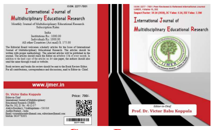
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THE DETOXIFICATION OF CHLORPYRIFOS-CONTAMINATED WASTEWATER USING BACTERIAL CONSORTIUM & RECOVERY OF *VIGNA RADIATA* SEEDLING GROWTH

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Abstract:

Chlorpyrifos (CP) and its persistent metabolite 3,5,6-trichloro-2-pyridinol (TCP) are two significant contaminants in agricultural wastewater and may seriously reduce pulse seed germination, root growth and biochemical stability. A mixed bacterial consortium consisting of Bacillus paramycoides BP-5 and *Pseudomonas sp.* were evaluated for this study. The TCP2 used for detoxification of CP/TCP-contaminated wastewater and evaluated the phytotoxicity by using *Vigna radiata* seedlings as biological indicators. Raw wastewater from Purba Medinipur contained CP at 0.85 ± 0.12 mg/L, TCP at 0.62 ± 0.09 mg/L, and COD of 1,250 mg/L, and caused marked toxicity, reducing germination to 42 ± 5%, root length to 1.8 ± 0.3 cm, plumule length to 0.9 ± 0.2 cm, germination index (GI) to 18.4 ± 2.1, and vigor index (VI) to 108 ± 12. However, the amount of phytotoxicity alleviation in the consortium-treated wastewater was found to be considerable as compared to the other treated wastewaters when the germination rate was restored to 91 ± 4%, root length to 4.2 ± 0.4 cm, plumule length to 3.2 ± 0.3 cm, GI to 85.6 ± 4.2 and VI to 672 ± 35. The dose response analysis revealed raw wastewater IC50 values of 42 ± 3.8% for germination and 38% for root growth while consortium treated wastewater did not exhibit any measurable inhibition up to 100% strength. Biochemical assessment of 14-day seedlings revealed that proline levels increased, malondialdehyde (MDA) concentrations rose, and the activity of antioxidant enzymes was down in raw wastewater, but near to the control level in consortium treated wastewater. The results show that BP5+TCP2 is an effective and sustainable method to remove pesticides from wastewater and allows for the reuse of treated water for irrigation of pulses without any danger.

Keywords: *Chlorpyrifos, TCP, bacterial consortium, wastewater detoxification, Vigna radiata, phytotoxicity, germination index, bioremediation.*

1. Introduction

Organophosphorus pesticides are still one of the most commonly used pesticides in agricultural systems due to their low initial cost and broad spectrum activity, however, the environmental persistence and toxic breakdown products of these chemicals pose significant concerns in relation to water quality and crop safety **[1,2]**. In this context, chlorpyrifos is particularly relevant, due to its common presence in agricultural effluents, irrigation drains, and water bodies that receive effluents from pesticide-intensive agriculture **[3]**. After exposure to the environment, chlorpyrifos breaks down into TCP, which is a more water-soluble and persistent metabolite that can cause effects on the next crop and associated microbiota, if present for long enough in aquatic and soil systems **[4]**.

The phytotoxic action of chlorpyrifos and TCP is well known in legumes and other crop plants. In *Vigna radiata*, exposure to chlorpyrifos has been linked to lower germination rates, root and shoot growth inhibition, disruption in elemental balance, and oxidative stress **[1,5]**. In addition to being a phenolic stressor, TCP induces production of reactive oxygen species, peroxidation of membrane lipids and suppression of antioxidant enzymes. Seedling growth assays are biologically relevant and useful for determining if a wastewater treatment process actually removes contaminants from the wastewater or simply lowers the analytical concentration of the contaminants, and are particularly sensitive to toxicants during the seedling stage **[3,6]**.

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Physicochemical conventional wastewater treatment technologies can help to decrease pesticide pollution, but are expensive, create sludge and incompletely mineralize metabolites **[7]**. Because of its ability to convert the contaminants into less harmful or completely mineralized products under relatively mild conditions, biological treatment is appealing. In monoculture systems, however, the intermediate compounds may get stuck at intermediate steps because one strain might be able to degrade the parent pesticide, while another step, such as the aromatic ring cleavage or dechlorination, is limiting **[3,7]**.In monoculture systems, however, intermediate compounds can get "stuck" at intermediate steps, because one strain may be able to degrade the parent pesticide, while another step such as aromatic ring cleavage or dechlorination is limiting.

As a result, mixed bacterial consortia are becoming more recognized as a more viable alternative to pesticide remediation due to the synergistic interactions among the bacteria, ecological stability, and tolerance to real wastewater conditions **[2,7]**. The present study led to the development of indigenous strains that were isolated from contaminated wastewater systems to develop a consortium named BP5+TCP2 **[7,4]**. *Bacillus paramycoides* BP-5 has been found to have a significant capacity for hydrolysis of chlorpyrifos, while *Pseudomonas sp.* has been found to have a negative capacity. TCP2 improves efficient TCP utilization, which makes it easier to achieve a complete detoxification pathway **[4,3]**. The optimized consortium was able to degrade 98.7 ± 0.9% of total degradation and 52.3 ± 3.1% mineralization under controlled conditions and was able to significantly improve the performance of BP-5 alone, and can be used as a foundation for phytotoxicity testing.

The test species chosen, *Vigna radiata*, is significant as it is a species of agronomic importance and is very sensitive. Mung bean is grown in a rice-fallow system and mixed-cropping system, and its establishment is influenced by its early seedling traits such as fast germination rate and vigorous root growth as well as good oxidative balance. This makes it an appropriate model for determining the feasibility of treated wastewater being used in agriculture. The raw wastewater, sterile wastewater, BP-5 treated wastewater and BP5+TCP2 treated wastewater were evaluated by using germination, seedling growth, dose-response and biochemical endpoints in this study.

The aim of the article was to find out if consortium treatment could transform chlorpyrifos contaminated wastewater from a phytotoxic matrix to a biologically safe irrigation water. This includes analytical removal data and plant-based toxicity endpoints, bridging the gap between microbial degradation performance and agriculture relevance.

2. Materials and Methods

2.1. Plant material and growth conditions

Parental *Vigna radiata* cv. seeds were certified Pusa Vishal were used for all phytotoxicity assays. Seeds were surface sterilised with 0.1% $HgCl_2$ for 1 min and then dipped in 70% ethanol for 30 s and rinsed 5 times with sterile distilled water before use. The 20 seeds per 9 cm Petri dish lined with two Whatman No. 1 filter paper layers and wetted with 5 mL test solution were tested according to OECD-type conditions for seedling bioassays. Plates were kept at 25 ± 2°C in the dark for 48 hours followed by a 16/8 light/dark cycle at 120 µmol m^{-2} s^{-1} photosynthetically active radiation until endpoint measurement was reached **[5]**.

2.2. Wastewater matrices and treatments

Four experimental matrices were used namely, untreated wastewater samples, raw wastewater, sterile wastewater, treated wastewater with consortium, and treated wastewater with BP-5. Optimized conditions described in the previous chapters were implemented in monoculture treatments with BP-5 and in consortium treatments with *B. paramycoides* BP-5 and *Pseudomonas sp.* TCP2 in a 3:1 ratio **[8]**.

2.3. The germination and growth measurements:

The percentage of germination, root length, plumule length, germination index (GI) and vigor index (VI) were measured on Day 7. Phytotoxicity (GI) and seedling performance (VI) were integrated and measured using these two tests. Germination time was followed for 7 days with cumulative germination recorded **[9,5]**.

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GI = S Σ[(germination rate on day *i* x day *i*)/total days]

Vigor Index (VI): VI = [mean length of seedlings (root + plumule, cm) x mean

2.4. Dose-response analysis

A series of wastewater dilutions (0–100% v/v) were made for raw wastewater, BP-5-treated wastewater and consortium-treated wastewater. Germination percentage, root length and GI were modeled with nonlinear sigmoid fits to determine IC50 values and to compare the toxicity threshold of different treatments **[7]**.

2.5. Biochemical analysis

The total chlorophyll, proline, malondialdehyde (MDA), and antioxidant enzymes (peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX)) were evaluated in the day-14 seedlings. The following parameters were used to evaluate oxidative stress, membrane damage and physiological recovery. The biochemical responses of these chemicals compared to those of untreated controls **[9,10]**.

2.6. Statistical analysis

All values are mean (SD). Analysis of variance (ANOVA) was used to test differences among treatments followed by post hoc comparison at $p < 0.05$. The dose-response curves were constructed using nonlinear regression, and where relevant, relationships between residual pesticide concentration and endpoints of phytotoxicity were explored **[11]**.

3. Results and Discussion

3.1. Germination and seedling growth

Table 1. Growth parameters Day 7 (mean ± SD, n= 80 seeds/treatment, 4 replicates).

Treatment	Germination %	Root length (cm)	Plumule length (cm)	GI	VI
UTC	98 ± 2	6.2 ± 0.5	3.8 ± 0.3	93.2	985 ± 45
Raw WW	42 ± 5	1.8 ± 0.3	0.9 ± 0.2	18.4 ± 2.1	108 ± 12
Sterile WW	45 ± 4	2.1 ± 0.4	1.1 ± 0.2	22.1	135 ± 15
BP-5 WW	72 ± 6	3.4 ± 0.4	2.1 ± 0.3	52.3	385 ± 28
BP5+TCP2 WW	91 ± 4	4.2 ± 0.4	3.2 ± 0.3	85.6	672 ± 35

V. radiata seedling establishment was severely inhibited by raw wastewater. Germination dropped to 42 ± 5%, root length to 1.8 ± 0.3 cm, plumule length to 0.9 ± 0.2 cm, GI to 18.4 ± 2.1, and VI to 108 ± 12, compared with 98 ± 2% germination, 6.2 ± 0.5 cm root length, 3.8 ± 0.3 cm plumule length, GI 93.2, and VI 985 ± 45 in the untreated control. The sterile wastewater showed similar behavior like that of the raw wastewater, suggesting that the toxicity was more of the residual chemical contaminants than that of the live wastewater microbiota.

Partial recovery was observed in BP-5-treated wastewater, with a germination rate of 72 ± 6% and an GI of 52.3, indicating that the hydrolysis of chlorpyrifos was not enough to remove all of the phytotoxicity. However, BP5+TCP2-treated wastewater enabled 91 ± 4% germination and 4.2 ± 0.4 cm of root length, 3.2 ± 0.3 cm plumule length, 85.6 ± 4.2 GI and 672 ± 35 VI, which showed a high detoxification and recovery in vigor of the seedlings.

This pattern was further confirmed by the time course germination profile. The untreated control and the consortium trajectory were very similar from Day 3 onwards; raw wastewater and intermediate BP-5 both showed a low level of

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germination. This indicates that consortium treatment not only increased final germination but also also brought back the natural germination and emergence rate of the seeds.

3.2. Dose-response behaviour and IC50:

Table 2. IC50 values for phytotoxicity endpoints (wastewater dilution, mean ±95%)

Endpoint	Raw WW IC50 (% dilution)	BP-5 WW IC50 (% dilution)	BP5+TCP2 WW IC50 (% dilution)
Germination	42 ± 3.8	65 ± 4.5	>100
Root length	38 ± 3.5	58 ± 4.0	>100
Germination index	35 ± 3.0	52 ± 4.2	>100

Dose response analysis indicated that raw wastewater was highly inhibitory even at low dilutions. The IC50 for germination was 42 ± 3.8% wastewater and 38% for root growth, indicating high phytotoxicity of the untreated matrix. The dose response curve for BP-5 treatment was shifted to less toxicity, yet germination inhibition was still observed, and was approximately 65-68% based on the interpretation of IC50 values.

No measurable inhibition was observed even at 100% concentration of the consortium treated wastewater in the assay window. The result is significant as it shows not only a decrease in toxicity, but a turning of the wastewater into a matrix that can be utilized biologically under the tested conditions. The significant difference between BP-5 and BP5+ TCP2 demonstrates that removal of TCP and downstream mineralization play critical roles in having the full alleviation of phytotoxicity.

3.3. Biochemical responses in seedlings

Table 3. Relative biochemical responses of 14-day seedlings

Parameter	UTC (% of control)	Raw WW (% of control)	BP5+TCP2 WW (% of control)
Total chlorophyll	100	38	92
Proline	100	280	120
MDA	100	350	120
POD activity	100	55	95
CAT activity	100	62	98
APX activity	100	48	96

A significant degree of oxidative stress was determined in seedlings exposed to raw wastewater using biochemical profiles taken on day-14. In comparison to untreated control, raw wastewater decreased total chlorophyll (38% of control), increased proline (280% of control) and increased MDA (350% of control) and decreased POD, CAT and APX (55%, 62% and 48% of control, respectively). All these changes are similar to those observed in chlorpyrifos/TCP-induced ROS generation, membrane lipid peroxidation, pigment degradation and disruption of antioxidant homeostasis **[12]**.

The total chlorophyll level was restored to 92% of control in the Consortium treated wastewater while proline and MDA were gradually reduced to the baseline and antioxidant enzymes were recovered to 95-98% of the control. The near normal biochemical profile suggests that the biochemical burden was significantly reduced by BP5+TCP2, thus explaining the better germination and growth rates in the bioassays.

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3.4. Biological interpretation

Table 4. Wastewater treatment summary

Matrix	CP/TCP status	Observed phytotoxicity	Interpretation
Raw WW	High initial residues	Severe germination and biochemical inhibition	Unsafe for irrigation
Sterile WW	Chemical load retained	Similar to raw WW	Toxicity mainly chemical
BP-5 WW	Partial detoxification	Intermediate recovery	Incomplete removal of toxic intermediates
BP5+TCP2 WW	Near-complete detoxification	Near-control recovery	Biologically acceptable under assay conditions

Results confirm a biologically viable mechanism, whereby raw wastewater is toxic to *V. radiata* due to a combination of pesticide and metabolite effects, whereas consortium treatment leads to a decrease in both chemical stress and downstream oxidative stress. The parent chlorpyrifos was likely removed from the water by BP-5, although some persistence of TCP may have continued to cause the phenolic toxicity and oxidative imbalance, which led to the partial recovery from the monoculture treatments. The superior performance of the consortium is in line with the complementary catabolic functions: one strain should be able to hydrolyze chlorpyrifos while the other efficiently consumes TCP and decreases accumulation of phytotoxic intermediates.

The results are found to be comparable with other microbial detoxification studies carried out by using *V. radiata* as a test organism. The results showed that the germination and biochemical balance recovered to near-control levels indicating that the treated wastewater may be used for agriculture in a substantially safer way than the untreated matrix .

4. Discussion

The study showed that raw wastewater contaminated by chlorpyrifos has a high degree of phytotoxicity on V. radiata and treatment with bacterial consortium has been able to significantly overcome the effects of chlorpyrifos. The inhibition pattern is similar to that known to occur in raw wastewater due to chlorpyrifos/TCP induced oxidative stress, pigment loss, and inhibited seedling development. Since the sterile wastewater showed almost an equivalent toxicity, the toxic effect is better attributed to toxic compounds in the wastewater than to living microorganisms in the wastewater **[1,3]**.

This is the difference between BP-5 monoculture and BP5+TCP2 consortium, which is of mechanistic importance. When used alone, BP-5 enhanced seedling survival and growth, suggesting that significant levels of the parent pesticide were removed **[13]**; however, these thresholds for growth and toxicity were not fully recovered, indicating the persistence of intermediates that were potentially toxic, such as TCP. The consortium treated effluent, however, demonstrated good recovery of the germination, plant vigor and biochemical balance similar to a more complete degradation pathway where TCP was also efficiently transformed. The consortium treated effluents, by contrast, showed good recovery of the germination, plant vigor and biochemical balance, similar to a more complete degradation pathway where TCP was also efficiently transformed.

The dose-response results support this conclusion, as the measurable inhibition pattern seen in raw wastewater was not seen in the consortium treated matrix. Biochemical restoration also showed that the treatment was not just masking symptoms but actually decreasing the oxidative injury. The seedlings treated with consortium wastewater exhibited near normal physiological recovery as indicated by the chlorophyll and antioxidant levels, indicating that the detoxification process was complete to achieve real physiological recovery.

These findings encourage the widespread application of mixed bacterial consortia for pesticide wastewater treatment, particularly when the safe application of these treatments is required to be proven by plant assays rather than relying on chemical tests. *V. radiata* offers a practical and agriculture relevant verification component to the treated wastewater reuse **[14]**.

5. Conclusion

The results show that the BP5+TCP2 consortium can detoxify chlorpyrifos polluted wastewater and significantly decrease the phytotoxicity to *Vigna radiata* seedlings. Consortium-treated wastewater restored all the parameters that were inhibited by raw wastewater to near control level while the raw wastewater caused strong inhibition of all the parameters. The mixed culture was able to provide a more complete biological recovery, signifying that TCP removal and mineralization are needed for safe wastewater use. The study indicates that application of indigenous mixed bacterial cultures is a sustainable method of pesticide wastewater remediation and bioassays using *Vigna radiata* can be used to validate the success of detoxification.

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