



Copper and synthetic pesticides are major drivers of soil bacterial and fungal communities

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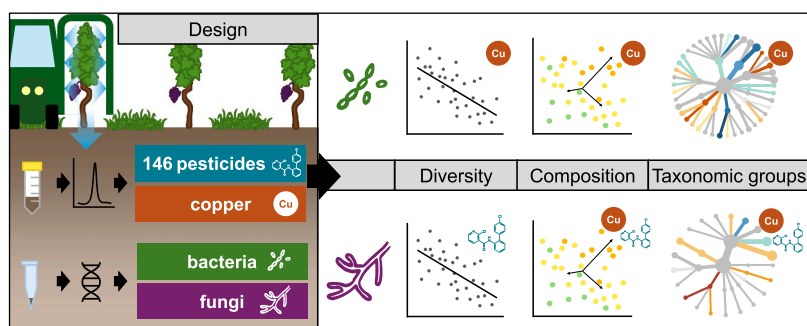
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HIGHLIGHTS

- Pesticide residues in intensive agriculture affect the soil microbiome.
- Copper strongly decreased bacterial diversity.
- Copper also strongly affected bacterial and fungal community composition.
- Synthetic pesticides were related to fungal diversity and community composition.
- Pesticide sensitive genera were identified as candidates for risk assessments.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Microbiome
16S and ITS rRNA
Multiresidue analysis
Heavy metals
Multiple stressors

ABSTRACT

Soil microbial communities play a key role in terrestrial ecosystems and provide important agricultural functions. While earlier studies have shown that soil properties, such as pH and soil organic matter, are major drivers of microbial community structure, the roles of copper and synthetic pesticides are poorly understood. Here we focused on intensively managed agricultural systems studying 61 conventional and organic vineyards from three distinct winegrowing regions in Switzerland. We identified the factors shaping bacterial and fungal communities, specifically assessing the importance of copper and synthetic pesticides. Both copper and pesticides were identified as major factors shaping microbial community structure. Copper led to a pronounced reduction in bacterial diversity. Moreover, it affected bacterial and fungal community composition, to a degree comparable to pH. The relative abundances of many large taxonomic groups and functional guilds were positively or negatively impacted by copper, indicating a strong variability in copper sensitivity. Synthetic pesticide concentrations were negatively associated with fungal diversity and community composition, indicating that they are an additional stressor for soil fungi. Finally, we identified several genera negatively related to synthetic pesticides, including

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<https://doi.org/10.1016/j.jhazmat.2026.141682>

Received 27 November 2025; Received in revised form 28 January 2026; Accepted 3 March 2026

Available online 5 March 2026

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the biocontrol fungus *Trichoderma*, that could be tested for inclusion in improved risk assessments. This is the first study comparing a wide range of field sites which documents consistent negative effects of copper and synthetic pesticides on soil microbial diversity and community composition. Our work points to the need for improved risk assessments, specifically including yet overlooked effects on soil microbes.

1. Introduction

Soils are extremely diverse ecosystems harboring as much as 59% of global biodiversity according to recent estimations [1]. Particularly microbes, which comprise the biggest share of soil biodiversity, are thought to play a key role in supporting soil health and consequently plant productivity [2]. In areas utilized for agriculture, soil biodiversity and functioning might be threatened by intensive management practices, such as tillage, fertilization, or the application of plant protection products (PPP) [3]. These practices can alter soil properties that are known to be important drivers of soil microbial communities, as for example pH, soil organic carbon (SOC), or contents of macro-nutrients [4]. Residues from PPP can directly affect microbes, however, their importance in influencing microbial communities is poorly understood thus far. This is problematic considering the growing body of evidence that documents the ubiquitous occurrence of PPP residues in agricultural soil in Europe [5–8] and other parts of the world [9,10], as well as natural areas [11,12]. Due to their inherent toxicity and widespread distribution, it is of utmost importance to obtain more knowledge about the potential impacts of PPP residues on soil microbial communities and ultimately on soil health.

Intensive PPP use is typically found in high value crops such as vegetables, orchards, and vineyards [13]. In the case of Switzerland, grapevines are the most intensively sprayed crop, receiving around 25 kg/ha of PPP per year on average [14]. Similar application rates can be found in other European countries, such as the major wine producers Spain, France, Italy, and Portugal, with viticulture consuming over 30% of total pesticide use in those countries [15]. Along with such elevated application rates, vineyard soils were also exposed to especially high synthetic pesticide concentrations compared to other crops in a Europe-wide study [16]. Our previous study has shown that vineyards are particularly at risk from copper and synthetic pesticides (herein after “pesticides”), due to frequent applications of these agrochemicals and accumulation over decades [15]. Current risk assessment approaches are based on a small number of model organisms and heavily biased towards earthworms and arthropods [17]. Microbes are only included through general nitrogen and carbon transformation tests. This strong simplification ignores many other ecological and agriculturally relevant functions provided by microbes, such as soil aggregation, pathogen suppression, or the provision of nutrients to plants through symbiotic interactions [18]. Recent initiatives advocate the inclusion of additional, more specific tests into pesticide risk assessments, covering microbial groups with pivotal functions, such as arbuscular mycorrhizal fungi (AMF) [19]. Such additional laboratory tests improve the evaluation of isolated pesticide effects towards different organism groups. However, field studies are required to attain a realistic assessment of copper and pesticide effects on soil microbial communities under the influence of various biotic, abiotic, and environmental factors.

For copper, few studies on individual fields or a small set of sites exist, however, large-scale field studies that link copper to microbial community structure are missing and the described effects on microbial diversity and community composition are inconsistent [20–26]. For instance, some studies reported negative effects of soil copper concentrations on bacterial diversity [20,21], whereas others found no [23,24] or even positive associations [25]. As soils are highly heterogeneous and different properties can strongly affect the bioavailability and toxicity of copper [27], larger studies, based on many fields from multiple locations, are required to make general statements about the impact of copper on soil microbiota. For pesticides, a limited number of field

monitoring studies is available. While some substance specific associations were reported, no effects of the general soil pesticide contamination status were found on microbial diversity and community composition in arable fields [28], vegetable fields [29], or paddy rice fields [30]. In arable fields, pesticide effects might be difficult to detect, as the applied quantities are rather small compared to horticulture [14] and other management practices, such as crop rotation, might overlay pesticide effects. Studying perennial crops, Lu et al. [31] observed a slight negative correlation between bacterial richness and pesticide sum concentration in soils of citrus orchards but not in vineyards. Although they often occur together, previous studies did not simultaneously assess the relative importance of copper and pesticides in affecting soil microbial communities under field conditions.

The aim of this study was to assess the effect of copper and pesticides on soil bacterial and fungal diversity, community composition, and potential functions under realistic field conditions, and identify potential test organisms for improved risk assessments. We focused on vineyards as a model system with intensive agricultural management and conducted a field study with 61 conventional and organic vineyards from three major winegrowing regions in Switzerland that differed in climate and pedogeological properties (Figure S1). We characterized bacterial and fungal communities through amplicon sequencing of the 16S and ITS regions of the rRNA genes, respectively. Additionally, we measured the concentrations of total and bioavailable copper and 146 different pesticides, including transformation products (TP), as presented previously [15]. This setting overcomes many of the above-mentioned limitations. Vineyards are a suitable system to study the effects of copper and pesticides due to their intensive use and the perennial cultivation of grapevines, which reduces variation from other management practices compared to crop rotation. With the size of this study, our observations could provide general insights into soil microbiota’s responses to copper and pesticide contamination in Europe and other temperate regions. To our knowledge this is the first study of this scale to jointly assess copper and pesticide effects on soil microbial communities.

2. Methods

2.1. Study design and sampling

Soil samples were taken from 61 vineyards in three distinct winegrowing regions of Switzerland and included conventional and organic farming. A detailed description of the study design and sampling can be found in Barmettler et al. [15]. Briefly, soil samples were collected from November to December 2020, which was 3–4 months after the last pesticide applications. The vine row and the inter-row sections of the vineyard parcels were sampled separately collecting ten soil cores of 3 cm diameter per section. The soil cores were split into a surface soil (0–5 cm) and a subsurface soil (5–20 cm) layer and thoroughly mixed with the other samples from the respective section and depth. The regions differed in climate and soil texture (Figure S1). Zurich is characterized by a cold and humid climate and clayey soils, whereas Valais has a warmer and clearly drier climate with more sandy soils, and the region Vaud is in between in both regards.

2.2. Pesticide, copper and physicochemical soil analyses

Measurement of soil inorganic properties was described in detail in Barmettler et al. [15]. Briefly, pesticides were extracted by an adapted

quick, easy, cheap, effective, rugged, and safe (QuEChERS) approach and analyzed by liquid chromatography coupled to triple quadrupole mass spectrometry with electrospray ionization (LC-ESI-MS/MS) as described in Rösch et al. [32]. This method covered 146 active ingredients and TP. Bioavailable copper was determined using the CAT-method which consists of an extraction with calcium chloride and DTPA and it was quantified by inductively coupled plasma optical emission spectroscopy (ICP-OES). All other physiochemical soil properties were analyzed using the reference methods of the Swiss Federal Agricultural Research Station (<https://www.agroscope.ch/referenzmethoden>).

2.3. DNA extraction and amplicon sequencing

DNA was extracted from 0.2 g of lyophilized soil using the NucleoSpin soil kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions but performing the extraction twice to attain more stable results. DNA concentrations were quantified with a Cary Eclipse fluorescence spectrometer (Agilent, Santa Clara CA, USA) and normalized to 1 ng/μL. To characterize the prokaryotic community, we amplified the V3–4 region of the 16S rRNA gene (herein after "16S") using the forward primer 341 F and the reverse primer 805 R [33] (see Table S1). For the fungal communities, we targeted the ITS2 region in the rRNA gene (herein after "ITS") using the forward primer mixture ITS3ngsmix1–5 and reverse primer ITS4ngsUni [34,35] (see Table S1). We added extra linkers, a frame shift to increase complexity, and the Nextera binding sequence to the primers. For the 16S PCR, initial denaturation (95 °C, 3 min) was followed by 20 cycles of denaturation (98 °C, 20 s), annealing (52 °C, 30 s) and elongation (72 °C, 30 s), and a final extension (72 °C, 2 min). The settings for the ITS PCR were slightly different with 30 cycles of denaturation (98 °C, 20 s), annealing (55 °C, 20 s) and elongation (72 °C, 30 s). Each 10-μL PCR reaction contained 1x Kapa Kapa HiFi HotStart DNA Polymerase ready-mix kit (Roche, Basel, Switzerland), 0.5 mM of each primer and 2 ng or 1 ng of template DNA for 16S and ITS, respectively. PCR was performed in triplicates and pooled products were purified using self-made SPRI Beads (GDC, Zurich, Switzerland) after visualization on 1% agarose gels. Index PCR was performed under the following conditions: 95 °C for 3 min, then 10 cycles (16S) or 5 cycles (ITS) of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and final extension at 72 °C for 5 min. Each 20-μL PCR reaction contained 1x Kapa HiFi HotStart DNA Polymerase ready-mix kit (Roche), 2 μL index primer 1 and index primer 2 from the Nextera XT 96 Index kit v2 Set A and D (Illumina, San Diego CA, USA), 1 μL dimethyl sulfoxide, and 4 μL cleaned PCR product. After beads purification and validation, DNA was quantified using the dsDNA BR Assay kit (Thermo Fisher, Waltham MA, USA). Separate 16S and ITS libraries were created by pooling all samples with equal amounts of DNA. The libraries were loaded on a 1% Agarose Gel and the fragments were cut from the gel and purified with the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) according to the manufacturer's instructions. Finally, the libraries were quantified using the QuDye dsDNA HS Kit (Lumiprobe, Hannover, Germany) and diluted to a final concentration of 4 nM for the sequencing run on the Illumina MiSeq Personal Sequencer (Illumina) using a 600 cycle v3 Sequencing kit (Cat. no. MS-102–3003), in paired-end 2 × 300 bp mode. The MiSeq libraries were prepared and sequenced at the Genetic Diversity Centre of ETH Zurich (<https://gdc.ethz.ch/>).

2.4. Bioinformatics

Processing of the raw 16S and ITS reads was performed using QIIME2 [36] and the R package DADA2 v.1.35.60.0 [37] in R v.4.3.3 [38] following the general analysis tutorial (<https://benjjneb.github.io/dada2/tutorial.html>). For the 16S data, the forward and reverse primer sequences were trimmed from the reads using the QIIME2 *cutadapt* plugin [39]. Forward and reverse reads were then trimmed to

270 and 175 base pairs, respectively to improve overall read quality and merging of the paired-end reads before denoising and merging into amplicon sequence variants (ASV) with the pool argument of the *dada* function set to *TRUE*. Chimeras were identified and removed using the *removeBimeraDenovo* function (*method="pooled"*). The ASV reference sequences were taxonomically assigned using the *classify-sklearn* command in QIIME2 with a 341F-805R region taxonomic classifier of the SILVA database v138.1 [40] obtained and processed with the *RESCRIPt* plugin [41]. Only ASVs assigned to the domains bacteria and archaea were retained. As < 1% of these were archaea, we use the term "bacteria" when referring to results from 16S sequencing.

The ITS data comprised two separate sequencing runs, as an inspection of sequence counts in Run 1 revealed very low counts for some samples which required resequencing. As a result, both ITS sequencing runs were processed independently by extracting the ITS2 subregion with the *ITSxpress* plugin [42] in QIIME2. Denoising and merging of the paired-end reads was performed on each run independently with DADA2 using the same settings as in the 16S data processing steps mentioned above. However, reads were not trimmed to a fixed length before denoising in order to preserve the natural length variation of the ITS2 region. The independently generated ASV tables from Run 1 and Run 2 were then combined using the *mergeSequenceTable* function. ASV counts from re-sequenced samples in Run 2 were summed with the counts in the originally sequenced sample from Run 1 using the *qiime feature-table group* command in QIIME2 (*-p-mode="sum"*). The ASV reference sequences were taxonomically assigned using the *classify-sklearn* command with a taxonomic classifier trained on the full length, dynamic sequences of the UNITE full eukaryotic database v10 [43] obtained and processed using *RESCRIPt*. ASVs that were not assigned to the kingdom of fungi were removed.

2.5. Data processing

The R package *phyloseq* v.1.52.0 [44] was used to compile the ASV and taxonomy tables and the sample data after removing samples with less than 12,000 sequences (three bacterial and one fungal sample). All samples were rarefied to equal depth using the lowest sequence number of the remaining samples (bacteria 13,321 and fungi 12,835 reads; Figure S2).

Diversity was characterized by computing the Shannon index [45], observed richness, and evenness [46]. For fungal evenness we used an ordered quantile normalization transformation to approach normal distribution. Differences in community composition were assessed using Bray-Curtis dissimilarities [47]. Functional annotation was carried out on genus level with the R package *microeco* v.1.15.0 [48]. For bacteria we used the FAPROTAX database [49] which resulted in annotations for 32% of ASVs which constituted 42% of total abundance. For fungi the FungalTraits database [50] was used that covered 67% of ASVs. As primary and secondary lifestyles were sometimes contradictory, we only considered primary lifestyles of genera with either no secondary lifestyle or a secondary lifestyle that was in accordance with the primary lifestyle (e.g. "arbuscular mycorrhizal" and "root associated").

To attain microbial data on parcel level, we averaged the read numbers and diversity indices across all samples of the same parcel. For community composition, we computed the centroids of each parcel using the *betadisper* function from the *vegan* package v.2.7–1 [51]. Parcels where one sample was removed due to low read numbers were retained. All pesticide sum concentration indices were log-transformed and individual pesticide concentrations were log(x + 1)-transformed due to the common occurrence of zeros. One of the initially 62 vineyards was excluded from the analyses, as it exhibited an extremely low bacterial and fungal evenness in the vine row samples, which was probably a result from strongly elevated sodium (>10x median) and potassium (>7x median) concentrations in these soil samples.

2.6. Statistical analyses

To test the effects of copper and pesticides on diversity, we used ANOVA testing different copper and pesticide contamination indices after correcting for region (Zurich/Vaud/Valais), soil, vegetation cover (full/inter-row/bare), and farming system (conventional/organic). Soil was a combination of pH and the first three axes of a centered log-ratio transformation that explained around 75% of variation in soil properties (*pcaCoDa* function from *robCompositions* package v.2.4.1 [52]). Vegetation cover was tested before farming system to correct for vegetation differences between conventional and organic vineyards. As farming system was strongly intertwined with pesticide contamination [15], we additionally included models where contaminants were tested before farming system. When testing individual pesticide concentrations, we included pesticide sum concentrations before and after the pesticide of interest to distinguish individual pesticide effects from general pesticide contamination effects. Only pesticides present in > 20 parcels were tested.

A general overview of the community structure was attained using principal coordinates analysis (PCoA). The corrected copper and pesticide effects on community composition were computed analogous to diversity with PERMANOVA using the *adonis2* function from the *vegan* package [51] and equal dispersion of categorical variable groups was tested with the *betadisper* function. We used distance-based redundancy analysis (dbRDA) to compare the relative importance of copper, pesticides, and other soil properties on community composition. For that we applied the *capscale* function from the *vegan* package [51] and conditioned it by region and vegetation cover, as those variables were found to be highly significant in the PERMANOVA. Thereby, the total variance was split into conditioned (explained by region and vegetation cover), constrained (explained by soil properties), and unconstrained (residual) variance. Soil properties with a correlation > 70% were excluded. The contribution of individual soil properties was tested using ANOVA. The variables were ordered according to their explanatory power when tested individually. Additionally, we tested each variable at the end of the model to compute uniquely explained variance. To test the robustness of these results, we applied a leave-one-out approach, where the same analyses were repeated removing each parcel once.

The effects of copper and pesticides on the abundance taxonomic and functional groups was computed through ANOVA, equivalent to diversity. As the distributions of these groups showed varying levels of skewness, we tested different transformations with increasing strengths: non-transformed, square root, cube root, fourth root, eighth root, logarithmic, and inverse. Due to the limitation to non-zero values in the logarithmic and inverse transformation, we added 1 if the respective data contained zeros. For each group, we tested model fit for all mentioned transformations and selected the weakest transformation that met the assumptions of normality [53] and homoscedasticity [54]. P-values from these analyses were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) procedure [55]. No suitable transformation could be found for a small number of taxonomic and functional groups, as indicated in the respective figures. For these groups generalized linear mixed-effect models (GLMM) with Poisson or negative binomial error distribution were fitted using the *glmer* and *glmer.nb* function from the *lme4* package v.1.1–37 [56] based on sample level count data and including vineyard as random effect. The significance of copper or pesticide effects was assessed using likelihood-ratio tests comparing a full model to a model without the respective contaminant. Model adequacy was evaluated using simulation-based residual diagnostics implemented in the *DHARMA* package v.0.4.7 [57]. These GLMM results were stated in the respective figure captions where relevant. All figures were produced using the *ggplot2* package v.3.5.2 [58]. All data transformations, statistical tests and figure generations were performed using R v.4.5.1 [59].

3. Results

Metataxonomic analyses of soil microbial communities yielded 62344 bacterial and 6810 fungal ASVs. The dominant bacterial phyla were Actinobacteriota (25%), Proteobacteria (25%), and Acidobacteriota (14%), while fungi were dominated by the phylum Ascomycota (78%), followed by Mortierellomycota (10%) and Basidiomycota (9%; Figure S3). Increasing copper concentrations were associated with decreasing bacterial diversity and shifts in bacterial and fungal community composition, whereas pesticide concentrations were negatively related to fungal diversity and community composition (Fig. 1, Fig. 2).

3.1. Bacterial and fungal diversity affected differently by copper and pesticides

Copper was strongly negatively correlated to bacterial diversity with an R^2 of 0.21 (Fig. 1a). When testing its effect in an ANOVA after correcting for region, soil composition, vegetation cover, and farming system, it remained highly significant ($p < 0.001$) explaining more variance than any correction variable (Fig. 1c). Copper had a particularly strong effect on bacterial evenness, where it accounted for around 30% of the observed variance, which was more than the combined explanatory variance of all correction variables (Figure S4). Bioavailable and total copper had similar effect sizes (Fig. 1c). When analyzing farming systems separately, the negative copper effect was more pronounced in conventional vineyards, despite conventional and organic vineyards having comparable copper concentrations (Figure S5). Pesticides showed no association with bacterial diversity.

In contrast, fungal diversity was more strongly influenced by pesticides, showing a strong negative correlation with log pesticide sum concentrations with an R^2 of 0.39 (Fig. 1b). In the corrected model, fungicide sum concentrations were particularly highly significant ($p = 0.003$) explaining approximately 7% of the variance in fungal diversity when tested before farming system (Fig. 1c). Fungicide sum concentrations explained more variance than farming system but lost significance when tested after, as farming system and pesticide concentrations were strongly intertwined [15]. The pesticide effect on fungal richness was even stronger with around 13% explained variance and remained significant even when tested after farming system (Figure S4). Conversely, farming system was not significant when tested after pesticide and fungicide sum concentrations (Figure S6). Unlike bacteria, fungal diversity was not significantly affected by copper. Notably, all observed effects of copper and pesticide concentrations were negative. In contrast to sum concentrations, no metrics counting the number of pesticides were significant except for a weak effect of the number of herbicides on bacterial richness (Figure S4).

Analysis of individual pesticide effects revealed that fungal diversity and richness was primarily impacted by fungicides, with nearly all significant relationships being negative (Figure S7). All individual pesticides explained less variance than pesticide sum concentrations and most became insignificant when tested after this metric. Bacterial diversity was only significantly associated with a few pesticides and responses were more variable (Figure S7). The marked regional differences in bacterial and fungal diversity are further elaborated in SI section 1.

3.2. Copper is a main driver of bacterial and fungal community composition

Analogous to diversity, we used PERMANOVA to assess copper and pesticide effects on bacterial and fungal community composition after testing correction variables. Copper was significant for both bacterial ($p < 0.001$) and fungal ($p < 0.01$) communities (Fig. 2a). Similarly to fungal diversity, pesticide and fungicide sum concentrations were significant for fungal community composition only when tested before

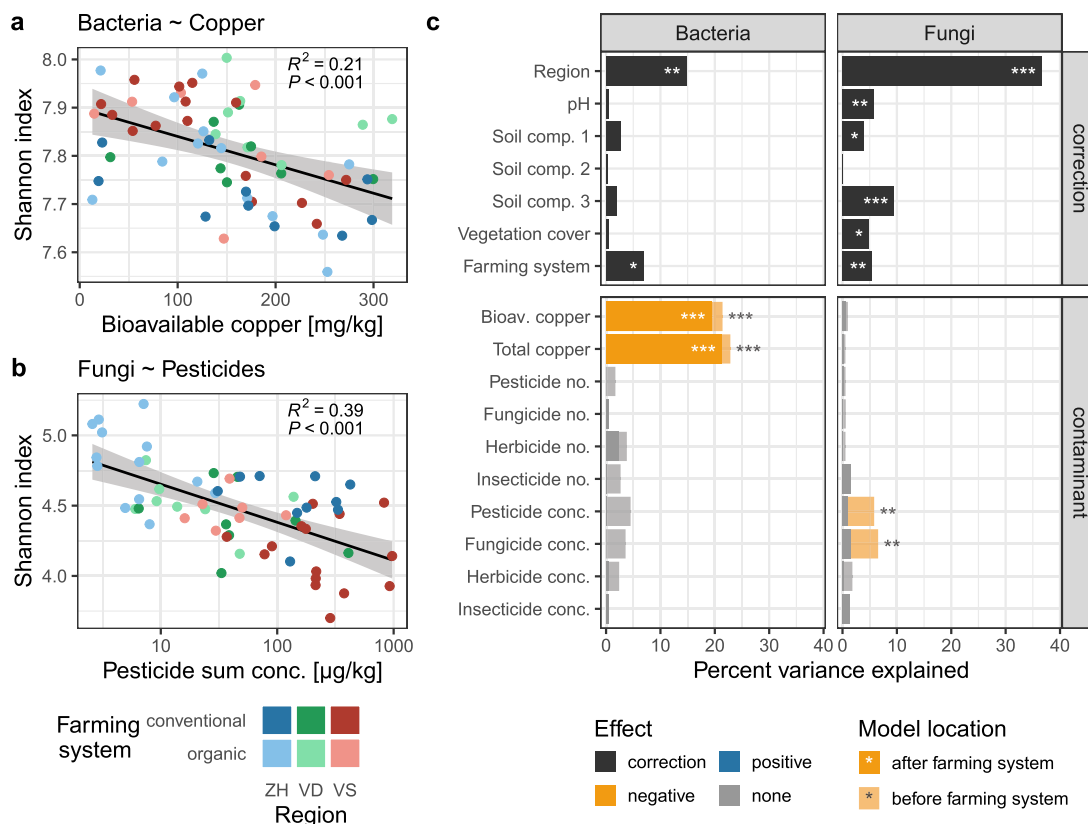


Fig. 1. Bacterial and fungal diversity in response to copper and pesticide contamination. a) Correlation plots between bacterial diversity and bioavailable copper and b) fungal diversity and pesticide sum concentration, colored by farming system and region (Zurich (ZH), Vaud (VD), and Valais (VS)). Diversity expressed as Shannon index. c) Percent variation explained by different variables. Correction variables were tested in the given order. Contaminant variables were tested individually after correction variables. A case of testing the contaminant variables before farming system was included. Significant explanatory variables are displayed using asterisks ($p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*)).

farming system and explained more variance (Fig. 2a). The PERMANOVA also revealed a strong region effect on microbial community composition, which was evidenced by clear regional clustering along the first axis of PCoA (Figure S8a,b; see also SI section 1). Further, bacteria seemed to be more strongly affected by pH and soil composition than fungi, which were more responsive to vegetation cover (Fig. 2a). Gradients in pH and copper concentration were apparent along the second and third PCoA axes for both bacterial and fungal communities (Figure S8c-f). Testing copper at the very end of the model might therefore underestimate its true driving force on soil microbial communities.

To analyze the effects of copper and pesticides in relation to soil properties, we performed a dbRDA that was conditioned by region and vegetation cover (variance removed). pH and copper emerged as the key driving soil properties of both bacterial and fungal community composition (Fig. 2b,c). Copper ranked second after pH amongst all tested soil properties in terms of explanatory power and explained even more unique variance than pH (Figure S9). The effect of fungicide sum concentrations on fungal community composition was apparent on the third and fourth canonical axes (Fig. 2d). Fungicide sum concentrations ranked fourth in their explanatory power for fungal community composition and stayed significant even when tested at the end of the model (Figure S9). Contrarily, herbicide and insecticide concentrations were not associated with fungal community composition, and bacteria were not responsive to any pesticide metrics (Fig. 2).

When testing the effects of individual pesticides on bacterial and fungal community composition, only a small number of fungicides were significant (Figure S10). Carbendazim and penconazole were significant for both bacteria and fungi and stayed significant even when tested after pesticide sum concentrations (Figure S10).

3.3. Copper affects many higher taxonomic groups

Given the strong influence of copper on microbial community composition, we further investigated the effect of copper on the abundances of different taxonomic groups. Copper was highly significant for many bacterial phyla (Fig. 3a,c). Proteobacteria, Acidobacteriota and Gematimonadota showed strong positive associations with copper concentrations, whereas Planctomycetota and Myxococcota were negatively related to copper.

Recognizing that responses within large phyla can be heterogeneous, we also assessed the effects of copper at the class level (Fig. 3a,c). Within the Actinobacteriota, the class Acidimicrobiia showed a strong negative association with copper. The positive relationship observed at the phylum level for Proteobacteria was mainly driven by Alphaproteobacteria, while Gammaproteobacteria showed no significant association. Similarly, within Acidobacteriota, the most abundant class Vicinamibacteria was unaffected by copper, whereas Blastocatellia showed a strong positive relationship. The phylum Chloroflexi contained both positively and negatively associated classes.

For fungi, copper had less significant effects at the phylum level (Fig. 3b,c). Nevertheless, strong negative associations were found for Chytridiomycota and Aphelidiomycota, and a weaker negative trend for Mortierellomycota. When examining the classes of the most abundant phylum Ascomycota, we found contrasting responses. Sordariomycetes and Pezizomycetes were negatively associated with copper, whereas Dothideomycetes and Eurotiomycetes showed positive associations. More detailed statistical information is shown in Figure S11 and copper effects on abundant bacterial and fungal orders are presented in Figure S12.

Analogous to copper, we assessed the effects of pesticide sum

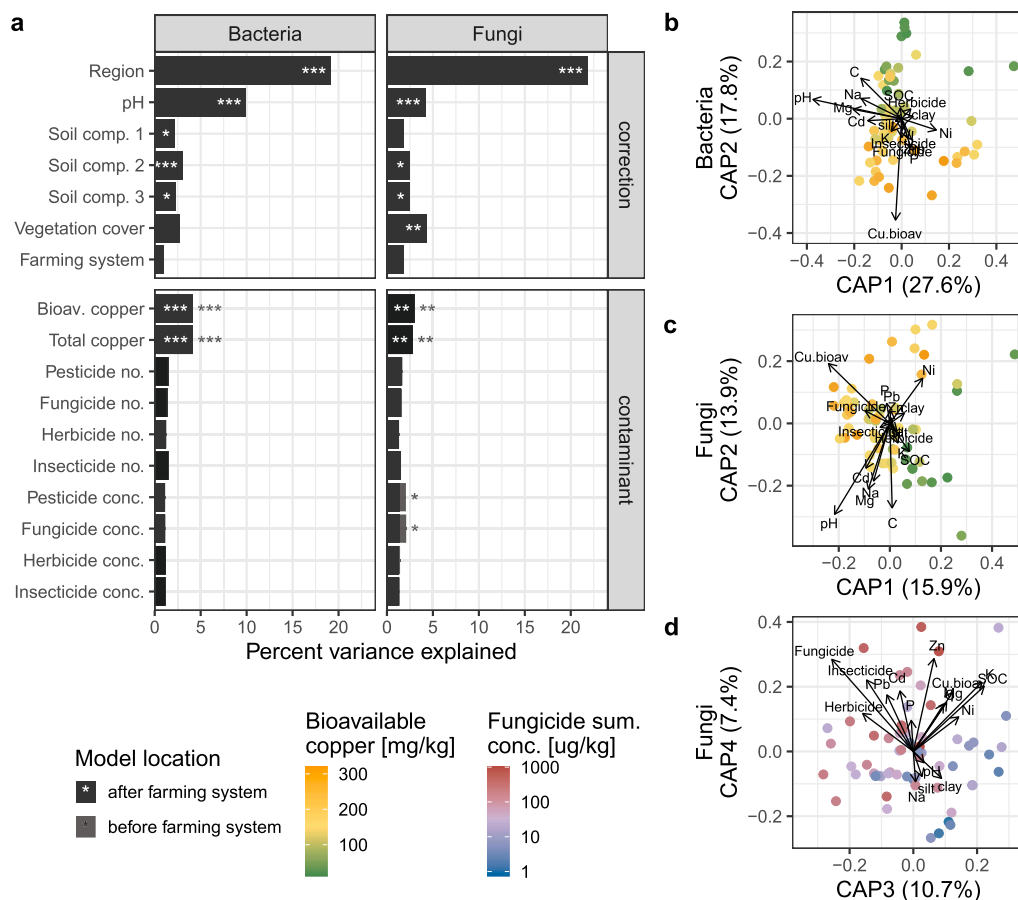


Fig. 2. Bacterial and fungal community composition in response to copper and pesticide contamination. a) Percent variation explained by different variables. Correction variables were tested in the given order. Contaminant variables were tested individually after correction variables. A case of testing the contaminant variables before farming system was included. Significant explanatory variables are displayed using asterisks ($p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*)). b-d) Ordination plots of distance-based redundancy analysis conditioned by region and vegetation cover. The color gradient represents copper concentration (first and second axes, panels b & c), or synthetic fungicide sum concentration (third and fourth axes, panel d; only displayed for fungal communities).

concentrations on bacterial and fungal taxa. While no associations were found at the phylum and class level, some fungal orders showed a trend to be affected by pesticide concentrations (adjusted p-value between 0.05 and 0.1; Figure S13). In Table S2 we provide a list of bacterial and fungal genera that were found to be sensitive to copper and pesticides. For example, the beneficial genus *Trichoderma* was negatively associated to pesticide concentrations ($p = 0.002$; p-adjusted = 0.077; Fig. 3d). There were strong negative correlations between pesticide concentrations and the relative abundance of *Trichoderma* ($R^2 = 0.28$) and its most abundant species *T. koningii* ($R^2 = 0.29$; Figure S14a,b). These negative effects remained even after correcting for farming system (Figure S14c).

3.4. Potential microbial functions are affected by copper

To assess possible effects of copper and fungicides on soil functioning, we assigned potential functions to bacterial and fungal ASVs using the FAPROTAX and FungalTraits databases, respectively. Copper was strongly negatively related to nitrate reduction and slightly to nitrite respiration (Fig. 4). Contrarily, nitrogen fixation was positively related to copper concentrations, which is in line with the positive association with the order Rhizobiales (Figure S12a). Bacterial functions associated with hydrocarbon degradation generally showed positive associations with copper. In contrast, several saprotrophic lifestyles of fungi were negatively related to copper exposure and there was a negative trend on the abundance of AMF (Fig. 4).

4. Discussion

Our study demonstrates that copper and synthetic pesticides both have a major impact on the diversity and composition of soil microbial communities. Bacterial communities were strongly affected by copper, while fungal communities were affected both by copper and pesticides (Fig. 1, Fig. 2). Earlier studies reported that pH, SOC content, and other soil physical and chemical properties have major impacts on microbial communities [4]. However, the strong effect of copper on microbial diversity and community composition has been overlooked and not studied in depth. The few field studies that investigated this so far have been based on a limited number of locations or single experiments with otherwise stable soil properties [20–25]. Our study covered a diverse set of soils and the effect of copper on bacterial and fungal community composition was comparable to the effect of soil pH, a well-known driver of microbial communities (Fig. 2b,c, Figure S9c,d). This further emphasizes the key role of copper as a driver of soil microbiota in intensively managed agricultural areas. We also documented a clear negative association between pesticide sum concentrations and fungal diversity and community composition (Fig. 1, Fig. 2), whereas previous field studies of similar scope hardly observed pesticide effects on soil microbes [28,29,31]. This supports that pesticides have relevant impacts on soil microbiomes that exceed acute short-term toxicity.

4.1. Copper has fundamental effects on soil microbial communities

Throughout all our analyses the effects of copper on microbial

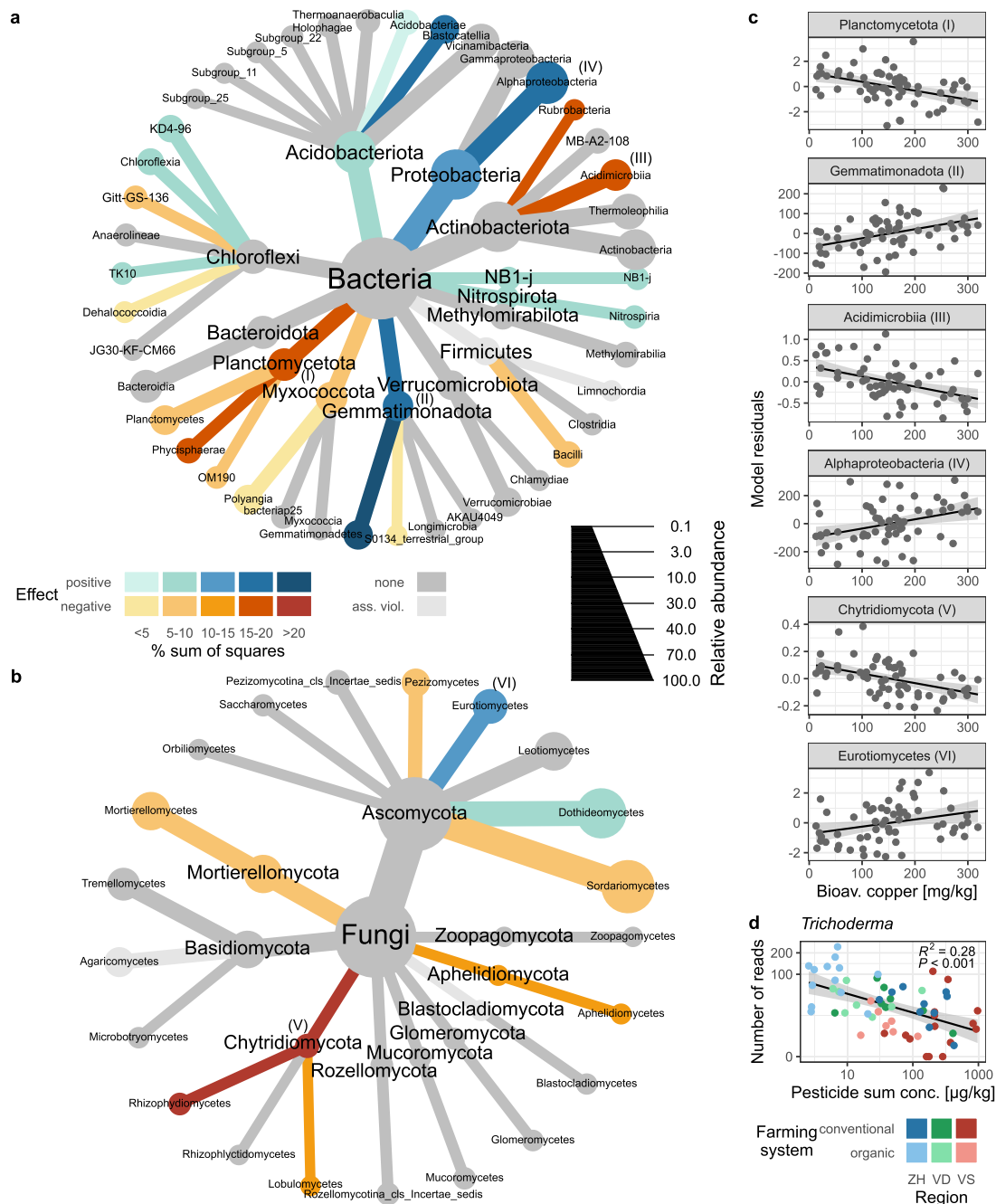


Fig. 3. Copper effects on bacterial and fungal phyla and classes. Heat trees of a) bacterial and b) fungal community displaying overall relative abundance of each group and copper effect on relative abundance. Percent of variance explained by copper was calculated after correcting for region, soil properties, vegetation cover, and farming system. Significant effects with FDR adjusted p-values < 0.05 are displayed in color. Phyla with abundance > 0.5% (bacteria) or > 0.1% (fungi) and their classes with abundance > 0.1% were included. The light gray nodes indicate that the model did not meet the assumptions of homoscedasticity and / or normality. More detailed information is presented in Fig. S11. c) Visualization of selected copper effects that were highly significant. Residuals of a model with only correction variables are displayed. d) Correlation between number of *Trichoderma* reads and pesticide sum concentrations colored by farming system and region (Zurich (ZH), Vaud (VD), and Valais (VS)).

communities were omnipresent. Copper was strongly negatively related to bacterial diversity (Fig. 1). Similar findings were described by Nunes et al. [20] and Li et al. [21] who studied individual fields with gradients in copper contamination. However, other comparable studies on single fields found no effects [23,24], or even positive effects of copper on bacterial diversity [25]. Contrarily, no effects of copper were found on fungal diversity (Fig. 1). This could indicate that fungi generally have a higher capacity to withstand negative copper effects than bacteria. This is in line with previous studies reporting a higher copper sensitivity of

bacteria compared to fungi in terms of abundance [60], growth [61], and diversity [26]. Zhang et al. [22] reported in their field experiment negative copper effects on fungal diversity only from very high concentrations above 1600 mg/kg that were not reached in our study.

Copper was also a major driver of bacterial and fungal community composition (Fig. 2). Previous studies described copper effects on microbial community structure as well [26]. Similarly to our study, Berg et al. [23] found that copper lead to an increase in the bacterial phyla Acidobacteria and Gemmatimonadota and a decrease in

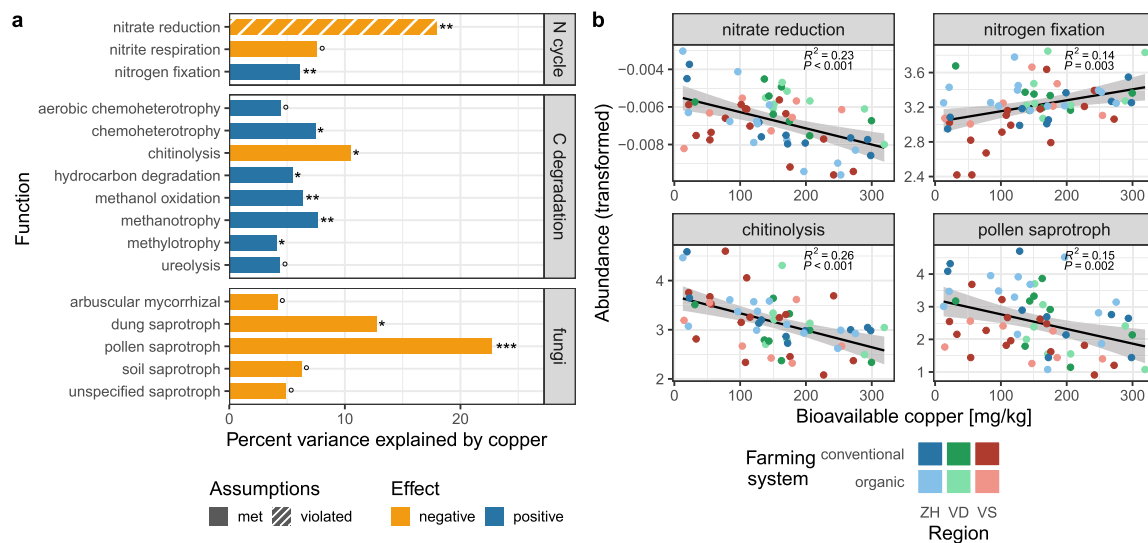


Fig. 4. Copper effects on bacterial and fungal functions. a) Percent of variance explained was tested after correcting for region, soil properties, vegetation cover, and farming system. Significance was assessed with FDR adjusted p-values and displayed using asterisks ($p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*), and $p < 0.1$ (°)). Functions present in less than 80% of vineyards and a mean number below 10 reads per parcel were excluded. For nitrate reduction, the assumption of homoscedasticity was violated. However, the negative association with copper was confirmed by a GLMM ($p < 0.001$). b) Correlation plots between the abundance of selected functions and bioavailable copper, colored by farming system and region (Zurich (ZH), Vaud (VD), and Valais (VS)).

Planctomycetes, as well as the class Acidimicrobiae (Fig. 3). They further found a decrease in Proteobacteria, Bacteroidota and Chloroflexi, which is in contrast to our findings. Li et al. [21] found analogous patterns for Gemmatimonadetes and Planctomycetes, but opposite trends for Acidobacteria. Regarding fungi, Zhang et al. [22] described a strong negative effect of copper on Chytridiomycota, which is in line with the current study (Fig. 3).

While earlier studies assessed the effects of copper on bacterial and fungal communities, they focused on individual fields or a small set of nearby sites. The reported results could, therefore, be location specific. For example, copper toxicity is known to be influenced by other soil properties, such as pH or SOC [27]. The results of the current study are more robust and can be better generalized, as we studied 61 vineyards from different climatic regions, on different soil types (e.g. pH ranged from 6.4 to 8.1; clay 7–34%; SOC 1.1–5.5%). Studies on individual locations also do not allow comparing effect sizes of different soil properties, as they are mostly stable. We found that the effect sizes of copper were similar to pH and much higher than most other soil properties (Fig. 2b,c, Figure S9). Also, so far, studies that characterized the relative importance of different variables (management, geography, soil characteristics) in driving microbial communities did not include copper and pesticide in the analysis [4,62]. Our large field study covering 61 vineyards from three distinct regions and on different soil types allows us to attain a general understanding of copper effects on soil microorganisms and indicates a profound impact of copper on bacterial and fungal communities.

Copper was further found to be associated with the abundance of certain functional groups (Fig. 4) and could, therefore, potentially alter soil functionality. Some nitrogen cycling functions, namely nitrate reduction and nitrate respiration, were negatively related to copper. Similarly, the meta-analysis of Elrys et al. [63] generally documented negative effects of copper contamination on nitrogen cycling functions such as potential nitrification. As an exception, nitrogen fixation and the abundance of the order Rhizobiales were positively related to copper contamination (Fig. 4, Figure S12a). Likewise, previous studies reported an increased abundance of the *nifH* gene in rhizosphere soils [64,65] and of taxonomic groups containing nitrogen fixing species [66,67] with elevated copper levels. Nitrogen fixation could, therefore, be more robust to soil copper contamination than other microbial processes.

Functions including hydrocarbon degradation were mainly positively related to copper (Fig. 4). This is in line with the study of Schneider et al. [60] that observed a shift towards saprotrophs with increasing copper contamination. Contrarily, many fungal saprophytic functions were negatively related to copper (Fig. 4). Saprophytic fungi are thought to be especially sensitive to heavy metals due to their dependence on extracellular enzymes for nutrient acquisition, which are often a target of heavy metal toxicity [68]. A slight negative association was also found for the abundance of AMF. In line with this, AMF were found to be much more sensitive to copper contaminated bulk soil than other fungi [69], and copper has been found to decrease AMF root colonization in grapevines [70]. It is important to note that functional annotations based on taxonomic information have many limitations and that metabarcoding studies only provide information about relative changes in microbial communities, which requires a careful interpretation of such data (see SI section 2).

4.2. Pesticides likely drive farming system differences in vineyard soils

Pesticide sum concentrations were negatively related to fungal diversity and community composition (Fig. 1, Fig. 2). This is a contrast to the studies of Walder et al. [28] and Beriot et al. [29] that found no effects of overall pesticide contamination on neither bacteria nor on fungi in arable and vegetable fields, respectively. The crop might play an important role in explaining this difference. Pesticides, especially fungicides, are used more intensively in viticulture than in arable farming and are, therefore, also expected to cause a stronger disturbance (e.g. in European viticulture the average yearly PPP use is typically 20–30 kg/ha, while cereal crops receive less than 3 kg/ha [14,15]). Fungicides are expected to be particularly detrimental for fungal communities. In line with this, fungicide sum concentrations had a higher explanatory power for fungal diversity and community composition than pesticide sum concentrations, and herbicides and insecticides did not show significant associations (Fig. 1c, Fig. 2a, Figure S9c,d). Compared to vineyards, arable and vegetable fields are also subject to more variable management practices that might increase variation and overlay pesticide effects. Crop rotation in particular increases variation over the years, as each crop requires targeted management interventions and different plants have specific effects on the soil microbiome [71].

Bacteria were much less responsive to pesticides than fungi. Also, the laboratory study of Riedo et al. [72] observed a much higher sensitivity of fungi compared to bacteria and Lu et al. [31] found no correlation between pesticide concentrations and bacterial diversity in vineyard soils.

Pesticide concentrations are strongly intertwined with farming system (conventional >> organic) [5,15,16]. This resulted in a strong reduction in explanatory power when farming system was tested before pesticides in the models for fungal diversity and community composition (Fig. 1c, Fig. 2a). However, pesticide and fungicide sum concentration consistently explained more variance and stayed significant for fungal richness when tested after farming system, whereas farming system lost significance when tested after pesticide sum concentration (Figure S6). This might indicate that farming system differences originated from pesticide contamination, but they could also be a result from other management differences between conventional and organic farming such as soil management including weeding or fertilization strategy. However, previous studies hardly found effects of soil disturbance on fungal diversity [73–76] and fertilization effects were mainly attributed to the amendment of organic matter [76,77] (see SI section 3). Furthermore, as soil disturbance and organic fertilizer use barely differed between the studied conventional and organic vineyards (Figure S15), their effects are expected to be negligible. Therefore, we argue that pesticides were likely the drivers of the observed farming system differences. Effects of individual pesticides on soil microbial community structure are discussed in SI section 4.

4.3. Stronger effects of copper – all clear for pesticides?

We found consistent effects of copper and pesticides on soil microbial communities despite the marked differences in climate and physicochemical properties across regions, which was reflected in a strong differentiation in soil microbiota (see SI section 1). This indicates that the observed trends were not region specific but could be applicable to other viticultural areas and beyond. Copper, overall, had much clearer effects on soil microbial communities than pesticides. One explanation of this could be that copper effects were easier to statistically isolate than pesticide effects, as copper was not confounded with correction variables and exhibited no difference across regions and between conventional and organic farming [15]. The stronger copper effects might also be explained by its much longer and very stable presence in the soil. The current copper concentrations are often a result of continuous copper accumulation over many decades [78]. Soil microbiota were, therefore, under constant influence of copper and shaped accordingly.

Pesticide contamination, on the other hand, is much more variable. Concentrations strongly fluctuate over the season with big peaks when applied and subsequent dissipation, which is also reflected in their toxicity [79]. We sampled 3–4 months after the last pesticide application to assess the long-term effects of pesticide use. At this timepoint, pesticide toxicity was probably also close to its minimum. Due to rapid dissipation, many pesticides might act as a temporal disturbance. This might partly explain why we found effects mainly on fungi but hardly on bacteria which generally grow faster [80] and might already have recovered at the time of sample collection. Multiple samplings across the season would be necessary to investigate the temporal effects of pesticides on microbial communities. An additional challenge in detecting pesticide effects is the broad selection of recommended products. While copper is a single compound found in all vineyards, the composition of applied pesticides varies across years and between farms, which led to unique mixtures of up to 60 different pesticides per vineyard [15].

4.4. The necessity of improved risk assessments

While it is difficult to disentangle effects from different contaminants in the field, improved risk assessments can help to identify substances that could be particularly problematic for soil organisms and their

functioning. Because different microbial groups respond so differently to copper and pesticide exposure (Fig. 3, Figure S13), general microbial indicators, such as the commonly used nitrogen mineralization test, are not sufficient to assess the risk of these PPP towards soil microbes. As with soil fauna, specific microbial groups that are known to perform key functions or to be particularly sensitive should be tested to avoid unintended effects on the soil microbiome. One widely accepted candidate to be included in risk assessments are AMF [19]. Several studies documented that AMF reacted sensitive to pesticide applications and residues, leading to reductions of root colonization [5], phosphorus transfer [81], and AMF diversity [76]. Although our study did not reveal a strong pesticide sensitivity of AMF, they are a key candidate for risk assessment studies, also in view of their ecological and agronomic relevance [82].

Amongst microbes that were found sensitive to pesticides (Table S2), *Trichoderma* and its most abundant species *T. koningii* were of particular interest (Figure S14). *Trichoderma* is widely distributed and well-known for its disease suppressive and plant growth promoting properties [83]. Hence, the application of pesticides could reduce the biocontrol ability of soils. *Trichoderma koningii* and other *Trichoderma* species are already used commercially as plant growth promoters and could be interesting candidates for risk assessments. Copper affected many bacterial and fungal genera (Table S2) but also higher taxonomic groups and its effects might be assessed on the abundance of certain phyla such as Planctomycetota or Chytridiomycota (Fig. 3).

The combination of copper and pesticides might increase the pressure on soil microbiota considering recent studies that show additive negative effects from multiple stressors [84,85]. In line with this, we found more severe copper effects on bacterial diversity in conventional vineyards than in organic ones (Figure S5). Microbes in conventional vineyards are expected to be exposed to more stressors due to the more intensive management including pesticide application, which might increase their copper sensitivity. Similarly, laboratory experiments have described synergistic negative effects of certain pesticide and heavy metal combinations [86]. While targeted trials can improve our general understanding about joint effects of copper and different pesticides on soil microbiomes, such studies should be complemented by field studies, taking into account the multitude of influencing factors under realistic conditions.

5. Conclusions

We documented that copper and pesticides are major determinants of soil microbial communities in vineyards. While copper was a strong driver of bacterial diversity and community composition, fungal communities were also affected by pesticides. Current risk assessments do not sufficiently capture the varying impacts of copper, pesticides, and other PPP on different taxonomic and functional groups of soil microorganisms. Therefore, research should develop and evaluate viable tests for extended risk assessments that allow a more detailed evaluation of PPP effects on soil microbiomes. While further research is also needed to disentangle effects from different management practices or specific pesticides and their interactions, our study suggests that conversion to organic viticulture can support soil microbial – and particularly fungal – diversity. This might be related to the dissipation of pesticides over time. In the long term, a shift towards management practices that allow copper-free viticulture is needed to prevent further copper accumulation in the soil. This applies equally to organic and conventional viticulture. Our study expands the current knowledge of the non-target effects from PPPs on soil microbiomes and highlights the need for action to maintain soil health in agriculture in the future.

Environmental implication

An increasing number of studies is documenting harmful effects of pesticides on non-target organisms and their widespread occurrence in

the environment, which could have negative consequences for biodiversity and ecosystem services. Pesticide effects on soil microbes are still poorly studied despite soil biota playing a central role in many environmental processes. Here we studied vineyards as a model system for intensive agricultural systems and observed that bacteria and fungi were negatively affected by copper and synthetic pesticides. Our findings provide an incentive for improved risk assessments including sensitive soil microorganisms and stricter policies to protect soils from unwanted pesticide effects.

Funding

This research was funded by Agroscope and did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Elias Barmettler: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Kyle Hartman:** Writing – review & editing, Data curation. **Alain Y. Valzano-Held:** Writing – review & editing, Methodology. **Lina Egli-Künzler:** Investigation, Data curation. **Pierre-Henri Dubuis:** Investigation, Data curation, Conceptualization. **Kathleen A. Mackie-Haas:** Writing – review & editing, Supervision, Conceptualization. **Thomas D. Bucheli:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Stefanie Lutz:** Writing – review & editing, Supervision, Project administration. **Marcel G.A. van der Heijden:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the farmers who provided management information and allowed us to sample in their vineyards. Also, we would like to thank C. Wenger, A. Schneller, and the members of the mycology group of Agroscope Changins who helped in the sampling campaign. Further, we thank A. Rösch, F. E. Wettstein, P. Sutter, D. Bürge, S. Pöschl, P. Peier and S. Gfeller for support in the laboratory and performing laboratory analyses. Library preparation and sequencing were carried out at the Genomic Diversity Centre (GDC) of ETH Zurich.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2026.141682](https://doi.org/10.1016/j.jhazmat.2026.141682).

Data availability

All sequences generated within this study are available at the Sequence Read Archive (SRA) database under BioProject accession number PRJNA1295439. Datasets and code for this manuscript are available on zenodo: <https://doi.org/10.5281/zenodo.18327657>.

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