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Revealing Fungal Diversity in Mesophilic and Thermophilic Habitats of Sewage Sludge Composting by Next-Generation Sequencing

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Abstract: The accumulation of sewage sludge is a severe problem in many countries. Its utilization through composting has the potential to become a widely applied technology. From this perspective, our study investigated the diversity of fungi in mesophilic and thermophilic habitats when composting biosolids, cow manure and wheat straw. It was conducted using a metagenomic approach and next-generation Illumina HiSeq2000 sequencing to reveal the fungal diversity. We found significantly enhanced microbial activity in the thermophilic phase. In contrast, the activity of enzyme β-glucosidase was 29% higher in the mesophilic zone. The range of α-diversity values was more pronounced in the mesophilic habitats than in the thermophilic habitats based on diversity indices. At the class level, the mesophilic fungi were represented by *Sordariomycetes*—58.7%, *Pezizomycetes*—15.1% and *Agaricomycetes*—12.3%, while the most abundant thermophilic fungi found were *Sordariomycetes*—39.5% and *Pezizomycetes*—9.8%. In the further clarification of genera diversity, it is striking that at 37.2 °C, *Psathyrella* was the most abundant with 35.91%, followed by *Chaetomidium* with 20.11%. Among the thermophiles, *Thielavia* and *Mortierella* were the most common. Further research on microbial diversity changes over time is needed to manage the metabolic processes in obtaining quality soil amendment.

Keywords: NGS; fungal diversity; mesophilic fungi; thermophilic fungi; composting; sewage sludge; biodegradable waste

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1. Introduction

The accumulation of biosolids is one of the main concerns of modern society [1]. Unfortunately, it is an increasing problem since the wastewater treatment plant (WWTP) is built without a permanent solution to the fate of activated sludge resulting from domestic wastewater treatment. One of the most environmentally friendly solutions is biosolids co-composting with other biodegradable wastes [2]. The compost contains many microorganisms capable of degrading natural biopolymers, such as proteins, lignin, and cellulose, among other organic molecules. The interactions in the microbial communities of composting are dynamic and dependent on many environmental and biochemical factors, which affect 'microorganism' diversity and activity. Over the past decades, composting has become increasingly widely used as a sensible alternative for waste management. As plants, insects, and human pathogens thrive in the compost, determining the species diversity of eukaryotes in the current stages of composting is crucial for their further use. The information known so far about the importance of various factors for selection in the microbial communities of the composting phases is insufficient. More information would allow us to regulate the factors, reduce the time of the process, and improve the final product's quality.

In a complex environment such as composting of sewage sludge and biodegradable wastes, microbial communities are composed of many genera that interact with one another in numerous ways that are not yet understood. The interplay between factors such as horizontal gene transfer, microbial interactions, cross-feeding, and genetic variation within

populations are just a few examples of what make those relations challenging to establish. Contaminated sites comprise environments of particular interest to microbiologists since they may be communities of microbes enriched with physiological characteristics of biotechnological interest. Still, much of their genetic diversity remains unexploited. However, with next-generation sequencing (NGS), whole-metagenome sequencing has recently emerged as a valuable method for determining taxonomic and functional relationships in compost metagenomes. Composting is a dynamic process in which microbial communities often change over successions due to changes in temperatures and transitions from mesophilic to thermophilic and vice versa. This leads to development in the ecological niches of the corresponding adapted communities able to survive in these conditions and metabolize the corresponding nutrients. NGS is a powerful method for establishing microorganism' type and genetic diversity in compost. In recent years, revolutionary NGS technologies have become widely used in various microbiological disciplines, including microbial taxonomy and ecology [3,4]. One of the major advances offered by this technique is the ability to generate large amounts of data cheaply and provide information on the impact of abiotic and biotic factors on microorganisms. Composting is a complex biochemical process that involves specific populations of microorganisms that play an essential role in the degradation of organic matter and the maturity of products in the composting process [5].

In the above context, the ascomycete fungi of *Thielavia* genus are known to contain both mesophilic and thermophilic species [6]. Robledo-Mahón and co-authors [7] reported on theremarkable lignocellulolytic activity of *Thielavia* sp. HM70 associated with five enzymes from a total of nine: ammonifying enzymes and tyrosinase, pectinase, protease, and cellulase, among others. Changes of physicochemical characteristics, composition, pH, temperature and humidity of biomass during composting strongly influence species biodiversity [2]. In this sense, Duan and co-workers informed that the temperature strongly influenced fungal diversity and nutrient transformation of wheat straw biochar during composting [8,9]. It is also known that microbial activity from conventional composting can lead to higher temperatures that affect other physicochemical characteristics [10]. In continuation, they found *Chytridiomycota*, *Ascomycota*, *Basidiomycota*, and *Mucoromycota* as most frequent phyla, while *Mucor*, *Batrachochytrium*, *Puccinia*, *Rhizophagus*, *Acidomyces*, and *Colletotrichum* were the most abundant genera in the compost. Zhang and co-workers found strong changes in community composition because of temperature fluctuations, which thus they modify fungal dynamics and activity [11]. Different authors used diversity indexes to describe community composition changes during their studies of high throughput analyses, such as Shannon (H′), Chao1, OTUs richness, etc. Tortosa and collaborators observed higher Shannon diversity indexes at the thermophilic phase than at the mesophilic one (4.29/2.77) during olive-mill composting [12]. In contrast, we found increased diversity indexes in the mesophilic phase of biowaste composting [4].

The present study examines mesophilic and thermophilic zones' microbial activity and interspecific diversity in a composting pile during the active biodegradation phase.

2. Materials and Methods

2.1. Composting Materials, Experimental Design, and Sampling

Biodegradable wastes and biosolids were mixed in order to study their valorization potential. Cow manure and straw were obtained from the local farmers of Kalekovec village in the Plovdiv district, while the biosolids were supplied from the WWTP of Plovdiv, South Bulgaria. Composting piles in three replicates on the concrete ground were made from 7.3 t of biosolids, 1.5 t of cow manure and 5.1 t of wheat straw setting a C:N ratio of 28:1. The initial dimensions of each pile were 5 × 3 m with 1.5 m height. Samples were taken several times to study changes in the organic matter and compost microbiome. The samples to study microbiome biodiversity were taken on day 20 from two pile depths (20 cm and 100 cm). The moisture content was maintained in the range of 55–65% during the experiment, while the piles were turned once every two weeks to insert oxygen and regulate the compost temperature. Samples for physicochemical and microbiological

analyses were taken from three places of both depths and mixed, yielding one sample for each composting depth.

2.2. Physicochemical and Microbiological Parameters

Pile temperatures were measured using a digital thermometer with a probe to reach the desired depth. The observation was made each day manually at both depths of the three piles. The CO_2 concentration emitted over the piles was measured daily for 5 min using portable equipment (Almemo 2590-3S, Ahlborn, Holzkirchen, Germany) and a chamber with a volume of 8000 cm^3 covering a pile surface of 226.86 cm^2. Samples were dispersed in water (1:10, *w*/*v*) and shaken, followed by sedimentation and measurement of compost pH and EC. Total nitrogen was measured using the Kjeldahl method. Ammonia (NH_4-N) and nitrate (NO_3-N) nitrogen were extracted using 2M KCl [13]. After centrifugation, the NH_4 was determined by NaOH distillation and titration with H_2SO_4. Nitrate concentration was calculated as the difference between the values of Zn-$FeSO_4$ and ammonium. The organic C was measured and calculated after the carbonization of the samples in a muffle furnace at 550 °C for 6 h [14].

The enzyme β-glucosidase is the limiting factor of microbial hydrolysis of cellulose to glucose. Its activity was assessed using the method described by Alef and Nannipieri (1995) [15]. Furthermore, another parameter that characterizes microbial activity in composts and soils is the released CO_2 flux resulting from organic matter degradation. This process, called microbial respiration from the substrate, is accepted as an indicator of the rate of microbial activity expressed as released CO_2 from one gram per hour. In our study, the indicator was assessed using the method described by Alef and Nannipieri [15].

2.3. Isolation of DNA from Compost and Qualitative Analysis

Two different samples were taken from the pile-one from 100 cm depth (with an average temperature of 58.1 °C) and the other from 20 cm depth (with an average temperature of 37 °C). Total genomic DNA was isolated from 50 mg of compost samples with a HiPurA™ Soil DNA Purification Kit, HiMedia (Himedia, Mumbai, India) according to the manufacturer's protocol. The quality of DNA was checked for the single intact band on 1% agarose gel (loaded 5 µL) stained with SafeView (NBS Biologicals) at 100 V for 50 min using a VWR Mini Electrophoresis System (Radnor, PA, USA) for gel visualization.

2.4. Isolation of DNA from Compost and Qualitative Analysis

NGS and analysis of the total sequences within the sample to uncover the microbial community composition within that particular sample, through a targeted approach via targeted metagenomics, were performed with the Illumina HiSeq 2000 platform (Eurofins Genomics Germany GmbH, Ebensburg, Germany). In this approach, a certain sub-group within the microbial community is targeted by first PCR amplifying sequences within the target group via a barcode gene unique to that sub-population, prior to sequencing those amplicons. For the metagenomics analysis of eukaryotes, the internal transcribed spacer (ITS) region of the eukaryotic rRNA operon has been applied. The ITS region is 330 bp long and is located between the 18S and the less variable 5.8S gene [16,17].

2.5. Sequence and Bioinformatics Analyses

Raw data were filtered, and the quality check was carried out by MG-RAST software version 3.6 before use in downstream bioinformatics applications [18]. Unassembled reads were submitted for automatic processing to the MG-RAST metagenomics analysis server version 4 using the default quality control pipeline [19,20]. The statistical analysis of these sequence reads is shown in Table 1. MG-RAST analysis was used to determine functional and taxonomic relationships between metagenomic sequence reads and database sequences. MG-RAST is an online annotation service that uses the SEED algorithm in a standardized pipeline analysis of metagenomic DNA sequences. We uploaded the two FASTQ files obtained from Illumina sequencing to the MG-RAST server and several

metagenomic assemblies in FASTA format. FASTQ reads were processed using the default MG-RAST parameters. Coverage of the assembled sequence, which enhances the MG-RAST analysis, was computed for each contig from the IDBA-UD contig output file using the formula: coverage = (number of reads on contig × 100 bp)/(contig length in bp). Microbial composition analysis was performed using the MG-RAST best hit classification tool, where reads were compared to the LSU-SILVA (non-redundant) database using a maximum e-value of 1×10^{-5}, a minimum identity of 97%, and a minimum alignment length of 60 bp, to generate taxonomic profiles. SILVA is a comprehensive web resource for up-to-date, quality-controlled databases of aligned ribosomal RNA (rRNA) gene sequences from the *Eukaryota* domains and supplementary online services [21]. Raw Illumina sequence data were uploaded to the MG-RAST server.

Table 1. Physicochemical parameters of the compost samples in thermophilic and mesophilic habitats. Results show the mean of 3 replicates ± standard error.

Parameters	Mesophilic	Thermophilic
Total organic carbon (%)	31.62 ± 2.1	32.19 ± 1.6
pH	7.81 ± 0.1	7.67 ± 0.1
EC (mS cm^{-1})	3.04 ± 0.12	2.76 ± 0.2
Total nitrogen (%)	1.31 ± 0.1	1.40 ± 0.09
N-NH_4 (mg kg^{-1})	1151.80 ± 31.1	1248.11 ± 26.3
N-NO_3 (mg kg^{-1})	108.14 ± 2.6	113.23 ± 3.2
C/N ratio	24.13	22.99

2.6. Alpha-Diversity and Principal Coordinate Analysis (PCoA)

The α-diversity of annotated samples can be estimated from the distribution of the species-level annotations. Shannon diversity is an average of the logarithmic values of the relative abundances of the annotated species. The species-level annotations were analyzed with annotation source databases in both MG-RAST platforms. The other indices Simpson, Chao1, ACE, and Good's coverage in compost samples, were calculated using QIIME2 (version 1.7.0, http://qiime.org/1.7.0/, URL accessed on 31 March 2023) and displayed with R software (version 2.15.3) [22]. The richness indices (ACE and Chao) and diversity indices (Shannon and Simpson) were calculated using the mothur program version 1.48.0 [23]. Principal coordinate analysis (PCoA) was obtained by principal coordinates and visualizing complex multidimensional data, based on Euclidean distance matrices of microbiomes of compost samples from the thermophilic and mesophilic phases.

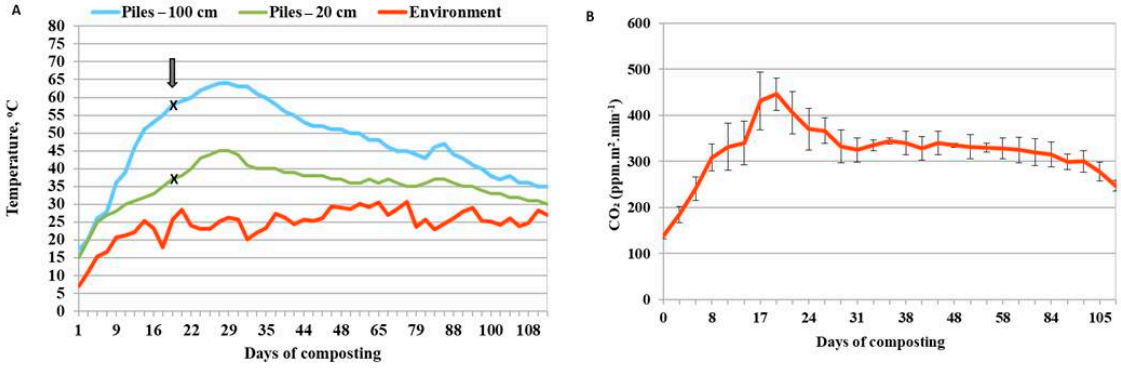
3. Results

3.1. Physicochemical and Microbiological Characteristics

The temperature course during the composting is shown in Figure 1A. At a depth of 100 cm, it was characterized by an initial rapid rise from 17 °C, to 50 °C by day 13, when conditions became thermophilic. At the same time, at the top of the pile at 20 cm from the surface, the temperature was 37.2 °C, i.e., with mesophilic characteristics. The samples taken for the microbiome analysis on the 20th day differed in temperature as at 20 cm, the composting material was 37.2 °C, while at 100 cm depth, it was 58.1 °C. Thus the microbiomes developing in both composting habitats were characterized as mesophilic and thermophilic. The pile temperature continued to increase until day 26, reaching 64 °C and 45 °C, respectively, at both depths. These changes were independent of the environmental temperature fluctuations since from the 15th day, it fluctuated within the limits of 25–30 °C with few exceptions.

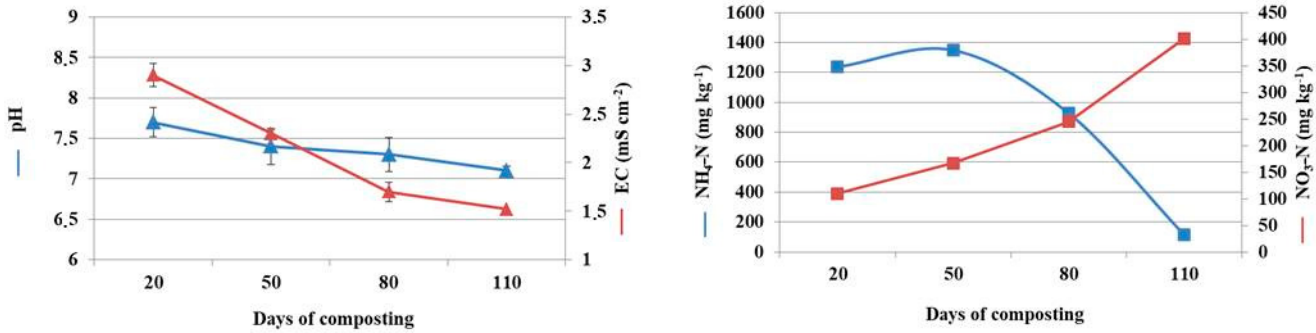
In the present research, the microbial activity was measured continuously by monitoring CO_2 was released from the compost pile by an infrared sensor (Figure 1B). CO_2 concentration followed the course of temperature, which is apparently one of the driving forces of microbial activity and the selection of mesophilic and thermophilic habitats. In addition, thermophilic microorganisms had a higher growth rate than mesophilic ones

had. On the other hand, the courses of pH and EC were similar. Since the beginning of composting, both parameters decreased—pH from 7.71 to 7.08, while the reduction in EC was much more significant, from 2.93 mS cm^{-1} to 1.54 mS cm^{-1} at the end of study (Figure 2A). The fluctuations in the concentrations of NH_4-N and NO_3-N were typical for biowaste composting. The ammonium concentration was initially quite high, but it decreased substantially after a slight increase. At the same time, the nitrate rose more than three-fold to 406.1 mg kg^{-1} (Figure 2B).



(**A**) (**B**)

Figure 1. Composting pile temperature ((**A**), 100 cm and 20 cm depth) and CO_2 emissions (**B**), during the course of process. The standard error was in the range of 5% for both pile depths.



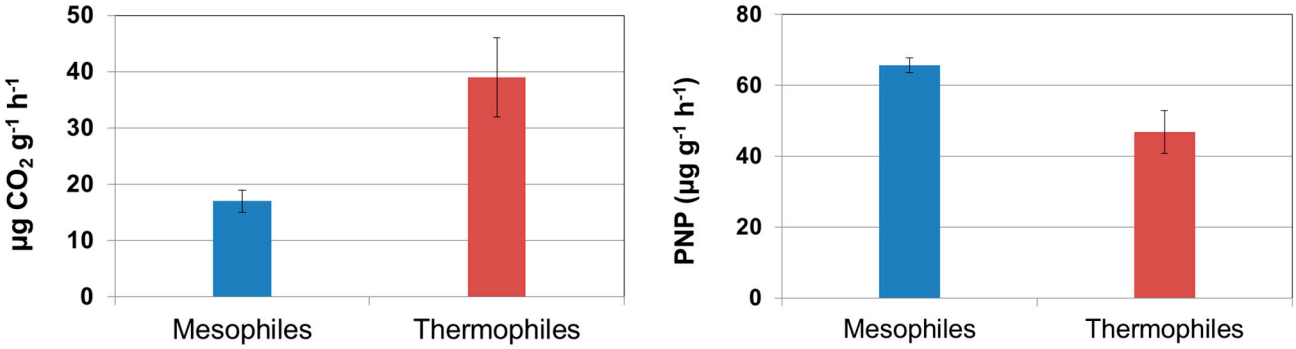
(**A**) (**B**)

Figure 2. Changes of pH EC (**A**), and NH_4-N and NO_3-N (**B**) during the course of composting. Data represent compost pile average. The standard errors were in the range of 5% in case of the forms of nitrogen.

In addition, the pH and EC values of the studied samples were lower in the thermophilic environment (Table 1). which was 1.31–1.4%, within the standard error. Comparing the water-soluble forms of nitrogen in the samples, a slight predominance of their concentrations in the thermophilic phase was found, and only in relation to ammonium nitrogen the differences were statistically significant. Based on the above results, C/N ratios were calculated in both habitats with predominance in the mesophilic zone.

Microbial emissions of CO_2 from compost are accepted as one of the most typical indicators of microbial activity. Their determination makes it possible to estimate the total microbial activity in the corresponding substrate, including the activity of microorganisms that are not cultivable by in vitro microbiological methods. In our case, the microbial respiration differed in both habitats of the composting pile. An increased respiration rate was found in the case of the thermophilic samples reaching 39 µg CO_2 g^{-1} h^{-1}, which was 1.3 fold higher than that in the mesophilic substrate (Figure 3A). Conversely, the activity

of the enzyme β-glucosidase was 29% higher in the mesophilic zone in contrast to the thermophilic zone (Figure 3B).



(**A**) (**B**)

Figure 3. Microbial activity expressed as released CO_2 (**A**) and β-glucosidase activity (**B**) for mesophilic and thermophilic habitats of the composting piles. Results show the mean of 3 replicates ± standard error.

3.2. Analysis of the Sequencing Data

In the present research, conventional analysis and high-throughput sequencing approaches were applied to assess the activity, diversity and composition of both prokaryotic and eukaryotic communities in compost produced at a commercial scale. The results showed microbial development in the compost samples, taken from two different habitats of the composting pile-thermophilic, at 58.1 °C and mesophilic, at 37 °C. The diversity of annotated samples can be estimated from the distribution of the species-level annotations in the combined MG-RAST data set.

The DNA extracted from the two samples taken from the pile was not completely amplified. After merging and quality trimming the raw data, the mesophilic eukaryotes contained 109,291 sequences to tailing 51,675,338 base pairs with an average length of 473 bps. Of the sequences tested, two sequences (0.00%) failed to pass the quality control (QC) pipeline (Table 2). Of the sequences that passed quality control, 107,530 (100%) contained ribosomal RNA genes, and no sequences (0.00%) contained predicted proteins with known functions. When thermophilic eukaryotes were assayed, they contained 3732 sequences to cover 50,707,302 base pairs with an average length of 344 bps. The mesophilic sample showed the content of 148,049 sequences to tailing 48,761,060 base pairs with an average length of 341 bps. After quality filtering, the minimum number of sequences of mesophilic microorganisms was 5518, while for thermophiles it was 3732. The reads of the soil metagenome exhibited an average GC content varying between 48 ± 5 and 49 ± 6 (Table 2). After alignment, the remaining operational taxonomy units (OTUs) had been clustered at a 3% distance.

Table 2. Metagenome sequences uploaded to MG-RAST for analysis with statistics of the sequence results are shown.

Parameters	Mesophilic	Thermophilic
Total starting base pairs	48,761,060	50,704,302
Base pairs after QC	1,879,114	1,283,854
Average sequence length	341 ± 56	344 ± 49
GC content, %	49 ± 6	48 ± 5
Post QC minimum number of sequence reads	3732	5518
MG-RAST identified rRNA features	1452	1650
MG-RAST identified protein features	5	2
α—Diversity from MG-RAST annotations, %	10	7

3.3. Analysis the Sequencing Data

The α-diversity of the fungal data set was 55 species. Regarding the different composting habitats (mesophilic and thermophilic), there was a difference in both observed OTUs and Shannon diversity index for the fungal communities. The Shannon diversity of the relative abundances of annotated species was much higher in the mesophilic habitats that in the thermophilic ones (Table 3, Supplementary Figure S1). Chao1 and ACE non-parametric indices of richness were highly sensitive to the number of species. The richness index (Chao1) of the fungal communities increased with a greater number of analyzed sequences and was higher in the case of mesophilic fungi (2623.109) than in the case of thermophilic ones. Similarly, the ACE index showed higher species diversity in the mesophilic than in the thermophilic environment (Table 3).

Table 3. The range of α-diversity values in the compost samples.

Sample Name	Shannon	Simpson	Chao1	ACE	Goods Coverage	PD Whole Tree
Compost at 37.2 °C	10.417	0.994	2623.109	2638.908	0.987	209.132
Compost at 58.1 °C	6.972	0.889	1654.224	1775.459	0.990	126.207

3.4. NGS Approach for Elucidation of Eukaryotes Biodiversity in the Compost

The NGS analysis of the sequenced 148,049 eukaryotic amplicons showed that in the initial phase of active degradation, the thermophilic fungal community was composed mainly of phyla the *Ascomycota* (89.96%) and *Basidiomycota* (3.36%). At the same time, in the mesophilic community, *Ascomycota* was less abundant, with 77.97% representation, while the *Basidiomycota* distribution was higher (Figure 4A). In both kinds of habitats, fungi of the class *Sordariomycetes* were at almost equal amounts of approximately 66.79–67.20%. These fungi participate most actively in both phases of organic matter degradation in composting. *Pezizomycetes* were represented by 12–18% of the fungi, with a slight advantage over thermophilic ones (Figure 4B). We have a greater difference between the two phases in the case of the *Agaricomycetes* class, where the mesophilic representatives are 21.7%, and the thermophilic ones are 13.2%.

The distribution of fungal genera is presented in Figure 5 and Supplementary Figure S2. The fungi from genera *Psathyrella* were the dominant populations in themesophilic habitats (35.91%), while in the thermophilic environment the abundance decreased by nine to 4.08% (Figure 5A,B). It seems that most of *Psathyrella* populations are mesophilic, as their representation was strongly reduced at a lower temperature. The three further most dominant fungal genera were *Chaetomidium*, *Mortierella*, and *Cheilymenia*. *Chaetomidium* increased to 20.11% in the 37.2 °C pile, dramatically reducing the population deep in the compost mound to 0.65%.

Similarly, the abundance of *Cheilymenia* decreased to 4.76% at 58.1 °C. However, the amount of *Mortierella* was increased in thermophilic habitats by 32.5% compared to with the mesophilic ones, reaching 22.91%. Furthermore, *Thielavia* sp. was predominant in the inner part of the compost pile, representing almost 55% of all genera where conditions were thermophilic. Both of them represented more than 78% of all thermophilic genera. With increasing depth and temperature, Penicillium and Verticillium frequency increased. Numerous mesophilic fungi such as *Acremonium*, *Fusarium*, *Microascus*, *Trichoderma*, and *Verticillium* proliferate during the cooling and curing phases. Many of the fungi we found are known to be widespread saprophytes on soil and dead plant tissue [23]. They occur in compost, given their ability to produce cellulolytic enzymes [24].

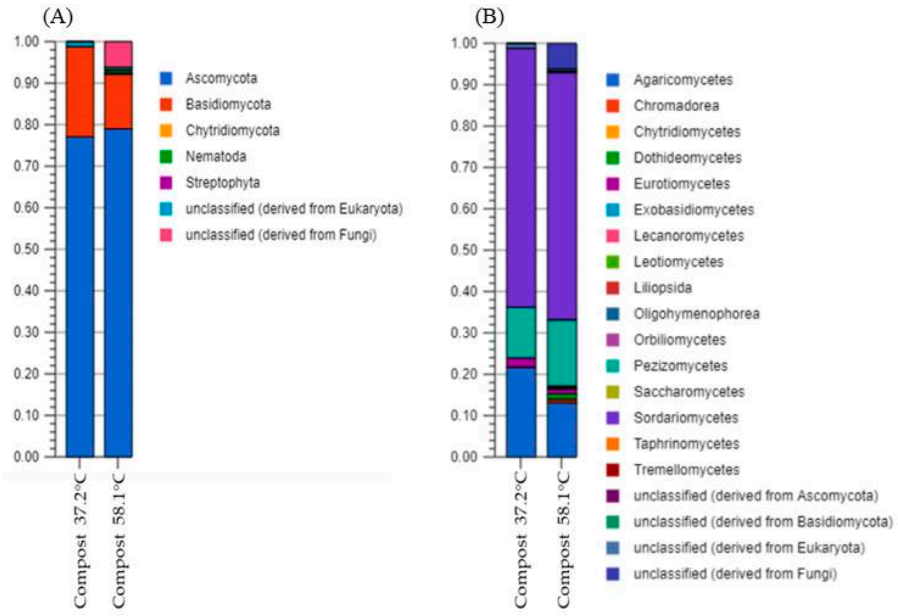


Figure 4. Microbial community composition with the number of OTUs identified by MG-RAST at the phylum level (**A**) and class level (**B**). Unassembled reads annotated on MG-RAST were analyzed using the classification tool based on RDP (97% identity; the e-value cutoff of 10,230) and M5NR (60% identity; the e-value cutoff of 1025) with a minimum alignment length of 50 bp. The figure displays the taxonomic distribution for the 10 most abundant phyla and classes.

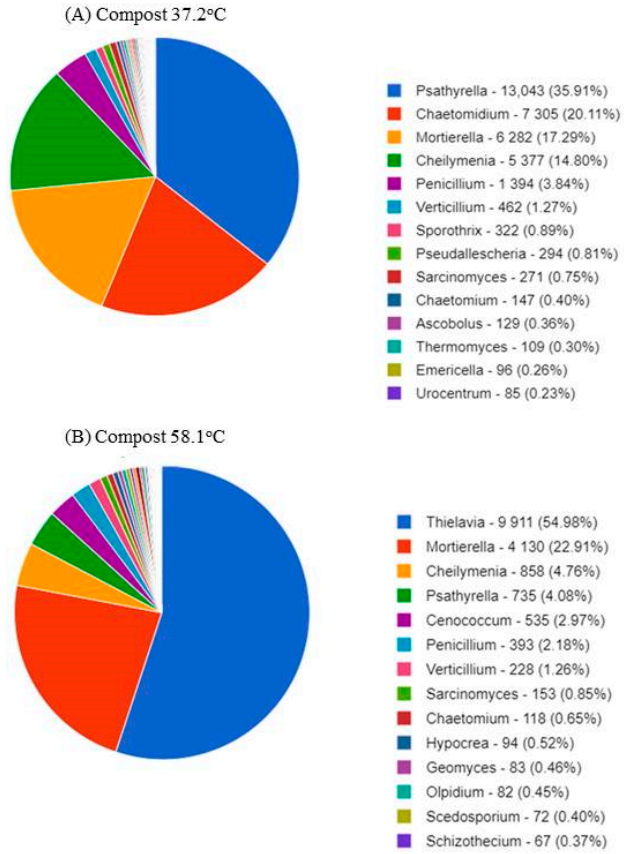


Figure 5. Analysis of taxonomic annotation with the number of OTUs identified by MG-RAST. (**A**) Mesophilic and (**B**) thermophilic habitats. Area of sector means respective proportion of different OTU annotation results in percent.

Figure 6 compares eukaryotic rRNA annotations with SSU, RDP, and ITS databases, filtered with MG-RAST software using a cut off of 97% identity, and E-value of 1×10^{-5} with an alignment length cutoff of 15. Visualization was restricted to eukaryotic sequences with an abundance more significant than 1% in the Illumina raw data. The most abundant fungal species in the mesophilic environment were *Pseudallescheria boydii*, and *Psathyrella corrugis*, followed by *Terfezia boudieri*. In our investigation, the fungus was found only in the mesophilic habitats.

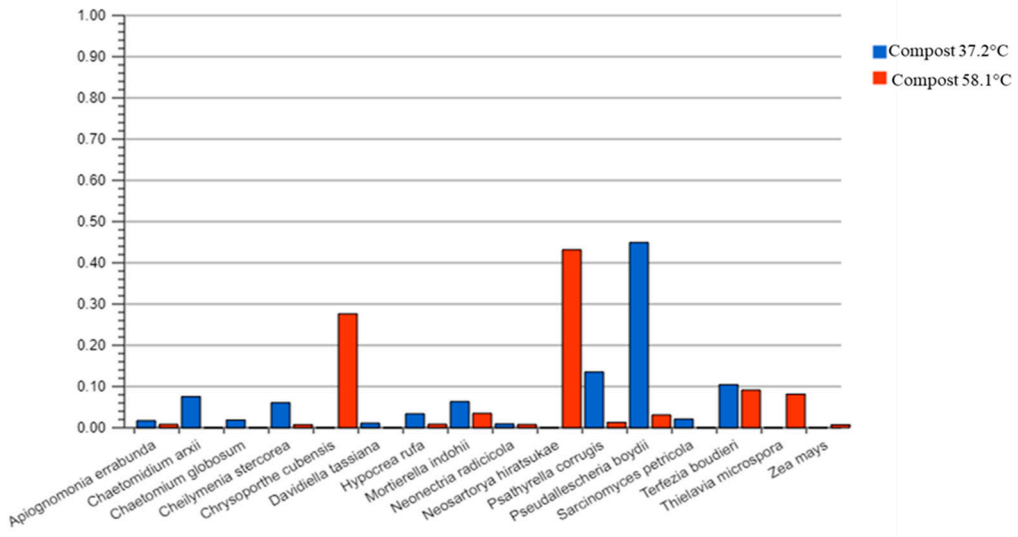


Figure 6. Taxonomic hits distribution of the eukaryotes under mesophilic and thermophilic conditions of compost pile.

On the other hand, in the thermophilic habitats, species such as *Neosartorya hiratsukae* and *Chrysoporthe cubensis* were taking dominance. The thermophilic fungus *Neosartorya hiratsukae* has been identified as a β-glucosidase capable of efficiently converting isoflavone glycosides into free isoflavones [25]

3.5. Biodiversity Curves

The rarefaction curves can directly reflect the sequencing data volume's rationality and indirectly reflect microbial community richness in the samples. In the current study, the rarefaction curves for both compost samples are based on the samples determined at a genetic distance of 3% using rRNA-related sequences extracted from the entire metagenomic sequence data set. The number of reads for the top-level sample at 37.2 °C (mesophylls) accounted for 127,697, and, for the compost sample in a thermophilic phase, there were 105,168 reported sequences. The curves in Figure 7 reached saturation, with the number of species sampled accordingly, and they supported the initial hypothesis that the composting process is characterized by enormous microbial diversity. Thus, mesophilic habitats possessed a 21.4% higher species richness than thermophilic habitats. This is in accordance with the data of Biyada and collaborators [3].

Figure 8 shows that compost microbial communities from thermophilic and mesophilic phases clustered separately when metagenomic functional categories were used for the analysis. Both components of the PCoA explained over 10% (PC1—10.35% and PC2—10.79%) of the variability between samples.

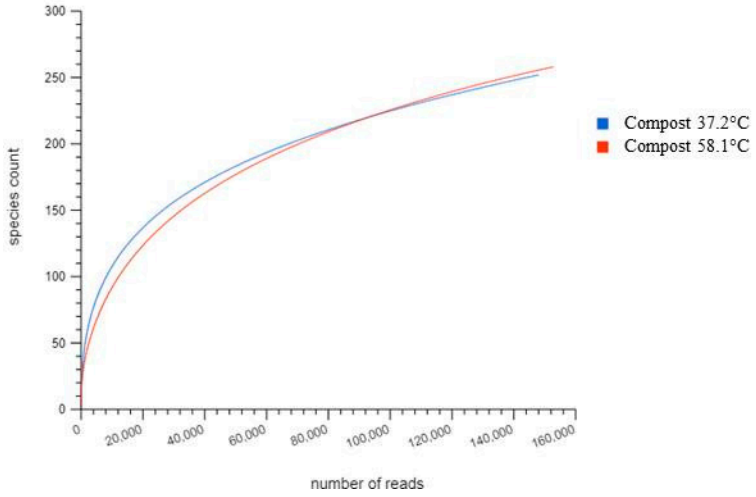


Figure 7. Rarefaction curves of sequencing data from the samples, counting the number of OTUs in both compost samples.

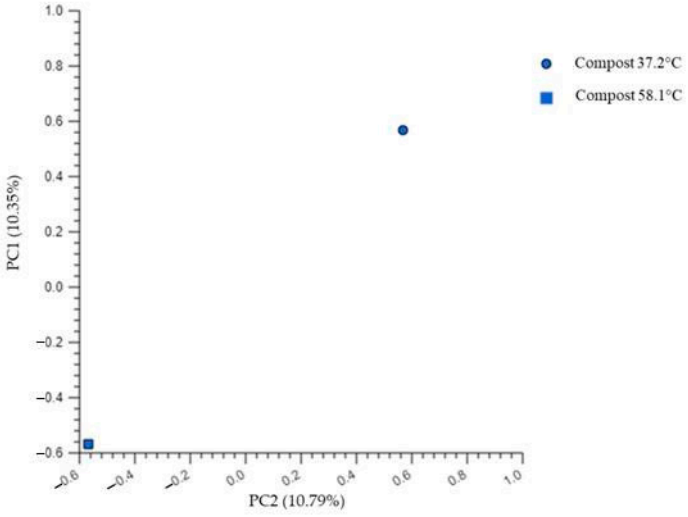


Figure 8. PCoA plot of metagenomic data based on Euclidean distance matrices of microbiomes of compost samples from thermophilic and mesophilic phases.

4. Discussion

Composting is an important process that ensures the recycling of organic matter and its transformation into a stable matrix that can be used as soil amendment. This approach complies with the principles of circular bioeconomy, which is the basis of the development of the EU in the foreseeable future. The presence of plant nutrients in the sludge, makes composting even more valuable as a recycling technology. Here we applied the NGS method to characterize fungal communities of thermophilic and mesophilic habitats of biodegradable waste and sewage sludge during composting. Fungi are the microbes having great importance for organic matter decomposition and transformation in nature, and thus are involved in nutrient cycling and solubilization [4,26]. The role of fungi involved in 'organics' decomposition is thought to be mostly restricted to the early decomposition stages because most endophytic fungi obviously depend on readily available sugars or easily degradable compounds such as cellulose [27]. *Chaetomidium arxii* and *Chaetomium globosum* have been analyzed to release different extracellular enzymes such as amylases, cellulases, esterases, lipases, proteases (gelatin and caseinase), pectinases, and cuticles [28]. Those saprophytic fungi primarily reside on plants, soil, straw, and dung. Endophyte *Chaetomium globosum* assists in the cellulose decomposition of plant cells [29].

We found higher β-glucosidase activity in mesophilic habitats than in thermophilic ones (Figure 3), but at the same time a higher rate of respiration in thermophiles. Simmons and collaborators found a lower transcriptional efficiency and lower fungal β-glucosidase abundance in thermophilic habitats compared to the bacterial communities [30]. Zang and co-authors (2017) reported on β-glucosidase gene representation in the bacterial phyla *Firmicutes*, *Actinobacteria*, and *Proteobacteria* and in fungal *Ascomycota* [31].

We found *Ascomycota* and *Basidiomycota* as the predominant phyla in both phases. These results are consistent with previous studies [32–34]. Thermophilic enzymes are favorable for the hydrolysis of isoflavone glycosides since high temperatures enhance the mass transfer rate, reduce the substrate viscosity, and reduce the risk of contamination [35]. In addition, *Sarcinomyces petricola* is amongst the most stress-resistant yeast known [36], and constitutive melanin synthesis from 1,8-dihydroxy naphthalene (1,8-DHN) monomers confers passive resistance toward environmental exposure to solar irradiation, dryness, temperature and pH extremes, hyperosmolarity, and even ionizing radiations [37]. Many of those fungi possess secondary metabolites, i.e. cell membrane-associated carotenoids and intracellular mycosporines and mycosporine-like amino acids. Most likely, adaptation to unfavorable conditions, active metabolism under mesophilic conditions, and a passive transport mechanism contribute to their distribution in the composted sludge. Current results showed higher fungal diversity of mesophilic species compared with the thermophilic phase.This is in accordance with previous investigations reported in the literature [8]. In addition, we found similar patterns of diversity between thermophilic and mesophilic fungi [4]. When the community succession passing to the thermophilic phase the dominance is taken over by the bacteria, especially in the late part of the phase. Early studies showed *Penicillium* and *Aspergillus* as the most dominant fungi during composting [38,39]. The functionality of microorganisms' systems is closely related to the nature of organic compounds, their stability and properties at the given temperature and pH. According to the knowledge accumulated over the years in microbial ecology, the existence and development at lower, higher or even at extreme conditions depend on the microorganisms' capability to maintain their inner environment at optimal conditions for systems functioning [40].

As confirmed by other studies [4,7], species biodiversity is more pronounced in mesophilic microorganisms than in thermophilic ones. This is a result of the species' ability to adapt to higher temperatures and the associated tools to overcome the consequences of these temperatures for the cell itself. With a sufficient increase in temperature, in the unadapted species, a disruption of the three-dimensional shape of the proteins is observed, followed by their denaturation and a decrease in enzyme activity. Likewise, lipids in cell membranes break down at high temperatures [40]. These complex adaptation mechanisms are part of the answer to the question of the reduced number of fungal species in thermophilic habitats compared with mesophilic ones [41].

In our research, 21.4% more mesophilic fungal species were found than thermophilic ones (Figure 7). In addition, the dominant genera in the inner part of the pile were *Thielavia,* with almost 55%, followed by *Mortierella,* with 32.5%. This is in accordance with the results of other researchers [7]. Zhu and collaborators [42] also reported higher diversity comprising of dominant mesophilic strains of fungi such as *Trichoderma*, *Aspergillus*, *Amorphotheca* and *Neurospora*. However, depending on habitats, climatic conditions and many more factors, Wang and co-authors [43] found *Mycothermus* as the most represented fungus in thermophilic habitats of composting material containing cow manure. Furthermore, from the results of taxonomic annotation, it can be seen that a large number of species were found below 5% (Figure 5).

Other researchers [34,44,45] also reported great fungal species diversity distributed unequally among different microhabitats of composting. The presence of *Sordariomycetes* and *Pezizomycetes* known as cellulose-degrading and lignin-degrading, in both samples is most likely due to the more drastic changes in the composting conditions. They play an essential role in nutrient and carbon cycling. *Sordariomycetes* are strong decomposers and are the most typical in compost ecosystems. According to Dang et al., 2021, *Sordariomycetes*

are considered biomarkers for compost in the soil [46]. This is in accordance with the result of Bonito et al. (2010), who published that most of the established fungi sequences closely follow the classes *Sordariomycetes* and *Pezizomycetes* after composting unclassified waste [47]. These findings are also partially supported by other researchers reporting the genera *Aspergillus*, *Trichoderma*, *Chrysosporium*, *Mortierella*, *Fusarium*, and *Penicillium* as the most often found fungi in composting [22,48].

5. Conclusions

Metagenomic sequencing leads to a more accurate and complete understanding of microbial diversity and interactions between individual species. It has the potential to elucidate the microbial base for the physicochemical results of composting. Our study is the first to provide a comprehensive analysis of fungal diversity in thermophilic and mesophilic habitats of composting piles under the conditions of Bulgaria. The mesophilic fungal community is distinguished by greater diversity, while the thermophilic community has a higher activity rate. Thus the genera *Psathyrella*, *Chaetomidium*, *Mortierella*, and *Cheilymenia* represent 85% of the mesophilic fungi, and *Mortierella* and *Thielavia*, with 78% are masters of thermophilic habitats.

Our research will further study the fungal enzymes in compost communities, allowing us to improve the compost quality, especially when applying sewage sludge. As recent EU directive changes restrict the use of sewage sludge compost in agriculture, process improvement will be conducted for safer application for recreational purposes, reclamation of disturbed areas, etc.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13095546/s1, Figure S1: Shannon diversity of the relative abundances of annotated species in both compost samples; Figure S2: Taxonomic hits of eukaryote distribution at the species level at 37.2 °C (A) and 58.1 °C (B).

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