Milunović, I.[1*], Svjetlana Lolić[1], V. Trkulja[2], Biljana Lubarda[1] Original scientific paper

The effect of earthworms *Lumbricus rubellus* on the total count of microorganisms in soil treated with pendimethalin

Abstract

This paper examines the modus in which earthworm Lumbricus rubellus with its activity affects the total count of different physiological groups of microorganisms in soil treated with herbicide pendimethalin. The experiment was carried out in ten glass containers with a soil substrate, whereby one of them was the control one, and each group of three containers was treated with different concentration of pendimethalin. A concentration of pesticide of 5 µl/kg, which is recommended by the declaration, one lower concentration of 3 µl/kg and one higher of 7 µl/kg were used. The number of physiological groups of microorganisms is determined from the three containers treated with different concentrations of pendimethalin 48 hours after the treatment, and from the other three containers 21 days after the treatment. Two days after the application of different concentration of pesticide, a significant statistical decrease was found in the number of fungi and cellulotical microorganisms in soil, while the total number of heterotrophic bacteria as well as microorganisms which participate in different stages of nitrogen metabolism increased. However, 21 days after the treatment a significant decrease in the total number of heterotrophic bacteria in all treated containers comparing to the control one was noticed, where earthworms with their activities partially attenuated the negative effect of pendimethalin on soil bacterial flora. They also had a positive effect on the number of fungi, actinomycetes and cellulolytic microorganisms while the presence of earthworms had no significant influence on the number of aminoautotrophs, oligonitrophils and free-living diazotrophs. It was shown that earthworms with their activities attenuate the negative effect of pendimethalin on bacterial flora, actinomycetes and fungi, which points out to their possible use in processes of bioremediation.

Key words: *Earthworms, Lumbricus rubellus, number of microorganisms in soil, pendimethalin*

Introduction

Soil represents one of the most important resources not only in agricultural production but also in terms of preservation of ecosystem as a whole. It is slowly formed in the long process of pedogenesis, but with inadequate usage it is often very quickly destroyed and damaged (Kljajić *et al.*, 2012). The quality of soil depends, to a significant extent, on the activity of microorganisms whose enzymes have a central role in the soil metabolism. The number of microorganisms ranges from few millions to few billions per gram of absolutely dry soil (Milošević, 2008). They are extremely significant for pedogenesis, the creation and maintenance of fertility of the soil. The total number of microorganisms in soil can be used as an indicator of its general biological activity (Milošević *et al.*, 2001; Imberger & Chiu, 2002; Radivojević *et al.*, 2003).

In intensive agricultural production, there is high usage of pesticides which come into the

[1] Doc. dr. sc. Igor Milunović, doc. dr. sc. Svjetlana Lolić, doc. dr.sc. Biljana Lubarda, Prirodno-matematički fakultet, Banja Luka, Dr. Mladena Stojanovića 2, 78000 Banja Luka, Bosna i Hercegovina

[2] Prof. dr. sc. Vojislav Trkulja, JU Poljoprivredni institut Republike Srpske, Knjaza Miloša 17, 78000 Banja Luka, Bosna i Hercegovina
Corresponding author: i.milunovic@inspektorat.vladars.net

soil where they affect the autochthonous soil microbiota in different ways. Pesticides affect the majority of populations of microorganisms in the manner that they change the number, activity and decrease or increase growth rate (Johnsen *et al.*, 2001). Some physiological groups of microorganisms have the ability of dissolving some pesticides and using them as a source of biogenic elements which leads to growth of microbic population (Janjić, 1996). Some microbes particularly bacteria may utilize pesticides as a source of nutrients facilitating their growth and survival, while sensitive species may be impaired or decimated by pesticides. These ecological alterations may trigger a cascade of indirect effects. For example, elimination or reduction of certain microbial populations by pesticides may release pesticide-tolerant microbes from competition for shared resources and thereby promote their growth and survival. Similarly, some protozoan species prey on bacteria and their suppression by pesticides may facilitate survival of bacterial prey. These processes may lead to dramatic shifts in microbial communities that may interfere or have little effect on microbial functions. On the other hand, the effect of pesticide on microorganisms can be lethal and can lead to change of biodiversity of normal microbiological community in soil (Muturi *et al.*, 2017; Jacobsen & Hjelmsø, 2014).

The impact of pesticides on a soil microbial community depends on their toxicity, which determines hazard, and on their fate in the soil, which is governed by several processes such as adsorption, leaching, run-off, degradation, volatilization, plant uptake, etc. (Pose-Juan *et al.*, 2017) and their activities thereby influencing the microbial ecological balance in the soil and affecting the productivity of soils (Kaur *et al.*, 2014)

The activity of soil invertebrates, especially earthworms, affects the number of microorganisms in soil, since in the process of digestion they grind and eject organic matter which creates larger superficial area for the action of microorganisms (Edwards & Bohlen, 1996).

The aim of this research was to determine impact of pendimethalin on the number of some physiological groups of microorganisms in soil in controlled laboratory conditions as well as to determine modus in which earthworm *Lumbricus rubellus* (Hoffmeister, 1843) affects the population of microorganism in treated soil.

Material and methods

Pendimethalin is a selective herbicide from the dinitroanaline group intended for pre-emergent control of annual grassy and broadleaf weeds (Tomlin, 2000). It is primarily used on different agricultural crops - 92 % of total usage (Janjić, 1996; Chikoye *et al.*, 2014; Sebiomo *et al.*, 2011), but in urban area as well. It is applied on lawns, paved areas, paths, yards, in industrial zones and on golf courses (WSDA, 2004). Its effect is such that it stops the process of cell division which consequently stops the growth of weeds in the stage of growing and germination.

Pendimethalin ($C_{13}H_{19}N_3O_4$) (Figure 1) is poorly volatile and mobile herbicide. Its persistence is influenced by the manner of cultivation, as well as conditions of moisture, temperature and the type of soil (Savage & Jordon, 1980; Schleicher *et al.*, 1995). It is the most persistent in the clay soil and is active longer if it is incorporated rather than applied on superficial soil where it gradually evaporates (Savage & Jordon, 1980; Zimdahl *et al.*, 1984). It is relatively stable and immobile in substrate, but it slowly degrades under water photolysis, with the decaying period of 21 days, while the aerobic soil metabolism lasts about 172 days. Pendimethalin is not toxic to bees, birds and mammalians but it is highly toxic to fish and water invertebrate (Zulalian, 1990) so it should not be used near streams and lakes.

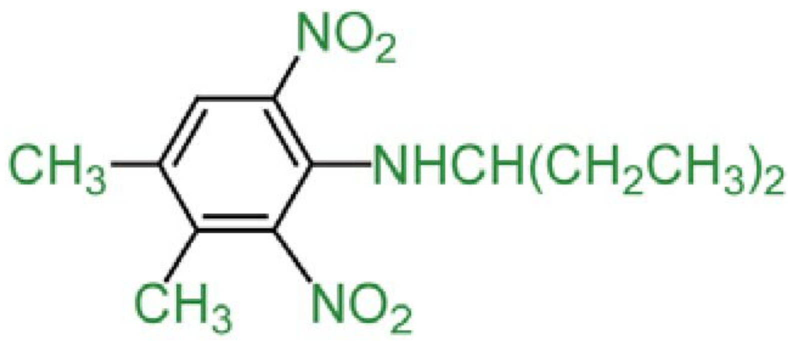


Figure 1 Pendimethalin structural formula (Tomlin, 2000)

Experiment was carried out in glass containers measuring 20x20x10 cm, which were divided with a glass barrier into two equal chambers. Each chamber was filled up with 1500 g of soil substrate prepared according to the ISO 17512-1 standard. The substrate consisted of 70 % kaolin clay (clay with 30 % of kaolin), 20 % quartz sand fine granulation and 10 % of peat. In order to determine the influence of pendimethalin on population of microorganisms, the soil in the chambers was treated with pendimethalin (herbicide Stomp 330 E manufacturer "BASF", Germany) with three different concentrations. The concentration of pesticide of 5 µl/kg, corresponded to the recommended application dose, and one lower concentration of 3 µl/kg and one higher concentration of 7 µl/kg were used. The number of individual groups of microorganisms is determined from three chambers treated with different concentration of pendimethalin 48 hours after the treatment, and from other three chambers 21 days after the treatment. In order to determine whether there is interaction between activity of earthworms and the number of microorganisms in pesticide contaminated soil 10 adult *Lumbricus rubellus* earthworms were placed in three chambers with mentioned concentrations of pesticide and analysis of the substrate was done 21 days after the treatment. A chamber containing soil substrate only, without application of herbicide and without presence of earthworms, was used as control. In all chambers even dispersion of herbicide and even level of moisture of substrate at 60 % soil water retention capacity was done with an electric mixer. The experiment was held in the laboratory of the Faculty of Natural Sciences and Mathematics in Banja Luka, and microbiological tests were done in the Laboratory for Microbiology at the Institute for Soil in Belgrade.

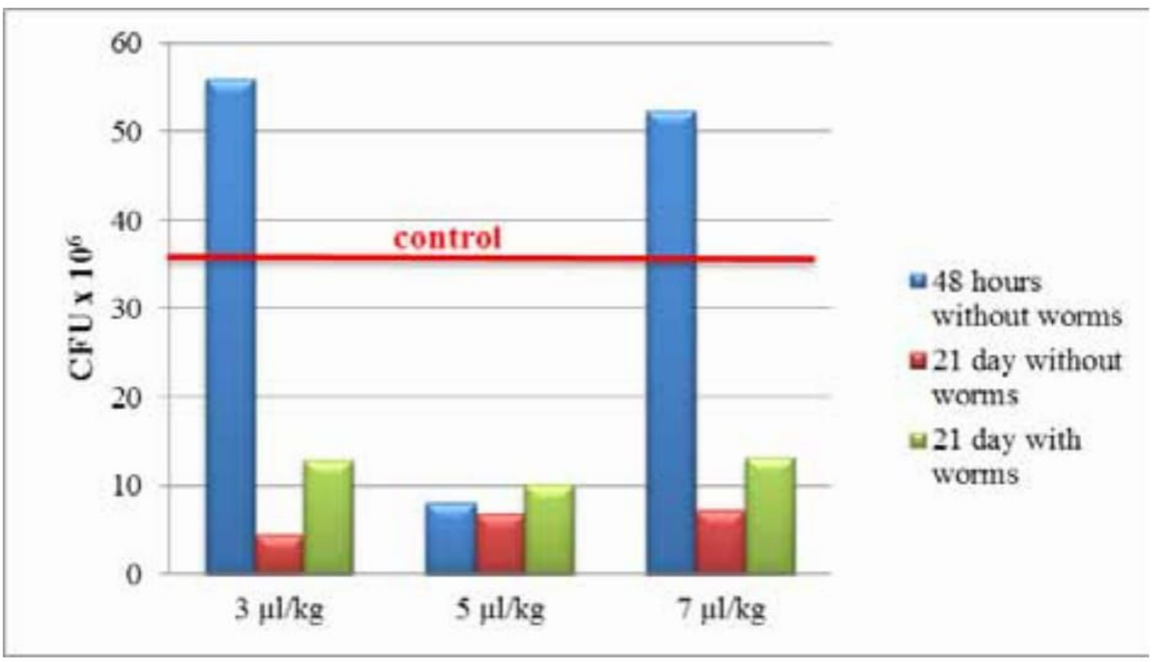
Sterile accessories were used to collect average specimen per treatment with the weight of about 300 g, which was formed from specimens collected from 5 points: one from the middle of chamber and four from the middle of diagonal lines, with one point that covers the entire depth of the chamber. For determination of the number of selected physiological groups of microorganisms indirect breeding method with three repetitions was used. The total number of heterotrophic microorganisms, number of fungi, actinomycetes, cellulolytic microorganisms, aminoautotrophs, oligonitrophils and free-living diazotrophs was determined (Sarić, 1992).

The total number of heterotrophic bacteria was determined by method of agar plates with soil extract. 1 ml of 10-6 dilution was cultured and after 7 days of incubation on 28°C all the grown colonies were counted. The number of fungi in soil was determined by indirect method of agar plates on Czapek agar. 1 ml of 10-4 dilution was cultured and after 3 days of incubation on 28°C all grown colonies of fungi were counted. Actinomycetes were determined by the method of agar plates on base with sucrose by Krasiljnikov (1965). 1 ml of 10-4 dilution was cultured and after 7 days of incubation on 28°C all grown colonies were counted.

The number of cellulolytic microorganisms was determined on Waksman-Carey`s nutrient media on which a sterile filter paper was placed as a source of carbon. The complete surface of filter paper was cultured with 0.5 ml of 10-4 dilution. The inoculated nutrient media had been incubating for 21 days in thermostat on the temperature of 28°C. The number of aminoautotrophic bacteria is determined on a starch-ammonium agar, where it is sown 1 ml of dilution of 10-5 and after 7 days of incubation at 28°C grown colonies were counted. The number of oligonitrophils was determined on Ashby`s nutrient media with mannitol by culturing with 1 ml of 10-5 dilution and the colonies were counted after 7 days of incubation at 28°C. The number of free-living diazotrophs was determined on Fyodor`s nutrient media (Govedarica & Jarak, 1996). A 0.5 ml of 10-5 dilution was cultured and 5 days after the incubation in thermostat at 28°C all grown colonies were counted.

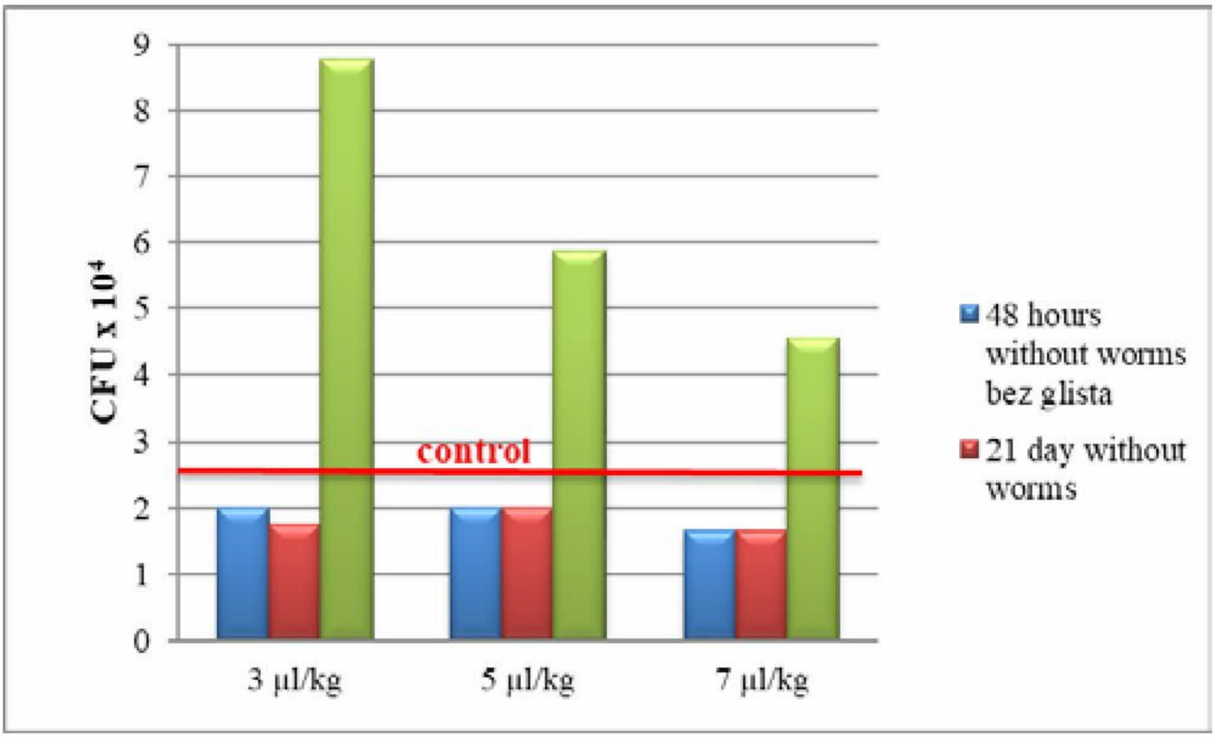
Results and discussion

The usage of pendimethalin has had a significant impact on the total number of heterotrophic bacteria in soil (Graph 1). There was a significant increase of the total number of heterotrophic microorganisms in soil 48 hours after the application of lower and higher concentration of pendimethalin with the recommended concentration. This was not the case in the control experiment where the soil was neither treated with herbicide nor it contained any earthworms. However, 21 days after the application of herbicide the total count of bacteria and fungi in all containers decreased considerably, regardless of applied concentration. Thereby in containers with earthworms, the number of total heterotrophs was significantly higher compared to containers without worms. Domsch & Grams (1983) showed that microorganisms had „benefit"of herbicide transformation at the beginning due to decaying process of complex molecules in which nitrogen and carbon are released. However, after the initial growth, many authors record decrease in the total number of microorganisms after the application of different kind of pesticide (Očinikova, 1976; Očinikova & Orlov, 1980; Đorđević *et al*., 1994; Govedarica *et al*., 1996; Tamburić & Lević, 1995; Radivojević, 1998). Still, the doubled total count of heterotrophic bacteria, which significantly affect the quality of soil itself, in the soil with earthworms compared to the soil without these organisms, shows that earthworms with their activities partially mitigate the negative effect of pesticide on microbiota.



Graph 1 The number of total heterotrophic bacteria in the soil depending on the concentration of pendimethalin and the presence of *Lumbricus rubellus*

The number of fungi in soil 48 hours after the treatment with the pesticide with the range of all three applied concentrations significantly decreased compared to the number of fungi in the control container (Graph 2). The highest decrease was noted during the usage of the highest concentration of pesticide. In soil without earthworms the number of these groups of organisms did not significantly change even after 21 days after the treatment was carried out. However, in containers with earthworms 21 days after pesticide treatment there was a significant increase in the number of fungi in soil. In the container in which the concentration of pendimethalin of 3 μl/kg was applied the number of fungi tripled, i.e. it increased from 26.700 CFU/g to 87.700 CFU/g. With the application of the recommended concentration of pesticide their number in soil doubled, and with the application of the higher concentration the number increased for 58% in relation to the number of fungi in the control container.

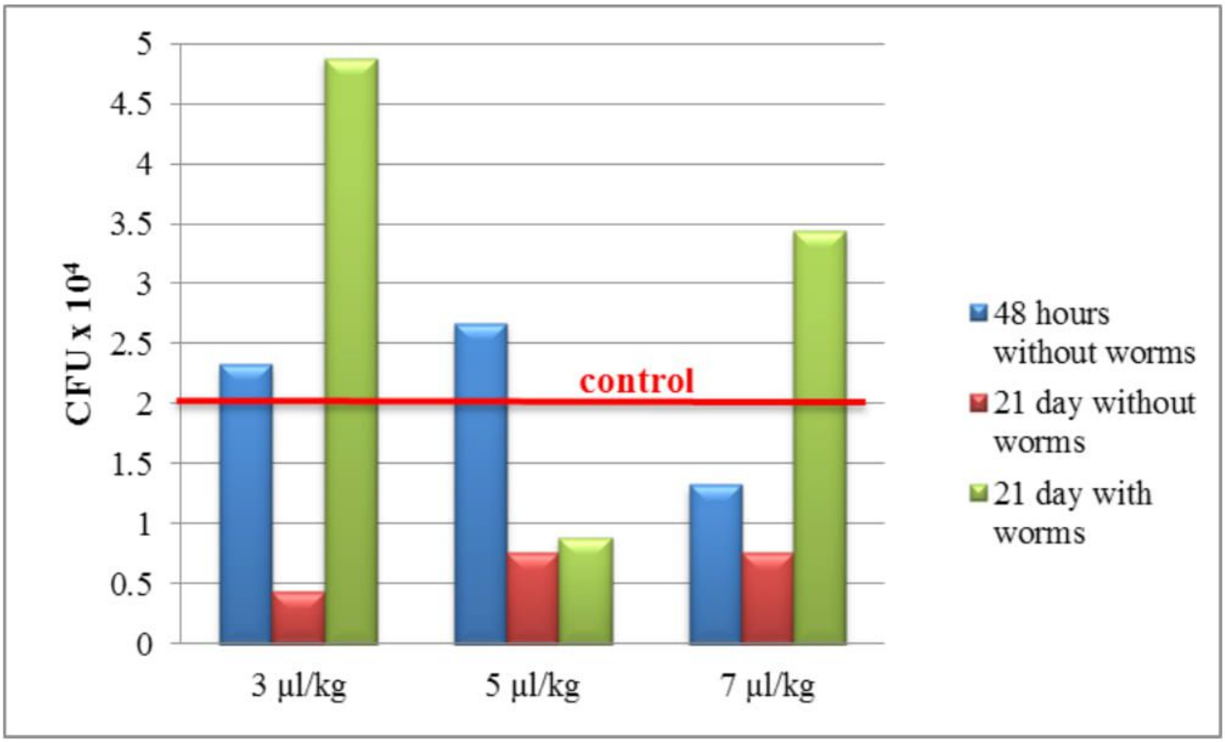


Graph 2 The number of fungi in soil depending on the pendimethalin concentration and the presence of *Lumbricus rubellus*

Grossbard & Devies (1976) state that fungi compared to other groups of microorganisms are more resistant to application of pesticide, primarily because they have enzyme systems which are capable of decaying molecules of pesticides and in that way they use it as sources of biogenic elements for their physiological processes. Also, the similar results were reported by Radivojević & Stanković-Kalezić (2000), who determined that after the initial inhibiting effect of pendimethalin there was a renewal of population of fungi because of their participation in the process of decaying of molecules of pendimethalin.

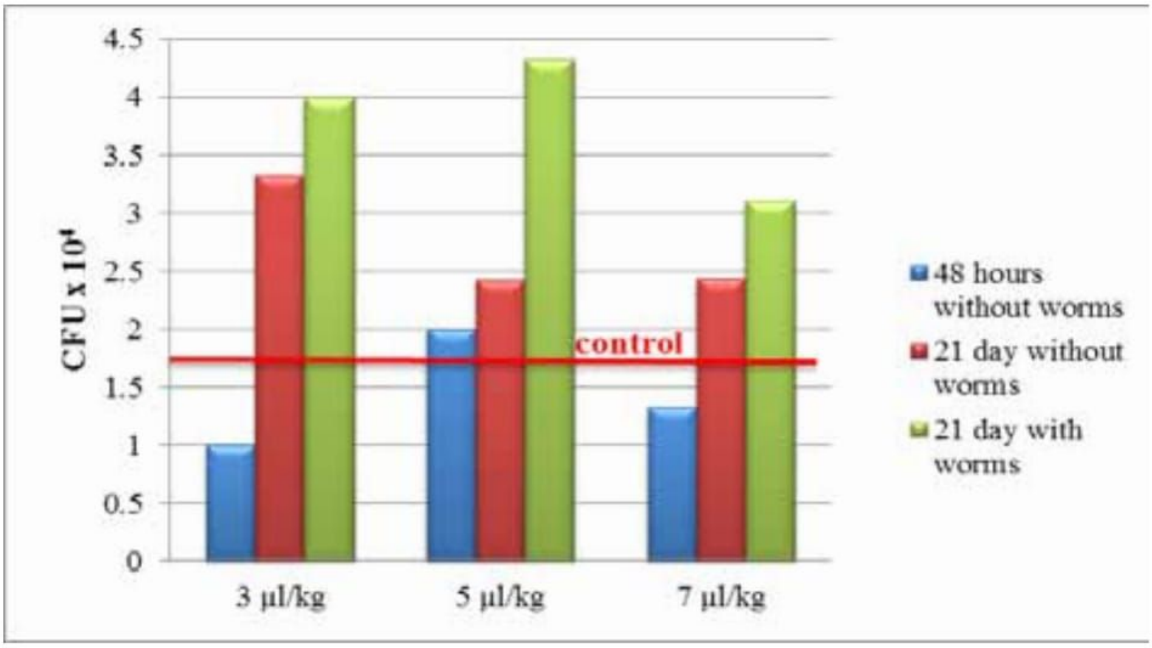
The number of actinomycetes in soil 48 hours after the pendimethalin treatment significant decreased only during the application of the highest concentration of pesticide (Graph 3). 21 days after its application there was a significant decrease in the number of microorganisms of this group in all containers without earthworms, regardless of the concentration of pesticide applied. However, in the containers with earthworms the number of actinomycetes was decreased twice as much only in containers in which recommended concentration of pendimethalin of 5µl/kg was applied, while the application of higher and lower concentration of pesticide brought about a significant increase in their number. Jarak & Govedarica (2003) point out that these groups of microorganisms have the capability of decaying even the most resistant types of humus, pectin, lignin, as well as pesticides and other xenobiotics. The conducted experiment shows that their activity depends on the concentration of applied pesticide, but also that earthworms with their activities have a positive effect on their metabolism, which results in increase in their number in soil and within it brings to improvement of quality of soil itself.

Similar results were obtained Kočárek *et al.* (2016) such as a concluded that the number of microorganisms (bacteria, fungi, and spores) at the end of the experiment ranged in a similar interval as at its onset and - the number of actinomycetes increased significantly at the end of the experiment, however, no difference was found between the treatments at the beginning as well as at the end of the experiment.



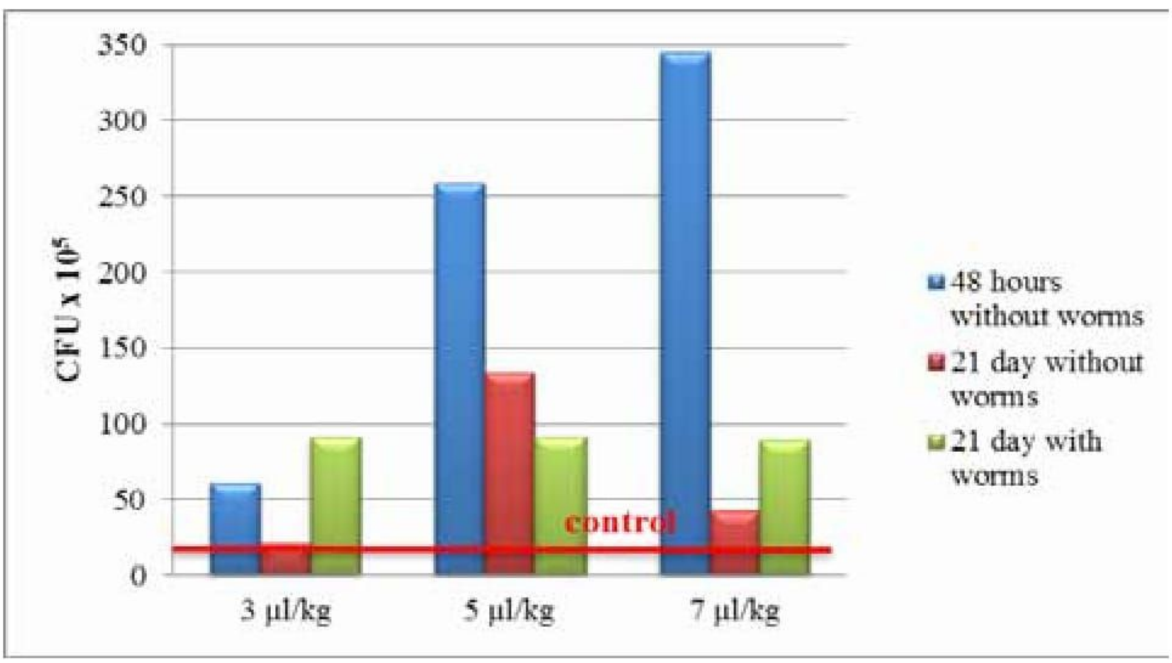
Graph 3 The number of actinomycetes in the soil depending on the concentration of pendimethalin and the presence of *Lumbricus rubellus*

The usage of pendimethalin in soil brought about an increase in the number of microorganisms which have the ability of decaying cellulose (Graph 4), compared to the control specimen. 48 hours after the application of the lowest concentration of pesticide their number decreased, while during the usage of higher concentrations a statistically significant divergence related to control specimen (p=0,05) was not noted. However, 21 days after the treatment there was a significant increase in their number, especially in containers which contained earthworms, in which the number of cellulolytic microorganisms doubled compared to the control container. Therefore, after the initial adjustment stage, pendimethalin had a positive effect on the number of cellulolytic microorganisms in soil, which was further enhanced by the activity of earthworms.



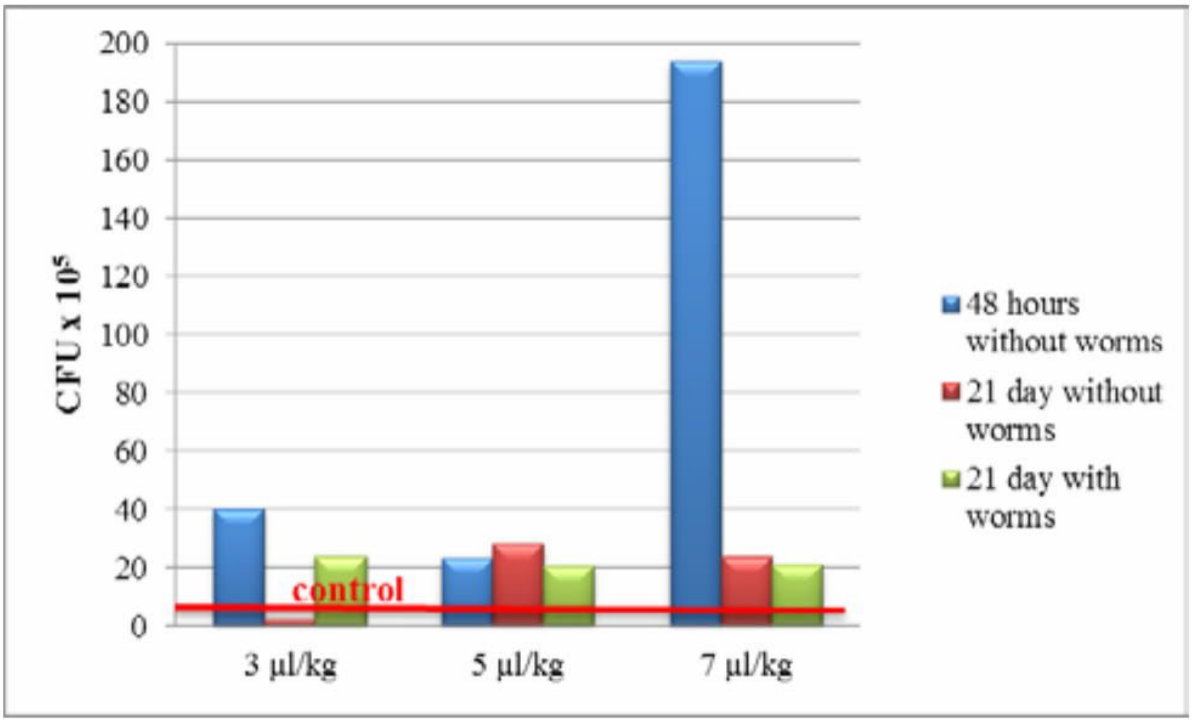
Graph 4 The number of cellulolytic microorganisms in the soil depending on the concentration of pendimethalin and the presence of *Lumbricus rubellus*

The number of aminoautotrophs which use nitrogen from inorganic compounds in their nutrition has statistically increased (p=0.05) in all containers compared to the control except during the application of the lowest concentration of pendimethalin 21 days after the treatment (Graph 5). The highest increase in the number occurred immediately 48 hours after the application of the pesticide, thereby the number of this group of bacteria went higher when higher concentration of pesticide was applied. Aminoautotrophs used mineral nitrogen which was released by decaying of pendimethalin, which brought to an increase in their number at the beginning of experiment. 21 days after, decreasing of the concentration of pesticide in soil decreased the number of these microorganisms, independently from the presence of earthworm in soil.



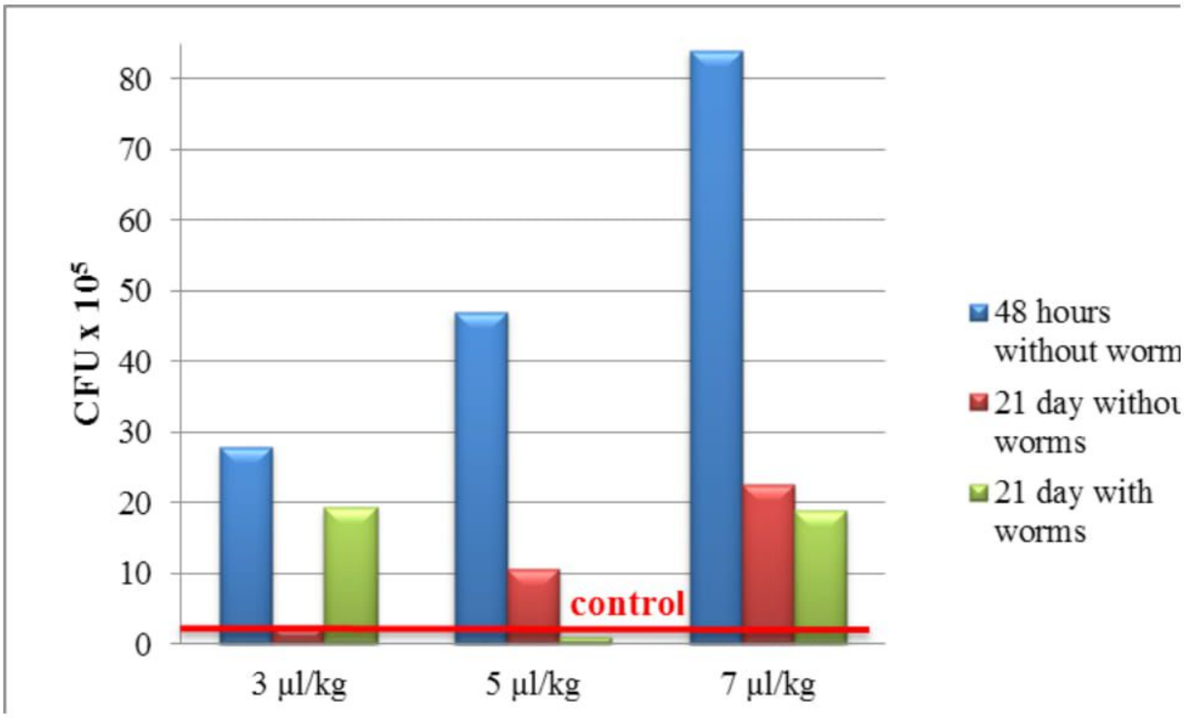
Graph 5 Amount of aminoautotrophs in soil depending on the concentration of pendimethalin and the presence of *Lumbricus rubellus*

The number of oligonitrophils in soil, which survive in environments with lower concentration of nitrogen due to their high ability to exploit nitrogen compounds, has the similar direction as the number of aminoautotrophs. In relation to the control container the number of oligonitrophils increased after the application of pendimetalin to more than 400 % in all test containers except during the application of the lowest concentration 21 days after the treatment (Graph 6). Similarly to aminoautotrophs, the number of oligonitrophils has a significant increase after the treatment itself, where their number increased even 34 times in the container with the highest concentration of pesticide. Also, the presence of earthworms had no effect on the number of these microorganisms.



Graph 6 The number of oligonitrophils in the soil depending on the pendimethalin concentration and the presence of *Lumbricus rubellus*

Exactly the same situation occurred with the free-living diazotrophs, e.g., bacteria which have the ability of non-symbiotic binding of elemental nitrogen and its transformation into mineral forms. Their number in soil considerably increased in 48 hours after the application of pendimethalin, whereby their number was higher as the concentration of pesticide was higher. 21 days after the treatment their number in soil decreased significantly, regardless to the presence of earthworms (Graph 7).



Graph 7 Number of free nitrogenous species in the soil depending on the concentration of pendimethalin and the presence of *Lumbricus rubellus*

The conducted experiment has shown that the usage of pendimethalin has a significant impact on the population of microorganisms in soil. Two days after the application of different concentration of pesticide a significant decrease in the number of fungi and cellulolytic microorganisms in soil was found while the total number of heterotrophic bacteria, as well as microorganisms that participate in different stages of nitrogen metabolism had considerably increased. Since the fungi mostly contain enzyme cellulase (Muntañola-Cvetković, 1990), and that the aminoautotrophs, oligonitrophils and free-living diazotrophs are mostly bacteria (Petrović *at al*., 1998), it can be said that the usage of pendimethalin brought about decrease in the number of fungi and increase in the number of bacteria in soil at the beginning. However, 21 days after the treatment, the total number of heterotrophic bacteria was significantly lower in all treated containers in relation to the control one. There was a positive interaction between the number of bacteria and the presence of earthworms in soil, since the total number of bacteria in containers which contained earthworms was higher in relation to the number of bacteria in containers without earthworms, regardless to the applied concentration of pesticide. Earthworms have a positive effect on the number of fungi in soil, since their number increased 21 days after the treatment in containers with earthworms compared to the control one, while the number of fungi was considerably lower in the containers without earthworms in relation to the control one. A positive effect of earthworms in the number of actinomycetes and cellulolytic microorganisms was noted while a significant impact of the presence of earthworms in the number of aminoautotrophs, oligonitrophiles and free-living diazotrophs was not recorded. Although the usage of pendimethalin after 21 days brought about an increase in the number of specific groups of bacteria (aminoautotrophs, oligonitrophils and free-living diazotrophs) in relation to the control container, the total number of heterotrophic bacteria in soil has a significant decrease. A negative effect of pesticide on whole soil bacteria flora, as well as in the number of fungi, was attenuated with activity of earthworms whereby their possible use in the process of *in situ* bioremediation of soil contaminated with this pesticide is pointed out.

Conclusion

Pendimethalin influences the bacterial flora to a significant extent as well as the fungi in soil, but the initial effect of this herbicide on these two groups is opposite: while the total number of bacteria had a significant initial growth, the number of fungi decreased. Pendimethalin has a positive effect on the number of aminoautotrophs, oligonitrophils and free-living diazotrophs, e.g., bacteria which participate in specific nitrogen metabolism, regardless to the presence of earthworms. However, longer exposure of soil to pesticide caused a decrease in the total number of bacteria which has direct negative effect on fertility of the soil itself. The activity of earthworms attenuates the negative effect of pendimethalin in soil bacteria flora and has a positive effect on the number of fungi in soil which indirectly increases the fertility of the soil and indicates its potential use in in the process of *in situ* bioremediation of soil contaminated with this pesticide.

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