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Effects of pesticide cocktails on soil life and soil functioning

GEO 511 Master's Thesis

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ABSTRACT

In the context of a growing world population and the raising demand in agricultural production pesticides seem indispensable and are often applied in large quantities to control pest, diseases and weeds and ultimately to maximize yields and economic return. However, the application of pesticides is raising concerns regarding their impact on the environment and in particular the negative effects on non-target soil microorganisms which are associated with the ecosystem services soils provide. While the effect of pesticides on non-target organisms are partially tested during legislation process, the application of pesticides as cocktails are not considered and also remain understudied in research. Even though pesticide mixtures are regularly applied in agricultural practices and potentially more harmful than pesticide single applications. In this thesis we conducted a greenhouse experiment with sequential harvest using intact soil cores to determine the effect of three herbicides and three fungicides applied alone and in cocktails on different soil microbial communities and important soil functions. For the greening of the soil cores we used lettuce as a model plant. We found the fungal abundance to be significantly affected by pesticide treatments conversely bacteria, protists and arbuscular mycorrhizal fungi remained unaffected. We found especially the herbicide mixture to a have a significant negative effect on fungal abundance. For the functional parameters we found a significant main effect of the treatments on the dry biomass and the phosphorous content of lettuce. Litter decomposition, potassium and nitrogen uptake seemed not to be significantly affected by the treatments. Even though we found only small indications for synergistic cocktail effects, further research is needed to understand the effects of pesticide cocktails on soil life in more detail for a judicious use of pesticides in an agricultural context.

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1 INTRODUCTION

The protection of crops from pests, weeds and diseases is a primary asset in agricultural production and shows the relevance of pesticides regarding the assurance of crop yield and its quality (FAO and ITPS, 2017). Especially when considering a continuously growing world population associated with an increasing need for agricultural products. Thus, the deployment of modern agrochemicals such as fungicides, insecticides and herbicides seem indispensable in ensuring the global food supply (FAO and ITPS, 2017; Filser et al., 2015; Sharma et al., 2019). Approximately 4.1 million tonnes of active ingredients were used worldwide in 2018, of which the largest share is taken by herbicides (47.1%) followed by insecticides (29.5%) and fungicides (17.5%) (FAO, 2018; Sharma et al., 2019). Even though the application of pesticides seem beneficial to crop production, the extensive use of large quantities have led to several concerns about their impact on the ecosystem (Böcker, T., Möhring, N., Finger, 2019; Sharma et al., 2019). The consequences associated with the application of pesticides in agricultural systems not only have attracted the interest of researcher but also has become increasingly more important in Switzerland's politics, where two initiatives were launched which aim reducing the use of synthetic pesticides (Verein Sauberes Wasser für alle, 2021; Vereinigung Für eine Schweiz ohne synthetische Pestizide, 2021). Simultaneously, the Federal Council introduced an action plan to reduce pesticide related risks and aims a sustainable use of pesticides, especially regarding towards maintaining soil fertility (Bundesrat, 2017).

Soils play an important role in sustaining ecosystem services, support plant roots, provide essential minerals and nutrients to crops and protect plants from erosion and other physical or chemical disturbances. Furthermore, soils provide a habitat for various types of living organisms such as insects and microorganisms (Kaviya et al., 2019). Already one single gram of soil accommodates tens of thousands microorganisms that are involved in multiple soil functions (Thiour-Mauprivez et al., 2019). Soil microorganisms are a diverse group of organisms that consist of bacteria, fungi, protists and viruses (Darine et al., 2015; Ockleford et al., 2017) and are regarded as essential drivers for almost all soil processes. Therefore, soil microorganisms have a crucial impact on soil formation (Kaviya et al., 2019), soil fertility, crop productivity and also diversity of the plant community (Bardgett and van der Putten, 2014; Bender et al., 2016). Decomposition of organic material, mobilization of nutrients, mineralization, biological nitrogen fixation and bioturbation are only a range of activities that influence the availability of nutrients for crop and thus agricultural production (Bünemann et al., 2006; Stanley and Preetha, 2016). Especially, arbuscular mycorrhizal fungi (AMF) play an important role in enhancing plant growth since they provide nutrients that are not accessible to plant roots (van der Heijden et al., 2015). Immobile soil nutrients such as phosphorous are efficiently captured with thin mycorrhizal hyphae and transported to the plant’s roots in return for carbohydrates. Therefore, microorganisms such as AMF but also bacteria enhance the nutrient availability to plants and act growth promoting. Furthermore, beneficial microbes supress plant pathogens and alleviate biotic stresses such as drought and salinity but also metal

toxicity in plant and soil (Stanley and Preetha, 2016). Additionally, soil microorganism are involved in the pesticide degradation (Stanley and Preetha, 2016), where pesticides are used as an energy source (Karpouzas et al., 2016). At the same time the application of pesticides in an agricultural context result in a serious threat to those microorganisms (Karpouzas et al., 2016; Thiour-Mauprivez et al., 2019).
Ideally, a pesticide should only be toxic to target organisms, be biodegradable and not leak into the groundwater, which unfortunately is rarely the case (Johnsen et al., 2001). Extensive management practices involving intensive, continuous and combined inputs of different pesticides have led to a pesticide contamination of the soil, surface water and groundwater through the applied products. Also, the application of pesticides lead to an accumulation of degradation products (Imfeld and Vuilleumier, 2012; Johnsen et al., 2001) that can cause substantial damage since some of the breakdown products may even be more harmful to non-target soil organisms than the parent compound (Stanley and Preetha, 2016). Riedo et al. (2021) point out that contamination of soils with pesticides can have long-term effects, especially in connection with the often underestimated persistence of pesticides in soil. In their study they were able to detect pesticides after more than 20 years of organic farming and even substances that have already been banned for several years (Riedo et al., 2021).

In summary the application of pesticides and their residues have the potential to induce the inhibition of microbial processes, reduce the population of specific microbial groups or the overall diversity of the microbial community (Karpouzas et al., 2016). This means the complex biological environment of soil microorganisms and therefore the ecosystem services soils provide such as primary production and water purification (Bünemann et al., 2018; Thiour-Mauprivez et al., 2019) are threatened by the application of pesticides (Devi et al., 2018; Imfeld and Vuilleumier, 2012; Johnsen et al., 2001; Stanley and Preetha, 2016). Nevertheless, the assessment for pesticide ecotoxicity before a commercial registration involves only the estimation of carbon and nitrogen mineralization (Johnsen et al., 2001) which is not suitable for an accurate estimation of the ecotoxicity of pesticides. There is a need to assess also the effects of pesticides on microbial abundance, diversity and activity (Thiour-Mauprivez et al., 2019). Additionally, the assessments of pesticide related environmental risks do not account for several stressors that have become more relevant in the recent years, like climate change, destruction of habitats, increasingly homogenized landscapes or different combinations of pesticides. All those factors can contribute to the intensification of the impact of pesticides on the environment (Topping et al., 2020).

2 PESTICIDES AND THEIR MODE OF ACTION

Pesticides represent a large group of organic and inorganic chemicals and can be distinguished according to their target organisms (e.g. insecticides, herbicides and fungicides), in addition pesticides do differ in their mode of action (MoA) (Bünemann et al., 2006; Imfeld and Vuilleumier, 2012). Many studies focussing on the effects of pesticides on soil microorganisms show opposing results (Puglisi, 2012). Furthermore the response of soil microorganisms to pesticides are not only dependent on the active substance applied but also can be influenced by additional factors. Different soil properties such as soil pH, temperature, organic matter content can modulate the behaviour of pesticides in soils (Chowdhury et al., 2008). In this thesis, the focus lies on three fungicides and three herbicides. Therefore, their mode of action and usage are discussed in this section.

2.1 FUNGICIDES

Fungicides are designed to interfere with critical cellular processes that inhibit fungal growth (FRAC, 2020). Fungicides control fungal pathogens by killing or inhibiting fungi or fungal spore germination. Fungicides not only have different modes of action but also differ in their systemicity. Generally, the MoA refers to a particular cellular process inhibited by a fungicide. Currently 11 MoA for fungicides are listed by the Fungicide Resistance Action Committee (FRAC) which involve the inhibition of sterol synthesis, respiration, site enzymes, nucleic acid and protein synthesis (FRAC, 2020). The systemicity of fungicides involve the uptake of a fungicide and its distribution within the plant. Additionally fungicides act curative or protective. In order for a fungicide to be curative, the active ingredient is translocated within a leave or the whole plant to inhibit fugal growth. A fungicide that is redistributed from the sprayed leave surface to unsprayed surface acts translaminar or locally systemic. A distribution of the fungicide in the whole plant means that a fungicide acts systemically. Systemic or locally systemic fungicides, therefore, have entered a plant and have a longer activity since they cannot be washed off. Protectant or contact fungicides remain on the surface of a leaf protecting the plant against the germination of fungal spores. Therefore, protective fungicides need to be applied before the infection (Teicher, 2017). A further possibility in order to control soil borne pathogenic fungi's is the direct application of fungicides to the soil (Stanley and Preetha, 2016).

Fungicides are thought to have greater effects on soil organisms than herbicides and insecticides as they directly inhibit fungal pathogens in plants and therefore could also affect soil fungi that are beneficial (Bünemann et al., 2006). Studies mostly observed from no significant effects to significant decreases or even significant increases in microbial biomass after fungicide application, but a different pattern can be observed at the microbial community level, where most studies found significant changes in community structure (Puglisi, 2012). Howell et al. (2014) found fungi and nematodes to be the most affected by fungicide application since they show a low initial resistance. However, they also observed a recovery where the biomass returned to comparable levels as in the control. Supposedly new growth

was promoted by utilizing dead biomass and filling a niche. In the following paragraphs the fungicides we used for this thesis are introduced.

2.1.1 Azoxystrobin

Azoxystrobin belongs to the class of strobilurins that bind to the cytochrome b complexes and as result inhibit mitochondrial respiration leading to the death of the target organism. It can be used as a foliar fungicide but also for seed treatment (Adetutu et al., 2008). Azoxystrobin has systemic and translaminar properties. It is recommended to apply azoxystrobin before the infection (Syngenta Agro AG, 2019a). Studies suggest that the half-life of azoxystrobin ranges between 14 days up to 6 months (Adetutu et al., 2008; Bending et al., 2007; United States Environmental Protection Agency, 1997).

2.1.2 Boscalid

Boscalid is a broad spectrum fungicide which belongs to the family carboxamide. It acts as a succinate dehydrogenase inhibitor in the complex II of the mitochondrial electron transfer chain and therefore, affects fungal cell respiration. The substance prevents the spore germination and the growth of the germ tube. Boscalid gets absorbed by the leaves and acts systemically (FRAC, 2020; Syngenta Agro AG, 2019b). The substance is a widely detectable pesticide in the environment with an estimated half-life ranging between 104 to 224 days based on a field experiment and 297-337 days in a laboratory experiment (Karlsson et al., 2016).

2.1.3 Epoxiconazole

Epoxiconazole belongs to the group of triazoles. The substance enters the leave rapidly and gets distributed through the whole plant, thus being a systemic fungicide. Epoxiconazole acts curative interfering with the biosynthesis of sterol, which is a key component of the fungal membrane. Hence, important functions of the fungal metabolism are disturbed. Epoxiconazole interferes with the mycelium growth and further the production of spores is limited (FRAC, 2020; Stähler Suisse SA, 2019) Half-life for epoxiconazole are reported to be over 1500 days, thus the substances are highly persistent in soil (Alexandrino et al., 2020).

2.2 HERBICIDES

The management of undesired plants and weeds involves the application of herbicides that interfere with the metabolism and other biochemical pathways of the target plant. Leading to irreversible damage, tissue injury and eventually to the elimination of the weed. Herbicides are applied either directly to the foliage or added to the soil, whereas herbicides can be absorbed over the shoots respectively the roots (Sherwani et al., 2015). Usually herbicides are applied when crops are absent or at an early growing stage, therefore the sprayed herbicides reach the soil and may affect soil microorganisms directly (Lupwayi et al., 2004; Pelosi et al., 2014). Herbicides are grouped according to their mode of action that include inhibition, interruption, disruption or mitigation of the regular plant growth (Sherwani et al., 2015). As herbicides target plant specific enzyme structures, they are thought to be less harmful to microorganisms than fungicides and insecticides (Johnsen et al., 2001). Lupwayi et al. (2004) reveal

that herbicides applied in recommended dosage in general do not show significant effects on soil microorganisms. Also Puglisi (2012) shows that most studies investigating the effects of herbicides on microbial biomass show no significant differences, but most studies showed significant changes in the microbial community structure. Furthermore, herbicides tended to decrease AMF diversity, however, it is unclear if the effect is due to a direct toxic effect on AMF or rather an indirect toxicity effect via the host plant (Thiour-Mauprivez et al., 2019).

2.2.1 Flufenacet

Flufenacet gets absorbed mainly over the roots but when applied post-emergence it can also be absorbed over the foliage. The substance affects the synthesis of very long fatty acids in the target plant (Bayer, 2020; HRAC, 2020). The target organisms are germinating weeds such as slender foxtail, annual meadow grass and loose silky-bent grass. The degradation of flufenacet is largely dependent on soil type and dosage (Milan et al., 2013), therefore, the half-life of flufenacet in different studies ranged between 8 to 12 days (Milan et al., 2013), 10 to 30 days (Gupta and Gajbhiye, 2002) or 44 to 66 days (Rouchaud et al., 2001).

2.2.2 Fluazifop-P-butyl

Fluazifop-P-butyl is a highly efficient post-emergent herbicide for the control of several perennial and annual grass weeds that inhibits the acetyl- CoA- carboxylase (ACCase). The inhibition of the enzyme ACCase results in a blocked biosynthesis of fatty acids in susceptible plants (Darine et al., 2015; HRAC, 2020; Kukorelli et al., 2013; Silveira et al., 2015). In the soil fluazifop P-butyl is rapidly hydrolyzed to fluazifop acid, which further gets degraded by microbes resulting in a half-live of the herbicide and its degradation products of two to five weeks (Negre et al., 1993; Smith, 1987).

2.2.3 Propyzamide

Propyzamide belongs to the benzonitrile amide herbicides that are applied pre-emergent or early post-emergent. Propyzamide is used for the control of weeds in a wide range of crops and gets mainly absorbed over the roots. The mode of action involves the inhibition of tubulin polymerization and therefore affects cell growth (HRAC, 2020; Syngenta Agro AG, 2018). The half-life of propyzamide ranges between 10 to 40 days (Travlos et al., 2017).

2.3 PESTICIDE MIXTURES

So far most studies have only focussed on single applications of a specific pesticide (Bünemann et al., 2006; Pelosi et al., 2014; Topping et al., 2020). Although single applications are in praxis only rarely applied and even not recommended due to possible resistance formation (FRAC, 2020; HRAC, 2020; IRAC, 2020). Additionally, several different pesticide treatments are usually applied during one cropping season. Hence, agricultural soils are exposed to cocktails of different pesticides and therefore, it is very unlikely that soil organisms are in contact with one single active ingredient (Topping et al., 2020). Literature about the impact of pesticides applied as cocktails on soil microorganisms is sparse so far. However, studies on the joint effect of different pesticides in aquatic systems demonstrated that the

effect of pesticides applied as cocktails had dramatic direct and indirect effects on aquatic communities. For example Relyea (2009) revealed that mixtures of common pesticides caused a mortality up to 99% in larval amphibians. Further, it has been demonstrated that multiple chemical stressors have complex and interacting effects on ecosystem functioning in a stream food web, suggesting that a mixture of pesticides exert a slightly higher toxicity compared to their single components on detritivores (Dawoud et al., 2017). Such results argue not only for more research on the impact of pesticide cocktails on aquatic communities but also the need to address the impacts on soil microorganisms. Rillig et al.(2019) revealed recently that a combination of multiple stressors on soil is of particular importance as the increasing number of stressors determines the impact on soil functioning, indicating the pressing need to investigate the impact of pesticide cocktails in more detail.

3 OBJECTIVES AND HYPOTHESIS

Since the abundance and activity of soil microorganisms are closely related to ecosystem functioning and stability (FAO and ITPS, 2017; Yang et al., 2018), it is important to assess the effects of pesticides on soil microorganisms. The objective of this thesis was to test if synergetic effects of pesticide cocktails can be observed on microbial soil life and soil functioning in a manipulative greenhouse experiment. We will test the effects of three fungicides and three herbicides applied individually and in different cocktails. The goal is to find out how pesticide cocktails affect non-target soil organisms compared to the application of the individual products. A particular interest lies in the effects of pesticides on AMF. Besides being beneficial for crop production, they have also been identified to be sensitive to agricultural management practices such as pesticide applications (Hage-Ahmed et al., 2019; Riedo et al., 2021; Rivera-Becerril et al., 2017). Therefore, the aims of this thesis are addressed answering the following research questions: (i) How do pesticides applied as cocktails affect soil microorganisms compared to single applications? (ii) Are herbicides and their cocktail more harmful to soil microorganisms than fungicides? (iii) Does the impact on microbial soil life increase when fungicides and herbicides are combined in a cocktail? (iv) Are arbuscular mycorrhizal fungi in particular sensitive to pesticide exposure?

We hypothesize that the effect of a pesticide cocktail on soil microorganisms and in particular to arbuscular mycorrhizal fungi (AMF) is stronger compared to single application, since different pesticides attack different biosynthesis pathways. Further, we hypothesize that protists and AMF are more sensible to pesticides than bacteria and fungi, therefore we expect the greatest effect within those two groups. Finally, fungicides are expected to have a larger influence on microbial soil life than herbicides, since fungicides are targeting fungi and therefore might affect soil fungi and particularly beneficial AMF.

4 METHODOLOGY

4.1 SOIL SAMPLING

Intact soil cores (25cm x 10cm) from a grassland located near Agroscope Reckenholz (47.434176, 8.511143) were sampled in May 2020 and stored at 4°C until the experiment began. The selection criterion for the field was that it had not been ploughed for at least a year. Since ploughing is known to negatively interfere with AMF and soil microorganisms (Hage-Ahmed et al., 2019; Krauss et al., 2020).

4.1.1 Assessment of soil parameters

Additional soil samples from the same grassland were collected and dried at 60°C for 24h. In order to determine the soil parameter we sieved the samples (<2mm). Soil pH, soil texture and organic carbon (OC) were analysed according to the Swiss reference methods of the Federal Agricultural Research Station (**Table 1)**. Further the bulk density was determined using samples collected in a 100cm^3 volumetric cylinder dividing the weight of the dry samples by the cylindric volume.

Table 1 Physicochemical soil properties of the grassland were the intact soil cores were sampled.

pH	Sand [%]	Silt [%]	Clay [%]	Humus [%]	Corg [%]	Bulkdensity [g/cm^3]
6.78	30.5	41.7	25.2	2.62	1.52	1.37

4.2 EXPERIMENTAL SYSTEM

The experiment was conducted in the greenhouse facility at Agroscope Reckenholz, where soil cores were used as terrestrial model ecosystems (TMEs) to investigate the effects of pesticide cocktails on soil life. TME's are regarded as reproducible, controlled systems in order to simulate processes in a portion of the terrestrial environment. TME's are a commonly used method to determine the effects of pesticides on soil microorganisms (Burrows and Edwards, 2004). The TMEs consisted of intact soil cores, which ensures that the experimental conditions are comparable to nature regarding the structure of the soil, and ensures the preservation of indigenous micro- and mesofauna. The soil cores were prepared to a length of 20cm prior to their installation into PVC tubes. We also removed the top with the grass and homogenized the top 3cm of each soil core with a fork. The PVC-tubes were 25 cm high and have a diameter of 10 cm. The bottom of the system has a drainage hole covered by a mesh and a 2cm-layer of sterilized sand for proper drainage. In total we set up 99 soil cores over a period of two months.

4.2.1 Plant growth condition

For the greening of the systems, we used leaf lettuce (*Lactuca sativa var. crispa)* having the advantage that it can be cut several times and regrows again. In total we planted three seeds and covered the system with plastic foil for better germination. After the germination we removed two plants so that the system only contained one plant. We let the plant establish for four weeks, than cut it down before applying the pesticides. To ensure that the plants did not suffer from water stress, we irrigated the soil cores with

rainwater two to three times per week depending on the temperature in order to maintain the systems at 75%-water-holding capacity (WHC). Furthermore, keeping the systems at 75%-WHC prevented overwatering and therefore the leaching of pesticides. To determine the 75%-WHC we saturated each soil core with water and weighted them after 24 hours, which represented the weight at saturation. Based on the bulk density we estimated the dry weight of the system to calculate the desired WHC.
During the time period of the experiment (July to October 2020) an average temperature of 22.2°C was measured in the greenhouse. A minimum temperature of 11.9°C was reached during the night and a maximum temperature of 40.2°C during the day. The relative humidity was on average 56.5%.

4.3 PESTICIDE TREATMENT

For the treatments, we decided to test six different active ingredients with different mode of action. We have selected commercially available products based on the criterion that each product contains only one active substance. In the following the active substances are listed with the respective product denoted in brackets. For the fungicides, we chose products that include the active substances azoxystrobin (*Amistar*), boscalid (*Filan*) and epoxiconazole (*Ombral*). As herbicides, we chose products with the active substances propyzamide (*GraminEx*), fluazifop-P-butyl (*Fusilade Max*) and flufenacet (*Cadou SC*) since they are suitable for lettuce. We applied the pesticides alone and in different mixtures (**Figure 1**) creating different levels of the pesticide combinations. For each treatment, nine replicates are necessary to account for the variability between the TMEs. The nine replicates were organized in nine blocks, where the systems with the different treatments were randomly arranged in each block.
The pesticide treatments involve all pesticides applied individually (Level 1), two treatments with either the fungicides or the herbicides applied as a triple-cocktail (Level 3) and one treatment containing all pesticides together (Level 6). Furthermore, one negative and one positive control plot was set up. In the negative control, we applied water, respectively N-P-K-fertilizer for positive control because fertilization is known to have a great impact on the soil microbiome and in particular on AMF (Li et al., 2020; Williams et al., 2017).

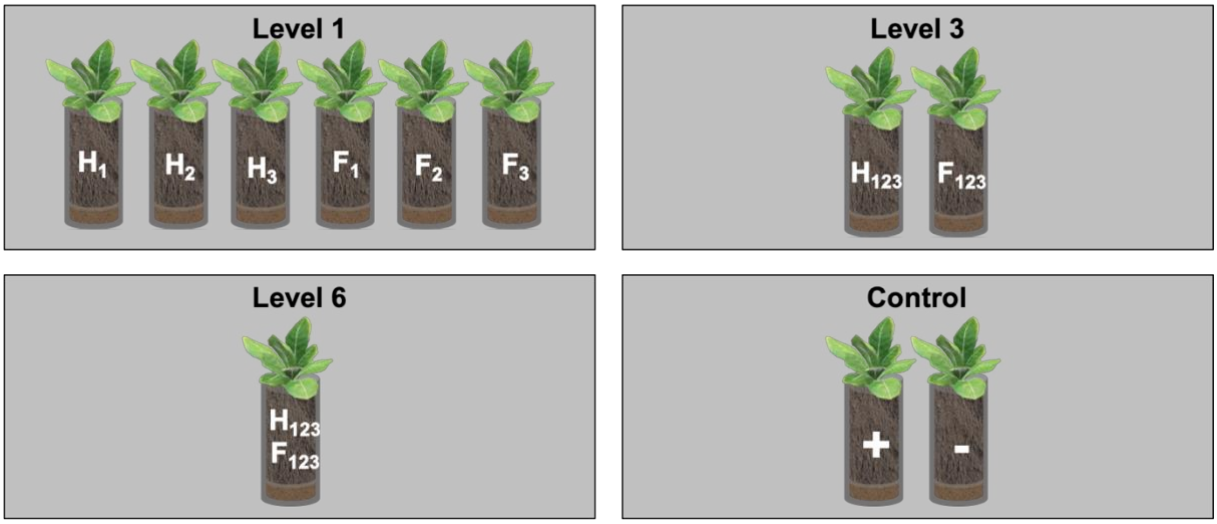

Figure 1 Pesticide treatments visualized according to their levels of application. *The treatments were replicated 9 times and randomly organized in 9 blocks.*

4.3.1 Pesticide Application

We applied the pesticides respectively the control treatments using a spraying chamber (Schachtner spray lab) in order to ensure a uniform distribution of the treatments on the surface of the soil cores. In order to apply the desired dosage spray pressure, spraying distance to the pots and velocity of the slide with the nozzle needed to be adjusted (**Table 2**). After each treatment the liquid tank and nozzle was cleaned carefully in order to avoid a contamination of the treatments.

Table 2 Settings of the Schachtner spray lab according to nozzle calculator for agricultural technology by Lechler.

Speed [km/h]	Spray Pressure [bar]	Spraying angle [°]	Spraying distance [cm]	Spraying width [cm]
2	2.3	80	30	50.4

4.3.2 Pesticide and Fertilizer Dosage

In order to create comparable pesticide dosages and to prevent that, pesticide cocktails have a higher active substance concentration; the dosages were adjusted. For the single applications each products was applied 6 times of the respective maximum recommended dosage (See **Table 17** and **Table 18** in the Appendix), which is in the range of what is applied on average per year in Switzerland for different crops (De Baan et al., 2015). In the triple-cocktails we applied each product twice the maximum recommended dosages. In the cocktail with six active substances we applied each product at the recommended dosage. Therefore, each treatment had the same relative dosage in the end. The negative control will be sprayed with water and the positive control with fertilizer containing N-fertilizer (50 kg/ha), P-fertilizer (4.4 kg/ha) and K-fertilizer (41.4 kg/ha). The dosage of the fertilizer is comparable to what is used on lettuce in the greenhouse (Neuweiler and Kraus, 2017).

4.4 HARVEST OF THE MICROCOSMS

In a non-destructive harvest 20 and 21 days after pesticide application, we sampled soil for analysis with a small soil corer (0.5cm diameter at 5cm depth). In order to standardize the harvesting process we used a template and took three samples in each pot. We mixed the soil samples before storing them at -20°C. Finally, we filled the holes with autoclaved soil from the same grassland where the soil cores originate from in order to keep the system intact. The destructive harvest, which included the sampling of soil and roots, took place 61, 62 and 63 days after pesticide application. Soil samples were taken the same way as in the first harvest using a stencil in order to avoid sampling the same space twice and therefore avoid sampling the newly introduced autoclaved soil from the first harvest.

4.5 SOIL BIOLOGY

4.5.1 DNA Extraction and Estimation of microbial biomass

Soil was lyophilised prior to the DNA extraction at 0.6 mBar at -30°C. The samples were covered by parafilm in order to avoid contamination during the lyophilization process. The reason for the lyophilization of the soil was the advantages in handling and homogenisation of the samples. For the DNA extraction, the manual of the NucleoSpin Soil kit (Macherery-Nagel) was followed. For the extraction 0.150g ±10mg of lyophilised soil is weighted into a bead solution tube provided by the extraction kit. For the cell lysis, lysing buffer SL1 and enhancer SX were used for high yield and purity. For each soil sample the sample lysis was repeated two times in order to enhance the yield and reduce variability between extraction. For three control samples, we did the extraction in duplicates to test for the variability between the samples. For ideal binding conditions, the amount of SB buffer had to be adjusted to the amount of the filtered lysate since the recommended amount was not sufficient.

We embodied the yield of DNA extraction to estimate the microbial biomass. Therefore, the DNA extracts were diluted 1:10 with ddtH_2O and mixed with PicoGreen dsDNA reagent and TE buffer to be analysed on VARIAN. The PicoGreen dsDNA reagent is an ultrasensitive fluorescent nucleic acid stain which specifically allows the quantification of double-stranded DNA (Invitrogen, 2018).

4.5.2 Quantification of different groups of the soil microbiome

Quantitative polymerase chain reaction (qPCR) is a highly sensitive method and well-established approach to quantify taxonomic and functional markers in soil samples based on the real-time detection of DNA amplification via the fluorescence of an added dye (Fierer et al., 2005; Holzapfel and Wickert, 2007). The basic principle of qPCR imitates the DNA-replication and involves three main steps: Denaturation of the DNA double string, annealing of the gene-based specific primers and elongation of the target sequence with the polymerase. This cycle is repeated several times following a temperature profile, which results in an exponential amplification of a gene sequence (PCR-product). The cycle proceeds until the reaction reaches a plateau when a reagent becomes limiting (Holzapfel and Wickert, 2007). For the quantification of the genes in the samples a standard is needed where the concentration

of the reference gene is known. The standard is added to the qPCR plate in an 1:10 dilution series. For each reaction the PCR amplification efficiency (E) and a threshold cycle (Ct) value is calculated. A standard curve can be created using the logarithmic concentrations and the E^Ct value. This allows the calculation of the copy numbers of a gene for each sample where each sample is normally measured in three replicates. In order to perform the qPCR the concentration of the DNA needed to be standardized to 5ng/µl. We used a ready-to-use solution including Eva Green® dye and a hot start polymerase, which means the reaction of the DNA polymerase is blocked at lower temperatures and can only be activated with heat. The components and their used quantities are summarized in **Table 3**.

Table 3 Set up of the qPCR reaction mix for one well in µl for each gene.

	16S	IT S	18S
No of reactions	1	1	1
Hot FIRE Pol + Eva Green® qPCR Mix	4	4	4
BSA (3%)	2	2	2
F-Primer	1	1	1
R-Primer	1	1	1
ddtH2O	11	11	11
Template (5 ng/ul)	1	1	1
Final Volume per well	20	20	20

4.5.2.1 Ribosomal genes

The ribosomal DNA (rDNA) gene-based specific forward and reverse primers are selected according to the taxonomic groups which get analysed (Shahsavari et al., 2016). We analysed three genes: 16S to identify bacteria, 18S for protists and ITS for fungi. The primer pairs and the ideal annealing temperatures are listed in **Table 4**. For 16S and ITS we used commercially available standards that contain plasmids from a pure culture.

For 18S we needed to extract a standard from our soil samples. Therefore, we performed a PCR with the respective primer pairs in order to amplify the 18S gene. The PCR product was afterwards run on a agarose gel in order to split up DNA and remove primer dimer. In our gel two bands were dominant, therefore both bands were cut out for the extraction. We extracted the DNA from the gel according to the manual of the NucleoSpin Gel and PCR Clean-up kit by Macherey-Nagel. Unfortunately, the primers were not ideal and did amplify two PCR products, therefore we decided to mix the extracted standards together. This gives an uncertainty regarding the calculation of the copy numbers, therefore, the values for protist are thought to only be an approximation.

Table 4 Ribosomal gene and their respective primer pairs, sequence and annealing temperature

Target group (*gene*)	Primer pair	Sequence (5' →3')	Annealing Temperature
Bacteria (*16S rDNA*)	338F	ACT CCT GGA GGC AGC AG	60°C
	518R	ATT ACC GCG GCT GCT GG	30sec
Fungi *(ITS rDNA*)	ITS1	CTT GGT TTA GAG GAH GTA	55°C
	ITS2R	GCT GCG TTC TTC ATC GAT GC	30sec
Protists (*18S rDNA*)	563F	GCC AGC AVC YGC GGT AAY	52°C
	1132R	CCG TCA ATT HCT TYA ART	30sec

4.5.3 AMF root colonization

To assess the AMF root colonization we sampled the roots in the last harvest, cut them into 1cm pieces and stored them in 70%-Ethanol. In a next step we rinsed the roots with distilled water, cleared them with 10% KOH incubating at 80°C in a water bath for 15min. The cleared roots were rinsed with distilled water again, stained with trypan blue, and incubated at 80°C for 15min. The coloured roots were stored in 50%-glycerol until they were prepared on a microscopic slide. On each microscopic slide 20-25 root pieces were placed in parallel and analysed under a microscope at 200-fold magnification. We scored the roots for the presence of hyphae, arbuscules and vesicles based on 100 line intersections (Liu et al., 2016). In order to prevent an observer bias the analysis was done blindly.

4.6 SOIL FUNCTIONING

4.6.1 Litter Decomposition

Litter decomposition was determined using tea bags that act as a simplified version of litterbags. The teabag index is currently used for a global survey including citizen science (Keuskamp et al., 2013). For the experiment, we used Lipton rooibos tea and Lipton green tea. Firstly, weighted green-teabags were buried at 8cm depth before pesticide application. In the first harvest, the green tea was retrieved from the soil and adhered soil particles were removed. A rooibos tea replaced the green tea and was removed during the last harvest. The rooibos tea stayed in the system for longer since it has shown a slower decomposition rate compared to green tea. The reason for not putting in the teabags at the same time as suggested by Keuskamp et al. (2013) was that the space in the system was limited. In a next step, we dried the teabags for 48h at 70°C before weighing them again. The loss of material in time is used as an indicator for the decomposition rate (Keuskamp et al., 2013). In order to determine the loss of material more precisely we weighted bag, label and tea separately for 10 replicates, to determine the average weight of label and bag.

4.6.2 Plant biomass and nutrient uptake

Before the pesticide treatment and during the two harvest we cut off and weighted the aboveground biomass. In a next step, we dried the plants at 60°C for 48h to determine the dry biomass. Furthermore, we determined the nutrient uptake of the plants from the last harvest since only those plants reached enough biomass for nutrient analysis. Therefore, the dried biomass was milled into a fine powder (~ 0.75 mm) using a vibrating mill (Retsch MM400) and analysed for the most common plant nutrients (N, P, K) according to the Swiss reference method of the Federal Agricultural Research Station.

4.7 STATISTICAL ANALYSIS

All the statistical analysis and the visualization of data was performed in the R environment (R Core Team, 2020). First the data was checked for normal distribution using the Shapiro-Wilks Test and a QQ-Plot. In case of a non-normal distribution the data was log-transformed. A repeated measure ANOVA was performed to reveal the effects of pesticide treatments for the variables that were measured two times in the curse of the experiment using the *lmer*-function (lme4 package). For the data which only reflected on one timepoint a linear model (*lm*-function) was created and analyzed with a two-way ANOVA. For the functional parameters a backward selection was performed to find the best fitting model including biological parameters, block and treatments. The ANOVA was followed by a post hoc pairwise comparison of the estimated marginal means to reveal significant differences between treatments (emmeans package). Further the effect size of each variable was determined using partial eta squared in order to evaluate how much of the variability is explained by the predictors. To unravel relationships between the biological endpoints and the soil functioning a Pearson's correlation test was conducted to examine how the different parameters do correlate. In all statistical analysis a significance level of $\alpha = 0.05$ was considered to represent significant differences.

5 RESULTS

5.1 SOIL BIOLOGY

A repeated measure ANOVA was conducted to compare the main effects of treatment and time of harvest as well as their interaction on the molecular parameters (DNA yield, ITS, 16S and 18S copy number per g soil).

5.1.1 DNA yield

For the DNA yield per g soil which acts as a proxy for microbial biomass we found a significant main effect for the time of harvest ($p<0.001$) which yielded in $\eta^2 = 0.10$ indicating that 10% of the variability of the DNA yield can be explained by the time point of harvest (**Table 6**). The microbial biomass significantly increased over time. However, there was no significant main effect of treatment nor the interaction between time point and treatment (**Table 5**).

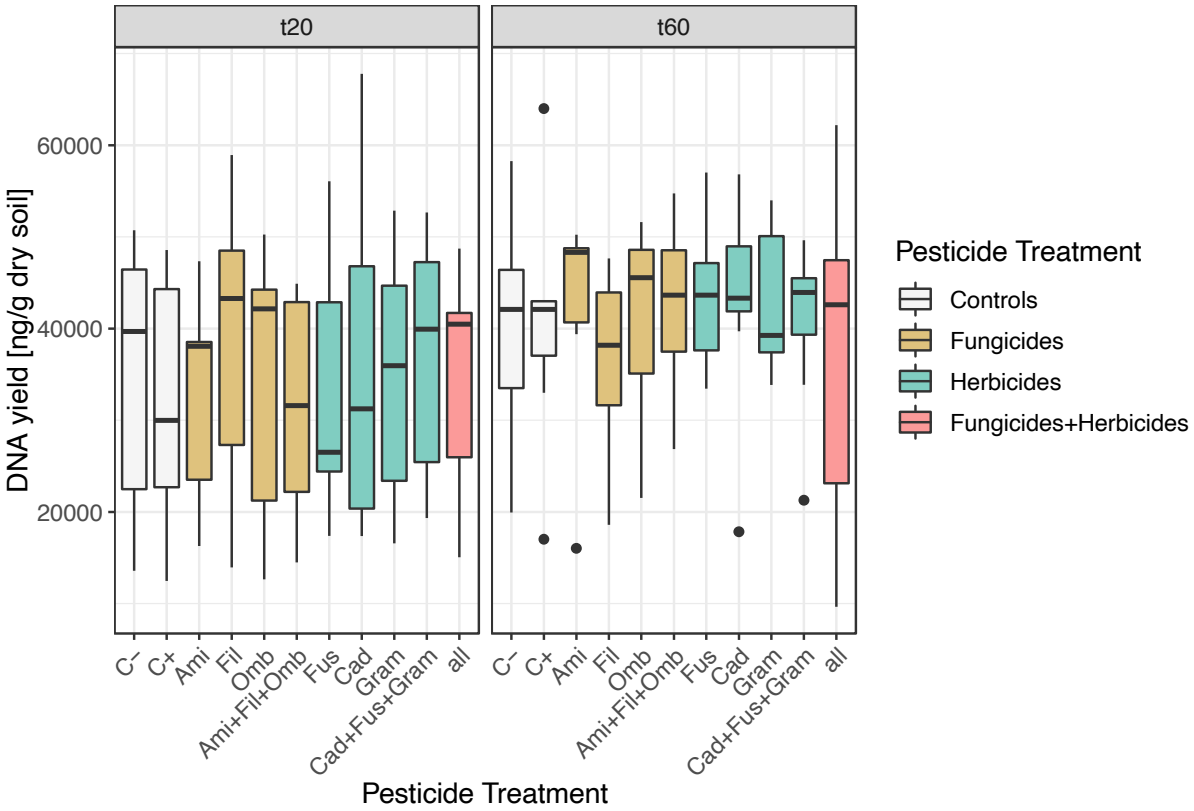

Figure 2 DNA yield per gram soil under different pesticide treatments. *The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. A significant effect of the timepoint of harvest was found ($F_{1,198}=19.5$, $p<0.001$), but there was no significant treatment effect ($F_{10,198}=0.208$, $p=0.995$).*

Although we have not reported significant differences between the treatments, the results suggest that the fungicide mix (31755ng/g dry soil ± 10895) resulted in marginally lower mean DNA yield compared to the negative control (34053 ng/g dry soil ±14969) as well as its components Amistar (32850 ng/g dry soil ± 11104) and Ombral (33393 ng/g dry soil ±14013) in the first harvest. The herbicide mix (36611 ng/g dry soil±12993) and the cocktail containing all pesticides (34770 ng/g dry soil ± 11861) resulted

in a slightly higher mean DNA yield. For the second harvest only the treatment with Filan (36405ng/g dry soil ± 10109), the cocktail containing all pesticides (38412 ng/g dry soil ±17084) and the positive control (40306 ng/g dry soil ± 12206) resulted in moderately lower microbial biomass than the negative control (40382 ng/g dry soil ±11366). Overall we can see that the variances for all the treatments were high in the first harvest and tended to decrease in the second harvest.

Table 5 Summary of Type III Analysis of Variance using Kenward-Roger's method for DNA yield as response variable.

	SumSq	MeanSq	NumDF	DenDF	F value	Pr(>f)	Sig
DNA yield							
t	2295716960	2295716960	1	87.721	19.5074	2.849e-05	***
treatment	245409244	24540924	10	79.765	0.2085	0.9950	
t:treatment	654199946	65419995	10	87.693	0.5559	0.8452	

*Signif. codes: ='***',0.001'**, 0.01 '*',0.05'.', 0.1' '1*

Table 6 Effect size for the parameter timepoint of harvest, treatment and their interaction explained by partial eta squared.

	η^2 (partial)	90% CI
DNA yield		
t	0.10	[0.04, 0.18]
treatment	0.01	[0.00, 0.00]
t:treatment	0.03	[0.00, 0.02]

5.1.2 Treatment Effect on the abundance of different microbial groups

5.1.2.1 Abundance of Fungi in the Soil

We found a significant main effect for the timepoint of harvest ($p < 0.001$) as well as a significant main treatment effect ($p=0.027$) on the fungal abundance (**Table 7**). The main effect of the harvest resulted in an effect size of $\eta^2 = 0.24$, therefore 24% was explained by the timepoint of harvest. The ITS copy numbers did significantly increase from the first to the second harvest. The treatment yielded in an effect size of $\eta^2 = 0.12$ implying that 12% of the variability was explained by the treatment (**Table 8**).The interaction effect was not significant ($p =0.50$) indicating that there was difference in the treatment effects between the harvests.

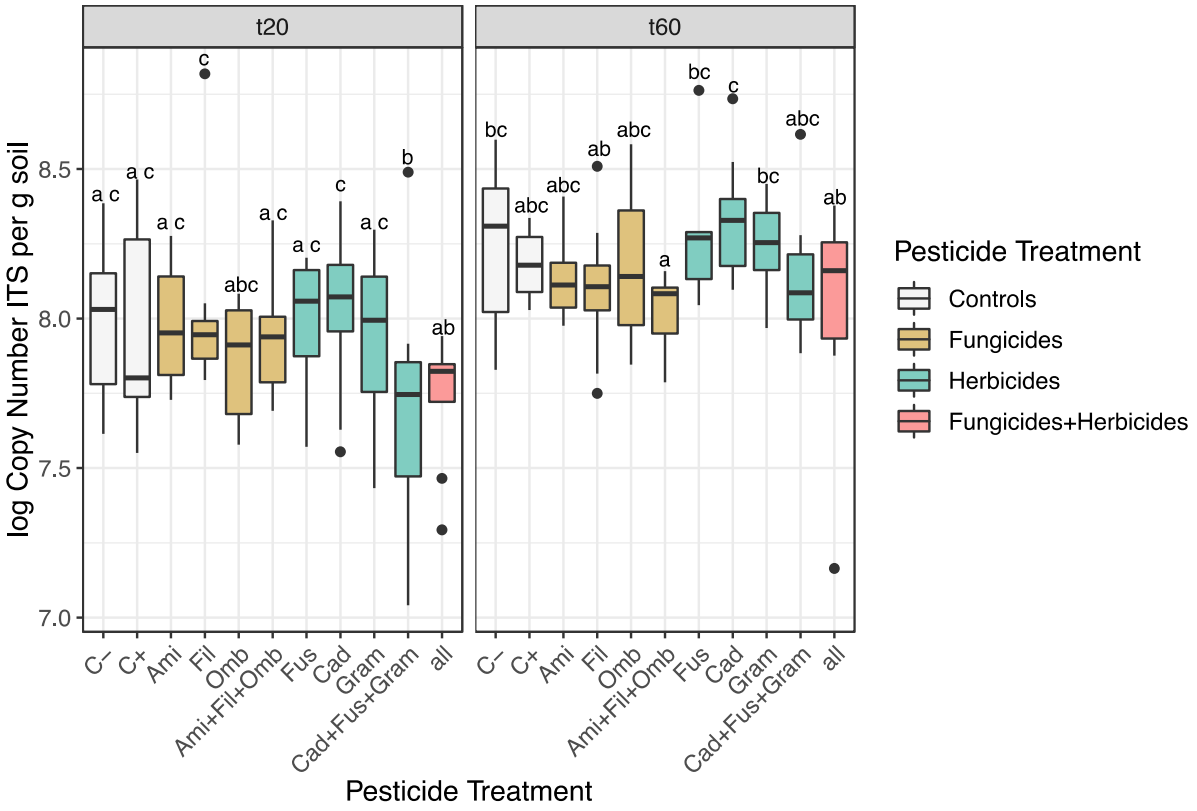

Figure 3 Fungal copy number per gram soil under different pesticide treatments for the first harvest (t20) and the second harvest (t60). *The variables are log transformed. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. There was a significant effect of the timepoint of harvest ($F_{2,196}$=54.04,, p<0.001) and the treatment ($F_{10,196}$=2.22,, p=0.025), there was no significant time-treatment interaction ($F_{10,196}$=0.96,, p=0.480). Different letters above the boxplots indicate a significant difference (P ≤ 0.05) among the means of ITS copy numbers. The letters of the harvest cannot be compared to each other.*

A pairwise comparison among the treatments of the fungal abundance in the first harvest revealed that only the herbicide mix resulted in significantly lower copy numbers than the negative control (p =0.022) as well as the positive control (p=0.021). This was not significant in the second harvest anymore. Further in the first harvest, the herbicide mix resulted in significantly lower ITS copy numbers than its components Fusilade Max (p=0.014), Cadou SC (p=0.004) and GraminEx (p=0.030), the fungicides Amistar (p= 0.015) and Filan (p=0.005) and the fungicide mix (p=0.025). Only Ombral and the cocktail containing all pesticides did not significantly differ from the herbicide mix.

The second harvest shows similar tendencies regarding the herbicide mix, however, the differences are not significant anymore. In the second harvest we found the fungicide mix resulting in significantly lower copy numbers than the negative control (p=0.046) and the herbicides GraminEx (p=0.042), Fusilade Max (p=0.029) and Cadou SC (p=0.006). We can observe a non-significant trend of the fungicide mix resulting in marginally lower copy numbers than its components.

The cocktail combining all pesticides tended to result in slightly lower copy numbers than its components in the first harvest and interestingly is always within the same range of the herbicide mix in both harvests.

5.1.2.2 Abundance of Bacteria in the Soil

We found a significant main effect of the timepoint on bacterial abundance (p = 0.004) explaining 5% of the variability for 16S copy number. The bacterial copy numbers did significantly increase from the first to the second harvest. However, no significant effect for treatment was found (p=0.246) nor for the interaction between treatment and timepoint of harvest (p=0.522)(**Table 7** and **Table 8**).

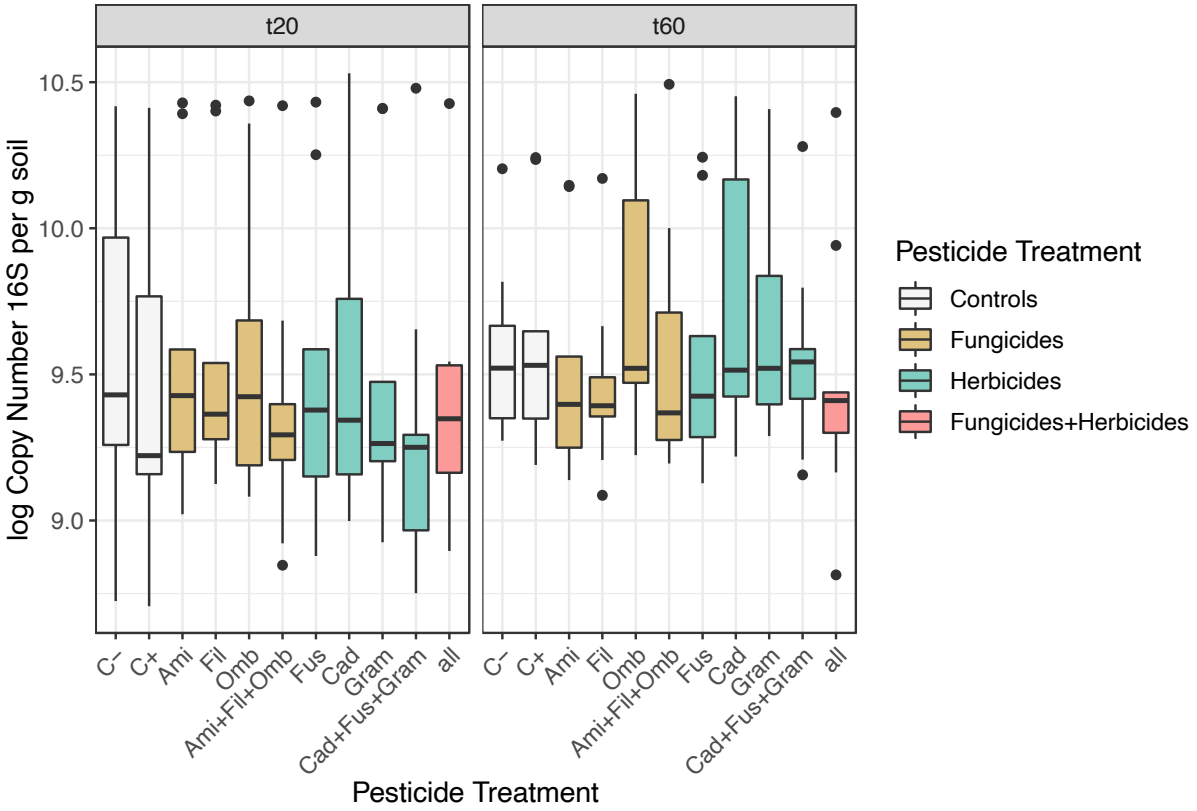

Figure 4 Bacterial copy number per gram soil under different pesticide treatments for the first harvest (t20) and the second harvest (t60). *The variables are log transformed. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. A significant effect of the timepoint of harvest was found ($F_{1,197}$=8.42, p=0.004), but there was no significant treatment effect ($F_{10,197}$=1.29, p=0.522) and the interaction between treatment and time remained unsignificant(($F_{10,197}$=0.91, p=0.004).*

Even though we found no significant effect of the treatments we found a tendency in the first harvest that the herbicide mix, fungicide mix and the cocktail containing all pesticides led to slightly lower mean copy numbers compared to their single components. Just looking at the cocktails the herbicide mix and fungicide mix tended to marginally lower bacterial abundance than the pesticide cocktail containing both pesticide classes. These trends are not significant and do not apply to the second harvest. In the second harvest the pesticide cocktail containing all products led to a nonsignificant decrease in copy numbers compared to the herbicide mix and the fungicide mix. It is to note that we found a lot of outliers for the16S copy numbers.

5.1.2.3 Abundance of Protists in the Soil

A significant main effect of time (p = 0.021) was found on the abundance of protists which yielded in an effect size of η^2 = 0.03, explaining 3% of the variance of 18S copy number per gram soil (**Table 7**

and **Table 8**) We found the copy number of protist to decrease over time. No significant main effect of treatment (p= 0.637) nor the interaction between treatment and time (p =0.766) was found.

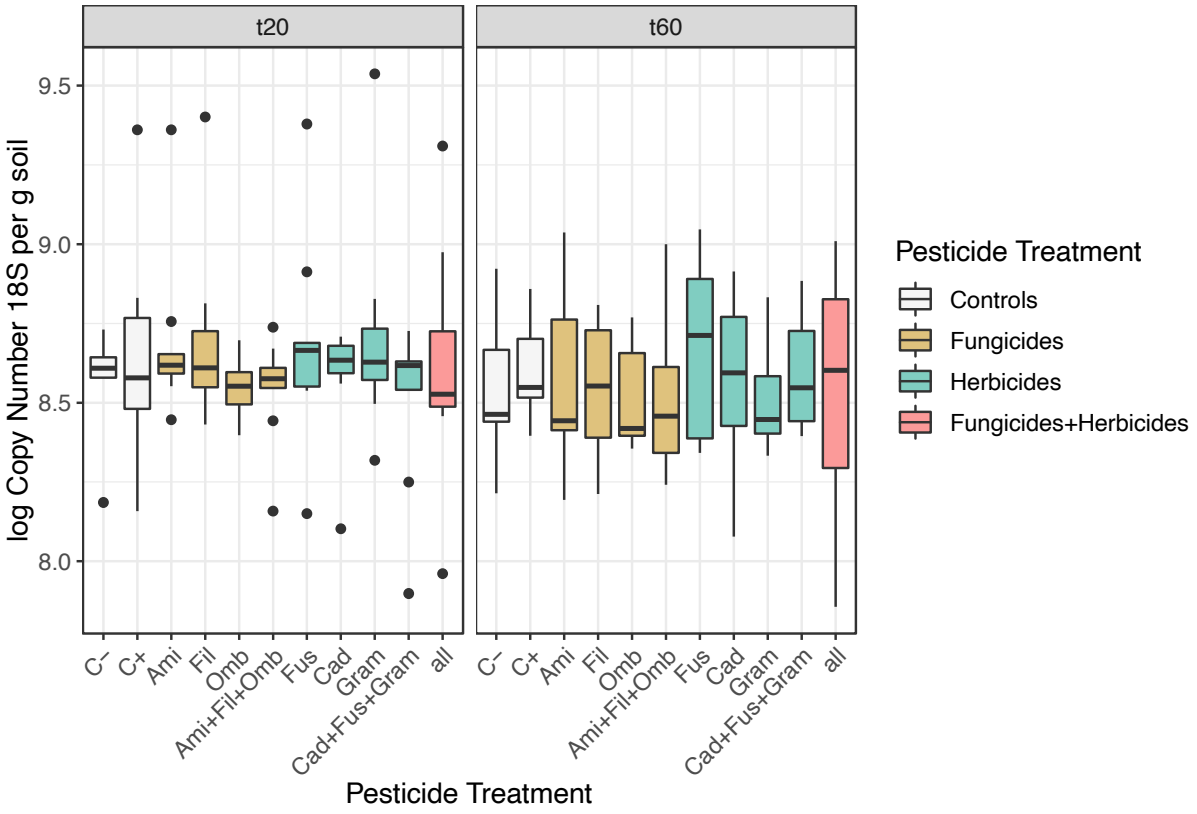

Figure 5 Protist copy number per gram soil under different pesticide treatments for the first harvest (t20) and the second harvest (t60). *The variables are log transformed. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. A significant effect of the timepoint of harvest was found ($F_{1,197}$=5.53, p=0.019), but there was no significant treatment effect ($F_{10,197}$=0.79, p=0.63) and the interaction between treatment and time remained unsignificant(($F_{10,197}$=0.65, p=0.762).*

In the first harvest we can find the indication that the pesticide cocktails result in marginally lower copy numbers than their components. In the second harvest a nonsignificant trend of the fungicide mix resulting in lower 18S mean copy number than its components can be observed.

Table 7 Summary of Type III Analysis of Variance with Kenward-Roger's method for copy number 16S, ITS and 18S per gram soil as response variables.

	SumSq	MeanSq	NumDF	DenDF	F value	Pr(>f)	Sig
log10(ITS)							
t	16.1618	16.1618	1	87.443	54.0449	9.857e-11	***
treatment	6.6267	0.6627	10	79.537	2.2159	0.02487	*
t:treatment	2.8836	0.2884	10	87.393	0.9643	0.48015	
log10(16S)							
t	3.0581	3.05807	1	87.718	8.4212	0.004689	**
treatment	4.7129	0.47129	10	79.747	1.2978	0.246147	

t:treatment	3.3265	0.33265	10	87.690	0.9160	0.522386	
log10(18S)							
t	1.1781	1.17812	1	165.98	5.5331	0.01983	*
treatment	1.6873	0.16873	10	165.97	0.7925	0.63608	
t:treatment	1.3861	0.13861	10	165.97	0.6510	0.7624	

*.Signif. codes: ='***',0.001'**, 0.01 '*',0.05'.', 0.1' '1.*

Table 8 Effect size for the parameter timepoint of harvest, treatment and their interaction explained by partial eta squared.

	η^2 (partial)	90% CI
log(ITS)		
t	0.25	[0.16, 0.33]
treatment	0.12	[0.01, 0.15]
t:treatment	0.05	[0.00, 0.06]
log(16S)		
t	0.05	[0.01, 0.11]
treatment	0.07	[0.00, 0.09]
t:treatment	0.05	[0.00, 0.06]
log(18S)		
t	0.03	[0.00, 0.09]
treatment	0.05	[0.00, 0.05]
t:treatment	0.04	[0.00, 0.03]

5.1.2.4 Effect of Pesticide Levels on Soil Microbial Groups

To get a deeper understanding how pesticide levels affected the microbial groups we also conducted a repeated measure ANOVA to unravel the effects of pesticide levels. The pesticide levels do refer to the different levels of pesticide combinations (e.g. Level 1 for only single substances, level 3 for the herbicide respectively the fungicide mix and level 6 for the cocktail combining all the pesticides) For the fungal abundance a significant main effect of pesticide level ($F_{6,196}$=3.54, p= 0.003) was found. The treatment-time-interaction remained not significant ($F_{6,196}$=1.26, p=0.283). A pairwise comparison of the different pesticide levels over both timepoints revealed that the level six pesticide cocktail (p=0.0146), and the herbicide mix (p=0.021) do significantly differ from the negative control. The mix containing herbicides and fungicides was significantly different to the herbicide (p<0.001) and fungicides (p=0.032) applied alone (**Figure 6**), however, did not significantly differ from the level 3 treatments. Also the herbicides applied as a mixture did significantly differ from herbicide's applied alone (p=0.001).

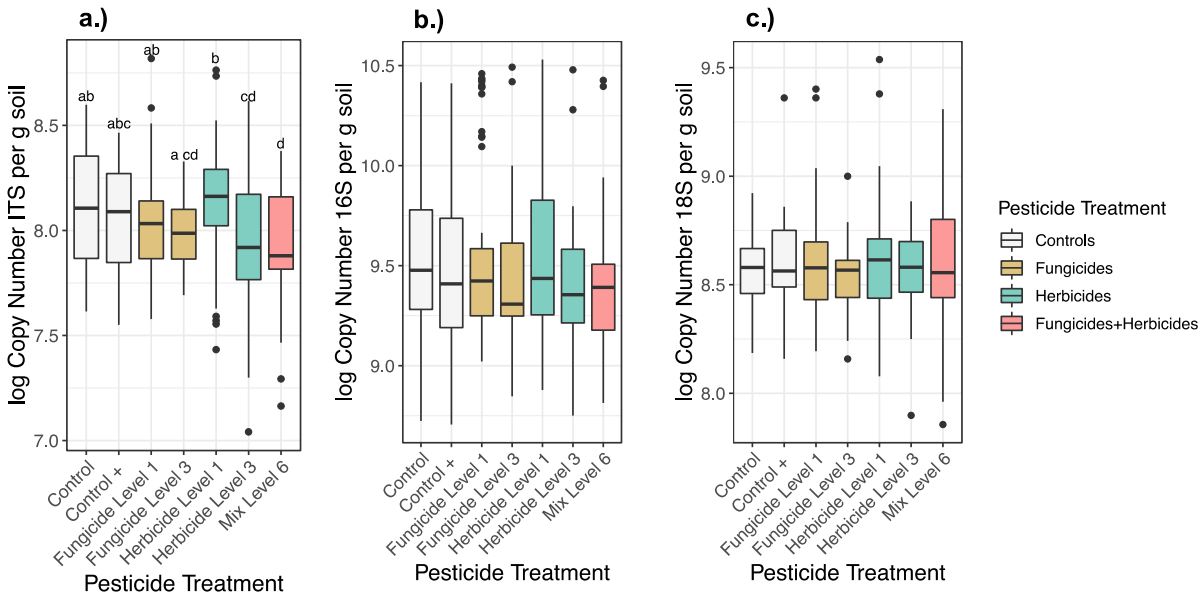

Figure 6 Microbial Gene copy number per gram soil for the different levels of treatments applied. *The variables are log transformed. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. The levels refer to the different pesticide combinations (Level 1 for the single applications, Level 3 for the herbicide or fungicide mix and Level 6 for the cocktail combining all the pesticides).* ***a.)*** *For fungal abundance we found a significant main effect of timepoint of harvest ($F_{1,196}$=45.114, p<0.001) and pesticide level ($F_{6,196}$=3.543 p= 0.003), the interaction between treatment and harvest remained unsignificant ($F_{6,196}$=1.26, p=0.283). Different letters above the boxplots indicate a significant difference (P ≤ 0.05) among the means of ITS copy numbers.* ***b.)*** *For bacterial copy number no significant effect of pesticide level ($F_{6,197}$=1.447, p=0.207) was found.* ***b.)*** *For protist copy number no significant effect of pesticide level ($F_{5,196}$ = 0.0247, p=0.6807) was found*

For 16S a significant main effect of the time of harvest was found ($F_{1,197}$=9.378, p =0.0047), but no significant effect of the pesticide level ($F_{6,197}$=1.447, p=0.207), nor the interaction ($F_{6,197}$ = 0.911, p= 0.491). For 18S neither a significant main effect of pesticide level ($F_{5,196}$ = 0.0247, p=0.6807), time of harvest ($F_{1,196}$ = 2.0606, p=0.1545) nor their interaction ($F_{5,196}$=0.782, p=0.565) was found.

5.2 AMF ROOT COLONIZATION

A two-way ANOVA was performed to compare the main effect of treatments and blocking for the AMF root colonization sampled in the second harvest. We examined the effects on the total root colonization, hyphal and arbuscular colonization and could not find a significant effect of the treatments. There was no significant main effect of the block on the total and the hyphal root colonization. For the arbuscular root colonization a significant main effect of the block was detected (p=0.037). The results for the ANOVA can be found in **Table 9** and the effect size of the tested predictors in **Table 10.**

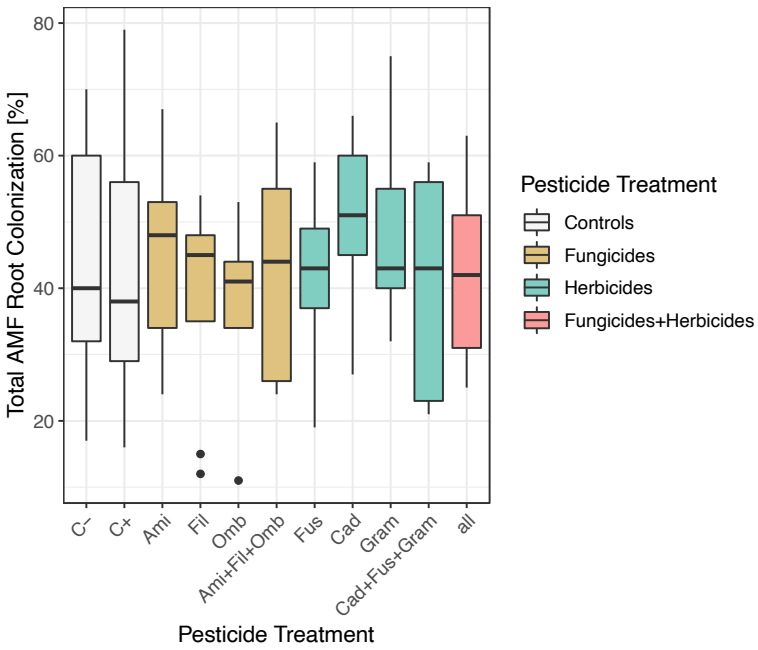

Figure 7 Total root colonization by AMF for the different treatments applied. *The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. No significant effect of treatment ($F_{10,99}$, p=0.890)) nor block ($F_{8,99}$, p=0.487) was found.*

Even though no significant effect of the treatments can be found slight trends are observable. The total colonization for the treatments is slightly decreasing from Amistar (44.4%±14.5) to Ombral (38.9%±12.6) to Filan (38.8%±15.4). The herbicide single applications GraminEx (47.6%±13.6), Cadou SC (49.4% ±13.8) and Fusilade Max (43%±11.9) tended to a slightly higher mean root colonization than the herbicide mix (41.9%± 13.3). We found a very high variability in the data which ranged from 11%-79% total colonization.

Table 9 Summary table of the two-way Analysis of Variance for the total root colonization, hyphal root colonization and arbuscular root colonization as response variables.

	DF	SumSq	Mean Sq	F value	Pr(>f)	Sig
Total colonization						
block	8	1773	221.7	0.942	0.487	
treatment	10	1160	116.0	0.493	0.890	
Residuals	80	18817	235.2			
Hyphal root colonization						
block	8	948	118.50	2.188	0.0369	*
Treatment	10	332	33.23	0.613	0.7981	
Residuals	80	4333	54.17			
Arbuscular root colonization						
block	8	659	82.36	0.612	0.765	

Treatment	10	1117	111.74	0.830	0.601
Residuals	80	10769	134.61		

*Signif. codes: ='***',0.001'**, 0.01 '*',0.05'.', 0.1' '1*

Table 10 Effect size for the parameter block and treatment explained by partial eta squared.

	η^2 (partial)	90% CI
Total root colonization		
block	0.09	[0.00, 0.12]
treatment	0.06	[0.00, 0.03]
Hyphal root colonization		
block	0.18	[0.01, 0.25]
treatment	0.07	[0.00, 0.06]
Arbuscular root colonization		
block	0.06	[0.00, 0.06]
treatment	0.09	[0.00, 0.10]

5.3 SOIL FUNCTIONING

To analyze the functional implication of pesticide treatments we applied a two-way ANOVA for the response variables teabags, plant biomass, and nutrient content per plant to unravel whether the pesticide application the blocking or any biological parameter did have a significant effect on the proxies for soil functioning.

5.3.1 Litter Decomposition

No significant main effect of treatment nor block was found for the decomposition of both kinds tea. The results of the ANOVA are summarized in **Table 11**. The effect size of the tested predictors can be found in **Table 12**. None of the biological parameters did exhibit a significant effect on the decomposition of green tea and rooibos tea and therefore were not included in the model.

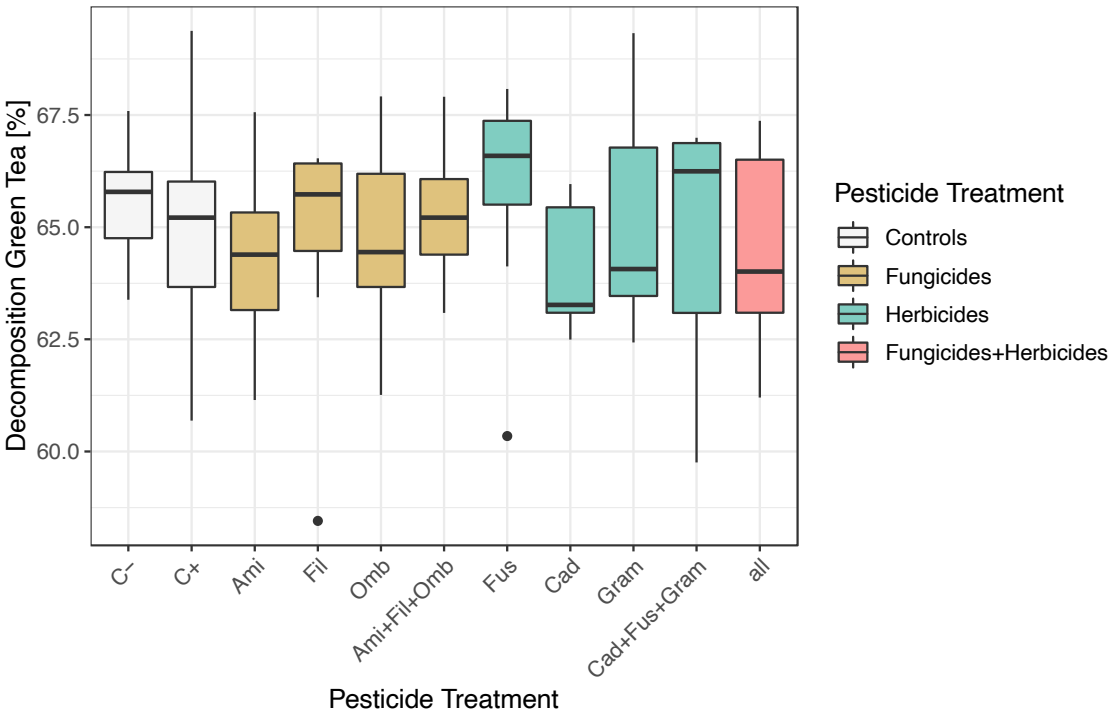

Figure 8 Decomposition of green tea under different treatments applied. *The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. No significant blocking effect ($F_{8,99}$ =1.26 , p=0.275) or treatment effect ($F_{11,99}$ = 0.54, p=0.858) was found.*

Even though no significant effect of the treatments were found, we observed that the decomposition of green tea in the negative control showed the highest mean decomposition (65.6% ± 1.29) whereas the single application of the herbicide Cadou SC showed the lowest mean decomposition (64.1% ± 1.43). The herbicide mix (64.9% ± 2.52) showed a slightly lower mean decomposition than the fungicide mix (65.4% ± 1.53). The pesticide cocktail containing herbicides and fungicides showed marginally lower mean decomposition (64.4 ± 2.24) than the other pesticide mixtures.

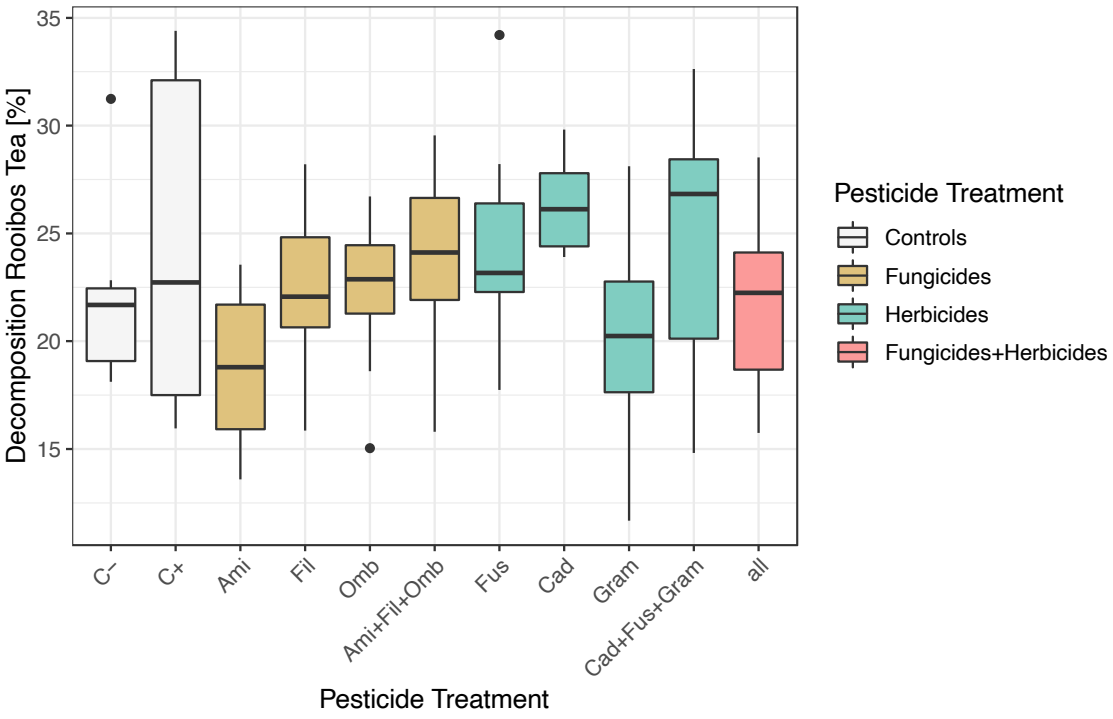

Figure 9 Decomposition of rooibos tea under different treatments applied. *The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. No significant effect of treatment ($F_{10,99}$=0.49 p=0.890)) nor block ($F_{8,99}$=0.94 p=0.487) was found on total root colonization*

Despite of having no significant treatment effect for rooibos tea, we observed that the fungicide mixture resulted in a slightly higher mean decomposition (23.4% ± 4.9) than its components Amistar (18.7% ± 3.93), Filan (22.3% ± 3.51) and Ombral (22.2% ± 3.55). A similar direction can be found for the herbicides, where the mix resulted in a moderately higher mean decomposition (24.5% ± 6.16) than its components Cadou SC (26.4% ± 2.23), Fusilade Max (24.6 ± 4.68) and GraminEx (20.1 ± 5.05). When comparing all the mixtures the cocktail containing both pesticide classes resulted in a moderately lower mean decomposition (21.5% ± 4.32) than the herbicide mix and the fungicide mix. The negative control showed a mean decomposition of (22% ± 4.12)

Table 11 Summary of the two-way Analysis of Variance table for the response variables decomposition green tea and rooibos.

	DF	SumSq	Mean Sq	F value	Pr(>f)	Sig
Green Tee						
Block	8	47	5.874	1.263	0.275	
treatment	10	25	2.504	0.538	0.858	
Residuals	80	372.1	4.651			
Roiboos						
Block	8	257.4	32.18	1.427	0.200	

treatment	10	347.6	34.76	1.542	0.142
Residuals	74	1668.5	22.55		

*Signif. codes: ='***',0.001'**, 0.01 '*',0.05'.', 0.1' '1*

Table 12 Effect size for the parameter block and treatment explained by partial eta squared.

	η^2 (partial)	90% CI
Green tea		
Block	0.11	[0.00, 0.16]
treatment	0.06	[0.00, 0.04]
Rooibos tea		
Block	0.13	[0.00, 0.19]
treatment	0.17	[0.00, 0.22]

5.3.2 Plant biomass and nutrient uptake

5.3.2.1 Above ground biomass

The distribution of the residuals for a repeated measures ANOVA were not normally distributed even after a log- transformation, therefore, dry biomass was analyzed by two individual ANOVAs for each timepoint since they individually met the normality assumption.

The dry biomass from the first harvest revealed a significant main effect of block and the treatment (**Table 13**) explaining 21% respectively 55% of the variance. A pairwise comparison did reveal a significant reduction of the dry biomass in the positive control (p=0.0001) the single fungicides Amistar (p <0.0001), Filan(p =0.044), Ombral (p<0.0001) and the fungicide cocktail (p=0.0017) compared to the negative control. For the single herbicide applications only Cadou SC (p<0.0001) resulted in significant lower biomass. The herbicide mixture (p=0.0001) as well as the cocktail combining fungicides and herbicides (p=0.0001) resulted in a significant decrease in biomass compared to the negative control. Further the herbicide mixture did significantly differ from its components. In total 9 plants needed to be replanted from a backup pot because they got eaten by a wire worm or did not survive the treatment application of fertilizer and Cadou SC.

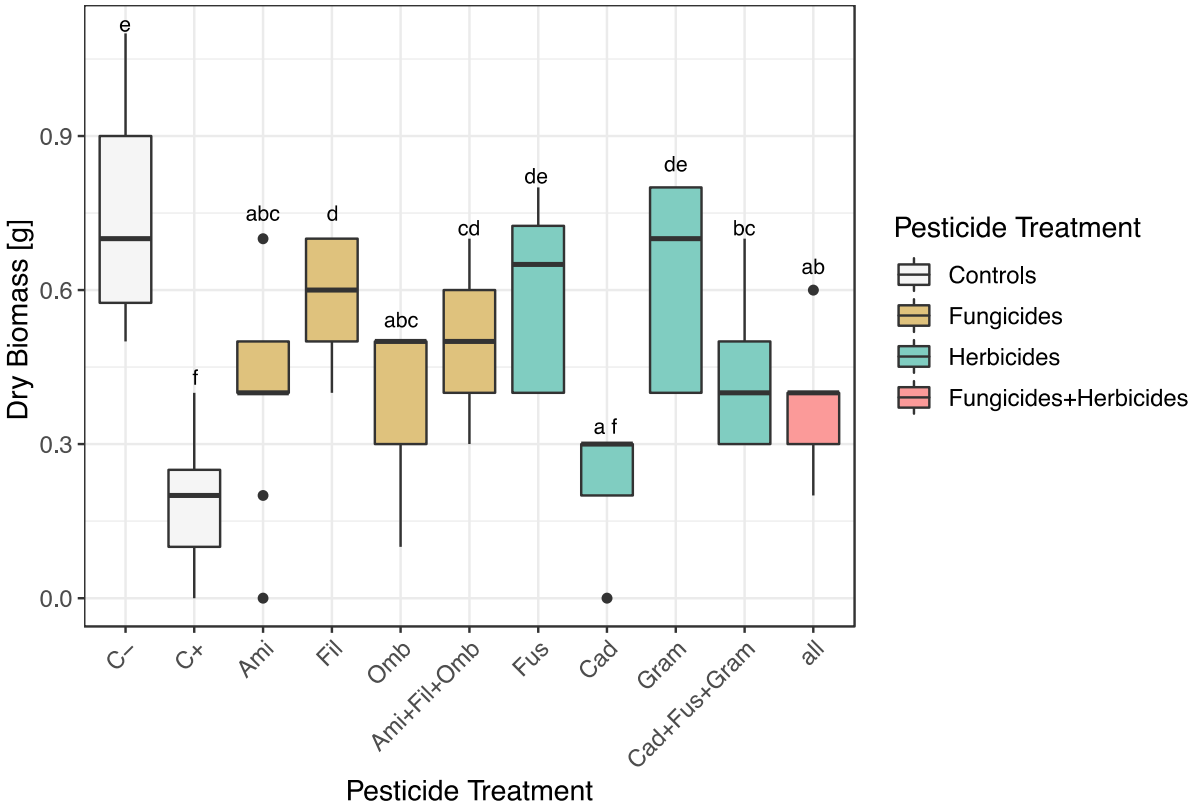

Figure 10 Biomass of lettuce at the first harvest. *The biomass is given in dry weight after 20 days of growth after application of different treatments. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. A significant main effect of block ($F_{8,98}$ = 2.39, p=0.024) and treatment ($F_{10,91}$ = 8.85 p<0.001) was found. In total 9 plants needed to be replanted and therefore are omitted in this analysis. Different letters above the boxplots indicate a significant difference (p≤ 0.05) among the means of dry biomass.*

The ANOVA for dry biomass from plants harvested at the second timepoint revealed a significant main effect of treatment and block (**Table 13**), where the treatment explains 59% of the variance of dry biomass (**Table 14**). A pairwise comparison between the treatments revealed a significant reduction in biomass for the plants treated with Cadou SC (p <0.0001) and Ombral (p <0.0001). Further the treatment Amistar (p= 0.0183), the fungicide mixture (p= 0.007), the herbicide mixture (p= 0.0191) and the cocktail containing all the pesticides (p= 0.0104) did result in significantly lower biomass than the negative control. Similar to the first harvest the application of fertilizer (p <0.001) led to significantly lower biomass than the negative control.

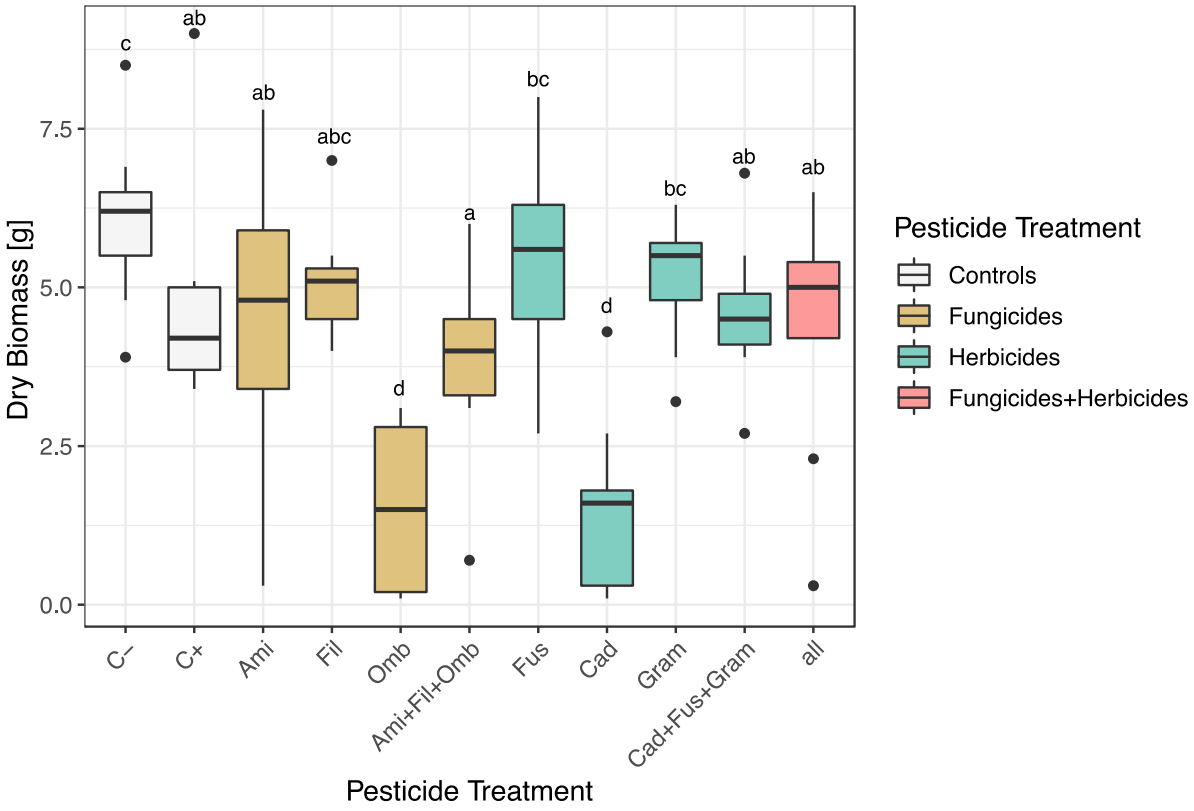

Figure 11 Biomass of lettuce at the second harvest. *The biomass is given in dry weight after 60 days of growth after application of different treatments. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. A significant main effect of block ($F_{8,99}$ = 4.51, p<0.001) and treatment ($F_{10,99}$ = 11.58, p<0.001) was found. Different letters above the boxplots indicate a significant difference (p≤ 0.05) among the means of dry biomass.*

Table 13 Summary of the two-way Analysis of Variance table for the response variable biomass for the first (t20) and the second harvest(t60).

	DF	SumSq	Mean Sq	F value	Pr(>f)	Sig
Dry biomass t20						
Block	8	0.4365	0.0546	2.387	0.0243	*
treatment	10	2.20231	0.20231	8.849	2.71e-09	***
Residuals	72	0.02286	0.02286			
Dry biomass t60						
Block	8	62.14	7.767	4.519	0.000146	***
treatment	10	199.05	19.905	11.582	5e-12	***
Residuals	80	137.49	1.719			

*Signif. codes: ='***',0.001'**, 0.01 '*',0.05'.', 0.1' '1*

Table 14 Effect size for the parameter block and treatment explained by partial eta squared.

	η^2 (partial)	90% CI

Dry biomass t20		
Block	0.21	[0.02, 0.29]
treatment	0.55	[0.38, 0.63]
Dry biomass t60		
Block	0.31	[0.12, 0.0]
treatment	0.59	[0.45, 0.66]

5.3.2.2 Pesticide effect on nutrient uptake

The nutrient uptake was only analyzed for plants which were harvested at the second timepoint, because the plants from the first harvest were too small to be analyzed for nutrient content. Further only two plants from the treatment Cadou SC and 4 from Ombral were big enough for nutrient analysis In total 84 plants were analyzed for nutrients.

The **nitrogen** content in plants was not significantly affected by the block (p=0.171), whereas the treatment almost resulted in a significant effect (p=0.059) (**Table 15Potassium content per plant under different treatments**. The boxplot displays the potassium content from lettuce harvested 60 days post application. The values got log transformed for the analysis. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. No significant differences between the ($F_{11,84}$=2.35, p=0.019) and blocks ($F_{8,84}$=2.12, p=0.046) were found.). 23% of the variance in the data can be explained by the treatment (**Table 16**). Even though no significant difference between the treatments was detected we observed that the fertilizer treatment did result in slightly higher mean N content in plants than all the other treatments. There were only marginal differences among the pesticide single treatments and their cocktails. In general the pesticide treatments tended to rather lower N content compared to the controls. We suggest that the plants treated with Cadou SC and Ombral resulted in the lowest N contents (**Figure 12**).

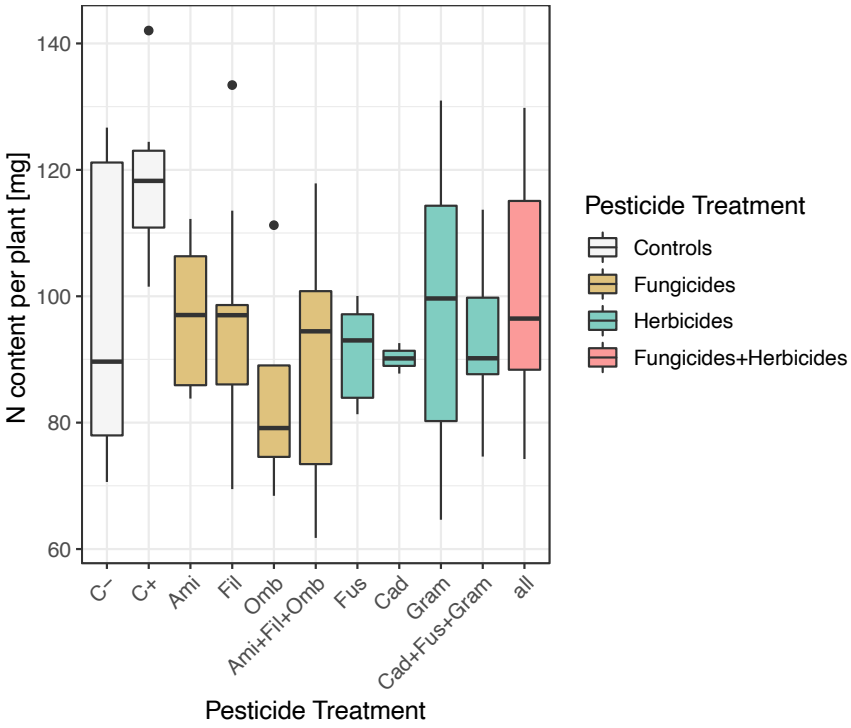

Figure 12 Nitrogen content per plant under different treatments*. The boxplot display the nitrogen content from lettuce harvested 60 days post application. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. No significant differences between the treatments ($F_{11,84}$=1.91, p=0.059) nor blocks ($F_{8,84}$=1.51, p=0.171) was found.*

The **phosphorous** content in plants was significantly affected by the treatment (p=0.019) and the block (p=0.046). We also found a significant effect of the 18S copy number (p=0.025) on the phosphorous content of lettuce (**Table 15Potassium content per plant under different treatments**. The boxplot displays the potassium content from lettuce harvested 60 days post application. The values got log transformed for the analysis. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. No significant differences between the ($F_{11,84}$=2.35, p=0.019) and blocks ($F_{8,84}$=2.12, p=0.046) were found.). A pairwise comparison revealed a significantly lower P content in the plants treated with fertilizer (p= 0.007) compared to the negative control. Obral (p=0.001) and Cadou SC (p<0.001) also resulted in significantly lower P content than the negative control. The fungicide treatments do not significantly differ from each other, nor does the fungicide mix. For the herbicide treatments only Cadou SC led to significantly lower P content than Fusilade Max (p=0.027) and GraminEx (p=0.021).

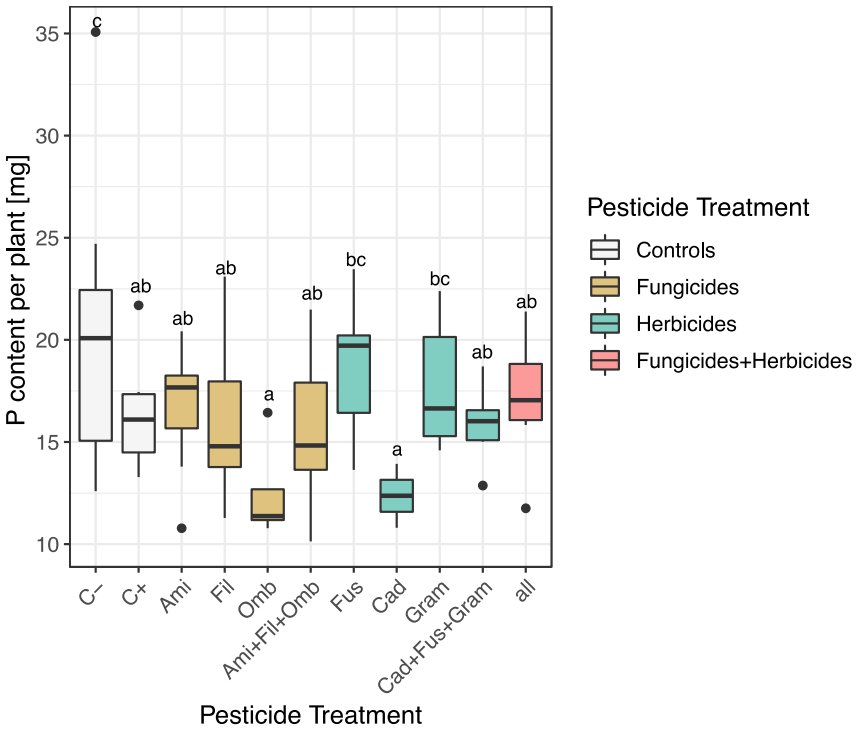

Figure 13 Phosphorous content per plant under different treatments. *The boxplot displays the phosphorous content from lettuce harvested 60 days post application. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. Significant effects were found treatments ($F_{11,84}$=2.5, p=0.013), 18S($F_{11,84}$=5.27, p=0.025) and blocks ($F_{8,84}$=2.26, p=0.034) were found. Different letters above the boxplots indicate a significant difference (p≤ 0.05) in the mean P content among different treatments.*

The **potassium** content in plants was not significantly affected by the treatment (p=0.008) nor by the block (p=0.502). Even though no significant differences between the treatments were detected, we found Cadou SC and Ombral to result in moderately lower K content compared to the other treatments. For the fungicides we observed a nonsignificant increase of K in the plants from the single application to the cocktail.

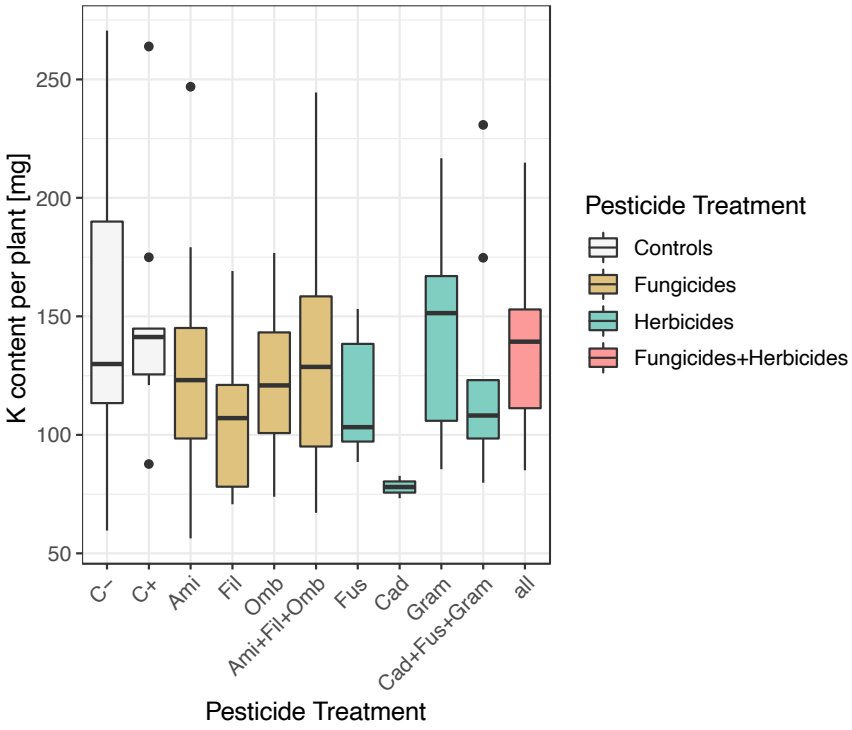

Figure 14 Potassium content per plant under different treatments. *The boxplot displays the potassium content from lettuce harvested 60 days post application. The values got log transformed for the analysis. The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red. No significant differences between the ($F_{11,84}$=2.35, p=0.019) and blocks ($F_{8,84}$=2.12, p=0.046) were found.*

Table 15 Summary of the two-way Analysis of Variance table for the response variables nitrogen, phosphorous, potassium.

	DF	SumSq	Mean Sq	F value	Pr(>f)	Sig
N (g)						
block	8	0.003336	0.0004171	1.508	0.1717	
treatment	10	0.005283	0.0005283	1.911	0.0594	.
Residuals	65	0.017523	0.0002738			
P (g)						
block	8	0.0001989	2.486e-05	2.262	0.0339	*
treatment	10	0.0002750	2.750e-05	2.503	0.0132	*
18S	1	0.0000579	5.791e-05	5.269	0.0250	*
Residuals	64	0.0007034	1.099e-05			
log(K g)						
block	8	1.066	0.1333	1.079	0.389	
treatment	10	1.163	0.1163	0.941	0.502	
Residuals	65	8.031	0.1236			

*Signif. codes: ='***',0.001'**, 0.01 '*',0.05'.', 0.1' '1*

Table 16 Effect size for the parameter block and treatment explained by partial eta squared.

	η^2 (partial)	90% CI
N (g)		
block	0.16	[0.00, 0.29]
treatment	0.23	[0.00, 0.29]
P g		
block	0.22	[0.00, 0.29]
treatment	0.28	[0.02, 0.34]
18S	0.08	[0.01, 0.20]
log(K g)		
block	0.12	[0.00, 0.23]
treatment	0.13	[0.00, 0.15]

5.4 RELATIONSHIPS BETWEEN BIOLOGICAL ENDPOINTS AND SOIL FUNCTIONING

Since many essential soil functions are driven by soil microbes we elaborated their relation using a Pearson's correlation test. The relationships between biological endpoints and soil functioning are summarized in a correlation matrix for the first harvest (**Figure 15Figure 8**) and the second harvest (**Figure 16**). In general there were only weak relationships between the biological endpoints and the functional parameters.

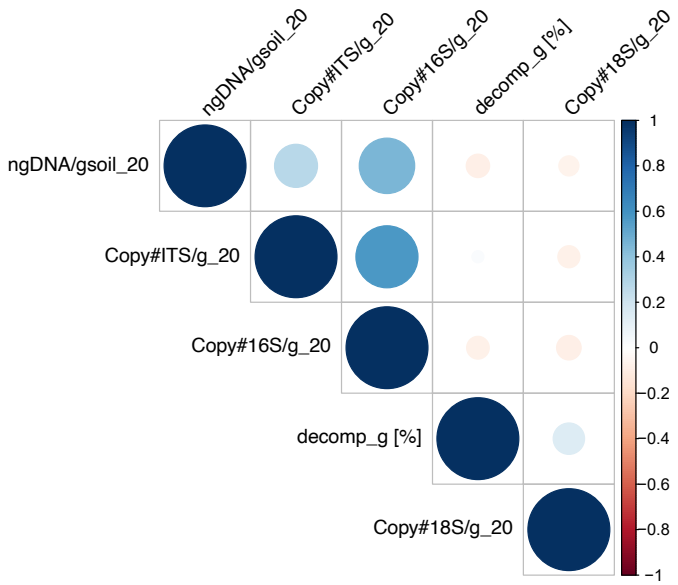

Figure 15 Correlation matrix between functional and biological parameter for the first harvest*. Positive correlations are shown in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients. Blank fields indicate that there was no linear relationship.*

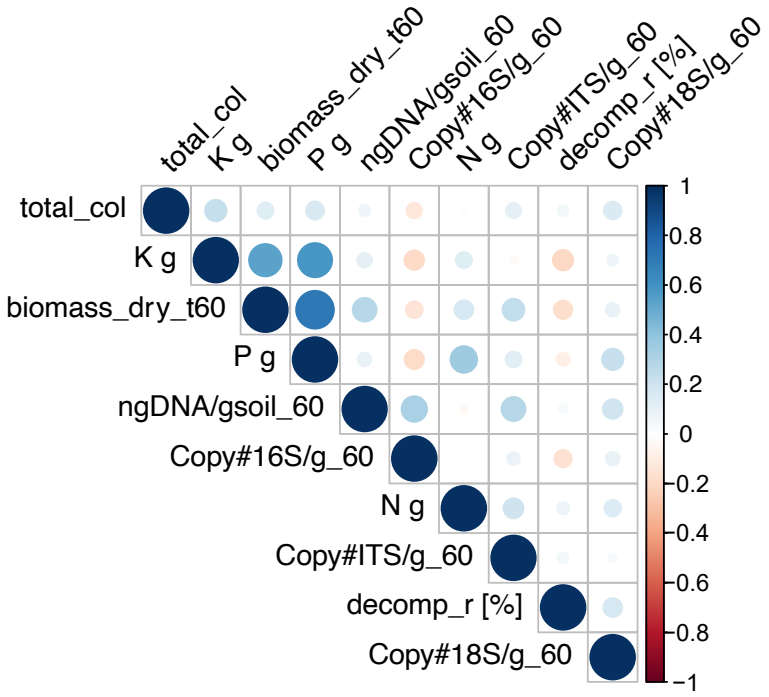

Figure 16 A Correlation matrix between functional and biological parameters for the second harvest. *Positive correlations are shown in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients. Blank fields indicate that there was no linear relationship*

Only weak correlations between the biological endpoints and the litter decomposition were found for both timepoints. Also only weak relationships were found between the lettuce biomass and the root colonization of AMF or the microbial groups that were studied.

Moderate to strong negative relationships were found between the plant biomass and the plant nutrients. The relationships between the nutrients and the dry biomass were significant ($p<0.0001$). The nutrients were moderately positively correlated among each other in the second harvest.

6 DISCUSSION

Pesticides are frequently used in agriculture with the potential to affect non-target soil microorganism. So far studies on the effects of pesticides on soil microorganism mainly focus on the application of single active ingredients. However, in the agronomical practice the application of only one substance is unrealistic and even not recommended. This leads to the fact that the application of pesticides as cocktails remain understudied, although it is on the agenda when it comes to the treatment of crops in reality. Therefore, this thesis aimed to find out how pesticides applied as cocktails affect soil microorganism compared to single applications in a short term experiment. We expected pesticide cocktails to have a greater impact on soil microorganisms since multiple stressors are introduced into the system which attack different biosynthesis pathways (Rillig et al., 2019; Topping et al., 2020). In order to answer the research questions we looked at different molecular and functional parameters, which could indicate changes in the soil microbiome induced by the different pesticide treatments.

6.1 PESTICIDE APPLICATION DID SIGNIFICANTLY AFFECT SOIL FUNGI BUT NOT BACTERIA OR PROTISTS

Our results show that the herbicide mix resulted in significantly lower ITS copy numbers than most of the treatments except for Ombral and the cocktail containing all pesticides. However, in the second harvest the fungal abundance seemed to be less affected indicating a certain degree of resilience against the herbicide cocktail effect. Further, we can conclude that the fungicide mix did decrease ITS copy numbers compared to the negative control in the second harvest. It is noticeable that the effect of the cocktail combining fungicides and herbicides is never greater than the effect of the herbicide mix indicating that the combination of the herbicides is driving the fungal response. This was surprising since we expected in particular fungicides to have a greater effect on soil fungi's since they target fungal pathogens directly (Bünemann et al., 2006).

We can conclude that neither the bacterial community nor protists were significantly affected by the treatments. The overall effects on 18S did not support our hypothesis, since we expected protists to be more sensible to pesticide application than bacteria or fungi. This result might be influenced by the fact that the employed primer pair for 18S did amplify two bands, even though it has been demonstrated that the primers 563F/1132R provided a good taxonomic coverage for eukaryotes and appeared to amplify less bacterial rDNA than other primer sets (Kounosu et al., 2019). Furthermore, our standard was obtained by our samples and not from a pure culture which might have influenced the results as well.

Although we could not find a significant treatment effect on the bacterial abundance we observed that the copy numbers of bacteria but also fungi did increase over time. This effect was expected since increasing plant biomass, rhizosphere and root exudates are known to enhance the microbial soil life (Haichar et al., 2008).

Nonetheless, we found slight trends of decreasing bacterial copy numbers for the fungicide mix and herbicide mix compared to their single components in the first harvest. In the second harvest especially

the cocktail combining all the pesticides did result in slightly lower mean copy numbers. Therefore, we suggest that the herbicides mix and fungicides mix tend to affect 16S copy numbers marginally more than the single applications. Tomkiel et al. (2019) investigated the effect of a mixture of the active ingredients flufenacet and isoxaflutole found in herbicides on microorganisms 30 and 60 days post application. They concluded that a mixture of herbicides evoked a decrease in population numbers of most of the analysed bacteria and fungi (Tomkiel et al., 2019). This is in accordance with our results since we observed tendencies of decreasing fungal and bacterial abundances in the cocktail treatments.

Herbicides applied in single applications did not significantly affect soil microorganisms in this thesis. However, Darine et al (2015) showed significant effects of fluazifop-P-butyl applied in usual field rates on the microbial community. They observed a shift in bacterial communities and a decrease of richness in cultivated soil. The effect was especially visible in the roots proximity. The same effect occurs in non-cultivated soil, however, to a lesser extent. Leading to the suggestion that roots play an important role in enhancing the effect of pesticide. Furthermore, the application of fluazifop-P-butyl even stimulated potential pathogens and inhibited bacteria that act growth promoting (Darine et al., 2015). We observed that Fusilade Max which contains fluazifop-P-butyl resulted in slightly lower 16S copy numbers than the other herbicides in the second harvest. However, we cannot make any propositions on changes at the community level. Another study shows that the single application of the herbicide propyzamide, which is contained in the product GraminEx we used, actually stimulated fungal growth on the one hand, leading to an increased total number of soil fungi, and reduced the actinomycete population on the other hand (Zaid et al., 2014).

We also could not find significant effects of the single fungicide applications on any biological parameters. On the contrary, Howell et al. (2014) reported that azoxystrobin significantly reduced fungal gene copy numbers resulting in significant changes in community structure and diversity. The bacterial copy number was not reduced by the azoxystrobin treatment as also shown in our results for the product Amistar which contains azoxystrobin. They observed that the effect was especially visible at higher concentrations (Howell et al., 2014). Another study by Bending et al. (2007) shows that azoxystrobin applied at the maximum recommended dosages did not show a significant effect on microbial biomass similar to our experiment. The DGGE analysis revealed that only a small number of bands which represented protozoa and fungi's were reduced or absent in soils treated with azoxystrobin indicating that the impacts on community structure were limited (Bending et al., 2007). Wang et al. (2017) found that different doses of azoxystrobin inhibited soil respiration, dehydrogenase activity and reduced the population of bacteria and actinomycetes to a small extent but did not show significant impacts on fungi. The population of bacteria and actinomycetes showed a stronger decrease with increasing concentration (Wang et al., 2018). Wang et al. (2018) hypothesized that the fungal population was not affected by the

treatment due to the low pH value of the soil (pH=5.22), which promotes fungal growth. Our soil had neutral pH of 6.78 therefore the pH might not be suitable to explain the effect on ITS copy numbers.
On the contrary, Baćmaga et al. (2015) revealed that increasing doses of azoxystrobin inhibits the growth of organotrophic bacteria and fungi. Further they observed changes in microbial biodiversity and activity of enzymes (Baćmaga et al., 2015). Riah-Anglet (2018) observed after the long-term addition of conzaole fungicides decreases in microbial biomass which might be mainly related to the decrease of fungal abundance but also to the reduction of some bacterial taxa, namely actinobacteria and bacteroidetes. Proteobacteria were insensitive or even stimulated by the fungicide treatment (Riah-Anglet et al., 2018)

In general there is a range of possible responses of soil microorganisms to pesticide exposure including alterations in structure and function. However, soil microorganisms have the capacity to return to their initial state (resilience), are able to maintain the population structure (resistance) or are able to support key soil functions even though the community composition is altered (Imfeld and Vuilleumier, 2012). For example 16S is widely present in all bacterial species, and therefore, those interwoven relationships of resilience, resistance and niche-filling are not necessarily visible in the pattern of the copy numbers. Therefore, a more in depth analysis via 'fingerprinting' of the different taxa belonging to the same microbial group could indicate changes in the diversity of the microbiome (Johnsen et al., 2001).
Furthermore, the response of the microbial groups are not necessarily observable in the microbial biomass. Therefore, the microbial biomass as DNA yield might not be a suitable method to explain the effect of the treatments on the soil microbiome. Even though we could not observe significant treatments effects on the DNA yield, we found that the fungicide mix slightly reduced the mean microbial biomass in the first harvest compared to their single components. The opposite was found for the herbicides where the single applications show slightly lower mean DNA yields compared to herbicide mix. This pattern is not as pronounced in the second harvest.

6.2 NO SIGNIFICANT EFFECT ON AMF ROOT COLONIZATION BY DIFFERENT PESTICIDE TREATMENTS

The non-target effects of pesticides on AMF is of particular interest in agroecosystems since negative effects might compromise the beneficial effects retrieved from AMF symbiosis in crop production (Entry et al., 2002). Our results reveal that the AMF root colonization was not significantly affected by any of the pesticide single treatments nor did the cocktails show remarkable negative effects. This does not correspond with the findings of Riedo et al. (2019) which show that the AMF root colonization was negatively driven by increasing number of pesticide residues in soil. Due to those results we would have expected that AMF might react more negatively to pesticide cocktails with increasing number of active substances. Also a study by Rivera-Beccerril et al. (2017) revealed that the exposure of a pesticide cocktail did lead to a decrease of AMF propagules, however, they also found a resilience of AMF a few

days after the application of the pesticide cocktail (Rivera-Becerril et al., 2017). Because of those findings, it could have been interesting to see if there was a different response in the first harvest for the root colonization since it is conceivable that the findings of the second harvest might indicate a resilience response as shown in the response of the ITS copy numbers. However, the AMF colonization seemed also not to be affected by the fertilizer treatment either. Even though AMF root colonization is usually decreasing with increased nutrient availability through fertilization, therefore we conclude that the AMF variable was flawed as a biological parameter in this experiment. Nonetheless, it is to note that the findings of the ITS copy numbers from the second harvest and AMF root colonization also sampled in the second harvest do correspond and show a similar pattern even though the variance within the treatments is remarkably greater for the AMF root colonization.

The single pesticide applications as well as the cocktail treatments seemed to rather stimulate the AMF colonization since a slightly higher mean root colonization compared to the negative control can be observed. Hage-Ahmed et al. (2019) conclude that herbicides applied at recommended dosages showed neutral or even positive effects on AMF in in-vitro studies. Further, they state that AMF can not only be exposed to active substances directly via their fungal structures but also indirectly via the host plant. Pesticide applications might alter the symbiosis between host and AMF an therefore, can result in either positive or negative effect on the root colonization. However, little is known about indirect effects which can alter the metabolism of the host plant and therefore, affect root colonization (Hage-Ahmed et al., 2019). The reported effects of fungicides in literature on AMF are in general very variable which can be explained due to differences in active substances used, host plants, soil properties and application techniques (Hage-Ahmed et al., 2019; Jin et al., 2013) For example the active substance azoxystrobin was found to negatively affect mycorrhizal activity to complete inhibition when applied in a soil drench on maize. However, the foliar application of azoxystrobin did not negatively affect the AMF symbiosis with maize (Diedhiou et al., 2004). Our results suggest that Amistar which contains azoxystrobin might even stimulated AMF since the mean total colonization was moderately higher compared to the negative control and also the other fungicides. It is also suggested that the effect of fungicides on AMF is species-dependent and therefore different species might react differently to different treatments (Jin et al., 2013). Jin et al. (2013) observed in their study that all the fungicides which negatively affected AMF colonization act systemically. Contact fungicides did not show an effect or even affected AMF positively, in contrast, already small amounts of systemic fungicides did result in a negative impact. Even though Jin et al. (2013) observed that effect for fungicides applied as a seed-treatment a similar trend can be observed in our data. Amistar which is a translaminar fungicide showed a higher mean colonization than the fully systemically acting fungicides Filan and Ombral. A possible explanation for this effect could be that foliar fungicides remain on the surface of the plant whereas systemic fungicides are transported and distributed in the whole plant and therefore, indirectly affect AMF.

6.3 PESTICIDE TREATMENT SIGNIFICANTLY AFFECTED PLANT BIOMASS

For the biomass we found a statistically significant main effect of the treatment for both time points. Both timepoints did also result in significant lower biomasses in the plants treated with fertilizer. We expected the plants treated with fertilizer to result in higher biomasses since we treated them with the amount of fertilizer that is recommended to grow lettuce in the greenhouse. In terms of the herbicide treatments especially Cadou SC did result in significant lower biomasses in both harvests. A possible explanation for the remarkably low biomass for Cadou SC and the fertilizer treatment in the first harvest, is that the treatments did harm the plant and therefore, negatively affected plant growth during the experiment. A direct application of fertilizer or pesticide to the foliage can result in a pesticide or fertilizer burn, this could have been avoided in spraying the plants with water after treatment which might also have altered the results. In general the plants treated with fungicides resulted in significant lower biomasses than the negative control in both timepoints. Especially Ombral which contains epoxiconazole showed a striking lower biomass in the second harvest. Opposite effects were found in a study where the foliar application of fungicides (epoxiconazole and isopyrazam in mixture) in absence of the disease pressure had a positive effect on photochemistry of winter wheat which is associated with positive effects on yield and biomass (Ajigboye et al., 2014). A study by Bertelsen et al. (2001) revealed that neither epoxiconazole nor azoxystrobin did influence above ground biomass in winter wheat. However, it is questionable to what extent these results can be compared to lettuce since to our knowledge there were no studies investigating the effects of fungicides on the above ground biomass of lettuce in absence of pathogens.

6.4 PHOSPHOROUS CONTENT WAS SIGNIFICANTLY AFFECTED BY TREATMENT BUT NOT NITROGEN AND POTASSIUM

In this thesis the nutrient content is used as an proxy for microbial functionality since microbes improve the bioavailability of nutrients in the soil (Jacoby et al., 2017). However, it is also to note that the nutrient content in plant is also driven by the biomass of the plant. In general the nutrient contents seemed to be rather negatively affected by the pesticide treatments which is in accordance with the results of the biomass. For potassium and nitrogen we could not find significant effects of the treatment or any of the biological parameters, therefore, they were excluded from the analysis. However, the phosphorus content was significantly affected by the treatment and the abundance of protists. The P content was rather negatively affected by the treatments, since most of the treatments resulted in lower P content compared to the negative control. Even though it is known that soil microbes play an important role in the uptake of nutrients and plants evidently shape microbiome structures, there is still a large knowledge gap regarding the mechanisms of these interactions and processes. Furthermore, very little is known about the specific microbial strains that are the key contributors to plant nutrition (Jacoby et al., 2017). Therefore, the qPCR assays in this thesis might not be sufficient enough to explain how pesticides altered the nutrient uptake in terms of the microbiome. Just looking at the pesticide treatments Majundar

et al. (2010) reported that nutrient (P,N,K and Zn) uptake by jute was significantly higher in herbicide and fungicide treatments compared to the control. Further, they identified that the uptake of N was significantly higher under fungicides than herbicides. Increasing the dose of the pesticides also resulted in significantly higher nutrient uptake (Majumdar et al., 2010). A possible explanation for this phenomena might be that with the pesticide inputs also the input of nutrients was enhanced.

6.5 NO SIGNIFICANT EFFECTS OF PESTICIDE TREATMENTS ON LITTER DECOMPOSITION

We did not find an overall significant treatment effect on the decomposition for both kinds of tea. Although we could not reveal any significant trends the cocktail containing all pesticides resulted in slightly lower mean decomposition compared to the herbicide mix and the fungicide mix. Which indicates that a combination of herbicides and fungicides might have a greater effect on decomposers in the soil.

As we expected the decomposition of green tea was higher than for the rooibos tea. The results we obtained are slightly higher than values found in literature, where green tea is expected to decompose about 50% of its initial weight depending on the soil properties and the microorganisms (Tresch and Fliessbach, 2016). This indicates that the decomposition process in our TMEs worked, however, the decomposition was only marginally affected by our treatments. Even though the tea bag index serves as a good proxy to determine decomposition activity in field studies (Tóth et al., 2017) or in citizen science projects, only a few studies used teabags in greenhouse experiments in combination with pesticides. A study by Zaller et al. (2016) investigated the effect of pesticide seed dressings on non-target soil organisms in a microcosm experiment using a substrate mix containing field soil. The seed dressings used in this study contained a mix of fungicides and insecticides and only fungicides. They have found that the seed dressings did significantly reduce the litter decomposition of the teabags. However, they did not find an additional effect on the decomposition rate when insecticides were combined with fungicides, a possible explanation was that the mesh size of the teabag hindered macrofauna taking part in the decomposition which might diminished the effect of the insecticide (Zaller et al., 2016). Glyphosate-based herbicide treatments in a greenhouse experiment using soil microcosms resulted in no significant effect on the decomposition rate (Gaupp-Berghausen et al., 2015) which is in accordance with the effects of the herbicides in our results.

6.6 INFLUENCE OF THE EXPERIMENTAL SYSTEMS ON OUR RESULTS

Despite of having the advantage of intact soil structures and the preservation of innate micro- and mesofauna, the application of TME's also come with limitations. First of all we observed our systems to dry out very fast which could be an additional stressor to the soil microbiome. Abiotic stressors such as droughts are often discussed in literature concerning the potential to negatively affect microbial

communities (Cavicchioli et al., 2019; Kundel et al., 2020). Nevertheless, we observed that the model plants in general grew well and the litter decomposition was in the expected range, therefore, we can conclude that the soil system was active.

Furthermore, the soil structure of soil cores can be very heterogenous which might have influenced our results since we generally found high variabilities within the treatments for the investigated parameters. Microscale studies have revealed that the microbial community is not evenly distributed inside the highly structured soil matrix which is known to challenge the extraction of the metagenome and the following analysis (Lombard et al., 2011). A spatial variability is also observable along field transects where the microbial community was found to be very variable across different samples (Nicol et al., 2003). For this reasons we can speculate that the variance between the different experimental systems was too high to find any significance. Therefore, it might be interesting to conduct a similar experiment using homogenized soils.

7 CONCLUSION AND OUTLOOK

We found the indication that the pesticide mixtures had a more negative effect on the different groups of the soil microbiome, however, this effect was only significant for the herbicide mix on fungal abundance. We cannot confirm that fungicides and their cocktails are in general more harmful to soil microorganisms than herbicides since it depends on the microbial group. On the contrary, our results even indicate that the herbicide mix were the most harmful cocktail for the fungal community, whereas the single fungicide applications seemed to have a marginally more negative effect on soil fungi than herbicides applied as single application. The impact did not significantly increase when herbicides and fungicides are combined in a cocktail. We found in general that the soil fungi were the most sensitive to pesticide application. But could not find a significant negative effect of pesticides on arbuscular mycorrhizal fungi and therefore state that in this experiment the AMF were not in particular sensitive to pesticide exposure. However, since also the fertilizer treatment did not cause a AMF response the experiment needs to be repeated to finally conclude on effects.

In terms of soil functions we could not find that the pesticide cocktails had a more negative effect than the single application. However, we found lettuce to be very sensitive to specific pesticide products which resulted in significantly lower biomasses suggesting that they negatively affected plant growth. Further the fungicide application also did affect plant growth to a greater extent than herbicides. On the contrary, the litter decomposition seemed rather to be positively affected by the pesticide treatments which is especially visible in the cocktails.

In further studies it might also be interesting to do a similar experiment but with different experimental systems, since soil microcosm are known to show a very high variability which could have influenced the outcomes to a certain degree. Furthermore, a long-term experiment with repeated pesticide inputs could be of interest since pesticides are known to be persistent in soil and therefore could enhance the effect of the cocktails. For receiving a broader understanding of the impact of pesticide cocktails insecticides could also be included in the treatments. Since we could not find significant differences for the copy numbers of 16S and 18S a next generation sequencing or a PLFA analysis could help to gain a deeper understanding on the effects of the pesticide treatments on different microbial taxa and microbial community composition.

In summary, further research is needed to better understand the interaction between pesticides and soil microorganisms to enable the judicious use of pesticides in agriculture and thus sustainable production in terms of maintaining soil health and fertility.

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APPENDIX

Table 17 Fungicide products listed with their respective active substances, mode of action according to FRAC (2020) and recommended dosage according to the manufacturer.

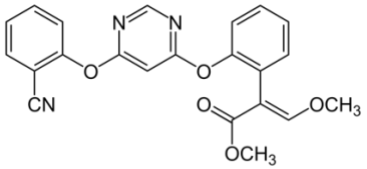
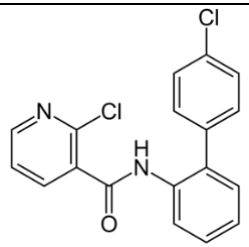
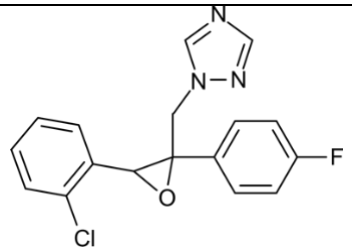
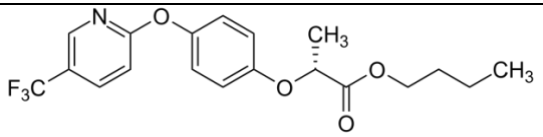
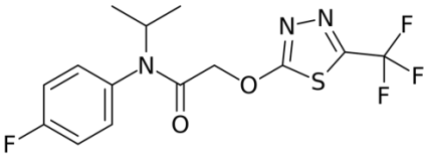
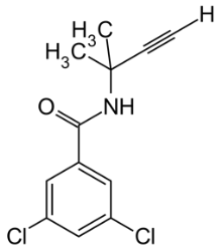
Product ***Active Substance***	**Structural formula**	**Mode of action**	**Mechanism**	**Recommended dosage**
Amistar *Azoxystrobin*		Inhibits cytochrome bc1 at Qo site and therefore, affects cell respiration	Systemic and translaminar characteristics, protective, good long-term effects	1 l/ha
Filan *Boscalid*		Succinate dehydrogenase inhibitor and therefore, affects cell respiration	Systemic	0.5 l/ha
Ombral *Epoxiconazole*		Blocks sterol biosynthesis in membranes	Curative stops pest up to 5 days after infection	0.5- 1l/ha

Table 18 Herbicide products listed with their respective active substances, mode of action according to HRAC (2020) and maximum recommended dosage according to the manufacturer.

Product ***Active Substance***	**Structural formula**	**Mode of action**	**Mechanism**	**Recommended Dosage**
Fusilade Max *Fluazifop-P-butyl*		Inhibits ACCase leads to blocked synthesis of lipids in susceptible plants	Post emergent Absorbed over the leaves	1.5 l/ha
Cadou SC *Flufenacet*		Inhibits very long chain-fatty acid synthesis	Mainly absorbed over roots and hypocotyl, when used post-emergence also in small quantities over the leaves	0.48 l/ha
GraminEx *Propyzamide*		Inhibits microtubule assembly	Mainly absorbed by weeds over the roots	2.5 - 3.75 l/ha

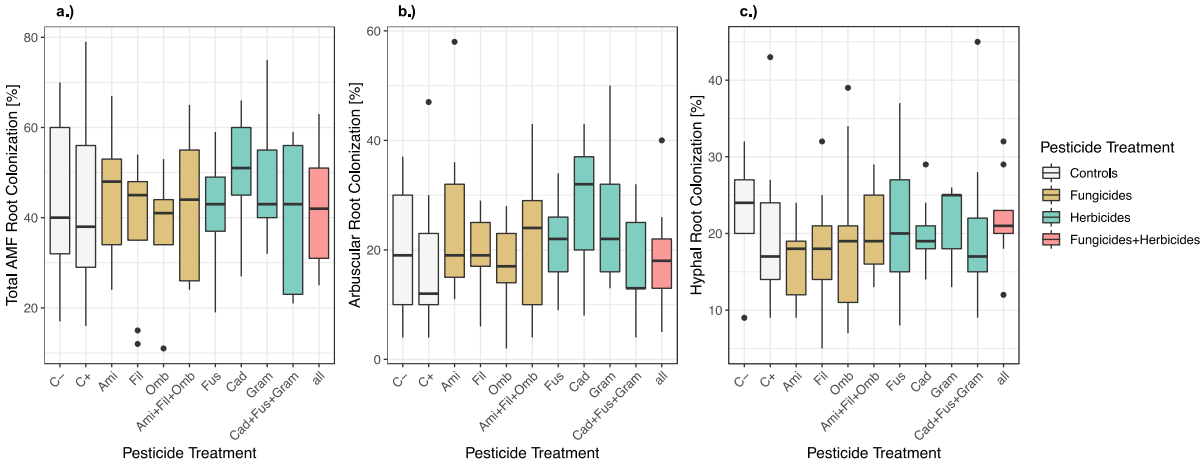


Figure 17 Root colonization by AMF under different pesticide treatments. *The control treatments are displayed in grey, the fungicide treatments are visualized in brown, the herbicide treatments in green and the treatment containing all the pesticides in red.* ***a.)*** *No significant effect of treatment ($F_{10,99}$=0.49 p=0.890)) nor block ($F_{8,99}$=0.94 p=0.487) was found on total root colonization* ***b.)*** *No significant effect of treatment ($F_{10,9}$=0.16, p=0.798)) but a significant effect of block ($F_{8,99}$=2.19, p=0.037) was found on hyphal root colonization.* ***c.*** *No significant effect of treatment ($F_{10,99}$=0.83 p=0.601)) nor block ($F_{8,99}$=0.61 p=0.765) was found on hyphal root colonization.*

PERSONAL DECLARATION

I hereby declare that the submitted thesis is the result of my own, independent work. All external sources are explicitly acknowledged in the thesis.

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Schindellegi, April 2021