Research Paper :

Influence of herbicides on cellulolytic, proteolytic and phosphate solubilising bacteria

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SUMMARY

Experiments were conducted at the Department of Agricultural Microbiology to study the effect of herbicides *viz.*, 2,4-DEE, butachlor, pretilachlor and pyrazosulfuron ethyl on soil microorganisms and enzyme activities in laboratory microcosms, *in vitro* effect on growth of pure cultures of *Azospirillum lipoferum* and *Bacillus megaterium* and their nitrogen fixation and phosphate solubilisation abilities and also on the effect of herbicide application to rice in pot culture inoculated with biofertiliser, Azophos. Soil microbiological, biochemical, chemical and agronomic variables were also studied in a permanent herbicide trial to study the impact of long term herbicide application in transplanted low land rice-rice system.In laboratory incubation studies, it was observed that butachlor was more inhibitory to microbial populations (7.85 to 34.20% reduction over control) and soil enzyme activities (5.03 to 19.11% reduction over control) when compared to 2,4-DEE, pretilachlor and pyrazosulfuron ethyl. Among the herbicides tested, the soil microbial population and enzyme activity inhibition followed a trend, butachlor > 2,4-DEE > pretilachlor > pyrazosulfuron ethyl.

Key words : Herbicides, Cellulolytic, Proteolytic, Phosphate solubilising bacteria

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The present day agriculture depends upon high yielding varieties, inorganic fertilizers and pesticides to achieve the increased food production required to keep pace with the increasing population. The progressive modernization of irrigated rice cultivation in India, using the above technologies has led to tremendous increase in rice production, which has more than doubled over the last 35 years, mainly driven by 85% increase in productivity.

In India, herbicides constituted only 15per cent of the total consumption of pesticides, compared to the worldwide consumption of 47.5 per cent. The herbicide consumption is expected to increase dramatically in future as the use of herbicides has been expanding more rapidly than that of the other pesticides (Bhan and Mishra, 2001). Herbicide usage, which was earlier confined to plantation crops, has now expanded to crops like wheat (42 per cent of the total consumption of herbicides) and rice (30 per cent) with the states of Punjab, Uttar Pradesh, Tamil Nadu and Andhra Pradesh leading in the consumption of more herbicides.

Since the herbicides are used when the crop is either absent as pre-emergence or at its early stage of growth as post-emergence, a high proportion of the herbicide reaches the soil and accumulates in the microbiologically active top layer of 0-15 cm soil. Herbicides being biologically active compounds, an unintended consequence of the application of herbicides could influence the microbial ecological balance in the soil leading to significant changes in the populations of microorganisms and their activities and affecting the productivity of soils (Boldt and Jacobsen, 1998). Hence, the increasing reliance of rice cultivation on herbicides has led to concern about their ecotoxicological behaviour in the rice field environment.

With this background, the present investigation was carried out with the main objective, to understand and predict the effect of herbicides *viz.*, 2,4-D-2ethylhexyl ester (2,4-DEE), butachlor, pretilachlor and pyrazosulfuron ethyl on rice soil microorganisms and their activities, which could lead to their judicious use and thereby to reduce their negative effects, if any on the environment.

MATERIALS AND METHODS

A laboratory incubation experiment was conducted using field soil obtained from wetlands of TNAU, Coimbatore, by devising microcosms to study the effect of different concentrations of herbicide formulations on cultivable microflora and potential enzyme

activities.

Soil microcosms :

Soil obtained from wetlands of TNAU, Coimbatore was air dried and passed through 2 mm sieve. The soil in portions equivalent to 250 g oven dry weight was placed in 500 ml beakers, adjusted to the required level of moisture in flooded condition and pre-incubated at 30 ± 1°C for 3 days for conditioning. Appropriate quantities of the herbicide formulations were added to the soil to maintain concentrations of herbicides at control, FR (field rate), 2FR (2 x field rate), 5FR (5 x field rate), 10 FR (10 x field rate) and 100 FR. Soil without herbicide application was also maintained as control. The field rates of application for different herbicides were 0.75 kg ai. ha^{-1} for 2,4-DEE, 1.0 kg ai. ha^{-1} for butachlor, 0.30 kg ai. ha^{-1} for pretilachlor and 25g ai. ha^{-1} for pyrazosulfuron ethyl. Water was adjusted to the same levels in all the treatments including control. A 3 cm depth of overlying water was maintained in all the treatments. The treated soils were then covered with plastic sheets having small holes and incubated at 30 ± 1°C in the dark for 30 days. For sampling the soil at different intervals, overlying water was carefully removed and the surface soil was collected from a depth of 0-3cm using a spatula (Saeki and Toyata, 2004). Samples were drawn at 0, 7, 15 and 30 days after application of herbicides and analysed for the effect of herbicides on soil microbial populations and enzyme activities.

Enumeration of cellulolytic, proteolytic and phosphate solubilising bacteria :

The population of cellulase producing bacteria was determined by plating soil dilutions on CMC agar medium (Henriksen and Breland, 1999). The plates were incubated in the dark at 25 ± 1°C for 5 days and then flooded with congo red (0.1% solution, 15 minutes), followed by the addition of 1M NaCl and 1M HCl for visualization of solubilization zones around the bacterial colonies (Teather and Wood, 1982).

Viable counts of protein degrading bacteria in soil were enumerated on skim milk agar medium (Aneja, 1996). The colonies that showed zones of solubilisation of casein were recorded as proteolytic bacteria.

The population of phosphate solubilising bacteria present in the soil samples was enumerated on sucrose – tri calcium phosphate agar medium (Pikovskaya, 1948). The bacterial colonies surrounded by clear zones were recorded as phosphate solubilizers.

RESULTS AND DISCUSSION

The effect of herbicides, 2,4-DEE, butachlor, pretilachlor and pyrazosulfuron ethyl at different concentrations and at various incubation times were studied using laboratory microcosms set up in the Department of Agricultural Microbiology.

The herbicides 2,4-DEE, butachlor, pretilachlor and pyrazosulfuron ethyl were applied at different concentrations to soil microcosms and the resultant differences in the population of cellulolytic bacteria were monitored (Table 1and Fig. 1). A comparison of the three factors revealed that butachlor among herbicides, 100FR among the concentrations and 15 days after application recorded significantly lower population (4.988, 4.900 and 5.027 log CFU g^{-1} soil, respectively). Cellulolytic bacterial population were significantly influenced by the interactive effects of herbicide x concentration. The highest population was observed in the control treatment of all herbicides and was comparable to the population in 1FR pretilachlor, 1FR pyrazosulfuron and 2FR pyrazosulfuron ethyl. Non significant effects were observed for herbicide x days and herbicide x concentration x days interaction.

Significant differences in the numbers of proteolytic bacterial population were registered in the presence of different concentrations of the herbicides when enumerated at 7, 15 and 30 days after herbicide application (Table 2). Significantly lower population (4.362 log CFU g^{-1} soil) was observed in the presence of butachlor compared to 2,4-DEE (4.395 log CFU g^{-1} soil), pretilachlor (4.421 log CFU g^{-1} soil) and pyrazosulfuron ethyl (4.427 log CFU g^{-1} soil). Higher concentrations of the herbicides were observed to lower the population of proteolytic bacteria, the 100 FR treatment recording the lowest population (4.321 log CFU g^{-1} soil). The influence of the interaction between herbicides and concentration significantly influenced the proteolytic bacterial

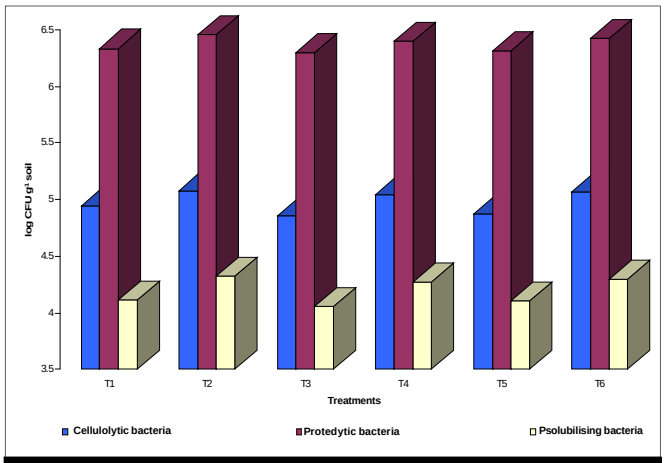

Fig. 1 : Impact of herbicide application on populations of cellulolytic, proteolytic and P solubilizing bacteria in the long term herbicidal trial

Table 1 : Population of cellulolytic bacteria as influenced by various herbicides under laboratory conditions

Herbicide concentration	Population of cellulolytic bacteria (x 10^4 CFU g^{-1} dry soil)												
	Days after 2,4-DEE application			Days after butachlor application			Days after pretilachlor application			Days after pyrazosulfuron ethyl application			Mean
	7	15	30	Mean	7	15	30	Mean	7	15	30	Mean	
1FR	11.62	12.82	13.94	12.79	10.48	12.06	13.72	12.09	12.84	13.05	14.16	13.35	13.42	14.04	14.32	13.93	13.04
	(5.059)	(5.107)	(5.144)	(5.107)	(5.024)	(5.083)	(5.141)	(5.083)	(5.108)	(5.116)	(5.151)	(5.124)	(5.131)	(5.152)	(5.156)	(5.148)	(5.115)
2FR	11.24	12.36	13.04	12.21	9.9[illegible]	11.12	12.95	11.33	11.98	12.98	13.84	12.93	12.98	13.89	14.00	13.62	12.52
	(5.053)	(5.093)	(5.114)	(5.087)	(4.993)	(5.043)	(5.115)	(5.050)	(5.076)	(5.114)	(5.139)	(5.110)	(5.120)	(5.143)	(5.147)	(5.137)	(5.096)
5FR	10.48	11.89	12.62	11.56	8.62	10.24	11.84	10.23	11.04	12.04	13.07	12.05	12.04	13.14	13.98	13.05	11.75
	(5.020)	(5.072)	(5.104)	(5.065)	(4.939)	(5.009)	(5.072)	(5.007)	(5.049)	(5.082)	(5.115)	(5.082)	(5.082)	(5.114)	(5.151)	(5.116)	(5.067)
10FR	8.24	10.62	11.84	10.23	7.25	9.32	10.26	8.94	10.62	11.83	12.62	11.69	11.00	12.26	13.12	12.13	10.75
	(4.918)	(5.013)	(5.076)	(5.002)	(4.862)	(4.967)	(5.004)	(4.944)	(5.024)	(5.079)	(5.102)	(5.068)	(5.040)	(5.090)	(5.123)	(5.084)	(5.025)
100FR	6.45	7.84	10.62	8.30	5.62	5.00	5.24	5.29	8.64	9.92	10.74	9.77	9.62	10.74	11.32	10.73	8.52
	(4.809)	(4.890)	(5.026)	(4.908)	(4.752)	(4.693)	(4.571)	(4.673)	(4.936)	(4.999)	(5.029)	(4.988)	(4.985)	(5.030)	(5.074)	(5.030)	(4.900)
Control	15.42	14.87	14.36	14.88	15.41	14.88	14.35	14.88	15.44	14.87	14.37	14.89	15.43	14.89	14.36	14.89	14.89
	(5.188)	(5.172)	(5.159)	(5.172)	(5.188)	(5.168)	(5.159)	(5.172)	(5.188)	(5.173)	(5.159)	(5.176)	(5.189)	(5.171)	(5.159)	(5.173)	(5.173)
Mean	10.58	11.73	12.74	11.68	9.55	10.44	11.39	10.46	11.76	12.45	13.13	12.45	12.42	13.16	13.60	13.06	11.91
	(5.008)	(5.058)	(5.105)	(5.057)	(4.960)	(4.994)	(5.010)	(4.988)	(5.064)	(5.094)	(5.117)	(5.091)	(5.091)	(5.117)	(5.136)	(5.115)	(5.063)
Factors	C.D. (P=0.05)				Factors				C.D. (P=0.05)				Factors			C.D. (P=0.05)	
Herbicides (H)	0.019				HXC				0.048				HXCXD			NS	
Concentration (C)	0.024				HXD				NS								
Days (D)	0.017				DXC				0.042								

Values in parenthesis are $\log_{10}$ transformed; Initial population of cellulolytic bacteria before herbicide application: 15.96 x 10^4 CFU g^{-1} dry soil NS-Non significant

populations. Butachlor at 100 FR had the lowest population (4.205 log CFU g^{-1} soil), while the highest population was observed in control and 1FR treatments of all herbicides.

Changes in the phosphate solubilizing bacterial population as a consequence of the application of herbicides at different concentrations were monitored at different days and are presented in Table 3. Among the herbicides, the lowest population was observed in butachlor (4.573 log CFU g^{-1} soil) followed by 2,4-DEE (4.591 log CFU g^{-1} soil), pretilachlor (4.599 log CFU g^{-1} soil) and pyrazosulfuron ethyl (4.609 log CFU g^{-1} soil). The highest concentration of herbicides (100FR) recorded the lowest population of 4.547 log CFU g^{-1} soil, compared to the other concentrations. Non significant interaction effects of the factors were observed for phosphate solubilising bacterial populations.

Weeds are the major biological constraint in most rice growing areas of the world. Unlike the periodic outbreaks of insect pests and plant diseases, weeds are ever present and threatening. Problems associated with weeds in rice cultivation are mounting dramatically in South and Southeast Asia because of the reduced availability of affordable labour. The lack of suitable weed control alternatives has led to the increasing reliance on herbicides in many rice growing areas and their use is increasing as they are less expensive and convenient than manual labour, very effective and easy to use. In India, herbicide consumption is expected to increase in future as the use of herbicides has been expanding more rapidly than that of the other pesticides used in pest and disease control (Bhan and Mishra, 2001).

Herbicides may affect non target organisms including microorganisms. Herbicide induced changes in abundance, diversity and activity of soil microbial communities may in turn influence microorganism mediated processes that are important to sustainable agriculture like recycling of plant nutrients. This is especially true of rice ecosystem which is composed of extremely large number of very diverse microbial subhabitats in space and time, and where productivity and environmental quality are both linked to microorganisms that are the main carriers of biocatalytic functions affecting nutrient supply to the crop and is viewed as a major supporter of the system's sustainability (Reichardt *et al.*, 1998).

Table 2 : Population of proteolytic bacteria as influenced by various herbicides under laboratory conditions

Herbicide concentration	Population of proteolytic bacteria (x 10^3 CFU g^{-1} dry soil)													
	Days after 2,4-DEE application			Days after butachlor application			Days after pretilachlor application			Days after pyrazosulfuron ethyl application			Mean
	7	15	30	Mean	7	15	30	Mean	7	15	30	Mean	7	15	30	Mean	
1FR	24.82	26.52	27.96	26.43	24.04	26.48	27.82	26.11	26.72	27.70	28.26	27.56	27.14	27.82	28.30	27.75	26.97
	(4.396)	(4.425)	(4.447)	(4.423)	(4.381)	(4.424)	(4.444)	(4.416)	(4.428)	(4.444)	(4.452)	(4.441)	(4.434)	(4.445)	(4.452)	(4.444)	(4.431)
2FR	23.89	25.48	27.04	25.47	23.24	25.92	26.76	25.31	26.00	26.88	27.82	26.90	26.42	27.18	28.14	27.25	26.23
	(4.380)	(4.408)	(4.433)	(4.407)	(4.367)	(4.414)	(4.428)	(4.403)	(4.418)	(4.430)	(4.446)	(4.431)	(4.422)	(4.434)	(4.449)	(4.435)	(4.419)
5FR	22.42	24.64	26.38	24.48	22.09	25.00	26.04	24.38	25.06	26.15	27.38	26.20	26.05	26.94	27.88	26.96	25.50
	(4.352)	(4.394)	(4.422)	(4.389)	(4.346)	(4.399)	(4.416)	(4.387)	(4.400)	(4.419)	(4.438)	(4.419)	(4.416)	(4.430)	(4.446)	(4.431)	(4.407)
10FR	21.30	23.26	25.32	23.29	20.84	19.26	20.12	20.07	24.02	25.12	26.52	25.22	24.26	25.68	27.12	25.69	23.57
	(4.329)	(4.368)	(4.405)	(4.367)	(4.320)	(4.285)	(4.304)	(4.303)	(4.383)	(4.402)	(4.425)	(4.403)	(4.385)	(4.410)	(4.433)	(4.409)	(4.371)
100FR	19.34	20.82	22.84	21.00	17.62	15.48	14.99	16.03	21.92	23.48	25.22	23.54	22.64	24.62	25.64	24.30	21.22
	(4.287)	(4.319)	(4.359)	(4.322)	(4.247)	(4.191)	(4.176)	(4.205)	(4.342)	(4.372)	(4.403)	(4.372)	(4.355)	(4.391)	(4.409)	(4.385)	(4.321)
Control	29.26	28.84	28.32	28.81	29.24	28.85	28.33	28.81	29.27	28.83	28.35	28.82	29.25	28.86	28.34	28.82	28.81
	(4.468)	(4.461)	(4.453)	(4.461)	(4.468)	(4.461)	(4.452)	(4.460)	(4.467)	(4.460)	(4.453)	(4.460)	(4.466)	(4.460)	(4.453)	(4.460)	(4.460)
Mean	23.51	24.93	26.31	24.91	22.85	23.50	24.01	23.45	25.50	26.36	27.26	26.37	25.96	26.85	27.57	26.79	25.38
	(4.369)	(4.396)	(4.420)	(4.395)	(4.355)	(4.362)	(4.370)	(4.362)	(4.406)	(4.421)	(4.436)	(4.421)	(4.413)	(4.428)	(4.440)	(4.427)	(4.401)

Factors	C.D. (P=0.05)	Factors	CD (P=0.05)	Factors	CD (P=0.05)
Herbicides (H)	0.021	HXC	0.051	HXCXD	NS
Concentration (C)	0.026	HXD	NS		
Days (D)	0.018	DXC	NS		

Values in parenthesis are $\log_{10}$ transformed; Initial population of proteolytic bacteria before herbicide application: 29.92 x 10^3 CFU g^{-1} dry soil NS-Non significant

Hence, there are concerns about the deleterious effects of herbicides on the rice ecosystems and such impacts cannot be overlooked when increasing attention is being focused on environmental issues.

The measurement of the microbial population and processes at a large number of concentrations is a better way to study the toxic effects of pesticides (Van Beelen and Doelman, 1997). The 10FR treatment is recommended in laboratory tests to assess the side effects of pesticides on soil microflora (Sommerville, 1987). Investigations performed with high concentrations like 100 times higher than field rates although not immediately relevant for agricultural management practice, may be useful for assessing environmental risk in those soils where monocultural practices require repeated application of the herbicide. Such positive control treatment which is already known to have an effect on microbes can also be used to validate the sensitivity of various measurements (Ahtiainen *et al.*, 2003).

The monitoring period is a most important part for the assessment of pesticide effects and a minimum of 30 days has been recommended for the recognition of persistent effects on soils. A delay of 30 days in the restitution of normality (recovery period) after herbicide application should be considered normal with ecological consequences being negligible, a delay of 60 days is not unusual, and the ecological consequences are tolerable and a delay of greater than 60 days is unusual with ecological consequences which may eventually be critical (Domsch *et al.*, 1983).

The results of this experiment revealed that the application of herbicides reduced the population of all the bacteria enumerated during the study with butachlor showing highest reduction in the populations. This effect was stronger with increasing concentration of the herbicides employed. However, the populations at 1FR (and also 2FR for pyrazosulfuron ethyl) concentrations recovered within 30 days to reach populations not significantly different from the control treatments.

Debnath *et al.* (2002) have reported that butachlor at 2 kg ai. ha^{-1} (equivalent to 1 µg g^{-1}soil) stimulated the population of phosphate

Table 3 : Population of phosphate solubilising bacteria as influenced by various herbicides under laboratory conditions

Herbicide concentration	Population of phosphate solubilising bacteria (x 10^3 CFU g^{-1} dry soil)													
	Days after 2,4-DEE application			Days after butachlor application			Days after pretilachlor application			Days after pyrazosulfuron ethyl application			Mean
	7	15	30	Mean	7	15	30	Mean	7	15	30	Mean	7	15	30	Mean	
1FR	39.16	40.12	41.86	40.38	38.24	40.26	41.54	40.01	40.16	41.54	42.15	41.28	41.12	41.84	42.28	41.75	40.86
	(4.592)	(4.599)	(4.621)	(4.603)	(4.584)	(4.607)	(4.619)	(4.603)	(4.599)	(4.622)	(4.624)	(4.617)	(4.617)	(4.626)	(4.626)	(4.622)	(4.611)
2FR	38.42	39.93	41.36	39.90	37.96	38.62	40.62	39.07	39.82	40.75	41.62	40.73	40.26	41.36	41.84	41.15	40.21
	(4.589)	(4.600)	(4.617)	(4.602)	(4.570)	(4.587)	(4.608)	(4.588)	(4.594)	(4.609)	(4.620)	(4.608)	(4.605)	(4.616)	(4.622)	(4.614)	(4.603)
5FR	36.89	38.04	39.82	38.25	35.82	36.62	38.53	36.99	38.16	39.84	40.24	39.41	39.28	40.79	41.04	40.37	38.76
	(4.568)	(4.582)	(4.598)	(4.583)	(4.557)	(4.562)	(4.583)	(4.567)	(4.582)	(4.598)	(4.601)	(4.594)	(4.597)	(4.611)	(4.607)	(4.605)	(4.587)
10FR	35.72	37.62	38.84	37.39	34.73	35.56	37.32	35.87	36.89	38.00	39.26	38.05	38.66	39.06	40.84	39.52	37.71
	(4.552)	(4.574)	(4.590)	(4.572)	(4.542)	(4.554)	(4.571)	(4.556)	(4.570)	(4.584)	(4.593)	(4.582)	(4.592)	(4.593)	(4.613)	(4.599)	(4.577)
100FR	33.84	35.42	37.15	35.47	31.42	30.26	30.99	30.89	34.75	36.26	38.64	36.55	36.24	38.62	40.37	38.41	35.33
	(4.534)	(4.550)	(4.570)	(4.551)	(4.494)	(4.481)	(4.497)	(4.491)	(4.537)	(4.565)	(4.585)	(4.562)	(4.561)	(4.584)	(4.609)	(4.585)	(4.547)
Control	43.26	42.88	42.32	42.82	43.27	42.86	42.32	42.82	43.25	42.86	42.32	42.81	43.24	42.87	42.33	42.81	42.81
	(4.643)	(4.632)	(4.625)	(4.633)	(4.631)	(4.631)	(4.625)	(4.631)	(4.634)	(4.636)	(4.625)	(4.632)	(4.633)	(4.618)	(4.627)	(4.627)	(4.631)
Mean	37.88	39.00	40.23	39.04	36.91	37.36	38.55	37.61	38.84	39.88	40.70	39.81	39.80	40.76	41.45	40.67	39.28
	(4.580)	(4.590)	(4.603)	(4.591)	(4.563)	(4.570)	(4.585)	(4.573)	(4.586)	(4.602)	(4.609)	(4.599)	(4.601)	(4.608)	(4.618)	(4.609)	(4.593)

Factors	C.D. (P=0.05)	Factors	C.D. (P=0.05)	Factors	C.D. (P=0.05)
Herbicides (H)	0.015	HXC	NS	HXCXD	NS
Concentration (C)	0.019	HXD	NS		
Days (D)	0.013	DXC	NS		

Values in parenthesis are $\log_{10}$ transformed; Initial population of phosphate solubilising bacteria before herbicide application: 43.93 x 10^3 CFU g^{-1} dry soil NS-Non significant

solubilising bacteria though a contrasting effect was observed in the present experiment. A reduction in the cellulose degradation was reported by the application of butachlor at 16 µg g^{-1}soil after four weeks of application (Katayama and Kuwatsuka, 1991) which was also observed in this experiment as a significant reduction in the population of cellulolytic bacteria at 100FR concentrations.

It was observed in this experiment that the detracting effects of herbicides towards all bacteria and enzyme activities decreased with time. Recovery of microbial populations and enzyme activities after initial inhibition may be due to microbial adaptation or most probably due to their degradation (Ismail *et al.*, 1998). 2,4-DEE with a half life of 18 days (Jayakumar and Ramulu, 1993) chloroacetamide herbicides like butachlor (half life of 19 days) and pretilachlor (17 days) degrade quickly in soil (Sahid and Yap, 1994, and Murato *et al.*, 2004). Both Gigliotti *et al.* (1998) and Saeki and Toyota (2004) have reported non-significant effects of the sulfonyl urea herbicide bensulfuron methyl on bacterial populations due to the fact that sulfonyl urea herbicides degrade rapidly in the soil. Pyrazosulfuron ethyl, the sulfonyl urea herbicide used in this study had been also reported to have half life of 16 days in an experiment conducted in Taiwan (Chu *et al.*, 2002). The initial decrease followed by small increases in populations could also be due to microbial multiplication on the increased supply of nutrients available in the form of microorganisms killed by herbicides (Boldt and Jacobsen, 1998).

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