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IN-VITRO EFFECTS OF HERBICIDES ON SOIL MICROBIAL COMMUNITIES

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ABSTRACT
Effect of six different herbicides representing four chemical families on soil microbial communities was studied using laboratory microcosm approach. The herbicides tested were isoproturon, metribuzin, clodinafop propargyl, atlantis (Mesosulfuron methyl 3% + Idosulfuron Methyl Sodium 0.6% WG) and sulfosulfuron applied at normal agricultural rates, and UPH-110 (Clodinafop propargyl 12% + Metribuzin 42% WG) tested at four different application rates. Microbial response to the applied herbicides was studied following cultivation dependent approach. The microbial community showed a mixed response towards applied herbicides. With a few exceptions, metribuzin displayed a negative, clodinafop a positive and sulphonylurea herbicides a neutral effect while as the effect of isoproturon was variable. Significant toxic impact of UPH-110 was mostly observed at higher concentrations (@ 600 and 1000 g ha^{-1}). The magnitude of hazard and duration of toxicity increased as the dose of UPH-110 increased. The influence whether positive or negative, was only transitory in nature and recovered to the level of untreated microcosms by or before 30th day of application. Among the microbial groups studied, fungal population was least affected at field rate, bacteria, actinomycetes and *Azotobacter* showed mixed response while as the phosphorus solubilizers population showed a tendency to increase in response to the applied herbicides.The herbicidal impact on soil microbial population was found to depend on the nature and dose of herbicide used and also the type of microbial group.

INTRODUCTION

Soil health with special reference to biological features maintaining the functionsof both natural and managed ecosystems, is essential for sustainable agricultural fertility and productivity (Enriqueta-Arias *et al*., 2005). The worldwide application of pesticides guarantees production capabilities, but their heavy use,persistence and transfer cross-ecosystems and into trophic foodwebs all cause major environmental contaminations (Pimentel, 1995; Ackerman, 2007). Several studies on widely-used pesticides have already shown that pesticide application leads to changes in soil nutrient levels and alterations to soil microbial activity, diversity and/or genetic structure (Girvan *et al*., 2004; Rose*t al*., 2006). Consequently, disturbances of microbial communities ensuring several key ecological processes in soil such as organic matter degradation and nutrient cycling, could harmfully alter soil fertility and sustainable agricultural productivity.

In India, over the past five decades pesticides have been increasingly added in the environment under intensively managed cultivation practices leading to contamination of natural bodies. Of late,there hasbeen increasing concern about the non-target effects of pesticides. Soil microorganisms are among the important non-target organisms most affected (Cycon *et al*., 2005; Ratcliff *et al*., 2006). Soil microbes undergo direct and indirect impacts of toxic substances entering the soil. As microbes form the life blood of soil system, it is therefore imperative that the impact on these organisms of any xenobiotic compound entering the soil be studied carefully. Both culture dependent and culture independent techniques can be used to study the response of soil microbes, but the cultivation dependent approach is more appropriate as it allows to study the impact of pesticides on culturable fraction of soil microbial community which is thought to play a more important role in biogeochemical cycling (Ellis *et al*., 2003).Side-effects of herbicides on soil microbial populations can be studied on both short and long-term basis. However, according to Haney *et al*. (2000), experiments conducted on a short-term basis may provide a more realistic evaluation of the effect of herbicides on soil microorganisms.

The studies on alterations in microbial activities and numbers brought about by pesticides have been undertaken by several authors (Pampulha and Oliveira, 2006; Sebiomo, *et al*., 2011; Cycon and Piotrowska-Seget, 2009; Lo, 2009; Valiolahpor, 2011). While most of the reports suggest that the application of these chemicals decrease the microbial population (Latha and Gopal, 2010; Newton, *et al.,* 2010), some are also in favour of increase in population when these products are applied to soil (Niewiadomska, 2004). With this back ground, the present investigation was undertaken with the objective to evaluate the eco-toxicity for soil microflora of six commonly used herbicides in cereal crop based cropping systems, representing several chemical families, modes of action and

different soil residual properties under microcosm conditions.

MATERIALS AND METHODS

The treatment details, methods, procedures and techniques adopted during the course of investigation are as follows:

Treatment details

The experiment was laid out in Completely Randomised Design (CRD) with a total of 10 treatments and three replications. The first five treatments were applied at normal agricultural rate while UPH-110 was tested at four different concentrations. The treatments were:

T_1; Isoproturon 75% WP @1333 g ha^{-1},T_2; Metribuzin 70%WP @ 300 g ha^{-1}, T_3; Clodinofop propargyl 15% WP @ 400 g ha^{-1}, T_4; Atlantis(MesosulfuronMethyl 3% + Idosulfuron Methyl Sodium 0.6%WG) @ 400 g ha^{-1},T_5; Sulfosulfuron 75% WG @ 33.33 g ha^{-1}, T_6; UPH-110 (Clodinafop propargyl 12% + Metribuzin 42% WG) @ 400 g ha^{-1},T_7; UPH-110 @ 500 g ha^{-1}, T_8; UPH-110 @ 600 g ha^{-1},T_9; UPH-110 @ 1000 g ha^{-1} and T_{10}; Control.

Soil sampling and processing

The soil used for experiment was procured from Norman E. Borlaug Crop Research Centre of G.B. Pant University of Agriculture and Technology, Pantnagar from 0-15cm layer of a field that had received no pesticides in the recent past. The soil was sandy loam in texture, neutral in pH, high in organic carbon, medium in N and K and low in P. It was thoroughly homogenised and passed through 2 mm sieve. Microcosms were prepared with 130 g soil samples (oven dry weight basis) placed in sterile conical flasks of 500 ml capacity. Moisture content was adjusted to field capacity using sterile ultrapure water. Soil samples were stabilised by keeping in dark for one week before exposing them to the treatments.Subsequently the samples were treated with herbicides as per treatment details. Control flasks received sterile water only. The mouth of flasks was loosely capped with the help of rubber corks to avoid excessive accumulation of CO_2 in the head space. The flasks were periodically weighed and compensation for any moisture loss was made as and when required. All the flasks were incubated at 28 ± 2^oC in dark. The samples were collected for analysis on 1st, 3rd, 7th, 15th, 30th, 45th and 60th day after the herbicide application and stored at 4°C in deep freezer until analysis.

Microbial population

The population count of microbes namely, bacteria, actinomycetes, fungi and two functional groups viz., *Azotobacter* and Phosphorus Solubilising Microbes was taken to evaluate the effect of pesticides on their respective populations. Plate Count Agar medium for bacteria, Martin's Rose Bengal medium for fungi, Kenknight and Munaier's medium for actinomycetes, Pikovskaya's medium for PSM and 'Azotobacter agar' medium for *Azotobacter* were used to raise the microbial cultures and serial dilution plate count method was used for enumeration of colony forming units (cfu) (Wollum,1982). The population counts were taken after an incubation period of 48 hours for bacteria, 48–72 hours for fungi, 96 hours for PSM, and one week for *Azotobacter* and actinomycetes.

Statistical analysis

Data was subjected to one way analysis of variance (ANOVA) for the significance of treatment effects and the mean values were compared using least significant difference (LSD) test. The analysis was done using R-software (R Development Core Team, 2008).

RESULTS AND DISCUSSION

Bacterial population

At field rate (FR), the soil bacteria showed a mixed response towards the applied herbicides. Bacterial population increased significantly in Clodinafop and is oproturon treated microcosms up to 3rd and 7th day, respectively, while as metribuzin caused significant decline in bacterial population upto 3 days after treatment. The population in sulfonylurea treated samples (T_4 and T_5) was not statistically different from that of control (Table 1). The population however bounced back to the normal levels after 3 to 7 days of application. UPH-110 didn't significantly alter the bacterial numbers at lower rates of application i.e. 400 and 500 g ha^{-1}, but as the dose increased, a significant shrinkage in the population was observed. In T_9, population decline was noted from 1st to 15th day of treatment while as in T_8, population decline was noticed from 3rd to 7th day. Thereafter, the bacterial population returned to normal in both treatments. Among all treatments, T_9 invariably exhibited the highest toxicity.

Actinomycetes population

Unlike the case of bacteria, the population of actinomycetes did not show a statistically significant increment with any type or concentration of chemicals employed (Table1). The population either remained unmoved or decreased. At FR, isoproturon (up to 15th day) and metribuzin (up to 7th day) decreased the actinomycete population while the other treatments did not alter the populations. The effect of UPH-110 at lower doses (T_6 and T_7) was non-significant; nevertheless a significant depression was observed at higher doses (T_8 and T_9), persisting respectively up to 7th and 15th day of application. The toxicity amplified progressively as the concentration increased.

Fungal population

The soil fungi were more or less resistant towards the herbicides applied at field rate except isoproturon which caused a significant depression in number of colony forming units (cfu) for first seven days (Table 1). In case of UPH-110, a reduction in the cfu count was registered for T_7 (up to 3rd day), T_8 (up to 15th day), and T_9 (up to 30th day). Among all treatments, the lowest population was nurtured by T_9 treated microcosms up to 30 days. Duration of hazardous impact also increased as the concentration increased.

The results indicated that Isoproturon enhances the population of bacteria and cause a decline in the population of

actinomycetes and fungi. Stimulation of bacterial and suppression of actinomycete and fungal population due to isoproturon was also reported by Nowak *et al*. (2004). Enhancement in bacterial population could be due to the possible metabolism of the compound by an array of bacteria as source of carbon and energy, favouring the enhancement in their population. Increase in bacterial count due to another phenylurea herbicide linuron was also reported by Cycon and Piotrowska-Seget (2007). Further Sorensen *et al*. (2003) and Breugelmans *et al*. (2007) argued that these herbicides are easily degradable by bacteria and serve as carbon and energy source. Mariusz and Zofia (2009) reported a noticeable increment in population of heterotrophic bacteria at field rate due to diuron. The negative effect of isoproturon on actinomycetes and fungi may be the handiwork of certain undesirable metabolic products released during the degradation of herbicides. Sorensen *et al*. (2003) showed that during the degradation of isoproturon, certain undesirable products accumulate in soil which could be more hazardous to non-target organisms than the herbicide itself. Such a response could also be attributed to the competition between higher bacterial population and relatively smaller fungi and actinomycete population for available carbon and energy sources. Negative effect of metribuzin on bacterial population is in accordance with the report of Sebiomo *et al*. (2011) who observed similar response for atrazine at field rate. Reduction in total population, actinomycete number and unaltered fungal populations due to metribuzin at field rate under laboratory conditions at 30°C was also reported by Radivojevic *et al*. (2003). The temporary rise in bacterial population following clodinafop application is most likely due to utilization of carbon and nitrogen present in it by the heterotrophic bacteria. Roy and Singh (2006) also confirmed the role of microbes in the dissipation of clodinafop. No significant change in actinomycete and fungal numbers due to clodinafop was observed in the current study. Similar results were found by Wardle and Parkinson (1990) who reported that bacterial propagules were temporarily enhanced while actinomycete and fungal propagule numbers were unaffected by glyphosate. From these findings we presume that actinomycetes and fungi

Table 1: Population of bacteria and actinomycetes at different time periods as influenced by various herbicides in microcosm

Code	Treatment details	Days of sampling						
		1st	3rd	7th	15th	30th	45th	60th
Bacterial population ($\times 10^7$cfu g^{-1} soil)								
T1	Isoproturon 75% WP	7.86	7.56	7.03	5.89	5.66	4.75	3.99
T2	Metribuzin 70%WP	6.53	6.45	6.45	6.04	5.46	4.55	3.95
T3	Clodinofop Propargyl 15%WP	7.63	7.58	6.45	6.15	5.44	4.57	3.89
T4	Atlantis	7.20	7.16	6.58	6.02	5.66	4.65	4.11
T5	Sulfosulfuron 75%WG	7.20	7.10	6.57	6.05	5.45	4.59	4.13
T6	UPH-110 @ 400 g ha^{-1}	7.09	7.04	6.6	5.86	5.33	4.73	4.08
T7	UPH-110 @ 500 g ha^{-1}	7.28	7.04	6.57	5.92	5.43	4.71	3.92
T8	UPH-110 @ 600 g ha^{-1}	7.18	6.61	5.75	5.96	5.30	4.70	3.88
T9	UPH-110 @ 1000 g ha^{-1}	6.46	6.40	5.54	5.25	5.38	4.71	4.08
T10	Control	7.27	7.09	6.55	5.99	5.48	4.65	4.03
	LSD p ≤ 0.05	0.26	0.34	0.30	0.35	0.27	0.26	0.25
Actinomycetes population ($\times 10^6$cfu g^{-1} soil)								
T1	Isoproturon 75% WP	10.98	12.70	12.54	12.41	12.80	11.97	10.92
T2	Metribuzin 70%WP	13.52	13.91	13.38	13.12	12.62	11.85	10.88
T3	Clodinofop Propargyl 15%WP	14.72	14.26	13.80	13.29	12.99	11.6	11.02
T4	Atlantis	14.30	14.54	14.11	13.34	12.67	12.11	11.20
T5	Sulfosulfuron 75%WG	14.50	14.51	14.00	13.52	13.04	11.78	11.31
T6	UPH-110 @ 400 g ha^{-1}	14.32	14.39	13.91	13.35	12.71	11.97	10.92
T7	UPH-110 @ 500 g ha^{-1}	14.49	14.42	13.83	13.80	12.49	11.56	10.74
T8	UPH-110 @ 600 g ha^{-1}	13.74	13.50	14.28	13.30	13.02	11.77	10.80
T9	UPH-110 @ 1000 g ha^{-1}	12.91	12.09	12.75	12.36	12.77	11.84	10.98
T10	Control	14.56	14.43	13.93	13.45	12.86	11.85	11.00
	LSD p ≤ 0.05	0.28	0.41	0.37	0.43	0.37	0.42	0.35

Table 2: Impact of various herbicides on fungal population at different time periods in microcosm

Code	Treatment details	Days of sampling						
		1st	3rd	7th	15th	30th	45th	60th
		Fungal population ($\times 10^4$cfu g^{-1} soil)						
T1	Isoproturon 75% WP	9.25	9.02	9.50	9.43	8.53	7.13	6.71
T2	Metribuzin 70% WP	10.55	9.90	10.01	9.83	8.02	6.99	6.49
T3	Clodinofop Propargyl 15% WP	10.89	10.24	10.23	9.53	8.48	6.86	6.50
T4	Atlantis	10.75	10.04	9.98	9.72	8.24	7.35	6.68
T5	Sulfosulfuron 75%WG	10.52	10.01	10.10	9.42	8.65	6.97	6.81
T6	UPH-110 @ 400 g ha^{-1}	10.48	9.94	10.09	9.92	8.54	7.08	6.54
T7	UPH-110 @ 500 g ha^{-1}	10.17	9.30	9.89	9.85	8.23	7.31	6.55
T8	UPH-110 @ 600 g ha^{-1}	9.75	9.05	9.55	9.31	8.50	7.37	6.79
T9	UPH-110 @ 1000 g ha^{-1}	9.18	8.33	9.10	8.65	7.72	7.00	6.41
T10	Control	10.62	10.38	10.15	9.65	8.36	7.30	6.63
	LSD p ≤ 0.05	0.41	0.51	0.29	0.33	0.35	0.44	0.41

Table 3. Impact of various herbicides on census of PSM and *Azotobacter* at different time periods

Code	Treatment details	Days of sampling						
		1st	3rd	7th	15th	30th	45th	60th
PSM population ($\times 10^4$cfu g^{-1} soil)								
T1	Isoproturon 75% WP	8.94	9.15	9.46	8.76	7.93	6.80	5.88
T2	Metribuzin 70% WP	8.46	8.72	8.85	8.43	7.88	6.90	6.02
T3	Clodinofop Propargyl 15% WP	9.00	9.61	9.25	8.87	8.16	6.73	6.11
T4	Atlantis	8.53	8.36	8.54	8.10	7.82	6.73	5.87
T5	Sulfosulfuron 75% WG	8.52	8.47	8.51	8.02	7.73	6.77	5.84
T6	UPH-110 @ 400 g ha^{-1}	8.34	8.37	8.37	8.25	7.91	6.54	6.26
T7	UPH-110 @ 500 g ha^{-1}	8.36	9.04	8.71	8.41	7.79	6.76	6.03
T8	UPH-110 @ 600 g ha^{-1}	8.82	9.25	9.49	8.79	7.85	6.96	6.23
T9	UPH-110 @ 1000 g ha^{-1}	8.99	9.77	9.93	9.13	7.81	6.73	6.18
T10	Control	8.42	8.52	8.66	8.27	7.95	6.88	6.05
	LSD $p \leq 0.05$	0.35	0.33	0.37	0.47	0.48	0.42	0.43
Azotobacter population ($\times 10^4$cfu g^{-1} soil)								
T1	Isoproturon 75% WP	11.29	11.18	11.08	10.56	9.99	8.28	7.20
T2	Metribuzin 70% WP	10.52	10.31	10.19	10.00	9.81	8.40	7.09
T3	Clodinofop Propargyl 15% WP	12.31	11.89	11.65	10.86	10.21	8.13	7.20
T4	Atlantis	12.24	12.00	11.92	10.14	9.83	8.42	7.35
T5	Sulfosulfuron 75% WG	11.88	11.76	9.95	10.62	10.09	8.08	6.83
T6	UPH-110 @ 400 g ha^{-1}	11.49	11.30	11.05	10.59	9.95	8.27	7.32
T7	UPH-110 @ 500 g ha^{-1}	11.12	10.69	10.03	10.73	9.89	8.32	7.22
T8	UPH-110 @ 600 g ha^{-1}	10.39	10.22	10.20	9.92	9.20	8.05	6.99
T9	UPH-110 @ 1000 g ha^{-1}	10.27	10.03	9.68	9.60	8.84	8.35	7.26
T10	Control	11.36	11.24	11.14	10.73	10.06	8.19	7.15
	LSD $p \leq 0.05$	0.33	0.33	0.42	0.35	0.30	0.39	0.46

are not as efficient as bacteria in utilizing herbicides to their advantage; in fact they are more vulnerable to the herbicide toxicity. The lack of interference with soil biological processes would suggest that sulfonylurea herbicides at FR have little or no harmful effect on soil health. The neutral effects of sulfonylurea herbicides on soil microbial population at FR and even at higher concentrations have been reported by many workers (El-Ghamry*et al.*, 2002, Radivojevic *et al.*, 2011). Thus,sulfonylurea herbicides can be considered as safe for soil microbes. For UPH-110, the duration of hazardous impact prolonged as the dose increased. This type of behaviour can be ascribed to the low toxicity and/or brief persistence of the compound used in small amounts. Drescher and Otto (1973); Marsh *et al.* (1978), and Gaynor and Hamill (1983) studied the persistence of a herbicide bentazon and observed that when concentrations of 10 ppm or less were used, the herbicide was no longer detectable after a few months, while as applications at high dose persisted for several months. Prakash and Suseela Devi (2000) reported that the limitation in the number of reaction sites in soils and toxic effect of a herbicide on microorganisms or enzyme inhibition could reduce its degradation rate at higher doses. Schuster and Schroder (1990) showed that increase in the dose of a herbicide amplifies its negative effect as well as duration of hazard.

The transitory nature of herbicidal effects observed during the study could be attributed to the higher levels of toxic compounds immediately after the application and reduction in their concentration over a period due to different modes of degradation. Radivojevic *et al.* (2004) also registered the toxic effect of herbicides immediately after the application when their concentration in the soil was higher and as the microbes degraded the toxic compounds, their concentration decreased and so did the toxic effect.

Functional groups

Phosphorus solubilising microbes (PSMs)

The PSMs did not experience any significant negative effects due to herbicides applied. The population either remained stable or amplified (Table 3). At FR, isoproturon and clodinafop significantly proliferated the phosphate solubilizers up to 15th day but the effect of remaining three herbicides was virtually non-significant. The PSMs responded positively to UPH-110, increasing the population significantly at different time intervals at all concentrations except the lowest (T_6). T_9 supported the highest populations up to 15th day and then the population reverted to normal level.

No reports dealing with the response of phosphorus solubilizers towards isoproturon and clodinafop or related herbicides were found by the authors. However, from their stimulatory impact on bacterial population (Table 1), the microbes could possibly exploit the carbon and nitrogen present in these chemicals. Response of these organisms towards metribuzin and two sulfonylurea herbicides is in line with the available research findings. Ahemad and Khan (2011) reported that metribuzin at FR didn't affect the phosphorus solubilization activity of *Klebsiella*sp. Strain PS19. (Dhagat and Verma, (2009) reported that sulfosulfuron did not have any significant effect on phosphorus solubilising fungi. UPH-110 significantly enhanced the population of PSM at higher doses though it invariably proved toxic to bacteria, actinomycetes and fungi. Such results point to possible metabolic diversity existing among various microbial groups. Hart and Brookes (1996) concluded that some microorganisms are indifferent to herbicides. They showed that application of glyphosate in soil reduced microbial biomass carbon but ammonification and nitrification increased as compared to control. From the finding that none of the herbicides caused a

decrease while many increased PSM population, it can be concluded that this microbial group has high capability of decomposing/ digesting the herbicides and use them as a source of bio-genous elements. The phosphorus solubilising organisms like *Aspergillus* sp., *Penicillium* sp., *Pseudomonas* sp. and *Bacillus* sp. have been reported as the intensive decomposers of herbicides by Nada *et al*. (2002).

***Azotobacter* population**

The reaction of *Azotobacter,* an asymbiotic nitrogen fixer, towards the applied herbicides was highly variable (Table 3). Some of the applied herbicides proved significantly toxic while certain others supported the growth and some didn't influence it at all. Also, an initial enhancement followed by depression was noticed in case of two sulfonylurea herbicides (T_4 and T_5). Isoproturon's influence was neutral throughout, however metribuzin significantly reduced the population up to 15th day while Clodinafop caused significant increment for initial seven days.Regarding UPH-110, at all the doses barring T_6, a fall in the *Azotobacter* population compared to control was observed at varying time intervals. In case of T_7, the *Azotobacter* count was well short of control at 3rd and 7th day of incubation. The inhibitory effect of higher doses (T_8 and T_9) was observable up to one month after which it neutralized and paralleled with control.

In harmony with present results about isoproturon, Lenart (2012) showed that linuron application didn't inhibit the growth of any of the fourteen strains of *Azotobacter chrococcum*. The results depicting negative impact of metribuzin in present study are in agreement with the findings of Radivojevic *et al*. (2003). The stimulatory impact of clodinafop on *Azotobacter* population suggests that these microbes exploited it favourably for their growth. Das *et al*. (2012) observed similar response for quizalofop. The initial decline followed by increment in *Azotobacter* population observed in case of both the sulfonylurea herbicides is in line with the results of He *et al*. (2006), who also noticed a similar response for metsulfuron-methyl. Dhagat and Verma, (2009) also observed a decline but no subsequent enhancement in *Azotobacter* population was noticed. Magnitude of toxicity and duration of hazard increased as the dose of UPH-110 increased. The lower concentration of UPH-110 might have been metabolized rapidly having no toxic effect while higher concentration might have persisted for longer period thereby inhibiting the *Azotobacte*r population. Similar results have been reported with bentazon that was not detected after a few months in soil when applied at 10 ppm concentration, whereas larger amounts persisted for several months (Drescher and Otto, 1973; Marsh *et al*. 1978 and; Gaynor and Hamill 1983). In the present study, free living diazotrophs were found to be more sensitive to the applied herbicide than phosphorus solubilising microbes. This difference in sensitiveness to the herbicide may be due to difference in morphological/ metabolic make up and growing habits of the microorganisms (Selvamani and Sankaran, 1993).

The results showed that microbial response to herbicides varies with the target group. Bacteria in general have a higher capability of decomposing/ digesting the herbicides and use them as a source of bio-genous elements as compared to actinomycetes and fungi. Phosphorus solubilizers are also very efficient decomposers of herbicides. The effect also depends upon the nature and dose of herbicide. The results also show that at doses tested, the impact on microbial populations is only transitory. In general, at field rate metribuzin was found to negatively affect the soil microbial populations the most while UPH-110 and sulfonylurea herbicides did not have a significant effect on the microbial populations. However, the effects were quite variable depending on the type of microbes investigated.This calls for in-depth analysis of specific microbial groups involved in key functions in the soil system.

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