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Toxicity of the Pesticides Hexachlorocyclohexane and Benomyl to Nitrifying Bacteria in Flooded Autoclaved Soil and in Culture Media

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ABSTRACT

The influence of the pesticides HCH and benomyl on nitrification in a flooded autoclaved soil was compared with that in culture media inoculated with Nitrosomonas *and* Nitrobacter *spp. The rate of nitrification was slow in autoclaved soil and was comparatively faster in the culture media, and both the pesticides inhibited nitrification at concentrations of 5 ppm and above. Commercial formulations of HCH and benomyl were more toxic to nitrifying bacteria than the technical grade, probably due to interactions of the carriers and pesticides in increasing the toxicity.*

INTRODUCTION

Extrapolation of results from pure microbial studies to the natural environment such as soil has often been questioned. However, most natural ecosystems are complex in nature with many natural factors interacting to control or monitor a single biological process. For example, in the nitrogen cycle of a flooded soil, it is difficult to determine whether the nitrification process is partially obscured by denitrifying organisms or by non-biological factors.

In a flooded soil system where rice is grown, although the bulk of the soil is reduced, the thin, oxidised surface layer at the top offers a favourable site for aerobic biochemical transformations such as nitrification (Mitsui, 1955). However, reports on the effects of pesticides on the nitrification in flooded soil are surprisingly limited, apart from a

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few publications on the inhibitory effect of benomyl (Ramakrishna *et al.*, 1979), HCH (hexachlorocyclohexane) (Ray *et al.*, 1980), etc. Since nitrification in a flooded soil can be undesirable from an agricultural viewpoint, the factors regulating this process need to be further clarified in order to assess the potential of nitrifying micro-organisms for practical purposes. In this paper, the effect of HCH and benomyl on the nitrifying characteristics of *Nitrosomonas* and *Nitrobacter* spp. were studied in artificial growth media and in a flooded autoclaved soil.

MATERIALS AND METHODS

An ammonium-oxidising bacterium, *Nitrosomonas* sp., and a nitrite-oxidising bacterium, *Nitrobacter* sp., isolated from an alluvial soil by an enrichment culture technique, were used in this study. *Nitrosomonas* sp. was grown in a mineral medium (Gowda *et al.*, 1977), i.e. $(NH_4)_2SO_4$, 1·0 g; K_2HPO_4, 0·5 g; NaCl, 2·0 g; $MgSO_4 . 7H_2O$, 0·2 g; $FeSO_4 . 7H_2O$, 0·5 g; $CaCO_3$, 6·0 g; distilled water, 1 litre. *Nitrobacter* sp. was grown in a mineral medium (Gowda *et al.*, 1977) , i.e., $NaNO_2$, 1·0 g; K_2HPO_4, 0·5 g; $MgSO_4 . 7H_2O$, 0·5 g; $FeSO_4 . 7H_2O$, 0·05 g; distilled water 1 litre; pH of the medium was adjusted to 7·6. The bacteria were transferred to fresh mineral media and grown for 10 days at 30 °C before they were used for inoculation of autoclaved soil or liquid media.

An alluvial soil (pH, 6·0; total nitrogen, 0·29 %; sand, 25·9 %; silt, 21·5 % and clay, 52·5 %) was used. The soil samples (10 g) were spread as a thin layer in 250-ml Erlenmeyer flasks and 2 ml of distilled water was added, before autoclaving the soil three times for 1 h over periods of 2 to 3 days. Technical HCH (1, 2, 3, 4, 5, 6-hexachlorocyclohexane) and benomyl (methyl 1-(butylcarbamoyl) benzimidazole carbamate), as well as commercial formulations of HCH (Hexamar, 5 % granules) and benomyl (Benlate, 50 % wettable powder) were obtained from Bharat Pulverising Mills and Hoechst Pharmaceuticals Ltd, Bombay, respectively. Commercial formulations of HCH and benomyl were incorporated into the sterilised soil samples in hexane and methanol, respectively, to give 5, 20 and 50 ppm active ingredient of the pesticides with respect to soil. The hexane and methanol were allowed to evaporate overnight and 18 ml of sterile distilled water containing millipore-filtered $(NH_4)_2 SO_4$ or $NaNO_2$ solution (40 mg per flask) were added to the sterilised soil samples. Flasks without pesticides, but with hexane or

methanol only (evaporated off) plus $(NH_4)_2SO_4$ or $NaNO_2$, served as controls. The soil samples were inoculated with 1·0 ml of the selected stock culture and swirled for a few seconds. The thin layer of soil in the 250-ml Erlenmeyer flasks flooded with 20 ml of distilled water provided a good model for the aerobic site of active nitrification in a predominantly anaerobic flooded soil (Ray *et al.*, 1980).

To test the effect of HCH and benomyl on autotrophic nitrification in culture media, commercial, as well as technical, grade HCH and benomyl were dissolved in hexane and methanol, respectively and then introduced into sterilised 100-ml Erlenmeyer flasks in 1-ml portions to provide final concentrations of 5, 20 and 50 ppm in 40-ml media. After evaporating off the methanol and hexane at room temperature, the sterile mineral salt media specific for *Nitrosomonas* and *Nitrobacter* spp., containing ammonium sulphate and sodium nitrite as nitrogen source, respectively, were dispensed in 40-ml portions to the flasks. After equilibration for 24 h in a Gallenkamp orbital shaker, the ammonium- and nitrite-containing media were inoculated with 10 day old cultures of *Nitrosomonas* and *Nitrobacter* spp., respectively. Media without pesticides but with hexane or methanol only (evaporated off) served as controls.

In soil incubation studies, nitrite and nitrate were extracted by shaking the soil with 50 ml N sodium sulphate solution (Onken & Sunderman, 1977) for 30 min on a wrist action shaker. After filtration, the clear filtrate was analysed for nitrite by diazotisation (Barnes & Folkard, 1951) and nitrate by the phenol disulphonic acid method (Bremner, 1965). In pure culture studies with autotrophic nitrifiers, nitrite in the mineral salt media was assayed colorimetrically by diazotisation after appropriate dilution of the media.

RESULTS AND DISCUSSION

Effect of HCH and benomyl on nitrification by *Nitrosomonas* and *Nitrobacter* spp. in autoclaved soil

Commercial formulations of HCH (granules) and benomyl (wettable powder) and commercial, as well as technical, HCH and benomyl, were used to study their effect on nitrification in the simulated oxidised surface layer of a flooded autoclaved soil and in culture medium, respectively,

inoculated with *Nitrosomonas* and *Nitrobacter* spp. (Tables 1 to 4). Oxidation of ammonium to nitrite by the inoculated *Nitrosomonas* sp. proceeded slowly in flooded autoclaved control soils. Similarly, the oxidation of nitrite to nitrate also proceeded slowly in the control soils inoculated with *Nitrobacter* sp. The addition of HCH and benomyl to these soil samples retarded ammonium oxidation (Table 1) and nitrite oxidation (Table 2) at a concentration of 5 ppm and more effectively at 20 and 50 ppm. Also, HCH was found to be more inhibitory than benomyl: it inhibited nitrite oxidation by 90% compared with 74% inhibition by benomyl during the 40-day incubation period (Table 2).

Earlier studies (Ray *et al.*, 1980) have shown significant inhibition of ammonium oxidation by HCH in a flooded soil at concentrations of 10 ppm and above. Thus, the results of the present study corroborated the earlier report. However, most of the studies concerning HCH reported that the insecticide was not deleterious to ammonium or nitrite oxidation in soil at rates close to those of field applications (3–5 ppm), whereas its application at concentrations as high as 100 ppm and above could lead to significant inhibition of oxidation of ammonium sulphate or nitrite (Bardiya & Gaur, 1970; Verstraeten & Vlassak, 1973; Gaur & Misra, 1977). In contrast, side effects of benomyl on nitrification in soil are somewhat conflicting. Hofer *et al.* (1971), in non-flooded soils, and Ramakrishna *et al.* (1979), in flooded soil, reported inhibition of nitrification by benomyl at the 100 to 1000 ppm levels. There have also

TABLE 1
Effect of HCH and Benomyl on Nitrification by *Nitrosomonas* sp. in Flooded Autoclaved Soil

Pesticide	*Concentration (ppm)*	*μg nitrite recovered per gram of soil Incubation (days)*		
		10	*25*	*40*
Control		13	20	36
HCH	5	10*	15	22
	20	5	11	18
	50	2	5	10
Benomyl	5	11*	20*	30
	20	7	10	15
	50	4	8	14

Means of three replicates.
* Not significantly different from control ($p = 0{\cdot}05$).

TABLE 2
Effect of HCH and Benomyl on Nitrification by *Nitrobacter* sp. in Flooded Autoclaved Soil

Pesticide	*Concentration (ppm)*	*μg nitrate recovered per gram of soil Incubation (days)*		
		10	*25*	*40*
Control		35	75	117
HCH	5	25*	54	88
	20	10	22	53
	50	1	8	12
Benomyl	5	27*	61	89
	20	17	27	70
	50	6	18	30

Means of three replicates.
* Not significantly different from control ($p = 0{\cdot}05$).

TABLE 3
Effect of HCH and Benomyl on Nitrification by *Nitrosomonas* sp. in Culture Medium

Pesticide		*Concentration (ppm)*	*μg nitrite recovered per millilitre of medium Incubation (days)*	
			5	*15*
Control			19	127
HCH	I. Technical	5	19*	128*
		20	10	116
		50	6	19
	II. Commercial	5	6	64
		20	3	18
		50	1	3
Benomyl	I. Technical	5	14*	122*
		20	9	118
		50	4	16
	II. Commercial	5	7	82
		20	3	16
		50	1	7

Means of duplicate.
* Not significantly different from control ($p = 0{\cdot}05$).

TABLE 4
Effect of HCH and Benomyl on Nitrification by *Nitrobacter* sp. in Culture Medium

Pesticide	*Concentration (ppm)*		*μg nitrite recovered per millilitre of medium Incubation (days)*	
			5	*15*
Control			337	10
HCH	I. Technical	5	446	136
		20	538	325
		50	727	675
	II. Commercial	5	628	515
		20	717	608
		50	928	825
Benomyl	I. Technical	5	444	170
		20	518	340
		50	617	428
	II. Commercial	5	545	217
		20	607	503
		50	827	613

Means of duplicate.
* Not significantly different from control ($p = 0{\cdot}05$).

been reports that nitrification was little affected (Helweg, 1973*b*) or slightly enhanced (Van Fassen, 1974) by applications of benomyl to non-flooded soils at concentrations ranging from 10 to 1000 ppm. In view of these reports showing largely innocuous or slightly stimulating effects of benomyl when applied at low levels, the present study of nitrification inhibition by benomyl at a concentration as low as 5 ppm (compared with the field application rates of 8 to 10 ppm) is noteworthy. However, such conflicting reports on pesticidal action on soil microorganisms and their activities are not uncommon in the literature. The reported discrepancies with respect to benomyl or HCH action on nitrification could, at best, be ascribed to their fluctuating persistence in soil, soil properties and different test conditions. Pesticides like HCH (Sethunathan, 1973) and benomyl (Helweg, 1973*a*) are readily biodegradable in soil and there is circumstantial evidence that bavistin, the degradation product of benomyl, is totally innocuous to nitrification in soil (Ramakrishna *et al.*, 1979). Since I have used autoclaved soil as the medium for studying nitrification, the microorganisms which could have degraded these pesticides were virtually absent from the soil. This factor possibly

stabilised the persistence of HCH and benomyl in the soil environment for maintaining continuous toxic action on the nitrifying organisms.

Effect of HCH and benomyl on nitrification by *Nitrosomonas* and *Nitrobacter* spp. in culture media

As compared with the soil system, oxidation of ammonium to nitrite in *Nitrosomonas* culture medium or nitrite to nitrate in *Nitrobacter* culture medium proceeded fairly rapidly in the unamended medium (Tables 3 and 4). The higher rate of ammonium or nitrite oxidation in unamended culture media than in flooded control soil was probably because of the ready availability of required minerals for optimum growth of *Nitrosomonas* or *Nitrobacter* spp. in culture media, whereas minerals other than ammonium or nitrite might be limiting in one phase or other for the growth of nitrifiers in the flooded control soils. The addition of HCH or benomyl (technical and commercial) at concentrations of 5 ppm and above inhibited the oxidation of ammonium to nitrite in *Nitrosomonas* culture medium and nitrite to nitrate in *Nitrobacter* culture medium. These results tallied with the previous reports which have shown that nitrite oxidation by *Nitrobacter agilis* was inhibited by HCH at 5 ppm and benomyl at 10 ppm, whereas ammonium oxidation by *Nitrosomonas* sp. was inhibited by either HCH or benomyl at concentrations of 10 ppm and above (Garretson & San Clemente, 1968; Ramakrishna *et al.*, 1979). Interestingly, HCH and benomyl were found to be more inhibitory in culture media than in the inoculated autoclaved soil. This was probably because a certain amount of the pesticide might have been sorbed by the soil, thereby depleting the amount of pesticide available to act on nitrifying organisms. In culture media, the sorption of pesticides was virtually absent (Sethunathan, 1973).

Also, commercial formulations of either HCH or benomyl were found to be more inhibitory to nitrification in culture media than the technical grade. Technical HCH or benomyl at the 20 ppm level reduced nitrification (compared with the control) in *Nitrosomonas* culture medium by 9 to 11 % compared with 93 % reduction by either formulated HCH or benomyl (20 ppm) at the end of 15 days' incubation (Table 3). However, this discrepancy in action between the technical and formulated HCH and benomyl is not understood. There are reports of significant interactions between the pesticides and carriers used in their formulations leading to increased/decreased toxicity to certain commercial formulations, over the

technical grade, to insects (Brattsten & Wilkinson, 1977) and biochemical transformations such as nitrification (Kuseske *et al.*, 1974) and sulphur oxidation (Ray & Sethunathan, 1980). Although it may be presumed that the metabolism of carrier compounds in the present study probably increased the toxicity to nitrifiers, over that of technical HCH or benomyl, this cannot be safely concluded without any valid data. Since commercial formulations of pesticides are used in agriculture, the inhibition of nitrification by formulated HCH and benomyl at field application rates, as reported in this study, may be of practical significance.

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