SCAP
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Remediation of a chlorpyrifos contaminated soil using novel bacterial strains and cyclodextrin: evaluation of its effectiveness by ecotoxicity studies

Recuperación de un suelo contaminado con clorpirifós usando nuevas cepas bacterianas y ciclodextrina: evaluación de su efectividad a través de estudios de ecotoxicidad

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ABSTRACT

Chlorpyrifos (CLP) is one of the most widely used insecticides in the world. However, it is highly toxic for living organisms and persistent in the environment. Biological treatment is considered as a good option to remediate polluted environmental areas. Two bacterial strains, *Bacillus megaterium* CCLP1 and *Bacillus safensis* CCLP2 were isolated from two selected agricultural soils (R and LL), using enrichment technique in presence of CLP as only carbon and energy source. These strains were able to remove in solution up to 99.1 and 98.9% of CLP after 60 d (initial concentration: 10 mg L^{-1}). Several treatments were performed in a soil artificially contaminated with CLP to enhance its remediation: 1. biostimulation, adding micro- and macronutrients; 2. bioaugmentation, inoculating *B. megaterium* CCLP1 or *B. safensis* CCLP2; 3. Addition of randomly methylated β-cyclodextrin (RAMEB), as bioavailability enhancement; 4. bioaugmentation + RAMEB. The best CLP biodegradation results were achieved when bioaugmentation and RAMEB were jointly applied. After biodegradation treatment, an ecotoxicological test was carried out to verify the effectiveness of the bioremediation strategy in soil. Results pointed out that bacteria individually inoculated into the soil were able to decrease the toxicity to undetectable levels.

Keywords: Chlorpyrifos, bioremediation, soil, *Bacillus*, cyclodextrin

RESUMEN

Clorpirifos (CLP) es uno de los insecticidas más usados en el mundo. Sin embargo, es altamente tóxico para los organismos vivos y persistente en el medio ambiente. El tratamiento biológico es considerado como una buena opción para recuperar áreas contaminadas. Dos cepas bacterianas, *Bacillus megaterium* CCLP1 y *Bacillus safensis* CCLP2 fueron aisladas a partir de dos suelos agrícolas a través de cultivos de enriquecimientos en presencia de CLP como única fuente de carbono y energía. Estas cepas fueron capaces de eliminar un 99.1 y 98.9% de CLP en solución tras 60 d de ensayo (concentración inicial: 10 mg L^{-1}). Varios tratamientos fueron aplicados en un suelo artificialmente contaminado con CLPpara su biorrecuperación: 1. bioestímulo, adicionando macro- y micronutrientes; 2. bioaumento, inoculando *B. megaterium* CCLP1 o *B. safensis* CCLP2; 3. Adición de β-ciclodextrina aleatoriamente metilada (RAMEB), como potenciador de la biodisponibilidad; 4. bioaumento + RAMEB. Los mejores resultados de biodegradación de CLP fueron obtenidos cuando bioaumento y RAMEB fueron aplicados conjuntamente. Tras el tratamiento de biodegradación, un ensayo ecotoxicológico fue realizado para verificar la efectividad de la estrategia de biorrecuperación en el suelo. Los resultados mostraron que las bacterias inoculadas individualmente en el suelo fueron capaces de reducir la toxicidad a niveles indetectables.

Palabras claves: Clorpirifos, biorecuperación, suelo, *Bacillus*, ciclodextrina

IINTRODUCTION

Chlorpyrifos (CLP) [O, O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is an organophosphorus insecticide. Currently, its use is banned throughout the European Union, but it is still used in South America and Asian countries, and with some restrictions in China and USA. It has been one of the most widely applied pesticides due to its low--cost and its high efficiency (Bose *et al*., 2021). CLP involves a serious risk for humans, since it is an acetyl cholinesterase (AChE) inhibitor, causing neurotoxic disorders. Nowadays its presence remains in water samples and soils even in those countries where CLP had been banned. Its continued application leads to its accumulation in soils, affecting their properties and productivity (Bose *et al*., 2021).

Microbial biodegradation is one of the best strategies to remove CLP from the environment. Biostimulation and bioaugmentation are used to enhance its bioremediation (Rayu *et al*., 2017). CLP is highly hydrophobic and persistent in soils, and bioavailability is an essential factor in efficiency of pesticide biodegradation in soils. For this reason, cyclodextrins (CDs) have been recognized as a tool for the elimination of pesticides in soils, since they are able to increase their water solubility, enhancing bioavailability to accelerate its biodegradation (Morillo *et al*., 2020).

The objective of this work was to bioremediate a soil contaminated with the insecticide CLP in presence of the bacterial strains *B. megaterium* CCLP1 or *B. safensis* CCLP2. Both strains were isolated from two agricultural soils treated with CLP for years. Several biodegradation treatments were performed: biostimulation, adding macro-and micronutrients solutions (NS), bioaugmentation, inoculating isolated bacterial strains, and addition of a CD as availability enhancer. Lastly, the feasibility of bioremediation strategy was verified through ecotoxicological studies at the beginning and after the CLP decontamination treatments.

MATERIALS AND METHODS

CLP was provided by Sigma-Aldrich (purity > 98%). Randomly methylated β-cyclodextrin was provide by Cyclolab (Budapest, Hungary). *B. megaterium* CCLP1 and *B. safensis* CCLP2 were isolated from agricultural soils by enrichment cultures with CLP. Soil properties of studied soil are show in Table 1.

Table 1 - Some properties of the soil used

Soil	pH	CO_3^{-2} (%)	OM (%)	Sand (%)	Silt (%)	Clay (%)
ALC	5.1	0.5	13.9	69.1	7.8	23.1
R	7.7	4.0	3.4	77.0	9.5	13.5
LL	7.8	4.0	0.9	79.6	9.3	11.1

Biodegradation experiments were carried out in 25 mL sterilized glass vials in triplicates. Each vial contained: 1 g of contaminated soil sample (50 mg kg^{-1} CLP) and the required volume of NS to reach 40% of the soil water holding capacity (73.4 ml per 100 g of soil) for biostimulation. Several biodegradation strategies were designed, where contaminated soil was treated with a solution of RAMEB (an amount corresponding to 10 times that of the CLP molar soil concentration initially added) and/or bioaugmented, inoculating the isolated degrading bacteria (10^8 CFU g^{-1}). Abiotic degradation control tests were also performed by adding 200 mg L^{-1} of $HgCl_2$. All assays were kept at 30°C under agitation at 180 rpm during 100 d. Samples were taken at different incubation periods to monitor the concentration of CLP. 1 g of soil sample was extracted with 5 mL of acetonitrile:water (90:10). The extraction process was: 1) 1 min under vortex mixer, 2) 10 min in an ultrasound bath, 3) 1 h of shaking at 100 rpm and 20 ± 1°C, and 4) 10 min centrifugation at 8000 rpm. Residual CLP was quantified by GC/MS. The separation was achieved with a 30×0.25 mm I.D. DB-5 MS (J&W Scientific, Agilent Technologies) column, which is covered with phenyl methylpolysiloxane 5%. The analytical method used was based on the method described by Ishag *et al*. (2016). Biodegradation curves were adjusted to three kinetic models: First Simple Order Kinetics (SFO), Hockey-Stick Model (HS) kinetics or Multicompartmental First Order Model (FOMC), following the indications of the Focus working group.

Microtox® Test System using *Vibrio fischeri* was employed to measure the toxicity of bioremediated soil. Briefly, 3 mL of NaCl at 2% were added to 2 g

of soil. These suspensions were shaken for 10 min, and centrifuged (2 min, 10000 rpm). Samples were serially diluted (1:2) with NaCl at 2% solution. After 15 min of exposure, the bioluminescence was read to calculate EC_{50} and TU. EC_{50} indicates the pollutant concentration (% v/v) that produce a reduction of 50% in *V. fischeri* luminescence, and the toxic units (TU) were calculated according to the equation $TU = 100/EC_{50}$.

Toxicity was measured at the beginning and at the end of the treatment and was analysed relatively to the control.

RESULTS AND DISCUSSION

CLP biodegradation in soil

Table 2 shows the different treatments conducted to achieve an effective remediation of the soil contaminated with CLP.

Table 2 - Treatments performed in this study

Treatments	
T1	Soil (non-inoculated) + $HgCl_2$
T2	Soil (non-inoculated) + NS
T3	Soil + NS + *B. megaterium* CCLP1
T4	Soil + NS + *B. safensis* CCLP2
T5	Soil + NS + RAMEB
T6	Soil + NS + RAMEB + *B. megaterium* CCLP1
T7	Soil + NS + RAMEB + *B. safensis* CCLP2

ALC soil from a Natural Park, was used to carry out biodegradation test. No significant biodegradation was observed in the non-inoculated soil in presence of its endogenous soil microbiota (0.3% after 100 d, Figure 1, T1). However, when NS was added to stimulate soil microbiota, a slight increase in the biodegradation percentage was observed (15.7%, T2), but DT_{50} value (required time for the pollutant concentration to decline to half of its initial value) was almost 2 years (Table 3). It indicates the high persistence of CLP in this soil. Based on these results, the application of bioremediation techniques such as bioaugmentation and the addition of RAMEB to improve the soil CLP biodegradation rate were considered.

B. megaterium CCLP1 and *B. safensis* CCLP2 were inoculated individually. After 100 d of inoculation, 60.6% and 64.8% of extent of degradation was reached, respectively (Figure 1, T3, T4). In addition, DT_{50} was significantly reduced from 696.6 d (only with biostimulation) to 44.6 and 47.1 d (Table 3) in the case of inoculation with B. *megaterium* CCLP1 and *B. safensis* CCLP2, respectively.

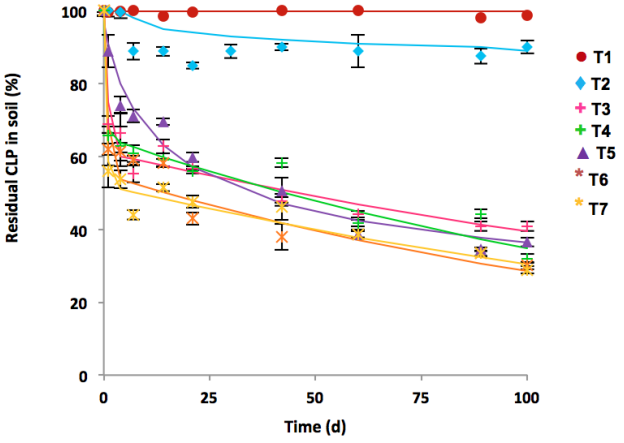

Figure 1 - CLP biodegradation curves in ALC soil after the application of different treatments.

Table 3 - Calculated kinetic parameters for CLP biodegradation in soil

Treatment	Kinetic model	DT_{50}	Extent of biodegradation (%)
T1	SFO	∞	0.3
T2	SFO	696.6	15.7
T3	HS	44.6	60.6
T4	HS	47.1	64.6
T5	FOMC	38.9	63.6
T6	HS	14	71.5
T7	HS	7.9	69.6

RAMEB was added to ALC soil (Figure 1, T5), achieving a 63.6% of biodegradation, and a decreasing in DT_{50} (38.9 d), regarding NS treatment. RAMEB would be provoking an increase of the CLP fraction in the soil solution, which involves an improvement in its extent and rate of biodegradation (Köse *et al*., 2022). CCLP1 or CCLP2 strains, jointly applied with RAMEB, were the most effective treatments (Figure 1, T6, T7). DT_{50} was reduced to 14 and 7.9 d, respectively (Table 3).

Toxicity analysis in soil samples

The toxicity of the CLP and their potential formed metabolites remaining in soil were assessed at the end of the bioremediation process. Results were compared according to classification proposed by Persoone *et al*. (2003) (Table 4). Non-inoculated contaminated soil (T1) showed a value of TU 5.6 (1 < TU < 10, acute toxicity). However, in presence of *B. megaterium* CCLP1, the toxicity was undetectable after 100 d. When *B. safensis* CCLP2 was inoculated, the level of toxicity decreased from acute toxicity to non-toxic (TU 0,004, TU < 0.4, non-toxic). These results confirmed its ability to degrade CLP to non-toxic substances. In addition, the effectiveness of bioaugmentation and RAMEB addition (T6 and T7 experiments) was demonstrated since no toxicity was detected in any case (Table 4). It is worth noting that, as far as we know, no study of CLP ecotoxicity in soil has been published previously.

Table 4 - Acute toxicity test towards *V. fischeri* before and after 100 d of incubation

Treatment	EC_{50} (%)	TU	Toxicity
T1	17,8	5,6	Acute
T3	-	-	Non toxic
T4	25363	0,004	Non toxic
T6	-	-	Non toxic
T7	-	-	Non toxic

CONCLUSIONS

B. megaterium CCLP1 and *B. safensis* CCLP2, isolated in our lab from two agricultural soils by enrichment cultures, in presence of CLP as the only carbon source, proved to be able to degrade CLP in soil. RAMEB was used as a bioavailability enhancer of CLP in soil due to its ability to form an inclusion complex. However, the most effective treatment was the joint application of bacterial strains and cyclodextrin. The feasibility of bioremediation strategy was checked using ecotoxicological studies, showing a decline in toxicity parameters in all cases where the selected bacterial strains were used. A complete elimination in the toxicity was achieved, when bioaugmentation and CD treatments were applied, reducing also the time required to remediate the soil to less than 14 d. It is concluded that, biodegradation should be considered as a useful process to degrade CLP, but the soil particular conditions must be adjusted to achieve the most suitable for bioremediation.

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