräume, die Weingärten bieten, zu schützen, müssen Wiederherstellungsmaßnahmen getroffen werden.

Natürliche, ökologische Bodenzugaben, wie Kompost und Holzkohle, können die Bioverfügbarkeit von Kupfer verringern, und somit deren Aufnahme durch Pflanzen und Tieren bremsen. Ein biologisch bewirtschafteter Weingarten in der Wachau wurde im Rahmen des KUSTAW-Projektes in mehrere Quadranten geteilt, und mit bestimmten Bodenzusätzen behandelt, um deren Wirksamkeit zu erkunden. Ziel dieser Masterarbeit ist die Kupferaufnahme durch zwei Begrünungspflanzen, dem Weißklee und einem Gras, und zusätzlich die Aufnahme durch den Regenwurm mittels Extraktion und Kupfergehaltsbestimmung mit Atomabsorptionsspektroskopie zu messen und die Verteilung des Kupfers mittels EDX (Energiedisperse-Röntgenstrahlung im Rasterelektronenmikroskop) innerhalb der Organismen zu erforschen.

Die Ergebnisse lassen darauf schließen, dass Klee mehr Kupfer aufnimmt, als Gras, doch die Werte des Regenwurms nicht übertrifft. Beim Vergleich der verschiedenen Boden-Additive, ist die Kupferverfügbarkeit in den mit Holzkohle bearbeiteten Quadranten am niedrigsten, doch der Kupfergehalt der Pflanzen und des Regenwurms zeigt keine signifikanten Unterschiede in den Quadranten, die mit Holzkohle oder Kompost behandelt wurden oder unbehandelt geblieben sind.

Introduction

The more precipitation a vineyard receives, the more fungicides have to be applied to save vine-stocks from fungal diseases, for example against the grapevine downy mildew, *Plasmopara viticola*, a globally- occurring plague. Due to the actual climate change, few but heavy rainfalls will impact agriculture, including vineyard management. Since improvements have been developed in every field of farming, viticulture had also extended and specialists found solutions to the most far-reaching problems, when they started applying copper based fungicides already in the 18th century. Over time this application was causing a heavy-metal pollution in the topsoils of vineyards (Vitanovic, 2012). Today copper is the only alternative to synthetic fungicides, making its usage inevitable for organic vineries.

Copper is an essential heavy metal. It is required for photosynthesis by chloroplasts of plants because it is a cofactor of plastocyanin, an essential electron carrier in the thylakoid lumen, and also animals need it for their metabolism and to maintain function of organisms. (Olivares and Uauy 1996; Aguirre and Pilon 2016). It is an integral part of antioxidant enzymes, particularly copper-zinc superoxide dismutase (Cu,Zn-SOD), and plays prominent roles in iron homeostasis. But at high concentrations copper is toxic, effecting microbes, plants, invertebrates and, via the food-net, possibly accumulates in vertebrates (Unrine et al., 2010). As ecosystem-engineers, earthworms are a key-species in healthy soils. With their burrowing they are shredding leaves and dead organic matter, renewing the soil, improving soil drainage. Thus, earthworms can be seen as an indicator species for fertile soils, contributing to a good soil quality (Edwards, 2004) The abundance of earthworms in vineyard soils is reduced by elevated copper levels (Paoletti et al., 1999). Also, the reproduction is limited (Spurgeon et al., 1994, Eijsackers et al., 2005). The grapevine itself is not affected, because its roots reach deep soil layers, which are not contaminated by copper (Besnard et al., 2001, Chopin et al., 2008, Provenzano et al., 2010). Instead shallow rooted plants, like most of the vineyard cover crops are directly influenced by the elevated copper concentrations in the topsoil (Wightwick et al., 2013). Furthermore, bacteria and fungi suffer under the presence of copper. Already low concentration of Cu fungicides may decrease their abundance in the uppermost soil layers (Fernández-Calviño et al., 2010; Ge and Zhang 2011).

Copper impact on soil organisms depends on the bioavailability of copper in the soil, which is controlled by heavy metal bioavailability, soil organic matter, ionic strength, the presence of iron and manganese oxides, and the pH (Arias et al., 2004, Devez et al., 2005, Soja et al., 2018). Moreover, the cation exchange capacity is said to be a crucial factor for the organic complexation of Cu, as described by Brun et al., (1998) and Vega et al., (2009).

Considering the bioavailability, Cu is most toxic in its mobile dissolved form as free Cu^{2+} ion. Sauvé et al., (1998) estimated that free Cu^{2+} activity between 10^{-7} and 10^{-4} µM Cu^{2+} correspond to a 50% reduction of crop yield, soil organisms and soil microbial processes.

There are several solutions to decrease the unwanted effects of copper. One option is to suppress the uptake of copper by organisms by decreasing its bioavailability. Three soil characteristics favour the stabilisation of copper: high cation-exchange capacity, high organic carbon values and alkaline pH (Soja et al., 2018). The soil amendment biochar can attribute to all these three soil features.

Biochar can enhance the soil structure, preventing it from erosion and increase the water holding capacity, reduce soil acidity through the exchanges of H+ because of its alkaline property, resulting in a raise of soil pH (Streubel et al., 2011, Pereira et al., 2015), improve the cation-exchange capacity (Mukherjee and Zimmerman, 2013) and the soil fertility. Additionally, it may increase the microbial biomass and it is said to overall immobilize copper and reduce its bioavailability to macro- and microorganisms by complexation of the heavy metal (Devez et al., 2005, Karami et al., 2011). Also, biochar is used for adsorbing pollutants, including copper (Namgay et al., 2010, Karami et al., 2011, Ippolito et al., 2012). Still there is a concern about not only withholding toxic substances, but also nutrients for plant uptake (Kuppusami et al., 2016). The agricultural amendment is made by pyrolysis to produce a lightweight, porous charcoal (Brewer and Brown, 2012; Glaser and Birk, 2012). The parent material can range from woods to straw or manure, while pyrolysis can differ in both duration and temperature, resulting in a great variety of end-products, which are all identified as biochar (Brewer and Brown, 2012).

For viticultural use another amendment is of great importance, namely compost. Compost is being seen to serve as a nutrient source, because it is increasing carbon, nitrogen and phosphorus in soils (Mays et al., 1973). Further it can stabilize soil aggregates and therefore also enhance the soil structure. In addition, it is stimulating microorganisms and boosting their turnover (Sizmur et al., 2011, Schultz et al., 2013). Navel and Martins (2014) even claim, that compost also reduces the Cu bioavailability, protecting microfauna and shallow rooted plants, and decreasing metal stress. According to Borchard et al. (2012), biochar-compost mixtures increase Cu sorption and reduce plant copper content. Therefore, the use of biochar, compost, and the combination of these two substances, for retaining copper in vineyard soils, will be analysed in this study.

The sampled vineyard is undergoing an experiment, called KUSTAW (German abbreviation for *Kupferstabilisierung in Weingartenböden*), the immobilization of copper in vineyard soils, a project coordinated by Professor Gerhard Soja, from the AIT (Austrian Institute of Technology) in cooperation with the BOKU Institute of Soil Research, LFZ Klosterneuburg (Viticultural School and Research Center) and the AGES, the Austrian Agency for Health and Food Safety, financed by the Federal Ministry of Agriculture, Forestry, Environment and Water Management. Aim of the project, which started 2014, is to minimize the bioavailability and ecotoxicological effects of the copper in vineyard soils. Therefore, eight focuses have been formed. The first was dealing mainly with research tasks, setting up the pilot plan, arranging vineyards for field-experiments, optimal soils for glass house trials and choosing right, high-quality soil-amendments. The second focus was on testing different application-techniques, to mix the additives well into the vineyard ground. Group 3 is dealing with copper immobilization in the soil by organic additives, group 4 focuses on the allocation of cupper in the grapevine plants, group 5 specifies into soil micro-organisms, group 6 reveals effects of soil additives on the grape, wine and phytopathology, group 7 worked on risk assessment and economic and legal aspects of the application of organic soil additives and finally the last focus, number 8, is on the coordination and publication of results. The experimental set-ups reach from lab analyses and pot experiments in the greenhouse to field experiments. Soils from regions of Burgenland, Lower Austria and Styria were selected for the experiments. Out of 3 application-methods two seem plausible (one distributing the additive homogenously, but only up to 7 cm depth, the other reaching up to 25 cm), the third focus is on the longevity of the amendments, and combined with package 4, the one where this thesis is added to, investigating which amendment is optimal for reducing copper-mobility (Soja et al., 2018)

Aim of this master thesis and research are the investigation of the effects of the listed amendments on two greening plants- the *Trifolium repens* and two grass species- *Elymus repens* and *Poa angustifolia*, and a soil organism, the *Lumbricus terrestris*. Focus is on the uptake and storage of Copper in different plant- and bodyparts.

Material & Methods

The Site

The organic vineyard is located south-east of Mautern an der Donau in Lower Austria, within the world heritage region of Wachau, which has been populated since ancient roman times. The site has a humid, for Austrian conditions warm continental climate, with oceanic influences and low annual rainfall rates, 515 mm in average.

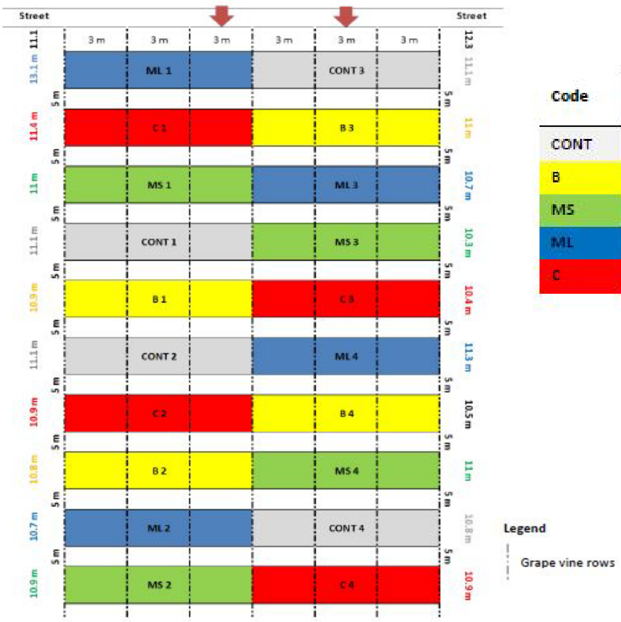
Geologically Mautern is shaped by young tertiary sediments, fluvial sediments and gravel and also loess deposits on the eastern slopes contribute to a fertile soil (Horvath 2015). Based on studies of the Ausrian Soil Classification, calcareous rigol soils are typical for the region around Mautern (BFW 2015). Rigol soils are highly cultivated soils with visible signs of mechanical turbation reaching from a depth of 40 cm up to 1 m. The investigated vineyard is even, with only a very slight slope towards north.

The vineyard has been managed organically since 1999, according to European Organic Agriculture Regulations, which prohibit the use of synthetic pesticides. Also, organic farming includes the greening of every second vineyard row. In this vineyard the REBENFIT seed mixture is used, which consists of *Camelina sativa*, ribwort (*Plantago lanceolata*), cornflower (*Centaurea cyanus*), crimson clover (*Trifolium incarnatum*), white clover (*Trifolium repens*) and yellow clover (*Medicago lupulina*). Due to its legume content, REBENFIT brings nitrogen to the soil and does not need fertilizers. A special feature is the ability to adapt to the water availability- in rainy seasons the mixture forms protein-rich organic matter, which is easily converted into nutrient-rich humus later. In summer the mulch prevents the soil from overheating and from drying out (http. I).

The grapevine grown there is *Grüner Veltliner*. From 2014 on the site has been treated with *Fungoran® progress*, a fungicide containing copper hydroxide. Before *Cuprocin®*, *Kocide® Opti* and *Cueva®*, have been used, all fungicides based on copper hydroxide. The fungicide has been applied 8-10 times a year in the period between May and August from 2014 on, whilst the years before only 4-5 applications have been noted. The average amount of kg copper/ha rose from 1,7 to 2,4 in the past few years.

Figure 1. Google Maps image of the vineyard

Regarding the size, the test site is 18 m wide, approximately 155 m long and covers a total area of 2790 m². It is reachable by a street, which is bordering the northern site of the vineyard. The rest is surrounded by other vineyards You can see the vineyard marked blue in Figure 1. Also, the difference between untreated and greened corridors is visible in the illustration. The site includes seven rows of vine stocks with a distance of approximately 3 m between the rows, which is visible in Figure 2.



Code	Additive	Ratio	Quantity [kg m^{-2} dw]	Refers to quantity [t ha^{-1} dw]
CONT	Control – no additive	-	-	-
B	Wood Biochar	-	4	40
MS	Biochar : Compost	1:1	4	40
ML	Biochar : Compost	1:1	10	100
C	Compost	-	4	40

Figure 2. Map of the different treatments on the experimental field of KUSTAW in Mautern. 5 treatments including a control plot with 4 replicates each.

Each of the 20 plots is 9 m wide and 10 m long. Between the plots there is a one-meter distance to avoid errors by mixing. The plots are reaching over three rows, but samples were only collected in two of the six rows, in those, marked with a red arrow (Fig. 2).

Treatments

The vineyard soil was treated with four different additives, each produced by the company *Sonnerde*. Together with the control plots each method has four replicates, all together there are 20 experimental plots on the site. The amendments were compost, wood chip biochar, compost to biochar 1:1, 4 kg/m² (Mix 4) and compost to biochar 1:1, 10 kg/m² (Mix 10). Variant Mix 10 is an extreme, and would not be applied usually, because according to the Austrian Compost Ordinance only a maximum of 40 t/ha compost are allowed for application on agricultural fields, which is surpassed by treatment Mix 10, with its 100 tons/ha. The additives were added in April 2015. All the samples were taken in 2017, two years after the amendment application.

Biochar

The biochars used in the field consist of wood chip and wheat husk, both produced by the company Sonnerde in Riedlingsdorf 7422, Austria. Wood chip biochar was made out of softwood with a pyrolysis temperature of 480°C. Wheat husk biochar was pyrolysed at 450°C.

Compost

Compost used in the experiments was also produced by the company *Sonnerde*. The quality refers to the Austrian classification A+, which conforms to Austrian organic standards and enables usage in organic agriculture.

Investigated Plants

Two common cover crops were collected and investigated: The white clover *Trifolium repens*, which also occurs in the seed mixture, and a grass species, first thought to be *Lolium perenne*, which is wide spread species, treadle-resistant and persistent. Later it turned out to be two different species: *Elymus repens* and *Poa angustifolia*. Anatomically grass consists of roots, stems, leaves and bloom, while white clover additionally has rhizomes and root-nodules.

Earthworms as indicator species

In this study earthworms represent the soil fauna. *Lumbricus* is a keystone species in the Edaphon, constructing habitats for many different species, shredding detritus and enrichening the soil with oxygen. These little ecosystem engineers live underground in tunnels, they burrow down to a depth of 2,5 metres. They are most active in spring and autumn. At too hot, and dry or too cold conditions during the summertime and winter period, they are inactive, hiding in the lower soil layers (Faber et al., 2013). These soil organisms serve ideally as bioindicators, because they are sampled easily, are immediately identifiable, have a wide distribution range, they strongly accumulate pollutants and their biological and ecological characteristics are already well known (Kizilkaya, 2004).

Lumbricus terrestris is a species in the phylum Annelida. Systematically earthworms, as all *Oligochaetae,* count to the *Clitellata*. Oligochates are full hermaphrodites. *Lumbrici* have a hydrostatical skeleton, a fluid-filled coelom, with which they maintain their structure. Annelids move through contraction of their giant circumferential and longitudinal muscles. Also muscles line the gut, and their actions move the digesting food toward the worm's anus, which is located at the last segment, also called the pygidium.

Chloragocytes are a special cell-type, which occur in and around the intestinal epithelium of annelid worms. The cells store fat and nitrogen products and help in the synthesis of glycogen. In addition, they play a central role in the immune system. It is suspected that earthworms store toxic products like heavy metals in chloragog cells (Stürzenbaum et al., 1998). In elevated concentrations fungicides, especially copper residues from copper sulfate, are highly toxic to earthworms and result in a decrease of earthworm abundance (Paoletti 1999).

Spermophorus clitellus

Additionally, to the above listed three species, that have been sampled, one organism has to be mentioned. During the dry summer months, when the soil is barely covered by vegetation, one can see a remarkable amount of ground squirrel (Spermaphorus clitellus) holes in the vineyard. Surveys show, that European ground squirrels occur frequently in organic or extensively treated vineyards (Hoffmann, 2005). These mammals are able to rapidly adapt to their, mostly anthropogenically changing environment (Hoffmann, 2002). A stable and water-permeable ground with not too dense vegetation and some bush-high plants, like the grapevine, to hide the ground squirrels from predators, results in a perfect habitat for the S*permophorus clitellus*.

After having set focus in the overall research field, the appropriate laboratory practices could be chosen.

Laboratory Analysis

All samples, except the earthworms, were collected on 2017.05.18. Colleagues of the AGES collected soil samples, Professor Soja took plant samples.

Soil was collected from 0-10 and 10-20 cm depth and then poured together. From each plot four samples were taken and pooled together in one plastic bag. The samples were then sieved and dried. When the laboratory work started in August, from each plot two soil samples were taken as follows: 20 grams of soil mixed with 20 ml of ammonium nitrate and shaken for 60 minutes before filtrating. These extracts where then taken to the AIT (Austrian Institute of Technology), where the copper-content was measured with atomic absorption spectroscopy (AAS). Aqua regia digests with the same soil samples were prepared and carried out by the AGES.

For the AAS a Perkin Elmer Atomic Absoprtion Spectrometer, PinAAcle 900T, was used This machine automatically dilutes the samples ten times. Further, the soil, root, rhizome, all the earthworm and some leaf samples had to be diluted another ten times, to be in the measurable range of the AAS. The spectrometer has two functions: flame absorption and furnace combustion. All the samples were measured with the furnace method.

Plants were collected by Professor Soja, on the 18th of May 2017, following the pattern of the former collections: The upper- above ground and lower- underground plant parts have been collected in separate bags. The stems and leaves were first cut with a scissor and put into a paper bag (see Fig. 3). Then at the same spot the roots were dug out with a dagger and put into a closable plastic bag. The samples were then frozen and stored in the AIT. The bags were taken over and transported to CIUS at the University of Vienna in August 2017.

Figure 3. Plant samples

Sample Preparation

First half of the plant material was gently washed in a sieve under running ionized water to remove coarse material and soil. Then the different plant parts were separated. The grass was sectioned into roots, stems and leaves, while the clover was separated into roots, rhizomes, stems, leaves and a few blossoms. The plants were washed again, put into petri-dishes and dried in the heating chamber Heraeus Labin, Type B 5050 E at 60°C for a minimum 24 hours. Then the dried samples were stored in paper bags until digestion.

For capturing earthworms, the still moist, early morning hours are ideal. The earthworms were collected in October. A mustard powder mixture, after Fründ and Jordan (2004), was prepared with 60 g of yellow mustard powder from the company Eder Gewürze to half a litre of water, left to soak overnight and then filled up to 10 L of water at the site. First a 50 cm² plot was marked and floated with the 10 L mustard solution. After five minutes earthworms began to appear and were captured by hand, and put into a glass jar. The glass jar tops were pierced, to allow the worms to breath. At home the worms were stored for six days in the fridge and on the balcony, where the temperature never exceeded 15°C. The animals were washed every two days and slowly some of the remaining gut content left. Then most of them were killed under hotboiled water and put into the heating chamber to dry. Three individuals were frozen with liquid nitrogen for cryo-fixation. Earthworm sampling happened seven times between September 30 and October 18, with 4-6 trials each time, from which only 1-3 plots brought success. Some plots had been tried to be sampled up to 3 times, still earthworms were not found in every plot.

For the digestion preferably 1 g of plant and *Lumbricus* tissue was weighed and mixed with 10 ml HNO_3. After one hour 4 ml of perchloric acid were added and the mixtures were left overnight. The next day the samples were heated on Severin KP 1091 heating plates, boiled and digested. The last step was to fill the extracts up with distilled water to a volume of 50 ml and filter through a Ø 150 mm Macherey-Nagel filter paper, which you can see in the illustration of the experimental set-up of the filtration process in Figure 4. If there was not enough dried plant or earthworm material, only 0,5 or less grams of material were weighed and the amount of acids was scaled down to 5 ml nitric and 2 ml perchloric acid.

Figure 4. Filtration

The extracts were then taken to AIT for Atomic Absorption Spectroscopy.

Microscopy

To have an overview of our plant organs, pictures were taken with the Olympus BX41 stereo microscope (Fig. 6) and the Olympus light microscope at CIUS and proceeded with the ZOOM program.

Also stubs for further EDAX-measurements were prepared under the light microscope.

Figure 5. Grass roots, leaves and stems prepared on “stubs”.

Figure 5 shows a table with stubs, where plant tissue sticks to. Before the analysis the samples had to be coated with carbon.

For the zoological analyses two earthworms were cut open and some of the chloragogenous tissue was dissected and placed on stubs.

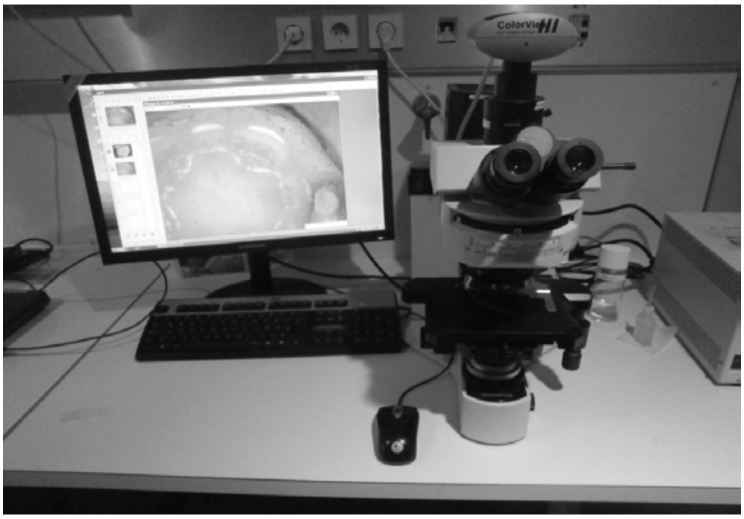


Figure 6. Olympus light microscope

On the computer screen visible in Figure 6 a clover section has been analysed when the photograph was taken. With the program ZOOM scale-bars were added.

EDAX Energy Dispersive X-ray Spectrometer / Electron Backscatter Diffraction

With EDAX Raster Electron Microscope (Fig. 7) one can determine the mass percentage and atomic composition of elements in plant or animal tissue in the Scanning Electron Microscope (SEM). Three to six sections per sample were observed with 1-6 replicates. Up to seven stubs fit into the operating chamber of this device, wich are inserted through the door visible in the front, of the picture below.

Figure 7. EDAX-Device in the CIUS Lab

Laboratory analysis

The copper content (Cu mg/kg) of the plant parts and the *Lumbrici*, which derived from the AAS measurements, were calculated including the weighed mass, the dilution, the volume and the copper values. Afterwards the results were illustrated with graphs in excel.

Statistics

Statistical analysis was carried out with the Statistica 7.1 program of the Statsoft software. Results of plant parts and the earthworm were calculated separately. First a test of normal distribution among the mean values of the different treatments has been run with the Kolmorogov-Smirnov test. If there was no significance, further analysis could be done, starting with a oneway ANOVA, which tests the variance for significance ($p < 0{,}05$). Further results could be visualised with the post hoc and Tukey test.

Results

First, we looked at the soil parameters, how much copper the soil in the different plots contained, and how much was bioavailable for the plants and earthworms, which could be found out with ammonium nitrate extracts, which were analysed through atomic absorption spectoscopy. Aqua regia digests of the AGES informed about the total amount of copper in the soils compared with ammonium nitrate digests, which revealed the bioavailability of the heavy metal for vineyard organisms.

Abbreviations: CT..control, CP..compost, BC..biochar, M4..Mix BC+CP á 4 kg, M10..Mix BC+CP á 10 kg/m²

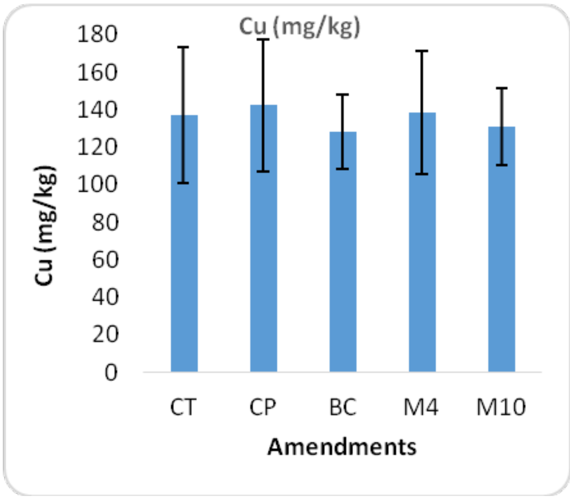


Figure 8. Mean Aqua regia digest values, each soil treatment pooled. (n=4)

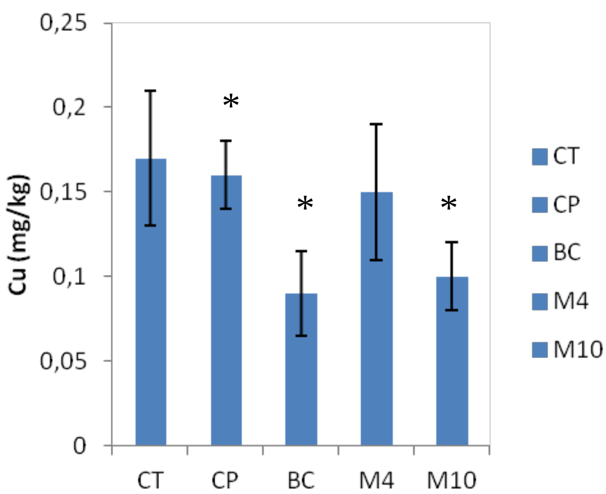


Figure 9. Mean bioavailable copper in soils with different treatments, values pooled. *marking significant differences

Table 1. Soil ammon nitrate extracts

Plots	Cu (mg/kg)	Plots	Cu (mg/kg)
CT 1	1,77	M 4 1	1,13
CT 2	1,184	M 4 2	1,69
CT 3	1,06	M 4 3	0,99
CT 4	1,97	M 4 4	2,01
CP 1	1,5	M 10 1	0,97
CP 2	1,6	M 10 2	1,19
CP 3	1,37	M 10 3	0,69
CP 4	1,73	M 10 4	1,17
BC 1	1,11		
BC 2	0,87		
BC 3	0,53		
BC 4	0,95		

Abbreviations: CT..control, CP..compost, BC..biochar, M4..Mix BC+CP á 4 kg, M10..Mix BC+CP á 10 kg/m²

Figure 8, the result of the Aqua regia digests, demonstrates that soils of all plots are more or less equally contaminated with copper. As one can see in Table 1 and Figure 9, about soil ammonium nitrate extracts, the copper concentration is the highest in the control plots. After that the compost amended soils have the second largest concentration, followed by the Mix 4 soils. As soon as the biochar concentration is higher, namely pure BC- and M 10 applications, the copper concentration sinks. The biochar plots have the least copper bioavailable, almost half the concentration of the control soils and results of those plots are also significantly different from the compost plots. In addition, the M 10 plots results are significantly lower compared to the control plots.

After having seen an overview of the theoretical copper availability, we wanted to know, how much copper has been taken up by the plants and the *Lumbricus*.

Grass species

For better understanding of the plants reactions to copper, the morphology and histology of the investigated species were studied first under the light- and stereo-microscope. Then the structures were recognized during the EDAX measurements, and spots of special interest were tested on element composition. In this chapter the microscopic and REM pictures are compared, before finally AAS results are revealed.

Figure 10. Grass type 1. *Poa angustifolia* habitus

Figure 11. Grass type 2. *Elymus repens* habitus

Poa angustifolia- Narrow-leaved meadow grass

Poa is a genus of about 500 species of grasses, native to the temperate regions of both hemispheres. Common names include meadow-grass, bluegrass and speargrass. As a common pasture plant, this species is also used by grazing livestock. Figure 10. demonstrates it's habitus. It is clearly visible that the roots are approximately as long as the leaves, and constructed very densely.

Elymus repens is a common perennial species native to most of Europe. It is often considered a weed, although it prevents erosion and can be used as forage.

Completely evolved, *Elymus* (Fig. 11) grows greater and higher than *Poa*. Its natural habitat are agricultural fields and gardens. *Elymus* leaves, compared to *Poa,* are broader, dryer and wear fine hairs. The Poa leaves are much softer and also the colour is slightly different, *Poa* appearing greener, and *Elymus* rather blueish-green. Figure 11 shows, that *Elymus* roots are relatively short and do not form a dense structure.

Grass roots

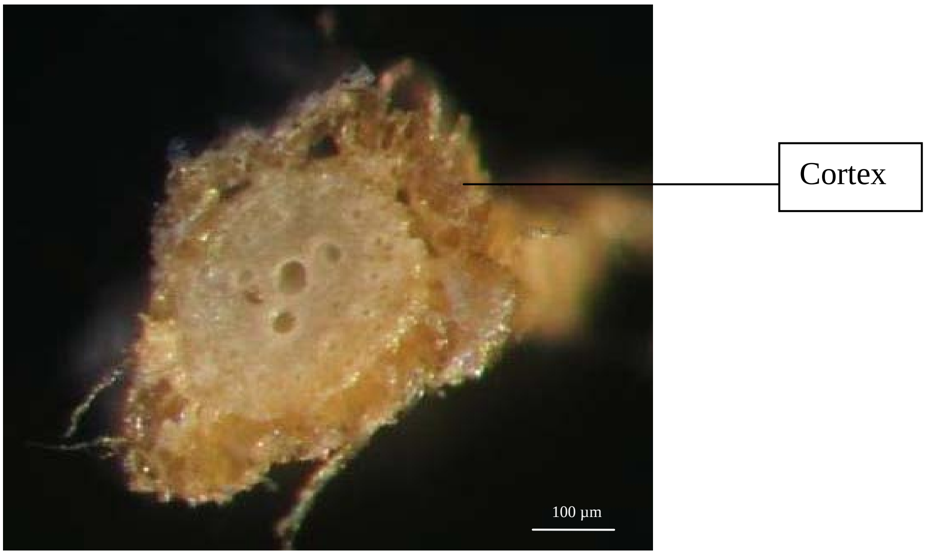


Figure 12. Grass root cross section under stereo microscope

Figure 12 and Figure 13 show that these grass roots have relatively wide cortices, with appendices, which could contain mycorrhiza.

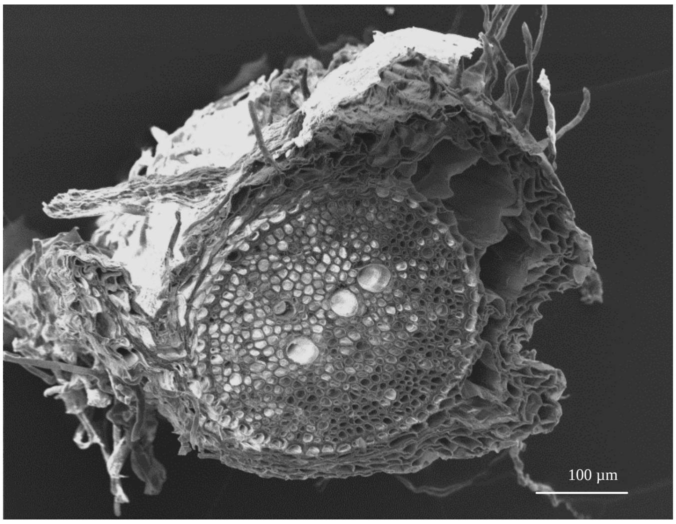


Figure 13. Grass root cross section with SEM

Grass roots have an anchor-type function to stabilise the plant. Another function of roots is nutrient-uptake, which is the most important role of these organs. Sometimes massive structures of mycorrhiza are amending roots to take up essential nutrients from the environment.

Stems

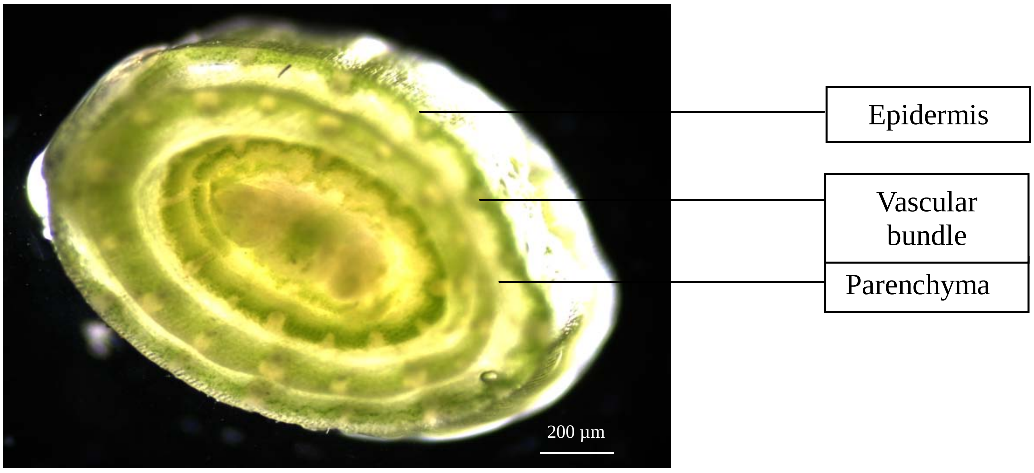


Figure 14. Grass stem cross section under light microscope

Hollow grass stems are wrapped around by leaves, causing a layered effect in sections, visible in Figure 14. The function of stems is to transport water and nutrients to the leaves. Again, the *Elymus* stems were longer and dryer, thus harder, whereas the *Poa* stems were soft and moist.

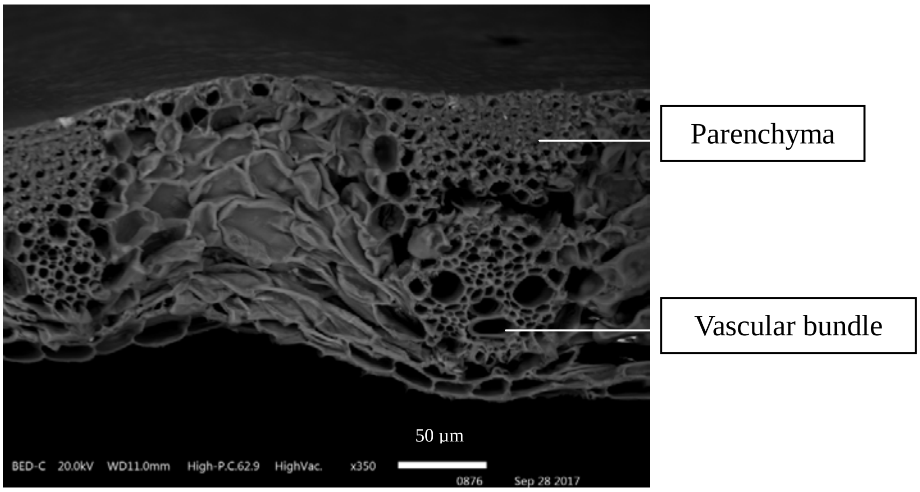


Figure 15. Grass stem cross section with EDAX, visualising vascular bundle in detail

The grass stem is hollow at the internodes with vascular bundles arranged in several rings. The epidermis is single layered, composed of compactly-arranged tabular cells with cuticularised outer walls (Fig. 15).

In the ground tissue sclerenchyma cells occur as patches in the subepidermal region. They are not arranged in a continuous band, but are interrupted by chloroplast- containing parenchyma cells here and there. The latter occur at the regions where stomata are present on the epidermis. The remaining portion of the ground tissue is parenchymatous, the cells having conspicuous intercellular spaces. The central portion is hollow.

Vascular bundles occur in two series. One ring of small bundles remains associated with subepidermal sclerenchyma, so that they appear to be embedded in sclerenchymatous patches. The second ring placed lower is composed of larger bundles. The bundles are collateral and closed. They usually remain surrounded by sclerenchymatous bundle sheath.

Grass Leaves

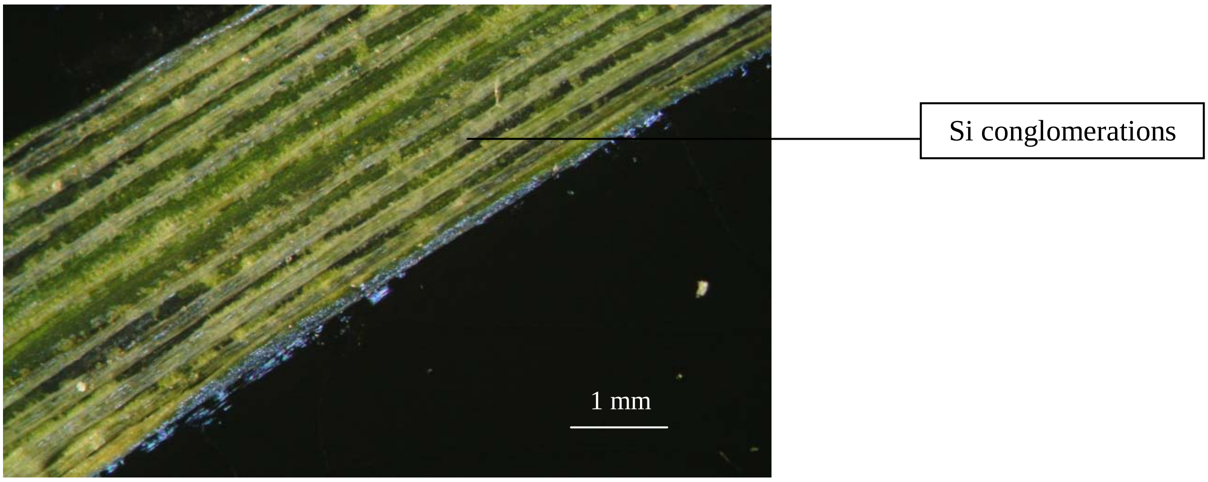


Figure 16. Abaxial side of a grass leaf with parallel veins under light microscope

In general, leaves store nutrients and keep gas-exchange with the environment. Gases exit leaves via stomata. Most of the photosynthesis takes place in the leaves, but all green plant parts, including the stems, are involved. Monocots, like *Poa* and *Elymus*, have parallel veins in the leaves (Fig. 16).

Grass leaves contain a high amount of silica, which functions as a defence against herbivores and insects (Massey et al., 2006), and can be even seen in Figure 18 as hairy-looking features. Neither grass inflorescense, nor clover blossoms are described in this work, because the samples contained very few. Clover blossoms were analysed with AAS, but the sample size (n=3) was too low to include them in the statistics. Nevertheless, a clover blossom sample was analysed in EDAX.

EDAX Results

The following results are from control plots.

Grass

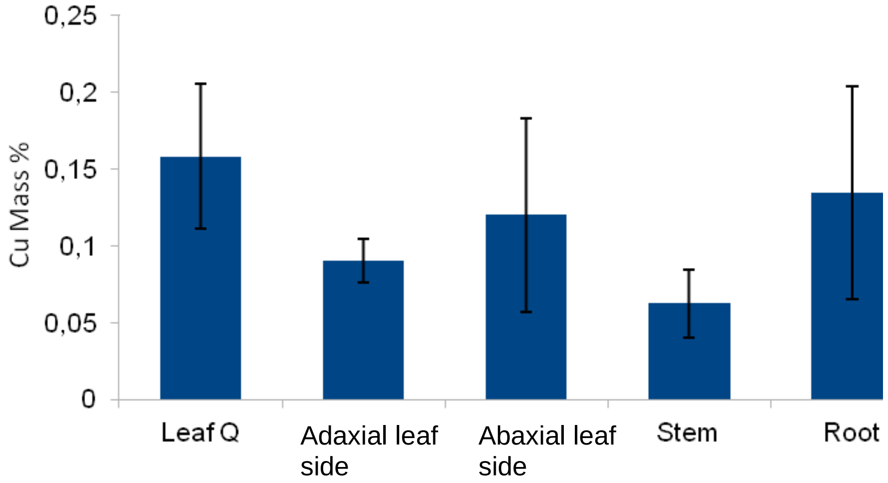


Figure 17. EDAX results for Grass tissue

Leaf Q n= 15, leaf upside n= 6, leaf downside n= 6, stem n=11, root n=7

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

The EDAX element analysis results of the grass tissue (Fig. 17.) show, that the leaf cross section had the highest copper mass percentage, followed by the roots, and the leaf downside. The upside of the leaf had lower copper values, and the stem the lowest.

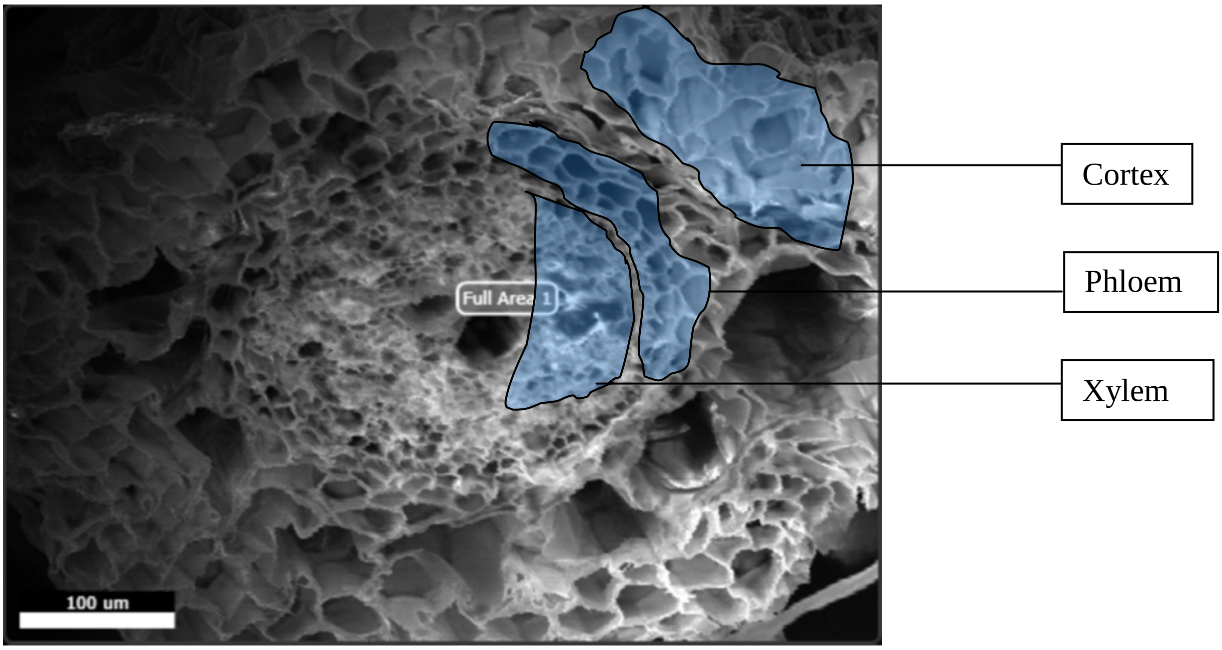


Figure 18. Grass root section under SEM, in TEAM viewer program for EDAX Analysis. Blue fields are freedraws, which can be analysed separately with the TEAM program.

The TEAM program, which was used to select spots for EDAX, has the option to draw sections, and investigate them separately. Figure 18 shows a grass root under REM, with selected areas of the cortex, phloem and xylem. The copper mass% is indeed varying, decreasing toward the inner parts of the root. While the cortex in this case has 0,15 copper-mass% and the phloem 0,12 mass%, the xylem has a value of 0,06 mass% (Appendix).

<u>AAS Results</u>

To continue the ongoing field studies of the AIT, Atomic Absorption Spectroscopy has been carried out not just on the soil, but also the plants, and soil-living organisms.

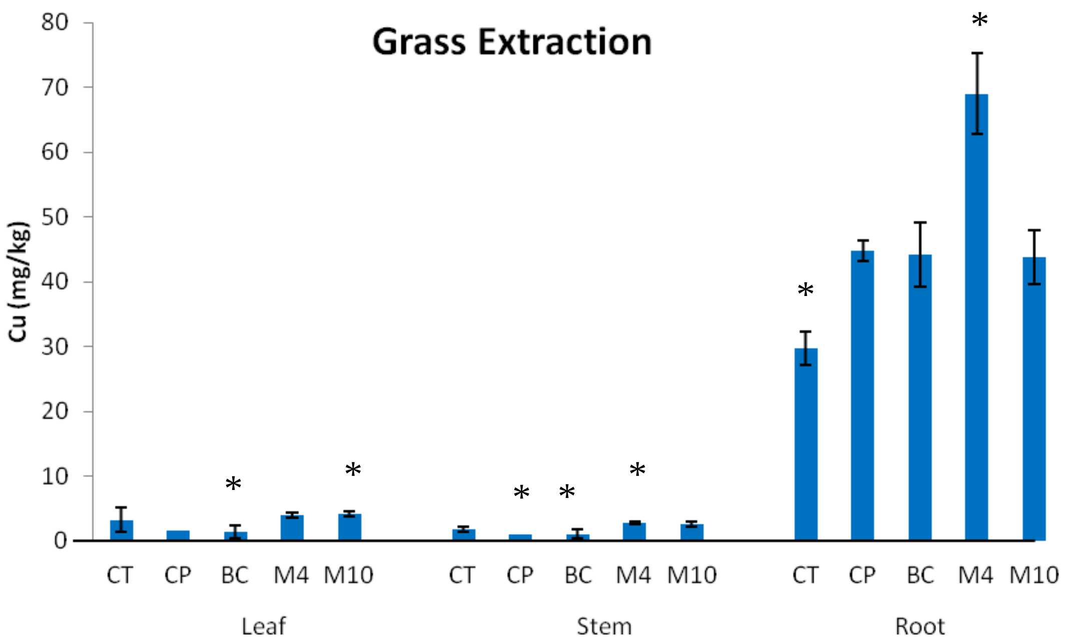


Figure 19. Copper in Grass. Results of AAS measurements. n=4 replicates/each, minus 4 outliers (Leaf CP, Stem CT, Stem M10, Root CP). * marking significances. <u>Abbreviations</u>: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Figure 19 illustrates the copper content (mg/kg) of the examined plant parts of the Grass.

Roots have remarkably higher copper concentrations, compared to the other two organs. The leaves and stems have less than 5 mg/kg copper content in average, whereas the roots concentration start around 30 mg/kg.

Comparing the plots, the M4 plots results in the grass lead with the highest copper concentrations. In the leaves and stems the M10 follows with the second highest concentrations and grass samples growing on biochar amended soils have the least copper uptake. Considering the roots also the M4 is leading, but in contrary to the other parts, the second largest concentrations are present in the BC roots. Here the control grass has the least uptake.

Table 2. Tukey Test on grass leaves

Tukey HSD Test; Variable Cu (mg/kg)				
Treatment	Cu (mg/kg) Mean	1	2	3
BC	1,38	****		
CP	1,55	****	****	
CT	2,98	****	****	****
M4	3,97		****	****
M10	4,20			****

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

You see in Table 2 the results of the Tukey Test, which reveals significant differences among the various treatments. These results show, that the leaves contained significantly more copper in the 10 kg/m² biochar-compost mixture. On the other hand, these two components, the biochar and the compost alone have significantly lower copper-uptake values in the grass leaves.

Table 3. Descriptive statistics on grass leaves

Table of descriptive statistics			
Treatment	Cu (mg/kg) Mean		Cu (mg/kg) SD
CT	3,0	±	1,0
CP	1,6	±	0,2
BC	1,4	±	0,7
M4	4,0	±	1,5
M10	4,2	±	0,7

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Table 3 visualises the descriptive statistics values on grass leaves, with the M10 plots reaching the highest averages around 4,2 mg Cu/kg and the BC plots having the lowest results with 1,4 mg/kg.

Table 4. Tukey Test on grass stems

Tukey HSD Test; Variable Cu (mg/kg)			
Treatment	Cu (mg/kg)	1	2
BC	1,02	****	
CP	1,04	****	
CT	1,77	****	****
M10	2,52	****	****
M4	2,80		****

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Table 4. proves the significant variances in the copper uptake of grass stems.

As one can see in comparison to the leaves the stems show only a marginal difference concerning the 4 kg/m² mixture and on the other side the biochar amended soil, which again shows the lowest copper values.

Table 5. Descriptive statistics on grass stems

Table of descriptive statistics			
Treatment	Cu (mg/kg) Mean		SD
CT	1,8	±	0,3
CP	1,0	±	0,6
BC	1,0	±	0,8
M4	2,8	±	0,7
M10	2,5	±	0,0

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Table 5. verifies that the copper is distributed evenly within the differently treated plots, which is resulting in a very low standard deviation.

Table 6. Duncan test on grass roots

Duncan Test			
Treatment	Cu (mg/kg)	1	2
CT	36,71	****	
CP	41,36	****	
M10	43,83	****	****
BC	44,17	****	****
M4	68,97		****

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Table 6. contains the results of the Duncan test, which had to be carried out after the Tukey-test, and did not show any obvious differences. Here the control and compost plots show lower results, than the M10 and biochar plots, which are not differing significantly, whereas the 4 kg/m² mixture shows significantly higher values.

Table 7. Descriptive statistics on grass roots

Table of descriptive statistics		
Treatment	Cu (mg/kg)	SD
CT	3,67	± 1,98
CP	4,14	± 0,72
BC	4,42	± 1,24
M4	6,90	± 1,65
M10	4,38	± 1,60

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Comparing the results of the grass roots with the values of the other plant parts, you can see that here we have greater values with higher deviations (Table 7). Summarizing this chapter, one can see through EDX measurements, that the copper mass% is shrinking towards the central cylinder, and the AAS results show clearly, that underground plant parts have higher Cu levels in the grass.

Trifolium repens

Figure 20. *Trifolium repens* habitus

The genum *Trifolium* consists of about 300 species. Clovers are perennial legumes within the family *Fabaceae*, often used as forage for animal grazing and pasture. While the genus has a cosmopolitan distribution, it is most frequent in the temperate northern hemisphere.

Trifolium repens, the white clover has a longer life-cycle, is a persistent grower and can reseed even under grazing and harsh conditions. Clovers are said to improve the soil, especially due to their nitrogen-fixing ability, which is why they are frequently used in seed-mixtures.

Opposite to the red clover, which is growing tall and upright, the white clover grows shorter in height and spreads laterally, as one can see in Figure 20. Red clover in contrary to the white, has elongated, sharp leaves.

Leaves

The leaves of *Trifolium repens* are trifoliolate, elliptic to egg-shaped and usually with light or dark markings. Quatrefoiled leaves occur rarely- then forming a symbol, called shamrock.

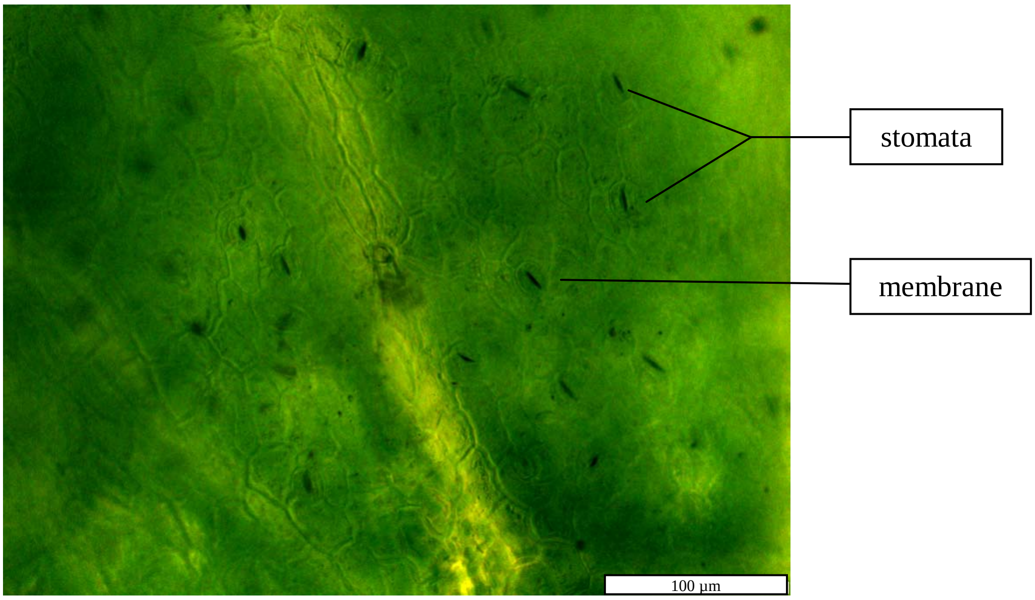


Figure 21. Abaxial leafside of the clover with visible cell membranes and stomata under stereo microscope

Figure 21. illustrates the visible stomata on the abaxial side of the leaf, where the gaseous exchange happens. Anatomically leaves are built with xylem and phloem in the vascular bundles, spongy mesophyll cells surrounding that, palisade mesophyll cells on the top side, the epidermis on both the top and the bottom, and finally enclosed by the cuticula. Compared to grass leaves, clover has softer, moister leaves and a stronger characteristic scent, which is caused by so-called green leaf volatiles, which arise under stress conditions.

Stems

This part of the plant is responsible of the water and nutrient transport towards the leaves. Nonetheless *Trifolium repens* stems function as stolons, so white clover often forms mats, with the stems creeping as much as 18 cm per year, and rooting at the nodes

Figure 22. Clover stem cross section under light microscope

Since clover is a dicot, sclerenchymatic cells of the stem form a continuous, radial band (Fig. 22).

Rhizomes

Rhizomes are anatomically equal to stems, but growing underground, thus seldom producing photosynthesis. The chlorophyll is missing or is very rare, painting the rhizomes light green. Also the structure of a rhizome is thicker and more stable, than a stem. The following two figures (Fig. 23, 24) visualise the difference in size and structure of a rhizome and a root.

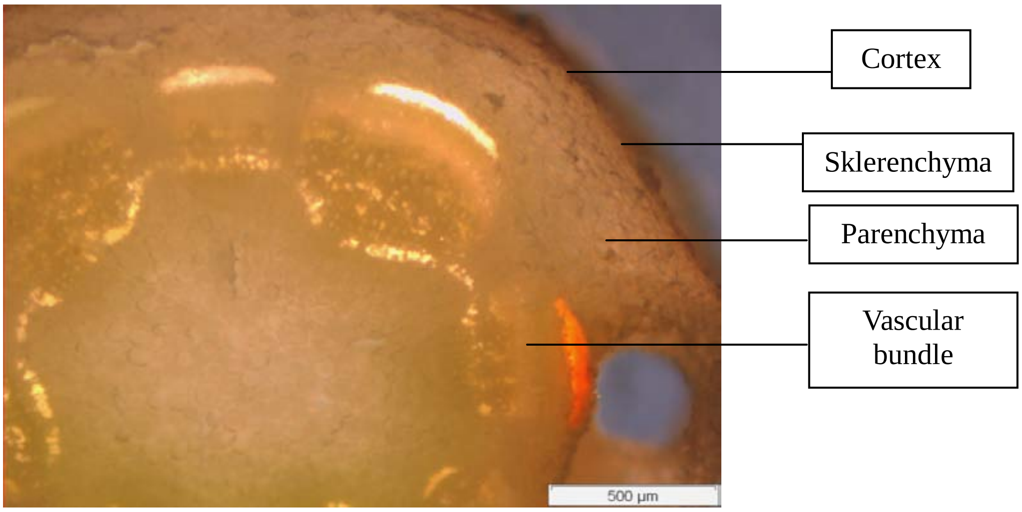


Figure 23. Clover rhizome cross section under light microscope

Roots

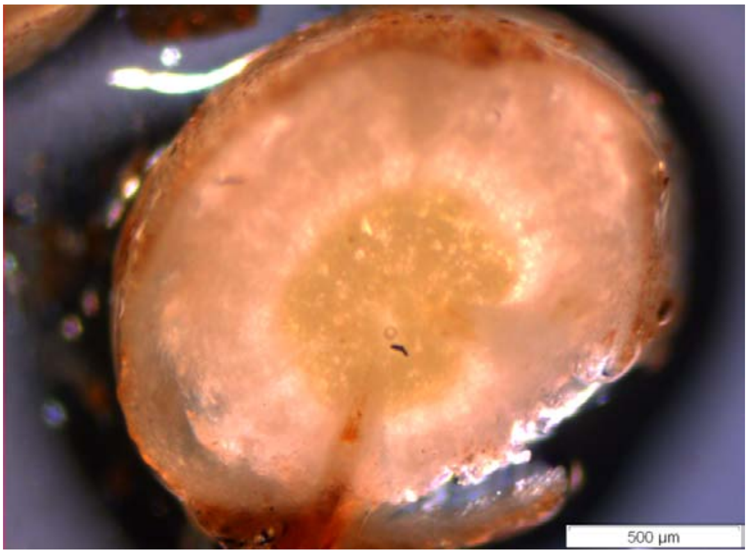


Figure 24. Clover root section under light microscope

In contrary to grass roots, clovers have a not as prominent cortex and the vascular bundles show a different pattern (Fig. 25).

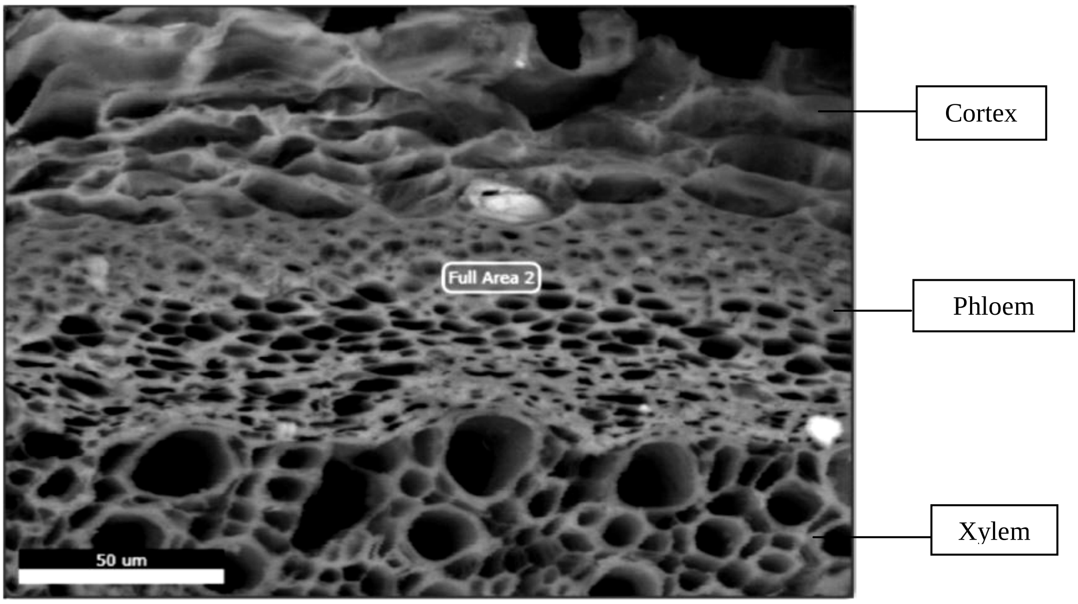


Figure 25. Clover root cross section in EDAX

Live maps

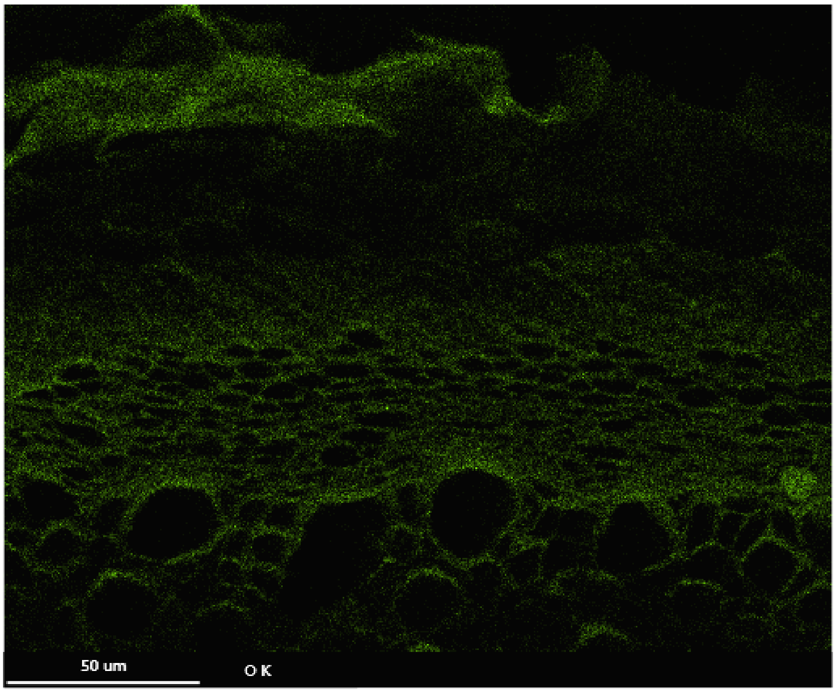


Figure 26. Clover root oxygen (mass%) distribution in EDX, Live map.

Live mapping is a tool, which helps finding points of interests, if one focuses on a special element. Compared to the more frequent element oxygen, here representing 34 mass% of the clover root, which`s distribution shows the pattern of the plant structure (Fig. 26), copper is represented much lower values (here Cu mass% at 0,12), and therefore not showing a recognizable pattern, which can be seen in Figure 27.

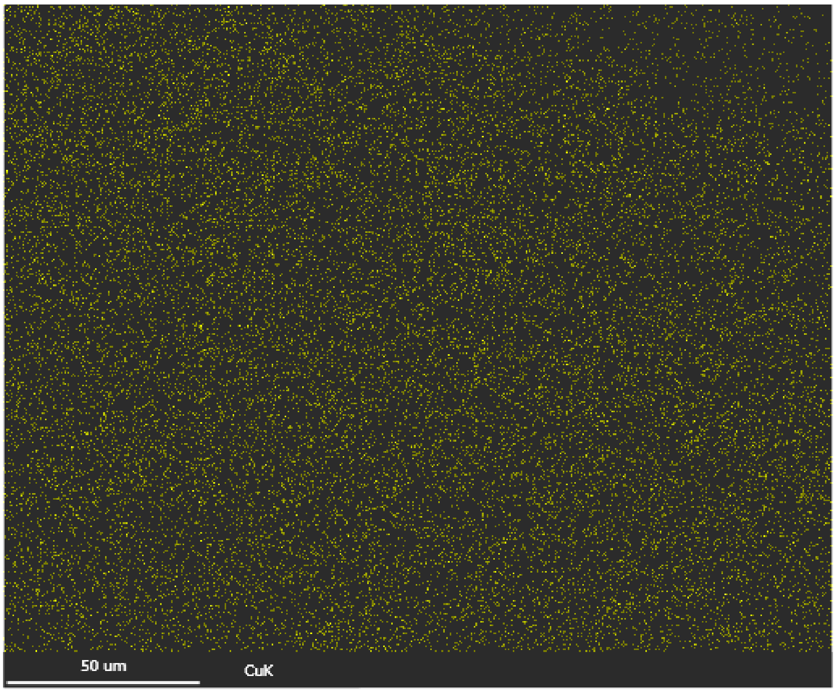


Figure 27. Distribution of Cu (mass%) in clover root analysed with EDX, Live map

Nodules

While grass is having a fungal symbiont, the mycorrhiza, clovers have an additional support, the root nodules. The root forms nodules, which associates with symbiotic nitrogen-fixing bacteria. In the centre of Figure 28 you can see the section of a nodule. On top there are two root sections visible, whereas Figure 29 shows a nodule under Scanning Electron Microscope.

Figure 28. Clover roots and nodules on a stub, prepared for EDAX measurements

Anatomically nodules are little, round shaped appendices on the fine root hairs. Their diameter exceeds the root hairs diameter multiple times.

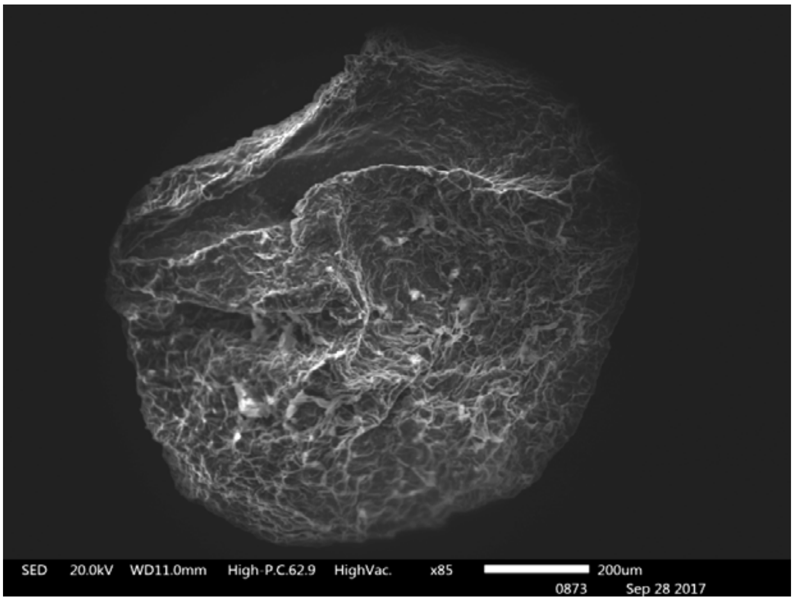


Figure 29. *Trifolium repens* root nodule in EDAX

EDX-Results

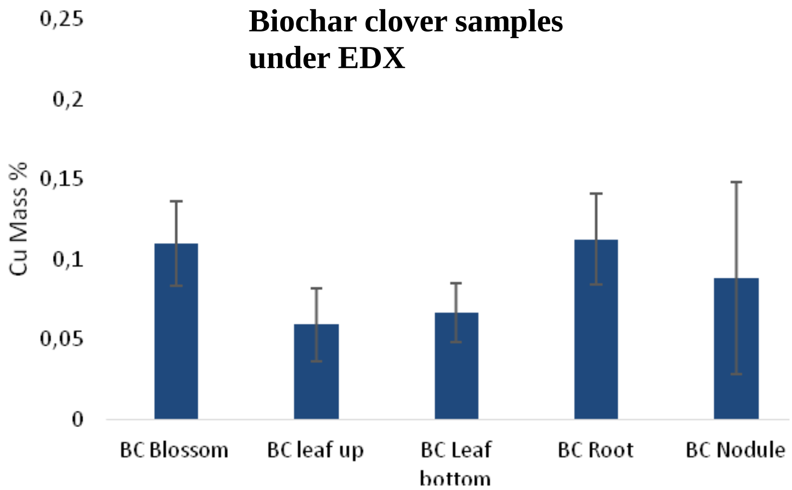


Figure 30. EDAX results for clover tissue from plots treated with biochar
Blossom n= 3, nodule n= 16, root n= 12, leaf bottom m=3, leaf upside n= 9

Clover results from the BC plots (Fig. 30) show high copper content in roots, blossoms and nodules and less in the leaves. Also here the upsides of the leaves had lower copper values in average and all in all the copper mass percentages are lower than the mass% of grass. Additionally the control plots results (Fig. 31) show that rhizomes have more or less equal copper values as roots. Similar to the grass results, stems show the least copper uptake. Compared to the BC plots the control plots clover nodules had much higher medium copper mass percentages- 0,15 compared to 0,08 %. The roots had equally 0,11 %, but the leaves show a great difference with almost twice the amount occurring in the control plots.

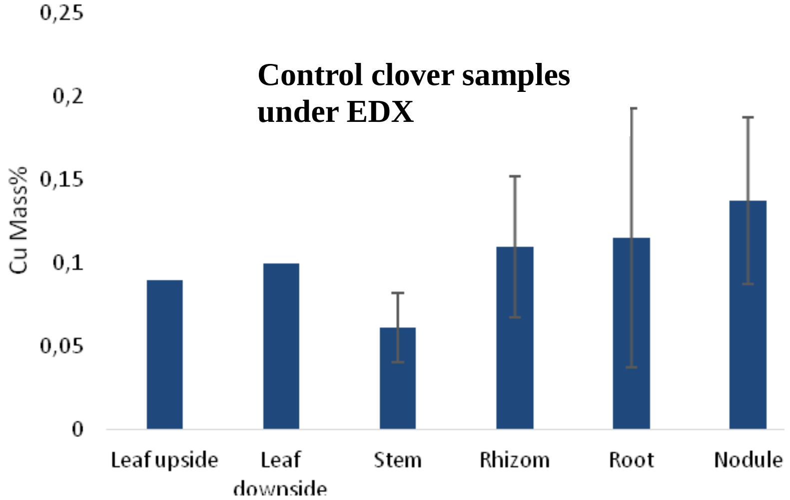


Figure 31. EDAX results for clover tissue from control plots

Leaf upside n= 3, leaf downside n= 1, stem n= 18, root n= 13, nodule n= 6 (1 outlier)

AAS-Results

To see a quantitative analysis, atomic absorption results follow.

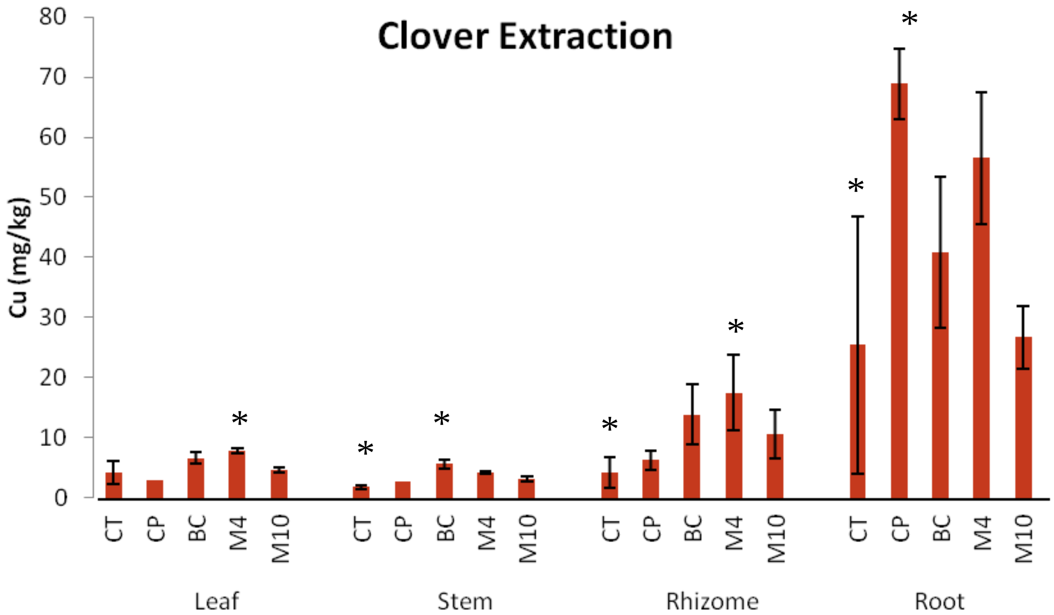


Figure 32. Copper in clover, pooled results from different treatments, measured with AAS. Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4kg/m², M10..BC+CP Mix á 10 kg/m²

Compared to the grass, the clover has additionally rhizomes, a fourth plant compartment, which had been analysed. As one can see in the Figure 32, the lower plant parts, covered by soil, have elevated copper concentrations. If we compare the plots regarding Figure 32, each plant part shows different results. Looking at the leaves, and the stems, the biochar clover has the highest copper concentration, whilst the rhizomes took up the most copper in the Mix 4 fields and the roots in the compost amended plots. In the lower parts you find the least copper concentrations in the control plots. On the other hand in the green plant parts the lowest concentrations are in the compost plots.

Table 8. Tukey Test on clover leaves

Tukey HSD Test; Variable Cu (mg/kg)			
Treatment	Cu(mg/kg)	1	2
CP	2,94	****	
CT	4,32	****	****
M10	4,72	****	****
BC	5,21	****	****
M4	7,80		****

Table 8 shows the variance of the different treatments on the uptake of copper by the leaves. Here the Mixture 4 values are significantly higher, and the control plots significantly lower than the other three treatments.. The control plots, biochar plots and mix 10 plots are indifferent concerning the significance.

Table 9. Descriptive statistics on clover leaves

Table of descriptive statistics			
Treatment	Cu(mg/kg)		SD
CT	4,3	±	1,9
CP	2,9	±	0,0
BC	5,2	±	3,0
M4	7,8	±	0,5
M10	4,7	±	0,4

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

As you can see in Table 9. the mean copper values reach from 2,9 mg/kg copper with a deviation of zero, in the Compost plots, to 7,8 mg/kg occurring in the Mixture 4 plots. A high range, but the low deviations show accuracy.

Table 10. Tukey Test on clover stems

Tukey HSD Test; Variable Cu (mg/kg)					
Treatment	Cu(mg/kg)	1	2	3	4
CT	1,84	****			
CP	2,48	****	****		
M10	3,10		****		
M4	4,23			****	
BC	5,62				****

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Table 10 shows a wide range of results. The Control plot´s values are significantly lower, than the Mixtures 10, 4 and the biochar values. The Control plot´s results do not differ significantly from the Compost values. The Compost values are also similar to the Mixture 10 results, but not the Mixture 4 and biochar values. The Mixture 4 values range high, but not as high as the biochar values, which are significantly the highest.

Table 11. Descriptive statistics on clover stem

Table of descriptive statistics			
Treatment	Cu(mg/kg)	±	SD
CT	1,8	±	0,5
CP	2,5	±	0,1
BC	5,6	±	0,7
M4	4,2	±	0,2
M10	3,1	±	0,4

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Table 11 contains the mean values and standard deviations of the clover stems descriptive statistics. Again the deviations are low.

Table 12. Tukey Test on clover rhizomes

Tukey HSD Test; Variable Cu (mg/kg)				
Treatment	Cu(mg/kg)	1	2	3
CT	4,69	****		
CP	6,31	****	****	
M10	10,59	****	****	****
BC	13,88		****	****
M4	17,48			****

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Regarding the Tukey Test´s results on the clover rhizomes (Tab. 12), one can say that the values of the Mixture 10 plots, which are significantly higher in other plant parts, show no significant differences to other plots in the roots. The Control plot`s results are significantly lower than the biochar and the Mixture 4 values. And the Mixture 4 plot´s values are significantly higher, than the Control plot´s and the Compost´s values.

Table 13. Descriptive statistics on clover rhizomes

Table of descriptive statistics			
Treatment	Cu(mg/kg)		SD
CT	4,7	±	2,7
CP	6,3	±	1,6
BC	13,9	±	5,0
M4	17,5	±	6,3
M10	10,6	±	4,1

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Table 13. shows the descriptive statistics results. The values are greater than in the leaves and stems and also the standard deviations vary in a higher range.

Table 14. Tukey Test on clover roots

Tukey HSD Test; Variable Cu (mg/kg)				
Treatment	Cu(mg/kg)	1	2	3
CT	25,3	****		
M10	26,74	****	****	
BC	40,91	****	****	****
M4	56,51		****	****
CP	68,85			****

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

As one can see in Table 14 the Tukey Test on *Trifolium* root results, the biochar amended plots show no significance towards the other treatments. The Control plots have significantly lower results than the Mixture 4 and Compost plots, and the Compost plot values are significantly superior than the Mixture 10 plot´s values.

Table 15. Descriptive statistics on clover roots

Table of descriptive statistics			
Treatment	Cu(mg/kg)		SD
CT	25,5	±	21,4
CP	68,9	±	7,2
BC	40,9	±	12,6
M4	56,5	±	11,0
M10	26,7	±	5,2

This last Table is about the descriptive statistics on clover roots, which show even higher values than the results seen in the rhizomes (Tab. 15).

The EDAX measurements prooved, that copper is evenly distributed within the plant tissue, in average slightly more present in the underground plant parts. Comparison of clover derived from biochar plots versus control plots showed, that copper is slightly less frequent in the biochar-amended clover leaves.

Lumbricus terrestris

As earthworms are soil organisms, they have direct contact to toxic substances, in this case fungicides. Several studies have revealed that an extraordinarily high copper concentration in the soil has a negative effect on the reproduction of earthworms (Belotti 1998). Values above

30 g copper/kg in the soil lead to an increased earthworm mortality. Also the *Lumbricus terrestris* is a keystone species, an ecosystem engineer and thus of great importance for the environment. By shredding down organic matter and renewing the soil day by day, enrichening it with oxygen, earthworms are essential for healthy soils. The worms form 12% of the edaphon (Paoletti et al.,1998) and are able to reproduce throughout the year, with a maximum in autumn and spring. At too hot, and dry or too cold conditions during the summertime and winter period, they are inactive, seeking shelter in the lower soil layers (Faber et al., 2013).

Lumbricus terrestris is a species in the phylum *Annelida*e. Systematically earthworms, as all *Oligochaetae*, count to the *Clitellata*. The clitellum is a thickened glandular and non-segmented section of the body wall in earthworms and leeches, that secretes a viscid sac in which the eggs are deposited. It is located near the anterior end of the body Oligochates are hermaphrodites.

Figure 33. Earthworms under light microscope

The parapodium, typical for polychates, is missing. *Lumbricus terrestris* has up to 180 segments, increasing with age. The cuticula is slightly pigmented at the upside, and the earthworms shape is round but pointed at the anterior edge. The first body segment features the mouth and the overhanging prostomium, which is used to chemically sense the surroundings. All organs can be found in the first half of the worm (Fig. 33). The growth is happening at the posterior end of the worm, and all the following new segments are comparted the same way. Each segment has its own pair of excretory nephridia and a nerve plexus. The nerve cord connects the nervous system of the segments. Also important to mention is the hydrostatical skeleton, fluid-filled coelom, with which the earthworms maintain their structure. Annelids move through contraction of their giant circumferential and longitudinal muscles. Muscles line also the gut, and their actions move the digesting food toward the worm's anus, which is located at the last segment, also called the pygidium (Storch & Welsch, 2009). You can see the habitus of an earthworm in Figure 35, and the illustration of a section, Figure 36.

The intestinal organs are surrounded by chloragogenous tissue, which has been investigated for metal-uptake in previous research.

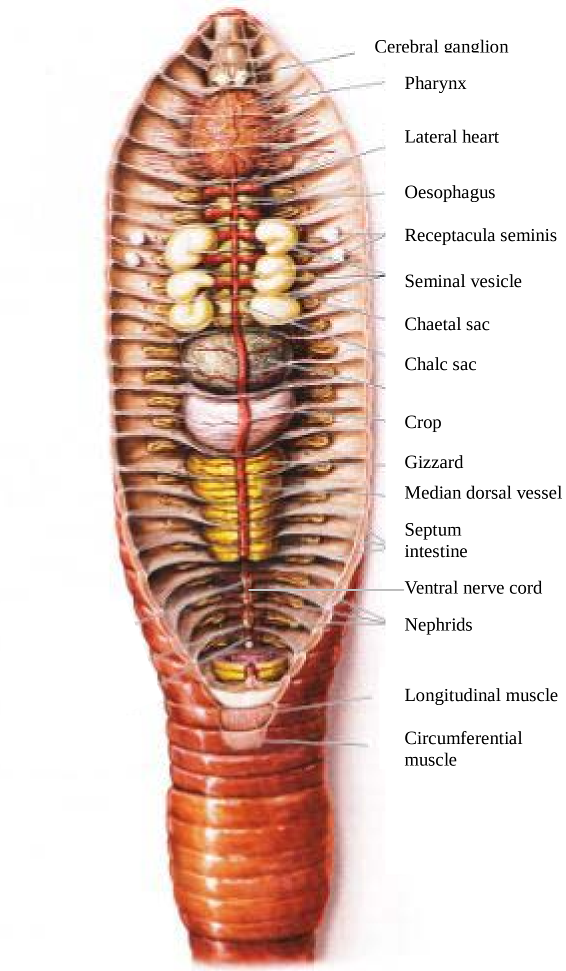


Figure 34. Earthworm section (Storch & Welsch, 2009 p.189)

Figure 35. *Lumbricus terrestris* cross section on stub

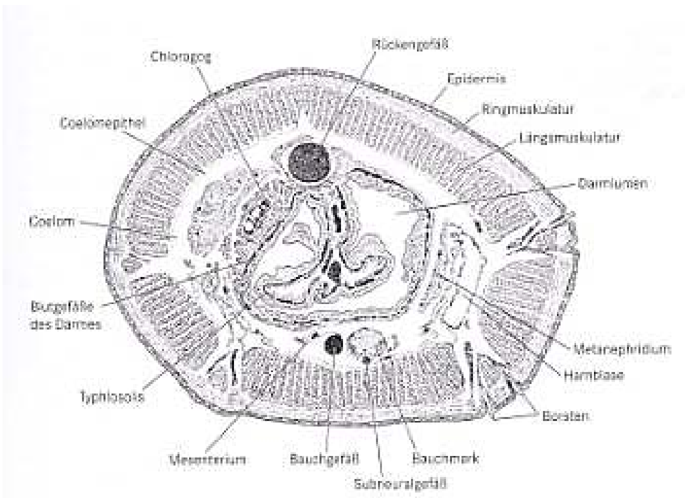


Figure 36. Earthworm cross section

By dissecting an earthworm, one can identify the chloragosoma as a yellow slimy substance, which is spread all over the stub on Figure 35. For comparison, see the anatomy, Figure 36. The cross section shows the muscles very well, but the inside parts of the worm are destroyed. Different muscle types and the epidermis are recognizable, also some setae were found (Fig. 38).

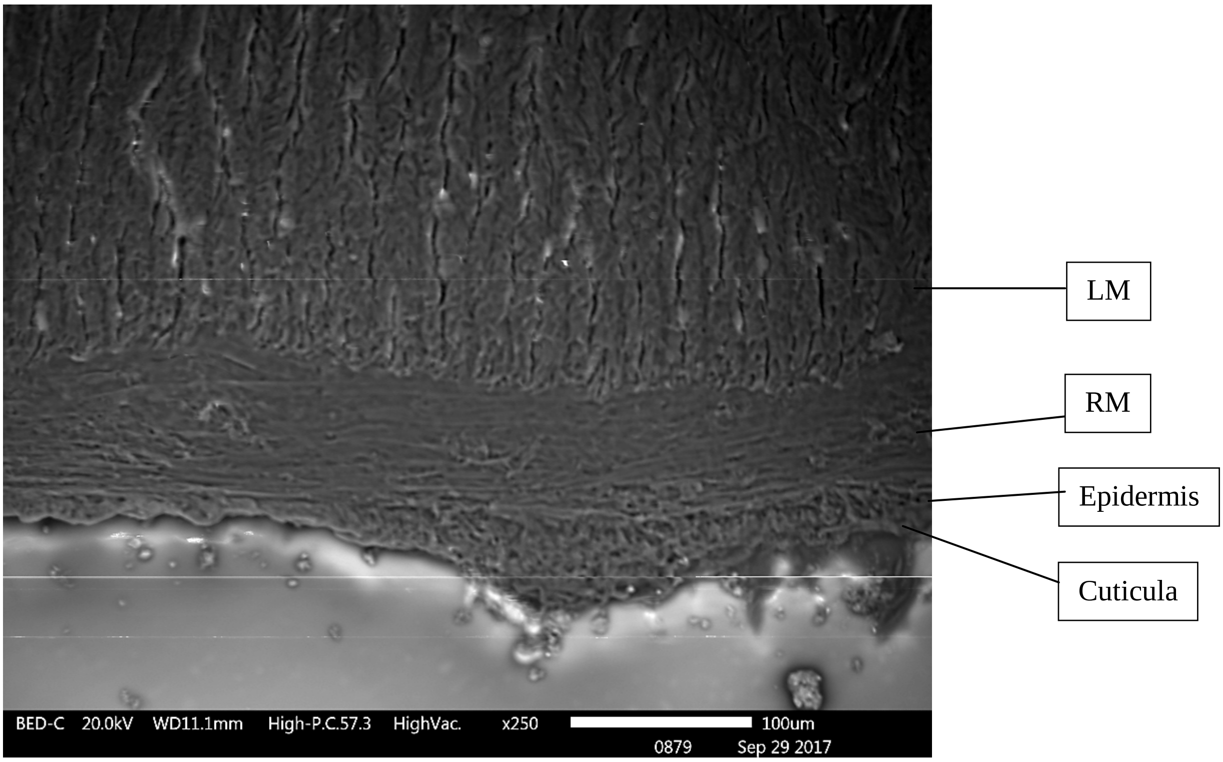


Figure 37. *Lumbricus terrestris* cuticula, circumferential(CM)- and longitudinal muscles (LM) under REM.

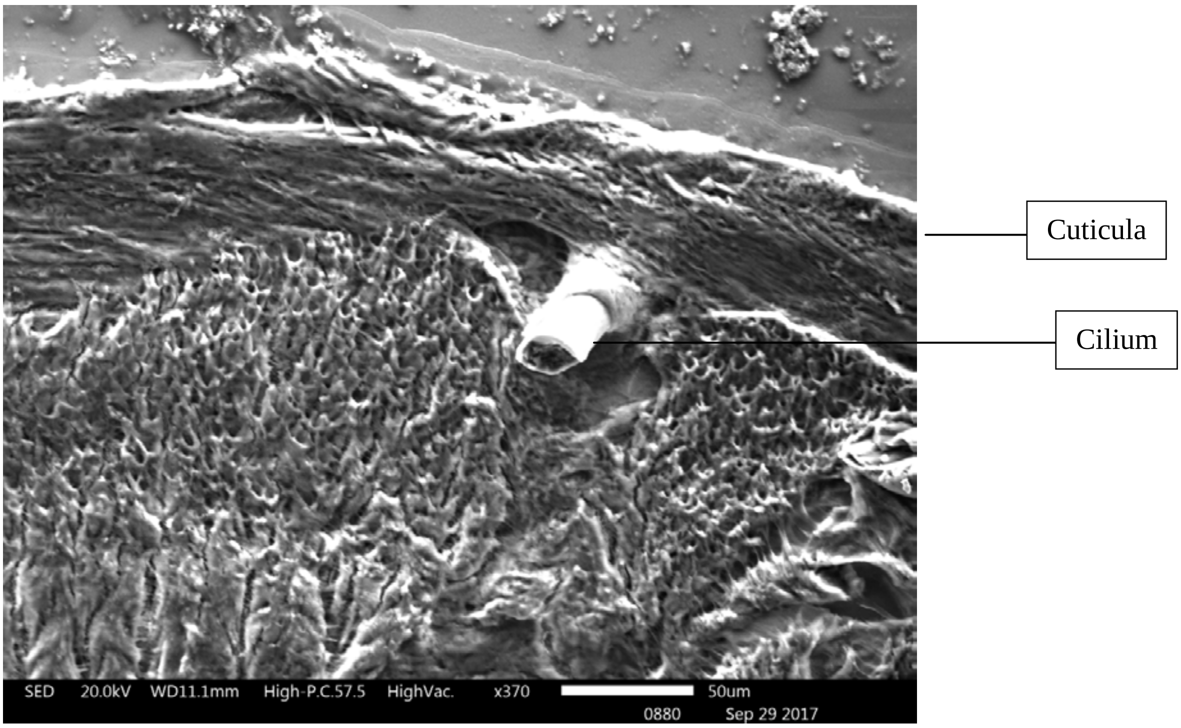


Figure 38. Earthworm cuticula with cilium under SEM

The earthworm consists mainly of muscle tissue, as one can see in the SEM picture above. It has a thick cuticula with setae (also called cilium) for sensing (figure 38). Underneath the cuticula one can see the epidermis, the circular muscle and the giant longitudinal muscles.

Lumbricus terrestris results

EDAX-Results

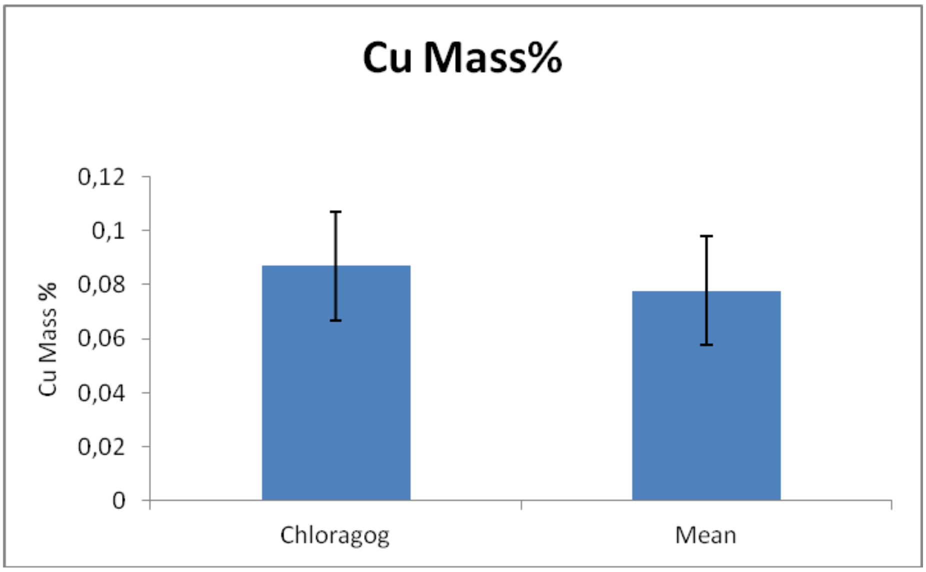


Figure 39. EDAX results for earthworm tissue . Sample derived from M4 plot

Chloragog n= 18, Mean n= 13

The earthworm analysis of the chloragogogenous tissue (Fig. 39) shows a higher copper mass percentage, than the mean tissue (Fig. 39). These two categories could be distinguished.

AAS-Results

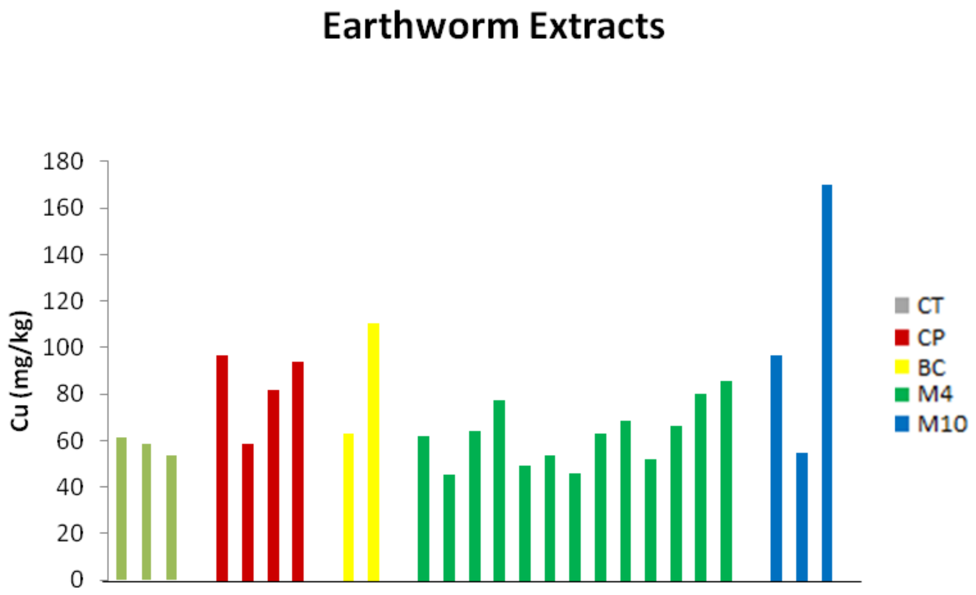


Figure 40. Earthworm copper concentration (mg/kg). Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Figure 40 confirms, that copper contents can vary much between single individuals. Each treatment is represented of at least 2 plots, which were successfully sampled. One blot in most cases represents a portion of earthworms, varying in size and individual number.

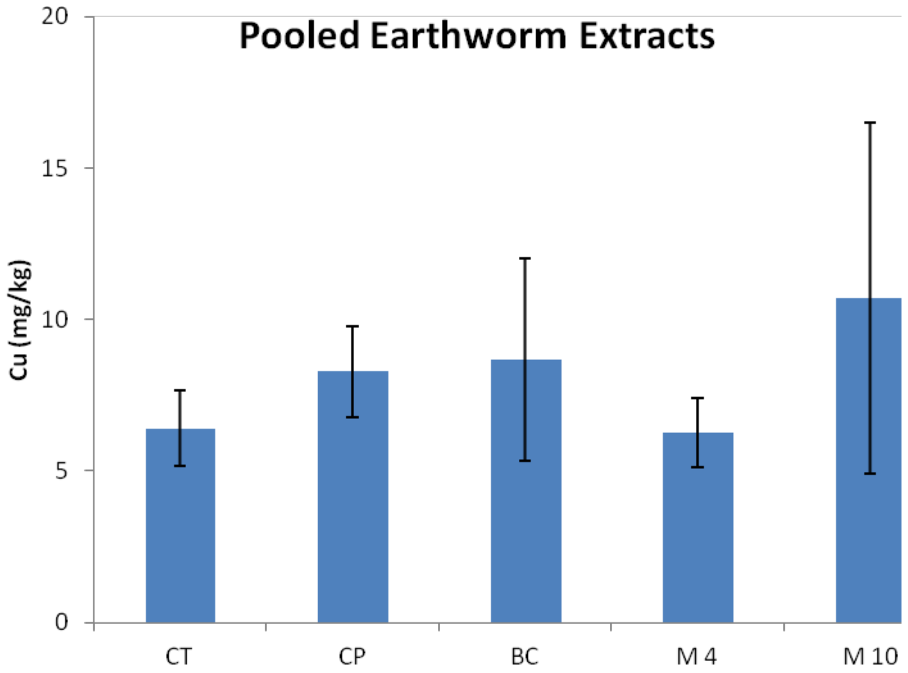


Figure 41. Pooled results of *Lumbricus* copper uptake. Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m². CT n=3, CP n=4, BC n=2, M4 n=13, M10 n=3

The results of the AAS of the earthworm samples (Figure 42.) show, that animals from the control plot were the least contaminated by copper. Also the Mix 4 plot results show low mean values, followed by the compost treatments. The mix 10 plot has the highest concentration with 10,7 mg Cu/kg.

Table 16. Tukey Test on earthworms

Tukey HSD Test; Variable Cu (mg/kg)			
Treatments	Cu(mg/kg)	1	2
CT	57,8246	****	****
M4	62,5966	****	
CP	82,7846	****	****
M10	10,71336	****	****
BC	86,5954		****

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

As one can see in Table 15. the earthworm values show no direct significance. The Biochar plots have a marginally higher values towards the other values and the M4 plots earthworms show marginally lower values.

Table 17. Descriptive statistics on earthworm values

Descriptive statistics			
Treatment	Cu(mg/kg)		SD
M4	62,60	±	13,07
CP	82,78	±	17,46
CT	57,82	±	3,88
BC	86,60	±	33,46
M10	107,13	±	58,53

Abbreviations: CT..control, CP..compost, BC..biochar, M4..BC+CP Mix 1:1 á 4 kg/m², M10..BC+CP Mix á 10 kg/m²

Where the values are higher, the standard deviation is also bigger (Fig. 17). From all organisms the earthworms show the highest copper uptake (Fig. 40).

Discussion

The results are interpreted based on literature and discussed in regard of the introduction. Considering the positive effects of biochar, mentioned in the introductive chapter, it is expected that plants of the plots treated with biochar have a lower Cu uptake, than the control plots (due to lowered bioavailability (Devez et al., 2005, Karami et al., 2011)). Also compost should restrict heavy metal uptake (Navel and Martins 2014).

Copper availability in the different plots

Regarding the copper values of the soils of different treatments, it is visible, that the bioavailability of copper sinks, if biochar is applied purely, or mixtures in high amounts (Fig. 8 and Fig .9). This occurrence may be explained by the biochars copper-stabilizing features: forming complexes with the copper, improving the cation-exchange capacity and nonetheless raising the pH. Streubel et al., 2011 reached an increase of soil pH of 0,1-0,9 units in all soil types, with the application of 9,8; 19,5; and 39,0 mg biochar/ha. They compared the use of four different biochars. The strongest pH increasing results were achieved by applying digested fiber origin generated biochar, followed by switchgrass and douglas-fir bark and the least effective was the wood pellets derived biochar. The pH in the Mautern vineyard indeed varies between the plots, the BC amended and Mix 10 containing plots having a pH above 7.4, whereas the other plots have a lower pH around 7.39 (Appendix). Here biochar made of softwood has been applied, which is good for plants, because plants need slightly acidic soil conditions for nutrient uptake. In disregard of the plants, other amendment sources could have been used, possibly resulting in a more effective elevation of the soil pH of this vineyard.

The compost did not lower the coppers bioavailability, which is underlined by the significant different to the biochar, which in contrast did lower the availability. That results of the amon nitrate extraction being about hundred times lower than the Aqua regia values, leads to the conclusion, that only up to one percent of the total copper in these soils is available. A figure matching Soja`s (2018) result of <1% bioavailability of Cu found in the investigated gardens. A toxicity level for copper is hard to determine, because the bioavailability depends on the acidity. According to the Environmental Federal Office of Germany plants take up copper only in acidic soils, which is why they consider the copper treshold-level for plants to be at 1.300 mg Cu/kg soil (http. II.)

Copper in grass

Before results of various measurements will be discussed, morphological specialities of the grass will be summarized. Anatomically the two grass species looked healthy. Very few samples had signs of a slight chlorosis. Arbuscular mycorrhiza is said to support the plants in many ways. Liao et al. (2003) found that elevated Cu concentrations show a reduction in beneficial mycorrhizal associations. Indeed mycorrhiza consists of fungi, which are known to be sensitive to copper. Under the microscope, grass roots had a wide coat potentially of mycorrhiza. Maybe these plants adapted to copper-based fungicides as a frequently recurring stress, and probably got resistant to its´ use.

Using the Jeol EDAX-Raster Electron Microscope one can discover structures, which are not visible with the stereo microscope. We found granules in grass tissue, which were then measured, to find out the elements composition, and indeed we found out with my coordinator, that those longitudinal granules are silica aggregates (Figure attached in Appendix). The EDAX measurements resulted in higher copper mass% values in leaves, second in roots and the least in found in stems. As leaves are known to store nutrients, obviously copper formed complexes and accumulated in leaves. Stems function as transporters, not as storage, so here the low copper content is with good reason. Roots are permanently surrounded by soil, which in these cases is slightly contaminated with copper. In addition besides leaves, also roots serve as nutrient storers. Thus the fact, that copper was found in roots is not surprising. The high standard deviation may arise because different spots, may reflect different organs, so the variance within the pooled investigations is relatively high.

Analysing the copper-uptake diagram, the outcome of the AAS process, on page 22 (Fig. 19.), the first thing that strikes the eye is the great difference between the uptake of underground and aboveground plant parts. Results of grass leaves unveil that the mixtures are contraproductive regarding the copper immobilization: Biochar and compost were more efficient to retain copper, when used pure. The two unmixed treatment-plots had significantly lower values than the mixtures. Mix 4 achieved the highest levels in all three plant parts. One ould interpret that the biochar : compost in legal masses is mobilising copper for plant-uptake.

Also interesting is the result of control plots` grass-root-uptake, which is the least among all treatments. Maybe the copper in this vineyard is already in its most passive form, and amendments are just mobilising it, to make complexes and get taken up by plants.

Copper in clover

The first anatomical property, which distinguishes white clover from red clover is not the blossoms colour, but the habit. *Trifolium repens*, the white clover is multiplying laterally through underground stems called rhizomes, forming mats. Typical for clover is that under the binocular and the stereo microscopes, but also with blank eyes root nodules are visible. Nitrogen fixating plants, typically *Fabaceae* are nodulating. Their size did not vary much, neighter their colour, reaching from orange-red/red to a pale brown and yellow. Recent studies show, that in nodules copper binds to cell walls (Sánchez-Pardo et al., 2012). Nodules were hard to cut into a nice shape, that remained for EDAX, because they dehydrated and contracted, causing uneven surfaces, not ideal for radiation, thus Sánchez-Pardos` statement, which refers to white lupin and soybean plant nodules, could not be confirmed to the white clover. *Trifolium* roots are several dozens of times smaller than the rhizomes, which is why roots had to be treated with special care at the extraction, with scaled down acids used. For visual analyses and EDAX, preferably thicker roots were used. Silica was also found in clover tissue, mostly in roots. Our guess is, that the samples were slightly polluted, still containing soil particles. Interestingly the "live map" of a root, revealed, that copper is scattered all over the sample, so no accumulation in an explicit plant organ could be found.

EDX measurement results of the clover grown in biochar-treated plots, vary between 0,05 and 0,12 mass% of copper. Unfortunately neither stems nor rhizomes from the BC plot were analysed. Instead blossoms, which were very rare in the samples, were taken into the EDAX check-up. Control plots results are relatively similar to the BCs outcome in height of the values. Regrettably many trials had to be cancelled, because the TOT-time did not suffice, no right areas could be found, the surface was uneven or the sample got damaged by radiation.

Regarding the AAS results of *Trifolium repens*, on page 35. the diagram reveals, that similar to the grass, in general roots show the highest Cu-levels, followed by rhizomes, and leaves, while stems only show a very low copper concentration. Analysing each plant part separately, we see that in the clover leaves the copper concentration is the highest in the Mixture 4 samples and the leaves grown in the compost plots` have a significantly lower copper uptake. In comparison to the grass leaves, which had a significantly low Cu concentration in the biochar plots, the biochar- clover leaves took up a much higher amount of Cu, second after the M 4-plots leaves. Looking at the stems, the control plot and the compost values are similarly low. The biochar stems had significantly higher copper concentrations, which is an unexpected outcome, because plants from biochar plots were expected to take up the least copper, due to the lower bioavailability (Devez et al. 2005). Rhizomes of the clover show that the Mixture 4 values are significantly the highest, and the control plots values the lowest. With the grass results, where M 4 results scored the highest copper concentrations, this result leads to the conclusion, that a moderate amount of a compost : biochar mixture mobilises copper for plant uptake. The roots show similar results to the rhizomes: also here the control plot has the significantly lowest results, whereas the compost plot shows the highest copper uptake in the clover roots. As stated before, the biochar and compost alone seem to be more effectively mobilising copper, which is proven by several results so far. In contrast to the diagram (Fig. 34), the statistics show high copper uptake in two biochar plots (clover stems, clover leaves) and in the compost amended plots the plants took up very high copper amounts. As roots take up more copper than stems, but not as much as roots, it is hard to tell wether white clover rhizomes rather fulfil the functions of stems or roots. Other plants have morphologically totally different looking rhizomes, for example the ginger, which in that case function more like a root, storing nutrients, and a great amount of benecifial bacteria. Here root nodules are hosting nitrogen fixating bacteria. Vantis and Bond (1950) found, that the addition of wood biochar to soils at a rate of 1 per cent resulted in a reduction in the number of nodules on clover. However, at higher rates of biochar (greater than 2 %) there were no effects of biochar on nodulation. Turner (1955) even found a significant increase in the number of root nodules in *Trifolium pratense*, but only after an initial reduction, compared to the control.

Von Chamier Glisczinski (2015), a master student investigating copper uptake in roots also in Mautern soils, noted, that roots growing in biochar amended soil represent a significantly lower copper uptake, whereas the Mix 4 amendments have led to a higher uptake, compared to the control plots. Among her samples the biochar-plots had the lowest results. No other similarities are to mention. Chen et al. (2015) investigated bamboo plant parts also sectioned into roots, stems and leaves and found the highest concentrations in the roots (178 mg/kg), but more in the stems (33 mg/kg) than in the leaves (16 mg/kg) (values from the experiment with soil containing 100 mg of copper per kilogram soil, whereas the vineyard is contaminated with 130-140 mg Cu/kg soil). Brennan et al. (2014) found, that biochar significantly lowers the Cu uptake into roots of *Zea mays*, but as Streubel et al. (2011) already stated, the origin of biochar matters.

Regarding the effects of biochar and compost listed in literature, the results of this master thesis lead to the conclusion, that biochar indeed does lower the bioavailability of heavy metals in ecological vineyards, but compost is less efficient, even contraproductive, showing high copper availability in the compost amended soils, similar to the control plots, which is approved by other results of the KUSTAW project (Soja et al. 2018).

Copper in earthworm

Although I was not satisfied with the low outcome of the earthworm sampling (see Fig. 41, not representative amount of earthworms in the control (n=2), biochar (n=3) and mix 10 (n= 3) plots) and some earthworms did not survive until the examination, at least some useful data could be collected. The AAS results show a high cupper-uptake of the earthworm. Much higher results in the animal tissue, compared to the plant parts, indicate, that the earthworm is more directly influenced by the heavy metal. Also Kizilkaya (2004) reported Cu uptake of *Lumbricus* higher, than the soil values, referring to the fact, that these bioindicators indeed are accumulating copper.

Considering the sample preparation, handling the cryostat is not an easy task, and while the section of an average garden-earthworm during the test phase of the experiment was marvel-lous, the more important worm sample from the vineyard was not so ideal to dissect and the organs harder to recognize on the microscope. The results of the EDAX analysis are hard to evaluate, because the chloragogeous tissue has been dissected manually, and the cross sec-tions were prepared differently, frozen by liquid nitrogen and the difference between chloragogenous tissue and overall *Lumbricus* tissue is minimal. No further distinction was possible, because at the EDX only muscle tissue and chloragocytes were identifiable.

Reviewing the sampling method, mustard extraction was an ideal choice, because it neither kills the animals, nor pollutes the soil with chemicals (Scheewe et al., 2010) and works on the basis of allyl isothiocyanate (further AITC), the active ingredient in mustard seeds, which compared to formalin is not carcinogenic, and works as an extractor because it is believed to irritate the earthworms (Valckx et al., 2009). The reason for the low earthworm output must be somewhere else. Scheewe et al. (2010) describe moisture, temperature, pH, soil type, organic matter and tillage as environmental factors influencing earthworm populations. Since the investigated site is relatively even, it is ideally accessible for agricultural tractors, which dense the soil and therefore hinder soil animals to move flawlessly. Also Scheewe (2010) was confronted with the problem of low earthworm abundance in orchards with compacted soils, which he reasons due to the suppression of the soil making it difficult for burrowing. Also in compressed soil the infiltration of water is disturbed, which is common in arable land (Larink & Schrader, 2003). Gerhard Soja (2018) mentions in the final protocol of the KUSTAW project, that the soil bulk density of the control plots, representing 1,2 g/cm³ was higher than in tillaged plots, which showed bulk densities of 1,0 g/cm³ four months after the incorporation of the amendments. Additional differences were noticed on site. The greened corridors in the vineyard of Mautern were visibly not driven on during the sampling period, but the samples were collected in the ungreened corridors.

Former studies revealed that copper leads to higher mortality than lead or zinc in *Eisenia fetida*, also from the family *Lumbricidae* (Spurgeon et al., 1994). Ma (1983) already stated in the 80ies, that heavy metals, especially copper, have a negative effect on the reproduction of *Lumbricus terrestris*. Morgan and Morgan (1988) claim, that the copper accumulation may be physiologically regulated by the earthworm. According to experiments of Morgan and Morris (1982) an excess of heavy metals in the *Lumbricus* is dealt with through two intracellular pathways: one involves the retention of certain metals in insoluble calcium phosphate granules, the chloragosomes, the other pathway is through sulphur donating ligands, which bind the metal within morphologically difficult to discern vacuoles, both techniques keeping metals insoluble such that they do not interfere with essential biochemical reactions in the cytoplasm. But 1990 Morgan and Morgan examined the distribution of metals in earthworm, including copper the first time, and found out, that copper is distributed fairly evenly in the investigated tissue fractions. In contrast to the other heavy metals investigated (Cd, Pb, Zn), no signs of sequestration were detected for copper, presuming that a lack of a Cu-detoxificaton strategy may contribute to susceptibility of earthworms to low environmental copper levels. They even noticed, that earthworms from sites of higher copper concentration, were markedly smaller. In this set-up, the earthworms were not measured, but this step could be added in future studies.

The effects of heavy metals on earthworms are a topic many authors set eyes on. But earthworms also have an effect on heavy metal mobility and bio-availability in soils, which are not examined yet efficiently (Sizmur and Hodson, 2009). *Lumbricus* burrows result not only in an amelioration of soil structure, for improved nutrient and water transport, but also pesticides get transported and spread more evenly (Edwards et al., 1993). Wen et al. (2004) carried out an experiment with a different earthworm species, *Eisenia fetida*, to prove the earthworms role in enhancing the bioavailability of metals. Therefore comparison of metal concentrations in the water-soluble fraction were made between soils with and without earthworms, and the authors observed a significant increase of water-soluble Cu in all investigated soils.

To sum up the role of earthworms as bioindicators I think, that they cause more benefits with their burrowing activity, constructing livable environments for other soil organisms, than disadvantages through enhancing the metals bioavailability, which to my mind needs to be approved with more scientific experiments.

Conclusion

The tested additives effectiveness strongly depends on soiltypes. One of the KUSTAW projects outcome is, that a reduction of soluble Cu was only observed in (mildly) acidic soils (Soja, 2018). But the speciation of Cu did prove the amendments success, through the reduced appearance of the ecotoxicologically most relevant form of Cu, Cu2+ (ionic Copper), which was also low in soils with neutral pH and was even confirmed in seepage water of pot experiments in the frame of one of the KUSTAW-glass house trials (Soja, 2018). Still the fact, that the proportion of ionic Cu was usually very small (at the most a few % of total Cu), and allegedly this reduction stays effective only temporarily during a few years, makes the need of remediation questionable. During the years of the KUSTAW- project, the envisaged immobilization of Cu became effective in the significant reduction of the Cu uptake of roots of annual green cover crops by a biochar additive without compost. But a pure biochar treatment without compost, which is effective because of the reduction of dissolved organic carbon in soil pore water, is no desirable management strategy in viticulture, especially not for organic wine-growers, because they depend on compost as the only nutrient source.

The difference observed within the organism groups, namely the huge copper values found in the earthworm tissue, not showing a great variance between the various treatment types, can be explained by the fact, that *Lumbrici* take up soil orally, therefore the soil`s sorption is not of relevance for them.

Future research

Avoiding the distribution of huge amounts of copper-sulfate, transition to *smart agriculture* could be a solution. Sensor systems to observe precipitation or drought are common already. Newest technologies, like the wireless sensor network system, called VineSens (Pérez-Expósito et al., 2017) has been created especially for vinegrowers to modify, and therefore reduce costs in their pesticide usage. In former times roses served as bioindicators at the edge of each vine stock, showing symptoms of fungal disease days before the grapevine. Nowadays vinegrowers are not checking their fields each day, so the first suggestion, installing a sensor-system in the vineyard would be the more appropriate decision.

Regarding soil bioindicators, the fact that Annelids seem to grow shorter in copper contaminated soils (Morgan and Morgan 1990) may make them usable for indicating, but since copper is distributed evenly in theirs tissues, a species, which accumulates the metal in a separate compartment, would be of greater interest. Woodlice need copper for moult, plus they tolerate some heavy metals by accumulating them in vesicles of the hepatopancreas (Paoletti and Hassal 1999), resulting to be potential bioindicators of copper pollution.

To seize another biological approach to decrease the copper concentration in organic vineyards, next to biological amendments, phytoremediation could be considered. Especially the phytoextraction could be handy (Andreazza et al. 2011). In this method plants extract pollutants from the ground, translocate them into the shoots which get harvested, so that in this case the coppers soil concentration gets decreased. One throwback is, that on one side there are hyper-accumulating plants which tolerate extraordinarily high levels of heavy metals, and are capable of accumulating them in their tissue, and on the other side fast growing crops, which produce big amounts of biomass, take up just a low percentage of metals which have to be removed. If these two traits would be combined, the ideal phytoextractor would be created. Clemens et al (2002) described the possibility of genetically engineer rapidly growing non-accumulator, so that they achieve properties of hyperaccumulators. Fatnassi et al. (2013) states, that legumes, including *Trifolium repens* may be efficiently used in phytoremediation of heavy metals polluted sites, although he refers on phytostabilization and the positive effects of revegetation rather than phytoextraction.

Another solution to increase the uptake of heavy metals is the use of chelators to mobilize the metals to the root zone (Madrid et al., 2003). Biodegradable chelates could be accepted in organic vineyards.

Back to the amendments of this site, studying the literature, one can find that there are several types both of biochar and compost available. Some authors find activated carbon more effective (Namgay et al. 2010, Beesley et al. 2011, Shaheen and Tsadilas 2015) and Mackie et al. (2015) suggest that the compost application should be repeated each year to maintain the positive effects, along with protecting soil microorganisms from toxic copper components. Since compost is degraded faster than biochar, an application each year seems to be worthy, if greening-plants, vineyard breeding birds and the microfauna are not damaged through the incorporation procedure. However in the case of biochar further application be is not needed, Soja et al. (2018) proved its longevity. Nevertheless since in this trial only the higher amount of applied biochar-compost mixture and the biochar alone show visible differences of copper-reduction in the results, but the fraction of bioavailable Cu is very low anyways, it is questionable, if this vineyard needs remediation at all.

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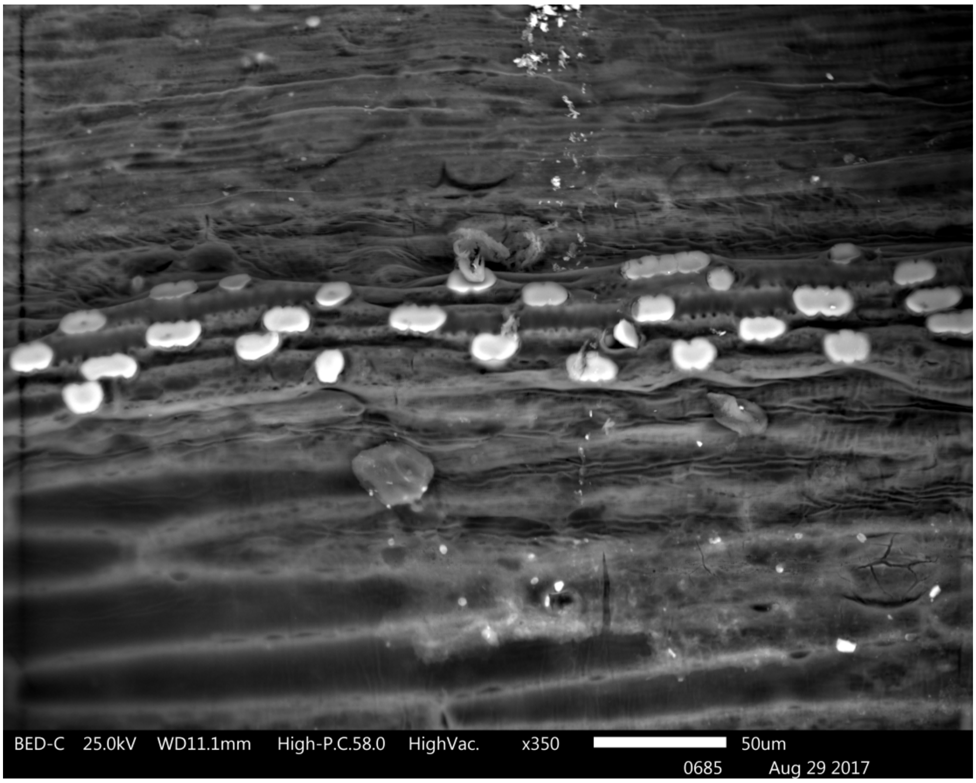
Appendix

1.AGES Soil pH

Table I.

Spalte1	Spalte3	Spalte4	Spalte5	Spalte6	Spalte7	Spalte8	Spalte9	Spalte10	Spalte11	Spalte12	Spalte13	Spalte14	Spalte15	Spalte16	Spalte17	Spalte18	Spalte24	Spalte25	Spalte26	Spalte30	Spalte31
		pH-Wert	Calciumcarb	Phosphor	Kalium	Magnesium	TOC	Humus	Stickstoff ge	Eisen	Mangan	Kupfer	Zink	Calcium	Magnesium	Kalium	CEC	Arsen	Blei	Kupfer	Nickel
		-	%	mg/kg	mg/kg	mg/kg	%	%	%	mg/kg	mg/kg	mg/kg	mg/kg	cmolc/kg	cmolc/kg	cmolc/kg	cmolc/kg	mg/kg	mg/kg	mg/kg	mg/kg
		CP-PHPH-V0	EL-KALKB00	EL-CAL--02	EL-CAL--02	EL-MG--01	EL-TOC--01	EL-TOC--01	EL-NGES-01	EL-SPUREN1	EL-SPUREN1	EL-SPUREN1	EL-SPUREN1	EL-KAT--02	EL-KAT--02	EL-KAT--02	EL-KAT--02	EL-SMGES01	EL-SMGES01	EL-SMGES01	EL-SMGES01
Auftragsnum	Externe Kennung																				
17056783	CONT1	7,31	8,2	340	541	133	1,84	3,2	0,179	97	399	172,4	16,4	17,82	1,71	1,54	21,08	13,07	20,8	305,21	32,89
17056783	CONT2	7,36	12,8	310	506	124	1,92	3,3	0,192	53	127	162,5	15,6	17,78	1,55	1,45	20,79	12,83	20,12	328,94	30,06
17056783	CONT3	7,38	8,7	375	613	158	1,92	3,3	0,195	95	348	95,6	14,9	16,84	2,01	1,71	20,57	10,04	26,81	173,06	31,78
17056783	CONT4	7,47	20,4	267	468	123	1,84	3,2	0,177	42	80	117,9	11,7	16,08	1,46	1,32	18,87	8,54	16,25	258,51	25,19
17056783	COMP1	7,32	6,8	446	667	157	2,2	3,8	0,216	326	468	186,7	19,1	18,88	2,08	1,83	22,81	11,54	21,26	305,37	33,46
17056783	COMP2	7,37	14,2	335	543	129	2,2	3,8	0,209	74	108	153,3	13,9	18,14	1,64	1,49	21,29	12,02	18,91	318,08	28,92
17056783	COMP3	7,44	14	465	600	156	2,35	4	0,208	137	125	115,8	13,2	18,63	2,01	1,68	22,33	9,43	20,45	237,67	28,65
17056783	COMP4	7,43	17,1	372	548	146	2,15	3,7	0,19	92	89	112,8	11,2	17,25	1,71	1,48	20,46	8,67	17,15	252,92	26,56
17056783	BIOCHAR1	7,41	8,9	338	591	145	5,03	8,7	0,21	74	288	156,1	17,2	17,51	1,79	1,58	20,89	11,49	18,85	284,63	30,05
17056783	BIOCHAR2	7,46	17,1	251	602	138	6,55	11,3	0,216	44	96	126,6	13,6	16,4	1,67	1,58	19,65	10,42	16,91	287,84	26,61
17056783	BIOCHAR3	7,42	6	421	734	170	5,74	9,9	0,203	125	462	111,8	17,8	17,49	2,18	2,05	21,73	9,52	19,28	186,97	31,29
17056783	BIOCHAR4	7,48	17,7	322	638	148	4,09	7	0,196	49	96	117,6	14,7	16,04	1,73	1,7	19,48	8,62	16,8	258,98	26,11
17056783	MIX 4-1	7,34	5,6	406	646	157	2,84	4,9	0,213	231	478	185,6	20,6	19,47	2,06	1,82	23,35	11,98	20,33	302,96	33,11
17056783	MIX 4-2	7,39	16,1	298	601	149	3,59	6,2	0,224	63	77	129,3	12,6	17,88	1,76	1,58	21,23	10,83	17,31	291,91	26,45
17056783	MIX 4-3	7,39	10,3	411	662	161	3,4	5,8	0,217	101	207	128,1	13,3	17,95	1,98	1,77	21,71	9,8	19,13	248,02	29,91
17056783	MIX 4-4	7,44	21	320	552	144	3,18	5,5	0,197	63	78	110,6	12,6	16,39	1,64	1,49	19,54	8,52	16,6	252,4	25,45
17056783	MIX 10-1	7,34	7,6	498	697	186	5,73	9,9	0,256	327	392	161,7	18,8	19,64	2,37	1,89	23,93	10,67	23,36	280,93	31,65
17056783	MIX 10-2	7,45	19,4	349	656	171	5,77	9,9	0,229	92	81	121,5	13,8	17,6	1,99	1,67	21,28	10,38	16,55	272,56	25,5
17056783	MIX 10-3	7,41	8,2	531	782	187	5,64	9,7	0,249	280	348	124,4	17,9	18,74	2,38	2,07	23,24	9,79	18,18	227,99	30,45
17056783	MIX 10-4	7,5	14,6	446	769	171	4,88	8,4	0,222	101	99	116,4	14,2	17,59	2,13	2,06	21,81	8,8	17,8	250,79	26,5
Anzahl: 20		20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
Minimum		7,31	5,6	251	468	123	1,84	3,2	0,177	42	77	95,6	11,2	16,04	1,46	1,32	18,87	8,52	16,25	173,06	25,19
Maximum		7,5	21	531	782	187	6,55	11,3	0,256	327	478	186,7	20,6	19,64	2,38	2,07	23,93	13,07	26,81	328,94	33,46
Mittelwert		7,41	12,7	375	621	153	3,64	6,3	0,21	123	222	135,3	15,2	17,71	1,89	1,69	21,3	10,35	19,14	266,29	29,03
Median		7,41	13,4	360	608	153	3,29	5,7	0,21	93	126	125,5	14,5	17,69	1,89	1,67	21,25	10,21	18,88	265,77	29,42



2.Grass stem detail: Silica granules in EDAX

Table II. Grass root section in EDX, cortex

eZAF Quant. Ergebnisse

Element	Gewicht%	Atom%	Net. Int.	Error %	Kratio	Z	R	A	F
C K	47.88	56.26	1419.42	5.71	0.2778	1.0289	0.9856	0.5638	1.0000
N K	2.36	2.38	13.12	99.99	0.0024	1.0046	0.9956	0.1027	1.0000
O K	44.68	39.42	937.73	9.66	0.0776	0.9834	1.0047	0.1765	1.0000
F K	0.05	0.04	0.96	99.99	0.0001	0.9137	1.0129	0.1260	1.0000
NaK	0.12	0.08	6.96	38.30	0.0004	0.8922	1.0276	0.3596	1.0020
MgK	0.14	0.08	15.15	18.39	0.0007	0.9071	1.0342	0.5317	1.0034
AlK	0.11	0.06	14.46	23.22	0.0007	0.8732	1.0403	0.6901	1.0059
SiK	0.21	0.10	32.49	11.07	0.0015	0.8922	1.0460	0.8131	1.0091
P K	0.12	0.06	16.92	19.92	0.0009	0.8567	1.0514	0.8987	1.0145
S K	0.09	0.04	13.61	22.88	0.0008	0.8733	1.0564	0.9546	1.0216
ClK	0.04	0.02	5.47	56.99	0.0003	0.8307	1.0612	0.9891	1.0334
K K	2.73	0.98	318.51	3.00	0.0239	0.8263	1.0697	1.0226	1.0371
CaK	1.17	0.41	114.93	6.21	0.0104	0.8413	1.0735	1.0155	1.0363
FeK	0.15	0.04	7.49	56.49	0.0013	0.7494	1.0883	1.0212	1.1864
CuK	0.15	0.03	5.32	58.16	0.0015	0.7168	1.0875	1.0167	1.3471

Table III. Grass root section in EDX, phloem

eZAF Quant. Ergebnisse

Element	Gewicht%	Atom%	Net. Int.	Error %	Kratio	Z	R	A	F
C K	47.60	55.55	5260.85	5.41	0.2800	1.0274	0.9866	0.5727	1.0000
N K	4.62	4.63	96.67	99.99	0.0049	1.0031	0.9966	0.1053	1.0000
O K	43.67	38.26	3271.63	9.44	0.0736	0.9819	1.0056	0.1717	1.0000
F K	0.05	0.03	3.33	99.99	0.0001	0.9123	1.0139	0.1262	1.0000
NaK	0.13	0.08	27.87	18.08	0.0004	0.8907	1.0285	0.3617	1.0019
MgK	0.08	0.04	29.99	17.31	0.0004	0.9056	1.0350	0.5344	1.0033
AlK	0.04	0.02	18.92	31.91	0.0002	0.8718	1.0411	0.6937	1.0057
SiK	0.07	0.03	38.13	14.36	0.0005	0.8907	1.0468	0.8170	1.0090
P K	0.20	0.09	105.05	6.33	0.0016	0.8553	1.0521	0.9034	1.0141
S K	0.10	0.05	57.33	10.30	0.0009	0.8719	1.0571	0.9576	1.0209
ClK	0.05	0.02	24.47	24.40	0.0004	0.8292	1.0618	0.9912	1.0322
K K	2.57	0.92	1100.22	2.28	0.0225	0.8249	1.0703	1.0237	1.0348
CaK	0.65	0.23	234.53	5.34	0.0058	0.8398	1.0741	1.0172	1.0387
FeK	0.06	0.01	10.47	56.11	0.0005	0.7480	1.0887	1.0222	1.2039
CuK	0.12	0.03	15.11	41.34	0.0012	0.7155	1.0879	1.0177	1.3840

Table IV. Grass root section in EDX, xylem

eZAF Quant. Ergebnisse

Element	Gewicht%	Atom%	Net. Int.	Error %	Kratio	Z	R	A	F
C K	49.86	57.18	3799.43	5.34	0.2975	1.0235	0.9892	0.5828	1.0000
N K	3.88	3.81	55.78	99.99	0.0041	0.9991	0.9991	0.1069	1.0000
O K	44.53	38.34	2350.33	9.41	0.0778	0.9780	1.0080	0.1786	1.0000
F K	0.03	0.02	1.35	99.99	0.0000	0.9085	1.0161	0.1270	1.0000
NaK	0.03	0.02	4.44	70.62	0.0001	0.8870	1.0305	0.3651	1.0017
MgK	0.08	0.04	20.42	21.18	0.0004	0.9017	1.0370	0.5398	1.0029
AlK	0.03	0.01	9.13	62.03	0.0002	0.8680	1.0430	0.6993	1.0050
SiK	0.14	0.07	54.59	11.28	0.0010	0.8868	1.0486	0.8222	1.0076
P K	0.15	0.07	51.99	9.24	0.0012	0.8515	1.0538	0.9068	1.0117
S K	0.07	0.03	26.97	16.48	0.0006	0.8680	1.0588	0.9607	1.0170
ClK	0.03	0.01	8.92	55.59	0.0002	0.8255	1.0634	0.9939	1.0258
K K	0.89	0.31	258.67	4.51	0.0078	0.8212	1.0718	1.0254	1.0394
CaK	0.16	0.05	39.14	18.41	0.0014	0.8360	1.0755	1.0268	1.0500
FeK	0.08	0.02	10.30	56.73	0.0007	0.7445	1.0897	1.0248	1.2592
CuK	0.06	0.01	6.18	59.10	0.0007	0.7121	1.0886	1.0188	1.4956

Table V Clover root section in EDX, Live map

Element	Gewicht%	Atom%	Net. Int.	Error %	Kratio	Z	R	A	F
C K	52.87	61.76	3184.76	5.42	0.3042	1.0263	0.9844	0.5605	1.0000
N K	5.46	5.47	53.99	99.99	0.0045	1.0043	0.9947	0.0821	1.0000
O K	33.66	29.52	1145.39	10.02	0.0398	0.9851	1.0039	0.1201	1.0000
F K	0.78	0.57	32.35	15.51	0.0008	0.9167	1.0125	0.1054	1.0000
MgK	0.19	0.11	49.09	12.82	0.0008	0.9139	1.0345	0.4500	1.0041
AlK	0.06	0.03	20.07	24.71	0.0003	0.8808	1.0410	0.6183	1.0071
SiK	0.09	0.05	41.35	13.80	0.0006	0.9008	1.0470	0.7636	1.0114
P K	0.10	0.05	43.31	13.72	0.0008	0.8659	1.0528	0.8726	1.0184
S K	0.54	0.24	256.53	3.42	0.0046	0.8835	1.0582	0.9457	1.0260
K K	4.72	1.69	1896.35	1.77	0.0422	0.8382	1.0729	1.0337	1.0318
CaK	1.32	0.46	444.93	2.62	0.0117	0.8542	1.0773	1.0074	1.0307
FeK	0.10	0.03	20.64	32.89	0.0009	0.7658	1.0978	1.0279	1.1601
CuK	0.12	0.03	17.74	36.03	0.0011	0.7367	1.1027	1.0243	1.3040

3. *Spermophorus clitellus*

Ground squirrel holes have been counted on June 29th, but no tendencies for a special soil amendment could be seen.

CT 1. 1 hole
CP 1. 8 holes
BC 1. 1 hole
M10 1. 15 holes
M10 2. 1 hole
M10 3. 10 holes
M 4 1. 8 holes

Note: The vegetation was still high and potentially additional holes were overseen.
The counting reveals that ground squirrels are not deranged by soil amendments.