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Influence of Pesticides on Methane Oxidation in a Flooded Tropical Rice Soil

S. Kumaraswamy, A. K. Rath, K. Bharati, B. Ramakrishnan, N. Sethunathan

Central Rice Research Institute, Cuttack, 753 006, India

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The atmospheric concentration of methane, an important greenhouse gas, is increasing by 0.7%.yr^{-1} (Houghton et al. 1995). The relative potential of methane for thermal absorption is 30 times greater than that of CO_2 (Bouwman 1990). The rice paddy ecosystem is one of the major sources of methane and also can serve as an effective sink for methane. In submerged rice paddies, the oxic surface soil-water interface modulates the methane flux to the atmosphere through microbial methane oxidation. Thus, methanotrophy can be important to the global methane budget (King 1992). About 80% of methane produced in anaerobic soil is oxidised to CO_2 in the aerobic thin surface layer (Conrad and Rothfuss 1991) and rhizosphere. Methane-oxidising bacteria, dominant in oxic zones of a flooded rice soil function as a biofilter for methane.

Microbial oxidation of atmospheric methane in a terrestrial enviromnent is controlled by many factors : land use and agricultural practices like fertilizer management and plant protection practices (Hanson and Hanson 1996). The effect of mineral fertilizers, land use practices and soil conditions on methane oxidation is well documented (Hutsch et al. 1994; Dunfield and Knowles 1995). Pesticides, insecticides in particular, are increasingly used in rice culture. Studies indicate that most pesticides used in agriculture and public health, at recommended levels and intervals, are seldom toxic to many transformations of importance to soil fertility and environmental safety. But, recent reports have shown that certain commonly used rice insecticides such as hexachlorocyclohexane (HCH) and carbofuran effect stimulation or inhibition of certain important biochemical transformations in flooded soil, even at recommended levels.

Application of HCH at close to recommended levels stimulated nitrogenase activity (Patnaik et al.1994) and inhibited nitrification in a rice soil (Ray et al. 1980). Ramakrishna et al. (1978) reported distinct stimulation of autotrophic nitrification by carbofuran in a rice rhizosphere soil suspension. The effect of xenobiotics on microbial methane oxidation in rice soils, however, is little understood.

Correspondence to: N. Sethunathan

Therefore, we studied the influence of commercial formulations of two widely used insecticides [hexachlorocyclohexane (HCH) and carbofuran] and a herbicide [2,4-dichlorophenoxyacetic acid (2,4-D)] on methane oxidation in a flooded soil.

MATERIALS AND METHODS

An alluvial soil, a Typic Haplaquept (deltaic alluvium) with a sandy clay loam texture (25.9% clay. 21.6% silt, 52.5% sand; pH 6.2, cation exchange capacity 15 mEq 100 g^{-1}, total C 0.76%, total N 0.09% electrical conductivity 0.6 dS m^{-1}) from the experimental farm of the Central Rice Research Institute, Cuttack, India was used. The soil was air-dried, sieved (<2 mm) and portions (10 g) of the soil were placed in 120 mL sterile serum bottles. Suspensions of 32% HCH granules [Hindustan Insecticides Ltd., Rune, India] in acetone, were added to the soils to provide concentrations of 1, 2, 5 and 10 µg active ingredient.g^{-1} soil. Likewise, a suspension of 75% carbofuran [Rallies India Ltd., Bangalore, India] in acetone, was added to the soil samples at 5 µg.g^{-1} soil, while aqueous suspensions of 2,4-D were added to the soil samples to provide concentrations of 5, 10, 25, 50, 75 and 150 µg.g^{-1} of air-dried soil. In all the cases, the volume of acetone solutions or of aqueous suspensions to provide different levels of pesticides to the soil was 1 mL. The serum bottles containing unamended soil receiving acetone or sterile distilled water alone served as controls. After keeping the serum bottles open overnight for acetone to get evaporated and also those treated with 2,4-D, the soil in each bottle was thoroughly mixed and flooded with sterile distilled water at 1 : 1.25 soil to water ratio and then allowed to equilibrate with ambient air for 3 days in a dark incubator at 30 ± 2 2°C. A time course study on methane oxidation was initiated by sealing the serum bottles with black rubber septa and injecting the headspace with 10 mL of methane (5%) in argon (to provide approximately 2200 µmol of methane.g^{-1} air-dried soil). Another set of soil samples (pesticide-treated and untreated) was exposed to acetylene, an inhibitor of methane oxidation (Oremland and Capone 1988) at a final concentration of 1% after addition of methane to ascertain the role of methanotrophs in the disappearance of methane from the headspace. Soil samples were incubated at room temperature (28 ± 2°C) with intermittent shaking on a rotary shaker for a period of 8 hr in a 24 hr cycle. On alternate days, headspace samples (0.2 mL) of the serum bottles were analysed for methane until 10 d by a Varian 3600 gas chromatograph fitted with flame ionization detector and a 5 A molecular sieve column (2 m x 0.3 cm stainless steel). The column and detector were maintained at 90°C and 100°C, respectively, and the carrier gas was argon with a flow rate at 1.0 kg cm^{-2}. Under these conditions, the retention time for methane was 1.5 mm and the detection limit was approximately 0.5 µg in a 1 mL gas sample. Triplicate incubation vessels of each treatment were sacrificed at each sampling. The mean values were analysed for statistical significance following Duncan's multiple range test.

Total bacterial populations in the soil samples were estimated by the standard dilution plate technique (Rand et al. 1975). Most probable number technique of Schmidt and Belser (1975) was followed to estimate the population of autotrophic ammonium oxidisers. Methane oxidisers with soluble methane monooxygenase (sMMO) activity were enumerated as described by Graham et al. (1992). Triplicate plates for each dilution were incubated in vacuum desiccators under the atmosphere of methane (5%)-air mixture, with replenishing the headspace atmosphere with methane on every four days, for 30 days in an incubator. The colonies which developed a colored complex with naphthalene and *O*-dianisidine (tetrazotized) were counted positive for methane oxidisers with sMMO.

RESULTS AND DISCUSSION

Methane oxidation, in terms of the decrease in the concentration of methane in the headspace of the incubation vessel, proceeded very rapidly in untreated (control) soil and in soil samples treated with HCH at 1 and 2 $\mu g.g^{-1}$ air-dried soil (Table 1). HCH inhibited methane oxidation significantly at 5 $\mu g.g.^{-1}$ soil and almost completely at 10 $\mu g.g^{-1}$ soil. Thus, the headspace concentration of methane reached very low levels (less than 0.5% of the original level) at 0, 1 and 2 μg HCH.g^{-1} soil in 8 days; in contrast, during the corresponding period, about 43% of the added methane was recovered from soil treated with HCH at 5 $\mu g.g^{-1}$ soil and above 70% from the soil treated with HCH at 10 $\mu g.g^{-1}$ soil. In samples (untreated or treated with HCH irrespective of concentration) amended with acetylene, an inhibitor of methane oxidation (Oremland and Capone 1988). there was no appreciable decrease in methane concentration in the headspace during an 8 d incubation period (data not provided) indicating the role of methane-oxidising bacteria in methane oxidation. In a flooded soil, HCH is known to inhibit both oxidation (e.g. nitrification; Ray et al. 1980) and reduction (e.g. iron reduction; Pal et al. 1980) reactions. Addition of HCH retarded the drop in redox potential and maintained the soil in oxidised state (Pal et al. 1980). Despite oxidised conditions in the flooded soil amended with HCH, methane oxidation monitored aerobically in the present study. was inhibited; probably, HCH is toxic to methane-oxidizing bacteria.

Most probable number estimates showed that HCH was inhibitory to the population of total bacteria, methanotrophs (sMMO) and autotrophic ammonium oxidisers: even at 2 $\mu g.g^{-1}$ air-died soil (Table 2). HCH, applied at 10 $\mu g.g^{-1}$ soil, suppressed the population of methanotrophs and autotrophic ammonium oxidisers totally. Autotrophic ammonium oxidisers have also been implicated in the oxidation of methane (Bedard and Knowles 1989). Evidently. inhibition of autotrophic ammonium oxidisers and other methane-oxidisers by HCH would explain the low methane-oxidising activity in soil samples amended with HCH, especially at 10 $\mu g.g^{-1}$ soil.

Table 1. Effect of commercial formulation of hexachlorocyclohexane (HCH) on the oxidation of methane* in a flooded alluvial soil

HCH ($\mu g.g^{-1}$ soil)	μmol of net methane oxidised.g^{-1} air-dried soil			
	Incubation (Days)			
	2	4	6	8
0	427^{ab}	1315^{a}	2125^{a}	2173^{a}
1	484^{a}	1153^{a}	1874^{b}	2099^{a}
2	265^{b}	955^{b}	1486^{c}	2030^{a}
5	28^{c}	217^{c}	430^{d}	644^{b}
10	2^{c}	198^{c}	274^{d}	395^{c}

Data represent mean values; in a column, means followed by a common letter are not significantly different at the 5% level by Duncan's multiple range test.

* The concentration of methane added to the headspace air was 2200 $\mu mol.g^{-1}$ air-dried soil.

Table 2. Influence of commercial formulation of hexachlorocyclohexane (HCH) on the populations (colony forming units.g^{-1} of air-dried soil) * of total bacteria, methanotrophs with sMMO activity and autotrophic ammonium oxidsers in a flooded soil

HCH ($\mu g.g^{-1}$ soil)	Total bacteria ($\times 10^{6}$)	Methanotrophs with sMMO activity ($\times 10^{4}$)	Autotrophic ammonium oxidisers ($\times 10^{2}$)
0	158	238	95
2	104	120	11
10	72	60	1

* Microbiological analyses of the soil samples were performed after 10 days of incubation for methane oxidation.

In another study, addition of 2,4-D, a herbicide, to the flooded soil, significantly retarded the oxidation of methane at concentrations of 50 $\mu g.g^{-1}$ soil and above (Table 3). Within 6 days, the headspace concentration of methane decreased to

negligible levels in untreated soil and in soil samples treated with 2,4-D at 5, 10 and 25 µg.g^{-1} soil. At 25 µg.g^{-1} soil, 2,4-D appeared to be stimulatory. In contrast, about 40% of the originally applied methane was recovered from soil samples treated with 2,4-D at 50 µg.g^{-1} soil and about 70% from soil samples at an abnormally high level of 150 µg.g^{-1} soil. This would indicate that methane oxidation is little affected at recommended levels of 2 to 5 µg.g^{-1} soil. Most probable number estimates showed that methane-oxidising bacteria were distinctly stimulated over that of control by 2,4-D at 25 µg.g^{-1} soil (Table 4). But, 2,4-D effected a >2-fold decrease in the population of methane-oxidising bacteria over that of control at 100 µg.g^{-1} soil. Evidently, the concentration-dependent effect of 2,4-D on methane oxidation was related to fluctuations in the density of methane oxidisers in the soil.

Table 3. Effect of 2,4-D on the oxidation of methane* in a flooded alluvial soil

2,4-D µg.g^{-1} soil	µmol of net methane oxidised.g^{-1} of air-dried soil		
	Incubation (Days)		
	2	4	6
0	518^{b}	1514^{b}	2188^{c}
5	495^{b}	1145^{b}	2193^{c}
10	418^{b}	1602^{b}	2200^{c}
25	1469^{c}	2198^{c}	2200^{c}
50	237^{a}	565^{a}	1083^{b}
75	0	0	135^{a}
150	0	0	179^{a}

Data represent mean value ; In a column, means followed by a common letter are not significantly different at the 5% level by Duncan's multiple range test.

* The concentration of methane added to the headspace air was 2200 µmol.g^{-1} air-dried soil.

In another experimen, the effect of HCH and carbofuran, applied singly and in combination, on methane oxidation in a flooded soil was studied. Carbofuran, applied singly at 5 µg.g^{-1} soil, appeared to stimulate methane oxidation over that of control (Table 5); but. the concentration of methane reached negligible levels within 6 days of incubation in both control and in soil samples treated with carbofuran. In contrast, HCH inhibited the oxidation of methane as noticed in the earlier experiment. Interestingly, the inhibitory effect of HCH could not be

Table 4. Populations (colony forming units.g^{-1} air-dried soil)* of total bacteria and methane-oxidizing bacteria with soluble methane monooxygenase in soil samples treated with 2,4-D at different concentrations

2,4-D ($\mu g \cdot g^{-1}$ soil)	Total bacteria ($x 10^6$)	Methantrophs with sMMO ($x10^4$)
0	45	185
10	98	170
25	130	330
100	69	80

* Microbiological analyses of the soil samples were performed after 10 days of incubation for methane oxidation.

Table 5. Methane oxidation* in flooded alluvial soil as influenced by the addition of carbofuran singly or in combination with commercial HCH

Treatment	µmol of net methane oxidised.g^{-1} air-dried soil Incubation (Days)			
	2	4	6	8
Control	416^{b}	1581^{b}	2052^{a}	2200^{a}
Carbofuran[1]	590^{a}	2121^{a}	2185^{a}	2200^{a}
HCH[2]	0	334^{c}	732^{c}	815^{b}
Carbofuran + HCH[3]	116^{bc}	434^{c}	1083^{b}	1314^{c}

Data represent mean values; In a column means followed by a common letter are not significantly different at the 5% level by Duncan's multiple range test.

* The concentration of methane added to the headspace air was 2200 µmol.g^{-1} air-dried soil.

[1] Added at 5 µg.g^{-1} soil; [2] Added at 5 µg.g^{-1} soil;

[3] Carbofuran (5 µg.g^{-1}) and HCH (10 µg.g^{-1}) in combination were added.

alleviated even when applied in combination with carbofuran. In a more recent study, application of HCH also inhibited net methane production in the laboratory-incubated flooded soil and methane emission from flooded rice

fields (Satpathy et al. 1997). Thus, HCH can be inhibitory to both methane oxidation and methane production.

To sum up, application of HCH to a soil, even at recommended levels, retarded methane oxidation while carbofuran stimulated it. Evidently, certain cultural practices used in rice cultivation, for instance pesticide use, can also serve as an effective means of regulating methane oxidation for mitigation of methane emission from rice fields.

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