Abrupt versus gradual application of pesticides: effects on soil bacterial and fungal communities

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Abstract

Pesticides are a major anthropogenic input to the environment and a factor in global change that puts pressure on soil microbial communities. However, the effects of different rates of pesticide application on soils remain poorly understood. This study investigates how abrupt versus gradual pesticide applications influence soil bacterial and fungal communities. Employing high-throughput sequencing, we examined the microbial diversity and community composition in response to ten commonly used pesticides. Bacterial communities exhibited minimal changes across treatments, whereas fungal communities responded strongly to pesticide exposure. Gradual applications reduced the relative abundance of dominant fungal taxa, resulting in an overall increase in community evenness. This effect was particularly pronounced for two herbicides and a triazole fungicide, which induced substantial shifts in fungal composition. Conversely, abrupt pesticide applications resulted in transient disruptions but did not promote the long-term proliferation of rare fungal phylotypes. These findings suggest that prolonged exposure to pesticides exerts strong selective pressures on fungi, potentially altering fundamental soil functions such as nutrient cycling and decomposition. Future research should focus on the long-term responses of soil microbial communities to pesticide application and the cumulative effects of chronic low-dose exposure, to provide a more comprehensive understanding of how they shape microbial communities.

Keywords

Soil microbiome, pesticide exposure, microbial diversity, gradual and abrupt stress, rate of change

Introduction

The importance of soil microbial communities to ecosystem functionality is well established. These communities mediate fundamental processes, including nutrient cycling, organic matter decomposition, and soil aggregate formation (Bardgett and van der Putten 2014; Wagg et al. 2014; Schimel 2016). They include diverse bacteria and fungi, which often perform complementary roles. Bacteria are dominant in the early stages of decomposition, while fungi contribute to aggregate stability and nutrient mobilization (Lehmann, Zheng, and Rillig 2017; Fierer 2017). A highly diverse microbial community is fundamental for the resilience of soil systems and can withstand environmental disturbances. Nevertheless, the integrity of these communities is increasingly threatened by various factors of global environmental change such as habitat loss, climate extremes, and chemical pollution, such as pesticides.

Pesticides are a ubiquitous global change factor and constitute the largest intentional inputs of hazardous compounds into soil systems, with global usage exceeding 2.5 million tons annually (FAO 2023). Within the European Union, there are over 400 active ingredients that have been registered for use (European Commission 2020). The off-target movement of these substances through drift, runoff, and volatilization following extensive use leads to the accumulation of residues in soils. Although pesticides are considered degradable, these residues can persist in the soil matrix and exert an impact on the surrounding environment (Tang and Maggi 2021; Riedo et al. 2022; Bošković et al. 2020). Studies targeting top soils from across Europe have revealed the prevalence of residues from a minimum of five pesticides in over half of the analyzed samples (Silva et al. 2019; Hvězdová et al. 2018; Riedo et al. 2021). These results indicate the widespread occurrence of multi-pesticide exposure and the considerable degree of anthropogenic pressure that could potentially be exerted on soil microbial communities.

Some microorganisms participate in pesticide degradation, thus reducing their concentrations in the environment (Mandelbaum, Wackett, and Allan 1993; Marrón-Montiel et al. 2006). It has been demonstrated, for instance, that bacterial genera such as *Achromobacter*, *Bacillus*, *Pseudomonas*, and *Rhodococcus* are particularly effective at breaking down pesticides. The transformation process is often enhanced by microbial co-metabolism, whereby byproducts from the metabolic transformations of one species can serve as substrates for others (Bælum, Jacobsen, and Holben 2010; Ermakova et al. 2010; Hou et al. 2011; Sandoval-Carrasco et al. 2013; Vargha, Takáts, and Márialigeti 2005; Y. Zhang et al. 2011). However, the metabolic adaptability exhibited by these bacterial genera does not mean all microbes are protected from pesticide-induced stress. Due to the sheer variety of active substances, their effects on soil microbial communities remain unclear and context-dependent. While some studies suggest that herbicides such as atrazine do not induce persistent changes in

microbial abundance, others report toxic effects, particularly from chlorinated herbicides such as diuron and linuron (Dennis et al. 2018; Prado and Airoldi 2002). Furthermore, the presence of pesticide residues in field sites has been shown to disrupt generic microbial metabolic pathways, reduce microbial biomass, and alter community composition (Ni et al. 2025; Riedo et al. 2021; Sim et al. 2022). It is thus clear that although some microorganisms can exploit pesticide residues as a resource, the extent to which pesticide accumulation and exposure alters microbial diversity and long-term community stability remains poorly understood (Rose et al. 2018; Banks et al. 2014).

The rate at which pesticides are introduced into soils, whether gradual or abrupt, is an underexplored factor potentially influencing microbial community dynamics. In real-world scenarios, pesticide accumulation frequently occurs in a gradual manner. However, most studies focus on abrupt applications, overlooking these exposure patterns, which better reflect environmental conditions (Golubeva et al. 2020; E. Li et al. 2021). Abrupt applications of pesticides have been demonstrated to potentially overwhelm microbial systems by introducing elevated concentrations of bioavailable compounds, thereby leading to oversaturation of the soil's sorption sites (Meidl et al. 2024). Conversely, the application of pesticides on a gradual basis might permit microbial communities adequate time to acclimate, although idiosyncratic responses have been observed (Golubeva et al. 2020). Understanding these dynamics is critical for sustainable soil management practices, as pesticide-driven shifts in microbial community composition might have long-lasting effects on soil function.

The present study investigates the influence of pesticide application rates, whether abrupt or gradual, on soil microbial communities, with a particular focus on fungi and bacteria. We addressed two hypotheses. i) Among pesticide classes, fungicides will demonstrate the most profound effects on soil microbial communities when compared to herbicides and insecticides. This is particularly evident for fungal communities, specifically designed to suppress fungi (Prudnikova, Streltsova, and Volova 2021). ii) Based on earlier results from this experimental setup, abrupt pesticide applications are hypothesized to have a more pronounced impact on soil microbial communities than gradual applications due to reduced adaptation time and increased bioavailability of the pesticides (Meidl et al. 2024).

Material and Methods

Experimental setup

A microcosm setup was employed to evaluate the impact of diverse pesticides and application rates on soil communities. Details regarding the experimental setup can be found in (Meidl et al. 2024). In brief, the top 10 cm of surface soil was collected from a local grassland at an experimental site of the

Freie Universität Berlin (52°46’60.67 N, 13°30’26.98 E). 30 grams of sieved soil (2 mm) was placed into micro-boxes (Deinze, Belgium; filter cover, 80 mm diameter, 40 mm height) and adjusted to a water holding capacity of 60%. The experiment was conducted over 50 days in a climatic chamber with a humidity level of 30% and a temperature of 20°C. The ten pesticides included in the setup were selected based on their high prevalence in pesticide screening studies carried out in the European Union and Switzerland (Pelosi et al. 2021; Riedo et al. 2021), encompassing two insecticides, four fungicides, and four herbicides. The applied pesticide concentrations were based on the maximum detected concentrations across all 280 field sites (Table S1). The analytical standards (Sigma-Aldrich, United States) were diluted in acetone to create the working solutions. Two distinct treatment regimens were tested: an abrupt application, whereby the entire pesticide load was applied in a single dose, and a gradual application, whereby the dose was administered at five separate time points. The treatments were replicated ten times each. To evaluate the potential impact of solvents, the final concentration of acetone was incorporated as an additional control treatment, in addition to the regular control comprising solely water. The treatments and controls were applied to the soil surface using a 1 mL glass syringe with a nasal spray pump (Teleflex Medical Europe Ltd., Ireland). The gradual treatments were applied at 10-day intervals, while the abrupt treatment was administered in a single dose on day 20. Following 50 days, soil samples were taken and stored in 2 mL Eppendorf tubes at -80°C until further analysis.

Molecular analysis (DNA extraction, PCR amplification, and Illumina sequencing library preparation)

The DNA was extracted from 250 mg of lyophilized soil using the DNeasy Power Soil Pro Kit (Qiagen, GmbH, Germany), following the manufacturer's instructions. The fungal sequences were amplified using the fITS7 and ITS4 primers, which when used in combination, yield amplicons that span the fungal ITS2 region (Ihrmark et al. 2012). Bacterial sequences were amplified from soil DNA extracts using the 515F-Y and 806R primers (Caporaso et al. 2011). The polymerase chain reaction (PCR) amplification and sequencing library preparation steps were conducted on an automated workstation (Biomek i7 hybrid, Beckman Coulter, United States), at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv, Berlin, Germany).

For the 16S target PCR, the reaction was carried out using Q5 High-Fidelity DNA polymerase (New England Biolabs, United States) in a 25-µl volume containing template DNA (10ng), 1.25 µl of both the 10 µM forward primer and 10 µM reverse primer, and 12.50 µl Q5 High-Fidelity 2X Master Mix. The target PCR comprised an initial denaturation step for 30 seconds at 98 °C, 30 cycles of a 10-second denaturation step at 98 °C, a 30-second annealing step at 57 °C, and an elongation step at 72 °C for 20 seconds. The final elongation step was conducted at 72°C for 120 seconds, after which the

temperature was maintained at 4°C. The same reagents and concentrations were employed for the indexing PCR as were used for the previous reaction. The PCR program was set to 30 seconds at 98 °C for initiation, followed by eight PCR cycles (98 °C for 10 seconds, 67 °C for 30 seconds, 72 °C for 30 seconds) and a final elongation step at 72 °C for 120 seconds.

For ITS, the target PCR was conducted using Kapa HiFi polymerase (Roche, Switzerland). The reaction volume of 25 µl contained template DNA (20ng), 0.75 µl of 10 mM dNTPs (200 µM), 5 µl of Kapa HiFi buffer, 0.75 µl of both the 10 µM forward primer and 10 µM reverse primer, 0.5 µl of Kapa HiFi polymerase (0.02 U/µl), and nuclease-free water. The target PCR involved an initial denaturation step for 180 seconds at 98 °C, followed by 30 cycles of a 20-second denaturation step at 98 °C, a 20-second annealing step at 58 °C, and an elongation step at 72 °C for 30 seconds. The final elongation step lasted 300 seconds at 72 °C, after which the temperature was maintained at 4 °C. The indexing PCR was conducted using 10 µl purified DNA, 0.5 µl of 10 mM dNTPs (200 µM), 1.25 µl of both forward and reverse primers, 5 µl Kapa HiFi buffer, 0.5 µl Kapa HiFi polymerase (0.02 U/µl), and nuclease-free water in a 25 µl reaction volume. The PCR program was set to 180 seconds at 98 °C for initiation, followed by eight PCR cycles (98 °C for 20 seconds, 52 °C for 20 seconds, 72 °C for 30 seconds) and a final elongation step at 72 °C for 300 seconds.

Following each PCR, products were purified using magnetic beads (CleanNGS, GCbiotech, the Netherlands) and to ascertain the quality and efficacy of the indexing PCR, a randomized comparison was conducted between a target and index sample, using a Tape station (Agilent Technologies, United States). The products of the second PCR were then pooled for sequencing on a MiSeq System (Illumina, United States) with the V3 chemistry (600 cycles) and paired-end dual indexing. Following the sequencing process, the reads were demultiplexed and assigned to fastq-files by their indexes, utilizing the MiSeq software (Illumina, United States).

Bioinformatics

The forward and reverse reads were processed separately using Dada2 v. 1.30.0 (B. J. Callahan et al. 2016), ShortRead v. 1.60.0 (Morgan et al. 2009), and Biostrings v. 2.70.1 (Pagès et al. 2021) to identify amplicon sequence variants (ASVs). The approach followed the Dada2 tutorial for ITS sequences (v1.8, https://benjjneb.github.io/dada2/ITS_workflow.html) and 16S sequences (v1.16, https://benjjneb.github.io/dada2/tutorial.html). First, primer sequences were removed from each read with Cutadapt v. 4.0 (Martin 2011). Reads were filtered to exclude any sequences under 50 base pairs (bp) in length and truncated where base quality scores dropped below a threshold of 10. Only reads with an expected error rate of 2 or lower were retained. Next, a machine-learning algorithm in Dada2 modeled the error frequency patterns across the filtered reads, and de-replication was used to identify unique sequence variants. The Dada2 sample inference algorithm then processed the reads

based on learned error patterns, defining ASVs. Forward and reverse reads were merged, and any chimeras were removed. Finally, the raw reads were mapped back to the ASVs to generate an ASV abundance table. Taxonomic identity was assigned using the naive Bayesian classifier method of the DADA2 package against the UNITE database 9.0 (Abarenkov et al. 2024) for fungi and the Silva reference database v123 (B. Callahan 2018) for bacteria.

Statistical analysis

All analyses were conducted using the R statistical computing environment, version 4.3.0. The data were organized and selected using the phyloseq package v1.46.0 (McMurdie and Holmes 2013), DESeq2 v1.42.1 (Love, Huber, and Anders 2014) and dplyr v1.14 (Wickham et al. 2023). All figures were generated using functions from the packages ggplot2 v3.5.1 (Wickham, Chang, and Wickham 2016).

The raw amplicon sequence variant (ASV) data were imported and subjected to preliminary processing, during which low-abundance ASVs and taxa not identified at the phylum level were excluded. The minimum sample depth was employed as the rarefaction threshold, with ASVs rarefied to this depth using the rarefy_even_depth function in phyloseq, with a fixed random seed (1782) to ensure reproducibility.

To evaluate microbial diversity within samples, alpha diversity metrics, including observed richness and inverse Simpson's dominance, were computed. To estimate the mean and 95% confidence intervals (CI) of the response metrics for each treatment, a custom bootstrap resampling procedure was employed, as described by (Rillig et al. 2019). For each treatment group, 1,000 bootstrap samples were generated through the resampling with replacement of the observed values. In each iteration of the resampling process, a mean was calculated, and the resulting bootstrap distribution was employed to derive the 2.5%, 50%, and 97.5% confidence intervals. Subsequently, a comparison was conducted between each treatment group and the control by subtracting the control means from the treatment means across the bootstrapped samples, to estimate the effect size distribution's 2.5%, 50%, and 97.5% percentiles. Furthermore, a significance level (p-value) was calculated with a Bonferroni correction applied to adjust for multiple comparisons. A density ridge plot was constructed to visually depict the raw data distributions, along with their bootstrapped confidence intervals, using the ggplot2 package.

The ASV data were subjected to a transformation process to facilitate the subsequent beta diversity analyses. A binary transformation (presence-absence) was applied to reduce the influence of dominant ASVs, thereby facilitating robust beta diversity analysis. A Jaccard dissimilarity matrix was then calculated for the transformed ASV data. A Principal Coordinates Analysis (PCoA) was conducted using the Jaccard distance matrix (command vegdist() in package vegan), and the ordination scores were extracted for the first two axes. The PCoA results were visualized with a scatter plot, with ellipses

representing the 95% confidence interval around each treatment group (control, gradual, abrupt). A PERMANOVA was performed to assess the differences between treatments and to determine whether these differences were due to differences in dispersion rather than true compositional differences, a dispersion test was performed using betadisper.

For the DESeq2 analysis, the data were filtered to exclude features with low counts (i.e., less than one count in over half of the samples) to reduce noise and focus on meaningful abundance patterns. Subsequently, the sample data were normalized using the internal methods of DESeq2 to account for discrepancies in sequencing depth. To identify significant differences in microbial abundance between treated and control samples, pairwise contrasts were performed. The resulting p-values were adjusted using the Benjamini-Hochberg procedure, and genera with adjusted p-values below 0.1 were considered significant, Heatmaps with the log2 fold change, relative abundance, and p-values were then created.

Results

Composition of the bacterial and fungal microbiota

A total of 4383 ASVs originating from the 16S marker were identified. Following the rarefaction process, 442 of these variants were excluded. Despite this loss, the rarefaction curves indicated that the number of ASVs per sample approached saturation for the majority of samples at the selected threshold. Of the 3941 16S-ASVs that remained, 3680 were classified as bacterial. The most prevalent bacterial phyla were *Actinobacteria* (32.3%), *Proteobacteria* (19.4%), *Chloroflexi* (9.12%), *Firmicutes* (8.5%), *Thaumarchaeota* (7.9%), *Acidobacteria* (5.7%), *Planctomycetes* (4.4%), *Gemmatimonadetes* (3.7%), *Cyanobacteria* (2.7%), and *Verrucomicrobia* (2.6%). For ITS, the bioinformatic process defined a total of 2,091 ITS2-ASVs. Rarefaction resulted in the exclusion of 226 variants. All of the remaining 1,865 IT2S-ASVs were confirmed to be of fungal origin. Fungal ASVs were predominantly distributed across five major phyla and were *Ascomycota* (58.4%), *Basidiomycota* (15.7%), *Mortierellomycota* (12.4%), *Chytridiomycota* (4.4%), and *Mucoromycota* (2.7%).

Effect of pesticide application on total bacterial and fungal diversity

Analysis based on the inverse Simpson's dominance index (iSD) revealed that the diversity of bacterial communities was not significantly different from the control treatment (Figure 1, A). The mean effect size difference between the application of the treatments (gradual vs. abrupt), and the difference of richness estimates of all treatment groups in relation to the control, were also not significant (Figure S1, A and Figure S2, A). Consequently, the remainder of this study will focus exclusively on the response of fungal communities.

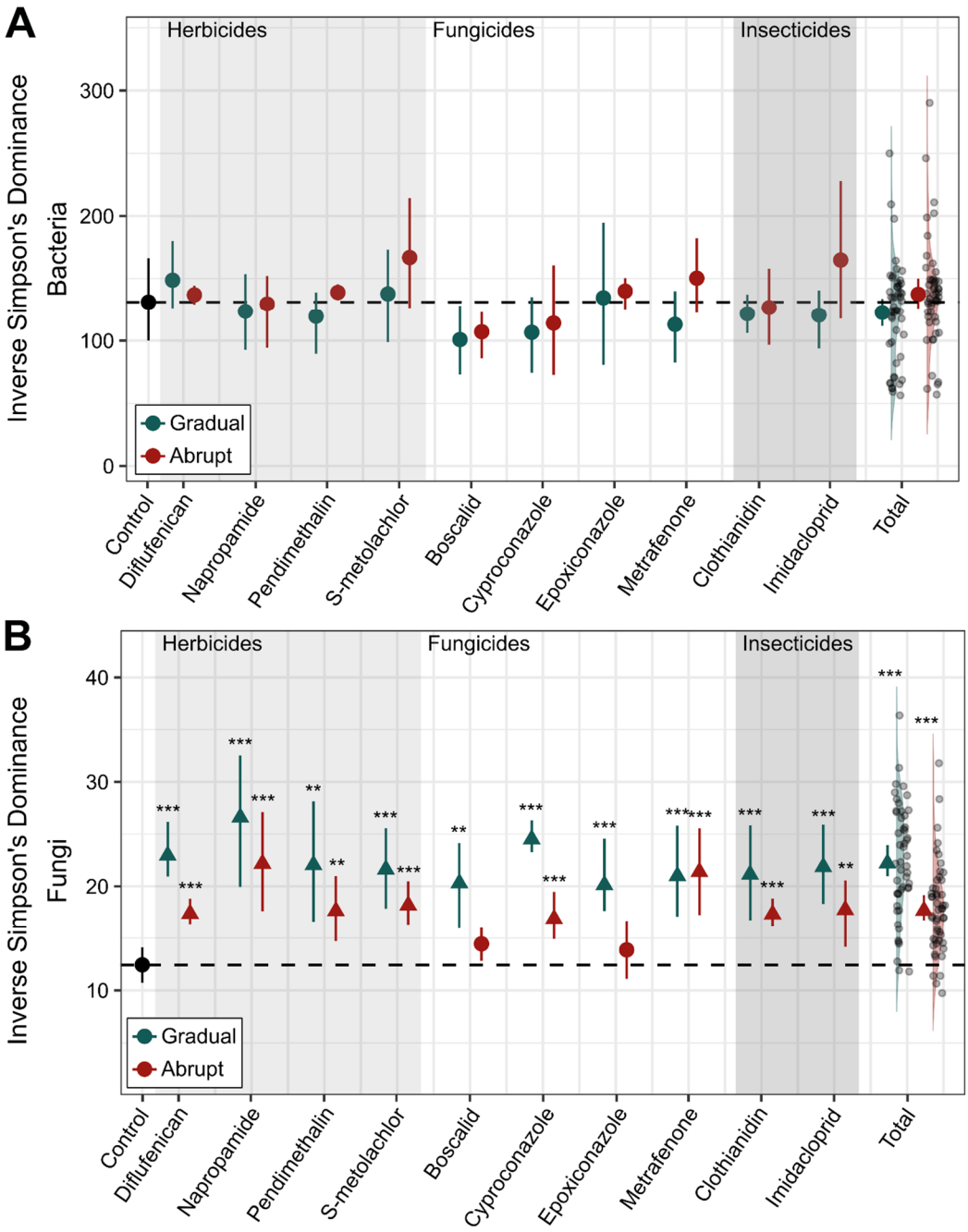

Figure 1: Individual effects of different pesticides (ordered by herbicides, fungicides, and insecticides) applied gradually (blue) or abruptly (red) on the inverse Simpson's dominance index. The circles and triangles, along with the error bars, represent the mean effect size and 95% confidence intervals (CIs), respectively. The circles represent neutral effects, while the triangles (with an arrow pointing up or down) represent significant positive or negative effects. The asterisks mark the degree of significance (*: P-value < 0.05, **: P-value < 0.01, ***: P-Value < 0.001). For bacteria (A), no significant change in Simpson's dominance index was observed for any of the ten treatments. For fungi (B), a significant increase in Simpson's dominance index was observed for all treatments except the abrupt application of boscalid and epoxiconazole. A higher Inverse Simpson's Dominance index reflects an increase in diversity and a more even distribution, reducing the dominance of few taxa.

The iSD of fungal communities significantly increased in almost all treatments, indicating an increase in the evenness of fungal communities (Figure 1, B). Exceptions to this pattern were observed for boscalid and epoxiconazole, where no significant changes occurred in the abrupt treatment compared to the control. Although generally, pesticide application regimes caused significant increases in iSD in

relation to the control group, differences in mean effect size between the application regimes (gradual vs. abrupt) could only be observed for diflufenican, cyproconazole, and epoxiconazole (Figure S2). Herbicides (diflufenican, napropamide, pendimethalin, S-metolachlor), lead to a significant increase in iSD compared to the control. Gradual application of herbicides consistently resulted in lower dominance than abrupt application. Similarly, gradual exposure to fungicides (boscalid, cyproconazole, epoxiconazole, metrafenone) resulted in significantly higher evenness than abrupt exposure, with the notable exception of metrafenone. The broad-spectrum triazole fungicides, cyproconazole, and epoxiconazole showed particularly pronounced effects with gradual applications. Insecticides (clothianidin, imidacloprid) elicited mixed effects on iSD, with both gradual and abrupt applications increasing evenness relative to the control. Also, within this group of pesticides, gradual applications consistently resulted in higher evenness than abrupt applications. The effects of pesticide treatments in general (summarized as total in Figure 1) showed increased iSD for both delivery methods.

Total fungal richness increments were observed only among four of the ten pesticides in the gradual treatment group, while only metrafenone showed a significant richness increase in the abrupt treatment group. Gradual delivery elicited significant richness increases across pesticide classes in relation to control (Figure S2, B). No notable patterns were observed regarding the relationship of fungal and bacterial communities with pesticide properties, such as DT_{50} or K_{fOC}, which are responsible for the persistence of applied concentrations or the availability of substances (Table S2; data not shown).

The impact of the rate of change on community diversity

Principal coordinate analysis (PCoA) revealed a clear clustering of treatment methods, indicating strong compositional dissimilarity between these two groups of communities, and the control (Figure 2). For herbicides, fungal communities under both gradual and abrupt treatments are distinctly separated from the control in all cases (P-values < 0.05), suggesting shifts in community composition (Table S3). For diflufenican, napropamide, and S-metolachlor the abrupt applications overlap with the gradual applications (P-values of 0.122, 0.149, and 0.055 respectively), indicating that there is no differing impact on fungal composition depending on the application method. With the exception of the abrupt epoxiconazole application (P-value = 0.161), fungicide treatments also induced clear shifts in fungal community structure, with gradual and abrupt applications forming distinct clusters separate from the control (P-values < 0.05). The broad-spectrum fungicide cyproconazole was the only fungicide treatment that elicited a distinct difference between the two treatment methods (P-value = 0.026). For the other fungicides, fungal communities under gradual applications overlap with those under abrupt applications (P-values > 0.05), indicating similar effects on the composition regardless

of the application method. Both insecticides also induced shifts in the fungal community composition, leading to a clear separation of the two treatments from the control (P-values < 0.05). For clothianidin, the gradual and abrupt treatment clusters are not different from each other (P-value = 0.09), suggesting a more consistent mode of action across application methods.

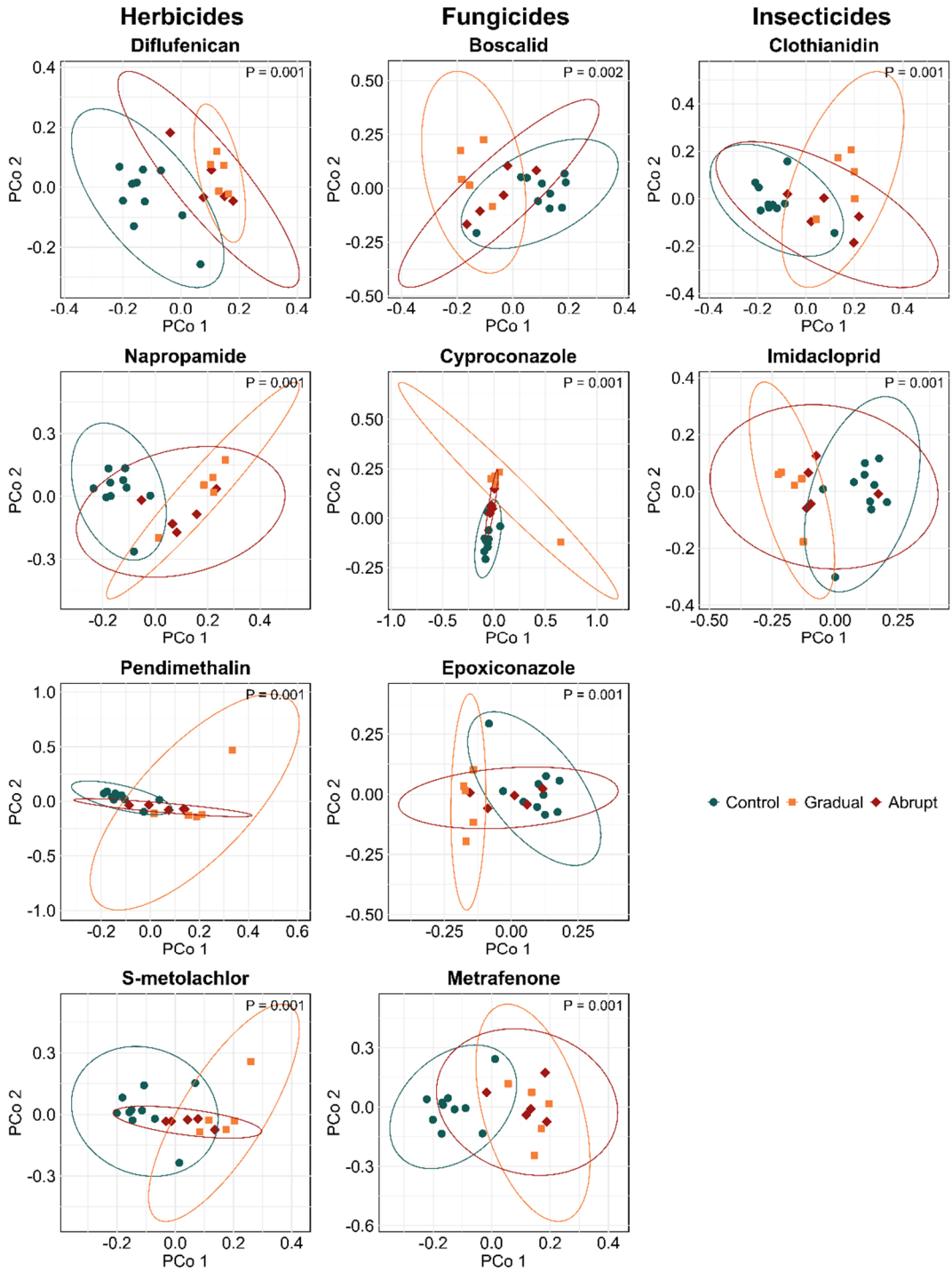

Figure 2: Principal coordinates analysis (PCoA) plot of the fungal species abundance based on the Jaccard distance of the control (blue dots), gradual application (orange squares), and abrupt application (red rhombuses). Each point represents a sample, and the distance between them indicates the compositional similarity. Ellipses correspond to clusters in each group with 95% confidence intervals. The P-value displayed on the plot represents the result of the overall PERMANOVA, indicating the significance of compositional differences between groups. A significant P-value (P < 0.05) suggests that treatment groups show distinct fungal community compositions.

Influence of the pesticides on the abundance of individual genera

Differential abundance testing (expressed as Log2 fold changes) generally revealed that the most abundant fungal genera decrease in abundance when exposed to pesticides, while rare genera increase (Figure 3). Gradual herbicide treatments consistently resulted in larger positive or negative log2 fold changes than abrupt applications. For instance, the gradual application of pendimethalin significantly increased the abundance of genera like *Cladosporium* (+3.11, P-value < 0.001) and *Alternaria* (+2.92, P-value < 0.001). Abrupt treatments produced smaller shifts in relative genera abundance. For example, under abrupt napropamide application, genera such as *Hymenoscyphus* experienced a decrease in abundance (-5.54, P-value < 0.001), but the overall impact on relative abundance among other genera was less severe compared to gradual napropamide application. Fungicides, again particularly broad-spectrum triazoles, had a pronounced effect on the relative abundance of fungal genera. For instance, under gradual cyproconazole treatment, *Tausonia* and *Enterocarpus* exhibited large relative abundance increases (+3.78 and +2.27, respectively, both P-value < 0.001). The supplementary heat map of relative abundances highlights the dominance of these genera (Figure S3). In contrast, abrupt fungicide treatments induced more transient disruptions, with smaller relative abundance shifts and fewer genera affected. Metrafenone, however, elicited consistent increases regardless of the application methods. Insecticides caused the least pronounced shifts overall, with fewer genera showing significant changes in abundance. Gradual insecticide applications resulted in modest shifts, with few genera responding to its presence. For example, under gradual clothianidin treatment, relative abundances across genera remained relatively stable. Abrupt insecticide application showed similarly limited effects on relative genera abundance.

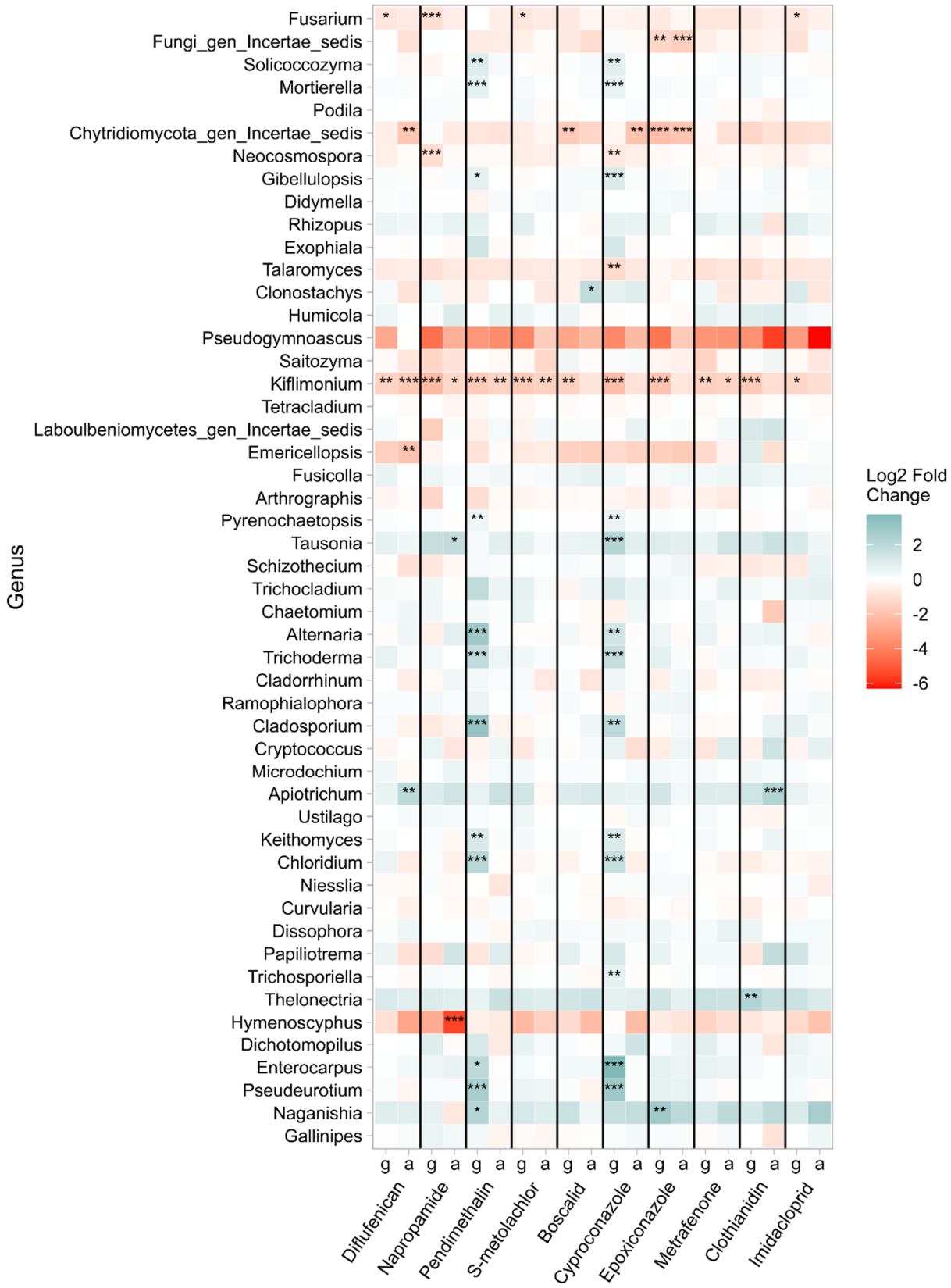

Figure 3: Heatmap showing the change in log2fold change of fungal genera as a function of pesticide application (g = gradual; a = abrupt) for the 50 most abundant genera, sorted by highest to lowest abundance. Red marks a decrease in abundance compared to the control and blue an increase. The asterisks denote the degree of significance (P-value < 0.05, **: P-value < 0.01, ***: P-value < 0.001) in change of abundance compared to the control.

Discussion

Understanding how pesticide applications influence microbial communities is crucial for assessing their long-term ecological impacts. Yet, the overall relationship between microbial communities and pesticide exposure remains complex and poorly understood. In this study, we found that while bacterial communities showed little to no response to pesticide applications, fungal communities showed a much greater sensitivity to their presence in soil. Furthermore, our findings provided mixed support for our hypotheses: while fungicides had strong effects on fungal communities, herbicides also caused considerable shifts, suggesting widespread non-target effects across various pesticide classes. Regarding application methods, gradual applications resulted in more pronounced changes in both alpha and beta diversity compared to abrupt applications.

In our first hypothesis, we assumed that fungicides would have the most pronounced effects on soil microbes of all pesticide classes. However, while this hypothesis considered microbial communities as a whole, our findings indicate that pesticide effects were largely restricted to fungi, with bacterial communities showing little to no response. This suggests that fungi are more sensitive to pesticide exposure, aligning with previous research (Astaykina et al. 2020; Streletskii et al. 2022). The lack of effects on bacterial communities might be due to the relatively short duration of the experiment. Previous studies have shown that bacterial communities can exhibit delayed responses, becoming apparent only after weeks or months (Santísima-Trinidad et al. 2018). This lag could be due to bacteria benefiting from carbon released during fungal degradation, with community changes occurring only after these resources become available (Howell et al. 2014; Das and Varma 2011). Our results for fungi on the other hand partially supported the expectation that fungicides would have the strongest effects, herbicides also had substantial impacts on fungal diversity and community composition. These observations suggest that pesticide impact cannot be solely attributed to differences in their mode of action (Prudnikova, Streltsova, and Volova 2021). For instance, the broad-spectrum fungicide cyproconazole led to pronounced shifts in fungal communities, in line with its known inhibitory effects on major fungal taxa (Vasilchenko et al. 2023). However, herbicides such as napropamide and pendimethalin elicited equally strong responses, including increased fungal evenness and changes in the relative abundance of key taxa. These findings align with prior studies showing that some herbicides can selectively stimulate fungal taxa by acting as a carbon source for fungi capable of their degradation (Crouzet et al. 2016). The observed increase in fungal diversity following herbicide treatments may indicate a compensatory response, where opportunistic fungi exploit ecological niches left by taxa sensitive to pesticide exposure. Since no plants were included in our system, the observed herbicide effects must have been direct, rather than indirectly mediated through plant-microbe interactions, indicating that certain herbicides may exert selective pressures on fungal

communities independent of their primary targets. This broader-than-expected consequence highlights the need to reconsider the assumption that non-fungicidal pesticides have insignificant effects on fungal communities.

Our second hypothesis predicted that abrupt pesticide applications would cause a more pronounced effect on microbial communities than gradual applications. However, contrary to this expectation, our results show that gradual pesticide application altered alpha diversity by increasing fungal evenness and or beta diversity by shifting fungal species composition, with more distinct effects than compared to abrupt application. This pattern likely stems from the ability of certain rare fungi to exploit selective conditions over time and thus adapt in the face of prolonged exposure. These opportunistic fungi may occupy the ecological niches left by dominant fungi whose abundance has declined, resulting in reduced competition (Senabio et al. 2024; Roman et al. 2021; Walder et al. 2022). The differential effects of gradual and abrupt applications were also evident in shifts at the genus level, with abundant species being particularly vulnerable to pesticide exposure, often showing declines in abundance. Gradual pesticide exposure resulted in more pronounced changes in both dominant and rare fungal taxa, whereas abrupt applications resulted in smaller shifts in relative abundance. This supports the theory that prolonged exposure creates a more selective environment, enabling certain taxa to thrive while suppressing others. Besides the better adaptability of rare taxa, this effect on abundant taxa may be due to their widespread distribution, increasing their likelihood of encountering pesticides, whereas rare taxa, being more sparsely distributed, may be less affected (Stanley and Preetha 2016). Additionally, the increase in fungal richness observed under some gradual treatments suggests that low pesticide doses may elicit hormetic effects, potentially stimulating sporulation and the proliferation of rare fungal variants (Y. Li et al. 2024; Agathokleous, Calabrese, and Veresoglou 2024). This phenomenon may further contribute to the observed shifts in fungal community composition, as certain taxa exploit pesticide-induced changes in resource availability or competition dynamics. Notably, the strongest responses were observed for the triazole fungicide cyproconazole and the herbicides napropamide and pendimethalin, suggesting that both pesticide class and application method shape fungal community dynamics.

Although Meidl et al. (2024) reported more substantial impacts on decomposition rates and physical soil properties resulting from abrupt pesticide applications, this application method did not promote greater fungal evenness or drive species composition shifts to the same extent as gradual applications. The immediate disruption caused by abrupt applications may overwhelm the adaptability of rare taxa while allowing partial resilience or recovery among abundant taxa. The impacts on individual fungal genera were generally also weaker and less consistent under abrupt exposure, likely due to the acute but short-lived nature of the disruption. The pronounced effects observed under gradual application may instead be attributed to repeated exposure, where both dosage and frequency influence

microbial responses while limiting the time available for recovery (M. Zhang et al. 2016; Druille et al. 2016). Additionally, the breakdown of pesticides over time may generate transformation products that could further contribute to shifts in fungal communities, either by acting as additional selective pressures or as alternative carbon sources for certain taxa (Kumar et al. 2021; Katsoula et al. 2020). These findings propose that abrupt applications do not exert the same selective pressures as prolonged exposures, leading to transient rather than persistent community changes. They align with previous research showing that repeated low-dose pesticide exposure can impose long-term selective pressures on microbial communities (Biache et al. 2017). While abrupt applications may cause short-term disturbances, their effects appear more transient, with microbial communities displaying greater resilience. However, it remains unclear whether fungal communities eventually return to their original state or remain altered after pesticide exposure ceases. This highlights the limitations of studies focusing solely on short-term and acute pesticide exposure, as they may underestimate the long-term ecological consequences of chronic, low-dose applications in real agricultural settings.

The pronounced fungal diversity and composition shifts observed under gradual pesticide exposure have important ecological implications. Fungal communities play key roles in soil health, including organic matter decomposition, nutrient cycling, and plant-microbe interactions (Bardgett and van der Putten 2014; Wagg et al. 2014; Schimel 2016). If certain fungi are disproportionately affected by long-term pesticide exposure, this could alter soil ecosystem functions in ways that are not immediately apparent but may have cascading effects over time. From an agricultural perspective, our findings highlight the need to consider application methods and frequency when assessing pesticide impacts. While current pesticide risk assessments often focus on single-dose applications, our results suggest that repeated low-dose exposures may have more persistent and selective effects on microbial communities. Incorporating gradual exposure scenarios into ecotoxicological studies could provide a more accurate representation of real-world pesticide dynamics.

Conclusion

This study emphasizes the influence of pesticide application methods on soil microbial communities. Our findings confirm that fungal communities are more sensitive to pesticide exposure than bacterial communities, exhibiting a more pronounced shift in both alpha and beta diversity. Gradual applications promoted the proliferation of stress-tolerant fungal taxa, particularly under certain fungicides and herbicides, while abrupt treatments caused more transient disruptions. These results suggest that the frequency of low-dosage pesticide exposure can drive long-term changes in fungal community composition, potentially impacting soil health and ecosystem functions. Long-term studies are needed to capture delayed bacterial responses and assess how chronic, low-dose pesticide exposure affects microbial resilience. Understanding whether microbial communities can recover or

remain permanently altered is essential for predicting long-term ecosystem consequences and informing sustainable soil management practices. Future research should also explore whether observed shifts in fungal diversity translate into functional changes, such as altered decomposition rates or nutrient cycling. Moving beyond taxonomic shifts to examine microbial functional traits could provide deeper mechanistic insights into how pesticides reshape soil ecosystems. An improved understanding of these dynamics is thus crucial for developing sustainable pesticide application strategies that minimize unintended ecological consequences.

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