Indicator Microbes of Chlorsulfuron Addition Detected by a Simplified Plate Counting Method

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Introduction

Effect of pesticides on soil biological processes

The intensification of agricultural practice has increased the application of various pesticides, among them the most frequently used herbicides. Apart from the desired toxic effects of some herbicides on weeds, however, the treatments may have a number of possible unfavourable „side effects" on organisms (AUDUS, 1964). One possibility is a direct action on one or more components of the complex populations of micro-organisms that inhabit the soil. Among them a great diversity of organisms can destroy the various types of herbicides (KECSKÉS, 1980; JOSEPOVITS et al., 1980). These include several orders of true bacteria (*Eubacteriales*), *Actinomycetes,* and the lower fungi (KUZMINSKAYA et al., 1983). Their degradative capacity, however, highly depends on the structure and the amount of the applied herbicides. According to these characters and the protected crops, some beneficial effects of herbicides (i. e. increased protein content of the crops) have also been reported (GRUZDEV & FOMIN, 1983; KECSKÉS et al., 1974).

An outstanding tendency nowdays is the development of a new herbicide family with less harmful effects in connection with a reduced applied concentration. GLEAN 75 DF (1986) – as a newly developed herbicide with a sulfon amide content – was found to be highly effective in the weed control of grain crops and linum. Although an approx. 10^{-2} times lower concentration is recommended for agriculture as compared to traditional herbicides, GLEAN 75 DF can still be 100 times more active against weeds. Soil biological adverse effects, on the other hand, have not really been considered yet.

An active role in the detoxification of chlorsulfuron by moulds and actinomycetes was reported in an earlier study (JOSHI et al., 1985). A low deleterious effect was also observed on nitrification processes and microbiological de-

hydrogenation (JUNNILA et al., 1996). In that case, however there was only a short applied period (24 hours) mentioned.

The effect of GLEAN 75 DF (chlorsulfuron) on the main microbiological groups of soil microbes is discussed in the present paper, estimated after short (3 weeks) and longer (3 months) periods of application.

Quantification of soil microorganisms

There are direct (by microscopes) and indirect (soil-dilution counting) methods for estimating special microbial groups from soils (SZEGI, 1979). Direct methods result in fast measurement, but it is impossible to clearly differentiate whether the cells are living or dead.

Recent developments of molecular techniques have enabled scientists to look at specific aspects of microbial biology and microbial genetics (PICKUP, 1991). The study of microbial ecology, however, still relies greatly on the use of the more traditional methods, such as dilution plating on agars, most probable number (MPN) assays, respiration measurements and perhaps the acetylene reduction test (nitrogen fixation).

A great diversity on the sensitivity of the various groups of microbes was found against some abiotic environmental factors (BIRÓ et al., 1995). It was also possible to find the most effective herbicide treatments for the crownvetch cultivation and a less sensitive inocula component after a detailed *in vitro* selection of about 200 crownvetch rhizobial strains (BIRÓ & KECSKÉS, 1984). In case of pesticides and other xenobiotics, it is especially recommended to use a rapid and simple *in vitro* method (such as plate counting) to estimate and select the various groups of tolerant microbes from the soil (KECSKÉS, 1980; HELMECZI et al., 1983).

Plate counting method on solidified media was first used by NOVOGRUDSKY (1949). Without a soil dilution, however, it was difficult to estimate the real number of various microorganisms due to the rapid growth around soil particles. Making a dilution series from the soil has led to a more accurate estimation of the real number of some microbes. Selective media were also found to be useful in the differentiation of the main soil microbiological taxa (SMITH & DAWSON, 1944; KRASILNIKOV, 1966). This estimation consists of three steps:

1. Preparing a dilution series from the soil (up to 10^{-8} as a function of the culturable microbe;
2. plating the suspensions on selective media;
3. counting the colonies after appropriate incubation.

According to the plating techniques for counting it is also possible to mix the suspensions thoroughly into the media, or spread (spray) them onto the surface. Occasionally, however it is difficult to predict the correct rate of dilution, therefore counting is impossible due to the low or high number of microbes on one unique plate.

A new simplified, rapid and more economic plating method is presented for estimating special groups of microbes from the soil, which were affected by various rates of chlorsulfuron addition.

Materials and Methods

Classical method (a) for plate counting. – 10 g of soil is mixed thoroughly by a laboratory shaker in 90 ml of sterile tap water for 20 minutes. Another 10 g of soil is dried and weighted for the calculations on the 1 gramm - dry soil basis. One ml of sample suspension is diluted with 9 ml of water and the procedure is repeated several times as a function of the countable microbes. Using a sterile glass-bar, 0.1 ml soil suspension is spread on the surface of an appropriate agar plate in three replicates (HORVÁTH, 1980). Petri dishes in an upside-down position are incubated for a few days till the appearance of the desired culturable microbes. After incubation, plates with less than 200 colonies are considered for counting.

Simplified method (b) for plate counting. – Dilution series was prepared according to the earlier method. Instead of plating 100 µl suspension onto the sur-

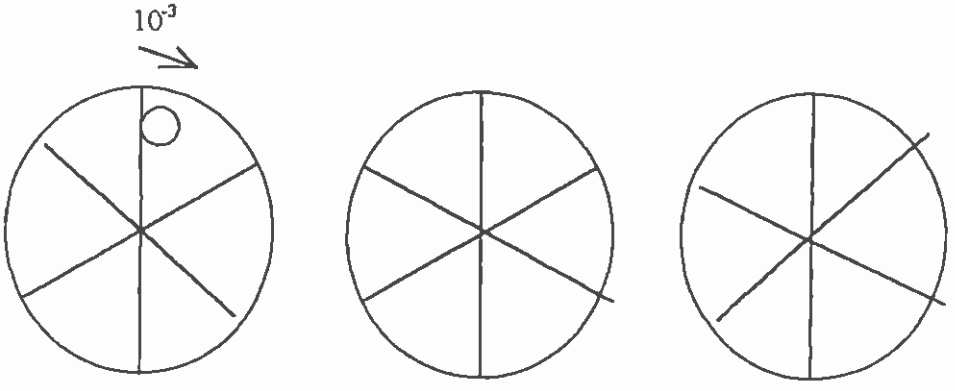

Plate 1:
Total number of microbes
(Nutrient agar)
dilution: 10^{-3}–10^{-8}

Plate 2:
Number of *Actinomycetes*
Arginine-glycerine agar
dilution up to 10^{-6}

Plate 3:
Free living N_2 fixers
Congored YEM
dilution up to 10^{-6}

Figure 1

Method for counting special groups of microbes in appropriate agar plates. A 20 µl dropplet of the soil suspension is plated in three replicates from each dilution step according to the figure. There are 6 segments in each Petri dish, therefore it is possible to cultivate 6 steps of soil dilution, using only one plate. The circle within the segment shows the size of the 20 µl dropplet (about 1 cm in diameter).

face of the agar plate, only 20 µl of suspension is used in this method. Application is possible by a fixed, standard micropipettor, changing the tip of every single dilution. It is necessary to place three dropplets from each single dilution into one segment of the Petri dish, which should be left open for drying (using a sterile box to keep them uncontaminated). Incubation is carried out as mentioned above (Figure 1).

According to the culturable group of soil microbes a special selective media is used for counting them (SKINNER et al., 1952; SZEGI, 1979).

Incubation of GLEAN 75 DF in model experiment in vitro. – GLEAN 75 DF herbicide (with a chlorsulfuron content) was applied at the dose (20 g ha^{-1}, equal to 0.001 mg kg^{-1}) recommended for agricultural practice on a calcareous chernozem soil (Nagyhörcsök). Other, higher doses (0.01, 1 and 10 mg kg^{-1}) were also tested (ANGERER et al., 1997). 200 g of soil was mixed with the herbicide in three replicates and incubated at 28 °C for three months, at 60% water holding capacity. The abundance of some desired groups of microbes was estimated after 3 weeks and three months.

Results and Discussion

Various microbial groups affected by GLEAN 75 DF

According to the total countable microbes on the nutrient agar plates, a cell reduction was detected even at the rate of chlorsulfuron application recommended for agricultural practice. After three months of cultivation, however, there was no significant difference between the control and the recommended rate. 0.01 mg kg^{-1} concentration, however, stimulated growth and increased the countable cell number by 2-3 ten-folds. Higher application rates resulted in a more pronounced reduction of the estimated colonies (Figure 2).

Regarding the sensitivity of some special taxa from the soil, the abundance of free living nitrogen fixers, the spore bearing *Bacillus* sp., and the group of *Actinomycetes* were considered (Table 1). Using two dates of the screening procedure, there was a variable effect found. According to the literature data the quantity of N_2-fixers, spore bearing and the *Streptomyces* family are 3 ten-folds less as compared to the total counts of bacteria. In case of all microbial taxa, however, there was a stimulative effect, mainly at the recommended rate or at the higher rates (0.001- 0.01 mg kg^{-1}). According to the short direct effect (three weeks) the free living nitrogen fixers proved to be the most sensitive group examined. Their abundance decreased from 6.9 to 0.55 x 10^{-3}, when GLEAN 75 DF was used, but increasing concentrations had no effect. The number of *Bacilli* and *Actinomycetes*, however, decreased simultaneously with the higher rates above the stimulative doses.

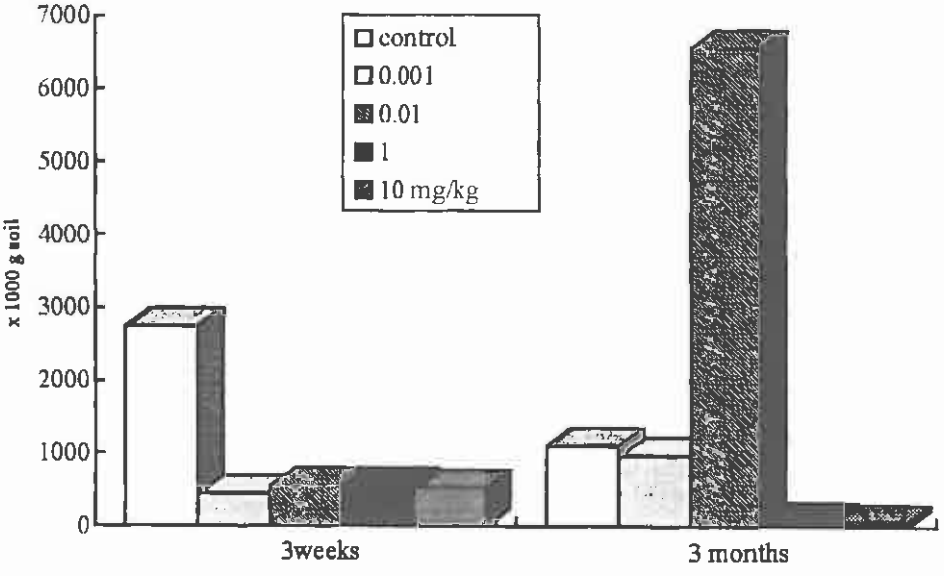

Figure 2
Various concentrations of GLEAN 75 DF herbicide affecting the total number of microbes after 3 weeks and 3 months of incubation

Table 1
Quantity of some microbial groups in a calcareous chernozem soil (Nagyhörcsök) affected by various rates of GLEAN 75 DF herbicide at sampling after 3 weeks and 3 months of incubation ($LSD_{5\%}$ 1.29)

Treatment (mg kg^{-1})	Total number of microbes (x 10^{-5})	Free living N_2 fixers (x 10^{-3})	*Bacillus* sp. (x 10^{-3})	*Actinomyces* sp. (x 10^{-3})
		3 weeks after incubation		
Control	27.55	6.90	0.20	0.76
0.001	4.53	0.55	0.15	0.31
0.01	5.55	0.53	0.66	0.56
1	5.60	0.51	0.09	0.27
10	5.22	0.37	0.11	0.02
Mean	9.69	1.77	0.25	0.38
		3 months after incubation		
Control	11.05	1.0	0.30	0.22
0.001	9.75	9.53	0.47	0.63
0.01	125.67	1.30	0.02	0.85
1	0.90	0.87	0.02	0.38
10	0.63	0.17	0.02	0.10
Mean	58.56	12.87	0.166	0.43

In accordance with literary data, three main groups of microorganisms were selected to estimate their abundance in an experiment with GLEAN 75 DF herbicide, using the agriculturally recommended and some higher doses. Although not a reduction, but mainly a growth stimulation was found at the applied rates, the considerable changes among the populations must be highlighted (ULASEVICH et al., 1980). Regarding all sample periods the free-living N_2 fixers were found to be the most sensitive group in the soil. Nitrogen fixing bacteria (*Rhizobium* microsymbionts) were also mentioned to be especially sensitive in connection with toxic metal applications in an earlier study (BIRÓ et al., 1995). The sensitivity of a special group of microbes, however, changes as a function of environmental conditions and the chemical structure of the applied herbicides. That is the explanation of the degradative capacity of the nitrogen fixing *Coronilla* rhizobia found by JOSEPOVITS et al. (1980) in case of aziprotrine herbicide, used in cabbage cultivation.

Before predicting the toxicity or the degradability of a certain herbicide (or other xenobiotics) it is recommended to consider both the short-term direct and the long-term effects in a given soil–plant environmental system.

Possibility of using the modified plate counting method

The main discrepancy of the traditional method for plate counting is that it is difficult to predict the correct step of dilution (to know prior to incubation which dilution step will be countable after a few days of growth). This prediction is much more difficult when using pesticides and other xenobiotics and when the rate of the effect (whether it is stimulative or depressive) is not really known.

The new simplified method presented in this study shows the effect of six dilution steps in 1 Petri dish. With the traditional method it was necessary to use 18 different Petri dishes to reach the same result. This modified plate counting procedure, therefore is much more economical and faster than the original method.

According to the literary data, the recommended dilution rates are: 10^2-10^4 for fungi, 10^3-10^7 for *Actinomycetes*, and up to 10^8 for the total counts of various microbes. All these dilution steps can be considered when using the simplified method.

Summary

Modifying the original plate countig method it was possible to estimate the abundance of various microbial groups from the soil in connection with the application of a newly developed herbicide (GLEAN 75 DF with chlorsulfuron content). Although the herbicide was not toxic, but stimulative at the rate recommended for agricultural practice, the modification of the community structure highlights the importance of a more reliable herbicidal application.

Using the simplified, (more economic and faster) plate counting method a more frequent monitoring is possible on the main, beneficial soil microbes in connection with the effect of various environmental pollutants.

Acknowledgement

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