European Journal of Biology
Eur J Biol 2024; 83 (2): 173–181
DOI: 10.26650/EurJBiol.2024.1484519

RESEARCH ARTICLE

Biodegradation of Chlorpyrifos in Paddy Rice Soil in the Mekong Delta, Vietnam

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ABSTRACT

Objective: Chlorpyrifos (CP) is an organophosphate insecticide. High toxicity makes it a potential source of soil pollution when used in agriculture. This study aimed to assess the anaerobic digestion of CP, the diversity of chlorpyrifos-degrading bacteria in soil, and the mechanism of CP decomposition.

Materials and Methods: Four soil samples were collected from paddy rice fields in Vietnam to evaluate the degradation of CP by anaerobic bacteria. The experiment was conducted in 50 mL microcosms containing 30 mL mineral salt medium, 10 g soil, and 35 mg/kg CP. The concentration of CP was determined using an high performance liquid chromatography.The intermediate products of CP were identified using a gas chromatography–mass spectrometry.

Results: Our bacterial communities in the soil samples anaerobically degraded CP. The rate of CP degradation was doubled after increasing the bacterial density during incubation. The percentage of CP degradation within a 4-month incubation period was significantly higher in the two bacterial communities isolated from alluvial soil than in acid soil. Four bacterial communities were found to degrade CP through the anaerobic reduction of chloride. The intermediate products resulting from the decomposition of CP by these soil bacterial communities were identified as O, O-diethyl-3, 6-dichloro-2-pyridyl phosphorothioate; 3,5,6-trichloro-2-pyridinol; O, O-diethyl-O (3,5,6-trichloro-2-pyridyl) phosphate. This shows the presence of the *Chloroflexi* bacterial phylum in the soil samples.

Conclusion: There is the presence of a group of anaerobic bacteria capable of decomposing CP in soil specialised in rice cultivation, opening up the potential to improve polluted soil by biological means.

Keywords: Anaerobic bacteria, Chlorpyrifos, *Chloroflexi*, DGGE, HPLC, GC/MS.

INTRODUCTION

Chlorpyrifos [*O*, *O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate-CP] is an organophosphate insecticide widely used by farmers for insect pest control in agriculture. It is also used, to a lesser extent, for indoor pest control in homes and buildings, and for soil treatment to manage subterranean termites.[1-7] Some reports indicate that CP bioaccumulate in blue-green algae,[8-10] mosquito fish,[11] aquatic plants, and goldfish.[12] In soil, slow resolution CP, soil persistence, and half-life (DT50) range from 60 to 100 days. The organic carbon adsorption coefficient of CP ranged from 652 L/kg for little soil organic matter (1.35% organic matter) to 30.381 L/kg for organic-rich soil (3.41% organic carbon).[13] The common use of CP caused air pollution, soil pollution, and contamination of surface water, rivers, streams, ponds, and lakes.[14] In soil, microorganisms play an important role in promoting CP breakdown. There have been many studies on the decomposition of CP, but most have focused on issues related to CP decomposition under aerobic conditions. However, the anaerobic bacteria can decompose CP in their respiratory process. In the world as well as in Vietnam, the anaerobic digestion of CP has not been studied. However, there are many studies on the anaerobic microbial decomposition of toxic substances containing -chlor, such as *Clostridium butyricum*, *Clostridium pasteurianum*, and *Citrobacter freundii*, which are capable of reducing -chlor hexachlorocyclohexane isomers.[15,16] The results of research on biodegradable dioxins showed reduced activity of some groups, and -chlor anaerobic bacteria play a crucial role in the decomposition of polychlorinated dibenzo-p-dioxins.[16] Other studies showed that soil samples collected from areas with more polluted areas in Vietnam had reduced activity of the chloro-good dibenzo-p-dioxin anaerobic bacteria.[17] In the Mekong Delta of Vietnam, the situation in the paddy soil is often favourable conditions for anaerobic bacteria involved in metabolic pathways and decomposition of soil organic toxins. Therefore, the

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Submitted: 15.05.2024 • **Revision Requested:** 09.07.2024 • **Last Revision Received:** 11.07.2024 • **Accepted:** 11.07.2024 • **Published Online:** 11.09.2024

theory is still capable of decomposing CP as well as other organic toxins when supplemented with organic acids. This is a matter of electronics. Bacteria can use it to remove chlorine from CP. Therefore, this study was performed to evaluate the decomposition of CP by anaerobic bacterial communities in paddy soil.

MATERIALS AND METHODS

Soil Samples

Soil samples were collected at a depth of 0–30 cm in paddy soils under flooded conditions after rice was harvested. The experiment was conducted with 4 soil samples coded as PH01, PH02, CL01, and CL02. The soil sample origins and characteristics are presented in Table 1. Each soil sample was a completely random layout including 5 treatments, each treatment was repeated 3 times, and the components of each treatment are presented in Table 2. Each soil sample was placed in a 50 mL glass vial and incubated. Before adding 10 g of soil to each vial, CP at a concentration of 35 ppm was dissolved in acetone, and 1 g of finely ground dry soil was added. The acetone was then allowed to evaporate naturally in a fume hood. Next, 30 mL of an anaerobic solution was added. This solution was prepared as described by Christoph et al.[18] and includes macro, trace, and essential vitamins that promote the activity of anaerobic Cl-reducing bacteria. These ingredients include D-biotin, folic acid, riboflavin, pyridoxine hydrochloride, vitamin B12, nicotinamide, and a mixture of organic acids such as pyruvic acid (pyruvate), acetic acid (acetate), butyric acid (butyrate), lactic acid (lactate), and propionic acid (propionate) at a concentration of 250 µM each. After mixing, the solution was aerated with N_2 and vacuumed to remove the O_2. The macromineral solution (1 L) should be supplemented with trace minerals (1 mL), vitamins (0.1 mL), and an organic acid mixture (4 mL). The anaerobic level of the solution can be checked by adding 1 mL of a resazurin anaerobic reagent. If the solution turns blue or purple-green, it indicates that the solution is indeed anaerobic (Figure 1). CP was added after 11 months of incubation: CP was added to the incubated samples to an enriched number of bacteria. 70 mg/L of CP was added to each incubated sample. CP was added for 1, 4, and 5 days. After 330 days, the incubated jars were supplemented with CP to enrich the bacterial population. The concentration of added drug was equivalent to the initial CP concentration of 35 ppm. Before adding the CP, an appropriate amount of anaerobic solution should be added to return the incubation bottle to its original volume of 40 mL. To add CP, a 3 mL medical syringe should be used to inject 1 mL of CP at a concentration of 1400 ppm into NT1, NT4, and NT5. no medicine should be added to live control treatments (NT2, NT3).

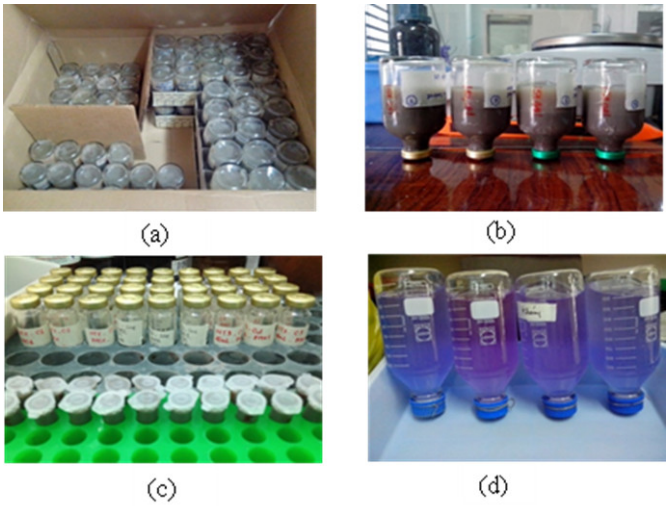

Figure 1. Experimental setup for anaerobic conditions. (a) Incubation vials are stored for measurements every 60 days, (b) Samples are incubated in 50 mL pi vials, (c) Chemical and biological measurements are taken, (d) Anaerobic solution is blue or blue-violet in color.

Samples were taken at 60, 120, 180, 300, 330, 350, and 450 days to determine the CP content, analyse biodiversity, and detect by products present.

Shake the incubation bottle well, use a sterile medical syringe to remove the fluid from the 1 mL incubation bottle, and put it into the 10 mL pi bottle. The syringe was blown with nitrogen gas to expel all the oxygen before sampling. The incubation bottle was gently shaken during the sampling process to make the sample more even and accurate.

The extraction was performed three times using a mixture of toluene and acetone at a ratio of 2:1. After extraction, the samples were allowed to naturally evaporate until they reached a volume of 1 mL. The resulting sample was then filtered and passed through an alumina column for further purification. The concentration of CP was determined using an HPLC (High-Performance Liquid Chromatography) machine equipped with a C18 column measuring 25 cm in length and 4.6 mm in diameter with a particle size of 5 µm. The mobile phase was a mixture of methanol and water in a ratio of 80:20, the detection wavelength was set to 230 nm, the flow rate was 1 mL, and the retention time was 17.7 min. The chromatograms of the baseline and standard are shown in Figures 2 and 3, respectively. The standard curve equation is $y = 20767x - 692.89$ with a linear range of 0.1-300 ppb. The limit of detection (LOD) is 0.1 ppm and the limit of quantification (LOQ) is 0.33 ppm.

The intermediate products of CP were identified using a Shimadzu GC/MS QP2010 Plus chromatography column, specifically, an Rxi 5SilMS 30 m x 0.32 mm chromatography column with a 0.25 µm film. The sample injection chamber temperature was set to 250 ℃, while the gas chromatography (GC)–mass spectrometry (MS) communication points and ion source were set to 250 ℃ and 200 ℃, respectively. A sample volume of 1 µL

Table 1. The origin and characteristics of soil samples used in the experiments.

Province	Code	Number crop/year	Depth (cm)	Soil characteristics			Organic content (%)	pH
				Clay (%)	Loam (%)	Sand (%)		
Tien Giang	CL01	2	0 – 20	67	31	2	7.62	5.70
	CL02	3						
Hau Giang	PH01	3		73	27	0.2	4.90	5.06
	PH02	3						

Table 2. Ingredient of treatments.

Treatments	Ingredient
1- Negative control (NT1)	Use 10 g of sterile soil, 70 mg/L of CP, 30 mL of mineral salt minimum
2 - Positive control (NT2)	Use 10 g of non-sterile soil, no additional CP, 30 mL of mineral salt minimum, vitamin supplements and an organic acid mixture
3 - Positive control (NT3)	Use 10 g of non-sterile soil, no additional CP, 30 mL of mineral salt minimum, no additional vitamin and organic acid mixture
4 - (NT4)	Use 10 g of non-sterile soil, 70 mg/L of CP, 30 mL of mineral salt minimum, vitamin supplements and an organic acid mixture
5 - (NT5)	Use 10 g of non-sterile soil, 70 mg/L of CP, 30 mL of mineral salt minimum, no additional vitamin and organic acid mixture

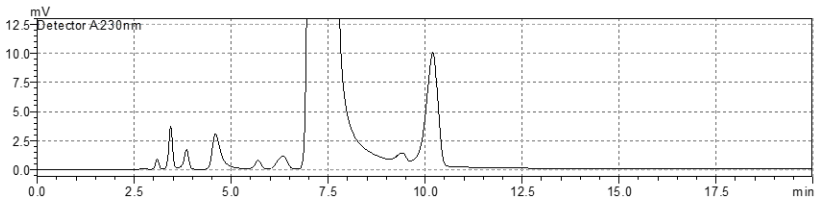

Figure 2. Chromatogram of baseline (blank sample).

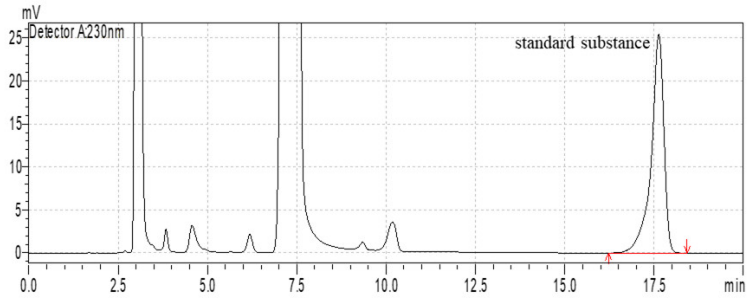

Figure 3. Chromatogram of standard substance.

was injected using the splitless injection mode. This method was selected for its accuracy in identifying intermediate CP products.

Soil samples of bacterial communities coded as PH01 after incubation for 2 months were used to extract DNA and perform PCR-denaturing gradient gel electrophoresis (DGGE). The DNA of bacterial communities was extracted using DNA extraction methods from the soil of PowerSoil (R) Isolation. The DNA product was amplified by PCR 2 times. The first PCR was performed using 338F/Chlor1101R primers.[19,20] This PCR product was generated using 341F-GC/534R primers.[21] The primer sequence is shown in Table 3. Then, the DGGE method was used to assess the diversity of the bacterial communities. To use the Cluster and Gel Compare software to analyse the electrophoresis images and compare similarities between bacterial communities.

Table 3. Oligonucleotide sequences of primer.

Gene	Primer sequence, 5'- 3'
338F	ACT CCT ACG GG AGG CAG CAG
Chl1101R	CTC GCK AGA AMA TKT AAC TAG CAAC
341F-GC	CGC CCG CCG CGC GC GGC GGG CGG GGC GG GGG CAC GG GGG CAC GGG GGG CCT ACG GGA GGC AGC AG
534R	ATT ACC GCG GCT GC TGG

Methods of Data Processing, Analysis, and Statistics

Microsoft Excel was used to calculate the percentage of decomposition and graph. The significance of differences was determined using one-way ANOVA and the Tukey test (p<0.05) with Minitab 16 software.

RESULTS

Evaluation of the Potential of Chlorpyrifos Degradation by Anaerobic Bacterial Communities

In the Hau Giang and Tien Giang provinces, four soil samples were collected from paddy soils and used in experiments to evaluate the degradation ability of CP by anaerobic bacterial communities. The results showed that all treatments were biodegradable for CP. After 2 months of incubation, the remaining concentration of CP in the treatment ranged from 3% to 45% of the initial concentration. The decomposition rate of CP increased after supplementation because the number of anaerobic bacteria increased. After 20 days of incubation, the remaining concentration of CP in the treatment ranged from 4.9% to 48.3% of the initial concentration. After 4 months of incubation, the remaining concentration of CP in the treatment ranged from 0.8% to 1.5% of the initial concentration (Figures 4 and 5).

On the other hand, at 4 months after the addition of CP, the decomposition rate CP of 2 bacterial communities was coded as CL01, and CL02 (the remaining CP from 1% to 1.5% compared to the control), which were higher than 2 communities were coded as PH01 and PH02 (the remaining of CP from 1% to 16% compared to the control).

Identification of Intermediate CP Products

The intermediate products generated during the decomposition of CP by soil anaerobic bacteria were identified.This results in the production of various metabolic byproducts, including a chlorine decomposition product at position 5 on the pyridine ring, namely O, O-diethyl-3,6-dichlo-2 pyridyl phosphorothioate. Another by-product formed is the ester radical decomposition product, which leads to the formation of 3,5,6-trichloro-2-pyridinol (TCP). Further analysis revealed the presence of O, O-diethyl-O (3,5,6-trichloro-2-pyridyl) phosphate (Chlorpyrifos oxon). Treatment without the addition of organic acids enhances the ability of bacteria to use CP for subproduct analysis. Specifically, the sample obtained at 60 days showed daughter products that were identical to those in the treatment with added organic acids (one-chlorine reduction product, TCP, and Chlorpyrifos oxon). However, the 330-day sample exhibited only the formation of chlorine-based decomposition products. In the analytical results, we were unable to determine the carbon cleavage product of CP. This shows that in a farming environment in which only CP is added to promote bacterial utilisation, dechlorination products are still formed. The content of dechlorinated CP was higher than that of TCP (Figure 6).

Anaerobic Bacterial Diversity in Soil

The results of PCR using 338F/Chl1101R primers showed that *Chloroflexi* phylum was present in soil samples. Besides, the results of PCR using 341F-GC/534R primers showed that all samples had a band of DNA at a position of 200 bp. The results of DGGE showed that each band on the gel represents a different bacterial species. To determine whether the chlor-reduction bacteria group in the experiment could be identified from the new DNA bands that appeared in samples supplemented with CP compared with the control treatment with no additional CP. Because the incubation period had passed, bacteria could increase the number. This leads to the formation of a new band in the treatments (Figure 7). The PCR-DGGE results showed that the structure of bacterial communities (was coded as PH01) diversity among treatment complements did not supplement the organic acid, and the control treatment (no additional CP) had a high degree of similarity, 90%-96% (Figure 8).

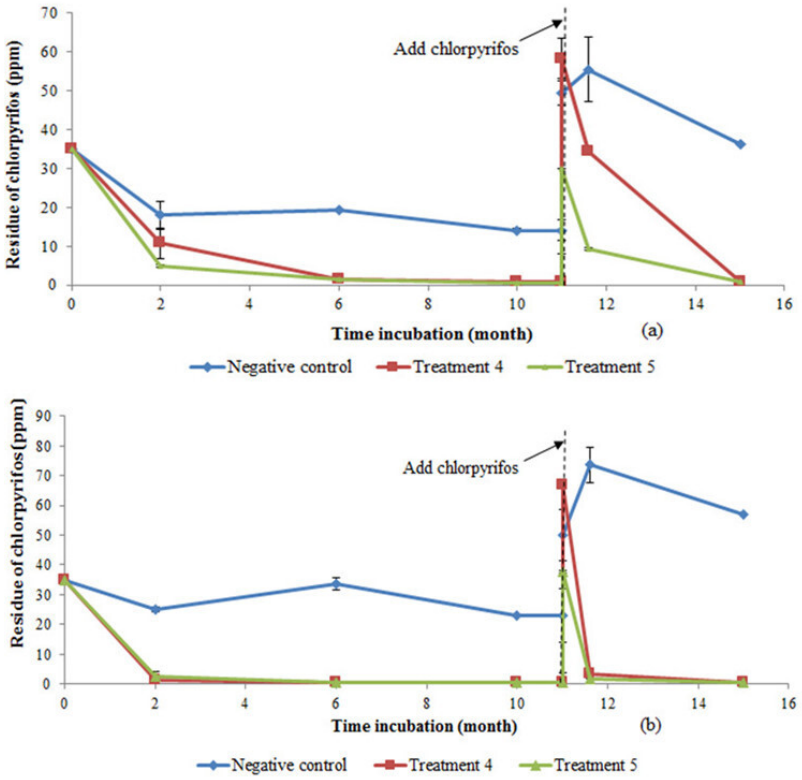

Figure 4. Chlorpyrifos degradability of bacterial comunities CL01 (a) and CL02 (b) (n = 3, standard error).

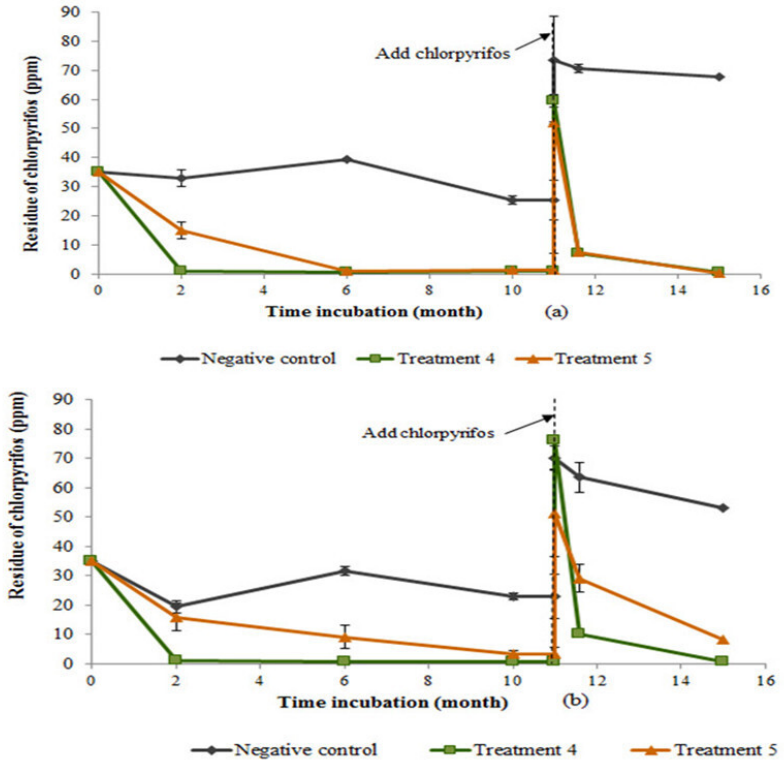

Figure 5. Chlorpyrifos degradability of bacterial comunities PH01 (a) and PH02 (b) (n = 3, standard error).

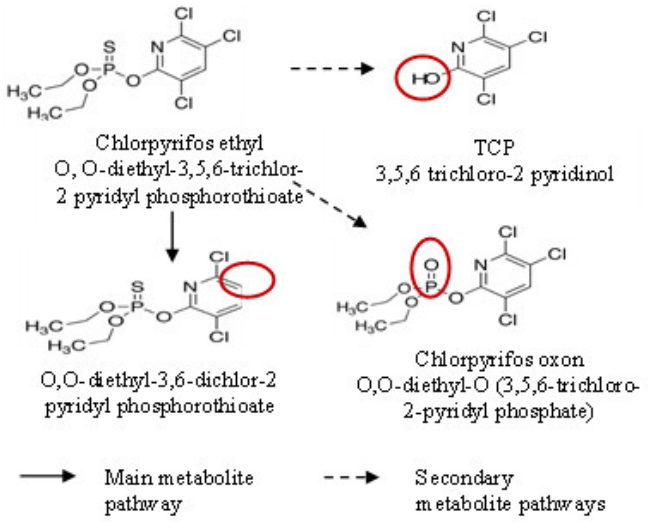

Figure 6. Metabolism diagram of chlorpyrifos in organic acid supplementation treatment.

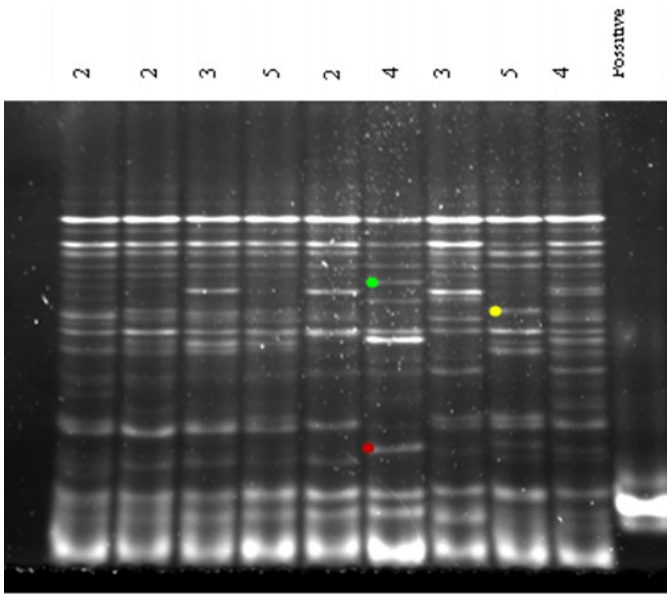

Figure 7. Electrophoresis map PCR-DGGE product of treatments in soil sample PH01.

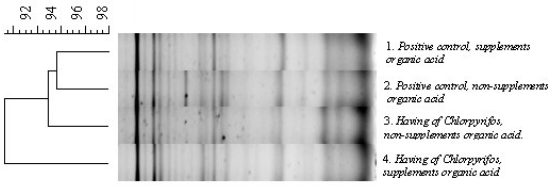

Figure 8. The similarity of the bacteria *Chloroflexi phylum* in soil samples PH01.

Thus, *Chloroflexi phylum* bacteria were present in 4 soil samples, and the bacterial communities had a high degree of similarity. This result is consistent with the test results for intermediate metabolic products of the CP decomposition route by anaerobic bacterial communities. Intermediate products generated during the decomposition of CP by soil bacterial communities were determined as O, O-diethyl-3, and 6-dichloro-2 pyridyl phosphorothioate.

DISCUSSION

In recent years, there has been a growing interest in the microbial biodegradation of pollutants as a means of finding sustainable ways to clean up contaminated environments. One effective approach is to enhance the growth of microbes that may already be present at the contaminated site and to introduce specialised microbes with the ability to degrade specific contaminants. This technique is known as bioremediation and has become increasingly common. One particularly efficient method is reductive dehalogenation, which rapidly detoxifies chlorinated compounds. However, the complete degradation of organochlorine contaminants under anaerobic conditions requires a consortium of many microorganisms to work together, with complex interrelationships.[22]

In the soil environment, CP can decompose and metabolise in various ways, including adsorption into the soil, infiltration into groundwater, evaporation into the air, and absorption and decomposition by soil microorganisms (both aerobic and anaerobic). In soil, CP decomposition via hydrolysis and photolysis is slow. Therefore, microorganisms play a crucial role in promoting drug decomposition. Although numerous studies have been conducted on the decomposition of CP, most have focused on aerobic conditions. Some notable studies on aerobic decomposition include Singh et al.,[23] who isolated *Enterobater* B14 strain, and Yang et al.,[24] who successfully studied the ability of *Alcaligenes faecalis* DSP3 strain to decompose both CP and its byproduct TCP. Ghanem et al.[25] isolated Klebsiella sp. for their study. Additionally, Anwar et al.[26] Isolated *Bacillus pumilus* C2A1 strains that decompose both CP and TCP. This strain decomposed up to 90% of TCP at a concentration of 300 ppm after only 8 days of incubation.

Numerous studies have been conducted on the decomposition of organic compounds containing chlorine in an anaerobic environment. Among these compounds, some are considered highly toxic, such as Dioxins. According to Duong et al.,[17] bacteria belonging to the genera *Clostridium, Chromium, Bacteroidetes*, and *Chloroflexi* can reduce chlorine content in these compounds. The *Chloroflexi* phylum is one of the most extensively studied genera due to its ability to degrade organochlorine compounds, which are highly toxic and persistent in soil and sediments. Within this phylum, *Dehalococcoides mccartyi* is strictly anaerobic bacteria that are well-known for their

ability to obtain energy through the reductive dehalogenation of organic chlorinated compounds. All known *D. mccartyi* species use hydrogen as an electron donor, acetate as a carbon source, and halogenated aliphatic or aromatic compounds as respiratory electron acceptors. For example, strain 195 can use perchloroethene (PCE), 1,2,3,4-tetrachlorodibenzo-p-dioxin, and hexachlorobenzene as electron acceptors in its respiratory process.[27-29] Similarly, strain CBDB1 has been shown to grow with hexachlorobenzene and dioxins as well.[30,31] For 1,2,3,4-TCDD, chlorine is reduced at both the parent site (para) and the branch site, resulting in the formation of products such as 1,2,3-trCDD, 1,2,4-trCDD, and diCDD. In the case of 2,3,7,8-TCDD, the product formed is 2,3,7-trCDD. The bacterial species examined in the experiment are considered quite diverse and differ from known chlorine-reducing bacterial species found elsewhere in the world. In addition to dioxins, other organic toxins have been successfully investigated. Jagnow et al.[15] Has shown that certain bacteria, such as *Clostridium, Bacillaceae*, and *Enterobacteriaceae*, can decompose γ-hexachlorocyclohexane (γ-HCH) under anaerobic conditions. Specifically, *Clostridium butyricum, C. pasteurianum*, and *Citrobacter freundii* were found to effectively reduce chlorine radicals attached to the gamma position within 4 to 6 days of incubation. However, other facultative anaerobic species, such as *Lactobacillaceae* and *Propionibacterium*, do not exhibit decomposition activity. The main byproduct of this process is γ-tetrachlorocyclohexene (γ-TCH), an important intermediate in the degradation of γ-HCH. Daniel (1990)[32] conducted a study on the degradation of polychlorinated biphenyls (PCBs). The focus of this research was on the biodegradation of PCBs under aerobic and anaerobic conditions. The PCB decomposition system includes aerobic bacteria living in oxidising environments and anaerobic bacteria living in oxygen-free environments, such as aquatic sediments. Drug decomposition experiments follow two different mechanisms. Aerobic bacteria can decompose PCBs by breaking their structure and opening carbon rings. On the other hand, anaerobic bacteria retain the biphenyl ring and reduce the chlorine radicals in the molecule, forming various derivatives of PCBs. These two natural processes complement each other and contribute to the decomposition of the most commonly used PCB mixtures. Other organophosphate pesticides, such as CP, have also been the subjects of research. The pesticide sumithion (o,o-dimethyl-o-(3-methyl-4-nitro) phosphorothioate) was tested for biodegradation using microorganisms isolated from sludge under anaerobic conditions. Analysis of drug concentrations using liquid and gas chromatography revealed that the bacterial system was able to decompose sumithion at concentrations of 20, 50, and 100 ppm in 15, 25, and 45 days, respectively. After 2 years of isolation, the bacterial strain was identified as SY, which can be easily produced and used to treat sumithion-contaminated soil.[33] Similar to other organic substances, CP also contains chlorine radicals attached to the aromatic rings. The successful decomposition of the aforementioned substances demonstrates the potential of the anaerobic decomposition of CP.

In the present study, after conducting the survey, all four bacterial systems (CL01, CL02, PH01, and PH02) effectively decomposed CP under anaerobic conditions. Upon the addition of CP, the bacterial systems could completely utilise the drug. Over 20 days, there was no significant difference in the rate of drug decomposition between the treatments with and without organic acid supplementation for all four bacterial systems (CL01, CL02, PH01, and PH02). However, the decomposition times of the four bacterial systems were shorter than those of the initial experimental setup, indicating an increase in the number of bacteria involved in the decomposition process. According to the National Registration Authority in 2000,[34] the hydrolysis of CP is slower in moderately acidic soils but faster in alkaline environments, because of having 2 types of soil in Tien Giang and Hau Giang provinces. In Tien Giang province, the soil has 7.62% organic matter content which is higher than the soil (having organic matter content of 4.9%) in Hau Giang province. Therefore, in the soil of Tien Giang province, the ability of CP hydrolysis and the decomposition of bacteria CP is better.

In this study, we examined the intermediate products produced during the decomposition of CP by anaerobic bacteria. Our findings revealed that the primary intermediate products were a chlorine decomposition product at position 5 on the pyridine ring, namely O, O-diethyl-3,6-dichloro-2 pyridyl phosphorothioate, TCP, and O, O-diethyl-O (3,5,6-trichloro-2-pyridyl) phosphate (Chlorpyrifos oxon). Chlorpyrifos oxon hydrolysis rate is significantly faster than that of the original CP properties. Therefore, the formation of chlorpyrifos oxons is a crucial intermediate step in promoting the decomposition of CP.[9] However, the concentrations of TCP and chlorpyrifos oxons were found to be very low, indicating that the main decomposition process is still the formation of dechlorination products. The findings of the investigation into the metabolism of CP are partially consistent with those of the Racke[35] study on the overall metabolic pathway of the compound. Both studies found that TCP and chlorpyrifos oxons are formed during metabolism. However, this study also discovered a new product, the dechlorination product at position 5 on the pyridine ring, which is the most abundant metabolite in the anaerobic digestion of CP. This product was not observed in the Racke[35] study on CP metabolic products. Thus, in each treatment with or without organic acids, bacteria used CP as an electron acceptor during respiration. Particularly for treatments without added organic acids, it is not possible to conclude from the analysis results whether or not the bacteria can use CP as their sole carbon source. Analysis of the control samples revealed CP in the soil samples used for testing. Of these samples, half showed evidence of TCP formation at low concentrations. The survey of CP decomposition activity and analysis of metabolic products indicated the presence of anaerobic bacteria in the natural environment that are capable of breaking down CP. Moreover,

the results of the analysis of bacterial diversity are consistent with the results of by-product testing using GC/MS. This shows that the presence of organic acid in the soil does not affect the products identified in the presence and absence of organic acid supplementation. This indicates that there was no difference in the drug decomposition mechanism between the two treatments, indicating a similar structure in the bacterial system. However, in treatments NT2 and NT3, only a small number of daughter products were detected, with only TCP appearing in half of the analysed samples. This could be due to the low concentration of the products, which renders them difficult to detect. Despite this, CP was still present, indicating the possibility of dechlorination activity in both treatments, regardless of the presence of additional organic acid sources. This finding could explain the high similarity between all treatments.

CONCLUSION

Analysis of CP during anaerobic incubation revealed that all four bacterial communities from the four soil samples possessed the ability to effectively decompose CP. Furthermore, the rate of degradation was found to double when the bacterial density was increased during incubation. Interestingly, the decomposition rate of CP over the 4-month incubation period was higher in the two bacterial communities from alluvial soil than in those from acid soil. It was observed that all four bacterial communities utilised anaerobic respiration to degrade CP by reducing –chlor. This shows the presence of *Chloroflexi* bacteria in the soil samples.

Ethics Committee Approval: Ethics committee approval is not required for the study.

Peer Review: Externally peer-reviewed.

Conflict of Interest: Author declared no conflict of interest.

Financial Disclosure: Author declared no financial support.

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How cite this article

Tat, TQ. Biodegradation of Chlorpyrifos in Paddy Rice Soil in the Mekong Delta, Vietnam. Eur J Biol 2024; 83(2): 173–181. DOI:10.26650/EurJBiol.2024.1484519