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Influence of Endosulfan on Microbial Biomass and Soil Enzymatic Activities of a Tropical Alfisol

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Abstract The effect of endosulfan at normal residue level (1 and 10 ppm) and elevated level (100 ppm) on microbial biomass and enzymatic activities of a tropical alfisol was studied. Dehydrogenase, Fluorescein diacetate hydrolase, acid phosphatase, aryl sulphatase activities and microbial biomass of the soil increased by 2.4, 1.7, 1.4, 1.8 and 3.7 times, respectively by the 14th day of incubation with 1 ppm endosulfan, indicating the possible involvement of soil microorganisms and their enzymes in degradation of endosulfan. Soil nitrogenase activity decreased by 8.0 times by the 14th day of application of 1 ppm endosulfan, suggesting that endosulfan or its metabolites may pose toxicological threat to nitrogen fixers in soil.

Keywords Endosulfan · Tropical alfisol · Soil enzymes · Microbial biomass carbon

Endosulfan (1,2,3,4,7,7-hexachlorobicyclo-2,2,1-heptene-2,3-bis-hydroxy methane-5,6 sulfite) is a broad spectrum cyclodiene insecticide that is used extensively worldwide to control the insect pests of a wide range of crops including cereals, tea, coffee, cotton, fruits, oil seeds and vegetables (Lee et al. 1995). Since a total replacement of endosulfan for pest control is not anticipated in near future, environmental impact of this chemical is receiving serious attention.

Since microorganisms form a vital part of the soil food web (Van Beelen and Doelman 1997), microbial biomass serves as a potential measure of ecosystem functioning (Rath et al. 1998). Soil Dehydrogenase is thought to be an indicator of overall microbial activity, because it occurs intracellulary in all living microbial cells and is linked with microbial oxydoreduction processes (Quilchano and Maranon 2002; Stêpniewska and Woliñska 2005). The hydrolysis of fluorescein diacetate has the potential to broadly represent soil enzyme activity (Schnuer and Rosswall 1982) and accumulated biological effects because Fluorescein diacetate is hydrolyzed by a number of different enzymes, such as protease, lipase and esterase (Green et al. 2006) further its hydrolysis has been observed among a wide array of the primary decomposers, bacteria and fungi (Lundgren 1981). Soil acid phosphatases are sensitive to contamination (Liu et al. 2004) and along with aryl sulphatase can serve as good indicators of alteration of soil quality (Balota et al. 2004). Pesticide contamination has been reported to have adverse effects on soil nitrogenases (Niewiadomska and Sawicka 2002). Thus soil enzyme activity is believed to be sensitive to pollution and has been proposed as an index of soil degradation (Gianfreda et al. 2005; Trasar-Cepeda et al. 2000). A combination of microbial biomass carbon and soil enzyme activities permits valid interpretation of effect of pesticide contamination on soil microbial activity and in turn on soil health. As few reports are available on the effect of endosulfan on soil microbes, the focus of the present study was to understand the influence of endosulfan on microbial biomass and soil enzymatic activities of a tropical alfisol.

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Materials and Methods

Soil samples used for the present study were of tropical alfisol collected from Kerala (India). Soil was collected from the surface 0–15 cm depth, dried in shade, ground to pass through 2 mm sieve, and was stored in polythene bags at room temperature. The physico–chemical characteristics of the soil were determined using standard analytical procedure: pH 5.2 measured at 1:1.25 soil-to-water ratio (Jackson 1967); organic carbon content – 0.85% by Walkley and Black method (Jackson 1967); soil mechanical fractions, sand – 66.0%, silt – 19.2%, clay – 14.8% employing the Bouyoucos hygrometer method (Black et al. 1965) and the electrical conductivity – 0.25 dS m^{-1} (Jackson 1967). Endosulfan was not present in detectable levels in the soil sample.

Endosulfan, technical grade (98%) was obtained from Hafed pesticides, a unit of the Haryana State Cooperative Marketing Federation Ltd., India. α-endosulfan and β-endosulfan are present in technical grade endosulfan in 7:3 proportion. The solvents used were of analytical grade and were purchased locally.

The soil samples were moistened to field capacity and incubated for a period of 7 days at 28 ± 2°C and used for further experiments. Each preincubated soil sample (500 gm, on air dry basis) was taken in earthen pots (1 L) and treated with different concentrations of endosulfan in 1 mL acetone separately to give concentration of 1, 10 and 100 ppm endosulfan in experimental soil. The untreated soil samples were also processed in a similar way by adding 1 mL of acetone and served as control. Treatments and control were set up in triplicates. The moisture loss during the incubation period was restored by weighing the soil samples every second day and replenishing the water lost due to evaporation. Triplicate samples of 15 g of soil were drawn on 0th and 1st day of incubation and thereafter in 7 days interval till 98th day of incubation to evaluate the enzymatic activities and the residual concentration of endosulfan.

Five gram of the withdrawn soil sample was shaken with hexane:acetone (9:1 v/v 50 mL) in a rotary shaker at 160 rpm for 30 min and was further allowed to remain with the extraction solvent for 1 h. The supernatant was passed through anhydrous sodium sulphate column. The extraction was repeated twice again with 25 mL portions of hexane: acetone (9:1 v/v). The organic solvent fractions from these extractions were combined and the solvent was removed by using rotary flash evaporator. The residue was redissolved in hexane and analyzed by GLC.

The samples were analyzed on a Hewlett Packard 5890A Series II Gas Chromatograph equipped with a methyl silicon column (10 m × 0.53 mm × 2.65 µm film thickness) packed with HP-1 and electron capture detector (ECD) ^{63}Ni. The oven, injector and detector temperatures were maintained at 200, 250 and 250°C, respectively and the flow rate of the carrier gar (N_2) was maintained at 60 mL min^{-1}. The detection limit for α-endosulfan, β-endosulfan and endosulfan sulphate were 0.05 µg g^{-1}, 0.04 µg g^{-1} and 0.02 µg g^{-1}, respectively and recovery from the soil at fortification levels of 0.1–100 µg g^{-1} soil was more than 90%.

The microbial biomass carbon in the soil was estimated by fumigation extraction method. Moist sample was taken in triplicate (to give approximately 1 g oven dry weight) in 50 mL glass beaker. Another set of soil was weighed in an aluminium can for moisture determination. Beaker with soil was kept inside vacuum desiccator and fumigated with fresh ethanol-free chloroform for 24 h and the excess chloroform was removed by repeated back suction (Black et al. 1965). The fumigated and non-fumigated soil was extracted with 25 mL of 0.5 M K_2SO4. Carbon content in the extracts was determined by following the modified procedure of Vance et al. (1987).

Soil dehydrogenase activity was estimated as per the method outlined by Casida et al. (1964). One gram of soil was taken in a screw cap vial and added with 3.0 mL of 2,3,5,triphenyl tetrazolium chloride solution (3% w/v) and 1.0 mL of distilled water with a thin layer of water above the surface of soil. The tubes were incubated in dark at 30 ± 1°C. After appropriate incubation (24 h), 25 mL of methanol was added in each tube, mixed on a vortex and left to stand for some time. Then it was filtered through Whatman No. 42 filter paper and optical density of the filtrate was read spectrophotometrically at 485 nm. Dehydrogenase activity was expressed in terms of triphenyl formazon (TPF) produced day^{-1} g^{-1} of soil with reference to a standard curve of TPF.

Fluorescein diacetate hydrolytic activity was estimated as per the method outlined by Green et al. (2006). The activity was estimated through the production of fluorescein from fluorescein diacetate by the action of hydrolytic enzymes in soil.

One milligram of soil was taken in a screw cap vial and added with 5.0 mL of 60 mM sodium phosphate and 10 µl of fluorescein diacetate (0.02%). The tubes were incubated at 37°C for 2 h. The reaction was stopped by adding 0.2 mL of acetone (5% v/v). The mixture was centrifuged at 800 rpm for 5 min. Then it was filtered through Whatman No. 2 filter paper and absorbance was read spectrophotometrically at 490 nm. Fluorescein diacetate hydrolase activity was expressed in terms of mg fluorescein released hr^{-1} g^{-1} of soil with reference to a standard curve of fluorescein.

Assay of acid phosphatase involved the colourimetric estimation of the *p*-nitro phenol released by phosphatase activity when soil was incubated with buffered (pH 6.5) sodium-*p*-nitrophenyl phosphate (PNP) solution and toluene at 37°C for 1 h as described by Tabatabai and Bremner

Table 1 Concentration of α-endosulfan, β-endosulfan and endosulfan sulphate in a tropical alfisol spiked with different concentrations of endosulfan at 1, 10 and 100 ppm

Incubation time (days)	α-Endosulfan (ppm)				β-Endosulfan (ppm)				Endosulfan sulphate (ppm)			
Concentration of applied endosulfan (ppm)	0	1	10	100	0	1	10	100	0	1	10	100
0	ND	0.7	7	70	ND	0.3	3	30	ND	ND	ND	ND
1	ND	0.69	6.18	69.64	ND	0.27	2.83	29.47	ND	0.02	0.45	0.45
7	ND	0.60	5.67	68.72	ND	0.23	2.62	28.91	ND	0.07	0.81	1.19
14	ND	0.45	5.41	64.79	ND	0.18	2.37	28.32	ND	0.12	1.09	3.45
21	ND	0.36	5.06	58.88	ND	0.13	2.04	26.26	ND	0.21	1.45	7.43
28	ND	0.27	4.59	52.76	ND	0.09	1.69	22.86	ND	0.29	1.86	12.19
35	ND	0.19	3.99	45.86	ND	0.06	1.36	17.74	ND	0.32	2.32	18.20
42	ND	0.12	3.09	34.32	ND	0.04	0.92	11.85	ND	0.39	3.00	26.92
49	ND	0.05	2.65	29.41	ND	ND	0.71	9.14	ND	0.42	3.32	30.73
56	ND	ND	2.12	24.54	ND	ND	0.63	7.22	ND	0.47	3.63	34.12
63	ND	ND	1.74	21.16	ND	ND	0.41	6.09	ND	0.46	3.93	36.38
70	ND	ND	1.32	18.25	ND	ND	0.22	5.89	ND	0.46	4.23	37.93
77	ND	ND	0.91	16.71	ND	ND	0.14	5.12	ND	0.45	4.48	39.09
84	ND	ND	0.83	14.27	ND	ND	0.07	4.72	ND	0.44	4.55	40.51
91	ND	ND	0.72	12.91	ND	ND	0.03	4.43	ND	0.42	4.63	41.33
98	ND	ND	0.63	11.03	ND	ND	ND	4.12	ND	0.39	4.69	42.43
CD 5%	–	0.02	0.19	0.36	–	0.01	0.04	0.14	–	0.02	0.07	0.18

(1969). One gram of soil was placed in a 50 mL Erlenmeyer flask. To this, 4 mL of modified universal buffer (pH 6.5), 0.25 mL of toluene and 1 mL of PNP solution were added. After mixing the contents, the flasks were stoppered and incubated at 37°C. After 1 h incubation, the stopper was removed and 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were added. The flasks were swirled for few seconds and filtered through Whatman No. 42. The colour intensity of the filtrate (yellow colour) was measured at 420 nm. The standard curve was drawn with a range of 0–50 µg of PNP. Phosphatase activity was expressed in terms of µg of *p*-nitrophenol released hr^{-1} g^{-1} of soil.

Assay of aryl sulphatase involved the colourimetric estimation of the *p*-nitro phenol released by sulphatase activity when soil was incubated with buffered (pH 5.8) sodium-*p*-nitrophenyl sulphate (PNS) solution and toluene at 37°C for 1 h as described by Tabatabai and Bremner (1970). One gram of soil was placed in a 50 mL Erlenmeyer flask. To this, 4 mL of modified universal buffer (pH 5.8), 0.25 mL of toluene and 1 mL of PNS solution were added. After mixing the contents, the flasks were stoppered and incubated at 37°C. After 1 h incubation, the stopper was removed and 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were added. The flasks were swirled for few seconds and filtered through Whatman No. 42. The colour intensity of the filtrate (yellow colour) was measured at 420 nm. The standard curve was drawn with a range of 0–50 µg of PNS. Sulphatase activity was expressed in terms of µg of *p*-nitrophenol released hr^{-1} g^{-1} of soil.

Nitrogenase activity (Postgate 1971) was measured using Gas Chromatograph (Shimadzu–GC–164A) equipped with a stainless steel column (183 cm × 0.32 cm) packed with Porapak N in 60–80 mesh and flame ionisation detector (FID). The oven, injector and detector temperatures were maintained at 80, 150 and 120°C, respectively and the flow rate of the carrier gar (N_2) was maintained at 30 mL min^{-1}.

The data was analyzed statistically using ANOVA for Completely Randomised Design method outlined in Statistical Procedures for Agricultural Research (Panse and Sukhatme 1978) and the test of significance was done at 5% level.

Results and Discussion

The persistence of α and β endosulfan and the formation of a recalcitrant metabolite viz., endosulfan sulphate in a tropical alfisol treated with different concentrations of endosulfan (1, 10 and 100 ppm) are presented in Table 1. The treatment with 100 ppm was the most persistent in the soil and the residues were detected even on 98th day of incubation. The treatment with 1 ppm endosulfan was the least persistent with the residues reaching undetectable levels by 56th day of incubation. Both α and β endosulfan exhibited similar trends of degradation. Endosulfan sulphate, the major

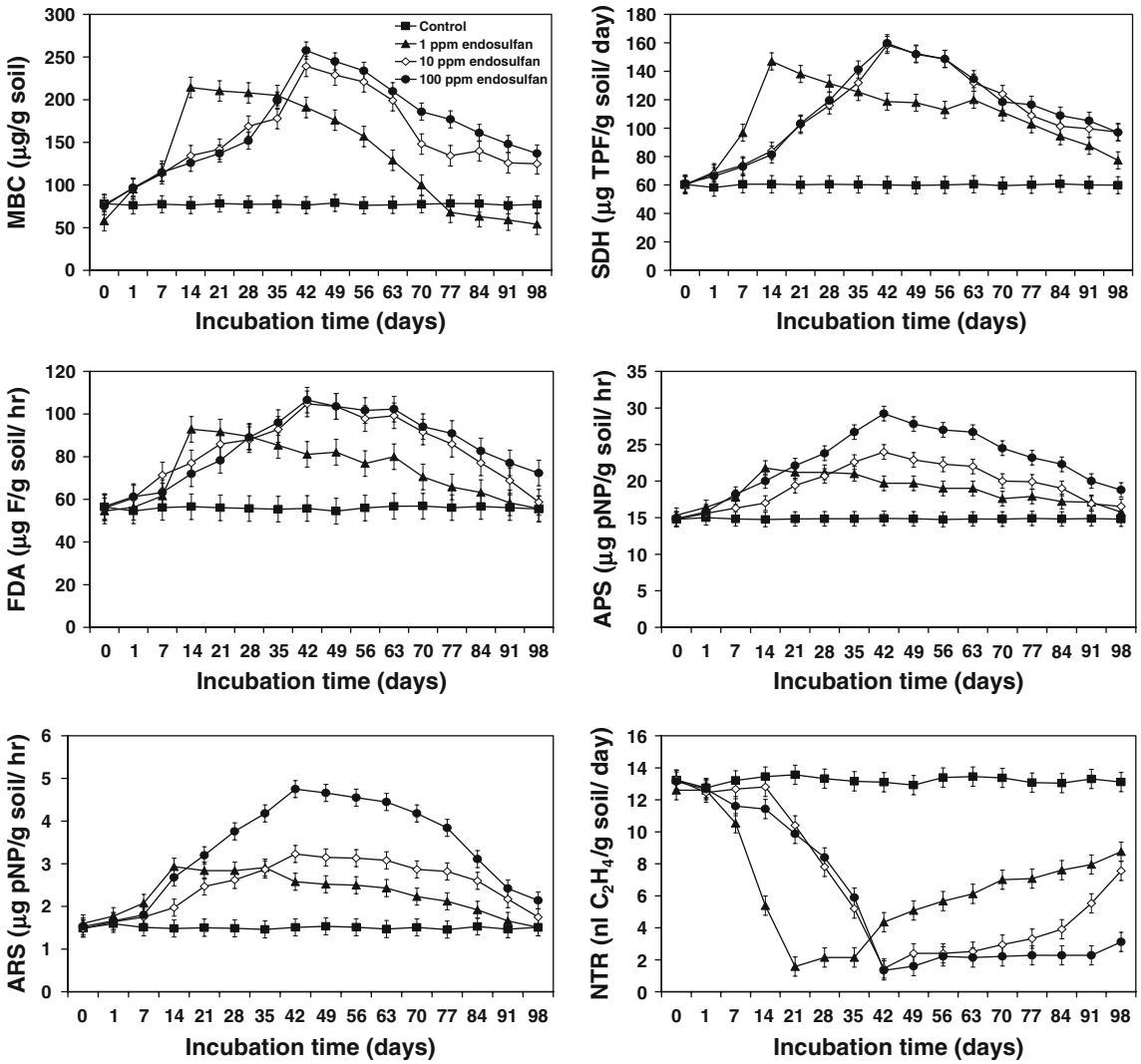

Fig. 1 Effect of endosulfan on microbial biomass carbon and soil enzyme activities of a tropical alfisol

product of degradation of endosulfan, was more persistent than the parent compound and degraded very slowly, an observation made by earlier reports as well (Keith and Telliard 1979; Reviejo et al. 1992).

In the present study, in all treatments microbial biomass carbon (MBC) increased significantly with the application of endosulfan (Fig. 1). For sample spiked with 1 ppm endosulfan, microbial biomass carbon reached its maximum value of 214.4 µg/g of soil by 14th day of incubation. For samples spiked with 10 and 100 ppm endosulfan, microbial biomass carbon reached its maximum value of 239.2 µg/g of soil and 257.7 µg/g of soil, respectively by 42nd day of incubation. Thus the change in microbial biomass followed the rate of depletion of applied endosulfan suggesting the possible proliferation of microorganisms using endosulfan as a nutrient source. The increase in microbial biomass carbon was temporary as evident from the decline in microbial biomass with the depletion of applied endosulfan. The short term effect of endosulfan may be due to degradation of endosulfan or gradual adsorption of endosulfan by the soil colloid making it unavailable for the microbes.

The activities of soil dehydrogenase (SDH), fluorescein diacetate hydrolase (FDA), acid phosphatase (APS) and aryl sulphatase (ARS) followed similar trends as microbial biomass carbon (Fig. 1). Dehydrogenase activity is present only in viable cells and it is a useful indicator of overall microbial activity in soil (Trevors 1984; Wei-xiang et al. 2004). The increase in soil dehydrogenase activity possibly indicated active metabolism of the compound by microbes either for use as nutrient source or for the detoxification of this compound. Pesticide hydrolysis by microbial enzymes can serve as a detoxification mechanism that can govern pesticide resistance and in turn determine the rate of pesticide biodegradation in the environment.

Endosulfan, an organophosphorous pesticide with a reactive cyclic sulfite diester group, may be degraded by

the action of esterases, phosphatases or sulphatases or a combination of these enzymes. An increase in fluorescein diacetate hydrolytic activity suggests the involvement of esterases in the metabolism of endosulfan as fluorescein diacetate is hydrolyzed by a number of different enzymes, such protease, lipase and esterases (Green et al. 2006) of which esterases are relevant to degradation of endosulfan. Arylsulfatase is the enzyme that is involved in mineralization of ester sulfate in soils (Tabatabai 1994) and Phosphatase catalyzes the hydrolysis of a variety of organic phosphomonoesters and has been widely implicated in the degradation of organophosphorous pesticides (Kanekar et al. 2004). The increase in the activity of these enzymes suggests the possible involvement of this group of enzymes in the degradation of endosulfan as well.

In all treatments soil nitrogenase activity (NTR) decreased significantly with degradation of endosulfan (Fig. 1). In soil spiked with 1 ppm endosulfan the soil nitrogenase activity reached the lowest value of 1.48 nl C_2H_4/g/day by 21st day of incubation, whereas in soils spiked with 10 and 100 ppm endosulfan the soil nitrogenase activity reached the lowest value of 0.92 nl C_2H_4/g/day and 1.08 nl C_2H_4/g/day, respectively by 42nd day of incubation. There was a gradual reversal from this drastic decrease, but the soil nitrogenase activity could not restored back to original levels even by the 98th day of incubation. These results suggest that endosulfan had deleterious effect on nitrogenase enzyme or nitrogen fixers in soil. Endosulfan could be adsorbed to the cell membrane of nitrogen fixers and bind nonspecifically to proteins thereby affecting the nitrogenase activity (Buff et al. 1992). Further, endosulfan is known to suppress the growth of nitrogen fixing bacteria (Tandon et al. 1988; Kumar et al. 2008). The suppression of growth of nitrogen fixing bacteria may be either direct or indirect due to the promotion of growth of non-nitrogen fixers that lead to competitive suppression of nitrogen fixers.

Endosulfan may accumulate in specific microhabitats, but it is highly unlikely that concentrations would ever reach effective levels after a single application of the pesticide at recommended dosage as microbial enzymes ensure rapid degradation of the pesticide. Multiple application of endosulfan or spillage above the prescribed dosages may have a short term/reversible detrimental effect on nitrogen fixers in soil. The influence of pesticide on soil is generally a long-term process and is closely related to soil characteristics. In addition, there may be many other pollutants like other pesticides and heavy metals entering the soil at the same time. Hence for obtaining a more realistic picture long-term field study is required.

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