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Bioremediation of a trifluralin contaminated soil using bioaugmentation with novel isolated bacterial strains and cyclodextrin

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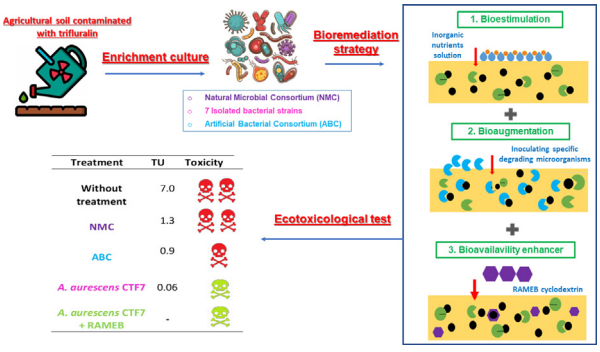
HIGHLIGHTS

- Bioaugmentation and cyclodextrin (CD) to bioremediate trifluralin (TFL) contaminated soils.
- *aurescens CTFL7* was described for the first time as an effective TFL degrader.
- CDs newly shown as bioavailability enhancers for TFL.
- *aurescens CTFL7* and CD, applied together increased soil TFL biodegradation.
- Toxicity was eliminated from soil after bioaugmentation and CD application.

GRAPHICAL ABSTRACT



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ABSTRACT

Trifluralin (TFL) is a highly persistent with a strong adsorption capacity on soil particles herbicide. This study was to isolate microbial consortia and bacterial strains from a soil with a historical application of pesticides to evaluate their potential to degrade TFL in soil. Different bioremediation techniques were considered for increasing the effectiveness of TFL degradation in soil. These techniques consisted of: i) biostimulation, using a nutrients solution (NS); ii) bioaugmentation, using a natural microbial consortium (NMC), seven individual bacterial strains isolated from NMC, and an artificial bacterial consortium formed by the seven TFL-degrading bacterial strains (ABC); iii) bioavailability enhancement, using a biodegradable compound, a randomly methylated cyclodextrin, RAMEB.

Biostimulation using NS leads up to 34 % of soil TFL biodegraded after 100 d. When the contaminated soil was inoculated with NMC or ABC consortia, TFL loss increased up to 62 % and 74 %, respectively, with DT_{50} values (required time for the pollutant concentration to decline to half of its initial value) of 5.9 and 11 d. In the case of soil inoculation with the isolated individual bacterial strains, the extent of TFL biodegradation ranged widely from 2.3 % to 55 %. The most efficient bacterial strain was *Arthrobacter aurescens* CTFL7 which had not been previously described in the literature as a TFL-degrading bacterium. Bioaugmentation with CTFL7 bacterium was also tested in the presence of RAMEB, provoking a drastic increase in herbicide biodegradation up to 88 %, achieving a DT_{50} of only 19 d. Cyclodextrins had never been tested before for enhancement of TFL biodegradation.

An ecotoxicity assay was performed to confirm that the proposed bioremediation techniques were also capable to reduce toxicity. A Microtox® test showed that after application *A. aurescens* CTF7 and *A. aurescens* CTF7 + RAMEB, the TFL-contaminated soil, which initially presented acute toxicity, became non-toxic at the end of the biodegradation experiments.

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1. Introduction

Trifluralin (TFL) is an herbicide that belongs to the dinitroaniline group, which has an aniline structure as a base, containing two NO_2 groups bonded to C 2 and 6 of the benzene ring and a trifluoromethyl group at position 4. TFL is a selective and pre-emergent herbicide widely applied worldwide since 1964 in crops such as carrot, lettuce, strawberry and sugarcane and acts by interrupting the mitosis process and inhibiting root development (Li et al., 2021). Due to the two nitro groups and the trifluoromethyl group, not frequently found in natural molecules, TFL is a poor substrate for naturally occurring microbial populations. Moreover, this compound is considered highly toxic to the environment and is included in the list of priority contaminants drawn up by the European Commission (Directive 2008/105/EC), with its use as herbicide forbidden in the European Union since 2008, but not in other countries (Coleman et al., 2020). According to the IUPAC Pesticides Properties DataBase (PPDB, 2018) TFL shows a low water solubility (0.221 mg L^{-1}) and a K_{ow} value of 5.27, which means that TFL has a marked hydrophobic character and indicates a high affinity for adsorption onto soil particles, decreasing the pollutant's mobility in soil and increasing the residual time (Epp et al., 2018), so TFL is classified as a very persistent pollutant. Malterre et al. (1998) observed that only 1 % of TFL was detected to leach out of the topsoil. Erguven et al. (2016) confirmed that at least 10 % of applied TFL remains in soils one year after application. In this sense, Johnston and Camper (1991) studied the removal of several pesticides (diuron, TFL and carbofuran) in contaminated soil, observing that only TFL was detected after many years. Although TFL is highly sensitive to ultraviolet radiation, suffering easily the photodegradation processes (Erguven et al., 2016), its average half-life in soils is increased due to its ability to be adsorbed strongly to soil particles (Li et al., 2021). According to Van Zwieten et al. (2016) TFL became the second herbicide most frequently detected in Australia, after glyphosate, in cereal crop soils, and Karasali et al. (2017) observed detectable concentrations of TFL in European soils 4 years after its ban.

Some studies using soil microcosms or at a field scale (Tiryaki et al., 2004; Querejeta et al., 2014) have revealed the loss of TFL in soils, but without defining if the loss process was due to abiotic or biotic degradation, immobilisation or volatilisation. However, studies carried out using ^{14}C-labeled TFL (Farenhorst, 2007) demonstrated that only a small percentage of TFL (< 3.2 %) was mineralised to $^{14}CO_2$.

In recent times, bioremediation methodologies, using microorganisms to degrade organic environmental contaminants into less toxic forms or compounds, have gained considerable attention, and they have proven to be an efficient tool to decontaminate pesticide-polluted sites (Morillo and Villaverde, 2017). To get microorganisms adapted to organic contaminants, enrichment cultures in presence of a high concentration of pollutants are used to isolate degrading microorganisms (Wu et al., 2021; Lara-Moreno et al., 2021). These isolated microorganisms, as pure culture or in the form of a microbial consortium, are inoculated into the soil in high concentrations (bioaugmentation) to remediate the contaminated environment (Villaverde et al., 2019). The application of microbial consortia may improve the efficiency of the bioremediation process in soils contaminated by pesticides (Morillo and Villaverde, 2017).

Several authors have improved the bioremediation using bioaugmentation in soils contaminated by different pesticides, such as phenylurea herbicides (Villaverde et al., 2012, 2018; Liu et al., 2019; Lara-Moreno et al., 2022, 2022b), organochlorine pesticides (Raimondo et al., 2020), MCPA (Dandan et al., 2022), or carbamates (kaur and Balomajumder, 2019; Mishra et al., 2021).

However, in the case of TFL, there are only a few studies that have confirmed its biodegradation in aqueous solution using TFL-specific degradation microorganisms isolated from agricultural soils via enrichment culture. Bellinaso et al. (2003) isolated *Klebsiella* sp., *Herbaspirillum* sp., and *Bacillus* sp. as well as an unidentified isolate that removed TFL in liquid medium. In the study performed by Erguven et al. (2016) microbial biodegradation of TFL was performed in a liquid media in presence of 11 different types of identified fungi and bacteria strains and their mixtures. The bacteria and fungi consortia achieved 69 % and 66 % TFL degradation, respectively. In the case of fungi, the best result was achieved by *Chlamydosporia* (80 %); in the bacteria studies, the best removal was achieved by *Bacillus simplex* (about 95 %). Ni et al. (2016, 2019) described two nitroreductases from the soil isolate *Bacillus subtilis* Y3 that converted in a cometabolic reaction the nitro-groups of TFL to amino groups. In addition, the oxidoreductase enzyme laccase immobilized on activated nanocellulose fibres showed 100 % degradation of trifluralin in 24 h in the presence of guaiacol as a mediator (Bansal et al., 2018).

However, as far as we know, studies on TFL biodegradation in soils by microbial consortia or isolated species are extremely scarce, despite its high persistence and toxicity. Fragoeiro and Magan (2008) studied the effect of fungal inoculants (*Trametes versicolor* and *Phanerochaete chrysosporium*) on the degradation of a mixture of TFL, simazine and dieldrin in soil microcosms. In the case of TFL, *T. versicolor* and *P. chrysosporium* improved the biodegradation regarding the effect of microbiota up to 17 % and 30 %, respectively.

The bioavailability of hydrophobic pollutants such as TFL adsorbed in soils is a problem for its degradation. For this reason, Mata-Sandoval et al. (2001) added a surfactant (Triton X-100) and a biosurfactant (Rhamnolipids) to enhance TFL biodegradation by a *Streptomyces* strain. TFL biodegradation was improved in some soil slurries but decreased when using other soils. Cyclodextrins (CDs) are proposed as a non-toxic and biodegradable alternative to organic solvents and surfactants for the removal of organic pollutants from contaminated matrices (Sánchez-Trujillo et al., 2013; Madrid et al., 2019, 2020). The ability of CDs to form inclusion complexes with a wide variety of hydrophobic guest molecules has been used in agriculture (Yáñez et al., 2012; Morillo et al., 2012; Villaverde et al., 2018; Godeau et al., 2021; Köse et al., 2022). Recently, CDs have been used in environmental applications to improve the remediation efficiency of contaminated soil, because CDs can increase the apparent water solubility of low-polarity organic compounds, which increases their availability for being biodegraded (Flaherty et al., 2013; Morillo et al., 2020).

Quantifying contaminant levels using analytical methods is relevant after the application of bioremediation treatments. However, a decrease in the pollutant concentration is not enough to confirm a reduction in toxicity, since toxic metabolites from the biodegradation process could be present (Jiang et al., 2016; Lara-Moreno et al., 2022b). For this reason, is interesting to assess toxicity after bioremediation treatment.

In this study, a natural microbial consortium (NMC) isolated from an agricultural soil treated for decades with a variety of pesticides, seven individual bacterial strains isolated from NMC and an artificial bacterial consortium (ABC) formed by these seven isolated bacterial strains were used as degrading microorganism for the same soil PLD artificially contaminated with TFL. Biodegradation strategies were based on biostimulation (employing nutrients solution (NS)), bioaugmentation (adding degrading microorganisms) and the use of cyclodextrins able to enhance significantly the water solubility of TFL and, therefore, its bioavailability. It is worth emphasising that the use of *A. aurescens* and CDs to bioremediate a TFL contaminated soil has never been described in the literature. Moreover, an ecotoxicological study was also conducted before and after TFL bioremediation process to check the feasibility of the treatment and the soil quality, which can be considered a novelty of the present research.

2. Materials and methods

2.1. Materials

Technical grade (>98 %) solid TFL ((2,6-dinitro-*N*,*N*-dipropyl-4-trifluoromethyl)aniline) was provided by Sigma Aldrich (Madrid, Spain). β-CD (BCD), hydroxypropyl-β-CD (HPBCD), randomly methylated-β-CD (RAMEB), (purity 97 %) were supplied by Cyclolab (Budapest, Hungary).

An agricultural soil was collected from Los Palacios (Seville, Spain) (PLD soil), that had been treated with organohalogenated herbicides for decades. TFL had not been applied in this soil. The physicochemical properties of the soil were organic matter, 1.67 %; $CO_3^{=}$, 9.7 %; pH, 8.24; sand, 47 %; silt, 18.5 %; clay, 34.5 % (clay loam).

2.2. Methods

2.2.1. Trifluralin solubility in the aqueous phase in the presence of different cyclodextrins

Solubility studies were performed according to the method reported by Higuchi and Connors (1965). An excess of TFL (5 mg) was added to aqueous solutions (20 mL) that contained various concentrations of CDs (0–0.012 M for BCD and 0–0.1 M for HPBCD and RAMEB). The flasks were shaken at 25 °C for 1 week. The suspensions were subsequently filtered through a 0.22 μm Millipore glass-fibre membrane. The concentration of TFL in the supernatant was analysed at different time points. The apparent stability constants of the different TFL − CD complexes (K_c) were determined from the straight line obtained in the phase solubility diagram according to the equation proposed by Higuchi and Connors (1965).

$$Kc = \frac{slope}{S_0(1 - slope)}.$$

where S_0 is the TFL equilibrium concentration in an aqueous solution in the absence of the CD, and the slope is the slope of the phase solubility diagram. Another parameter that can be obtained from the data of the solubility study is the solubilisation efficiency (S_e), which is defined as the increment of TFL apparent solubility at the highest CD concentration studied regarding its water solubility.

The concentration of TFL was analysed using GC/MS (GC; Agilent GC 6890 N) connected to a mass spectrometer (MS; Agilent MD 5975B) according to California Department of Food and Agriculture (2009). The separation was achieved with a 30 × 0.25 mm I.D. DB-5 MS (J&W Scientific, Agilent Technologies) column, which was covered with phenyl methylpolysiloxane 5 %. The oven temperature ramp was from 80 °C (the temperature at which it was maintained for 1 min) to 180 °C, increasing 15 °C per minute and remaining at this temperature for 3 min. It then increased to 300 °C at a rate of 15 °C per minute, maintaining this final temperature for 3 min. The sample injection was carried out in splitless mode. The carrier gas was helium, with a flow of 1 mL min^{-1}. Injector, transfer line and ion source temperatures were 250, 280 and 200 °C, respectively. To increase the sensitivity and specificity of the method, the quantification was carried out in SIM mode (Selected Ion Monitoring), using different ions *m/z* 264, 290, 306, selecting the ion 306 to quantify the TFL concentration. Calibration curves were linear ($R^2 > 0.996$) from 10 to 500 μg L^{-1}. The limit of detection (LOD, 1.12 μg kg^{-1}) and the limit of quantification (LOQ 3.73 μg kg^{-1}) were calculated as three and ten times the signal-to-noise ratio, respectively.

2.2.2. Enrichment and isolation of TFL-degrading consortium and TFL degrader strains

A TFL-degrading microbial consortium was isolated from PLD soil adding TFL as the only source of carbon and energy. Enrichment was performed in 250 mL glass flasks (autoclave Auster-G, P-Selecta with one cycle at 120 °C, inlet pressure of 103 kPa, for 20 min) in which 10 g of fresh soil was added to 80 mL of a Mineral Salt Medium (MSM, g L^{-1}): 4.0 Na_2HPO_4; 2.0 KH_2PO_4; 0.8 $MgSO_4$; 0.8 NH_4SO_4, containing a TFL final concentration of 0.5 g L^{-1} (Lara-Moreno et al., 2021), together with a solution of micronutrients (MNS, mg L^{-1}): 75.0 $MnCl_2$ $4H_2O$; 37.5 $FeSO_4$ $7H_2O$; 25.0 $SnCl_2$ $2H_2O$; 12.5 $ZnSO_4$ $7H_2O$; 12.5 $Al_2(SO_4)_3$ $18H_2O$; 12.5 $NiCL_2$ $6H_2O$; 12.5 $CoCl_2$ $2H_2O$; 10.0 $CaSO_4$ $2H_2O$; 3.75 KBr; 3.75 KCl; 2.50 LiCl (Fenlon et al., 2011). The mix of MSM + MNS (50:1) is named NS. The culture was incubated with orbital shaking (150 rpm) at 30 °C, and every 7 d, 10 mL of the culture was transferred to a new flask containing the same sterile MSM and incubated again. This process was repeated 4 times and the natural TFL-microbial consortium (NMC) was obtained.

Individual bacterial strains were isolated directly from NMC. The consortium was inoculated on MSM agar plates supplemented with TFL (0.5 g L^{-1}) as the only added source of carbon and energy for 7 d at 30 °C. Successive isolations were performed recognising and selecting different colonies according to their size, colour, edge and elevation. Selected isolated strains were streaking in LB plates and incubated at 30 °C in aerobic conditions. Once purified they were stored in cryovials (Microbank™ 2D) at −80 °C.

2.2.3. Isolated strain identification with 16S rDNA amplification

DNA was extracted from a liquid culture of each strain using the G-spinTM total DNA Extraction Kit (iNtRON Biotechnology), then the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using a high-fidelity polymerase (VELOCITY DNA polymerase-Bioline) with universal oligonucleotides primers: 16F27 (annealing at position 8–27 *E. coli* numbering) and 16R1488 (Lane, 1991). Finally, the PCR product was purified using PCR clean-up Gel Extraction kit NucleoSpin® Gel and PCR clean-up (Macherey-Nagel) to be sequenced. Direct sequencing of the PCR products was performed by the Sanger method and the results were obtained by Sequencing Genetic Analysis Software, provided by Stab Vida company (Caparica, Portugal). The reverse strand sequence obtained was treated with the Reverse Complement DNA tool, using the BioEdit application, to have accurate results since reverse and complementary sequences were aligned with their corresponding forward strand, which allowed the overlap of those two sequences. This achieved one more complete sequence ready to be compared with gene sequences in Genbank database by submitting the sequences one by one to BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), then the closest available database was determined. The combination of highest identity, total score and query cover values were used to attribute the suggested species.

2.2.4. Inoculum preparation

Before each experiment, *microbial consortium NMC, the seven strains isolated from NMC (CTFL1, CTFL2, CTFL3, CTFL4, CTFL5, CTFL6 and CTFL7)* and the consortium ABC composed of the sum of the seven individual degrader bacteria *were inoculated in* Luria−Bertani (LB) medium. The *cultures were incubated with orbital shaking (130 rpm) at 30* °C. Microorganisms were harvested and centrifuged at the end of the exponential phase and subsequently washed twice using MSM, to remove any possible LB residues, before initiation of the experiments. In the case of the isolated strains, the final density of the inoculum added was 10^8 CFU mL^{-1}. The NMC inoculum was prepared to achieve a $OD_{600} = 1$. ABC inoculum was a mixture of the isolated strains formed by the same volume of each one with $OD_{600} = 0.15$, in order to reach a final $OD_{600} = 1$, and a final density about 10^8 CFU mL^{-1}.

2.2.5. Biodegradation experiments

TFL photodegradation in solution was carried out in triplicate using 25 mL sterilized glass vials during 60 d of assay. Vials were contaminated with 10 mg L^{-1} of TFL and completed to 15 mL with MSM. Flasks were kept shaking at room temperature (30 ± 1 °C). Samples were taken at the initial time and at different incubation times (0, 1, 3, 7, 14, 21, 30 and 60 d).

TFL biodegradation experiments in the soil were performed in triplicates in 250 mL autoclaved amber colour sterilized glass bottles containing 25 g of soil (25 % of the total soil used), and 10 mL of a 250 mg L^{-1} TFL stock solution in acetone was added. The solvent was allowed to evaporate for 24 h, and the remaining uncontaminated 75 g of soil was added and mixed to avoid damage to indigenous soil microbiota, obtaining a final concentration of 50 mg kg^{-1} TFL.

After that, 10 mL (40 % of the soil water holding capacity) of NS was added (Villaverde et al., 2018). Different soil biodegradation treatments were designed: (*i*) Contaminated soil + NS, used as biostimulation control; (*ii*) Contaminated soil + NS + RAMEB (RAMEB solution with an amount corresponding to 10 times the concentration of TFL (9.8×10^{-2} mol RAMEB); (*iii*) contaminated soil + NS + NMC; (*iv*) contaminated soil + NS + ABC; (*v*) contaminated soil + NS + each individual bacterial strain; (vi) contaminated soil + NS + RAMEB + CTFL7 (the most effective TFL degrader, *Arthrobacter aurescens* CTFL7). In the case of individual bacterial strains and ABC, 0.62 mL of bacterial culture (10^8 CFU ml^{-1}) was added to 100 g of soil, and a final bacterial concentration 6.2×10^5 CFU g^{-1} was reached. For NMC, a bacterial suspension equivalent to $OD_{600} = 1$ was

inoculated. In parallel, abiotic TFL degradation controls were performed by adding 200 mg L^{-1} of $HgCl_2$.

Residual TFL in soil was analysed after the different sampling times (6, 14, 26, 42 and 100 d): 1 g of contaminated soil was extracted using 10 mL of hexane as an organic solvent. The mixture was homogenised, mechanically shaken for 20 min plus 1 min of vortex agitation and sonicated in a water bath for 10 min. Both steps (sonication and shaking) were repeated twice and then centrifuged at 11000 rpm for 10 min. Recovery experiments of soil samples spiked with TFL were carried out to assure extraction method quality. A recovery of 93.6 % (Std. Dev. 7.6) of the original contamination was obtained. The concentration of the supernatant was analysed using GC/MS (method shown above).

2.2.6. Biodegradation kinetic models

The curves were fitted to three kinetic models: a simple first-order model (SFO), a biphasic first-order sequential model (Hockey-Stick, HS) and a first-order multi-compartment model (FOMC). These models were selected based on their relative simplicity and their potential to best fit the measured loss kinetic datasets for TFL herbicides that appear to be monophasic or biphasic. The selection of the best kinetic model was carried out according to the FOCUS (2006) workgroup. To facilitate kinetic analysis of the degradation of the parent compound using rate curves, the Solver tool (included in Microsoft Excel statistical software) and the following equations were used depending on the kinetic model that fits the best to each experimental set of data:

$$[C]_t = [C]_0 e^{-kt} \text{ (SFO)}$$

$$DT_{50} = \ln 2/K \text{ (SFO)}$$

$$[C]_t = [C]_0 e^{-kt\,tb} e^{-k2(t-tb)} \text{ (HS)}$$

$$DT_{50} = \ln 2/k_1, \text{if } DT_{50} \leq tb \text{ (HS)}$$

$$DT_{50} = (\ln 2 - k_1 tb)/k_2, \text{if } DT_{50} \geq tb \text{ (HS)}$$

$$M = M_0/((t/\beta) + 1)^{\alpha} \text{ (FOMC)}$$

$$DT_x = \beta\left[(100/100 - x)^{(1/\alpha)} - 1\right] \text{ (FOMC)}$$

$$DT_{50} = \beta\left(2^{(1/\alpha)} - 1\right) \text{ (FOMC)}$$

where $[C]_t$ and $[C]_0$ are the concentrations of biodegraded TFL at time t and just after spiking the soil (initial), respectively (mg kg^{-1}), and k is the rate constant of biodegradation (d^{-1}). In the HS model, k_1 and k_2 are the rate constants of biodegradation for the fast and the slow fractions, respectively, and tb is the time at which the rate constant changes. In the FOMC model α is a shape parameter determined by the coefficient of variation of k values, and β is a location parameter. The Chi-square (χ^2) was used to estimate the appropriateness of the models and to assess the accuracy of each resulting fit. This test considers the deviations between observed and estimated values (numerator) for each model relative to the uncertainty of the measurements (denominator). The chi-square (χ^2) test was calculated as an indicator of the goodness of fit (χ^2 values should be <15 to mean a good fit).

2.2.7. Toxicity analysis

The Microtox® Test System, based on the bioluminescence of the marine bacterium *Vibrio fischeri* after 15 min of exposure, was used to measure the toxicity of the soil, according to the standard protocol for the Microtox® basic test ISO NF EN 11348-3 (2019). Soil toxicity was estimated through the determination of the calculated effective concentration EC_{50} of the soil lixiviate, which is a hypothetical value that represents leachate concentration (% v/v), that would produce a 50 % reduction in luminescence in *V. fischeri*. Briefly, 2 g of each soil sample was added to 3 mL of NaCl in a 2 % solution. These suspensions were incubated in an orbital shaker for 10 min, centrifuged for 2 min at 10000 rpm and serially diluted (1:2) with NaCl at 2 % solution (50 %, 25 %, 12.5 % and 6.25 % (v/v)) and compared with the control (Molina et al., 2009). Freeze-dried *V. fischeri* were rehydrated forthwith before use in testing. Tests were performed in a temperature-controlled photometer at 15 °C (Microbics Corporation, 1992). It was assumed that the control containing only *V. fischeri* is regarded to have 0 % of inhibition, and the samples containing NaCl (2 %) are considered to have 100 % inhibition. EC_{50} parameter is given by the Microtox® Text System for each sample analysed, and the toxic units (TU) were estimated using the formula $TU = 100/EC_{50}$, according to Persoone et al. (2003).

3. Results and discussion

3.1. Isolation and identification of TFL-degrading bacteria from the NMC consortium

Soil bacterial strains were isolated from the TFL-degrading NMC consortium. Specific TFL-degrader strains were selected using a minimal medium containing TFL as the only added source of carbon and energy. CTFL1, CTFL2, CTFL3, CTFL4, CTFL5, CTFL6 and CTFL7 were identified as *Bacillus aryabhattai, Pseudomonas guariconensis, Pseudomonas brassicacearum, Bacillus circulans, Bacillus safensis, Bacillus maritimus and Arthrobacter aurescens,* respectively. In Table S1 the phylogenetic affiliations of the seven bacterial strains obtained are shown. All of them presented 100 % similarity, except in the case of CTFL1, it was 99.92 %. Gram-positive bacilli were the most abundant taxa and that coincided with the fact that other authors described strains of the genus *Bacillus* as dinitroaniline degraders (Erguven et al., 2016; Ni et al., 2016; Ni et al., 2019). *Arthrobacter aurescens,* another Gram-positive bacterium and two bacterial strains belonging to the *Pseudomonas* genus (Gram-negative) were isolated from the microbial consortium.

3.2. Biodegradation of trifluralin by microbial consortia in soil

TFL biodegradation using the microbial consortia NMC, the individual isolated bacteria and the artificial bacterial consortium ABC were carried out directly on soil because TFL in aqueous solution is highly sensitive to ultraviolet radiation (Le Person et al., 2007; Caoa et al., 2019). It was observed that the concentration of TFL (10 mg L^{-1}) in solution decreased by 92 % after 60 d (Fig. S1 in supplementary data) when it was not protected from daylight, confirming its photodegradation. However, when TFL is in contact with soil it can be adsorbed onto soil particles, especially on soil organic matter, which is inhibitory for its photodegradation (Coleman et al., 2020; Chowdhury et al., 2021). This fact can be observed in Fig. 1 where TFL biodegradation curves in PLD soil are shown, and TFL

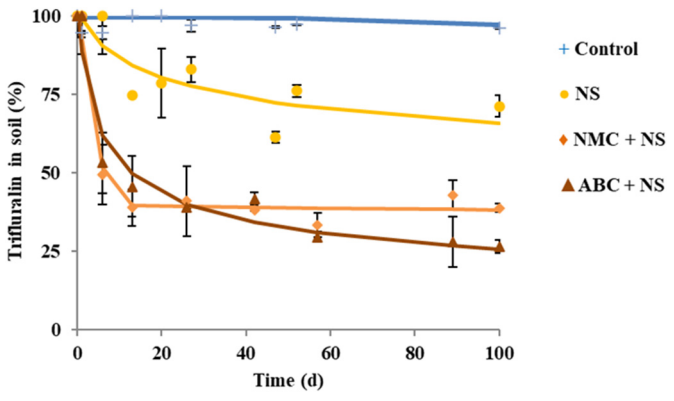

Fig. 1. Biodegradation curves of trifluralin in non-sterile soil (100 d) without treatments (+), and in presence of: nutrients solution (NS) (●), a natural TFL-degrader microbial consortium isolated from a soil + (NS) (♦) and an artificial bacterial consortium formed by 7 TFL-degrading bacterial strains (ABC) + NS (▲). Solid lines show model fitting to the experimental results (symbols). Vertical lines show standard deviation calculated using triplicates.

Table 1
Kinetic parameters (*) calculated after different biodegradation treatments applied in trifluralin contaminated soil.

Treatment	Kinetic model	K_1 (d^{-1})	K_2 (d^{-1})	Tb (d)	α (d^{-1})	β (d^{-1})	DT_{50} ± SD (d)	Extent of biodegradation (%)	χ^2
Control	SFO	2.3×10^{-4}	–	–	–	–	2325 ± 102	1.8	1.3
Nutrients solution (NS)	FOMC	–	–	–	1.4×10^{-1}	4.3	644 ± 83	34	6.7
Natural Microbial consortium (NMC) + NS	HS	1.2×10^{-1}	4.5×10^{-4}	8.3	–	–	5.9 ± 1.7	62	0.3
Artificial Bacterial consortium (ABC) + NS	FOMC	–	.	–	3.4×10^{-1}	1.6	11 ± 1.0	74	9.4
B. aryabhattai CTFL1	SFO	3.5×10^{-4}	–	–	–	–	1933 ± 139	2.3	1.1
P. guariconensis CTFL2	SFO	6.4×10^{-3}	–	–	–	–	125 ± 22.7	48	4.6
P. brassicacearum CTFL3	FOMC	–	–	–	3.3×10^{-1}	11.1	79 ± 14.2	47	3.1
B. circulans CTFL4	SFO	8×10^{-3}	–	–	–	–	98 ± 15	48	1.1
B. safensis CTFL5	SFO	1.0×10^{-3}	–	–	–	–	678 ± 146	18	2.9
B. maritimus CTFL6	SFO	1.2×10^{-3}	–	–	–	–	579 ± 165	17	1.1
A. aurescens CTFL7	SFO	8.2×10^{-3}	–	–	–	–	84 ± 24	55	1.7
RAMEB	HS	4.3×10^{-1}	1.9×10^{-3}	1.5	–	–	19 ± 2.3	56	5.6
RAMEB + *A. aurescens* CTFL7	HS	1.7×10^{-1}	1.7×10^{-2}	2.4	–	–	19 ± 0.1	88	5.1

(*) K, α, β: mineralization rate constants; tb: Time at which rate constant changes; DT_{50}: Time required for the concentration to decline to half of the initial value; SD: Standard deviation of replicates ($n = 3$).

biodegradation kinetic parameters determined from the kinetic models are shown in Table 1. In the control experiment when no treatments were added, TFL presented a loss of only 1.8 %. $HgCl_2$ was added to sterilise the soil, discarding abiotic processes (data not shown).

Clear differences were observed among some of the treatments studied. In presence of NS (biostimulation), the soil microbiota was stimulated to check its ability to biodegrade the herbicide, and after 100 d of assay 34 % of the concentration initially added was removed. Experimental data fitted well with FOMC kinetic model, calculating that the initial concentration would be reduced by 50 % after >600 d. In the case of inoculation with the natural microbial consortium NMC, all the biodegradation parameters were improved. The biodegradation curve was fitted to a biphasic kinetic model, HS. After 100 d of experiment, the extension of biodegradation increased up to 62 % and a DT_{50} of only 5.9 d was determined. The biodegradation curve of the NMC consortium did not show a lag phase (Fig. 1), which means that the microbial consortium did not require an adaptation period to start using TFL as a carbon source. These data confirm that, effectively, bioaugmentation using the autochthonous soil microbiota obtained from a TFL enrichment culture increases the loss of TFL at 100 d by 27 %, concerning the biostimulation treatment (NS) as the only assisted natural attenuation tool. Something similar could be observed when the artificial consortium ABC was used as soil bioaugmentation. At the end of the assay, the ABC was able to degrade 74 % of the initial TFL concentration (Table 1), a percentage even higher than those observed with the NMC. However, DT_{50} is higher (11 d). With both consortia, a complete TFL biodegradation was not achieved. This fact could be due to the formation of metabolites from the biodegraded herbicide, which could be toxic for inoculated microorganisms causing inhibition of TFL biodegradation (Ni et al., 2016; Villaverde et al., 2018). Studies have shown that the main metabolites (2-ethyl-7-nitro-5-(trifluoromethyl)benzimidazole, 2-amino-6-nitro-4-(trifluoromethyl)phenyl)propylamine, α, α, α-trifluoro-2,6-dinitro-7-(3-hydroxypropyl)-p-toluidine formed by degradation of TFL are toxic for aquatic organisms and mammals (Chan et al., 2013; Han et al., 2019) and although high toxicity has not been observed for microorganisms present in soil, deviations from respiration and nitrogen transformation of up to 25 % have been described (EFSA, 2009). There is also the possibility that TFL became bio-unavailable after a period in contact with soil due to a very strong sorption on it (ageing process) (Semple et al., 2004). The degradation of the herbicide TFL may be affected by both processes: the presence of metabolites more toxic than parent compound and the possibility of an ageing course.

3.3. Biodegradation of trifluralin by pure cultures of degrading bacteria in soil

Seven potential TFL degrader strains found in the studied soil were selected to perform TFL biodegradation assays in soil: *Bacillus aryabhattai* CTFL1, *Pseudomonas guariconensis* CTFL2, *Pseudomonas brassicacearum* CTFL3, *Bacillus circulans* CTFL4, *Bacillus safensis* CTFL5, *Bacillus maritimus* CTFL6, and *Arthrobacter aurescens* CTFL7.

TFL biodegradation curves obtained after inoculation with the isolated strains are shown in Fig. 2 and biodegradation kinetics results are in Table 1. Three species of the genus *Bacillus*, *B. aryabhattai* CTFL1, *B. safensis* CTFL5 and *B. maritimus* CTFL6 achieved the lowest biodegradation percentages (2.3 %, 18 % and 17 %, respectively). These values were even lower than the result obtained by the stimulation of the soil microbiota in presence of only NS (extent of biodegradation 34 %, Table 1), and for some of them, a longer time was also necessary to reach 50 % of the biodegradation (DT_{50} = 1933 d, 678 d, for CTFL1 and CTFL5, respectively). Observing these results, the selection of a good bacterial strain to be used as the bioaugmentation is important since it is not possible to know initially the behaviour of a particular strain and its interaction with the endogenous microbial population (Singer et al., 2005; Herrero and Stuckey, 2015; Bose et al., 2021). Among the biotic factors affecting bioaugmentation, the most important seems to be the interactions between autochthonous and inoculated microorganisms, such as predation and the competition for nutrients and niches.

The best results were obtained with *P. guariconensis* CTFL2, *P. brassicacearum* CTFL3 and *Bacillus circulans* CTFL4, reaching about 47–48 % in the extent of biodegradation, and particularly with *A. aurescens* CTFL7, which increased up to 55 % the biodegradation of TFL. In addition, the DT_{50} values obtained were lower (125, 79, 98 and 84 d, respectively) than those of the control (>6 years) or the system biostimulated with NS (almost 2 years). Given the results obtained,

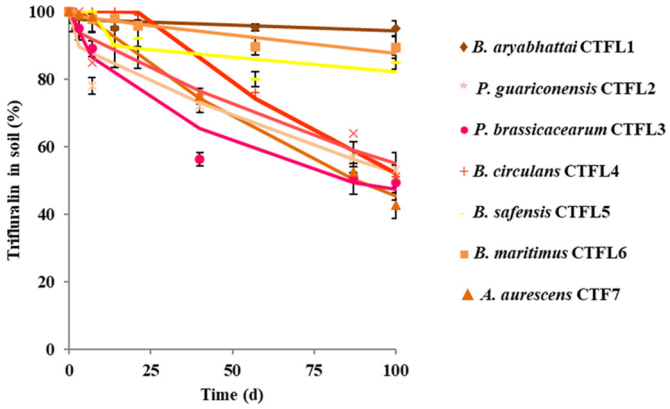

Fig. 2. Biodegradation curves (100 d) of trifluralin herbicide in non-sterile soil in the presence of 7 isolated bacterial strains + nutrients solution (NS): (♦) *B. aryabhattai* CTFL1, (✳) *P. guariconensis* CTFL2, (+) *B. circulans* CTFL3, (–) *B. safensis* CTFL4, (■) *B. maritimus* CTFL5, (●) *P. brassicacearum* CTFL6 and (▲) *A. aurescens* CTFL7 in PLD soil. Solid lines show model fitting to the experimental results (symbols). Vertical lines show standard deviation calculated using triplicates.

A. aurescens CTFL7 was selected as the most suitable bacterial strain to continue with TFL soil bioremediation studies.

Few bacterial species have been described in the literature as TFL degraders, such as strains belonging to the *Bacillus* (Bellinaso et al., 2003; Erguven et al., 2016) and *Pseudomonas* genera (Carter and Camper, 1975; Sato, 1992). However, all these studies published have been carried out in aqueous systems. It is noteworthy that *A. aurescens* and the rest of bacterial strains isolated in the present study had never been previously described as TFL-degrading bacteria in both aqueous solution and soil.

3.4. Application of CD in a TFL contaminated soil

The low water solubility of TFL and its high hydrophobicity can limit its availability to microorganisms, which is a potential problem for the bioremediation of soils contaminated by such hydrophobic compounds. Biodegradable complexing agents are used to achieve an improvement in the water solubility of the contaminant, thus increasing its bioavailability (Morillo et al., 2012). CDs have been selected as bioavailability enhancers for improving the aqueous solubility of TFL and its removal from contaminated soils. To increase TFL hydrosolubility three CDs were studied and their phase solubility diagrams are shown in Fig. 3. No studies had been previously conducted on the effect of CDs on TFL. A solubility limit could not be obtained in the range of CD concentrations used in the case of BCD and HPBCD, which is in line with an inclusion complex with 1:1 stoichiometry (straight lines of the phase solubility diagrams with slope < 1, A_L diagram according to Higuchi and Connors, 1965). However, in the case of RAMEB, a diagram type B_s was observed since the solubility increased linearly until it reached a plateau. The plateau area shows that the herbicide solubility has reached its maximum value, and subsequent additions of RAMEB will lead to a precipitation of the formed complex. The major driving forces for the formation of CD inclusion complexes are hydrophobic and Van der Waals interactions between the inner surface of the CD ring and the hydrophobic sites of the guest molecule (Wenz et al., 2006). The complexation parameters for all CDs tested are shown in Table 2. K_c values showed that HPBCD achieved an increase in solubility, but the highest values were obtained when RAMEB was used, with an increase of the solubilisation efficiency (S_e) of 3153 times higher than the aqueous solubility of TFL, and the lowest S_e and K_c values corresponded to BCD. Based on the results obtained, RAMEB was selected to be used to potentially increase TFL bioavailability in the TFL biodegradation tests in soil.

RAMEB addition caused an improvement (almost double) in the extent of biodegradation (56 %, Fig. 4) as well as a drastic decrease in DT_{50} (19 d) in comparison to the treatment with only biostimulation (NS, Table 1, 34 % and DT_{50} 644 d). CD application could have increased the TFL bioavailable fraction in the soil solution, which would have accelerated its biodegradation by the endogenous microbiota of PLD soil (Villaverde et al., 2018).

Table 2
Trifluralin apparent stability constants (K_c)[a] and solubilisation efficiency (S_e)[b] obtained for TFL-CDs complexes from their phase solubility diagrams.

	S_e	k_c (M^{-1})	R^2
RAMEB	3153	487599	0.89
HPBCD	487	7993	0.96
BCD	13	5	0.96

[a] Calculated according to Higuchi and Connors (1965).
[b] Increment of TFL apparent solubility at the highest CD concentration studied regarding its water solubility.

On the other hand, the result of combining the bacterial strain that achieved the best biodegradation results in the previous study, *A. aurescens* CTFL7, and RAMEB was also evaluated (Fig. 4). The degradation curve obtained was fitted to the HS model. The biodegradation percentage increased considerably (88 %), although DT_{50} was the same as in the treatment with only RAMEB (19 d, Table 3). Therefore, the combined use of *A. aurescens* CTFL7 and RAMEB promotes the bioremediation process of studied soil contaminated with TFL.

The higher TFL degradation observed based on solubility results could be caused by the ability of the RAMEB to form an inclusion complex with TFL, improving its extraction from the soil and its bioavailability and consequently the biodegradation rate. On the other hand, RAMEB might also stimulate the soil microbial community and *A. aurescens* CTFL7, since it is an oligosaccharide and may be attractive as a carbon source for microorganisms present in the soil (Fenyvesi et al., 2005). The biostimulant effect of RAMEB on *A. aurescens* was confirmed and the results are shown in Table S2.

3.5. Ecotoxicology

Quantifying contaminant levels using analytical methods is relevant after the application of bioremediation treatments. However, a decrease in the pollutant concentration is not enough to confirm a reduction in toxicity, since toxic metabolites from the biodegradation process could be present (Jiang et al., 2016; Lara-Moreno et al., 2022b). In the Microtox Test System, the reduction in the luminescence of *V. fischeri* bacteria is used to measure the toxicity of an environmental sample, which is translated into toxic units (TU) and the concentration of the pollutant that produces a 50 % reduction in initial luminescence (EC_{50}) (Madrid et al., 2019).

TFL toxicity was evaluated in the PLD soil artificially contaminated with TFL (50 mg kg^{-1}), following the protocol established by Molina et al. (2009). In all cases, after the application of the different treatments, a very large decrease in the toxicity at the end of the experiment was observed for the contaminated soil before bioremediation treatments

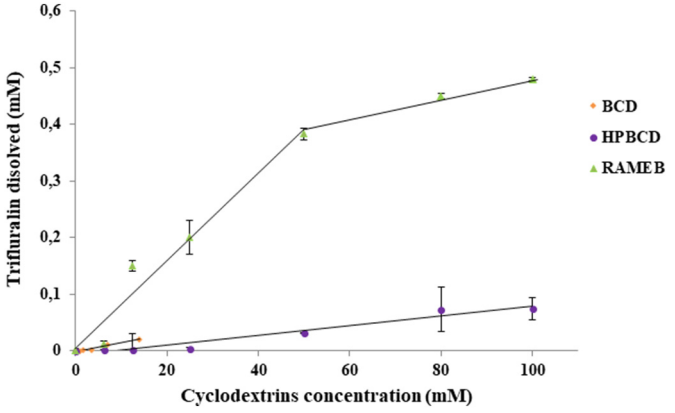

Fig. 3. Phase solubility diagrams of trifluralin in the presence of the cyclodextrins studied. Solid lines show model fitting to the experimental results (symbols). Vertical lines show standard deviation calculated using triplicates.

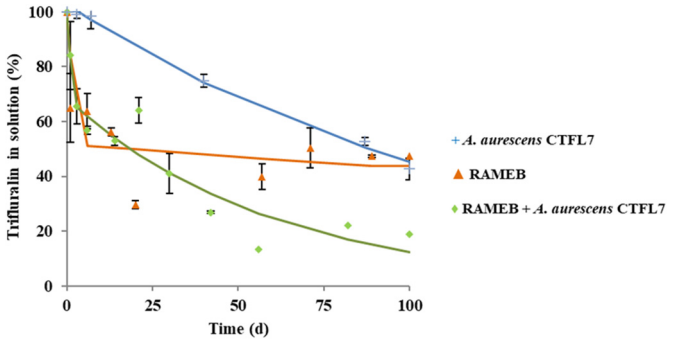

Fig. 4. Biodegradation curves (100 d) of trifluralin herbicide in non-sterile soil + nutrient solution (NS) in presence of *A. aurescens* CTFL7 (+), RAMEB (▲) and *Arthrobacter aurescens* CTFL7 + RAMEB (♦). Solid lines show model fitting to the experimental results (symbols). Vertical lines show standard deviation calculated using triplicates.

Table 3
Acute toxicity test towards *V. fischeri* before and after 100 d of incubation of PLD soil contaminated with TFL (50 mg kg^{-1}) with NMC, ABC, *Arthrobacter aurescens* CTFL7 and RAMEB.

Treatment	EC_{50} (%)*	TU	Toxicity**
Without treatment	14	7.0	Acute toxicity
NMC	98	1.3	Acute toxicity
ABC	110	0.9	Slight acute toxicity
A. aurescens CTF7	1679	0.06	Non-toxic
A. aurescens CTF7 + RAMEB	ND***	–	Non-toxic

* For each soil sample the EC_{50} value corresponds to the soil extract concentration (% v/v) having a toxic effect on 50 % of the bacterial population.
** According to Persoone et al. (2003).
*** No detectable.

(Table 3), which presented a value of 7.0 TU (1 < TU < 10, acute toxicity). When bioaugmentation techniques were applied by inoculating NMC, ABC or the bacterial strain *A. aurescens* CTFL7, lower TU values were obtained (1.3, 0.9 and 0.06 TU, respectively). However, in the case of NMC, although a considerable reduction in the TU value was determined, an acute toxicity level remained at the end of the treatment, probably due to the formation of toxic metabolites in the biodegradation process (Klupinski, 2003; Ni et al., 2016). When ABC was inoculated, a TU value of 0.9 (slight acute toxicity, 0.4 < TU < 1) was obtained according to the Persoone classification. The most satisfactory results were reached in presence of *A. aurescens* CTFL7, with a TU value of 0.06, reaching a non-toxic level in the soil (TU < 0.4) and when RAMEB was simultaneously applied with *A. aurescens* CTFL7 since, at the end of the biodegradation assay no toxicity was detected.

Ecotoxicological studies of TFL in soil using Microtox® have not been previously described in the literature. However, several authors have employed the Microtox® bioassay to determine TFL residual toxicity in water. Dimou et al. (2004) observed a decrease in toxicity from 17 % of inhibition to 2 % after 40 min when contaminated water samples with TFL were irradiated. In another study, Trajkovska et al. (2009) carried out a toxicological study of numerous pesticides (chlorothalonil, cyprodynil, dichlobenil, pendimethaline, trifluraline, and α-endosulfan) in precipitations of Paris. Oxidation kinetics of TFL in water was also studied by Chelme-Ayala et al. (2011), and the end products were analysed with Microtox®. After oxidation a decrease in toxicity was detected.

4. Conclusions

In conclusion, the endogenous microbiota of PLD soil was not able to achieve a significant percentage of biodegradation of TFL after stimulation using a nutrient solution (34 %). However, when the studied soil was bioaugmented with its endogenous microbial consortium (NMC), isolated by enrichment culture, an improvement in TFL biodegradation was obtained (62 %), as well as when the contaminated soil was inoculated with ABC consortium (7 individual bacterial strains isolated from NMC), reaching a global extent of biodegradation of 74 %. Among the bacterial strains that constitute ABC, *A. aurescens* CTFL7 showed to be the most suitable for carrying out bioremediation assays. The absence of toxicity in the soil after the bioremediation assay was achieved. In addition, an improvement in the results was observed when bioaugmentation with CTFL7 strain was combined with the addition of the cyclodextrin RAMEB (88 % degradation), being the best combination to remediate the contaminated soil. RAMEB could be acting as a bioavailability enhancer due to its capacity to form an inclusion complex with the herbicide and as a biostimulant, since it could be used as a carbon source by microorganisms. It should be noted that the *A. aurescens* strain had not been previously described as a TFL degrader; moreover, so far, no author has published the use of CD in TFL contaminated soil to improve the bioremediation strategy.

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CRediT authorship contribution statement

Alba Lara-Moreno: Literature revision, Investigation, Design of experiments, Methodology, Writing an original draft. **Esmeralda Morillo:** Writing - review & editing, Funding acquisition. **Francisco Merchán:** Investigation and Supervision. **Fernando Madrid:** Investigation, methodology, review. **Jaime Villaverde**: Conceptualisation, Design of experiments, Writing - review & editing, Funding acquisition, Supervision.

Declaration of competing interest

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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