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Effects of the Pesticides Benomyl, Metribuzin and Imidacloprid on Soil Microbial Communities in the Field

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Abstract: Pesticide preparations based on benomyl, metribuzin and imidacloprid were used on a potato plot for three years. Every year soil samples were taken at 0, 7, 14, 28, 56 and 84 days after treatment. For all samples, the pesticide residues in the soil were measured. A study of the soil's bacterial and fungal community structures was preformed using next-generation sequencing. It was found that the rate of decomposition of the pesticides increased every year, while the pesticides affected only certain phylogenetic groups of microorganisms. The most significant effect was the decrease in the proportion of Ascomycota. Additionally, in the field, as well as previously in the laboratory, pesticides have had an impact on the relative abundance of the genera *Haliangium*, *Solicoccozyma* and *Humicola*. It can be assumed that the repeated application of pesticides does not have a significant effect on the microbial communities of soils, provided that they are applied according to the application rate.

Keywords: pesticides; bacteria; fungi; next generation sequencing; soil microbial community

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1. Introduction

The impacts of pesticides on many non-target organisms, such as aquatic organisms, are being actively investigated through registration trials. With regard to the effects of pesticides on soil organisms, the previous research has been focused mainly on soil mesofauna, such as earthworms. The evaluations of the effects on soil organisms in registration trials are limited to carbon and nitrogen cycle tests [1,2]. These integral indicators are not sensitive enough to assess changes in the structures of the bacterial and fungal communities in the soil. Working with soil, it is also necessary to consider that pesticides and products of their metabolism can potentially accumulate and have additive and chronic effects [3]. On the other hand, many pesticides are actively sorbed or destroyed in the soil [4], thereby reducing their potential impact on non-target organisms [5].

At present, the scientific community has accumulated some data and proposes to expand the standard methods for assessing the impact of pesticides on soil microorganisms. For example, it is proposed to use separate bio-indicators, such as ammonia-oxidizing microorganisms (AOM) and arbuscular mycorrhizal fungi (AMF) [6]. The statement of the main task of research being the search for bioindicators indicates the need to study the effect of not only individual pesticides but also their complexes. The study of pesticide complexes is also relevant because pesticides are used in the field in the form of mixtures of preparations for various purposes [7]. Currently, there are not many field studies on the effects of pesticides on soil microbial communities; mainly, these studies concern long-standing pollution [8]. Many studies, including those performed with the involvement of molecular genetic approaches, show certain effects of pesticides on individual phylogenetic or ecological groups of microorganisms [9–11]. However, the scale of the impact of pesticides on agricultural soils in a global sense has yet to be assessed. Often, the changes in soil microbiota caused by pesticides are short-term [12].

For benomyl (and its major metabolite, carbendazim), imidacloprid and metribuzin, which are investigated in this study, a certain amount of data have now been accumulated. Imidacloprid can have a negative effect on nitrifying and nitrogen-fixing bacteria, substrate-induced respiration and the activity of several enzymes [13]. In another study, no significant effect of imidacloprid on the richness and functional diversity of the soil microbial community was found [14]. In saline soils, conflicting results have been obtained, depending on the degree of salinity [15]. Carbendazim was able to have an effect on the structure of the bacterial community up to 126 days in a laboratory experiment [16] or 360 days in the field [17]. Benomyl also inhibits the growth of arbuscular mycorrhizal fungi [18]. Metribuzin does not have a significant effect on the soil microbial community in greenhouses, provided that the application rate is observed [19]. Metribuzin is able to influence the enzymatic activity of the soil, particularly for dehydrogenase and alkaline phosphatase [20]. In Russia, at least 226 tons of preparations with benomyl, 1085 tons with imidacloprid and 336 tons with metribuzin are used (information according to private companies).

A separate issue is the extent to which pesticides can affect soil microorganisms in real field conditions. We were interested in whether there would be any chronic effect on the microbial community with repeated use for three years of the pesticide complex, provided that the application schedule was followed. In addition, we tried to identify the most sensitive groups of microorganisms. Pesticide residues were also measured to assess the rate of decomposition and the possibility of their accumulation in the soil.

2. Materials and Methods

2.1. Soil Sampling and Chemical Substances Used

The field experiment was conducted in the Odintsovo district of the Moscow region (55°41′ N, 38°05′ E). The soil contained Umbric Albeluvisols (IUSS Working Group WRB, 2014) [21]. The main soil properties were given in the article with a laboratory experiment [22]. During the period of the experiment every year (for sampling and growing potatoes), the temperature does not fall below 15 degrees but periodically rises to 30 [23].

The experiment used formulations containing metribuzin (a herbicide, CAS No. 21087-64-9), imidacloprid (an insecticide, CAS No. 138261-41-3) and benomyl (a fungicide, CAS No. 17804-35-2). More details about the properties of the active ingredients are given in [22].

2.2. Experimental Design

The field experiment was a continuation of a laboratory experiment that was carried out with the same soil [22]. Laboratory experiments were carried out in soil microcosms with treatments of the pesticide formulations imidacloprid, benomyl and metribuzin at single and ten-fold application rates. The soil for the laboratory experiment was taken from the site where the described field experiment was then carried out. The duration of the field experiment was three years (2018–2020). Two plots planted with potatoes were used, namely control and treated with pesticides according to different application rates. Processing was carried out on the day of planting potatoes by spraying the soil with a knapsack sprayer (DAEWOO DSA 6 Li). The application rate for the herbicide was 1.4 L/ha (0.98 kg/ha metribuzin), for the insecticide was 0.1 L/ha (0.02 kg/ha imidacloprid) and for the fungicide was 3 kg/ha (1.5 kg/ha benomyl) on 25 m^2 plots. The potatoes were planted in June and harvested in September. Every year, starting from the day of pesticide treatment (it was also the day of planting potatoes), soil samples were taken at 0, 7, 14, 28, 56 and 84 days from the upper horizon of 0–10 cm. Soil samples after 1, 2 and 3 years of pesticide processing were tested for pesticide residues, for third-year soil samples high-throughput sequencing on the Illumina MiSeq platform of the V4 (16S rRNA) and ITS1 (18S rRNA) was carried out. The soil sampled within 0 days was not analyzed using the NGS (next-generation sequencing) method, since on the day of planting potatoes and carrying out agrotechnical measures, the microbial community could get out of equilibrium and it

would be difficult to interpret the data obtained. At the same time, it was unreasonable to expect the influence of pesticides to appear directly on the day of application.

2.3. DNA Extraction and High-Throughput Sequencing

The soil was taken from five points with a drill, then the mixed soil was taken from the ridges next to the plants. The mass of each combined sample was about 500 g. Two samples were taken for sequencing. The isolation of the total DNA was carried out from 0.5 g samples of soil using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA). The amplification of the variable region V4 (16S rRNA) was carried out in one round using the forward and reverse primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) [22,24] with dual-index sample multiplexing. These primers are specific to both bacteria and archaea. The amplification of the variable region ITS1 (18S rRNA) was carried out using the primers BITS (5′-CTACCTGCGGARGGAT CA-3′) and B58S3 (5′-GAGATCCRTTGYTRAAAGTT-3′) [24]. The amplification for the V4 and ITS1 regions was performed in two replicates. The PCR products were purified using the Cleanup Mini Kit (Evrogen, Moscow, Russia) for DNA isolation. The concentrations of the obtained libraries of 16S rRNA and ITS1 in solution were measured on a Qubit® fluorometer (Invitrogen, Waltham, MA, USA) using a Quant-iT® dsDNA High-Sensitivity Assay Kit. The purified amplicons were mixed in an equimolar way in accordance with the concentrations obtained, and the quality of the resulting library prepared for sequencing was assessed via agarose gel electrophoresis. Further sample preparation and sequencing of the pooled samples were carried out using a MiSeq sequencer (Illumina, San Diego, CA, USA) and the MiSeq Reagent Kit v2 (500 cycles).

A bioinformatic analysis of the sequencing data was carried out in Mothur v.1.44.3 [25].

The analysis of bacterial 16S rRNA gene V4 fragments in general was performed according to the standard operating procedure (SOP) [26,27] using SILVA SEED v132 for alignment and SILVA v138 for taxonomic classification [28], but singletons were excluded from the analysis using the remove.rare and split.abund functions.

The fungal ITS fragments were also analyzed using the MiSeq SOP, although the alignment of the ITS sequences was performed using MAFFT v.7 [29], the taxonomic classification was made using UNITE ITS database v.8.0 with dynamic clustering thresholds [30] and singletons were excluded in the same way as for bacterial genes.

For bacterial and fungal sequences, both the alpha and beta diversity indices, AMOVA, PCoA, NMDS, Spearman correlations of different taxon abundance levels with PCoA and NMDS axes and tree construction were carried out via Mothur v.1.44.3 [25]. The sequencing data were rarified according to the values of the samples with the lowest reads counts.

The relative abundance of different taxa as well as alpha diversity indices were non-parametrically compared using a Kruskal–Wallis ANOVA. After obtaining a significant Kruskal–Wallis ANOVA, the Mann–Whitney U test was performed for direct comparisons between every two groups. Comparisons were made between groups differing in the time of sampling and the presence of pesticides, and groups differing only in the time of selection and only in the presence of pesticides. These samples were grouped in three ways: by two factors (pesticides and time), by the time factor and by the presence of pesticides. Differences were considered significant at $p < 0.05$. These comparisons were performed in Statistica 8.0 (TIBCO Software, Palo Alto, CA, USA). The data visualization was performed using Microsoft Office Excel (Microsoft, Redmond, WA, USA).

Since the singletons excluded for the analysis of the environmental samples can provide a number of advantages as well as disadvantages for the sample's discrimination and as the most proper approach is still under discussion [31–33], some analyses were repeated in a variant without singleton exclusion, and the main trends in microbial communities were checked and confirmed.

2.4. Assessment of the Pesticides' Residual Quantities

For the analysis of pesticide residues, 3 averaged samples were taken. The residual quantities of the pesticides were measured using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) system with a diode array detector (DAD) (wavelengths: carbendazim—280 nm; metribuzin—290 nm; imidacloprid—270 nm). The analytical standards of active substances manufactured by Dr. Ehrenstorfer GmbH (Augsburg, Germany) were used for the quantitative determination. Benomyl was not determined because it is extremely unstable [34] and almost immediately turns into carbendazim in soil. An absolute calibration with analytical standards was used for quantitation. The correlation coefficient was 0.999. More details on the chromatographic conditions are given in [22]. The DT50 values were calculated every year using the first-order, single-phase kinetic equation [35]. Uncertainties were calculated as two-sided confidence intervals obtained through the standard error of the regression.

3. Results

3.1. Analysis of Pesticides Residual Amounts

Residual amounts of the pesticides were analyzed at 0, 7, 14, 28, 56 and 84 days for every year. The DT50 values are shown in Figure 1. In general, the data obtained are consistent with those obtained during registration tests of the active substances [34]. Every year the rate of decomposition of all studied pesticides increases. The residual amounts of benomyl (as carbendazim) and metribuzin measured at day 84 also decrease every year, and the concentration of imidacloprid practically does not change (Table 1). There is no significant accumulation of active substances every year.

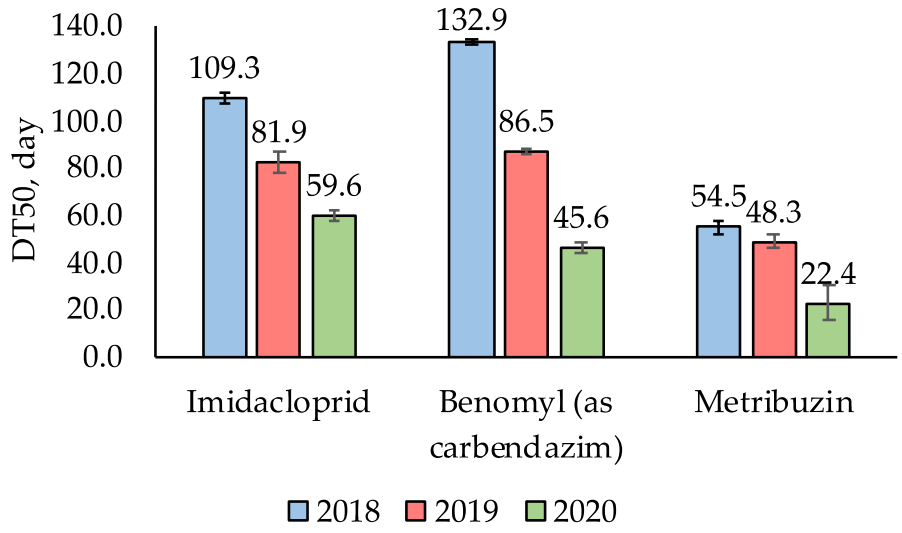

Figure 1. Half-life ($t_{1/2}$) values of active substances with the number of days (n = 3). Error bars show the confidence intervals with 95% probability.

Table 1. Residual amounts of pesticides with standard deviations in mg/kg (n = 3).

Substance	2018 Year	2019 Year	2020 Year
Imidacloprid	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.004
Benomyl (as carbendazim)	1.50 ± 0.12	0.54 ± 0.11	0.43 ± 0.04
Metribuzin	0.16 ± 0.03	0.11 ± 0.02	not detected

3.2. Bacterial Community Structure Changes

During the prokaryotic library sequencing, 676,298 paired-end reads were obtained, from 9405 to 21,258 reads per analytical replicate (average 16,907 per replicate). After quality filtering, removal of chimeras, non-target taxa and singletons, 411,089 sequences were obtained, from 5696 to 13,726 sequences (average 10,277) per replicate.

The sequencing coverage for all soil samples was high and was at least 90%. The differences in the number of detected OTUs (operational taxonomic units) in the samples were insignificant. There were no significant differences in the values of the inverse Simpson index between the control and treated samples at the same sampling time. At the same time, the index values significantly increased both in the control and experimental samples on the 56th day of sampling relative to the 7th day. There were no statistically significant differences in the values of the Chao index between all variants of the experiment (Table 2).

Table 2. Bacterial alpha-diversity indices.

Variants	Coverage	Number of OTU	Invert Simpson Index	Chao Index	Shannon Index
C 7	91.3 ± 0.5%	1018 ± 47.5	119.5 ± 23.1	1725.8 ± 122.5	5.95 ± 0.11
E 7	91.2 ± 0.7%	1021 ± 33.3	130.6 ± 15.4	1762.2 ± 152.4	5.97 ± 0.06
C 14	91.5 ± 0.4%	996 ± 37.6	143.4 ± 12.4	1813.4 ± 130.1	5.98 ± 0.07
E 14	90.3 ± 0.2%	1105 ± 10.8	166.8 ± 6.2	1959.5 ± 92.3	6.14 ± 0.02
C 28	90.9 ± 0.4%	1070 ± 33.1	149.2 ± 8.6	1827 ± 69.3	6.09 ± 0.05
E 28	90.7 ± 0.4%	1092 ± 21.8	157 ± 11.4	1841 ± 72.8	6.12 ± 0.04
C 56	90.6 ± 0.4%	1127 ± 39.5	179.5 ± 8.8	1883 ± 57.4	6.2 ± 0.05
E 56	90.3 ± 0.3%	1135 ± 4.6	189.8 ± 12.9	1991.9 ± 100.4	6.23 ± 0.04
C 84	90.6 ± 0.5%	1079 ± 39.1	167.5 ± 5.9	1935.1 ± 86	6.12 ± 0.04
E 84	90.4 ± 0.8%	1094 ± 40.4	178.6 ± 21	1972.3 ± 260	6.14 ± 0.09

Errors—standard deviation; C—control; E—experiment (pesticide treatment); numbers—days of sampling.

The Shannon diversity index values ranged from 5.95 to 6.23, and similarly to the inverse Simpson index, it reached its highest values on the 56th day (statistically significant differences between the values of the Shannon index on the 56th day compared to the 7th day were revealed only in the variants with pesticide addition). The evenness index values ranged from 0.836 to 0.864, which indicates a close representation of different taxa in the studied samples. In the control and experimental soil samples on the 56th day, the evenness was significantly higher than on the 7th day, which indicates similar trends in successional transformations of the community structure over time. In general, both in the control and with pesticide treatment, there was an increase in the alpha diversity indices for the period from 7 to 84 days, while there were no differences between the control and the treatment at the same time of sampling. When considering biodiversity indices for the presence of pesticides, no significant differences were found.

3.2.1. Bacterial Phylum-Level Community Structure

In all studied samples, Pseudomonadota (33.1–39.4%), Actinobacteriota (14.0–23.8%) and Acidobacteriota (11.2–16.7%) dominated at the phylum level. Representatives of the phyla Verrucomicrobiota (4.6–7.9%), Gemmatimonadota (4.6–6.2%), Bacteroidota (4.0–5.9%) and Myxococcota (2.2–3.8%) also accounted for a significant proportion. The representatives of the phyla, as a rule, made up no more than 3% (with the exception of Bacillota in the control soil sampled on the 14th day at 4.9%), while other phyla made up no more than 1% (Armatimonadota, Nitrospirota, Desulfobacterota, Cyanobacteria, Bdellovibrionota, Elusimicrobiota, WPS-2, Latescibacterota, Dependentiae, RCP2-54, Abditibacteriota, Campilobacterota, MBNT15, Patescibacteria, Methylomirabilota, FCPU426, Spirochaetota, Fibrobacterota, Sumerlaeota, WS2, Deinococcota, Caldatribacteriota, Hydrogenedentes) (Figure 2).

Grouped according to two factors (pesticides and time), there were no differences in the relative abundance levels of phyla between the control and experimental (treated with pesticides) samples at the same sampling time.

Grouped by time factor, differences in the relative abundance of bacteria of some dominant phyla between the first and the last samplings analyzed (i.e., between days 7 and 84) were revealed. Representatives of the Pseudomonadota phylum reduced their share in the community from 38.7% to 33.9%, while the percentage of Acidobacteriota, on the

contrary, increased from 12.1% to 15.7% and Verrucomicrobiota increased from 5.7% to 7.4%. Changes in the relative abundance of Pseudomonadota, Actinobacteriota, Acidobacteriota, Verrucomicrobiota, Gemmatimonadota, Bacteroidota, Myxococcota, Planctomycetota and Bacillota were also noted when comparing 7 and 56 days.

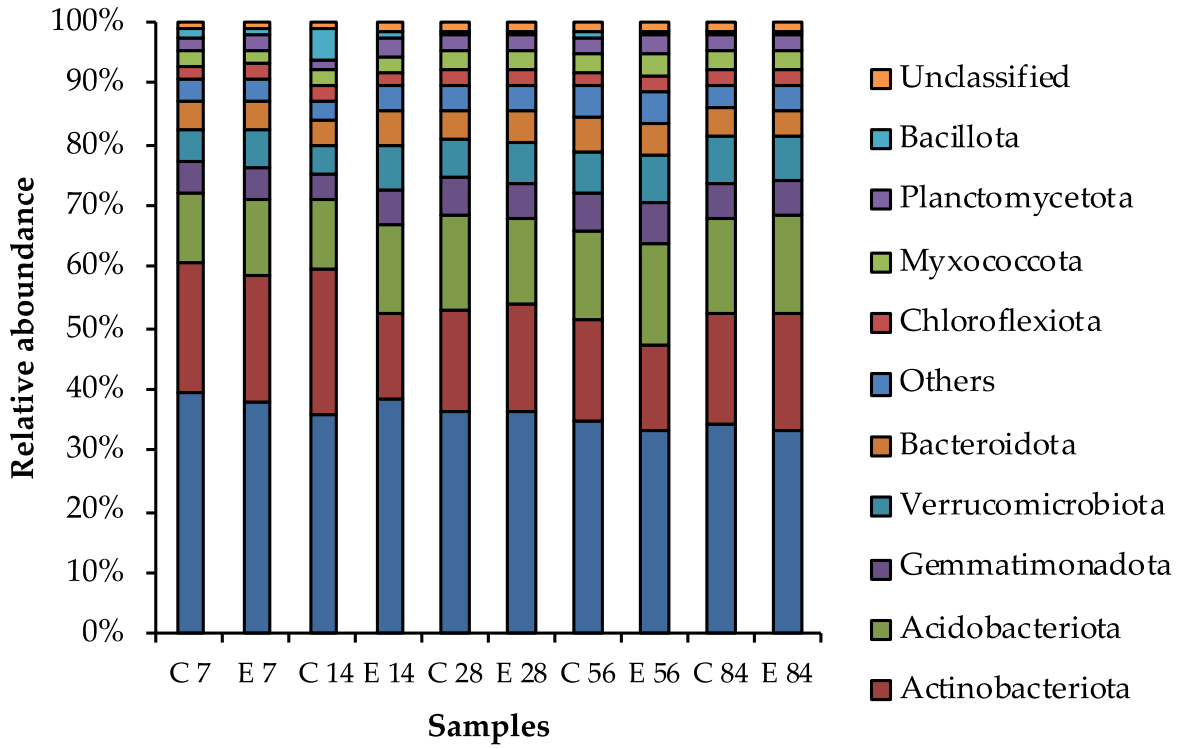

Figure 2. Phylum-level bacterial community structure. C—control; E—experiment (pesticide treatment); numbers—days of sampling.

The grouping by the presence of pesticides revealed differences in the relative abundance levels of representatives of the phyla Verrucomicrobiota (6.1% in control, 7.0% with pesticides), Planctomycetota (2.4% in control, 2.6% with pesticides) and Bacillota (1.6% in control, 0.8% with pesticides).

3.2.2. Bacterial Genus-Level Community Structure

When grouped by two factors, there were practically no differences between the control and experimental samples of the same sampling time, with the exception of *Bryobacter* on the 14th day (Figure 3). In some cases, differences were found between samples taken at different times. Thus, the results differed between *Sphingomonas* (experimental plots 56th–7th days, experimental plots 84th–7th), Candidatus_*Udaeobacter* (control plots 84th–7th, control 84th–14th days), *Solirubrobacterales* (E7–E14), *Vicinamibacterales* (C84–C14, C84–C7, E84–E7) *Gaiellales* (E84–E14); *Pseudarthrobacter* (E56–E7); *Massilia* (C84–C7, E84–E7), *Mycobacterium* (E56–E7) and *Comamonadaceae* (E84–E14).

By grouping the data according to the presence of pesticides, differences in relative abundance levels were revealed only for Candidatus_*Udaeobacter*, *Pseudarthrobacter* and *Bryobacter*.

Grouping by the time factor revealed differences in the relative abundance levels of bacteria from 6 dominant genera between the beginning (7 days) and end (84 days) of the experiment: *Sphingomonas*, Candidatus_*Udaeobacter*, *Gemmatimonadaceaea*_uncultured, *Vicinamibacterales*, *Pseudarthrobacter* and *Massilia*. The obtained results indicate the predominant influence of the sampling time factor on the structures of communities, rather than the presence of the studied pesticides in the soil.

Genus	C7	E7	C14	E14	C28	E28	C56	E56	C84	E84
Sphingomonas	7.1%	6.9%	5.5%	6.0%	6.2%	6.0%	5.2%	4.4%	4.8%	4.0%
Candidatus_Udaeobacter	3.0%	3.8%	2.9%	4.1%	3.7%	4.1%	3.5%	4.0%	4.7%	4.3%
Acidobacteriales_uncultured_ge	3.2%	3.6%	3.1%	4.0%	4.5%	3.9%	3.6%	4.1%	3.8%	4.2%
Gaiellales_uncultured_ge	3.5%	3.4%	3.9%	2.6%	3.0%	3.1%	2.9%	2.5%	3.1%	3.5%
Gemmatimonas	2.5%	2.5%	2.0%	2.9%	3.0%	2.5%	2.7%	3.0%	2.3%	2.5%
Xanthobacteraceae_unclassified	2.9%	1.9%	1.6%	2.4%	2.6%	2.6%	2.4%	2.4%	2.3%	2.4%
Gemmatimonadaceae_uncultured	2.0%	2.0%	1.9%	2.2%	2.4%	2.3%	2.4%	2.6%	2.6%	2.7%
Xanthobacteraceae_uncultured	2.1%	2.3%	2.0%	2.0%	2.1%	2.3%	2.3%	2.1%	2.5%	2.4%
Solirubrobacterales_67-14_ge	2.4%	2.9%	2.2%	1.7%	1.8%	2.0%	1.9%	1.8%	2.1%	2.2%
Vicinamibacterales_uncultured_ge	1.5%	1.5%	1.6%	1.8%	2.0%	1.9%	2.2%	2.4%	2.8%	2.7%
Gaiellales_unclassified	1.6%	1.9%	2.1%	1.4%	1.5%	1.6%	1.5%	1.5%	1.6%	2.0%
Pseudarthrobacter	2.6%	1.8%	1.9%	1.1%	1.4%	1.4%	1.5%	0.8%	1.3%	1.2%
Nitrosomonadaceae_Ellin6067	1.1%	1.3%	1.2%	1.8%	1.6%	1.4%	1.6%	1.7%	1.6%	1.5%
Massilia	3.1%	2.0%	1.0%	1.6%	1.5%	1.3%	1.8%	1.0%	0.7%	0.6%
Candidatus_Solibacter	1.2%	1.4%	1.2%	1.5%	1.6%	1.3%	1.6%	2.0%	1.5%	1.3%
Bacteria_unclassified	1.1%	1.2%	1.0%	1.5%	1.6%	1.4%	1.6%	1.6%	1.5%	1.6%
Mycobacterium	1.6%	1.7%	1.6%	1.2%	1.3%	1.4%	1.2%	1.0%	1.3%	1.4%
Pseudolabrys	1.2%	1.5%	1.4%	1.4%	1.3%	1.4%	1.1%	1.3%	1.5%	1.4%
Bryobacter	1.1%	1.2%	1.0%	1.5%	1.4%	1.4%	1.4%	1.7%	1.3%	1.3%
Comamonadaceae_unclassified	1.6%	1.1%	1.2%	1.4%	1.2%	1.3%	1.6%	1.2%	1.0%	0.7%

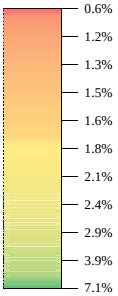

Figure 3. Genus-level bacterial community structure. Twenty of the most abundant genera are presented. C—control; E—experiment (pesticide treatment); numbers—days of investigation.

3.2.3. The Relative Abundance of Indicator Taxa and Statistical Analysis

Several hundred genera's relative abundances correlated with the PCoA and NMDS axes (Figures 4 and S1–S3); therefore, for a comparison of relative abundance levels, genera with significant correlations with both axes and with the largest vector lengths were selected.

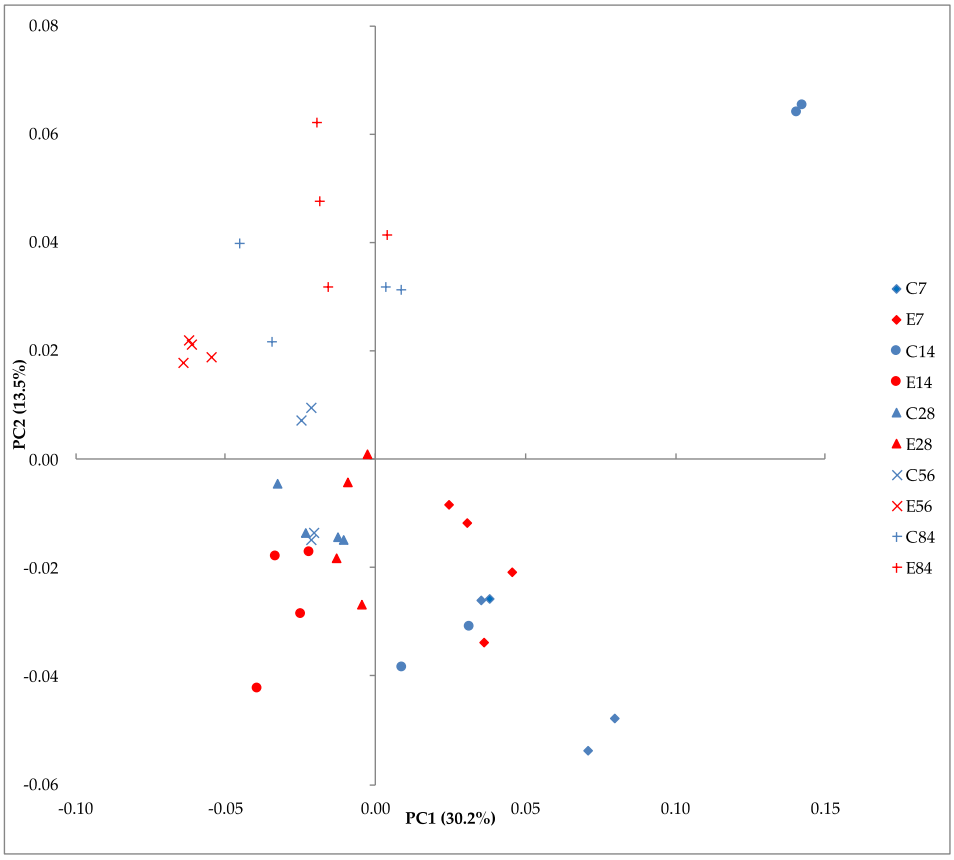

Figure 4. PCoA results based on the UniFrac weighted distance matrix for bacterial communities. C—control; E—experiment (pesticide treatment); numbers—days of sampling.

When grouped by two factors, no significant differences were found in the relative abundance levels of the representatives of these genera. When grouped by the presence of pesticides, significant differences in relative abundance levels were found for representatives of the genera *Terracidiphilus*, *Paenarthrobacter*, *Haliangium* and *Dyella* and unidentified genera from the Bacillacea and Micropepsaceae families.

When grouped by time, differences between the start and end points were found only for the proportion of bacteria of the genus *Haliangium*.

When grouping by the presence of pesticides, differences were found in the relative abundance levels of the following families: Micrococcaceae, Bacillaceae and Planctomycetota_OM190, WPS-2.

Comparing control samples on day 7 with control samples from other sampling times using an AMOVA (Tables A1 and S1), an increase in Fs values can be observed with increasing time. Thus, the microbial community differs more and more from the initial one precisely with the course of observation time. The dynamics of the structure of the microbial communities of the experimental (treated with pesticides) samples is similar; however, on day 84, the Fs value decreases. The Fs values between control and treated soils of the same sampling times were relatively low, and the smallest contribution of intergroup variability can be observed between the control and experimental samples taken on day 84 (Fs = 2.15, $p = 0.0292$). This indicates that the course of bacterial succession is common to all samples.

The PCoA reveals that the two principal components account for 43.7% of the variance in total (Figure 4). The microbial communities sampled on day 84 are grouped separately from the communities sampled on days 7–28.

3.3. Fungal Communities Structure Changes

During the sequencing of the fungal ITS gene fragment libraries, 895,864 paired-end reads were obtained from 15,579 to 32,688 reads per analytical replicate. After filtering for quality and removing chimeras, non-target taxa and singletons, 743,765 sequences were obtained from 12,610 to 28,176 sequences (average 18,594) per replicate.

The coverage for all samples was high and amounted to at least 99%. No differences were found in the presence or absence of pesticides for all biodiversity indices. Differences in the time of sampling were revealed, as the number of OTUs on the 84th day was significantly lower than on the 7th and 14th; the inverse Simpson index significantly increased by the end of the experiment; and the Chao index on the 84th day was significantly lower than on the 7th, 14th and 28th days. The evenness on days 14 and 56 was significantly lower than on day 84, which indicates the completion of successional processes in the community. Analyzing the simultaneous influence of two factors (pesticides and time), differences were found only in evenness.

3.3.1. Fungal Phylum-Level Community Structure

At all sampling periods, both in samples with pesticides and without them, Ascomycetes (27–49%), Basidiomycetes (14–26%) and fungi, which could not be attributed to any of the phyla (15–44%), dominated. Representatives of the phyla Mortierellomycota and Chytridiomycota also accounted for significant shares in the communities, as 7–20% and 0.5–6%, respectively. The total percentage range of other phyla (Olpidiomycota, Glomeromycota, Mucoromycota, Rozellomycota, Zoopagomycota, Kickxellomycota, Basidiobolomycota, Monoblepharomycota) was 1–4% (Figure 5). According to the presence of pesticides, the relative abundance rates of Ascomycetes significantly differed (decrease from 42.9% in the control to 35.3% with pesticides, $p = 0.013$).

The presence of pesticides significantly affected the relative abundance of ascomycetes (decrease from 42.9% in control to 35.3% with pesticides, $p = 0.013$). The main differences in the structure of fungal communities are due to the time factor; in the course of the experiment, increases in the proportions of Ascomycetes (from 32.6% to 46.3%) and

Mortierellomycota (from 7.9% to 16.1%) and a decrease in Chytridiomycota (from 6.0–8.0% up to 3.9%) were observed. Analyzing the two factors, no differences were found.

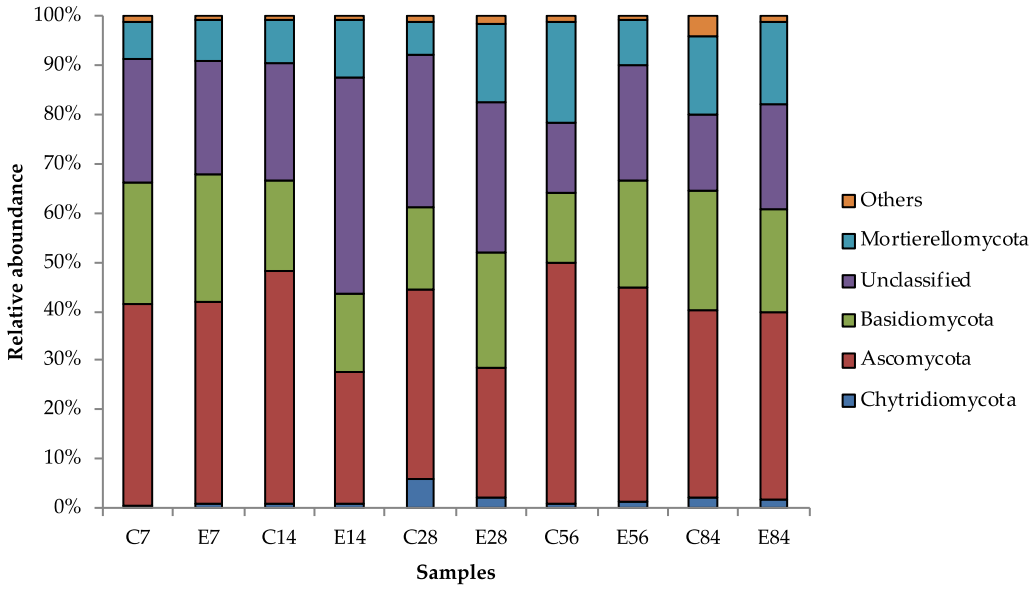

Figure 5. Phylum-level fungal community structure. C—control; E—experiment (pesticide treatment); numbers—days of sampling.

3.3.2. Fungal Genus-Level Community Structure

At the level of genera, 20 genera of fungi were found, which were characterized by the largest percentage in the communities, reaching up to 70–80% of the communities (Figure 6). At the genus level, the presence of pesticides in the soil led to an increase in the relative abundance of representatives of the genus *Phialocephala* (from 0.3 to 1.6%, respectively) and a decrease in the relative abundance of representatives of the genera *Solicoccozyma* (4.5% in the control vs. 3.4% with pesticides), *Humicola* (from 3.6 to 1.7%), *Penicillium* (from 2.2 to 1.4%) and *Cladosporium* (from 2.0 to 0.8%).

Genus	C7	E7	C14	E14	C28	E28	C56	E56	C84	E84
Fungi_unclassified	23.7%	30.7%	14.5%	24.9%	15.5%	44.2%	30.5%	23.6%	22.9%	21.6%
Mortierella	8.6%	6.1%	18.5%	7.3%	15.2%	11.0%	15.2%	8.5%	8.1%	16.4%
Saitozyma	6.5%	6.0%	4.9%	7.7%	5.5%	4.9%	6.2%	3.9%	7.1%	8.6%
Solicoccozyma	4.5%	4.2%	2.8%	5.5%	5.4%	2.5%	4.2%	2.1%	4.1%	4.3%
Lectera	1.3%	0.8%	15.0%	1.4%	0.8%	0.9%	1.1%	11.7%	0.3%	4.7%
Humicola	3.3%	6.6%	3.4%	3.5%	1.3%	2.1%	1.9%	1.5%	2.2%	1.0%
Hypocreales_unclassified	1.9%	1.7%	1.8%	2.1%	4.9%	3.2%	5.0%	1.1%	3.3%	2.7%
Metarhizium	0.9%	1.2%	0.7%	1.8%	0.9%	1.2%	1.6%	0.7%	7.1%	2.1%
Apiotrichum	1.8%	1.9%	0.2%	2.2%	4.9%	3.3%	1.5%	0.9%	1.8%	1.9%
Penicillium	2.5%	2.7%	1.0%	2.9%	1.9%	1.2%	2.2%	1.0%	1.4%	1.3%
Ascomycota_unclassified	1.4%	3.1%	1.3%	1.8%	3.2%	1.0%	0.9%	1.4%	1.7%	2.0%
Boeremia	0.1%	0.5%	4.3%	0.2%	0.6%	0.6%	0.2%	7.7%	0.2%	0.5%
Cladosporium	1.9%	0.9%	1.5%	3.7%	2.2%	0.8%	0.4%	0.9%	0.5%	1.1%
Fusarium	4.1%	1.2%	1.4%	1.3%	0.4%	0.5%	0.2%	0.5%	1.7%	0.5%
Sordariomycetes_unclassified	1.4%	0.6%	0.9%	1.6%	2.5%	1.2%	1.0%	1.5%	1.1%	1.2%
Plectosphaerella	2.0%	0.7%	1.3%	1.3%	1.4%	0.5%	0.6%	0.8%	0.8%	1.7%
Helotiales_unclassified	0.8%	0.7%	0.9%	1.1%	0.8%	1.3%	1.2%	0.6%	0.9%	3.3%
Erythrobasidiales_unclassified	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.3%	0.0%	7.4%	0.0%
Tausonia	0.2%	0.1%	0.1%	0.2%	3.4%	0.4%	0.2%	5.6%	0.3%	1.0%
Phialocephala	0.3%	0.7%	0.0%	0.2%	0.0%	1.2%	0.1%	0.4%	4.4%	1.6%

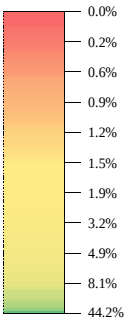

Figure 6. Genus-level fungal community structure. Twenty of the most abundant genera are presented. C—control; E—experiment (pesticide treatment); numbers—days of investigation.

3.3.3. Relative Abundance of Fungal Indicator Taxa

The indicator taxa were selected based on correlations with the PCOA and NMDS axes (Figures 7 and S4–S8), as well as on the basis of laboratory experiment data [22]. When grouping by the presence of pesticides, a significant difference was found for the genera *Candida*, with an increase from 0.1 to 0.5%, and *Fusicolla*, with a decrease from 0.9 to 0.1% in the presence of pesticides. When grouping by time and by two factors, no differences were found. When grouping by the presence of pesticides, a significant difference was found for Rhynchogastremataceae family (0.03% (control) and 0.11% (pesticides)). When grouped by time, differences were found for Didymellaceae and Plectosphaerellaceae, differing at the 56th day from all others, and Rhynchogastermataceae, differing at days 7 and 14 from day 56. For the two-factor analysis, no difference was found. The two principal components (Figure 7) accounted for 30.3% of the variance in total. The microbial communities selected on day 84 were grouped separately from the communities selected on days 7–28.

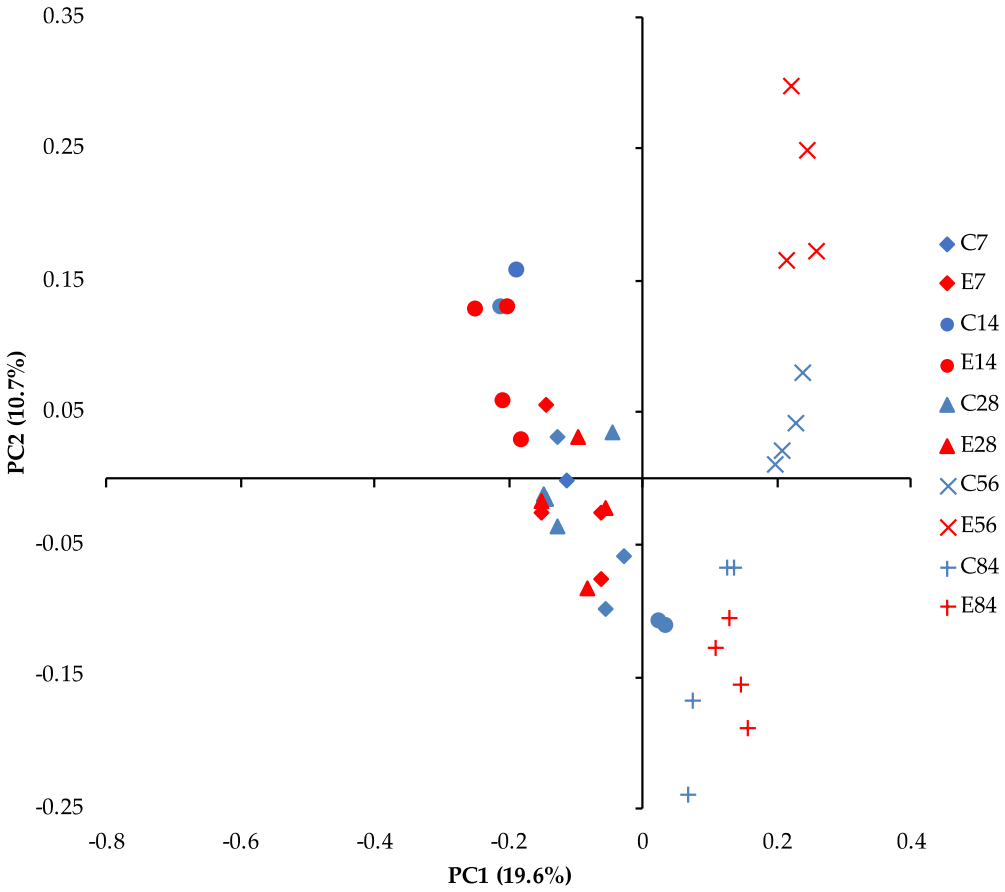

Figure 7. PCoA results based on the UniFrac weighted distance matrix for fungal communities.

A comparison was performed of the control samples on day 7 with the control samples from another sampling time using an AMOVA (Tables A2 and S2). Additionally, in the case of bacteria, the results show that the Fs increases with time, i.e., the microbial community becomes increasingly different from the original; at the same time, the maximum differences were observed on day 56 (on day 84, the Fs decreased). When comparing control and experimental samples from the same sampling time, the variability was also higher than within groups but the variability between groups was not as great as the variability that occurred with a change in the time of sampling. Notably, in some pairs the *p*-value was >0.05, although in most cases the results were statistically significant.

4. Discussion

Since the pesticides did not have a significant effect on the alpha diversity indices, it can be stated that there was no effect on the structure of the bacterial and fungal communities as a whole. The sampling time factor had a more significant effect on the structure of the soil microbiota. Thus, such natural factors as the temperature, humidity, time and agrotechnical measures in the field mainly determine the structure of the soil microbial community, and the influence of pesticides in the recommended application is relatively small.

An acceleration of the rate of decomposition of the pesticides in the soil with repeated applications was observed earlier [36,37]. This indicates that the microbial community of the soil is adapting to the application of pesticides [38]. At the same time, there was no significant restructuring of the microbial community, since pesticides were introduced in small quantities and did not cause a sharp increase in the relative abundance of specific decomposers. It has already been shown earlier that at relatively low application rates, one should not expect a significant restructuring of the soil microbial community [39]. The half-life times of the pesticides in the field and laboratory experiments were comparable [22], which indicated relatively normal soil conditions during the field experiment.

In a laboratory experiment with imidacloprid, metribuzin and benomyl, as well as in a field study, the pesticides had an effect on individual phyla and genera of bacteria and fungi according to a pairwise analysis and correlations with PCoA axes. The abundance of taxa as affected by the pesticides was also assessed according to a laboratory experiment [22]. Both in the laboratory and field experiments, a decrease in the proportion of ascomycetes under the influence of pesticides was observed. This was the most significant effect that the pesticides had on the microbial community. It is likely that a decrease in the proportion of Ascomycetes can serve as a marker of the impact of pesticides, especially fungicides. Additionally, a decrease in the proportion of Ascomycetes in the soil under the influence of pyrethroid insecticides was previously noted [40]. According to laboratory and field experiments, the pesticides affected the genera *Haliangium*, *Solicoccozyma* and *Humicola*. *Haliangium* is involved in the soil nitrogen cycle. Cloransulam-methyl has been shown to reduce the relative abundance of this genus [41]. An increase in the relative abundance of *Solicoccozyma* under the influence of pesticides has been previously found [42]; representatives of this genus have been shown to be able to degrade glyphosate [43]. The relative abundance of *Humicola* decreased under the influence of difenoconazole [44]. A number of genera were also found to be affected by pesticides in the field experiment but not in the laboratory. These were Candidatus *Udaeobacter*, *Pseudarthrobacter*, *Bryobacter*, *Phialocephala*, *Paenarthrobacter*, *Penicillium*, *Cladosporium*, *Terracidiphilus*, *Haliangium*, *Dyella*, *Candida* and *Fusicolla* and unidentified genera from Bacillacea and Micropepsaceae families. No single taxonomic group has been found to appear or practically disappear under the influence of pesticides. This also indicates a low influence level of pesticides on the microbial community of the soil as a whole.

It should be noted that the soil on which the experiment was carried out is characterized by a low carbon content and relatively low microbiological activity. In soil with a higher content of organic matter and more taxonomically diverse microbial communities, more sensitive taxa could probably be identified. On the other hand, in such soils, greater binding of pesticides or faster decomposition could be expected [45].

Apparently, when applying pesticides in the field without exceeding the recommended application rates, one should not expect significant changes in the structure of the microbial community. For example, for glyphosate, which has a high solubility rate in water, and as a result good availability for microorganisms, no effect on bacteria or fungi in the rhizosphere was found when applied to corn [46].

5. Conclusions

Based on the results of both field and previous laboratory experiments, it could be stated that pesticides studied, when applied in accordance with the regulations, do not cause rearrangement of the bacterial and fungal communities of the soil upon long-term

application. We assume that this will be true for the vast majority of modern pesticides. Additionally, in a warmer climate, the negative impact of pesticides will be even less significant due to the higher rate of their decomposition. In a sense, the conditions under which the experiment was conducted are the "worst case". The presence of winter periods with freezing of the soil can allow pesticide residues to accumulate [47]. Only certain taxa are subject to the effects of pesticides. Thus, if we talk about studies of the toxicity of pesticides for soil microbiota, then at the moment a possible list of biomarkers indicating no effect of pesticides is already being formed. However, it also remains to be seen how large the effects must be to be considered significant. Apparently, the first stage of studies of the toxicity to microorganisms of new pesticides is sufficient to conduct short laboratory experiments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13071330/s1. Figure S1: PCoA results based on the UniFrac weighted distance matrix. Figure S2: PCoA results based on the UniFrac weighted distance matrix. Figure S3: PCoA results based on the UniFrac weighted distance matrix. Figure S4: PCoA results based on the UniFrac weighted distance matrix. Figure S5: PCoA results based on the UniFrac weighted distance matrix. Figure S6: PCoA results based on the UniFrac weighted distance matrix. Figure S7: PCoA results based on the UniFrac weighted distance matrix. Figure S8: PCoA results based on the Yue and Clayton measure of dissimilarity matrix. Table S1: An AMOVA of bacterial communities. Table S2: An AMOVA of fungal communities.

Author Contributions: Conceptualization, R.S. and A.A.; methodology, R.S. and V.G.; software, R.S. and V.C.; validation, A.A. and V.C.; formal analysis, V.C., R.S. and A.B.; investigation, R.S. and A.A.; resources, R.S. and V.G.; data curation, A.B.; writing—original draft preparation, R.S.; writing—review and editing, V.G. and A.B.; visualization, V.C. and A.B.; supervision, V.G.; project administration, R.S. and V.G.; funding acquisition, R.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Raw sequence reads have been submitted to GenBank under the BioProject accession number PRJNA822791.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. An AMOVA of bacterial communities, showing pairwise Fs values (under the diagonal) and *p*-values (above the diagonal).

Samples	C 7	C 14	C 28	C 56	C 84	E 7	E 14	E 28	E 56	E 84
C 7	-	0.028	0.020	0.032	0.028	0.023	0.031	0.029	0.020	0.023
C 14	3.76	-	0.031	0.028	0.026	0.148	0.029	0.028	0.028	0.026
C 28	9.94	4.80	-	0.029	0.018	0.027	0.030	0.032	0.018	0.024
C 56	9.46	5.08	4.15	-	0.019	0.023	0.027	0.027	0.027	0.026
C 84	17.89	5.15	5.46	5.09	-	0.015	0.020	0.017	0.015	0.029
E 7	4.10	2.63	6.96	8.62	9.79	-	0.021	0.027	0.020	0.023
E 14	13.16	5.55	4.40	4.54	5.83	6.15	-	0.031	0.024	0.026
E 28	11.03	3.95	3.45	4.54	3.81	4.02	2.79	-	0.025	0.028
E 56	26.83	8.69	8.82	4.50	4.22	18.78	4.88	7.44	-	0.026
E 84	19.53	5.35	6.96	6.11	2.15	9.82	5.17	4.88	3.81	-

C—control; E—experiment (pesticide treatment); numbers—days of sampling.

Table A2. An AMOVA of fungal communities, showing pairwise Fs values (under the diagonal) and *p*-values (above the diagonal). Non-significant values are shown in bold.

	C7	C14	C28	C56	C84	E7	E14	E28	E56	E84
C7	-	**0.344**	0.048	0.007	0.030	**0.059**	0.031	0.029	0.025	0.021
C14	1.22	-	**0.252**	0.026	0.026	**0.346**	**0.139**	**0.082**	0.023	0.025
C28	1.94	1.22	-	0.029	0.029	**0.089**	0.030	0.032	0.028	0.026
C56	21.34	5.86	10.27	-	0.027	0.027	0.009	0.025	0.024	0.028
C84	4.93	2.62	4.35	8.71	-	0.030	0.031	0.029	0.028	0.030
E7	2.16	1.20	1.59	8.68	3.84	-	0.027	0.027	0.029	0.030
E14	11.03	2.41	3.55	79.92	11.03	2.60	-	0.031	0.021	0.029
E28	4.09	2.19	2.76	25.10	5.25	2.18	7.18	-	0.026	0.028
E56	8.54	3.89	5.64	3.84	5.05	5.05	12.92	8.84	-	0.025
E84	7.30	3.23	5.97	9.06	2.57	4.70	21.04	8.51	6.03	-

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