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IMPACT OF PESTICIDES ON SOIL MICROBIAL BIOMASS AND ENZYME ACTIVITY

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Abstract:

Pot experiments were conducted to determine effects of trifluralin and 2,4-D applied at five rates (0, 0.1, 1, 10 and 100 mg/kg soil) on total organic carbon (TOC), carbon dioxide (CO_2) production, microbial biomass carbon (MBC), enumeration of aerobic heterotroph bacteria, filamentous fungal population, cellulose-decomposers and enzymatic activities (fluorescein diacetic acid (FDA) and dehydrogenase activities) in "kovarvany" brown forest (KBF, Nyíregyháza) and meadow chernozem (MC, Szeged) soil types incubated for 7 days at temperature 28°C. Results showed that trifluralin was more toxic than 2,4-D at higher rates. The data of investigated parameters in MC soil type were higher than those obtained from KBF soil type under the stress of two tested herbicides. It was found that 10 mg/kg concentration more effective on the aerobic heterotroph bacterial population and cellulose-decomposers than fungal population, FDA and dehydrogenase as well as TOC and CO_2 release. Results indicated that the herbicidal treatments at the tested levels were not deleterious to soil microbial and enzymatic activities which are important to soil fertility. This study has shown the microbial activity increased as a result of adaptation to the herbicides during incubation; also, demonstrated a potential capacity for adaptation of the microorganisms in soils when large amounts of herbicides are added. Dehydrogenase can consider as sensitive bioindicator of the microbial activity response to herbicidal amendment.

Keywords:

Herbicides - enzymatic activities - microbial properties - soil carbon mobilization

INTRODUCTION

Soil microbial processes are an integral part of soil quality and soil health, and a better understanding of these processes is needed. There are reports of beneficial and adverse effect of herbicides and fungicides on growth and activities of beneficial microorganisms in soil (Das and Debnath [1], Jastrzebska and Kucharski [2], Sukul [3]). Microbial biomass being an important attribute of soil quality [4] is also a potential source of enzymes in soil and acts as a sink/source of plant nutrients. Analysis of soil enzymatic activity is one of the microbiological indicators of soil quality [5]. Among the different enzymes in soil, dehydrogenase, urease, phosphatases and fluorescein diacetate hydrolyzing activity (FDHA) are more important in the transformation of various plant nutrients in the soil. Basal soil respiration rate is the most important tool for assessment of side effects of chemicals such as heavy metals, pesticides etc. [6,7]. On the other hand, the FDHA is considered as a suitable tool for measuring the early detrimental effect of pesticides on soil microbial biomass, as it is a sensitive and non-specific test able to depict the hydrolytic activities of soil microbes [8]. Pesticides are important tools in modern agricultural protection that reduce economic losses caused by weeds, insects, and pathogens. They enter the soil *via* spray drift during foliage treatment, wash-off from treated foliage, release from granulates or from treated seeds in soil. Some pesticides such as soil fumigants and nematocides are applied directly into soil. The transport, persistence or degradation of pesticides in soil depends on their physical, chemical and biological properties and soil, too. Most of active pesticides ingredients are synthetics. 2,4-dichlorophenoxyacetic acid (2,4-D) is a postemergence systemic and selective herbicide kills broad-leaved but not grasses or conifers. It has growth-

regulating (plant hormone: auxins). Commercially, 2,4-D formulations consist of the more soluble forms such as alkali salts, amine salts, or esters. These are combined with solvents, carriers, or surfactants and are marketed in the form of dusts, granules, emulsions, or oil and water solutions in a wide range of concentrations [9]. Typically, the ester and amine forms of 2,4-D are expected to degrade rapidly to the acid form. Soil half-life values have been estimated at 10 days for the acid, diethylamine salt, and ester forms. Another study estimated a soil half-life for the ester form ranging from 1-14 days with a median half-life of 2.9 days. In aerobic mineral soils, a half-life of 6.2 days was estimated [10]. The physico-chemical properties of 2,4-D and its formulations have an important effect on its behaviour in environmental compartments. It has half-life in soil is less than 7 days [11]. 2,4-D is relatively non-toxic to soil microorganisms at recommended field application rates. It does not persist in soil because of its rapid degradation by soil microorganisms [12]. Persistence of 2,4-D in soil is determined by microbial activity [13] and biochemical reactions [14]. Although 2,4-D accumulation is not a problem at application recommended rate (1 kg a.i./ha), its indiscriminate use may lead to residue accumulation in the soil [15]. Long persistence herbicides adversely affect the soil microflora as 2,4-D is mainly adsorbed on the soil organic fraction [16]. The 2,4-D bioavailability and uptake by terrestrial organisms is strongly influenced by organic matter (OM) content of soils, microbial activity, and by environmental conditions such as temperature and pH. In aerobic soils, with a high OM content, and at high pH values and temperatures, the toxic effects are limited because of rapid 2,4-D degradation. Some microorganisms are capable of utilizing 2,4-D as their sole C source. Repeated application to soil stimulates the number of organisms capable of degrading the compound. No effect of 2,4-D was observed on respiration of either sandy loam or clay loam soils at concentrations up to 200 mg/kg. In the range of 25.2 to 50.4 mg/litre, 2,4-D was inhibitory to all types of soil fungi. Mukhopadhyay [17] measured the bacterial, fungal, and actinomycete populations of soils supporting rice or maize plants which had been treated with various herbicides. There was no effect of 2,4-D, applied at the recommended rate, either on soil microbial numbers or on the evolution of carbon dioxide (CO_2) by soil cultures. On the same way, Huber et al. [18] examined the effect of 2,4-D at 66, 44 and 22 mg/l on seven cultures of soil microorganisms. Results showed no effect on the growth of *Nocardia sp., Pseudomonas fluorescens* in both aerobic and anaerobic culture *Bacillus subtilis* and *Ustilago maydis.* Trevors & Starodub [19] measured respiration in 2,4-D sandy and clay loam treated soils. There was no effect on soil respiration, monitored either as O_2 consumption or CO_2 evolution, at any of 2,4-D concentrations (0, 10, 25, 50, 75, 100, or 200 mg/kg) in either soil. Mineralization appeared to be the main process limiting 2,4-D availability, with each soil containing its own 2,4-D decomposers [20]. Soil organic matter (SOM) has a wide range of physical, chemical, and biological characteristics. It has been demonstrated that amount and composition of OM had large impact on pesticides sorption. For example soil rich on humus content are more chemically reactive with pesticides than non-humified soil [21]. Trifluralin is a selective preemergence, soil-incorporated herbicide [22]. Helling [23] have summarized the mobility, persistence, and degradation or metabolism of dinitroaniline herbicides as a group. Trifluralin is of moderate to high persistence in the soil environment, depending on conditions. It is subjected to the degradation by soil microorganisms and may be decomposed by UV light or may volatilize. Degradation of the herbicide was rapid in flooded (half lives, 33 and 45 days) than in non-flooded (half lives, 52 and 67 days) conditions in non-sterile and sterile soils, respectively. Reported half-lives of trifluralin in the soil vary from 45 to 60 days [11] to 6 to 8 months [24]. Trifluralin is readily degraded under sunlight in all media, with half-lives of minutes to several months, depending on the substrate. Losses of trifluralin from treated soils occur by both volatilization [25] and biological [23] processes. Estimated half-lives of trifluralin under a variety of agronomic conditions range from 25 to > 201 days, thus categorizing its persistence from moderate to persistent. Trifluralin at lower concentrations from 0.5 mg/kg dry soil to lower than 10.0 mg/kg dry soil appeared to stimulate the growth of soil bacteria, actinomycetes, mould, and the pure cultures of *B. japonicum* and *A. chroococcum*. The observation that soil microorganisms could use trifluralin as sole C resources and could decompose trifluralin [26]. Laboratory tests were conducted

with different herbicides and trifluralin to determine any serious effects on microbial and enzymatic activities related to soil fertility in loamy sand soil at the rate 10 μg/g. Some herbicides showed an effect on bacteria and fungi for the first week of incubation, but, subsequently, the populations returned to levels similar to those obtained in the controls. O_2 consumption was increased significantly after 96 hr incubation with atrazine. The soil dehydrogenase activity was inhibited by ethalfluralin treatment respectively for 1 week, and *p*-nitrophenol liberation was inhibited for 2 hrs by all herbicide treatments [27]. Because dehydrogenase activity is implied in initial splitting of organic substrates, the dehydrogenases are frequently used as indicators of soil biological activity [28]. These enzymes are important endocellular enzyme catalyzing ATP-producing metabolic reactions; they exist in intact cells and have not soil extracellular accumulation. Also, biochemical indicator is often used as a measure unit of pesticide caused destructions [29]. In particular, this study focuses on herbicides impacts on various microbiological and some biochemical parameters of two different soil types.

MATERIALS AND METHODS

Soil characterization and sampling

Bulk soil samples were obtained from farmland surface layer (0-250 mm) that, for many years, was not treated with pesticides. The laboratory pot experiments were laid out with two soil types: kovarvany brown forest and meadow chernozem collected from of University of Debrecen, Centre of Agricultural Sciences, Operating area of brown forest soil of Research Centre of Nyíregyháza and Institute of Cereal Research, Szeged, respectively, Hungary. Some selected physico-chemical parameters of the two soil types are given in Table 1.

Table 1. Some physico-chemical properties of investigated soil types

Parameters	**Soil samples**	
	Kovarvany Brown Forest	**Meadow Chernozem**
Topsoil profile	Sandy loam	Clay loam
$pH_{(KCl)}$	5.78	6.20
Humus content [%]	2.54	3.55
NO_3-N [mg/kg]	2.30	3.90
NH_4-N [mg/kg]	5.60	4.50
Ca [mg/kg]	893	1136
Mg [mg/kg]	214	257
Na [mg/kg]	64	53
P_2O_5 [mg/kg]	318	378
K_2O [mg/kg]	412	428
Zn [mg/kg]	1.70	1.10
Cu [mg/kg]	1.40	2.4
Mn [mg/kg]	55	61
Fe [mg/kg]	945	1094
Cd [mg/kg]	1.70	1.02
Pb [mg/kg]	1.30	0.96

Herbicides

Two herbicides 2,4-D (2,4-Dichlorophenoxyacetic acid) and trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toiuidine) belonging to phenoxys and dinitroanilines, respectively were used as commercial formulations of Dikamin D of 40% active ingredient and Treflan EC with 26% active ingredient, respectively. Herbicide was added to soil at five rates (0, 0.1, 1, 10, and 100 mg/kg).

Soil amendment with herbicides

Fresh soil samples were air dried, sieved through a 4 mm sieve and placed in polyethylene bags. Soil samples were stored at 4°C until used. Before use, soil samples were kept for air dry environment at room temperature (approximately 28°C) for 24 hours. The two herbicides were used as commercial formulations and added to soil at 5 rates. Different herbicidal concentrations were prepared by dilute the herbicide with sterile distilled water on the basis of the active ingredient to reach 0.1, 1, 10 and 100 mg/kg and applied to the soil samples as a part of the moisture required to adjust the soil to 45% of their water holding capacity. The herbicidal solution was sprayed onto the soil surfaces by means of a syringe that dispensed very small droplets. Pots of 1 kg of untreated control and herbicide amended soil samples were incubated at 28°C for 1 week. Loss of water by evaporation was compensated daily to avoid dryness.

Measurement of microbial biomass carbon

Soil microbial biomass carbon (MBC) content was determined using the chloroform fumigation-extraction method [30,31], which is based on fumigation of the soil sample with ethanol free chloroform for 24 hrs and subsequent extraction with 0.5 M K_2SO_4 of both fumigated and unfumigated samples, using a K_{EC} (conversion factor: extractable part of microbial biomass C after fumigation) = 0.38 [31]. So, MBC was calculated as follows: MBC = E_C/K_{EC}, where E_C = ($DOC_{\text{extracted from fumigated soil}}$) − ($DOC_{\text{extracted from non-fumigated soil}}$). The K_{EC} factor was used to account for the efficiency of extraction for MBC.

Dynamics of organic carbon

Total organic carbon (TOC) was analyzed by dichromate ($K_2Cr_2O_7$) oxidation and titration with ferrous ammonium sulphate [32]. In this reaction C is oxidized by the dichromate ion and the excess of dichromate ion is then back titrated with ferrous ion.

Evaluation of soil respiration

Soil respiration rates were determined in control and soil treated with herbicide according to the method of Anderson [33], 500 g of each of soil samples were placed in hermetically sealed glass bottle moistened at 45% of water holding capacity and incubated in the dark at 28°C for 7 days. The amount of CO_2 released and absorbed in vials containing 50 ml of 1 M NaOH placed inside bottle. The emitted CO_2 formed Na_2CO_3 which was measured by titration with 1 M HCl.

Determination of enzymatic activities

Fluorescein diacetate (3',6'-diacetyl-fluorescein, FDA) hydrolyzing activity of the soil sub-samples were determined by measuring the released fluorescein at 490 nm according to Schnürer & Rosswall [34]. The activity was expressed as mg fluorescein diacetate hydrolyzed/kg soil dry weight/h. Dehydrogenase activity was determined by the method of García et al. [28] and expressed as mg INTF/kg dry soil.

Enumeration of some microbial population densities

The enumeration of soil microbiota was done by the serial dilution spread-plate technique at the start and end of incubation period of pot experiment and expressed in the form of total colony forming units (CFU) of aerobic heterotroph bacterial and fungal populations in nutrient agar (NA) with

cycloheximide (100 µg/ml) as antifungal growth inhibitor and Difco Czapek-Dox agar and potato dextrose agar (PDA) media were used for fungal population, respectively. Rose Bengal (RB) was added to Czapek-Dox agar and PDA at 65 ppm as a bacteriostatic agent. Enumeration of cellulose decomposers was determined according to Hendricks et al. [35].
Cellulase activity in herbicide-treated and untreated control soil was assayed using carboxymethyl cellulose (CMC) as a substrate in NA and RB-Czapek-Dox agar and RB-PDA media were used for cellulose decomposers of bacterial and fungal populations, respectively. For quick identification of cellulose decomposed clear zone, Congo red indicator was added to the NA and RB-Czapek-Dox agar and RB-PDA media. The plates were incubated at 28°C and microbial population densities were calculated per 1 g dried soil. Due to the addition of hydrochloric acid, this stopped the cellulase enzyme production and a blue-violet clear zone surrounding the cellulose-decomposing colonies were developed [36].

Data analysis

Data are shown as average of triplicates. ANOVA was used to determine the statistical differences among treatments and LSD at $P < 0.05$ was calculated.

RESULTS AND DISCUSSION

Soil pollution by application of pesticides in modern agriculture is a serious worldwide problem and can be potentially harmful to human health *via* the food chain. In recent years, the intensive use of herbicides has increasingly become a matter of environmental concern, partially because of the adverse effects of these chemicals on soil microorganisms. The hydrolysis of FDA, related to several soil hydrolases, has been utilised to estimate the potential microbial activity of soil freshly amended with a herbicides and compared to the size and activity of soil microflora, measured by the MBC and CO_2 evolution, respectively. The results of pot experiment illustrated the followings:

Microbial biomass carbon

In the present investigation, the amounts of MBC were measured in two various soil types treated with 2,4-D and trifluralin at different rates are shown in Figure 1. The effect of herbicides treatments on MBC was significant at the 5% level.

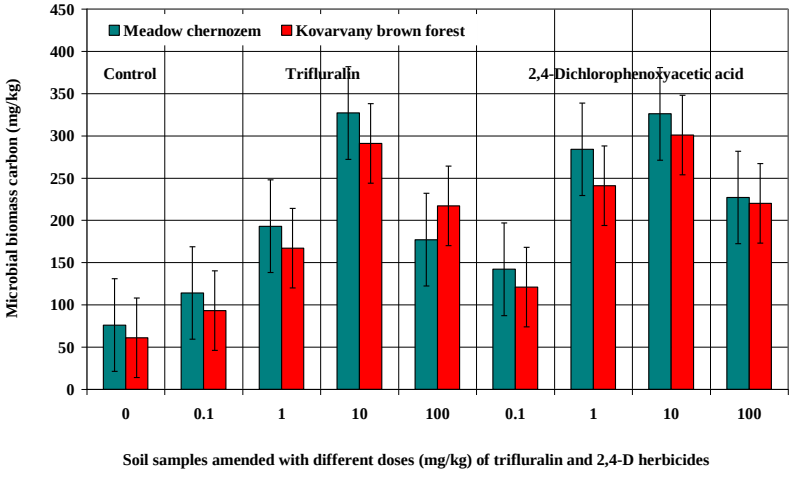

Figure 1: Microbial carbon biomass influenced by the herbicidal application in different soil types

MBC ranged from 93.1 to 327.4 mg C_{mic}/kg in kovarvany brown forest soil and 114.7 to 301.5 mg C_{mic}/kg in meadow chernozem soil after one week incubation at 28°C with soil moisture of 45%. Before incubation, soil was amended with 0, 0.1, 1, 10 and 100 mg/kg of 2,4-D or trifluralin. Results showed the stimulation effects of the two tested herbicides on the MBC in comparison with untreated control soil samples. It was found that soil samples treated with 2,4-D or trifluralin significantly reach a maximal MBC when amended with 10 mg herbicide/kg soil. Also, 100 mg/kg had increased the MBC than those treated with 0.1 mg/kg. Meadow chernozem soil had greater MBC content than the kovarvany brown forest soil. Soils treated with the highest concentration of herbicides had always lower soil MBC content, but higher than control soil (76.3 and 61.7 mg/kg, respectively). The actual interest in the effects of herbicides on soil microbial biomass is driven by the awareness of the importance of soil microorganisms in controlling C, N, P and S flows in soil through decomposition, mineralization and immobilisation processes [37]. In addition, a severe decrease in OM could occur, causing an impoverishment of the physical, chemical and microbiological properties of soils under modern agricultural management [38]. Soil C reflects the available level of substrate to allow the system to function. Soil microbial parameters, such as microbial C to TOC or basal respiration to microbial biomass, are reliable indicators for describing changes in ecosystems [39]. Soil microbial biomass is the labile fraction of the SOM, an agent of transformation of added and native OM and acts as a source and sink of plant-available nutrients [40]. The results obtained in this investigation are supported by the recent work, in which the MBC increased with increasing the applied rates of herbicides are depended on other soil ecological factors. Also, our suggestion is confirmed by the conclusion of Wardle & Parkinson [41] where the effects of 2,4-D addition on the microbial variables tested, even when significant, were typically small and probably of little ecological consequence especially when spatial and temporal variation in these variables is taken into account. Although, our results are confirmed by Devi et al. [42] who indicated that 2,4-D does not persist in the paddy field beyond 30 days after spraying. In case of trifluralin, our observation can be confirmed by Dzantor & Felsot [43] when applied concentrations of trifluralin and other herbicides did not have any pronounced effect on bacterial and fungal populations and the activities of soil dehydrogenase and esterase. At 10 mg/kg, all the herbicides degraded by 80 to 90% within one year. Lewis et al. [44] mentioned that herbicides did not affect respiration, assayed by CO_2 evolution and dehydrogenase activity, in either silty clay loam or loamy sand. Dumontet & Perucci [45] indicated that trifluralin showed a toxic effect on both the respiration and the size of microbial biomass than acifluorfen when applied at field recommended and 10-fold higher rate in a soil incubated for 5 weeks. Wiren-Lehr et al. [46] observed that mineralization of this herbicide is related to the both the activity and biomass of soil microorganisms. Microbial degradation is considered to be the most important of the transformation processes that determine the persistence of herbicides in soil [47].

Total organic carbon (TOC)

Figure 2 demonstrates that TOC (C_{org}) in kovarvany brown forest and meadow chernozem soil samples received different concentrations (0, 0.1, 1, 10 and 100 mg/kg) of trifluralin or 2,4-D. The herbicidal received soil samples of 45% soil moisture were incubated for one week at 28°C. After determination the TOC, results represented in Figure 2 show that the untreated soil samples had a lowest values of C_{org}, it was 0.43% in brown forest and 0.64% in meadow chernozem. After one week incubation, it was found that the ranges of TOC in kovarvany brown forest soil samples treated with trifluralin were 0.79 to 3.45% and the ranges of C_{org} in soil samples amended with 2,4-D were extend from 0.91 to 3.89%. While the percentages of TOC in meadow chernozem soil samples received trifluralin were ranged from 0.88 to 4.04% and for those treated with 2,4-D were ranged from 1.23 to 5.39%. In general it was found that by increasing the herbicidal concentrations in soil samples, the TOC values significantly ($P > 0.5$) increased. The effect of both herbicides on C_{org} was significant. The untreated control soil had lowest Corg content in comparison with two treated soil samples. Reductions of TOC were not observed, however, a significant stimulation effects on TOC content in

soil samples were found at the maximum with the highest concentration (100 mg/kg) of trifluralin especially for meadow chernozem soil was 4.05% and for kovarvany brown forest soil samples was 3.45% but, in cease of soil samples treated with 2,4-D, it was found 5.39 and 3.89%, respectively.

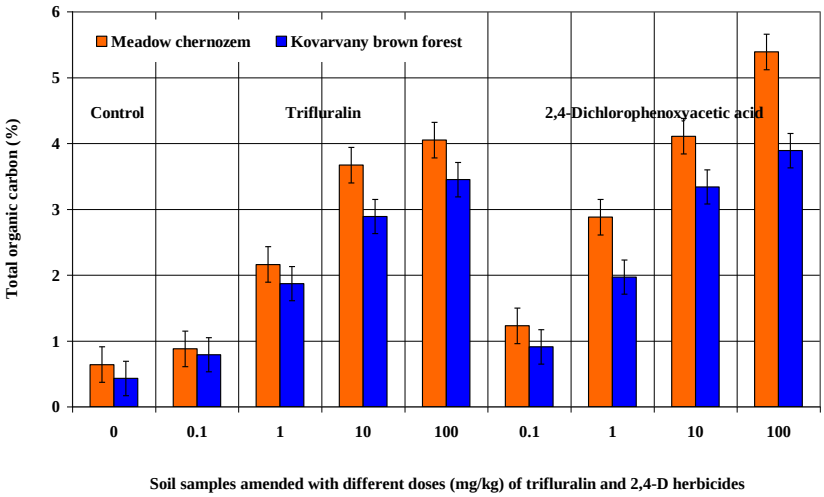

Figure 2: Effects of herbicidal treatment on total organic carbon in various soil types

The build-up of SOC and total N is determined by the amount and quality of OM and its decomposition rate (Koenig and Cochran 1994). During decomposition of OM, the majority of the C is evolved as CO_2. Xu et al. [48] mentioned that the sorption of 2,4-D, atrazine, and trifluralin was positively correlated to TOC, EXCAT, and CEC but negatively correlated to % clay. Sağlıker [49] mentioned that trifluralin is used as a C source by soil microorganisms. The herbicide trifluralin was degraded completely in the cotton field but a small fraction remained in the virgin field. This result can be explained by the cotton field soil having both more active microbial populations and more microorganisms adapted to the trifluralin applications than the virgin field. This conclusion can explain the increasing of TOC in treated soil is may be due to the increasing in the microbial population. Several studies indicate that 2,4-D sorption in soil is a function of soil pH, and to some extent also on SOM content [50]. On these bases, we selected 2,4-D as a model pesticide for our study because of its unique behaviour in soil.

CO_2 production

The overall microbial activity and C mineralization measured by CO_2 evolution was unaffected by trifluralin or 2,4-D in the investigated soil types (Figure 3). Soil respiration is the biological oxidation of OM to CO_2 by aerobic organisms, notably microorganisms. It was expected that comparisons of the rate and extent of CO_2 production by treated and untreated soil samples would differentiate stable from unstable and toxic from non-toxic compounds. Substances that were degraded microbiologically would increase CO_2 production, and the magnitude of the increase would be a function of the extent to which the C of the pesticidal molecule was oxidized completely. Toxic substances would depress CO_2 production and the effect would be transient or persistent, depending on the stability of the compound. Moreover, the sensitivity of microorganisms to toxicants is under nutritional and environmental control and may be influenced by an exogenous supply of carbohydrate. Some substances inhibit respiration and act on resting cells, but others interfere with biosynthetic and genetic mechanisms. For compounds of the latter type to exert an influence, a system that is nutritionally complete and able to support cell growth and reproduction is required. The results of analyses of CO_2 production revealed

patterns predictable from the principles presented above. In this regard, the effects of 2 herbicides summarized in Figure 3 which indicates that 100 mg/kg of both herbicides had little negative influence on soil respiration.

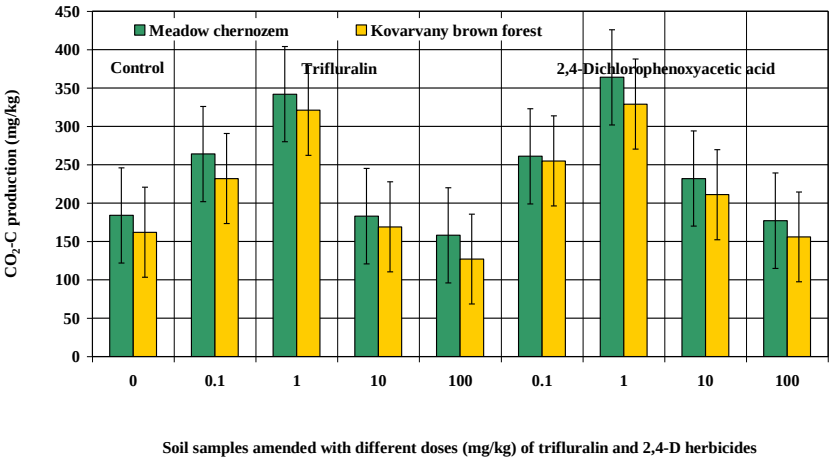

Figure 3: CO_2 production in different soil types amended with herbicides

The lower concentration of 2,4-D (10 mg/kg) had a stimulative effect in comparison with control (0 mg herbicide/kg soil). Trifluralin at 10 mg/kg had no effect on CO_2 production, but the magnitude of the stimulative effect was higher at 1 mg/kg of both herbicides than 0.1 mg/kg in both soil treated with herbicides, but it was lower in kovarvany brown forest soil and higher in meadow chernozem soil type. After one week incubation at 28°C, observation showed the toxicity as function of highest herbicides concentration (100 mg/kg). In general, trifluralin was more depressed the CO_2 production more than 2,4-D. The rate and magnitude of increased CO_2 production varied greatly, but maximal values were obtained from soil treated with 2,4-D. Frequently, the initial increase and subsequent decrease in CO_2 production by soil were both related directly to the concentration of herbicide applied to the ecosystem. Since many explanations are possible for this deviation from the more conventional patterns of CO_2 production by soil, evidence that would enable the more reasonable interpretations to be accepted or rejected was sought by examining model ecosystems having established activities. The results illustrated in Figure 3 were obtained by treating kovarvany brown forest soil and meadow chernozem soil type with various concentrations of 2,4-D and trifluralin. A pattern as mode of action was similar in the magnitude. For this purpose, it is necessary to assume that one of the following two possibilities is operative in soil: (i) a herbicide lacking antimicrobial activity is oxidized in part and transformed to a stable and non-toxic product; or (ii) a herbicide is selectively non-toxic. In the beginning, it promotes CO_2 production by sensitive microorganisms but is subject to oxidation, without detoxification, by other members of the microbial population that are resistant to its action. In each case, some of the C added to soil as herbicide will be oxidized completely, causing an increase in CO_2 production. This is represented by the uppermost curve in Figure 3. CO_2 production by sensitive soil organisms will be decreased by the addition or the formation of a toxicant by increasing the applied dose to 100 mg/kg; this is represented by the lowest curve in Figure 3. The activity of the total microbial population of treated soil is equal to the algebraic sum of these two effects, as illustrated by the centre curve in Figure 3. Generally higher numbers of bacteria were found in these herbicide-treated soil samples than in the untreated control samples, indicating that a higher proportion of inactive cells were present in the treated samples. Inoculation of herbicide-treated soil with these organisms resulted in an increased CO_2 output and the detoxification of the herbicidal compound.

Aerobic conditions are essential for the bacterial decomposition of 2,4-D. The bacterial oxidation of herbicides was attributed to adaptive enzyme formation. Respiration experiments showed that the oxidation of 2,4-D or trifluralin, but no intermediary metabolites have as yet been established for 2,4-D [51]. The microbial activity is an important factor in the behaviour of herbicide in the soil. According to Heinonen-Tanski [52] soils with high microbial activity favor the fast biodegradation of glyphosate, and our results for herbicide degradation as measured by CO_2 evolution correlate with our results for soil microbial activity. Trifluralin applied with various rates generally had no effect on fungal or bacterial populations in either the two dark brown chernozem soils. CO_2 evolution was unchanged when trifluralin was added to the soil. Also, there were no significant effects of trifluralin (1 kg/ha) on soil fungi and bacteria. These studies indicate that trifluralin is unlikely to cause changes in the numbers of soil microorganisms when used at recommended levels [53]. These results are in agreement with our observations.

Enzymatic activity

Several factors may affect directly or indirectly the activities of enzymes in soil. Soil enzyme activities may integrate information about microbial population densities and activities and soil (bio)chemical properties and may be used as a useful indicator of soil sustainability and fertility.

Fluorescein diacetic acid activity

Fluorescein diacetic acid activity (FDA) was measured as mg hydrolyzed fluorescein/kg soil. In the present experiment, FDA activity was studied so as to determine effect of soil-treated herbicides trifluralin and 2,4-D on the biochemical activities encountered in total biological activity flow in the amended-soil samples compared with untreated soil samples. The analysis of variance of the results obtained from the experiment showed that all factors (herbicide doses and the quantity of fluorescein) significantly influenced enzyme activities (Figure 4) in different soil samples of different treatments.

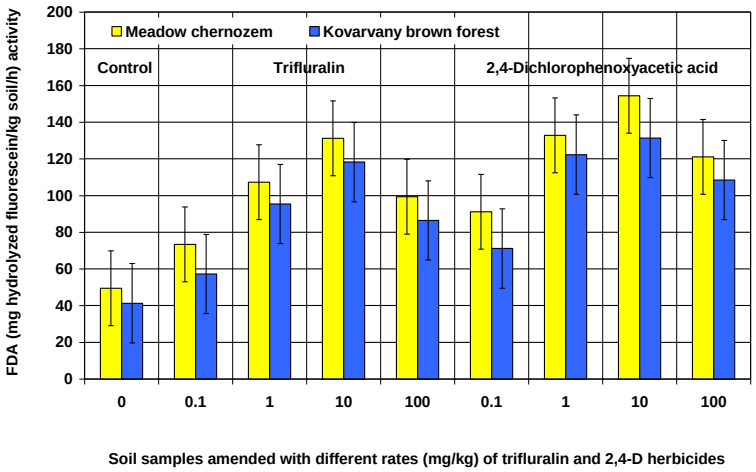

Figure 4: Trifluralin and 2,4-D affect the hydrolysis of fluorescein in various soil types

The results in Figure 4 demonstrated that the amounts of hydrolyzed fluorescein measured in trifluralin treated soil types at different concentrations had significant difference with control except at 0.1 mg trifluralin/kg soil. However, in case of 2,4-D amended soil samples, all measured quantities of hydrolyzed fluorescein were positively significant with the untreated control 0 mg/kg soil types at all concentrations except the measured amount of hydrolyzed fluorescein at 100 mg 2,4-D/kg soil sample. Maximal quantities of hydrolyzed fluorescein were measured at 10 mg trifluralin/kg soil sample and

the measured hydrolyzed fluorescein quantities at 1 mg trifluralin/kg soil sample of both soil types were higher than those measured at 100 mg/kg soil. However, this trend with similar pattern was found in the case of soil samples of both soil types when the soil received various doses of 2,4-D. Soil treatment with 2,4-D increased the quantities of hydrolyzed fluorescein higher than those treated with trifluralin. Also, results illustrated that herbicide-treated meadow chernozem soil samples had higher hydrolyzed fluorescein than those measured in the herbicide-amended kovarvany brown forest soil samples. Soil enzymes are the catalysts of important metabolic processes including the decomposition of organic input and the detoxification of xenobiotics [54]. Vekemans et al. [55] and Perucci [56] reported a close relationship between FDA-hydrolysis kinetics and soil microbial biomass. This result is in agreement with those found by Perucci & Scarponi [57]. Our results were in opposite to the recent observation and this may be due to the herbicidal properties.

Dehydrogenase activity

The experimental data showed that dehydrogenase activity was greater in meadow chernozem soil type than in kovarvany brown forest soil type. It was found that dehydrogenase activity did not inhibit by the application of both herbicides (Figure 5) in comparison with its activity in untreated soil samples. Also, 2,4-D treated soil samples of the two types had higher dehydrogenase activity than those amended with trifluralin. Regarding to the dehydrogenase activity, results illustrated significant differences between the untreated soil types and those applied with 10 and 100 mg/kg of trifluralin and 1, 10 and 100 mg/kg of 2,4-D. In both soil types, maximum dehydrogenase activity was found at 10 mg/kg. Enzyme activity per unit microbial biomass may provide a better understanding of the link between enzyme activity and microbial biomass. The higher ratios of dehydrogenase:MBC after forest floor removal may indicate the increasing enzyme production and enzyme release by microorganisms. In the case of enzymes maintaining their activity in the extracellular environment, Landi et al. [58] have suggested that calculating the ratio between the measured enzyme activity and the microbial biomass would provide more meaningful information on the location of the measured enzyme activities.

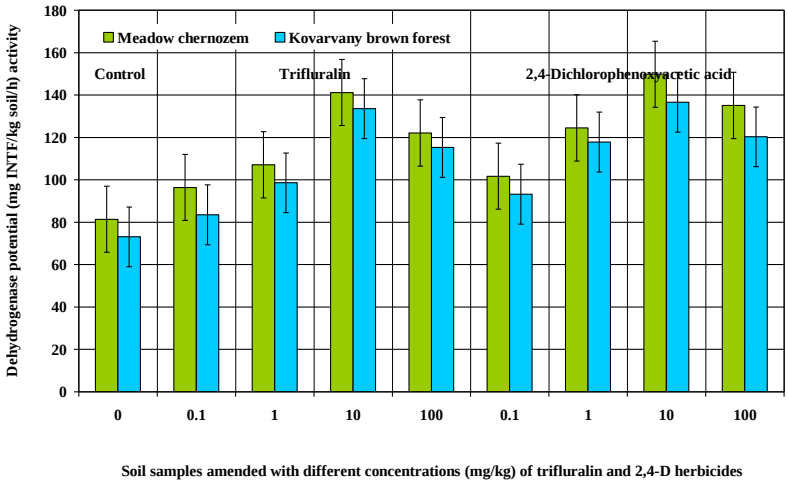

Figure 5: Impacts of application of herbicides on dehydrogenase activity in different soil types

Majumdar et al. [6] established that the enzyme activities (dehydrogenase and, FDA hydrolyzing activity), MBC and basic soil respiration rate content in herbicides and fungicides treated plots started recovering after 15 days of their application and recovered almost to the extent of respective initial level at harvest. Among the herbicides and fungicides, trifluralin 0.75 kg ai/ha was the safest regarding

soil quality next to hand weeding with higher fibre yield and residual nutrient availability. Dehydrogenase plays an important role in the initial oxidation of SOM and occurs only in viable cells; therefore, it is believed that dehydrogenase is an intracellular enzyme involved in microbial respiratory processes [59]. Here, we can give an attention point that in our observed data show a direct proportional relationship between the most of measured parameters, e.g., between the enzyme potential activity and MBC, TOC, etc.

Enumeration of microbial counts

The effect of soil treatment with the tested herbicides on soil bacteria, fungi and cellulose-decomposers is given in Figures 6, 7 and 8. The low application rate of the herbicide 2,4-D consistently increased the total population numbers of soil bacteria, fungi and cellulose-decomposers. The effect of the low application rate of trifluralin was of a similar effect but it was rarely significant. On the other hand, the high application rate of both herbicides used was not of inhibitory effect on total counts of soil bacteria, fungi and cellulose-decomposers after one week incubation period. The effect of pesticide application on population counts of soil bacteria and fungi was extensively examined. Our results indicated that both herbicides increased population counts of soil bacteria and fungi at the lower application rates but depressed at the higher levels. In agreement with our findings, Fletcher [60] found that at the rates used in agricultural practice, the 2,4-D, has no adverse effects on the total number of soil microorganisms, but the higher application rate (100 mg/kg) reduced the bacterial numbers.

Bacterial population

For both investigated herbicides, it was found that on the basis of the bacterial count (Figure 6), there were significant differences between bacterial enumeration in soil samples amended with various rates of herbicides and herbicide untreated soil (control), and also between bacterial count in soil samples treated with 1 mg/kg and 100 mg/kg. Maximum bacterial enumerations were found in soil amended with 1 mg/kg of trifluralin or with 2,4-D. Furthermore, there were no inhibition effects of both herbicides on the total viable bacterial count in both soil types. Higher bacterial enumeration was recorded in meadow chernozem soil type than in kovarvany brown forest soil type. Also, the bacterial counts in all herbicide-treated soil samples were significantly positive with the bacterial counts in untreated soil samples. Meanwhile, results demonstrated that there were no significant differences in between the bacterial count in between the herbicide-amended soil samples or soil types.

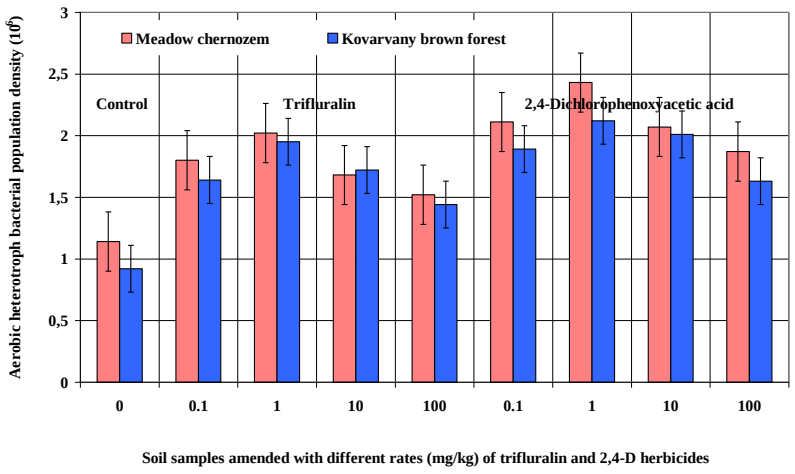

Figure 6: Influence of soil amendment with herbicides on aerobic heterotroph bacterial enumeration

Sebiomo et al. [2011] mentioned that this study indicated significant response of soil microbial activity to herbicide treatment and increased adaptation of the microbial community to the stress caused by increase in concentration of the herbicides over weeks of treatment. Our results give an outline that the incubation interval for 7 days was useful to produce adapted bacterial forms to soil applied herbicides.

Fungal population

Soil microorganisms, mainly bacteria and fungi, and their extracellular enzymes, mostly of microbial origin are responsible for the biological transformations that make nutrients available to plants and for sustaining soil functions. Since soil microbial communities play a critical role in the recovery of a soil from a disturbance, measurements of the characteristics of the microbial community provide invaluable information for soil quality and for a sustainable management of agricultural soils. However, after 7 days of incubation, the results indicated that there was an increase in the bacterial population in all treatments suggestive of dissipation of the herbicide. Higher doses of 2,4-D led to a corresponding increase in the fungal colonies and the maximum population was noticed at 10 mg/kg (Figure 7) for both applied herbicides in both soil types. These results indicated that fungi may use herbicide as a source of nutrient and energy. This may consequently explain the increase in microbial populations obtained in this study. Fungal population showed a significant increase with herbicidal concentration, mainly in the presence of trifluralin. The results demonstrated that meadow chernozem soil had higher fungal population than kovarvany brown forest soil. Our results did not show any significant differences between the counted population of filamentous fungi in controls of both soil types and with the both herbicides amended soil types except those at the rates of 1 and 10 mg herbicide/kg soil.

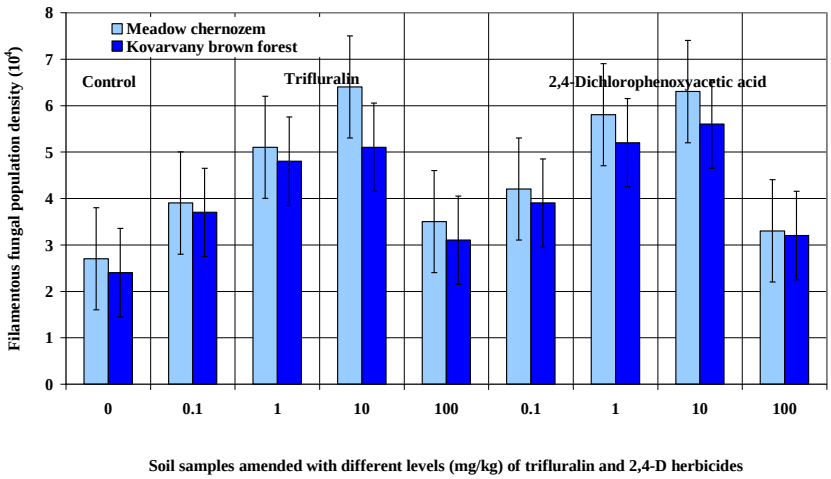

Figure 7: Filamentous fungal count in different soil types treated with different herbicides

Application of 2,4-D benefited soil fungi while the bacterial populations were depressed initially. By 30 days after spraying, the populations in the treated and untreated plots were similar and this period coincided with the disappearance of the 2,4-D residues in the soil. The duration of effectiveness depends upon the chemical, the rate and method of application, and specific environmental conditions. Herbicides are, as a rule, applied at only a few l or kg/ha. Although such small doses may have a profound influence upon sensitive plants, microorganisms or microbial transformations generally seem little affected by these low levels. For instance, lack of microbial inhibition by field applied levels has been reported for 2,4-D [60]. Van Schreven et al. [61] mentioned that in several cases higher numbers

of bacteria were found for a longer or shorter period in soil treated with herbicides. This study has shown that there exists positive correlation between microbial population and soil organic matter and that the variation in soil microbial activity represents the capacity of microorganisms to respond to inputs of herbicides. Microbial activity increased as an adaptation to the stress caused by increase in concentration of the herbicides over days of treatment. The results obtained demonstrate a potential capacity for adaptation of the microorganisms in soils when higher rates of herbicides are amended to the soil.

Cellulose-decomposers

Microbial biomass in relation to soil bioload and pesticidal contamination is a significant component of the soil quality, and probably the content of OM in the soils favoured the aerobic heterotrophic bacteria and fungi. Most of the earlier information on the effects of pesticides on non-target soil microorganisms comes from observations in upland temperate soils [62]. So, the investigation of the side-effect of trifluralin and 2,4-D on cellulose-decomposers in two different soil types was studied in pot experiment. The results showed that the enumeration of these microorganisms had reach the maximal values at 1 mg/kg herbicide rate of trifluralin and 2,4-D applied to the different soil samples. Comparatively, it was found that the enumeration of cellulose-decomposers in two different soil types at all applied rates of herbicides were higher than the untreated control soil samples and over 1 mg/kg of herbicide, the enumeration of cellulose-decomposers decreased but still higher than the enumeration at control.

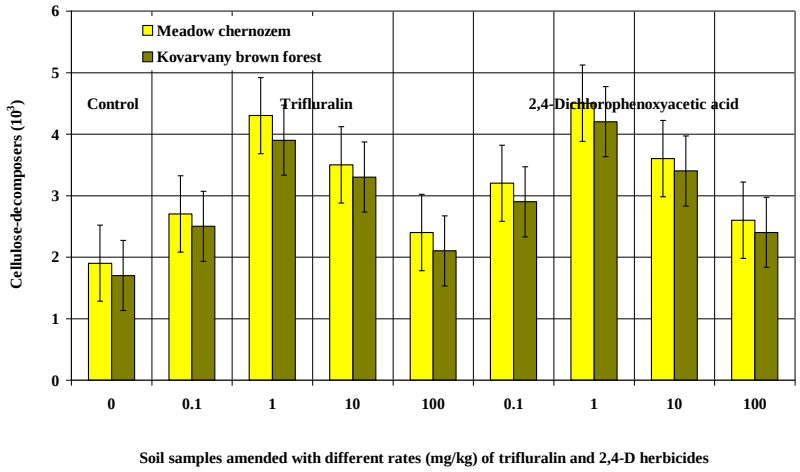

Figure 8: Enumeration of cellulose-decomposers in various soil types amended with herbicides

Frioni [63] mentioned that the dehydrogenase activity did not show conclusive effects of herbicide (atrazine, linuron and 2,4-D) action. The microbial population of cellulose decomposers was very sensitive to herbicides. This restriction seems to depend on unfavourable food conditions for these microorganisms in a soil without weeds, or it is due to enzyme inhibition by pesticides. Our observation showed that the lowest counting of the decomposers were found at 100 mg/kg concentration, but is higher than the control. Here, we conclude that the enumeration of cellulose-decomposers is depended on the applied rate of herbicide and the amount OM as a substrate in the soil. Voets et al. [64] found that the herbicide atrazine reduced the number of cellulose decomposers in soil. Also, similar results were obtained by Abdel-Mallek [65], Abdel-Kader et al. [66]. We conclude that the viable count of cellulose-decomposers in soil is depending on soil characteristics and the chemistry of the herbicide. The use of biological indicators has the problem of knowing which

indicator responds to a specific soil treatment or contaminant. Therefore, the use of multiple biological and biochemical properties is often suggested. General biochemical properties such as MBC [67], or ecophysiological quotients [68], as well as specific biochemical properties such as hydrolytic soil enzymes related to C, N and P cycles [69] are suggested. Persistence of herbicides, in general, is influenced by soil type, soil temperature, soil moisture and cultivation practices. Pesticides presenting a chemical structure similar to natural products are biodegradable, and their use therefore seems a reasonable compromise between crop protection and environmental concerns. Furthermore, it is possible that these biotic and environmental variables appear correlated with each other merely because they are both jointly affected by other factors. 2,4-D in the two soils was found to be mineralized by microorganisms. Mineralization appeared to be the main process limiting the availability of this chemical. Hence, while 2,4-D is one of the most mobile herbicides, its very rapid mineralization (50% of the applied dose in 10 days) lessens some of its potentially adverse effects on the environment.

CONCLUSIONS

The present study shows that MBC, CO_2, bacterial, fungal and cellulose-decomposers populations and the activities of FDA and dehydrogenase and TOC were positively significant affected by all the rates of trifluralin and 2,4-D amended to meadow chernozem and kovarvany brown forest soil types. Enzymatic activities were more sensitive than microbial activities to the applications of the herbicides. Enzymes may respond to changes in soil more quickly than other soil variables and therefore might be useful as early indicators of biological changes. Monitoring soil quality by means of bioindicators can be help for the management and sustainability of soils that received pesticidal application. Future study is clearly required to characterize the significant long-term ecological effects of the complex interactions between soil microbial activities during the biodegradation of investigated herbicides.

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