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Soil microbiome signatures are associated with pesticide residues in arable landscapes

Florian Walder [a,*], Marc W. Schmid [b], Judith Riedo [a,c], Alain Y. Valzano-Held [a], Samiran Banerjee [a,d], Lucie Büchi [e], Thomas D. Bucheli [f], Marcel G.A. van der Heijden [a,c]

[a] Research Group Plant-soil Interactions, Agroscope, Zurich, Switzerland
[b] MWSchmid GmbH, Glarus, Switzerland
[c] Department of Plant and Microbial Biology, University of Zurich, Zurich, Switzerland
[d] Department of Microbiological Science, North Dakota State University, ND, USA
[e] Natural Resources Institute, University of Greenwich, Chatham Maritime, United Kingdom
[f] Research Group Environmental Analytics, Agroscope, Zurich, Switzerland

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ABSTRACT

Pesticides are widely applied in agriculture to combat disease, pests, and weeds, leading to long-lasting contamination of agricultural soils with pesticide residues. While classical risk assessment experiments have repeatedly addressed immediate pesticide effects, we employ an ecological approach to investigate how pesticide residues persisting in soils influence the soil microbiome under realistic agricultural conditions. We assessed a wide range of soil characteristics, including the occurrence of 48 widely-used pesticides in 60 fields under conventional, no-tillage and organic management. We then tested which factors best explain soil microbiome traits. Environmental factors, including climate, geography, and soil characteristics, were the soil microbiome's leading drivers. Remarkably, of all management factors, pesticide residues showed the strongest associations with soil microbiome traits, which were even more pronounced than the effects of cropping systems. Pesticide residues were almost exclusively positively associated with the relative abundance of 113 bacterial and 130 fungal taxa, many of them being assigned to taxa of known pesticide degraders. While fungal diversity and abundance were primarily positively associated with pesticide residues, bacterial diversity and abundance of the gene *nifH* - essential for biological nitrogen fixation - were negatively linked to the concentration of individual pesticide residues. Our results suggest that pesticide residues alter the soil microbiome, with potential long-term implications for the functioning of agricultural soils.

1. Introduction

Pesticides have become an indispensable part of modern agriculture. They reduce yield losses caused by disease, pests, and weeds, securing agricultural production (FAO and ITPS, 2017; Savary et al., 2019). However, the frequent use of pesticides may lead to the environment's contamination, raising several ecological and societal concerns (Möhring et al., 2020; Topping et al., 2020). So far, much attention has been paid to the impact of pesticides on freshwater systems (Malaj et al., 2014; Stehle and Schulz, 2015), and various studies linked the occurrence of pesticides in the environment to declining insect, pollinator and bird populations (Gill et al., 2012; Hallmann et al., 2014, 2017; Sánchez-Bayo and Wyckhuys, 2019).

The environmental consequences of pesticides in soils, however, have largely been neglected. Agricultural soils are particularly at risk since 30–50% of the applied pesticides may not reach their targets and end up on the soil surface (Rodríguez-Eugenio et al., 2018). Recent screenings of agricultural soils have revealed widespread yet hidden contamination with multiple pesticide residues - notably in the surface layer (Hvezdova et al., 2018; Silva et al., 2019; Riedo et al., 2021). Pesticide residues may also be common in organically managed soils (Humann-Guilleminot et al., 2019; Geissen et al., 2021), where synthetic pesticides are no longer applied. Even after 20 years of organic management, up to 16 different pesticide residues were found (Riedo et al., 2021). Consequently, agricultural soils are contaminated in the long term, with numerous pesticide residues posing an ecological risk to the

* Corresponding author. Agroscope, Reckenholzstrasse 191, CH-8046, Zürich, Switzerland.
E-mail address: florian.walder@agroscope.admin.ch (F. Walder).

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soil ecosystem (Vasickova et al., 2019).

Soils are one of the largest reservoirs of biodiversity on earth, harboring myriads of microorganisms, contributing fundamentally to soil functioning and fertility (Bardgett and van der Putten, 2014; Bender et al., 2016; Orgiazzi et al., 2016). Particularly important are bacteria and fungi, which function together as a microbiome and, for example, largely control soil nitrogen cycling (Kuypers et al., 2018) - one of the most limiting nutrients for plant growth on Earth (Galloway et al., 2008). It is thus essential to know how the widespread contamination of agricultural soils with pesticide residues affects their microbiome and soil fertility.

Many studies examined the immediate effects of pesticides and their metabolites on the soil microbiome in classical risk assessment experiments (e.g. Karpouzas et al., 2014; Ju et al., 2017; Vasileiadis et al., 2018; Yu et al., 2020). These experiments typically apply individual compounds and assess the microbiome's response in subsequent weeks or months. For instance, such studies consistently reported adverse effects of pesticides on the soil nitrogen cycle (Karpouzas et al., 2014; Karas et al., 2018), while effects on abundance, diversity or composition of soil microbial communities often remained transient (Feld et al., 2015; Storck et al., 2018).

Effects on the soil microbiome are not unexpected, as pesticides are, by definition, bioactive, toxic substances. However, pesticides aim at interfering with specific processes in the target organisms to minimize such side effects. For instance, herbicidal compounds targeting enzymes involved in photosynthesis or the synthesis of plant-specific amino acids should theoretically be specific to plants. However, several herbicides also act on targets shared by plants and soil microbes (Thiour-Mauprivez et al., 2019). In contrast, fungicides are designed to suppress fungi, potentially affecting non-target soil fungi (Johnsen et al., 2001). Yet, adverse effects on a wide range of non-target soil microbes - not just fungi - have been observed in pesticide risk assessment studies in simplified model systems (Bünemann et al., 2006; Stanley and Preetha, 2016). Interestingly, it is not uncommon to find that pesticides also promote the occurrence of particular microbial taxa (Imfeld and Vuilleumier, 2012; Itoh et al., 2014). The latter is often attributed to microbes' ability to degrade pesticides as a source of energy and nutrients (Singh et al., 2016; Douglass et al., 2017; Gallego et al., 2019), and microbes are even used for bioremediation of contaminated soils (Singh and Walker, 2006). In addition, it was suggested that pesticides alter competitive interactions among microbes - e.g. if specific microbes are suppressed, others benefit and increase in abundance (Johnsen et al., 2001). Therefore, pesticides do not necessarily reduce the abundance or diversity of microbial populations but may instead lead to shifts in microbial communities with potential consequences for soil functioning.

While research has focused so far on prospective risk assessment following the application of specific pesticides, far less attention has been paid to the role of the widespread pesticide residues that persist in agricultural soils for years or even decades (Bünemann et al., 2006; Vasickova et al., 2019). Prospective risk assessment experiments are well suited to investigate the causal effects of a particular pesticide on the soil microbiome but only test pesticide effects in isolation without considering other key factors (e.g. climate, soil properties, soil management) that regulate microbial community composition under realistic agricultural conditions. Moreover, such experiments are usually performed over a relatively short period and cannot uncover the effects of long-term exposure, as occurs in agroecosystems. In an earlier study, we employed an ecological approach to investigate such long-term effects by linking environmental concentrations of pesticide residues to microbial biomass and the abundance of beneficial soil fungi, indicating adverse effects on microbial soil life (Riedo et al., 2021). However, it is not clear to which extent pesticide residues also affect the composition and structure of the soil microbiome and whether these residues threaten critical soil functions such as nitrogen cycling across arable landscapes.

To advance our knowledge of the impact of pesticide residues on the soil microbiome, we utilized an earlier study in which we assessed soil fertility and crop yield in a farmnetwork across Switzerland's arable landscape, evaluating 60 wheat fields (Büchi et al., 2019). We compared three cropping systems: 20 wheat fields that were managed conventionally with tillage, 20 conventionally without tillage (no-till), and 20 organically managed wheat fields without synthetic pesticide application. We analyzed the concentration of 48 residues of widely-used pesticides – including 26 herbicides such as glyphosate and their transformation products, 17 fungicides and seven insecticides-in soil samples of these 60 fields (Riedo et al., 2021). Here we characterized the bacterial and fungal microbiota and the abundance of genes central to soil nitrogen cycling in the same soil samples. We then assessed whether pesticide residues' concentrations were linked to different traits of the soil microbiome in the surface and topsoil layer.

We specifically asked: (i) which factors best explain soil microbiome traits across the arable landscape. (ii) What is the relative importance of pesticide residues compared to other management factors such as cropping systems? (iii) Do fungicides indicate a more pronounced effect on microbial communities - especially fungal communities - than herbicides? (iv) Are microbial functional genes such as those involved in soil nitrogen cycling particularly sensitive to pesticide residues? (v) Are there specific microbiome traits (e.g. abundance of specific taxa) that can act as indicators for the pesticide contamination of arable soils?

2. Materials and methods

2.1. Farm network

We established a network of 60 farms across Switzerland organized in two hubs (one in the northeast and one in the southwest of Switzerland) in 2016 (Fig. S1A); all soils were classified as Cambisol. We selected one field cultivated with winter wheat on each farm. The fields were farmer-managed under the following three cropping systems: (i) conventional agriculture with tillage, (ii) conventional agriculture with continuous no-till practice, (iii) and organic agriculture with tillage. Conventionally and no-till managed fields applied synthetic pesticides and fertilizers and were managed according to the guidelines of the "Ecological Performance Certificate" of the Federal Office for Agriculture, Switzerland (www.blw.admin.ch). Organically managed fields received no pesticides or synthetic fertilizers and were managed according to the association of Swiss organic farmers (www.bio-suisse.ch). In all groups, the investigated cropping system had been implemented for a minimum of five years.

Details on each field's management were collected directly from the farmers through a questionnaire about the last five years before sampling (for details, see Büchi et al. (2019)). This survey gave us management indices for crop diversification, soil tillage, synthetic nitrogen fertilizer input and organic amendments.

2.2. Soil sampling and physicochemical analysis

We designated an area of approximately 300 m^2 of at least 20 m distance from the field's edge as the sampling area on each field. Sampling took place between April 20 and May 27, 2016. Composite soil samples were collected by sampling 20 soil cores (0–20 cm depth) along two perpendicular transects crossing in the centre (approximately at a 45° angle to the seeding rows) with a hand auger. After separating them into the surface- and topsoil layers (0–5 and 5–20 cm, respectively), we pooled soil samples to obtain a representative composite sample for each soil layer and field, yielding a total of 120 samples. Thus, we only analyzed one composite sample for each layer per site, as our aim was not to assess within-site variability but rather the general patterns that soil microbiome exhibit concerning pesticides and other management factors across the 60 sites in an arable landscape (Lauber et al., 2008; Delgado-Baquerizo et al., 2018). Besides, five cylindrical soil cores (100 ml volume and 5.1 cm in diameter) were collected at two depths (2.5

and 12.5 cm) for soil bulk density estimates of each soil layer. We stored all soil samples at 4 °C and no longer than one week before further processing.

Composite soil samples were mixed by passing through an 8 mm sieve, cleaned of plant residues and animal debris and dried at 4 °C in open boxes until soil moisture allowed sieving to 2 mm. A subsample of approximately 50 g fresh weight was stored at −20 °C until further processing for molecular analysis. A further subsample of 150 g was dried at 60 °C and used to determine soil physicochemical properties. The soil physicochemical properties texture, pH, organic carbon (C), and soil nutrients (phosphorous (P), potassium (K), calcium (Ca) and magnesium (Mg)) were determined according to the Swiss reference methods of the Federal Agricultural Research Stations (Agroscope, 1996). Briefly, organic C was determined with the Walkley-Black method, total nitrogen with the Dumas method, texture with a pipetting method with the following cut-offs (clay <2 μm, silt 2–50 μm and sand >50 μm), K, Ca and Mg were extracted with 0.5 M ammonium acetate-EDTA solution at pH 4.65. Total P was extracted by ashing the soil sample and using 0.6 M HCl as extractant, and pH was determined in water. To obtain soil bulk density, the cylindrical soil samples were dried at 105 °C for at least 72 h before weighing.

2.3. Pesticide residue analysis

Concentrations of pesticide residues in the surface layer soil samples were quantified as reported in Riedo et al. (2021). Briefly, pesticides and their transformation products were selected for analysis based on amount and frequency of usage in Switzerland, persistence in soil, bioavailability, ecotoxicological relevance and analytical determinability. The in Riedo et al. (2021) described multi-residue method allows quantifying 16 herbicides, eight herbicide transformation products, 17 fungicides and seven insecticides, yielding a total of 46 compounds. Accelerated Solvent Extraction was used to extract the pesticide residues from the soil. Pesticides were quantified by high-performance liquid chromatography coupled to a triple quadrupole tandem mass spectrometer (LC-MS/MS). The limit of detection was between 0.037 and 36 μg/kg, depending on the pesticide. For details on quality assurance and figures of merit of the validated method, see Riedo et al. (2021).

The herbicide glyphosate and its metabolite aminomethylphosphonic acid (AMPA) were quantified by LC-MS/MS-based on their pre-column derivatization with fluorenylmethyloxycarbonyl chloride and their enrichment by solid-phase extraction as described earlier (Daouk et al., 2013). The limit of quantification was 10 ng/L, and possible matrix effects were calculated and taken into account by analyzing spiked soil solution.

Out of the 48 pesticides and transformation products assessed, only compounds occurring in more than ten samples were considered for the statistical analysis of individual pesticide residues (Table S1). We refer to both the pesticides and the transformation products as pesticides for simplicity.

2.4. DNA extraction and quantitative PCR

We extracted DNA from each soil sample of each layer and site on the basis of 0.5 g of the composite sample stored at −20 °C using the NucleoSpin soil kit (LS2 Buffer, Macherey-Nagel GmbH & Co. KG, Duren, Germany) according to the manufacturer's instructions. The absolute abundance of bacterial 16S, fungal ITS and microbial genes central to soil nitrogen cycling (*nirK, nosZI, nosZII, amoA* of ammonia-oxidizing bacteria (AOB) and archaea (AOA), and *nifH*) was determined by quantitative polymerase chain reaction (qPCR) using the C1000 Thermal Cycler (Bio-Rad Laboratories AG, Cressier, Switzerland). The primers used are given in Table S2. Each 20 μl reaction mixture contained 10 ng of DNA template, 4 μl 5xHOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Tartu, Estonia), 500 nM of each primer, 0.3% Bovine Serum Albumin (BSA) and the remaining volume nuclease-free water. For nosZII, we used a different qPCR mix, namely Luminaris HiGreen qPCR Master Mix, Low ROX (Fisher Scientific). Each reaction for nosZII contained 10 ng of DNA template, 10 μl 2x Luminaris HiGreen qPCR Master Mix, 1000 nM of each primer, 0.3% BSA, and the remaining volume of nuclease-free water. Thermal cycling conditions for each gene are listed in Table S3. Standard type, strain and fragment size, and cloning kit and vector size are given in Table S4. We assessed the specificity of the amplified products by melting curve analysis. All samples and standards were run with three replicates.

2.5. Amplicon sequencing

The 16S rRNA gene amplicon library was generated using the PCR primers 341F (Thijs et al., 2017) and 805R (Klindworth et al., 2013), amplifying the V3–V4 region. The ITS amplicon library was generated using the PCR primers ITS1F1 (Gardes and Bruns, 1993) and ITS2 (McGuire et al., 2013), amplifying the ITS region 1. Thermal cycling conditions for 16S and ITS genes are listed in Table S5. The primers were extended at the 5′end with an error-tolerant barcode for multiplexed library sequencing. We refer to Supplementary Methods for details in PCR setup, cycling conditions, and library preparation protocol. The libraries were sequenced on a MiSeq Instrument (Illumina, San Diego, USA).

Short reads generated in this study were deposited at NCBI Sequence Read Archive (SRA, www.ncbi.nlm.nih.gov/sra; accession number PRJNA682947). We describe details of sequence data processing in the Supplementary Methods. In brief, we generated operational taxonomic units (OTUs) with USEARCH (Edgar, 2013) after removing sequencing adapters, primers, and low-quality and chimaeric sequences. The remaining sequences were sorted according to length and clustered with a minimal identity threshold of 99% using search (Edgar, 2013). OTU sequences were taxonomically annotated with the Ribosomal Database Project (bacterial sequences (Cole et al., 2014)) and UNITE (fungal sequences (Nilsson et al., 2019)). We removed OTUs with less than 50 (16S) or 10 (ITS) counts or occurring in less than five samples from all further analyses to avoid sequencing artefacts.

2.6. Diversity indices and community structure

To assess the impact of the environmental and management factors on bacterial and fungal diversity, we analyzed the variation in effective species richness (exponent of the Shannon index (Magurran, 2004)) and evenness (Pielou, 1975). The impact on bacterial and fungal community structure was first inspected by principal component analysis (PCA) and then assessed by performing permutational multivariate analysis of variance (PERMANOVA) using manhattan and UniFrac (Lozupone et al., 2011) distances on log2(x+1) transformed OTU counts. As input for the UniFrac distances, we used a mid-point rooted tree of OTU sequences. The genetic tree was obtained with QIIME (Caporaso et al., 2010) with the scripts "align_seqs.py -m muscle" and "make_phylogeny.py -t fasttree". For the analyses with the diversity indices and the community structure, data were rarefied to the sample with the lowest number of counts (e.g. 16′753 for bacteria and 2′328 for fungi) in the data set.

2.7. Identification of differentially abundant OTUs and enrichment of taxonomic groups

Variation in relative abundances of individual OTUs and enrichment of bacterial or fungal taxonomic groups were analyzed on non-rarefied rarefied (Weiss et al., 2017), but with DESeq2 (Love et al., 2014) normalized and log2(x+1)-transformed OTU counts. Variation in relative abundances of individual OTUs was analyzed with the same models used for the biodiversity indices and community compositions described below, i.e., using the regular ANOVA and linear model functions in R. For a given term in the model, P-values from all OTUs were adjusted for

multiple testing (Benjamini and Hochberg, 1995). OTUs with an adjusted P-value (false discovery rate, FDR) below 0.05 were considered to be differentially abundant.

To test for enrichment of bacterial or fungal taxa occurrences in a given set of OTUs, we constructed a contingency table for each taxon and tested for significance with Fisher's exact test. P-values were adjusted for multiple testing (Benjamini and Hochberg, 1995), and phyla, order and genus with an adjusted P-value (FDR) below 0.05 were considered to be significantly enriched.

2.8. ANOVA and PERMANOVA models

We performed ANOVA, and PERMANOVA models to test which factor(s) significantly explained microbiome traits (e.g. microbial diversity). The structure of all models followed general design principles, as described in Schmid et al. (2017). In each model, factors were fitted sequentially (type I sum of squares), and the terms "field-ID" and "field-ID:layer" were used as random terms employing appropriate error terms and denominator degrees of freedom (Hastings et al., 1947). We first fitted individual geography, climate, soil variables and hub to account for variation explained by these factors as we were not interested in these effects per se (details on the model structure and compilation of the environmental variables are given in Fig. S1B). For simplicity, we merged these environmental variables by summing up the sum of squares and degrees of freedom, calculating the mean sum of squares and only reporting the overall terms geography, climate, and soil in the main figures. The variables of interest were the assessed pesticide residue concentrations, the number of herbicides, fungicides, and insecticides and the total number of pesticide residues found per soil sample. Also, we tested whether other management characteristics (e.g. crop diversification, soil tillage, synthetic nitrogen fertilizer and organic amendments) explained microbiome traits. As these management practices differ between cropping systems, we tested each variable of interest either after the cropping system or separately without the cropping system. We reported both results to provide a range of possible effects.

Relative importance was expressed as the percentage sum of squares explained by a term in the model. The direction of an effect, i.e., if a response variable increases/decreases with an increase/decrease of an explanatory term, were extracted from the model coefficients based on standardized variables. We ran the same models after winsorizing (i.e., limiting extreme values to the 5- and 95-percentile) all numeric data to ensure that outliers did not drive effects. Overall, the effects were robust, and the results without winsorizing were more conservative than the original data (data not shown). All analyses were carried out with R 3.6.3 (R Development Core Team, 2020). Base R was used for the graphical work, except for the heatmap in Fig. 4B, which was created with the *gplot* function of the *sna* package (Butts, 2020).

3. Results

3.1. Composition of the bacterial and fungal microbiota

Amplification of 16S rRNA and ITS gene fragments yielded an initial set of 11,600 and 1764 OTUs, respectively. After removing low-abundant OTUs, 8375 16S- and 1444 ITS-OTUs remained. Of the 8375 16S-OTUs, 7065 were classified as bacteria, six were classified as archaea, and 1304 remained unclassified or unknown. Within the bacterial domain, the six most frequent phyla accounted for 88.7% of all 16S-OTUs; they were Proteobacteria (9.7% Alpha-, 9.6% Beta, 6.2% Gamma-, 8.4% Delta- and 1.5% other Proteobacteria, respectively), Acidobacteria (19.7%), Bacteroidetes (12.6%), Verrucomicrobia (9.2%), Actinobacteria (8.9%), and Planctomycetes (2.9%).

Of the 1444 ITS-OTUs, 717 were classified as fungi, and 727 remained unclassified or unknown. The six most frequent phyla accounted for 96.5% of all fungal ITS-OTUs; they were Ascomycota (52.2%), Basidiomycota (12.7%), Chytridiomycota (12.1%), Mortierellomycota (8.8%), Glomeromycota (6.4%), and Rozellomycota (4.3%).

3.2. Environmental determinants of the soil microbiome

Of the factors tested, soil, climate and geographic variables primarily determined the soil microbiome, while the effect of agricultural management was relatively small in comparison. Soil characteristics were the most important driver for fungal and bacterial diversity indices. They significantly explained between 14 and 31% of the variation in richness and evenness (Fig. 1, Tables S6–9), with texture emerging as the prominent factor (Table S10). Geography was also a significant predictor of effective species richness of bacteria and fungi (explaining between 15 and 22% of the variation), where spatial location explained more than altitude (Table S10). Climate as a whole term was significant for bacterial 16S effective richness (12–14%) but explained a smaller proportion of variance than soil characteristics and geography (Fig. 1, Tables S6–9). Of the climate variables, precipitation explained the most significant share of variation for the diversity indices (Table S10).

Besides the effects on bacterial and fungal diversity, we assessed the factors' impact on the absolute abundance of the 16S and ITS genes. The abundance of the 16S gene was primarily affected by geography and, to a lesser extent, soil and climate (Fig. 1C). In contrast, the abundance of the ITS gene was most affected by soil.

The PCA of the bacterial and fungal communities depicts the strong influence of environmental determinants, indicating a solid gradient for soil pH but no grouping according to the cropping system (Fig. 2). The first two PCA components explained 56.4% and 49.8% of the overall variances in the 16S- and ITS-OTUs, respectively. The PERMANOVA results for the impact of the different environmental characteristics on the bacterial and fungal community composition and the phylogenetic distance proved the importance of the environmental terms (Table S11/S12). For the 16S-OTUs, the soil had a significant impact on community composition. In contrast, geography was only significant in the case of the phylogenetic distance between bacterial communities. For the ITS-OTUs, only soil significantly affected the community composition but not on fungal communities' phylogenetic distance. Within the environmental terms, the most important terms for both OTU types appeared to be the spatial variation in the coordinate system (geography), average temperature six months before sampling (climate), and pH (soil; Table S10).

In the next step, we tested whether environmental determinants affect the abundance of individual OTUs. For the 16S-OTUs, the soil had the most considerable impact, with 58.9% of all OTUs significantly influenced by soil characteristics (Fig. 3A, Table S13). Soil characteristics explained up to 70% of the variation in individual OTU abundances of more than half of 16S-OTUs. Geography and climate also significantly influenced specific OTUs, with 18.6% and 12.6% of all OTUs being significant and explaining up to 50% and 40% of the variation in individual OTU abundances. For the ITS-OTUs, the results were similar, although much smaller than the impact on 16S-OTUs, with 5.9%, 4.6%, and 0.5% significant OTUs for soil, geography, and climate, respectively (Table S14).

3.3. Influence of agricultural management and pesticide residues on microbial communities

We then assessed the effects of different management factors on the different soil microbiome traits. Several pesticide residues were associated with bacterial and fungal diversity indices. For instance, 2-hydroxyatrazine, glyphosate, AMPA, and carbendazim negatively correlated with bacterial diversity indices (Fig. 1, Fig. S4/S5, Table S6/S7). In contrast, pesticide residues showed negative and positive associations with fungal diversity indices. 2-Hydroxyatrazine was negatively associated with fungal evenness, whereas S-metolachlor was positively associated with fungal diversity and evenness (Fig. 1, Fig. S6, Table S8/

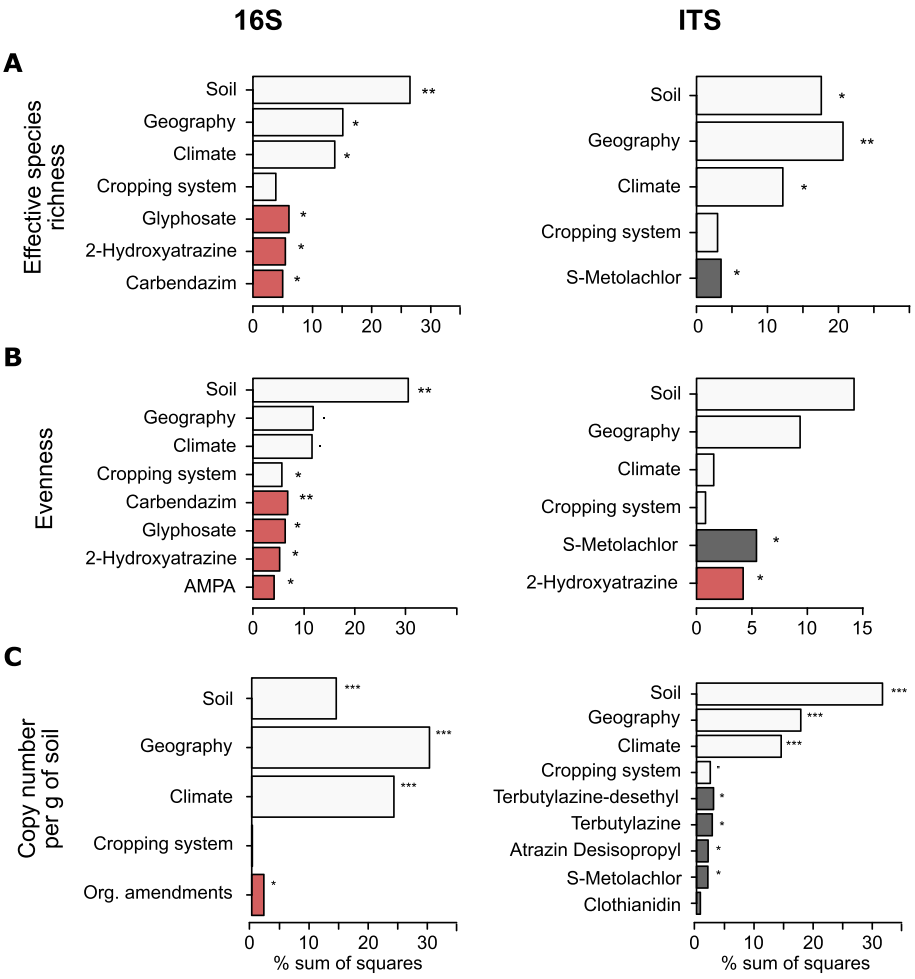

Fig. 1. Relative importance of environmental characteristics (soil, geography and climate), cropping system and management factors including pesticides in explaining bacterial (left) and fungal (right) species richness (A), Pielou's evenness (B), and 16S or ITS gene copy number (C; expressed as percent sums of squares (SS)). For reference, the correction terms (soil, geography, and climate) and the cropping system are always shown; otherwise, only the terms that were significant when fitted after the correction variables are shown. Colors indicate the direction of pesticides' effect on bacterial and fungal characteristics (orange for a negative, dark grey for a positive effect, and white for when we could specify no direction, e.g. for summarized correction terms and cropping system). Significance is given in the labels to the right of the bars. Labels are: •:P < 0.1, *:P < 0.05, **:P < 0.01, ***:P < 0.001. The relative importance of management terms fitted after cropping system is shown in Fig. S2. Results, including the interactions with layer, are shown in Fig. S3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

S9). Notably, pesticide residues' relative importance in explaining bacterial and fungal diversity was similar whether they were fitted separately or after the cropping system, respectively (Fig. 1 & S2, Tables S6–9). The interactions with the soil layer were evident, but they were at most equal and usually weaker than the main effects of the pesticide residues (Fig. S3).

The cropping system was only a significant predictor of bacterial evenness (Fig. 1, Table S7). The management indices crop diversification, soil tillage, synthetic nitrogen fertilizer input and organic amendments were not significantly linked to bacterial or fungal diversity indices (Tables S6–9). While individual pesticide residues showed a clear association with bacterial and fungal diversity indices, the number of herbicides, fungicides, insecticides and the total number of pesticide residues did not show any significant relationship (Tables S5–S8). Bacterial and fungal community composition did not reveal any significant association with cropping system, management indices, or pesticide residues (Table S11/S12).

The relative importance of management effects on absolute 16S and ITS gene abundances was also relatively small compared to the responses of the diversity indices (Fig. 1C). Of the tested agricultural management characteristics, only organic amendments affected the absolute abundance of the 16S gene, and the effect was negative. In contrast, the abundance of the ITS gene showed a positive association with several pesticide residues and tended to be affected by the cropping system (Fig. 1C).

3.4. Pesticide residue-sensitive microbial taxa

We then tested whether cropping systems, management indices and pesticide residues are linked to the relative abundance of the 8375 16S-OTUs and 1444 ITS-OTUs (Fig. 3). Specific pesticide residues showed a significant association with 113 individual 16S-OTUs, with carbendazim, tebuconazole, glyphosate, and 2-hydroxyatrazine correlating to more than ten 16S-OTUs (Fig. 3A, Table S9/S10). Except for the association of 2-hydroxyatrazine and several 16S-OTUs, pesticide residues were almost exclusively positively associated with individual OTU abundances (Fig. 3B/C). The associations with specific pesticide residues were positive overall for 71.7% of the significantly associated 16S-OTUs. Similarly, 130 ITS-OTUs showed a significant – 97.7% positive - association with specific pesticide residues, where linuron, S-metolachlor and deethylatrazine were among the top residues. Pesticide residues could explain up to 32% of the variation in individual OTU abundances (Fig. 3A), and these pesticide residue-sensitive OTUs were neither rare nor dominant (Fig. S7). Interestingly, the abundance of fungal ITS-OTUs was more frequently correlated to pesticide residues than the abundance of 16S-OTUs (8.9% for fungi vs 1.3% for bacteria across all residues).

In contrast, the cropping system was only associated with 41 16S- and three ITS-OTUs (Table S13/S14). From the management indices, only soil tillage had a significant association on a few 16S-OTUs and ITS OTUs; however, only if fitted without accounting for cropping system effects.

The pesticide residue-sensitive OTUs were broadly distributed across the phylogenetic tree and found in all major phyla (Fig. 4 and S8). Several genera were enriched with pesticide-sensitive 16S-OTUs and consistently showed positive associations with various pesticide residues. Prominent examples of positive responding prokaryotic genera are *Mucilaginibacter, Niastella, Edaphobacter, Polaromonas* and *Burkholderia* -

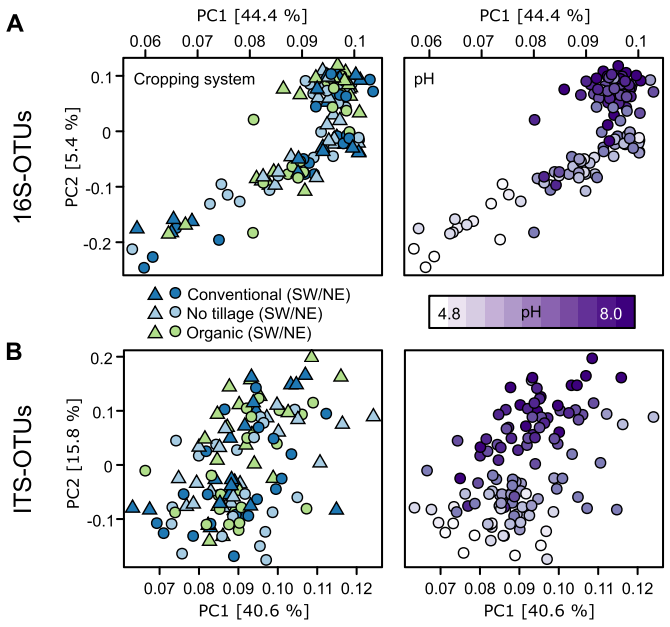

Fig. 2. Principal component analysis (PCA) of bacterial and fungal communities. PCA was performed on normalized operational taxonomic unit (OTU) abundances of all samples. For the 16S-OTUs (A), the first two axes explained 40.6% and 15.8% of the variance, respectively. For the ITS-OTUs (B), the first two axes explained 44.4% and 5.4% of all variance, respectively. Samples are colored according to the cropping system (left) and pH (right). Symbols indicate the different network hubs (NE: northeast, SW: southwest; see Fig. S1 for details).

most associated with 2-hydroxyatrazine or carbendazim (Fig. 4, Table S15). A few genera contained significantly more pesticide-sensitive 16S-OTUs showing negative associations, notably *Skermanella* and *Pedomicrobium*. Among the fungal genera significantly enriched with pesticide-sensitive OTUs are *Spizellomyces* of the phylum Chytridiomycota, *Volvopluteus* and *Heterobasidion* of the phylum Basidiomycota and several genera of the phylum Ascomycota such as *Aspergillus, Beauveria, Candida* and *Fusarium*, associated among others with the residues deethylatrazine, cyprodinil and S-metolachlor (Fig. S8, Table S16).

3.5. Pesticide residues associated with soil nitrogen cycling

Finally, we evaluated whether agricultural management and the occurrence of pesticide residues correlated with the absolute abundance of genes central to soil nitrogen cycling (Fig. 5). All genes except *nirK* were significantly associated with at least one pesticide residue when the statistical model first accounted for the cropping system. The abundance of *nifH* involved in nitrogen fixation was exclusively negatively associated with several pesticide residues, including glyphosate and its metabolite AMPA (Fig. 5). The *amoA* of AOA was also negatively associated with the pesticide residues S-metolachlor and terbuthylazine. However, the *amoA* gene of AOA and AOB was positively associated with other pesticide residues.

Similarly, *nosZI* was positively and negatively associated with concentrations of several pesticide residues, while *nosZII* was only positively associated with metalaxyl. The relative importance of pesticide residues in explaining these functional genes' absolute abundances was relatively high, ranging between 4.0 and 9.6% sum of squares. Of all other management variables, only the cropping system showed a significant association with the abundance of the *amoA* gene of AOA.

Our results indicate that pesticide residues influence arable soil microbiomes. We observed clear associations between the concentrations of pesticide residues and microbial diversity, the relative abundance of specific microbial taxa, and the absolute abundance of functional genes related to nitrogen cycling. The observed associations were highly compound-specific, while the effect of indices representing aggregated pesticide contamination, including the number of residues, herbicides etc., per sample, was less evident. Similarly, we found no evidence that specific pesticide classes (e.g. fungicide, herbicide or insecticide) or mode of action explained soil microbiome characteristics (Table S17). However, the associations of pesticide residues with microbiome traits were more robust than other management characteristics and even exceeded the cropping system's effect.

4. Discussion

Pesticides are an integral part of modern agriculture but contribute to widespread and long-term contamination of agricultural soils. While a range of studies investigated the immediate effects of pesticide application on soil microbial communities in prospective risk assessments, no study has yet examined the extent to which pesticide residues persistent in agricultural soils affect the microbiome. Our work sheds first light on this critical question by linking residues of 48 widely-used pesticides with soil microbiome traits in soils across an arable landscape. Our results suggest that pesticide residues impact microbiome structure and functioning in arable soils more than other management characteristics such as cropping system, fertilization, and tillage.

The link between pesticide residues and soil microbiome traits was highly variable and ranged from strictly negative to mostly positive. At the level of individual OTUs, most bacterial and all fungal OTUs were positively associated with pesticide residues, suggesting a stimulating effect. A possible direct effect that could explain such a positive association of microbial taxa with pesticide residues is biodegradation - the process where pesticides are metabolized by microbes and serve as a resource (Johnsen et al., 2001; Singh and Walker, 2006). Although our data do not provide direct evidence, our observations indicate that biodegradation played a role, as outlined by the associations with atrazine and its metabolites.

Atrazine degradation by fungi leads to the two primary metabolites, deethylatrazine and deisopropylatrazine (Kaufman and Blake, 1970). We found that the abundance of several fungal OTUs positively associated with both metabolites, for instance, within the genus *Aspergillus* harboring fungi that have shown to be able to degrade atrazine (Fig. S8) (Kaufman and Blake, 1970; Fan and Song, 2014). Conversely, bacteria generally initiate atrazine degradation leading to the metabolite 2-hydroxyatrazine (Sene et al., 2010). Indeed, we found bacterial OTUs abundance only associated with increased 2-hydroxyatrazine, but not with the former two metabolites (Fig. 4). In particular, OTUs belonging to *Mucilaginibacter* were positively associated with 2-hydroxyatrazine, which has been earlier identified as a dominant genus in a community of aerobic atrazine-degraders (Douglass et al., 2017).

Further indications of biodegradation in our data are the enrichment of the genus *Burkholderia* under elevated tebuconazole concentrations, as it has already been shown experimentally that *Burkholderia* bacteria degrade triazole fungicides (Fig. 4) (Satapute and Kaliwal, 2016; Han et al., 2021). Several other bacterial and fungal genera, such as *Polaromonas, Fusarium*, and subdivisions of *Acidobacteria*, among which pesticide degraders have been identified, were also positively associated with pesticide residues (Kaufman and Blake, 1970; Xie et al., 2011; Jiang et al., 2018). Earlier work showed that such an increase in the abundance of specific microbial groups is linked to their capability to produce specialized enzymes to degrade these pesticides (Itoh et al., 2014; Rousidou et al., 2017). In this sense, additional metagenomic analyses could provide a more direct understanding of the importance of biodegradation for the assembly of microbial communities in arable soils.

Pesticide residues, thus, may represent new niches for specialized microbial taxa in agricultural soils that could increase overall microbial diversity. However, we only see indications for stimulating effects on the

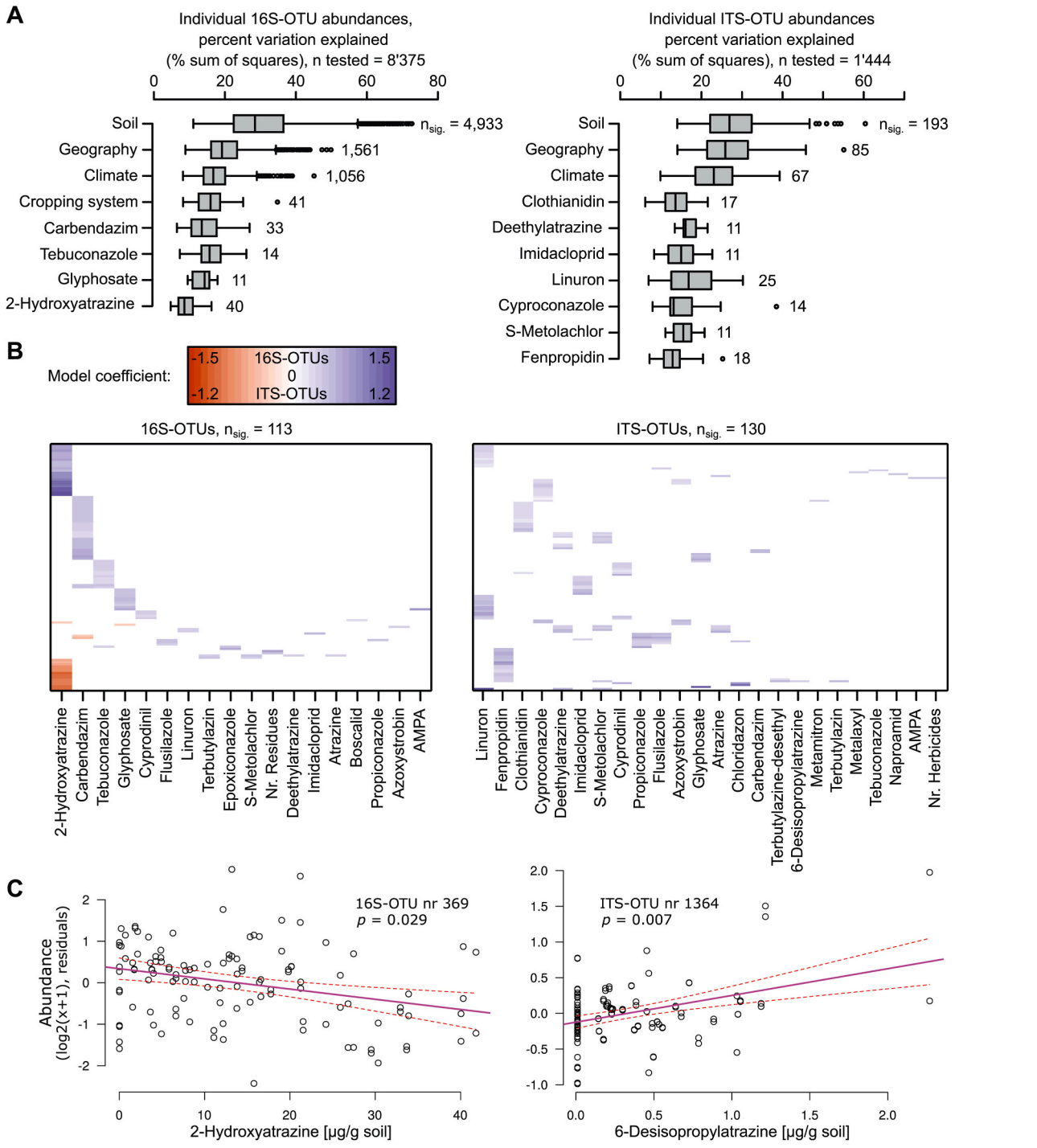

Fig. 3. Impact of pesticide residues on the abundance of individual OTUs. A) Relative importance (expressed as percentage sum of squares) of environmental characteristics (soil, geography and climate), cropping system and pesticide residues in explaining the abundance of the 8375 16S-OTUs and 1444 ITS-OTUs. The number behind each bar shows the number of taxa significantly affected by the variable shown (n_{sig}). For the correction variables (soil, geography and climate) and cropping systems, the results are shown for a model that was fitted first in the model. Only results from the analysis that first accounted for correction variables and cropping system are shown for pesticides and management strategies. Only model terms with more than ten significant OTUs are shown (FDR <0.05 and %-SS > 1; numbers are given in Table S13/S14). B) Heatmap of the pesticide-sensitive taxa – the 113 16S-OTUs and 130 ITS- OTUs that significantly changed their abundance in response to pesticide residues. Shown are the model coefficients-blue and red are OTUs that were positively or negatively associated with pesticides, respectively. Only pesticide residues with significant OTUs are shown. C) Example of the relationship between a pesticide-sensitive 16S- and ITS-OTU and the concentration of the corresponding pesticide residue in the soil. The values on the y-axis refer to the residuals extracted after fitting the correction variables and the cropping system. The pink line shows the fitting line (residuals ~ pesticide) with 95% confidential intervals. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

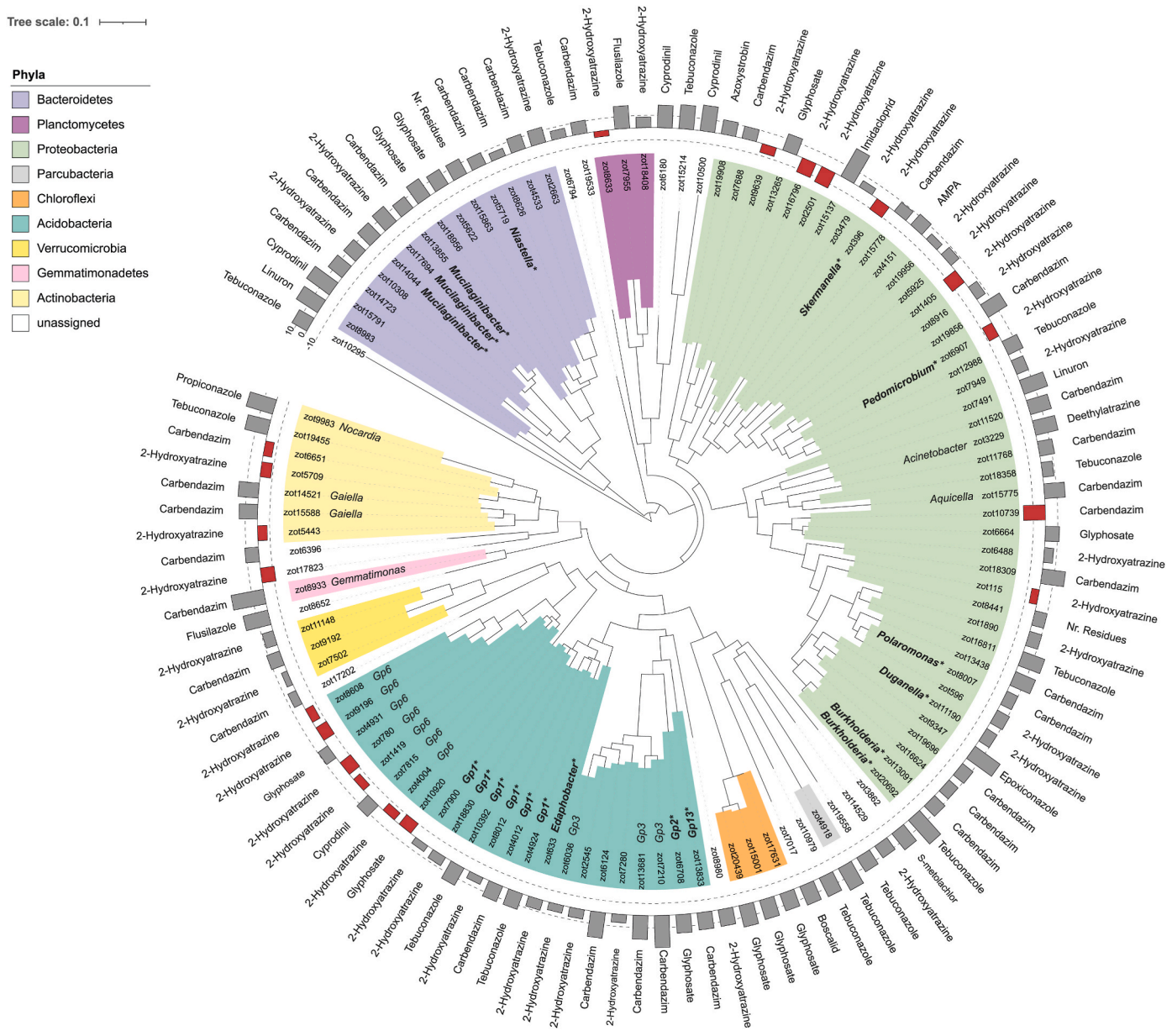

Fig. 4. Bacterial taxa sensitive to pesticide residues. Taxonomic tree showing prokaryotic OTUs associated with pesticide residues. Bar graphs show the relative importance of a particular pesticide residue - the identity of the residue is given in the outer text ring - in explaining the abundance of the corresponding OTU, expressed as a percentage sum of squares. Colors indicate the direction of pesticides' effect (orange for a negative, dark grey for a positive effect). The assigned genus level is indicated in the inner text circle. If the genus name is in bold, the genus is enriched with pesticide-residue sensitive OTUs based on contingency table analysis (see Table S15). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

diversity of fungi (Fig. 1). In contrast, pesticide residues were consistently negatively associated with bacterial diversity indices. Likely, such an impact on bacterial diversity can be attributed to the residues' potentially toxic effects, which threaten the abundance of susceptible bacterial taxa. In line with this, we found that carbendazim, 2-hydroxyatrazine and glyphosate - the residues associated with reduced bacterial diversity - also showed the most pronounced negative associations with the relative abundance of several bacterial OTUs (Fig. 3). Such negative associations appeared particularly often in the bacterial genera *Skermanella* and *Pedomicrobium* and OTUs of Acidobacteria subdivision 6 (Fig. 4), whose susceptibility to pesticides has already been indicated in previous studies (Ezeokoli et al., 2020; Yu et al., 2020; Baćmaga et al., 2021). Therefore, these microbial groups could constitute promising microbial indicators for pesticide stress in agricultural soils (Giesy et al., 2000). However, further experimental studies need to be conducted to confirm whether pesticides directly threaten these taxa.

It is worth noting that we found far more positive than negative associations with OTUs. One reason for this could be that biodegradation of residues relies on compound-specific enzymes (Singh and Walker, 2006) - only if a microbe has the appropriate enzyme, it can thrive in the residue's presence - leading to specific responses in abundance. In contrast, toxicity to microbes results from inhibition of essential processes common to most bacteria or fungi but with varying degrees of susceptibility. This leads to more gradual and widespread responses that may be less evident in the relative abundance of OTUs. Therefore, it is possible that negatively associated taxa were only incompletely captured in the present analyses and are consequently underestimated.

The marked influence of pesticide residues on bacterial and fungal microbiota can likely not be entirely attributed to the direct effects of toxicity and biodegradation and is driven by indirect effects. Although

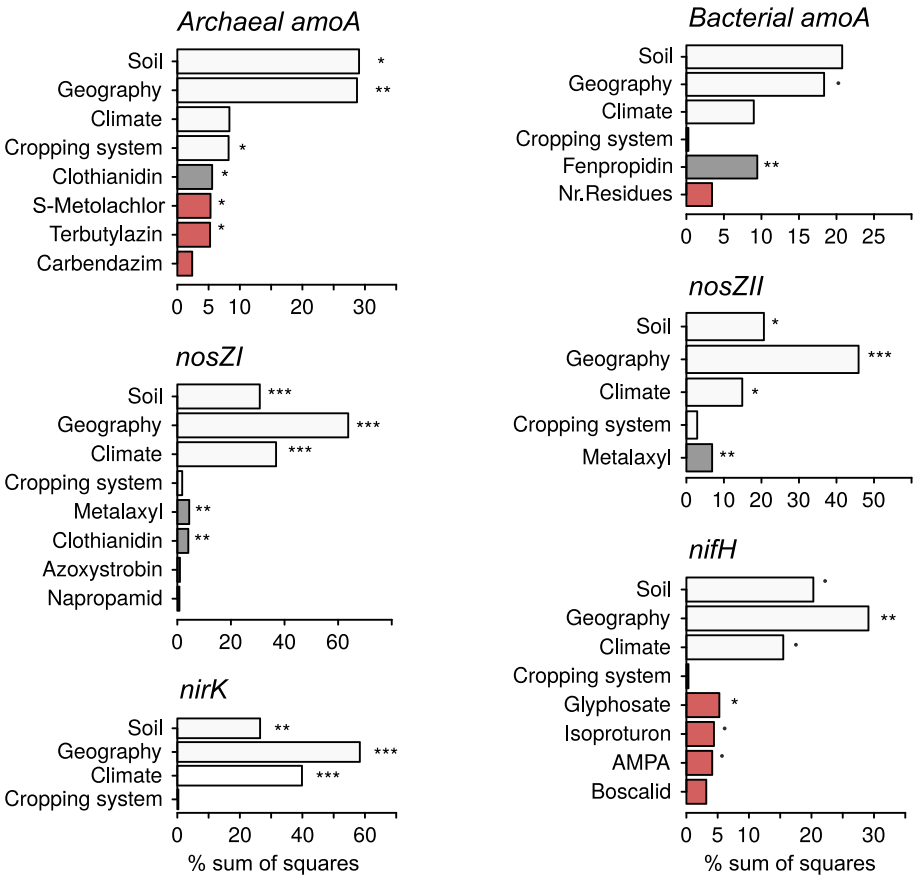

Fig. 5. Relative importance of environmental characteristics (soil, geography and climate), cropping system and management factors, including pesticides, in explaining the abundance of different functional genes in the N-cycle (expressed as percent sums of squares). The genes are markers for soil nitrogen cycling: *amoA* genes for nitrification, *nirK* and *nosZ* genes for denitrification and *nifH* for nitrogen fixation. For reference, the correction terms (soil, geography, and climate) and the cropping system are always shown; otherwise, only the significant terms were fitted after the correction variables. Colors indicate the direction of the coefficient (orange for a negative, dark grey for a positive effect). We could specify no direction of effects for correction terms and cropping system. Significance is given in the labels right of the bars. Labels are: •: $P < 0.1$, *:$P < 0.05$, **: $P < 0.01$, ***:$P < 0.001$. The relative importance of management terms fitted after cropping system is shown in Fig. S9. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

our findings do not provide direct evidence, it is known that pesticides can affect the competition for ecological niches by giving advantages to less susceptible microbes and enabling displacing niche competitors (Johnsen et al., 2001; Foit et al., 2012). Pesticides were also shown to accelerate predator-prey and host-pathogen relationships when the prey/host was stressed by the pesticides (Liess et al., 2013; Lu et al., 2019). Besides these indirect effects and the direct toxic effects, one could also speculate that the dominance of a few pesticide-degrading bacterial groups leads to a subsequent decline in the overall bacterial diversity.

Our data suggest, in contrast, that fungal populations performed better and increased in diversity and abundance (Fig. 1). However, we have expected particular firm adverse effects of fungicides on soil fungi, as reported in earlier work investigating isolated fungicide impact (Thirup et al., 2001; Howell et al., 2014). Surprisingly, we found only weak associations with fungicides on fungal diversity and other traits and, if any, positive. It remains unclear why we did not find any negative associations with fungicides. However, more herbicides than fungicides were analyzed (Riedo et al., 2021), so this study may have underrepresented fungicide effects. Note that the primers we used to detect fungi only marginally cover arbuscular mycorrhizal fungi (Glomeromycota), whose abundance was found to be negatively associated with the number and concentration of pesticides in the soil (Riedo et al., 2021). A recent study also demonstrated that fungicide application was negatively linked to the diversity of arbuscular mycorrhizal fungi and reduced their ability to acquire phosphorus for plants (Edlinger et al., 2022).

Although the effects of microbial diversity appear striking, such data do not directly indicate to which extent soil functioning is affected. Therefore, we investigated more direct indicators for soil functioning through the abundance of microbial genes central to nitrogen cycling. We only found negative associations between pesticide residues and the gene *nifH*, which is common to nitrogen-fixing bacteria (Fig. 5). Our findings complement earlier studies, which reported adverse effects of pesticides on biological nitrogen fixation, rhizobia recruitment and root nodule formation (Fox et al., 2007; Angelini et al., 2013; Zhang et al., 2016) and imply that pesticide residues may pose a long-term risk to biological nitrogen fixation in agricultural soils.

We could further link pesticide residues to genes involved in nitrification but with varying compound-specific outcomes, while in earlier work, soil nitrifiers were predominantly negatively affected (Munoz-Leoz et al., 2013; Zhang et al., 2016; Karas et al., 2018). In contrast, we found both *nosZ* genes central for converting N_2O into N_2 in denitrification (Fig. 5) and the genus *Niastella* (Fig. 4), harboring N2O consuming bacteria (Nishizawa et al., 2014), positively associated with different pesticide residues. These results imply that, depending on the compound, pesticides may also mitigate deleterious soil processes such as nitrification and N_2O emissions by influencing specific functional groups of microbes. It remains to be clarified whether the soil's nitrogen cycle is sensitive because only a few taxa can fulfil these functions or because the enzymes are inherently sensitive to chemical perturbations. It is also unclear whether pesticide residues directly affect the nitrogen-converting microbes or shift the nitrogen pools in the soil and have indirect effects. Yet, we can reinforce recent results of prospective risk assessment that soil nitrogen cycling appears to be particularly sensitive to pesticide residues and thus can serve as microbial indicators for pesticide stress (Karpouzas et al., 2014; Crouzet et al., 2016; Karas et al., 2018).

The relationships between pesticide residues and microbiome traits were evident even after accounting for differences between cropping systems, e.g. the difference between conventional and organic farming. This underlines that our results go beyond cropping system effects. Notably, the impact of the cropping system and management indices on the soil microbiome was not as pronounced as recently reported in field

trials (Hartmann et al., 2015; Degrune et al., 2017; Hartman et al., 2018; Schmidt et al., 2018). Agricultural management in field trials compares sharply defined and contrasting treatments on the same soil type, while our on-farm study included many different farms, each with a unique set of management practices under a wide range of pedoclimatic conditions. The less clearly defined management and the different environmental conditions may have led to less pronounced differences between cropping systems. Our analysis also confirmed that environmental determinants such as soil, geography and climate characteristics are the most important factors explaining soil microbiome traits at landscape scales in line with many studies (Lauber et al., 2008; Dequiedt et al., 2009; Rousk et al., 2010; Xue et al., 2018; Crowther et al., 2019). By accounting first for the variation explained by environmental determinates in our statistical models, we were nevertheless able to focus on the effects of agricultural management.

Our statistical analysis revealed clear associations between specific pesticide residues and microbiome traits. We must mention that we found the strongest associations with microbiome traits for the pesticide residues most frequently present in the soils studied (Table S1). The statistical power to detect links might be too weak for residues with a low occurrence. It is also unclear how pesticide residues differ in their bioavailability, significantly affecting exposure (Gevao et al., 2000). In this context, it is also important to note that the statistically inferred effects were not based on isolated pesticide residues but complex mixtures, as up to 32 different residues were found per sample (Riedo et al., 2021). For these reasons, no conclusions can be drawn about the individual residues' ecotoxicity from this setting.

Furthermore, this study lacks detailed information on the pesticide application scheme in different fields. Therefore, the potential effects statistically inferred here are either long-lasting effects of a previous, high exposure immediately after application or a response to a current, but low exposure due to the remaining residues. Therefore, it would be desirable to include such critical information about pesticide application schemes in future studies to highlight the importance of different exposure pathways. Ecological risk assessments, such as this study, can only indicate ecotoxicological issues. Therefore, these observations need to be complemented by controlled experiments in a prospective risk assessment to confirm the causality of the purported relationships.

5. Conclusion

We report that pesticide residues influence the soil microbiome's diversity and functioning. Interestingly, statistical analysis indicates that the effect of pesticide residues on the soil microbiome exceeds that of the cropping system's impact. These findings suggest that the widespread and chronic contamination of arable soils with pesticide residues could impair bacterial diversity, particularly nitrogen-fixing bacteria - critical properties of fertile soils (Vance, 2001; Wagg et al., 2019; Delgado-Baquerizo et al., 2020). Our results complement previous work suggesting adverse effects of pesticide residues on beneficial soil fungi and earthworms (Pelosi et al., 2021; Riedo et al., 2021). The present work further indicated that many microbial taxa, particularly fungi, responded positively to pesticide residues, probably due to biodegradation processes, revealing that pesticide effects on the soil microbiome are complex.

Currently, regulatory assessments of pesticides only test the immediate response of a single microbial indicator – soil N mineralization (Bünemann et al., 2006; Ockleford et al., 2017). Our work points to the need to expand efforts in the assessment process by increasing the number of microbial indicators and assessing the long-term effects of pesticide mixtures. Such efforts should focus on organisms responsible for beneficial soil functions (e.g. biological nitrogen fixation or uptake of nutrients by soil fungi). Improved risk assessment is also relevant to the need to develop sustainable agroecosystems that rely much more on natural processes and aim to reduce synthetic inputs (Power, 2010; Pretty et al., 2018).

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Authors' contributions

MvdH and FW conceived and designed the study. FW and LB conducted the sampling. JR conducted the organic trace analysis with advice from TB. AV performed the molecular work with advice from FW and SB. MS and FW performed statistical analyses. FW wrote the manuscript with substantial contributions from MS, SB, TB and MvdH; all authors edited the manuscript.

Availability of data and materials

The raw sequencing data are available from Sequence Read Archive (accession ID PRJNA682947) at the time of acceptance.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marcel van der Heijden reports financial support was provided by Swiss National Science Foundation.

Data availability

The raw sequencing data are available from Sequence Read Archive (accession ID PRJNA682947) at the time of acceptance.

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Appendix A. Supplementary data

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