Bull. Environ. Contam. Toxicol. (2004) 73:358–363
© 2004 Springer Science+Business Media, Inc.
DOI: 10.1007/s00128-004-0436-7

Bulletin of Environmental Contamination and Toxicology

Disappearance of Chlorpyrifos from Cultures of *Chlorella vulgaris*

I. Mukherjee,[1] M. Gopal,[1] D. W. Dhar[2]

[1] Agricultural Research Service, Division of Agricultural Chemicals, Indian Agricultural Research Institute, New Delhi 110012, India
[2] National Center for Conservation and Utilization of Blue Green Algae (NCCUBGA), Indian Agricultural Research Institute, New Delhi 110012, India

Received: 15 April 2003/Accepted: 14 May 2004

Chlorpyrifos [O, O-diethyl-O-(3,5,6-trichloro-2-pyridinyl)-phosphorithioate, I] is known to be an effective insecticide for the control of insect pests in crops and vegetables (Rouchaud et al. 1991). It is used both as foliar spray and soil treatment. Chlorpyrifos is recommended for the control of soil borne insect pests in pulses, oil seeds and vegetables. It is persistent in nature (Chapman et al. 1984) as its residues were detected in soil even after 3 months. Due to its widespread use in agriculture, occurrences of its residues in food and in different environmental compartments have been reported (Neidert et al. 1994; 1994; KAN-DO, 1995).

The metabolism of chlorpyrifos by microorganism in soil has been reported by Rouchaud et al. (1991). It gets oxidized to the oxon analog [O, O-diethyl -O-(3,5,6-trichloro-2-pyridinyl) phosphate, III] of the insecticide and finally into 3,5,6-trichloropyridinol (II). The insecticidal activity of the latter is 20 percent of chlorpyrifos. There is a growing concern about the toxicological and environmental risk associated with chlorpyrifos residues, which demands a more comprehensive monitoring. The persistent nature of the pesticide is a health hazard (Cochran et al. 1995) and there is need to detoxify this moiety. Meghraj et al. (1987) has been able to degrade monocrotophos and quinalphos successfully by the use of both green and blue-green algae. Chlorpyrifos has been effectively degraded by two soil fungi, *Trichoderma viride* and *Aspergillus niger* (Mukherjee and Gopal 1996). This paper presents a novel route for the detoxification of chlorpyrifos using unicellular green algae.

MATERIALS AND METHODS

Axenic cultures of *Chlorella vulgaris* (ARC 1) were obtained from Division of NCCUBGA, IARI, New Delhi. The study was carried out using BG11 broth medium (Stainer et al. 1971) in which the algae were incubated at 28 ± 1°C temperature and 3000-lux light intensity (16 hr / 8 hr, light dark regime). The experiment was carried out in triplicate. One mL of 25 μg and 50 μg of chlorpyrifos dissolved in acetone was added to eighty-four flasks under sterilized conditions of Laminar flow hood and allowed to evaporate under room temperature. To all the one hundred and five conical flasks (150 mL capacity), 50 mL of the BG11 medium was added and the flasks were plugged

Correspondence to: I. Mukherjee

with cotton. The flasks were autoclaved at 15 atms pressure for 30 min. Four sets of the experiment were maintained, set I: BG11 medium + algal culture, set II and set III: BG11 medium + pesticide (of two different concentrations), set IV and set V: BG11 medium + algal culture + pesticide. The sets III, II and I served as control experiments. The set IV and set V served as treated. To each flask (of set

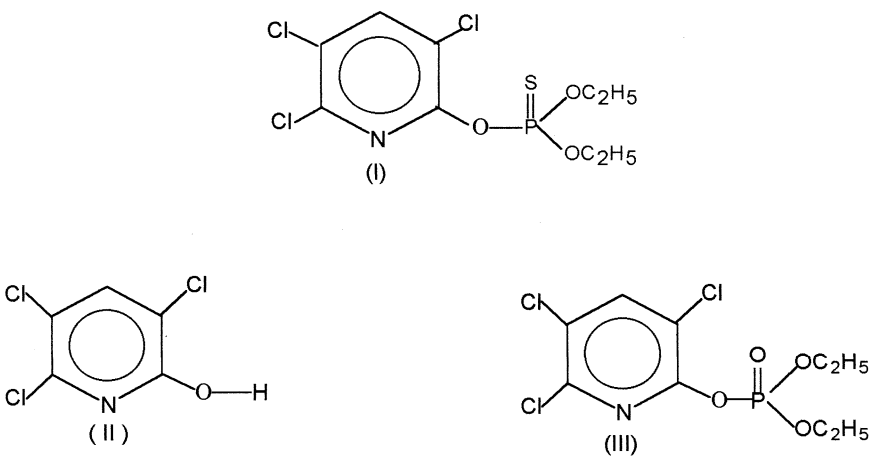

Figure 1. Chlorpyrifos(I), 3,5,6-trichloropyridinol (II) and Chlorpyrifos oxone (III)

I, IVand V) 5 mL of the exponentially growing algal culture was added but not in flasks of set II and III. The pH of the medium recorded after the addition of algae and pesticide was about 7.5. The flasks were taken out periodically on day -0, 1, 3, 5, 7, 15 and 20 for the estimation of chlorpyrifos. The medium was centrifuged and then filtered. The filtrate was subjected to liquid-liquid partitioning thrice with hexane (30 mL). The algal residue left in the tube was transferred to a conical flask, to which hexane (20 mL) was added and shaken vigorously for 15 min in a shaker. The solvent was filtered through anhydrous sodium sulfate and stored. The process was repeated again with 20 mL hexane. The combined organic layer was dried and evaporated under reduced pressure to remove all the traces of hexane. The sample was made up in hexane (10 mL) and analyzed by GLC using a Hewlett Packard 5890 series II instrument. The column used was a megabore HP I (10 m long × 0.25 μm film thickness × 0.52 μm). The column temperature was maintained at 190 °C while the injector port and the detector were set at 210 °C and 250 °C, respectively. The carrier gas nitrogen flow was maintained at 30 mL min^{-1}. The retention time of chlorpyrifos was 2.71 min. The identity of the pesticide was confirmed by carrying out the GLC using a column of alternate polarity, on glass column SE30 (2 m x 2 mm ID) coated on Chromosorb WHP. The column temperature was maintained 190 °C and the injector port and the detector were set at 210 °C and 250 °C, respectively. The nitrogen gas flow was 32 mL min^{-1}. The retention time of the pesticide under these conditions was 3.01 min.

The metabolite of chlorpyrifos (I), 3,5,6-trichloropyridinol (II) was prepared in the laboratory to serve as authentic standard sample. Chlorpyrifos (500 mg) was dissolved in ethanol (30 mL) and subjected to alkaline hydrolysis with 10 percent potassium hydroxide (15 mL). The reaction mixture was refluxed for 4 hour and worked up by neutralizing with dilute hydrochloric acid and further partitioning into dichloromethane thrice (3x 30 mL). The organic solvent was removed and 3,5,6-trichloropyridinol (II) obtained was recrystallized from ethanol as white crystals, mp 68 °C, Rf 0.26 (10% acetone -benzene), IR ν KBr cm^{-1} (3150); NMR CCl_4 : δ 8.20 (s 1 H, Ar-H) (Figure 1).

The oxon metabolite of chlorpyrifos, O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphate (III), was prepared by stirring chlorpyrifos (100 mg) dissolved in ethanol with 10 % aqueous solution of potassium permanganate for 4-5 hr. The organic solvent present in the reaction mixture was evaporated and the compound was subjected to column chromatography over silica gel. The oxon metabolite (III) of chlorpyrifos was obtained as an oil, Rf 0.54 (10% acetone -benzene), IR ν KBr cm^{-1} (1580) ; NMR CCl_4 : δ 2.21 (t, 3H, CH_3), 3.98 (d, 2H, CH_2), 8.20 (s, 1 H, Ar-H). This was used as a reference standard for quantification of the presence of the 3,5,6-trichloropyridinol and the chlorpyrifos oxon formed during the dissipation experiment.

RESULTS AND DISCUSSION

The average percent recovery from three replicates spiked at 0.1 and 1 μg recorded was 92. The data in Table 1 show that the percent dissipation by day -1 of the pesticide is 5.80 and 6.93 in the control - II (medium+ pesticide) and in the treated sample IV (medium + algae + pesticide), respectively and the corresponding percent dissipation recorded at higher concentration were 6.09 in control (III) and 5.59 in treatment V. The initial loss of the pesticide, though is not significant, by day-3. The rate of dissipation increased to 20 percent in the treated sample as compared to only 15 percent in control-II and 7.92 percent in control III. The algae started metabolizing only after its incubation period of 7-10 days. At the end of 15 days incubation period *Chlorella vulgaris* affected significant degradation of chlorpyrifos, compared to control II and III, where percent loss was only 36 and 37. By day-20 the pesticide dissipated to 51.94 and 68.23 percent in the control-II and treated set IV samples, respectively. The set I acted as a reference control to eliminate any interfering peaks arising due to the co-extractives from algae. The 3,5,6-trichloropyridinol (II) and the chlorpyrifos oxon (III) were not detected (TLC) in the samples indicating that chlorpyrifos dissipated under experimental conditions. Racke et al. (1990) has also, reported that chlopyrifos mineralised under similar conditions. The possibility of adsorption of chlorpyrifos on the algal surface is negligible as the remaining algal mass was further subjected to extraction process in order to minimize any loss.

The effect of pesticides on the physiological properties of algae has been extensively studied (Pipe 1992), but there are very few reports available about the

Table 1. Dissipation of chlorpyrifos with *Chlorella vulgaris*.

Sampling days	Control I (M+A)	Control II (M+P) mg/kg	Control III (M+P) mg/kg	Treated Set IV (M+P+A) mg/kg	Treated Set V (M+P+A) mg/kg
0	-	22.93 * (±0.002)	49.2 (±0.002)	23.36 (±0.003)	48.3 (±0.003)
1	-	21.60 (±0.002)** (5.80)***	46.2 (±0.002) (6.09)	21.74 (±0.004) (6.93)	45.6 (±0.001) (5.59)
3	-	19.42 (±0.003) (15.30)	45.3 (±0.003) ((7.92)	18.67 (±0.002) (20.07)	40.6 (±0.001) (15.9)
5	-	17.23 (±0.001) (24.85)	38.9 (±0.001) (20.9)	15.98 (±0.003) (31.59)	33.6 (±0.001) (30.4)
7	-	16.42 (±0.004) (28.39)	35.2 (±0.003) (28.4)	14.67 (±0.004) (37.20)	30.9 (±0.001) (36.0)
15	-	14.58 (±0.002) (36.41)	30.7 (±0.002) (37.6)	11.58 (±0.005) (50.42)	23.2 (±0.001) (51.9)
20	-	11.02 (±0.002) (51.94)	26.3 (±0.003) (46.5)	7.42 (±0.001) (68.23)	16.3 (±0.001) (66.2)

* Each value is a mean of three replicates, ** SD ***, % dissipation

effect of algae on the degradation of pesticides. The agricultural applications of synthetic pyrethroids have been shown to have no affect on algae, microorganisms, annelids, gastropods and fish (Hill 1989). There are however,

Table 2. Regression equation and half life.

Treatment	Regression Equation, y=	Half life (days)	Coefficient of correlation r^2
Medium + pesticide(II)	1.33-0.014×	21.5	0.94
Medium + pesticide + *Chlorella vulgaris*(IV)	1.34-0.022×	13.6	0.96
Medium + pesticide(III)	1.67-0.013x	30	0.97
Medium + pesticide + *Chlorella vulgari s*(V)	1.34-0.022x	15	0.98

some reports that the application of organophosphorus insecticide, fenitrothion prevented the occurrence of normal mitoic divisional processes (Kent and Weinberger 1991). Herbicides at higher concentrations were found to inhibit phosphate assimilation than carbon fixation, whereas ammonium assimilation was inhibited at intermediate concentrations (Brown and Lean 1995).

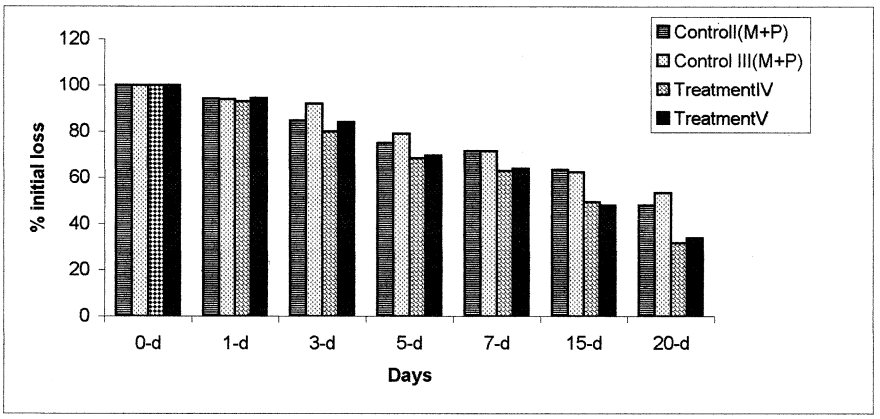

Figure 2. Percent initial loss of chlorpyrifos with *Chlorella vulgaris*.

Half-life recorded in Table 2 clearly establishes the ability of *Chlorella vulgaris* to degrade chlorpyrifos faster. This experiment indicates (Figure 1 and Table 1) that the green algae may be used to detoxify a persistent pesticide like chlorpyrifos. The results of the present study suggest that the algae are effective in detoxifying the pesticides when they are present in microgram amounts in the water and soil environment. Further, screenings of different algal cultures need to be carried out to evolve a suitable detoxification technique for soil and for bioremediation.

Acknowledgments We thank Dr.B.S.Parmar, Head Division of Agricultural Chemicals and Dr.P.K.Singh, Project Director, NCCUBGA for providing the facilities for the work. Contribution No. 646 Division of Agricultural Chemicals.

REFERENCES

Brown LS, Lean DRS (1995) Toxicity of selected pesticides to lake phytoplankton measured using photosynthetic inhibition compared to maximal uptake rates of phosphate and ammonia . Environ Toxicol Chem 14: 93-98

Chapman RA, Harris CR, Svec HJ, Robinson JR (1984) Resistance and mobitily of granular insecticides in an organic soil following furrow application for onion maggot. J Environ Sci Health B19: 259-270

Cochran RC, Kishiyama J, Aldous C, Carr NC, Pfeifer KF (1995) Chlorpyrifos hazard assessment based on a review of the effects of a short term and long term exposure in animals and humans. Food Chem Toxicol 33: 165-172

Hill IR (1989) Aquatic Organisms and pyrethroids.Pesticide Sci 27:429-457

KAN –DO (office and Pesticide team) (1995) Accumulated pesticide and industrial chemical findings from a ten-year study of ready to eat foods. J AOAC Int 78: 614-631

Kent R A, Weinberg P (1991) Multibiological level response of freshwater pyto-Plankton pesticide (fenitrothion) stress. Environ Toxicol Chem 10: 209-216.

Meghraj M, Venkateswarlu K , Rao A S (1987) Metabolism of monocrotophos and quinalphos by algae isolated from soil. Bull Environ Contam Toxicol 39: 251-256.

Mukherjee I, Gopal M (1996) Degradation of chlorpyrifos by two soil fungi *Aspergillus niger* and *Trichoderma viride*. Environ Toxicol Chem 37: 145-151.

Niedert E, Tritman RB, Saschen Brecken PW (1994) Levels and incidence of residues in selected agricultural food commodities available in Canada. J Assoc Anal Anal Chem Int 77: 18-33.

Pipe P (1992) Pesticide effects on the soil algae and cyanobacteria. Rev Environ Contam Toxicol 127: 97-170.

Racke K D, Lakowski DA, Schultz MR (1990) Resistence of chlorpyrifos to enhanced biodegradationin soil. J Agric Food Chem 33: 1430-1436.

Rouchaud J, Gustin F, Vanparys L (1991) Influence of slurry and ammonium nitrate fertilizations on soil and plant metabolism of chlorpyrifos in field cauliflower. Bull Environ Contam Toxicol 46: 705- 712.

Stanier RY, Kunisama R, Mandel M & Cohen Bazire G (1971) Purification and properties of unicellular blue green algae (order Chroococcalus). Bacterial Rev 35: 171-205.