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*Original Research Article*

# SHORT-TERM INFLUENCE OF GLYPHOSATE ON MICROORGANISMS IN BACKYARD COMPOST

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A b s t r a c t

The composting process is a natural method of waste disposal. Decomposition of organic matter occur with the participation of various groups of microorganisms. Using glyphosate-based herbicides aims to reduce agricultural losses. Biodegradation of glyphosate in soil is obtain with bacteria and fungi and may affect their functioning. The purpose of this study was evaluating the effects of glyphosate on bacterial diversity during composting and to monitor potential changes in community structure and species abundance. The addition of glyphosate did not affect the morphology of the tested groups of bacteria and did not cause changes in the morphological structure of fungi. Sampling after 10 minutes, 24 hours, 48 hours and 72 hours showed that the abundance of bacterial colonies changed over time compared to the control groups. However, the results suggest that the addition of glyphosate is insufficient to influence the composting process, thus disturbing the specific biocenosis.

Key words: compost, microorganisms, glyphosate, herbicide, soil

## 1. INCRODUTION

Microorganisms such as bacteria and fungi are responsible for the decomposition of organic matter. Composting process is an aerobic decomposition. Microorganisms determinate transforming biological waste into humic material [25]. Final product should not contain pathogens, and at the same time the mass should be stable and suitable for use as a soil additive [14]. In a typical composting process, both bacteria and fungi are present and active [18]. The main groups of bacteria at the beginning of the composting process are mesophilic bacteria such as *Lactobacillus sp*. and *Acetobacter sp*. Later, at the stage of increased temperature, Gram-positive bacteria such as *Bacillus sp*. and *Actinobacteri*a become the dominant microflora [3]. When the compost heats up above 40°C, thermophilic bacteria are most numerous. When conditions become unfavorable, the bacteria form spores that are resistant to the stress caused by excessive heat. When the compost cools down, mesophilic bacteria again dominate. The number and types of mesophilic microorganisms recolonizing the compost depend on the organisms

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present in the compost, as well as in its immediate environment. In general, longer compost maturation phase determinate a more diverse microbial community [10].

Fungi are microorganisms that effectively promote the decomposition of cellulose or lignin. Fungi include microorganisms classified as molds and yeasts [23]. They are responsible for the decomposition of many natural plant polymers. In compost, they break down organic pollutants, allowing bacteria to continue the decomposition process [38]. They spread and grow by decomposing organic compounds that are too dry, acidic, or low in nitrogen to be broken down by bacteria. Fungal species are abundant during both the mesophilic and thermophilic phases of composting [16]. All groups of microorganisms need each other in the composting process. The most efficient composting process is achieved with mixed composting, involving both bacteria and fungi [7].

Knowledge of microbes at different stages of composting is crucial to understanding and optimizing the process [8]. Adding agrochemicals disrupts the balance in the food chain. As a result, residues destined for composting will decompose more slowly [4]. This is due to their effect on biochemical mechanisms. Thus, soil enzymatic activity and the number of plant growth regulators (gibberellin synthesis, indoleacetic acid transport) are affected [15]. Soil microorganisms influence soil structure, function, and fertility [20]. They primarily decompose organic matter, but also perform many other functions, such as providing nitrogen (N), phosphorus (P), and potassium (K) to the soil [19]. The microbial population is influenced by many factors such as temperature, oxygen, moisture, nutrients, and pH. Characterization of microbial communities during the composting process can provide important information on the development of the compost biodegradation process and the maturity of the final product [26].

Glyphosate-based preparations are the most widely used herbicides in the world [30]. Glyphosate is the primary ingredient in non-selective herbicides used to protect crops from weeds. Glyphosate is the only herbicide that affects 5-enolpyruvate shikimate 3-phosphate synthase (EPSPS) and blocks the biosynthesis of aromatic amino acids in the shikimate pathway. Consequently, inhibition of EPSPS delays the synthesis of essential proteins [18]. For this reason, it is possible that the use of glyphosate causes selection pressure on soil microorganisms, which in turn may affect the dynamics of soil processes. High solubility in hydrophilic solvents, especially in water, and high mobility allow glyphosate to quickly leach into the soil, which also leads to water pollution [24]. Despite the widespread use, only a small part of the applied herbicides have a direct effect on weeds and is only marginally removed to the ground [6]. Leakage of herbicides into the ground and water reduces crop yields, poor quality of agricultural products, deterioration of soil fertility, water pollution, which indirectly endangers animals and humans [21]. There are studies that suggest that glyphosate-based herbicides may cause increased oxidative stress, lower testosterone levels and delayed female development in many bird species [29]. Potential associations between glyphosate exposure and immunoendocrine changes have been studied in humans health. Results indicate that living in areas exposed to high levels of sprayed herbicides may be correlated with an increased incidence of various neurological disorders such as Alzheimer's disease, Parkinson's disease, and ADHD [13].

## 2. MATERIAL AND METHODS

An environmental sample of compost from a home composter was taken for microbiological testing in the winter (January 2023). The sample contained decomposable material (grass cuttings, leaves, plant peelings). In order to obtain homogeneous compost, the components were used in the ratio of one-third nitrogen-based compost (green compost), for every two-thirds carbon-based compost (brown compost).

### Conditions and methods of culture

The compost sample was divided into two and diluted 1:10 in 0.85% salt solution for microbial culture using standard Petri dishes. Flask 1 served as a control, while 1 ml of the prepared 1 mg/L glyphosate solution was added to flask 2. The experiment was conducted for 72h at a room temperature of about 21°C. The first tests were performed 10 minutes after the addition of glyphosate, and then at 24-hour intervals for both trials. Inoculations were carried out in 3 replicates. Mesophilic, thermophilic, and saprobic bacteria were analyzed. In addition, the total number of fungi was determined. Mesophilic and thermophilic bacteria were determined by depth inoculation on agar. For this purpose, the prepared dilutions were placed in Petri dishes in a volume of 1 ml and inoculated with dissolved agar at about 50°C. Petri dishes were then thoroughly agitated before the agar solidified. For the determination of spore-forming bacteria, 4 ml of dilution was taken from each sample and then pasteurized for 15 minutes at 80°C on a shaker. After this time, inoculation was performed in the same way as for the other groups of bacteria on agar. To obtain fungal growth, Czapek-Dox medium was used. Surface inoculation was performed in a volume of 0.1 ml. Sterile plate spreaders were used to accurately spread the material on the prepared medium. The prepared plates were incubated in incubators using appropriate culture conditions for each group of microorganisms. Mesophilic and spore-forming bacteria were incubated at 37°C for 24 hours each time. The same incubation time was used for thermophilic bacteria, which were incubated at 55°C. Fungi were incubated for 5 days at 26°C.

### Examination of the morphology of bacteria

Gram staining is used to differentiate intact, morphologically similar bacteria into two groups based on the color of the cell after staining. This method is useful for visualizing the shape of the cell under study, determining its size and other structural details. It is a source of preliminary taxonomic information about the strain under study [12]. In order to observe bacterial morphology under a light microscope, standard Gram staining was performed. The technique used allows the bacteria to be distinguished into Gram-positive (purple) and Gram-negative (pink) based on the characteristics of their cell wall. For this purpose, a small amount of bacterial colony was taken from each Petri dish, from each day of incubation and repetition, and a smear was made on a basic slide. After fixation of the slides, standard staining was performed, and observations were made on the morphology of the bacterial cell wall.

### Determination of the dry weight gain of fungi

Liquid fungal medium was poured into 100-milliliter Erlenmeyer flasks and inoculated with 0.5 ml of an aqueous suspension of fungal spores. The suspension was prepared from a 7-day-old fungal culture on an agar slant by rinsing it with 5 ml of sterile water. After 10 days of culture at 22°C, the mycelium was separated from the medium on a blotting paper filter, washed thoroughly with water and dried to a constant weight at 80°C.

### Fungi identification

Fungi were identified based on their cultural morphology and microscopic features of their spores and hyphae according to a simplified key to fungal classes (Class: Mycota) and chlorophyll-free fungal-like organisms.

# 3. RESULTS

## 3.1 Number of bacterial colonies grown

After the incubation time, colonies were counted in 3 replicates for each group of bacteria. This enabled the calculation of the arithmetic mean and the presentation grown colonies on the graphs.

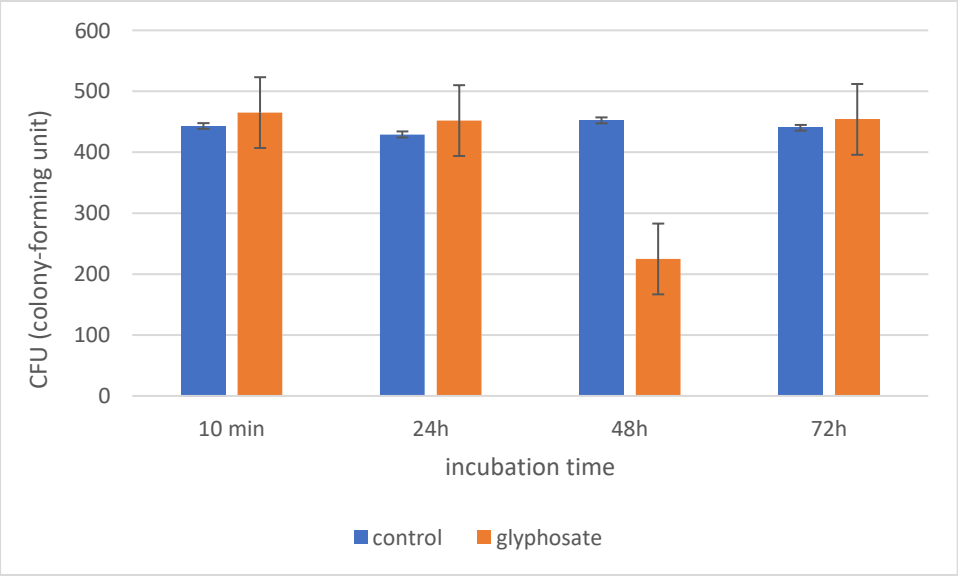



Fig. 1. Average number of mesophilic bacteria

A comparison of the variation in mesophilic bacteria counts at successive time intervals is shown in Figure 1. The number of mesophilic bacterial colonies in the herbicide-treated groups after 10 minutes, as well as on the first day of incubation, increased slightly compared to the groups without the addition of glyphosate. For mesophilic bacteria, significant differences were observed compared to the control group on the second day of incubation (a 51% decrease compared to the control). After a period of decline, the number of bacteria increases after another day of incubation with glyphosate in the compost.

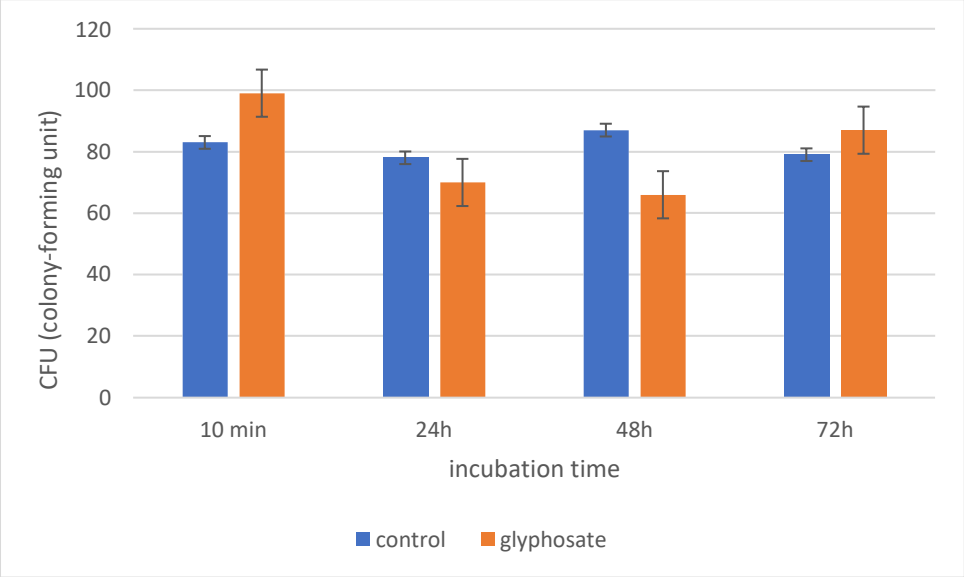



Fig. 2. Average number of thermophilic bacteria

A comparison of the variation in the number of thermophilic bacteria at successive time intervals is shown in Figure 2. In the case of thermophilic bacteria count determinations, a decreasing population of bacterial colonies is observed for the first 2 days of incubation. An increase in the number of colonies occurs, as with other groups, on the 3rd day of incubation. Such fluctuations in abundance may be influenced by the short-term pasteurization process. As temperature and exposure time increase, glyphosate degradation increases, resulting in a decreasing effect [31].

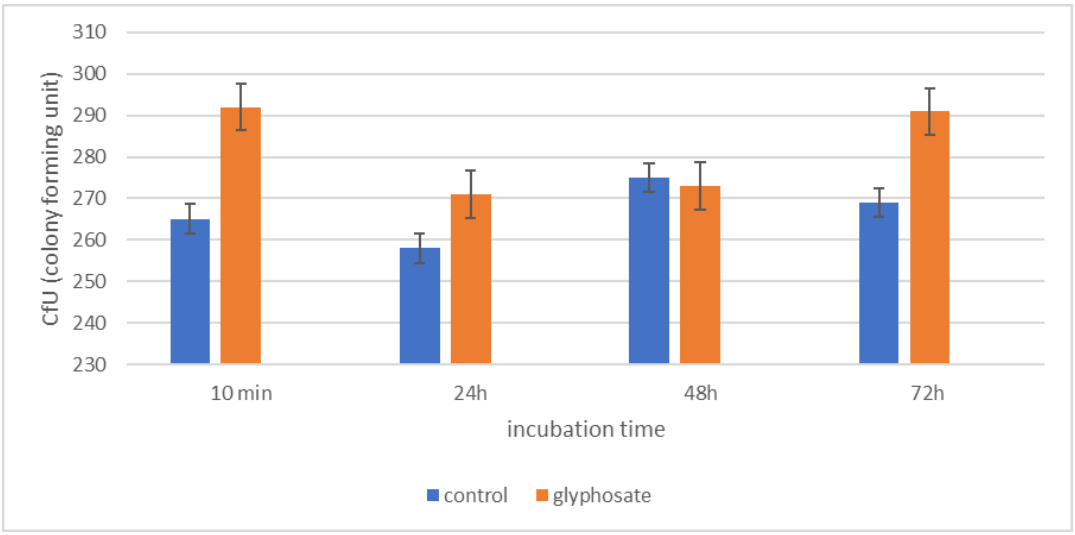



Fig. 3. Average number of saprobic bacteria

A comparison of the variation in the number of saprobic bacteria at successive time intervals is shown in Figure 3. In this group, an increase in the number of bacterial colonies is observed after 24h of incubation and on day 3 of incubation. The period of decrease in the number falls at 48h of incubation.

No significant differences were observed for saprobic bacteria compared to the control group (8% decrease). The higher quantitative stability may be due to the ability of sporulating bacteria to produce spore forms, which allow the bacteria to survive adverse environmental conditions. These microorganisms can become resistant to external influences by reducing the permeability of the cell wall or changing the enzymatic binding sites of EPSPS. On the third day of incubation, the bacteria in the experimental groups again reached an abundance that exceeded that of the control groups.

## 3.2 Microscopic observations of bacteria

For light microscopy observations, bacteria were stained using standard Gram staining methodology and then observed under an oil immersion objective.

**Observation of mesophilic bacteria treated with glyphosate**.

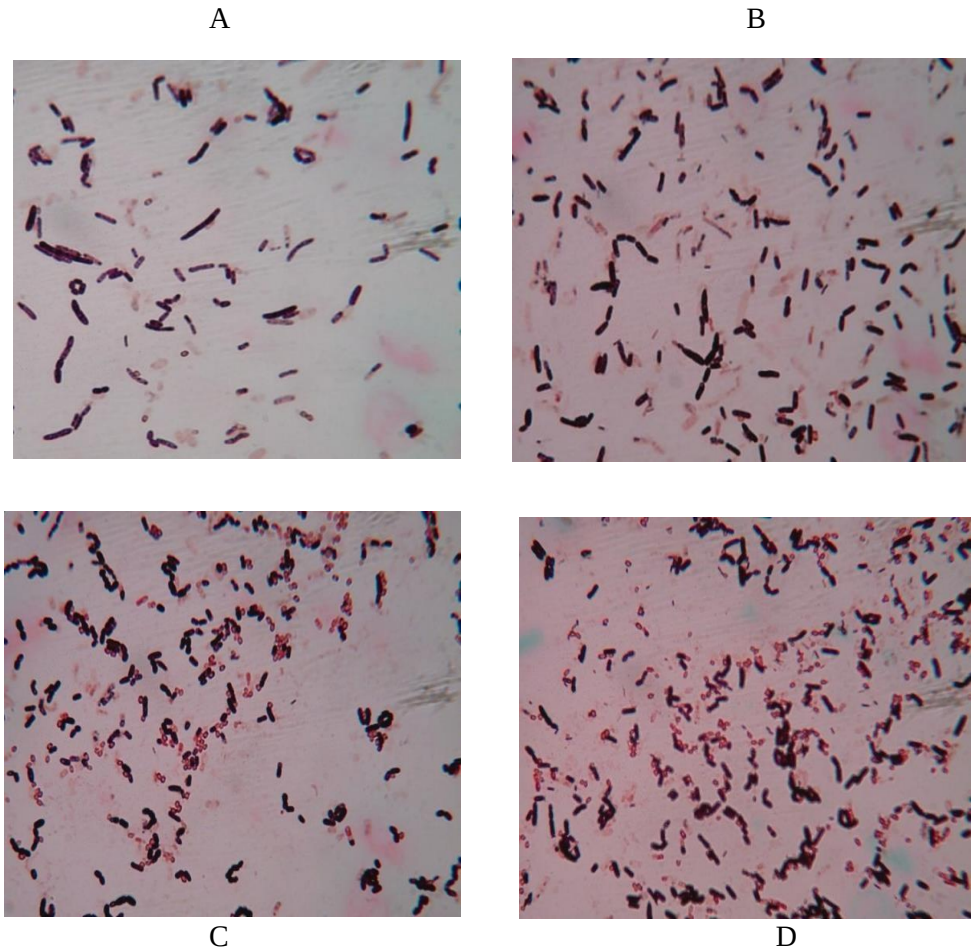



Fig. 4. Bacteria treated with glyphosate (A-compound incubated 10 minutes, B-24h, C-48h, D-72h)

Light microscope images showing the morphological structure of mesophilic bacteria at different incubation times are shown in Figure 4. Microscopic observations using an immersion objective showed no significant differences in the morphological structure of bacteria classified as mesophilic bacteria.

Bacteria of the genus Bacillus are the predominant form of mesophilic bacteria. No changes in cell wall fragmentation or deformation of morphological forms were observed in Gram-positive bacteria. Due to the specific structure of the cell wall of Gram-positive bacteria, a direct effect on the morphological structure of the cell wall is considered unlikely.

**Observation of thermophilic bacteria treated with glyphosate.**

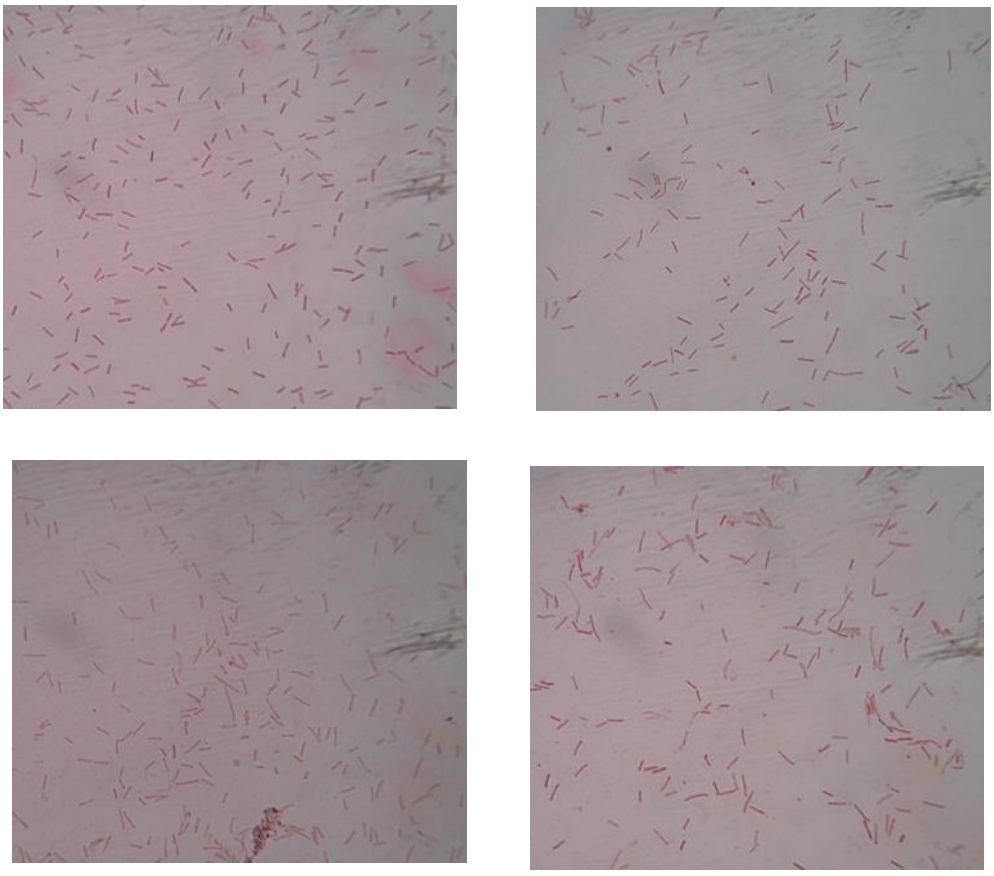



Fig. 5. Bacteria treated with glyphosate (A-compound incubated 10 minutes, B-24h, C-48h, D-72h)

Light microscope images showing the morphological structure of thermophilic bacteria at different incubation times are shown in Figure 5. Microscopic observations using an immersion objective showed no significant differences in the morphological structure of bacteria classified as thermophilic. No changes in cell wall fragmentation or deformation of morphological forms were observed in Gram-negative bacteria. Due to the obesity of the outer membrane (OM) of Gram-negative bacteria, a direct effect on the morphological structure of the cell wall is considered unlikely. It is an additional permeability barrier for the entry of hydrophilic compounds, preventing changes that affect membrane integrity.

**Observation of saprobic bacteria treated with glyphosate.**

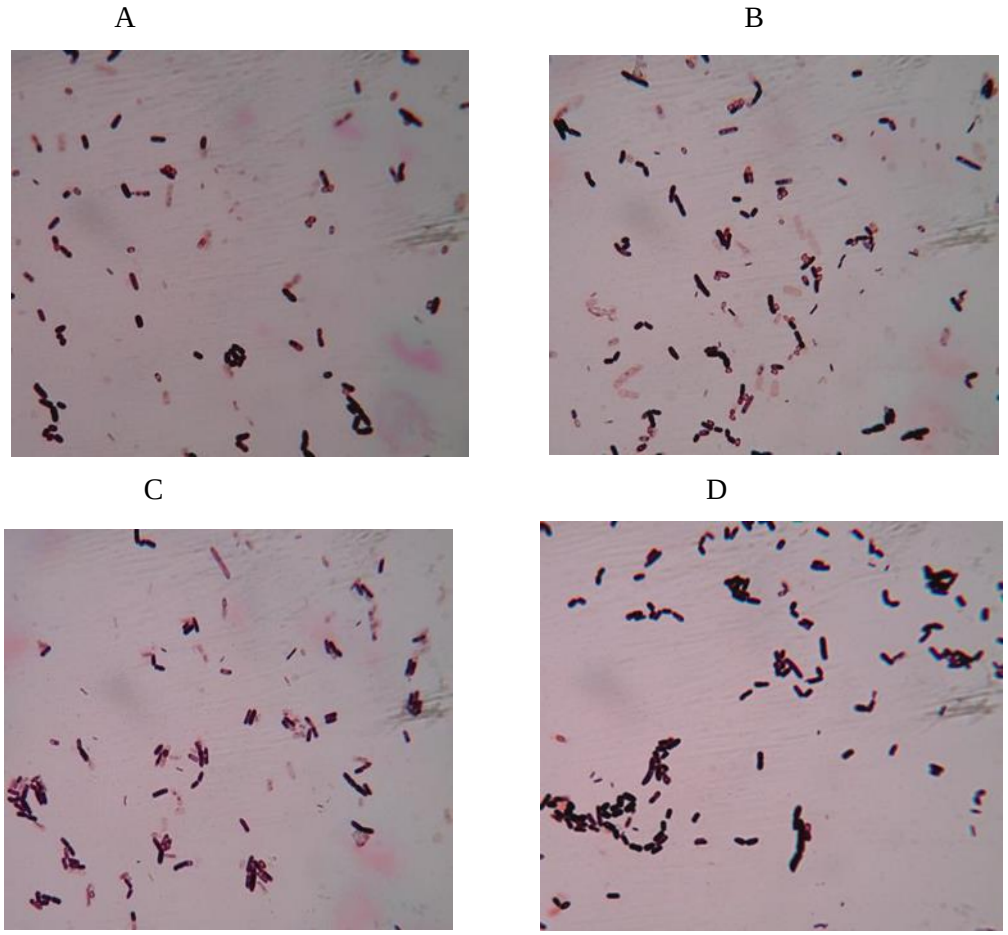


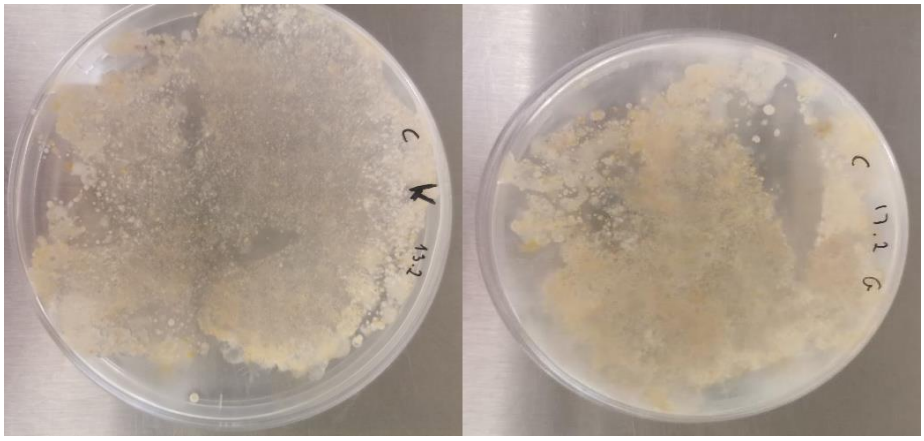

Fig. 6. Bacteria treated with glyphosate (A-compound incubated 10 minutes, B-24h, C-48h, D-72h)

Light microscope images showing the morphological structure of saprobic bacteria at different incubation times are shown in Figure 6. Microscopic observations using an immersion objective showed no significant differences in the morphological structure of bacteria classified as sporulating. No changes in cell wall fragmentation or deformation of morphological forms were observed in Gram-positive bacteria. The obesity of the survival forms, which consist of the outer spore (OSC) and inner spore (ISC), cortex, germ cell wall and medulla. The OSC contains alkaline fractions that determine alkali resistance, while the ISC contains alkali-soluble proteins. OSC and ISC are believed to be important barriers to the entry of many substances.

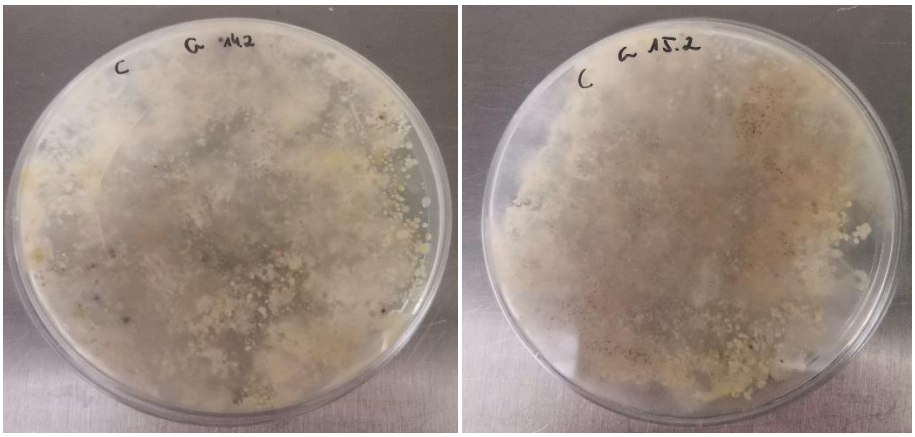
## 3.3 Microscopic observations of fungi

Microscopic observations of fungi were conducted using Lugol's solution under a 40x magnification objective.

A B



C D



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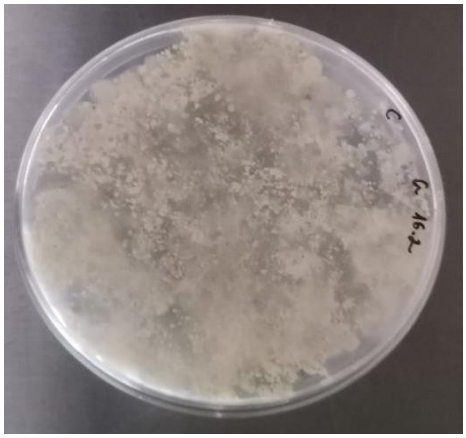

Fig. 7. Fungi treated with glyphosate (A- control group, B-compound incubated 10 minutes, C-24h, D-48h, F-72h)

Microscopic observations of mesophilic fungi did not show any changes in the morphological structure of the recognized species (filaments and spores). Determination of the dry weight gain of fungi allowed to conclude that the addition of glyphosate and the process of accumulation affect the dry weight gain of fungi. The data showed that the presence of glyphosate reduced biomass production compared to the control ($p < 0.05$), causing an inhibition of fungal growth ranging from 4,7% (48h incubation) to 8,6% (72h incubation).

## 4. DISCUSSION

In ecosystems there is a flow of energy and circulation of matter, including nutrients. Microorganisms are a vital component of biodiversity and play a key role in maintaining soil processes [1]. Many species of microorganisms, due to their occupation of the first trophic level in the food chain, are a key element of nutrient cycling in the ecosystem [22]. Glyphosate increases microbial activity as microbes break it down as a source of carbon, nitrogen, and phosphorus. However, this is a short-lived effect [14]. Many studies conclude that glyphosate affects the number of bacteria, fungi and *Actinomycetes*, causing an increase in colony numbers. However, some studies suggest that glyphosate may have indirect effects on soil microorganisms and soil structure in agricultural ecosystems. A study conducted by Newman et al. demonstrated the effect of glyphosate on soil bacteria, resulting in a reduced number of Acidobacteria in corn and soybean crops [17]. In the long term, the decreasing number of bacteria may weaken the ability of soil microorganisms to carry out specific biogeochemical transformations that determine the growth of soil fertility [22]. Some studies show that glyphosate alters soil structure and microbial diversity, reducing microbial populations but increasing phytopathogenic fungi. This may be because the addition of glyphosate significantly increases the rate of microbial respiration [26]. There is evidence of increased fungal activity and populations in glyphosate-treated soil. This stimulation may be since fungi are the main degraders of glyphosate [5].

Profiling of bacterial communities isolated from compost samples was aimed at determining the effect of glyphosate on species diversity and community composition as a function of incubation time with glyphosate. The analyses conducted showed no effect of glyphosate on species diversity, as the types and genera present in the control and glyphosate-treated groups were the same. However, differences were found in quantitative fluctuations between incubation times. In the conducted study, it was observed that the number of bacterial populations (mesophilic, thermophilic and spore-forming) fluctuates. The direct addition of glyphosate increased the growth of all groups of microorganisms compared to the control groups [2].

For mesophilic and saprobic bacteria, the results show similar relationships between glyphosate dose and incubation time. In these groups, an increase in bacterial colonies is observed after 24h incubation and on the 3rd day of incubation. The period of decline in abundance is 48h of incubation. In the case of mesophilic bacteria, significant differences were observed compared to the control group (a decrease of 51%, and in the case of saprobic bacteria by 8%). The higher quantitative stability may be due to the ability of the sporulating bacteria to produce surviving forms, which allow the bacteria to survive adverse environmental conditions. These microorganisms can become resistant to external influences by reducing cell wall permeability or altering the enzymatic binding sites of EPSPS [33]. Sensitivity or resistance can vary within the same bacterial taxon, even at the species level. Bacteria can easily become resistant to glyphosate as a result of a single mutation in the EPSPS active site, which would result in a higher proportion of glyphosate-resistant bacteria in glyphosate-exposed environments [28]. On the 3rd day of incubation, bacteria in the experimental groups again reach an abundance that

exceeds that of the control groups. After a period of decline, the number of bacteria increases after another day of incubation with glyphosate in the compost. This relationship can be explained by the ability to break down glyphosate into simpler compounds and use it as a source of an important biogenic element - phosphorus [3]. The effect of glyphosate on bacterial abundance is dose-dependent, and the increased effect can be explained by the rapid enrichment of bacteria using this compound as a nutrient. [20] For thermophilic bacterial abundance determinations, a decreasing population of bacterial colonies is observed for the first 2 days of incubation. And an increase in colonies occurs such as in the case of other groups, on the 3rd day of incubation. Such fluctuations in abundance may be influenced using a short-term pasteurization process. Studies report that temperature affects the uptake and/or translocation of glyphosate by many species of microorganisms. With increasing temperature and exposure time, the degradation of glyphosate increases which results in a diminishing effect [32]. The effect of glyphosate on bacterial abundance is dose-dependent, and the increased effect can be explained by the rapid enrichment of bacteria that use the compound as a nutrient [33]. In this case, the application of glyphosate can change (increase) the microbial activity of the soil. Increased microbial activity can be beneficial or detrimental to plant growth, soil microbial ecology and soil quality [11]. Beneficial effects include optimal growth of many plant species due to nutrient availability as a result of glyphosate mineralization via soil microorganisms. Increased microbial activity may be responsible for increased susceptibility to diseases and pests, caused especially by the growth of excessive fungi with phytotoxic properties [37].

Microbiological analyses of fungi identified species of the genus *Saccharomycetales*, including *Galactomyces geootrichum* and *Candida.* In addition, species belonging to the genera *Fusarium* and *Cladosporia* were identified in the compost. Microscopic observations of fungal microorganisms showed no significant differences in fungal morphology; however, it was observed that the addition of glyphosate would affect the decrease in fungal dry weight with longer exposition time. Some studies suggest negative effects of glyphosate in high doses on soil fungal biomass [9]. The high variability of soil fungi often gives inconclusive results depicting the impact of glyphosate herbicides. It is worth mentioning that the high variability of soil fungi often yields inconclusive results showing the impact of glyphosate herbicides. In one study by van Bruggen et. al, it was observed that the addition of glyphosate affected the decrease in fungal dry weight [35]. Over a two-year period, no effect of glyphosate was observed on fungal biomass in the soil. However, a more thorough analysis made it possible to detect temporal changes in fungal biomass after herbicide application.[31] A negative effect on fungal biomass was observed for species present in low abundance. Also, a study conducted on *Purpureocillium lilacinum* showed comparable results, suggesting a negative effect of glyphosate. After four weeks, it was found that the presence of glyphosate significantly reduced biomass production compared to the control causing an inhibition of fungal growth in this species ranging from 39.3% to 84.1% [34].

## 5. CONCLUSION

The study, conducted under controlled conditions, shows the changes in the bacterial community that occurred during composting in the presence of glyphosate. The results show that glyphosate had no significant effect on the diversity and species composition of the isolated communities. This suggests that abiotic conditions, such as temperature, pH, and decomposition stage, are more important than glyphosate in driving succession dynamics. Compost is an extremely rich environment, both in terms of available nutrients, the various physicochemical conditions created and the diversity of microorganisms. As such, it may contain species capable of degrading glyphosate that have not yet been characterized. The diversity and repetition of functions performed by microorganisms may also provide some

resilience to the microbial ecosystem, which manages to conduct organic matter degradation despite the presence of the contaminant. The increasing use of agrochemicals continues to raise concerns about negative biological effects in agricultural crops. The nature of microbial succession determines all soil processes, its current state, and properties [36]. Therefore, the elimination or reduction of a certain physiological group of microorganisms can cause changes in the biological balance, which in turn can affect its biological balance [27].

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