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Developing Specialized Bacterial Consortia for Enhanced Biodegradation of Chlorpyrifos in Dairy Farms in Nakuru County

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Abstract

Chlorpyrifos (CP), a widely used organophosphate in Kenya, poses significant human health risks due to its high solubility. This study aimed to isolate CP-degrading bacteria from dairy farm soils in Nakuru County and develop bacterial consortia for efficient degradation. Soil samples were collected from six sub-counties: Molo, Njoro, Rongai, Subukia, Gilgil, and Naivasha. The enrichment culture technique using minimal salt medium (MSM) was employed to isolate CP-degrading bacterial strains. Seven bacterial strains were identified: *Alcaligenes faecalis*, *Bacillus weihenstephanensis*, *Bacillus toyonensis*, *Alcaligenes* sp., *Pseudomonas* sp., *Pseudomonas japonicum*, and *Brevundimonas diminuta*. *Bacillus weihenstephanensis* exhibited the highest individual degradation efficiency at 79.8%. Consortia treatments demonstrated enhanced degradation, with consortia M1, M2, and M3 achieving removal efficiencies of 91.3%, 92.48%, and 93.76%, respectively, and M5 achieving 95.32%. The study also monitored 3,5,6-trichloro-2-pyridinol (TCP), a CP metabolite, showing that consortia treatments significantly reduced TCP levels to near initial concentrations. Kinetic analysis revealed consortia had significantly shorter half-lives for CP degradation compared to individual isolates, indicating improved bioremediation potential. The findings underscore the potential of using tailored bacterial consortia for effective bioremediation of CP-contaminated environments. Future research should focus on field trials to validate these laboratory findings under real-world conditions and explore the genetic and enzymatic mechanisms underlying CP degradation.

Keywords: chlorpyrifos, bioremediation, bacterial consortia, environmental contamination, organophosphate degradation

1. Introduction

1.1 Introduction to the Problem

Chlorpyrifos (CP) is an organophosphate pesticide widely used in Kenyan agriculture, particularly in dairy farming. Its extensive application has led to significant environmental contamination and poses serious risks to human health due to its high solubility and persistence in the environment (George et al., 2014). This study addresses the problem of CP contamination in dairy farm soils in Nakuru County, Kenya, where its usage is prevalent for controlling pests in livestock. The problem is crucial due to the toxic effects of CP on non-target organisms, including humans, and the contamination of water, soil, and food resources (Ambreen & Yasmin, 2021). The high solubility and persistence of CP result in long-term environmental and health impacts, making its degradation a priority for environmental safety.

Previous studies have demonstrated the potential of microorganisms to degrade CP, utilizing it as an energy and carbon source, thus offering a viable bioremediation strategy (Asamba et al., 2022). However, there is need to explore the specific bacterial strains capable of CP degradation in the Kenyan context, as well as to develop effective consortia for enhanced degradation. The primary objective of this study is to isolate and identify CP-degrading bacteria from dairy farm soils in Nakuru County and to develop bacterial consortia for efficient CP

degradation. The secondary objective is to understand the degradation kinetics and the impact of these bacterial consortia on CP and its primary metabolite, 3,5,6-trichloro-2-pyridinol (TCP).

1.2 Importance of the Problem

Given the serious effects it has on the environment and human health, further study is necessary to address the issue of chlorpyrifos (CP) pollution in agricultural soils. According to George et al. (2014), CP is frequently used in pest control but lingers in the environment and contaminates food, water, and soil, endangering human health. According to previous research, microbial degradation is a viable bioremediation technique (Chen et al., 2012). Nonetheless, additional research is required due to inconsistent degradation efficiencies and a lack of knowledge regarding bacterial interactions (Rayu et al., 2017). Developing efficient bioremediation methods depends on resolving these problems. The objective of this work is to create bacterial consortia for improved degradation as well as to isolate and characterize CP-degrading bacteria from Kenyan soils. The results will advance our understanding of bioremediation overall and provide workable ways to reduce CP contamination and enhance environmental safety. The advancement of sustainable farming methods and the preservation of public health depend on this research (Ambreen & Yasmin, 2021; Bose et al., 2021).

1.3 Relevant Scholarship

Recent studies have highlighted the potential of specific bacterial genera, including *Pseudomonas*, *Bacillus*, and *Alcaligenes*, in degrading chlorpyrifos (CP) and its metabolites (Chen et al., 2012; Bose et al., 2021). These bacteria exhibit robust enzymatic pathways enabling the breakdown of CP into less harmful compounds. Previous research has primarily focused on individual strains, revealing significant degradation capabilities but with varying efficiencies (Sun et al., 2020). Notably, microbial consortia have demonstrated superior degradation performance due to synergistic interactions among different bacterial species (Rayu et al., 2017). This study builds on existing knowledge by isolating CP-degrading bacteria from Kenyan soils and forming effective consortia. Furthermore, while several studies have reported CP degradation, there is limited research on its application in the Kenyan context, making this study crucial for local environmental management (Asamba et al., 2022). The findings aim to advance bioremediation strategies and enhance environmental sustainability (Ambreen & Yasmin, 2021).

1.4 Research Hypotheses

The study hypothesizes that specific bacterial strains isolated from dairy farm soils in Nakuru County can effectively degrade chlorpyrifos (CP) and that bacterial consortia will enhance this degradation efficiency. These hypotheses are grounded in the theoretical framework that microbial consortia possess a broader enzymatic repertoire, facilitating more comprehensive degradation of contaminants (Rayu et al., 2017).

Primary Hypothesis: Isolated bacterial strains from Nakuru County soils can degrade CP effectively.

Secondary Hypothesis: Bacterial consortia will enhance CP degradation efficiency compared to individual strains.

Prior studies have shown that individual bacterial strains can degrade CP, but their efficiency varies (Chen et al., 2012; Bose et al., 2021). The combination of different strains into consortia is expected to enhance degradation due to synergistic interactions and a wider range of enzymatic activities (Sun et al., 2020).

2. Method

2.1 Study Area Description

Nakuru County (Latitude: 0°29′59.99″N; Longitude: 36°00′0.00″E), Kenya, was the location for collection of soil samples for analysis (Figure 1). Nakuru has a temperate climate that is normally warm, and it is located 1871 meters above sea level. There is roughly 762 mm of annual rainfall and 17.5 °C on average. The laboratory experiments were carried out at Mount Kenya University, Thika, Kenya. The bacterial DNA samples were shipped to Macrogen laboratories, the Netherlands for 16S rDNA gene sequencing.

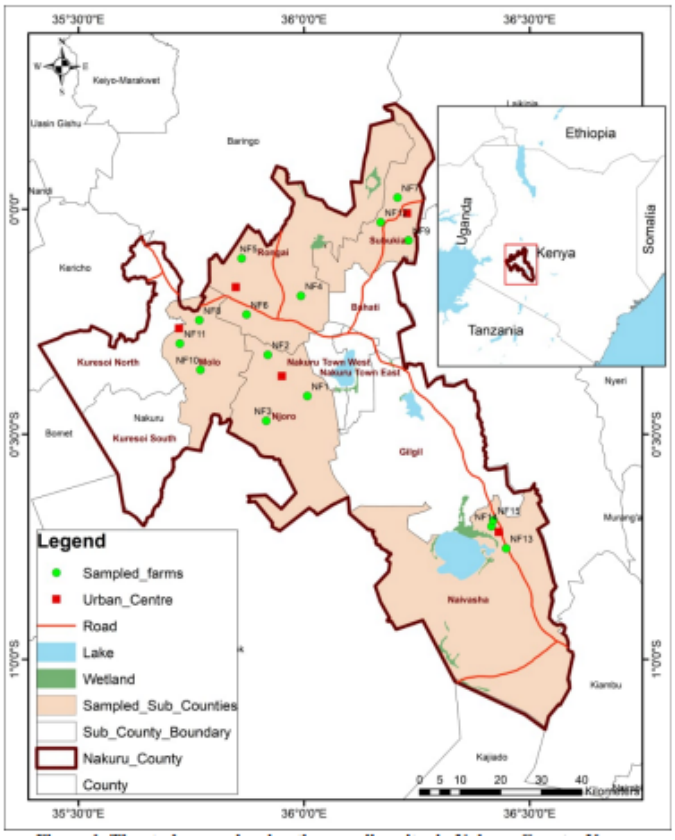


Figure 1. Map showing the study area in Nakuru County

2.2 Bacterial Isolation and Selection

2.2.1 Collection of Soil Samples

The study's soil came from dairy farms close to cow units, quite close to the locations where acaricide treatments (spray races and dips) are applied to the animals. Ten distinct farms in Nakuru County provided soil samples, which were taken and kept in sterile, sealable plastic zipper bags at 4 °C. A depth of 10 cm was used to collect the soil. Physicochemical analysis was conducted for the soil because the source of inoculum is related to bacterial properties. Properties of the soil that were measured included pH, electrical conductivity, soil water content, soil texture, and soil organic matter. In the laboratory, soil samples were air-dried and sieved before analysis.

2.2.2 Isolation of CP-Degrading Bacterial Strains

The study used the enrichment culture technique with minimal salt medium (MSM) to isolate bacteria capable of degrading chlorpyrifos (Barathidasan et al., 2014). The MSM included ammonium nitrate, hydrated calcium nitrate, hydrated magnesium sulphate, potassium chloride, hydrated iron (II) sulphate, dipotassium phosphate, and potassium dihydrogen phosphate. We adjusted the pH using a benchtop pH meter. Commercial-grade chlorpyrifos was added as the sole carbon source at concentrations of 5 ppm, 10 ppm, and 40 ppm to simulate soil exposure.

Soil samples were mixed with distilled water and serially diluted. The sixth dilution was inoculated into the MSM broth, which was incubated on a rotary shaker at 150 rpm and 30 °C for 21 days (Eissa et al., 2014) under aerobic conditions. Bacterial growth was monitored by measuring turbidity (OD600) with a UV-VIS Spectrophotometer every five days. After 21 days, 1 ml aliquots were spread on MSM agar plates containing 10 ppm chlorpyrifos. Isolated colonies were further cultured in basal MSM with chlorpyrifos for 14 days to confirm their phosphorus-utilizing capabilities. These cultures were then streaked onto Nutrient Agar (NA) and Tryptic Soy (TS) plates with chlorpyrifos and incubated at 30 °C. Cycloheximide was added to inhibit fungal growth (Maarten et al., 2007).

2.2.3 Preliminary Identification of Bacteria From Chlorpyrifos Contaminated Soil

Preliminary identification of chlorpyrifos degrading bacteria from soil samples was done using morphological and biochemical techniques. Morphological identification of bacteria isolates involved the use of colony characteristics such as colony colour, elevation, margin, and size observed using a microscope. Pure isolates obtained after streaking on nutrient agar medium was subjected to gram stain and observed under the oil immersion ×100 lens. Other biochemical test such as Methyl-Red Test, Catalase Test, Oxidase Test, Urease Test, Citrate Utilization Test, Lactose Utilization Test, and production of Hydrogen sulphide were also conducted to enhance the identification process. Identification of bacteria following morphological, Gram staining and biochemical test was aided with the use of Bergey's Manuel of Systemic Bacteriology for biochemical characterization.

2.3 Molecular Characterization of Bacterial Strains

We characterized the isolated bacteria using 16S rDNA gene sequencing. The process involved four main steps: extracting genomic DNA, performing PCR, conducting agarose gel electrophoresis, and sequencing the genes (Clarridge, 2004; Daneshparvar et al., 2017; Galal & Seufi, 2020). For DNA extraction, we used the Zymo Quick DNA Miniprep Kit. Bacterial isolates were suspended in saline, vortexed, and centrifuged. The supernatant was discarded, and the pellet was mixed with genomic lysis buffer. After incubation, the lysate was processed through a Zymospin column, washed, and the DNA was eluted and stored at -20 °C (Wei et al., 2020; Cao et al., 2018). PCR amplification followed Lorenz's protocol (2012) using primers 1492r and 27f. The PCR cycle included denaturation at 95 °C, annealing at 62 °C, and extension at 72 °C. We then separated the PCR products via agarose gel electrophoresis, visualized them under UV light, and documented the results (Franco-Duarte et al., 2019). The purified PCR products were sent to Macrogen Europe Laboratory for Sanger sequencing, using an ABI 3700 DNA sequencer and Big Dye Terminator Kit (Johnson et al., 2019). This method ensured accurate sequencing and identification of the bacterial species.

2.4 Consortium Development

The optimum conditions for degradation for each of the seven isolates were determined in our previous study (Wepukhulu et al., 2024). In the current study, insights from the study were used to inform the development of the consortia for optimum degradation. The optimum degradation for individual isolates are as presented in the Table 1.

Table 1. Optimum degradation conditions for Individual Isolates

Isolate	Ph	CP conc (mg/L)	Temperature (°C)
MW1	7	2	25
MW2	5	5	30
MW3	5	5	25
MW4	5	5	25
MW5	5	5	25
MW6	7	100	25
MW7	5	100	25

Based on these optimum conditions, different consortia were developed as described below. The first experiment involved the determining degradation potential of individual isolates The experiment was coded Mo. Next, all the seven isolates were mixed and cultured in MSM supplemented with 25 mg/l of CP, at pH of 5 and temperature of 25 °C. The experimental setup was named as M1. The third experiment (M2) included a combination of MW1 and MW6 at 25 mg/L CP, pH 7, 25 °C. Experiment 4 (M3) had MW4 and MW7 at 25 mg/L CP, pH 5, 25 °C. Experiment 5 (M4) had MW1 and MW6 at 100 mg/L CP, pH 7, 25 °C. Experiment 6 (M5) had MW4 and MW7 at 100 mg/L CP, pH 5, 25 °C. Besides, there was a positive control (*Pseudomonas putida* strain TCA1) (Asamba et al., 2022) (M6) and negative control (uninoculated CP to measure natural degradation) (M7). This experimental setup is summarized in the Table 2.

Table 2. Experimental setup

Experiment	Combination	CP conc (mg/L)	Ph	Temperature (°C)
Mo	Individual isolates	varied based on optimum per isolate (Table 1)	varied based on optimum per isolate (Table 1)	varied based on optimum per isolate (Table 1)
M1	All seven isolates	25	5	25
M2	MW1 + MW6	25	7	25
M3	MW4 + MW7	25	5	25
M4	MW1 + MW6	100	7	25
M5	MW4 + MW7	100	5	25
M6	Pseudomonas putida TCA1	-	-	-
M7	Negative Control	-	-	-

After inoculation, the setups were incubated for a total of 30 days. All the samples were incubated on rotary shakers at 100 rpm. Periodically, starting with day zero, in an interval of four days, 0.1 ml of the inoculated media was pipetted and subjected to degradation assays using HPLC.

2.5 Degradation Assays

2.5.1 High-Performance Liquid Chromatography

HPLC with a Shimadzu system, two LC-10AT VP pumps, a variable wavelength UV detector, and a reversed-phase C-18 column was used to monitor degradation (Shaker & Elsharkawy, 2015). It used Shimadzu's Lab Solutions software. The mobile phase, 75% acetonitrile and 25% P_O4 buffer, was supplied at 1 ml/min at 30 °C with UV detection at 230 nm.

The extraction method was adapted from Fagnani et al. (2011). After defrosting, 1 ml of each sample was put in a screw-top centrifuge tube with 5 ml acetonitrile/phosphate buffer (90/10, pH 4.5). The mixture was gently swirled, sonicated for 10 minutes, vortexed for 15 minutes at 1200 rpm, and centrifuged for 5 minutes at 2000 rpm. The supernatant was double-extracted with 5 ml of buffer solution in another tube. The extract was filtered into an HPLC vial using a 0.45 µl Teflon syringe filter (Harshit et al., 2017).

To analyze, 20 µl of each sample was loaded into the HPLC apparatus. Retention times and peak regions were compared to reference standards to identify and quantify chemicals. Sigma-Aldrich chlorpyrifos standards were dissolved in acetonitrile and serially diluted to 10-ppm solutions (Chen et al., 2014). The Dichloroquant approach in the machine software was used for batch analyses. In each sample, the peak area was measured and a calibration curve for concentration against response area/peak area was plotted. Precision was achieved with external standards with a standard deviation below 0.05. Figure 2 shows the calibration curves for CP and TCP.

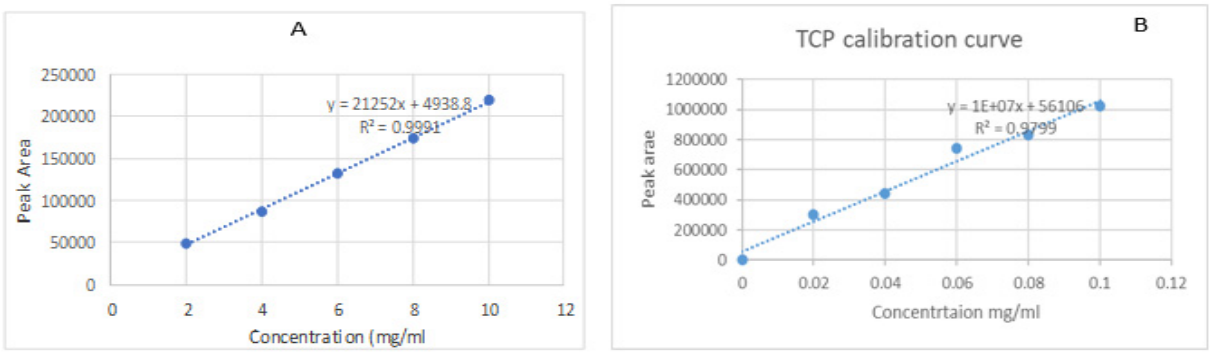


Figure 2. HPLC calibration curve of (a) chlorpyrifos standard and (b) TCP

2.5.2 Biodegradation Kinetics

Kinetic analysis is a method commonly used to determine the rate of biodegradation. In this study, only one reactant was used in the first order reaction, the CP. The concentration of chlorpyrifos at any given time tt during the degradation process can be described using the equations:

$$\ln(C) = a + k1t \quad (1)$$

$$C = b + k0t \quad (2)$$

Here, C represents the concentration of chlorpyrifos at time tt. The specific growth rate, denoted as μ, can be determined using the equation:

$$\ln(A_t/A_0) \quad (3)$$

The half-life, which is the time needed to reduce the concentration of the contaminant by 50%, is calculated using the equation from Denga et al. (2015):

$$t_1/2 = \ln(2)/k \quad (4)$$

For calculation of removal efficiency, Equation 7 was used (Jiang et al. 2015).

$$\text{Removal efficiency (\%)} = 100 \times (C_\text{influent} - C_\text{effluent})/C_\text{influent} \quad (5)$$

Here, C_influent is the initial concentration of chlorpyrifos, and C_effluent is the concentration at the end of the experiment.

2.6 Data Analysis

The data was subjected to inferential statistics analysis using SPSS software (version 20), including analysis of variance (ANOVA), t-test, Chi, and Pearson correlation analysis. To separate the means, Tukey's honest significant difference (HSD) test was used. Using the National Center for Biotechnology Information's (NCBI) public databases as a reference, the sequences were compared using the BLAST search software in order to facilitate molecules analysis. The Tree View function of the MEGAX program was utilized to compute and display sequence similarity matrices and a phylogenetic tree (Kumar et al., 2018). The sequences have been added to the NCBI GenBank collection.

3. Results

3.1 Morphological and Biochemical Characteristics of Bacteria From Chlorpyrifos Contaminated Soil

Based on morphological, cultural and biochemical studies, seven distinguishable bacterial isolates were obtained from the soil samples coded MW1-MW7 (Figure 3). All isolates were Gram negative. The isolates vary in size, shape, margin, elevation, surface texture, pigmentation, opacity, and several biochemical properties. They are mostly Gram-negative and demonstrate diverse enzyme activities. Catalase and oxidase tests reveal mixed results with most being catalase positive and oxidase results varying. Methyl red and indole tests show negative results for most, with only MW4 testing negative for indole. Isolates show limited urease activity, and the H_2S production test is negative across all isolates.

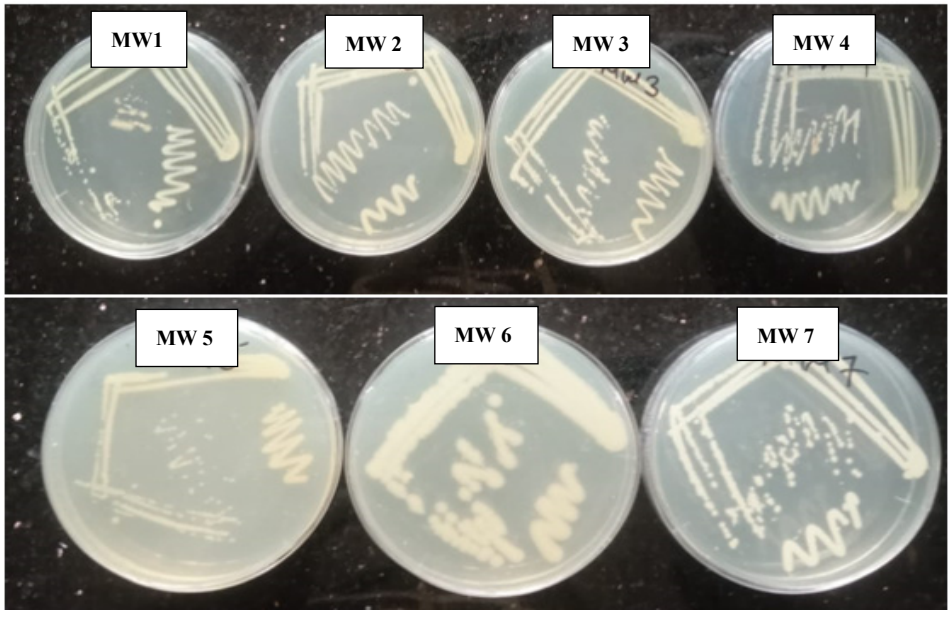


Figure 3. Colony morphology of the isolated bacteria

In the above figure: MW1 is *Alcaligenes faecalis* UWI9, MW2 is *Bacillus weihenstephanensis* FB25M, MW3 is *Bacillus toyonensis* 20SBZ2B, MW4 is *Alcaligenes* sp. SCAU23, MW5 is *Pseudomonas* sp. P_B845W, MW6 is *Pseudomonas japonicum* strain M5-MRL, and MW7 is *Brevundimonas diminuta* CB21.

3.2 Molecular Characterization of Bacteria Isolates

Following NCBI GenBank blasting of the 16S rDNA sequences of the bacteria isolates, all the isolates showed significant similarity of 90-100% in the GenBank with query coverage ≥ 98%. The 16S rDNA gene sequencing showed that the study isolates belong to 7 different bacterial strains. The isolates were identified to belong to the genera *Pseudomonas*, *Alcaligenes*, *Bacillus* and *Brevundimonas* (Table 3).

Alignment and sequence comparison of the 16S rDNA gene study sequences with the obtained known bacterial sequences in the NCBI database showed that the study isolates were closely related to known bacterial lineages. BLAST revealed close similarity of the sequences with; *Alcaligenes faecalis*, *Bacillus weihenstephanensis*, *Bacillus toyonensis*, *Alcaligenes* sp. strain SCAU23, *Pseudomonas* sp. strain PB845W, *Brevundimonas diminuta* and *Pseudomonas japonica* strain M5-MRL.

Table 3. Phylogenetic neighbors of bacteria on the basis of similarity to the 16S rDNA sequence

Bacteria isolate	GenBank accession number	Identity	% similarity	Query coverage
MW1	MZ359822.1	*Alcaligenes faecalis* UWI9	96	100
MW2	MZ359823.1	*Bacillus weihenstephanensis* FB25M	99	100
MW3	MZ359824.1	*Bacillus toyonensis* 20SBZ2B	99	100
MW4	MZ359825.1	*Alcaligenes* sp. SCAU23	96	98
MW5	MZ359826.1	*Pseudomonas* sp. P_B845W	93	99
MW6	MZ359827.1	*Pseudomonas japonica strain* M5-MRL	90	99
MW7	MZ359828.1	*Brevundimonas diminuta* CB21	97	100

Figure 4 shows that strains with comparable sequences were clustered together and likely were close cousins. Maximum Likelihood phylogenetic trees using the isolated isolates' 16S rDNA genes and their closest relatives. Also included were 1000-replication bootstrap values. The tree scaled at 0.05, with branch lengths in the same units as the evolutionary distances used to estimate the phylogenetic tree. The Maximum Likelihood technique based on the Tamura-Nei model inferred evolutionary history. The tree was scaled, with branch lengths measured in substitutions per site. In the investigation, 14 nucleotides were examined. We removed any gaps and missing data. The final dataset has 1557 locations.

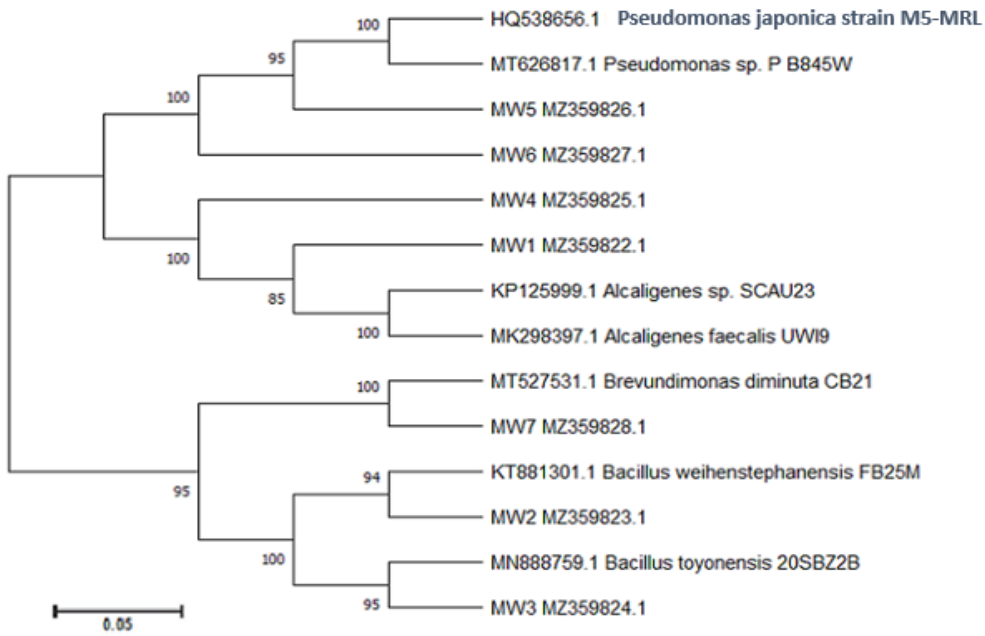


Figure 4. Phylogenetic tree showing relationship of the isolates (given in MW numbers) to closely related bacteria. Reference type strains of corresponding bacteria are involved in the tree

3.3 Chlorpyrifos Degradation

Table 4 presents the CP degradation data with the average concentration values and their respective standard deviations at different time points:

Table 4. CP degradation within the 24-day period

Isolate	0 day	4th day	8th day	12th day	16th day	20th day	24th day
MW1	24.95±0.01	24.7±0.01	22.92±0.26	15.38±0.03	12.35±0.0	10.99±0.0	9.19±0.0
MW2	24.73±0.07	23.15±0.14	20.63±0.07	10.23±0.0	8.65±0.0	6.54±0.0	4.97±0.0
MW3	25.0±0.07	24.91±0.0	24.71±0.08	21.78±0.02	18.95±0.0	15.8±0.0	11.9±0.0
MW4	24.59±0.07	24.51±0.07	23.35±0.0	18.95±0.0	14.13±0.0	11.45±0.0	9.67±0.0
MW5	99.82±0.01	98.42±0.02	94.81±0.0	76.0±0.0	55.2±0.31	34.02±0.08	25.13±0.03
MW6	99.85±0.07	93.58±0.01	91.88±0.07	85.46±0.0	70.23±0.31	54.31±1.72	44.72±0.56
MW7	99.43±0.14	95.72±0.0	83.76±0.0	65.7±0.0	50.7±0.0	38.9±0.33	30.17±0.08
M1	24.93±0.01	23.9±0.07	16.32±0.04	13.13±0.0	8.97±0.0	5.57±0.0	2.18±0.0
M2	25.0±0.0	22.85±0.05	18.01±0.01	13.22±0.0	7.9±0.02	4.16±0.0	1.88±0.13
M3	25.0±0.0	23.02±0.01	18.0±0.0	14.0±0.06	8.18±0.0	3.69±0.03	1.56±0.02
M4	99.01±0.01	97.42±0.07	84.01±0.0	63.57±0.11	44.91±0.0	28.32±0.13	13.77±0.12
M5	100.0±0.0	95.57±0.07	89.01±0.01	65.28±0.0	34.94±0.0	15.82±0.0	4.68±0.0
M6 (Positive)	100.0±0.0	91.08±0.0	77.9±0.0	55.9±0.16	40.0±0.17	14.98±0.08	7.12±0.0
M7 (Negative)	100.0±0.0	99.76±0.28	99.07±0.08	98.9±0.0	98.58±0.18	98.29±0.06	98.0±0.01

Note. MW1 is *Alcaligenes faecalis* UWI9, MW2 is *Bacillus weihenstephanensis* FB25M, MW3 is *Bacillus toyonensis* 20SBZ2B, MW4 is *Alcaligenes* sp. SCAU23, MW5 is *Pseudomonas* sp. P_B845W, MW6 is *Pseudomonas japonicum* strain M5-MRL, and MW7 is *Brevundimonas diminuta* CB21. Consortia M1 includes all seven isolates, M2 includes *Alcaligenes faecalis* UWI9 and *Pseudomonas japonicum* strain M5-MRL, M3 includes *Alcaligenes* sp. SCAU23 and *Brevundimonas diminuta* CB21, M4 includes *Alcaligenes faecalis* UWI9 and *Pseudomonas japonicum* strain M5-MRL, M5 includes *Alcaligenes* sp. SCAU23 and *Brevundimonas diminuta* CB21, M6 is the positive control, and M7 is the negative control.

Differences between isolates or consortia's CP degradation at various time points were determined through paired sample t-tests. Additionally, a post hoc test, Bonferroni test, was used to adjust the significance level and control the probability of the Type I error rate. Therefore, the correction was executed, and this set the significance threshold at 0.008 (0.05/6). The reason is that 6 comparisons were made, namely, Day 0 versus Day 4, Day 4 versus Day 8, Day 8 versus Day 12, Day 12 versus Day 16, Day 16 versus Day 20, and Day 20 versus Day 24. In this case, a p-value equal to or less than 0.008 was considered statistically significant. The results are presented in Table 5.

Table 5. CP degradation paired sample t-test results

		Paired Differences					t	Df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Day0-Day4	2.409	2.598	.694	.908	3.909	3.468	13	.004
Pair 2	Day4-Day8	5.301	4.638	1.240	2.623	7.979	4.276	13	.001
Pair 3	Day8-Day12	10.491	8.261	2.208	5.722	15.261	4.752	13	.000
Pair 4	Day12-Day16	10.272	9.014	2.409	5.067	15.477	4.264	13	.001
Pair 5	Day16-Day20	9.346	8.568	2.290	4.400	14.293	4.082	13	.001
Pair 6	Day20-Day24	5.564	4.444	1.188	2.998	8.130	4.685	13	.000

The results indicated that CP degradation increased significantly from Day 0 to Day 4 ($p = 0.004$), Day 4 to Day 8 ($p = 0.001$), Day 8 to Day 12 ($p = 0.000$), Day 12 to Day 16 ($p = 0.001$), Day 16 to Day 20 ($p = 0.001$), and Day 20 to Day 24 ($p = 0.001$). Therefore, the findings affirm that there is a progressive and statistically significant increases in CP degradation over time.

3.4 Degradation Efficiency

Table 6 shows the analysis of degradation efficiency, including the rate constant and the half-life for each isolate and consortia.

Table 6. Degradation analysis of chlorpyrifos (CP) using various isolates and consortia

Isolate/consortia	Initial CP Concentration (mg/L)	Time (days)	pH	Temp (°C)	Removal Efficiency (Degradation) (%)	Rate Constant (k)	Half-Life (t_{1/2}) (days)
MW1	25.00	24	7	25	63.1	0.1	6.93
MW2	25.00	24	5	30	79.8	0.12	5.78
MW3	25.00	24	5	25	52.3	0.08	8.66
MW4	25.00	24	5	25	60.6	0.09	7.70
MW5	100.00	24	5	25	74.8	0.07	9.90
MW6	100.00	24	7	25	55.7	0.05	13.86
MW7	100.00	24	5	25	69.6	0.06	11.55
M1	25.00	24	5	25	91.3	0.15	4.62
M2	25.00	24	7	25	92.1	0.16	4.33
M3	25.00	24	5	25	92.0	0.17	4.08
M4	100.00	24	7	25	80.1	0.11	6.30
M5	100.00	24	5	25	92.3	0.13	5.33
M6	100.00	24	5	25	92.9	0.14	4.95
M7	100.00	24	5	25	1.99	0.01	69.31

Note. MW1 is *Alcaligenes faecalis* UWI9, MW2 is *Bacillus weihenstephanensis* FB25M, MW3 is *Bacillus toyonensis* 20SBZ2B, MW4 is *Alcaligenes* sp. SCAU23, MW5 is *Pseudomonas* sp. P_B845W, MW6 is *Pseudomonas japonicum* strain M5-MRL, and MW7 is *Brevundimonas diminuta* CB21. Consortia M1 includes all seven isolates, M2 includes *Alcaligenes faecalis* UWI9 and *Pseudomonas japonicum* strain M5-MRL, M3 includes *Alcaligenes* sp. SCAU23 and *Brevundimonas diminuta* CB21, M4 includes *Alcaligenes faecalis* UWI9 and *Pseudomonas japonicum* strain M5-MRL, M5 includes *Alcaligenes* sp. SCAU23 and *Brevundimonas diminuta* CB21, M6 is the positive control, and M7 is the negative control.

The degradation efficiency of Chlorpyrifos (CP) by individual isolates over 24 days varies greatly. MW2 (*Bacillus weihenstephanensis* FB25M) degraded CP the most at 79.8% at pH 5 and 30 °C. MW5 (*Pseudomonas*

sp. P_B845W) degraded 74.8% and MW7 (*Brevundimonas diminuta* CB21) 69.6% at pH 5 and 25 °C. MW1 (*Alcaligenes faecalis* UWI9), MW4 (*Alcaligenes* sp. SCAU23), and MW6 (*Pseudomonas japonicum* strain M5-MRL) degraded moderately at 63.1%, 60.6%, and 55.7%, respectively. At 52.3%, MW3 (*Bacillus toyonensis* 20SBZ2B) degraded the least across isolates (Figure 5). At 96.0%, the positive control (M6) degraded faster than the negative control (M7) at 1.99%. Isolate MW2 (*Bacillus weihenstephanensis* FB25M) was particularly more efficient in degradation as compared to the others.

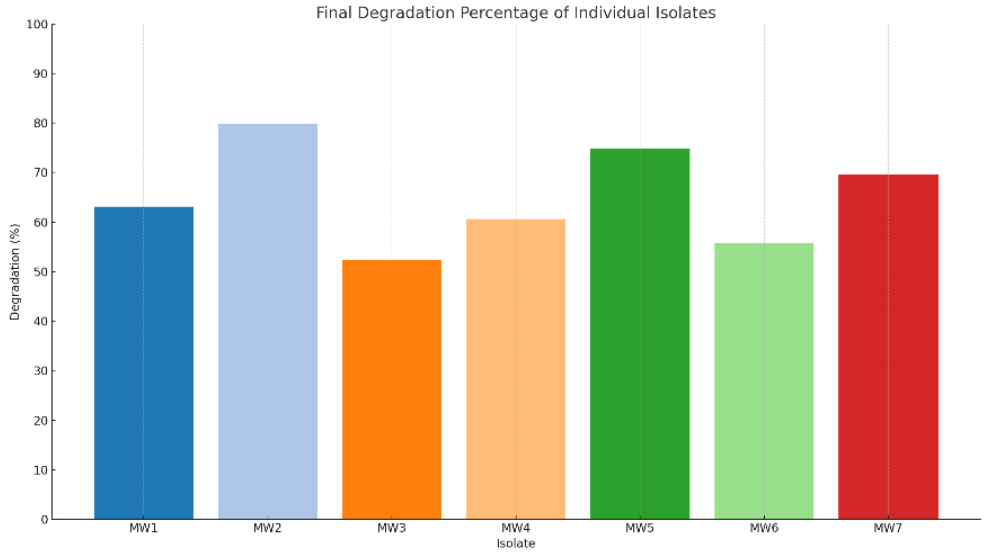


Figure 5. Final degradation percentage of individual isolates

Chlorpyrifos (CP) breakdown by consortia over 24 days is more efficient than by isolates. Under pH 7 and 25°C, consortium M5 (Alcaligenes sp. SCAU23 + Brevundimonas diminuta CB21, higher CP) degraded the most at 92.3%, followed by M2 (Alcaligenes faecalis UWI9 + Pseudomonas japonicum strain M5-MRL) and M3 (Alcaligenes sp. SCAU23 + Brevundimonas diminuta CB21) at 92.1% and 92.0%. M1, containing all seven isolates, degraded 91.3% (Figure 6). M4 (Alcaligenes faecalis UWI9 + Pseudomonas japonicum strain M5-MRL, greater CP) degraded 80.1%. Degradation efficiency was 92.9% for the positive control (M6) and 1.99% for the negative control (M7). Under specific pH and temperature circumstances, consortia outperform individual isolates in CP breakdown, with M5, M2, and M3 showing the most promise.

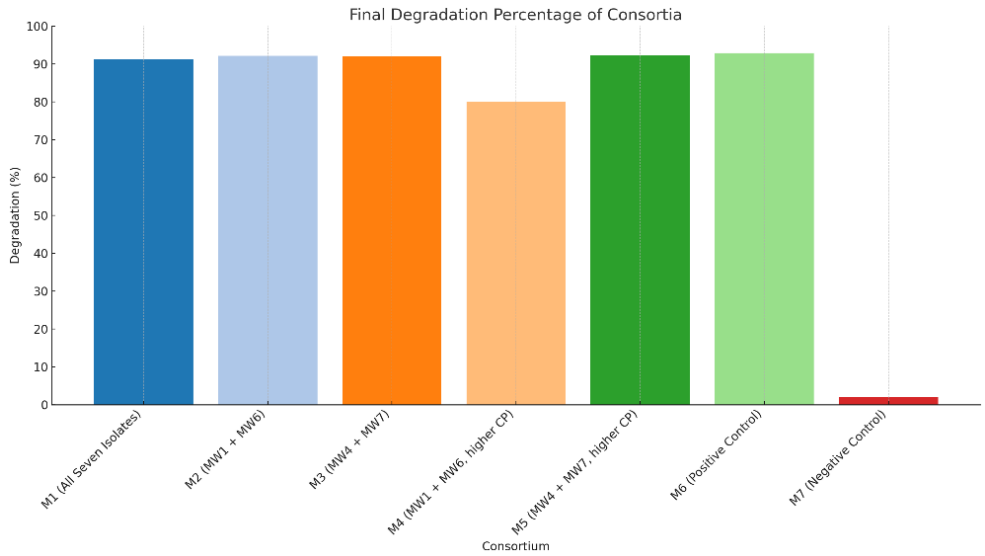


Figure 6. Final degradation percentage of bacterial consortia

Moreover, the half-lives of isolated microorganisms and consortia varied significantly in the study. MW1 (*Alcaligenes faecalis* UWI9) had a half-life of 6.93 days, while MW2 (*Bacillus weihenstephanensis* FB25M) showed a shorter half-life of 5.78 days, indicating a faster degradation rate. MW3 (*Bacillus toyonensis* 20SBZ2B) and MW4 (*Alcaligenes* sp. SCAU23) had half-lives of 8.66 and 7.70 days, respectively. MW5 (*Pseudomonas* sp. P_B845W) and MW6 (*Pseudomonas japonicum* strain M5-MRL), with higher initial concentrations, exhibited half-lives of 9.90 and 13.86 days. MW7 (*Brevundimonas diminuta* CB21) degraded half of its concentration in 11.55 days. Consortia M1, M2, and M3 had similar short half-lives of 4.62, 4.33, and 4.08 days, respectively, showing enhanced degradation. M4 and M5, with higher CP, had half-lives of 6.30 and 5.33 days. The positive control M6 showed a half-life of 4.95 days, while the negative control M7 had an extremely long half-life of 69.31 days. The half-lives of CP are significantly shorter in the consortia compared to the individual isolates ($p<0.01$).

A repeated measures ANOVA was conducted to examine the effect of different time points on the concentration of chlorpyrifos (CP) degradation by various isolates and consortia. The time points included 0-day, 4th day, 8th day, 12th day, 16th day, 20th day, and 24th day. The analysis revealed a significant effect of time on CP concentration ($p < 0.001$). The single isolates MW1, MW2, MW3, MW4, MW5, MW6, and MW7 showed varying degradation rates over time. Notably, MW2 and MW5 demonstrated higher degradation efficiencies compared to the other isolates. By the 24th day, MW2 reduced CP concentration from 24.6840 mg/ml to 4.975 mg/ml, and MW5 reduced CP concentration from 99.8331 mg/ml to 25.11 mg/ml. The consortia M1 (all seven isolates), M2 (MW1 + MW6), M3 (MW4 + MW7), M4 (MW1 + MW6 at higher CP), M5 (MW4 + MW7 at higher CP), M6 (positive control), and M7 (negative control) showed significant degradation capabilities. Consortia M2 and M3 were particularly effective, with M2 reducing CP concentration from 25.0000 mg/ml to 1.78 mg/ml and M3 from 25.0006 mg/ml to 1.5433 mg/ml by the 24th day. Moreover, the analysis showed that the interaction between time and type of isolates/consortia was significant ($p < 0.05$), which suggests that the degradation pattern over time varied among the isolates and consortia.

3.5 TCP Production and Degradation

The study also evaluated the production and degradation of 3,5,6-trichloro-2-pyridinol (TCP) over a 24-day period for both individual bacterial isolates and consortia. The data presented in Table 7 demonstrates the dynamic changes in TCP concentrations, starting with an initial increase, reaching a plateau, and eventually showing significant degradation, especially in consortia treatments. The consortia showed enhanced degradation, with TCP levels reducing to near initial levels by the end of the study period.

Table 7. TCP production and breakdown over the 24-day period

Isolate/Consortia	0 day	4th day	8th day	12th day	16th day	20th day	24th day
MW1	0.00	0.13	0.24	0.25	0.25	0.20	0.15
MW2	0.00	0.18	0.23	0.29	0.33	0.30	0.20
MW3	0.00	0.23	0.35	0.38	0.39	0.35	0.25
MW4	0.00	0.10	0.21	0.24	0.31	0.28	0.20
MW5	0.00	0.10	0.12	0.15	0.18	0.17	0.12
MW6	0.00	0.29	0.43	0.47	0.54	0.50	0.35
MW7	0.00	0.12	0.02	0.54	0.82	0.80	0.70
M1	0.00	0.05	0.18	0.34	0.32	0.22	0.06
M2	0.00	0.11	0.16	0.19	0.26	0.24	0.05
M3	0.00	0.13	0.19	0.24	0.25	0.22	0.06
M4	0.00	0.10	0.16	0.21	0.24	0.20	0.05
M5	0.00	0.05	0.08	0.09	0.25	0.23	0.05
M6	0.00	0.18	0.25	0.29	0.32	0.28	0.07
M7 (Negative)	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Note. MW1 is *Alcaligenes faecalis* UWI9, MW2 is *Bacillus weihenstephanensis* FB25M, MW3 is *Bacillus toyonensis* 20SBZ2B, MW4 is *Alcaligenes* sp. SCAU23, MW5 is *Pseudomonas* sp. P_B845W, MW6 is *Pseudomonas japonicum* strain M5-MRL, and MW7 is *Brevundimonas diminuta* CB21. Consortia M1 includes all seven isolates, M2 includes *Alcaligenes faecalis* UWI9 and *Pseudomonas japonicum* strain M5-MRL, M3 includes *Alcaligenes* sp. SCAU23 and *Brevundimonas diminuta* CB21, M4 includes *Alcaligenes faecalis* UWI9 and *Pseudomonas japonicum* strain M5-MRL, M5 includes *Alcaligenes* sp. SCAU23 and *Brevundimonas diminuta* CB21, M6 is the positive control, and M7 is the negative control.

Differences between isolates or consortia's TCP production and breakdown at various time points were determined through paired sample t-tests. The Bonferroni test was used as the post hoc test. In this case, the significance threshold was set at 0.008 (0.05/6) because a total of 6 comparisons was made. The pairs included Day 0 versus Day 4, Day 4 versus Day 8, Day 8 versus Day 12, Day 12 versus Day 16, Day 16 versus Day 20, and Day 20 versus Day 24. Any p-value that was equal to or less than 0.008 was regarded as statistically significant. Table 8 summarises the results.

Table 8. TCP production and breakdown paired sample T-Test results

		Paired Differences					t	Df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Day0-Day4	-.126	.075	.020	-.170	-.083	-6.299	13	.000
Pair 2	Day4-Day8	-.061	.063	.017	-.097	-.024	-3.596	13	.003
Pair 3	Day8-Day12	-.076	.133	.036	-.153	.001	-2.124	13	.053
Pair 4	Day12-Day16	-.056	.079	.021	-.101	-.010	-2.655	13	.020
Pair 5	Day16-Day20	.034	.023	.006	.020	.047	5.367	13	.000
Pair 6	Day20-Day24	.120	.061	.016	.085	.155	7.343	13	.000

The results indicated that TCP production and breakdown advanced significantly from Day 0 to Day 4 ($p = 0.000$) and Day 4 to Day 8 ($p = 0.003$). However, TCP production and breakdown between Day 8 and Day 12 ($p = 0.053$), and between Day 12 and Day 16 ($p = 0.020$), after Bonferroni correction, did not exhibit any significant changes. Therefore, it is found that although there were periods of significant TCP production and breakdown, the changes were not uniform across all the time points.

Significant trends over the course of the 24-day observation period are highlighted in the study's findings on TCP production and degradation. TCP concentrations initially increased in both consortia and individual bacterial isolates, indicating the start of the manufacturing phase. TCP concentrations in the isolates, including MW1 (*Alcaligenes faecalis* UWI9) and MW2 (*Bacillus weihenstephanensis* FB25M), increased moderately and peaked on the twelfth day before gradually declining. On the other hand, MW6 (*Pseudomonas japonicum* strain M5-MRL) showed a sharper early rise, reaching its peak on day 16, and then markedly declining by day 24. The

pattern of initial buildup and subsequent breakdown points to bacterial metabolic adaptability and the activation of degradation mechanisms (Uniyal et al., 2021).

When compared to individual isolates, consortium treatments—specifically, M1, M2, and M3—exhibited superior TCP degradation. The TCP concentrations in these consortia were initially modest, rose between days 12 and 16, and then rapidly dropped to almost initial levels by day 24. For example, M1 demonstrated a high concentration on day 12 and a considerable decline the following day, suggesting improved collective metabolic capacity for TCP degradation. This illustrates the effectiveness of consortia in bioremediation, likely due to the synergistic relationships between several bacterial species that promote faster and more thorough breakdown of TCP. These findings highlight the potential of bacterial consortia for the efficient bioremediation of polluted settings with chlorpyrifos and its metabolites.

4. Discussion

Nakuru County, Kenya is an agricultural region which is subject to intensive application of agrochemicals, including organophosphates such as chlorpyrifos. Hence, there have been great concerns over environmental consequences and safety. Previous studies have shown that microorganisms can be used for bioremediation of xenobiotics such as CP (Uniyal et al., 2021). This study aimed to isolate chlorpyrifos (CP)-degrading bacteria from dairy farm soils in Nakuru County, Kenya, and develop consortia for enhanced degradation. The results supported the primary hypothesis that specific bacterial strains isolated from these soils can effectively degrade CP. Furthermore, the secondary hypothesis that bacterial consortia would enhance CP degradation efficiency compared to individual strains was also supported.

This study successfully isolated seven bacteria strains capable of degrading chlorpyrifos (CP). They belong to the genera *Pseudomonas*, *Bacillus*, *Alcaligenes*, and *Brevundimonas*. These genera have been well-documented in previous studies for their biodegradation capabilities (Bose et al., 2021). For instance, *Pseudomonas* species have been shown to degrade various organophosphates effectively due to their versatile metabolic pathways and robust enzyme systems (Chen et al., 2012). Similarly, *Bacillus* and *Alcaligenes* have also demonstrated significant potential in breaking down CP and its metabolites (Conde-Avila et al., 2021). This corroborates our findings with other studies that have highlighted their role in bioremediation of pesticide-contaminated environments (Bose et al., 2021). Sun et al. (2020) and Patel and Kumar (2016) found that *Bacillus* and *Pseudomonas* species degrade CP and its major metabolite, 3,5,6-trichloro-2-pyridinol (TCP), which is more persistent and hazardous than CP. *Brevundimonas* and *Alcaligenes* also break down organophosphates, improving bioremediation (Asamba et al., 2022). This suggests that these bacterial strains can be used for bioremediation, especially in CP-contaminated areas. Our findings match those in the literature, demonstrating the reliability and promise of these bacteria in sustainable and effective bioremediation methods (Wu et al., 2013; Fang et al., 2019).

The degradation efficiency of chlorpyrifos (CP) by individual bacterial isolates and consortia under aerobic conditions was a focal point of this study. Among the individual isolates, *Bacillus weihenstephanensis* FB25M (MW2) demonstrated the highest degradation efficiency, reducing CP levels by 79.8% over 24 days. This high degradation rate can be attributed to the robust enzymatic systems of *Bacillus* species, which are known for their ability to degrade various pollutants through diverse metabolic pathways (Fang et al., 2019; Feng et al., 2017; Sun et al., 2020). The moderate degradation rates observed in other isolates, such as MW1 (*Alcaligenes faecalis* UWI9), MW4 (*Alcaligenes* sp. SCAU23), and MW6 (*Pseudomonas japonicum* strain M5-MRL), ranging from 55.7% to 63.1%, highlight the varying capabilities of different bacterial species to degrade CP.

Most importantly, the study found that combinations of different bacterial isolates significantly enhanced CP degradation compared to individual strains. Consortia M1, M2, and M3 showed superior degradation efficiencies, with M1 (comprising all seven isolates) achieving a degradation rate of 91.3%. M2, combining *Alcaligenes faecalis* UWI9 (MW1) and *Pseudomonas japonicum* strain M5-MRL (MW6), and M3, combining *Alcaligenes* sp. SCAU23 (MW4) and *Brevundimonas diminuta* CB21 (MW7), demonstrated even higher efficiencies of 92.1% and 92.0%, respectively. The current study is the first one to demonstrate the effectiveness of these consortia, comprising of the combinations of isolated bacteria, under optimized conditions, as determined previously in our earlier study (Wepukhulu et al., 2024).

Synergistic bacterial interactions boost these consortia's performance. Each bacterial consortium member's metabolic and enzymatic capacities enhance degradation. *Pseudomonas* species may break down several organic contaminants because to their varied metabolic pathways (Patel & Kumar, 2016). The consortium can degrade CP more efficiently and completely than either species alone when paired with *Alcaligenes* species, which also have bioremediation capability. The greater enzymatic activities in consortia also contribute to their improved

performance. Consortia can use many degradation pathways simultaneously, reducing intermediate product accumulation that could slow degradation. M2 and M3 degraded CP faster and more completely due to the combined enzymatic activity of *Alcaligenes faecalis* and *Pseudomonas japonicum* in M2 and *Alcaligenes* sp. and *Brevundimonas diminuta* in M3. These findings suggest bacterial consortia can bioremediate CP-contaminated settings. Using several bacterial species together improves degrading efficiency and provides a more sustainable pesticide pollution remedy (Abraham & Silambarasan, 2016; Bhatt et al., 2021).

The kinetic parameters, including rate constants (k) and half-lives (t{1/2}), provide essential insights into the efficiency of CP degradation by individual isolates and consortia. The study revealed that individual isolates exhibited varying half-lives, with *Bacillus weihenstephanensis* FB25M (MW2) showing the shortest half-life of 5.78 days, indicating a rapid degradation rate. In contrast, *Bacillus toyonensis* 20SBZ2B (MW3) had the longest half-life of 8.66 days among the isolates, reflecting its lower degradation efficiency. Other isolates like MW1 (*Alcaligenes faecalis* UWI9) and MW6 (*Pseudomonas japonicum* strain M5-MRL) had moderate half-lives of 6.93 and 13.86 days, respectively. By contrast, the shorter half-lives of bacterial consortia showed noticeably quicker rates of disintegration. With half-lives of 4.62, 4.33, and 4.08 days, respectively, consortia M1, M2, and M3 demonstrated their improved capacity for degradation as a result of their combined actions and wider range of enzymatic activity. The efficiency of utilizing mixed cultures for bioremediation is highlighted by the notable decrease in CP half-life in consortia as compared to individual isolates. According to these results, consortia are a better option for cleaning up CP-contaminated areas since they guarantee a more thorough breakdown of CP while also accelerating degradation of TCP.

Analysing the hazardous nature of the intermediate metabolites created during CP degradation is essential for the successful application of the microbial bioremediation technique. Toxic metabolite (TCP) is produced during the biodegradation of CP insecticides (Rayu et al. 2017; Verma et al. 2020). TCP has a higher water solubility than the parent chemical and is more persistent in soil (60-360 days) (Rayu et al. 2017; Mali et al., 2022; Asamba et al., 2022). In both individual bacterial isolates and consortia, our study found an early rise in TCP concentrations, signifying the start of CP breakdown. Examples of isolates exhibiting modest TCP production are MW1 (*Alcaligenes faecalis UWI9*) and MW2 (*Bacillus weihenstephanensis FB25M*), which peaked at the twelfth day and then gradually declined. On the other hand, TCP levels in MW6 (*Pseudomonas japonicum strain M5-MRL*) showed a more pronounced early rise, peaking on day sixteen and then sharply declining by day twenty-four. According to Uniyal et al. (2021) this pattern of first accumulation and then breakdown implies that these bacteria have the metabolic pathways needed for both the conversion of CP into TCP and the subsequent breakdown of TCP itself. Previous studies have found bacteria strains that can degrade CP, but only a few degrade TCP (Verma et al., 2020). Hence, this study contributes significantly to the body of knowledge concerning both CP and TCP degradation. Although the current study did not empirically determine the degradation pathway, insights from the findings, together with previous literature (Sasikala et al., 2011; Uniyal et al., 2021), reveal the degradation pathway to be as shown in the Figure 7.

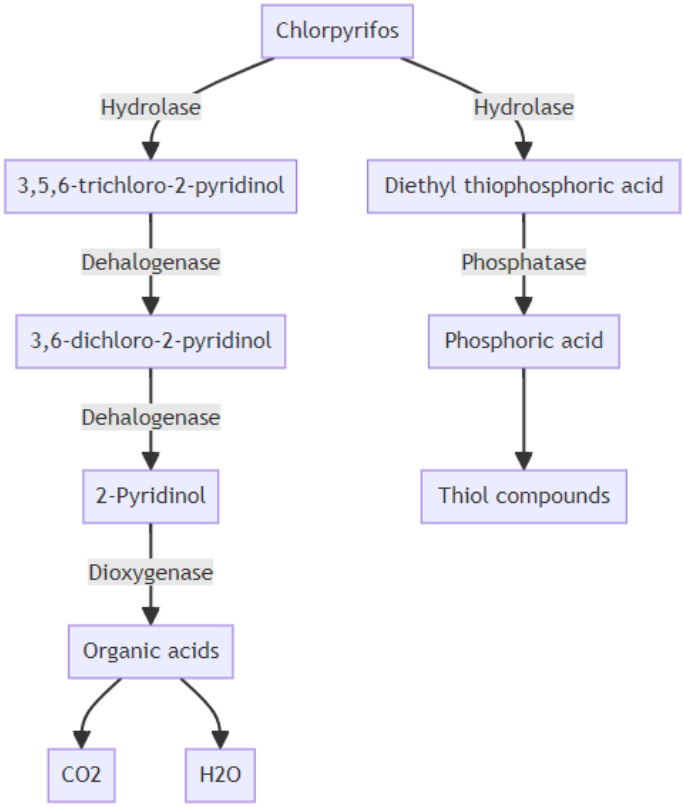


Figure 7. Proposed degradation pathway (Huang et al., 2021)

The improved performance of consortia is evident when comparing the TCP degradation capabilities of individual isolates with the latter. Compared to individual isolates, consortia like M1, M2, and M3 showed more effective and quick reductions of TCP levels. For instance, M1, which consists of all seven isolates, demonstrated a notable drop in TCP levels soon after peaking on day 12, underscoring the consortium's overall metabolic capacity. Significant TCP reduction was also seen by M2 (*Alcaligenes faecalis* UWI9 + *Pseudomonas japonicum* strain M5-MRL) and M3 (*Alcaligenes* sp. SCAU23 + *Brevundimonas diminuta* CB21), which reached nearly initial levels by day 24. Consortia's quick TCP degradation highlights their metabolic adaptability and the synergistic interactions between several bacterial species, which allow for a more thorough and efficient breakdown process. These findings highlight the potential of bacterial consortia in bioremediation. By utilizing their combined enzymatic capabilities, they can break down CP and its metabolites more quickly and thoroughly, improving the overall effectiveness of bioremediation techniques (Bose et al., 2021; Conde-Avila et al., 2021).

Despite promising results, various limitations must be noted. First, controlled laboratory circumstances may not fully imitate natural habitats' complexity and variety. Soil composition, temperature, and field microbial competition may affect bacterial consortia effectiveness. Second, the study focused on a limited number of bacterial strains, which may have excluded other potent CP degraders. Isolation methods may miss some soil CP-degrading bacteria. The study did not objectively determine degradation pathways, instead inferring processes from literature. Third, while instructive, kinetic analysis could benefit from more extensive modeling to better understand deterioration processes. Metagenomics could illuminate CP degradation's genetic and enzymatic pathways.

This study's findings have important theoretical and practical ramifications. The potential of generating local bioremediation solutions is demonstrated by the effective isolation and characterization of CP-degrading bacteria from Kenyan farm soils contaminated with organophosphates. Instead of using chemical remediation techniques, which can be expensive and have unintended environmental effects, bacterial consortia for CP degradation provide a sustainable and eco-friendly solution. The study emphasizes to legislators the significance of funding bioremediation technology research and development. By making investments in these technologies, one can lessen the hazards that pesticide use poses to the environment and human health, resulting in safer agricultural practices and better public health results.

To ensure real-world application, further study should validate these findings with field testing. Field trials would evaluate the bacterial consortia in various environments and uncover implementation difficulties. Exploring the genetic and enzymatic pathways of CP breakdown may help optimize bioremediation techniques. Scaling up bacterial consortia in large-scale bioremediation operations should be studied. This may involve establishing cost-effective bacterial inoculant production technologies and testing their field stability and performance.

In conclusion, this study successfully recovered seven bacterial strains that were able to degrade chlorpyrifos (CP). These isolates showed notable capacities for CP breakdown and were identified as members of the genera *Pseudomonas, Bacillus, Alcaligenes* and *Brevundimonas*. Up to 92% degradation was attained by consortium treatments; consortia demonstrated higher degradation efficiency than individual isolates. The identification of these bacteria was verified by the 16S rDNA gene sequence analysis. The consortia's combined potential for bioremediation was demonstrated by their increased degradation rates and shorter half-lives. Finally, the results highlight the possibility of specialized bacterial consortia for efficient bioremediation of CP-contaminated farm soils. This strategy may provide prospects that greatly enhances environmental health and safety by providing a long-term and economical solution for controlling organophosphate pesticide contamination. The use of mixed bacterial cultures improves the bioremediation processes, guaranteeing a more thorough and effective breakdown of chlorpyrifos and its byproducts.

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Authors Contributions

Ms. Miriam Wepukhulu was responsible for study design, data collection, data analysis, and manuscript writing. Dr. Peter Wachira was responsible for supervision, study design, editing, and proofreading. Dr. Paul Sifuna Oshule was responsible for project funding, supervision, and study design. Dr. Suliman Essuman was responsible for the Microbiology aspects in the project closely monitoring laboratory experiments.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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