Effects of Chlorpyrifos on Bacterial Community Diversity and Structure in Cotton Rhizosphere Soil: Postprint

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Abstract

Through indoor pot experiments simulating natural environmental conditions and using high-performance liquid chromatography (HPLC) and terminal restriction fragment length polymorphism (T-RFLP) techniques, we investigated the effects of chlorpyrifos applied to soil at the recommended dose (5 $mg \cdot kg^{-1}$) and at two, three, and four times the recommended dose (10 $mg \cdot kg^{-1}$, 15 $mg \cdot kg^{-1}$, 20 $mg \cdot kg^{-1}$) on the diversity and structure of bacterial communities in cotton rhizosphere soil, with soil not treated with chlorpyrifos serving as the control. The results showed that the half-lives of chlorpyrifos in soil at 5 $mg \cdot kg^{-1}$, 10 $mg \cdot kg^{-1}$, 15 $mg \cdot kg^{-1}$, and 20 $mg \cdot kg^{-1}$ were 10.04 d, 11.36 d, 11.55 d, and 12.16 d, respectively, and it was essentially completely degraded by 60 d. After 60 d of chlorpyrifos treatment, cotton biomass was significantly reduced; the higher the chlorpyrifos concentration, the lower the cotton biomass. Under chlorpyrifos-free conditions, no significant differences in rhizosphere bacterial diversity were observed among different sampling times; in the chlorpyrifos treatment groups, bacterial diversity was significantly reduced during the first 30 d, but had recovered to normal levels by 60 d. The study found that higher chlorpyrifos concentrations caused more significant inhibition of bacterial diversity and slower recovery. Principal component analysis revealed that bacterial community structure differed significantly between the chlorpyrifos treatment groups and the control group at 10 d, 30 d, and 60 d, with the 20 $mg \cdot kg^{-1}$ chlorpyrifos treatment group showing the most significant difference at 60 d; even when chlorpyrifos in the soil was completely degraded, the rhizosphere bacterial community structure still did not return to normal levels. At 60 d, bacteria suppressed by chlorpyrifos included Nitrospina sp. and Cellulophaga sp., while those activated included Bacillus sp. and Streptomyces sp. Thus, the introduction of chlorpyrifos reconstructed the soil bacterial community structure, significantly affected cotton growth, and caused considerable impact on

the microecological environment of cotton rhizosphere soil, warranting serious attention to its ecological safety.

Full Text

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Changes in Cotton Rhizosphere Soil Bacterial Community Diversity and Structure Induced by Chlorpyrifos*

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Abstract

We conducted indoor pot experiments to simulate natural environmental conditions and investigated the effects of chlorpyrifos on bacterial community diversity and structure in cotton rhizosphere soil using high-performance liquid chromatography (HPLC) and terminal restriction fragment length polymorphism (T-RFLP) techniques. The experiment included four chlorpyrifos application rates: 5 mg·kg^{-1} (recommended dose), 10 mg·kg^{-1}, 15 mg·kg^{-1}, and 20 mg·kg^{-1}, with a no-chlorpyrifos treatment serving as the control. Diversity indices (including Shannon-Weiner and Simpson indices) were used to evaluate bacterial community diversity, principal component analysis (PCA) was performed to assess changes in bacterial community structure, and the MiCA web tool was employed for taxonomic interpretation of terminal restriction fragments (TRFs).

The results showed that the degradation half-lives of chlorpyrifos at doses of 5 mg·kg^{-1}, 10 mg·kg^{-1}, 15 mg·kg^{-1}, and 20 mg·kg^{-1} were 10.04 d, 11.36 d, 11.55 d, and 12.16 d, respectively, with nearly complete degradation occurring by 60 days. At the end of the experiment (60 days after treatment), cotton biomass decreased significantly with increasing chlorpyrifos dose, and root growth was also significantly inhibited. Compared with the control, bacterial community diversity in the rhizosphere under chlorpyrifos treatments decreased significantly after 10 and 30 days, though there was no significant difference in diversity indices between the 10-day and 30-day samplings across all chlorpyrifos treatments. By 60 days, bacterial community diversity in the chlorpyrifos treatments had almost recovered to normal levels. However, higher chlorpyrifos concentrations caused more significant inhibition of bacterial diversity and required longer recovery times.

PCA revealed that bacterial community structure differed significantly between chlorpyrifos treatments and the control at 10, 30, and 60 days after treatment, even though chlorpyrifos was almost completely degraded by 60 days. The divergence between chlorpyrifos treatments and the control increased over time, with

the 20 mg · kg^{-1} treatment showing the most significant difference at 60 days. At 60 days, *Nitrospina* sp. and *Cellulophaga* sp. were inhibited by chlorpyrifos, whereas *Bacillus* sp. and *Streptomyces* sp. were enhanced. These findings demonstrate that chlorpyrifos application restructured the rhizosphere soil bacterial community, significantly affecting cotton growth and exerting substantial impacts on the cotton rhizosphere microecosystem, warranting serious attention to its ecological security.

Keywords: Chlorpyrifos; Half-life; Cotton biomass; Rhizosphere soil bacteria; Community diversity; Community structure

Introduction

Chlorpyrifos is a broad-spectrum organophosphorus insecticide, nematicide, and acaricide containing chlorine. Since its introduction in 1965, it has been widely used worldwide for pest control in crops such as cotton and rice, as well as in lawns and ornamental plants. Due to its low cost and easy availability, its sales volume exceeds that of other pesticide types. According to a 2012 report on China' s pesticide market, global demand for chlorpyrifos is increasing at an annual rate of 10%. In 2015, China' s monthly chlorpyrifos production reached 4,000 tons, with continued increases expected. Chlorpyrifos adsorbs strongly to soil particles, organic matter, and clay minerals, exhibiting poor mobility and persistent efficacy. Its half-life varies depending on application rate, soil type, soil microorganisms, and climatic conditions, ranging from a few weeks to as long as four years. Long-term repeated use leads to substantial accumulation, potentially threatening the stability of soil microbial communities and functions, sustainable agricultural development, and human health.

Soil microbial communities are essential components of farmland ecosystems, participating in the transformation of mineral nutrients and playing crucial roles in soil nutrient cycling and maintaining normal crop growth. They represent indispensable actors in maintaining farmland soil ecosystem stability and serve as reliable indicators for monitoring soil quality and sustainability. Currently, research on chlorpyrifos both domestically and internationally has focused primarily on its effects on soil microbial biomass, respiration, enzyme activities, and mineral cycling, as well as on screening for chlorpyrifos-degrading bacterial strains. For example, studies have shown that 10–300 mg · kg^{-1} chlorpyrifos significantly inhibits aerobic nitrogen-fixing bacteria, total bacterial populations, and nitrogen fixation in soil. Shan et al. reported that 2–10 mg · kg^{-1} chlorpyrifos significantly inhibited total bacterial populations and enzyme activities in the short term, with higher concentrations causing stronger inhibition that persisted longer. The recommended field application rate of chlorpyrifos (5 mg · kg^{-1}) significantly inhibited dehydrogenase activity and Fe^{3+} reduction in loamy sand soils. Application rates of 4–12 mg · kg^{-1} suppressed soil microbial community diversity within two weeks. Various bacterial species including *Al-*

caligenes sp. JAS1, *Bacillus pumilus* strain C2A1, *Acinetobacter calcoaceticus*, and *Streptomyces* sp. have demonstrated chlorpyrifos degradation capabilities.

Most previous studies have examined chlorpyrifos effects on non-rhizosphere soils without planted crops. To date, few studies have investigated its impacts on crop rhizosphere microbial communities. In China, chlorpyrifos is primarily used for pest control in important crops such as cotton, yet research on its effects on cotton rhizosphere soil microbial community diversity and structure remains scarce. Therefore, investigating the impacts of chlorpyrifos application on farmland crop rhizosphere soil microbial community diversity and structure holds significant importance.

Current methods for analyzing environmental microbial community structure and diversity include phospholipid fatty acid (PLFA) analysis, terminal restriction fragment length polymorphism (T-RFLP), ribosomal intergenic spacer analysis (RISA), denaturing/temperature gradient gel electrophoresis (DGGE/TGGE), single-strand conformation polymorphism (SSCP), and amplified ribosomal DNA restriction analysis (ARDRA). Among these, T-RFLP is widely applied in microbial community analysis due to its speed, high reproducibility, and compatibility with the RDP database for comparative analysis.

This study employed indoor pot experiments to simulate natural environmental conditions and used HPLC and T-RFLP techniques to analyze the effects of different chlorpyrifos application rates on cotton rhizosphere soil bacterial diversity and structure. Our objectives were to reveal whether chlorpyrifos poses hazards to the cotton rhizosphere microbial ecological environment and to provide technical support for safety evaluation of chlorpyrifos use.

Materials and Methods

Materials

The cotton cultivar used was 'Jimian 169'. Chlorpyrifos (emulsifiable concentrate formulation with 480 $g \cdot L^{-1}$ active ingredient) was obtained from Suzhou Jiahui Chemical Co., Ltd., Jiangsu Province.

Soil Collection and Preprocessing

Experimental soil was collected from a test field at the Shanghai Academy of Agricultural Sciences (121.48 E, 30.95 N) that had not received pesticide applications for nearly 10 years. Soil was sampled from five points, with the surface layer (0–10 cm) discarded and soil from 15–30 cm depth collected. The soil was air-dried at room temperature, thoroughly mixed, and passed through a 2 mm sieve before being placed in PVC pots (17 cm diameter, 13.5 cm height).

Pot Experiment Design

Cotton seeds were surface-sterilized by rinsing twice with 70% ethanol for 30 seconds each, followed by washing with sterile distilled water. After germination induction, ten seeds were sown per pot. The pots were placed in an illuminated incubator at 23°C with a 12-hour daily photoperiod. Sterile water was applied to maintain soil moisture at approximately 60% of field capacity. After seedlings developed two cotyledons, excess seedlings were removed, leaving two uniform seedlings per pot. The experiment included four chlorpyrifos concentration treatments and a control without chlorpyrifos. The four treatments received chlorpyrifos at 5 $mg \cdot kg^{-1}$ (recommended dose), 10 $mg \cdot kg^{-1}$, 15 $mg \cdot kg^{-1}$, and 20 $mg \cdot kg^{-1}$. Each treatment had three replicates, totaling 45 pots. The experiment lasted 60 days, with soil samples collected at 0, 10, 30, and 60 days for subsequent analysis of soil bacterial community diversity and structure and for chlorpyrifos residue detection.

Cotton Biomass Measurement

At the end of the experiment (60 days after treatment), three cotton plants were harvested from each treatment group to measure biomass parameters indicating plant growth status, including plant height, stem fresh/dry weight, and root fresh/dry weight.

Soil Chlorpyrifos Residue Detection

High-performance liquid chromatography (HPLC) has been shown to accurately detect chlorpyrifos residues in soil and other samples. Soil samples were air-dried for 1 hour, and 10.0 g was placed in a 250 mL Erlenmeyer flask with 20 mL acetonitrile, then shaken at 120 $r \cdot min^{-1}$ for 30 minutes. The extract was vacuum-filtered, and the filtrate was transferred to a 250 mL flask, concentrated to near dryness, and reconstituted with acetonitrile before filtration through a 0.22 m organic membrane. HPLC detection conditions were as follows: Agilent 1100 HPLC with UV detector, COSMOSIL 5C18-MS-II reversed-phase column (5 m, 4.6 mm I.D. × 250 mm), mobile phase of acetonitrile:water = 70:30 (v/v), flow rate 1.0 $mL \cdot min^{-1}$, detection wavelength 230 nm, column temperature 25°C, and injection volume 20 L. The retention time for chlorpyrifos was 18.3 minutes.

T-RFLP-Based Bacterial Community Analysis

Total DNA was extracted from rhizosphere soil using the PowerSoil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA). Bacterial 16S rRNA genes were amplified using universal bacterial primers 27f (5 -AGAGTTTGATCCTGGCTCAG-3) and 1492r (5 -GGT TACCTTGTTACGACTT-3), with the forward primer 27f labeled at the 5 end with 6-carboxyfluorescein (6-FAM). Amplification was performed using Touch-Down PCR conditions. The PCR products were digested with restriction enzymes HaeIII and HhaI

at 37°C for 3 hours, and the digested products were analyzed by T-RFLP to obtain profiles.

T-RFLP Data Processing and Analysis

T-RFLP profiles were analyzed using GeneMapper. Terminal restriction fragments (TRFs) smaller than 50 bp or larger than 500 bp were excluded. TRFs present only once across three replicates were discarded; only TRFs repeated in at least two of three replicates (2/3 or 3/3) were used for subsequent analysis. TRFs with relative abundance less than 1% were also excluded, as they do not significantly influence community characteristics. Past 2.02 software was used for principal component analysis (PCA) and diversity index calculations to assess microbial community diversity and structure. TRFs were compared against the RDP database using the MiCA3 website (http://www.mica.ibest.uidaho.edu/) to determine microbial taxonomic changes.

Results

Soil Chlorpyrifos Residues

The HPLC chromatogram of blank soil samples showed no detectable chlorpyrifos [Figure 1: see original paper], confirming the suitability of the collected soil for this study. Chlorpyrifos degradation in soil followed first-order kinetics according to the equation $Ct = C_0e^{-}$. The degradation kinetic equations for the 5 $mg \cdot kg^{-1}$, 10 $mg \cdot kg^{-1}$, 15 $mg \cdot kg^{-1}$, and 20 $mg \cdot kg^{-1}$ treatments were $Ct = 5e^{-0}.^{069}$, $Ct = 20e^{-0}.^{061}$, $Ct = 15e^{-0}.^{06}$, and $Ct = 10e^{-0}.^{057}$, respectively, with half-lives of 10.04 d, 11.36 d, 11.55 d, and 12.16 d. At 60 days, residual concentrations were 0.08 $mg \cdot kg^{-1}$, 0.33 $mg \cdot kg^{-1}$, 0.41 $mg \cdot kg^{-1}$, and 0.51 $mg \cdot kg^{-1}$, respectively. The degradation curves are shown in [Figure 2: see original paper].

Effects of Chlorpyrifos on Cotton Growth

After 60 days of treatment, all four chlorpyrifos treatments significantly reduced both above-ground and below-ground cotton biomass . No significant differences in biomass were observed between the 5 $mg \cdot kg^{-1}$ and 10 $mg \cdot kg^{-1}$ treatments. Between the 15 $mg \cdot kg^{-1}$ and 20 $mg \cdot kg^{-1}$ treatments, only plant height differed significantly, while other organ biomass parameters showed no significant differences. Higher chlorpyrifos concentrations caused more pronounced growth inhibition, resulting in lower cotton biomass.

Effects of Chlorpyrifos on Cotton Rhizosphere Bacterial Community Diversity

Both enzyme digestion profiles and diversity indices showed that HhaI-digested samples exhibited slightly higher bacterial diversity than HaeIII-digested sam-

ples; therefore, subsequent analyses used HhaI digestion results. The diversity indices of bacterial communities in HhaI-digested samples are presented in . Under control conditions without chlorpyrifos, rhizosphere bacterial diversity showed no significant differences across sampling times. In contrast, all chlorpyrifos treatments exhibited significantly reduced bacterial diversity at 10 and 30 days compared to the control, though no significant differences existed between the 10-day and 30-day samplings within each treatment. However, diversity differed significantly between 10 days and 60 days, as well as between 30 days and 60 days, with lower diversity indices observed in the earlier stages. At the same sampling time, higher chlorpyrifos concentrations resulted in lower bacterial community diversity indices, indicating that greater concentrations caused stronger inhibition of rhizosphere microbial community diversity and required longer recovery periods. By 60 days, bacterial community diversity in all chlorpyrifos treatments had recovered to normal levels.

Effects of Chlorpyrifos on Cotton Rhizosphere Bacterial Community Structure

To improve PCA accuracy, only TRFs present in all three replicates were used for analysis. The PCA results for HhaI-digested bacterial communities are shown in [Figure 3: see original paper], with principal components 1 and 2 accounting for 67.5% and 15.0% of the variation, respectively, summing to over 80%. Based on proximity, the 16 sample groups clustered into three major groups: control samples from 0, 10, 30, and 60 days formed one cluster; all chlorpyrifos treatments at 10 and 30 days formed a second cluster; and all chlorpyrifos treatments at 60 days formed a third cluster. These results indicate that both chlorpyrifos and plants exerted selective pressures on rhizosphere bacterial community structure evolution, with chlorpyrifos showing a more prominent selective effect by 10 days and plants (control group) showing their effect by 30 days. At 10, 30, and 60 days, all chlorpyrifos treatment groups were distant from the control group. At 60 days, the distance from the control increased with chlorpyrifos concentration in the order: 5 $mg \cdot kg^{-1}$, 10 $mg \cdot kg^{-1}$, 15 $mg \cdot kg^{-1}$, and 20 $mg \cdot kg^{-1}$, demonstrating that higher concentrations exerted greater selective pressure on rhizosphere bacterial communities with no trend toward recovery to normal levels over time.

TRF Species Classification

To improve taxonomic resolution and simplify results, only TRFs present in all three replicates were used for species classification analysis. The T-RFLP profiles from HhaI digestion of control and chlorpyrifos treatments (5, 10, 15, and 20 $mg \cdot kg^{-1}$) at 60 days yielded 39 distinct TRFs. The distribution and relative abundance of different TRF lengths are shown in . TRFs inhibited by chlorpyrifos included fragments of 68, 89, 94, 98, 175, and 350 bp, while activated TRFs included fragments of 79, 91, 93, 215, and 366 bp. Comparison of these TRFs against the RDP database using MiCA3 identified the bacterial genera affected

by chlorpyrifos at 60 days . Inhibited bacteria included *Nitrospina* sp. and *Cellulophaga* sp., while activated bacteria included *Bacillus* sp., *Streptomyces* sp., and *Magnetococcus* sp.

Discussion and Conclusion

Chlorpyrifos is the most widely used organophosphorus pesticide for pest control in cotton and other crops in China. Rhizosphere bacterial communities play vital roles in maintaining farmland ecosystem stability and nutrient cycling. Investigating the effects of chlorpyrifos on soil microbial community diversity in cotton farmland ecosystems is therefore of great significance.

Our results showed that chlorpyrifos at 1–4 times the recommended application rate had half-lives of 10.04 d, 11.36 d, 11.55 d, and 12.16 d in soil, with nearly complete degradation by 60 days. Application at these rates significantly inhibited rhizosphere bacterial community diversity during the first 30 days, with recovery to normal levels by 60 days. Higher concentrations resulted in slower recovery of bacterial diversity. Similar findings have been reported, showing that 4–12 $mg \cdot kg^{-1}$ chlorpyrifos significantly inhibited soil microbial community diversity for two weeks, with higher concentrations causing stronger inhibition and longer recovery times, though diversity eventually returned to control levels. Pandey et al. also found that chlorpyrifos had transient inhibitory effects on soil microbial community diversity that later recovered to control levels. This pattern likely occurs because residual chlorpyrifos initially suppresses bacterial community diversity, which gradually recovers as the pesticide dissipates.

At 60 days, when chlorpyrifos was nearly completely degraded, the 1–4 times recommended rate treatments still showed significant differences from natural conditions in rhizosphere bacterial community structure, with greater differences observed at higher concentrations. The 4× recommended rate treatment showed the most significant divergence. Under natural conditions, planting cotton caused only minor differences in rhizosphere bacterial community structure across sampling times. Bulgarelli et al. similarly found that plants exerted only minor influence on rhizosphere soil bacterial community formation. In the chlorpyrifos treatments, even when the pesticide was nearly completely degraded by 60 days, rhizosphere bacterial community structure remained significantly different from natural conditions. TRF species analysis revealed that the relative abundance of some bacteria changed significantly after 60 days of chlorpyrifos treatment, indicating that chlorpyrifos application induced restructuring of soil bacterial communities. Azarbad et al. reported that long-term heavy metal pollution significantly affected bacterial community structure without impacting bacterial diversity. Newman et al. noted that chemical pesticides significantly increased the relative abundance of Proteobacteria while decreasing Acidobacteria in the rhizosphere. This may occur because soil bacterial communities can respond to environmental changes by altering their structure to adapt to new conditions.

Studies have shown that microbial pesticides such as *Pseudomonas fluorescens* 2P24, *P. fluorescens* CPF10, *Trichoderma atroviride* SC1, and *Bacillus thuringiensis* preparations only temporarily affect rhizosphere soil microbial communities, which later recover to normal levels. In contrast, chlorpyrifos exerts much greater impacts on the rhizosphere soil microecological environment, possibly because its application alters soil structure or physicochemical properties. Delmont et al. suggested that soil microbial community structure changes in response to variations in soil physicochemical properties, and Bulgarelli et al. similarly concluded that soil structure primarily determines rhizosphere and bulk soil microbial community composition.

T-RFLP molecular technology has been widely applied to analyze various environmental samples due to its speed, high reproducibility, and compatibility with RDP database comparison. However, as a PCR-based method, T-RFLP primarily amplifies dominant bacteria in soil DNA and can only annotate to the genus level in the RDP database, preventing investigation of functional gene changes at the species level.

In conclusion, chlorpyrifos application at the recommended rate or higher significantly affected cotton growth and restructured soil bacterial community composition. Even when chlorpyrifos was nearly completely degraded, the newly established bacterial community structure did not return to normal levels, with higher concentrations creating greater divergence from the natural state. Chlorpyrifos exerts substantial impacts on the cotton rhizosphere soil microecological environment, and its ecological security deserves serious attention. This study explored the effects of chlorpyrifos on cotton rhizosphere soil bacterial community diversity and structure. Future research should employ high-throughput sequencing to investigate the long-term effects of chlorpyrifos in soil, revealing its impacts on species composition and functional genes in plant rhizosphere microbial communities to provide critical information for assessing the ecological safety of chlorpyrifos and ensuring sustainable development of farmland ecosystems.

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Note: Figure translations are in progress. See original paper for figures.