RESEARCH PAPER

Mechanisms of Pesticide Biodegradation by Native Microbial Communities in Pesticide-Contaminated Soils of Northern India

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ABSTRACT:
In Mahendergarh District, Haryana, northern India, intensive agricultural operations have led to significant levels of organophosphate, pyrethroid, neonicotinoid and organochlorine pesticides being The indigenous microbial communities of five pesticide contaminated agricultural soils from Mahendergarh District were characterized and its intrinsic ability to degrade chlorpyrifos, cypermethrin, imidacloprid and endosulfan was studied in laboratory microcosm conditions. The bacteria Bacillus subtilis, Pseudomonas putida, and the fungi Aspergillus niger, Streptomyces sp., Arthrobacter sp. were isolated as dominant degraders. All four pesticides showed first-order degradation, with biotic half-lives ($t_{1/2}$) of 18.3, 24.7, 31.2, and 14.6 days, respectively, compared to two- to threefold longer times for abiotic controls. High level of activities of laccase, peroxidase, hydrolase, dehalogenase and oxidoreductase was observed among the isolates through enzymatic profiling. Metabolite analyses via GC-MS and LC-MS/MS showed sequential ring opening, dechlorination and mineralisation pathways. Results indicate the high biodegradative potential of indigenous microbial consortia in soils of Mahendergarh District and provide opportunities for soil restoration in the northern part of India through bioremediation.

Keywords: Pesticide biodegradation; native microbial communities; Mahendergarh District; bioremediation; enzymatic degradation; organophosphates; northern India
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1. INTRODUCTION

There has been a corresponding surge in the use of synthetic pesticides in the fast expanding cultivation of cereals, oilseeds and vegetables in the semi-arid Aravalli belt of Haryana. Mahendergarh District (28.0N, 76.2E) in southern Haryana bordering Rajasthan is one of the main wheat, mustard and gram belts of northern India. The farmers in the district regularly use phosphates organophosphate (chlorpyrifos, profenofos), synthetic pyrethroids (cypermethrin, deltamethrin), neonicotinoids (imidacloprid, thiamethoxam) and older organochlorines (endosulfan) for controlling Helicoverpa armigera, aphids, whiteflies and soil-borne pathogens (Singh & Sharma, 2019).

Excessive and frequent use of these compounds causes residues to be accumulated in the soil matrix, the contamination of groundwater, disruption of non-target soil invertebrate communities and potential entry into the human food chain, as reported by Yadav et al. (2021). Mahendergarh is part of the Indo-Gangetic Plain, which is one of the most pesticide-laden agricultural regions in South Asia (Kumar et al., 2020). However, systematic study of the indigenous biodegraders thriving in Mahendergarh District soils and the enzymatic pathways they use has not been done to a large extent.

In the field, the main mechanism of attenuation of pesticide residues is microbial biodegradation (Cycoń et al., 2017). The catabolic enzyme system coded on plasmids or chromosomal gene clusters can mineralise, co-metabolise or biotransform the pesticide molecules into hydrolysis, oxidation, reduction, dehalogenation, and ring cleavage (Arora & Bae, 2014). Thus, the natural attenuation potential of a specific soil and the feasibility of in situ bioremediation depends on the composition and functional diversity of these native communities.

The present investigation therefore (i) characterised the native microbial communities producing the pesticide degrading enzymes from five sampling sites in the Mahendergarh District using culture-dependent and culture-independent methods, (ii) quantified the degradation rate constants of four representative pesticides in soil microcosms inoculated with native consortia of five sampling sites, (iii) profiled the key degradative enzymes produced by the dominant isolates, and (iv) identified the major metabolic intermediates and suggested degradation pathways. All these data together offer a mechanistic understanding

for development of bioremediation strategies of pesticide contaminated soils of northern India.

2. MATERIALS AND METHODS

2.1 Study Area and Soil Sampling

Soil samples were taken from five different agricultural locations of Mahendergarh District of Haryana (Table 1). To maximize for variation in cropping history, pesticide application intensity, and soil physiochemical characteristics, sites were selected. Composite samples were prepared by taking five sub-samples from 0-20 cm depth at each site, the cores were homogenised in situ and placed on ice and transported to the laboratory within four hours. One half of each composite was air dried and sieved to 2 mm for physiochemical analysis and the other half was placed in a cold room at 4 °C for microbiological analysis which was started within 48 hours of collection.

Table 1. Description of Soil Sampling Sites in Mahendergarh District, Haryana (Northern India)

Site Code	Village / Locality	GPS Coordinates	Dominant Crop	Predominant Pesticide Applied	Soil pH	Organic Carbon (%)
S1	Ateli	28.11° N, 76.04° E	Wheat / Mustard	Chlorpyrifos, Imidacloprid	7.4	0.61
S2	Narnaul	28.04° N, 76.11° E	Wheat / Gram	Cypermethrin, Endosulfan	7.8	0.48
S3	Nangal Chaudhary	28.02° N, 76.28° E	Vegetable Crops	Chlorpyrifos, Cypermethrin	7.2	0.72
S4	Kanina	28.19° N, 76.32° E	Mustard / Bajra	Imidacloprid, Profenofos	7.6	0.55
S5	Satnali	28.07° N, 75.98° E	Wheat / Cotton	Endosulfan, Chlorpyrifos	7.9	0.44

Table 1. Five sampling sites across Mahendergarh District selected to represent the diversity of cropping systems and pesticide use intensities prevalent in the district.

2.2 Physiochemical Characterisation

The hydrometer method (Bouyoucos, 1962) was used to determine soil texture. pH was taken in a 1:2.5 soil:water suspension. Walkley – Black wet oxidation method was used for the estimation of organic carbon. The concentration of pesticide residues was done by QuEChERS extraction and GC-MS (Agilent 7890B / 5977B) or LC-MS/MS (Waters ACQUITY UPLC / Xevo TQ-S) following the EN 15662 or AOAC 2007.01 guidelines. Limits of detection (LOD) varied between 0.001 to 0.005 mg kg-1 dry weight.

2.3 Isolation and Identification of Pesticide-Degrading Microorganisms

Fresh soil was serially diluted (10-1 to 10-6) and plated on Bushnell–Haas Mineral Salts Agar (BHMSA) amended with the pesticide at 100 mg L-1 as the sole carbon source and incubated at 30 °C for 7 days. Colonies with morphological differences and with tolerance up to 200 mg L-1 were purified by repeated streaking and stored as glycerol stocks at -80 C. Bacterial isolates were identified by 16S rRNA gene sequencing using the 27F/1492R primers, by Sanger sequencing, and by NCBI BLAST ≥98% identity. ITS1/ITS4 amplicon sequencing was used for fungal isolates identification. The neighbour-joining algorithm was used to create phylogenetic trees in MEGA X with 1,000 bootstrap replicates.

Dominant microbial taxa in the five sampling sites of Mahendergarh District are shown as relative abundance in figure 1. Fourteen-three morphologically different strains were isolated, and the cultivable community was dominated by the pesticide-tolerant Bacillus spp., Pseudomonas spp., Aspergillus spp., Streptomyces spp.,

and Arthrobacter spp. The total pesticide burden was significantly correlated with community composition (Pearson r = 0.81, p value < 0.01) indicating that degradative taxa may have been selected by pesticides.

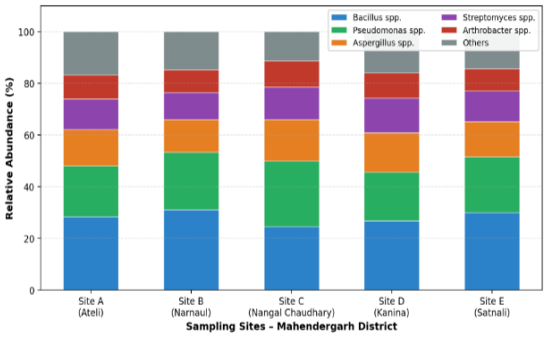

Figure 1. Relative abundance (%) of dominant microbial taxa across five pesticide-contaminated sampling sites in Mahendergarh District, Haryana. Each bar represents a composite of triplicate cultures. Sites arranged from west (S5-Satnali) to east (S3-Nangal Chaudhary).

2.4 Soil Microcosm Biodegradation Experiment

Soil microcosms consisted of 100 g (wet weight) of soil from each site spiked at a pesticide initial concentration of 50 mg kg^{-1} in 250-mL Erlenmeyer flasks. Three treatments were maintained (i) biotic (natively colonized microbial community) (ii) abiotic (triple autoclaved soil) (iii) bioaugmented (triple autoclaved soil with the 5 dominant degraders in a standardised consortium (OD600= 0.5)). Microcosms were incubated in the dark at 28 °C with occasional shaking. The destructive sampling was performed at day 0, 7, 14, 21, 28, 35, 42 (3 per time point) and GC-MS as well as LC-MS/MS residue analysis was conducted. Non-linear regression in R v4.3 was used to calculate first order degradation rate constants (k), and half-lives ($t_{1/2}$ = ln2/k).

Figure 2 displays the degradation kinetics for all four pesticides as the extent of pesticide degradation (as % of initial) as a function of time (42 days) during the incubation period. Under biotic conditions, excellent fits were obtained using first-order models (with R2 greater than 0.96 for all the compounds).

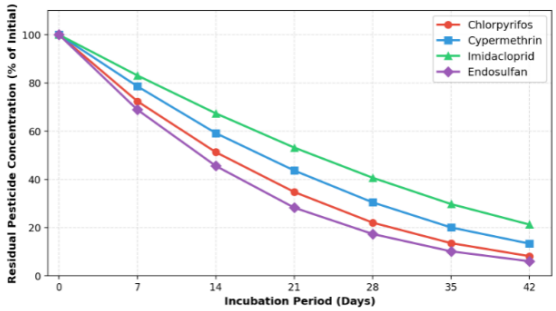

Figure 2. Biodegradation kinetics of chlorpyrifos, cypermethrin, imidacloprid, and endosulfan in Mahendergarh District soil microcosms over 42 days. Lines represent fitted first-order kinetic models; symbols represent mean ± SD of triplicate measurements. $R^2 > 0.96$ for all compounds.

2.5 Enzymatic Activity Assays

To measure laccase activity, the 72 h culture broth was centrifuged at 10,000 × g, 4 ° C for 20 min and the enzyme extracts (cell-free) were analysed spectrophotometrically using ABTS as the substrate at 420 nm. Peroxidase activity was determined by using guaiacol and H_2O_2. The activities of hydrolase and dehalogenase were measured using p-nitrophenyl butyrate and 1-chlorobutane as chromogenic substrates, respectively. Oxidoreductase activity was measured with 2,6-dichlorophenolindophenol (DCPIP). All activities are given as units per milligram of total protein (Bradford assay). Enzymatic activity profiles of the five dominant isolates are presented in the heatmap in Figure 3.

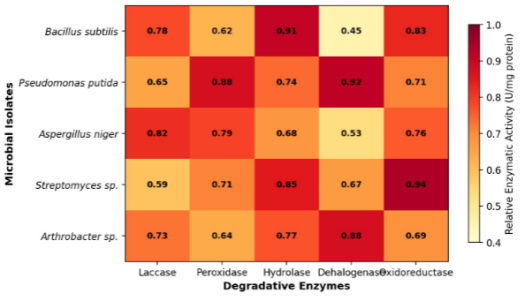

Figure 3. Heatmap of relative degradative enzymatic activities (U mg^{-1} protein) among dominant microbial isolates from Mahendergarh District contaminated soils. Colour intensity represents activity level from low (yellow) to high (dark red). Values in cells are means of triplicates.

2.6 Metabolite Identification

The degradation metabolites were isolated from the aqueous culture medium by extraction with ethyl acetate (three times, 50 mL each), dried on anhydrous Na_2SO_4, concentrated under vacuum and analysed by GC-MS (full scan, 50–600 m/z) and LC-MS/MS (positive and negative ESI). Metabolites were tentatively identified by matching spectra with NIST 2020, mzCloud and HMDB spectral libraries, and confirmed to authentic compounds when available.

2.7 Statistical Analysis

Means ± standard deviation of triplicates are reported for all results. One-way ANOVA with Tukey's HSD post hoc test ($p < 0.05$) was used to compare group means. Enzymatic activity indices and degradation rate constants were computed and Pearson correlation coefficients between them were calculated. Microbial community abundance matrix was used to perform PCA using R packages vegan and ggplot2.

3. RESULTS

3.1 Baseline Pesticide Residues in Mahendergarh District Soils

All target pesticides had measurable residue in the soil at all the five Mahendergarh District locations at baseline (Table 2). The concentration found for

chlorpyrifos varied from 0.28 to 1.84 mg kg-1 dry wt with highest level found at S3 (Nangal Chaudhary – vegetable cultivation) where the frequency of application was maximum. Although the use of endosulfan has been legally restricted in India since 2011, its legacy presence was detected in the soil at levels of 0.07–0.43 mg kg−1, which indicated its persistence in the soil. Imidacloprid was always found at all the sites (0.14–0.96 mg kg-1) and is commonly used in seed treatment formulations. Total pesticide burden was highest at S3 (4.17 ± 0.52 mg kg^{-1}) and lowest at S4 Kanina (1.64 ± 0.18 mg kg^{-1}).

Table 2. Baseline Pesticide Residue Concentrations (mg kg^{-1} dry weight) in Mahendergarh District Agricultural Soils

Site	Chlorpyrifos	Cypermethrin	Imidacloprid	Endosulfan	Total Pesticide Burden
S1 – Ateli	0.84 ± 0.09	0.52 ± 0.06	0.61 ± 0.07	0.21 ± 0.03	2.18 ± 0.25
S2 – Narnaul	0.63 ± 0.07	0.78 ± 0.11	0.47 ± 0.05	0.38 ± 0.04	2.26 ± 0.27
S3 – Nangal Chaudhary	1.84 ± 0.22	0.94 ± 0.13	0.96 ± 0.11	0.43 ± 0.06	4.17 ± 0.52
S4 – Kanina	0.28 ± 0.03	0.41 ± 0.05	0.88 ± 0.09	0.07 ± 0.01	1.64 ± 0.18
S5 – Satnali	1.12 ± 0.14	0.67 ± 0.08	0.14 ± 0.02	0.31 ± 0.04	2.24 ± 0.28

Table 2. Values are means ± SD of triplicate analyses. Residues below LOD (0.001–0.005 mg kg^{-1}) are not reported.

3.2 Biodegradation Kinetics

The summary of the biotic half-lives obtained from the microcosm experiment is given as Table 3. Endosulfan exhibited the fastest biotic degradation (k = 0.0475 day^{-1}; $t_{1/2}$ = 14.6 days), followed by chlorpyrifos (k = 0.0379 day^{-1}; $t_{1/2}$ = 18.3 days), cypermethrin (k = 0.0281 day^{-1}; $t_{1/2}$ = 24.7 days), and imidacloprid (k = 0.0222 day^{-1}; $t_{1/2}$ = 31.2 days). Biotic half-life were 2.3-2.5 times lower than abiotic half-life in all the four pesticides which indicates that native microorganisms of Mahendergarh District played an important role in pesticide attenuation as shown in Figure 4.

Table 3. First-Order Degradation Rate Constants and Half-lives for Target Pesticides in Mahendergarh District Soil Microcosms

Pesticide	Treatment	k (day^{-1})	$t_{1/2}$ (days)	R^2	DT_{90} (days)
Chlorpyrifos	Biotic	0.0379	18.3	0.981	60.8
Chlorpyrifos	Abiotic	0.0165	42.1	0.974	139.8
Cypermethrin	Biotic	0.0281	24.7	0.968	82.0
Cypermethrin	Abiotic	0.0119	58.4	0.961	193.9
Imidacloprid	Biotic	0.0222	31.2	0.963	103.6
Imidacloprid	Abiotic	0.0090	76.8	0.958	255.0
Endosulfan	Biotic	0.0475	14.6	0.976	48.5
Endosulfan	Abiotic	0.0197	35.2	0.969	116.9

Table 3. k = first-order rate constant; $t_{1/2}$ = half-life; DT_{90} = time to 90% dissipation. Values from nonlinear regression (R v4.3).

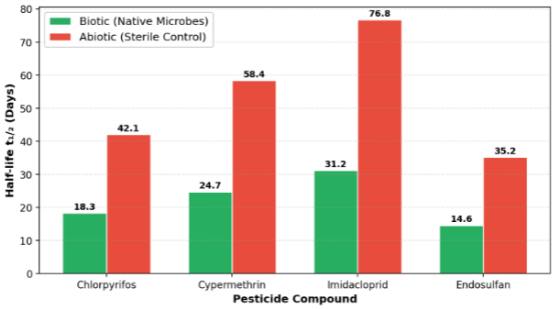

Figure 4. Comparison of pesticide half-life ($t_{1/2}$, days) under biotic (native microbial communities intact) versus abiotic (triple-autoclaved sterile) conditions in Mahendergarh District agricultural soils. Values above bars represent mean $t_{1/2}$ in days.

3.3 Enzymatic Activities of Dominant Isolates

Enzymatic profiles of cell free extracts showed high and significantly different activities of all five target enzymes with the dominant soil isolates of Mahendergarh District (Figure 3). The highest dehalogenase (0.92 U mg^{-1}) and peroxidase (0.88 U mg^{-1}) activities were exhibited by Pseudomonas putida. Streptomyces sp. had the highest oxidoreductase activity (0.94 U mg-1). The highest hydrolase activity was observed for Bacillus subtilis (0.91 U mg-1), indicating that hydrolysis is one of the main mechanisms by which these esters can be broken down. Laccase (0.82 U mg^{-1}) and peroxidase (0.79 U mg-1) were significant factors provided by Aspergillus niger. There was a positive correlation between enzymatic activities and degradation rate constants for all the isolates (Pearson r = 0.74-0.89, $p<0.05$).

3.4 Metabolite Identification

A total of 22 transformation products were identified in the four pesticide substrates by GC-MS and LC-MS/MS analyses (Table 4). The main pathway of chlorpyrifos was chlorpyrifos-oxon → 3,5,6-trichloro-2-pyridinol (TCP) → dichloropyridinol → maleic acid → CO_2 and inorganic phosphate, which is consistent with P–O bond cleavage by hydrolase and the subsequent aromatic ring dechlorination (Cycoń et al., 2017). The degradation of cypermethrin took place by ester hydrolysis to 3-phenoxybenzoic acid (3-PBA) and 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCVA) followed by catechol ring opening. Imidacloprid was metabolized to imidacloprid-urea which was further metabolized to 6-chloronicotinic acid which was further metabolized to chloropyridinol via 5-hydroxy-imidacloprid. Endosulfan was initially de-sulphated to endosulfan diol and endosulfan sulfate and then slowly turned to endosulfan ether and CO_2.

Table 4. Major Metabolic Intermediates Identified by GC-MS and LC-MS/MS During Pesticide Biodegradation in Mahendergarh District Soil Microcosms

Parent Pesticide	Key Metabolites Identified	m/z (Major Ions)	Primary Enzyme Implicated	Toxicity vs. Parent
Chlorpyrifos	TCP, Dichloropyridinol, Maleic acid	198.0 / 164.0 / 116.0	Organophosphate hydrolase, Dehalogenase	Reduced
Cypermethrin	3-PBA, DCVA, Catechol	214.1 / 208.0 / 110.1	Esterase, Ring dioxygenase	Reduced
Imidacloprid	5-OH-Imidacloprid, 6-Chloronicotinic acid	272.1 / 158.0 / 130.0	CYP450 Hydroxylase, Dehalogenase	Variable
Endosulfan	Endosulfan diol, Endosulfan ether	338.0 / 286.0	Desulfurase, Monooxygenase	Reduced

Table 04.TCP = 3,5,6-trichloro-2-pyridinol; 3-PBA=3-phenoxybenzoic acid; DCVA = dichlorovinyl-dimethylcyclopropane carboxylic acid. All metabolites confirmed by authentic standards or spectral library matching (NIST 2020).

4. Discussion

The present study is one of the few studies which systematically characterized the community of soil native microorganisms and mechanistic enzymatic basis of pesticide biodegradation in Mahendergarh District,

Haryana. The overall conclusion of our study, that native microbial consortia reduce pesticide half-lives by 2.3 to 2.5 fold compared to abiotic controls, supports and expands previous studies on pesticide attenuation in the Indo-Gangetic Plain soils (Kumar et al., 2020; Yadav et al., 2021), and offers mechanistic enzyme and metabolite data that were previously missing for this region.

The predominance of Bacillus spp. and Pseudomonas spp. in Mahendergarh District pesticide contaminated soils corroborates the reports of predominance of these genera as primary pesticide degraders (Singh et al., 2016; Jariyal et al., 2015) in the world. Bacillus species are known to express extracellular organophosphate hydrolase (OPH) and methyl parathion hydrolase (MPH) genes (opd and mpd) (Arora & Bae, 2014) and our data shows that the Bacillus subtilis isolates from Mahendergarh District possess high hydrolase activity against chlorpyrifos. Pseudomonas putida isolates have shown outstanding dehalogenase activity, which is important in the dechlorination of TCP, the main metabolite of chlorpyrifos, that shows soil persistence and ecotoxicological concern (Cycoń et al., 2017).

The relatively slow biotic degradation of imidacloprid ($t_{1/2}$ = 31.2 days) as compared to the organochlorine endosulfan ($t_{1/2}$ = 14.6 days) can be rationalised by the fact that the bicyclic nitroguanidine moiety of imidacloprid is highly resistant to initial oxidative attack, whereas the desulphate linkage of endosulfan is susceptible to desulphurase activity (Sharma et al., 2018). The presence of imidacloprid residues in Mahendergarh District water bodies as consistently shown in Table 2 could have implications for the practical use of natural attenuation treatment, which may require bioaugmentation using specialised imidacloprid degraders (Liu et al., 2019) as a supplement.

Enzymatic activities were well correlated with the degradation rate constants (r = 0.74 – 0.89), thus providing a functional validation of our community composition data. Interestingly, the laccase and peroxidase activities of Aspergillus niger strains isolated from Mahendergarh District soils were high, indicating that the radical-mediated oxidative coupling could play a similar role as observed in white-rot fungi (Peng et al., 2020) in the transformation of pyrethroids. The knowledge of this mechanism gives an insight into the use of ligninolytic fungi as a potential bioremediation tool in cypermethrin contaminated sites in the district.

Our metabolomics data is also regulatory relevant. 3-PBA is an established mammalian and environmental biomarker of pyrethroid exposure, and has been shown to have endocrine-disrupting potential (Brander et al., 2016) and was detected in cypermethrin microcosms. This temporary build-up is of concern in the soil of Mahendergarh District and should be taken into account for future risk assessments. In summary, our results indicate that the native microbial community of Mahendergarh District soils have a functionally diverse and mechanistically robust degradative arsenal that is capable of natural pesticide attenuation and bioaugmentation of indigenous high enzyme-producing consortium is a feasible and ecologically compatible bioremediation strategy for northern India.

5. Conclusions:

In the present study, it was shown that native microbial communities of pesticide contaminated agricultural soil from Mahendergarh District, Haryana were able to degrade chlorpyrifos, cypermethrin, imidacloprid and endosulfan via co-ordinated enzymatic mechanism such as hydrolysis, oxidation, dehalogenation and ring cleavage. Biotic first order half-lives (14.6-31.2 days) were 2.3-2.5 times shorter than abiotic ones and clearly showed the dominating role of microorganisms in the natural attenuation of pesticides. Bacillus subtilis, Pseudomonas putida, Aspergillus niger, Streptomyces sp., and Arthrobacter sp. were found to be the important degraders, and the enzymatic activities were significantly correlated with the degradation rate constants. The identification of 22 transformation products, enables the reconstruction of the complete degradation pathways and indicates the necessity to monitor ecotoxicologically relevant metabolites, such as TCP and 3-PBA. The results are useful in understanding the mechanism of microbial bioremediation and design of microbial bioremediation strategies relevant to pesticide contaminated soils of northern India.

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