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Changes in Bacterial and Fungal Community of Soil under Treatment of Pesticides

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Abstract: Experiments were carried out in soil microcosms with the treatment of pesticide formulations—imidacloprid, benomyl, and metribuzin in single and tenfold application rates. For additional stimulation of microorganisms, a starch–mineral mixture was added to some variants. For all samples, high-throughput sequencing on the Illumina MiSeq platform of the V4 (16S rRNA) and ITS1 (18S rRNA) fragments was carried out. As a result, it was possible to establish the characteristic changes in the structure of the soil fungal and bacterial communities under pesticides application. The application of pesticides was accompanied by dramatic shifts in alfa-diversity of the fungal community. The phylum Basidiomycota was likely to be involved in the degradation of pesticides. The changes in the relative abundance of the genera *Terrabacter*, *Kitasatospora*, *Streptomyces*, *Sphingomonas*, *Apiotrichum*, *Solicoccozyma*, *Gamsia*, and *Humicola* can be proposed as an indicator of pesticide contamination. It is suggested to use these markers for large-scale assessment of the effect of pesticides on soil microbial communities instead of classical integral methods, including within the framework of state registration of pesticides. It is also recommended to research the effect of pesticides on the soil microbiome during artificially initiated successions using the additional source of carbon.

Keywords: pesticide; bacteria; fungi; NGS; soil microbial community

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1. Introduction

The study of the effect of pesticides on the soil microbial community is an important issue, which, however, is associated with methodological difficulties, since the assessment is aimed at an extremely complex system. Until recently, methods were not available to capture the full microbial diversity of the soil. From the middle of the 20th century, the studies on the possible harm of pesticides to soil microbial community (Bacteria and Fungi) have been relying on the use of integral indicators such as soil respiration and nitrification, as well as microorganism seeding on solid media. Any effects have been observed only at very high concentrations and have been difficult to explain. For example, DDT (insecticide) applied at a concentration of 0.1% [1] has not affected nitrogen fixers, nitrifiers, ammonifiers, and sulfur-oxidizing bacteria; however, it has led to an increase in the total amount of microorganisms. Aldrin (insecticide) has also led to an increase in the number of bacteria, enhanced soil respiration, and either stimulated or suppressed nitrification and ammonification depending on the type of soil [2]. An assessment of the influence of 29 pesticides on respiration and nitrification has shown both positive and negative effects depending on the pesticide and the time of exposure [3]. All effects have been observed at concentrations well above field application rates. The herbicides metribuzin and glyphosate are capable of suppressing carbon dioxide emissions from soils with low carbon content when applied at a level of 100 mg/kg [4]. A study of the effect of pyrethroid insecticides on nitrification, respiration, and dehydrogenase activity has shown that pesticides did not have a statistically significant long-term effect [5]. For the fungicide

benomyl, which is also used in our study, no significant effect on microbiota was found by the method of seeding and measuring soil respiration [6]. A laboratory experiment with a set of pesticides designed to simulate comprehensive plant protection has shown the likelihood of synergistic effects. To assess the state of the microbial community, researchers also actively use indicators of enzymatic activity as well as dynamics of microbial biomass in terms of carbon emission [7]. The most significant effects were exerted by preparations containing the fungicides captafol and triadimefon, but only after the third treatment. Often, individual phyla of microorganisms can be stimulated by pesticides, for example, the insecticides hexachlorocyclohexane and forate at the recommended application rate had positive effects on microorganisms involved in the nitrogen and carbon cycles [8]. Positive effects on bacteria and fungi in the rice rhizosphere as a result of insecticide application have been documented in a field experiment using the plate method and counting Colony Forming Units (CFUs) [9].

It is also possible to study the effect of pesticides on microorganisms in pure cultures. For example, the herbicides glyphosate and hexazinone have been found to have a significant negative effect on ectomycorrhizal fungi in experiments in a liquid nutrient medium [10]. However, it is not clear to what extent these results relate to soil; moreover, only a small fraction of soil microorganisms can be efficiently grown in vitro. The study of the effects of herbicides using BIOLOG methodology has not shown a significantly higher sensitivity of this method compared to the classical methods for assessing the mineralization of carbon and nitrogen [11].

At present, studies using classical methods are still ongoing. For example, the most popular fungicides, carbendazim and tebuconazole, have been shown to exert no effect on soil respiration and enzyme activity; the effects manifest themselves only at a concentration of 100 mg/kg [12]. Imidacloprid (insecticide) has been shown to inhibit soil respiration and enzymatic activity even when applied at a single application rate in loamy sand [13]. Respiration and enzymatic activity of tropical soils are less susceptible to the influence of imidacloprid; at a single application rate, changes are weakly expressed [14].

The use of molecular genetic methods has made it possible to dramatically increase the volume and quality of the data. For instance, the use of genetic fingerprinting methods to separate the products of amplifications of the 16S rRNA region from total soil DNA has revealed significant changes in the structure of the bacterial community under the influence of herbicides [11,15]. An analysis of phylotypes based on the 16S rRNA genes of the total soil DNA has shown the effect of methylparathion (insecticide) on the soil microbial community that is manifested in the replacement of phylotypes [16]. As the results of amplification of the V4 (16S rRNA) region followed by TGGE and band sequencing show, carbendazim (fungicide) reduces the microbial diversity expressed as the Shannon index [17]. The fungicides penconazole and cyprodinil have a temporary effect on nitrification in vineyard litters, but the changes in the abundance of archaea and ammonia-oxidizing microorganisms caused by them persist for a much longer time; it should be noted that ammonia-oxidizing bacteria are more sensitive to these fungicides [18]. The faster recovery of nitrification can be explained by the excess microbial functions in the soil. Structural changes have been identified using PCR-DGGE with DNA and cDNA fragments. RNA-based analysis was found to be more informative. Ammonia-oxidizing Archaea (AOA) and Bacteria (AOB) and are likely to be suitable target groups for monitoring the effects of pesticides on the soil microbiota [19]. An analysis of fatty acid profile (PLFA) has shown that imidacloprid has a temporary effect on the structure and abundance of fungal and bacterial communities in the soil when applied at the rate of application. These data have also been confirmed by DGGE [20]. The use of pyrosequencing has allowed identifying changes in the structure of the soil microbiome under the influence of neonicotinoids, in particular, concerning some families of Proteobacteria and Actinobacteria, which are involved in the biodegradation of neonicotinoids [21].

The use of high throughput sequencing (NGS) and Taxon XOR analysis (Taxon Exclusive Or) to assess the state of the soil microbial community under the influence of

organochlorine pesticides (OCPs) (insecticides) has made it possible to determine that some species are absent in contaminated soils and their absence can serve as a marker of contamination [22]. Other species, on the contrary, are characteristic of soils contaminated with OCPs and are probably involved in the bioremediation of pollution. *Escherichia* and *Mortierella* are the main genera were found inside the contaminated soil. The low abundance of *Nitrospirae* species and the disappearance of *Glomeromycota* in contaminated soil indicate that the use of OCPs leads to serious toxicological consequences. Sometimes during the joint application of pesticides, certain smoothing of the effects is possible. For example, soil microbial activity and functional diversity based on NGS data have shown a tendency to suppression after carbendazim (fungicide) application and a tendency to suppression–restoration–stimulation when treated with chlortetracycline (antibiotic) and carbendazim simultaneously [23]. At the same time, repeated treatments with carbendazim do not lead to an increase in the resistance of the microbial community to it. The genera of bacteria most sensitive to carbendazim are *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Mycobacterium*, *Streptococcus*, *Burkholderia*, and *Corynebacterium*.

High throughput sequencing of the 16s rRNA has shown that the number of OTU significantly decreased in the samples of soils treated with imidacloprid in comparison with the control samples and amounting to 31,173 and 21,909. The genus *Gemmata* has completely disappeared in the soil treated with imidacloprid, whereas the species belonging to the genus *Prevotella* (class Phycisphaerae) have been identified in treated samples [24].

Approximately 10 years ago, a consensus began to emerge regarding the assessment of methods for determining the effects of pesticides on the soil microbial community. The researchers came to realize that integral methods are not informative enough and that there is a need for the use of DNA sequencing [25], as well as for the combined application of classical and molecular methods. At the same time, the need to use new tools to assess the effect of pesticides on soil microorganisms to satisfy the corresponding state regulations was also discussed [26]. Currently, according to the Organization for Economic Co-operation and Development (OECD) and EU rules, only nitrogen and carbon mineralization tests are used to assess the effect of pesticides on soil microorganisms in framework of pesticides registration [27–29]. Moreover, although scientific research has advanced significantly in recent years, nevertheless, in the field of government regulation in the OECD countries, there are no new standards.

A multilevel approach for assessing the effect of pesticides on microbial communities has also been proposed; it includes the analysis of fatty acids and potential enzymatic activity, as well as qPCR for individual phylogenetic groups [30].

A separate direction of research is the assessment of enzymatic activity by determining the expression of the corresponding genes, which is probably more informative than the direct determination of enzyme activity [31]. In addition, specific enzymes associated with the destruction of herbicides have been proposed to be used as biomarkers of the effect of the latter on the soil microbiome [32]. The genes encoding these enzymes are located inside of the plasmids, who allows them to actively spread among bacteria. However, this method does not provide any information about the ongoing changes in the structure of the soil microbiome.

In our opinion, an insufficient number of informative studies aimed at the identification of the most sensitive components of the microbiome has yet been carried out using high-throughput sequencing allowing us to assess changes in the structure of the bacterial community in response to pesticide treatment. To detect these components, it is important to assess the effects of pesticides both individually and in the combined application. At the first stage, it is preferable to carry out these assessments in laboratory conditions, where the problem of sample homogeneity is not so acute.

Our task is to identify the most sensitive taxa to the introduction of pesticides. These taxa can be used as universal indicators of pesticide contaminations in a soil.

2. Materials and Methods

2.1. Chemical Substances

The experiment used formulations produced in local factories containing metribuzin (an herbicide, CAS № 21087-64-9), imidacloprid (an insecticide, CAS № 138261-41-3), and benomyl (a fungicide, CAS № 17804-35-2). Benomyl converts very quickly into the main metabolite of carbendazim (CAS № 10605-21-7) in the soil. The main characteristics of these pesticides are listed in Table 1. Metribuzin has a low sorption capacity (K_{oc} = 38) and a rather high solubility (~10.7 g/L), which determines its bioavailability. Imidacloprid and carbendazim (the main metabolite of benomyl), due to their high persistence in soil (DT_{50} > 60 to 120 days), can act on soil microorganisms for a long time. These three pesticides can be used together in real field conditions, for example, on potatoes.

Table 1. Physical/Chemical Properties of pesticides.

Active Substances	Chemical Formula	Molecular Weight	Solubility—In Water at 20 °C (mg/L)	Octanol–Water Partition Coefficient at pH 7, 20 °C, log P	Vapor Pressure at 20 °C (mPa)
Metribuzin	$C_8H_{14}N_4OS$	214.29	10,700	1.75	0.121
Imidacloprid	$C_9H_{10}ClN_5O_2$	255.66	610	0.57	4.0×10^{-7}
Benomyl	$C_{14}H_{18}N_4O_3$	290.32	2	1.4	0.005
Carbendazim	$C_9H_9N_3O_2$	191.21	8	1.48	0.09

2.2. Soil and Experimental Design

Umbric Albeluvisols (IUSS Working Group WRB, 2014) soil was air-dried and sieved through a 1 mm sieve. The main properties of the soil are presented in Table 2.

Table 2. Main soil properties.

Parameter	Value
Organic matter (%) 0–20 cm	1.5 ± 0.04
pHwater	5.6 ± 0.2
Texture class (USDA) 0–25 cm	Silty loam
Soil bulk density, g/cm^3	1.2 ± 0.1

Firstly, 100 g of soil was mixed in a mortar with 20 mL of water (control variants) or with 20 mL of water supplemented with pesticide formulations or their mixture. The application rate for the herbicide was 1.4 L/ha (0.98 kg/ha metribuzin), for the insecticide 0.1 L/ha (0.02 kg/ha imidacloprid), and for the fungicide 3 kg/ha (1.5 kg/ha benomyl). There were also experimental variants with a 10-fold rate of application. This was necessary to simulate a "worst-case" scenario where the concentration of pesticides was locally overdosed (long-term pesticide application) or an overestimated rate was applied.

After that, the soil samples were placed in 250 mL glass jars. Umbric Albeluvisols soil is rather poor (C_{org} = 1.5%); therefore, microbiological processes go on in it rather slowly, which can complicate monitoring the state of the microbial community. Because of this, we decided to include in the experiment variants with the initiation of succession due to the addition of an additional carbon source. Preliminary experiments with measuring carbon dioxide emissions showed that starch in an amount of 5 g/kg of air-dry soil was best suited for this purpose. Water-soluble cellulose, glucose, sucrose were also tested. Along with the starch, we also introduced mineral salts into the soil in order to avoid NPK limitation. Moreover, K_2HPO_4 and $(NH_4)_2SO_4$ were introduced in an amount of 1 g/kg. The design of the experiment was as follows in Table 3. Glass jars were placed in the thermostat at 20 °C.

Table 3. Experimental design.

Experiment Option	Individual Application of Pesticides		Mix of Pesticides Application	
	Substrate Induction	Sampling Time, Days	Substrate Induction	Sampling Time, Days
control	yes	14	yes	7, 14, 28, 56
control	no	14	no	7, 14, 28, 56
1-fold	yes	14	yes	7, 14, 28, 56
1-fold	no	14	no	7, 14, 28, 56
10-fold	yes	14	yes	7, 14, 28, 56
10-fold	no	14	no	7, 14, 28, 56

2.3. Sampling and DNA Extraction

Soil samples were extracted with a micro-drill from microcosms, so as to collect the soil layer completely to the very bottom. Then the soil samples collected in this way were placed in 1.5 mL test tubes and frozen.

Isolation of total DNA was carried out from 0.5 g samples of soil using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA).

2.4. Assessment of Pesticide's Residual Quantities

The analytical standards of active substances manufactured by Dr. Ehrenstorfer GmbH (Augsburg, Germany) was used for quantitative determination. Benomyl was not determined because it is extremely unstable [33] and almost immediately turns into carbendazim in soil. Absolute calibration with analytical standards was used for quantitation. The correlation coefficient was 0.999.

Residual quantities were measured using an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) system with DAD. Wavelength: carbendazim—280 nm, metribuzin—290 nm, imidacloprid—270 nm. Chromatographic column was Phenomenex Synergy Polar-RP 80A C18 150 × 4.6 mm, 4 µm. Eluents are water (A) and acetonitrile (B) with the addition of acetic acid (0.1%) in a gradient mode (0 min—20% «B»; 12 min—80% «B»). Extraction of pesticides was carried out from 5 g of soil with a mixture of acetonitrile and water (95:5) for 15 min in 50 mL centrifuge flacks on a vibration platform at 2400 rpm with an amplitude of 4 mm, followed by sonication in an ultrasonic bath for 5 min at 160 kHz. To increase the efficiency of extraction on the platform, three metal balls with a diameter of 5 mm were added to each tube. Successively the extracts were centrifuged for 5 min at 13,400× *g*, the supernatant was additionally filtered through a filter with a pore diameter of 0.45 µm. The extraction was performed twice and the extracts were combined and subjected to evaporation until dry on a rotary evaporator at 40 °C. The extract was dissolved with 1 mL of acetonitrile, treated for 30 s in an ultrasonic bath, and transferred into chromatographic vials. The analysis was carried out for three independent samples.

The soils samples were analyzed with the addition of analytical standards of active ingredients at the level of 0.01 and 0.1 mg/kg to determine the metrological characteristics of the method. The efficiency of extraction was for metribuzin—93 ± 1%, carbendazim—90 ± 1%, imidacloprid—91 ± 1%.

Moreover, some samples were analyzed using an Agilent 1200 series HPLC with a quadrupole time-of-flight mass spectrometric detector (6520 Accurate-Mass Q-TOF LC/MS, ionization source electrospray (Agilent Technologies, Santa Clara, CA, USA), column Phenomenex Hydro-RP C18 4 µm 4.6 × 100 mm, the mobile phase was water and methanol with the addition of formic acid (10 mM), the volume of the injected sample is 5 µL to search for metabolites.

2.5. Amplification and DNA Sequencing

Amplification of the variable region V4 (16S rRNA) was carried out in one round using forward and reverse primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R

(5′-GGACTACHVGGGTWTCTAAT-3′) [34] with dual index sample multiplexing. These primers are specific to both bacteria and archaea. Amplification of the variable region ITS1 (18S rRNA) was carried out using the following primers BITS (5′-CTACCTGCGGARGGAT CA-3′) and B58S3 (5′-GAGATCCRTTGYTRAAAGTT-3′) [33]. The PCR products were purified using the Cleanup Mini Kit (Evrogen, Moscow, Russia) for DNA isolation. The concentration of the obtained libraries of 16S rRNA and ITS1 in solution was 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 with agarose gel electrophoresis. Further samples 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).

2.6. Sequencing Data Processing

Bioinformatics analysis was carried in R version 3.6.3 (R Core Team, 2018) using the package DADA2 (Divisive Amplicon Denoising Algorithm). For fungal 18S rRNA gene sequencing, forward and reverse reads have been pre-merged at the initial step using MeFiT tool because of high diversity of the amplicon lengths. The derived RSV (Ribosomal Sequencing Variants) lengths were about 253 bp (with minimal variability) for bacterial 16S V4 fragments and 140 to 343 for fungal ITS1 (18S rRNA) regions.

In contrast to Operation Taxonomic Units (OTUs), the current analysis, based on RSVs (also referred to as Amplicon Sequence Variants (ASVs) or Exact Sequence Variants (ESVs)), does not imply merging of closely related amplicon variants (<3% differences) into a single consensus sequence (i.e., OTU), and, therefore, can distinguish single-nucleotide differences between species [35].

For taxonomic annotation of the derived RSV sequences, we also used the DECIPHER Bioconductor package supplied with SILVA v132 reference database [36] and the UNITE ITS database v. 8.0 [37]. RSVs annotated as chloroplasts, mitochondria, cercozoa, etc., have been removed. Raw data loaded in NCBI (BioProject ID PRJNA723173). The derived read counts data were normalized between the samples using the number of reads annotated at the Kingdom level.

The remaining analysis was also conducted in R environment (version 3.6.3). To assess alpha diversity, we calculated Shannon, Simpson, Chao1, and ACE indexes using fossil 0.4.0 and vegan 2.5–6 packages. When calculating these indices, the read counts data were rarefied to match the sample with the minimum number of reads. For beta diversity analysis, we used Bray–Curtis and Jaccard metrics. The remaining analyses and visualization were performed using phyloseq 1.30.0, plotly 4.9.2.2, phytools 0.7–47, pheatmap 1.0.12, ggplot2 3.3.3 packages. To visualize the differences between samples, metrical and non-metrical multidimensional scaling (MDS) was applied. The association analysis between period time and taxon abundances was performed using Spearman and Pearson correlations. For non-paired comparison of two groups, we used the Mann–Whitney U test, and for paired comparisons, we applied the Wilcoxon test. FDR was calculated using Benjamini–Hochberg adjustment for the derived *p*-values.

3. Results

3.1. Analysis of Pesticides Residual Amounts

Pesticides residues were analyzed on days 0, 7, 14, 28, and 56 [38]. The DT_{50} of the three pesticides were calculated using the first order single phase kinetic equation [39], (Table 4).

Table 4. Half-life ($t_{1/2)}$ of active substances with standard deviation, day (n = 3).

	Benomyl (Carbendazim)	Imidacloprid	Metribuzin
1-fold −SMM	86 ± 4	104 ± 7	41 ± 1
10-fold −SMM	52 ± 5	57 ± 3	20 ± 0.3
1-fold +SMM	78 ± 7	102 ± 8	37 ± 1
10-fold +SMM	50 ± 2	42 ± 2	20 ± 1

At a tenfold application rate, the pesticides were decomposed much faster, which was probably due to that they were not adsorbed and remained available to microorganisms. The addition of starch did not affect the rate of decomposition of the pesticides.

On the 56th day of the experiment, the residual amounts of pesticides were benomyl (by carbendazim)—1.57 mg/kg at a single (1×) dose rate and 12.51 mg/kg at a 10-fold (10×) dose rate, which corresponds to 63 and 50% of the first application; imidacloprid—0.057 mg/kg at 1× dose rate and 0.44 mg/kg at 10× dose rate, which corresponds to 69 and 53% of the application level; metribuzin—0.52 mg/kg at 1× dose rate and 3.65 mg/kg at 10× dose rate, which corresponds to 45 and 31% of the application level.

3.2. Alpha Diversity of Soil Prokaryotic and Fungal Communities

The study showed that pesticides have practically no effect on the alpha diversity of the soil bacterial community, in both situations, with the addition of a starch–mineral mixture (SMM) and without SMM (Table 5). There was a very small decrease in the Shannon and Chao1 indexes (by 10–15%), but it was not statistically significant (p = 0.1 . . . 0.2).

Table 5. Indices of alpha diversity of the bacterial community at the genus level in all variants of the experiment.

Treatment, and Sampling Time, Days	Bacteria −SMM		Bacteria +SMM	
	Shannon	Chao1	Shannon	Chao1
Control 7 d	3.17	237.5	2.98	178.8
Mix 1–7 d	2.98	224.6	2.04	98.0
Mix 10–7 d	3.35	229.1	2.24	147.8
control 14 d	3.19	164.5	2.45	140.1
mix 1–14 d	2.99	180.1	2.67	159.0
mix 10–14 d	3.12	152.4	2.28	136.6
control 28 d	3.36	169.3	2.97	195.0
mix 1–28 d	3.16	136.8	2.58	145.4
mix 10–28 d	3.31	178.5	2.61	214.3
control 56 d	3.63	172.6	3.08	222.6
mix 1–56 d	3.38	135.7	3.03	194.6
mix 10–56 d	3.40	162.0	2.39	207.9
control 14 d	3.15	170.2	3.10	180.1
imidacloprid 1–14 d	2.90	119.5	2.98	143.4
imidacloprid 10–14 d	-	-	2.96	145.3
Benomyl 1–14 d	3.05	100.0	2.80	151.6
benomyl 10–14 d	2.93	111.1	2.52	141.4
metribuzin 1–14 d	2.91	103.0	3.10	177.6
metribuzin 10–14 d	-	-	2.97	149.0

However, the application of the SMM itself resulted in a decrease in alpha diversity—at genus level, and the average Shannon index decreased from 3.1 to 2.04 (p-value < 0.001 for

Welch's *t*-test, Mann–Whitney U test, paired Wilcoxon test) (Table 5). The same observation was made at RSV level too ($p < 0.01$ for all these tests).

By the 56th day, the alpha diversity in both variants (with and without SMM) of the experiment increased according to the Shannon index, but decreased according to the Chao index. Most likely, this indicates that the composition of the bacterial community became more balanced over time (more taxa began to have a significant proportion in the bacterial community), but many minor taxa that were originally present in the soil die with time. This trend was observed both for control samples and for samples treated with pesticides. At the same time, in general, temporary succession made a significantly greater contribution to the structure of bacterial communities than treatment with pesticides; the addition of SMM produced an even greater effect. It should be noted that alpha diversity was more affected by pesticides when we added the starch–mineral mixture. However, these observations are only trends; no statistically significant differences were noticed here. Surprisingly, we also noticed a strong positive correlation between the rarefied Chao1 and ACE, Jackknife indices and initial read counts derived for samples.

The analysis of alpha diversity indices for soil fungal communities showed that the addition of SMM led to a decrease in diversity at genus level. For instance, at the genus level, the average value of the Shannon index decreases from 2.07 to 1.15 ($p < 0.001$ for Welch's, Mann–Whitney and Wilcoxon tests) (Table 6). The same is applied for Chao1 index (decrease from 52 to 33 at genus level; $p < 0.01$ for all listed tests) (Table 6). These differences were also observed at RSV level and were also statistically significant.

Table 6. Indices of the alpha diversity of the fungal community at the genus level in all variants of the experiment.

Treatment and Sampling Time, Days	Fungi −SMM		Fungi +SMM	
	Shannon	Chao1	Shannon	Chao1
control 7 d	2.60	63.0	1.43	20.0
mix 1–7 d	1.58	31.0	1.80	36.0
mix 10–7 d	2.05	59.0	1.80	77.8
control 14 d	1.68	46.0	0.33	15.0
mix 1–14 d	2.55	59.0	0.87	28.5
mix 10–14 d	2.08	66.3	1.88	81.8
control 28 d	1.98	60.0	0.20	16.0
mix 1–28 d	2.43	52.0	1.44	30.0
mix 10–28 d	2.12	50.0	1.58	43.5
control 56 d	2.47	58.0	0.35	13.0
mix 1–56 d	1.55	38.0	0.51	15.3
mix 10–56 d	2.00	47.3	1.23	13.0
control 14 d	2.30	53.0	0.98	30.0
benomyl 1–14 d	1.55	40.0	0.68	19.0
benomyl 10–14 d	2.12	68.3	2.22	59.8

At the same time, in the variant with SMM, treatment with pesticides led to increase in diversity from 0.20 to 1.88 according to the Shannon index compared to the control variants (Table 6). The application of pesticides (at 10× concentrations; both benomyl and a mixture of three pesticides containing benomyl) was accompanied by dramatic shifts in the composition of the fungal community, the elimination of dominant species, and, as a consequence, a striking increase in the proportion of many other taxa and the resulting increase in diversity according to the Shannon index. Generally, the SMM was causing an avalanche-like restructuring of the entire fungi community. For example, *Humicola*, which was actively growing on starch and accounted for 60% of the total fungal community (without SMM—only 5%), was up to 300 times reduced under treatment with benomyl. The effect of pesticides (partially, benomyl) was less noticeable in the variants without SMM.

3.3. Taxonomic Structure of Soil Bacterial and Fungal Communities

The addition of SMM led to a change in dominants and a restructuring of the bacterial community. For the control, the dominants were the phyla Proteobacteria, Actinobacteria and Firmicutes (Figure 1) and the genera *Thermomonas*, *Sphingomonas*, and *Pseudoarhtrobacter* (Figure 2).

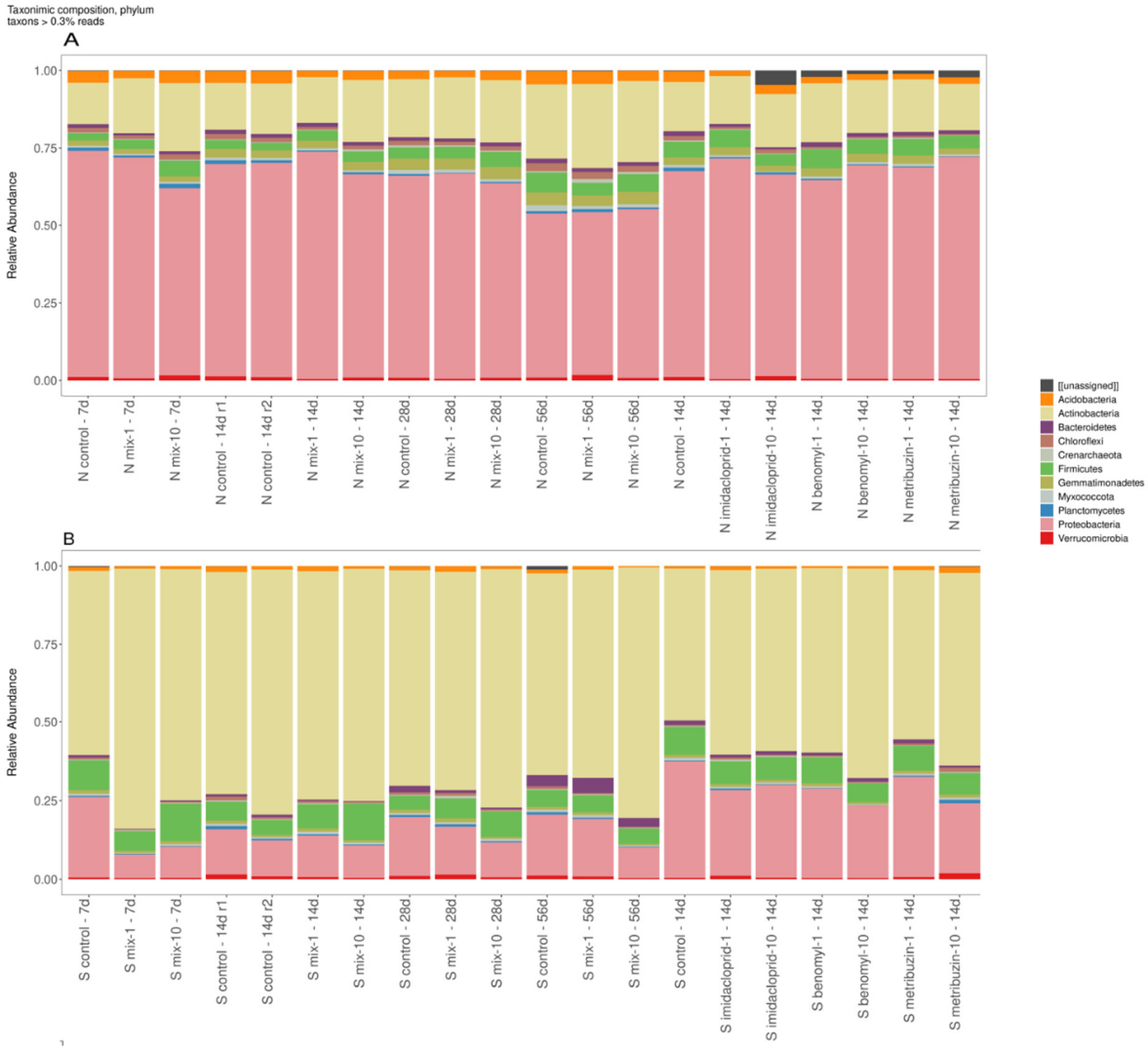

Figure 1. Bacteria's phyla in all variants of the experiment without SMM (**A**) and with SMM (**B**).

After the application of SMM, the representation of Actinobacteria sharply increased while the representation of Proteobacteria decreased (Figure 1).

The genus *Streptomyces* sharply increased in the variant with SMM, while genus *Thermomonas* decreased in the variant with SMM (Figure 2).

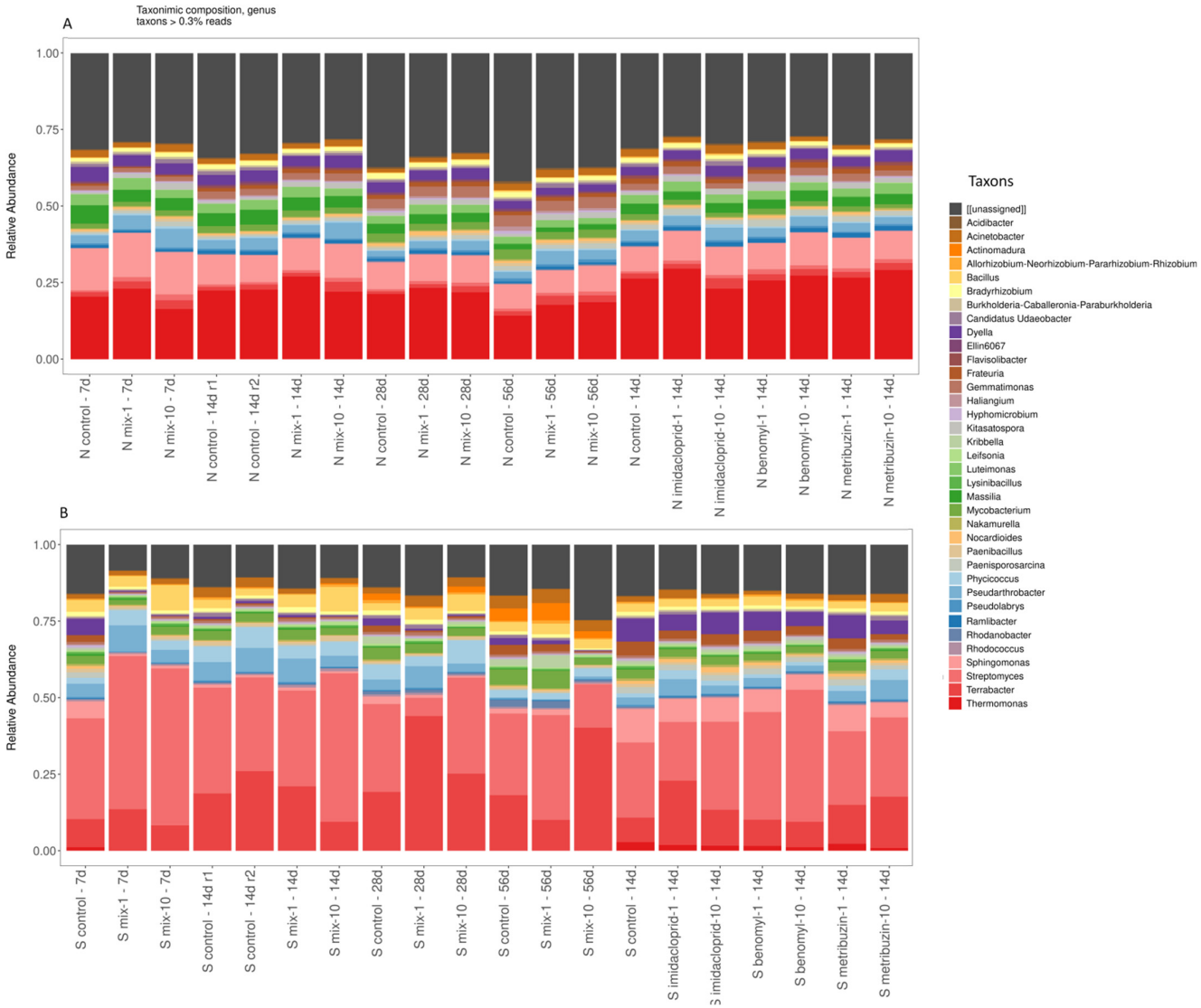

Figure 2. Bacteria's genus in all variants of the experiments without SMM (**A**) and with SMM (**B**).

The observed changes were expected since the proportion of bacteria actively decomposing complex substrates increased. The application of pesticides led to a change in the dominants of the bacterial community both in the control and in the soil supplemented with SMM.

A pairwise comparison using the Wilcoxon test showed an impact of the addition of pesticides on the abundance of several bacterial phyla ($p \leq 0.05$). The pesticide application with SMM led to a reduction in the relative abundance of bacterial phyla Myxococcota, Bacteroidetes, Gemmatimonadetes, Proteobacteria. The relative abundance of Acidobacteria, Chloroflexi, and Planctomycetes was also decreased in the variant without SMM. The phylum Actinobacteria increased both in with and without SMM addition, which was associated with the high hydrolytic activity of this bacteria (Table 7).

Treatment with pesticides (at 1× and 10× concentrations) affected 29 genera in the variant without SMM and 28 genera in the variant with SMM; among them, *Catenulispora*, *Sphingomonas*, *Terrabacter*, *Haliangium*, *Bradyrhizobium*, *Burkholderia*, and *Mycobacterium* were affected by pesticides in both variants (Table 8).

Table 7. Pairwise comparison of abundances of various bacterial phyla between control and treated soil samples.

Phylum	Reads (Average)	Mean LogFC	Wilcoxon p	Log2(Fold Change)													
				Mix1_CTRL 7 d	Mix10_CTRL 7 d	Mix1_CTRL 14 d	Mix10_CTRL 14 d	Mix1_CTRL 28 d	Mix10_CTRL 28 d	Mix1_CTRL 56 d	Mix10_CTRL 56 d	Imi1_CTRL 14 d	Imi10_CTRL 14 d	Ben1_CTRL14 d	Ben10_CTRL 14 d	Met1_CTRL 14 d	Met10_CTRL 14 d
Without SMM																	
Acidobacteria	908	−0.54	0.0009	−0.65	0.05	−0.98	−0.41	−0.42	0.15	−0.06	−0.40	−0.96	−0.22	−0.75	−0.85	−0.93	−0.69
Actinobacteria	5970	0.14	0.002	0.41	0.72	−0.08	0.35	0.08	0.11	0.18	0.13	−0.05	0.11	0.26	0.10	0.09	−0.09
Bacteroidetes	380	−0.27	0.0009	−0.69	−0.31	0.01	−0.10	−0.13	0.08	−0.29	−0.29	−0.57	−1.24	−0.04	−0.26	−0.26	−0.39
Chloroflexi	407	−0.59	0.0009	−0.41	0.20	−0.89	−0.39	−0.31	−0.03	−0.09	−0.35	−1.03	−0.12	−1.17	−1.46	−1.11	−1.51
Myxococcota	270	−0.38	0.0006	−0.07	0.16	−0.18	−0.46	−0.34	−0.58	−0.90	−0.75	−0.43	−1.79	−0.18	−0.24	−0.11	−0.52
Planctomycetes	201	−1.03	0.004	−0.83	0.45	−1.35	−0.63	−1.44	−0.49	0.39	−0.68	−1.38	−0.50	−1.21	−1.82	−1.80	−2.61
Verrucomicrobia	297	−0.33	0.09	−0.61	0.48	−1.31	−0.33	−0.63	−0.01	0.82	−0.20	−1.33	0.33	−1.05	−1.07	−1.10	−1.23
With SMM																	
Actinobacteria	21,392	0.22	0.0009	0.50	0.32	−0.03	−0.01	0.02	0.15	0.05	0.31	0.28	0.26	0.28	0.46	0.16	0.34
Bacteroidetes	423	−0.82	0.01	−2.32	−1.03	−0.82	−1.74	−1.19	−1.82	0.42	−0.34	−0.66	−0.33	−0.61	−0.32	−0.18	−1.14
Gemmatimonadetes	276	−0.23	0.04	−0.70	−0.23	0.16	−0.28	0.29	−0.64	0.05	−0.82	−0.11	−0.37	−0.10	−0.57	−0.02	0.00
Myxococcota	209	−0.36	0.02	−1.03	−0.23	0.47	0.16	0.02	0.06	−0.27	−1.06	−0.68	−0.38	−0.32	−1.00	−0.38	−0.37
Proteobacteria	6242	−0.51	0.0002	−1.76	−1.36	0.04	−0.31	−0.30	−0.75	−0.07	−0.97	−0.44	−0.33	−0.38	−0.69	−0.22	−0.73

Explanation: Mix—the mixture of three pesticides; 1—the recommended application rate; 10—the ten-fold application rate; CTRL—the control; 7, 14, 28, 56 days—sampling time; Imi, Ben, Met—imidacloprid, benomyl, metribuzin; SMM—the starch–mineral mixture. Abundances of microbial phyla with positive scores are marked with red color, abundances with negative scores—with blue color. The degree of difference is shown by the brightness of the color.

Table 8. Pairwise comparison of abundances of various bacterial genera between control and treated soil sample.

Genus	Reads (Average)	Mean LogFC	Wilcoxon p	Log2(Fold Change)													
				Mix1_CTRL 7d	Mix10_CTRL 7 d	Mix1_CTRL 14d	Mix10_CTRL 14d	Mix1_ CTRL 28d	Mix10_CTRL 28d	Mix1_CTRL 56d	Mix10_CTRL 56d	Imi1_CTRL 14d	Imi10_CTRL 14d	Ben1_CTRL14 d	Ben10_CTRL 14 d	Met1_CTRL 14 d	Met10_CTRL 14 d
Without SMM																	
Actinoplanes	31	−0.22	0.05	−0.39	−0.57	0.15	−0.30	−0.01	0.12	0.00	−0.08	−0.06			−0.70	−0.68	
Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium	28	−0.72	0.02	0.06	0.03	−0.20	−0.03	0.22	−1.02			−1.37	−0.65	−3.45	−1.43	−1.31	−1.28
Arenimonas	40	−0.58	0.0001	−0.55	−0.59	−0.47	−0.93	−0.68	−1.34	−0.26	−0.49	−0.42	−0.52	−1.35	−0.40	−0.14	−0.75
Bradyrhizobium	383	−0.19	0.004	−0.03	0.17	−0.28	−0.21	−0.31	−0.23	−0.50	−0.40	0.07	−0.19	−0.19	−0.15	0.00	−0.28
Bryobacter	102	−0.17	0.01	−0.32	−0.07	−0.17	−0.18	−0.23	−0.06	−0.21	0.05	0.31	−0.42	−0.61	−0.19	−0.17	−0.09
Burkholderia–Caballeronia–Paraburkholderia	185	0.20	0.009	−0.11	0.05	0.03	0.16	0.41	0.31	−0.14	0.51	0.04	0.49	0.43	0.41	0.07	0.13
Candidatus Solibacter	20	−0.59	0.03	−0.52	−0.58	−0.51	−0.81								−0.64		−0.46
Catenulispora	43	0.45	0.0007	0.52	0.50	0.23	0.08	0.55	0.39	0.38	0.24	−0.29	0.42	1.33	0.90	0.68	
Cohnella	20	−0.45	0.03		0.48			−0.71	−0.59	−1.07	−0.61	−0.32		−0.41	−0.40	−0.39	−0.15
Ellin6067	126	−0.30	0.02	−0.44	−0.60	−0.50	−0.64	−0.14	−0.23	−0.45	−0.75	0.26	0.36	−0.38	0.08	−0.27	−0.04
Gaiella	50	−0.31	0.003	−0.04	0.16	−0.03	−0.35	−0.16	−0.17	−0.74	−0.77	−0.02	−2.82	−0.21	−2.82	−0.25	−0.33
Haliangium	132	−0.40	0.002	−0.20	−0.10	−0.10	−0.61	−0.12	−0.93	−0.90	−0.97	−0.36	−4.08	−0.40	−0.13	0.24	−0.23
Hyphomicrobium	126	−0.36	0.02	−0.44	−0.21	−0.05	−0.04	−0.29	−0.55	−1.16	−0.53	0.40	−3.92	−0.20	−0.45	−0.84	0.68
Janthinobacterium	19	−0.81	0.03	−1.47	−1.25	−0.30		−0.55	−0.86			−0.42					
Kitasatospora	464	1.03	0.0001	1.11	1.73	1.20	1.35	0.63	0.70	1.09	1.03	0.93	1.03	1.40	0.90	1.01	0.63
Marmoricola	45	0.91	0.01	0.64	1.12	1.30	0.89	0.07	−0.94		1.12		1.36	1.30	1.23		0.54
Massilia	953	−0.12	0.02	−0.59	−0.61	−0.04	−0.26	0.01	−0.09	−0.21	0.03	−0.34	0.00	−0.20	0.07	−0.12	0.07
Mycobacterium	570	−0.22	0.001	−0.13	0.12	0.02	−0.11	−0.34	−0.21	−0.45	−0.32	−0.21	−0.43	−0.01	−0.28	−0.12	−0.58
Occallatibacter	39	−0.73	0.005	−0.43	0.13	−1.57	−0.49	−0.72	−0.17	−0.59	−0.62	−1.03	0.70	−1.73	−1.07	−0.61	−1.74

Table 8. *Cont.*

Genus	Reads (Average)	Mean LogFC	Wilcoxon p	Log2(Fold Change)													
				Mix1_CTRL 7d	Mix10_CTRL 7 d	Mix1_CTRL 14d	Mix10_CTRL 14d	Mix1_ CTRL 28d	Mix10_CTRL 28d	Mix1_CTRL 56d	Mix10_CTRL 56d	Imi1_CTRL 14d	Imi10_CTRL 14d	Ben1_CTRL14 d	Ben10_CTRL 14 d	Met1_CTRL 14 d	Met10_CTRL 14 d
Paenisporosarcina	393	0.35	0.002	0.54	1.31	0.51	0.61	0.19	0.61	−0.22	0.20	0.40	−0.08	0.54	0.27	0.23	0.04
Phycicoccus	154	0.39	0.01	0.67	0.97	−0.40	0.34	0.32	0.74	0.72	0.14	−0.38	1.38	0.47	0.03	0.14	0.30
Ramlibacter	289	0.25	0.02	−0.51	−0.34	−0.01	0.02	0.52	0.11	0.51	0.10	0.11	0.42	0.41	0.34	0.50	0.50
Sphingomonas	2893	0.16	0.001	0.10	0.00	0.17	0.27	0.01	0.02	−0.09	0.09	0.30	0.28	0.13	0.46	0.36	0.26
Streptacidiphilus	55	0.65	0.0005	0.30	0.79	0.26	0.54	0.79	0.25	0.69	0.96	0.83		1.22	0.84	0.47	
Streptomyces	325	0.74	0.0001	1.33	1.69	0.34	0.91	0.53	0.76	0.13	0.58	0.65	0.55	1.14	0.75	0.72	0.77
Terrabacter	592	0.62	0.0009	0.67	0.92	−0.34	1.12	0.51	1.20	1.03	0.58	−0.10	1.09	0.31	0.35	0.19	0.54
Terracidiphilus	48	−0.52	0.05	−0.77	−0.17	−0.96	0.08	−0.28	0.53	0.61	−0.20	−1.38	−3.53	−0.32	−1.22	−3.53	0.06
Thermomonas	6779	0.12	0.05	0.19	−0.33	0.29	0.00	0.16	0.06	0.35	0.42	0.20	−0.12	−0.02	0.09	0.06	0.20
Xylophilus	31	0.17	0.01	0.27	0.30	0.43	0.06	0.12	−0.06			0.19		0.29	−0.02	0.13	
With SMM																	
Aminobacter	58	−0.33	0.05	−1.39	−0.87	−0.10	−0.25	0.73	−0.45	0.16	0.01	−0.48	−0.47	−0.28	−0.01	−0.46	−0.81
Arachidicoccus	17	−2.78	0.008					−0.08		−1.06	−4.13	−3.34	−1.71	−3.34	−3.34	−3.34	−3.34
Bradyrhizobium	318	−0.28	0.02	−0.78	−0.50	0.63	−0.14	0.10	−0.89	0.07	−0.88	−0.23	−0.17	−0.09	−0.60	−0.15	−0.25
Bryobacter	32	−0.50	0.01	−0.90	−1.03	0.62	−0.45	0.24	−0.43	−0.26	−1.53	−0.91	−0.07	−0.65	−1.90	−0.22	−0.08
Burkholderia–Caballeronia–Paraburkholderia	109	−0.31	0.02	−0.74	−0.79	0.26	−0.61	−0.19	−1.00	0.07	−1.72	0.02	−0.24	0.18	−0.05	−0.08	−0.44
Catenulispora	25	−0.55	0.04				0.84		−0.01			−1.46	−0.34	−0.59	−0.72	−0.44	−1.17
Conexibacter	56	−0.29	0.03	−0.24	−0.23	−0.12	−0.56	−0.39	−0.40	−0.58	−1.30	−0.28	0.03	−0.13	−0.64	0.26	0.51
Devosia	56	−0.55	0.01	−1.02	−0.99	0.08	−0.21	−1.29	−0.73	0.59	−0.84	−0.59	−0.32	−0.26	−0.24	−0.26	−1.23
Dyella	862	−1.35	0.0001	−4.42	−4.79	−0.08	−1.56	−2.15	−2.79	−0.92	−3.78	−0.51	−0.10	−0.28	−0.67	0.00	−0.76
Edaphobacter	15	0.51	0.03							1.78		0.41	0.17	0.06	0.36	0.31	
Frateuria	504	−1.31	0.001	−6.47	−6.47	−0.03	−1.47	−2.01	−1.96	−0.48	−3.50	−0.74	−0.43	−0.41	−0.72	−0.41	−1.34
Gemmatimonas	191	−0.21	0.05	−0.91	−0.21	0.24	−0.27	0.36	−0.54	−0.03	−0.70	−0.11	−0.43	−0.03	−0.50	−0.07	0.04
Hyphomicrobium	93	−0.46	0.02	−1.06	−0.88	0.28	−0.11	0.52	−0.22	−0.62	−1.00	−0.95	−0.32	−0.39	−1.07	0.17	−0.24
Janibacter	30	0.84	0.02	3.37		−0.05	−0.31	0.76	0.51			1.97	0.86			0.86	0.98
Jatrophihabitans	63	−0.93	0.002	−3.74	−0.59	0.18		−3.61	−1.04	−0.53	−2.12	−0.54	0.08	−0.72	−0.43	−0.02	−0.71
Kribbella	246	−0.97	0.02	−2.32	−3.47	−0.99	−0.62	−6.59	−1.41	0.90	−2.41	−0.67	−0.10	0.10	0.08	−0.38	−0.90
Leifsonia	171	0.22	0.02	0.19	0.54	0.24	0.88	0.18	0.20	0.21	0.96	0.08	0.22	0.00	−0.16	0.29	−0.46
Luteimonas	33	−0.48	0.006	−1.12	−0.09	0.12		−0.60	−1.31	−0.84	−1.98	−0.55	0.06	−0.40	−0.34	0.13	−0.18

Table 8. *Cont.*

Genus	Reads (Average)	Mean LogFC	Wilcoxon p	Log2(Fold Change)													
				Mix1_CTRL 7d	Mix10_CTRL 7 d	Mix1_CTRL 14d	Mix10_CTRL 14d	Mix1_ CTRL 28d	Mix10_CTRL 28d	Mix1_CTRL 56d	Mix10_CTRL 56d	Imi1_CTRL 14d	Imi10_CTRL 14d	Ben1_CTRL14 d	Ben10_CTRL 14 d	Met1_CTRL 14 d	Met10_CTRL 14 d
Marmoricola	33	−0.62	0.05	−3.44	−0.10	−0.70		−0.39				−0.45	0.02	0.37	−2.12	−0.34	−0.86
Mycobacterium	841	−0.29	0.009	−0.75	−0.08	0.19	−0.28	−0.33	−0.61	0.17	−1.16	−0.24	−0.12	−0.42	−0.47	−0.06	−0.29
Nakamurella	154	−0.42	0.02	−0.79	−0.73	0.63	−0.42	−0.70	−0.99	−0.40	−1.24	−0.04	−0.03	−0.27	−0.48	0.30	−0.32
Ochrobactrum	49	0.50	0.05	0.36	0.11	−0.80	0.81	0.01	0.69	1.04	1.57						
Paenarthrobacter	62	0.44	0.05	1.47	0.89	0.55	−0.10	0.95	0.85	−0.90	−0.85	0.74	0.36	0.25	−0.24	0.17	1.36
Paenisporosarcina	278	−0.28	0.01	−1.92	−1.69	0.71	−0.11	0.03	−0.64	−0.54	0.23	−0.16	−0.22	−0.09	−0.36	−0.03	−0.67
Pseudolabrys	102	−0.24	0.05	−0.97	−0.37	0.30	−0.36	0.49	−0.35	−0.22	−0.89	−0.12	−0.12	−0.10	−0.49	−0.18	−0.12
Ramlibacter	38	−0.25	0.05	−2.88	−0.19	0.54	−0.01	0.38	−0.52			−0.02	−0.32	−0.62	−0.64	−0.38	−0.14
Sphingomonas	1073	−1.08	0.0006	−2.92	−2.62	0.22	−0.40	−1.37	−2.12	0.18	−2.61	−0.52	−0.48	−0.57	−1.12	−0.38	−1.19
Thermomonas	228	−0.64	0.004	−2.54	−2.72	0.22	−0.18	0.22	−0.53	−0.16	−0.26	−0.55	−0.77	−0.81	−1.27	−0.30	−1.61

Explanation: Mix—the mixture of three pesticides; 1—the recommended application rate; 10—the ten-fold application rate; CTRL—the control; 7, 14, 28, 56 days—sampling time; Imi, Ben, Met—imidacloprid, benomyl, metribuzin; SMM—the starch–mineral mixture; Abundances of microbial genera with positive scores are marked with red color, abundances with negative scores—with blue color. The degree of difference is shown by the brightness of the color.

Note that the genus *Terrabacter* increased while *Haliangium*, *Bradyrhizobium*, and *Mycobacterium* decreased in both situations, with and without SMM addition. The genus *Sphingomonas* slightly increased in the variant without SMM; after the addition of SMM, its share decreased by about 1.5 times.

The addition of SMM led to a radical restructuring of the soil fungi community. Mucoromycota practically disappeared while the share of Ascomycota, on the contrary, increased (Figure 3).

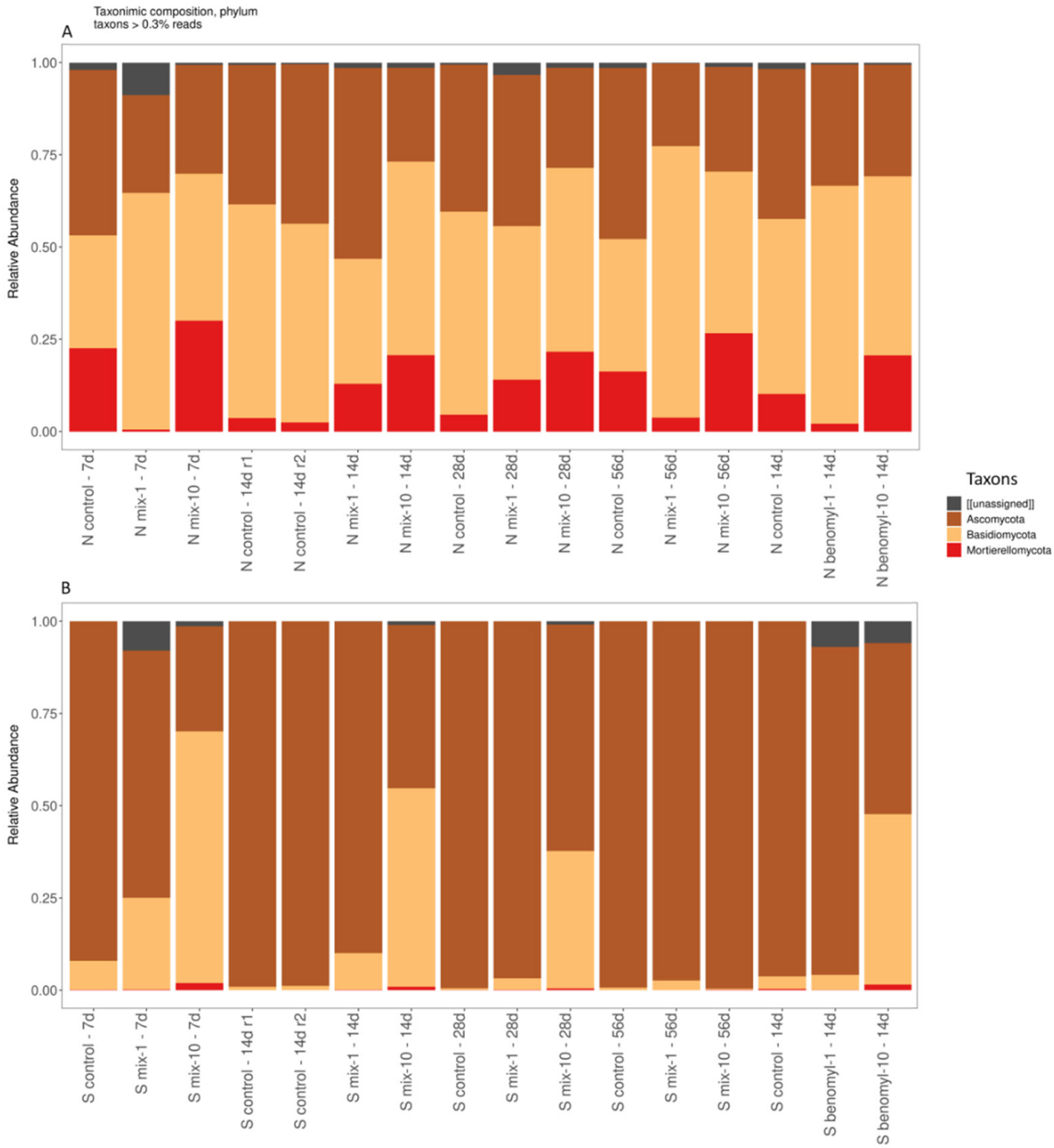

Figure 3. Fungi's phylum in all variants of the experiments without SMM (**A**) and with SMM (**B**).

In the control, the dominants were the genera *Mortierella*, *Saitozyma*, *Apiotrichum*, *Humicola*, *Solicoccozyma*, *Fusarium*, and *Trichoderma* (Figure 4).

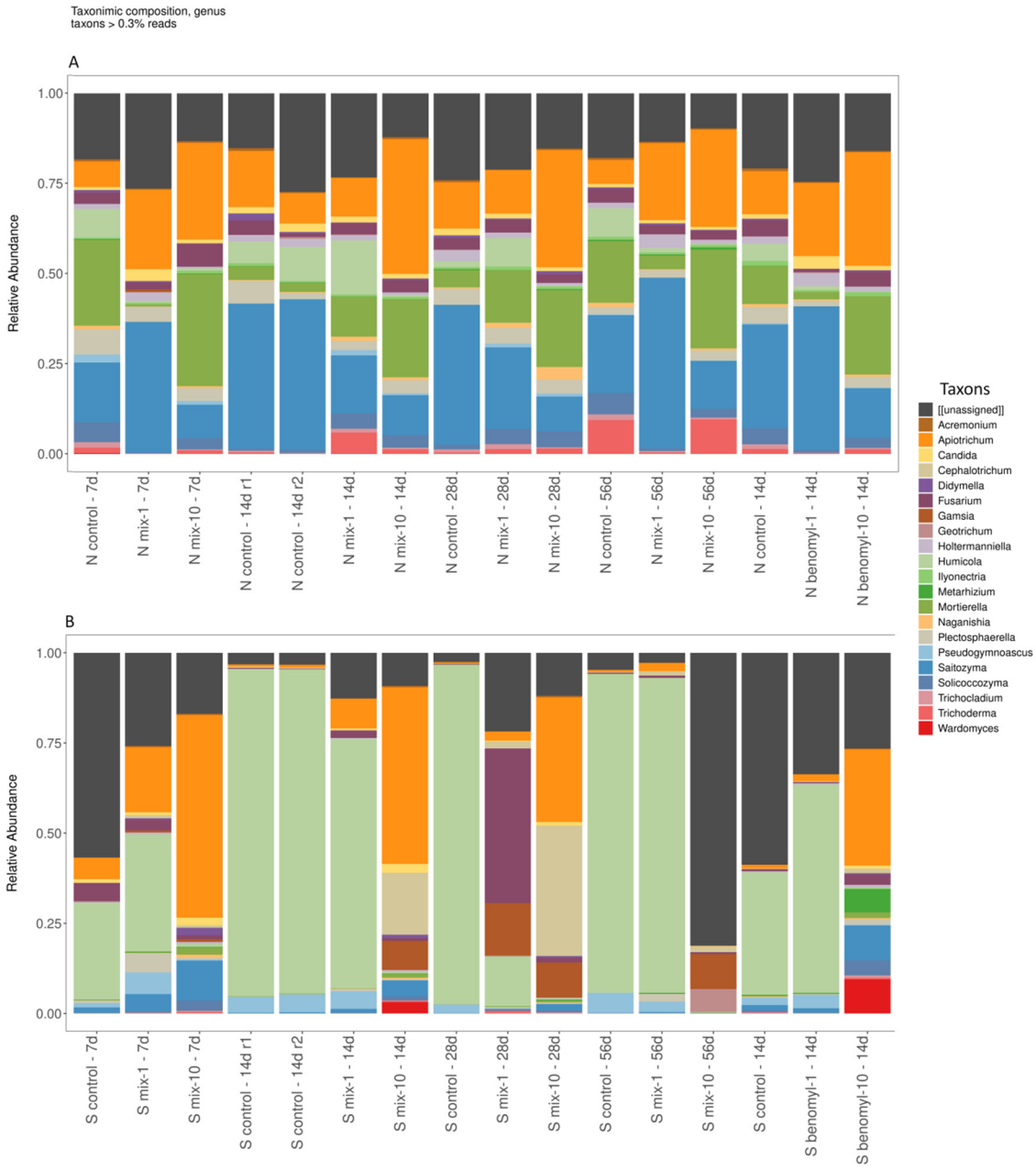

Figure 4. Fungi's genus in all variants of the experiments without SMM (**A**) and with SMM (**B**).

After the application of SMM, the share of *Humicola* in the soil significantly increased with a sharp decline in the representation of *Mortierella*, *Saitozyma*, and *Solicoccozyma*. The genus *Trichoderma* completely disappeared (Figure 4). The dominants of the fungal community turned out to be susceptible to the influence of pesticides, in particular, benomyl, especially a 10× dose rate (Figure 4).

Treatment with pesticides led to an increase in the relative abundance of Basidiomycota with a decrease in the share of Ascomycota, which was confirmed by paired samples Wilcoxon test ($p \leq 0.05$; Table 9).

The results of this test have shown that the effect of pesticides on the fungal community was significantly higher with SMM at the phylum level.

A pairwise comparison using the Wilcoxon test ($p \leq 0.05$) showed that exposure to pesticides in the variant without SMM affected the genus *Apiotrichum* (Table 10).

Table 9. Paired comparison of abundances of various fungal phyla between control and treated soil samples.

Phylum	Reads (Average)	Mean LogFC	Wilcoxon p	Log2(Fold Change)									
				Mix1_ CTRL 7 d	Mix10_ CTRL 7 d	Mix1_ CTRL 14 d	Mix10_ CTRL 14 d	Mix1_ CTRL 28 d	Mix10_ CTRL 28 d	Mix1_ CTRL 56 d	Mix10_ CTRL 56 d	Ben1_ CTRL14 d	Ben10_ CTRL 14 d
Without SMM													
Ascomycota	11,935	−0.49	0.02	−0.73	−0.59	0.34	−0.67	0.04	−0.55	−1.05	−0.71	−0.31	−0.43
With SMM													
Ascomycota	27,377	−0.46	0.004	−0.46	−1.70	−0.14	−1.16	−0.04	−0.70	−0.03	0.00	−0.11	−1.06
Basidiomycota	5978	2.77	0.004	1.67	3.12	3.22	5.66	2.53	6.12	1.90	−1.26	0.27	3.77
Mucoromycota	136	2.09	0.05	0.38	3.64	1.93	4.56	1.37	3.16			−1.94	2.05

Explanation: Mix—the mixture of three pesticides; 1—the recommended application rate; 10—the ten-fold application rate; CTRL—the control; 7, 14, 28, 56 days—sampling time; Ben—benomyl; SMM—the starch–mineral mixture; Abundances of microbial phyla with positive scores are marked with red color, abundances with negative scores—with blue color. The degree of difference is shown by the brightness of the color.

Table 10. Paired comparison of abundances of various fungal genera between control and treated soil samples.

Genus	Reads (Average)	Mean LogFC	Wilcoxon p	Log2(Fold Change)									
				Mix1_CTRL 7d	Mix10_CTRL 7 d	Mix1_CTRL 14d	Mix10_CTRL 14d	Mix1_CTRL 28d	Mix10_CTRL 28d	Mix1_CTRL 56d	Mix10_CTRL 56d	Ben1_CTRL14 d	Ben10_CTRL 14 d
Without SMM													
Acremonium	139	−0.87	0.002	−0.67	−0.33	−1.03	−0.06	−1.71	−0.62	−0.90	−0.81	−1.21	−1.36
Apiotrichum	6385	1.30	0.010	1.73	1.96	−0.22	1.59	−0.10	1.34	1.74	2.05	0.79	1.35
Didymella	150	−1.02	0.04	−0.81	−1.96	−2.51	−1.31	−1.19	0.36	0.44	−0.77	−4.43	0.02
Gibberella	23	−2.20	0.05	−0.11	−3.21	−3.17	−3.17			−1.33	−3.67		2.77
Plectosphaerella	1171	−0.40	0.03	−0.62	−0.89	−0.69	−0.18	−0.05	−0.23	0.11	0.48	−1.51	−0.64
Trichocladium	232	−1.29	0.05	−3.10	−2.23	1.53	0.37	0.81	−1.20	−1.95	−1.53	−2.96	−1.65

Table 10. *Cont.*

Genus	Reads (Average)	Mean LogFC	Wilcoxon p	Log2(Fold Change)									
				Mix1_CTRL 7d	Mix10_CTRL 7 d	Mix1_CTRL 14d	Mix10_CTRL 14d	Mix1_CTRL 28d	Mix10_CTRL 28d	Mix1_CTRL 56d	Mix10_CTRL 56d	Ben1_CTRL14 d	Ben10_CTRL 14 d
With SMM													
Acremonium	49	3.15	0.02	3.98	4.17	2.26	3.15	2.38	3.70	−0.75			3.43
Apiotrichum	4627	3.11	0.004	1.60	3.15	3.54	6.04	2.62	6.38	2.07	−1.83	0.91	4.95
Candida	204	1.48	0.05	−0.51	0.87	2.10	4.12	1.71	3.73	0.25		−0.85	2.19
Cephalotrichum	1346	4.80	0.006	5.44	5.09	−3.53	6.09	6.55	10.74	2.95	3.50	2.92	5.89
Didymella	93	3.57	0.03	2.33	4.50	2.81	5.40		4.46				1.94
Fusicolla	82	3.61	0.02	2.86	5.17	3.48	4.04	4.73	5.50			−3.33	1.37
Gamsia	961	7.60	0.03	4.83	5.06		8.54	9.45	8.85		8.88		
Holtermanniella	77	1.78	0.02	1.37	1.29	2.14	2.73		3.47			0.66	1.38
Humicola	13,471	−3.86	0.03	0.27	−5.24	−0.39	−7.64	−2.79	−8.08	−0.01	−8.96	0.77	−6.99
Metarhizium	183	1.99	0.02	1.37	0.95		0.92		2.76	3.93		0.32	4.70
Mortierella	134	2.08	0.05	0.38	3.61	1.93	4.51	1.37	3.16			−1.94	2.02
Plectosphaerella	274	1.90	0.02	2.91	−0.53	2.32	1.24	2.30	2.01	6.71		0.12	2.43
Saitozyma	828	2.05	0.01	1.74	2.83	1.95	3.96	2.08	4.35	1.20	−0.02	−0.22	2.67
Solicoccozyma	224	1.94	0.04	0.31	3.77	1.93	4.19	1.60	3.17	0.00		−1.80	2.81

Explanation: Mix—the mixture of three pesticides; 1—the recommended application rate; 10—the ten-fold application rate; CTRL—the control; 7, 14, 28, 56 days—sampling time; Ben—benomyl; SMM—the starch–mineral mixture; Abundances of microbial genera with positive scores are marked with red color, abundances with negative scores—with blue color. The degree of difference is shown by the brightness of the color.

In the variant with SMM, exposure to pesticides affected the genus *Solicoccozyma*. The genera *Acremonium*, *Apiotrichum*, *Plectosphaerella*, and *Didymella* were affected by pesticides in both variants (Table 10).

At the same time, 10× dose rate of a mixture of three pesticides resulted in an increase in the relative abundance of the fungal genus *Gamsia*. Thus, the effect of pesticides under study on the fungal community was significantly higher than on the bacterial one and was manifested already at the phylum level, affecting the taxonomic structure of the entire community as a whole.

Multidimensional scaling (MDS) analysis performed at various taxonomic levels for the fungal community showed that samples with 10× dose rate of pesticides (both benomyl and a mixture) formed a distinct cluster and had consistent differences in the structure of microbial community from other samples (control and with 1× dose rate) (Figure 5).

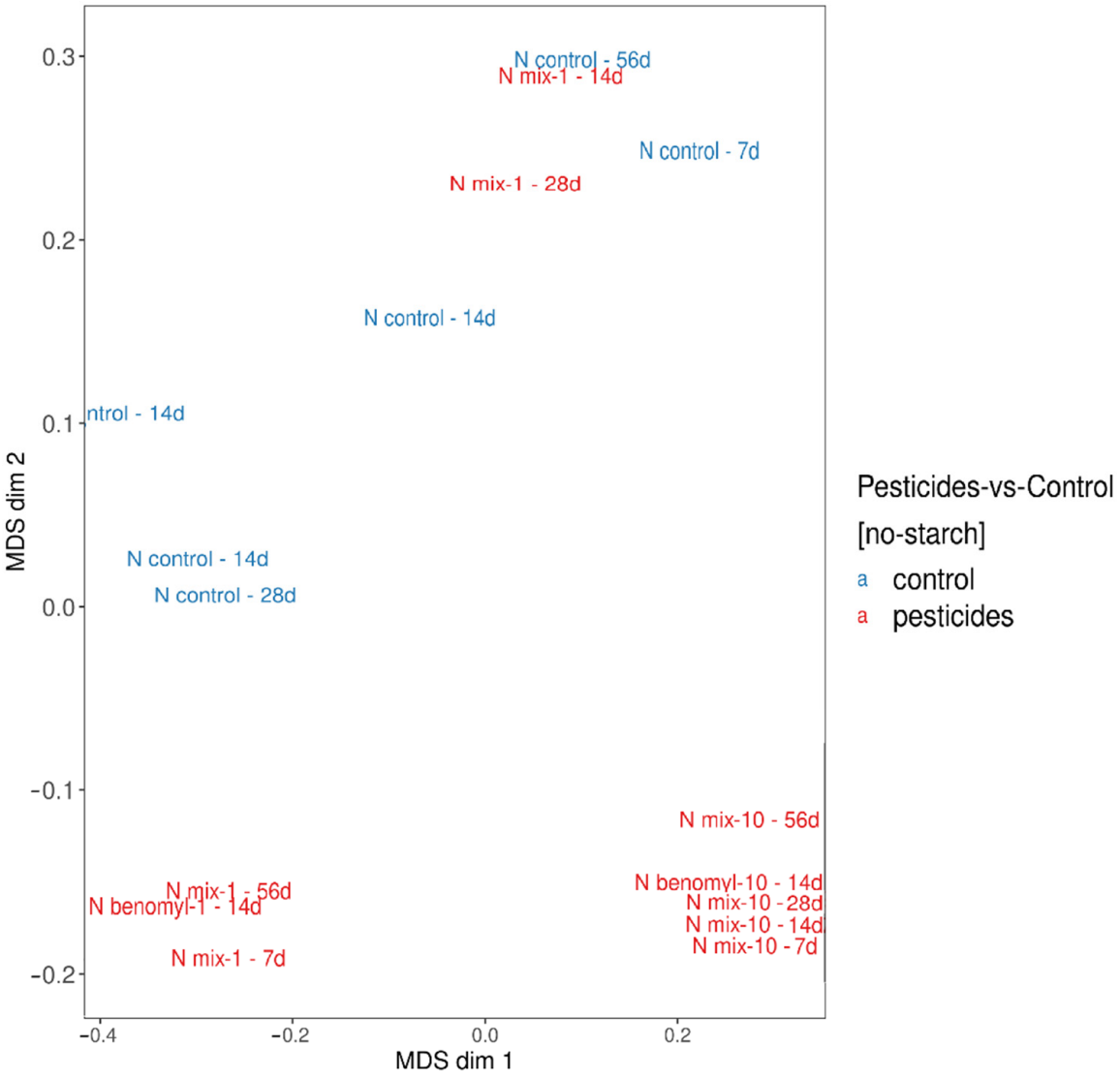

Figure 5. MDS plot representing the beta diversity of fungal community compositions (at genus level) in soil samples (without SMM) treated with pesticides (red) and control ones (blue). Samples treated with 10× application rate concentration form a separate cluster, distant from the other samples.

4. Discussion

The decrease in the diversity according to the Shannon index with the addition of SMM was caused, first of all, by an increase in the proportion of hydrolytic bacteria, such as Actinobacteria. Despite this, after the addition of SMM, a larger number of bacterial genera

were affected by pesticide treatment. In general, the spectra of bacterial and fungal genera reacting to pesticides, as well as the dynamics of this reaction, are significantly different in the variants without SMM and with SMM. This is quite expected since the addition of carbon source triggers the succession, which is visible in the microbiome structure in the control samples. The starch led to changes in the microbiome structure that persisted throughout the entire duration of the experiment. This substrate is rather slowly degraded in comparison with, for example, such a universal source of carbon in microbiological experiments as glucose [27]; at the same time, it is accessible enough to initiate a succession in a short time. Note that the soil, for the most part, is practically "dead mass", and only in some zones (hotspots) is there a high activity of microorganisms [40], provided that readily available substrates are supplied. In laboratory experiments, the soil is dried, sieved, and homogenized, which, on the one hand, makes it homogeneous, and on the other hand, completely destroys the hotspots. It cannot be argued that starch is an ideal substrate for initiating succession. Moreover, additional research is needed to select the optimal concentration. To select the optimal substrate, separate studies should be carried out using DNA-sequencing. Stimulation of actinomycetes with starch somewhat blur the picture, but at the same time long-term changes in the structure occur, which corresponds to our goals. In fungi, pesticide treatment without SMM reduces Ascomycetes; with the SMM addition, the same tendency was observed, but Basidiomycetes increase statistically significantly as well. Basidiomycetes were likely to be involved in the degradation of pesticides, which was confirmed by other studies, for example, where it has been shown that Basidiomycetes can degrade organochlorine pesticides [41,42].

The increase in the genus *Terrabacter* can also be explained by the involvement of these bacteria in the degradation of pesticides [43]. *Kitasatospora* and *Streptomyces* increase their relative abundance upon treatment with pesticides. The genera of Actinobacteria are also degraders; strains of these genera isolated from paddy soils have been shown to be capable of degrading ipconazole [44]. It is noteworthy that although the total abundance of Actinobacteria increases upon treatment with pesticides, no significant differences from the control were found for the genera *Kitasatospora* and *Streptomyces* after the addition of SMM. This is probably due to the fact that actinomycetes increase their share by actively utilizing starch and the stimulating effect of pesticide application in relation to some genera is lost. As noted above, polymeric substrates applied together with pesticides mask the increase in the share of degraders due to the pesticide treatment, which has to be borne in mind when conducting a search for pesticide-degrading strains. It probably makes sense to use lower concentrations of substrates, as well as to use mixtures of them.

Regardless of the SMM addition, the relative abundance of bacteria of the genera *Burkholderia* and *Mycobacterium* decreases under the influence of pesticides; as shown previously, these genera are sensitive to carbendazim [23]. The share of *Sphingomonas* slightly increases in comparison with the control variants without SMM and decreases with the addition of SMM. The increase in the share may be associated with the involvement of representatives of this genus in the degradation of pollutants [45]. Note that representatives of this genus are characterized by many important properties in the soil, such as stimulation of plant growth under stress conditions and the synthesis of phytohormones [46].

Among the representatives of the fungal community, yeast fungi of the genera *Apiotrichum* and *Gibberella* are susceptible to the influence of pesticides without SMM addition; in the case of SMM addition, positively affected by the pesticide application and the same influence was observed for the genera *Candida* and *Saitozyma*. The relative representation of the genus *Apiotrichum* increases upon treatment with pesticides, which is much more pronounced with the addition of SMM. The increase in the proportion of yeast fungi is likely to be associated with vacating the niches due to a decrease in the representation of other species. This is more noticeable with the addition of SMM, since the decomposition of starch results in the appearance of glucose, which is readily assimilated by copiotrophs.

5. Conclusions

In conclusion, it is also noteworthy that the analysis of the structure of microbial communities based on the 16S and 18S rRNA gene sequencing provides information only about the relative proportion of different microorganisms; it should be remembered that the absolute content of microorganisms between different samples (for example, with SMM and without SMM) can significantly differ. Further research is needed with the addition of carbon sources to establish optimal conditions for such experiments. The changes in the relative abundance of the genera *Terrabacter*, *Kitasatospora*, *Streptomyces*, *Sphingomonas*, *Apiotrichum*, *Solicoccozyma*, *Gamsia*, and *Humicola* can be proposed as an indicator of pesticide contamination. It is proposed to use these signs for large-scale assessment of the effect of pesticides on soil microbial communities instead of classical integral methods, including within the framework of state registration of pesticides. It is also proposed to conduct research on the effect of pesticides on the soil microbiome during artificially initiated successions using the additional source of carbon.

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